Non-Small Cell Lung Cancer Spheroids to Investigate Factors in Genome Stability

DEPARTMENT OF
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Preliminary Report

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Abstract

Lung cancer is by far the leading cause of cancer death in the US, accounting for about 1 in 5 of all cancer deaths according to the American Cancer Society. Approximately 80% of lung cancer cases are classified as non-small cell lung cancer (NSCLC), a condition marked by significant clonal and morphological diversity within a tumor. When it comes to *in vitro* models that can accurately mimic the microenvironment of NSCLC, spheroids are a promising approach. This is because current two-dimensional models have limited biological relevance to the tumor microenvironment, often failing to replicate the complex cell-cell interactions found *in vivo*. On the contrary, animal models can provide the most clinically relevant responses but are a time commitment, costly, and involve complex interactions that are difficult to study all at one time. Spheroids can be cultured from a variety of sources including primary isolated tumor cells or continuous cancer cell lines. NSCLC spheroid models can provide a more accurate analysis of intercellular interactions, oncogenic signaling, and anti-cancer drug effects, making them a helpful model in improving the therapies associated with NSCLC. Our findings may support the claim that there are statistically significant genetic expression profiles between 2D and 3D cancer models, establishing the need for an optimized spheroid formation protocol that can be accurately stained for DNA double-stranded breaks ahead of a genome wide CRISPRi screen to find tumor-specific factors that regulate genome stability.

Introduction

Non-small cell lung cancer (NSCLC) has a 28% 5-year survival rate, and continues to be one of the most challenging cancers to treat [1]. A major obstacle in treating NSCLC is the continuous evolution of cancer genomes, which leads to frequent mutations, clonal diversity, and ultimately, resistance to therapeutic treatment. Additionally, factors such as immune evasion, an abundance of extracellular matrix (ECM), and hypoxic conditions further complicate treatment options and are often linked to poor patient outcomes [2]. Accurately replicating these characteristics in tumor models is necessary for refining existing therapies and advancing new treatment approaches. For this reason, we have selected the A549 cell line to use in our 3D spheroid model. A549 cells are well-suited for spheroid formation due to their strong ability to aggregate and self-organize into three-dimensional structures as seen in figure 1. They exhibit excellent adherent characteristics and key features of lung adenocarcinoma, including epithelial characteristics and drug-resistant properties. When quantifying gene expression within the A549 spheroid model, an increase in SOX2 has often been associated with stemness and tumor progression. 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 [3]. SOX2 can be quantified using qPCR by extracting RNA from the sample, converting it to cDNA via reverse transcription, and amplifying the SOX2 gene using a human specific primer and a TaqMan probe to measure its expression level. Once measured, the upregulation of SOX2 would confirm the data seen in other NSCLC spheroid models such as Guo et al [4].

Furthermore, the use of γH2AX staining has been important in understanding the mechanisms of DNA double-strand breaks (DSBs) in 3D spheroid models. γH2AX is a phosphorylated variant of the histone H2AX, which serves as a sensitive marker for DNA double-strand breaks (DSBs) [5]. The phosphorylation of H2AX occurs rapidly in response to DNA damage, making it an essential marker for monitoring the integrity of the genome [6]. To induce DSBs, etoposide can be introduced into the spheroids. Etoposide is a chemotherapeutic drug that works as a topoisomerase II inhibitor, preventing the re-ligation of DNA strands during replication [7]. It has been seen that compared with 2D cancer cell cultures, spheroids are more resistant to cytotoxic drugs like etoposide [2]. Measuring DNA damage using a 3D γH2AX staining protocol will allow for better understanding of how disruptions in the DSBs repair contribute to NSCLC progression, providing a greater understanding of how 2D and 3D environments vary in cancer modeling. This process of forming A549 spheroids, performing qPCR for stemness, and staining for γH2AX will provide the backbone for a CRISPRi genome wide screen to find tumor-specific factors that regulate genome stability.

