Overview of 3D Cell Culture Model Systems & Validating Cell-based Assays for Use with 3D Cultures

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Outline

• Justification for using 3D culture models
• Overview of 3D culture methods
• Unmet need for validated assays
• Example assays and how they work
• Path forward
• The “take home” message
Justification for Using 3D Culture Models

Progression Toward More Physiological Relevant (i.e. Predictive) Assay Approaches

Biochemical

Cell-based

Animal models

Primary cells

Co-cultures of different cell types

Three dimensional (3D) models

Devices with microfluidic control

Human clinical trials
Justification for Using 3D Culture Models

- 3D culture models mimic tissue-like structures more effectively than monolayer cultures
- 3D cultures can exhibit differentiated cellular function absent in 2D cultures
- 3D cultures hold promise for being more predictive of in vivo responses to drug treatments
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General Classification of 3D Culture Methods

Scaffold
- Hydrogels
- Inert matrix

Scaffold-free
- Low adhesion plates
- Micropatterened surfaces
- Hanging drop
- Suspension using methyl cellulose, rolling vessel or magnetic levitation
Hydrogel Examples

Animal-derived

- Matrigel®
- Collagen

Alginate / Agarose (Plant-derived)

Synthetic

- QGel® Matrix
- 3-D Life Biomimetic
- Puramatrix
**Matrigel® Hydrogel**

- Most widely used hydrogel that represents a reconstituted basement membrane
- Extract from Engelbreth-Holm-Swarm (EHS) mouse sarcoma
- Contains: Laminin, Collagen IV, Entactin, growth factors
- Generally provided at 8-11 mg/ml
- Forms a gel when shifted from 4°C to 37°C
- Developed at NIH
- Several commercial vendors
  - [http://wwwbdbiosciences.com](http://wwwbdbiosciences.com)
Collagen Hydrogel

- Collagens are the most abundant class of ECM proteins
- Several types of collagen from various tissues
- Purified collagens are used to coat plastic surfaces or form gels to support or embed cells in culture
- RGD amino acid sequence interacts with integrin receptors on cells
- Commercial sources and description of use of collagen
  - [https://www.millipore.com/publications.nsf/a73664f9f981af8c852569b9005b4eee/3a3d5e1f86ebeed98525730600724f30/$FILE/ECM675.pdf](https://www.millipore.com/publications.nsf/a73664f9f981af8c852569b9005b4eee/3a3d5e1f86ebeed98525730600724f30/$FILE/ECM675.pdf)
“RAFT” System using Collagen Hydrogel

- RAFT (Real Architecture for 3D Tissue) System from TAP Biosystems
- Cells suspended in dilute collagen are dispensed into a culture plate
- Liquid is absorbed from well with absorbent plug resulting in concentrating cells and collagen gel
- Overcomes problem of detachment and shrinking of collagen gels as cells grow and differentiate
RAFT System Example using Collagen Hydrogel

RAFT Process Simulation - Producing collagen-based 3D cell cultures in under one hour

3D cell culture creation
As liquid is absorbed, the concentration of collagen and cells increases.
A range of in vivo cell and collagen concentrations can be formed.

Stage 1 - Set up and incubation (0 - 15 mins)

Stage 2 - 3D cell culture formation commences (30 mins)

Stage 3 - 3D cell culture creation (30-45 mins)

Stage 4 - 3D cell culture formation complete (45 mins)

Stage 5 - 3D cell culture ready to use (process complete in <1 hour)

https://www.raft3dcellculture.com/
Hydrogels (Animal-derived)

Advantages:
- Long history of use of collagen and Matrigel®
- Natural biological ligand for integrins
- Often contains “magic potion” ingredients similar to serum
- Pre-coated surfaces are commercially available

Disadvantages:
- Animal derived / Growth factor contamination
- Lot-to-lot variability
- Challenging to handle at low temperatures
- May need special equipment (e.g. for RAFT system)
Alginate Hydrogels (Plant-derived)

