3-Dimensional Cell Culture Systems, Novel Technologies, and Their Use at the Laboratory Bench

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This work was completed in part by Shurong Hou, Franck Madoux and Louis Scampavia

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http://hts.florida.scripps.edu/
Introduction to Scripps Molecular Screening Center

Where robotics, chemistry and biology join forces to help discover new drugs

• SRIMSC began in 2005
• Biologists, Biochemists, Programmers, Chemists & Engineers (currently >600 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
Pancreatic cancer remains a leading cause of cancer-associated death, with a 5-year survival rate less than 10%.

Genetic KRAS (Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) mutations are found in more than 90% of pancreatic cancer patients.

Genetic alterations in tumor suppressors TP53, CDKN2A, SMAD4, ARID1A and MLL3 also accumulate in the pancreatic cancer patient.

Stage at diagnosis
2002-2008

- 12% Localized
- 8% Regional
- 53% Distant
- 27% Unknown

5-year relative survival rate (%)
2002-2008

- 23% Localized
- 9% Regional
- 2% Distant
- 6% All stages

R. Siegel, et.al. CA CANCER J CLIN 2013, 63: 11-30
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. 

<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>hM1-2D</td>
<td>a resected metastatic lung lesion</td>
<td>Kras(^{G12D}), p53(^{R175H}) (epithelial-like)</td>
</tr>
<tr>
<td>hF2-2D</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>hT1-2D</td>
<td>resected primary tumors</td>
<td>Kras(^{G12V}), P53(^{loss}), SMAD4(^{loss}), CDKN2A(^{hom,del}) (epithelial-like)</td>
</tr>
</tbody>
</table>

- Targeted sequencing analysis of human pancreatic organoids reveals their genetic makeup.
Why Culture Cells in 3D?

• Evaluation of drug cytotoxicity traditionally relies on use of cell monolayers.

• Three-dimensional (3D) cell models have been described as more physiologically relevant for a variety of applications.

• Numerous methods have been developed to produce spheroids.

• We are funded to help develop the HTS 3D formatted technologies
  • NCI IMAT R33 CA206949

M. Zanoni, et.al. Scientific Reports, 2017, 6:19103
H. Tseng, et.al. Scientific Reports 2017, 5:13987
Prototype Ultra-Low Attachment T-25 Spheroid Microcavity Flask.
Microcavity size and numbers: 500 µm in diameter and depth; 157 microcavities/cm², a total of 3300 microcavities/flask.
Gravity, in conjunction with the ULA surface, and rounded pit-bottom geometry, work to promote spheroid formation.

From Corning spheroids T-25 microcavity flask insert
Initial Test Goals

☑  Aim #1: Verify ability to grow 3D hT29 spheroids in microcavity T25 flask

☑  Aim #2: Detect shape and size of hT29 spheroids in microcavity flask and spheroid plates

☑  Aim #3: Assess cavity-to-cavity variability
2.6M HT29 cells were seeded in T25 microcavity flask (800 cells/cavity) and normal T25 flask.

Images from standard microscopy reveals that HT-29 cells regroup in dense structure over 48hrs and spheroid size increases from 48hrs to 96hrs.
Monitoring 3D hT29 spheroid size over time (slide 1 of 2)

Images from bright field microscope (4X Objective) of Corning 384-well spheroid plate

<table>
<thead>
<tr>
<th>Time</th>
<th>Spheroids from microcavity flask</th>
<th>Spheroids in spheroid plate seeded at 800 cells per well</th>
</tr>
</thead>
<tbody>
<tr>
<td>1h after seeding</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>+24h</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>+48h</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>+72h</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>+96h</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND= not done to avoid disruption of spheroids

→ As point of comparison 800 HT29 cells per well (matches microcavity method) were seeded in Corning 384 spheroid plate and compared with spheroids grown in microcavity flask.
   → Spheroids grown in microcavity flask were transferred to Corning 384 spheroid plate for imaging at 72hrs and 96hrs post-seeding.
→ Standard microscopy shows that the size of spheroids from microcavity flask is smaller than spheroids seeded at 800 cells per well in spheroid plates.
Images taken of spheroids in Corning 384 well plate
Our imaging instruments confirmed the spheroidicity of the spheroids grown in microcavity flask: Thermo CellInsight (A) 5X Objective, and Scripps HIAPI Imager (B).

