

DEVELOPING ORGANOIDS FOR PRE-CLINICAL DRUG TESTING

Submitted in partial fulfillment of the requirements for the award of
Bachelor of Technology degree in Biomedical Engineering

By

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SCHOOL OF BIO AND CHEMICAL
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SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

Accredited with Grade "A++" by NAAC

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Ms. Fazila A (40240009)** and **Ms. Najeeba M (40240021)** who have done the project work as a team and carried out the project entitled "**Developing Organoids for Pre-Clinical Drug Testing**" under our supervision from July 2023 to April 2024

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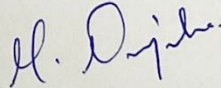
DECLARATION

We, **Ms. Fazila A (40240009)** and **Ms. Najeeba M (40240021)** hereby declare that the Project Report entitled "**Developing Organoids for Pre-Clinical Drug Testing**" was done by us under the guidance of **Dr. T. Sudhakar** (Internal Guide) and **Dr. S. Johnson Retnaraj Samuel** (External Guide) is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in Biomedical Engineering.

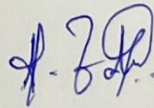
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Organoids are 3D in-vitro models of organs. Developing a 3D organoid is a complex process and the costs associated with entire steps are

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We would like to express our sincere and deep sense of gratitude to our Project Guide **Dr. T. Sudhakar**, Head of the department, Dept. of Biomedical Engineering, and our External Guide **Dr. S. Johnson Retnaraj Samuel**, Associate Professor – Research, Centre for Molecular & Nanomedical Sciences, for their valuable guidance, suggestions, and constant encouragement paved the way for the successful completion of my phase-2 project work.

We wish to express our thanks to all Teaching and Non-teaching staff members of the Department of Biomedical Engineering who were helpful in many ways for the completion of the project.

We would like to express our thanks to our parents for their unwavering support to us during this year.

DEVELOPING ORGANOIDs FOR PRE-CLINICAL DRUG TESTING

ABSTRACT

Organoids are 3D in-vitro models for biomedical research. Developing a 3D organoid is a complex process and the costs associated with entire steps are expensive. Specialised instruments are required for culturing organoids/ Cell aggregates. Here in this study, we have developed a novel cost effective method for developing 3D organoid/ cell aggregate with C2C12 cells and HT-29 cell lines. Integrity and live death nature of integral cells was assessed by AO-EB staining method. Morphology of the cell aggregate was observed and documented using microscopes. Internal cell aggregate architecture was assessed by Cryomicrotome. Immunofluorescence technique was used to assess the proliferation of cells with Actin, Histone H3 and PCNA antibodies. Based on the results we have successfully developed organoid/ 3D cell aggregate, cost effectively and standardised the parameters like cell numbers, volume of medium, rpm and time. Further experiments are necessary to confirm this finding.

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LIST OF ABBREVIATIONS

ABBREVIATION	EXPANSION
3D	Three dimensional
CF	Cystic Fibrosis
μl	Microliter
ml	Millilitre
RPM	Rotation per minute
DMEM	Dulbecco's Modified Eagle Medium
GFP	Green fluorescent protein
RFP	Red fluorescent protein
PBS	Phosphate-buffered saline
AO	Acridine orange
EB	Ethidium bromide
C2C12	Cardiomyoblast cell
HT29	Human colon cancer cell
ATP	Adenosine Triphosphate
DILI	Drug-induced liver injury
ORC	Organoid raft cultures
PDXs	Patient-derived xenografts
NSCLC	Non-Small Cell Lung Cancer
IHC	Immunohistochemistry
IH	Immunohistology

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CHAPTER 1

INTRODUCTION

For many years, researchers were fascinated by the intricate procedures that govern the development and operation of human organs. These intricate structures, which are made up of many cell types that are methodically organised, offer crucial information to comprehending numerous diseases and researching new treatments. However, prior research approaches frequently depended on models of animals or trials involving entire organs, which had limits because of ethical considerations and precisely mimicking the human environment.

Organoids are unique lab-grown tiny bodies that have the potential to revolutionise medical study and therapy. These originate from diverse stem cells and are meticulously cultured in an environment that is three-dimensional to facilitate self-organisation and differentiation into intricate arrangements. These miniature organs closely mirror their counterparts in the real world in terms of shape, functioning, and cell type. Researchers employ organoids for a variety of revolutionary purposes. They give frameworks for disease modelling, which gives a closer look at how illnesses evolve and how various cells are influenced. Organoids are invaluable instruments for testing drugs, allowing for the assessment of novel medicines in a human-relevant setting. Furthermore, by creating particular to the patient organoids, investigators can investigate the possibilities of customised medicine by trying several treatment choices on a microscopic replica of an individual's own tissue before deciding on the most beneficial method of treatment. Organoids have enormous potential to revolutionise our method of treating diseases, speed drug development, and eventually pave the door for better, more tailored treatments as technology advances.

Organoids are extremely complicated in addition to their basic structure. They can duplicate important features of the functioning of organs, such as colon organoids propensity to absorb nutrition or brain organoids primitive neuronal function. This distinguishing feature renders them essential for investigating complex physiological processes that are challenging to witness outside of a living body. Organoids are also excellent at modelling complex disorders with different kinds of

cells and connections. Cancer organoids produced from tumour samples, for example, can aid researchers in understanding how cancers communicate with their surroundings and may suggest new treatment targets.

In the past few years, a groundbreaking technology has arisen, providing an effective fresh instrument for unravelling the unsolved mysteries of organ growth and function, which is organoids. These three-dimensional in nature miniature organs created in laboratory conditions have an opportunity to transform our comprehension regarding human biology and disease.

1.1 APPLICATIONS OF ORGANOID

1.1.1 Disease modelling: Organoids are used by scientists to examine the beginnings and stages of diseases such as cancer, cystic fibrosis (CF), neurological conditions, and many more by simulating both healthy and unhealthy tissues. They shed light on the ways in which illnesses modify cellular functions and molecular routes.

1.1.2 Drug testing: By acting as substrates for drug assessment, organoids help scientists assess the toxicity and efficacy of possible treatments in a model that is applicable to humans.

1.1.3 Personalised medicine: Customised treatment decisions are made possible by the use of patient-derived organoids, which allow physicians to test various medications or therapeutic modalities on a small replica of the individual's own tissue.

1.1.4 Developmental biology: Researchers studying stem cell activity, tissue creation, and possible birth abnormalities can benefit from the use of organoids, which can simulate various phases of the growth of organs.

1.1.5 Closing the Separation Between Animals and Individuals: Although models of animals have been invaluable in biomedical studies, the biology of humans is not always well-represented in them. Organoids are a more pertinent

and morally sound option for researching illnesses and processes unique to humans as they are made from cells from human beings.

1.2 THE TRANSFORMATIVE POTENTIAL OF ORGANOIDS

Organoids have enormous potential to increase the comprehension of human biological science and transform medicine. Their impact is projected to rise in fields such as:

- Addressing Complicated Disorders.
- Advancing the creation of drugs.
- Introducing an entirely novel phase of individualised diagnoses and treatments.
- Developing tissues and tiny organ-like constructs for transplanting.

CHAPTER-2

LITERATURE SURVEY

2.1 EXPLORING DIVERSE APPROACHES TO ORGANOID CULTURES FOR PRECLINICAL DRUG EVALUATION

Isabel Auge *et al.*, (2023) states that the generation and culture of hiPSC-derived renal organoids, Determines cell viability using a luciferase ATP assay. The paper establishes a fluorescent whole-mount approach for nuclear and membrane staining in renal organoids. The organoids demonstrated a dose-dependent decline in cell viability in response to drug treatment.

Julia Y *et al.*, (2023) Use of primary human intestinal organoids as an in vitro model system. The paper proposes the use of primary human intestinal organoids for inclusive preclinical research. The authors call for an industry-wide effort to incorporate diversity into preclinical drug programs.

Mustafa Karabicici *et al.*, (2023) States conventional hepatic cell lines and animal models. 3D organoids derived from inducible pluripotent stem cells or adult stem cells. The paper reviews current preclinical drug-induced liver injury (DILI) assessment tools. The paper discusses the potential of organoid technology for in vitro detoxification capacities.

