CRISPRi Screening in Cancer Spheroids

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BME 400 Capstone Design

PROBLEM STATEMENT

- 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]
- The team was tasked with developing a 3D spheroid formation protocol compatible with genome-wide CRISPR interference (CRISPRi) screening
- Next semester, the team will develop γH2AX staining protocol to identify sources of DNA double-strand breaks (DSB)
- Following γH2AX staining, a genome-wide CRISPRi screen will identify factors involved in genome stability

BACKGROUND

- 2D monolayer models have helped study cancer proliferation and therapy responses [2]
- 2D models do not accurately replicate the 3D tumor environment [2]

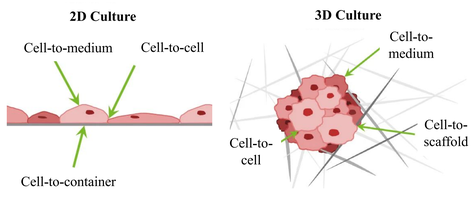

Figure 1: 2D vs 3D culture [3]

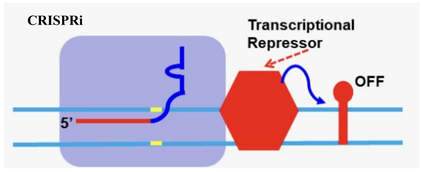

Figure 2: CRISPRi gene knockdown via transcriptional repressor [5]

- CRISPRi via lentivirus allows precise gene repression using deactivated Cas9 (dCas9) without cutting the DNA [4]
- γH2AX initiates a signaling cascade that recruits DNA repair proteins and facilitates genomic stability [7]

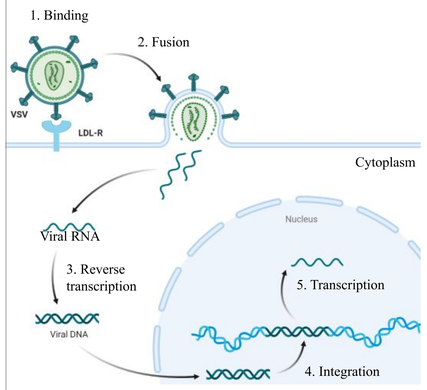

Figure 3: Gene manipulation via lentiviral infection [6]

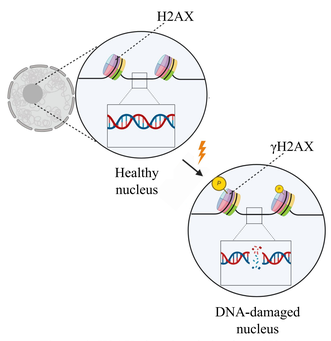

Figure 4: H2AX phosphorylation into γH2AX during DNA breaks [8]

DESIGN CRITERIA

- ✔ Select suitable cancer cell line
- ✔ Select and optimize spheroid formation protocol
- ✔ Scale 3D culture to prepare for genome-wide CRISPRi screen
- ✔ Adhere to all Biosafety Level 2 (BSL-2) standards
- ✔ Budget of $1000

FINAL DESIGN

Cell Line: A549

- Non-small cell lung cancer (NSCLC) [9]
  - Adenocarcinoma
  - Adherent
  - 50 µm cell diameter
- Maintenance
  - Doubling time: 22 hours
  - Confluency 5,000,000 cells/10 mL
- Cells provided by Hess Lab

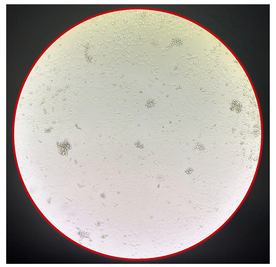

Figure 5: 10x Brightfield image of A549 Passage 5 cells taken at 200% confluency.

