CRISPRi Screening in Cancer Spheroids to Investigate Factors in Genome Stability

Final Report

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Abstract

Current two-dimensional (2D) models have limited biological relevance to the tumor microenvironment, often failing to replicate the complex cell-cell interactions found *in vivo*. The objective of this project is to select a suitable cancer cell line to develop and refine a three-dimensional (3D) cell culture model that better recapitulates the tumor microenvironment. Toward this end, A549, a non-small lung cancer line (NSCLC) with mutations to tumor protein 53 and a relative sensitivity to the three main cancer drugs, was cultured and a doubling time of 23 hours was established. An optimal static spheroid formation protocol was engineered and tested by seeding spheroids with A549s at various densities. BioTek cytation images of thes5e spheroids were taken and processed using ImageJ and MATLAB. From this data it was determined that ideal A549 aggregates were formed at a seeding density of 50k-75k.

In the future, the relevance of this information can be applied to scale up spheroid formation to prepare for γH2AX —a sensitive marker for DNA double-strand breaks (DSBs)— staining and a high-throughput genome-wide CRISPRi screen. The team will provide valuable insights that bridges the gap between in vitro studies and clinical applications leading to more effective cancer therapies and improved patient outcomes.

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1. Introduction

1.1. Motivation and Societal impact

Cancer impacts millions of lives each year, with an estimated 2.2 million new cases and 736,790 deaths reported in the United States in 2024 [1], and not mentioning those still living with cancer. In the past, using 2D monolayer with CRISPR screening has provided extensive knowledge on what drives cancer cell high growth rate and what therapeutic treatments they are susceptible to. However, it fails to accurately represent the 3D tumor environment, leaving out important factors such as genome stability and DNA damage regulatory genes. Providing a replicated 3D tumor environment that is compatible with CRISPR screening can help identify sources of DNA damage not found with 2D cultures. This can lead to early detection, new therapeutic tools, and possibly genomic therapies.

1.2. Problem Statement

Although previous CRISPR screening in 2D monolayers has provided useful knowledge on cancer drivers and therapeutic susceptibilities, it lacks an element of biological relevance to an in vivo environment. Therefore, our team was tasked with developing a cell culture method that is compatible with a 3D environment and CRISPRi screening in order to identify sources of DNA mutations in the tumor environment. To achieve this goal, the team must select a viable cell line for the screen, create and optimize a spheroid formation protocol, and develop a protocol to stain for γH2AX: a histone variant that is a sensitive marker for DNA damage.

2. Background

2.1. Background Research

2.1.a. *3D spheroids*

The study of cancer and its progression has evolved significantly, with advancements in genetic and molecular biology providing deeper insights into the mechanisms underlying tumorigenesis. In this context, 3D spheroids have emerged as a superior model compared to traditional 2D monolayer cultures. 2D cultures are limited in their ability to replicate the complex cellular interactions and microenvironment characteristic of actual tumors, which can lead to poor results in drug response and tumor behavior studies [2]. In contrast, 3D spheroids, composed of cancer cells aggregated in a spherical configuration, better mimic the architecture and growth phenotypes observed in tumors as seen in Figure 1 [2]. This structural arrangement facilitates critical cell-cell and cell-matrix interactions, which are essential for maintaining cellular functions and responses to therapeutic agents [3]. From these interactions, more DNA damage is observed in 3D spheroid cultures compared to 2D monolayers due to the hypoxic and nutrient-deprived microenvironment, which generates reactive oxygen species (ROS) and impairs DNA repair pathways [4]. Consequently, the use of 3D spheroids enhances the fidelity of experimental models, allowing for a more accurate investigation of tumor structure, cancer progression, and the efficacy of potential treatments.

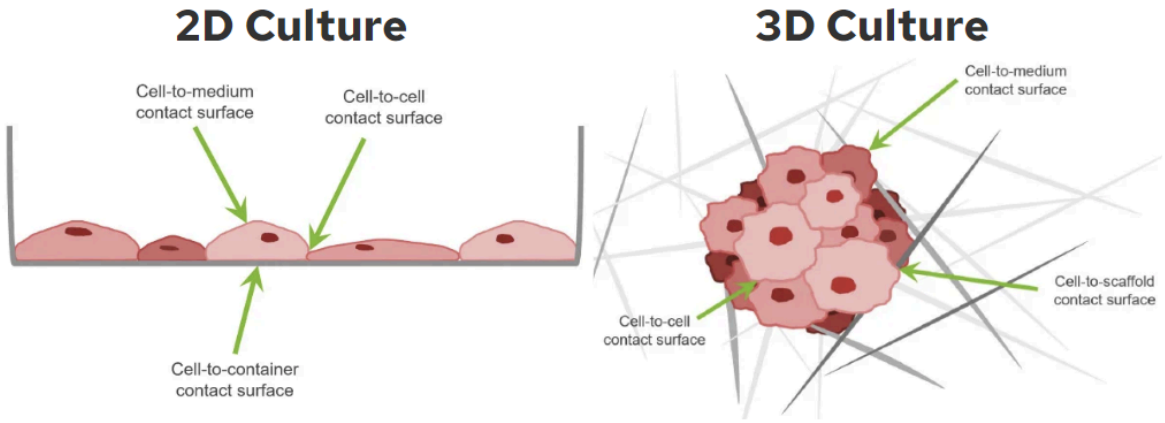

Figure 1: Comparison of 2D versus 3D tissue culture [5].

2.1.b. *Cell line*

Creating a cancer cell line is a multifaceted process that begins with the collection of tumor tissue from a cancer patient, typically obtained through biopsy or surgical resection. Once the tissue is collected, it undergoes processing to isolate individual cancer cells, often involving enzymatic digestion or mechanical disruption to break down the extracellular matrix and liberate the cells [6]. These isolated cells are then placed in a nutrient-rich culture medium designed to support their growth, containing essential nutrients, growth factors, and an appropriate pH. As the cells proliferate, certain cancer cells may demonstrate faster growth rates; these cells can be selected for further cultivation. Once cultivated, a cell line is then defined as continuous (infinite) or finite. For the purposes of this design project, both infinite and finite cell lines have been considered. After establishing a stable culture, the cell line undergoes thorough characterization to confirm its cancerous properties, assessing factors such as cell morphology, growth rate, and genetic markers. Finally, once a stable and well-characterized cell line is established, it is cryopreserved in liquid nitrogen for long-term storage [7]. The cell lines considered must be able to display a 3D tumor environment upon spheroid formation, be compatible with CRISPRi screening, and express γH2AX.

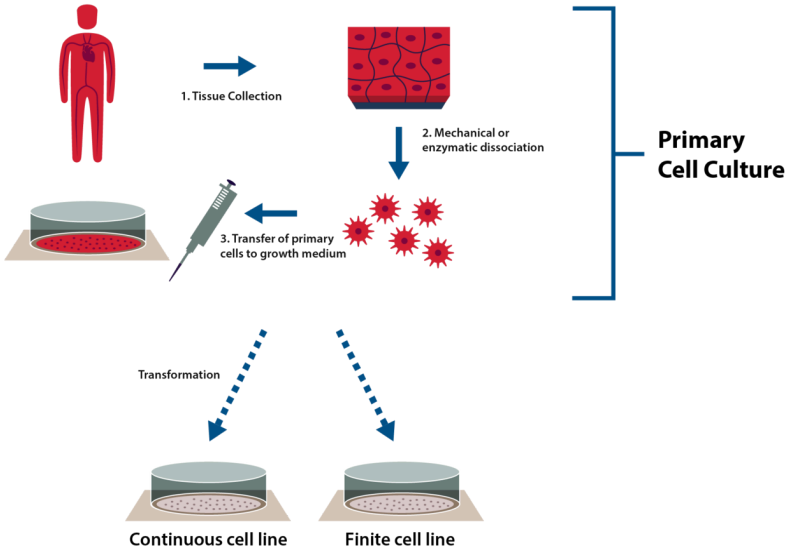

Figure 2: Cell line creation [6].

2.1.c. *CRISPRi Screening*

CRISPR interference (CRISPRi) is a powerful tool for gene regulation that allows for the targeted repression of genes without directly inducing double-strand breaks in the DNA. This technique utilizes a catalytically inactive Cas9 (dCas9) protein fused to transcriptional repressors, enabling precise control over gene activity [8]. In this project, lentiviral vectors are employed to deliver the CRISPRi components into the target human lung cancer cell line, which is A549. The use of lentivirus is advantageous due to its ability to efficiently transduce a wide range of dividing and non-dividing cells, ensuring stable expression of the dCas9 and guide RNA (sgRNA) constructs necessary for effective gene repression [8]. For instance, SOX2 is a transcription factor associated with stemness and tumor progression in non-small cell lung cancer (NSCLC). It regulates self-renewal, differentiation, and pluripotency, making it a key driver of cancer stem cell (CSC) properties. SOX2 is frequently co-expressed with surface markers such as CD133 and CD44, which are commonly used to identify CSCs. CD133, a marker of tumorigenicity, is often upregulated in SOX2-expressing cells and is linked to enhanced self-renewal and tumor initiation [9]. Similarly, CD44, which plays a role in cell adhesion and migration, is associated with SOX2 in promoting metastasis and resistance to therapies [9]. These surface markers, in conjunction with SOX2, delineate a subpopulation of CSCs that contribute to lung cancer progression, recurrence, and therapeutic resistance, making them a target for gene therapies in NSCLC.

By applying CRISPRi in a 3D spheroid model, the team's design aims to better mimic the in vivo environment for the screen, which is essential for understanding the complex interactions that drive cancer progression and treatment resistance. As stated, this approach facilitates the identification of gene targets that contribute to DNA damage responses and cellular survival in a more physiologically relevant context compared to traditional 2D cultures.

2.1.d. *γH2AX*

γH2AX is a phosphorylated variant of the histone H2AX, which serves as a sensitive marker for DNA double-strand breaks (DSBs) [2]. The phosphorylation of H2AX occurs rapidly in response to DNA damage, making it an essential marker for monitoring the integrity of the genome [3]. In cancer research, γH2AX staining is utilized to assess the extent of DNA damage and the efficacy of DNA repair mechanisms.

γH2AX allows for the quantification of DNA damage induced by various genetic suppression within the 3D spheroid model. γH2AX staining is more challenging in a 3D environment due to the compact and multilayered structure of spheroids creating physical barriers that hinder the diffusion of antibodies into the inner core. This lack of uniform antibody penetration can result in incomplete staining, where only the outer layers of the spheroid are adequately labeled, leading to a skewed representation of DNA damage throughout the entire structure.

If the γH2AX staining is done correctly in a CRISPRi screen within 3D spheroids, it enables accurate assessment of DNA damage across the entire structure, including inner cell layers, providing a comprehensive view of the effects of gene knockdowns on genome stability. This ensures that the screening results are not biased toward cells in the outer layers and allows for the identification of key genetic contributors to DNA repair and damage pathways in 3D. The response of dsDNA breaks can effectively evaluate how specific gene targets contribute to genomic stability, tumor biology, cancer progression, and therapeutic vulnerabilities.

It is important to choose a cell line that has been proven to be sensitive (or has a baseline of sensitivity) to DNA damage for successful γH2AX staining. One way to determine this criteria is to see whether that cell line is sensitive to drugs that can induce DNA damage. The drugs chosen for this purpose include cisplatin, oxaliplatin, and bleomycin.

Cisplatin is a platinum-based chemotherapeutic agent widely used in the treatment of various solid tumors, including lung, ovarian, and bladder cancers. Its mechanism involves the formation of DNA cross-links, which interfere with DNA replication and transcription, leading to cell cycle arrest and apoptosis [10]. Another platinum-based compound with similar anticancer mechanisms is oxaliplatin, which is more effective in some forms of cancer such as colorectal cancer [11]. On the other hand, bleomycin, an antitumor antibiotic, induces DNA damage through the generation of free radicals, leading to both single and double-strand DNA breaks. Unlike platinum-based drugs, bleomycin's mechanism of action makes it suitable for combination therapy, targeting cancer cells through a different pathway [12].

Drug sensitivity is quantitatively measured by Z-score, which measures drug sensitivity of a specific cell line to a specific drug in comparison to other cell lines. Negative Z-scores reflect sensitivity, with lower values indicating greater susceptibility to the drug's cytotoxic effects, while positive Z-scores indicate resistance [13]. A Z-Score of ± 1 is considered significant [14]. These differential responses across cancer types and drug classes highlight the complexity of cancer treatment and the need for tailored therapeutic approaches based on specific tumor characteristics.

2.1.e. *Previous Literature*

In previous research, 3D cancer spheroid models have been utilized for CRISPR-Cas9 screens with and without using an exogenous extracellular matrix (ECM).

Takahashi *et al*, 2020 utilized 4% Matrigel, an ECM based approach, as bio-scaffolding to plate A549 cell spheroids. They concluded that their seeded spheroid models *in-vivo* interactions between cells and the ECM were more biologically relevant than former 2D studies [15]. The study's CRISPRi library gene list as well as its phenotypes have a strong overlap with those from Han *et al*, 2020, suggesting that inner cells of ECM-based 3D spheroids may be similar to ECM-free 3D spheroids.

Han *et al*, 2020 devised an ECM-free scalable method to propagate 3D spheroids [16]. H23 cells were seeded on pre-treated ultra-low attachment or poly-HEMA-covered plates in RPMI 1640 medium with 0.75% methylcellulose. Similar methods have been found to be implemented in other studies, such as by Ferrarone, *et al* (2024) and by Stiff, *et al* (2024) [17], [18]. A total of around 210,000 single guide RNAs (sgRNAs) were designed for the CRISPRi library with around 10 sgRNAs per gene [16]. Samples were used to calculate growth and/or tested for drug resistance [16].

3D stem cell models have also been genomically edited via CRISPR-Cas9 to better model various disease phenotypes. Cells can be assembled with or without supporting scaffolds. Some example scaffolds include laminin-rich Matrigel, collagen matrix, or those that are naturally derived from decellularized tissues [19].

2.2. Client Information

The clients, Ms. Carley Schwartz and Dr. Gaelen Hess are researchers at the Hess Laboratory within the Wisconsin Institutes for Medical Research (WIMR). The group's research focus is on high-throughput functional genomics to investigate DNA repair and pathogenic effectors [20]. Dr. Hess is an assistant professor in the Department of Biomolecular Chemistry and Center for Human Genomics and Precision Medicine [20]. Ms. Carley Schwartz is the lab manager and a research intern at the Hess Laboratory.