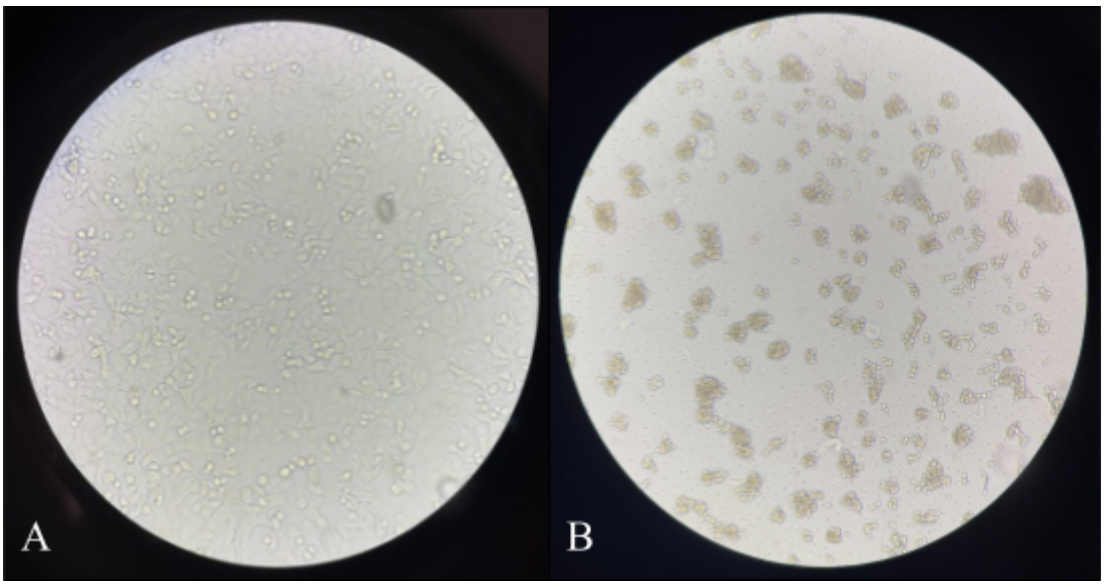

Figure 1. A) WT A549 cells at 147% confluence under 20x, brightfield microscopy, B) WT A549 seeded at 75k cells/cm^2 spheroids under 10x, brightfield microscopy.

Materials and Methods

Cell Thawing

The thawing protocol for the WT A549 cell line was developed by Hess Lab [8]. Cell pellets are thawed in the cryovial by slow mixing of complete DMEM media (high glucose Gibco™ DMEM) (Thermo Fisher Scientific, USA, 11965118) supplemented with 1% v/v Gibco™ Penicillin-Streptomycin (Thermo Fisher Scientific, USA, 15070063) and 10% v/v Gibco™ Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, USA, A5256701). Once thawed, cells are pelleted and resuspended in fresh complete DMEM media.

Cell Passaging

Cell passaging protocol, as outlined by Hess Lab, is based on passaging in a T75 flask (VWR, USA, 10062-860). Cell cultures were incubated at 37°C and 5% CO_2. During passaging, media was first removed then cells were washed once with Gibco™ pH 7.5 phosphate buffered saline (PBS) (Thermo Fisher Scientific, USA, 10010023). Cells were detached from the culture vessel by addition of Gibco™ Trypsin-EDTA (0.05%), phenol red (Thermo Fisher Scientific, USA, 25-300-120) and incubation at 37°C, followed by inactivation of Trypsin/EDTA via addition of complete DMEM media. Cells were passaged every 2-3 days for up to 20 passages, and were frozen down within the first 2 passages. Number of cells was determined using the Beckman Coulter CytoFLEX Flow Cytometer. In each passage, cells were reseeded at a density of 2 - 2.5 x 10^4 cells/cm^2.

Cell Freezing

Cell freezing protocol was outlined by Hess Lab. Fresh cells (cells that underwent 2 or fewer passages) were used for the freezing process. 1 x 10^6 A549 cells were pelleted and then added in freezing media, composed of FBS and 10% v/v Anhydrous DMSO (Thermo Fisher Scientific, USA, D12345). Cells were first frozen at -80°C for one day before being moved to -160°C in liquid nitrogen.

Spheroid formation

Spheroid formation protocol was based on Han et al, 2020 [9]. Cells were seeded at multiple densities and concentrations to determine the optimal condition for high spheroid numbers, uniform spheroid size, and high percent viability. Cells were seeded in polyHEMA (SigmaAldrich, USA, P3932) coated tissue culture plates (VWR, USA, 76446-962). Cells were seeded at two different densities, 50,000 (or 50k) cells/cm^2 and 75,000 (or 75k) cells/cm^2 and three different methylcellulose (SigmaAldrich, USA, M0512) concentrations, 0.75%, 1%, and 1.25% with media volume of 500 µL/cm^2. FBS was 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 were split every 3-4 days, and can be dissociated into single cells using Accutase (ThermoFisher, USA, A1110501) and can be reseeded into new spheroids.