Prachi Bajpai *et al.*, (2022) Proposed development of organoid raft cultures (ORCs) from patient-derived xenografts (PDXs). Testing the efficacy of an investigational drug, Navitoclax (ABT-263), in ORCs. The paper developed a 3D tumour organoid model called organoid raft cultures (ORCs). The ORCs were used to evaluate the efficacy of an investigational drug, Navitoclax (ABT-263) .

Ruoshi Shi *et al.*, (2023) Proposed organoid culture establishment from NSCLC patient tissue and PDX models. Histological and molecular characterization, exome sequencing, RNA-sequencing analysis, drug testing. NSCLC organoids closely recapitulate the genomics and biology of patient tumours. NSCLC organoids can be used for drug testing and biomarker validation.

Lee B *et al.*, (2023) In-vitro screening platform for pre-evaluating the efficacy of immunotherapeutic drug candidates. Creation of ODISEI platforms to recreate interactions between tumour organoids and immune cells. The ODISEI platform was developed as an effective evaluation tool for immunotherapy drugs. The platform was validated using different drug candidates and immune cells to assess their efficacy.

Amy Westerling-Bui *et al.*, (2022) Development of a novel approach for pharmacodynamic (PD) studies using transplanted, perfused human kidney organoids. Performance of pharmacokinetic (PK) studies with an investigational new drug (GFB-887) in athymic rats that had undergone organoid transplantation.

Sandra Carvalho *et al.*, (2020) Development of a low-cost microfluidic device for culturing and maintaining organoids on-chip. Optimization of dimensions, geometry, and flow rates to prevent high shear flows. A low-cost microfluidic device was designed for culturing and maintaining organoids on-chip. Tumour cell spheroids derived from colorectal cancer cell line SW480 were successfully generated on-chip.

Tepon P J *et al.*, (2023) Bioprinting of tumour organoids. High-speed live cell interferometry imaging with machine learning-based quantitation. The pipeline successfully generates bioprinted tumour organoids with unaltered histology and gene expression profiles. The strategy identifies organoids sensitive or resistant to specific therapies.

Ruoshi Shi *et al.*, (2020) Development of short-term and long-term organoid cultures from NSCLC patient and PDX tumour tissue. Histological and molecular characterization of organoids through cytology, histology, exome sequencing, and RNA-sequencing analysis. The established organoids were used for drug testing and biomarker validation.

Jan Markus *et al.*, (2021) States that organotypic epithelial tissue models were produced by seeding small intestinal epithelial cells onto cell culture inserts and culturing at air liquid interface (ALI) for up to 14 days. The models expressed efflux

transporters and drug-metabolising enzymes and were confirmed using functional assays. The paper discusses the potential of in vitro small intestinal tissues as a preclinical research tool and an alternative to animal testing. MatTek's intestinal tissue model has been extensively used by the pharmaceutical industry for various studies.

Dirk Schumacher *et al.*, (2019) Targeted proteomics was used to analyse MAPK signalling in organoid cultures. Panel sequencing was used to gather mutation information in organoid cultures. Heterogeneity in MAPK signalling and drug response observed in colorectal tumour-derived 3D cultures. Separate testing of drug effects in multiple subpopulations may improve therapy response prediction.

Hirosumi Tamura *et al.*, (2018) Patient-derived tumour organoids (PDOs) were established from human lung, ovarian, and uterine tumour tissues. Comparative histological and comprehensive gene expression analyses were performed. F-PDOs are superior to conventional cell lines for identifying therapeutic targets. F-PDOs can be used for drug testing and personalised medicine approaches.

2.2 FROM CELLS TO ORGANOIDS: A SPECTRUM OF DEVELOPMENTAL TECHNIQUES

Franziska Lampart *et al.*, (2023) States organoid cultures for high-throughput drug screening. Bulk and single-cell omics approaches for studying tissue interaction and disease progression. The paper discusses advancements in organoid protocols for high-throughput and high-content screenings. Organoids can be made accessible for drug screening and imaging analysis.

Zixuan Zhao *et al.*, (2022) Proposed organoid engineering strategies for culture, growth, differentiation, and maturation. Considerations for cell isolation, matrix selection, and physical cues. Development of organoids and methods for controlling their cellular environment. Discussion on limitations and priorities in organoid engineering.

Max A *et al.*, (2021) Suggested cell tracking techniques using 3D time-lapse microscopy and automated image analysis. Use of fluorescent markers to study differentiation events and cellular processes. The paper discusses the potential of cell tracking approaches for organoid science. It highlights the technical challenges associated with cell tracking in organoid biology.

Allen Thayakumar Basanthakumar *et al.*, (2022) Suggested pluripotent stem cells and embryonic stem cells are utilised for organoid development. Patient-Derived Organoids (PDO) were developed to overcome limitations of other cell lines. The paper discusses the evolution of organoids in oncology research. It highlights the latest technologies developed specifically for organoids.

Justyna Augustyniak *et al.*, (2019) Proposed organoid generation from adult SCs and pluripotent SCs. Characterization of species-specific organoids from tissue-specific SCs. The paper discusses the use of organoids as a powerful model for in vitro toxicology. The paper presents the state of the art of organoid generation and characterization.

Zhisong He *et al.*, (2020) Inducible lineage recording system with reporter barcodes and inducible CRISPR/Cas9 scarring. Single-cell transcriptomics and lineage-coupled spatial transcriptomics to analyse lineage relationships. Lineage-coupled single-cell and spatial transcriptomics reveal distinct lineage compositions in organoid brain regions. iPSC clones used to initiate organoids tend to accumulate in specific brain regions.

Giuseppe Pettinato *et al.*, (2021) Suggested rapid organoid formation using microfabricated cell-repellent microwell arrays. Real-time label-free Raman spectroscopy and confocal light scattering spectroscopic microscopy. The paper introduces a new approach for rapid organoid formation. The approach allows for monitoring live differentiating organoids without sacrificing a sample.

Tomoki Kodera *et al.*, (2023) Machine learning, genetic and viral engineering, and optical imaging. Interdisciplinary approaches integrating stem cell biology with other research fields. The paper discusses the use of interdisciplinary approaches

in brain organoid biology. It highlights the potential applications and future perspectives of interdisciplinary research in organoid biology.

Bojana Radojevic *et al.*, (2021) Proposed dissection method of developing retinal organoids. Suspension method of developing retinal organoids. Retinal organoids developed using the suspension method showed delayed development and disorganised retinal layers compared to dissected organoids. Omitting the re-plating of embryoid bodies resulted in numerous optic vesicles that matured along a retinal lineage.

Natasha Arora *et al.*, (2017) Two-sample t-test was used to compare pre organoids and other spheroids. Spheroids were stained with propidium iodide to identify dead cells. 13% of spheroids matured into intestinal organoids. Spheroids with diameter >75µm and inner mass are enriched for pre-organoids.

Rosa Villanueva *et al.*, (2023) Tridimensional cultures of human induced pluripotent cells (iPSCs). Brain organoids made from iPSCs of patients with psychiatric disorders. Brain organoids can recapitulate early developmental stages of nervous tissue differentiation. Brain organoids have provided insights into the pathophysiology and treatment of psychiatric disorders.

Jeonghwan Youk *et al.*, (2021) Whole-genome amplification, microdissection of clonal patches, and in vitro clonal expansion. In vitro clonal expansion via adult stem cell-derived organoid culture technologies. The paper provides an overview of three approaches for detecting somatic mutations in single cells. The combination of organoid and genome sequencing technologies yields sensitive and precise mutation detection.

2.3 OPTIMISING ORGANOID PROTOCOLS FOR PRECLINICAL DRUG DEVELOPMENT AND SCREENING

Wijler L *et al.*, (2023) Colorectal, breast, lung, pancreatic, ovarian, cervix, and melanoma PDO models were selected. Experiments were executed using automated liquid handling equipment to standardise procedures. The assay-ready organoid technology showed high reproducibility and predictability. The platform allowed for short timelines, large panel screening, and patient stratification.

