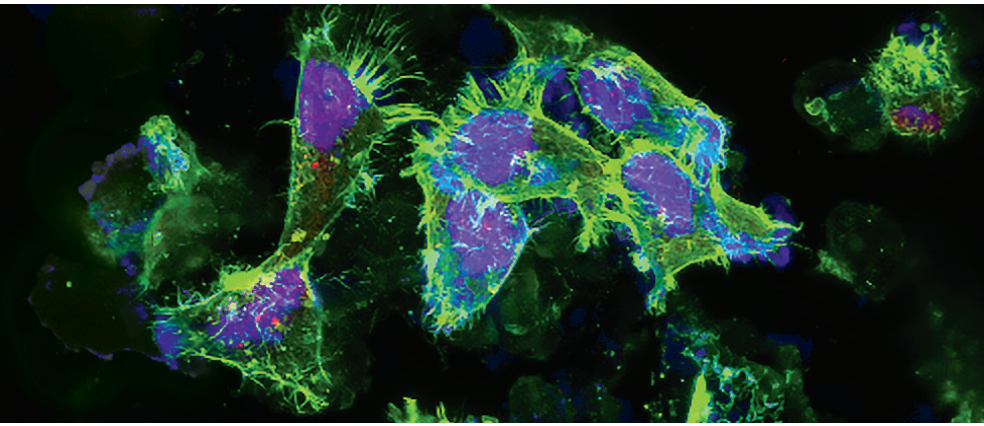
Cell-Mate 3D™

TIPS on Visualizing your Cell-Mate3D™ Cultures



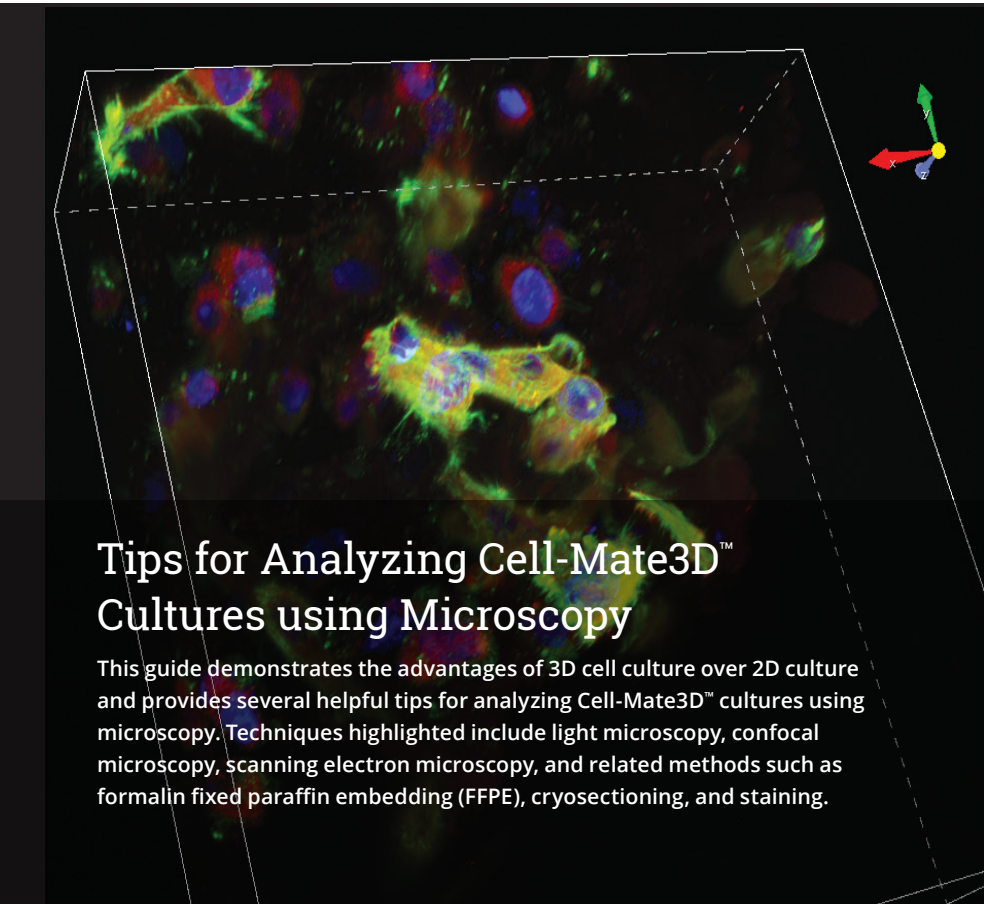
BRTI™ Life Sciences

Cell-Mate3D™ Cultures

Cell-Mate3D™ is a chemically defined, tissue-like, and injectable three-dimensional (3D) cell culture matrix that supports many cell types and encourages complex, 3D structures not observed in two-dimensional (2D) culture.

Cell-Mate3D™ Is Ideal For:

- Cancer Cells
- Fibroblasts
- Co-cultures
- Stem Cells
- Hepatocytes



Tips for Analyzing Cell-Mate3D™ Cultures using Microscopy

This guide demonstrates the advantages of 3D cell culture over 2D culture and provides several helpful tips for analyzing Cell-Mate3D™ cultures using microscopy. Techniques highlighted include light microscopy, confocal microscopy, scanning electron microscopy, and related methods such as formalin fixed paraffin embedding (FFPE), cryosectioning, and staining.

2D vs. 3D culture

Advancements in two-dimensional (2D) cell culture techniques have elucidated many aspects of human disease over the past few decades. However, most diseases are complex and are not captured in their entirety with this approach. Cancer cells and liver cells grown in 2D often have altered phenotypes and gene expression patterns, behaving differently than they do *in vivo*.[1–4] Three-dimensional (3D) culture creates a complex microenvironment, elucidating aspects of cellular behavior not seen before. [1,5–7]

There is a large disparity between therapeutics that demonstrate efficacy in *in vitro* and in animal models, and those that translate to clinical relevancy.[8–10] While there are some animal models available, many drugs generate different reactions, or none at all, in human disease states.[9] Furthermore, the mechanisms underlying the differences are not usually well understood, but differences in gene expression and tissue composition across species are likely to play a role.