BioTek Cytation Imaging

In summary, open BioTek Gen5 3.14 application [10], place plate in BioTek with A1 well in on the bottom right of the holder, create 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. The protocol procedure was set as follows: temperature at 37°C; middle imaging; deselect auto exposure; select plate type; and set imaging settings to 4x magnification, Brightfield, FVOW, set z-stack, and distance. Then, run the experiment trial.

Cell-Titer Glo

For full protocol see CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corporation, USA, G7570) user manual [11]. Briefly, remove CellTiter-Glo reagent from -30°C freezer storage and let it acclimate to room temperature. Make sure the CellTiter-Glo remains covered in aluminum foil to reduce light exposure. Add 100 uL of 1X of CellTiter-Glo reagent to 96 well plate prepared with 100 uL of cell solution media and cover the wells with aluminum foil. Insert 96 well plate with lid into BioTek Gen5 3.14 with A1 well in on the bottom right of the holder and create a new experiment. Input pre-made protocol with three directives: first, Mix plates for 2 minutes at RT on an orbital shaker; second, incubate plates for 10 minutes at room temperature; and third, measure luminescence of ATP with chemiluminescence setting on BioTek. Hess lab protocol, analyze and plot data by normalizing replicate cell viability numbers for each condition to vehicle only numbers.

ImageJ Analysis of BioTek Cytation Images

To begin, choose the most in-focus image for the desired well and upload it to an image analysis tool named ImageJ version 1.54p. 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.

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’.

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.

Results

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

During the imaging process, the image for the 4th well for the 75k cells/cm^2, 0.75% methylcellulose concentration did not have good quality, resulting in the number of spheroids captured by ImageJ to be lower than actual. Thus, data regarding this well was excluded for data analysis as an outlier.

Regarding spheroid area, overall, the average spheroid area per well of each condition is similar among each other (Figure 2). The 50k cells/cm^2 seeding density had an average of 6006.751 μm^2 and the 75k cell seeding density had an average of 6265.328 μm^2 (Figure 3). The 0.75% methylcellulose concentration had an average of 6248.067 μm^2, the 1% methylcellulose concentration had an average of 5893.392 μm^2 , and the 1.25% methylcellulose concentration had an average of 6292.092 μm^2 (Figure 3).

50k cells/cm^2 seeding density and 0.75% methylcellulose concentration condition had an average of 6085.596 $\mu m^{2>}$ (Figure 3). 50k cells/cm^2 seeding density and 1% methylcellulose concentration condition had an average of 5721.736 μm^2 (Figure 3). 50k cells/cm^2 seeding density and 1.25% methylcellulose concentration condition had an average of 6250.038 μm^2 (Figure 3). 75k cells/cm^2 seeding density and 0.75% methylcellulose concentration condition had an average of 6410.537 μm^2 (Figure 3). 75k cells/cm^2 seeding density and 1% methylcellulose concentration condition had an average of 6065.047 μm^2 (Figure 3). 75k cells/cm^2 seeding density and 1.25% methylcellulose concentration condition had an average of 6334.146 μm^2 (Figure 3).

