3D Primary Cancer Models for Phenotypic Drug Discovery Applications

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AACR Exhibitor Spotlight Presentation:
Magnetic 3D Bioprinting, High-Throughput Screening Platform to Advance Cancer Research
April 3rd, 2019

http://hts.florida.scripps.edu/
• SRMSC HTS Operations Overview

• 3D Research Approach
  ▪ Monoculture vs. 3D Spheroid/Organoid:
    ➢ 3D Structure Confirmation
    ➢ Compound Response to 2D vs. 3D
  ▪ 3D Pancreatic Cancer Screen
    ➢ 3D Technology and HTS Protocol
    ➢ Dose Response results
  ▪ Current 3D efforts and future directions
    ➢ Precision Oncology
    ➢ Glioblastoma cells isolation from patients

• Summary
SRMSC began in 2005
Biologists, Biochemists, Programmers, Chemists & Engineers (currently >500 employees)
Industrial scale HTS lab with Kalypsys/GNF automated platform
• >646K Proprietary (largest in academia, ~30k unique compounds, focused sub-libraries, professionally curated)
• >360K Public Domain (NIH)
Funding is driven by NIH grants and Collaborations with Pharma and Biotech
HTS-Lead Identification occurs early in drug discovery; it can save $ and speed up the overall drug-discovery process.
1.7m Arm Incubators Transfer Dispense/Wash PE Suite FLIPR

- Built specifically for 1536-well plate screening (384-well plate screening also possible)
- Capable of over one million wells screened in a 24-hr period (1536-well format)
- Patented lid prevents evaporation of plate contents
- Long (>96 hr) plate incubation protocols possible
- Plate capacity >1,500 (>2.3 million wells in 1536-well plate format)
- Plate incubation from 4°C to 50°C, 0-100% rH, any gas (CO₂, N₂, Ar, etc.)
- 1536-well plate washers and transfer pipettor enable heterogeneous assays/fixing steps
- Luminescence, BRET, Absorbance, Fluorescence Intensity, FP, TRF, FRET, TR-FRET, AlphaScreen, AlphaLISA, FLIPR, High-Content...
Cancer is among the leading causes of death worldwide

Survival generally ranges about 14-18 months, although about 10% of patients live 5 years or longer.

Most common cause of cancer death

Remains a leading cause of cancer-associated death, with a 5-year survival rate that still remains less than 10%.

Primary Cell lines
Resected tumors from human samples

Wikipedia: Häggström
Cells are grown in flasks as 2D monolayer, harvested using sterile technique transferred into plates in 3D spheroid/organoid format.
1. Cell Culture

2D cell culturing not good predictor of in vivo Biology

2. Animal Testing

Poor results, costly with ethical issues of animal testing NOT human cells

3. Humans

$$$$$$$$$$
and too long

Why 3D?
3D organoid models are driving the new approach in research to recapitulate the body functions and mimic the physiological processes in the human tissue.

Our Research is using Pancreatic Cancer Patient-derived Primary Cell Lines

• Primary human pancreatic ductal cells hM1-2D, hF2-2D, hT1-2D, and cancer-associated fibroblasts hM1-CAF and hT1-CAF, were generated from tissues of pancreatic cancer patients in the laboratory of Dr. Tuveson, M.D. at CSHL.

• 2D monolayer cells were conditioned from established pancreatic 3D organoids.


• Targeted sequencing analysis of human pancreatic organoids reveals their genetic makeup.