[8] For many cancers, there simply is not a comparable animal model available.[9] An even higher number of therapeutics demonstrate efficacy in two-dimensional culture systems, and have little to no effect in human clinical trials.[11–13] This can be attributed in part to the differences in cell morphology and phenotype observed in two- and three-dimensional culture.[14] Cells tend to divide at a slower rate in 3D culture, and these cells demonstrate an increased drug resistance.[15] There are differential expression patterns of drug transporters in cells cultured in two versus three dimensions, which may contribute to the decrease in drug efficacy.[10–12,16] By developing a more accurate, 3D culture system for cancer cells, high-throughput drug screens could be conducted before reaching the costly clinical trial stage.[12,17] In addition, therapeutics targeting genes that are differentially expressed between 2D and 3D culture, such as those involved in integrin signaling and cell-cell contact may be tested with this approach.[5,17]

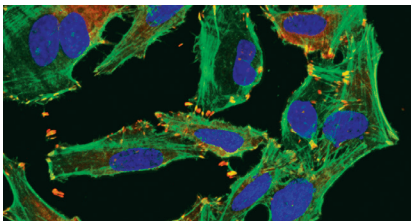
3D cell culture provides biological relevance to biomedical research. Differences between 2D and 3D culture include alterations in cell-cell and cell-extracellular (ECM) adhesion, mechanical signaling through tension and stiffness, and chemical and gas diffusion gradients. These aspects drastically affect gene expression, cellular morphology, and function.[1] 3D cell culture better mimics the *in vivo* environment and therefore lends itself to new and more predictive discoveries. Images of cells cultured in 2D and in Cell-Mate3D™ shown in this guide clearly demonstrate changes in gene expression, cellular morphology, and cellular behavior, which can affect research outcomes and downstream analysis.

