Development of a FRET-based 3D in vitro breast tumor model and its application in evaluating drug-induced apoptosis

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**Development of a FRET-based 3D *In Vitro* Breast Tumor Model
and Its Application in Evaluating Drug-induced Apoptosis**

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School of Chemical and Biomedical Engineering
Doctor of Philosophy
2017

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School of Chemical and Biomedical Engineering

A thesis submitted to Nanyang Technological University
in partial fulfillment of the requirement for the degree of

Doctor of Philosophy

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Preface

This thesis is submitted for the degree of Doctor of Philosophy to the School of Chemical and Biomedical Engineering, Nanyang Technological University, under the supervision of Professor Teoh Swee Hin and Professor Kathy Qian Luo. No part of this thesis has been submitted for other degree at other universities and/or institutions. To the author's best knowledge, all the work presented in this thesis are original unless reference is made to other works. Parts of this thesis have been published in international journals and conferences listed in the List of Publications.

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Journal Publications

1. James L Hickey, Anand Padmaja, Brian W Skelton, Allan H White, Fu Afu, Kathy Qian Luo, Murray V Baker. Cationic, linear Au (I) haloalkyl N-heterocyclic carbene complexes: synthesis, template reactivity and anti-cancer properties. (*manuscript in preparation*).
2. Anand P, Fu A, Teoh SH, Luo KQ. 2015. Application of a fluorescence resonance energy transfer (FRET)-based biosensor for detection of drug-induced apoptosis in a 3D breast tumor model. *Biotechnology and Bioengineering* 112(8):1673-1682.
3. Bhav MS, Hassanbhai AM, Anand P, Luo KQ, Teoh SH. 2015. Effect of heat-inactivated *clostridium sporogenes* and its conditioned media on 3-dimensional colorectal cancer cell models. *Scientific Reports* 5: 15681

Scientific Conferences

1. Padmaja Anand, Afu Fu, James L Hickey, Swee H Teoh, Murray V Baker, Kathy Qian Luo. Detection of Drug-induced Apoptosis in a 3D Breast Tumor Model using FRET Imaging. Joint Research Day - Clinical Tissue Engineering Symposium 2016, 29th September 2016, Singapore. (Poster Presentation, **Young Investigator Award**)

2. **Padmaja Anand**, Afu Fu, James L. Hickey, Swee H Teoh, Murray V. Baker, Kathy Q. Luo. FRET-Based Method for Detection of Drug-Induced Apoptosis in a 3D Breast Tumor Model. BES10SM, 14th May 2016, Singapore. (Oral Presentation, **Bronze Merit Award**)
3. **Padmaja Anand**, Afu Fu, Swee-Hin Teoh, Kathy Luo Qian. FRET-Based Analysis for Detection of Drug-Induced Apoptosis in a 3D Breast Tumor Model. ASCB Meeting, 12-17th Dec 2015, San Diego, California, USA. (Poster Presentation)
4. **Padmaja Anand**, Swee-Hin Teoh, Kathy Luo Qian. Development of Multicellular Fluorescent Resonant Energy Transfer (FRET)-Based 3D Tumor Model for Anti-Cancer Drug Testing. 7th APOCB Congress, 24-27th Feb 2014, Singapore. (Poster Presentation)
5. **Padmaja Anand**, Terence Aik-Huang Tan, Swee H Teoh. Polycaprolactone-Tricalcium Phosphate Scaffolds for In Vivo Tumor Modeling: Investigating Scaffold Stability under Simulated Tumor Microenvironment. BoneTec 2013, 16-19th Dec 2013, Singapore. (Poster Presentation)
6. Terence Aik-Huang Tan, **Padmaja Anand**, Jing Lim, Zhuo Wang, Swee-Hin Teoh. Tumor Reverse Bioengineering: Radio-opaque Polycaprolactone-tricalcium phosphate Scaffolds for Implantation in Tumor Microenvironments. Clinical Translational Workshop – Bone Tissue Engineering, 9th Dec 2012, Singapore. (Poster Presentation)
7. Terence Aik-Huang Tan, **Padmaja Anand**, Jing Lim, Zhuo Wang, Swee-Hin Teoh. Tumor Reverse Bioengineering: Radio-opaque Polycaprolactone-

tricalcium phosphate Scaffolds for Implantation in Tumor Microenvironments.

International Conference on Cellular and Molecular Bioengineering 3, 8-10th

Dec 2012, Singapore. (Poster Presentation)

Summary

Tumorigenesis is a dynamic process involving growth, proliferation, heterotypic cell-cell and cell-ECM interactions and migration. Having traditionally relied on two-dimensional (2D) cultures for cancer research, it has been well-proven that these systems are unable to recapitulate the complexity and heterogeneity of *in vivo* tumors effectively. Compelling evidence from various studies have shown that three-dimensional (3D) systems can overcome these limitations and can bridge the gap between 2D and animal models. These 3D models would be able to provide a deeper understanding of cancer pathogenesis and drug responses. Therefore, 3D spheroids of breast cancer were developed in less than 24 h in this thesis using the non-adhesive coating method. Expectedly, these breast cancer cells were able to form tight cluster of cells that simulated *in vivo* tumors in its architecture (the necrotic core) and expression of tumorigenic markers better than 2D monolayers.

Three-dimensional models have been extensively used to determine *in vivo*-like responses of investigational drugs and provide additional information on the efficacy of the drug in a more physiological system than 2D monolayers. However, many of the currently available techniques to assess drug-induced effects require dissociation of spheroids, large number of samples or complex technologies to visualize the drug responses making them destructive, time-consuming, and lacking real-time analysis. This has created a need for a physiologically relevant model that at the same time provides rapid, non-invasive and longitudinal analysis of drug-induced effects. Therefore, in this thesis, a novel fluorescence resonance energy transfer (FRET)-based 3D *in vitro* model has been developed to evaluate drug-

induced apoptotic responses in a real-time manner. In this work, the FRET-based C3 biosensor, which detects the activation of caspase-3 enzyme during apoptosis, was employed. Therefore, when breast cancer cells were transfected with this biosensor (MCF7-C3), real-time information about the cell status under FRET imaging was achievable. Subsequently, on subjecting these spheroids to chemotherapeutic agent paclitaxel, cells at the rim of the spheroid displayed a reduced FRET effect (47 % reduction for 2000 nmol/L paclitaxel treatment), in a dose- and time-dependent manner. These results provide a proof-of-concept of using FRET-based biosensors in 3D models to detect and evaluate drug response in a non-invasive and time-series manner.

Subsequently, this biomimetic 3D platform of MCF7-C3 cells was employed to screen various Au (I) *N*-heterocyclic carbene (NHC) complexes to assess their anti-cancer properties. The dose response curve and mitochondrial membrane potential analyses suggested that gold-complexed compounds had anti-cancer properties and the monomer compounds acted as controls not having any apoptosis-inducing activity. Furthermore, it was confirmed by FRET imaging that these five gold-complexes were able to activate caspase-3 enzyme, thus triggering apoptosis, in less than 72 h at a concentration of 5 μ M on 2D. However, when these five compounds were tested in our 3D biomimetic model, the complexes were unable to elicit a response equivalent of their 2D response. This was further substantiated with the evaluation of FRET ratio of gold-complexed compounds and their corresponding monomer. This result demonstrates the applicability of using our 3D biomimetic

system as an intermediate drug screening platform to bridge the gap between 2D and animal models for faster and efficient drug efficacy analysis.

It is well-documented that cancer cells are not the only entities that constitute *in vivo* tumor. Cell types like fibroblasts, macrophages etc., interact with the cancer cells, in addition to providing physical support (dense ECM), thus contributing to the tumor microenvironment. As a step towards recreating an accurate tumor model *in vitro*, we have incorporated fibroblasts into our model system. In 24 h, a 3D co-culture system comprising of FRET-based breast cancer cells and fibroblasts was formed. Further histological analysis have shown that this 3D co-culture system grown for a week resembled *in vivo* tumors more closely than their 3D mono-spheroid. It is believed that the additional involvement of fibroblasts in this FRET-based 3D co-culture model would enable better accuracy in screening and evaluating responses of investigational compounds.

In conclusion, this thesis demonstrates that FRET-based 3D *in vitro* breast tumor models would serve as a novel platform for non-invasive imaging and longitudinal monitoring of drug-induced effects. Screening of NHC complexes as potential anti-cancer agents stand as a culmination of development of this 3D *in vitro* model. Additionally, the role of fibroblasts in tumor progression has been explored by incorporating them in our previously established 3D mono-spheroid model.

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List of Abbreviations

2D	Two-dimensional
3D	Three-dimensional
APH	Acid Phosphatase
ATCC	American Type Culture Collection
ATF4	Activating Transcription Factor 4
BCS	Breast Conserving Surgery
bFGF	Basic Fibroblast Growth Factor
C3	Caspase-3
CAF	Cancer-Associated Fibroblast
cDNA	Complementary Deoxyribonucleic Acid
CFP	Cyan Fluorescent Protein
DAB	3, 3'-Diaminobenzidine
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EMT	Epithelial-Mesenchymal Transition
FBS	Fetal Bovine Serum
FDA	Food And Drug Administration

FRET	Fluorescence Resonance Energy Transfer
FU	Fluorouracil
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
H&E	Haematoxylin & Eosin
HEMA	(2-Hydroxyethyl Methacrylate)
HUVEC	Human Umbilical Vein Endothelial Cells
IACUC	Institutional Animal Care And Use Committee
IORT	Intra-Operative Radiotherapy
LAMP3	Lysosomal-associated Membrane Protein 3
LDH	Lactate Dehydrogenase
LSFM	Light Sheet based Fluorescence Microscopy
MCTS	Multicellular
MIBG	Metaiodobenzylguanidine
mMMP	Mitochondrial Membrane Permeabilization
MMP	Matrix Metalloproteases
mRNA	Messenger Ribonucleic Acid
MTT	(3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide)
NAF	Normal Fibroblasts
NCI	National Cancer Institute
NHC	N-Heterocyclic Carbene

NMR	Nuclear Magnetic Resonance
NRDO	National Registry Of Diseases Office
OCT	Optimum Cutting Temperature
OCT4A	POU Domain Class 5 Transcription Factor 1
PBS	Phosphate Buffered Saline
PCL	Polycaprolactone
PDX	Patient-Derived Xenografts
PEG	Polyethylene Glycol
PERK	PKR-like Endoplasmic Reticulum Kinase
PLA	Polylactic Acid
PLGA	Poly(Lactic-Co-Glycolic Acid)
PVA	Poly(Vinyl Alcohol)
RGD	Arginylglycylaspartic Acid
ROI	Region Of Interest
RT	Room Temperature
RT-qPCR	Quantitative Reverse Transcription Polymerase Chain Reaction
SOX2	SRY-Related HMG Box Protein
TMRE	Tetramethylrhodamine Ethyl Ester Perchlorate
TrxR	Thioredoxin Reductase

TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
UV	Ultra-Violet
VEGF	Vascular Endothelial Growth Factor
YFP	Yellow Fluorescent Protein
$\Delta\Psi_m$	Mitochondrial Membrane Potential

Chapter 1: Introduction

1.1 Breast Cancer

Cancer is a leading cause of death worldwide with approximately 8.2 million cancer-related deaths in 2012 [1]. Among the different cancers, breast cancer is the most recurrent form of cancer diagnosed in women, with estimated 1,383,500 new cases each year [2]. It is predicted that one in 8 women would be diagnosed with breast cancer in their life in the United States [3]. In Singapore, more than 25 % of cancers diagnosed in women are breast cancers (Figure 1.1) making it the most frequent cancer in females according to the interim report released by the National Registry of Diseases Office (NRDO) for 2010 to 2014 [4]. In addition, breast cancer incidence rate compiled from 1992-2002 registry, is high especially for women in the age group 35-49 (98.9 %) [5].

Figure 1.1: Ten most frequent cancers (%) in females, 2010-2014. Adapted from [6].

1.2 Breast Cancer Treatments

1.2.1 Surgery and Radiation

The primary therapies for breast cancer include surgery and radiation. Nowadays, breast conserving surgery (BCS) or mastectomy is conducted depending on the stage of breast cancer and its severity. It is observed that combination of BCS followed by radiation to the localized region is equivalent in its long-term survival as a total mastectomy [7]. BCS may include (i) lumpectomy or wide local excision, wherein the tumor and a little surrounding breast tissue is excised, or (ii) partial mastectomy or quadrantectomy, where up to a quarter of the breast is removed. The volume of breast tissue to be removed would depend on the type of cancer, the size of tumor and its location on the breast, and the breast size of the patient and the volume of normal breast tissue. Usually, BCS is accompanied with radiation therapy or systemic therapies. On the other hand, a mastectomy would involve the removal of all the breast tissue, including the nipple. If no obvious signs of cancer spreading to the lymph nodes is observed, the patient undergoes a mastectomy. While it is observed that the cancer has spread to the lymph nodes, an additional surgical excision of the lymph nodes from the axilla under your arm would be recommended [4].

Radiotherapy utilizes radiation to kill cancer cells. It is a common treatment for patients who have undergone BCS/mastectomy. This treatment regime lasts from 4 weeks to 6 weeks depending on the patient. National Cancer Centre Singapore offers intra-operative radiotherapy (IORT), a new treatment technique, for patients. This treatment includes a single session of radiation administered to the patient during

surgery for ~40 mins, replacing the earlier radiotherapy which lasts for 6 weeks. This IORT has similar efficacy to radiotherapy and additionally protects surrounding normal breast tissues and underlying organs like heart, lungs from exposure to high energy X-rays [4].

However, these therapies provide a cure only if breast cancer is diagnosed in its early stage and is still localized to its primary site. Once the cancer has metastasized (secondary malignant tumors), these therapies are less effective. Therefore, surgery needs to be complemented with additional systemic therapies like chemotherapy, hormone therapy and targeted therapy.

1.2.2 Hormone Therapy and Targeted Therapy

Hormone therapy is a systemic therapy, which would be effective in patients tested positive for the hormone receptors and they have no effect on hormone receptor-negative patients. The mode of action of hormone therapy is by two ways: i) by reducing the concentration of estrogen in the body, or ii) by blocking the action of estrogen on breast cancer cells [8]. There are multiple types of medicines for this treatment including selective estrogen receptor modulators [9], estrogen receptor down-regulators and aromatase inhibitors [10]. The introduction of hormone therapy post-surgical treatment or in conjunction with cytotoxic therapy, has reduced disease relapse and increased survival rates. For instance, tamoxifen has been the adjuvant therapy of choice for more than three decades leading to significant reduction in disease recurrence and favorable effects on bone metabolism [11]. However, tamoxifen can pronounce adverse effects on the patients including increased risk of

endometrial cancer, vaginal bleeding and thromboembolic disease due to its estrogenic activity in remaining tissues [12, 13]. Therefore, other agents have been developed to eliminate these side effects and enhance the effectiveness of the treatment.

On the other hand, targeted therapy (cytostatic) works differently than chemotherapeutic drugs (cytotoxic - attacks all cells). These classes of drugs target specific proteins or genes [14]. They can be effective, even when traditional chemotherapies have failed to cause any response. It is mostly combined with other traditional therapies, and rarely used as a single treatment option. These targeted drugs kill cancer cells with specific changes that promote abnormal growth and doubling of cells. They can either block chemical signals that enhance growth from reaching the cancer cells [15], prevent angiogenesis to cut off blood supply [16], stimulate the immune system [17], or carry toxins that specifically target cancer cells.

1.2.3 Chemotherapy

Chemotherapeutics are required as an adjuvant therapy to help patients in their advanced stages of cancer [18, 19]. Some currently available chemotherapeutics are largely palliative. Many drugs which target various signaling pathways in cancer do provide a treatment for uncontrollable cancer growth, but their effective therapeutic window is time-constrained [20-22]. Although many drugs prevent relapse of cancer, new therapeutic options are necessary.

