CORNING

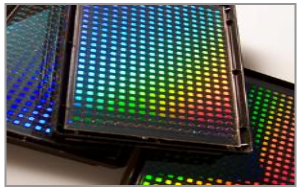
Rock the Science of 3D! Corning Life Sciences Exhibitor Tutorial

Thomas Villani, Ph.D.
Chief Science Officer and Co-Founder
Visikol Inc.

Ann E. Rossi, Ph.D.
Applications Lab Manager
Corning Life Sciences

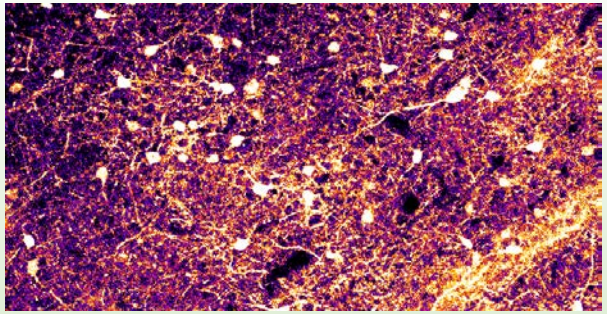
SLAS | February 2018

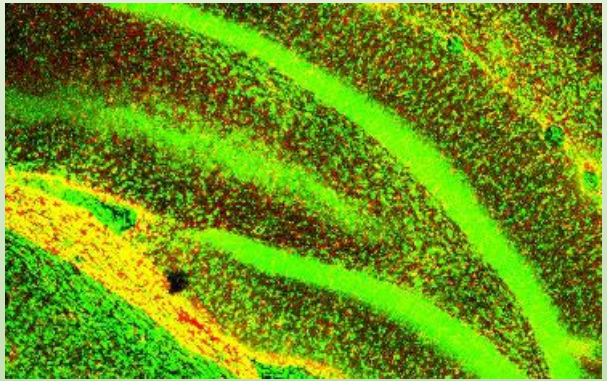
Corning Life Sciences:
An Innovator in Laboratory Products for Discovery Research

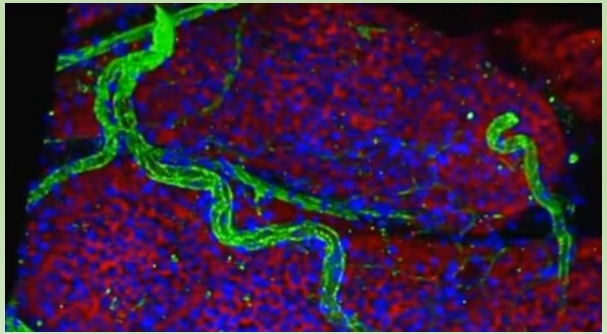


- Business Overview:
  - Market leader for over 100 years
  - Significant R&D investment for continuous innovation
  - Global manufacturing and distribution
  - Sales representatives worldwide including Technology and Field Applications Specialists

- Laboratory Products and Solutions for:
  - Cell Culture and Bioprocess
  - Drug Discovery
  - ADME/Tox
  - Genomics
  - Chemistry
  - Microbiology
  - General Laboratory Products



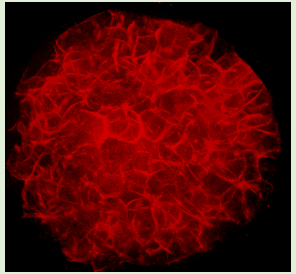




3D Image-Based Characterization of 3D Cell Culture Models Generated Using Spheroid Microplates

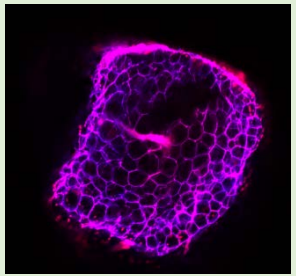
Tom Villani, PhD
Chief Science Officer
Visikol Inc.

Who is Visikol?



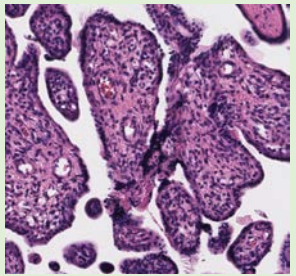
In Vitro Screening

- 3D Models
- High content confocal imaging
- Drug discovery assays



Whole Mount Analysis

- Vasculature mapping
- Whole brain imaging
- Whole mount IF, FP, ISH



Digital Pathology

- Slide scanning
- Quantitative analysis
- Feature extraction

- Biotechnology company that spun out of Rutgers University in 2016.
- Contract research organization focused on quantitative and 3D tissue imaging.
- Sells suite of patented Visikol® HISTO™ reagents for use in research.

VISIKOL™

Tutorial Outline

Introduction to 3D Cell Culture

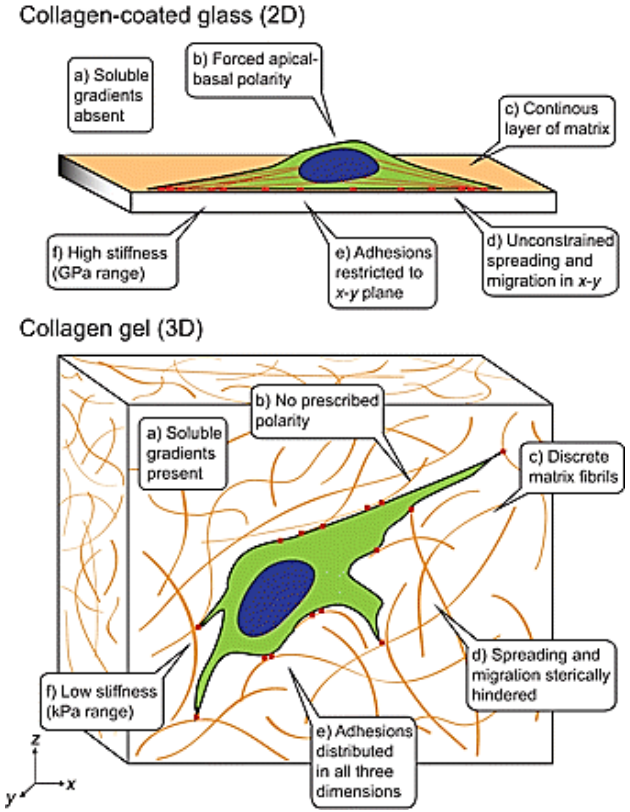
Corning® Ultra Low Attachment Spheroid Microplates