<table>
<thead>
<tr>
<th>Name</th>
<th>Tissue</th>
<th>Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>hM1-CAF</td>
<td>a resected metastatic lung lesion, same as hM1</td>
<td>Kras^{wt} (fibroblast, SV40 immortalized)</td>
</tr>
<tr>
<td>hM1</td>
<td>a resected metastatic lung lesion</td>
<td>Kras^{G12D}, p53^{R175H} (epithelial-like)</td>
</tr>
<tr>
<td>hF2</td>
<td>a fine-needle aspiration biopsy of a metastatic lesion</td>
<td>Kras^{G12V}, SMAD4^{loss}, CDKN2A^{loss} (epithelial-like)</td>
</tr>
<tr>
<td>hT1-CAF</td>
<td>resected primary tumors, same as hT1</td>
<td>Kras^{wt} (fibroblast, SV40 immortalized)</td>
</tr>
<tr>
<td>hT1</td>
<td>resected primary tumors</td>
<td>Kras^{G12V}, P53^{loss}, SMAD4^{loss}, CDKN2A^{hom del} (epithelial-like)</td>
</tr>
</tbody>
</table>
# Examples of Commercially Available 3D Spheroids Production Methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Example of Product (Manufacturer)</th>
<th>Single spheroid per well</th>
<th>Miniaturizable to 1536?</th>
<th>Spheroid centered within well?</th>
<th>Homogeneous in format?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular matrices</td>
<td>Matrigel (Corning) Qgel (Qgel)</td>
<td>✗</td>
<td>✓</td>
<td>✗</td>
<td>✓</td>
</tr>
<tr>
<td>Hanging drops</td>
<td>GravityPLUS hanging drop system (InSphero) Perfecta3D (3D BioMatrix)</td>
<td>✓</td>
<td></td>
<td>Potentially difficult</td>
<td>✗</td>
</tr>
<tr>
<td>Micro-patterned surfaces</td>
<td>AggreWell (StemCell Technologies) 3D micro pattern culture plate (Diagenode)</td>
<td>✗</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Ultra-low attachment surfaces and flat-well geometry</td>
<td>Cell repellent microplates (Greiner Bio-One) Corning flat ULA microplates (Corning) Nunclon Sphera microplates (Thermo)</td>
<td>✗</td>
<td>✓</td>
<td>✗</td>
<td>✓</td>
</tr>
<tr>
<td>Ultra-low attachment surfaces with round-well geometry</td>
<td>GravityTrap ULA plates (InSphero)</td>
<td>✓</td>
<td>✓</td>
<td>✗</td>
<td>✗</td>
</tr>
<tr>
<td></td>
<td>ULA spheroid Microplates (Corning)</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Magnetic 3D bioprinting</td>
<td>NanoShuttle (Nano3D) w/Greiner Bio-One cell repellent plates</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
</tbody>
</table>

## Diagrams

- Matrigel-embedded culture
- Hanging drop culture
- Round-bottomed microwell culture
- Magnetic 3D bioprinting
**2D Monolayer vs. 3D Spheroid/Organoid Screen**

**Capability to form spheroids or Organoids**

**4X objective, wide field**
- PANC-1
- hT1

**20X objective, Z-stack**
- PANC-1
- hT1

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**2D Monolayer vs. 3D cell model**

**2D HTS**
- 4X objective

**3D HTS**
- 20X objective
- Z stack Confocal Image

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**Hill Slope**
- DOX: 1.47
- GEM: 0.93
- SN-38: 0.47

**IC50**
- DOX: 3.28E-07
- GEM: >9.98E-06
- SN-38: 3.68E-08

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**Log[Compound], M**
- % Inhibition

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**Hou et al SLAS Discovery, 2018, 23 (6): 574-584**

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**Tuveson et al Cell, 2015, 160 (0): 324-338**
Cell repellent plates readily form spheroids using cells either combined with Nanoshuttle when forced into shape using a magnet.
Concentrating Cells Magnetically

0 to 10 minutes of levitation

Cells come together rapidly

Levitation
Large size samples

Magnet Top

Cells

Bioprinting
High-throughput
Smaller Samples
Automation

Cells

Magnet Bottom

Souza et al. Nature Nanotechnology, 2010
Timm et al. Scientific Reports, 2013
Tseng et al. Scientific Reports, 2015
1536-well Assay

Add nanoshuttle to cells in flask (0.6 mL per T175), incubate cells overnight | 37°C 5%CO₂ 95% RH

Harvest cells, seed 1250 cells in 5 μL culture medium to 1536 Greiner cell repellent plate