Additionally, over the years, drug resistance has caused serious issues to the overall efficacy of the drug, requiring rapid translation of inhibitors and lead

compounds into clinics as biomarkers for diagnosis or targets for treatment. Given the large number of anti-cancer therapeutics that are in development for prolonged survival, reduced recurrence and the many potential clinical scenarios including broad range of cancer models for their use, robust and efficient drug development strategies are needed.

1.2 Assessment of Efficacy and Safety of Chemotherapeutics - Drug Development Process

Drug development including optimization of dose, formulation, pharmacokinetic and pharmacodynamic studies is a prolonged and expensive process [23]. After screening numerous compounds and identifying the lead compounds, they are extensively tested in a preclinical setup (*in vitro* and animal studies). Upon further narrowing down the lead compounds that have a larger likelihoods of functioning in humans, these are then moved to the next step – phase I clinical trial (dosage identification) and then to phase II (safety and efficacy). The drugs successfully entering and completing phase III (study in larger groups) are then available for clinical use after obtaining FDA approval [24]. The preclinical testing begins with 2D cultures and is subsequently tested on animals as elaborated below:

1.2.1 Two-dimensional (2D) Monolayers

The standard *in vitro* 2D cultures are the first line of experiments conducted to assess the effectiveness of drug candidates. These 2D cultures are convenient to use, straightforward in their culturing technique and are easy to maintain. A major

drawback of the 2D cultures would be their sheet-like appearance on the petri dish indicating their poor depiction of *in vivo* structural architecture. In addition, the biological complexities of cell-cell and cell-extracellular matrix (ECM) interactions and their effect on molecular pathways are masked in such 2D cultures. And, it has been proven that the malignancy of cancer cells when cultured on 2D is altered thereby generating a huge gap between 2D cultures and animal models for the analysis of drug efficacy [25, 26].

Often, results obtained for 2D are not translatable to *in vivo* models [27]. While cells cultured in 2D are uniformly exposed to oxygen and nutrients, in solid tumors, the cells are arranged in multilayers and thereby have gradients of chemical and biological signals, which may have a pronounced effect on tumor progression (either stimulatory or inhibitory) [28]. Lastly, the lack of ECM network in monolayer cultures can affect drug testing results. When the drugs are incubated with cells cultured in 2D, it is readily available to all cells without any physical barriers. However, in *in vivo* situations, a different environment exists that can significantly restrict the penetration and availability of the drugs to all cells [27].

1.2.2 Animal Models (Preclinical Study)

After screening out the compounds at the 2D level, the hit compounds which caused significant anti-cancer effects would then be tested on mouse models. Mouse cancer models are well established and are frequently used for studying drug efficacy and tumorigenesis. Although a wealth of information has been provided by the physiologically relevant animal models, some facets of tumorigenesis are difficult to

capture [29]. For example, in xenograft tumors in mice, the host cells provide the ECM, thus raising concerns on species specific tumor-stroma interaction studies as well as cell-signaling mechanisms [26, 30]. Although majority of the characteristics of a subtype of cancer are preserved in mouse xenograft tumors, no individual model can recapitulate all the expression profiles of the cancer subtype. Therefore, the platform to study human breast cancers in murine models may not be accurate [31].

The high costs, ethical issues, lack of real-time observations in addition to unfortunate deaths of animals during the course of the experimental studies add to the challenges of using animal models. In addition, the mice used for research are mostly immuno-compromised, which do not represent the environment of solid tumors *in vivo* [32-34]. Another challenge during drug efficacy treatment in animal models is the observation of tumor growth or regression on application of drug at weekly intervals.

1.2.3 Pitfalls and Limitations in Drug Discovery Process

Unfortunately, several studies have shown that drug development costs are becoming increasingly expensive [35, 36]. The maintenance cost of the animals adds to a large proportion in the drug development costs [37]. The current and maximum-possible success rates of investigational drug candidates by therapeutic class have been provided in Table 1.1 (adapted from DiMasi, J.A., *et al.*, [38]). It can be observed that the proportion of drug candidates tested for antineoplastic studies is higher than the rest of the therapeutic classes (~20%). Although the percentage of drugs cleared from Phase I to Phase II/III studies is noteworthy (~70%), the probability of these

drugs accepted by the US FDA is very low (7%) [38]. This can be attributed to uncertainty in science knowledge base and discrepancies in their efficacies.

Table 1.1 Current success rates by therapeutic class for compounds first tested in humans from 1993 to 2004. Adapted from [38] with permission.

Therapeutic Class	N	Approved molecules	Current success rate to enter clinics (%)
Antineoplastic/Immunologic	254	18	7.1
Cardiovascular	134	4	3.0
Central Nervous System	235	9	3.8
Gastrointestinal	120	4	3.3
Respiratory	88	4	4.8
Systemic anti-infective	122	19	15.6
Musculoskeletal	88	8	9.1
Miscellaneous	189	21	11.1

Furthermore, the high failure rates, poor safety and limited efficacy of the tested investigational drug candidates in oncology have given rise to many alternate approaches to the existing drug development process. New strategies have been incorporated in the drug development process in order to hasten the process and deliver efficient drugs to the clinic. One such possibility is to employ 3D cultures to bridge the gap between *in vitro* 2D systems to discover and screen multiple compounds and *in vivo* experiments for safety and efficacy testing.

1.3 Adopting Three-dimensional Models – Speedy Discovery of Drugs and Cost-effective

Three-dimensional (3D) cultures are increasingly being recognized as important tools for advanced studies, complementing the existing techniques necessary for bridging the knowledge gap between 2D and animal models [25]. The complexity and better resemblance to *in vivo* tumors make 3D cultures an advanced tool to 2D cultures. These models provide an *ex vivo* approach to understanding the interactions and responses of cells grown in a tissue-specific architecture. Thereby, they would provide significant information on drug efficacy and also on tumorigenesis better than 2D. The advantages this system offers are numerous as listed below:

1.3.1 3D Models – Altered Proliferation Profile and Cell Morphology

The native cellular microenvironment *in vivo*, which plays a vital role in cell functioning, cell shape and expression thereby regulating the drug resistance and response, is mirrored in 3D cultures. Usually in 2D cultures, all cells grow uniformly across the surface. Within *in vivo* tumor, sub-populations of cells exist based on the mass transport of nutrients. The region with no nutrient exchange consists of necrotic cells and the region with minimal/ inefficient nutrient exchange attributes to cells in the quiescent stage. The cells at the periphery would be actively proliferating. This multi-layered arrangement can be observed in 3D MCTS as depicted in Figure 1.2 [39]. Cells grown as monolayers tend to be flat as they adhere to the surface of the tissue-culture treated plates and have no chance for cellular contact resulting in morphological changes to the cell, subsequently modifying its cellular function [40].

Encapsulating prostate cancer cells in Matrigel™ revealed spheroidal or stellate morphology of the cells, suggestive of differences in invasive nature [41]. Conversely, similar experiments conducted for colon and endometrial cancer cells did not generate any trend of invasive behavior [42, 43]. These variations can reveal that cells' response to 3D culture is variable and how different culturing method can produce more *in vivo*-like observations. Thus, 3D models are suitable to understand tumor morphology and differences in proliferation of different cells (tumor and normal) of different organs and tissues.

Figure 1.2: “Microenvironments inside a spheroid. Tumor spheroids exhibit spherical geometry with a concentric organization of proliferating, quiescent and dead cells. The formation of multi-layered arrangements attributes to the insufficiency of mass transport through cellular barriers” Reproduced from [39] with permission.

1.3.2 3D Models – Phenotypic Heterogeneity

Heterogeneity in any population of cells is marked by differences in proliferation, expression profiles, morphological and functional variations [44]. It has been revealed that within a spheroid or *in vivo* tumor, a population of cells exists that

exhibit stem cell-like properties like decelerated proliferation rate, undifferentiated phenotype and self-renewable capacity [45-47]. An increased expression of stemness genes has been observed when cells were cultured as 3D spheroids as against 2D monolayers [48, 49]. However, the expression of stemness markers do not define the existence of cancer stem cells. But, these results show the ability of 3D spheroids to replicate stem cell-like population like *in vivo* tumors, which plays a key role in drug resistance.

1.3.3 3D Models – Varied Gene Expression and Behavior of Cells within the Spheroid

The 3D culturing renders different cell migration and adhesion mechanisms on the cells in comparison to the 2D monolayers. This will be helpful in assessing extravasation and intravasation of tumors in healthy tissues and obtaining insights on integrin-mediated drug resistance. Many reports have confirmed that the activation of oncogene or the suppression of tumor suppressor gene are markedly different when cells are cultured in different microenvironments [50]. Furthermore, the signaling mechanisms are different when cells are cultured on 2D and 3D [51, 52]. For example, Mina Bissell's group had conducted the first comparative study between the transcription profiles of different breast cancer cell lines grown as 3D cultures and observed that the adopted morphology of the cell lines regulated their gene and protein expression profiles [53].

1.3.4 3D Models – Mimics Tumor Microenvironment

As co-cultivation of cells is possible in 3D cultures, various interactions and heterologous cross-talks can be studied in detail in a more realistic environment [54]. Many studies have explored this possibility by introducing cancer-associated fibroblasts to the culture of cancer cells [55-57] or understanding the cross-talk between immune and malignant cells [58, 59]. In the tumor microenvironment, in addition to the cancer cells interacting with one another, they interact with their surrounding non-malignant cells, secreted factors, chemokines, ECM. These complex interactions and forces and gradients present within the solid tumors contribute to their behavior and progression of cancer [60, 61].

1.3.5 3D Models – *In vivo*-like Drug Response

The well-established multicellular tumor spheroid (MCTS) cultures are able to mimic the *in vivo* situation more closely and are accepted as essential complex tools for determining the efficacy of drugs as well as to study interactions and response between heterotypic cells. Furthermore, *in vitro* 3D spheroid models provide a link between 2D systems and animal models for screening drugs and testing drug delivery efficacy. This has been shown by Mairs, R., *et al.*, [62], where neuroblastoma spheroids and xenograft models were subjected to radioisotope iodine-131-metaiodobenzylguanidine ($[^{131}\text{I}]$ MIBG) without any added carrier. The spheroid response to the treatment correlated with the animal data of reduced tumor growth and reduced tumor burden respectively. However, it was observed that smaller sized spheroids (~250 μm diameter) were resistant to the treatment and larger sized

spheroids (~400 μm diameter) were vulnerable to the treatment. These experiments have great clinical implications for employing radiation therapy for cancer treatment of micro metastases [63].

Taken together, these 3D cultures have the potential to be considered as an advanced research tool in determining drug resistance and understanding various mechanisms associated with tumorigenesis. This has been further tabulated as shown in Table 1.2 below:

Table 1.2: Comparison between 2D monolayers, 3D models and In vivo models.
(summarized from previous reviews [25] [51, 64])

Measures	2D Monolayers	3D models	<i>In vivo</i> models
Growth Substrate	Cells cultured on tissue culture plastic surface	Varies depending on method	Varies depending on tissue
Matrix Generation	No	Yes	Yes
Nutrient/oxygen gradient	No gradient	Depends on diffusion of molecules	Depends on vascular supply
Interaction with stromal cells	Can be achieved in co-culture but limits biochemical signaling	Can be achieved by co-culture allowing biochemical and migration analyses	Interact with stromal cells comprising of endothelial, fibroblast, immune cells in a complex microenvironment
Drug Response	High chemo-sensitivity	Decreased sensitivity to drugs	Low sensitivity due to complex microenvironment

1.4 Assays to Evaluate Drug Sensitivity in *In Vitro* (2D and 3D) Cultures

Expectedly, 3D cultures differ in their drug response from 2D. For example, endometrial cancer cells cultured in 2D and 3D, were subjected to doxorubicin and

cisplatin to assess the drug resistance [65]. Although the effects of the drugs were dose-dependent, the 3D cells showed higher viability as opposed to their corresponding 2D cultures. A similar observation was noted in a study conducted by Wen, Z., *et al.*, where both 2D and 3D systems were subjected to drug testing and the drug efficacy was evaluated using acid phosphatase (APH) assay [66]. This assay measures the cytosolic acid phosphatase and gives a good measure of viable cells in 3D. Many studies have utilized cell viability/cellular apoptosis reagents to determine the effect of drug exposure to cells in spheroids [67, 68]. For example, alamar blue reagent has shown to provide a quantitative analysis on drug resistance evaluation in heterospheroids, homospheroids and monolayers [37]. For more advanced analysis, flow cytometry has been employed to segregate the viable, apoptotic as well as necrotic cells [69-72].

While the above methods provide quantitative data about the drug responses from 2D and 3D, a spatial and temporal responses of the action of the drugs is lacking. These spatiotemporal effects can be addressed by employing sensitive and rapid live cell imaging systems like biosensors, which additionally would represent information about microenvironment- dependent effects of drugs or lead compounds.

1.5 FRET Biosensor

In this thesis, a FRET-based biosensor for apoptosis has been employed to provide information on effects of drugs in a non-invasive and longitudinal manner, especially in 3D. Apoptosis is characterized by the activation of the caspase cascade. In

particular, caspase-3 plays a vital role in regulating programmed cell death on drug treatment. In order to facilitate the detection of apoptosis in cells, Fluorescence Resonance Energy Transfer (FRET)-based biosensor probe has been used in this study. This probe combines two fluorophores – Cyan Fluorescent Protein (CFP) and a Yellow Fluorescent Protein (YFP) linked by a 16 amino acid peptide linker which contains the cleavage sequence (DEVD) of caspase-3 protease. This construction is depicted in the Figure 1.3 below. The CFP acts as the donor chromophore whereas YFP acts as the acceptor chromophore. On exciting the FRET probe at an excitation wavelength of 433 nm (CFP), the energy is transferred from CFP to YFP upon close proximity of the two proteins. Therefore, the emission wavelength is at 526 nm corresponding to YFP. When cells undergo cell apoptosis, the caspases are activated which then cleave the site that link the two proteins of CFP and YFP, thereby separating the donor and acceptor. This subsequently eliminates the FRET effect. Therefore, when these dying cells are excited at 433 nm, the light that is emitted comes majorly from CFP protein rather than YFP protein when the cells were viable. Therefore, this probe provides us a way to monitor activation of apoptotic pathway [73-75].

Figure 1.3: Principle of FRET Biosensor. Reproduced from [76] with permission.

1.6 Objective and Specific Aims

The current methods for drug efficacy studies in 3D *in vitro* systems largely rely on end-point assays like cell viability reagents/flow cytometry [37, 67], and immunostaining [77]. Despite their accuracy, these assays do not allow longitudinal and real-time monitoring. Therefore, our approach to overcome this limitation, is to use biosensors for live-cell imaging that provide sensitive, rapid and non-invasive readouts for drug-induced effects in different microenvironments. This may be achieved via FRET-based biosensor that help us detect early apoptosis in live cells. In addition, this model would provide a proof-of-concept for utilizing biosensors for imaging cells in 3D.

Therefore, the objective of this thesis is to develop a novel FRET-based 3D *in vitro* breast cancer platform to evaluate drug-induced apoptotic effects. In line with this aim, the following specific aims was investigated:

1. Establish a suitable FRET-based 3D breast tumor model to evaluate drug-induced apoptosis of breast cancer cells in 2D and 3D
2. Validation of the novel platform for assessing anti-cancer properties of N-heterocyclic carbene (NHC) complexes
3. Establish a 3D co-culture system for studying the interplays between breast cancer and stromal cells

1.7 Significance of Work

Currently, patient-derived xenografts (PDX) and organoids have gained importance for personalized treatment of patients, leading to US National Cancer Institute (NCI) to phase out NCI-60 panel of 60 cancer cell lines. PDXs are derived by implanting human tumors in mice, and they have proven to mimic the heterogeneity and molecular signatures of the clinical tumor better than cell lines or genetically modified mouse models [78, 79]. Similarly, tumor organoids have also been established from patient tumor specimens [80]. Although PDX and organoids have become popular in recent times, the limitations of PDX include lack of dose response curve, low throughput for broad drug testing, conducted in mice which lack normal immune response, require optimization of protocol and high cost [79]. Therefore, we believe the proposed 3D spheroid model system in this thesis would provide us with the knowledge and expertise to handle more complex systems like patient-derived

organoids and PDXs. Critically, we believe that this FRET-based model system can act as an intermediate step and screen drugs before they are tested on PDX models and eventually bigger animals.