Actin & Focal Adhesion Expression

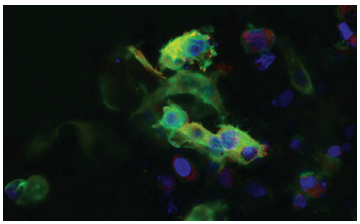
In two-dimensional culture, cells adherent to plastic or glass surfaces are subjected to basal strain tangential to the cell surface beyond physiological levels, and are only permitted to deform in one direction.[1] However, 3D culture systems provide more physiologically relevant structural support, allowing for a more accurate representation of native mechanical forces, perpendicular to the cell membrane.[1,2]

Actin (phalloidin) staining is homogenous in 2D cultures, such that the cells are flat, geometric, and spread out with relatively smooth edges (Fig.1A). In Cell-Mate3D™ cultures, however, we observed various types of morphologies such as migrating cells that are polarized and irregularly shaped as well as resting cells in the background that appear rounded (Fig.1B).

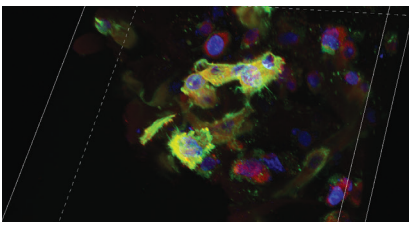
In 2D cultures, focal adhesions complexes and actin fibers were observed only on the plane of the culture dish. However, in Cell-Mate3D™ cultures, we observed contrasting actin and vinculin staining patterns such that focal adhesions formed on multiple planes of the matrix (Fig.1). In addition, we imaged thick 38 micron Z-sections and observed cells projecting prominent lamellipodia that appear to be interacting with neighboring cells and the surrounding Cell-Mate3D™ matrix (unstained) in multiple directions and planes (Fig.1C).



A. 2D Culture



B. Cell-Mate3D™ Culture



C. Cell-Mate3D™ Culture Volume view

Figure 1. Cells stained with vinculin (red), phalloidin (green), and DAPI (blue) in 2D culture (A) or in 3D culture in Cell-Mate3D™ (B and C).

Primary Cells & Cell Morphology

Cell viability and cell morphology is different in 3D culture compared to 2D culture. For example, when primary human hepatocytes were cultured in Cell-Mate3D™ and stained with mitotracker (mitochondria-green), lipid tox (lipid droplets-red) and DAPI (blue) in 3D culture we found that cells survived for over 15 days compared to just 2-3 days in 2D culture.

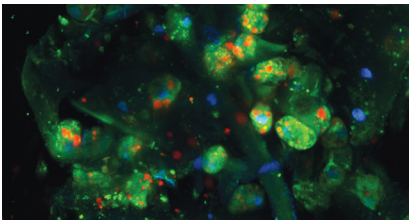


Figure 2. Primary human hepatocytes from Triangle Research Labs were grown in a Quasi Vivo® fluidics system for 7 days. The culture was stained with mitotracker (mitochondria-green), lipid tox (lipid droplets-red) and DAPI (blue) and imaged using inverted confocal microscopy. Quasi Vivo is a registered trademark of Kirkstall Ltd.

Oxygen Gradients

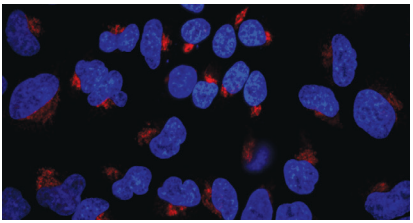
Oxygen gradients play an important role in developmental and cancer biology. For example, during cell growth cycles, cells respond to signal gradients directing their cell fate toward one type or another.[1] Cancerous tumors are heterogeneous tissues that contain complex and dynamic microenvironments. Such microenvironments create oxygen gradients where regions of well-oxygenated (normoxic) tissue and poorly-oxygenated (hypoxic) tissue exist. Hypoxia in tumor cores is a main regulator of survival and apoptotic pathways, and is thought to be the driving force in the formation and promotion of potent cancer stem cells (CSCs). CSCs are known to be a major cause of cancer following relapse because there are currently no treatments that target CSCs.[18] Furthermore, it has been demonstrated that tumor cells in hypoxic regions distant from blood vessels show resistance to chemo- or radiotherapy.[19,20]