2.3. Design Specifications

The project aims to develop a 3D cell culture method compatible with CRISPRi screening to identify sources of DNA mutations in tumors, utilizing γH2AX as a marker for DNA damage. The client requires the team to select an appropriate human lung cancer cell line, to which A549 and NCI-H23 meet that criteria [21]. This protocol must focus on optimal seeding density, targeted at 50,000 cells/cm², and viscosity adjustments through methylcellulose to ensure uniform spheroid size and maximal cell growth [16]. Additionally, a γH2AX staining protocol needs to be established following the guidelines of scalability, and be reproducible with optimized antibody concentrations and reagent volumes.

With the use of human contaminants, safety measures are to adhere to all Biosafety Level 2 (BSL-2) standards, implementing containment protocols and proper waste disposal [22]. The accuracy and reliability of the spheroid formation process will be monitored through live-cell imaging to confirm a ~30% cell death rate, and at least three staining experiments will be conducted to establish statistical confidence in the results.

For life in service, cells will require approximately two months in culture for protocol optimization, spheroid formation following the culture, and a minimum of six days post-infection with the CRISPRi library. To ensure long-term viability, cells will be cryopreserved at temperatures below -135°C. Operating conditions will be maintained at 37°C, 5% CO_2, and 90–95% humidity [23].

The design will prioritize ergonomic efficiency in the experimental setup, aiming to enhance throughput while accurately mimicking in vivo conditions. Spheroids are targeted to be ~500 µm in diameter, and accurate assessments of mass and density will be facilitated through specialized equipment [24]. The project budget is capped at $1,000, with a focus on utilizing existing lab materials to keep costs manageable.

Lastly, the team will conduct a competitive analysis of existing 3D cancer models, focusing on methodologies that incorporate ECM-free approaches. Key milestones include cell line creation and protocol development by October 2024, optimization of spheroid formation by November 2024, completion of the γH2AX staining protocol by December 2024, execution of high-throughput CRISPR screening by February 2025, followed by analysis of phenotypic differences before May 2025. For more detailed information see Appendix 12.1.

3. Preliminary Designs

3.1. Cell Lines

3.1.a. *NCI-H23*

NCI-H23 is an epithelial-like cell that was isolated from the lung of a Black, 51-year-old, male patient with adenocarcinoma. H23 is a non-small cell lung cancer (NSCLC) with a doubling time 38 hours [25]. Although H23 is considered an adherent cell line, it is known to have weaker cell-cell interactions due to the lack of E-cadherin at the cell membrane [26]. H23 exhibits TP53, KRAS-12, epidermal growth factor receptor (EGFR), L-dopa decarboxylase-negative, and a high degree of c-myc DNA amplification (20-fold) but no detectable amplification of c-myc RNA [25]. Additionally, these cells have a reported colony forming efficiency of 9.7% in soft agarose [2]. H23 is very sensitive to bleomycin (Z-score average of -1.22), somewhat sensitive to cisplatin (-0.73), and somewhat resistant to oxaliplatin (+0.41) [27]. One vial of these cells from ATCC is $555.00 [25].

3.1.b. *A549*

A549 is an adherent cell line isolated from the lung tissue of a White, 58-year-old male with lung cancer [28]. This NSCLC adenocarcinoma has a doubling time of 22 hours [28]. A549 exhibits mutations for TP53, EGFR, PIK3CA, ALK, and PTEN [29]. A549 is very sensitive to

bleomycin (Z-score average of -1.04), somewhat sensitive to cisplatin (-0.12), and very sensitive to oxaliplatin (-1.45) . One vial of these cells from ATCC is $555.00 [28].

3.1.c. *OVCAR-5*

OVCAR-5 is a cell line established from ascites fluid from a 67 year old, female, non-treated patient with a metastatic gastrointestinal cancer that has presented as advanced-stage ovarian carcinoma [30]. The doubling time for this cell line is 27 hours [31]. OVCAR-5 expresses mutations for KRAS-12, EGFR, and CLAUDIN-4 [30]. This ovarian cancer cell line is somewhat resistant to bleomycin (Z-score average of +0.67), somewhat resistant to cisplatin (+0.52), and somewhat sensitive to oxaliplatin (-0.18) [32]. This cell line is not currently available for the team to purchase [30], [33].

3.2. Spheroid Formation Protocol

3.2.a. *Treated Tissue Culture Plates*

Treated tissue cultures method is a scaffold-free method [34]. It uses low attachment plates, which are coated with a hydrophilic polymer (such as hydrogel or poly-HEMA) to prevent specific and nonspecific cell adhesion to the culture vessel [34]. Thus, high cell-to-cell interactions allow cells to cluster together, form cell aggregates and eventually into 3D spheroids (Figure 3) [35]. The number and size of spheroids formed depend on the shape of the wells, with U-bottom wells creating a single and well-defined spheroid for each well (Figure 3) [35]. The whole process usually takes up to 4 days [35].

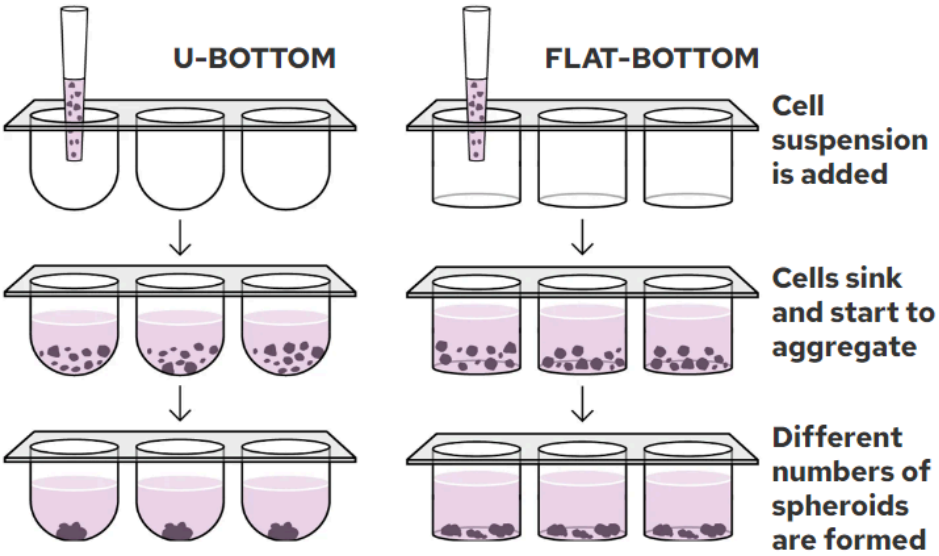

Figure 3: Spheroid formation by treated tissue culture plates/low attachment plates with U-bottom wells (left) and flat-bottom (right) [34].

3.2.b. *Hanging Drop*

Similar to the treated tissue cultures method, the hanging drop method is also anchorage-independent [34]. Currently, two methods to form hanging drops exist. The first method is the traditional method, involving dispensing small amounts of cell suspension on a surface, usually the underside of a petri dish, then flipping the dish upside down to form hanging drops (Figure 4) [36]. However, this method has a high risk of droplet fusion (if space is limited), loss of droplet shape (because of frequent media change), and droplet evaporation [37], [38]. The second method using hanging drop plates negates these risks [38]. Each plate consists of a lid to maintain sterility and a main component, which has a water reservoir to prevent evaporation and access holes on top where cells can be added to form hanging drops on the bottom (Figure 5a) [38]. Hanging drops create a flexible spheroid cell-to-liquid interface, allowing cells to gravitationally aggregate and form because of high cell-to-cell interactions (Figure 5b) [34]. The whole process usually takes up to 4 days [35].

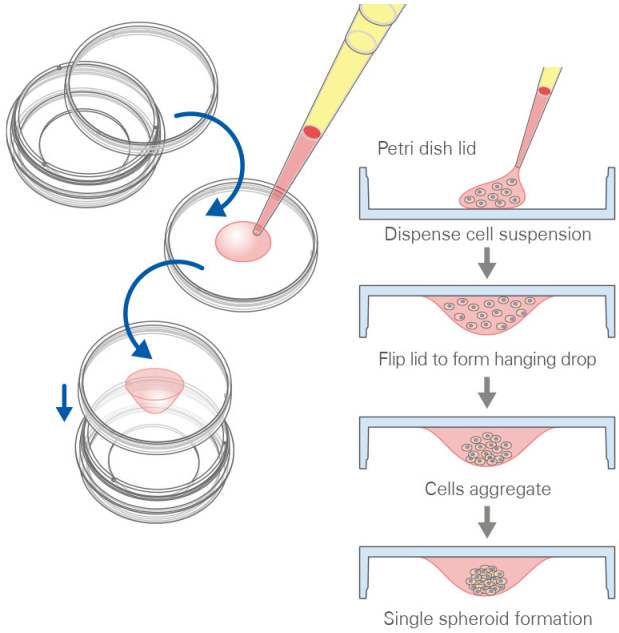

Figure 4: Spheroid formation by flipped hanging drop method [36].

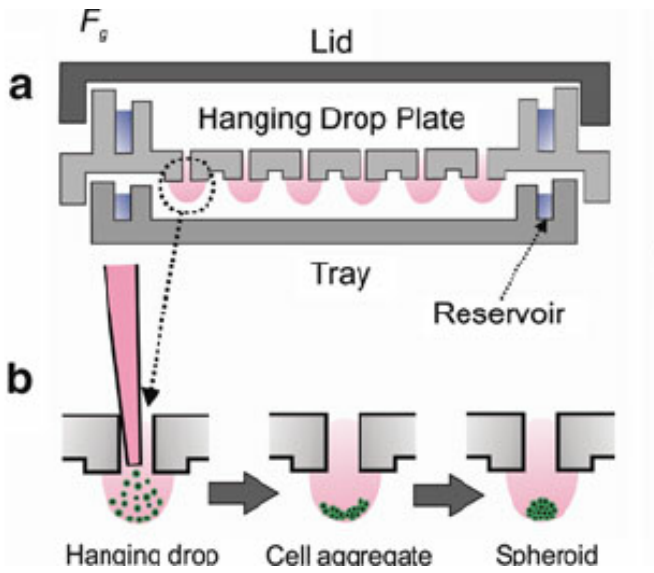

Figure 5: Spheroid formation by hanging drop plate [34].
a. Composition of a hanging drop plate, b. Spheroid formation.

3.2.c. *Matrigel*

The Matrigel method is a scaffold-dependent method [34]. Cells are cultured in low attachment plates for 4 days to form aggregates, then Matrigel is added to encourage cell-to-ECM interactions which will form spheroids after another 2 days (Figure 6) [39].

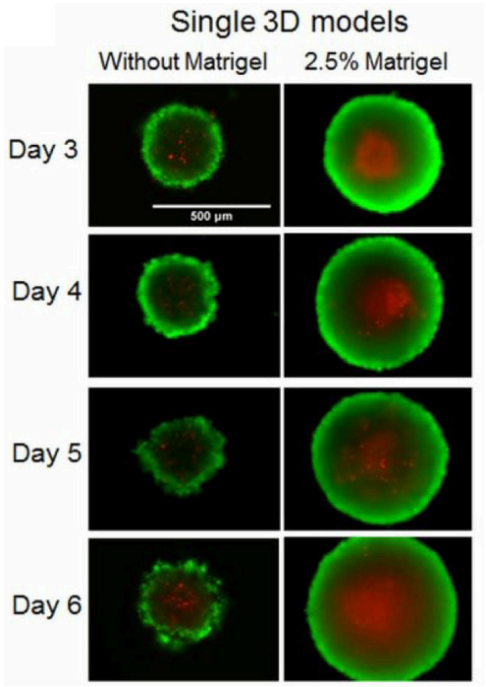

Figure 6: Comparison of single 3D spheroids grown without and with Matrigel [40].
Green indicates live cells and red indicates dead cells.

4. Preliminary Design Evaluation

4.1. Design Matrix for Cell Line

Table 1. Design matrix for Cell Line.

Design Criteria (weight)	NCI-H23		A549		OVCAR-5	
	Score X/5	Weighted score	Score X/5	Weighted score	Score X/5	Weighted score
Viability (20)	4	16	5	20	3	12
Adhesion (20)	3	12	4	16	4	16
Reproduction Speed (20)	3	12	5	20	4	16
Drug Sensitivity (15)	4	12	5	15	3	9
Genetic Mutation (15)	5	15	3	9	2	6
Ease of Procurement (10)	5	10	5	10	5	10
Total Score (100)	77		90		69	

Determination of Criteria and Weights & Justification of Assigned Scores: After discussion about the important criteria to evaluate the initial designs against our Product Design Specifications, the following categories were chosen.

Viability (20):

Cell viability is a measure of the proportion of live, healthy cells within a population [41]. For this category, viability is a measure of how robust the cell is and how well it can withstand various stresses. The highest ranked cell line is predicted to be the healthiest after fluid shear stress, transduction, and cisplatin treatment.

NCI-H23 cells were assigned a score of 4/5 for its and >20% cell death after 72 hours when subjected to [3 μM] of cisplatin and its sensitivity to cisplatin treatment [42]. The NCI H23 cell line showed a higher expression level of DNA repair proteins after cisplatin treatment compared to A549 cells. In previous experiments for both NCI-H23 and A549, a negative correlation between cell viability and DNA damage induction upon cisplatin treatment has been noted [42]. A549 cells were assigned a score of 5/5 for its >20% cell death after 72 hours when subjected to [3 μM] of cisplatin and its greater resistance to cisplatin treatment compared to NCI-H23 cells [42]. OVCAR-5 scored a 3/5 for its severe morphological changes (decreased

spheroid-forming capacity, reduced cytoskeleton organization, and chromosomal instability) after exposure to <1 dyne/cm^2 fluid shear stress and >50% cell death after 72 hours when subjected to [2.5 μM] cisplatin [43], [44].

The type of transduction done to these three cell types may either help or hinder the cell health depending on the transduction virus used (lentivirus or siRNA)and what it is used in conjunction with (Nrf2, cisplatin, or both). The trend in scientific literature indicates that these three cell types have the same viability, within 7±3%, when transfected under the same conditions [42], [43], [44]. Since the vectors encoding the components necessary for CRISPR/Cas are large, they result in low transduction efficiency and cell viability [45]. To overcome those obstacles, the team can add exogenous small plasmids that increase transduction efficiency up to 40-fold and cell viability up to 6-fold [45].