Background on Tissue Clearing

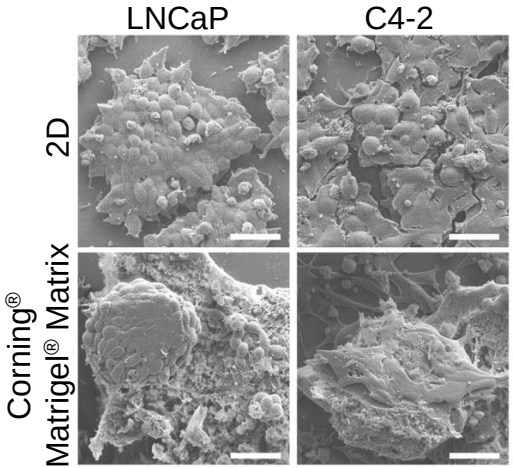
Limitations of High Content Screening

HepG2 Spheroid Application

Cells in 3D Culture More Closely Mimic *In Vivo* Physiology



J Cell Sci 2012;125(Pt 13):3015-24.

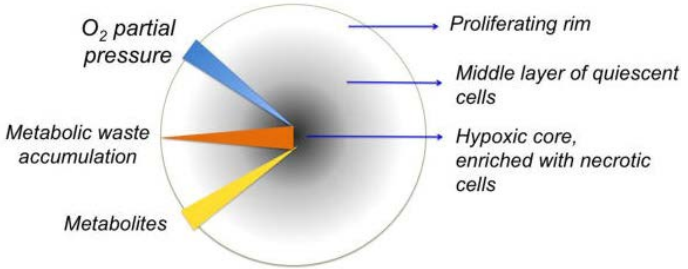


Adv Healthc Mater 2012;1(5): 590–99.

