

Validation of a Novel Tumoroid-Based Cell Culture Model to Perform 3D *in vitro* Cell Signaling Analyses

Brad Larson¹, Grant Cameron², Nicolas Pierre³, and Peter Banks¹

¹BioTek Instruments, Inc., Winooski, Vermont, USA • ²TAP Biosystems, Royston, Hertfordshire, UK

³Cisbio US, Inc., Bedford, Massachusetts, USA

cisbio
Bioscience
Member of IBA group

TAP
Biosystems

BioTek

Introduction

A central focus for improving drug efficacy in clinical trials over the last decade has been to increase the biological relevance of assays performed early in the drug discovery process. Yet it remains difficult to simulate an *in vivo* response to drug using an *in vitro* assay, where the cells are grown on hard plastic or glass substrates, in a two-dimensional (2D) format which is not representative of the *in vivo* cellular environment¹. When examining cells within a tissue, it can be observed that cells interact with neighboring cells, and with the extracellular matrix (ECM) to form a communication network. This communication controls a number of cellular processes including proliferation, migration, and apoptosis². However, most of the tissue-specific architecture, cell-cell communication, and cues are lost when cells are grown in a more simplified 2D manner. Therefore, more advanced cell culture methods are required to better mimic cellular function within living tissue. 3D cell culture serves to meet this demand by providing a matrix that encourages cells to reorganize into a structure more indicative of an *in vivo* environment; thereby allowing normal cell-cell and cell-ECM interactions to develop in an *in vitro* environment.

Here we demonstrate an *in vitro* HTRF[®] microplate assay that can quantify total, as well as phosphorylated eIF4E. The assay used a novel 3D culture system called RAFT[™] to create cell accumulations termed tumoroids in a cell/collagen hydrogel mix. In addition, the assay was also performed with cells cultured using traditional 2D methods for comparison. Dispensing and removal steps were performed by the MultiFlo[™] FX Microplate Dispenser, including cell/collagen mix, medium, and reagent dispensing, as well as removal of spent medium and compounds. Detection of the fluorescent signals from the HTRF assay, as well as tumoroid imaging, was performed by the Cytation[™] 3 Cell Imaging Multi-Mode Reader. Validation data generated using RAFT confirms the robustness of the 3D system. Pharmacology comparison data from both methods also demonstrates the validity of using 3D cell culture for cell signaling analyses.

BioTek Instrumentation

MultiFlo FX Microplate Dispenser. MultiFlo FX is a fully modular, automated reagent dispenser for 6- to 1536-well plates offering BioTek's unique Parallel Dispense[™] technology – both peristaltic and syringe pump operation down to 500 nL. An available microplate wash module adds multi-purpose functionality. The instrument was used to dispense the cell/collagen mix for hydrogel creation and all assay components, in addition to removal of spent medium and test compounds.

Cytation 3 Cell Imaging Multi-Mode Reader. Cytation 3 combines automated digital widefield microscopy and conventional microplate detection. The instrument includes both high sensitivity filter-based detection and a flexible monochromator-based system for unmatched versatility and performance. The upgradable automated digital fluorescence microscopy module provides researchers rich cellular visualization analysis without the complexity and expense of standard microplate-based imagers. The filter-based system was used to detect the 665 nm and 620 nm fluorescent emissions from the HTRF[®] eIF4E assay chemistry. Imaging was also performed using the microscopy capabilities to confirm cellular lysis and assess cytotoxic effects from test compounds. Gen5[™] Data Analysis Software was used for initial data analysis.