Adhesion (20):

This criteria refers to the ability of cells to attach to the surface of the culture vessel or substrate they are grown in, as well as to attach to other cells. For this criteria, cell lines were therefore judged on whether they were considered an adherent cell line and on their strength of cell-cell interactions. Because adhesion is an important factor in cells being able to effectively form spheroids, this criteria was given a high weight for scoring.

Although NCI-H23 is considered an adherent cell line [25], it is known to have weaker cell-cell interactions due to the lack of E-cadherin at the cell membrane [46]. Additionally, NCI-H23 has presented difficulty in forming spheroids in previous literature, particularly using the Matrigel method [26]. For these reasons, NCI-H23 scored 3/5. Similarly, although A549 and OVCAR-5 are considered adherent cell lines [28], [47], [48], it is also known to have a lack of E-cadherin and thus weaker cell-cell interactions [46], [49]; thus, A549 and OVCAR-5 scored 4/5.

Reproduction Speed (20):

Reproduction Speed is the time it takes the cells to grow and reproduce. In order to look specifically at the cell line, and exclude any factors caused by the spheroid formation protocol, the team used the cell lines' doubling speed. The doubling speed is the amount of time it takes for a cell population to double in number. Essentially, it is how quickly a cell line replicates itself in culture [50].

As each cell line has a range of doubling times depending on the culture medium used, the most typical doubling times were used to determine this category's score. With the longest doubling time of about 38 hours [25], [51], NCI-H23 scored the lowest at a 3/5. A549 is the fastest of the three cell lines with a typical doubling time of about 22 hours [28], [51], scoring a 4/5. Lastly, OVCAR5 has a doubling time of 27 hours [31], [51] scoring a 4/5.

Drug Sensitivity (15):

Gamma-H2AX foci staining is used for this project to assess the extent of CRISPR-Cas9-based DNA damage. Cell lines that are particularly sensitive to drugs that induce DNA damage will have a baseline of DNA damage to refer to, making them appropriate for this project.

Z-scores for NCI-H23, A549, and OVCAR-5 are obtained from the Genomics of Drug Sensitivity in Cancer Project [52]. NCI-H23 has a Z-score average of -1.22 for bleomycin, -0.73 for cisplatin and +0.41 for oxaliplatin [27]. A549 has a Z-score average of -1.04 for bleomycin, -0.12 for cisplatin, and -1.45 for oxaliplatin [53]. OVCAR-5 has a Z-score average of +0.67 for bleomycin, +0.52 for cisplatin, and -0.18 for oxaliplatin [32]. A549 has 2 significant negative Z-scores, scoring a 5/5. NCI-H23 has a negative Z-score for 1 of the drugs, scoring a 4/5. OVCAR-5 has none, scoring a 3/5.

Genetic Mutation (15):

Analysis of genetic mutations inherent to various cell lines is crucial in determining the ideal cell line for our project. A high score in this category means genetic mutations that are helpful must work with for any step in the adhesion process, Cas9 attachment, CRISPR edit, or screen. Using Han's ten selected lung cancer cell lines, a set of ideal cell line mutations were created including: TP53 (tumor protein p53), a gene that encodes a tumor suppressor protein containing transcriptional activation, DNA binding, and oligomerization domains [54]; EGFR, a cell surface protein that binds to epidermal growth factor leading to cell proliferation [55]; and KRAS, Kirsten rat sarcoma viral oncogene homolog causes malignancies based on an error encoding a protein that is a member of the small GTPase superfamily [56]. These mutations may either cause or enhance the malignancy of existing cancer cells. Since TP53 is the most frequently mutated gene in human cancer, the presence of this mutation is crucial for biological relevance to half of all cancer cases [57].

NCI-H23 has all of the key mutations including TP53, KRAS, EGFR, a high degree of c-myc DNA amplification, and no counterproductive mutations, so it was given the highest score of 5/5 [25]. A549 scored second highest with 3/5 because it contains EGFR and TP53 which are key, but also PIK3CA, ALK, and PTEN which aren't key but won't impede the success of the project [29]. The protein encoded by PIK3CA, phosphatidylinositol 3-kinase, is oncogenic and represents the catalytic subunit, which uses ATP to phosphorylate PtdIns, PtdIns4P and PtdIns(4,5)P2 [58]. ALK, anaplastic lymphoma receptor tyrosine kinase, a chromosomal rearrangement which results in creation of multiple fusion genes in tumorigenesis. PTEN guards the genome by controlling multiple processes of chromosome inheritance [59]. Although OVCAR-5 has KRAS, a key mutation, it was ranked the lowest at 2/5 due to the lack of essential protein TP53 and the presence of additional mutation CLDN4, which is a mutation of integral membrane proteins [30], [60], [61].

Ease of Procurement (10):

This criteria assesses how easy it is to obtain the cell line. It is expensive to purchase an entirely new cell line from ATCC, Sigma-Aldrich or similar sources. Therefore, the chosen cell line should be either already available in Hess Lab or can be obtained from other labs, preferably within Wisconsin Institutes for Medical Research (WIMR); however, that process can be time-consuming.

All 3 chosen cell lines are readily available in Hess Lab, both wild-type and genetically-modified, and therefore all scored a 5/5.

4.2. Design Matrix for Spheroid Formation

Table 2. Design matrix for Spheroid formation.

Design Criteria (weight)	Treated Tissue Culture Plates		Hanging Drop		Matrigel	
	Score X/5	Weighted score	Score X/5	Weighted score	Score X/5	Weighted score
Ease of Fabrication (30)	5	30	4	24	3	18
Scalability (20)	4	16	3	12	5	20
Uniform properties (20)	4	16	3	12	2	8
Cost (15)	3	9	4	12	2	6
Safety (15)	5	15	5	15	5	15
Total Score (100)	86		75		67	

Determination of Criteria and Weights & Justification of Assigned Scores: After discussion about the important criteria to evaluate the initial designs against our Product Design Specifications, the following categories were chosen.

Ease of Fabrication (30):

This criteria assesses the complexity of the 3D spheroid formation protocol, which is one of the important factors when choosing an appropriate fit-for-purpose 3D culture model [62]. Specifically, this criteria assesses whether additional specialized training or equipment will be needed for the protocol, on top of those necessary for 2D traditional cell culture methods. Also,

the time needed to complete the protocol once will also be considered, as it can directly affect the length of the project due to the large number of cells needed for genome-wide CRISPR screening.

Treated tissue culture plates method scores a 5/5 as the cell seeding method is not particularly labor-intensive, only requires 1 media change and takes the least time (up to 4 days). Hanging drop method also takes the same amount of time but the cell seeding method requires more careful technique to ensure hanging drop viability and daily media change, thus scoring a 4/5. The Matrigel method does not require extensive cell seeding technique and daily media change but requires longer time (6 days), scoring a 3/5.

Scalability (20):

This criteria assesses the plausibility to use these methods to generate a large amount of viable spheroids. A high-throughput method is preferred because, per the client, each CRISPR screen would require 100-150 million cells per replicate to generate a sufficient amount of spheroids [16]. Because a high-throughput method is very important to successfully completing a spheroid CRISPR screen, this criteria was given a high weight for scoring.

The treated tissue culture plates method is known for its high-throughput capabilities because treated plates have a non-adherent coating that promotes rapid cell aggregation into spheroids [63]. This method also allows for a greater amount of spheroids to be made in a single plate because it can accommodate large volumes of media [64]. For these reasons, this method was given a score of 4/5 for scalability. The Hanging Drop method, in contrast, is considered to be low-throughput in nature. One reason for this is because the method requires a lot of manual handling and is prone to error when setting up and transferring the drops [65]. Additionally, scalability is limited with the Hanging Drop method because each tissue culture plate can only hold a limited amount of drops, and each drop only tends to form one spheroid [37]. For these reasons, this method was given a score of 3/5 for scalability. Lastly, the Matrigel method is considered high-throughput because, like the treated tissue culture plates method, it can allow for a comparatively larger volume of media to generate spheroids compared to the Hanging Drop method. Additionally, this method involves polymerizing a layer of Matrigel matrix onto tissue culture plates to promote spheroid formation [26], and unlike the other two methods, has been shown to improve the growing environment of spheroids due to its mimicry of in-vivo tumor conditions [40]. For these reasons, this method was given a score of 5/5 for scalability.

Uniform Properties (20):

This criteria outlines the reliability of the spheroid formation to produce uniform properties. The ideal formation protocol has no batch-to-batch variability. Minimal variation in size, shape, and porosity is expected so the experiment will have a higher chance of reproducibility and fewer outliers. It is important to note that research has shown that differences in spheroid formation arise from the differences in cell-biomaterial interactions rather than due to differences in cell viability or proliferation [66].

The treated tissue culture plates scored the highest in this category, 4/5, due to their minimal variation between batches. Additionally, treated tissue culture plates have a larger amount of spheroids per batch (see *Scalability* section), making it easier to harvest a uniform size or shape [62]. Due to the low-throughput nature of Hanging Drop, making it harder to harvest uniform spheroids, and due to its 10% to 15% size variation between drops, this method scored a 3/5 [67]. Matrigel, animal-based basement membrane extract, has a batch-to-batch variation that can hinder reproducibility, so a score of 2/5 was assigned to it [66]. Cells seeded in matrigel also tend to form large cell aggregates with looser morphology rather than spheroids with round, regular shape [66].

Cost (15):

This category is scored based on the expenses of the materials needed to perform the spheroid formation protocol. While the client stressed the importance of a working protocol they also expressed their desire to keep costs to a minimum, as to be able to repeat the protocol and perform it on a larger scale.

Explain scoring:

Treated tissue culture plates scored a 3/5 in this category. Generally, the culture plates range from $0.22-3.78 per well [68], with a need for an abundance of wells. The cost of treated tissue culture plates can vary depending on the manufacturer, the number of wells needed, and the volume of cell culture media required. The Hanging Drop method is rather inexpensive as it does not require specialized equipment or reagents. At around $4 [69], [70] per 100 mm plate the Hanging Drop method is the most cost effective method out of all the options and scored a 4/5 [71]. Lastly, Matrigel scored a 2/5 in this category as the Matrigel itself is rather expensive, at $380 for 10 mL of Matrigel [66], [72].

Safety (15):

This criteria assesses the ability of the method to be used in a laboratory environment without posing significant health risks to the user or the experimental results. This includes evaluating the potential hazards associated with the materials/reagents and processes used, the ease of handling, and the effectiveness of protective measures (e.g., PPE, ventilation). A method should be dependable, minimizing the risk of contamination or adverse effects that could compromise the safety of the personnel and the integrity of the experiments.

All of the methods proposed were able to obtain a 5. This is due to none of the methods using harmful reagents in the process of fabrication. All of the cell lines pose similar contamination risk but can be lowered considerably with proper PPE use.

4.3. Proposed Final Design

A549 was the cell line selected based on the team's decision matrix (Table 1). A549 is an adherent cell line which exhibits the most useful mutations (EGFR, TP53, PIK3CA, ALK, and PTEN) [28], [29]. Since this cell line has a doubling time of approximately 22 hours, the team will need to passage the cells 2-3 times a week [73]. Hess lab recommends a maximum of 20

passages be done on each cell line, so, once thawed, each cell line will have approximately 7 weeks before it must be bleached and another cell line thawed [73].

Treated tissue culture plates, or low attachment plates method was the selected spheroid formation protocol selected based on the team's decision matrix (Table 2). The process includes using hydrophilic polymer-coated plates [34], which are not labor intensive to make, and spheroid formation takes around 4 days [35], which is not time-consuming. Furthermore, as this method does not require a scaffold, it does not run the risk of high batch-to-batch variability due to using animal-derived matrices [34].

5. Design Process

5.1. Doubling time

5.1.a. *Calculations used*

See Appendices 12.2 and 12.3 for the full cell line maintenance and passaging protocols. Doubling time of cultured A549 cells was determined based on a Monday-Wednesday-Friday passaging schedule using equation 1 below:

$$t_d = \frac{ln(2)}{\mu} \cdot 24h$$

Equation 1. Equation for calculating a cell line's doubling time. t_d *= doubling time (days) and* μ *= growth rate (1/day).* μ *was obtained from the CytoFLEX cell confluency reading [74].*

Once doubling time was determined, the volume of cells to passage was calculated using equation 2 below:

$$\frac{\#\ cells\ in\ culture\ solution}{10\ mL} = \frac{\#\ cells\ needed\ to\ passage}{volume\ needed\ to\ passage}$$

Equation 2. Equation for calculating the required volume for passaging. The number of cells needed to passage was estimated by looking at previous passage values and their resulting doubling times.

5.1.b. *CytoFLEX Protocol*

Comprehensive CytoFLEX protocol can be found below and in Appendix 12.3. Briefly, ensure daily clean has been run, load a well vortexed sample, alter settings to fast, click run, auto set axes from properties menu, gate a representative live cell population, and save file to BME team folder.

5.1.c. *Final established doubling time*

The cultured A549 cells' average established doubling time (for cells derived from second cryovial thawed) was 43.94 hours. When cutting down to 1.75 million cells for a two-day

passaging period (Monday to Wednesday; Wednesday to Friday), the doubling time was closer to the doubling time of 22 hours observed in literature [28], averaging at 22.93 hours. However, when cutting down to below 1.75 million cells for a 3-day passaging period (Friday to Monday) the doubling time was much higher, averaging at 52.95 hours. It is also important to note that there was significant variability in measured doubling times with cells derived from the first cryovial of the semester, with some doubling times reaching up to 72 hours. A suspected reason for this variability was that step 8) in Appendix 12.3 was not followed consistently by all team members. This likely caused the CytoFLEX cell confluency reading to be underestimated for these passages and, as a result, the doubling times to be higher than expected. See Appendix 12.4 for the cell line maintenance benchling data.

5.2. Spheroid Protocol Selection

Once treated tissue culture plates were selected for our fabrication method, additional research was done to compare the client's spheroid formation protocol to protocols from other literature (section 2.1e). With the client's assistance, the selection of techniques was narrowed down to K. Han *et al.* and J. R. Ferrarone *et al.*. Both protocols were fairly similar in terms of complexity and required materials, but differed greatly in terms of time and scalability (table 3). Overall, a modified version of K. Han *et al.* was used for preliminary spheroid formation and seeding density testing. The main modifications made include using DMEM + 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin rather than the RPMI, using different TC plates, different poly-HEMA and Methylcellulose preparation methods, and exact seeding density. K. Han *et al.* used RPMI to prevent excessive aggregation of cells in the spheroid, but that should not be a concern for the team in this early testing phase. Other alterations made to the protocols will be discussed in the testing and discussion sections respectively. Deciding on an exact preliminary protocol to follow was essential for materials selection and ordering.