Cell Morphology

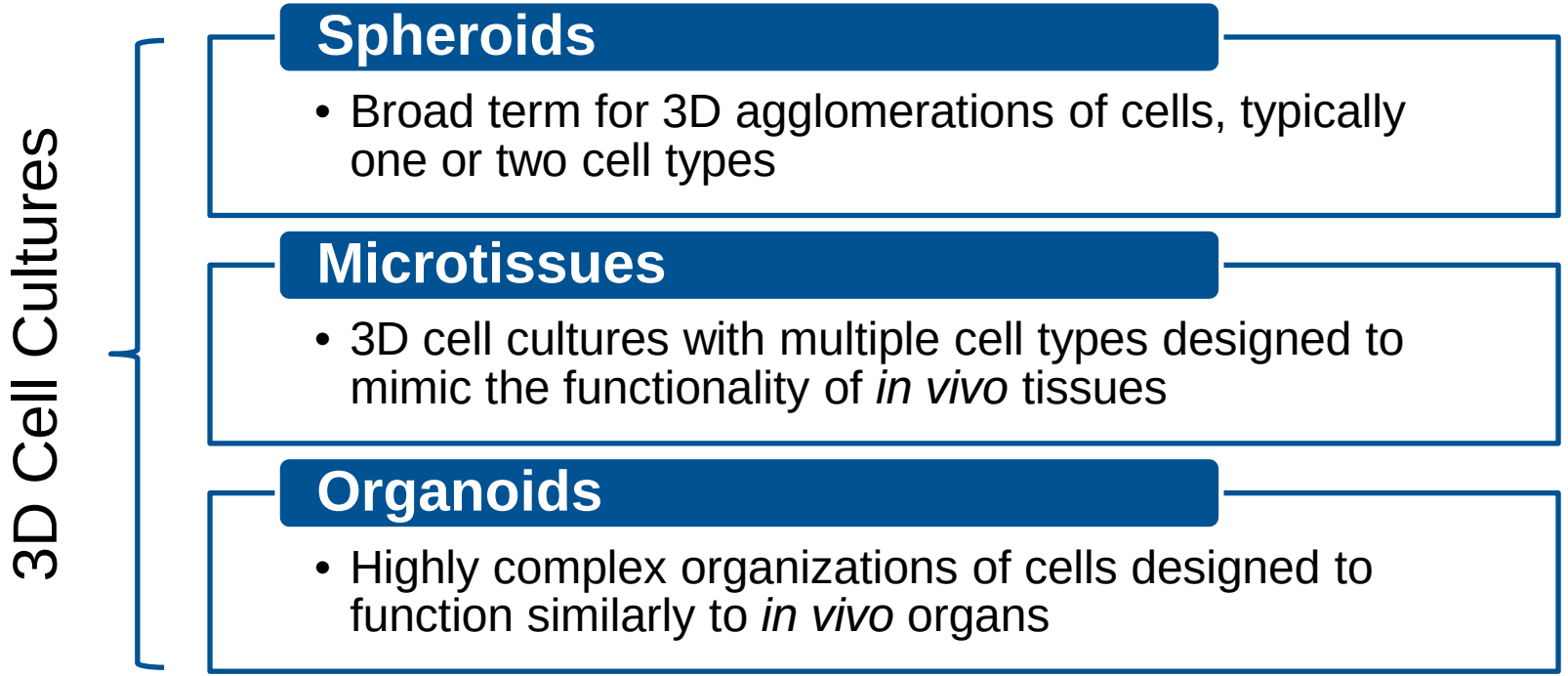
Structural Complexity

Phenotype

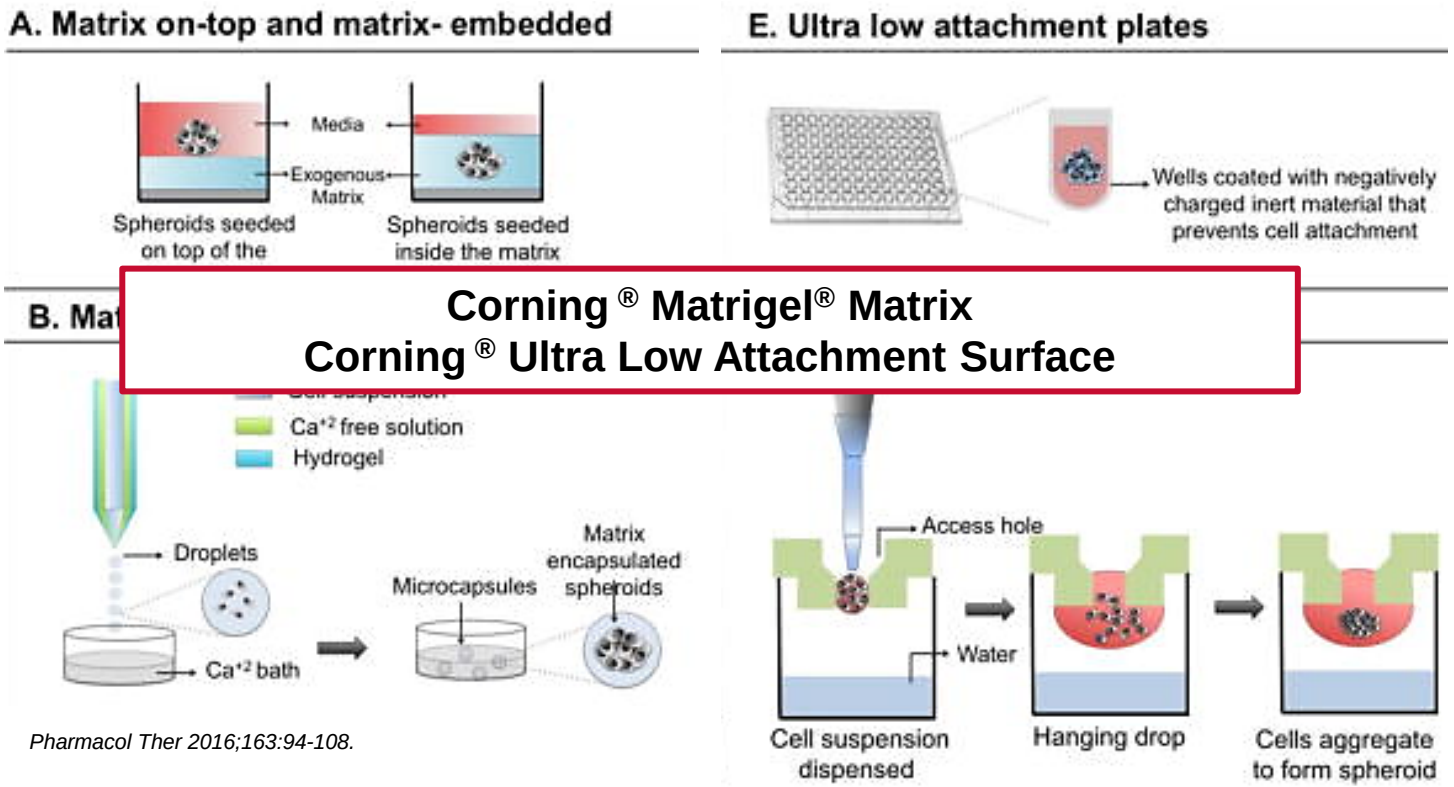


Pharmacol Ther 2016;163:94-108.

Spheroids, Microtissues, and Organoids



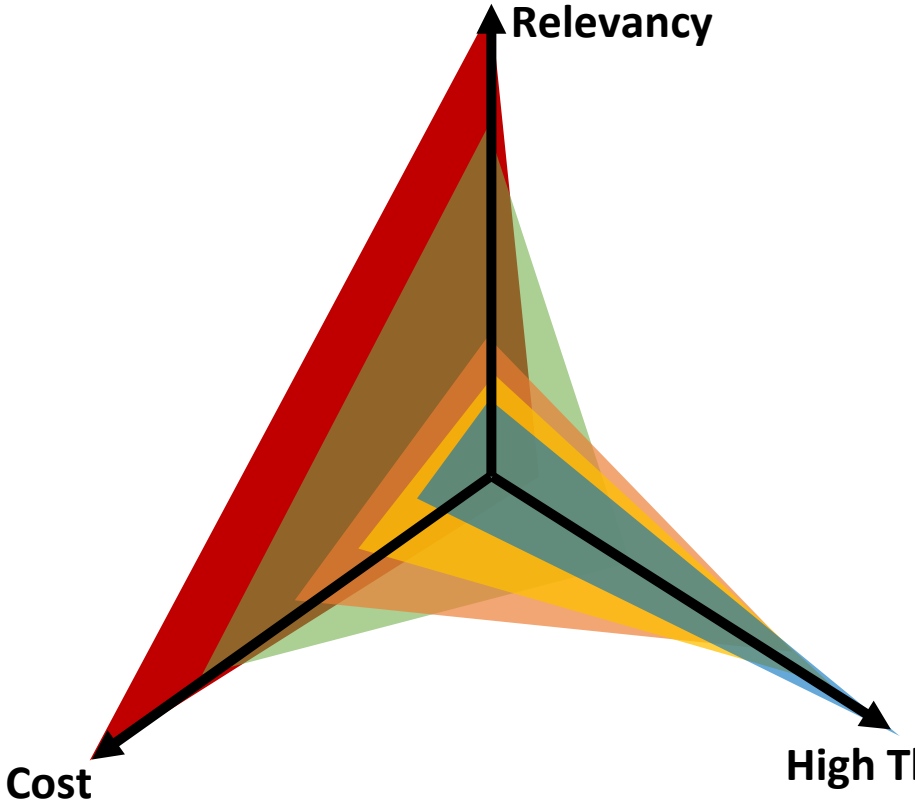
Common Techniques for Generating 3D Cultures



Corning ® Matrigel® Matrix
Corning ® Ultra Low Attachment Surface

Pharmacol Ther 2016;163:94-108.