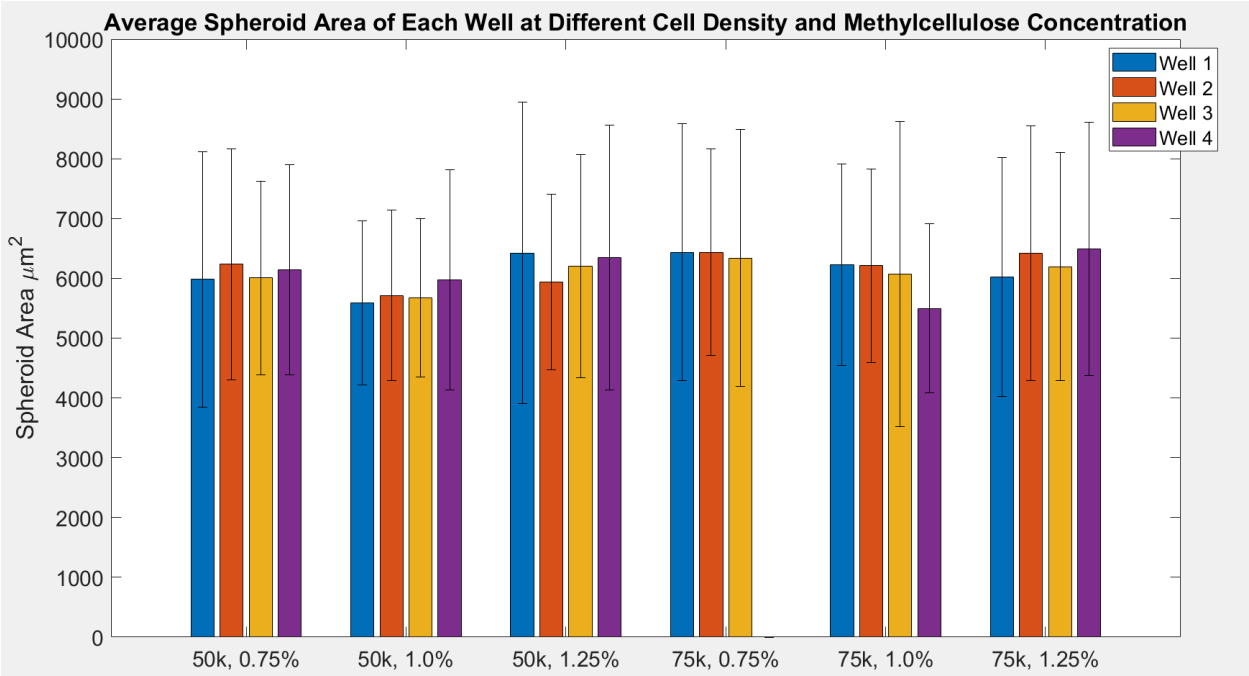

Figure 2. Average spheroid area of each well at different cell density and methylcellulose concentration. Error bars represent standard deviation. Condition notations are shortened: 50k and 75k stand for the corresponding cells/cm^2 seeding density; 0.75%, 1.0%, and 1.25% stand for the corresponding methylcellulose concentration. Each condition has 4 replications or 4 wells. Data for the 4th well of 75k cells/cm^2 seeding density and 0.75% methylcellulose concentration is excluded.