1.8 Scope of Thesis

Chapter 1 introduces briefly the need for 3D *in vitro* models and also lists the specific aims of this thesis.

Chapter 2 thoroughly surveys the literature to review methods to generate 3D spheroidal models. Their application in tumorigenesis and discusses current approaches to evaluate drug responses in 3D.

Chapter 3 describes the development of a FRET-based 3D breast tumor model and demonstrates the ability of FRET sensor for real-time and non-invasive readouts in 3D.

Chapter 4 demonstrates the applicability of FRET-based 3D breast tumor model in screening novel anti-cancer compounds.

Chapter 5 discusses the development of a 3D co-culture model and its characterization in terms of tumorigenic markers.

Chapter 6 presents conclusions of this thesis with future work recommendations.

Chapter 2: Literature Review

2.1 Reductionist Approach to Study Pathophysiology of Breast Cancer and the Relevance of Tumor Microenvironment

Breast cancer manifests itself when normal breast cells start to divide and grow uncontrollably owing to epigenetic transformations and/or in response to stimuli from the microenvironment [81]. These breast cancers can be benign, wherein the cancerous cells proliferate locally and are confined within the breast tissue. When these cancer cells begin invading neighboring tissues like the lungs and bones etc., the cancer is classified as malignant breast cancer.

The breast tissue consist of ducts and lobules. These are lined by two membranes: the inner epithelial layer and the outer myoepithelial layer, which runs along the basement membrane. As seen in Figure 2.1, breast cancer progresses with the help of the stromal microenvironment surrounding it. From ductal carcinoma *in situ*, where the breast cancer cells are confined to the region within the basement membrane to invasive ductal carcinomas, wherein the breast cancer cells invade surrounding tissues and establish inter-cellular contacts, the stromal microenvironment plays a pivotal role by secreting multiple cytokines, growth factors and ECM [82].

Some studies have explored micro-environmental cues that promote breast tumor progression. For example, hypoxia in *in vivo* tumors, influence responses to treatment options like radiotherapy [83] and chemotherapy [84] and is associated with the metastatic cascade [84, 85]. Similarly, physical properties of the ECM in the microenvironment [86] has also been studied extensively to determine if cancer

can be reversed by developing biologically-inspired materials that mimic the embryonic microenvironment [87].

Breast cancer cells themselves secrete multiple factors and ECM degrading enzymes [88], in addition to inducing stromal elements to secrete growth factors and cytokines [89, 90]. These secreted factors are responsible for intercellular contact, adhesion, motility, and invasion [91, 92]. These factors are capable of enhancing the metastatic ability of cancer cells. For example, it was observed by Wang, T.N. *et al.*, that inhibition of MMP-9 synthesis by stromal cells prevented matrix degradation, a key step for tumor invasion and metastasis [91]. It has also been evident that these growth factors and chemokines are able to trigger signaling cascades in breast tumor cells, thus promoting breast cancer cell survival and resistance to chemotherapeutic agents [93].

Most studies employ the reductionist approach by focusing on the cancer cells and their genetic changes. Although these studies provide basic information, it is too simple. Cancer cells co-exist with the non-tumorigenic cells to form the tumor microenvironment. Many of the cells that constitute the microenvironment were considered silent spectators of tumor progression until it was proven that bidirectional signaling between tumorigenic and non-tumorigenic cells was critical for breast cancer progression [94-96].

However, majority of these studies are conducted using 2D monolayer cultures. The drawback of using 2D systems is that they do not enable the study of functional behaviors of cells in a physiologically mimicking environment. Therefore, 3D cultures, especially multicellular spheroid models, have been explored as a system

of intermediate complexity between 2D and animal models. These would serve as a platform to obtain important insights in a variety of areas of tumor biology, when used in conjunction with experiments of higher complexities in other systems [97].

Figure 2.1: (a-d) Progression of mammary ductal carcinoma. Reproduced from [98] with permission.

2.2 Importance of 3D Spheroids as *In vitro* Models

Three-dimensional cultures are indispensable tools in further enhancing our knowledge on tumorigenesis. They provide a platform for assessing drug efficacy, heterologous cross-talk and other facets of tumor progression. As illustrated in the Figure 2.2, they represent higher complexity than the standard 2D monolayers and enable better imaging and analysis. In addition, they can reduce the dependence on animal testing for drug testing and also provide a model for screening of drug candidates with higher efficacy and tissue penetration [99].

Figure 2.2: “3D cultures recreate the complex cellular microenvironment more precisely than traditional 2D cultures, due to the incorporation of multiple physical, mechanical and chemical cues that arise from ECM–cell and cell–cell interactions. At the opposite end of the experimental continuum, animals do not capture important facets of human behavior and they are not feasible for HTS applications. Therefore, 3D cultures can bridge the gap between 2D cultures and animal models”. Reproduced from [99] with permission.

Spheroids, cellular clusters formed by self-assembly of cells, are popular and commonly used for generating 3D models to simulate *in vivo* tumors [100]. Differently sized spheroids provide flexibility in generating models for avascular tumors (development of necrotic cores, presence of quiescent cells) and for

metastatic tumors by incorporating other cell types. In addition, these 3D spheroids have been used to study drug efficiency and resistance, as they are less sensitive to such treatments than their 2D counterparts [101]. Not limited to drug screening and testing, these spheroids are advantageous in understanding tumorigenesis in terms of growth constraints like nutrient consumption and oxygen tension, cellular interactions, mechanical properties, radiation effects and angiogenesis [102-104].

These 3D spheroids are valuable tools for evaluating drug efficiency testing for the following reasons:

- 1) These 3D cultures are able to simulate *in vivo* architecture of tumors such as multicellular arrangement, ECM deposition and cellular interactions. Therefore, drug response closer to real situations can be assessed better than 2D monolayers [25, 99].

- 2) Diffusion limitations like nutrients, waste, oxygen gradients can be observed within a spheroid. This would prove useful in determining the penetration of the drug and its corresponding efficiency at each of these gradient zones [105].

- 3) Other cell types can be incorporated in this model, thereby mimicking the heterologous cross-talk between different cells in a real tumor. This helps us identify various aspects of cell-cell interactions responsible for drug resistance [106].

- 4) Expression of stem cell markers and maintenance of stem cells in these cultures is possible in 3D spheroid cultures, thereby facilitating studies of targeting these specific rare cells with drugs [107].

As a result of diffusion limitations like nutrient distribution, waste accumulation, oxygen diffusion, spheroids are able to recreate *in vivo* tumor architecture with

proliferating cells at the peripheral, with viable but quiescent cells beneath that layer of proliferating rim and a necrotic core well within the spheroid at the center [39, 108]. These gradients in this model have been valuable in understanding therapeutic problems. For example, the inherent gradients present in spheroids have increased our knowledge about the response of hypoxic cells to chemotherapy and radiation therapy [109, 110]. In addition, these diffusion limitations coupled with complex cell-cell and cell-ECM interactions lead to varied protein expression, thereby affecting treatment responses to therapy [50, 111]. Taken together, this spheroid model can serve as a good platform for testing drugs.

2.3 Methods to Generate Spheroids

Spheroids have been recognized as good 3D models to understand various facets of tumor progression and for drug testing [28]. However, there are many challenges to generate and maintain these spheroid models, so that they can be adapted for studying drug delivery extensively. The possible methods to produce spheroids are illustrated in Figure 2.3 and further explained in the following sections:

2.3.1 Hanging Drop

Hanging drop is a well-known method and is extensively used for homospheroids or co-culturing two cell-types. This technique utilizes the effect of gravity to assemble the cells at the bottom of the droplet and aggregate together without the need for any additional ECM components or scaffolds. However, the disadvantage of medium

exchange and sometimes, generation of irregularly shaped spheroids and the labor-intensive approach when scaling up make this technique not dependable at all times.

In 2010, Yi-Chung Tung *et al.* [112], developed a platform based on hanging drop technique for generation of uniform spheroids. This platform allows efficient drug screening of physiologically relevant spheroids. They tested the spheroids generated by their method with an anti-cancer drug, 5-Fluorouracil (5-FU) and a cytotoxin, tirapazamine. 5-FU targets the proliferating cells whereas the cytotoxin targets the hypoxic core. The combination therapy provided an additive effect on the inhibition of growth of spheroid. However, this system was unable to sustain the spheroids beyond 14 days owing to its susceptibility to mechanical shocks [28].

2.3.2 Scaffolds

Many scaffolds including natural components like Matrigel™, collagen [113-115] etc., and synthetic components like PCL, PLGA [116-118] etc., have been used to generate 3D cultures. Dhiman, H.K., *et al.*, have investigated the possibility of using chitosan scaffolds for *in vitro* model of MCF-7 cells [119, 120]. Similarly, biodegradable microparticles of PLA coupled with composites PVA and chitosan has been explored for the development of cancer models [121]. A combination of both natural and synthetic components like collagen-PEG, developed by Liang, Y., *et al.*, has shown to promote spheroidal growth. In addition, by controlling the stiffness of the scaffold, they were able to regulate the malignancy in the liver spheroids [122].

2.3.2.1 Microencapsulation

Cell microencapsulation is a promising technology for delivering therapeutics, without invoking an immune response from the host. They have also been explored for developing 3D cancer models to study various drug responses and mechanisms [123, 124]. Alginates consists of both β -D-mannuronic acid (M) and α -L-guluronic acid (G) monomers linked to each other. The divalent cations determine the gel forming capacity of these alginates. Various factors including the composition of alginate monomers, additional outer membrane, liquefaction of core influence their elasticity and strength [125, 126]. Although alginates are considered ECM-mimicking component, they are typically lack cellular attachment. Therefore, RGD peptides are chemically incorporated to provide a microenvironment closer to *in vivo* tumors [127].

Figure 2.3: Methods to generate 3D spheroids. (A) Hanging drop technique. (B) 3D scaffolds. (C) Bioreactors – (i) spinner flask cultures (ii) rotary vessel cultures. (D) Non-adhesive coating technique. Adapted from [39] with permission. (E) Microfluidic devices. Adapted from [128] with permission.

2.3.3 Use of Bioreactors to Generate Spheroids

Large bioreactors like spinner flasks, rotary vessels, and perfusion chambers have been used to induce spheroid formation. These promote cellular aggregation by preventing cells to settle down and attach [46, 129, 130]. These bioreactors are successful in generating mass production of spheroids and are suitable for long-term cultures. However, the need for specialized equipment and production of irregular and non-uniform spheroids make them unsuitable for drug testing applications. As uniform size would enable size-dependent drug response studies more reproducible, it becomes critical to have systems that can generate spheroids of uniform size for repeatability of results.

2.3.4 Microfluidic devices

Recently, many advanced techniques employ micro- and nano-technologies to develop spheroids. These techniques enable control over spheroid size, cell composition and may be eventually used for high-throughput analysis. Many groups have employed microfluidic wells to contain the spheroids, others have utilized such systems to develop microspheres of natural ECM containing cancer cells [131-133]. For example, embryoid bodies were generated from embryonic stem cells by forcing their aggregation on micro-patterned surfaces [134]. Similarly, other groups have developed 3D scaffolds, surface-modified substrates to promote spheroid generation [135-139]. Photolithographic techniques have helped us generate molds of customizable volumes which help us confine cells within the microwells and guide

their reorganization into spheroids [140, 141]. Although these techniques have generated 3D spheroid models, they are complicated in nature requiring special skills to fabricate and operate these microfluidic devices. Prolonged culture and medium exchange to perform drug screening and testing are problematic in such systems. In addition, it is always argued if these materials are compatible with the hydrophobic compounds, thereby questioning their applicability in drug delivery testing.

2.3.5 Non-adhesive Coating/ Liquid Overlay Technique

This technique of non-adhesively coating the surface promotes cellular aggregation, thereby facilitating strong cellular interactions. Subsequently, these aggregates come together to form tight structures which proliferate and represent the morphological features of *in vivo* tumors [142, 143]. The non-adhesive can either be agar or poly-HEMA or pluronic F-127 coating. This technique is simple and rapid. The shift to large scale production of spheroids for high-throughput analysis is smooth, adding to its advantages. Additionally, breast cancer metastatic spheroids and a model of epithelial-mesenchymal transition (EMT) has been established by Li, Q., *et al.*, using this technique [144]. The liquid overlay technique employs agarose to form a concave surface in a well plate, and shares the same principle as non-adhesive coating technique. Pancreatic spheroids generated were able to mimic *in vivo* tumor more closely than 2D monolayers in their drug response [66].

2.4 Application of Spheroids in Various Facets of Tumor

Tumor spheroid models are able to recreate *in vivo* tumor architecture, and therefore are employed to study different facets of cancer (Figure 2.4).

Figure 2.4: Use of tumor spheroids in studying various tumor-related parameters.

2.4.1 Migration and Invasion

The step-by-step process of metastasis visualized in 3D models is due to the presence of non-cellular components of the tumor microenvironment like hypoxia and ECM that drive tumor progression [85, 145, 146]. In addition, it has been shown that the ECM laydown pattern in 3D spheroids is similar to *in vivo* tumor [147]. Various studies have used spheroids to study migration and invasion of cancer cells in addition to evaluating the effect of drugs with anti-migratory properties [148-150]. Invasion of cells grown as 3D spheroids has been studied when embedded in matrices [151, 152] or seeded on layer of ECM [153] and also in the presence of other cell types in the spheroid model.

2.4.2 Stemness

These 3D cultures are known to enhance the population of cancer stem cells, which otherwise aren't present in 2D monolayers [107]. Research by Su, G., *et al.*, has shown that 3D sphere formation induced by low-attachment surface induced cells to express stemness properties [48]. The significance of these stem cells in cancer has been researched by several groups, providing evidence of their influence on therapeutic efficacy, metastasis and angiogenesis [45, 82]. Oncospheres generated from breast cancer cells supported their metastatic ability by producing tenascin C, an ECM component of stem cell niche. Tenascin C was able to enhance the expression of stem cell markers [154].

2.4.3 Hypoxia studies

The importance of hypoxia in tumor has gained interest in the last few years. Hypoxic environments in within tumor has influenced sensitivity of the cells, tumor progression and cancer stem cells maintenance and migration [85, 155, 156]. For example, in the study conducted by Nagelkerke, A., *et al.*, it was concluded that hypoxia-mediated cellular migration and invasion in spheroids (Figure 2.5) [157]. It is suggested that these models would be useful to study the efficacy and response of hypoxia-targeting drugs in these spheroid cultures. Furthermore, in recent times, bacterial cells have gained importance for anti-cancer properties by accumulating in hypoxic environments within solid tumors to regress tumors [158-160]. Therefore, some studies have utilized 3D spheroidal models to determine the penetration and activity of these bacteria [161, 162].

Figure 2.5: (A) Breast cancer spheroid stained with hypoxia marker pimonidazole. Scale bar 100 μ m. (B) Relative mRNA expression of PERK, ATF4 and LAMP3 in breast cancer spheroids compared against 2D monolayers. (C) Migration ability of cells grown as 2D and 3D spheroid cultures. (D) Gap closure assay at 16 h for cells grown as 2D and 3D spheroid cultures. Reproduced from [157] with permission.

2.4.4 Metabolic changes

Multicellular spheroids have been employed to understand the energy metabolism in solid tumors [163, 164]. It was identified that metabolic readjustment occurred when the proliferative conditions began to stagnate [164]. NMR studies have also been conducted to understand the influence of spheroid age on glucose metabolism and phosphate metabolite concentrations [165]. Another study led by Mueller-Klieser,

W. *et al.*, has observed that a large proportion of the cells in the spheroid are capable of adapting their metabolic rates according to the different glucose concentrations [166].