3D Cell Culture: Not a One-Size-Fits-All Solution



- Cell-based Assays
- 2D Cell Culture
- 3D Cell Culture
- Animal Models
- Clinical Testing

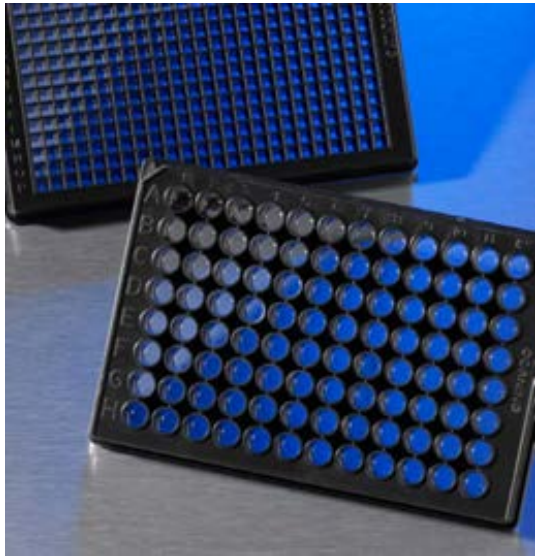
Realizing the Benefits of 3D Cell Culture can be Challenging

3D Strengths	3D Weaknesses
More physiological than 2D • Cell morphology • Cell phenotype • More predictive of in vivo efficacy/toxicity	Culture conditions • Variability, non-reproducible conditions with biological matrices • Hanging drop not amenable to high throughput without automation
Heterogenous cell populations	→ Culture and maintaining uniform size
Customizable environment	• Transfer necessary for assay, depending upon method
Growing body of work on 3D models, especially in oncology and toxicology	Assay methods/parameters not as well developed as 2D
Reagents specific for 3D	→ Imaging can be limited by 3D nature of samples
	Scalability

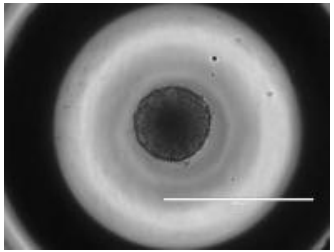
Corning® Spheroid Microplates

Corning Ultra-Low Attachment surface and unique round well-bottom design enable the formation and growth of a single, uniform spheroid per well with reproducible size.

Standard ANSI/SBS footprint dimensions for 96- and 384-well formats



Clear bottom for visualization and imaging



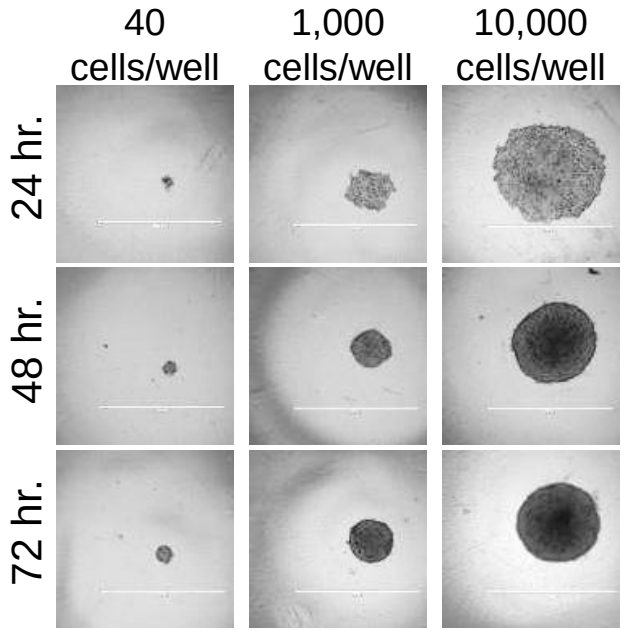
Black sidewalls to reduce cross-talk and background noise in fluorescent- and luminescent-based assays

Corning Spheroid Microplate User Guide: Corning document CLS-AN-235

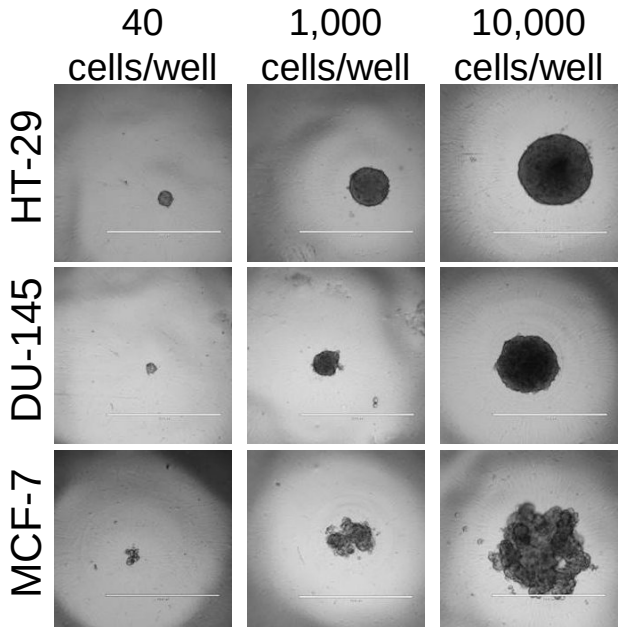
Reproducible and Routine Culture of Uniform Spheroids in Corning Spheroid Microplates