RAFT 3D Cell Culture System

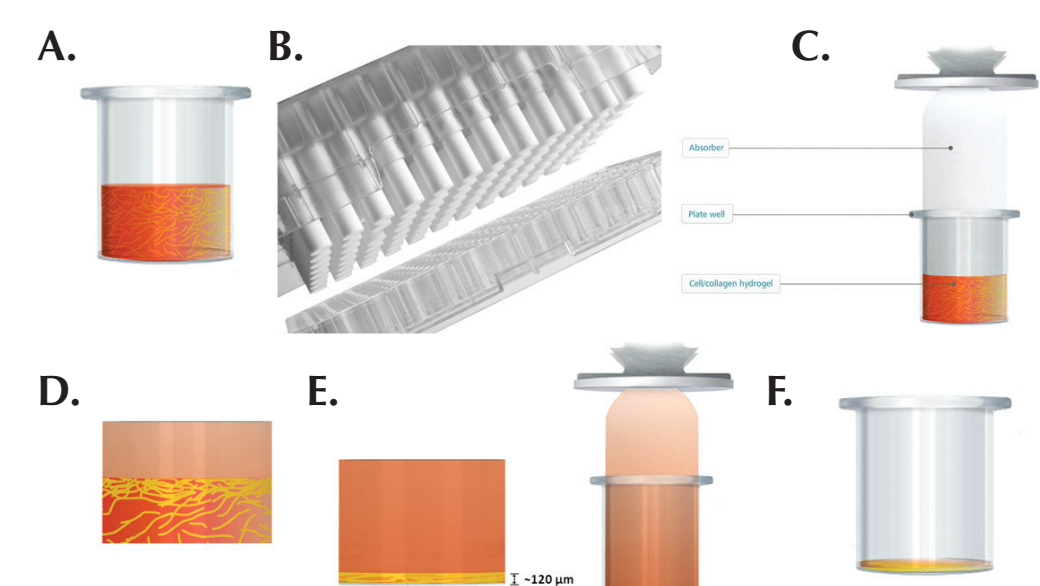


Figure 1 – Creation of 3-Dimensional Cell/Collagen Hydrogel using RAFT System. (A) Cell/collagen mix dispensed to wells of 96 well culture plate. (B) 96 well RAFT plate containing individual sterile absorbers. (C) Absorber insertion into plate well. (D) Absorption of medium, concentrating collagen and cells to *in vivo* strength. (E) Completion of absorption process creating 120 µm thick hydrogel. (F) Removal of absorber prior to dispense of fresh cell medium.

HTRF Phosphorylated and Total eIF4E Assays

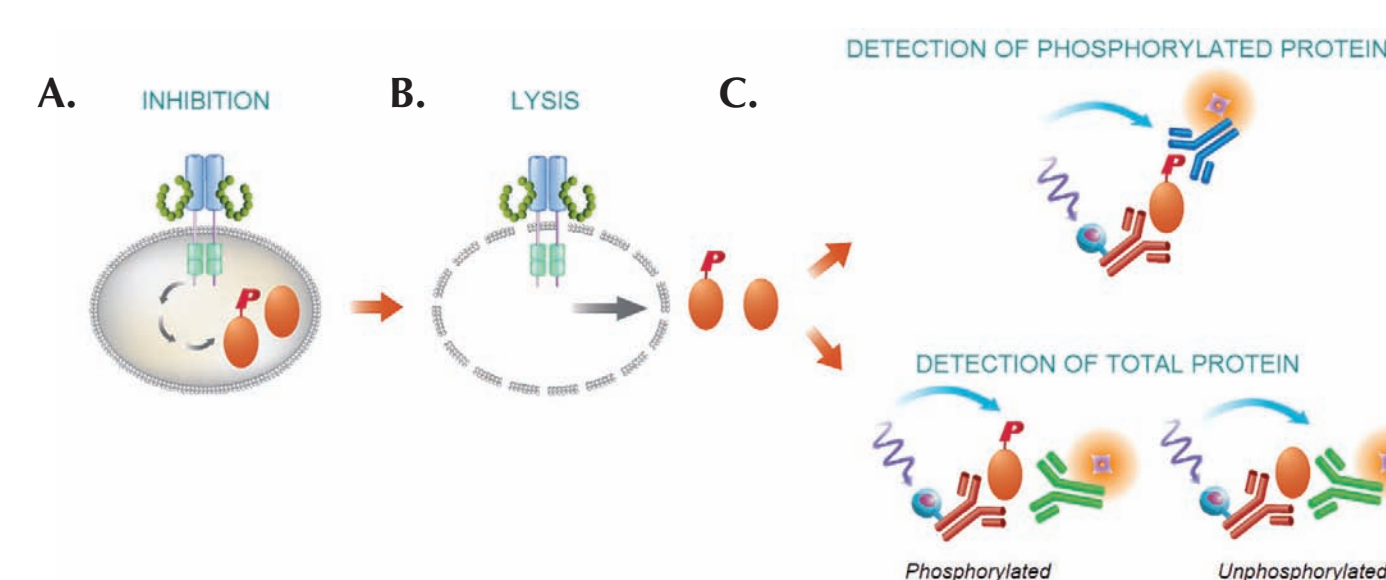


Figure 2 – Two-Plate HTRF Human eIF4E Assay. In the assay, phosphorylated and total eIF4E protein levels are measured using sandwich immunoassays involving two monoclonal antibodies. phospho-eIF4E assay: anti-peIF4E-K (Ab1) labeled with Eu-Cryptate and anti-eIF4E-d2 (Ab2) labeled with d2; Total-eIF4E assay: anti-eIF4E-K (Ab1) labeled with Eu-Cryptate and anti-eIF4E-d2 (Ab2) labeled with d2. The two antibodies for each respective assay may be pre-mixed and added in a single dispensing step, to further streamline the protocol. The assay is run in three steps. (A) In the inhibition step, cells are incubated with inhibitor compounds. (B) In the lysis step, cells are lysed, releasing the phosphorylated initiation factor eIF4E. (C) In the detection step, lysate is then transferred to a second plate, followed by antibody addition.