2.4.5 Penetration studies

Solid tumors are characterized by leaky vasculature, hypoxic environments, increased interstitial fluid pressure and the presence of dense ECM [167-169]. These, in turn, significantly limit the delivery of drugs to the tumor environment from the blood vessels and thereby compromising on the efficiency of drug administered. In addition, the penetration of the drugs are influenced by their shape, charge, and size [170-172]. Although *in vivo* models are extensively used to understand the pharmacokinetics of these drug compounds [173], *ex vivo* approaches like spheroid cultures are developed to minimize the burden on animal models. Huo, S., *et al.*, has shown that multicellular 3D spheroids have provided an *ex vivo* approach to determine the penetration properties of differently sized gold nanoparticles for drug delivery [174]. Similarly, extracellular accumulation and penetration of doxorubicin in a linker-engineered spheroid was found to be dose-dependent as shown in Figure 2.6 [175].

Figure 2.6: Penetration of doxorubicin (red) in 2D monolayers (a-c), mature linker-engineered spheroids (d-f), mature naturally formed spheroids (g-i) at 24 h. Scale bar 20 μm Reproduced from [175] with permission.

2.4.6 Radiation Therapy

This multicellular spheroid model is well-suited to study the effects of ionizing radiation because the tumor sensitivity to radiation therapy largely depends on cell-cell contact and communication, oxygen gradients and apoptosis stimulation [176]. It has concluded from several studies that spheroids are able to accurately model sensitivity to radiation therapy better than 2D monolayers, as it resembles *in vivo* like response [177, 178]. The resistance to radiotherapy in these *in vitro* 3D spheroidal cultures has been attributed to the hypoxia [179] and intercellular contacts [177]. Ferrante, A., *et al.*, has shown that mature spheroids, having stronger cellular

contacts, are more resistant to radiation therapy than the early spheroids which were more susceptible [180].

2.4.7 Chemotherapy

Over many decades, it has been firmly established that these 3D spheroidal cultures have been able to recreate *in vivo* tumor *ex vivo* and mimic the drug sensitivity/resistance more accurately than the conventional 2D cultures. Experts in this field have time and again recommended the use of this model for drug screening purposes [181]. Numerous studies have evaluated the efficacy of multiple therapeutics in these models and compared them against the response from 2D monolayer cultures [101, 106, 182-184]. It has been found that the observed effects of the chemotherapy was equal to or more sensitive than their corresponding 2D studies (Figure 2.7) [185, 186]. Together, these studies demonstrate the potential of employing spheroid cultures for an accurate representation of investigational drug activity *in vivo*. In addition, the uniform size, easy handling and scale-up make spheroidal cultures powerful tools for drug screening studies [187].

Figure 2.7: Cell viability of different pancreatic ductal carcinoma cell lines grown as 2D monolayers and 3D cultures after gemcitabine treatment. Reproduced from [186] with permission.

2.4.7.1 Detection Techniques

Different methods are used to determine the drug efficacy in three dimensions including immunostaining [188] and flow cytometry. The relevance of caspases in assessing apoptosis in monolayers, spheroids and xenograft models was established using immunohistochemistry methods by Bressenot *et al.* [77]. In another study conducted by Wan Yong Ho *et al.* [67], on obtaining the spheroids, three different methods (MTT, LDH and flow cytometry analysis) to determine the cellular viability

was carried out and the performance of these methods was compared. The cytotoxicity determined from MTT and flow cytometry were comparable, whereas the LDH didn't correlate to this data. The author's attributed to this discrepancy to the necrotic core already present within the spheroid which in turn generates a basal LDH value. For more advanced analysis, flow cytometry has been employed to segregate the viable, apoptotic as well as necrotic cells [69-72].

Table 2.1 Limitations of the current techniques used to assess cytotoxic activity of drugs/investigational compounds in 3D cultures

Methodology	Limitations	Ref
<ul style="list-style-type: none"> • Hemocytometer counting • Cell – viability reagents • Apoptosis quantification (TUNEL assay, LDH etc.) • Flow cytometry • Immunostaining 	<ul style="list-style-type: none"> • High cost • Pre-processing steps • Post-processing steps • End-point analysis • Large number of samples required 	[66, 67, 77, 189, 190]

From the Table 2.1, it is clear that the current methods used to evaluate drug response in 3D cultures are extensive and lack longitudinal and non-invasive analysis. The kits that help with these quantitative assays and qualitative analysis are expensive and are destructive analysis. They require the dissociation of the spheroids to individual cells before proceeding for any analysis.

2.5 FRET Biosensor: Proposed Approach

As elaborated in the earlier section 1.5, FRET-based biosensor C3 was transfected in our breast cancer cells to enable real-time and non-invasive tracking of cell fate. The use of this C3 biosensor has been demonstrated in endothelial cells for screening vascular disrupting agents. In this study conducted by Zhu, X *et al.*, it was reported that HUVEC cells transfected with C3 biosensor (HUVEC-C3) enabled non-invasive detection of caspase-3 activation during apoptosis [75]. In addition, it was proven that the sensor was specific to apoptosis and would not detect necrosis as illustrated in the Table 2.2. The reduction in YFP/CFP (Y/C) emission ratio was evident for agents that induced apoptosis (paclitaxel, UV irradiation), but not more agents that induced necrosis in cells (H₂O₂ and Triton-X).

Table 2.2: Y/C emission ratio and cell viability readouts when HUVEC-C3 was exposed to different agents. Reproduced from [75] with permission.

Group	Y/C emission ratio	Cell viability (%)
Control	5.75 ± 0.63	100.0
UV-1 h	5.76 ± 0.46	90.8 ± 19.2
UV-3 h	4.93 ± 0.33	75.3 ± 12.5
UV-6 h	3.11 ± 0.23	59.0 ± 6.3
UV-9 h	2.23 ± 0.16	45.2 ± 8.6
UV-12 h	2.09 ± 0.11	15.0 ± 0.7
UV-24 h	2.02 ± 0.19	16.1 ± 1.1
H ₂ O ₂ -1 h	5.74 ± 0.56	17.4 ± 4.8
H ₂ O ₂ -3 h	5.32 ± 0.61	8.1 ± 3.6
H ₂ O ₂ -6 h	5.37 ± 0.77	-1.1 ± 0.2
Triton X-100-1 h	6.02 ± 0.46	0.6 ± 0.1
Triton X-100-3 h	6.19 ± 0.39	0.4 ± 0.1
Triton X-100-6 h	6.28 ± 0.41	0.4 ± 0.1

Similarly, this sensor has been used in cancer cells to screen herbal compounds for their anti-cancer activity [191]. Furthermore, these sensor transfected endothelial cells have also been used to assess the effect of inhibitors to the differentiation of HUVEC-C3 in a direct co-culture model with HepG2 liver cancer cells and when seeded on Matrigel™ [192].

2.6 Co-culture studies

Cancer cells co-exist with stromal elements in the tumor microenvironment and the bidirectional signaling between these different cells influence and regulate key mechanisms and promote tumor progression. It is known that the tumor microenvironment plays a vital role in influencing radiotherapy and chemotherapy resistance to cancer cells [193, 194].

For ductal carcinomas, the tumor microenvironment is abundant in myofibroblasts. It has been clinically observed that the presence of such fibrotic focus is correlated to poor survival of invasive ductal carcinoma patients than without fibrosis [195]. The presence of fibroblasts has shown to positively regulate metastasis and angiogenesis in invasive ductal carcinoma patients [196, 197]. As mentioned earlier, the fibroblasts are known to secrete oncogenic signals which promote cell migration and invasion of cancer [91, 198].

Together, it is understood that stromal fibroblast cells are not bystanders and provide physical support and play a functional role in tumorigenesis [199]. The heterologous cross-talk between the cells are considered vital for breast cancer

progression. Therefore, these findings suggest the need to establish *in vitro* models that can help us examine and understand these interactions in breast cancer.

Many 3D models have been developed to evaluate the regulatory mechanisms that are altered when the breast cancer cells and fibroblasts are co-cultured and in turn influence angiogenesis, invasion and treatment response [200-204]. Table 2.3 illustrates some of these models which are look into aspects of cancer cell migration, invasion and drug delivery systems.

Table 2.3: 3D Co-Culture Models of Cancer Cells and Fibroblasts

Cells	Study	Key features	Conclusion	Ref
MCF10A, MCF10AT + NAF/CAF (E:F:: 2:1 / 1:3)	Effect of CAFs on proliferative ability of benign breast tumor	Transfection of MCF10A to develop new cell line- MCF10AT	NAF suppress breast epithelial growth.	[205]
T47D + Immortalized human mammary fibroblasts	Effect of Sdc1 shedding by stromal fibroblasts on breast cancer cells	Elucidate the partial links in stromal and cancer cell interactions via Sdc1 shedding	Paracrine activity of heparin sulphate GAG – growth stimulating effect on breast cancer cells	[206]
A549 lung cancer + NAF/CAFs	Characterization of tumor cell-stromal interaction morphologically and at molecular level	3 sets of patient matched primary fibroblasts for co culture	No changes in morphology. CAFs- enhanced contractile property; cancer cell invasion.	[207]
YD-10B, YD-38 oral epithelial cancer + NIH 3T3 fibroblast	Effect of fibroblasts on cancer cell invasion	Direct and indirect effects was analyzed. Cathepsin D analysis	Fibroblasts secrete MMP-2, MMP-9 and stimulate cathepsin D secretion >Promoting invasiveness of cancer	[57]
MCF-7 + primary human dermal fibroblasts	Cellular uptake of nanocarriers in co-culture systems	Various experiments to validate their claim	The np internalization higher in malignant cancer cells than in fibroblasts	[208]
MCF-7 + fibroblast from reduction mammoplasties	Development of 3D morphogenesis model and understanding the influence of ECM and fibroblasts		Stromal elements are essential for individual and collective cell survival and phenotype	[209]

Cells	Study	Key Features	Conclusion	Ref
HeLa Cell lines and human dermal fibroblasts	Develop model to study the interaction between tumor and stromal cells	Study direct interactions between already formed aggregates	Model developed is poorly analyzed	[210]

Chapter 3: Materials and Methods

3.1 Cell Culture

The plasmid DNA for C3 biosensor, containing the CFP-DEVD-YFP fusion gene, was generated and characterized by our group previously [211]. The breast cancer cell lines, MCF-7 (non-metastatic cell line) was purchased from ATCC and MDA-MB-231 cells (metastatic cells) were provided by Prof Xiaofeng Le from the Department of Experimental Therapeutics at the University of Texas M.D. Anderson Cancer Centre, Houston, TX, USA, and later transfected with this C3 biosensor plasmid to generate two stable cell lines designated as MCF7-C3 and 231-C3 respectively in this study. MCF-7, MDA-MB-231, MCF7-C3 and 231-C3 cells were cultured in Dulbecco's Modified Eagle's medium – high glucose (Gibco, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) and 100 units per mL penicillin plus 100 mg/mL streptomycin (Gibco, USA). The cells were incubated at 37°C with an atmosphere maintained at 5% CO₂. For 3D culture, the cells were grown in DMEM supplemented with 20% FBS and 1% PS. The lung fibroblasts (IMR90) were purchased from ATCC (Manassas, VA) and were also cultured with DMEM complete growth media.

3.2 Generation of Multicellular Tumor Spheroids (MCTS)

3.2.1 Microencapsulation

2% (w/v) alginate solution and 2% (w/v) gelatin was mixed at 1:1 ratio thoroughly and then mixed with 1,000,000 cells/mL of MCF7-C3 cells. To generate the beads, droplets of cell suspension in ECM-mimicking alginate-gelatin mixture was slowly extruded into 100 mmol/L CaCl₂ using a 30-gauge needle. The beads were allowed

to gel completely for 5 min before incubating them in their complete growth media (DMEM + 20% FBS and 1% penicillin-streptomycin). The average size of the beads ranged from 2,400 -2,600 μm and about 10,000 cells were encapsulated in each bead (Figure 4.1).

3.2.2 Hanging Drop Method

MCF7-C3 cells grown on tissue culture flasks were trypsinized using 0.25% trypsin-EDTA (Gibco, USA) to obtain single cell suspension. Then, the spheroids were generated as described previously [143]. Briefly, 30 μL of cell suspension was dropped onto the lid of a 100 mm diameter petri dish. 30 droplets were placed per petri dish to prevent any coalescence. The lid was placed on the petri dish containing 10 mL PBS to ensure hydration. The spheroids were generated after 2 days incubation at 37°C and 5% CO_2 (Figure 4.3).

3.2.3 Non-adhesive Coating

Low adherent round-bottom 96 well plates were employed to generate spheroids. The non-adhesive coating was generated by using 1% pluronic F-127 (Sigma, USA). The cell suspension is diluted to obtain 5,000 cells in 200 μL of medium. The plate was then centrifuged at 1,000g for 5 min [142]. Twenty-four hours incubation at 5% CO_2 and 37°C was provided to complete the compaction of single cells to spheroids (Figure 4.4).

3.3 Generation of 3D Co-culture Spheroid Model

The MCF7-C3 spheroids (2000 cells) were generated as mentioned in the earlier chapters. The 3D co-culture (2000 cells) consisting of the breast cancer cells (either MCF7-C3 or 231-C3) and fibroblasts (IMR90) were mixed in the following ratios: 1:1, 1:2, 1:5 and 2:1 respectively; before seeding them into a 96 well plate coated with a non-adhesive agent. The plate was centrifuged at 1000 rpm for 5 minutes to facilitate the spheroid formation. These spheroids were maintained in DMEM combined with 20% FBS and 1% PS and medium was changed every two days.

3.3 Drug Treatment

For 2D monolayer, 10,000 MCF7-C3 cells were seeded on a 96 well plate and incubated overnight. For 3D culture, spheroids using 5,000 cells in 200 μ l media were generated as described before. Then, paclitaxel, a chemotherapeutic agent, was added into growth media at the concentrations of 20, 200 and 2000 nmol/L. The duration of drug treatment was 72 h. For both culturing methods, untreated controls were cultured in parallel.

3.4 Imaging Microscopy Techniques

3.4.1 Apoptosis Detection using FRET Imaging

MCF7-C3 cells and 231-C3, cultured in monolayers and spheroids, were subjected to either different concentrations of paclitaxel or NHC complexes (courtesy of Prof Murray V Baker's lab from the School of Chemistry and Biochemistry, The

University of Western Australia, Perth, Australia), to examine the responses to drug treatment based on different culturing methods. After treatment with paclitaxel or compounds, fluorescent images were obtained using an inverted fluorescence microscope (Zeiss Axiovert 100, Germany). The cells were excited at 436 ± 10 nm. The corresponding YFP and CFP images were obtained using 535 ± 15 nm and 480 ± 20 nm filters and recorded using a SPOT CCD camera (Diagnostic Instruments, USA).

3.4.2 Confocal Microscopy

In order to visualize fibroblasts in the co-culture spheroids as they are not fluorescent, these fibroblasts were stained with Hoechst 33342 for 10 minutes before live cell imaging was conducted using a confocal microscope (LSM 710, Carl Zeiss, Germany). Green fluorescence signal for breast cancer cells and blue fluorescence for nuclei staining of the fibroblasts were visualized.

3.5 Image-based Analysis of FRET Emission Ratio

The images were quantified using ImageJ software. Briefly, the spheroids were partitioned as center (80%) and rim (20%) of the spheroids and their corresponding YFP and CFP images were quantified individually for their fluorescence intensities. A region of interest (ROI) was marked in these assigned portions of the spheroids (center and rim) and the Y/C emission ratio was obtained by using the following formula: $\text{FRET emission ratio} = (\text{YFP}_{\text{spheroid}} - \text{YFP}_{\text{background}}) / (\text{CFP}_{\text{spheroid}} -$

CFP_{background}) [212]. The plot profile for the individual YFP and CFP channels were also obtained for the control and drug-treated spheroids using the software. While for the compound screening studies, the spheroids were subjected to 5 μ M of the all compounds and YFP and CFP images were recorded. The spheroids were considered as a circular entity and the FRET ratio was calculated and plotted versus time for the compound(s).