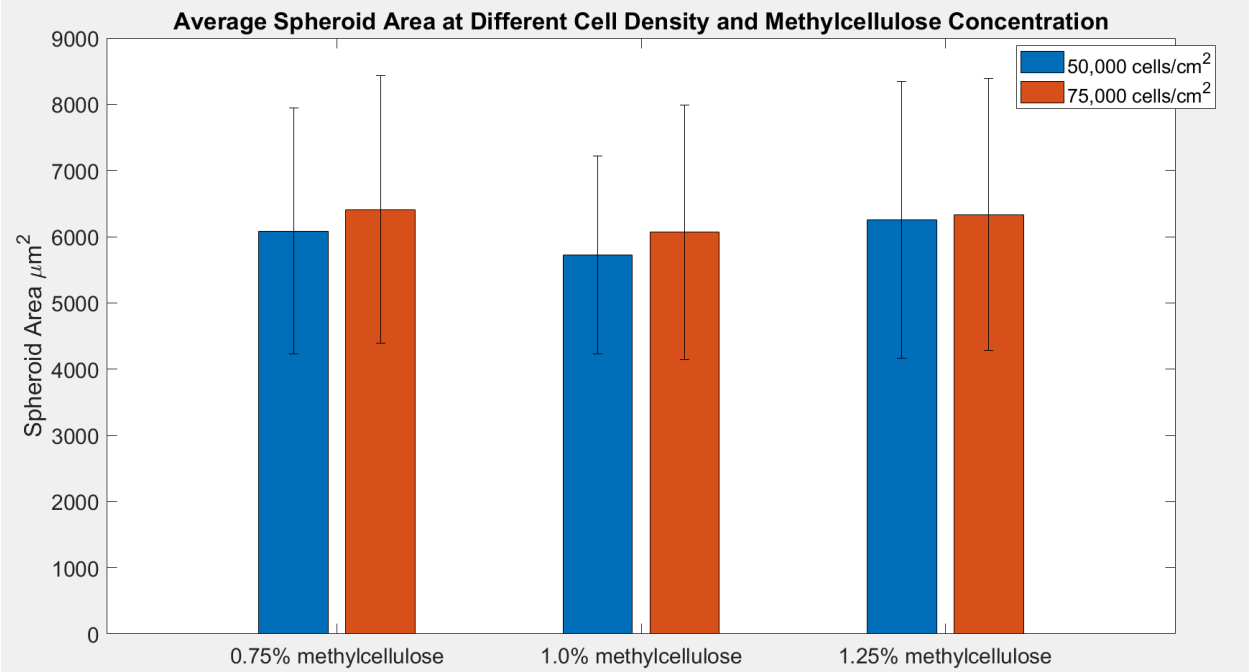

Figure 3. Average spheroid area of each different cell seeding density and methylcellulose concentration. Error bars represent standard deviation. Data for the 4th replicate of 75k cells/cm^2 seeding density and 0.75% methylcellulose concentration is excluded.

Regarding spheroid count, the 50k cells/cm^2 seeding density had an average of 70.3 spheroids and the 75k cells/cm^2 seeding density had an average of 94.3 spheroids (Figure 4). The 0.75% methylcellulose concentration had an average of 89.3 spheroids, the 1% methylcellulose concentration had an average of 85.1 spheroids , and the 1.25% methylcellulose concentration had an average of 72.4 spheroids (Figure 4).

50k cells/cm^2 seeding density and 0.75% methylcellulose concentration condition had an average of 84.5 spheroids (Figure 4). 50k cells/cm^2 seeding density and 1% methylcellulose concentration condition had an average of 70.75 spheroids (Figure 4). 50k cells/cm^2 seeding density and 1.25% methylcellulose concentration condition had an average of 55.5 spheroids (Figure 4). 75k cells/cm^2 seeding density and 0.75% methylcellulose concentration condition had

an average of 124 spheroids excluding the replicate with poor image quality (Figure 4). 75k cells/cm^2 seeding density and 1% methylcellulose concentration condition had an average of 99.5 spheroids (Figure 4). 75k cells/cm^2 seeding density and 1.25% methylcellulose concentration condition had an average of 89.3 spheroids (Figure 4).

