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Dedication

For my parents who has endlessly supported my path, my colleagues Punkaj Ahuja and Zhehao Zhang, my undergraduate students Youjoung Kim and Grace Nemckey, For my friends in 4th floor Wickenden Dr. David Prabhu, Dr. Zheng Han, Dr. Da Sun and Dr. Nadia Ayat
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Multicellular Tumor Hemi-Spheroid: A Novel *In Vitro* 3D Model Platform

For Accelerated Drug Development

Abstract

by

KIHWAN KIM

Multicellular tumor spheroid (MCTS) is currently the most accepted 3D *in vitro* tumor model. MCTS can mimic *in vivo* avascular tumor and often used in drug development studies before the *in vivo* animal experiment. Despite of its recognized values, MCTS is still not widely implemented in drug development pipeline. This is because culturing MCTS to an appropriate size (above 300µm) is often difficult and assays to analyze drug penetration in MCTS relies on the information of lumped cell death and biomarkers after exposure to drugs. We have developed a novel 3D *in vitro* model called Multicellular tumor hemi-spheroid (MTHS). MTHS shares same nutrient and oxygen distribution with MCTS due to similarity in the geometric shape. Also the flat equatorial surface of the MTHS can act as a window to its inner region. With this key feature cell viability and drug distribution can be mapped and oxygen distribution quantified non-invasively. This thesis describes the process of MTHS culture using an agarose scaffold approach, characterization of MTHS and viability and drug penetration assays using MTHS.
Chapter 1 Multicellular Tumor Hemi-Spheroid a novel in vitro model of tumor capillary microenvironment

1. Introduction

1.1 3D in vitro tumor models

Today, in vitro 3D tumor models are becoming widely adopted in tumor system biology, pharmaceutical drug discovery and screening and personalized medicine. This is because 3D tumor models can recapitulate the in vivo phenotype of a solid tumor such as cell morphology, cell-to-cell signaling and cell–to–extracellular matrix (ECM) interaction, in an in vitro setting (Leoni A. Kunz-Schughart, Freyer, Hofstaedter, & Ebner, 2004; X. Xu, Farach-Carson, & Jia, 2014). In tumor system biology 3D tumor models can provide insight into the pathobiology of human cancer, close to in vivo cancer (Weiswald, Bellet, & Dangles-Marie, 2015). For drug discovery and screening, an in vitro 3D tumor model can be used to predict therapeutic efficacy of the drug under development (Shin, Kwak, Han, & Park, 2013). Finally, in personalized medicine, a combination of the patient’s genetic information and 3D-cultured primary cells in vitro can be used to predict treatments from existing anti-cancer drugs that will yield improved therapeutic efficacies (Ma et al., 2012).

1.2 Multicellular tumor spheroid model

The most commonly used 3D tumor model is the Multicellular Tumor Spheroid (MCTS) model. MCTS is an in vitro 3D tumor model that is a compact aggregate of growing tumor
cells that resemble a sphere. The significance of MCTS is that the model can recapitulate initial avascular stages of solid tumor (L. A. Kunz-Schughart, Kreutz, & Knuechel, 1998). As the tumor tissues grow, tumor cells close to vessels exhibit high proliferative activity, while cells 100 – 200 µm distance away from the vessels form quiescent and necrotic cells (L. A. Kunz-Schughart et al., 1998). This is recapitulated within MCTS when it grows ≥ 250µm in diameter. Three distinct layers are manifest in MCTS: a) proliferating peripheral cell layer, b) intermediate quiescent cell layer and c) necrotic core at the center (Dubessy et al. 2000; Ivascu and Kubbies 2006; Mikhail et al. 2014). These distinct layers (or regions) are formed because nutrients and oxygen are not evenly diffused and distributed due to high consumption of rapidly proliferating cells. In fact oxygen and nutrient gradients established in both nascent tumor and MCTS lead to hypoxia and acute acidosis because metabolic waste products are accumulated and not cleared (Mueller-Klieser 1987; Rotin, Robinson, and Tannock 1986; Thoma et al. 2014). Therefore, due to similarities between MCTS and tumors in terms of morphology, distinct differentiated cell populations from proliferating to necrotic cells and mass transport features, MCTS can be applied in various experimental therapy studies using radiotherapy, chemotherapy, photodynamic treatment, antibody-based immunotherapy and hyperthermia (Hirschhaeuser, Leidig, Rodday, Lindemann, & Mueller-Klieser, 2009; Leoni A. Kunz-Schughart et al., 2004).

1.3 MCTS culture methods

There are four main methods to culture MCTS (Breslin and O’Driscoll 2013; Hirschhaeuser et al. 2010; Mueller-Klieser 1987). MCTS are cultured using static liquid based techniques such as 1) hanging drop and 2) liquid overlay methods, or flow based
liquid techniques such as 3) spinner flask and 4) microfluidic methods (Breslin and O’Driscol 2013; Mueller-Klieser 1987). Static liquid based techniques have the advantage of generating a large number of uniformly sized MCTS. Hence high throughput screening is possible (Breslin and O’Driscol 2013; Hirschhaeuser et al. 2010; Mueller-Klieser 1987). However the process of culturing and handling is rather difficult with the static liquid based techniques because the culture medium has to be changed frequently, and some cell lines form loose aggregates which are not close to the in vivo tumor. Flow based techniques require less maintenance for culturing MCTS because fresh culture medium is continuously provided using a peristaltic pump (microfluidics). In addition a large number of compact MCTS can be formed (spinner flask). Nevertheless, the sizes of MCTS are not uniform and shear flow may be problematic for mechanically fragile cells (Breslin & O’Driscoll, 2013). Figure 1 summarizes each of the techniques and their applicability.
Figure 1-1: Multicellular tumor spheroid (MCTS) generation methods (Katt, Placone, Wong, Xu, & Searson, 2016).

1.4 MCTS endpoint assays

Today, MCTS is accepted as the most favored 3D tumor model in research and pharmaceutical drug development. Companies such as Insphero and Perkin Elmer are producing multi-well plates that can culture 96 or 356 MCTS for high throughput screening. Integrating image based techniques into high throughput screening system, an image based assay platform has been built to assess MCTS by: 1) morphological change of MCTS before and after drug treatment, 2) tumor cell viability in MCTS using Alamar blue assay, and 3) immunostaining to observe the manifestation of hypoxia inducing factor (HIF) and
certain protein expressions in MCTS (LaBarbera, Reid, & Yoo, 2012). All these are important assays required to quantify and predict the therapeutic efficacy. However, MCTS has distinct differentiated cell populations from proliferative to necrotic cells. In addition its inner region has hypoxia and acute acidosis as oxygen, nutrient and metabolic gradients are established. It is important to evaluate drug response at these sub-population of cells because hypoxia and acute acidosis can neutralize the drug before it reaches the target tumor cells (Gandolfo, Kyle, & Minchinton, 2016; Wojtkowiak, Verduzco, Schramm, & Gillies, 2011). Also quiescent tumor cells that survived drug treatment can lead to recurrence of tumor and treatment resistance which is a serious adverse factor in tumor drug treatment (Wang & Lin, 2013). Therefore an assay that can quantify drug response at the inner regions of MCTS can lead to more efficient design and development of drugs and predict their therapeutic efficacy in clinical trials.

1.5 Multicellular tumor hemi-spheroid

With high end microscopy, the inner region of MCTS can be visualized and its treatment response activity quantified using fluorescence measurement. Confocal microscope and multi-photon microscope can optically slice and image one horizontal section of MCTS. Yet the so called “optical sectioning” method can suffer from the problem of poor image quality if MCTS size gets larger over time. The larger the MCTS (beyond 300µm), the higher the light scattering, absorption and reflection effect which leads to deteriorated image quality (Fennema, Rivron, Rouwkema, van Blitterswijk, & de Boer, 2013; LaBarbera et al., 2012). Hence to visualize and analyze inner-region drug response with high image quality, a novel approach to designing in vitro 3D platform relevant to MCTS
is necessary. Previously in Dr. Miklos Gratzl’s lab, a novel *in vitro* 3D tumor model based on MCTS was developed. This novel 3D tumor model called “multicellular tumor hemispheroid” (MTHS) is basically a MCTS cut in half.