- Timing, seeding conditions dependent upon cell type, spheroid size

HT-29 Spheroids



Multicellular Spheroids



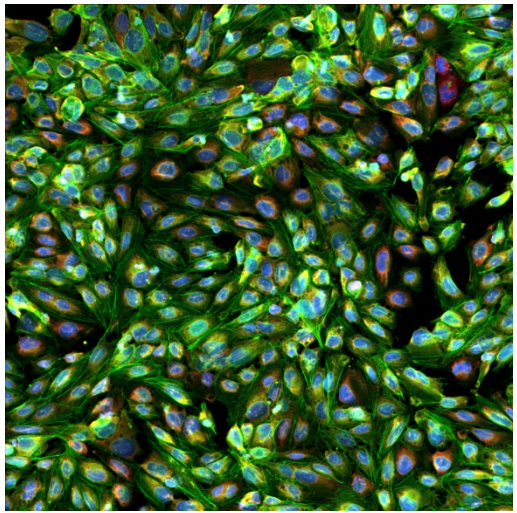
Scale bar = 1 mm

Traditional Methods for Interrogation

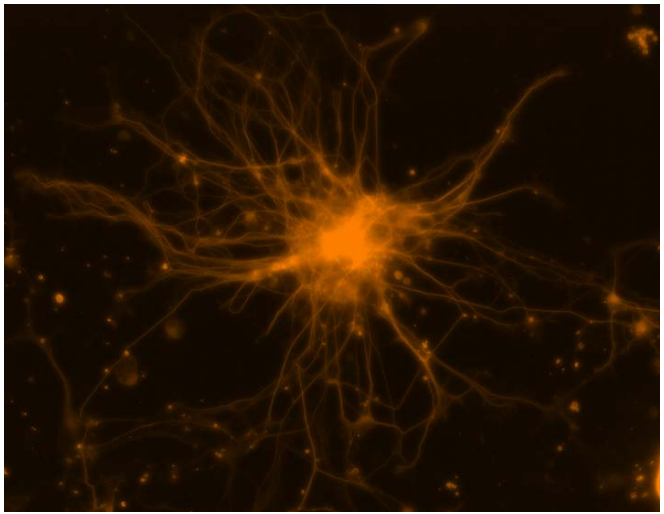
	Cost Per Well	Throughput	Limitations
Dissolution Assays	$	High	Loss of spatial information
Chemical Analysis	$	High	No spatial or cellular information
Widefield Imaging	$$	High	Only characterizes periphery of models
Histopathology	$$$	Low	Tedious and low throughput
Confocal Microscopy	$$$	Medium	Imaging depth limited to a few cell layers

High Content Screening (HCS)

- Phenotypic screening conducted on cells in well-plate format
- State of art has been to acquire single colorimetric readings, images or confocal z-stacks



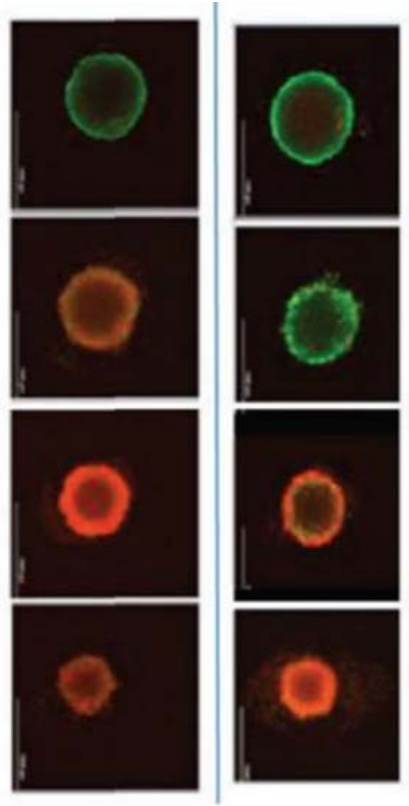
U2OS actin, tubulin at 40X



Stitched image of neuron at 20X

Limitations in Imaging 3D Cell Cultures

- Opacity of 3D cell-cultures causes light scattering which limits imaging to ~50 µm deep, i.e. first 2-3 layers of cells
- Causes “eclipse” images common in microtissue confocal images
- Imaging bias: Majority of cells surveyed are the outer layer – most proliferative, most exposed to drug, oxygen, nutrients
- Histological sectioning of microtissues is cumbersome, not high throughput
- Many imaging endpoints currently assay total microtissue, unable to differentiate between cell populations
  - Diameter, total fluorescence, ATP-luminescence assays
- Myth: microtissue centers cannot be imaged because labels cannot penetrate to center



Tissue Clearing

- Numerous methods for clearing tissues have been developed over the last several years

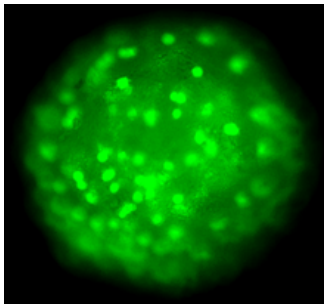
Type of Technique	Technique	Refractive Index	Processing time for spheroids	Immuno-labeling Compatibility	Fluorescent Protein Compatibility	Tissue Integrity	Compatibility with well-plates	Clearing is reversible
Protein Hyper-hydration	Scale	1.38	Days	NO	YES	Expansion, very fragile	YES	NO
	ClearT2	1.44	Hours	YES	YES	Fragile	YES	NO
	CUBIC	1.38 - 1.48	Hours	YES	YES	Expansion, very fragile	YES	NO
Solvent Based	BABB	1.55	Minutes	YES	NO	Shrinkage	NO	NO
	i/3DISCO	1.56	Minutes	YES	NO	Shrinkage	NO	NO
	Visikol® HISTO-M™	1.51	Minutes	YES	YES	No change	YES	YES
Aqueous RI Matching	FocusClear™	1.47	Hours	YES	YES	No change	NO	NO
	SeeDB/FRUIT	1.48	Hours	NO	YES	No change	YES	NO
Hydrogel Embedding	PACT/PARS	1.38 - 1.48	Hours/Days	YES	YES	Expansion	NO	NO
	CLARITY	1.45	Hours/Days	YES	YES	Expansion	NO	NO

Limitations of Known Clearing Techniques

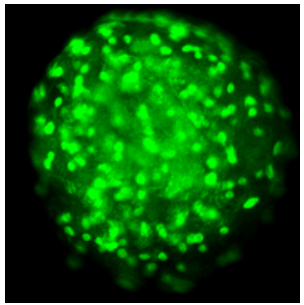
- CLARITY + Hydrogel embedding techniques
  - Slow: requires days to weeks to clear even small tissues
  - Requires complicated processing technique – not amenable to medium-to-high-throughput
  - Not easily amenable to well-plate format
- Protein hyperhydration (CUBIC, Sca*l*e, SeeDB)
  - Slow: require days to weeks for clearing
  - Hyperhydration of proteins severely reduces tissue integrity
  - Not compatible with immunolabeling techniques
- Organic solvent-based (BABB, iDISCO, 3DISCO)
  - Use of highly lipophilic organic solvents not compatible with plastic well-plates