Table 3. Additional design matrix for spheroid formation protocols.

Design Criteria	1: K. Han *et al.*	2: J. R. Ferrarone *et al.*
Cost	Medium	Low
Spheroid Formation Time	Overnight*	24 hours
Scalability -cells/spheroid -spheroid #	20k-150k cells/cm2	1k cells/well
Required machinery & materials	1) 500uL media containing 0.75% methylcellulose 2)RPMI 1640 growth medium 3)96-well poly-HEMA TC plates	1) 80 µL of methylcellulose-containing medium 4)RPMI 5) V-bottom 96-well plates

		(low-attachment)

*time does not include Methylcellulose, poly-HEMA, media, or plate preparation.

6. Methods

6.1. Materials

Materials listed are required for culturing and passaging A549 cells and are already available in Hess Lab and thus require no purchasing. One 1 mL cryovial of A549 can be used up to 20 passages and 3 passages will be performed for each week of the project. In total, two cryovials of A549 were used, and a total of 20 passages (15 for the first cryovial and 5 for the second) were performed.

Table 4. Materials List.

Material	Part Specification	Quantity	Price
Already available in Hess Lab, requires no purchasing			
T-75 Flasks	Catalog #156800 - Thermo Fisher Scientific	1 for up to 20 passages	$381.65 / case of 100 [75]
A549	CCL-185 - ATCC	N/A	$555 / vial [28]
DMEM (high glucose)	Catalog #11965118 - Thermo Fisher Scientific	1x500 mL bottle 10 mL / passage (used in solution with Pen/Strep and FBS)	$264.65 / 10 x 500 mL [76]
Penicillin-streptomycin (Pen/Strep)	Catalog #15070063 - Thermo Fisher Scientific	1% in solution with DMEM and FBS	$23.65 / 100 mL of 5,000 U/mL [77]
Fetal Bovine Serum (FBS),Value	Catalog #A5256701 - Thermo Fisher Scientific	10% in solution with DMEM and Pen/Strep	$314.37 / 500 mL [78]
PBS, pH 7.4	Catalog #10010072 - Thermo Fisher Scientific	2 mL / passage	$244.00 / 6 x 1000 mL [79]
Trypsin-EDTA (0.05%), phenol red	Catalog #25300120 - Thermo Fisher Scientific	3 mL / passage	$287.65 / 20 x 100 mL [69]
VWR® Flat-bottom 96-well	Catalog #76446-962 - VWR	2 plates	$494.24 / 100 plates [80]

polystyrene assay plates			
Ethanol	Catalog #BP28184 - Fisher Scientific	33 mL	$1,595.00/ (4 x 4L) [81]
Requires purchasing			
Poly-HEMA	Catalog P3932-10G - SigmaAldrich	1.3 g	$238 / 10 g [82]* 1.3 g = $30.94
Methylcellulose	Catalog M0512-100G - SigmaAldrich	2 g	$51.40 / 100 g [83] * 2g = $1.03
Accutase	Catalog A1110501 - ThermoFisher	Has not been used	$60.65 / 100 mL [84]
		Total:	$92.62

6.2. Protocols

6.2.a. *Hess Lab Cell Line Maintenance*

Thawing Protocol: This protocol is developed by Hess Lab [85]. New media is prepared in a 15 mL tube. Slowly mix the media in the cryovial to thaw cell pellets. Once thawed, cells are pelleted by centrifugation and resuspended in fresh media. Cells need to be grown for typically 3 days before passaging further. See Appendix 12.2 for detailed protocol.

Passaging Protocol: This protocol is developed by Hess Lab [85]. This assumes passaging in a T75 flask (is using a T150, double the volume). Media is removed via aspiration. Wash cells with PBS then detach cells from the culture vessel with trypsin, then media is re-added to inactivate the trypsin. Cells are moved to a 15 mL conical tube, pelleted by centrifugation, and resuspended in new media. Add cell suspension to a new flask and add new media, enough to cover the cells. See Appendix 12.2 for detailed protocol.

6.2.b. *Spheroid formation protocol*

Spheroid formation protocol is based on Han *et al,* 2020 [16]. This semester, cells will be seeded at multiple densities to determine the optimal density for high spheroid numbers, uniform spheroid size, and high percent viability (see section 7).

To prepare 2% methylcellulose solution stock, for spheroid formation, 2 g methylcellulose is dissolved in 100 mL deionized water at 80°C. Then, the solution will be autoclaved then mixed overnight at 4°C. Solution can be kept at 4°C.

To prepare poly-HEMA stock solution to coat plates, 1.3 g of poly-HEMA is dissolved in 33 mL 99% ethanol by mixing overnight at 37°C. The solution is then filtered through a 0.22 µm filter and can be stored at room temperature (20°C). To coat plates, 50 µL of poly-HEMA stock solution is added to each well of a 96-well plate. Plates will be left to dry overnight at room

temperature in an open tissue culture BSC hood for ventilation, and should be partially-closed instead of fully-closed. After drying, poly-HEMA-coated plates can be stored at 4°C for up to one month.

Seed cells at 4 different densities (25k, 50k, 75k, and 150k cells/cm^2) in serum-free DMEM medium containing 0.75% methylcellulose, with 6 wells for each density. FBS is not added to the growth medium because it will promote cell attachment to the vessel wall and thus prevent spheroid formation. The methylcellulose will prevent excessive aggregation of cells in spheroid culture and maintain even spheroid size. Spheroids should be split every 3-4 days, and can be dissociated into single cells using Accutase (see section 6.2.c) and can be reseeded into new spheroids. See Appendix 12.2 for detailed protocol.

6.2.c. *Spheroid Cell Dissociation*

This protocol is based on Honeder *et al*, 2021 [86] and Han *et al*, 2020 [16], and it accounts for dissociation of 6 wells for seeding densities 1-4 (see section 6.2b). Spheroids can be dissociated after 3-4 days, and dissociated cells can be used to form new spheroids to determine the percent viability (see section 7.2). Firstly, 24 1.5 mL Eppendorf tubes are prepared and labeled accordingly, with 6 tubes for each seeding density group. Seeded contents from each well are transferred into their corresponding Eppendorf tubes and diluted with 480 µL PBS. Spheroids are collected via centrifugation at 800g for 15 min, and resuspended in 150 µL Accutase. Spheroids are incubated at 37°C for 10 minutes, and 850 µL serum-free DMEM is added to bring the final volume to 1 mL. See Appendix 12.7 for full protocol.

6.2.d. Lentiviral infection protocol

This protocol was developed by Hess Lab [87]. First, one should count and plate the number of cells listed above for each infection in a well of a 24-well plate (see Appendix 12.9). After filtering with a 0.45µm filter, 1-2 mL of virus(es) are added to each well and left to incubate for 24 hours at 37°C. After incubating, viral media is aspirated from each well and replaced with 1 mL of proper media. 48-72 hours later, the cells should be ready for analysis by fluorescence or selection with antibiotics. See Appendix 12.9 for detailed protocol.

6.2.e. *High-throughput CRISPRi screen protocol*

High-throughput, pooled CRISPRi-dCas9 loss-of-function screening protocol is based on Mathiowetz *et al*, 2023 [88] and may be modified to better suit this project's needs. Constitutive Cas9-expressing cells are first generated through lentiviral transduction. A dose response analysis is then performed to determine the concentration of cytotoxic compounds. For antibiotic selection, this analysis can either be a drug-resistance screen or a drug-sensitivity screen. Next, the dynamic range of the reporter for fluorescence-based assays is determined (aim for a greater distance between high and low fluorescence intensity bins). The sgRNA library is prepared, either by amplification and packaging into lentivirus or purchased as pre-packaged in lentivirus. The sgRNA is introduced via lentiviral transduction (note: this step can be done either together

or separately from the Cas-9 introduction step). sgRNAs present in each cell population are then identified by extracting genomic DNA (gDNA) from frozen cell pellets and amplified by polymerase chain reaction (PCR). The pooled sgRNA library is sent for deep sequencing and readings are analyzed using casTLE. See Appendix 12.10 for detailed protocol.

6.3. Final Prototype

6.3.a. A549

A549 is an adherent cell line isolated from the lung tissue of a White, 58-year-old male with lung cancer [28]. This NSCLC adenocarcinoma has a doubling time of 22 hours [28]. A549 exhibits mutations for TP53, EGFR, PIK3CA, ALK, and PTEN [27]. Since TP53 is the most frequently mutated gene in human cancer, the presence of this mutation is crucial for biological relevance to half of all cancer cases [57]. A549 is very sensitive to bleomycin (Z-score average of -1.04), somewhat sensitive to cisplatin (-0.12), and very sensitive to oxaliplatin (-1.45) [27], [32], [53]. Although one vial of these cells from ATCC is $555.00, Hess lab provided 2 vials for this project [28]. Due to their 50 µm cell diameter, confluency in a T-75 flask was defined as 5,000,000 cells/10 mL.

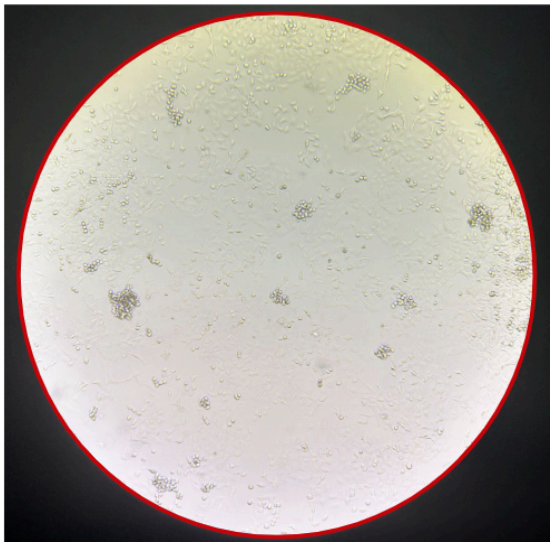

Figure 7: 10x Brightfield image of A549 Passage 5 cells taken at 200% confluency.

6.3.b. Poly-HEMA Plates

For full poly-HEMA stock solution plate preparation see Appendix 12.5.b. Briefly, add poly-HEMA to 99% ethanol, mix solution overnight at 37°C, filter solution into a glass bottle, and keep solution at room temperature until needed.

6.3.c. Spheroid Formation Protocol

For full materials and protocol see Appendix 12.5. Briefly, prepare methylcellulose stock solution, seed cells at multiple densities ranging from 200,000-150,000 cells/cm² (with 500 µL

growth medium/cm²) in Poly-HEMA coated 96-well VWR plate, centrifuge spheroids, and leave in incubator for 3-4 days.

7. Testing

7.1. BioTek Cytation Imaging

In summary, Open BioTek Gen5 3.14 application, place plate in BioTek with A1 well in on the bottom right of the holder like pictured in Appendix 12.6, select new experiment and new protocol making sure that the experiments and photos are saved in the same location and transferred together otherwise both will be lost, set protocol and imaging settings, and click run.

7.2. Percent Viability after Spheroid Formation Determination

The purpose of a spheroid cell dissociation experiment is to determine percent cell viability across seeding densities 1-4. Results from this experiment were meant to inform future percent live/dead experiments using mCherry for alive cells and Sytox Green for dead cells, with the aim to determine the seeding density showing a ~30% peak cell death rate during the initial growth phase of the spheroids [16]. Although this experiment was delayed to next semester due to an unforeseen maintenance issue with the Hess Lab's flow cytometry equipment, the protocol is outlined briefly here. After 1 mL of dissociated cells are obtained (see section 6.2.c), the cellular concentration and number of live and dead cells are counted using the CytoFLEX. The percent cell viability can be determined by comparing the number of live cells to the total number of cells (live and dead cells combined). See Appendix 12.8 for full protocol.

7.3. ImageJ Analysis of BioTek Cytation Images

To begin, choose the most in-focus image (figure 8a) for the desired well and upload it to an image analysis tool named ImageJ. Measure the scale bar in the corner of the image using the line tool and measurement feature. Set the scale under 'Analyze' → 'Set Scale' by setting the 'distance in pixels' to the measured amount of pixels from the images scale bar length. Then set the known length to the scale bar length in μm, and changing the 'Unit of length' to read um in order to output results in micrometers. Select the 'Global' box to retain the scale bar incase of needing to restart the analysis process with an image, then select 'OK'.

Starting image analysis, change the image type to a 16-bit under 'Image' → 'Type' → '16-bit' in order to use the 'Threshold' feature. Next, select 'Image' → 'Adjust' → 'Threshold'. Uncheck the box titled 'Dark background' if selected, then move the ranges until the desired section shows the spheroids highlighted in red, as seen in figure 8b, then hit 'Apply'. Threshold recognizes pixels containing cells, or in this case spheroids. Next, go to 'Process' → 'Binary' → 'Watershed', which separates any spheroids that were close together but were recognized as one by the 'Threshold' feature. In order to block out any background noise from the edges of the well use the ellipse tool to encircle only the center of the well being analyzed (Figure 8c).

To analyze the highlighted spheroids click 'Analyze' → 'Analyze Particles'. Set the size range to 4147-infinity, as to ensure only spheroids of 20 or more cells were being analyzed. In

the dropdown menu under ‘Show:’ select ‘Outlines’ then underneath select the ‘Display results’, ‘Clear results’, and ‘Exclude on edges’ boxes before hitting ‘OK’ (Figure 8d).

To save the results one of two windows will pop up. 1. A window asking to save measurements or 2. A results window. Should option 1 appear, select save measurements, but if option 2 appears, select ‘File’ → ‘Save As’ then save results as an excel file to a secure and known location. Combine the excel files into one master document for data analysis.