![Figure 1-2](image)

**Figure 1-2:** Concept of multicellular Tumor Hemi-Spheroid (MTHS) from a MCTS after cut (Sheth D.B., 2011)

The flat equatorial plane of half cut MCTS can be placed on an impermeable substrate. Such a MTHS then can be studied as if it were uncut MCTS because transport paths of nutrients, oxygen, waste and drug molecules do not change by the cutting due to spherical symmetry. Therefore a “window” into the interior of the MCTS is created that makes it possible to visualize and analyze drug response in inner regions of MCTS without the risk of deteriorating image quality as well as perform noninvasive analytical measurement of the gradient (oxygen, pH, glucose, ATP).
1.6 MTHS culture – scaffold based approach

MTHS, a depth resolved in vitro 3D tumor model, has the advantage of being able to visualize inner regions of MCTS as well as quantifying oxygen, metabolic and nutrient distribution within inner regions of MCTS. However MTHS is difficult to be implemented in large scale culture which in turn constraints its use for research or low throughput drug screening, because it requires significant labor and skills to culture and cut one MCTS and place the cut MCTS on a well plate. Yet MTHS can be cultured in an alternative approach that is based on natural or synthetic scaffolds. Scaffold is a polymeric structure that provides proper morphology and stability and mimics the native extracellular matrix of biological tissues. The key advantages of scaffold based 3D tumor construct are that: 1) scaffold can mimic extracellular matrix of solid tumor, 2) provide uniform and defined architecture and morphology of 3D tumor tissue and 3) amenable for large scale 3D culture and high throughput screening (X. Xu et al., 2014). Although natural scaffolds are the most preferred in tissue engineering because they are similar to biological extracellular matrix (extracellular matrix is composed of collagen, hyaluronic acid, proteoglycans, glycosaminoglycans, and elastin), there are drawbacks due to auto-fluorescence and they have poor batch-to-batch consistency, along with less controllable, and less reproducible characteristics such as matrix mechanical properties, porosity, degradation profile and shape (G. Xu et al., 2014; X. Xu et al., 2014). Synthetic scaffolds on the other hand provide more flexibility in controlling the biophysical and biochemical characteristics of the scaffold. They have improved batch-to-batch consistency and also biocompatibility making it available alternative to natural scaffolds. However synthetic scaffolds may lack one of the major components of extracellular matrix: collagen, hyaluronic acid,
proteoglycans, glycosaminoglycans, or elastin. Also some synthetic scaffolds may not fit for use depending on the pH, ionic strength and cross-linking chemistry of the synthetic scaffolds (Wolfe, Sell, & Bowlin, 2011; G. Xu et al., 2014).

1.7 Agarose as a scaffold for MTHS

In order to culture MTHS with reduced amount of labor, in this work a natural scaffold approach was chosen. Among the natural scaffolds available, agarose was selected as ideal scaffold for culturing MTHS. Agarose is a marine polysaccharide that consists of thick bundles of agarose chains linked by hydrogen bonds with large pores holding water (Narayanan, Xiong, & Liu, 2006). The pore size of agarose is 100 – 700nm with a mean of 400nm (Lee et al., 2013). Compared to natural scaffolds, agarose is more stable in terms of pH, ionic strength, cross-linking chemistry and temperature and biocompatible (G. Xu et al., 2014). Agarose is utilized in bone cartilage tissue engineering by seeding chondrocyte stem cells in liquid agarose. Agarose can recapitulate the in vivo phenotype of cartilage tissues and is therefore widely used for cartilage tissue engineering (Awad, Quinn Wickham, Leddy, Gimble, & Guilak, 2004). In this work agarose MTHS can be made when tumor cells are homogenously suspended in aqueous agarose and microliter volume of agarose + cell droplets are deposited on top of a cold hydrophobic surface. Due to low surface tension at the hydrophobic surface, droplets can form hemi-spherical shape while gelling if the surrounding temperature around the agarose +cell droplets is below 10° C. The gelled agarose + cell droplets are then incubated at 37° C with 5% CO₂ to promote proliferation and growth of cells inside the scaffold for three to five days.
In this work we present the detailed tissue engineering design of making scaffold for MTHS model. We evaluate the size, cell viability, morphology and cell packing density of the scaffold based MTHS model.

2. Experimental Methods

2.1 Materials

Agarose type VII-A was purchased from Sigma Aldrich (St Louis, MO). DMEM cell culture medium, fetal bovine serum (FBS), Dulbecco’s phosphate buffer saline (DPBS) and 0.25% Trypsin EDTA were purchased from Life technologies (Carlsbed, CA). Accutase and Accumax were purchased from Innovative cell technologies (San Diego, CA). Ultra Low Retention (ULR) pipette tips were purchased from BrandTech (Essex, CT). Lyophilized beta agarase solution was purchased from Sigma Aldrich (St Louis, MO). Mouse Mammary HER-2 positive cells (NF639) were obtained from Dr. Norbert and Stefanie Avril’s laboratory.

2.2 Multicellular tumor hemi-spheroid culture

NF639 cells were maintained and cultured in DMEM containing 10% fetal bovine serum (FBS) only in T75 flask. Cells are incubated at 37°C in 5% CO2 environment. 80-90% confluent NF-639 cells in T75 flasks are then detached by adding 3mL of accutase solution and collected in a sterile 15ml conical tube. 3ml of sterile DPBS is added to make the final volume in 15ml conical tube to be 6mL. 100µl of sample from the 15ml conical tube is
extracted and pipetted in 1.5ml vial containing 650µl of DPBS. The 1.5ml vial is then used to calculate the cell density and the total number of cells in 15ml conical tube by cell counting using a hemocytometer or a Vi-Cell counter (Beckman Coulter). Cells in the 15ml conical tube are spun down in a centrifuge (Sorvall ST40) at 1400 rpm for 5min. Supernatant medium in the 15ml conical tube is aspirated leaving cell pellets at the bottom. 1% agarose type VII-A (gelling temperature at 26 °C and melting temperature above 66 °C) is then added to the cell pellet and mixed to achieve a density of 0.2million cells per µl. The conical tube with cell-agarose mixture is then sonicated for 2 min to suspend cells homogenously in liquid agarose solution. The conical tube after sonication is kept in sterile water bath at 35° C to prevent the cell-agarose mixture from gelling. 1µl droplets of cell suspension in liquid agarose are deposited onto an ice cold sterile non-tissue-culture petri dish using 0.1 – 2.55 µl pipette and ULR pipette tips. Liquid droplets with the suspended cells in them form hemi-spherical droplets due to hydrophobic property of the polystyrene petri dish and low temperature, and quickly gels in less than few seconds. 20µl of DMEM + 10% FBS are deposited on top of the cell-agarose hemi-spheroids using 10-100µl pipette and the petri dish carrying the model cell-agarose hemi-spheroids is carefully placed in an incubator at 37° C (in humidified air with 5% CO2) and cultured for 1-5 days. Henceforth, model agar hemi-spheroids will be referred to here multicellular agar suspended hemi-spheroid or MTHS.
2.3 MTHS analysis: cell number estimate using automatic cell counter

Total cell number in MTHS was quantified using combinations of cell dissociation solution and agarose degrading enzyme solution. Each MTHS was carefully detached and transferred to a 1.5ml vial using a 1ml pipette. Supernatant medium was aspirated exposing only MTHS at the bottom of the vial. Pre-warmed 50µl of 30units/ml of beta agarase was added immediately after aspiration and the MTHS structure was mechanically disrupted by pipetting 20 times using a 100µl pipette. After mechanical disruption of MTHS, the vial was placed in water bath (T = 38° C) for 5min. Pre-warmed 100µl accutase or accumax solution was then added after 5 min and the vial was left in warm water bath again for 15 min. 20 min later, the vial was taken out from the water bath and additional 350µl of DPBS solution was added to slow down both beta agarase and accutase or accumax activity. The 500µl solution was homogenously mixed using 1ml pipette and transferred to a cell counter cuvette. The cell counter cuvette was then inserted to an automatic cell counter (Vi-Cell
counter) and the total cell number in MTHS was displayed on the corresponding cell counter’s software.

2.4 MTHS analysis: cell number estimate using volume based approach

The total cell number in MTHS was estimated also using volume based approach. A simple digital goniometer was designed according to a setup published by Lamour et al (Lamour et al., 2010).

![Figure 1-4: A simple digital goniometer to measure contact angle of MTHS (Lamour et al., 2010)](image)

MTHS was detached and carefully placed on top of a microscope glass slide using a spatula. The glass slide with MTHS on top was then fixed at a specific position which is shown in figure 1-2. Once the glass slide was placed, pictures were taken using Nikon DSLR camera and MTHS contact angle pictures were evaluated using image J software. Using the contact
angle and the diameter obtained, we calculated the volume of MTHS. Finally using the estimated volume result of MTHS, estimated cell number was calculated by multiplying the estimated volume to estimated cell seeding density.