Limitations of Imaging Spheroids

Wide Field Microscopy



Non-cleared
57 cells detected

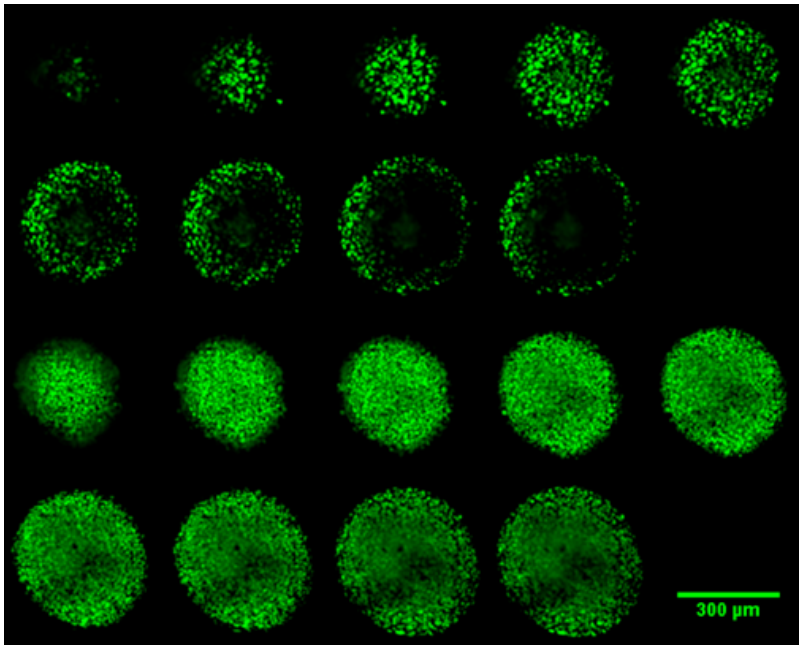


Cleared
151 cell detected

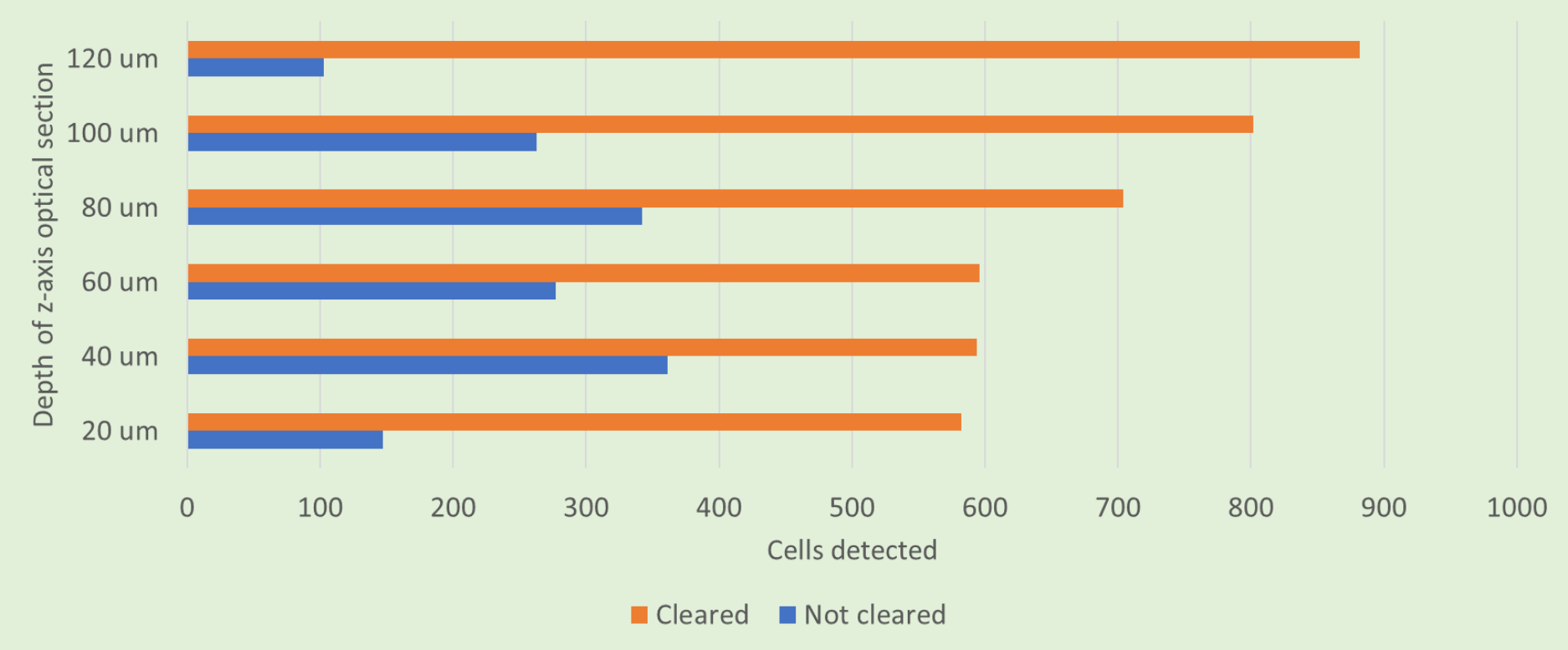
Confocal Microscopy

Non-cleared

Cleared



Clearing Allows Detection of Every Cell



Practical Considerations for Assaying Spheroids

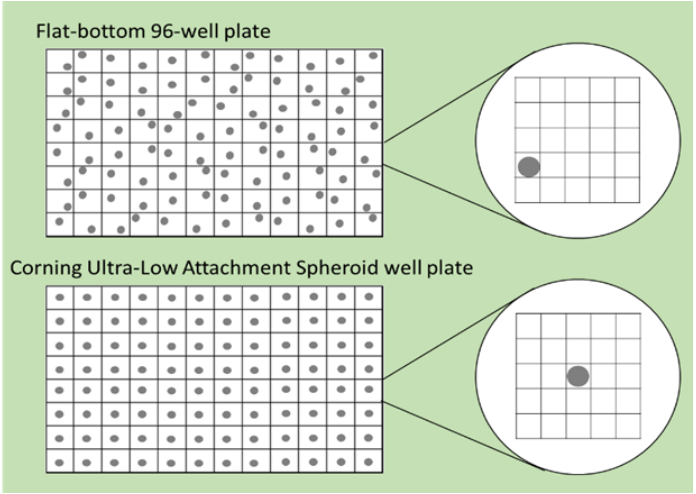
- Generation of Spheroids
  - Choice of cell line
  - Plate choice
- Labeling techniques
- Considerations for High Content Imaging
  - Localizing spheroids
  - LED vs. Laser
  - Air objective vs. immersion objective
  - Confocal vs. widefield
  - Z-projections vs. Z-stacks