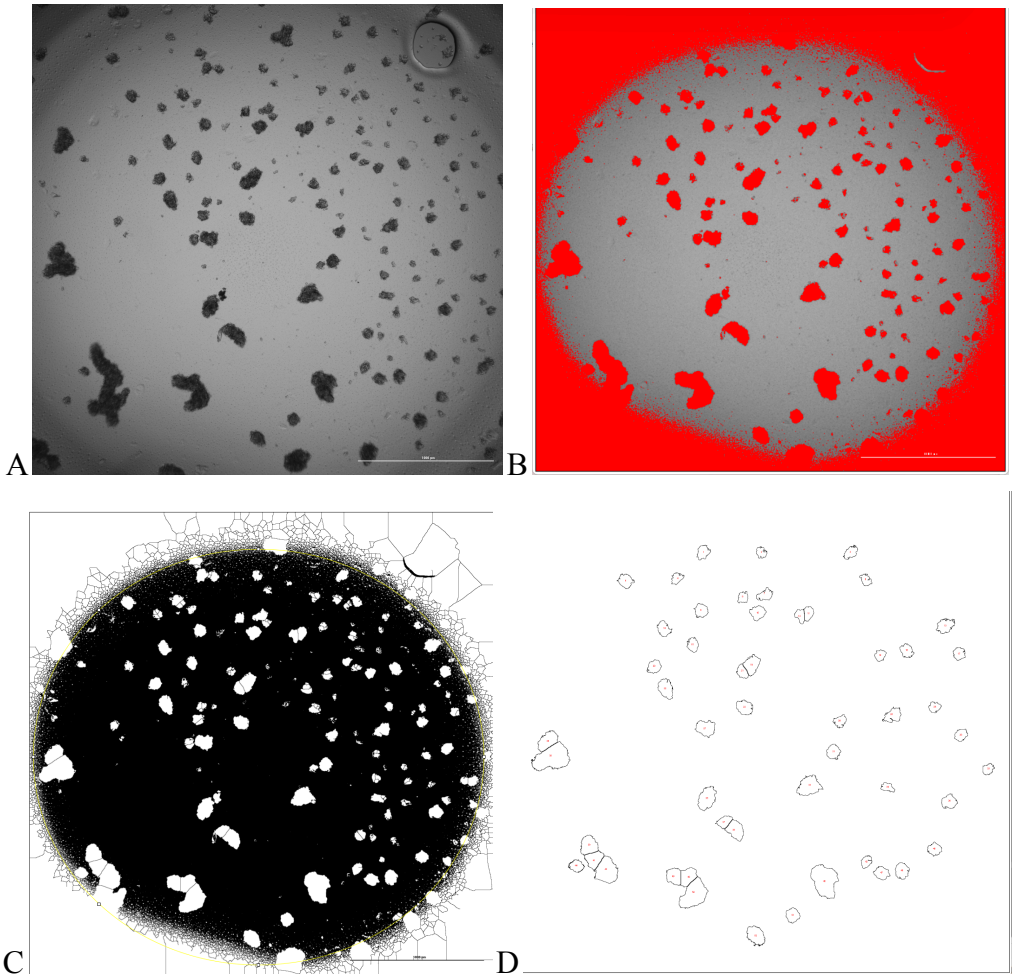

Figure 8: A. Initial image of well G1 from BioTek Cytation imager B. Threshold applied to image C. Binary→Watershed applied to the image with a thin yellow ellipse enclosing the well space D. Outlines of all spheroids analyzed for well G1

7.4. qPCR for Stemness Markers

This protocol was developed by Hess Lab [89]. A quantitative polymerase chain reaction (qPCR) will be performed to test for the expression level of stemness marker genes indicative of spheroid formation. For the purposes of this project, SOX2 and KLF4 will be the marker genes selected due to their association and upregulation in A549 spheroid cells [9].

First, RNA is extracted using the Qiagen RNAeasy Kit. cDNA template is then synthesized using extracted RNA. Primers for gene(s) of interest are designed, along with

preparing a loading control primer. qPCR is then performed using the Taqman method. CT values obtained for experimental samples compared to control are analyzed to determine if the expression of gene(s) of interest has been perturbed. See Appendix 12.11 for detailed protocol.

8. Results

The results from the ImageJ analysis were compiled into one master document to further analyze. The average number of spheroids per density and the average spheroid size per density were taken.

First the average spheroid size per density was taken by averaging all the data from the six wells of each density. There appeared to be no apparent correlation between size and density as the average was 5675, 8558, 6301, and 9232 μm^2 for 25k, 50k, 75k, and 150k cells/cm^2 respectively.

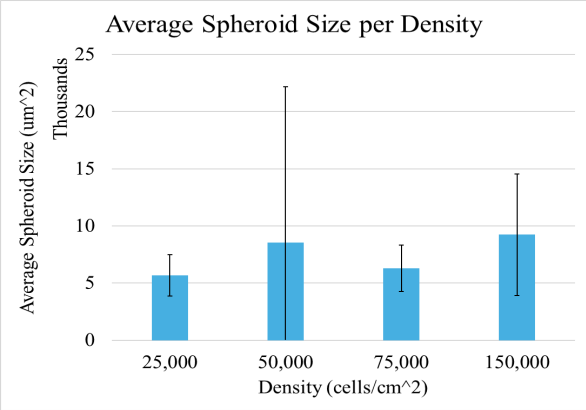

Figure 9: Average Spheroid Size per Density

Secondly, the average number of spheroids per density was taken by once again averaging all the data from the six wells of each density. There appeared to be a positive correlation between an increase of spheroid number to an increase in density. The average were 4, 23, 39, and 60 spheroids for 25k, 50k, 75k, and 150k cells/cm^2 respectively.

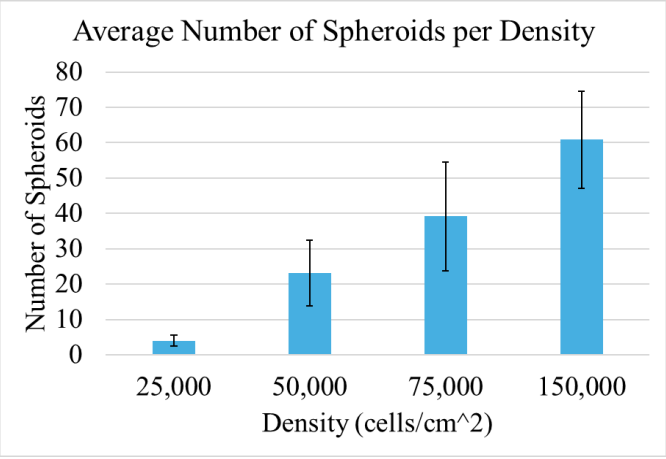

Figure 10: Average Number of Spheroids per Density.

9. Discussion

The passaging protocol for A549 cells was optimized to reflect the established doubling time of approximately 23 hours. This optimization ensured consistent cell viability and growth, avoiding over-confluence that could disrupt spheroid formation and cellular responses. By implementing a schedule of one passage every 48-72 hours, the team maintained cellular integrity, aligning with literature values and reducing variability caused by inconsistent handling. During the spheroid formation process, the results demonstrated a linear increase in spheroid number with seeding density. The average spheroid size remained within the 50–800 μm diameter range reported in the literature. Towards the end of the semester, the team could have made more spheroids to further back the results seen in figure 10, but issues with the CytoFLEX led to a change in plans. Next semester a repeat trial of seeding density variations using a live/dead cytometry assay will confirm the preliminary findings regarding spheroid viability and optimal seeding conditions. Additionally, in creating uniform spheroids, the methylcellulose concentration for each well is then considered optimized.

While the team was successful in creating spheroids, performing qPCR for SOX2 will have to be done next semester. It is hypothesized that gene expression differences are to be seen when comparing 3D spheroids and 2D monolayer cultures, with the idea that a 3D model is better at mimicking in vivo tumor microenvironments. SOX2 amplifies the ability of cancer cells to sustain growth and resist apoptosis, which is why it is a significant measurement of tumorigenicity. The SOX2 qPCR would provide valuable information pre-CRISPRi screen. For instance, the CRISPRi screen could knockdown a gene such as SOX2, allowing for repression at the transcriptional level to see how the gene played a role in growth and survival. The more favorable a gene is, the more DNA damage will be seen due to its repression. While in hindsight this process seems to be moving in the right direction, there is currently no data on CRISPRi screening within a A549 spheroid model, which leaves the results and processes involved open to discussion.

Lastly, the final future work aspect of this project involves testing the processes of dissociation, staining, and fixing the spheroid cells for γH2AX. This will help determine how the specific genes targeted respond to the repression from a CRISPRi screen. γH2AX is a marker for DSBs, which will either be increased or decreased as a result of gene repression. DSBs are one of the more severe forms of DNA damage, where both DNA helix strands are broken simultaneously. These breaks can be the result in many cases from ionizing radiation, reactive oxygen species, or chemotherapeutic agents for cancer treatment. By utilizing γH2AX staining in a 3D environment, more accurate predictions can be made about what genes contribute to tumor progression and metastasis. The goal is to refine these methods and achieve these significant milestones by May 2025.

10. Conclusions

This semester the team was able to select a viable adenocarcinoma non-small cell lung cancer cell line: A549. The cell line was chosen based on characteristics such as: doubling time, adherence properties, and key tumor protein 53 mutation. The cell line was maintained and counted to stay at a consistent 60-80% confluency, which for A549 cells is 3 to 4 million cells per vial. For spheroid formation, poly-HEMA treated plates were chosen due to their cost-effectiveness and ability to produce a consistent size and shape of spheroids. The crosslinking of poly-HEMA prevents cell attachment and promotes cell-cell aggregation into 3D spheroids that are better at mimicking an *in vivo* environment than a traditional 2D structure. Current research has an optimal spheroid size for the A549 cell line around 500μm, so testing data from next semester will be used to compare against this value. By optimizing a spheroid formation protocol, the team will be able to begin working on a γH2AX staining protocol at the beginning of next semester.

11. References

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12. Appendix

12.1. Product Design Specifications

Created: September 19, 2024

Function: Although previous CRISPRi screening in 2D monolayers has provided useful knowledge on cancer drivers and therapeutic susceptibilities, it lacks an element of biological relevance to an *in vivo* environment. Therefore, our team was tasked with developing a cell culture method that is compatible with a 3D environment and CRISPRi screening in order to identify sources of DNA mutations in the tumor environment. On a high scale, the team must select a viable cell line for the screen, create and optimize a spheroid formation protocol, and develop a protocol to stain for γH2AX: a histone variant that acts as a sensitive marker for DNA damage.

Client requirements:

1. The team must choose an appropriate human cell line for the project, ensuring to maximize cell spheroid viability.
2. The team must develop a spheroid formation protocol for the chosen cell type or line that optimizes seeding density and viscosity.
3. The team must optimize the cancer spheroid characteristics and their formation protocols for CRISPR screens.
4. By May 2025, the team must develop a protocol to stain for γH2AX: an indicator for the location of maximal damage due to DNA double-strand breaks (DSBs).
5. By May 2025, the team must perform high throughput genome-wide CRISPR screening to check for the effects of different genes on the amount of γH2AX detected.
6. Future steps post-design project include performing an analysis to determine the phenotypic differences between cells grown in monolayers (2D) versus cells grown as spheroids (3D).

Design requirements:

1. Physical and Operational Characteristics
    a. *Performance requirements:*
        i. Spheroid formation protocol should be scalable, repeatable, and optimized regarding:
            1. Seeding densities to ensure only 1 single guide (sgRNA) will enter each cell.
            2. Viscosity of growth medium (by changing the amount of methylcellulose) to ensure homogenous spheroid size.
            3. Optimizing reagent percentages of growth medium to ensure maximum cell growth.

ii. γH2AX staining protocol should be scalable, repeatable and optimized regarding the antibody used and amount of reagents used.

b. *Safety:*

i. Biosafety Level 2 (BSL-2) is necessary for work with agents associated with human tissue and disease including the human lung cancer cell line which the team will be using [1]. The following universal precautions for BSL-2 are used to reduce the risk of bloodborne pathogens:

1. Laboratory personnel receive specific training in handling pathogenic agents and are supervised by scientists competent in handling infectious agents and associated procedures [1].
2. Access to the laboratory is restricted when work is being conducted [1].
3. All procedures in which infectious aerosols or splashes may be created are conducted in BSCs or other physical containment equipment [1].

ii. Cultures, tissues, specimens of body fluids, or potentially infectious wastes are to be placed in a container with a cover that prevents leakage during handling, processing, and storage [2].

iii. Although the team does not anticipate the need to remove our cells or cancer spheroids from Hess Lab, the following BSL-2 guideline must be followed.

1. Biological agents prepared for transport must be placed in a secondary leak-proof, unbreakable carrier. Carriers must have the biohazard label, including the identity of the agent, affixed to the outer surface of the transport container [2].

iv. If genome editing leads to the inactivation of tumor suppressors or activation of oncogenes in human cells, then oncogenesis becomes a possibility in the event of exposure [3]. This is a concern when:

1. Genome editing components are delivered by a method that can enter human cells (e.g., viral vectors that can infect human cells).
2. All components required for genome editing are likely to be introduced in a single exposure event, such as a needle stick or mucosal splash.
3. Genome editing is designed to target human oncogenes/tumor suppressors or can target human oncogenes/tumor suppressors due to homology.

v. A risk assessment and containment preparations for research with lentiviral vectors should consider (1) the nature of the vector system, (2) transgene insert, and (3) type of manipulations involved [4]. Either BL-2

or enhanced BL-2 will be appropriate for these such experiments. The major risks to be considered for research with HIV-1 based lentivirus vectors are (1) potential for generation of replication-competent lentivirus (RCL) and (2) potential for oncogenesis [4]. These risks can be mitigated by the nature of the vector system (and its safety features) or exacerbated by the nature of the transgene insert encoded by the vector [4].

c. *Accuracy and Reliability:*
    i. A starting cell seeding density of 50,000 cells/cm² will be used. Cells at these densities will be monitored for growth and death rates on a live-cell microscope for 60 hours to confirm a ~30% cell death rate during the initial growth phase of the spheroids [5].
    ii. Cell seeding densities must be such that only 1 single guide (sgRNA) enters each cell to allow for analyzable results. Therefore, a total of 100-150 million cells will be required for each genome-wide CRISPR screen [6].
    iii. A minimum of 3 γ-H2AX staining experiments will be performed to establish confidence in results.

d. *Life in Service:*
    i. Before preparing them for CRISPRi screening, cells will need to be in culture for approximately 2 months to allow for optimization of the spheroid formation protocol, including the spheroids' size and cell quantity. This time period may consist of thawing a new vial of cells for continuing cell culture if the passage number exceeds 25 [7].
    ii. After infection with the selected genome-wide library, a minimum of 6 days will be required before the cells can be further utilized [5].
    iii. After generating spheroids from these cells, a minimum of 21 days will be required for the genome-wide screening [5].