- Alginate is a polysaccharide found in the cell walls of brown algae
- Alginate monomers can be cross-linked to form hydrogels and entrap cells
- ECM proteins may be added during cell immobilization
- Cell-matrix interactions can be modified using peptide-coupled alginates
Alginate Hydrogels *(Plant-derived)*

**Alginate entrapment procedure**

- Suspend cells in sodium alginate
- Drip into calcium chloride solution
- Gel forms by $\text{Ca}^{+2}$ crosslinking monomers to entrap cells
- Size of drips and cell number can be controlled
- Cells and multi-cellular structures can be recovered intact by dissolving gel using divalent chelators (e.g. EDTA and citrate)
Alginate Hydrogels

Advantages:

- No contamination with animal proteins
- Controlled stiffness
- Controlled size of spheres
- Can recover cells

Disadvantages:

- Artificial substrate
- Not HTS friendly
Synthetic Chemically Defined Hydrogels

QGel® Matrix

- Composed of synthetic PEG-based molecules that can be modified to contain bioactive domains (e.g. RGD sites)
- Polymerized to form a gel to mimic ECM

3-D Life Biomimetic Synthetic Hydrogel

- Dextran, polyvinyl alcohol, or PEG-based
- Polymers can be modified with RGD or protease sites
  - [http://www.cellendes.com/](http://www.cellendes.com/)
**Synthetic Chemically Defined Hydrogels**

**PuraMatrix™ Peptide Hydrogel**

- Monomer is repeating amino acid sequence of Arginine-Alanine-Aspartic Acid-Alanine prepared in an aqueous solution
- Peptide self-assembles into nanofiber structures that form a hydrogel that resembles native ECM
- Can be further functionalized by adding specific biologically relevant amino acid sequences
  - www.puramatrix.com

Chemically Modified Alginate Hydrogels

- Alginate can be chemically modified
- Covalently attached peptide (Gly-Arg-Gly-Asp-Ser-Pro) facilitates cell binding and signaling via integrin-RGD interaction.
- NovaMatrix®-3D w/NOVATACh pre-formed dried alginate foam discs
- Cell suspension in alginate monomer is added to foam disc

www.NovaMatrix-3D.com
Hydrogels (Synthetic)

Advantages:

• Chemically defined with lot-to-lot consistency
• Polymer is usually biologically inert
• Can be modified to contain RGD ligand for integrins or other amino acid sequences
• Control over gel stiffness

Disadvantages:

• Artificial substrate
• Some are not HTS friendly
Inert Matrix Scaffold

Alvetex (from Reinnervate)

- Highly porous 200µm thick polystyrene scaffold
- Pore sizes of 36-40 µm
- Available as inserts for 6-24 well plates or welded in the bottom of 96 & 384 well plates

http://www.reinnervate.com
Inert Matrix Scaffold

Alvetex Workflow:

• Rehydrate (70% EtOH) and rinse wells with PBS
• Seed cells on Alvetex scaffold
• ECM components can be added (optional)
• Cells migrate into pores and assume morphology representative of *in vivo*
• Detection reagents can be added directly to wells for assay
Inert Matrix Scaffold

Advantages:

• Thin (200µm) scaffold so oxygen and nutrient diffusion is less limited
• Use with 96 or 384 well plates can be automated

Disadvantages:

• Unable to view cells in wells with microscope
• Wetting and rinsing required
• Biomass may overwhelm some assay chemistries
Scaffold-free Methods for 3D Culture

- Low adhesion plates
- Micropatterned surfaces
- Hanging drop methods
Low Adhesion Plates

- Plastic is coated with hydrophilic polymer to prevent cells from sticking to surface
- Cells stick to each other, form clusters and generate their own extracellular matrix
- Flat bottom plates or flasks result in many irregular size clusters
- U- or V-bottom wells can generate a single cluster of cells in 2-4 days (dependent on cell type)
Low Adhesion Plates

Example sources:

• Corning (Ultra-Low Attachment)

Well Shape Promotes Cell Aggregation

- Example well shapes for 96 and 384 well plates from Sumitomo Bakelite
- Different cell types may form more intact spheroids in V-vs. U-bottom wells