Put the plate atop of the spheroid drive, incubate the plate on drive for 4 hrs

Incubate cells for 24 hrs | 37°C 5%CO₂ 95% RH

Pintool transfer 10 nL compounds

Incubate cells for additional 3 days | 37°C 5%CO₂ 95% RH

Image spheroids using HCS

Dispense 5 μL CellTiter-Glo 3D reagent, shake for 10 mins, incubate for 60 min at RT, Read on ViewLux

Final volume = 5 μL

Cell Viability Assay

Example of DMSO Plate

High Control

Low Control: Cells + DMSO

Low Control

\[ Z' = 1 - \frac{3SD \text{ of Low Control} + 3SD \text{ of High Control}}{(\text{Low Control} - \text{High Control})} \]

% inhibition = 100 \times \left(1 - \frac{\text{Test Well} - \text{Median High Control}}{\text{Median Low Control} - \text{Median High Control}}\right)
Outcomes of Oncology Drug Screen

1 - Trametinib
2 - Romidepsin
3 - Bortezomib
4 - Carfilzomib
5 - Homoharringtonine
8 - Doxorubicin
97 - Gemcitabine

Hou et al SLAS Discovery, 2018, 23 (6): 574-584
Activity of approved drugs against both 2D and 3D models

% Inhibition (hT1-2D) vs. % Inhibition (hT1-3D)

- Disulfiram

Outcomes of Approved Drug Screen
Drug Resistance Factor (the ratio of IC_{50} between 2D and 3D assay)

<table>
<thead>
<tr>
<th>Drug</th>
<th>hT1</th>
<th>hT1-CAF</th>
<th>hM1</th>
<th>hM1-CAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bortezomib</td>
<td>4.0</td>
<td>5.9</td>
<td>7.9</td>
<td>4.4</td>
</tr>
<tr>
<td>Carfilzomib</td>
<td>3.3</td>
<td>8.1</td>
<td>3.2</td>
<td>7.3</td>
</tr>
<tr>
<td>Trametinib</td>
<td>&lt;0.001</td>
<td>&lt;0.3</td>
<td>6.1</td>
<td>&lt;0.6</td>
</tr>
<tr>
<td>Romidepsin</td>
<td>24.0</td>
<td>3.3</td>
<td>28.8</td>
<td>1.5</td>
</tr>
<tr>
<td>Disulfiram</td>
<td>&lt;0.03</td>
<td>/</td>
<td>0.3</td>
<td>&gt;32.9</td>
</tr>
</tbody>
</table>

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• Scalable

• Automation-friendly

• Single spheroid per well

• Minimize peripheral devices - i.e. adapters

• Miniaturizable to 1536-well plate format

• Spheroid to be centrally located within the well

• Well-to-well uniformity of spheroid size and morphology

• Homogenous format (no transfer of spheroids or aspiration step required)
Magnetic 3D bioprinting technology has been successfully integrated into our GNF/Kalypsys automation platforms.
Both lab adapted and primary tumor cells readily form 3D structures which we confirmed using Z-stack confocal analysis.

HT-29, PANC-1 and CAFs formed compact spheroids, while the primary cancer cells grew as organoids. (confirmed by Dr. Tuveson at CHSL)
Homogeneity of Organoids in 1536

A

Cells per well at seeding

500  1000  1500  2000  2500  3000  3500  4000  4500  5000

B

C

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This assay showed excellent Z’ values.
CRC of control compounds are consistent from batch to batch.
150K library primary screen vs. hT1-3D was complete.
Identified 735 hits will be directly proceeded to dose response test.
### 3D hT1 Titration Results