3.6 Cytotoxicity Assay Using MTT

For measuring cell viability, 5000 cells of 231-C3 were seeded in each well on a 96 well plate. After overnight incubation, the cells were subjected to serial dilution of the compounds ranging from 5 – 0.08 μ M. The cell viability was measured by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay [213]. Briefly, the absorbance of the control group at 72 h (C_{72}), and the compound-treated groups at 72 h (T_{72}), were measured using spectrophotometer at 595 nm. The 72 h cell viability was calculated using the following formula: $(T_{72}/C_{72}) * 100\%$.

3.7 Determination of Morphology and Membrane Potential ($\Delta\psi_m$) of Cancer Cells

To detect mitochondrial integrity, we have adopted the use of fluorescent dyes tetramethylrhodamine ethyl ester perchlorate (TMRE) and MitoTracker Red (CMXRos) that bind in a manner dependent on the mitochondrial membrane potential. Briefly, for the qualitative analysis, the 231-C3 cells were seeded at $4 - 9 * 10^4$ cells/mL on poly-lysine coated 8-well chamber (Ibidi, Germany). The

following day, cells were incubated with the NHC complexes for 30 h before subjecting them to 0.1 μ M MitoTracker and 1 mg/mL Hoechst 33342 for 10 min. After washing the cells thrice with PBS, live cell imaging was performed using confocal microscope (LSM 710, Carl Zeiss, Germany). To measure mitochondrial membrane potential, 5000 cells of 231-C3 were seeded on a 96-well plate. After 24 h, the media was gently aspirated and the cells were incubated with the test concentration diluted in fresh medium at 10 μ M for each compound. Following three days of treatment, the cells were incubated with TMRE at a final concentration of 100 nM at 37°C for 20 min. Afterwards, the solution was aspirated and replaced by PBS. Fluorescence was measured at RT on a fluorescence plate reader (SpectraMax® M5, Molecular Devices, California) set to 540 nm excitation and 590 nm emission [214]. Data obtained was represented as normalized values to the control to eliminate any variability between the independent experiments.

3.8 *In vivo* MCF-7 Tumor Model

All experiments on animals were conducted in accordance to and approved by the Institutional Animal Care and Use Committee (IACUC) of Nanyang Technological University (NTU). One estrogen pellet (50 mg/pellet, Innovative Research of America, USA) was implanted subcutaneously to an eight-week-old female BALB/c nude mouse. Four days later, 1 million of MCF-7 cells suspended in 100 μ L Matrigel™ (BD Biosciences, USA) were subcutaneously injected into the mammary fat pad of nude mouse (purchased from BioLasco Company, Taiwan). Eight weeks later, the xenograft tumors were removed from the animal and the tumor tissues were

immediately frozen in the liquid nitrogen and then stored at -80°C. The tumors were later subjected to H&E staining.

3.9 Sample Fixation, Paraffin Embedding and Hematoxylin and Eosin Staining

The spheroids after five days of culture were fixed with 4% paraformaldehyde at 4°C for overnight. The fixed samples were processed and embedded in paraffin wax. The paraffin blocks were sectioned at 3 µm thicknesses using a rotary microtome and collected in a clean Polysine® slides (Thermo Scientific, USA). For the xenograft tumor, the samples were fixed in Leica OCT Cryocompound tissue-freezing medium (Leica Microsystems, Germany) and cryosectioned at 5 µm thickness using a cryostat and collected in a clean Polysine® slides (Thermo Scientific, USA). Subsequently, sectioned samples were stained with haematoxylin and eosin (H&E) staining and mounted with a coverslip using mounting media.

3.10 Immunohistochemistry

Spheroids cultured for one week (for the 3D co-culture model, breast cancer cells and fibroblasts were seeded in a ratio of 1:1) were fixed with 4% paraformaldehyde at 4°C for overnight. The fixed samples were embedded in paraffin wax. The paraffin blocks were sectioned at 5 µm thickness using a rotary microtome and collected in a Polysine® slides (Thermo Scientific, USA). Subsequently, H&E staining was conducted and samples were mounted using mounting media. Furthermore, immunohistochemical analyses were conducted on these sections for fibronectin and N-cadherin. The samples were rehydrated before blocking endogenous peroxidase

activity with hydrogen peroxidase. Following antigen retrieval using sodium citrate (pH 6) at 100°C for 5 min, immunostaining was conducted using streptavidin-biotin complex method. The slides were incubated with the primary antibody overnight at 4°C. Subsequently, biotinylated secondary antibody was added for 30 min, following streptavidin enzyme conjugate for 10 min. The chromogen used was 3, 3'-Diaminobenzidine (DAB) and the slides were counterstained with haematoxylin. The immunostained slides were viewed by light microscopy.

3.11 Gene Expression Analysis

Gene expression analysis was carried out using quantitative real-time polymerase chain reaction (RT-qPCR) which was used in our previous study [213]. Briefly, total mRNA was collected using TRIzol reagent (Life Technologies, USA) after dissociating the spheroids using StemPro® Accutase® (Life Technologies, USA). The total mRNA was purified using RNeasy Plus Mini Kit (Qiagen, Valencia, CA) and subsequently reverse-transcribed to cDNA. Afterwards, qPCR was carried out using iQ SYBR green supermix and iQ qPCR system (Bio-Rad, USA). For quantification, gene expression relative to the house-keeping gene, GAPDH, was calculated and represented using the comparative method, $2^{-\Delta\Delta CT}$. Table 3.1 lists the primer sets used in this experiment (AIT Biotech, Singapore). All the reagents necessary for reverse transcription and qPCR was purchased from Promega, USA and Bio-Rad, USA, respectively. All the qPCR reactions were conducted in triplicates.

Table 3.1: qPCR forward and reverse primer sequences for tumorigenic and internal markers

Gene	Accession number/ reference	Primer sequence (5'→ 3')
MMP-9	NG_011468	F: GGGCTTAGATCATTCTCAGTG R: GCCATTCACGTCGTCCTTAT
SOX2	[107]	F: CCCCTTTATTTCCGTAGTTGTATTT R: GATTCTCGGCAGACTGATTCAA
OCT4A	[107]	F: CCCCTGGTGCCGTGAAG R: GCAAATTGCTCGAGTTCTTTCTG
GAPDH	[88]	F: CGGAGTCAACGGATTTGGTCGTAT R: AGCCTTCTCCATGGTGGTGAAGAC

3.12 Statistical Analysis

All the experiments were performed independently for three times and data was presented as mean $\pm$ SD. Five spheroids from one experiment making it 15 spheroids from three independent experiments were used for analysis. Statistical significance were determined using student t-test or linear regression fit or one-way ANOVA as applicable and * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ were used to indicate the levels of statistical significance.

Chapter 4: Development of FRET-based Breast Cancer Spheroids for Detection of Drug-induced Apoptosis[#]

[#]peer reviewed and published in 'Biotechnology and Bioengineering' journal

4.1 Introduction

As elaborated in Chapter 1, breast cancer in women is the leading cause of cancer death globally with 1,383,500 estimated new cases each year [2]. This has generated an interest to obtain insights on breast tumorigenesis and also to develop drugs that effectively combat the disease. Conventionally, petri dish cultures and small animal models have been used to evaluate the efficacy of drug treatment and to determine the factors responsible for tumor progression. However, these two-dimensional (2D) monolayers differ from *in vivo* tumors in their cell-cell and cell- ECM interactions, and are therefore unsuitable platforms for understanding *in vivo* tumor responses. Additionally, the malignancy and the signaling pathways are masked in such cultures [25, 52, 215]. Owing to the lack of suitable *in vitro* platforms, animal models are the primary choice for conducting cancer research, even though they have differences in terms of physiology and microenvironment, and high cost [29, 30]. More importantly, not every type of human cancer cells can form solid tumors in nude mice. Therefore, there is an urgent need to develop 3D *in vitro* models which better recapitulate breast tumor for drug efficacy and mechanism studies.

Currently, multicellular tumor spheroids are well-established and accepted as advanced tools for cancer drug screening. In this respect, many techniques are available to generate such spheroids: hanging drop [216], microcarrier beads [217], and rotary cultures [218]. However, these methods have limitations which can be overcome by the non-adhesive coating technique enabling the formation of spheroids in uniform size. Moreover, this method is straightforward, does not use any

chemicals, and is reproducible [142]. Recently, Guo *et al.*, developed a magnetic 3D spheroid platform by using non-adhesive plates [219].

Along with the establishment of *in vitro* 3D tumor models, techniques of evaluating cellular responses to drugs were developed. Current techniques include dissociating the spheroids for manual counting or cell viability assessments using commercial reagents [67], by quantification using flow cytometry [220], or by immunostaining techniques [77]. Immunostaining techniques require fixation and pretreatment steps for antigen retrieval and may cause damage to tissue morphology, making it difficult to identify distinct cell types and tissue structures [221]. Large number of samples are required to carry out cell viability measurements using reagents or flow cytometry. Despite their accuracy, these assays do not allow real-time monitoring and longitudinal follow-up. On this note, a potential solution may be live-cell imaging using biosensors that provide sensitive, rapid and non-invasive readouts for drug-induced effects in different microenvironments. FRET has been extensively used to map protein interactions by distant-dependent energy transfer from donor chromophore to acceptor chromophore. It is highly sensitive and robust. Our group has previously established the use of FRET for monitoring the activation of caspase-3 enzyme [211], which can be activated by both intrinsic and extrinsic pathways in apoptosis. Previously, our group has applied this FRET-based caspase-3 (C3) biosensor in HeLa cells to assess a potent anti-cancer compound from herbal plant [212].

In this study, we have analyzed different 3D culturing methods and established that multicellular tumor spheroids obtained with non-adhesive coating technique

provided high reproducibility, better handling and is physiologically representative of *in vivo* tumors. In addition, we have applied this FRET-based biosensor for obtaining *in situ* assessments on microenvironment-based drug effects on live cells. The breast tumor spheroids, in combination with the sensitive and specific C3 biosensor, provide a proof-of-concept of live-cell imaging in three dimensions.

4.2 Results

4.2.1 Comparison of 3D Culture Models for the Generation of Spheroids

To establish a three-dimensional system that closely mimics the *in vivo* situation of tumors, three different methods were compared to culture cells in 3D for their robustness and suitability for drug testing. In microencapsulation technique, the distribution of the bead diameter was evaluated using microscopy and ImagePro Plus software. It was found that the diameter of the microcapsules ranged between 2400 – 2600 μm and averaged at 2537.55 μm (Figure 4.1B (ii)). Although cells in these microcapsules had prolonged viability, cellular attachment was lacking. Even when seeding densities higher than the other two methods were used, spheroidal growth was not observed (Figure 4.2). The hanging drop method formed irregular spheroids after 2 days. In addition, difficulty in medium exchange posed a problem in maintenance of these spheroids for long-term studies (Figure 4.3).

Figure 4.1: (A) Schematic of generation of alginate-gelatin microcapsules. (B) (i) Alginate-gelatin beads and (ii) their distribution of microcapsules diameter. Average bead diameter = 2537.55 μm .

Figure 4.2: Proliferation profile of MCF7-C3 cells encapsulated in alginate-gelatin microcapsules over 5 days. Scale bar: 500 μm

Figure 4.3: (A) Schematic of hanging drop method (B) Hanging drop method using 5,000 MCF7-C3 cells to obtain spheroids after two days of incubation. Scale bar: 100 μ m.

Using a cell suspension combined with centrifugation, spheroids were generated in 24 h with uniform size and in large quantity (Figure 4.4). Since the advantages of the third method can support the use of spheroids in high throughput drug screening, we chose this non-adhesive coating technique over the other two approaches in the subsequent experiments.

Figure 4.4: Non-adhesive coating of 96 round-bottom well plates to generate MCF7-C3 spheroids of 5,000 cells. Scale bar: 100 μ m.

4.2.2 FRET-based MCF7-C3 Cells Demonstrated Real-time Live/Dead Readouts in both 2D and 3D Cultures

To visualize cell apoptosis in real-time, C3 sensor proved to be advantageous as it has shown to be responsive to various apoptotic inducers including UV-light and anticancer drugs [211, 212]. The principle of which is clearly illustrated in the earlier section – literature review. As shown in Figure 4.5, MCF7-C3 cells seeded directly on petri dishes proliferated and formed monolayers. On the other hand, suspended cells formed spheroids with tight cell contacts. 2D cultures validated the real-time observation of apoptotic cells (blue fluorescence) and live cells (green fluorescence) (Figure 4.5A and B). Importantly, the 3D spheroids also allowed for the visualization of live cells (green fluorescence). The application of this sensor in our study is suitable as many chemotherapeutic drugs activate apoptotic cascades in neoplastic cells [222].

Figure 4.5: (A) Morphological variation of MCF7-C3 cells when grown as a confluent monolayer and as a 3D tumor spheroid using non-adhesive coating method. The YFP and CFP images were individually taken and then overlaid to obtain the merged FRET image. Scale bar: 100 μm . (B) Enlarged fluorescent images of MCF7-C3 cells under YFP and CFP channels from 2D and also the merged FRET image from 2D and 3D, where live cells appeared in green (yellow arrow) and apoptotic cells appeared in blue (red arrow). Scale bar 50 μm .

4.2.3 Spheroid Diameter of MCF7-C3 and 231-C3 Cells Dependent on Initial Seeding Density

Different concentrations of cells ranged from 2,500 to 10,000 were used to form the spheroids. A strong correlation between the number of cells and the size of spheroids ($n = 5$ spheroids) was observed for both MCF7-C3 ($R^2 = 0.994$) and 231-C3 cells ($R^2 = 0.935$) (Figure 4.6B). The size of 231-C3 spheroids (ranging from 575 to 1,023 μm) was two times larger than the diameter of MCF7-C3 spheroids (ranging from 349 to 560 μm) (Figure 4.6B). However, 231-C3 cells formed looser aggregates, whereas MCF7-C3 cells formed tighter spheroids which should be similar to the compact cellular structure in a tumor. Henceforth, MCF7-C3 cells were used for further evaluation.

4.2.4 Development of Necrotic Core in Spheroidal Cultures

The inefficient transfer of nutrients and wastes beyond 150-200 μm through cellular barrier results in low oxygen centers, leading to nuclear fragmentation and necrosis within the spheroid. This was visualized by H&E staining of a spheroid which was generated by seeding 10,000 MCF7-C3 cells and cultured for 5 days (Figure 4.7). The absence of the hematoxylin staining of the nuclei in the core of spheroid indicated the presence of necrotic cells in the central region. Furthermore, the presence of hematoxylin staining in the peripheral region of the spheroid ($\sim 200 \mu\text{m}$) suggests the presence of nuclei in these cells. Additionally, the morphological features and the necrotic core observed in the histological sections of the xenograft tumors further demonstrated that the spheroids were able to represent the histological state of tumors *in vivo*. It can also be observed that from both conditions, the cells at the periphery are more closely packed than the cells located between the necrotic core and periphery.

Figure 4.6: Formation of spheroids of varying initial seeding densities on non-adhesive coated plates. (A) 2,500, 5,000, 7,500 and 10,000 cells of MCF7-C3 or 231-C3 were seeded in round-bottom well plates and observed after 24 h to determine the correlations between seeding density and size of spheroids in these two cell lines (YFP images). Scale bar 100 μm . **(B)** Diameter of each spheroid was measured using ImagePro Plus software. The mean diameter averaged from 15 spheroids generated from three independent experiments was plotted against cell seeding density to determine the R^2 value in a linear regression curve.

Figure 4.7: Hematoxylin and eosin staining of MCF7-C3 spheroids and xenograft tumor of MCF-7 cells. Enlarged images of the spheroid and xenograft tumor from the boxed regions. Scale bar: 50 μ m.

4.2.5 Up-regulation of Tumorigenic Markers in Spheroids

Overexpression of certain biomarkers such as matrix metalloproteases (MMPs) mediates tumor metastasis *in vivo*. MMPs are essential in degrading ECM to facilitate invasion and metastasis and is highly expressed in metastatic breast tumors [223]. Real-time qPCR analysis revealed that the transcriptions of MMP-9 in MCF7-C3 spheroids were significantly increased by 1.89 ± 0.73 -fold relative to the same cells in monolayers (Figure 4.8). It has been reported previously that elevated expression of SRY-related HMG box protein (SOX2) and POU domain class 5 transcription factor 1 (OCT4A) were representative of an acquired malignant phenotype in a breast cancer *in vitro* model [107]. Similarly, tumorigenic markers of SOX2 and OCT4A were upregulated by 6.25 ± 1.36 and 2.30 ± 0.54 fold respectively in MCF7-C3 spheroids (Figure 4.8), thereby demonstrating that 3D spheroids are a

better *in vitro* model than 2D monolayer in revealing the expression of tumorigenic markers.