PolyHEMA-coated Plates

- PolyHEMA stock solution
  - 1.3 g poly-HEMA
  - 33 mL 99% ethanol
- Add 50 µL of PolyHEMA stock to each well in a 96-well flat-bottom plates, left dry overnight

Spheroid Formation

- In each polyHEMA-coated well, seed cells in 320 µL of serum-free DMEM with 0.75% methylcellulose/cm^2
  - 4 densities: 25k, 50k, 75k, and 150k cells/cm^2
  - 6 wells/seeding density → 24 total
- Spheroids will form after 3-4 days
- Spheroids can be dissociated with Accutase (150 µL/well) [10]

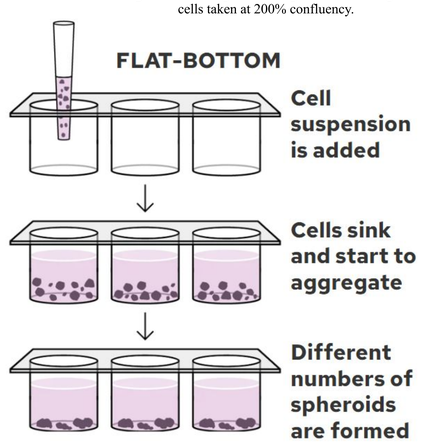

Figure 6: Process of spheroid formation after cell seeding in flat-bottom wells. Picture adapted from Ashgar *et al*, 2013 [11].

IMAGING

- Used BioTek Cytation microscope to image wells individually
- 5 layers/well → 120 total images

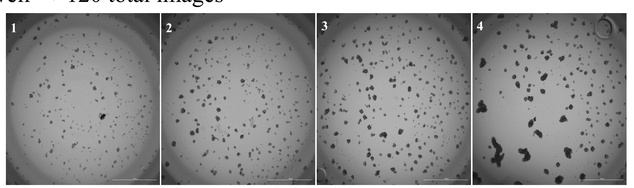

Figure 7: Representative brightfield images of wells 3 days after initial cell seeding for densities 1-4 (25k, 50k, 75k, and 150k cells/cm², respectively).

REFERENCES

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ANALYSIS AND RESULTS

ImageJ Analysis

- Used most in-focus images (of 5 layers) and changed to 16 bit
- Applied threshold feature to identify pixels representing cells
- Applied watershed feature to separate clumped spheroids
- Analyzed spheroids with ≥20 cells (~4147 µm²) [12]

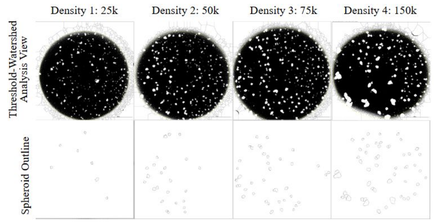

Figure 8: ImageJ analysis of spheroids across seeding densities 1-4 (25k, 50k, 75k, and 150k cells/cm², respectively). Images in top row are in threshold-watershed view and images in bottom row are in spheroid outline view.

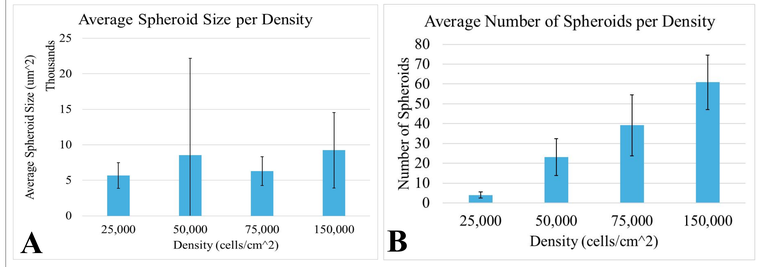

Figure 9: Spheroid analysis across seeding densities 1-4 (25k, 50k, 75k, and 150k cells/cm²). **A:** Average spheroid size (in µm²) across seeding densities (in cells/cm²). **B:** Average number of spheroids across seeding densities (in cells/cm²). Error bars in **A** and **B** display standard deviation.