Labeling Techniques for Cleared Tissues

- Fluorescent probes
  - Work very well, very rapid in most cases
- Immunolabeling
  - Requires permeabilization to allow antibody penetration
  - Can label > 2 mm deep into brain tissue
  - Very powerful: label virtually any target in wild-type or genetically modified models
  - 1000s of antibodies available from vendors qualified for IF/IHC
- FISH
  - Requires protease pre-processing
  - Powerful tool to characterize gene expression
  - Expensive, often requires customized probes
- Fluorescent proteins
  - Excellent tool for interrogating gene expression, localization of target protein, etc.
  - Clearing provides excellent way to visualize fluorescent protein distribution

Considerations for Imaging: Localization

- Localization within the well
  - Flat-bottom plates cause spheroids to have random placement
  - Scan of entire well: ~72 hr. per 96-well plate
- Corning plates localize tissue to center of wells, drastically improving image throughput
  - Scan of entire plate: ~12 hr. per 96-well plate
  - Nearly 6x increase from U-bottom plates localizing tissue!



Considerations for Imaging: LED vs. Laser

- High content imaging systems utilize either LED or laser to excite fluorescent dyes
- Perkin Elmer Opera/Operetta, GE InCell 6000/6500, and Thermo CX7 LZR utilize laser illumination
- Molecular Devices, Thermo CX5/CX7 utilize LED

- Laser: better resolution, better penetration of light into tissue, no “foreshadowing” – slower, more expensive to maintain
- LED: lower resolution, causes “foreshadowing” – faster, less maintenance

Considerations for Imaging: Air Objective vs. Immersion

- For imaging spheroids, air objectives are perfectly compatible
- 10x air objective gives field of view (FOV) requiring ~36 tiles to cover well
  - Higher magnification requires even more scanning
- Immersion objective with high numerical aperature (NA) not required for routine spheroid work
- Myth: immersion objectives allow visualization of center of non-cleared spheroids
- Fact: spheroids must be cleared to visualize interior cells

Considerations for Imaging: Confocal vs. Widefield

- Confocal imaging limits out-of-focus light, resulting in sharper images with very narrow (~5-10 µm) depth of field
  - Volume images obtained by taking z-slice at various depth
  - With clearing, allows for interrogation of entire cell population
  - Much slower than widefield
  - Much crisper, sharper images than widefield
  - Smaller pinhole = sharper resolution = less light hitting detector = lower intensity image
- Widefield imaging
  - No depth information
  - With clearing, cross section of cell population can be observed
  - Much faster than confocal
  - Poorer image quality
  - Far more light hits detector, can detect weaker signals

Considerations for Imaging: Z-projections vs. Stacks

- Z-projections are a merge of images in a z-stack
  - Far smaller file size (2MB vs 50MB)
  - Loss of z-spatial information
  - Easier and much faster to analyze
  - Poorer quality than z-stack, better than widefield
- Z-stacks
  - Full set of coordinates for every detected cell
  - One image for each plane throughout spheroid
  - Very high image quality
  - Far more light hits detector: can detect weaker signals
  - Enables spatial response profiling

Data Processing: Open Source Software

- ImageJ: Open source image processing platform built on Java, from NIH
  - Clunky and difficult to learn, but **extremely powerful!**
  - Used for general processing (e.g. contrast adjustment, cropping, merging channels, etc)
  - Easy to process huge numbers of image files (>10k)
  - Easy to program using macros
- CellProfiler: Open source image processing platform for analyzing sets of images, from Broad Institute
  - Very powerful platform for cell-counting, colocalization, and other quantitative analyses of image sets obtained from high content imaging

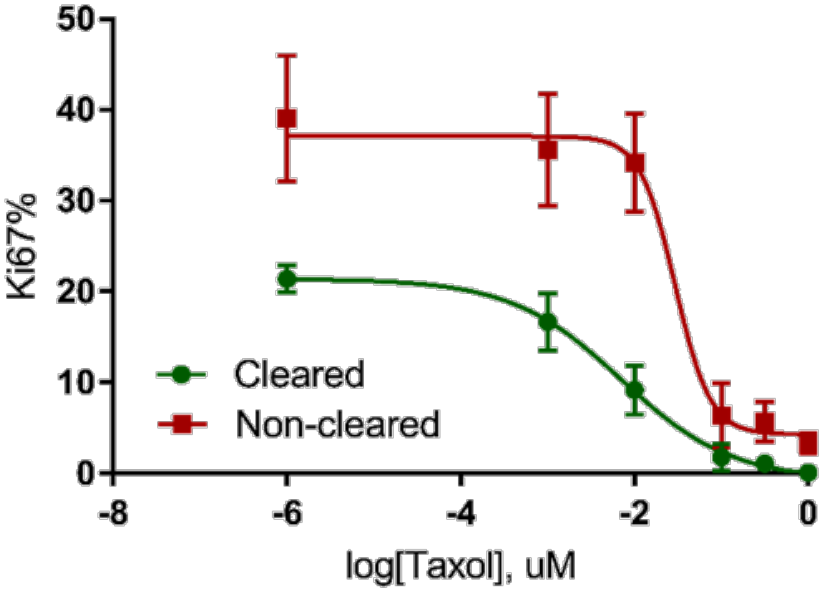
Specific Application: HepG2 Cell Proliferation