Stoneman Paul *et al.*, (2022) Patient-derived organoid models were characterised for growth and expansion capacity. An automated high-throughput screening platform was developed and validated. The paper describes the development of an automated platform for screening patient-derived organoid models. The platform was validated by screening a panel of patient-derived colon adenocarcinoma organoids against a library of oncology drugs, demonstrating robust performance.

Lee. B. E *et al.*, (2022) Development of an in vitro screening platform for evaluating immunotherapeutic drug candidates. Creation of a patient-specific immune system using tumour organoids and PBMC. The ODISEI platform was developed for efficacy evaluation of immunotherapy drugs. The platform was validated using PD-1/PD-L1 blocker antibodies and multiple drug candidates.

Annamaria Rapisarda *et al.*, (2023) High-throughput screening (HTS) with patient-derived organoid models and NCI-60 cell lines. Screening system and data analysis software platform used for testing. The paper describes the development of a screening platform using patient-derived organoid models for drug discovery and development. The organoid models are being screened with a library of FDA-approved oncology drugs.

Susanne Ramm *et al.*, (2022) Live-cell brightfield imaging for monitoring organoids and spheroids over time. Fixation and staining protocol for detailed morphological analysis of 3D structures. The paper presents a fixation and staining protocol that preserves the live-cell imaging results. The protocol allows for high-throughput screening and phenotypic characterization of organoids.

Karsten Boehnke *et al.*, (2016) Establishment of an automated platform in 384-well format for three-dimensional organoid cultures derived from colon cancer patients. Validation of assay robustness and reproducibility via plate uniformity and replicate-experiment studies. The paper describes the establishment and validation of an automated drug sensitivity platform using patient-derived organoid cultures. The study demonstrates the feasibility of using patient-derived tumour samples for high-throughput assays in drug discovery.

CHAPTER-3

AIM & SCOPE

3.1 AIM

This research paper presents a systematic exploration of various methodologies aimed at developing organoids utilising cost-effective machinery and minimising the requisite number of cells.



Fig 3.1: Aim of this research

3.2 SCOPE

This project seeks to investigate innovative approaches to organoid culture that can reduce the financial burden associated with traditional methodologies while also maximising the efficiency of cell utilisation. By employing novel techniques and leveraging affordable equipment, the scope of this study encompasses the development of organoids across various tissue types and applications. Through rigorous experimentation and optimization, the goal is to establish streamlined protocols that enable efficient organoid generation with minimal resource requirements, ultimately advancing the accessibility and practicality of organoid research across diverse scientific disciplines.

CHAPTER-4

MATERIALS, METHODS AND ALGORITHMS USED

4.1 CELL LINE: C2C12

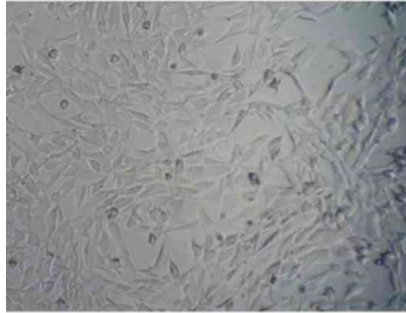


Fig 4.1: C2C12 cells

4.1.1 Small Petri Plate

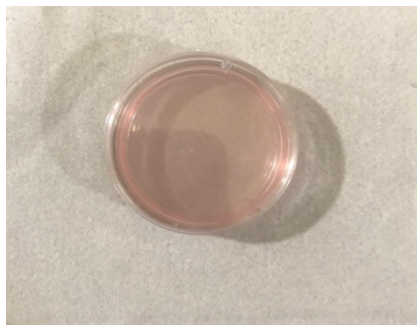


Fig 4.2: Petri plate with media and cells

Preparation of 1% Agarose Solution with distilled water

- Step 1: Take 0.3gram of agarose
- Step 2: Dissolve it in 30ml of distilled water
- Step 3: Autoclave the solution

Preparation of 1% Agarose Solution with 1X PBS

- Step 1: Take 0.3gram of agarose
- Step 2: Dissolve it in 30ml of 1X PBS
- Step 3: Autoclave the solution

Petri plate capacity 3-4 ml.

- Step 1: Adding 2 ml 1% agarose in the petri plate.

Step 2: Allow it to solidify.

Step 3: The next process involves well cutting utilising a variety of implements such as tip ends and syringes.

Step 4: Adding media to the petri plates.

Step 5: Further adding 2000 cells to the wells using microliter pipettes.

Step 6: Keep the petri plate in a CO₂ incubator to maintain temperature and humidity levels.



Fig 4.3: Cells in Petri plate (10x, 20x, 40x)

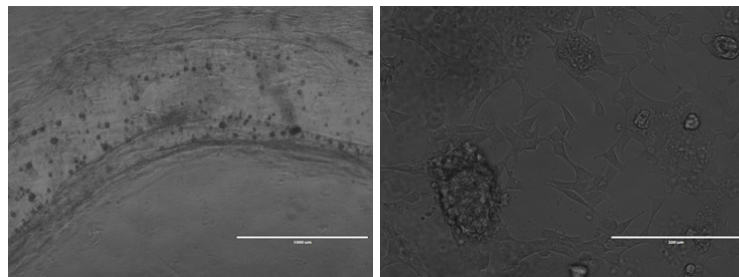


Fig 4.4: Cells in Agarose with 1X PBS (4x, 20x)

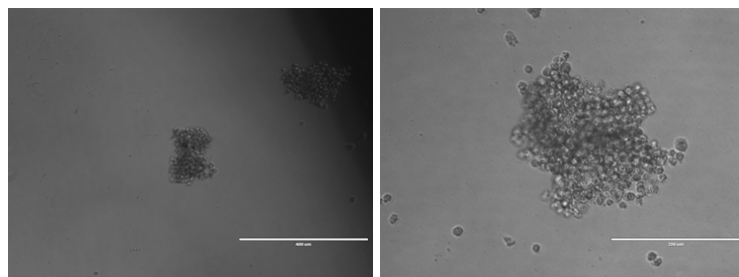


Fig 4.5: Cells in Agarose with Distilled Water (10x, 20x)

Disadvantage:

The formation of the aggregate getting attached to the petri plate and the agarose

4.1.2 96-well plate

Per well capacity 300 microlitre (μl) of growth medium.

Adding cells in 1st 12 wells in different concentrations. Starting with 5000 cells (1 microliter = 1000 cells approx). Adding the cells in the concentration of 100 microliter per well.

Well 1: 5 μl of cells + 95 μl of media

Well 2: 6 μl of cells + 94 μl of media

Well 3: 7 μl of cells + 93 μl of media

Well 4: 8 μl of cells + 92 μl of media

Well 5: 9 μl of cells + 91 μl of media

Well 6: 10 μl of cells + 90 μl of media

Well 7: 11 μl of cells + 89 μl of media

Well 8: 12 μl of cells + 88 μl of media

Well 9: 13 μl of cells + 87 μl of media

Well 10: 14 μl of cells + 86 μl of media

Well 11: 15 μl of cells + 85 μl of media

Well 12: 16 μl of cells + 84 μl of media

Position the instrument within the CO₂ incubator subsequent to the seeding of cells into the wells, and subsequently place the 96-well plate within the setup.

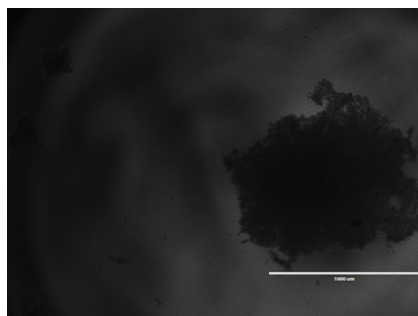


Fig 4.6: Cell aggregate in 96-well plate (4x)

Instrument used: Microplate shaker

Shaker RPM: 300

Disadvantage:

- Most of the cells are getting attached at the bottom of the well.
- Cells are not in a floating state as required.

AO-EB Staining for (96-well plate)

Step 1: Remove the old media from the well

Step 2: Adding 100 μ l of 1xPBS + 4 μ l of AO-EB mixture.