Low Adhesion Plates

Advantages:
- Can generate single spheroid per well in U- or V-bottom 96 or 384 well plates (HTS friendly)
- Can generate large numbers of spheroids in flasks

Disadvantages:
- Variable size range spheroids form in flat bottom vessels
- Difficult to select uniform size and dispense from flasks
- Cell type dependent variability in forming spheroids
- May have mixture of spheroid and attached cells
Micropatterned Surfaces

Plastic surfaces can be modified to provide a micropattern or microwells to influence cell growth

Example Micropattern:

- Scivax NanoCulture® Plate multi-well plate for 3D spheroid cell culture
- Square or honeycomb pattern on plastic surface induces cells to grow as clusters
Micropatterned Surfaces

Example Micropattern:

- Photolithography process used to create specific areas for cell attachment on surface of plates
- Spheroids form on surface of plate (800 x 100µm circles/well of 96 well plate)
- Cell-able™ System from Transparent Inc.

Micropatterned Surfaces

Example Micropattern:

- CYTOO Adhesive Micropatterns
- Growth of micropatterned spheroids in 96P

Micropatterned Surfaces

- Example images of “spheroids” grown on CYTOO plate

*Biphoton image courtesy of CYTOO SA
http://www.cytoo.com/*
**Micropatterned Surfaces**

**Microwell Example:**

- Elplasia™ Microspace Cell culture
- Microwells provide confined spaces
- Various sizes available divided by walls
- Many microspaces in each well of multiwell plate
- Can be coated with polyHEMA to modulate attachment
  - [http://www.elplasia.com/](http://www.elplasia.com/)
Micropatterned Surfaces

Advantages:

• Can generate multiple spheroid structures per well
• Can make many small (controlled size) spheroids

Disadvantages:

• May be difficult to generate uniform size spheroids for some technologies dependent on uniform settling of cells
• Medium change may lead to loss of spheroids
• Biomass of multiple spheroids may overwhelm assay chemistry
**Hanging Drop Method**

- Addition of ECM is not required
- Cells produce endogenous extracellular matrix
- Cell-cell and cell-ECM interactions form naturally
- Size and composition of spheroid is defined by initial cell number and proliferation

Diagram:

- **Seeding**
  - Medium/air interface

- **After 1 hour**
  - Cells

- **After 2-4 days**
  - Medium

- **Tumor tissue**
Hanging Drop Method

Ways to Form Hanging Drop Cultures

1. Home brew method
   - Add suspension of cells in a drip on lid of petri dish or multiwell plate
   - “Gently” turn it upside down
   - Hope the drip doesn’t move or fall off

2. Use commercially available devices designed to form hanging drop cultures
Perfecta3D® Hanging Drop Plates

Dispense Cell Suspension  →  Forming Hanging Drop  →  Hanging Drop  →  Cell Aggregate  →  Spheroid
Perfecta3D® Hanging Drop Plates

Spheroids can be transferred to microwell plate
GravityPLUS™ 3D Cell Culture Kits

http://www.insphero.com
Example Spheroids Resulting from Seeding Different Numbers of Cells

1mm diameter bottom of GravityTRAP™ Plate is useful for estimating size of spheroids
Advantages of Hanging Drop Method

Advantages:
- One spheroid per well
- Automatable using liquid handling systems
- Size can be controlled and is reproducible well-to-well
- Can mimic hypoxic condition using large spheroids
- Offers flexibility to mix cell types at beginning or add cells later to form microtissues

Disadvantages:
- Challenging to image center of very large spheroids
- Cell number can be too low for some assays (e.g. MTT)
Which 3D Model System Should I Use?

Depends on…

• Experimental model system
• Goals of experiment
• Number of samples…
Limitations of 3D Culture Models

- They are still just a model
- Size limitations lead to:
  - Heterogeneity of cell population within spheroids
  - Oxygen and nutrient diffusion limits

Modified from Metha et al., Journal of Controlled Release 164: 192, 2012
Limitations of 3D Culture Models

• They are still just a model
• Size limitations lead to:
  • Heterogeneity of cell population within spheroids
  • Oxygen and nutrient diffusion
• **Availability of validated assay methods to monitor biology**
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**Unmet Need for Assay Methods to Interrogate Markers in 3D Culture Models**

Most existing cell-based assays were designed for monolayer or suspension cultures.