3. Results and Discussion

3.1 MTHS culture with different agarose concentrations

MTHS was cultured using an agarose scaffold. Agarose type VII-A was chosen for this work. Agarose type VII-A dissolves in DPBS solution above its melting temperature (above 66°C). The liquid agarose solution maintains liquid phase above 24-28°C. Below 24-28°C, liquid agarose gels. Because agarose type VII-A can maintain liquid state above 28°C, cells can be suspended in liquid agarose solution at 37-39°C and be viable. This was verified by comparing the percent cell viability before and after adding warm liquid agarose using a 0.4% Trypan blue assay from an automated cell counter (Table 1).
A.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Viable cells</th>
<th>Viability (%)</th>
<th>Total cells /ml (x10^6)</th>
<th>Viable cells /ml (x10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF639 cell count1</td>
<td>167</td>
<td>94.0</td>
<td>0.30</td>
<td>0.28</td>
</tr>
<tr>
<td>NF639 cell count2</td>
<td>161</td>
<td>92.0</td>
<td>0.30</td>
<td>0.27</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Viable cells</th>
<th>Viability (%)</th>
<th>Total cells /ml (x10^6)</th>
<th>Viable cells /ml (x10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose + cell_1</td>
<td>79.0</td>
<td>90.8</td>
<td>0.15</td>
<td>0.13</td>
</tr>
<tr>
<td>Agarose + cell_2</td>
<td>52.0</td>
<td>89.7</td>
<td>0.10</td>
<td>0.09</td>
</tr>
<tr>
<td>Agarose + cell_3</td>
<td>80.0</td>
<td>88.0</td>
<td>0.15</td>
<td>0.14</td>
</tr>
<tr>
<td>Agarose + cell_4</td>
<td>81.0</td>
<td>94.2</td>
<td>0.15</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Table 1: NF639 cells percent viability A: from initial cell count, B: after homogenously suspending in warm agarose solution

We chose to maintain the temperature of our cell + agarose mixture at 36° C. 2-3° C temperature above this point will not influence cell viability, but the mixture would take a longer time to gel once it is deposited on bottom of a petri dish. To form a hemi-spherical shape with agarose, surrounding ambient and plate temperature should be below 10° C before the droplet would begin to spread. Also the water content in agarose can evaporate faster if gelling time is long. If that happens, it will cause cell death by dehydration. However cells + agarose mixture is already highly viscous at 36° C, and deposition using a standard 0.5-2.5µl pipette tip is often difficult and may take longer time to deposit especially when depositing these mixtures in lower than 1µl volume. We utilized specialized pipette tip (0.5-2µl) that was designed to deposit viscous liquid. The ultra low retention pipette tip that we used is coated with a chemical that reduces surface energy.
relative to the conventional polypropylene pipette tips. With surface coated tip we deposited 4 x 1µl cell + agarose mixture consecutively on the bottom of a petri dish without difficulty. Since the petri dish was placed on top of a cold ice pack (temperature 20° C measured on the surface of the ice pack using a thermometer), the deposited mixtures gelled within 10 sec and formed MTHS.

Once we deposited at least 16 MTHS, they were then cultured for 1-5 days. NF639 MTHS growth were recorded each day by taking a phase contrast image of MTHS using an inverted light transmission microscope at 4x magnification. Figure 1-3 shows typical NF639 MTHS culture and growth from day 1 to day 5.

![Image of NF639 MTHS culture and growth from Day1 to Day5]

**Figure 1-5**: NF639 MTHS culture and growth from Day1 to Day5

Some of NF639 cells located at the periphery of the MTHS detached from the MTHS and was attached to the bottom of the petri dish at Day1. At Day 3 MTHS became compact while cells that were detached from the MTHS and attached to the bottom of the petri dish proliferated. At Day 5 the cells attached to the bottom of the petri dish proliferated more and were attached to the periphery of the MTHS. MTHS became more compact compared to day 3 and its cells at the periphery looked connected to cells grown attached to the bottom of the petri dish.
3.2 MTHS size

One of the advantages of using a scaffold based 3D tumor model is that the construct’s size and shape can be controlled and uniform. This is important because experiments with 3D tumor spheroids can be reproducible with low variance. Thus the model can be well suited for high throughput drug screening purposes. In our experiment, we evaluated the average diameter of MTHS in 4 petri dishes (n=48) for 1µl deposition by taking pictures of MTHS after they were transferred from the Teflon sheet to a petri dish. Using image J, we determined the MTHS average diameter to be 1450µm with a standard deviation of 200µm. The minimum diameter of the MTHS and its maximum was 1100µm and 1900µm. There seemed to be some variation with MTHS diameter. However two petri dishes (each 12 MTHS) had standard deviation of 160µm with average diameter of 1430µm and 1380µm and one petri dish had standard deviation of 55µm with average diameter of 1300µm. The other remaining petri dish had standard deviation of 120µm and average diameter of 1700µm. This may indicate that MTHS diameter is consistent within each petri dish because ULR tips are not reused but changed after depositing all 12 MTHS in one petri dish. Then the difference in MTHS diameter between the different petri dishes can be partly attributed to two different factors: viscosity of cells + agarose mixture, and low temperature around the tip containing the mixture. However it is still unclear whether both factors or one of the factors contribute to the inconsistency in inter-petri dish MTHS diameter size.
3.3 MTHS cell retrieval

Another advantage of using agarose scaffold to construct 3D tumor model is that cells in the agarose scaffold can be retrieved using mechanical and biochemical disruption of the agarose scaffold. Individualize cells from differentiated cell subpopulations can be obtained and evaluated using biochemical assays. In addition it is feasible to estimate the proliferation rate of the cells as well as its necrosis or apoptosis rate by comparing the number of cells seeded initially and the number of cells after 1 – 14 days period of culture through biochemical assays. Therefore here we tested different methods of cell retrieval from agarose scaffold MTHS and compared the estimated cell numbers to the measured cell numbers. To determine the initial estimated cell number, we looked into the initial cell seeding density in 1µl of liquid agarose. The initial cell seeding density beginning of this project started as 0.3million cells/µl, but 0.15million cells/µl was chosen and set because 0.15million cells/µl was easier to pipette. Initial cell seeding density was determined right after the total cell number estimation using either an automated cell counter or a hemocytometer. From the estimated total number of cells, a known agarose solution volume was added and mixed using a pipette. To verify our initial cell seeding density, we disintegrated MTHS using beta agarase and/or accutase or accumax cell detachment solution to extract individual cells and quantified them using the cell counter. The result we obtained had varying number of cell counts. The average measured cell count was 63,000 cells, median was 62,000 cells and standard deviation was 24,000 cells. This was expected since MTHS had variance in diameter and size after deposition. Hence to further verify that our cell seeding density was consistent between MTHS, we measured MTHS
diameter, contact angle and its volume to quantify total cell number in each MTHS and compared it to measured cell number obtained from the cell counter.

We have previously measured MTHS diameters. We measured contact angle of MTHS using a simple digital goniometer and image J software. The average contact angle for 5 MTHS was 51°. This was surprising because this showed that MTHS was not perfectly hemi-spherical in shape. Non-tissue culture petri dish or polystyrene based petri dish did not have very low surface energy to make agarose + cell deposit into a hemi-spherical shape. Although our MTHS was not perfectly hemi-spherical, it resembled a spherical cap. To estimate the volume of our model, a spherical cap equation was applied

$$V_{cap} = \pi \left( 2 - 3\sin(\alpha) + \sin^3(\alpha) \right) \frac{R^3}{3}$$  \hspace{1cm} (1)

$$R = \frac{a}{\cos(\alpha)}$$  \hspace{1cm} (2)

$$\alpha = 90° - \theta$$  \hspace{1cm} (3)

All 48 MTHS volumes were calculated based on each MTHS corresponding diameter. Contact angle of 51° was chosen for all the volume calculation.

![Figure 1-6: Spherical volume parameters for volume calculation in eq. 1-3](image-url)
Once we obtained the volume for 48 MTHS, initial cell number estimate in MTHS was easily calculated by multiplying estimated volume to estimated initial cell seeding density. All 48 MTHS had varying cell numbers with average cell number to be 40,000 cells, median 38,000 cells and standard deviation of 17,000 cells. Compared to hemi-spherical volume calculation, spheroid cap volume calculation had less variance in estimated cell number count.