DISCUSSION & FUTURE WORK

Discussion

- Cultured viable, adherent human cell line A549 under BSL-2 guidelines
- Established passaging protocol reflective of A549 doubling time
- Average spheroid size remained steady and average spheroid number increased linearly with seeding density
- Largest average spheroid size was within the 50-800 µm diameter range typically observed in literature [13]

Future Work

- Repeat trial of seeding density variation with % live/dead cytometry assay to confirm preliminary results
- Optimize methylcellulose concentration for spheroid formation
- Perform qPCR for SOX2 to confirm gene expression changes for 3D (spheroids) vs. 2D [14]
- Optimize dissociation step in γH2AX staining protocol

ACKNOWLEDGMENTS

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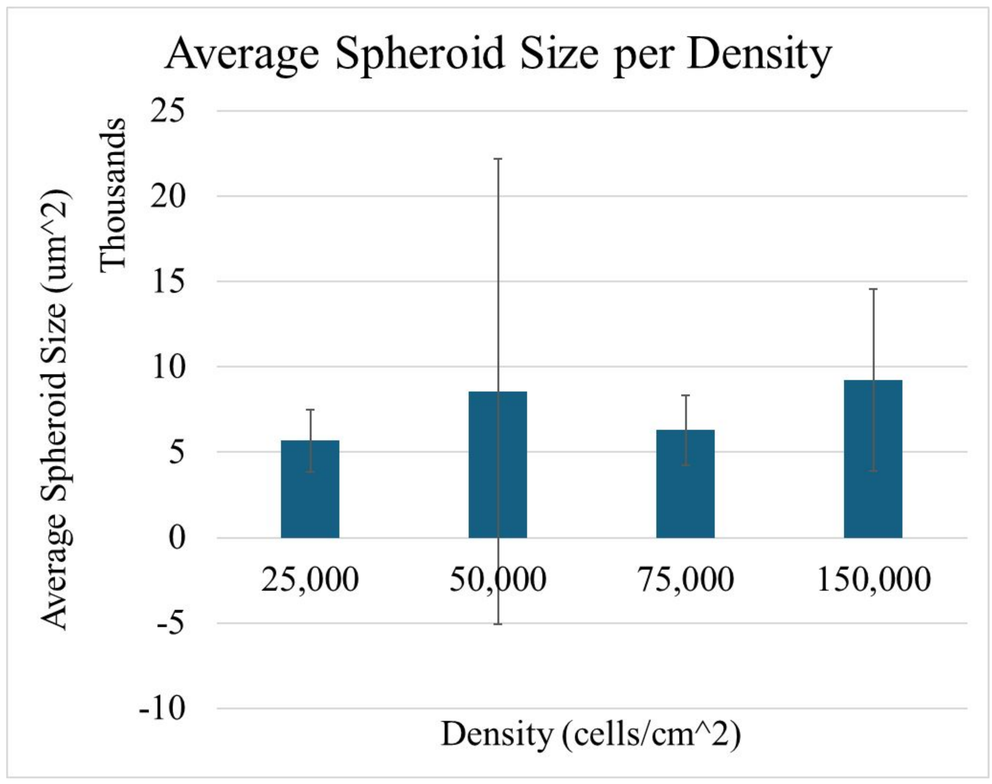
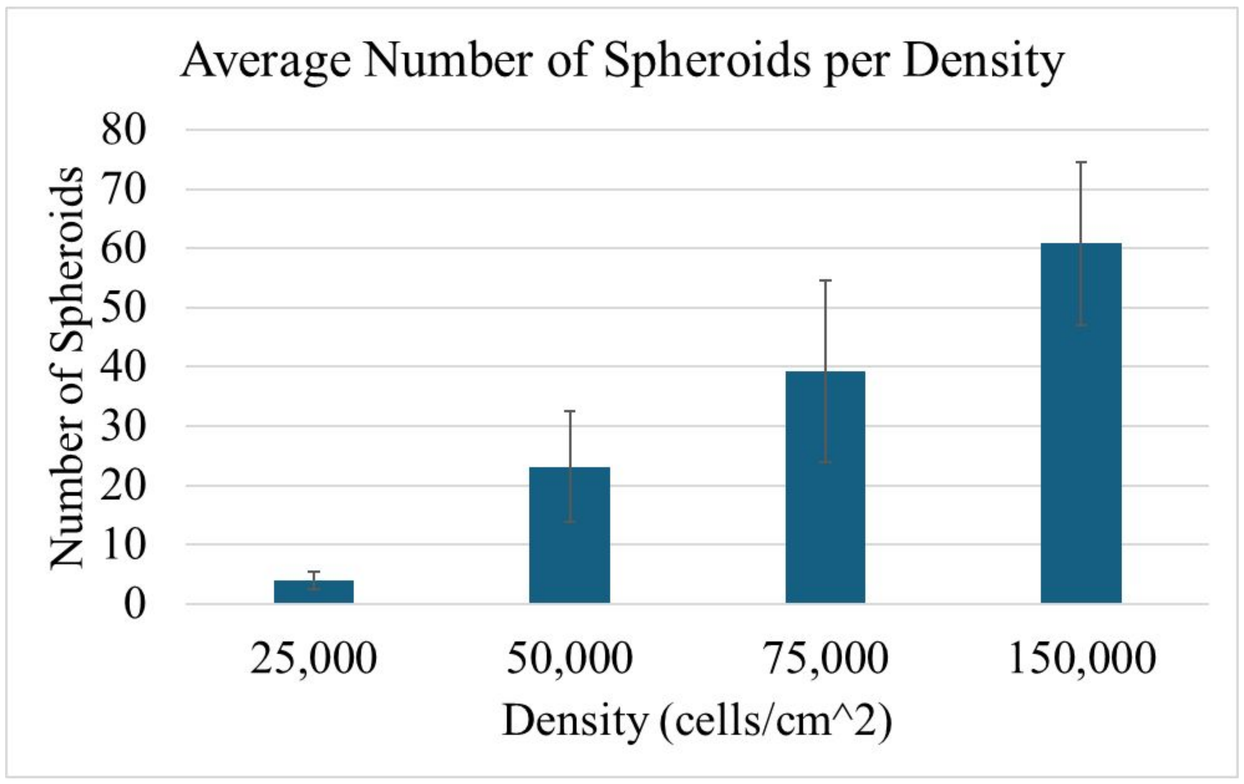