Modeling a tumor microenvironment is not achievable using (normoxic) two-dimensional culture systems as oxygen is uniformly distributed throughout the surrounding medium.[3] *In vitro* studies utilizing 2D culture are unable to replicate sustained signal gradients, as the nutrients, soluble factors, and gases quickly equilibrate, resulting in a homogeneous response

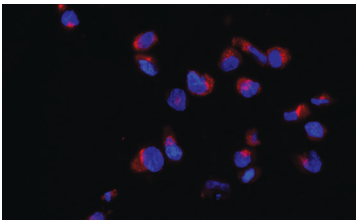
Oxygen Gradients

to their introduction to the cellular environment.[1] Because gradients cannot be achieved in 2D culture, the predictive nature of these cultures in drug screening is severely limited. 3D culture offers a more realistic environment for drug screening and takes into account the hypoxic gradient present in solid tumors.

HIF-1α is a transcription factor that is expressed when cells are experiencing a hypoxic environment.[21] Physiologically relevant hypoxic regions are created in Cell-Mate3D™ cultures, demonstrated by HIF-1α staining below. In normoxic 2D cultures, HIF-1α is sequestered to a site peripheral to the nucleus (Fig.3A). However, after 7 days of culture, HIF-1α is recruited into the nucleus and its expression is increased. This is observation indicates that culturing cells in Cell-Mate3D™ creates hypoxic environment and more closely mimics a solid tumor microenvironment compared to 2D culture (Fig.3B).



A. 2D Culture



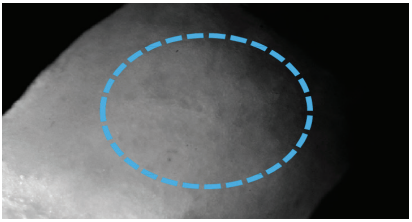
B. Cell-Mate3D™ Culture Day 7

Figure 3. HeLa cells were cultured in 2D on coverslips (A) or in Cell-Mate3D™ for 7 days and then cryosectioned and stained (B). Slides were stained with HIF-1α (red) and DAPI (blue) for nuclei.

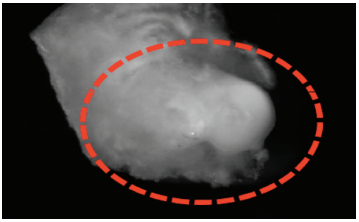
Light Microscopy Tips

Cell-Mate3D™ is an opaque, tissue-like matrix. We recommend using a darkfield technique combined with the use of an oblique white light, or a stereoscope to better visualize your cultures. Although cells deep within the gel cannot be visualized, certain gross characteristics may be observed on the matrix periphery with light microscopy.

In some cases, such as with MCF-7 cells (an epithelial invasive ductal adenocarcinoma cell line), nodules form and protrude out of the matrix (Fig.4B,C). Interestingly, normal MCF10A cells (a non-malignant ductal cell line) do not exhibit this phenotype (Fig. 4A).

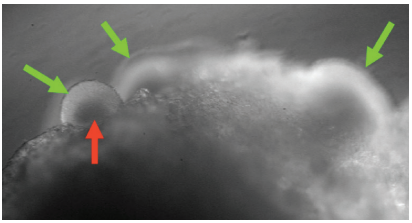


A. MCF-10 A



B. MCF-7 large nodule

Figure 4. Images of Cell-Mate3D™ cultures taken with dark field microscopy combined with the use of an oblique white light. MCF10A cells (A) and MCF-7 cells (B and C) were cultured in the Cell-Mate3D™ Matrix for 30 days. MCF-7 nodules were seen in as little as 6 days (B and C). Courtesy of Tim Lyden, University of Wisconsin, River Falls. Presented at the Wisconsin Science and Technology Symposium 2015.