We calculated the relative percent error between the measured cell count and estimated cell count to see if there was significant difference in both approach:

\[
Error \% = \left(\frac{ECC - MCC}{ECC}\right) \times 100
\]  

(4)

where ECC is estimated cell count and MCC is measured cell count. The result is plotted in histogram shown in figure 1-5.
Figure 1-7: Relative error percent distribution between measured and estimated cell count

The mean average of our error percentage was -5.7%, median was -1% and standard deviation was 10.4%. From the distribution of error percentage calculation, we can conclude that our initial cell number count between the estimated and measured approach was low and measured cell count relatively had more cells compared to the estimated cell count approach. This means that initial cell count can be retrieved through volume analysis of the MTHS, and can be used to predict accurate proliferation rate and necrotic rate as MTHS is cultured for a long time. Although it is not known what causes the variance in this calculation, we can assume that it can be caused by a) errors during pipetting, b) some minor variation of contact angles between MTHS and c) imperfect in disintegration of MTHS.
3.4 Agarose concentration for optimal scaffold structure

MTHS was cultured using 1wt% concentration of agarose dissolved in DPBS. We also cultured MTHS with different agarose concentrations (ranging from 1.5wt% to 0.25wt%). With lower agarose concentration, dissociation and disintegration of MTHS structure was easier, to count cell numbers, growth and viability rates using hemocytometer or cell counter. Higher agarose concentration can generate mechanical stresses to the cells that can change cell signaling (Fang et al., 2016). Figure 1-4 displays MTHS fabricated with agarose concentration lower than 1wt%.

![0.25wt% agarose scaffold](image1)  ![0.50wt% agarose scaffold](image2)  ![0.75wt% agarose scaffold](image3)

**Figure 1-8**: NF639 MTHS cultured with different agarose concentrations

From this experiment, we observed that agarose concentration lower than 0.75wt% conveyed poor structural integrity because the gelled agarose was not strong enough to immobilize the cells. For 0.25wt% MTHS as soon as 20µl medium was dropped on top of the MTHS, MTHS disintegrated and released the cells in the drop of medium. The same was seen for 0.5wt% MTHS but structural disintegration occurred less frequently (6 out of 12 MTHS remained structurally intact while 3 out of 12 for 0.25wt%) than 0.25wt% MTHS. 0.75wt% MTHS remained structurally stable for all 12 MTHS that was made. Nevertheless gelling time for 0.75wt% was 3-4 seconds longer than 1wt% MTHS because 0.75wt% had less thermos-reversible crosslinking than the 1wt%. Other than that there was no significant
difference between 0.75wt% and 1wt% MTHS regarding deposition viscosity, shape and cell viability. We also made 1.5wt% and 2.0wt% MTHS and both had very stable structure. However cell proliferation was very slow in 2.0wt% MTHS due to high mechanical stiffness of the agarose scaffold (Pernodet, Maaloum, & Tinland, 1997). 1.5wt% MTHS also behaved similarly with 1wt% MTHS, but it was difficult to extract cells even after mechanical and biochemical disintegration of the MTHS.

4. Conclusion and future works

We have characterized a novel in vitro 3D tumor model called Multicellular Tumor Hemi-Spheroid as an intermediate pre-clinical tumor model to be used between common in vitro monolayer and in vivo animal model. We have established that agarose type VII-A is the choice for our 3D tumor tissue scaffold because of its stability and biocompatibility relative to natural scaffolds. Using agarose, we were able to culture MTHS of different sizes and grow them within a week. Although degradation of agarose using agarose degrading enzyme still has to be further tested, agarose degrading enzyme would help to retrieve differentiated type of tumor cells such as proliferating, quiescent and necrotic cells from agarose scaffold based MTHS for further studies to elucidate drug responses in these three cell subpopulations. Yet more work has to be done to culture MTHS with an ideal hemispherical shape. Recently we started growing MTHS on Teflon sheet and measured its contact angle. The contact angle was between 70-80°, which was a significant improvement from the previous contact angle that we obtained. However a downside with culturing MTHS on Teflon sheet is that MTHS has to be transferred to a transparent substrate to assess its features. This would also add difficulty in culturing MTHS using
agarose scaffold approach, but can be overcome with a thin transparent Teflon sheet embedded on top of a well plate or petri dish.

MTHS culture using agarose can be implemented for high throughput screening because agarose structures are reproducible on a large scale. In addition co-culturing fibroblasts and stromal cells with tumor cells can mimic the *in vivo* solid tumors more accurately as well as its corresponding tumor microenvironment. This co-cultured MTHS would be ideal for use in pre-clinical drug screening and for predicting clinical therapeutic efficacy more accurately because MCTS are difficult to co-culture.
5. Reference


Figure 6-1: Multicellular tumor hemi-spheroid fabrication process starting from T75 flask
**Figure 6-2:** Sample layout of MTHS deposition on non-tissue culture petri dish
Figure 6-3: NF639 MTHS cultured on Teflon sheet for a week. The periphery resembles granular shape.
Figure 6-4: NF639 MTHS cultured on Teflon sheet for a week 10x magnification
Figure 6-5: NF639 cells that were cultured with MTHS forming circular construct of their own
Chapter 2 Characterization of Multicellular Tumor Hemi-Spheroid

1. Introduction

1.1 Tumor microenvironment

A tumor microenvironment is a tumor cellular environment that is composed of 1) fibroblasts, 2) stromal cells 3) endothelial blood vessels, 4) lymphocytes and 5) tumor cells (Xu, Farach-Carson, & Jia, 2014). The complex interaction between tumor cells and fibroblasts as well as different inhibitory and stimulatory cell signaling triggers growth and malignancy of tumor tissue (Breslin & O’Driscoll, 2013; LaBarbera, Reid, & Yoo, 2012; Xu et al., 2014). It is important to mimic solid tumors with their complex tumor microenvironment in the in vitro setting in order to better predict clinical therapeutic efficacy. With conventional in vitro 2D model, result of screening from drug treatment can be misleading because tumor cells are equally exposed to drugs. In vivo however drugs have to penetrate and interact with complex microenvironment before they can reach their target (Xu et al., 2014). This can be realized with in vivo animal model where physiological and chemical barriers closely mimic those in humans. However key components of tumor microenvironment in animal model such as fibroblasts and stromal cells are that of the animal. The animal model represents a different tumor microenvironment than that of human tumor (Jung, 2014). Therefore a tumor model that mimics the complex human tumor microenvironment in the in vitro setting can often better predict therapeutic efficacy in clinical setting (Leoni A. Kunz-Schughart, Freyer, Hofstaedter, & Ebner, 2004).
1.2 Multicellular tumor spheroid model and its screening assay

Multicellular tumor spheroid (MCTS) is an in vitro 3D tumor model that mimics the in vivo phenotype of an initial avascular nascent solid tumor (L. A. Kunz-Schughart, Kreutz, & Knuechel, 1998). The tumor microenvironment of these nascent tumors comprises of distinct subpopulation of cells that are made up of 1) proliferative cells at the periphery, 2) necrotic cells at the center and 3) quiescent cells in between the periphery and the center (Hirschhaeuser et al., 2010; L. A. Kunz-Schughart et al., 1998; Leoni A. Kunz-Schughart et al., 2004). Because oxygen and nutrient delivery are limited to within 100-200 µm depth away from the periphery of the nascent tumor, cells beyond the 100-200µm depth become deprived of oxygen and nutrients and therefore become hypoxic and acidic (Grimes, Kelly, Bloch, & Partridge, 2014; Hirschhaeuser et al., 2010; L. A. Kunz-Schughart et al., 1998). MCTS has concentric distribution of these subpopulation of cells as well as concentric oxygen and nutrient gradients that is similar to the nascent tumor.

1.3 Overview of MCTS assays

It is important that these cell subpopulations and gradients can be accessed and evaluated to comprehend the drug penetration process. Using currently available assays, it is possible to map cell viability, hypoxia, level of glucose and ATP with in MCTS (Gong et al., 2015; Grimes et al., 2014). Cell viability can be mapped using fluorescent cell viability markers called Calcein AM and Ethidium homodimer-1 (Gong et al., 2015). Once MCTS are stained with these markers and imaged using a confocal microscope, images of live and dead cells in MCTS can be captured and overlapped to form a single image that displays
regions of live and dead cells in a MCTS. Regions of hypoxia can be mapped in the same manner using fluorescent biomarkers or through immunostaining in which cells in MCTS are stained with immune antibodies to express hypoxia relevant proteins and imaged under the microscope (Grimes et al., 2014; Laurent et al., 2013). Other than the image based assays inner regions of MCTS can be analyzed using an invasive approach. Hypoxia in MCTS can be measured with an invasive polarographic oxygen electrode in which a micro-needle oxygen electrode is inserted into the MCTS and oxygen concentrations are measured at different depths (Raleigh, Dewhirst, & Thrall, 1996).