Materials and Methods

The Nuclear-ID[™] Blue/Red Cell Viability Reagent (Catalog No. ENZ-53005) from Enzo Life Sciences (Plymouth Meeting, PA) is a mixture of a blue fluorescent cell-permeable nucleic acid dye and a red fluorescent cell-impermeable nucleic acid dye that is suited for staining dead nuclei. Nuclei from viable cells will stain blue. As cell viability decreases, their membranes lose integrity and the red fluorescent dye is then able to stain the nucleus.

The CellTox[™] Green Express Cytotoxicity Assay (Catalog No. G8731) from Promega Corporation (Madison, WI) measures changes in membrane integrity that occur as a result of cell death. A proprietary asymmetric cyanine dye is used which is excluded from viable cells, but preferentially stains dead cell DNA. When the dye binds DNA in compromised cells, the fluorescent properties are substantially enhanced. The dye can also be diluted in culture medium and delivered directly to cells at dosing, allowing for kinetic cytotoxicity measurements.

RAFT 3D Assay Procedure

Day 1: HCT116 cells (Catalog No. CCL-247), from ATCC (Manassas, VA), were added to the prepared collagen solution. The mixture was then dispensed to the 96 well plate in a volume of 240 µL per well⁴. The final cell concentration equaled 25,000 cells/well. The cell plate was then incubated at 37 °C/5% CO₂ for 15 minutes, followed by addition of the absorbers in the RAFT plate, and a second 15 minute incubation at 37 °C/5% CO₂ during which the RAFT process increases the collagen density to a physiologically relevant strength. The absorbers were then removed and 100 µL of new medium was then added to the concentrated cell/collagen hydrogel⁴. The plate was once again incubated at 37 °C/5% CO₂ for three days to allow the tumoroids to form.

Day 4: Following the incubation period, spent medium was removed⁵, and 100 µL of small molecule inhibitor was dispensed back to the plate⁴. The cells and compounds were incubated together for 60 minutes at 37 °C/5% CO₂. The aspirate and dispense procedure was repeated to remove each inhibitor concentration⁶ and add 75 µL of lysis reagent⁴. This was followed by a 60 minute incubation at room temperature with shaking. 16 µL lysate aliquots were transferred to a separate low volume 384-well plate. 4 µL of the appropriate HTRF antibody mix for the phospho or total eIF4E assays were then added to the lysate aliquots⁴, and the plate incubated for 4 hours before reading.

*Steps performed by MultiFlo FX peristaltic pump (A) and wash module (B) during automated assay procedure.

RAFT 3D Assay Optimization and Automation Validation

Cell Concentration and Incubation Time Optimization

Multiple assay parameters were optimized to ensure robust and accurate pharmacology results using the pelf4E assay. Initial tests included cell concentration and inhibitor compound incubation time analyses. Ensuing experiments examined lysis reagent incubation times with cells in the hydrogel matrix. Dose response curves of two eIF4E pathway inhibitor compounds, including cercosporamide (Catalog No. 4500), a known blocker of eIF4E phosphorylation, and the mTOR inhibitor PI 103 (Catalog No. 2930) from R&D Systems (Minneapolis, MN), were analyzed to determine the most appropriate conditions to use for subsequent experiments.