Step 3: View under microscope in GFP & RFP filter for live dead cells.

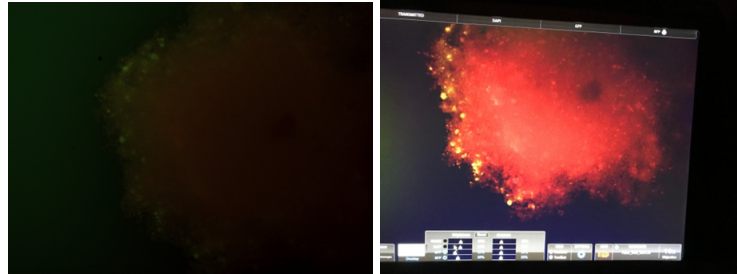


Fig 4.7: AO-EB staining for cells in 96-well plate

4.1.3 6-well plate

Per well capacity 1-3 ml of growth medium.

Adding 2 ml of DMEM media in first 3 wells

Well 1: 1 lakh cell (approx) + 2ml media

Well 2: 1.20 lakhs cell (approx) + 2ml media

Well 3: 1.20 lakhs cell (approx) + 2ml media

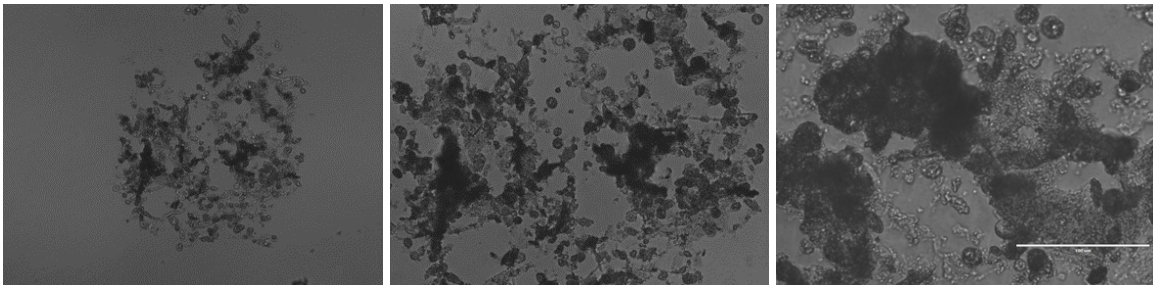


Fig 4.8: Cells in 6-well plate (10x, 20x, 40x)

Instrument used: Microplate shaker

Shaker RPM: 300

Position the instrument within the CO₂ incubator subsequent to the seeding of cells into the wells, and subsequently place the 6-well plate within the setup.

Disadvantage:

- The spillage of media is attributed to the substantial circumference of the well.
- As a consequence of the spillage of media, there exists a pronounced risk of contamination.

AO-EB Staining:

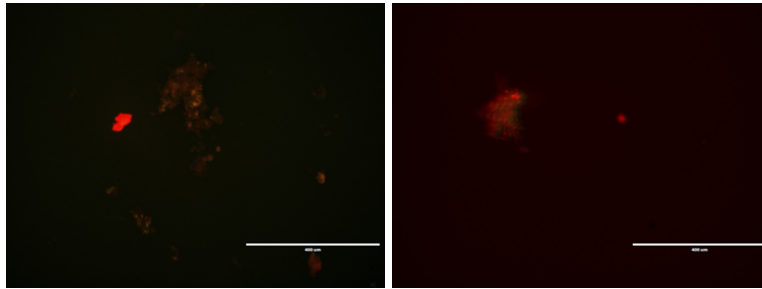


Fig 4.9: AO-EB staining for cells in 96-well plate (10x)

4.1.4 12-well plate

Per well capacity 1-2 ml of growth medium.

Adding 2 ml of media in each well

Well 1: 50,000 cells + 2ml media

Well 2: 60,000 cells + 2ml media

Well 3: 70,000 cells + 2ml media

Well 4: 80,000 cells + 2ml media

Well 5: 90,000 cells + 2ml media

Well 6: 1,00,000 cells + 2ml media

Well 7: 1,10,000 cells + 2ml media

Well 8: 1,20,000 cells + 2ml media

Well 9: 1,30,000 cells + 2ml media

Well 10: 1,40,000 cells + 2ml media

Well 11: 1,50,000 cells + 2ml media

Well 12: 1,60,000 cells + 2ml media

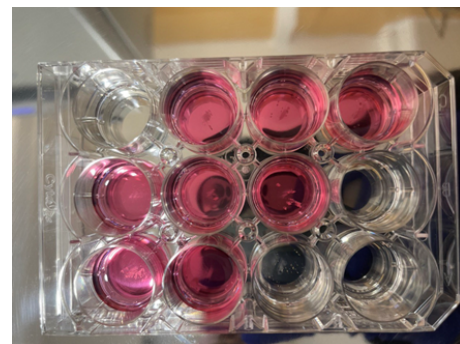


Fig 4.10: 12-well plate with cells and media

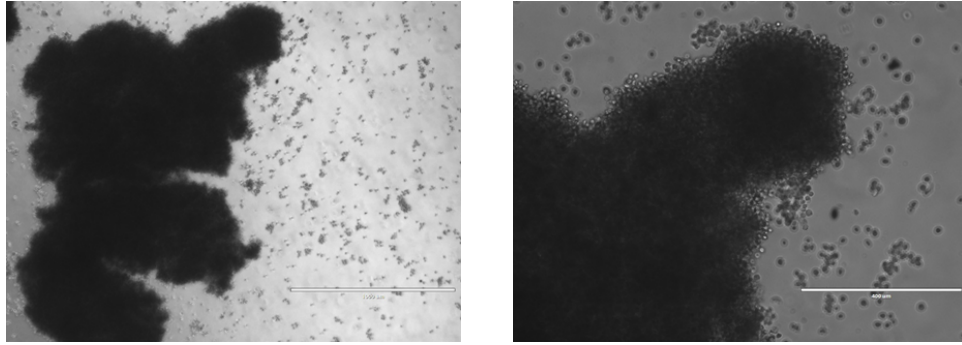


Fig 4.11: Cell aggregate in 12-well plate (4x, 10x)

Instrument used: Shaker

Shaker RPM: 18

Position the instrument within the CO₂ incubator subsequent to the seeding of cells into the wells, and subsequently place the 12-well plate within the setup.

Advantage:

- Visible aggregate formation in a free floating state.
- After the formation of the aggregate, the cells within the clump remained viable.

AO-EB Staining (12-well plate)

Step 1: Remove the old media from the well

Step 2: Adding 100µl of 1xPBS + 4µl of AO-EB mixture.

Step 3: View under microscope in GFP & RFP filter for live dead cells.

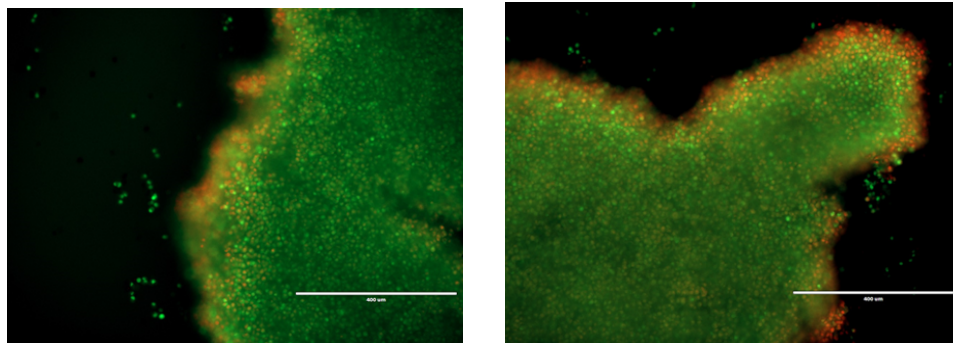


Fig 4.12: AO-EB staining for cells in 12-well plate (10x)