Figure 2-1: Fluorescent hypoxia biomarker used to map regions of hypoxia in MCTS (Grimes et al., 2014)
1.4 Problems with current MCTS assays

Despite the improvements in MCTS culture and its assays, MCTS are not widely adopted in the HTS system. To be suitable for HTS system, the assays have to be systematically managed by robots and automatic plate readers. Most MCTS assays that are developed and used are image based assays for fast endpoint analysis suitable for HTS system. Although image based assays to analyze cell viability and hypoxia can be obtained using high end microscope, the quality of the images obtained vary depending on the size of the MCTS. Small size MCTS (200-300µm in diameter) can produce high quality images compared to MCTS size of 600 – 800µm that cannot be imaged due to higher degree of light scattering, absorption and reflection from the growing tumor cells (LaBarbera et al., 2012). Immunostaining poses a different problem in which the sample has to be stained, sectioned and placed on a microscopic slide for observation (Laurent et al., 2013). The process requires labor, and skills to section small size MCTS and prepare it on a microscopic slide for view. Lastly invasive polarographic microelectrode can give direct measurement of oxygen concentration at different depth, but it is difficult to measure in MCTS of smaller sizes. Therefore a new in vitro 3D tumor model relevant to the avascular nascent tumor has to be developed. This new model should also be compatible with current image based assays with better results, and perform noninvasive analytical measurements of the essential gradients (oxygen, pH and glucose). This new model will then be used for HTS system for standardized drug screening with better therapeutic efficacy.
Figure 2-2: Live/dead cell staining of MCTS using calcein AM (green) and ethidium homodimer-1 (red). Cells that are viable appear green and nonviable red (Gong et al., 2015)

1.5 Multicellular Tumor Hemispheroid (MTHS)

Multicellular tumor hemi-spheroid (MTHS), developed in Dr. Miklos Gratzl’s laboratory, is a half spheroid model derived from MCTS. The flat plane of half spheroid in MTHS when placed on top of an impermeable transparent substrate, the substrate can act as a window into the MCTS. Such a MTHS then can be studied as if it were uncut MCTS because transport paths of nutrients, oxygen, waste and drug molecules do not change by the cutting due to spherical symmetry. With MTHS analytical measurement of oxygen, nutrient and pH can be obtained by placing MTHS on top of an optode or a micro-electrode platform. A student in Dr. Gratzl’s lab developed a platform to analytically quantify oxygen partial pressure at different location and depths of 3D tumor model using MTHS model (Sheth D.B, 2011). The schematic in figure 3 shows how MTHS and oxygen sensing
electrode platform were used to quantify oxygen partial pressure at different location and depth of the MTHS.

![Microelectrode Platform Diagram](image)

**Figure 2-3:** Oxygen sensing microelectrode platform and oxygen sensing scheme using MTHS model (Sheth D.B., 2011)

With MTHS, it is feasible to obtain high quality images with MTHS size larger than 300µm because the flat plane of MTHS gives direct access to the inner regions of the construct. MTHS can be suitable for image based assays as well as instrument based analytical assays with reduced constraints. Hence MTHS can be implemented for HTS because it provides simple endpoint assays in drug penetration and binding of cells in proliferative and quiescent cells in MTHS as well as viability mapping after the drug treatment. In addition drug kinetics can be analyzed and its effective diffusion coefficient can be found.
In this chapter we will develop an image based assay to map cell viability in MTHS using 0.4% Trypan blue solution. Trypan blue is the most common cell viability dye in which dead cells are stained in blue. With MTHS concentric cell viability map can be obtained by staining the flat plane of MTHS. Through this experiment we will test that MTHS model can form concentric cell viability map similar to that of MCTS.

2. Experimental

2.1 Materials

Agarose type VII-A and Trypan blue were purchased from Sigma Aldrich (St Louis, MO). DMEM cell culture medium, fetal bovine serum (FBS), 0.25% Trypsin EDTA and Dulbecco’s Phosphate Buffer Saline (DPBS) were purchased from Life technologies (Carlsbed, CA). Accutase and Accumax were purchased from Innovative cell technologies (San Diego, CA). Ultra Low Retention (ULR) pipette tips were purchased from BrandTech (Essex, CT). Mammary gland breast tumor cells (mcf7) were obtained from Dr. Nicole Steinmetz lab.

2.2 Multicellular tumor hemi-spheroid fabrication

Mcf7 cells are maintained and cultured in DMEM containing 10% fetal bovine serum (FBS) only in T75 flask. Cells are incubated at 37° C in 5% CO2 environment. 80-90% confluent mcf7 cells in T75 flasks are then detached by adding 3mL of accutase solution and collected in a sterile 15ml conical tube. 3ml of sterile DPBS is added to make the final volume in 15ml conical tube to be 6mL. 100ul of sample from the 15ml conical tube is extracted and pipetted in 1.5ml vial containing 650ul of DPBS. The 1.5ml vial is then used
to calculate the cell density and the total number of cells in 15ml conical tube by cell counting using a hemocytometer or a Vi-Cell counter (Beckman Coulter). Cells in 15ml conical tube are spun down in a centrifuge (Sorvall ST40) at 1400 rpm for 5min. Supernatant medium in the 15ml conical tube is aspirated leaving cells pellets at the bottom. 1% agarose type VII-A (gelling temperature at 26 °C and melting temperature above 66 °C) is then added to cell pellet and mixed to achieve density of 0.15million cells per µl. The conical tube with cell-agarose mixture is then sonicated for 2 min to suspend cells homogenously in liquid agarose solution. The conical tube after sonication is kept in sterile water bath at 35 °C to prevent the cell-agarose mixture from gelling. 1ul droplets of cell suspension in liquid agarose are deposited onto an ice cold sterile non tissue culture petri dish using 0.1 – 2.55 µl pipette and ULR pipette tips. Liquid droplets with the suspended cell in them form hemi-spherical droplets due to hydrophobic property of the polystyrene petri dish, and quickly gels in less than few seconds. 20µl of DMEM + 10% FBS are deposited on top of the cell-agarose hemi-spheroids using 10-100 µl pipette and the petri dish carrying the model cell-agarose hemi-spheroids is carefully placed in an incubator at 37° C (in humidified air with 5% CO2) and cultured for 1-5 days. Henceforth, model agar hemi-spheroids will be referred to multicellular agar suspended hemi-spheroid or MTHS.
2.3 MTHS staining using 0.4% Trypan blue solution

0.4% Trypan blue solution was prepared by dissolving trypan blue powder in DPBS solution. Non tissue culture petri dish containing model MTHS was taken out from incubator after 3-5 days of culturing in the incubator at 37°C in 5% CO2 environment. Cell culture medium droplets capsuling MTHS was aspirated exposing MTHS in dry air. 1-2 ml of DPBS was added and aspirated twice to wash MTHS from the remaining medium. Then MTHS were simply lifted off from the petri dish through an air flow action using 10-100 μl pipette.
Figure 2-5: Detachment of MTHS from the substrate and adding 0.4% trypan blue dye solution

Once all MTHS were suspended in 1-2ml DPBS, DPBS was then aspirated again. 2ml of 0.4% trypan blue solution was added after DPBS was aspirated. MTHS were suspended in 0.4% trypan blue solution for 4-5min. and washed with DPBS solution (x4) and observed under an inverted transmission microscope.