e. *Shelf Life:*
    i. When not in culture or experimental use, cells will be stored using cryogenics for long-term use. Frozen cells will be kept in cryovials with medium containing a cryoprotective agent like DMSO. These vials will be immersed in liquid nitrogen at a temperature below -135℃ [8].

f. *Operating Environment:*
    i. Cells needed to generate spheroids for this project will be kept in standard incubation conditions: 37℃, 5% CO_2, and 90–95% relative humidity [9].

ii. Cells in culture will be maintained in a cell culture medium supplemented with fetal calf/bovine serum to deliver essential growth factors. The medium will contain phenol red, which will indicate the need to replace it if it turns yellow (acidic) or purple (alkaline) [9].
iii. For preparation for CRISPRi screening, spheroid cells will be exposed to a pooled single-guide RNA (sgRNA) library and CRISPR associated protein 9 (Cas9) via lentiviral vectors [10].

g. *Ergonomics:*
   i. Protocols should create a more efficient and accurate experimental system that better mimics *in vivo* tumor conditions, while proving the effects of γH2AX in DNA damage.
   ii. The design of cell culture methods should be optimized by selecting appropriate cell lines and developing correct spheroid formations to enhance the accuracy, relevance, and scalability of CRISPRi screening in a 3D tumor model.
   iii. The γH2AX staining protocol should be optimized for improved precision. Additionally, γH2AX foci counting should be performed via high-throughput screening for efficiency.

h. *Size:*
   i. Spheroids will be generated in 60 mm tissue culture plates [5].
   ii. Cell seeding density for spheroids will be restricted to 50,000 cells/cm² to ensure appropriate CRISPRi screening [5].
   iii. To adhere to a spheroid size suitable for γH2AX staining, spheroids will be kept to ~500 μm in diameter [11].

i. *Weight:*
   i. Although solutions have been presented for single-cell analysis, literature does not cover this aspect for 3D models such as spheroids [12]. Tools like a flow-apparatus are needed for the accurate measurements of mass density, size and weight of such spheroids [12].

j. *Materials:*
   i. In order to leave room for the engineering design element, the client did not give specific requirements for materials other than cell type to be used for the project.
   ii. The client has recommended that the team select a human lung cancer cell line that (1) adheres well to spheroids and (2) has a high percentage of viable cells.

k. *Appearance:*
    i. Since the spheroids should have an overall appearance that will accurately replicate the 3D tumor microenvironment, the spheroids must be: translucent, porous, uniformly spherical, and have a stiffness analogous to the lung cancer cell line selected [13].

2. Production Characteristics
    a. Quantity:
        i. By the end of this semester, the team will design, fabricate, and develop two appropriate protocols for the chosen cell line, one for spheroid formation and one for γH2AX.
        ii. The final protocols should be capable of accurately mimicking the 3D tumor environment for CRISPR screening, including features for effective spheroid formation and reliable γH2AX staining to assess DNA changes.
        iii. The protocols can be adapted to other similar human cancer cell lines; however, some of the characteristics may be subjected to change.

    b. *Target Product Cost:*
        i. The budget for the project is $1,000. Some of the materials and services that the team expects to use are listed below; however, many are already available in Hess Lab and will not need to be purchased.
            1. Biological materials such as reagents (for various media), antibodies (for γH2AX staining) and lentivirus for gene insertion.
            2. Obtaining new cell lines if needed.
            3. Designing of CRISPR library.
        ii. This project is a part of an emerging field, so similar products on the market are not available for non-clinical purchasing purposes. Therefore, the target cost of the final prototype is to remain within the budget proposed.

3. Miscellaneous
    a. *Standards and Specifications:*
        i. Cell lines used for this project should comply with the requirements outlined in part 1b - *Safety* above. Specifically, cell lines used and their associated reagents and protocols will need to comply with requirements for BSL-2 labs; meaning, they are or will contain bacteria, viruses, and organisms that pose a moderate safety hazard to healthy adult humans [14].

ii. The use of human cell lines and other organisms in research for this project is outlined in various standards and regulations including Section 29 CFR 1910.1030 *(OSHA Bloodborne Pathogens),* Section 21 CFR 876.5885 *(Tissue culture media for human ex vivo tissue and cell culture processing applications),* and a comprehensive cell line guideline by Geraughty, *et al* (2014):
    1. Bloodborne pathogens are microorganisms in the blood that can infect and cause diseases in humans [15].
    2. Human cell lines are designated by the FDA as Class II (Special Controls) [16].
    3. If applicable, genetic modification processes on the cell line must be detailedly-noted and kept track of, including but not limited to, sequence details, insertion vectors and modes, and antibiotic resistance markers [17]. Following modification using lentivirus, cell lines may be additionally tested to confirm non-infectivity [17].

iii. Human cell lines that may be needed for this project are already available in the client's lab, or can be obtained from another lab, meaning they already comply with the requirements listed above. In the case that biological research materials are obtained from another lab or institute, an MTA (material-transfer agreements) must be signed by both the provider and recipient to define their rights regarding the materials [17].

iv. In the unlikely case that new human cell lines must be acquired, additional ethical and authentication considerations must be taken into account:
    1. Ethical considerations are outlined in Part 3c - *Patient-related concerns* below.
    2. For cell line authentication, short tandem repeat (STR) is the recommended profiling method, outlined in ASN-0002 *(Authentication of Human Cell Lines: Standardization of STR Profiling)* provided by the American National Standards Institute (ANSI) and the American Type Culture Collection (ATCC). STR is a DNA-based sequence method that allows for detection of mutations and adventitious agents, as well as confirmation of the origin of the interested cell line [18].

b. *Patient-related concerns:*

i. As cell lines and other tissue materials needed for this project are either already available in the client's lab or in another lab, their ethical and other patient-related considerations have already been considered.

ii. In the unlikely case that a new cell line is obtained from patients or donors, the Patient Consent Form and associated Patient Information Sheet will be needed to explain the need for the specimen and the purpose and importance of the research [17]. Patient/donor and original tissue information such as age, sex, clinical history, site of origin, nature of tissue specimen, stage and grade of cancer or other disease/ pathology, and other important information must also be recorded.

iii. For maximal biological relevance to patient *in vivo* tissue, the team will ensure our spheroids are designed based on critical 3D parameters: stiffness analogous to lung cancer tissue, replication of the complex cell-cell interactions and cell-extracellular matrix (ECM) interactions, and increased accuracy in CRISPRi screening environment to better detect precancerous cells [19], [20].

c. *Competition:*

i. 3D cancer spheroid models that can be utilized for CRISPR-Cas9 screens have been implemented in previous research, with and without using an exogenous extracellular matrix (ECM).

1. Takahashi *et al*, 2020 utilized 4% Matrigel™ as a bio-scaffolding to plate A549 and H1437 cells spheroids, which can better model *in-vivo* interactions between cells and the ECM [21]. The study's CRISPRi library gene list as well as its phenotypes have a strong overlap with those from Han *et al*, 2020 (described below), suggesting that inner cells of ECM-based 3D spheroids may be similar to ECM-free 3D spheroids.
2. Han *et al*, 2020 devised an ECM-free scalable method to propagate 3D spheroids [5]. H23 cells were seeded on pre-treated ultra-low attachment or poly-HEMA-covered plates in RPMI 1640 medium with 0.75% methylcellulose. Similar methods have been found to be implemented in other studies, such as by Ferrarone, *et al* (2024) and by Stiff, *et al* (2024) [22], [23]. A total of around 210,000 single guide RNAs (sgRNAs) were designed for the CRISPRi library with around 10 sgRNAs per gene [5]. Samples were used to calculate growth and/or tested for drug resistance [5].
3. 3D stem cell models have also been genomically edited via CRISPR-Cas9 to better model various disease phenotypes. Cells can be assembled with or without supporting scaffolds. Some example scaffolds include laminin-rich Matrigel, collagen matrix, or those that are naturally derived from decellularized tissues [24].

ii. γH2AX has been used in various research as a marker to monitor DNA damage and repair, such as in understanding the toxicity of cigarette smoke or in understanding the effects of chemotherapeutic treatments in cancer patients [25], [26]. High throughput systems have also been developed to aid with counting γH2AX foci [26].

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12.2. Hess Cell Line Maintenance Protocol

Thawing

1) Prepare 10mL of the appropriate media in a 15ml tube.
2) Using a P1000 slowly mix the media in the cryovial to thaw the pellet of cells.
3) Set the pipette to something below 1000uL, maybe 900uL to avoid clogging the filter while resuspending
4) Once thawed, add to the remaining media and spin down at 1400 rpm for 5 min.
5) Resuspend the cells in fresh media and add to a flask to incubate for typically 3 days before passaging further.

Passaging Adherent (eg. A549) Cell Line

1) This assumes passaging in a T75 flask, if using a T150, double the volumes)
2) Remove all media with Pasteur pipette and vacuum
3) Add 2ml of PBS to remove remaining media and remove with vacuum
4) Add 2mL trypsin and wait five min at 37C
5) Add 6mL media to neutralize trypsin while mixing to remove cells from the bottom of the flask (at least 3x the volume of trypsin used)
6) Wash the flask with the cells in media, remove and add to a 15ml conical tube
7) Return enough media to the flask so cells will be confluent when next checked
8) Add additional media (eg. 10 ml)
9) The media just needs to be enough to cover the cells, provide nutrients and won't dry out. Adding more media doesn't change the confluence of the cells.

Counting Cells

10) Add 100uL of cells in media to 900ul PBS in a microcentrifuge tube.
11) This may not be necessary for less confluent cells, but highly dense cells, especially adherent cells, tend to clump together and throw off the count.
12) Measure 10uL on the cytometer and gate for the live population of cells
13) Use the "live" number of events to calculate the confluence and total number of cells you have.
14) Since the cells have been diluted 1:10, the "live" events gives you the number of cells per uL, multiply this by 1000 to get the number of cells per mL.

References

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12.3. CytoFLEX Operation

Once the CytoFLEX and its corresponding computer have been turned on, the following steps must be followed:

1) In the top left click Cytometer > Daily clean
    a) Load Blue tube and press run (3 minutes)
    b) Load DI water and press run (1 minute)
2) To create a new experiment click file> new> from template>Browse
    a) Click into BME Team cell counting file on computer drive Documents>CytoFLEX>BME Team
3) Name file after current date and passage number
4) Vortex sample
5) Uncap sample in 1 mL Eppendorf container and place in CytoFLEX
6) Select fast in settings on left side
7) Click record
8) Once a dot plot of the events has been created right click on the plot>properties
    a) Auto set x-axis
    b) Auto set y-axis
9) Right click on graph and select polygon tool
10) Gate the cells in a oblong hexagon fashion trying to bin all relevant events (general note: live cells are larger than dead cells)
11) Save file
12) Rerun daily clean (step 1)

12.4. Benchling Confluency Tracker

A549 Confluency Tracker

	Date	Passage Number	Cytoflex Confluency	Cells per mL	Total cells in solution	Doubling time	Cut Back To
1	10/11/24	0 (thaw)	N/A	N/A	1000000	Thaw	
2	10/14/24	1	5309	530900	5309000	28.2	1000000
3	10/16/24	2	5094	509400	5094000	20.4	750000
4	10/18/24	3	1956	195600	1956000	36.2	1200000
5	10/21/2024	4	2393	239300	2393000	72.3	2000000
6	10/23/2024	5	9156	915600	9156000	21.9	1500000
7	10/25/2024	6	3147	314700	3147000	44.9	1750000
8	10/28/2024	7	3708	370800	3708000	54.0	1875000
9	10/30/2024	8	8804	880400	8804000	21.1	1750000
10	11/1/2024	9	4187	418700	4187000	42.9	1650000
11	11/4/2024	10	4506	450600	4506000	46.9	1750000
12	11/6/2024	11	8117	811700	8117000	21.7	1700000
13	11/8/2024	12	4208	420800	4208000	35.7	1650000
14	11/11/2024	13	3305	330500	3305000	68.4	1725000
15	11/13/2024	14	8124	812400	8124000	21.5	1710000
16	11/15/2024	15	4973	497300	4973000	33.1	1650000

A549 Confluency Tracker1

	Date	Passage Number	Cytoflex Confluency	Cells per mL	Total cells in solution	Doubling time	Cut Back To	mL to Keep
1	11/15/2024	0 (thaw)	N/A	N/A	1000000	Thaw	1000000	
2	11/18/2024	1	9296	929600	9296000	22.4	1750000	1.9
3	11/19/2024	2	3535	353500	3535000	23.7	1750000	5.0
4	11/20/2024	3	3426	342600	3426000	22.7	1500000	4.4
5	11/22/2024	4	2985	298500	2985000	50.4	1600000	5.4
6	11/25/2024	5	3934	393400	3934000	55.5	1700000	4.3

12.5. Spheroid formation protocol

12.5.a. *Methylcellulose Stock Solution Preparation*

Materials:

- 2 g Methylcellulose
- 100 mL deionized water (diH_2O)
- 1x beaker that can hold 100 mL
- 1x heated stirrer plate
- 1x magnetic stir bar
- 1x 500 mL glass bottle with screw cap

Steps:

- Add stir bar into glass bottle
- Add methylcellulose and diH_2O into glass bottle
- Mix solution at 80°C until homogeneous
- Autoclave solution using Liquid 2 (L2) setting with screw cap loose
- Move solution into walk-in fridge and mix overnight at 4°C
- Keep solution at 4°C until needed

12.5.b. *Poly-HEMA Stock Solution Preparation*

Materials

- 1.3 g poly-HEMA
- 33 mL 99% ethanol
- 1x beaker that can hold 33 mL
- 1x heated stirrer plate
- 1x magnetic stir bar
- 1x 0.2 μm filter (Nalgene) or something similar
- 1x 250 mL glass bottle with screw cap

Steps:

- Add poly-HEMA to 99% ethanol
- Mix solution overnight at 37°C
- Filter solution into glass bottle
- Keep solution at 20°C (room temperature) until needed

12.5.c. *Cell Seeding*

Materials:

- Methylcellulose stock solution
- Poly-HEMA stock solution
- Cells collected after passaging
- Serum-free DMEM
- 15 mL conical tubes
- Eppendorf tubes
- 96-well plates

Procedure:

1) Collect cells from that day's passage and obtain cell concentration using the CytoFLEX to obtain cell concentration (cells/mL).
2) Obtain a 1.5 mL cell solution of 500,000 cells/mL (750,000 cells in total)
    a) Volume needed to obtain 750,000 cells: 750,000 (cells) / cell concentration from CytoFLEX (Step 1, cells/mL)
    b) Obtain the volume calculated from the T75 flask and transfer into a 15 mL conical tube.
    c) Spin down the the tube at 200g for 5 minutes, then remove supernatant to collect cells using vacuum filter
    d) Resuspend cells in 1.5 mL of serum-free DMEM.
3) Prepare master mix of cell solutions for spheroid formation for 4 different densities. The master mix can be used for 6 wells in a 96-well plate and can be scaled to adjust for different numbers of wells if needed.
    a) Density 1: 25,000 cells/cm^2
        i) Into a new 1.5 mL eppendorf tube, add:
            (1) 100 µL of 500,000 cells/mL cell solution (Step 2)
            (2) 525 µL of serum-free DMEM
            (3) 375 µL of 2% methylcellulose stock
        ii) Mix gently by pipetting up and down, make sure the solution is well-mixed
    b) Density 2: 50,000 cells/cm^2
        i) Into a new 1.5 mL eppendorf tube, add:
            (1) 200 µL of 500,000 cells/mL cell solution (Step 2)
            (2) 425 µL of serum-free DMEM
            (3) 375 µL of 2% methylcellulose stock
        ii) Mix gently by pipetting up and down, make sure the solution is well-mixed
    c) Density 3: 75,000 cells/cm^2
        i) Into a new 1.5 mL eppendorf tube, add:

(1) 300 µL of 500,000 cells/mL cell solution (Step 2)
(2) 325 µL of serum-free DMEM
(3) 375 µL of 2% methylcellulose stock

ii) Mix gently by pipetting up and down, make sure the solution is well-mixed

d) Density 4: 150,000 cells/cm^2

i) Into a new 1.5 mL eppendorf tube, add:
(1) 600 µL of 500,000 cells/mL cell solution (Step 2)
(2) 25 µL of serum-free DMEM
(3) 375 µL of 2% methylcellulose stock

ii) Mix gently by pipetting up and down, make sure the solution is well-mixed

4) Aliquot the prepared master mix into each well: add 160 µL of cell mixture into each well.
5) Grow spheroids in 37C, 5% CO2 incubator. Spheroids are split every 3-4 days.