**Legend:** 3D-hT1, 3D-hT1-CAF, 3D-hM1, 3D-Hepato

<table>
<thead>
<tr>
<th>Curve Class</th>
<th>Inhibition Profile</th>
<th>Selection Parameter</th>
<th>Example</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Selective for 3D hT1, little activity in 3D hT1 CAF or 3D HepatoCells</td>
<td>• &gt;50% in 3D hT1 (red)</td>
<td><img src="image1.png" alt="Graph" /></td>
<td>6     (0.8%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• &lt;50% in 3D hM1 (green)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• &lt;50% in 3D hT1-CAF (blue)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• &lt;50% in 3D HepatoCells (grey)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• 2 compounds with &gt;1.5 fold CC50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Selective for 3D hM1, little activity in 3D hT1 CAF or 3D HepatoCells</td>
<td>• &gt;50% in 3D hM1 (green)</td>
<td><img src="image2.png" alt="Graph" /></td>
<td>7     (1%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• &lt;50% in 3D hT1 (red)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• &lt;50% in 3D hT1-CAF (blue)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• &lt;50% in 3D HepatoCells (grey)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• 5 compounds with &gt;1.5 fold CC50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Selective for 3D hT1 or 3D hM1, less activity in 3D hT1-CAF, inactive in 3D HepatoCells</td>
<td>• &gt;50% in 3D hT1 (red)</td>
<td><img src="image3.png" alt="Graph" /></td>
<td>5     (0.7%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• &gt;50% in 3D hM1 (green)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• &lt;50% in 3D hT1-CAF (blue)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• &lt;50% in 3D HepatoCells (grey)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• 2 compounds with &gt;3 fold CC50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
New molecules have become discovered that show efficacy in the cancers but normal cells and hepatocells are unaffected!

SR-963 shows <80nM potency in 14 different pancreas cancer organoids in the Tuveson Lab!
Cancer-associated fibroblasts (CAFs) are cellular components of the desmoplastic stroma characteristic to the tumor that contributes to this treatment resistance.

D. Ahrens, et. al. Journal of Hematology & Oncology, 2017, 10: 76

Fig. 1 Pancreatic ductal adenocarcinoma with desmoplasia. The PDAC tumor microenvironment is comprised of cellular and acellular components including CAFs, immune cells, and extracellular matrix.

Co-culture of cancer cells and fibroblasts (hT1 + hT1-CAF)
Co-culture using Magnetic 3D Technology

2500 hT1-CAF

2500 hT1

Co-culture (2500:2500)

hT1-3D monoculture

Coculture

hT1CAF-3D monoculture

→ Co-culture of hT1-CAF and hT1 formed a more compact structure but not as tight as CAFs alone.
→ Co-culture format more closely approximates the hT1 cancer cell outcome alone...

Octadecyl Rhodamine B Chloride (R18)
CellTracker green

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High Grade Gliomas

- 3D HTS targeting primary tumors presents an attractive approach to improve treatment outcome in GBM

(Classical established tumor cell lines HTS campaign haven’t historically shown a good correlation with clinical outcome)

Characterization of GBM6 in 1536-wells

- GBM6-spheroids presents an homogenous distribution in size and number after GSC seeding in 1536-wells.
**Run Statistics (n = 3 plates)**
- 3290 compounds tested at 2 μM nominal concentration, 1X
  - Ave $Z'$ = 0.77±0.02
  - Ave S:B = 163.52±7.49
  - Hit cutoff (Ave+3SD) = 32.41%
  - Hits = 66, Hit Rate = 2.0%

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**In vivo studies**

**C. Intracranial GBM6 tumor growth**

It was administered as a continuous intravenous infusion (CIVI) over 72 h in 21-day treatment cycles.

**D. Survival curve**

Vehicle

YM155

*
Aim to isolate cells from brain tumors collected from patients that are positive for GBM.

For therapeutic applications and determine physiological relevance toward humans.

**PDAC derived cells**

**Glioblastoma derived cells**
Protocols to isolate the GBM6 PDX tumor cells were successfully implemented for this purpose.

Successfully disaggregated tumor biopsies to isolate cells from samples.

Cell Isolation

**BRRH**: Biopsy of tumor brain from patient. Collect and perform pathology and send to Scripps.

**SCRIPPS**: In the tissue culture biosafety hood, mince the tumor with the help of scalpels.

- Make at least three rounds of mincing in 5-10ml of medium (10%FCS is ok) taking the medium with cells (leaving the chunks) and adding 5-10ml of fresh medium.