3. Results and Discussion

10 mcf7 MTHS was stained with trypan blue after MTHS (2 is shown here and 3 is shown in appendix), which was attached to the bottom of the petri dish, was lifted. After 5min of staining, we observed a circular blue colored area appeared at the center of MTHS.
Figure 2-6: 0.4% trypan blue staining of MTHS before and after

Figure 2-6 shows the result of staining MTHS using trypan blue solution. As it can be observed, trypan blue has stained the cells located at the core and not the cells at the periphery of MTHS. The MTHS diameter presented in figure 2-6 was about 1100µm. The diameter of the Trypan blue stained area was 530µm. To determine how many cells were stained, we calculated the fraction of dead cells in 1100µm diameter size MTHS. This was determined by calculating the estimated number of cells and the estimated number of stained cells in MTHS using volumetric estimation approach (chapter 1). For this mathematical approach, spherical cap equation was applied

\[
V_{\text{cap}} = \pi \left(2 - 3\sin(\alpha) + \sin^3(\alpha)\right) \frac{R^3}{3}
\]  

(1)

\[
R = \frac{a}{\cos(\alpha)}
\]

(2)

\[
\alpha = 90^\circ - \theta
\]

(3)
in which contact angle (\(\theta\)) of 50° was chosen for all the volume calculation.
Thus the estimated total number of cells in MTHS was calculated to be 21,000 and the estimated number of stained cells in MTHS was 2,300. By dividing the number of stained cells to the total number of cells in MTHS, we calculated the fraction of dead cells in the 1100µm size MTHS to be 11%.

We also stained mcf7 MTHS with diameter size of 1350µm. Figure 2-8 (right image) displays comparison results between 1100µm size MTHS (left) and 1350µm size MTHS (right).

**Figure 2-8:** 0.4% trypan blue staining of two different size MTHS. MTHS on the left has smaller staining area than the MTHS on the right. The estimated total number of cells in 1350µm size MTHS using spheroid cap calculation was 37,500 and the estimated number of cells stained was 13,500. The fraction of dead cells in 1350µm size MTHS was 36%. From this result, we observed that 1100µm size
MTHS had 11% of its cells dead, while 1350µm size MTHS had 36% of its cells dead. The difference in dead cell fraction is relative to the distance that nutrients and oxygen can diffuse. The smaller the MTHS size, the smaller the number of dead cells because nutrient and oxygen can diffuse further just like the 1100µm size MTHS (figure 2-8 left).

4. Conclusion and Future works

Cell viability mapping of MTHS was performed using 0.4% Trypan blue solution. Trypan blue solution stained dead cells blue, and did not stain viable cells. Thus the region of dead cells and live cells can be distinguished. Agarose scaffold MTHS was cultured for three days and was immersed in 0.4% Trypan blue solution for 5 min. All stained MTHS was then observed under the light microscope. Here we found that 1100µm size MTHS had dead cell fraction of 11% and 1350µm size MTHS had dead cell fraction of 36%. The smaller size MTHS had low number of dead cells because nutrients and oxygen can diffuse easily in smaller size MTHS. However more tests have to be done with different cell lines and sizes in order to verify that region of stained cells in our assay is independent of the cell line characters and is dependent on the sizes of the MTHS.

We have developed a simple assay that can map cell viability in large (600 – 1000µm diameter) size 3D tumor model without the use of high end microscope. This assay can be implemented in high throughput screening in which multiple MTHS can be assayed with different fluorescent biomarkers before and after the drug treatment. Regions of cells viability can be mapped using both bright field and epi-fluorescent microscope.
5. Reference


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6. Appendix

**Figure 6-1:** 0.4% trypan blue staining of 3D in vitro tumor tissue fabricated from mcf7 and 1wt% agarose scaffold. Trypan blue is stained in the middle of the structure.
Figure 6-2: 0.4% trypan blue staining of MTHS that has elliptical shaped flat plane. The center stained with trypan blue indicates nonviable cells.
Figure 6-3: 0.4% trypan blue staining of MTHS
Chapter 3 Doxorubicin delivery in the Multicellular Tumor Hemi-Spheroid

1. Introduction

1.1 Preclinical anti-cancer drug discovery stage

Today’s anti-cancer drug discovery and development mostly relies on target based approach (Hoelder, Clarke, & Workman, 2012; Suggitt & Bibby, 2005; Terstappen, Schlüpen, Raggiaschi, & Gaviraghi, 2007; Wilhelm et al., 2006). With the combination of genomics and proteomics, certain target molecules such as cellular receptors, enzymes or hormones that transduce a specific signaling pathway to elicit a cellular response (ex: apoptosis) can be discovered. The discovered target that elicit a cellular response is then validated through in vitro and in vivo assays using cell lines or genetically engineered mouse model (GMM) by overexpressing or inhibiting the target in its signaling pathway (Hoelder et al., 2012; Wilhelm et al., 2006). Once it is confirmed that the target causes the desired cellular response, drug molecules are formulated and screened to find the “hit” molecules that interacts with the discovered target through high throughput screening (HTS) system using biochemical and cell based (monolayer) assays (Balas, 2002; Teicher & Andrews, 2004; Wilhelm et al., 2006). Once the hit drug molecules are identified by screening about 100,000 drug molecules, they are chemically optimized to become the lead drug molecules. This process involves optimizing drug molecules’ parameters to achieve initial pharmacologic parameters. The lead drug molecules are then screened again in HTS monolayer culture model to validate its optimization (Burger & Fiebig, 2014; Hoelder et al., 2012). The lead drug molecules that show promising activities in cell based HTS will
then progress to *in vivo* animal studies to further optimize the pharmacokinetics and pharmacodynamics of the lead drug molecules (Burger & Fiebig, 2014; Hoelder et al., 2012; Narang & Desai, 2009). The success up to this stage will eventually lead to human clinical trials which focuses on identifying the safe dose level and the spectrum of anticancer activity of the drug molecules before being licensed (Narang & Desai, 2009).

**Figure 3-1:** Target oriented drug discovery process (Hoelder et al., 2012; Wilhelm et al., 2006)

1.2 *In vitro and In vivo based models in drug screening*

A small number of drugs successfully screened through monolayer based assays enter *in vivo* preclinical and clinical trial because monolayer based model do not recapitulate *in vivo* phenotype of human tumor. Monolayer model lacks cell-to-cell signaling, cell-to-extracellular matrix interaction and extracellular matrix composition (Breslin & O’Driscoll, 2013; Fitzgerald, Malhotra, Curtin, O’ Brien, & O’ Driscoll, 2015; HogenEsch & Nikitin,
If the drug molecule is validated using monolayer based assays, *in vivo* animal (murine) model, is the essential model used at the last stage of preclinical drug development (Breslin & O’Driscoll, 2013; Xu, Farach-Carson, & Jia, 2014). *In vivo* animal model provides the complexity of the tumor morphology and its microenvironment that are missing in the monolayer model. However the tumor microenvironment of *in vivo* animal model lacks and differs in some features of the human tumor microenvironment such as stromal cells, endothelial vasculature and immune system. In addition, due to expensive cost in maintaining the animals with experienced personnel and strict animal testing based regulation, *in vivo* animal model is used mainly for low throughput screening process (Breslin & O’Driscoll, 2013; HogenEsch & Nikitin, 2012; Kamb, 2005; Xu et al., 2014). Still animal model is valuable to determine initial dosage and toxicity as well as to predict therapeutic efficacy in the first phase of clinical trials. Yet due to the differences in tumor compositions in animal models, the accuracy of therapeutic efficacy can be poor when comparing it to the actual human tumor.

1.3 *In vitro* 3D tumor model in pharmaceutical industry

*In vitro* 3D tumor model is now becoming the necessary intermediate model between the monolayer model and the animal model. The extra dimension added to the monolayer model results in similar solid tumor characteristic features in terms of 1) extracellular matrix formation, 2) cell-to-cell signaling, 3) cell-to-extracellular matrix interaction and 4) morphology (Breslin & O’Driscoll, 2013; Hirschhaeuser et al., 2010). Also with the capability of translating *in vitro* 3D culture to a HTS system, large number of *in vitro* 3D tumor models can be screened for large number of drug molecules with reduced time (Atefi,
Lemmo, Fyffe, Luker, & Tavana, 2014; Deiss et al., 2013). More importantly 3D tumor models can be made with human primary tumors, which can lead to recapitulation of *in vivo* human tumor features at *in vitro* and provide better therapeutic efficacy than that of the *in vivo* animal models.

1.4 Multicellular tumor spheroid (MTCS)

Multicellular tumor spheroid (MCTS) is the widely adopted *in vitro* 3D tumor model currently for various types of drug treatment studies (Durand & Olive, 2001; Tung et al., 2011). This is because MCTS mimics the characteristics and the gradients of initial avascular solid tumor with the capacity for large scale production and screening for HTS (Ateti et al., 2014; Breslin & O’Driscoll, 2013; Tung et al., 2011). MCTS is assayed with drugs and the drugs’ efficacy are quantified 1) comparing the volume of MCTS before and after adding drugs using phase contrast microscopy and 2) mapping the distribution of fluorescent cell viability markers before and after adding drugs in MCTS using high end microscopy (Mehta, Hsiao, Ingram, Luker, & Takayama, 2012; Tung et al., 2011).