Average spheroid diameter shown in Figure A was calculated using Thermo CellInsight software.

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### Monitoring 3D hT29 spheroid size over time (slide 2 of 2)

<table>
<thead>
<tr>
<th></th>
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<th>+48h</th>
<th>+72h</th>
<th>+96h</th>
</tr>
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<tbody>
<tr>
<td><strong>A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spheroids from microcavity flask</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Spheroids in spheroid plate seeded at 800 cells per well</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tbody>
<tr>
<td><strong>B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spheroids from microcavity flask</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
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ND= not done to avoid disruption of spheroids or in case of plate based method it was early and deemed not necessary.
The spheroids in most of the cavities are in similar shape and size (A). However, spheroids in some cavities are quite different in size (B). In order to transfer spheroids for analysis in 384 well microplates the microcavity flasks had to be manipulated (i.e. shook and tilted). After manipulating, spheroids in microcavity flask were dislodged, resulting in one or (C) several spheroids in one cavity even (D) zero.
Initial Test Summary and Next Steps

**Conclusion**
- Spheroids grows well in Ultra-Low Attachment T-25 Spheroid Microcavity Flasks.
- Our imaging system can detect shape and size of spheroids in microcavity flask and spheroid plates
- Most of the spheroids are in the same shape and size. However, some of cavities did not grow or grew spheroids in different sizes.

**Next**
- **✓** Aim #1: Verify the ability of other cell lines to grow 3D spheroids in microcavity T25 flask
  - Assess the variability: cell line-to-cell line; cavity-to-cavity; flask batch-to-batch
- **✓** Aim #2: Evaluate HTS readiness by dispensing whole spheroids into 1536 well plate
  - Confirm the 3D structure of dispensed spheroids
  - Identify possible issues and solutions
2.6M HT29 or PANC-1 cells, and 0.8M hT1-2D (primary pancreatic cancer cells) were seeded in T25 microcavity flask (~800 cells/cavity).

Images from standard microscopy reveal that HT-29 and PANC-1 cells form dense spheroids in microcavity flasks, while hT1-2D could not.

The difficulty of hT1-2D to form spheroids in flask is anticipated, as this cell line could not form typical spheroids using other technologies, including Corning spheroid plate, and n3D bioprinting.
2.6M HT29 or PANC-1 cells, and 0.8M hT1-2D were seeded in T25 microcavity flask (800 cells/cavity).

Most of the formed spheroids are in similar shape and size (Top images). However, spheroids in some cavities are quite different in size and numbers (Bottom images).

Air trapped in cavities when seeding cells in flask could cause heterogeneity of spheroids.
Spheroid dispense using solenoid valve dispenser

Spheroid Culture and Dispense

Seed ~2.6M cells per T25 spheroid flask (800 cells per cavity)

Incubate for 4 days | 37°C 5%CO₂ 95% RH

“Tip and Tap” to harvest the spheroid

Let spheroids settle for ~5 min, aspirate off top media. Bring up to the estimated # of spheroids per well

Dispense 5 µL spheroids into 1536 ULA plates (1 or 4 spheroids per well)

→ Estimate 3000 spheroids per T25 flask, and diluted them with fresh medium, so that proper dispense would get either 1 or 4 spheroids per well.
To evaluate its compatibility for HTS application, HT29 or PANC-1 spheroids cultured in T25 microcavity flask were harvested and dispensed using Scripps solenoid valve dispensers into 1536 ULA plate.