Passage the chunks through 70 μm cell strainer to get the maximum amount of cells.

Spin down for 5min at 320g. The cells are going to be the pellet.

Count and seed using StemPro NSC SFM (Life Technologies).

**StemPro NSC SFM**

Developed for the growth and expansion of neural stem cells maintaining their potential to differentiate into neurons and glial cells.

After cell isolation procedure, the cells were counted:

- Cell Count: $4.2 \times 10^6$ cells/mL
- Total yield: ~20M*

*Note: These counts represent a mix population of all cells after isolation.

**BRRH 003 Tumor Specimen**

Tumor weight ~0.5g

Before mincing

After centrifugation

Cell Pellet
GBM Human Patient Cells Growth In vitro

→ GBM cells are seeded in flasks with Serum free media to select for the growth of cancer cells.
→ The formation of spheres is clearly observed.
→ Center of the spheroid shows a possible “necrotic core”
3D spheroid formation was confirmed by Z stack confocal analysis.

Images were taken using a INCell 6000 confocal microscope. Slices every 4 nm increments are showing below (24 slices total)

→ 3D spheroid formation was confirmed by Z stack confocal analysis.
Future Directions: Drug Screening

Cancer markers:
Confirm type of cancer
GBM: ex. EGFRvIII, aneuploidy, 5-ALA

Cellular Heterogeneity:

ECM Deposition:
e.g. Collagen, cell adhesions, cell to cell contact

Cell Growth Conditions:
EGF, FGF, N2 and B27 supplement
Glutamax, MEM-NEAA, Y27632

3D Drug Screening:
Determine potential candidates (SAR)
Establish possible drug gene therapies
Improve clinical outcome correlation
Challenge: Able to expand the human cancer cell lines in 3D format

Necrotic Zone “Core” (Low oxygen levels)
Senescence Zone (quiescent zone)
IF with Hoechst and anti-ΔEGFRvIII-Alexa 488

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3D Cell Titer Glo was used to determine the cell viability of the cells.

Preliminary studies show that the ATP concentration increases based on the cell linearity test.
Day 0
Seed >100 cells in 5 µL

Allow the spheres to form for 2 days in incubator (37°C, 5%CO₂, 99% RH)

Day 2
Transfer compounds using 10 nL pintool

Maintain in incubator for 3 days (37°C, 5%CO₂, 99% RH)

Day 5
Add 5 µL CellTiterGlo

Incubate 60 min at RT

Read plates using ViewLux
**Pharmacology of control matches the expected.**

**Next: Data results from NCI Approved pilot screen as CRC**
Screenshot of the active hits found from the NCI oncologic CRC library pilot screen.

11 of the 128 drugs have potency <5uM and some of them are extremely potent

Next: BRRH 003 vs. NCI Approved Oncology Dose Response pilot screen
Screenshot of the active hits found from the NCI oncologic CRC library pilot screen.

→ 15 of the 128 drugs have potency <5uM and some of them are extremely potent.

Next: Update the team
Key takeaways

- Evidence that 3D technologies are rapid, cost effective and homogeneous solutions for physiologically relevant HTS

- A comparison of both 2D and 3D cell culture models vs large approved drug libraries testing primary pancreatic tumors including their cancer associated fibroblasts

- Co-culturing models of pancreatic tumors and CAFs in HTS behave differently

- 3D modeling of brain tumors (Glioblastoma) in HTS vs. Drugs or pre-IND molecules is an effective process

- Direct evidence to support 3D models exhibit outcomes that are clinically relevant

- Rapid 3D technological implementation directed towards large scale HTS and precision medicine has been developed.

2. Singhera F, Cooper E, Scampavia L, Spicer T. **Using bead injection to model dispensing of 3-D multicellular spheroids into microtiter plates.** Talanta. 2017 Sep. PMID:29108585


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