- Will reagents effectively lyse 3D structures?
- Will reagents penetrate to center of 3D spheroids?
- Will mass of cells block/quench signal before it reaches detector?

Questions prompted collaborations with 3D culture system providers to evaluate effectiveness of various assay chemistries.
First Approach Chosen was…
Hanging Drop Method & ATP Assay for Viability

Hanging drop method
• Single spheroid per well
• Matrix free
• Size can be controlled
• Can easily estimate size with InSphero plates

ATP Assay
• Most widely used assay for viable cells
• Fastest and most sensitive method
• Strong lytic capacity enabled by stabilized luciferase
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Why Not Use MTT or Resazurin Assays?

- MTT and resazurin assays have been used for 3D culture models (e.g. MTT used in MatTek's multilayered keratinocyte EpiDerm™ System)
- Little information is available on penetration of reagents
- MTT and resazurin have been observed to be toxic to cells (Assay Guidance Manual: http://www.ncbi.nlm.nih.gov/books/NBK144065/)
- Sensitivity of MTT assay limits signal to background ratio
Toxicity of Balb 3T3 Cells Caused by Treatment with Resazurin for 4 Hours

Time Zero

4 hours

Images captured by Tracy Worzella using Incucyte instrument from Essen Biosciences
Comparison of Signal:Background Among Three Cell Viability Assays Measuring Spheroids

![Graph comparing Signal:Background among three cell viability assays](image-url)
ATP Assay for Cell Viability (How it Works)

- Lysis Solution
- ATPase Inhibitors
- Luciferin
- UltraGlo Luciferase

ATP Assay Reagent

Viable Cell

ATP

Luciferin + Luciferase

Light

Dead Cell

ADP

No Reaction

X
**DNA Dye Staining to Detect Dead Cells (How it Works)**

- **Non-permeable DNA dye**
  - Staining of dead cells results in a fluorescent signal that is stable.
  - **Viable Cell**
    - Dye is excluded from live cells
  - **Dead Cell**
    - DNA dye only stains nucleus of "dead" cells or debris
Critical Experiment that Prompted Effort to Validate Assays

Observation of Lytic Efficiency of ATP Assay Reagents

- Spheroids grown to ~350μm using hanging drop method
- Add ATP assay reagents + DNA dye to indicate lytic effectiveness
- Photograph using laser confocal microscopy

ATPLite 1-Step Reagent

CellTiter-Glo® Reagent

~350 μm spheroids
Evidence Suggests Large Spheroids Require Mixing to Extract ATP

HCT116 cells grown for 4 days

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Data suggested protocol may need to be modified to capture higher % of ATP.
ATP Assay Improvements for 3D Cultures

CellTiter-Glo® 3D Assay

• Higher concentration of detergents to more effectively lyse cells in larger 3D structures

• Protocol modification to incorporate
  • Physical disruption (shaking for 5 min)
  • Longer incubation with lysis buffer (30 min)

• Has been used with hanging drop, inert scaffold, low binding plates, Matrigel® and collagen models.
ATP Recovery from Cells Cultured Four Days with Matrigel®: Comparison of ATP Assays

HCT116 - 2,000 cells
RPMI +10% FBS
CellTiter-Glo 3D = 110.4%
ATPlite 1Step = 46.1%

A549 - 5,000 cells
F12K +10% FBS
CellTiter-Glo 3D = 107.7%
ATPlite 1Step = 19.9%

HepG2 - 5,000 cells
DMEM +10% FBS
CellTiter-Glo 3D = 86.6%
ATPlite 1Step = 15.1%

DU145 - 5,000 cells
MEM +10% FBS
CellTiter-Glo 3D = 98.3%
ATPlite 1Step = 32.3%

MCF7 - 5,000 cells
MEM +10% FBS
+10μg/ml insulin
CellTiter-Glo 3D = 87.3%
ATPlite 1Step = 31.0%