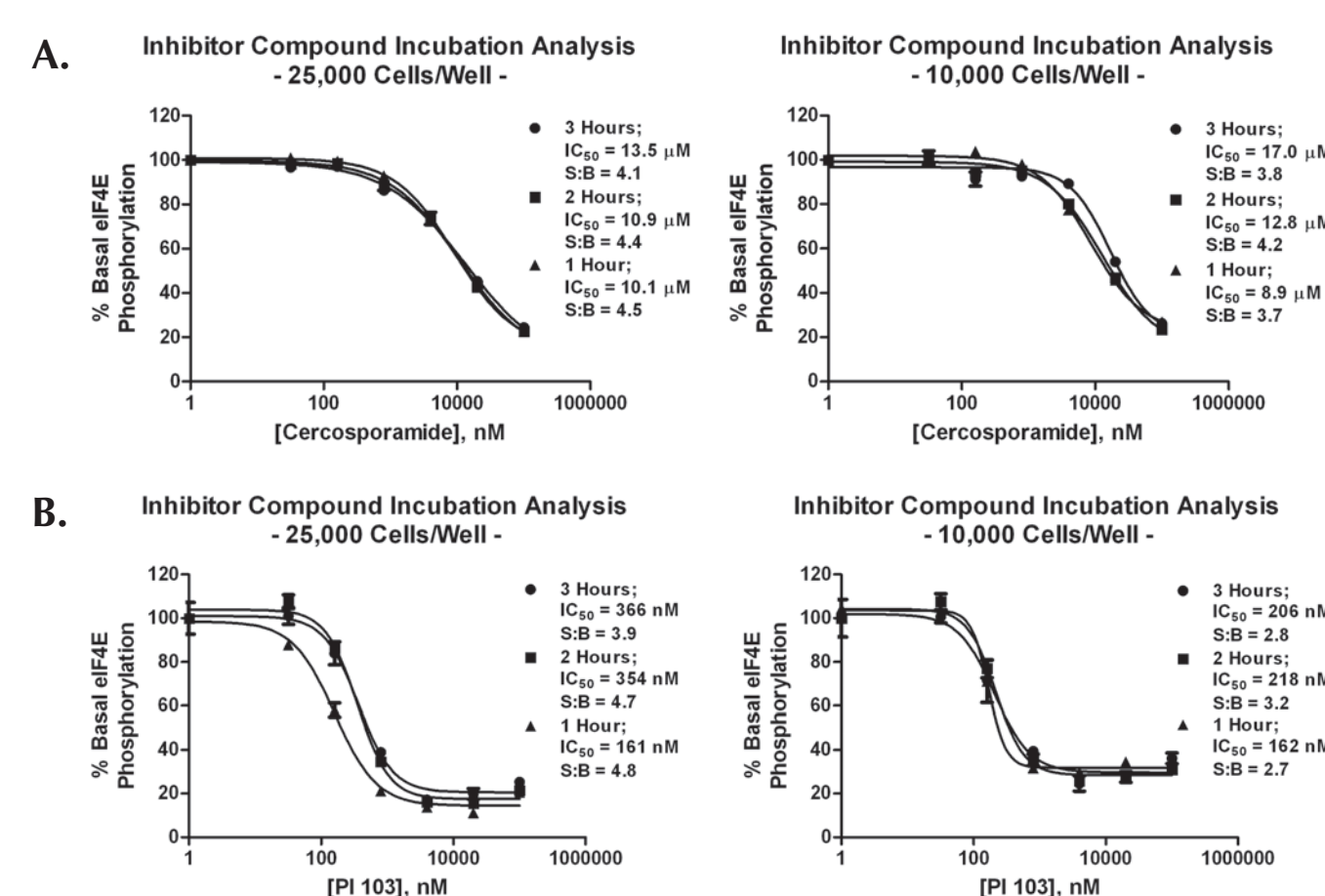


Figure 3 – Cell Concentration and Inhibitor Incubation Optimization Data. Dose response curves generated using (A) cercosporamide, and (B) PI 103, representing percent of uninhibited basal eIF4E phosphorylation levels. Tests performed using 25,000 or 10,000 cells/well, and a 1, 2, or 3 hour incubation time of inhibitor compound with cells.