C. MCF-7 small nodules (high mag)

Inverted Confocal Microscopy Tips

Inverted Confocal Microscopy is one of the quickest and easiest ways to obtain additional information about your Cell-Mate3D™ cultures (Fig.5).

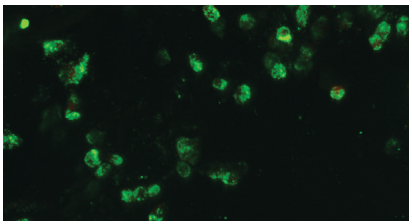
Both chemical and antibody stains can be performed directly in the matrix without the need for sectioning, and some stains take as little as 30 minutes to complete.

For Example:

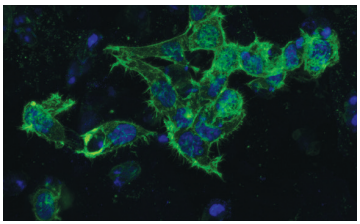
Live/dead staining (Calcein AM/Ethidium Homodimer 1) and various chemical stains such as nuclear (NucBlue), membrane, lipid droplets (LipidTox), mitochondria (MitoTracker), and actin (Phalloidin) can be performed. Cell embedded matrices may also be stained with antibodies.

Inverted Confocal Microscopy Tips

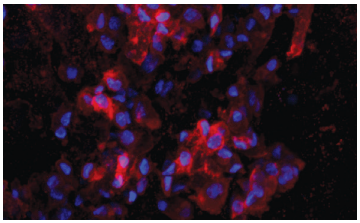
- After staining, simply place the gel on a cover slip and image.
- Higher blocking concentrations are required for antibody staining.
- For live/dead and antibody staining, be sure to follow BRTI's online protocols as staining times, dilutions, and blocking reagents are slightly different compared to standard protocols.



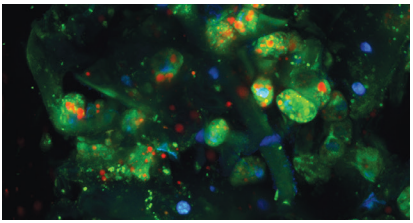
A. Live/Dead



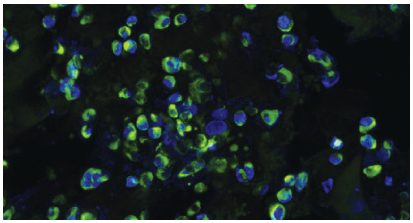
B. Phalloidin (actin)



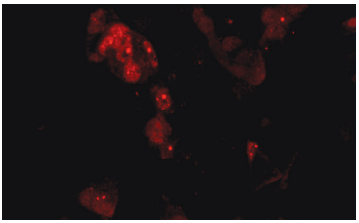
C. Plasma membrane



D. Lipids Droplets and Mitochondria



E. Vimentin (antibody)



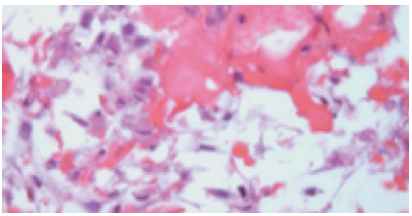
F. 53BP1 (nuclear antibody)

Figure 5. Using inverted confocal microscopy to visualize Cell-Mate3D™ cultures. HeLa cells (A,B,C,E,F) or primary human hepatocytes (D) were cultured in Cell-Mate3D™, stained, and imaged using inverted confocal microscopy. A small portion of the matrix was either stained live (A, D) or was fixed and stained (B, C, E, F). Samples were then placed on a cover slip for imaging. Viability staining using Calcein AM (green-live) and Ethidium homodimer 1 (red-dead) 20X (A). Phalloidin (green) staining actin cytoskeleton and NucBlue (blue) nuclear stain (60X) (B). CellMask™ Deep Red Plasma membrane stain (red) and NucBlue (blue) nuclear stain 60X (C). Lipid Tox Stain (red), Mito Tracker (green) and NucBlue (blue) 60X (D). Vimentin (green) antibody stain with NucBlue (blue) nuclear stain 20X (E). 53BP1 (red) nuclear antibody stain (F).