Average Spheroid Size per Density
Average Spheroid Size (um^2)
Thousands
25
20
15
10
5
0
-5
-10
25,000
50,000
75,000
150,000
Density (cells/cm^2)


Average Number of Spheroids per Density
Number of Spheroids
80
70
60
50
40
30
20
10
0
25,000
50,000
75,000
150,000
Density (cells/cm^2)

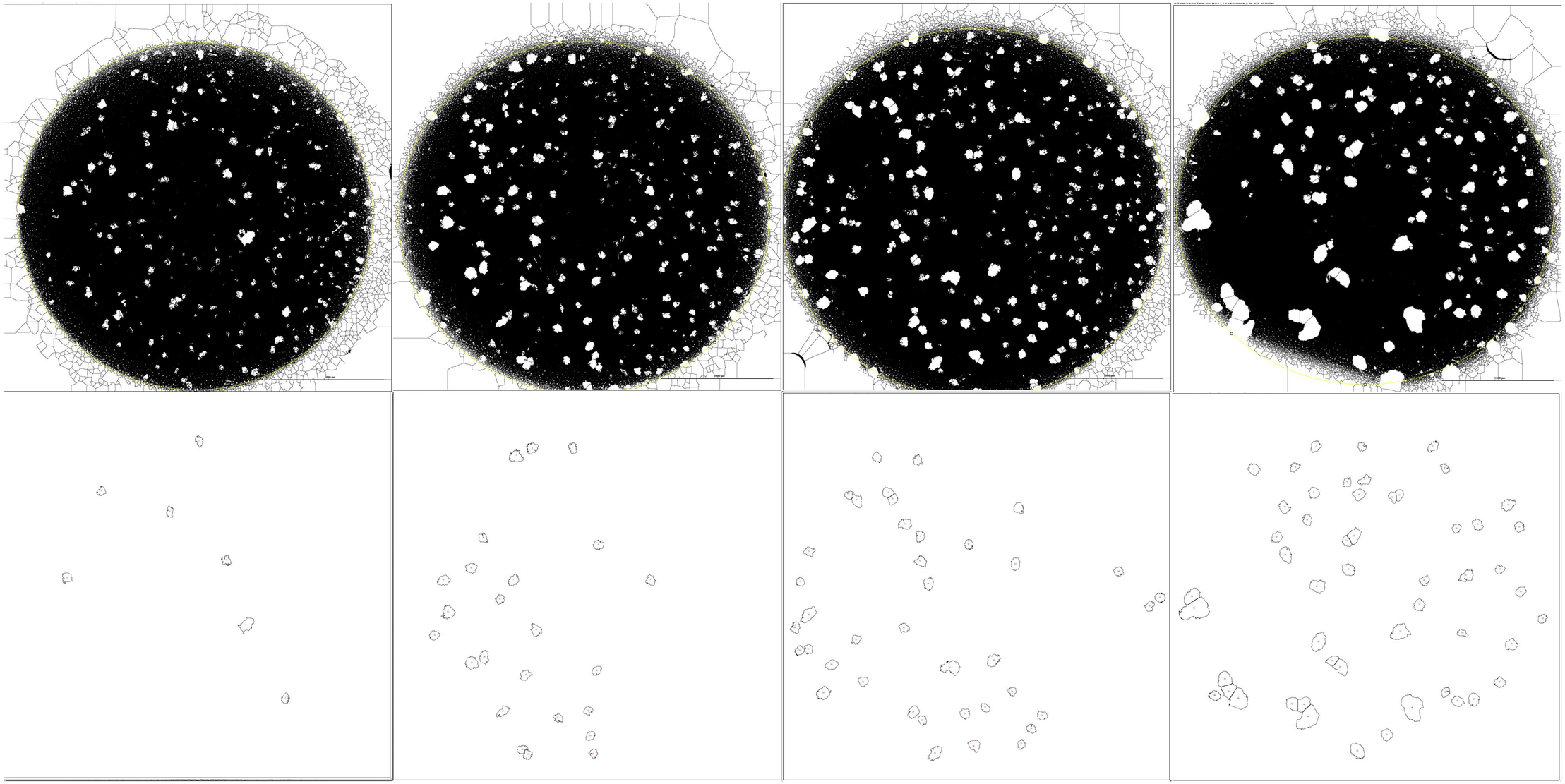
Density 1: 25k

Density 2: 50k

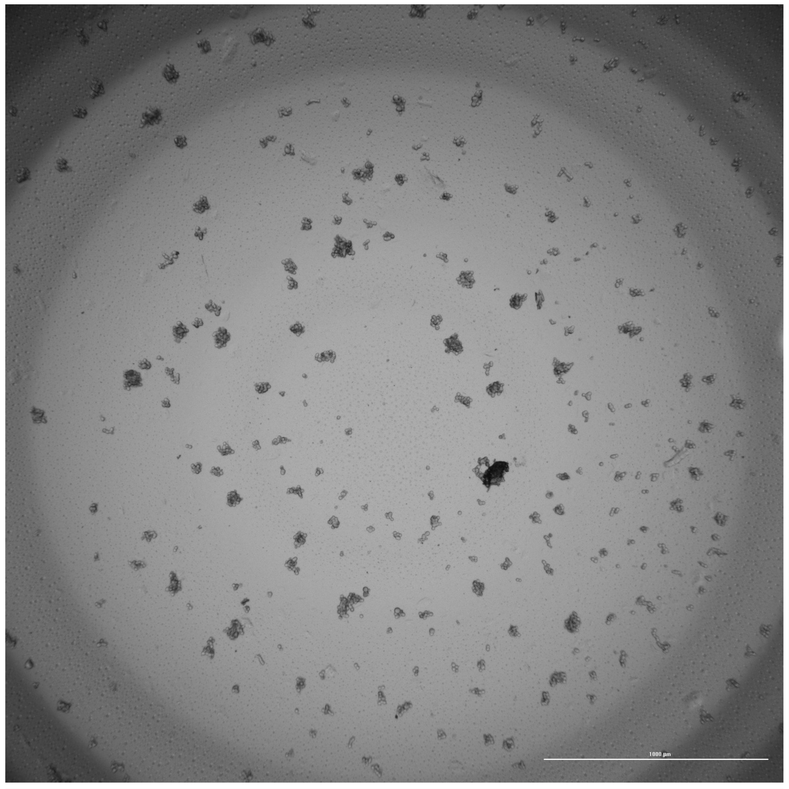
Density 3: 75k

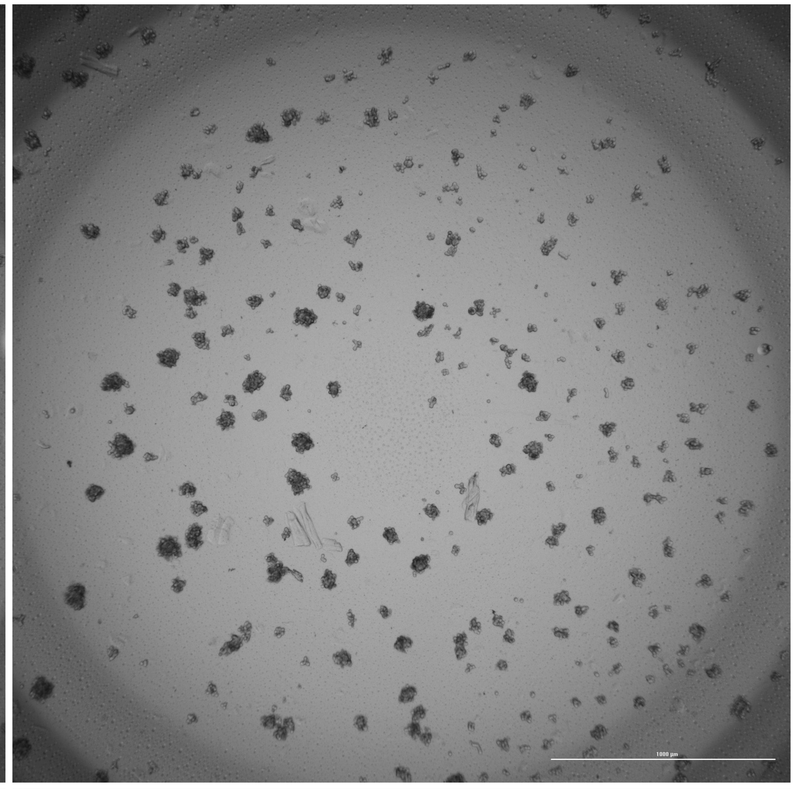