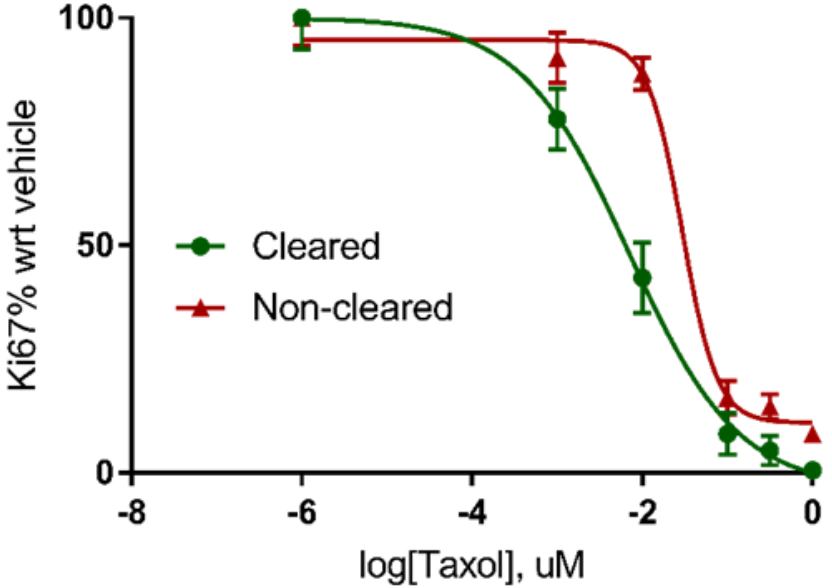
- HepG2 spheroids were seeded at 1000 cells/well in Corning ULA U-bottom 96-well plates
- Grown for 2 days prior to dosing. Dosing day = day 0
- Dosed with paclitaxel at 1 µM, 500 nM, 100 nM, 10 nM, 1 nM, as well as one row for vehicle control at day 0 and 2
- On day 4, spheroids were fixed with 10% neutral buffered formalin for 5 minutes

HepG2 Cell Proliferation: Processing

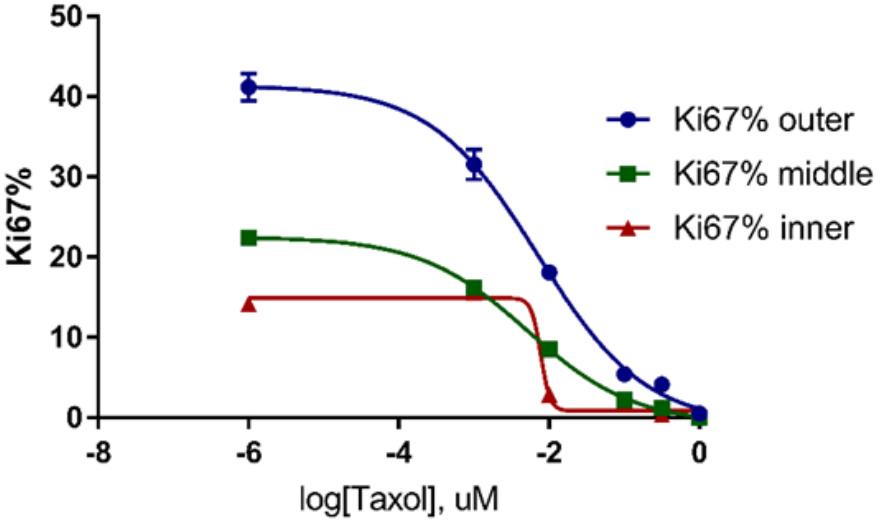
Pre-treatment (20 min.)	• Treatment with methanol/DMSO • Treatment with PBS/Triton X-100
Labeling (1 hr.)	• Blocking • Primary antibody (mouse anti-human Ki67) • Secondary antibody (goat anti-mouse AlexaFluor488)
Clearing (5 min.)	• Dehydration • Clearing with Visikol

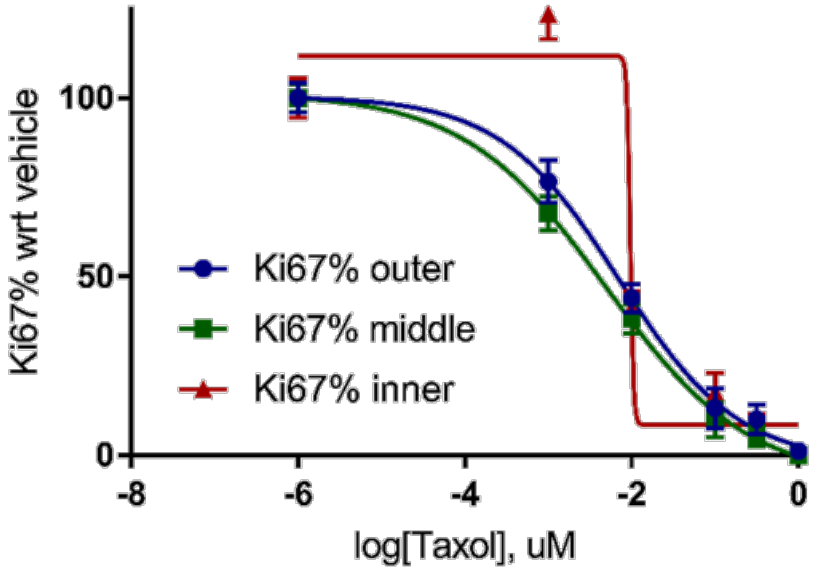
HepG2 Cell Proliferation: Dose Response

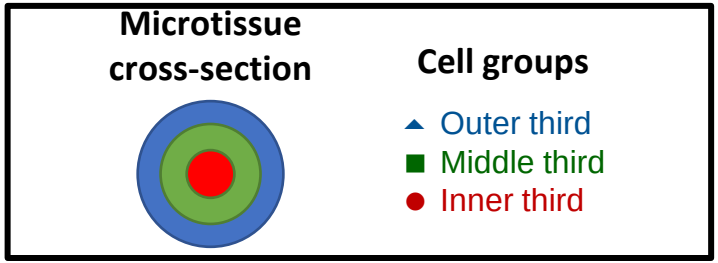




HepG2 Cell Proliferation: Spatial Dose Response







Questions?

Poster #1036-B Today 5-6PM

Visikol Exhibit #1829

Corning Technical Program Handout

Visit Corning at Exhibit #1029 and

Rock the Science of 3D!

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