4.2 CELL LINE: HT-29

Human Colon Cancerous cells

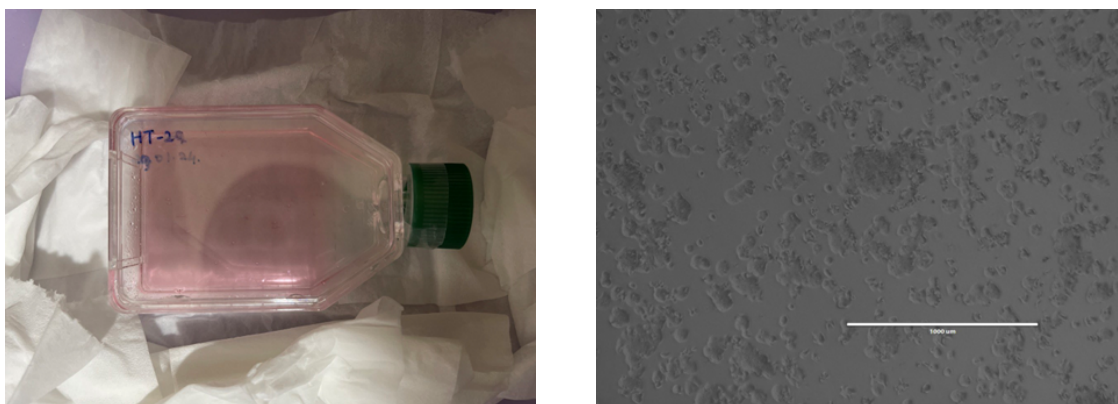


Fig 4.13: T25 flask, and HT-29 cells (4x)

4.2.1 12-well plate

Per well capacity 1-2 ml of growth medium.

Adding 2 ml of media in each well

Well 1: 50,000 cells + 2ml media

Well 2: 60,000 cells + 2ml media

Well 3: 70,000 cells + 2ml media

Well 4: 80,000 cells + 2ml media

Well 5: 90,000 cells + 2ml media

Well 6: 1,00,000 cells + 2ml media

Well 7: 1,10,000 cells + 2ml media

Well 8: 1,20,000 cells + 2ml media

Well 9: 1,30,000 cells + 2ml media

Well 10: 1,40,000 cells + 2ml media

Well 11: 1,50,000 cells + 2ml media

Well 12: 1,60,000 cells + 2ml media

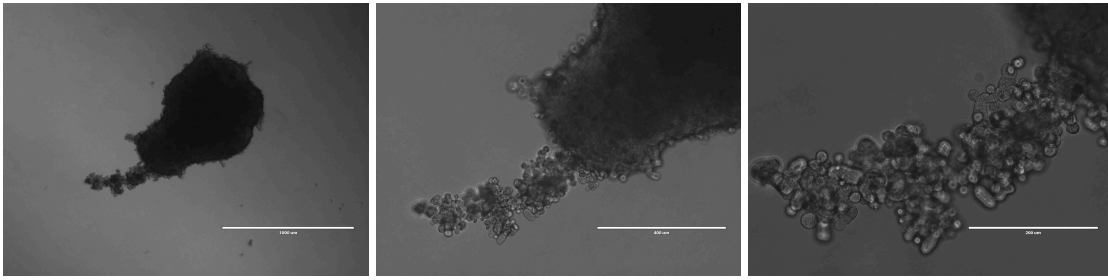


Fig 4.14: Cell aggregate in 12-well plate (4x, 10x, 20x)

Instrument used: Shaker

Shaker RPM: 18

Position the instrument within the CO₂ incubator subsequent to the seeding of cells into the wells, and subsequently place the 12-well plate within the setup.

Advantage:

- Visible aggregate formation in a free floating state.
- After the formation of the aggregate, the cells within the clump remained viable.

Note: Need to change the culture media every four days, due to the event of substantial debris accumulation, conduct a single wash with 1X PBS followed by the addition of fresh media.

AO-EB Staining for (12-well plate)

Step 1: Remove the old media from the well

Step 2: Adding 100μl of 1xPBS + 4μl of AO-EB mixture.

Step 3: View under microscope in GFP & RFP filter for live dead cells.

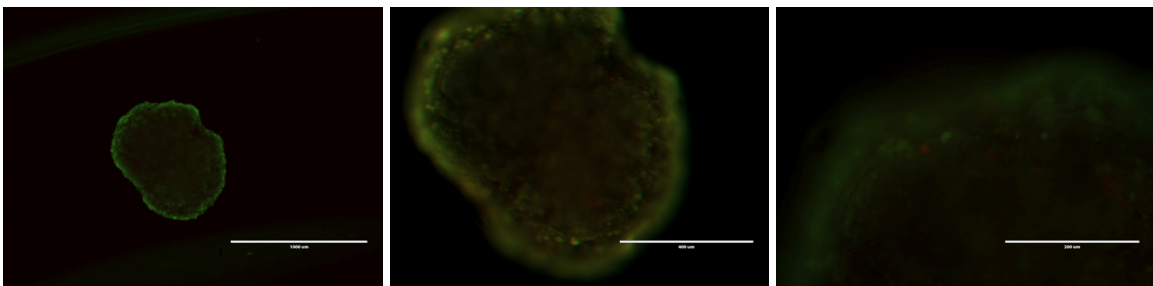


Fig 4.15: AO-EB staining for cells in 12-well plate (4x, 10x, 20x)

4.2.2 Cryostat Microtome Sectioning



Fig 4.16: Cryostat Microtome

Step 1: Prior to sectioning, apply a coating of 0.1% gelatine onto the microscope glass slide and allow it to air dry.

Step 2: After removal from the media, the aggregate should be rinsed with 1xPBS solution.

Step 3: For enhanced dye penetration within the aggregate, immerse the aggregate in OCT compound (freezing medium) supplemented with 1xPBS for a designated period.

Step 4: Retrieve the cellular section using a Cryostat microtome.

Step 5: Position the cellular section onto the gelatine-coated glass slide and allow it to stay at room temperature for 5-10 minutes.

Step 6: Subsequently, proceed with additional procedures such as Immunohistochemistry (IHC) and Immunofluorescence (IF)



Fig 4.17: Cell Aggregate



Fig 4.18: Ultra Freezer

4.2.3 Immunohistochemistry (IHC)

Hematoxylin and Eosin staining Procedure

Step 1 : 95% Ethanol (10 dips)

Step 2 : 10% Formalin in Phosphate Buffer (10 dips)

Step 3 : Distilled water (10 dips)

Step 4 : Hematoxylin (30 seconds)

Step 5 : Distilled water (10 dips)

Step 6 : Distilled water (10 dips) - optional

Step 7 : 95% Ethanol (10 dips)

Step 8 : Eosin (15 seconds)

Step 9 : 95% Ethanol (10 dips)

Step 10 : 95% Ethanol (10 dips) - optional

Step 11 : 100% Ethanol (10 dips)

Step 12 : 100% Ethanol (10 dips) - optional

Step 13 : Xylene (10 dips)

Step 14 : Xylene (10 dips) - optional

Step 15 : Mount with DPX mount. Observe under a microscope.