References

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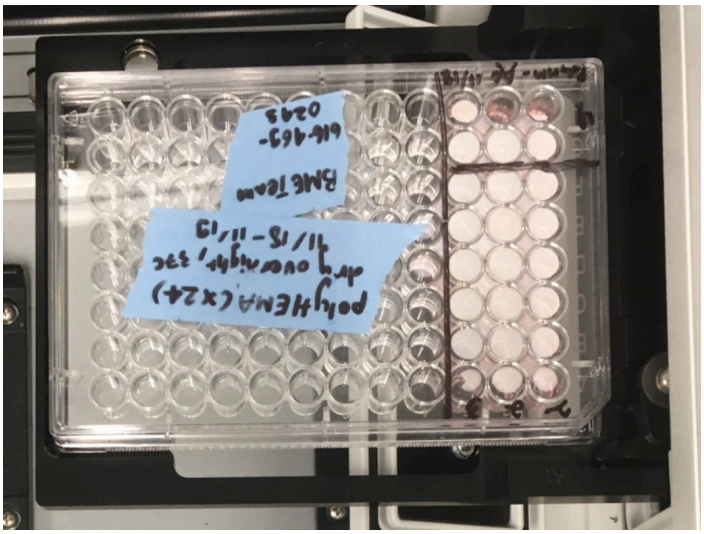
12.6. BioTek Cytation Imaging Protocol

1. Turn on and sign in to BioSpa associated computer.
2. Open BioTek Gen5 3.14 application
3. Place plate in BioTek like in the following image, make sure that the A1 well is on the bottom right of the holder.
    a. 
4. Select new experiment and new protocol
    a. Name with experiment type and date
5. Set protocol procedure
    a. Set temperature to 37°C
    b. Middle imaging (images taken from middle of well)
    c. Deselect auto exposure
    d. Choose plate type: Falcon® 96-well flat-bottom 300 µL
6. Imaging settings
    a. 4x magnification
    b. Brightfield
    c. FVOW
7. Click microscope image
    a. Find well and click auto exposure
    b. For z-stack: set focus distance from bottom
    c. Set z-stack = 5, distance = 80 µm
    d. Select which wells to image
8. Save images and experiment directly to computer
    a. Save > Options > save options > experiment > images
    b. This PC > Users > Public > documents > protocol > Hess lab > BME Team
    c. This PC > Users > Public > documents > experiment > Hess lab > BME Team
9. Run experiment trial by clicking “play” button

Please note:

- Experiments and photos must be saved in the same location and transferred together otherwise both will be lost.
- Complete set up and run takes around 30 minutes for 24 wells.

12.7. Spheroid Dissociation

Materials

- Eppendorf tubes
- P200, P1000 pipettes, 15 mL serological pipets
- Accutase
- Full DMEM (DMEM + 10% FBS + 1% Pen/Strep)
- Formed spheroids (see Appendix 12.5)

Protocols:

1. Prepare 24 eppendorf 1.5 mL tubes (for dissociation of 24 wells). Label them accordingly.
2. Transfer the media (with cancer spheroids inside) from the wells into the according eppendorf 1.5 mL tubes using a 15 mL serological pipet.
3. To each eppendorf 1.5 mL tube, add 480 µL of PBS.
4. Pellet the spheroids via centrifugation at 800g, 15 min, then remove the supernatant using a pasteur pipet.
5. Resuspend the pellets in 150 µL Accutase using a P200 pipette. Thoroughly mix the solution by pipetting up and down 10 times using a P200 pipette set to 100 µL.
6. Incubate the tubes in 37°C for 10 minutes.
7. Pipette the solution in each tube up and down 10 times using a P200 pipette set to 100 µL.
8. Add 850 µL of DMEM so that the final volume reaches 1 mL.

References:

[1] K. Han *et al.*, “CRISPR screens in cancer spheroids identify 3D growth-specific vulnerabilities,” *Nature*, vol. 580, no. 7801, pp. 136–141, Apr. 2020, doi: 10.1038/s41586-020-2099-x.

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12.8. Percent Viability after Spheroid Formation Determination

Materials

- Eppendorf tubes of 1 mL dissociated cells from spheroids (see Appendix 12.7)
- Cytoflex machine

Protocols:

1. Run the Cytoflex for each tube (see Appendix 12.6).
2. Gate (/draw polygon) areas where there are alive cells, record the number shown on the screen.
    a. Cell concentration = number shown on screen * 10,000 (cells/mL)
    b. Total number of live cells = cell concentration (cells/mL) * 1 mL (volume in eppendorf tube)
3. Gate (/draw polygon) areas where there are dead cells (smaller scatter than live cells), record the number shown on the screen.
    a. Cell concentration = number shown on screen * 10,000 (cells/mL)
    b. Total number of dead cells = cell concentration (cells/mL) * 1 mL (volume in eppendorf tube)
4. Calculate percent viability: total number of live cells / (total number of live cells + total number of dead cells) * 100%

12.9. Lentiviral infection protocol

Small scale infection of mammalian cells with Lentivirus: Adherent Cells Version

Materials:

- ThermoFisher cell culture numbers for adherent cells:
- https://www.thermofisher.com/us/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/cell-culture-useful-numbers.html

Infection Procedure:

Cell Line	# of cells (24 Well)
293T	50,000
HeLa	20,000
HAP1	20,000

1) Count and plate the # of cells listed above for each infection in a well of a 24 well plate. (If you want to plate the cells the day before, plate half as many as listed above)
2) Sterile filter with 1 0.45µm filter. Add 1-2 mL of virus(es) to each well and leave to incubate for 24 hours at 37C.
3) After incubating, aspirate the viral media from each well. Add 1 mL of proper media to the cells.

4) 48-72 hours later, the cells should be ready for analysis by fluorescence or selection with antibiotic.

References

[1] “Small scale infection of mammalian cells with Lentivirus: Adherent Cells Version · Benchling.” Accessed: Oct. 05, 2024. [Online]. Available: https://benchling.com/uw_hesslab/f/lib_jUyOwxBE-tissue-culture-protocols/prt_Sdt7mJ5N-small-scale-infection-of-mammalian-cells-with-lentivirus-adherent-cells-version/edit

12.10. High-throughput CRISPR screen protocol

This protocol was originally written for use with HuH7 and U-2 OS cells. However, the protocol steps to test dose-response cytotoxicity can be applied to any cell type, including A549 cells which will be used for our project.

Materials: See “key resources” table in protocol [1] for a more detailed description of necessary reagents for the CRISPR screening process.

Procedure:

12.10.a. *Generate Cas9-Expressing Cells*

The Cas9 endonuclease and targeting sgRNAs can be introduced together or separately through lentiviral transduction. This integrates Cas9 and sgRNAs non-specifically into the genome, which is appropriate for almost all CRISPR screens in immortalized cells. Through this protocol, constitutive Cas9 expression is induced through lentiviral transduction. Cas9 is then stably expressed and remains active throughout the screening process.

1) Plate 300,000 HEK293T cells into one well of a 6-well culture plate in 1 mL DMEM + 10% FBS such that they are at ~50% confluence 24 h later.
2) After 24 h, transfect HEK293T cells with Mirus LT1 transfection reagent, 500 ng 3rd generation lentiviral packaging vector mix (equal parts pMDLg/pRRE [Addgene # 12251], pRSV-Rev [Addgene # 12253], and pMV2.g [Addgene # 12259]), and 500 ng pLenti-Cas9-blast (Addgene # 52962) at a 3:1 Mirus:DNA ratio. Follow Mirus Bio’s “TransIT-LT1 Full Transfection Protocol” [2].

   > **Critical:** To ensure sufficient viral titers are reached, all library and packaging plasmids should be endotoxin-free and the packaging HEK293T cells need to be healthy prior to transfection.

3) Incubate cells for 72 h.

4) After 72 h, collect viral supernatant through a 0.45 μm filter. Use immediately or store at 4°C for up to one week or −80°C for up to six months.

    Critical: When working with lentivirus, bleach all media and supplies and turn on ultraviolet light in the biosafety cabinet for thirty minutes to inactivate viral particles.

5) Plate 100,000 HuH7 cells into two wells of a 6-well tissue culture plate in DMEM + 10% FBS such that cells are at ~80% confluence 24 h later.
6) After 24 h, introduce 0.5 mL fresh DMEM + 10% FBS and 0.5 mL pLenti-Cas9-blast lentivirus-containing medium to the cells in one well with 8 μg/mL polybrene. Incubate for 24 h.

    Note: Keep one well uninfected as a control for the antibiotic selection.

7) After 24 h, remove viral media and replace with DMEM + 10% FBS.
    a) Expand cells for 24 h and then begin antibiotic selection with 4 μg/mL blasticidin.

        Note: With the amount of lentivirus added in step 6, ~30%–50% of cells should be infected. Cells can therefore start selection at high confluency and not overgrow the plate.

    b) Replace the selection media every 3–4 days and split cells as necessary until all control cells have died.

        Note: The concentration of selection antibiotic is dependent on the cell line. This concentration should be determined in advance with antibiotic kill curves.

8) Once all control cells have died, replace media for Cas9 cells with fresh DMEM + 10% FBS without antibiotics to allow cells to recover. These are now your “Cas9” cells.

    Note: Cas9 pools or clonal cells can be used for CRISPR screening. To avoid clonal bias from the genetic background, this protocol calls for screening pools of Cas9 cells and does not select monoclonal cells.

9) Validate Cas9 expression by Western blot.
10) Freeze cells at −80°C and store in liquid nitrogen.

    Note: It is useful to expand these cells and store in excess since they can be used for subsequent screens or individual gene knockouts.

    Note: After introduction into cells, it is important to make sure that Cas9 is active (steps 11-16). To test Cas9 activity, independently infect cells with a lentiviral plasmid encoding: 1. mCherry plus a non-targeting sgRNA (control) and 2.

mCherry plus an mCherry-targeting sgRNA. Employ flow cytometry to measure mCherry expression. Cells expressing active Cas9 will cleave the mCherry DNA and appear as an mCherry negative population. Conversely, cells lacking active Cas9 will fail to cleave the mCherry DNA and appear as an mCherry positive population. Due to the long half-life of mCherry, it may take up to 1–2 weeks to distinguish the active Cas9 (e.g., mCherry-negative) cells.

11) Repeat steps 1–4 to make lentiviral media containing a control sgRNA or mCherry-targeting sgRNA (see "key resources" table [1] for sequences) cloned into pMCB320 lentiviral vector (Addgene # 89359).
12) Plate 100,000 HuH7 Cas9 cells into three wells of a 6-well plate in DMEM + 10% FBS so that cells reach ~80% confluence 24 h later.
13) After 24 h, introduce viral media containing the control sgRNA or the mCherry-targeting sgRNA cloned into the pMCB320 lentiviral vector with 8 μg/mL polybrene to two of the wells. Incubate for 24 h.
14) After 24 h, remove viral media and replace with DMEM + 10% FBS. Expand cells for 48 h and then begin antibiotic selection with 2 μg/mL puromycin (see note above on antibiotic concentrations). Replace the selection media every 3–4 days until all control cells have died.
15) Once all control cells have died, replace media with fresh DMEM + 10% FBS without antibiotics to allow cells to recover.
16) Measure mCherry fluorescence by flow cytometry to validate Cas9 activity.

12.10.b. *Dose Response Analysis to Determine Concentration of Cytotoxic Compounds*

An optimal concentration of your choice compound to induce cell death is crucial to achieving the maximum dynamic range of the screen readout. For a drug-resistance screen, a sublethal concentration of drug that causes very modest cell death (~5%) in 24-48 h should be determined. Presumably, the depletion of a drug resistance factor will lead to a substantial increase in the sensitivity to the drug, leading to a depletion of the sgRNA over time. For a drug-sensitivity screen, an initial drug concentration that causes ~50% cell death should be determined. However, as pools of surviving cells from the initial selection will become resistant to cell death induced by the drug, a slightly higher concentration may be required for each subsequent treatment cycle to achieve ~50% death.

Note: This is a specific example for identifying ferroptosis resistance factors using known ferroptosis inducing compounds. However, this protocol can be extrapolated to any treatment or condition that provides a selective pressure on cell viability.

17) On day 0, seed ~5,000 U-2 OS cells in each well of a 96-well plate such that the final volume per well is 200 µL.
18) On day 1, aspirate the media from the 96 well-plate and replace it with 100 µL fresh media.
19) Prepare a 2× final solution of the compound at varying concentrations by serial dilution in media containing 60 nM SYTOX Green Dead Cell Stain.