Figure 4.8: Relative expression of tumorigenic markers between 2D and 3D. Expression levels of MMP-9, SOX2, and OCT4A were compared between MCF7-C3 cells in 3D spheroids and 2D monolayers by qRT-PCR. Results are shown as mean $\pm$ S.D. GAPDH was employed as an internal control.

4.2.6 Differential Response of MCF7-C3 Cells to Paclitaxel in 2D and 3D

In order to compare the drug response between 2D and 3D, high concentration (2000 nmol/L) of paclitaxel was added to the MCF7-C3 cells for 72 h. As expected, 2D monolayer cultures displayed high percentage of cell death (70%) to high dose of paclitaxel, which was determined by the FRET images of over 400 cells. In contrast, 3D spheroids showed different results, with the cells in the rim region displayed blue color indicating the cells died of apoptosis, while most of the cells in the central region appeared green in color indicating those cells were resistant to drug treatment (Figure 4.9A). This was further substantiated by measuring the fluorescence intensity of MCF7-C3 spheroids under YFP and CFP channels using the plot profile

function in ImageJ software (Figure 4.9B). It can be observed that in the central region of both control and drug-treated spheroids, the emission intensity of YFP is much higher than the intensity of CFP which resulted in a high emission ratio of YFP/CFP (1.99 – 2.20). Interestingly, at the rim region of the drug treated group, the intensity of CFP was elevated which resulted in a reduction of the YFP/CFP emission ratio to 0.90 – 0.91. As the emission ratio of YFP/CFP can be used to represent the FRET effect, this result indicated that these sensor cells can detect apoptosis in a 3D set up. More importantly, in comparison to the 2D monolayer culture, the application of these sensor cells in 3D allowed us to evaluate the spatial response of the cells to the drug treatment.

Figure 4.9: Apoptotic effects of paclitaxel. (A) 10,000 MCF7-C3 cells seeded in 96-well plate (2D) were treated with high concentration (2000 nmol/L) of paclitaxel and observed after 72 h incubation. Similar treatment conditions were applied to spheroids of 5,000 MCF7-C3 cells. Scale bar 100 μ m. (B) The intensity of YFP and CFP across the spheroid was measured separately using the plot profile function in the ImageJ software. The yellow line represents the profile along which the measurement was made. Rim region counts for 20%, while the center accounts for the 80% of the spheroid.

4.2.7 *In Situ* Assessment of Paclitaxel-induced Apoptosis in MCF7-C3 Spheroids

Since FRET-based C3 sensor enables real-time and non-invasive detection of apoptosis, longitudinal studies of spheroid integrity with and without drug treatment were conducted. From panel A in Figure 4.10, it was observed that after 48 h of paclitaxel treatment, the onset of apoptosis was visualized. After 72 h, the dose-dependent effect of the drug on the spheroids was observed, with more apoptotic cells appeared in the rim region when the drug concentrations were increased. But more live cells appeared in the central region as evident by the strong green fluorescence in those cells. This was further substantiated by measuring the FRET effects at different regions of the spheroids such as the rim and the center (Figure 4.10B and C). It can be observed from Figure 4.10 B that the FRET effect was reduced by 47% between the control and 2,000 nmol/L of paclitaxel at 72 h. However, much less FRET reduction (25%) was detected between the same two groups at the same time (Figure 4.10C). Taken together, these results indicate that these sensor cells-based 3D models can be used to detect the apoptotic effect of anti-cancer drugs in situ and in real-time.

Figure 4.10: In situ visualization of spheroids. (A) Effects of drug treatment at different concentrations of paclitaxel on spheroid integrity captured spatially and temporally without disrupting the spheroid. The yellow arrow points to the intact circumference of the control spheroid whereas the red arrow points to the apoptotic mass at the rim of the drug-treated spheroid at 72 h. Scale bar 100 μ m. **(B)** Relative YFP/CFP emission ratio quantification for different paclitaxel concentrations over time at the rim of the spheroid. **(C)** Relative YFP/CFP emission ratio at the center of the spheroid.

4.3 Discussion

Three-dimensional *in vitro* cell culture has been extensively employed in advancing our knowledge on tumorigenesis and also as a platform to test different treatment modalities. This can be attributed to the better tumor biomimetic ability of these 3D models with respect to 2D [25].

On this note, majority of the available 3D models have been able to recapitulate tumor architecture that is analogous to biological tumors, where a proliferating rim is supported by a necrotic core due to the lack of oxygen and nutrient diffusion. In our results, we have demonstrated a similar tumor architecture (Figure 4.7), that was previously illustrated by Brahimi-Horn *et al* [85]. This technique of generating spheroids has been shown to be reproducible, and is advantageous to other methods such as microencapsulation (Figure 4.1). Through the formation of tight spheroids with intimate cell-cell interactions, ECM laydown may be similar to physiological conditions, which may be substantiated by the necrotic core. In addition, we attribute the formation of loose aggregates of 231-C3 and not tight spheroids to the metastatic ability of the MDA-MB-231 cells. These loose aggregates can be disintegrated by mechanical agitation and therefore, may not qualify as a good platform for drug testing unlike the tight robust spheroids formed by MCF7-C3 cells.

In this study, we observed the upregulation of SOX2 and OCT4A and MMP-9, key proteins in ECM degradation which further facilitate tumor metastasis [107]. The physiologically relevant architecture and hypoxic core in the spheroids lead to the modulation of genes associated with metastasis and tumorigenesis [48]. A similar expression pattern has been reported by Chen Lei *et al.*, using breast tumor cells

cultured on 3D collagen scaffolds, suggesting the enhanced tumor stem cell properties in 3D [107]. Together, this demonstrated that 3D cultures are more physiologically relevant as compared to their 2D counterparts in their morphology, multi-layered arrangement and gene expression, making it a superior platform to study *in vivo*-like responses to drug candidates.

Evaluation of cell fate on drug treatment is carried out using methods ranging from histology [77] to biochemical assays [67] which are inherently destructive and time-consuming. In addition, real-time dynamics of microenvironment-dependent effects of drug treatment cannot be obtained. Real-time assessments provide precise tracking of treatment response and provide biologically relevant understanding of tumor progression and/or mechanism of drug action. In this regard, FRET-based biosensors can provide high-resolution real-time monitoring of protein interactions in cells [224]. In addition, chemotherapeutic drugs are known to kill tumor cells by activating apoptotic signaling pathways [225]. Therefore, by utilizing the C3 biosensor, a rapid and sensitive evaluation of apoptosis can provide ample information on chemotherapy-induced cell death and apoptosis [75, 211, 212]. This study proved the feasibility of FRET in assessing longitudinal analysis of the drug-induced effects in a real-time and non-invasive manner, without any post-processing requirements in three dimensions.

Our results clearly demonstrated that spheroids were more resistant to drug treatment than monolayers, which correlated with similar observations with other drugs [190, 215]. This resistance can be due to the poor penetration within the spheroids, and/or mechanism of action of paclitaxel on proliferating cells rather than

quiescent cells [226]. Another factor would be heterogeneity in the spheroids which make some cells intrinsically drug resistant and fail to activate apoptotic machinery [225]. Further studies may be required to understand if combinational therapy may provide synergistic effects in eliminating this drug resistance.

4.4 Conclusion

This study was aimed at developing a novel platform for real-time and non-invasive imaging of three-dimensional spheroids using FRET imaging. Our results have demonstrated that FRET-based biosensor may be useful for obtaining *in situ* information of apoptotic effects induced by chemotherapeutics in a 3D biomimetic system. The results obtained by assays such as histology and gene expression together with cell imaging analysis showed that spheroids generated using the non-adhesive coating plus centrifugation method were more physiological than 2D cultures. Taken together, these results show the potential of these FRET sensor-based 3D spheroids serving as an *in vitro* tumor model for longitudinal monitoring of drug-induced apoptotic effects. More importantly, this 3D biomimetic spheroid model has been subsequently employed to screen compounds for their anti-cancer activity, thus validating the application of this FRET-based 3D system for drug screening.

Chapter 5: Application of FRET-based Breast Cancer Spheroids for Evaluation of Anti-cancer Activity of Novel Compounds

5.1 Introduction

Having developed a 3D model for non-invasive assessment of apoptotic effects in chapter 3, this chapter aims to utilize this model to evaluate anti-cancer properties of novel compounds. This thesis has established the necessity of new chemotherapeutic options to allow complete remission of cancer and to decrease the toxicity to normal cells. Most chemotherapeutics kill susceptible cancer cells by inducing apoptosis [222, 225, 227, 228]. However, many cancer cells are able to evade apoptosis and become resistant to the treatment and therefore, stimulation of apoptosis in these cells would be regarded as an effective treatment [229]. In addition, the discovery of anti-tumor activity of cisplatin in 1969 [230] invoked an interest in researchers to understand if other metal-complexes would possess similar anti-tumor properties.

Fischer and his students first introduced carbenes into organometallic and inorganic chemistry in 1964 [231]. Since then, NHCs have been extensively studied for their ease in preparation and their property to form stable bonds with metals than their phosphine ligand alternatives [232-234]. After Arduengo *et al.*, was able to isolate a free NHC in 1991 [235], many NHC-metal complexes have been widely synthesized and explored for biomedical uses.

Gold-complexes have been emerging as effective compounds for biological applications. For example, Auranofin, 2',3', 4',6'-tetra-*O*-acetyl- β -D-glucopyranosyl-1-thiolato Au(I) phosphine complex, has been used for treating rheumatoid arthritis patients for twenty years [236, 237]. This Au(I) phosphine complex has shown to disrupt the regulation of mitochondrial membrane permeabilization (mMMP), resulting in loss of membrane potential, mitochondrial

membrane permeability transition and swelling [238]. Following which many studies have presented mitochondrial damage caused by NHC-gold (I) complexes [239, 240]. For example, six of the seven dinuclear gold (I)-carbene complexes could induce Ca^{2+} -sensitive mMMP causing mitochondrial swelling. This was further confirmed when the mitochondrial swelling was reversed by the addition of cyclosporine A [241]. Another research by the same group has proven that lipophilicity of the complex can have an effect on its selectivity and cytotoxicity to cancer cells alone leaving the normal cells unaffected [242]. Importantly, these compounds were selective to mitochondrial selenoproteins like thioredoxin reductase (TrxR), paving way for mitochondria-dependent chemotherapeutics.

In the studies mentioned above, use of 2D cultures is popular in identifying and understanding the mechanism of action of the cytotoxic compound. Low cost and high speed qualities attribute to 2D cultures being the primary step in many drug development processes [243]. However, the restrictions of 2D culture have been increasingly recognized. As a result, many of these drug testing processes using only 2D cultures have often provided misleading and non-predictive data for animal models [50, 244, 245]. We hypothesize that this multi-layered arrangement of cells in this FRET-based 3D breast tumor model [76] can provide a more physiologically relevant analysis of NHC-gold complexes' cytotoxic effect than the 2D conventional monolayers.

Herein, the results from the anti-cancer studies of gold (I)-NHC complexes against FRET-based breast cancer cells cultured in 2D and 3D have been reported. These gold complexes have varying degrees of anti-cancer activity based on their

alkyl linker length and the halogen atom attached to the end of these chains. In addition, all these Au (I) complexes have a detrimental effect on mitochondria and its membrane potential. However, upon testing the efficacy of the complexes in 3D, the cytotoxic effect was less evident than their corresponding 2D culture effect.

5.2 Results

5.2.1 Using Breast Cancer Cells To Screen NHC Complexes for Anti-Cancer Effects

The gold (I)-NHC complexes (complexed dimers) and the monomers used in this study are shown in Figure 5.1. These complexed dimers are synthesized from imidazole precursors which are then alkylated to form imidazolium ions which are able to form Au-NHCs in the presence of gold using a base to eliminate the hydrogen at the carbon position between the two nitrogen atoms (compounds **4, 5**). The gold-NHCs with alkyl groups attached at the end are synthesized from their N-haloalkylimidazolium salts. However, the functionality of these novel compounds (**6-8**) are yet to be explored. The monomer compounds **1-3** are the imidazolium salts which are not complexed.

Figure 5.1: Structures of the compounds 1-8.

Cell shrinkage and detachment are predominantly the characteristics of an apoptotic cell and breast cancer cells (231-C3) treated with compounds **4-8** exhibit these traits under phase microscopy (Figure 5.2).

Figure 5.2: 231-C3 cell morphology during the course of treatment with compounds 1, 4-8 at 10 μM at 72 h. Scale bar 100 μm .

5.2.2 FRET-based Breast Cancer Cells Can Detect Compounds that Activate Caspase-3 and Trigger Apoptosis

In order to rapidly determine anti-cancer properties and eliminate compounds which do not target the apoptotic pathway, FRET imaging of 2D monolayer cultures was conducted. Under normal growth conditions, 231-C3 and MCF7-C3 cells emit green fluorescent light because of efficient energy transfer from CFP to YFP. On treatment with the eight compounds, it can be observed that over a period of time the compounds **6-8** (Figure 5.3 and 5.4) and compounds **4** and **5** (data not shown) were able to trigger apoptosis in breast cancer cells, as visualized by the change in fluorescence of 231-C3 and MCF7-C3 cells from green to blue. A closer look at the FRET images indicate that compound **8** had the strongest effect in killing 231-C3 cancer cells and compounds **7** and **8** had the strongest effect in MCF7-C3 cancer cells.

Figure 5.3: Time-series dynamic FRET changes in 231-C3 cells after treatment with compounds 1, 6-8 at 10 μM . The YFP and CFP images were separately taken before merging them to obtain the FRET image as shown.

Figure 5.4: Time-series dynamic FRET changes in MCF7-C3 cells after treatment with compounds 1, 6-8 at 5 μM . The YFP and CFP images were separately taken before merging them to obtain the FRET image as shown.

5.2.3 Evaluation of Cell Viability of Apoptosis-inducing Compounds

Having established from FRET imaging that gold - complexed compounds have the ability to cause apoptosis in cancer cells, we went on to understand their structural and functional correlation. Their cellular viability was evaluated at varying concentrations from 5 – 0.08 μ M using MTT assay and has been summarized in Table 5.1. The results indicate that the compounds **8**, **7** and **4** have the strongest inhibitory effect on 231-C3 cells than the remaining compounds, causing > 50 % cell death. From Figure 5.5A, it is evident that Au - complexed compounds had an apoptotic effect on cancer cells against their monomers (**2** and **3**), which had negligible effect (cell viability > 80 %). It is interesting to note from Figure 5.5 B that amongst the Au-NHCs, the longer the alkyl chains, the higher the inhibition effect. For example, compound **4** treatment, which has a longer alkyl chain, resulted in 42.02 ± 7.02 % cell viability, which is lower than compound **5** treatment (63.37 ± 3.28 %). In addition, the halogen (chlorine) attached to the ends of one of the alkyl chains (compounds **7** and **8**) had a greater inhibitory effect on 231-C3 cells than compound **4** with similar structure but no halogen attached (Figure 5.5C and D). Although the monomers were significantly different from the control (no compound treatment), the Au-complexed dimers were able to elicit a significantly much stronger anti-cancer effect.

Figure 5.5: (A) Dose-dependent cytotoxic effect of compound 6 compared with monomer compound 2 and 3 after 72 h treatment. *** $p < 0.001$ compared with compound 2 and 3. (B) Dose-dependent reductive effect of compounds 4 and 5 compared against compound 3 at 72 h. *** $p < 0.001$ compared with compound 3. (C and D) Dose-reductive effect of compounds 4, 5, 7 and 8 could be observed at 72 h and was compared against monomer compound 1 with not pronounced anti-cancer effect. *** $p < 0.001$ compared with compound 1 (one-way ANOVA, Bonferroni post hoc test).