- Cellular debris was obvious but still get spheroids.
- It is difficult to homogeneously dispense same or even similar number of spheroids into each well.
- Z-stack confocal images confirmed the 3D structure of hT29 and PANC-1 spheroids after dispense, but the harvest and dispense procedure may affect spheroid integrity.
Conclusions:
- 3 cell lines were tested to evaluate the ability of Microcavity flasks to grow spheroids.
  - hT29 and PANC-1 cells formed nice 3D spheroids in Ultra-Low Attachment T-25 Spheroid Microcavity Flasks.
  - Same as using other technologies, hT1-2D has difficulty to grow spheroids in the spheroid flask.
  - Issues:
    - Air trapped in cavities greatly affected spheroid formation, and the suggested protocol could not remove them.
  - Scripps is able to dispense whole spheroids into 1536 well plate using solenoid valve dispensers.
  - Issues:
    - It is hard to homogeneously dispense spheroids into wells.
    - The procedure of harvest and dispense could affect the integrity of whole spheroids.

Next:
- **Aim #1**: Try spheroid dispense using acoustic dispenser
- **Aim #2**: Evaluate HTS readiness by dispensing whole spheroids into 384 well plate
  - Compare manual dispense vs. WellMate dispense
  - Evaluate the homogeneity of spheroid dispense
- **Aim #3**: Test cytotoxicity of known compounds on PANC-1 spheroids
  - Side-by-side comparison of CRC of 5 drugs on spheroids transferred from flask and spheroids cultured in plate
Spheroid Culture and Dispense

Seed ~2.6M PANC-1 cells per T25 spheroid flask (800 cells per cavity)

Incubate for 2 days | 37°C 5%CO₂ 95% RH

“Tip and Tap” to harvest the spheroid

Let spheroids settle for ~5min, aspirate off top media. Bring up to the estimated # of spheroids per well

Dispense 30nL (1 drop) or 300nL (10 drops) into 384 ULA plates (1 or 10 spheroids per well)

Add 25µL medium into each well

→ Diameter of PANC-1 spheroids cultured for 2 days is ~180 μm, and the volume would be ~30 nL, we should be able to get a 30 nL drop to include 1 spheroid.
→ Tried to dispense 1 drop per well, or 10 drops per well for well drain test, so that proper transfer would get either 1 or 10 spheroids per well.

This paper used acoustic dispenser for individual cell dispense
PANC-1 spheroids cultured for 2 days in T25 microcavity flask were harvested and dispensed using Scripps Acoustic dispensers into 384 ULA plate.

Dispensing 1 drop or 10 drops of spheroids did not achieve 1 or 10 spheroids per well.

Acoustic dispense procedure disrupted spheroid integrity.
Spheroid dispense using WellMate dispenser and cytotoxicity test

Spheroid Dispense and Drug Test

Seed ~2.6M PANC-1 cells per T25 spheroid flask (800 cells per cavity)

Incubate for 4 days | 37°C 5%CO₂ 95% RH

“Tip and Tap” to harvest the spheroid

Let spheroids settle for ~5 min, aspirate off top media. Bring up to the estimated # of spheroids per well

Dispense 25 μL spheroids into 384 ULA plates (1, 3 or 10 spheroids per well)

Transfer 50nL compounds into each well

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

Dispense 3D CellTiter-Glo and read plates

→ Estimate 3000 spheroids per T25 flask, and diluted them with fresh medium, so that proper dispense would get either 1, 3 or 10 spheroids per well.

→ To evaluate the homogeneity of spheroid dispense, 3D CellTiter-Glo was added immediately after spheroid dispense.
To evaluate its compatibility for HTS application, PANC-1 spheroids cultured for 4 days in T25 microcavity flask were harvested and dispensed using WellMate into 384 ULA plate.

It is difficult to homogeneously dispense same or even similar number of spheroids into each well. WellMate dispense performed similarly with manual dispense (multichannel pipetting). %CV decreased as the dispensed spheroids # per well increased.
To test the cytotoxicity of known compounds on spheroids, PANC-1 spheroids cultured in T25 microcavity flask were harvested at 4 days post seeding and dispensed using WellMate dispenser into 384 ULA plate (aiming for 8 spheroids per well), then incubated for 3 more days after drug addition.