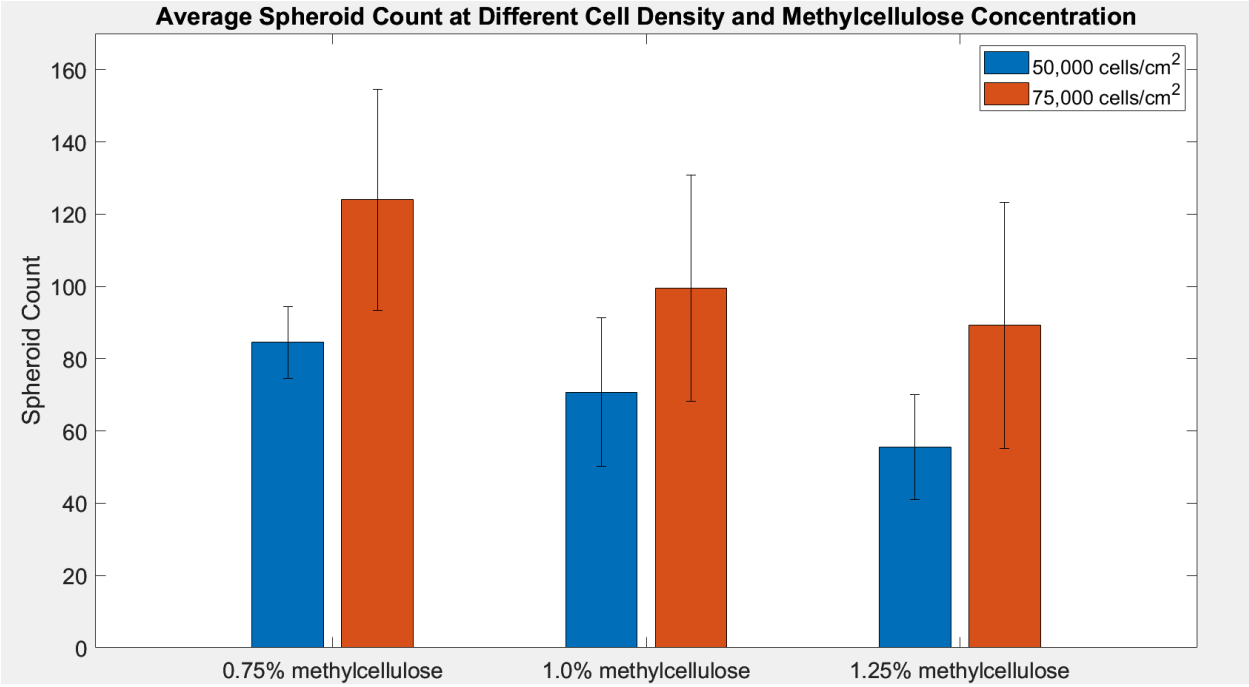

Figure 4. Average spheroid count of each different cell seeding density and methylcellulose concentration. Error bars represent standard deviation. Data for the 4th replicate of 75k cells/cm^2 seeding density and 0.75% methylcellulose concentration is excluded.

Results from the Celltiter-Glo assay were normalized by subtracting the average luminescence of just the media the spheroids were seeded into (no cell control) from the average luminescence of each treatment condition. The 75k cells/cm^2 seeding density and 0.75% methylcellulose concentration condition had the highest luminescence data meaning the condition had the highest amount of ATP (Figure 5).

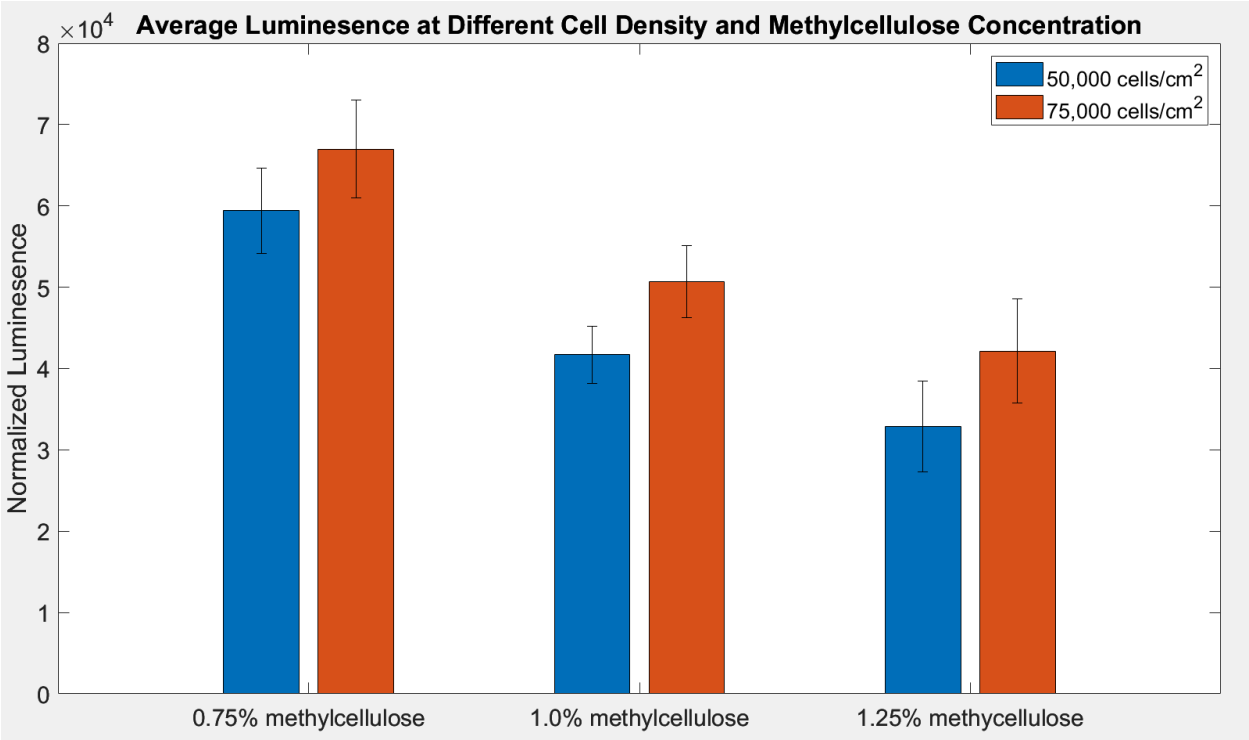

Figure 5. Average luminescence data of each different cell seeding density and methylcellulose concentration from CellTiter Glo assay. Error bars represent standard deviation.