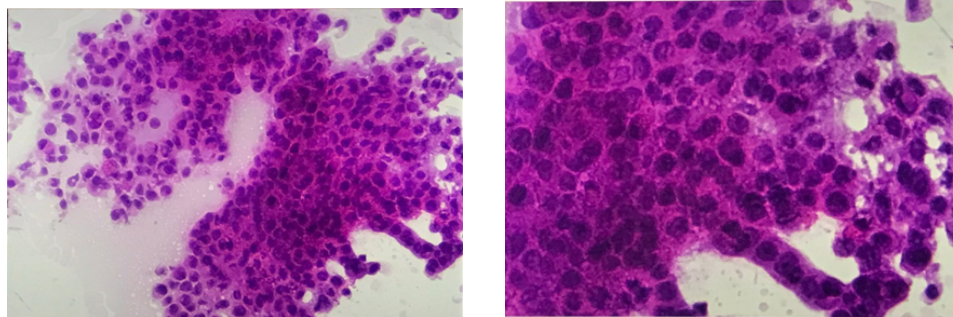


Fig 4.19: Day 1 of H & E staining

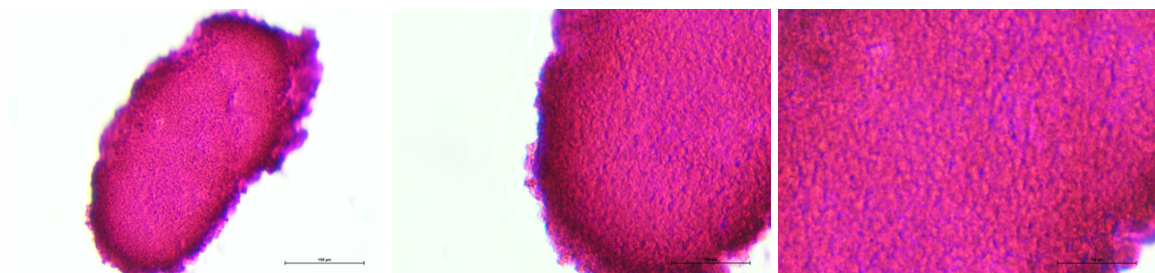
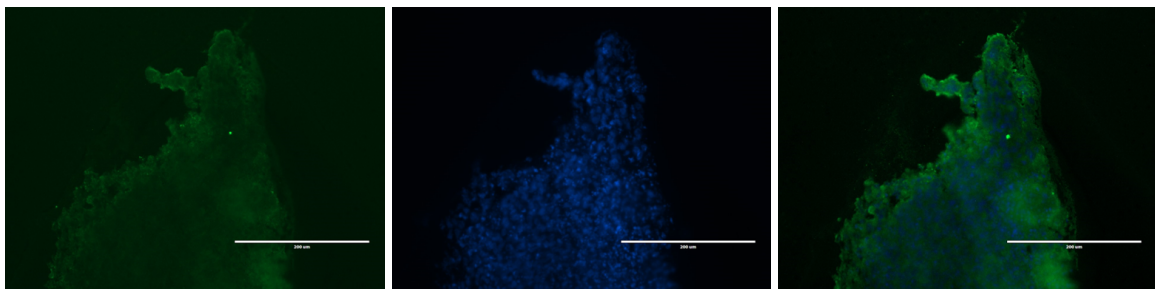


Fig 4.20: Day 14 of H & E staining

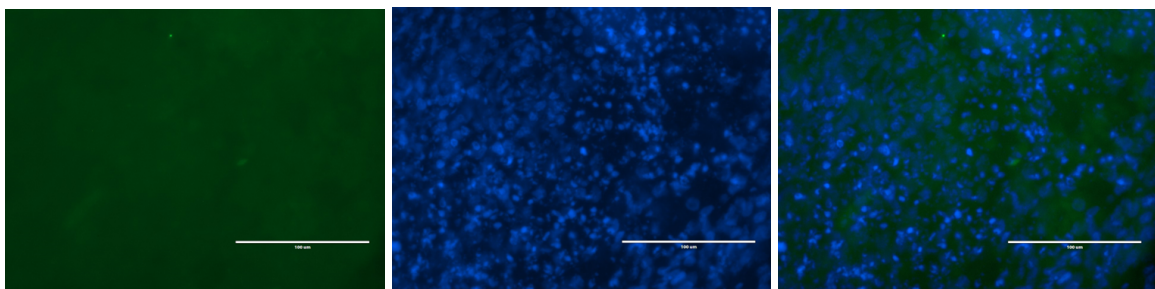
4.2.4 Immunofluorescence (IF)

- Step 1 : 95% Ethanol (10 dips)
- Step 2 : 10% buffered formalin (10 dips)
- Step 3 : PBST (2-5 mins incubation / wash)
- Step 4 : Normal water (1 dip)
- Step 5 : PAP marking
- Step 6 : Blocking (2 hours)
- Step 7 : Primary antibody incubation overnight at 4°C
- Step 8 : PBST (2-5 minutes incubation)
- Step 9 : Secondary antibody incubation (2 hours) Alexa 488
- Step 10 : PBST wash (2 minutes)
- Step 11 : DAPI (10 minutes)
- Step 12 : PBS wash (2 minutes)
- Step 13 : Mount with DPX mount. Observe under a microscope.

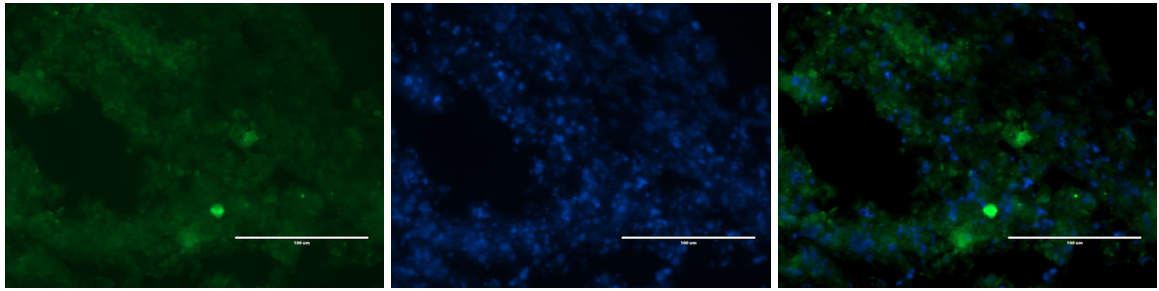
Here we used Actin (AC026), PCNA (18197), Histone H3 (1791) as primary antibodies



***Fig 4.21: Immunofluorescence - Actin AC026
GFP, DAPI, Overlay (20x)***



***Fig 4.22: Immunofluorescence - PCNA 18197
GFP, DAPI, Overlay (40x)***



**Fig 4.23: Immunofluorescence - Histone H3 1791
GFP, DAPI, Overlay (40x)**

4.2.5 AO-EB Staining

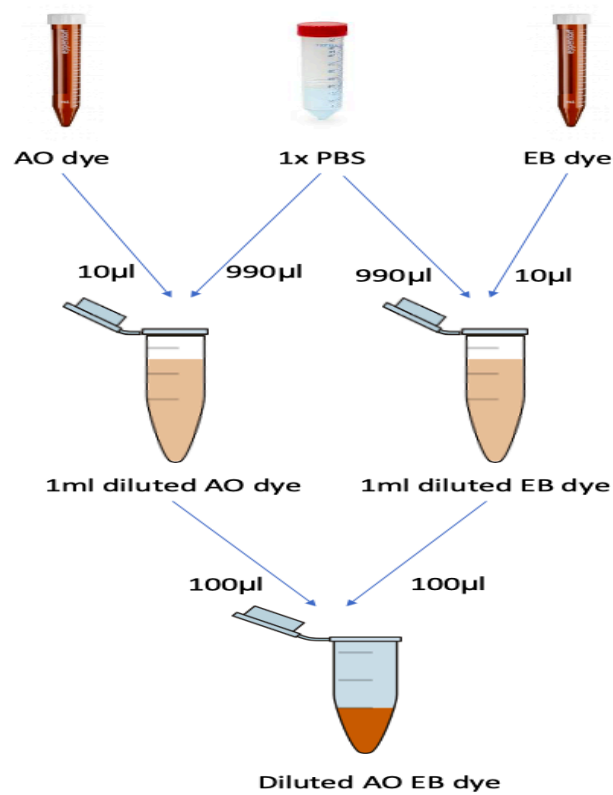


Fig 4.24: AO-EB Staining

Dilution of the AO-EB dyes:

AO : 10µl of AO dye + 990µl of 1xPBS = 1 ml

EB : 10µl of EB dye + 990µl of 1xPBS = 1 ml

Mixture of AO-EB : Adding 100µl of diluted AO-EB dyes to a new eppendorf tube

Procedure:

Step 1: Remove the media from the well

Step 2: Addition of 1X PBS into the well. If necessary, a washing with 1X PBS to eliminate debris should be performed prior to the addition of the 1X PBS buffer.

Concentration of 1X PBS with diluted AO-EB mixture

When introducing 25µl of 1X PBS into the well, subsequently incorporating 1µl of AO-EB mixture is recommended.