Table 5.1: Apoptosis-inducing effects of tested compounds at 5 μM after 72 h of treatment

Relative apoptotic effects of compounds	Compounds	Cell viability (%)
++	8	21.99 ± 1.9
	7	27.62 ± 3.4
	4	42.02 ± 7.0
+	6	50.16 ± 1.9
	5	63.37 ± 3.3
—	1	89.55 ± 3.4
	2	83.15 ± 0.5
	3	80.57 ± 0.5

'++' suggests significant apoptosis with cell viability < 50 %, '+' suggests moderate apoptosis with cell viability between 50 – 80 %, and '-' suggests low percentage of apoptosis (cell viability ≥ 80 %) for 231-C3 cells at 5 μM concentrations treated for 72 h.

5.2.4 Mitochondrial Function of 231-C3 Cells Were Severely Affected By NHC Complexes

To study whether the compounds have any effect on mitochondrial morphology, the mitochondria of the 231-C3 cells incubated with the compounds were stained with 0.1 μ M Mitotracker CMXRos. It was observed that after treatment with compounds **4-8**, the morphology of the mitochondria drastically changed from being filamentous structures as seen in the control to swollen aggregates suggesting mitochondrial damage. The mitochondria of the cells treated with compounds **1-3** resembled similar structures as the control owing to their lack of anti-cancer activity. In addition, as nucleus damage is a marker for late apoptosis, changes in the nucleus morphology was not observed using the Hoechst 33342 staining at 30 h for all conditions treated (Figure 5.6).

Figure 5.6: Compounds 4-8 induces mitochondrial damage in 231-C3 cells at 10 μM . The cells were visualized at 30 h by incubating the cells with 0.1 μM fluorescent dye MitoTracker Red and recording the images using a confocal microscope.

To test whether these compounds could depolarize $\Delta\Psi_m$, we examined the effects of all the compounds at 10 μM at 72 h. As shown in the Figure 5.7, significant reduction in $\Delta\Psi_m$ can be observed in 231-C3 cells after treatment with compounds **4-8**. Compounds **4** and **8** was able to cause $16.67\pm3.34\%$ and $16.68\pm2.41\%$ reduction in the membrane potential respectively as compared against the control. Compounds **1-3** had no effect on $\Delta\Psi_m$, thus providing convincing evidence that these monomers do not have potential to trigger apoptosis via mitochondrial damage in breast cancer cells.

Figure 5.7: Effect of compounds 1-8 on the amount of tetramethylrhodamine ethyl ester (TMRE) sequestered within the mitochondria as a measure of mitochondrial membrane integrity, after 30 h incubations. Data is normalized to control (fluorescence of non-treated cells set to 100%).

5.2.5 Response of MCF7-C3 Cells in a 3D Biomimetic System

To test the effect of these compounds in a more physiological setup, we employed our previously established 3D FRET-based biomimetic model of breast cancer cells (Chapter 4) to visualize the cytotoxic effects. As the 231-C3 cells were unable to form compact rigid spheroids as observed earlier, MCF7-C3 3D model was employed for future studies. It can be observed that from our day 3 FRET image analysis, that the monomer compound **1** was ineffective in causing any cytotoxic effect while the Au-complexed compounds **6-8** resulted in reduction in spheroid size relative to the control untreated spheroid and appearance of blue apoptotic cells (Figure 5.8). Also, green fluorescence from live cells in the spheroids, which are unaffected by the compound treatment can also be viewed in Figure 5.8. Although the compounds **7** and **8** killed >80 % of cells in 2D, in 3D, live cells emitting green fluorescence was still visible suggesting that the 3D architecture enables resistance to the cells interior of the spheroid.

Figure 5.8: Apoptotic effects of MCF7-C3 cells cultured in a 2D and as a 3D spheroid when treated with compounds 1, 6-8 at 5 μ M after 72 h. Scale bar 100 μ m.

To further substantiate this result, image-based analysis of the fluorescent intensities for all the compounds was conducted. As expected, the monomer compounds **1-3** didn't have any significant reductive effect on the MCF7-C3 spheroids. However, the Au-complexed compounds elicited a cytotoxic activity on these spheroids (Figure 5.9), but was not equivalent to their 2D activity. From our FRET-image analysis, it can be seen that complexes **4, 7** and **8** had a larger apoptotic effect than the remaining compounds. In addition, the FRET emission ratio for compounds **4, 7** and **8** was 0.69, 0.71 and 0.74 respectively compared against the control spheroids. For complexes **5** and **6**, the FRET emission ratio was found to be ~0.8 and ~0.83 respectively compared against control spheroids. This trend is consistent with our 2D studies where compounds **4, 7** and **8** were able to exert higher cytotoxicity effect than other compounds.

Figure 5.9: Relative Y/C emission ratio for all compounds at 5 μM at 72 h. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with control. Grey bars indicate the monomer compounds and the patterned bars indicate the Au-NHCs.

The advantage of using FRET-based 3D cultures is that time-series analysis of the compounds' anti-cancer effects on 3D spheroid integrity would be feasible. From Figure 4.9, it can be seen that within 24 h, appearance of some blue cells can be visible for spheroids treated with compounds **7** and **8**. In addition, over the duration of 72 h of treatment, spheroid size decreases and larger proportion of cells undergo apoptosis as visible from FRET imaging in comparison to the other compounds. However, beneath the layer of apoptotic cells at the rim, cells which fluorescence green in color indicating their viable status can also be observed for all the spheroids treated with complexes. Furthermore, a time-dependent effect of the compounds **6-8** is observed in Figure 5.10, wherein the compound **1** doesn't exhibit any inhibition

to spheroidal growth. It can also be seen from the FRET images and emission ratio quantification, that compound **6** doesn't significantly affect spheroid integrity. This suggest that this compound may not be as effective as compounds **7** and **8** in an *in vivo* setup.

Additionally, the longitudinal analysis for compounds **1**, **6**, **7** and **8** in terms of emission ratio was calculated and plotted in Figure 5.11, by determining the fluorescent intensities of YFP and CFP signals individually. The treatment with compound **8** has resulted in a decrease of FRET emission ratio by 26% when evaluated against the monomer compound **1** at 72 h. The FRET emission ratios for compounds **7** and **8** are similar, while for compound **6** the ratio is much higher in comparison to compounds **7** and **8**. Taken together, these results clearly indicate that FRET-based 3D models provide *in situ* and longitudinal analysis of effects of compounds in a physiologically relevant environment. Thereby, these models help assist in validating efficacy of compounds and also eliminate compounds that fail to evoke a satisfactory response in a complex setup.

Figure 5.10: Longitudinal analysis of apoptotic effects of compounds 1, 6-8 at 5 μ M in a spheroidal breast cancer model.

Figure 5.11: Relative Y/C emission ratio for compounds 1, 6, 7 and 8 over 72 h.

5.3 Discussion

Gold compounds have been extensively studied and have been identified to possess medicinal properties [246, 247]. Thus, a variety of gold (I) and gold (III) compounds stabilized either by phosphine or nitrogen atoms were prepared and were shown to possess anti-cancer activity *in vitro* and *in vivo* [238, 239]. These compounds were able to induce cellular apoptosis in cancer cells, thus paving way for new chemotherapeutics [248]. Many of these *in vitro* cytotoxicity analysis have been carried out by conventional cell viability assays, caspase activity assays etc., and require multiple samples and are invasive techniques. To overcome these limitations, a FRET-based sensor developed by Luo K.Q., *et al.*, has been used in this study as a powerful tool to identify compounds that cause apoptosis in live cells [73, 75]. With the use of FRET-based technology, we have been able to non-invasively identify the complexes capable of triggering apoptosis (by the appearance of blue cells) in our breast cancer cells (Figure 5.3 and 5.4). Consistent with literature, in this study, Au-complexes have significantly inhibited the proliferation of breast cancer cells in a dose-dependent manner (Figure 5.5).

Several evidences have suggested a profound difference in the mode of action of these gold complexes apart from DNA binding activity (exhibited by successful metal-complex anti-cancer compound – cisplatin) which could be responsible for their cytotoxic effect on the cancer cells [249, 250]. Mitochondria are responsible for 80 % of the energy required to carry out cellular activities and also play a vital role in cell death pathways. Therefore, to gain insight into the mechanism of action of these compounds, mitochondrial-mediated apoptosis was assessed. The loss of

mMMP was evident from our results showing mitochondria damage (Figure 5.6) and decrease in mitochondrial membrane potential (Figure 5.7), which correlated with the mode of action of various other NHC complexes synthesized [241, 242].

From our studies, the inactivity of compounds **1-3** further ascertain the importance of the gold center for biological activity irrespective of the alkyl chain attached. Additionally, these gold-NHCs are novel interesting novel compounds as they provide us the feasibility to vary ligand substituents, thereby fine tuning them to be selective to tumor cells. In this regard, this study has looked at novel complexes synthesized from *N*-haloalkylimidazolium salts for their anti-mitochondrial activity in cancer cells. In the study conducted in 2011, it was observed that the substituents at the aromatic rings of the NHC complexes played a significant role in determining their cytotoxic activity [251]. For example, the F-substituents at the ortho-position resulted in an increase in inhibitory activity against MCF-7 cells unlike the methoxy substituents which did not show any increased activity. Similarly, compounds **7** and **8** which possessed a longer alkyl chain with a pendant chlorine attached seemed to increase the inhibitory activity against breast cancer cells as opposed to complexes **4** and **5** with no halogen attached (Figure 5.5). This substantiates that the insertion of a chlorine at the end of an alkyl chain would influence its anti-cancer properties. As for future work, it would be interesting to study if having other halogen groups attached to the end of alkyl chain would also have any impact on the anti-cancer property.

Currently, three-dimensional models are increasingly employed to study different treatment modalities as they are able to simulate *in vivo* tumors more closely than

conventional 2D systems [25]. Therefore, we have used our established FRET-based 3D model to evaluate these compounds and determine their response in a more physiological setup [76]. As expected, compounds **1–3** did not affect the spheroids as evident from FRET images and emission ratio quantification (Figure 4.8). Although compounds **4–8** caused inhibition of spheroidal growth, it was clearly visible that viable cells (green cells) were trapped within the rim of apoptotic cells (blue cells; Figure 5.10). In addition, compounds **5** and **6** were unable to elicit a response equivalent to compounds **7** and **8** suggesting their cytotoxic activity to be poor (Figure 5.7). This resistance to treatment (live cells) was visible in this 3D set up rather than on 2D systems suggesting that a 3D *in vitro* model would be helpful in assessing the efficacy and screening out compounds true to its effect in a real tumor.

5.4 Conclusion

In conclusion, this findings obtained in this chapter suggest two outcomes: 1) the potential of using NHC complexes as anti-mitochondrial agents, and 2) application of a FRET-based 3D model to screen compounds based on their cytotoxic ability. The mode of action of these compounds was to cause mitochondrial swelling and reduce mitochondrial membrane potential, thereby eventually resulting in apoptosis (as visualized by appearance of blue cells). Although the 3D model supported the visualization of compound-induced apoptosis, it further provided us valuable insights on the compound response in a multi-cellular layered setup. Therefore, the use of this FRET-based 3D *in vitro* model is recommended for its potential to identify

lead compounds that cause apoptosis and evaluate its cytotoxic response (via FRET emission ratio quantification). To add more physiological relevance to this current model and obtain accurate drug responses, fibroblasts have been incorporated in our established 3D MCF7-C3 spheroids and these 3D co-culture spheroids have been characterized for their tumorigenic properties in the following Chapter 5.

Chapter 6: FRET-based 3D Co-culture Model as a Physiologically Relevant Tool for Anti-cancer Drug Screening

6.1 Introduction

Having validated the use of FRET-based spheroids for screening novel compounds in chapter 4, we went on to develop a more physiologically relevant model by incorporating fibroblasts to the breast cancer spheroid. Fibroblasts, immune cells, adipose cells, vasculature, growth factors and ECM constitute the stromal microenvironment, which is known to play a significant role in regulating tumorigenesis [252-254]. This regulatory role has been well documented with clinical evidence [255, 256] and cancer mouse models [257]. For example, it has been reported that radiation treatment resulted in changes in the tumor stromal microenvironment which in turn promoted tumor progression *in vivo* [258]. As a component of this stromal environment, fibroblasts are associated with all stages of cancer including initiation, progression and invasion (metastasis) [259-261].

Fibroblasts form a large part of tumor stroma and are shown to promote tumorigenesis of initially non-tumorigenic prostate cells [262] and also cells of breast and colon tissue in animal models [263]. They secrete cytokines and growth factors, produce excessive ECM and also support the recruitment of endothelial cells for angiogenesis [89, 95]. In recent times, Vermeulen, L., *et al.* demonstrated that the tumor microenvironment by their interaction via β - catenin pathway with the colon cancer cells was responsible for increasing stemness in colon cancer [264]. In addition, this heterologous cross-talk of cancer cells with fibroblasts confers resistance to the cancer cells, making them less susceptible to a variety of available treatment methods [203]. Therefore, understanding the interactions between tumor

stroma and cancer cells will help to discover new agents and evaluate their efficacy as potential chemotherapeutics.

Despite the fact that stromal elements contribute to tumor progression and they co-exist with tumor cells in 3D, majority of these studies were conducted in 2D monolayers. Although culturing cancer cells in 2D provided initial insights on basic cell biology and tumorigenesis, it couldn't encompass physiological parameters that affect tumor progression like cell-cell and cell-ECM interactions, biochemical and mechanical cues and tissue architecture. Therefore, studies have shown that cells grown in 3D are able to recapitulate *in vivo* tumors more closely than 2D monolayers [55, 265]. Additionally, conventional assays are used to test the efficacy of chemotherapy and radiation therapy in these spheroids.

Thus, in this study, we combined 3D spheroidal cultures (as discussed in chapter 3) and co-cultures into one experimental setup. The use of FRET-based cancer cells and non-fluorescent fibroblasts provided a useful approach to test tumor specific responses to drugs. In addition, using histological and gene expression analyses, we were to show that 3D co-cultures are much better models than the 3D monocultures.

6.2 Results

6.2.1 Generation of 3D Co-culture Model with MCF7-C3 and IMR90 Cells

Breast cancer MCF7-C3 or 231-C3 and fibroblasts IMR90 cells at different ratios of 1:1, 1:2, 1:5 and 2:1 were seeded to generate a 3D co-culture spheroids. Of all the different starting seeding densities tested, a linear correlation was seen between

initial seeding density and the diameter of the spheroid for all ratios (Figure 6.1). In order to observe the distribution of the cells in the spheroid, the spheroid seeding density was maintained at 2000 cells (Figure 6.2). MCF7-C3 cells formed tighter spheroids when they were co-cultured with IMR90 fibroblasts, whereas 231-C3 cells formed much looser spheroids with irregular shapes at all four ratios tested (Figure 6.2). Thus, we used MCF7-C3 and IMR90 cells to conduct the rest of 3D study in this Chapter.

Figure 6.1: Linear dependence of initial seeding density and mean diameter of 3D co-culture spheroid of MCF7-C3 and IMR90 cells at all ratios. Linear regression analysis was conducted and the R^2 value for 1:1, 1:2 and 2:1 was 0.99, while for 1:5 it was 0.88.

6.2.2 Longitudinal Analysis and Confocal Imaging of 3D Co-culture Spheroids

As FRET-based sensor enables real-time and non-invasive readouts of cell condition, we were able to monitor the viability of the cells in the co-culture spheroids of MCF7-C3 and IMR90 longitudinally. FRET images showed that almost all the visible MCF7-C3 cells appeared in green color during the course of 5 days incubation under all the various ratios (Figure 6.3). There is a slight decrease in spheroid diameter from day 3 to day 5, which may be attributed to the compaction of the spheroids owing to the necrotic core formation resulting in a mass of dead cells at the core. This result indicates that at least for the MCF7-C3 cells located at the outer layer of the spheroid, they can live in this co-culture environment with IMR90 fibroblasts.