Density 4: 150k

Threshold-Watershed Analysis View



Spheroid Outline


1000 µm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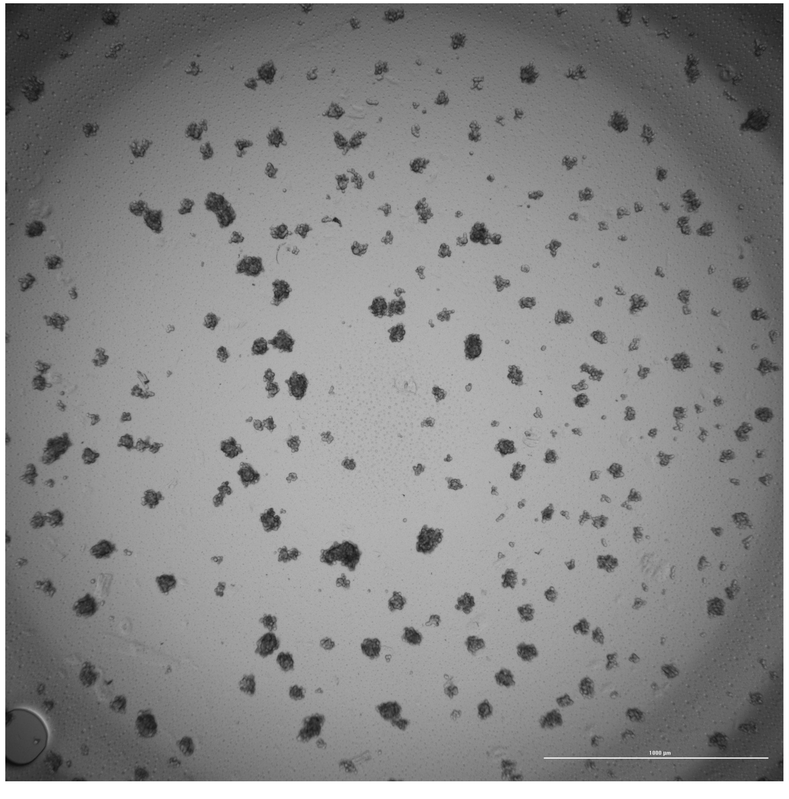
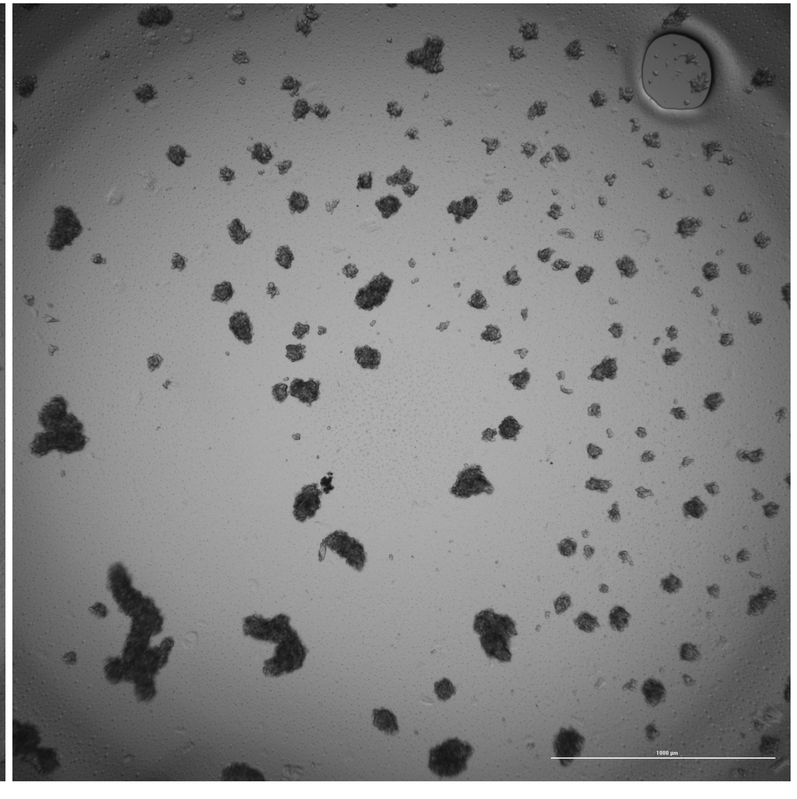

1000 µm


1000 µm