Step 3: Add 100µl of 1X PBS + 4µl of AO-EB mixture.

Step 4: View immediately under the microscope in GFP & RFP filter, for live dead cells.

CHAPTER-5

RESULTS AND DISCUSSION, PERFORMANCE ANALYSIS

An important source of information on a cell's physiological state, behaviour, and reaction to experimental settings is its morphology. The purpose of this study was to examine the morphology of cells over two weeks in a shaker. The cells were cultured in DMEM at 37°C with 5% CO₂ concentration, following standard methods. The cells were not given any particular therapies before being observed. The digitally inverted EVOS microscope evaluates the morphology of the cells.

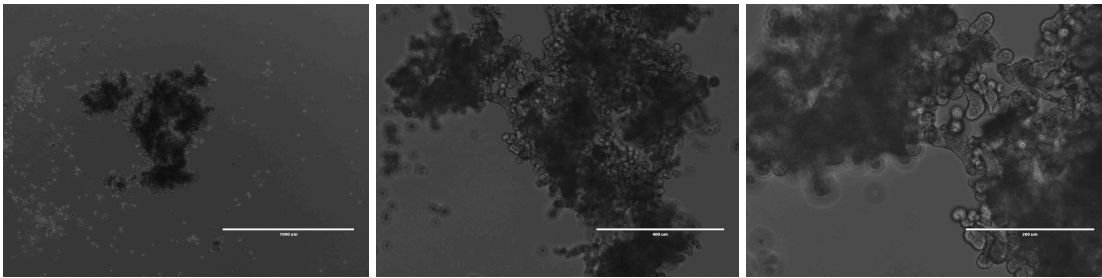


Fig 5.1: Day 1: Cells in shaker (4x, 10x, 20x)

The morphological characterization of organoid cells on day 1 provides valuable insight into the early stages of organoid formation and development. These observations lay the groundwork for further investigations into the dynamics of organoid growth and behaviour over time.

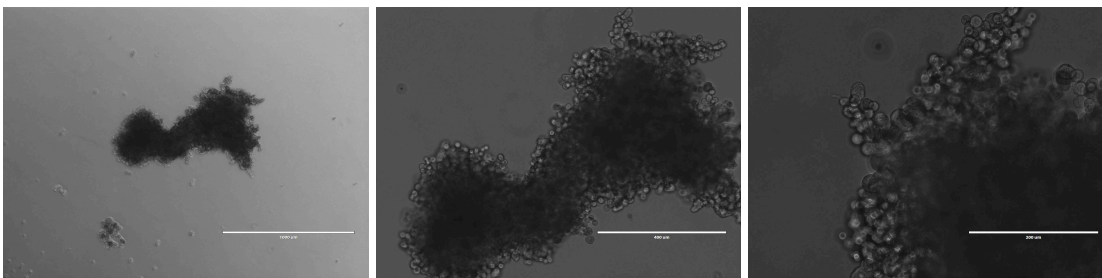


Fig 5.2: Day 2: Cells in shaker (4x, 10x, 20x)

Day 2 organoids morphology demonstrates their potential as a reliable and physiologically accurate model system for researching intricate cellular reactions and processes. These findings lay the groundwork for additional research on organoid biology and its potential uses in tissue engineering, regenerative medicine, and drug screening.

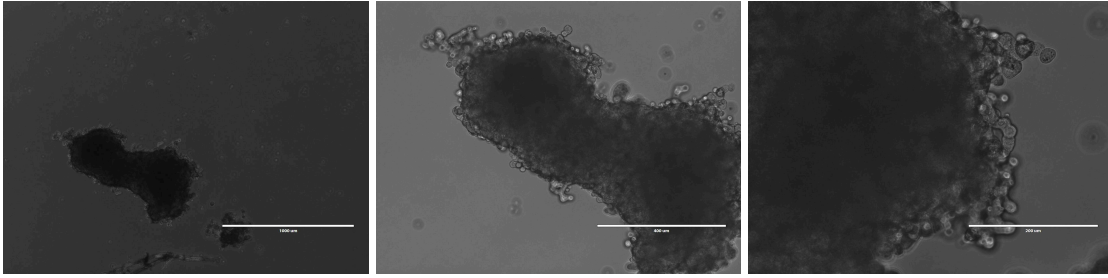


Fig 5.3: Day 3: Cells in shaker (4x, 10x, 20x)

The morphological characterization of organoid cells on day 3 provides valuable insights into their structural organisation and growth dynamics within the three-dimensional culture system. These observations support the utility of organoid models for studying complex cellular interactions and responses in vitro.

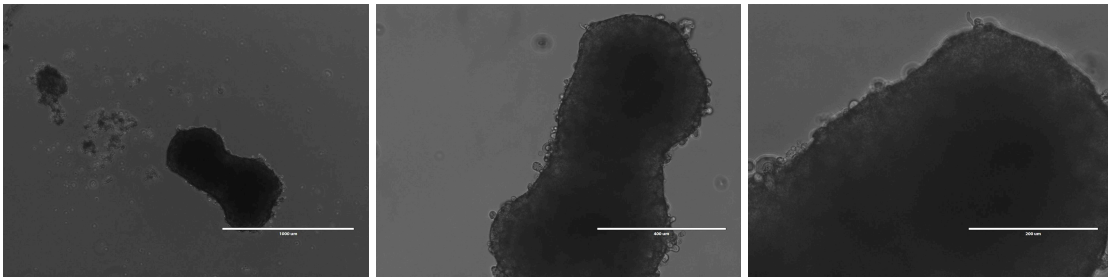


Fig 5.4: Day 4: Cells in shaker (4x, 10x, 20x)

On day 4th organoid cell morphology shows that, in the given experimental conditions, organoid creation and growth were effective. The organoids compact and rounded shape, along with their remarkable cellular viability, point to stable and robust organoid cultures. These results highlight the usefulness of organoid models for investigating intricate cellular responses and behaviours in a setting more similar to the human body.

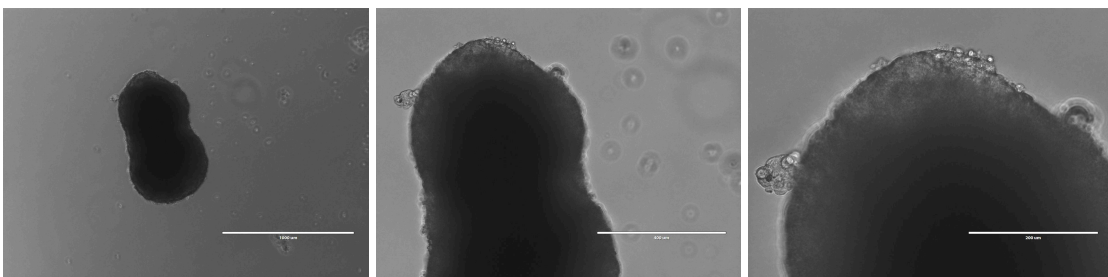


Fig 5.5: Day 7: Cells in shaker (4x, 10x, 20x)

The majority of the cells were organised into monolayers, with each cell tightly adhering to its neighbouring cell. Nevertheless, there were also sporadic regions

with poor cell distribution, which may indicate uneven cell proliferation. Numerous cellular characteristics, such as tiny cytoplasmic vesicles and fine cytoplasmic extensions, were discovered in addition to the distinctive size and structure of the cells. There were no obvious indications of cellular damage or stress.