Although these are important assays to validate the efficacy of drugs overall, these assays do not provide information on drug response in the inner region of MCTS where distinct differentiate cell subpopulations (quiescent cells and necrotic cells) are located (Hirschhaeuser et al., 2010; L. A. Kunz-Schughart, Kreutz, & Knuechel, 1998; LaBarbera, Reid, & Yoo, 2012; Mueller-Klieser, 1987). Different types of cells and gradients can affect the result of therapeutic outcomes. Oxygen and pH gradients can destabilize weak acid and base type of drugs by deprotonating the drug’s chemical structure and quiescent cells can become multidrug resistant (MDR) (L. A. Kunz-Schughart et al., 1998; Leoni A. 
Kunz-Schughart, Freyer, Hofstaedter, & Ebner, 2004; Mellor, Ferguson, & Callaghan, 2005; Tredan, Galmarini, Patel, & Tannock, 2007). Therefore mapping and quantifying drug distribution inside the MCTS can lead to detailed evaluation of drug response for better prediction of drug’s efficacy.

1.5 MCTS drug penetration assay using image based approach

Today with the power of high end microscopy such as confocal and multiphoton microscope and fluorescently labeled drugs, drug penetration inside MCTS can be mapped and quantified using optical sectioning technique (Masson et al., 2015). Optical section technique images a horizontal section of MCTS. However mapping and quantifying drug penetration using high end power microscope has its constraints especially if the MCTS size grows more than 300µm (LaBarbera et al., 2012). As MCTS grow, more cells are accumulated and build up inside and cause increased light scattering, reflection and absorption (LaBarbera et al., 2012). This then can cause degradation or artifact in the image and lead to misleading screening results. Evaluating drug treatment in MCTS size greater than 300µm is important because differentiated cell subpopulations and gradients are established beyond MCTS size greater than 300µm (Breslin & O’Driscoll, 2013; Leoni A. Kunz-Schughart et al., 2004). Analyzing drug treatment MCTS size less than 300µm is also important because the model still retains important in vivo characteristics (extracellular matrix, cell-to-cell signaling, cell-to-extracellular matrix interaction).
**Figure 3-2:** MCTS cultured in 96 well plate and its volume evaluated using phase contrast microscopy (Friedrich et al. 2009).

### 1.6 Multicellular Tumor Hemi-Spheroid in drug screening assay

With the difficulty in visualizing and mapping drug penetration and diffusion in MCTS with size greater than 300µm, a novel *in vitro* 3D model has to be developed that makes it feasible to analyze inner regions of MCTS. Multicellular tumor hemi-spheroid (MTHS), developed in Dr. Miklos Gratzl’s laboratory, is a spheroid cut in half and is derived from MCTS. Once a MCTS is cut in half, the flat equatorial plane of MCTS can be placed on an
impermeable substrate. Such a MTHS then can be studied as if it were uncut MCTS because transport paths of nutrients, oxygen, waste and drug molecules do not change by the cutting due to spherical symmetry. Therefore a “window” into the interior of the MCTS is created that makes it possible to visualize and analyze drug response in the inner region of MCTS without the risk of deteriorating image qualities. With MTHS it is possible to analyze and map the drug penetration without the constraints of the MTHS size. More importantly, it is possible to identify which region and cell subpopulation showed effective as well as ineffective results. In addition, oxygen, pH and glucose gradients can be directly monitored during drug penetration by performing simultaneous parallel experiments on MTHS by placing the flat plane of MTHS on top of optodes and oxygen microelectrodes. All these information can lead to detailed analysis of the drug penetration and give better prediction of therapeutic efficacy.

In this chapter, we cultured MTHS using agarose as the tissue scaffold. Agarose provides controlled biochemical properties and defined shape for MTHS culture. It is also biocompatible. More importantly, agarose scaffold MTHS can lead to HTS because, similar size and shape of MTHS can be replicated at large scale and used for different types of drug penetration assays (image based and analytical based). Here, a confluent MTHS was immersed in 10µM doxorubicin (DOX) solution. DOX penetration was mapped in MTHS and its response was quantified using epi-fluorescent microscope.
2. Experimental setup:

2.1 materials

Agarose type VII-A and Trypan blue were purchased from Sigma Aldrich (St Louis, MO). RPMI-1640 cell culture medium, fetal bovine serum (FBS), 0.25% Trypsin EDTA and Dulbecco’s Phosphate Buffer Saline (DPBS) were purchased from Life technologies (Carlsbed, CA). Accutase and Accumax were purchased from Innovative cell technologies (San Diego, CA). Ultra Low Retention (ULR) pipette tips were purchased from BrandTech (Essex, CT). Prostate cancer cells (pc3) were obtained from Dr. Susann Brady-Kalnay.

2.2 Multicellular tumor hemi-spheroid fabrication

Pc3 cells are maintained and cultured in RPMI1640 containing 10% FBS only in T75 flask. Cells are incubated at 37° C in 5% CO2 environment. 80-90% confluent pc3 cells in T75 flasks are then detached by adding 3mL of accutase solution and collected in a sterile 15ml conical tube. 3ml of sterile DPBS is added to make the final volume in 15ml conical tube to be 6mL. 100µl of sample from the 15ml conical tube is extracted and pipetted in 1.5ml vial containing 650µl of DPBS. The 1.5ml vial is then used to calculate the cell density and the total number of cells in 15ml conical tube by cell counting using a hemocytometer or a Vi-Cell counter (Beckman Coulter). Cells in 15ml conical tube are spun down in a centrifuge (Sorvall ST40) at 1400 rpm for 5min. Supernatant medium in the 15ml conical tube is aspirated leaving cells pellets at the bottom. 1% agarose type VII-A (gelling temperature at 26 °C and melting temperature above 66 °C) is then added to cell pellet and mixed to achieve density of 0.15million cells per µl. The conical tube with cell-agarose mixture is then sonicated for 2 min to suspend cells homogenously in liquid agarose.
solution. The conical tube after sonication is kept in sterile water bath at 35° C to prevent the cell-agarose mixture from gelling. 1µl droplets of cell suspension in liquid agarose are deposited onto an ice cold sterile non tissue culture petri dish using 0.1 – 2.55 µl pipette and ULR pipette tips. Liquid droplets with the suspended cell in them form hemic-spherical droplets due to hydrophobic property of the polystyrene petri dish, and quickly gels in less than few seconds. 20µl of RPMI+10% FBS (for pc3) are deposited on top of the cell-agarose hemi-spheroids using 10-100 µl pipette and the petri dish carrying the model cell-agarose hemi-spheroids is carefully placed in an incubator at 37° C (in humidified air with 5% CO2) and cultured for 1-5 days. Henceforth, model agar hemi-spheroids will be referred to multicellular agar suspended hemi-spheroid or MTHS.

2.3 Doxorubicin delivery experiment

The well plate containing both mcf7 and pc3 MTHS are filled with RPMI-1640 (pc3) cell culture medium carefully in order not to dislodge the MTHS from the bottom. An inverted epi-fluorescent microscope (Leica DMI-6000) is used to observe the MTHS first in phase contrast and then with Texas Red fluorescent filter under 10x magnification. The excitation and emission wavelength of Texas Red filter is 596nm and 615nm, respectively. A humidifier chamber at 37° C in 5% CO2 is placed on top of the well plate to prevent MTHS from drying. MTHS are visualized using phase contrast image initially at 10x magnification. The fluorescence imaging is controlled through Metamorph (Molecular Devices, USA) software. After positioning the MTHS such that the entire MTHS is seen, it is switched to fluorescence via Metamorph and time-lapse fluorescence images are acquired. The exposure time is set for 70ms, with gain of 2, 2x2 binning and digitizer of
10MHz. First images are taken for 3 min. experiment. This time-lapse image is labeled as MTHS before Dox is added. After the first time-lapse, next time-lapse configuration is set from 3 min. to 90 min. with same 10 sec. time interval.