Figure 6.2: FRET imaging of 3D co-culture spheroids of breast cancer MCF7-C3 or 231-C3 cells with fibroblasts IMR90 seeded together in different ratios and observed after 24 h for MCF7-C3:IMR90 co-culture and after 48 h for 231-C3:IMR90 co-culture. Scale bar 100 μ m.

In order to visualize the fibroblasts cells in these co-culture spheroids, Hoechst 33342 staining was conducted. A total of 8 confocal z-stacks are shown in Figure 6.4, where the blue-colored nuclei represent the fibroblasts; and the green-colored cytoplasm, plus blue-colored nuclei indicate breast cancer cells. The enlarged image in Figure 6.5 clearly illustrates that physical interactions between both cell types are established in the 3D co-culture spheroid. As penetration depth of the confocal microscopy is limited, after a certain depth no signal could be obtained from the center of the spheroid. All the stacks have been aligned and shown in Figure 6.5. Based on our preliminary study and other published reports, 1:1 ratio between the two cell types was used for evaluation of gene expression and histological studies [57, 190].

Figure 6.3: Time-series FRET imaging of MCF7-C3 and IMR90 3D co-culture spheroid for ratios 1:1, 1:2, 1:5 and 2:1. Scale bar 100 μ m.

Figure 6.4: Live cell z-stack imaging of 3D MCF7-C3 and IMR90 co-culture spheroid (1:1) grown for 7 days after staining the nucleus with Hoechst 33342 dye. The non-fluorescent fibroblasts have their nuclei stained blue and the breast cancer cells appear green (because of the FRET sensor) with nuclei stained blue. Scale bar 50 μ m.

Figure 6.5: Representative confocal z-stack images of MCF7-C3 and IMR90 spheroids seeded at 1:1 ratio and grown for 7 days. Scale bar 50 μ m.

6.2.2 Tumorigenic Markers were Up-regulated in 3D Co-cultures

ECM-degrading enzymes, MMPs, and stem cell-like markers such as SOX2 and OCT4A are often enriched in the cancer cells [107]. In this study, we used real-time qPCR analysis to compare the expressions of stem cell-like markers between mono

and co-culture in 3D format. The results showed that the mRNA levels of SOX2 and OCT4A were 6.4 ± 0.78 and 2.3 ± 0.66 times higher in the 3D co-culture than the 3D mono-culture of MCF7-C3 cells (Figure 6.6). Similar data was observed for the MMP-9 expression (2.4 ± 1.6). These results suggests that 3D co-cultures can better represent the tumor characteristics under *in vivo* conditions than the 3D mono-culture model.

Figure 6.6: Relative mRNA levels of tumorigenic markers of hMMP9, SOX2 and OCT4A compared between 3D mono-culture and 3D co-culture spheroids. GAPDH was employed as internal control.

6.2.3 Presence of Necrotic Core and Expression of Fibronectin and N-cadherin in 3D Spheroids

In the center of a solid tumor with a diameter larger than $\sim 200 \mu\text{m}$, due to the lack of nutrients and oxygen, cancer cells often die via necrosis resulting in the formation of necrotic core [266]. To evaluate the presence of such necrotic core in our 3D

spheroids, hematoxylin and eosin (H&E) staining was employed. The H&E staining results showed that cells in the center of the 3D co-culture spheroid stained with pink-colored eosin, but not purple colored hematoxylin indicating these cells are dead. As the size of the 3D mono-culture spheroid is smaller, a large necrotic core isn't visible (Figure 6.7). This result indicates that co-culture model is able to recreate tumor architecture under *in vitro* situation.

Figure 6.7: Haematoxylin and eosin staining of 3D spheroids of (A) MCF7-C3 and (B) MCF7-C3 and IMR90 cells cultured for 7 days.

ECM secretion and its accumulation are important for breast tumor progression. Major ECM components include fibronectin, tenascin, collagen and laminin [267]. In our study, we have observed higher secretion of fibronectin in our 3D co-culture model as compared to 3D mono-culture (Figure 6.8). The *in vivo* xenograft sample also expressed fibronectin in the regions devoid of cells as seen in Figure 6.8, which was recreated in the 3D co-culture.

Figure 6.8: Histological cross-sections of 3D spheroid models of (A) MCF7-C3, (B) co-culture of MCF7-C3 and IMR90 cells at day 7, and (C) *in vivo* MCF-7 tumor were immunostained for fibronectin. Scale bar 50 μ m.

Similarly, higher expression of N-cadherin has shown to promote migration, invasion and metastasis in breast cancer [268, 269]. Therefore, our 3D co-culture spheroid model was assessed for N-cadherin expression. It was observed from immunohistochemical analysis that 3D co-culture model showed the presence of N-cadherin expression similar to the *in vivo* xenograft sample (Figure 6.9). Although little expression can be detected in the 3D mono-culture spheroid, it is mild. A more quantifiable assay may help us to understand the expression of N-cadherin in our 3D cultures. Together, these results confirm that the 3D co-culture model is able to resemble key properties of *in vivo* tumors by expression of ECM and metastatic markers with the addition of stromal cells.

Figure 6.9: Histological cross-sections of 3D spheroid of (A) MCF7-C3, (B) co-culture of MCF7-C3 and IMR90 cells at day 7, and (C) *in vivo* MCF-7 tumor were immunostained for N-cadherin. Scale bar 50 μ m.

6.3 Discussion

The lack of availability of experimental cancer models that can predict the outcome of investigational drugs similar to *in vivo* tumors is considered one of the many barriers in drug development process. While animal studies have provided some insights on drug response, these models are time-consuming and expensive [32]. Therefore, there is a need to develop *in vitro* models that can better represent tumor tissues, while providing responses closer to the real situation in shorter time. Many three-dimensional models have been developed over the past decade to study cellular behaviors like cell-cell and cell-ECM interactions and cellular responses [216, 265, 270-272]. In addition, the stromal environment surrounding the tumor cells are considered vital in promoting tumor progression by sustaining cell-cell interactions, secretion of ECM molecules, growth factors and cytokines [253, 260, 273]. Thus, it is important to incorporate stromal components in these 3D breast tumor models.

Fibroblasts, form a large proportion of breast tumor microenvironment, and are responsible for providing multiple growth factors, proteoglycans and enzymes [89,

95]. As they are the primary source for collagenous ECM, they contribute to the decreased uptake of drugs in tumor cells, thereby playing a significant role in tumor sensitivity to the drugs [274]. Therefore, it becomes essential to include fibroblasts in 3D *in vitro* models to test drugs and evaluate their responses. In this study, we have used the non-adhesive coated technique to generate 3D spheroids by directly co-culturing breast cancer cells and fibroblasts. In addition, this simple technique is able to generate robust models of 3D *in vitro* breast tumors containing the important stromal cells (Figure 6.1 and 6.2).

The method of forming tumor spheroids is simple and does not require the addition of expensive ECM matrices such as Matrigel™ rather it can produce its own ECM from the fibroblast over time (Figure 6.8). The use of Matrigel™ and other scaffolds take a longer time to recapitulate *in vivo* tumor phenotypically [275, 276]. Another study by Szot, C.S., *et al.*, has shown the development of large co-culture breast cancer models using collagen I hydrogels [277]. Although, the use of external ECM-mimicking scaffolds is feasible, they may not always accurately represent the tumor environment. For example, the use of Matrigel™, an animal-derived ECM may vary in the proportion of endogenous factors in each batch. They might influence drug penetration and efficiency studies.

Many clinical observations have suggested that the overexpression of SOX2 and OCT4A at the transcription levels indicates poor prognosis for cancer patients [278-280]. As anticipated, the stemness of the 3D co-culture spheroids was elevated in comparison to 3D mono-culture spheroids as indicated by their gene expression analysis. Hazan, R.B., *et al.*, had observed that the expression of N-cadherin is

correlated with the upregulation of MMP9 [268]. Additionally, it has been reported that tumors with high fibronectin expression were associated with poor survival rates, higher chances of metastasis and also higher levels of MMP expression [281, 282]. A similar observation was seen in our study, wherein the expression of N-cadherin in the 3D co-culture was correlated with that of MMP9. Together with the expression of ECM marker fibronectin, this 3D co-culture model has demonstrated its potential as a promising platform to study *in vivo*-like drug responses.

In addition, the breast cancer cells are transfected with a FRET sensor that enables real-time and non-invasive readouts of cell viability. In this study, the FRET sensor was able to provide information about the distribution of the breast cancer cells in a 3D co-culture spheroid and ascertained that the physical contact between both cell types were established and maintained over five days. Having previously established that FRET-based biosensor provides *in situ* information of drug-induced effects in a 3D biomimetic model [76], we believe that in this 3D co-culture model, tumor specific responses can be obtained upon treatment with the testing agents.

6.4 Conclusion

We have employed a direct co-culture technique to develop 3D co-culture spheroids in 1-7 days. Mature 3D co-culture spheroids grown for 7 days have exhibited ECM and tumorigenic properties better than 3D mono-culture spheroids suggesting that they may be able to mimic the tumor tissues containing the tumor microenvironment. In addition, they have also shown the presence of a central necrotic region which

resembles the tumor architecture *in vivo*. This study provides a platform for development of improved biomimetic *in vitro* model for cancer research and drug screening and testing.

Chapter 7: Concluding Remarks

7.1 Conclusion

This thesis aims to develop a novel FRET-based 3D *in vitro* model to bridge the gap between 2D monolayers and animal models and utilize it as a platform to screen and test efficacy of investigational drugs. The application of FRET biosensor in our 3D model enables it to be more advantageous (non-invasive and real-time) over current techniques to evaluate drug response. The inherent properties of this model – multilayered arrangement, diffusion limitation and gene expression; augments its suitability as a platform for *in vitro* drug testing. **Chapter 2** has reviewed the importance of 3D spheroids and previously established spheroid models to study tumorigenesis and treatment efficacy, while also elaborating on the co-culture 3D models and use of NHC complexes for biomedical applications. The FRET-based 3D breast cancer spheroid developed in **Chapter 4**, has proved to be a suitable model for longitudinal analysis of drug-induced apoptotic effects non-invasively, thus addressing the **Specific Aim 1** of this thesis. **Specific Aim 2** has been addressed in **Chapter 5**, by demonstrating the application of FRET-based breast cancer spheroid to screen complexes for their efficacy in a physiologically relevant platform. Moving forward to further enhance the applicability of the model closer to *in vivo* tumor as hypothesized in **Specific Aim 3**, stromal cells were incorporated to form a 3D co-culture model and has been characterized in **Chapter 6**.

In conclusion, this thesis has proved the applicability of FRET-based biosensors in 3D imaging. The development of a 3D spheroid model has shown to better mimic *in vivo* tumor architecture better than 2D monolayers. More importantly, the use of FRET-based cancer cells has enabled real-time and longitudinal monitoring of drug-

induced apoptotic effects. With regards to its application, this 3D spheroid platform has demonstrated its potential in identifying complexes that can stimulate apoptosis in cancer cells and also screen multiple compounds rapidly. This thesis has also explored the involvement of stromal elements in tumorigenesis, in terms of expression of stem cell markers, ECM and tumorigenic markers. Together, these FRET-based spheroid models would be useful to hasten the process of drug screening to be able to deliver drugs to the clinics.

7.2 Future Outlook

This thesis has provided a novel 3D *in vitro* platform that bridges the gap between 2D monolayers and animal models and has shown the applicability of employing FRET-based biosensors to screen and test drug compounds. In addition to these results, more work can be done to improve the properties of this platform and diversify its applications.

7.2.1 Influence of Fibroblasts on Drug Response in 3D Co-Cultures

It can be observed that stromal elements interact with the tumor cells and aid in tumor progression and increase resistance to chemotherapy. Despite the extensive studies conducted in 2D [89, 260, 283], the readouts may not mirror the drug responses of clinical tumors. In addition, the signaling mechanisms are deregulated when cells are cultured in monolayers [25, 284]. Fibroblasts are found in abundance in breast tissue, which can be transformed to myofibroblasts/cancer-associated fibroblasts, which can

then advance tumorigenesis by secreting growth factors, chemokines and ECM remodelling enzymes (like MMP-9, as shown in our 3D co-culture model). Therefore, we hypothesize that the fibroblasts would increase resistance to drug treatment and provide apoptotic responses milder than 3D mono-cultures.

7.2.2 Chemotherapeutic Drug Penetration Studies

In order to classify drugs as most effective, they should be able to penetrate the tumor tissue, accumulate mainly in the tissue at a concentration sufficient to exhibit its therapeutic effect. However, it is well known that this displacement of the drug in the tumor tissue is incomplete [285, 286]. As 2D cultures form monolayers, all the cells are exposed to the drug making it an ineffective model to determine diffusion limits. Therefore, studying drug penetration in *in vitro* spheroid models can help us predict the diffusion of the drug in this multi-arrangement of cells and determine its efficacy. For example, Lu, M., *et al.*, has observed that cells grown in 3D cultures altered the penetration dynamics of the chemotherapeutic drug [287]. Our FRET-based 3D model can help us determine the efficacy of the treatment by evaluating the penetration kinetics and also the cell fate of this penetration non-invasively. This can be further diversified to determine the penetration kinetics for drug delivery systems as well as done previously by other groups [105, 288, 289].

7.2.3 Introduction of Dynamic Environments to Assess Chemo-sensitivity of Spheroids

As reviewed earlier, it is evident that tumor microenvironment is heterogeneous and dynamic in nature [290, 291]. Several biological processes including proliferation, invasion, angiogenesis, metastasis, shear stresses etc. contribute to this dynamic microenvironment [292]. Micro-technologies have been able to precisely control multiple factors like flow, shear stresses; requiring smaller volumes and have been used to recreate tumor microenvironments and or tumor processes like intravasation, extravasation etc [293-296].

It is well-documented that tumor fluid environment has an impact on drug delivery and response. The fluid environment is characterized by uneven cytokine gradients and irregular flow shear stresses [297]. Innovative microfluidic devices have been used in the past to study the influence of hydrodynamic stresses on therapy response [298]. For example, research by Yu, J.Q., *et al.*, has shown that detection of apoptosis was possible using the FRET-based sensor for endothelial cells in a microfluidic chip which was able to mimic the pulsatile flow in blood vessels [299]. Therefore, we hypothesize that the incorporation of hemodynamic shear stress can enable us to improve the existing FRET-based 3D model to provide better *in vivo*-like response.

7.2.4 High-throughput Screening (HTS) Test Systems

The current strategy is to develop HTS-amenable test systems with better pathophysiological mimicry of solid tumors to optimize selection of most-active

molecules from a large pool of lead compounds against tumors [108]. Indeed, it has been demonstrated in this thesis and from other studies, that 3D cultures reflect *in vivo* conditions behavior better than 2D monolayers for most cell types [76, 215, 300-302]. However, these models are yet to be fully incorporated in the drug development operations. This can be attributed to the lack of rapid and standardized analytical tools to evaluate the efficacy.

Fortunately, it is to be noted that the several screening methods used for 2D monolayer drug screening in a multi-well plate format can be adapted for 3D spheroids as well, such as cytotoxicity, proliferation, apoptosis etc. Commercially automated microscopic systems can be configured to enable rapid detection of drug effects on spheroidal growth (shrinkage of spheroids). A recent technique for toxicological testing with spheroid cultures called the bio-hybrid sensor system, detects impedance measurements on the spheroid surface [303-305]. Although the above mentioned HTS methods may help in the initial screening test, the recorded response would be an average of the cells in the spheroid.

Multiple technologies have been developed over the years to optimize automated 3D culture readouts. However, many of them have not met industrial standards. For example, an emerging technology is the light-sheet based fluorescence microscopy (LSFM), which selectively illuminates the focal plane with a light-sheet attained by a cylindrical lens [306]. This method is able to process large sample sizes with little preparation, rapid recording speed with high resolution and multi-fluorescence imaging. Therefore, we hypothesize that the integration of LSFM with our existing platform would help us record fluorescent signals rapidly for each focal plane and

for a large size to produce an image in 3D. In addition, combination of this documented data coupled with high end computational algorithms would enable faster analysis of drug effects on spheroids.

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