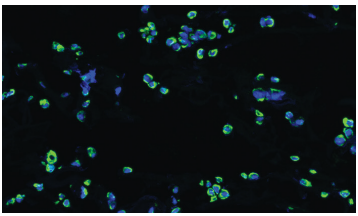
FFPE & Cryosectioning/Staining

Cell-Mate3D™ cultures can be embedded in paraffin, sectioned, and stained, or cryosectioned and stained (Fig.6). Slides can be imaged using epifluorescence or confocal microscopy.

- When performing FFPE, samples should be hand processed. Automated machines may break up the Cell-Mate3D™ matrix, making analysis difficult.
- Antigen retrieval may be required depending on the antibody.
- Be sure to follow BRTI's online protocols as staining times and blocking reagents are slightly different compared to standard protocols.



A. FFPE H&E Stain



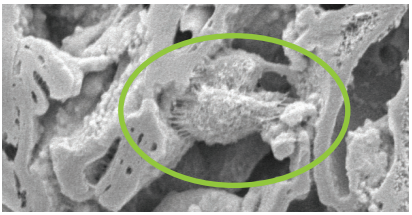
B. Cryosectioning/Staining (CD44)

Figure 6. FFPE and Cryosectioning/Staining. hMSCs (A) or HeLa cells (B) were cultured in Cell-Mate3D™. Cultures underwent routine FFPE, sectioning, and H&E staining (A) or Cryosectioning and staining for CD44-A488 (green) (B).

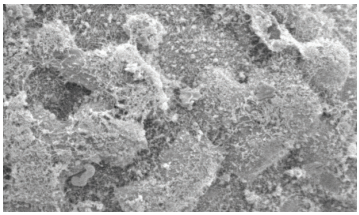
Scanning Electron Microscopy

Scanning electron microscopy enables visualization of Cell-Mate3D™ cultures at the nanometer scale, enabling observation of detailed cell morphology. Cell-Mate3D™ cultures are processed for scanning electron microscopy similarly to a tissue sample. In order to observe cells within the matrix, you may prepare the matrix as follows:

- After dehydrating through successive gradients of ethanol, and once the specimens are in 100% ethanol, immerse the specimen in liquid nitrogen.
- Place the specimen on a brass surface submerged in liquid nitrogen, and fracture it into smaller pieces using a pre-chilled wooden dowel.
- Return the specimen to 100% ethanol, and process in a critical point dryer.
- Continue specimen preparation as you would a piece tissue.



A. Inside Surface



B. Outside Surface

Figure 7. Cell-Mate3D™ cultures containing HeLa cells were prepared for Scanning Electron Microscopy using the guidelines described above. Both the inside surfaces (A) and the outside surfaces (B) of the 3D cultures were visualized. Images taken at 14 days (A) and 21 days (B) are shown.

Find Cell-Mate3D™ Microscopy Protocols and Resources Online. Other applications include:

RNA Isolation ○ Protein Isolation ○ Cell Retrieval

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Recommended Stains & Antibodies

- Viability-LIVE/DEAD® Viability/Cytotoxicity Kit.
  (Thermo Fisher# R37601)
- Nucleus-NucBlue (Fixed Cell or Live) ReadyProbes Reagent.
  (Thermo Fisher# R37606 or R37605)
- Actin-Phalloidin Alexa Fluor® 488.
  (Thermo Fisher# A12379)
- Plasma Membrane-CellMask™ Deep Red Plasma Membrane Stain.
  (Thermo Fisher# C10046)
- Lipids-HCS Lipid Tox™ Deep Red Neutral Lipid Stain.
  (Thermo Fisher# H34477)
- Mitochondria-MitoTracker Orange
  (Thermo Fisher# M7511)
- Antibodies: CD44-A488 *(VWR# 103016-BL),* 53BP1 *(Millipore, MAB3802)*, Vimentin-A488 *(BD Pharmingen #562338)*

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