Figure 3-3: Preparation and staging of pc3 MTHS on Leica inverted fluorescence microscope

2.4 Data analysis

Time-lapse images are taken from Metamorph software are imported to Image J software to evaluate average intensity distribution of MTHS. The images were scaled in Image J according to pre-scaled value setting in Metamorph software under 10 x magnification. A unit pixel was equal to 0.78 µm. A region of interest (ROI) of 78 x 78 µm square is highlighted to extract average intensity increase over time. Then 890 x 78 µm rectangle box is highlighted at each 15 min. time frame to quantify average intensity increase over distance at 15 min. frame. These data are assessed through matlab software.
3. Results and discussion

Dox is a chemotherapeutic drug that has its own inherent fluorescence due to central anthracycline chromophore group. Because of this visualization of Dox distribution in tumor cells or tissues can be achieved with fluorescent microscope (Mohan & Rapoport, 2010). Dox can penetrate in agarose hemi-spheroid (MTHS without cells) in 5min because cells are not present to uptake the Dox molecules. Here we performed three Dox penetration assays using two pc3 agarose scaffold MTHS and 1 mcf7 agarose scaffold MTHS. In this work, we show the result of pc3 MTHS dox penetration. Dox penetration and diffusion into MTHS was imaged for every 10s during 90 min period of experiment using epi-fluorescent microscope. Since no fluorescent intensity was observed in MTHS except the intensity of the background, the initial 3 min. images were averaged and labeled as the baseline image. The baseline image was subtracted from the rest of the images. Figure 3-1 shows the baseline subtracted MTHS Dox penetration images with 15min time interval.

![Image of MTHS Dox penetration images](image)

**Figure 3-4:** MTHS in RPMI-1640 medium with 20µL of 10µM Dox injected and observed for 90 min in a humidifier chamber.
From figure 3-1, there was no signs of fluorescence from the cells in MTHS 15 min after MTHS was immersed in 10µM of Dox solution. 30 min later after exposed to 10µM Dox solution, we observed first sign of fluorescence of cells around the periphery region of MTHS. At the end of the 90 min experiment, most of the cells at the periphery of MTHS fluoresced. The distance that the cells’ fluorescence covered was 150 µm at the end of the experiment. From figure 3-1, we observed that the Dox solution did not diffuse all the way into the center of MTHS at the end of the experiment. However when plotting a fluorescent intensity profile at three different regions in MTHS against the time and plotting another intensity profile across the MTHS diameter throughout the experiment, we observed that there was a penetration of Dox into the center of the MTHS at the end of 90min of experiment. Figure 3-2 shows the first intensity profile plot of MTHS at three different regions labelled as region a, region b and region c. The size of each region was 78 x 78 µm. About 16 cells can be fit in the designated square region since each cell in MTHS is about 22 µm size. As can be seen, region a which is located at the periphery of MTHS, had a significant increase in average intensity at 30 min compared to region b and region c. By the end of the experiment, the average intensity at region a was 50% higher than region b and 70% higher than region c. However from this intensity profile, we noticed that both region b and region c showed a steady increase in intensity throughout the experiment in which region b intensity was 40% higher than that of region c.
Figure 3-5: Normalized intensity vs time of MTHS at three different region: region a (350µm away from the center), region b (175µm away from the center) and region c (at the center).

Another intensity profile that quantifies average intensity over the distance showed an increase in average intensity at the periphery of the MTHS throughout the experiment. Figure 3-3 shows a sharp intensity peak located ±350µm from the center. In addition the intensity profile at ±200µm showed a significant increase from 60 to 75min, but was not high compared to average intensity values at ±350µm
**Fig 3-6:** Normalized intensity vs distance of MTHS during 90 min of Dox penetration experiment.

From these data, we can map a concentric Dox penetration into MTHS using epi-fluorescent microscope. The first sign of cell fluorescent showed 30 min after 10µM of Dox was added to a medium containing 700µm diameter size MTHS. Dox uptake and binding in cells is a rapid process. However there are many binding sites in cell nucleus in which Dox has to bind and since there are about 0.2 million cells in MTHS (cell density of 0.2million for 1µl cells +agarose deposition), it is possible that it took 30 min for cells to show its first sign of fluorescence. This would also partly explain increase in intensity around the center of MTHS. Dox penetration and diffusion at the center of MTHS did occur, however due to low concentration of Dox (10µM) and large number of cells exposed to Dox initially, few Dox molecules penetrated and diffused to the center and bind to many binding sites in cells.

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4. Future works and Conclusion

Dox diffusion and penetration was mapped and quantified using MTHS as the in vitro 3D tumor model. We have performed three drug penetration experiments using three MTHS (two pc3 MTHS and 1 mcf7 MTHS). MTHS was cultured using agarose scaffold approach. From the data we obtained, Dox penetration and diffusion into the MTHS was radial and showed high fluorescent intensity at the periphery of the MTHS at the end of the experiment. However looking at two different intensity profile plot, Dox did diffuse into the center of MTHS but its intensity was lower than the intensity at the periphery of MTHS. This is because most of the Dox concentration was taken by cells located at the periphery initially and had time to bind to many of the cells binding site. As time passed remaining Dox solution diffused into the center of the MTHS but it was not enough to bind many of the cells binding site and elicit fluorescent response.

Drug penetration and diffusion assay can be translated to a HTS using agarose scaffold MTHS model. The advantage of scaffold approach is that similar size and shape of MTHS can be generated to a large scale and screened with different assays. Agarose scaffold MTHS is not only favorable for HTS, it is also favorable for detailed analytical measurement to provide better therapeutic efficacy than the MCTS. This is because the flat plane of MTHS can act as a window to the inner region of the model in which distinct cell populations and gradients can be quantified and assessed. For drug development, it is important that the drug reacts to all subpopulation of cells in tumor because the surviving tumor cells from the drug treatment can become resistant to the drug. Also gradients play a significant role in drug treatment or nanomedicine because low oxygen and pH can affect drug stability or can be used as a release mechanism for nanocarriers carrying the drug.
5. Reference


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6. Appendix

Figure 6-1: 10μM Dox penetration in pc3 MTHS with 650μm diameter size
Figure 6-2: 10μM Dox penetration in mcf7 MTHS with 1300μm diameter size: a: 1 min, b: 15 min, c: 30 min, d: 45 min and e: 60 min
Figure 6-3: MTHS without cells. 10μM Dox diffused 5min in MTHS without cells because there was no cells to uptake the Dox
Conclusions

We have developed and cultured a novel *in vitro* 3D tumor model called Multicellular Tumor Hemispheroid (MTHS) using agarose scaffold approach. MTHS was initially developed in Dr. Miklos Gratzl’s laboratory. This work expands on the characterization of the agarose scaffold MTHS and testing the model to observe regions of cell viability and drug penetration. This is an important part of the work because using agarose scaffold, similar sizes of MTHS can be cultured at a large scale and screened for standardized testing which is favorable for high throughput screening (HTS) system.

From chapter 1’s work, we found the optimal agarose concentration to be used as scaffold was 1wt%. We evaluated the sizes as well as the shapes of the agarose scaffold MTHS. Also we were able to retrieve individual cells from MTHS through the combination of biochemical and mechanical disintegration.

In chapter 2, we developed and tested cell viability assay for the agarose scaffold MTHS. We used 0.4% Trypan blue to distinguish the region of dead cells from live cells. From this experiment we found that the smaller size MTHS had smaller fraction of dead cells relative to bigger size MTHS.

Finally in chapter 3, we developed and tested drug penetration assay for the agarose scaffold MTHS. We used chemotherapeutic drug doxorubicin because uptake of doxorubicin can be visualized under a fluorescent microscope. We saw that cells at the periphery of the MTHS showed first signs of doxorubicin uptake. At the end of the experiment, cells at the periphery of MTHS had higher fluorescent intensity compared to the cells located near the center of MTHS.
Future works

Chapter 1

More work has to be done in obtaining MTHS that has high contact angle close to 90 degrees. With conventional petri dish, it is difficult to obtain contact angle close to 90 degrees. Currently a transparent Teflon sheet with 12.7 µm thick can be used as an alternative. The Teflon sheet can be cut and attached to the bottom of the petri dish. Then agarose + cell droplets can be deposited on top of the transparent Teflon sheet layer and produce higher contact angle than that of the petri dish itself.

Chapter 2 and Chapter 3

MTHS has to be attached to the bottom of the petri dish before the trypan blue or drug penetration assays can be done. If the MTHS is suspended in the medium, it can lead to misleading results. Therefore a method for attaching MTHS has to be developed in order to perform the assays.

MTHS is a promising model that can change the current HTS assays for in vitro 3D models. Although further testing with different types of cell lines, primary tumors and co-culturing has to be achieved and developed, MTHS provides an alternative in vitro 3D tumor model that the researchers can explore.
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