800 PANC-1 cells per well were seeded into Corning Spheroid Plate, and cultured for 4 days before drug addition, then incubated for 3 more days.

Due to the difficulty to homogeneously dispense similar number of spheroids into each well, CRC data for spheroids transferred from flask was more variable than CRC for spheroids cultured in Corning Spheroid Plate (n=12 per concentration).

ViewLux images of 384 ULA plates dispensed with ~8 spheroids per well (A) or 384 spheroid plates seeding with 800 cells per well (B), treated with different concentrations of 5 compounds and detected by 3D CellTiter-Glo.

Col1: Medium only; Col2: Cells + 33µM DOX; Col23&24: Cells + DMSO

<table>
<thead>
<tr>
<th>Compound</th>
<th>LogIC50</th>
<th>HillSlope</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXA</td>
<td>-3.734</td>
<td>-0.4086</td>
<td>0.0001844</td>
</tr>
<tr>
<td>DOX</td>
<td>-5.488</td>
<td>-0.9703</td>
<td>3.248e-006</td>
</tr>
<tr>
<td>GEM</td>
<td>-6.603</td>
<td>-1.186</td>
<td>2.497e-007</td>
</tr>
<tr>
<td>5-FU</td>
<td>~ 1.706</td>
<td>-0.5368</td>
<td>~ 50.79</td>
</tr>
<tr>
<td>SN-38</td>
<td>-6.885</td>
<td>-0.7871</td>
<td>1.302e-007</td>
</tr>
</tbody>
</table>
Summary for 3rd Round of Tests

Conclusion

• Same issues existed when using acoustic dispenser to dispense spheroids.

• Spheroid dispense using WellMate dispenser worked better than solenoid valve and acoustic dispenser.
  • Homogeneity is still the biggest issue.

• Cytotoxicity of known compounds on spheroids: PANC-1 vs. 5 drugs (oxaliplatin, doxorubicin, gemcitabine, 5-Fluorouracil, SN-38)
  • Due to the difficulty of homogeneous dispense, large variability was observed in CRC data for spheroids cultured in flasks and transferred into 384 ULA plates.
• Corning 384 or 1536 well spheroid plates may be the better option for compound screening application at this time

A 1536-Well 3D Viability Assay to Assess the Cytotoxic Effect of Drugs on Spheroids

Franck Madoux, Allison Tanner, Michelle Vessels, Lynsey Willetts, Shurong Hou, Louis Scampavia, Timothy P. Spicer

First Published January 13, 2017 | research-article
The new 1,536-well 3D spheroid plates appear to work!
Well to Well Consistency-1536

HT-29 cells per well at seeding

HT-29 spheroid volume overtime

10 mm

5 mm

TSRI © 2017. All rights reserved.
3D spheroid cytotoxicity assay characterization

- HT29 cells used in 1536 format throughout this analysis
- 3D CTG works well but 1 hour incubation is optimal
- Control compounds elicit a cytotoxic effect—Panobinostat designated for future studies
- Spheroid volume is linear in terms of signal output at 72 hours post seeding
- Plate to plate response reproducibility is excellent
2D vs 3D 1536 HTS Testing Approved Drugs

- 1536-well spheroid cytotoxicity pilot screen results demonstrate clear differences in outcomes between 2D and 3D HTS
- Z's >0.72 in either format
Bead Injection Technology:

- 40-80 micron beads have been dispensed using Scampavia designed channels with a measure, rotate, and flush approach (see video)

Mantis based technology:

- Using a loaded tip coupled to chambered chip based peristaltic pump approach
• HTS involves testing very large numbers (e.g., millions) of compounds in order to identify biologically active molecules
• HTS utilizes automation, cutting-edge technology and highly miniaturized assays to test large #s of compounds in a cost-effective way
• Adaptability, scale-up and implementation hurdles are key considerations
• Corning 1536 well plates have met with preliminary success
  • Other technologies have as well (N3D and Greiner BioOne)
• Corning spheroid flasks also meet with success but require further implementation for HTS dispensing
SRIMSC Team-Thank you!!!