Discussion

To confirm preliminary findings regarding A549 spheroid viability, optimal seeding density, and optimal methylcellulose concentration, we formed spheroids using polyHEMA-coated plates at six different conditions: 50k cells/cm² seeding density and 0.75% methylcellulose; 50k cells/cm² seeding density and 1% methylcellulose; 50k cells/cm² seeding density and 1.25% methylcellulose; 75k cells/cm² seeding density and 0.75% methylcellulose; 75k cells/cm² seeding density and 1% methylcellulose; and 75k cells/cm² seeding density and 1.25% methylcellulose. We then analyzed average spheroid size for each condition via ImageJ and spheroid cell viability via a CellTiter-Glo® Luminescent Cell Viability Assay.

Results indicate that the 75k cells/cm² seeding density and 0.75% methylcellulose condition presented a seeding density and methylcellulose concentration that allowed for optimal spheroid formation (Figure 4), while average spheroid area did not seem to be drastically impacted across conditions (Figure 3). Overall, the trend observed for average spheroid count consisted of a decrease with increasing methylcellulose concentration as well as with decreased cell density. A significantly higher spheroid count was observed with the 75k cells/cm² seeding density and 0.75% methylcellulose condition compared to the 50k cells/cm² seeding density and 0.75% methylcellulose condition. Moreover, spheroid cell viability was observed to be significantly higher for the lower methylcellulose concentration and higher seeding density, with the highest viability (measured via normalized luminescence) observed in the 75k cells/cm² seeding density and 0.75% methylcellulose condition (Figure 5). It is important to note that no seeding density nor methylcellulose condition resulted in poor spheroid cell viability. Based on these findings, the 75k cells/cm² seeding density and 0.75% methylcellulose condition was selected as the seeding density and methylcellulose concentration to be used moving forward for spheroid formation because it would ensure an adequate spheroid size, count, and viability while allowing us to conserve methylcellulose reach a cell density scale required for genome-wide CRISPRi screening and related experiments.

Future work will include performing a reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) for SOX2 using the selected seeding density and methylcellulose concentration above. 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 the in-vivo tumor microenvironment. SOX2 is a transcription factor that regulates self-renewal, differentiation, and pluripotency, making it a significant marker for stemness and

tumorigenicity that is known to be upregulated in non-small cell lung cancer lines such as A549 [12],[13]. Not only would a RT-qPCR for SOX2 in our A549 spheroids provide confirmation of this gene expression increase between 3D and 2D culture, but it would also provide valuable information prior to CRISPRi screening. For instance, the CRISPRi screen could knock down a gene such as SOX2, allowing for transcriptional repression and resultant DNA damage, to investigate the importance of that gene's role in growth and survival. Due to limited previous work on CRISPRi screening with A549 cells, RT-qPCR findings for SOX2 within our spheroid model could provide further evidence for the statistically significant differences in genetic expression profiles between 2D and 3D cancer models.

Additional future work will involve optimizing the dissociation, fixation, and staining process for γH2AX in our A549 spheroid system using the selected seeding density and methylcellulose concentration as discussed above. This will help determine how specific genes targeted respond to repression from a CRISPRi screen. γH2AX is a phosphorylated variant of the histone H2AX that serves as a sensitive marker for DNA double-strand breaks (DSBs), which are a severe form of DNA damage where both DNA helix strands are broken simultaneously [14]. Aside from gene repression mechanisms observed in cancer, DSBs can result from ionizing radiation, reactive oxygen species, or chemotherapeutic agents for cancer treatment [15],[16]. Due to time constraints, we will begin this γH2AX staining optimization by inducing DSBs in A549 cells via introduction of etoposide, a chemotherapeutic agent. Eventually, this optimization will be performed using a A549 CRISPRi cell line in preparation for genome-wide screening. Ultimately, by employing γH2AX staining in a 3D environment, more accurate predictions could be made about genes contributing to tumor progression and metastasis.

Acknowledgments

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Detailed Methods

Cell Maintenance

Cell 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. WT 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 a 1.5 mL eppendorf tube
11) Measure 10uL on the cytometer and gate for the live population of cells
12) Use the "live" number of events to calculate the confluence and total number of cells you have.

13) 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.

CytoFLEX Cytometer

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)

Spheroid Formation Protocol

Methylcellulose Stock Solution

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

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

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

[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.