PC3 - 5,000 cells
F12K +10% FBS
CellTiter-Glo 3D = 117.7%
ATPlite 1Step = 29.6%
HCT116 cells were cultured in InSphero GravityPLUS™ 3D Cell Culture system for 4 days to form ~350 μm microtissues. Samples were treated with CellTox™ Green and panobinostat for 48 hr. After recording fluorescence, an equal volume of CellTiter-Glo® 3D Reagent was added, plate was shaken for 5’, and the luminescence was recorded after a 30’ incubation.
**Homogeneous Caspase Assay (How it Works)**

Caspase Detection Reagent

- Lysis Solution
- Z-DEVD-aminoluciferin
- **Stable Luciferase**
- ATP

Viable Cell

- Inactive Pro-Caspase
- Reagent
- No Rxn

Apoptotic Cell

- Active Caspase
- Reagent
- Luminescence

Dead Cell

- Inactive Pro-Caspase
- Reagent
- No Rxn
Caspase Assays of 3D Spheroids

• Stability of caspase limits ability to adjust detergent formulation used in reagent designed for 2D cultures
• Stability of luciferase in reagent is not an issue
• Caspase-Glo® 3/7 protocol was modified to incorporate
  • Physical disruption (shaking for 30 min)
  • Includes longer incubation with lysis buffer
Caspase Assay Protocol Optimization to Facilitate Enhanced Spheroid Cell Lysis

• HCT116 cell spheroids grown to ~330µm using hanging drop method
• Add Caspase-3/7 assay reagent + DNA dye to indicate lysis
• Shake with assay reagent for 5 or 30 min
• Image with confocal after a total of 30 min incubation with reagent

Increased shake time results in near complete spheroid cell lysis
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Path Forward

• Validation of additional cell-based assays
  • Oxidative stress markers (GSH & ROS)
  • Stress response reporter assays
• Demonstrating additional multiplex assay combinations
• Improving lysis of large spheroids
For GSH assay, the medium was removed from 4-day ~350 μm HCT116 spheroids treated for 48 hr with buthionine sulfoximine. 50μl of total glutathione lysis reagent was added and the plates shaken for 30’. 50μl of luciferin generation reagent was then added, and after a 30’ incubation, 100 μl of luciferin detection reagent was added. After a final 15’ incubation, luminescence was recorded. ATP was assayed from parallel samples using the CellTiter-Glo® 3D Assay.
HCT116 cells expressing NanoLuc luciferase under a constitutive promoter (A) or a HIF-1 promoter (B) were cultured in InSphero GravityPLUS™ 3D Cell Culture system for 4 days to form ~200-700 mm microtissues. An equal volume of NanoGlo Reagent or CellTiter-Glo® 3D Reagent was added to each well, the plate shaken for 10’, and luminescence recorded after a total of 30 min incubation.
Measurement of Luciferase Reporter Activity from Different Size Spheroids

HCT116 cells expressing NanoLuc luciferase under a constitutive promoter (A) or a HIF-1 promoter (B) were cultured in InSphero GravityPLUS™ 3D Cell Culture system for 4 days to form ~200-700 mm microtissues. An equal volume of NanoGlo Reagent or CellTiter-Glo® 3D Reagent was added to each well, the plate shaken for 10’, and luminescence recorded after a total of 30 min incubation.
Path Forward...
Improving Lysis of Large Spheroids

General Approach

• Change detergent formulation when possible
• Longer incubation in lysis solution
• Incorporate mixing or physical disruption step
Physical Disruption Options

- Plate shakers to mix 96 or 384 well plates (motion and speed matter)
- Pipetting sample up-and-down can be used instead of shaking, but our results show greater variability among replicates
- Ultrasonic treatment (potential for future)
**Take Home Message**

- There is a growing number of approaches to choose from for generating 3D spheroids
- Most *in vitro* cell-based assays were originally designed for 2D monolayers or cells in suspension
- Validation of assays with each 3D model is recommended
- Size / mass of 3D structure may limit efficient lysis of all cells or overwhelm assay chemistry
- Optimizing incubation time with lysis solution and mixing or physical disruption are recommended first steps for validating assays
Thank you for attending!

Questions are Welcome