    Note: 8–12 different concentrations are recommended to ensure that the optimum concentration is within the standard curve. It is recommended to begin with a 10-point, 5-fold dilution series.

20) Slowly add 100 µL compound containing media back to each well so that the final volume of media in each well is 200 µL with 30 nM SYTOX Green Dead Cell Stain.
21) Monitor cell death using an Incucyte Live-Cell Analysis System (Essen Biosciences), taking images every 2 h for 24–48 h total. Dead cells will be SYTOX green-positive.
22) On day 2 or 3, determine the percentage of cell death by dividing the number of dead cells (SYTOX green-positive) by the total number of cells (visualized by phase imaging).

    Note: Due to some limitations of the Incucyte system and the dramatic difference in cell morphology, thresholding and automatically counting total cell number using phase images can sometimes be difficult and inaccurate. Generating a cell line that stably expresses mCherry or using a genetically encoded live-cell nuclear marker (e.g., Incucyte Nuclight reagents) greatly improves the accuracy of the counting for live cells.

    Note: If an Incucyte Live-Cell Analysis System is not available, a CellTiter-Glo 2.0 Cell Viability Assay can be used to determine the sub-lethal dose of the drug.

23) Choose a concentration of drug that results in ~5% cell death. Use this concentration for the CRISPR screen.

12.10.c. Determine the Dynamic Range for Fluorescence-Based Assays

The confidence of screen results depends on the dynamic range of the fluorescence reporter. A greater distance between the high and low fluorescence intensity bins will result in less biological noise and will increase the confidence of positive results and reduce the occurrence of false positives and negatives [3]. When possible, it is useful to determine the dynamic range of a cell population using a positive control prior to screening to ensure that cells with altered phenotypes can be accurately sorted by FACS.

Note: Fluorescence can arise from a fluorescent reporter protein or a fluorescent dye. To obtain the highest dynamic range from a reporter protein, it may be useful to sort cells to

obtain a population with uniform fluorescence levels. For fluorescent dyes, test multiple concentrations and incubation times.

Note: It is important to establish the timeframe and treatment conditions before performing the screen itself. For example, it may take several days for a genetic perturbation or drug to produce a measurable effect on a fluorescent reporter. Cells may also need to be differentiated or pretreated with drugs or nutrients. Therefore, optimize conditions and establish a timeline for seeding cells, inducing genetic perturbations, differentiating (if applicable), and treating cells, and carry it over to the screen to yield the most robust results.

24) Choose a positive control gene (if possible) that is known to influence levels of the fluorescent reporter. Generate a knockout cell line or treat cells with a drug targeting the positive control protein. Confirm that the expected increase or decrease in fluorescence is detectable by flow cytometry.
25) Measure fluorescence by flow cytometry to validate that a change in fluorescence is detected and to determine the dynamic range of your assay.
    a) In this example, HuH7 cells were treated with 1 µg/mL triacsin C or 100 µM oleic acid to deplete or increase neutral lipid storage, respectively. Cells were treated with 1 µg/mL BODIPY 493/503 to label neutral lipids and fluorescence was measured by flow cytometry. The 10× decrease and 5× increase in fluorescence intensity will be the target dynamic range of fluorescence for this CRISPR screen.

        Note: Fluorescence intensity can diminish over time. Incubate cells on ice for multiple hours (as long as the FACS sort will be) and check that fluorescence does not change during sorting. We have not found this to be an issue with GFP-based reporters. If necessary, cells can be fixed prior to FACS to ensure fluorescent marker stability over time.

        Note: In some cases, there are no drugs or known regulators to manipulate or validate the system. In the absence of a positive control to validate the fluorescence reporter, it is possible to move directly to the screen.

12.10.d. *Prepare sgRNA Library*

Many genome-scale and small-scale libraries are deposited on Addgene. This protocol used the Bassik Human CRISPR Knockout Library (Addgene # 101926-101934), which is composed of 9 sublibraries, or the custom Human Lipid Droplet and Metabolism Library (Addgene # 191535). Each sgRNA library will need to be amplified and packaged into lentivirus. Alternatively, pre-packaged lentivirus can be purchased directly from Addgene.

26) Follow the Bassik Lab's "Liquid Culture Library Plasmid Re-amp Protocol" [4].

27) Measure DNA concentration using the Qubit dsDNA HS Assay.

12.10.e. *Preparing for Deep Sequencing*

To identify the sgRNAs present in each cell population, genomic DNA (gDNA) is extracted from frozen cell pellets and guide sequences are amplified by PCR.

> **Note:** This protocol modifies the above protocol for increased yield. For the DNA precipitation step, increase the spin time if centrifuging at a slower speed to fully precipitate DNA. Elute with Qiagen Buffer EB (10 mM Tris-Cl, pH 8.5; Cat # 19086) instead of Buffer AE. Spin at 4,500 × g for 5 min. Elute 2–3 times with new Buffer EB each time (do not reload eluate).

28) Extract gDNA from cell pellets using Qiagen QIAamp DNA Blood Midi Kit (Cat # 51183) according to the manufacturer's instructions [5].
29) Measure the gDNA concentration by nanodrop. 100 µg gDNA per 20 × 10^6 cells are typically obtained.
30) Amplify the integrated sgRNA (PCR1) with the following reagents and reaction program:

> **Critical:** To make sure the diversity and coverage of sgRNAs is not lost during PCR1, multiple 100 µL PCR reactions are required for screen preparation. Empirically, we recommend 1 PCR reaction for every 2,500 sgRNA in the library (e.g., For a customized library that has 25,000 sgRNAs, at least 10 PCR reactions are needed, and therefore a minimum of 100 µg gDNA is required).
>
> **Note:** Although this protocol calls for 10 µg of genomic DNA per 100 µL PCR1 reaction, DNA input can be decreased to 5 µg or less if necessary.

Table 1. PCR1 reaction master mix reagents and volumes.

Reagent	Volume (µL)
gDNA template (10 µg)	x
Herculase II polymerase	2
oMCB_1562 (100 µM)	1
oMCB_1563 (100 µM)	1
5× Herculase buffer	20

dNTPs (100 nM)*	1
ddH2O	75-x

*25 nM per dNTP.

Table 2: PCR1 cycling conditions.

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	2 min	1
Denaturation	98°C	30 s	18
Annealing	59.1°C	30 s	
Extension	72°C	45 s	
Final Extension	72°C	3 min	1
Hold	4°C	∞	

Note: As the amount of genomic DNA collected from cell samples can be limited, especially for FACS-based screens, it is highly recommended to run a single (or "pilot") PCR1 to ensure all conditions are correct and yield an amplified fragment.

31) Pool and mix all amplicons of the PCRs from the same gDNA sample. Add Illumina sequencing indexes with the following reagents and reaction program:

Table 3: PCR2 reaction master mix reagents and volumes.

Reagent	Volume (µL)
PCR1 Product	5
Herculase II polymerase	2
oMCB_1440 (100 µM)	0.8
oMCB_1439 (100 µM)	0.8
5× Herculase buffer	20
dNTPs (100 nM)*	2
ddH2O	69.4

Table 4: PCR2 cycling conditions.

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	2 min	1
Denaturation	98°C	30 s	20
Annealing	59.1°C	30 s	
Extension	72°C	45 s	
Final Extension	72°C	3 min	1
Hold	4°C	∞	

Note: Though PCR1 uses 18 cycles, it was empirically determined that 20 cycles for PCR2 resulted in the best signal-to-noise ratio.

Note: Selecting index adapters with diverse sequences for pooled libraries is CRITICAL: for successful sequencing and data analysis. For information on how to optimize the color balance of the index adapters see the "Index Adapters Pooling Guide" published by Illumina.

Note: Only one 100 µL PCR2 reaction is sufficient to achieve sequencing depth.

32) Load PCR products onto 2% TBE-agarose gel.
33) Run the sample at 120 V for 50 min. Excise the brightest band.

Note: The size of the band is expected to be 280 bp but may run higher due to overloading of the gel.

34) Purify DNA products using QiaQuick Gel Extraction Kit (Cat # 28706) according to the manufacturer's instructions [6].

Note: We slightly modified this protocol for increased yield. When dissolving the gel, add 4 volumes Buffer QG instead of 3. For the DNA precipitation step, add 3 M sodium acetate pH 5.2 at a 1:100 ratio. For the wash step, wash with Buffer PE two times instead of once. Elute DNA in Buffer EB, not water.

35) Check DNA concentration by Qubit dsDNA HS Assay. We typically obtain 30 ng/mL DNA.
36) Verify DNA quality using a fragment analyzer. DNA fragments should run as a single band at ~300 bp.
37) If the DNA runs as a single band at ~300 bp without contamination at other sizes, pool equal amounts of DNA from each screen sample so that the final concentration is 3 nM. Send the pooled library for deep sequencing.

Note: The molecular weight of the DNA can be calculated based on the nucleotide sequence.

a) 50 µL of the pooled library at 3 nM and 30 µL of the Bassik custom sequencing Illumina sequencing primer (oMCB1672_new10gCRKO) at 100 µM are typically sent. Both are sent in Qiagen EB (10 mM Tris, pH 8.5).

38) Analyze deep sequencing data using casTLE.

References

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[3] T. Nagy and M. Kampmann, "Crispulator: A discrete simulation tool for pooled genetic screens," *BMC Bioinformatics*, vol. 18, no. 1, Jul. 2017. doi:10.1186/s12859-017-1759-9.
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12.11. qPCR Testing Protocol

Materials: Qiagen RNAeasy Kit
(https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/rna-purification/total-rna/rneasy-kits?catno=74104)

Procedure:

12.11.a. *RNA Extraction with Qiagen RNAeasy Kit*

1) Clean bench and spray down with RNaseZap.
2) Make RLT buffer (350µL per sample) by adding β-mercaptoethanol (10µL for every 1 mL of RLT buffer) into a separate tube inside the fume hood.
3) Note: β-mercaptoethanol should be added freshly before each set of preparations.
4) Prepare 1-5 million cells and spin down at 500g for 5 minutes. Aspirate supernatant and resuspend cells in 350µL of prepared RLT buffer.
5) Pipet cell solution onto a QiaShredder column and spin for 2 minutes at 14000 rpm. ***Discard column, not flow-through!***
6) Add 350µL of 70% ethanol to flowthrough. Transfer mixture to RNAeasy spin column and spin for 30s at 10000rpm. Discard flow-through.
7) Prepare Dnase solutions by adding 10µL of DNase I stock solution to 70µL of RDD buffer for each column. Mix well.
8) Add 80µL of DNase I + RDD mix onto the column and let sit at room temperature for 15 min.
9) Add 350µL of RW1 buffer to column and spin 30s at 10000rpm. Discard flowthrough.
10) Add 500µL of RPE buffer to column and spin 30s at 10000rpm. Discard flowthrough.
11) Add 500µL of RPE buffer to column and spin 2 minutes at 14000rpm. Discard flowthrough.
12) Place Column into new 1.5mL tube and elute with 30-100µL of RNase-free water. Let sit for >5 minutes and spin down for 1 minute at 10000 rpm. Nanodrop to determine concentration.
13) Purified RNA can be stored at -80 C for 6 months, -20 C for 1 month.

12.11.b. *cDNA Synthesis with RNAeasy purified RNA*

1) This protocol is for 20µL/2µg of RNA protocol. Prepare in PCR tubes:

RNA (2µg)	x
Oligo dT (0.5µg/µL)	2
H_2O	8-x

Note: If using a transcript specific primer, Oligo concentration should be 0.5-1 µM in final RT reaction. I usually use 2µL of 10µM stock.

2) Heat sample at 70°C for 5 minutes. Place heated sample on ice for 5 minutes.
3) Add 10µL of following mix:

5X AMV RT Buffer	4
10mM dNTPs	1
RNAsin	0.25
AMV RT	0.75
H_2O	4

4) Incubate at 42°C for 2 hours.
5) If going into a PCR, add 50 uL of Milli-Q-H2O and proceed to set up qPCR reaction.

12.11.c. *Setting up qPCR: TaqMan Method*

Note: Check out more information on TaqMan method here: https://tools.thermofisher.com/content/sfs/manuals/cms_041280.pdf

1) **Designing the experiment**: For these experiments, you require a housekeeping gene as a loading control in addition to your genes of interest. We generally order these conjugated to VIC and our target gene conjugated to FAM.

Name	**Cat No.**
ACTB_VIC	
GAPDH_VIC	

a) There are a number of ways to set up this experiment. You can use the $\Delta\Delta C_T$ calculation. This works since the primer sets have been properly optimized. *Alternatively, you can set up the experiment as described in the for SYBR Green*. You will need the following samples. where you will need the following controls.

Experimental sample is measured at 1:10 dilution in **quadruplicate** with gene expression assay and housekeeping gene (both are in a single well)

Note: these are only if you are making a standard curve:

Primer curve of control template to be done (1 well each dilution) for each primer set consisting of:

No dilution
1:5 dilution

1:25 dilution
1:125 dilution
1:625 dilution
1:3125 dilution
Empty sample

2) Setup the Following Master Mix.

Reagent	**1 X**	**5X**
20X Taq Man Gene Expression Assay Target Gene	1	5
20X Taq Man Gene Expression Assay Housekeeping Gene	1	5
2x Taq Gene Expression Master Mix	10	50
cDNA Template (1:10 dilution)	4	20
RNAse Free water	4	20
	20	100

3) Put these 20 uL reaction into a 96 well plate.
4) Setup the following protocol:

95ºC 10min
95ºC 15s
60ºC 60s
plate read
Go to 2 39 times

5) **Analysis:** Use the relative quantification (ΔΔCt) method to analyze results. The general guidelines for analysis include:
    a) View the amplification plot; then, if needed:
        i) Adjust the baseline and threshold values.
        ii) Remove outliers from the analysis.
    b) In the well table or results table, view the Ct values for each well and for each replicate group.
    c) Perform additional data analysis using any of the following software:
        i) Relative Quantification application thermofisher.com/connect
        ii) ExpressionSuite™ Software[1] thermofisher.com/expressionsuite

References:

[1] qPCR Protocol · Benchling, Accessed: Oct. 10, 2024. [Online]. Available: https://benchling.com/uw_hesslab/f/lib_xtPaCTIU-extraction-and-staining-protocols/prt_Va3aG4Cc-qpcr-protocol/edit.