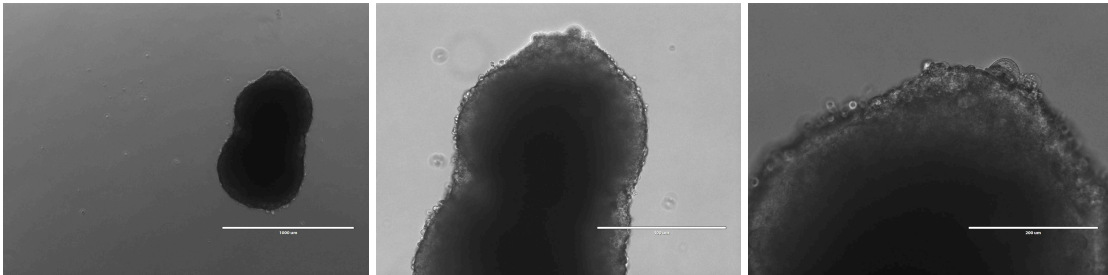


Fig 5.6: Day 8: Cells in shaker (4x, 10x, 20x)

In brief, cells were sown in 12-well ultra-low attachment plates and given seven days to develop into organoids. Organoids were gathered for morphological examination on day eight. A 4x, 10x, and 20x objective on an inverted microscope was used to do microscopic imaging. Organoids demonstrated successful organoid production and maintenance throughout the culture period on day 8 when they manifested as compact, spherical aggregates with well defined boundaries.

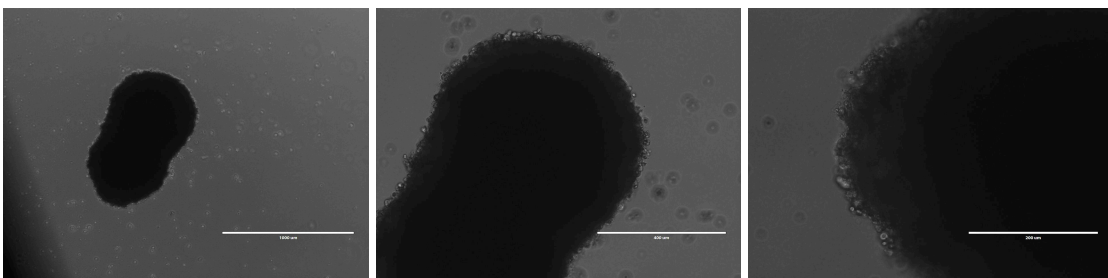


Fig 5.7: Day 10: Cells in shaker (4x, 10x, 20x)

Organoid structures were easily visible at 4x magnification; they appeared as smooth-surfaced, compact, spherical aggregates. The size distribution of the organoids was somewhat variable, with diameters ranging from about 100 to 300 micrometres. At this magnification, there were no visible internal structures, which is consistent with the dense cell packing within the organoids.

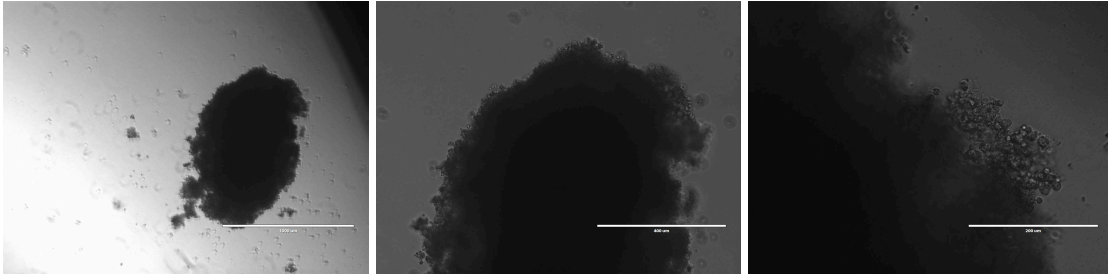


Fig 5.8: Day 11: Cells in shaker (4x, 10x, 20x)

The rapid changes in morphology and cellular organisation that occur after cell splitting are highlighted by the morphological characterisation of organoids on day 11th. It is probable that variations in cell proliferation, migration, and differentiation within the culture account for the observed heterogeneity in organoid size and shape. The scattered cells that encircle the centre mass indicate that the organoids are still remodelling and maturing. This could have an impact on the organoids' functional characteristics and potential uses in regenerative medicine and research.

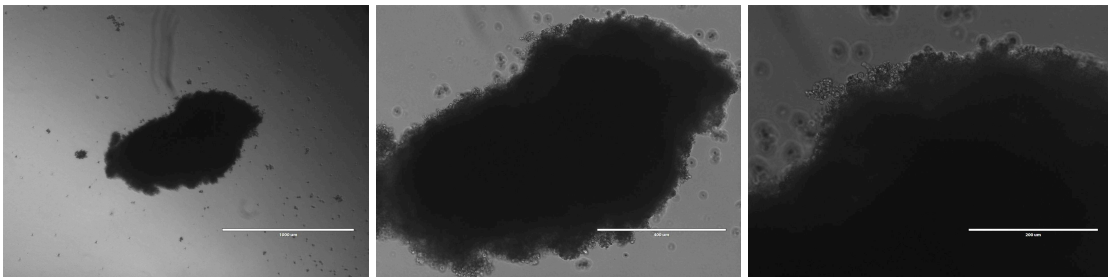


Fig 5.9: Day 14: Cells in shaker (4x, 10x, 20x)

In conclusion, day 14th morphological examination of the organoids sheds important light on their intricate cellular dynamics and structural complexity. The morphological, size-variation, and cell-detachment changes that have been seen highlight the dynamic character of organoid cultures and have consequences for using them as model systems in a range of scientific applications.

CHAPTER-6

SUMMARY AND CONCLUSIONS

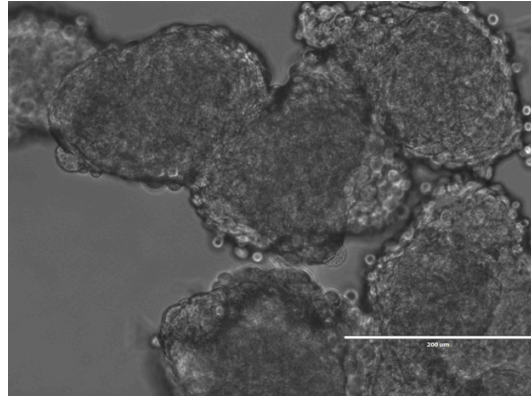


Fig 6.1: C2C12 cells aggregate (20x)

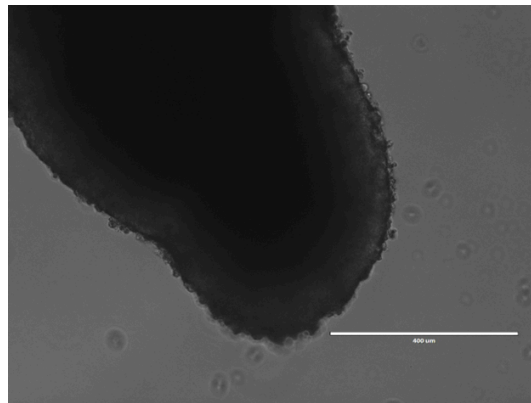


Fig 6.2: HT-29 cell lines aggregate (10x)

The development of organoids made with low cell populations and low-cost equipment is an essential turning point in biomedical research, providing a transformative avenue for investigating human physiological processes and diseases. By avoiding the constraints of standard organoid culture methods, this novel approach not only improves accessibility and scalability, but also promotes increased inclusivity and diversity throughout the field of science.

Furthermore, the price and simplicity of manufacturing organoids with fewer cells expands the range of applications outside well-funded research institutes. Facilities with few assets can now conduct sophisticated organoid studies, allowing scientists in resource-constrained areas to participate significantly in global scientific projects.

In addition, the downsizing of organoids provides new opportunities for high-throughput testing as well as customised therapy. With the capacity to create huge quantities of organoids from small cell populations, investigators may perform comprehensive drug screenings and personalise treatment to specific patients, creating unparalleled prospects for personalised healthcare methods.

As this innovation develops, co-operation among engineers, scientists, and doctors will be critical to realising its maximum potential. By combining expertise from various fields, we can speed up the conversion of organoid results from studies into practical applications, getting us closer to realising regenerative therapies and tailored treatments for a wide range of disorders.

Finally, the development of organoids with low cell counts and low-cost equipment is creating an exciting era of connectivity, creativity, and collaboration in biomedical research. Organoid technology, with its prospects for transforming drug discovery, disease modelling, and personalised treatment, has the potential to make major improvements to the well-being and health of people in the years that follow.

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