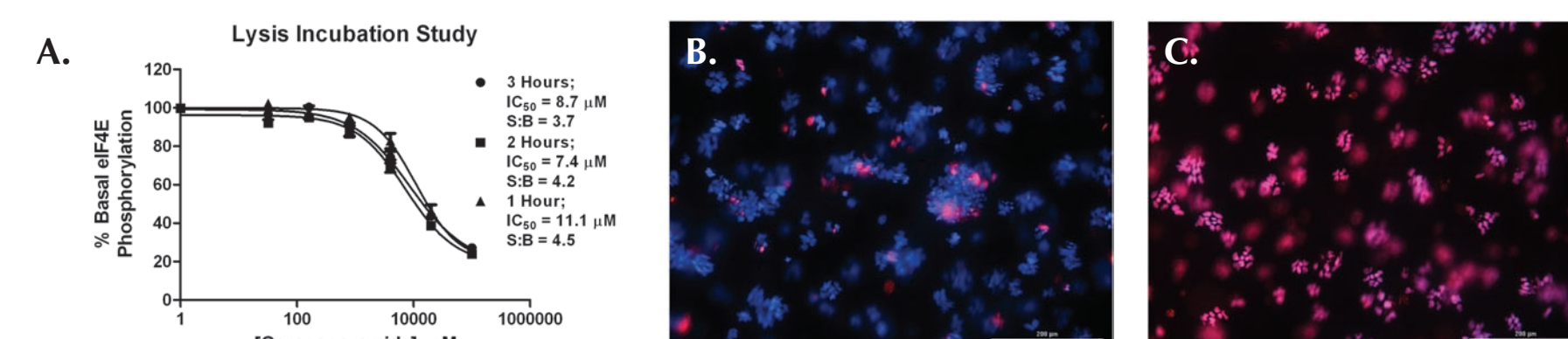


Figure 4 – Lysis Reagent Incubation Time Analysis Results. (A) Cercosporamide dose response curves generated from assays run using a 1, 2, or 3 hour incubation time following lysis reagent addition. 10x images also captured with Cytation 3 of tumoroids stained with Nuclear-ID Blue/Red Cell Viability Reagent before (B) and after (C) 1 hour lysis incubation.

The results of the cell concentration analysis (Figure 3) confirm that the use of 25,000 cells/well consistently delivers a larger assay window, specifically when looking at the PI 103 data. The same can be said for the one hour compound incubation time when using the higher cell concentration. The combination ensures that small changes in phosphorylation levels can be distinguished in subsequent inhibition studies. Finally the outcome of the lysis incubation experiment (Figure 4A) illustrates that extended incubation times do not improve assay results. The efficacy of the lysis reagent, when combined with the cells for one hour, was also confirmed via imaging. Images taken before lysis (Figure 4B) show limited cytotoxicity, while those captured following the incubation period demonstrate loss of membrane integrity in almost all cells imaged. These findings validate the effectiveness of the reagent to lyse cells within one hour, which also serves to create a shorter total time to complete the assay.

Automated Assay Validation

The 3D automated assay workflow was run with the MultiFlo FX using 0 and 100 µM concentrations of cercosporamide as positive and negative controls, respectively in a Z'-factor experiment to measure the ability of the instrument to perform accurate and repeatable aspirate and dispense steps. Z'-factor is a measure of assay robustness, and takes into account the difference in signal between positive and negative controls as well as the signal variation amongst replicates³. A scale of 0-1 is used, with values greater than or equal to 0.5 indicative of an excellent assay. Forty replicates of each compound concentration were included.

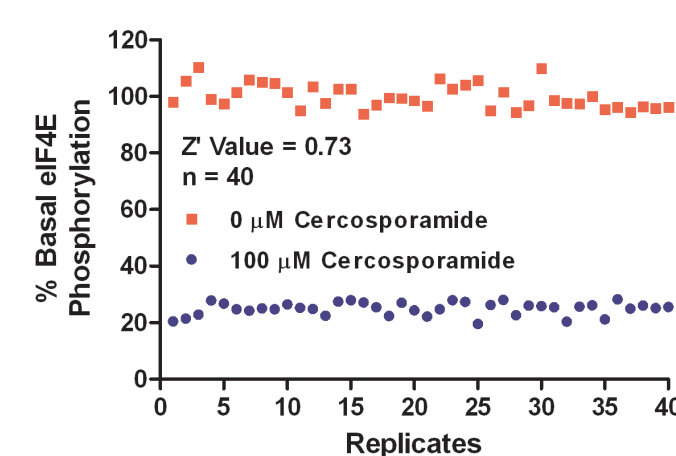


Figure 5 – Z'-factor results from automated 3D assay performed with MultiFlo FX.

The Z' value of 0.73 which was generated, as explained previously, is indicative of an excellent, robust assay procedure incorporating the RAFT cell culture system and MultiFlo FX.

Focus Stacking of 3D Tumoroid Images

Manual 20x images were taken at various z planes of tumoroids stained with three fluorescent probes; including a DAPI nuclear stain, Alexa Fluor[®] 488 phalloidin, and CellMask[™] Orange plasma membrane stain. The images were exported from the Gen5 software and imported into the CombineZP image stacking freeware package. The program serves to create a single "stacked" image with all planes in focus. The final image was then imported back into the Gen5 Data Analysis Package. This process enables phenotypic image-based analysis of tumoroids formed in the hydrogel.

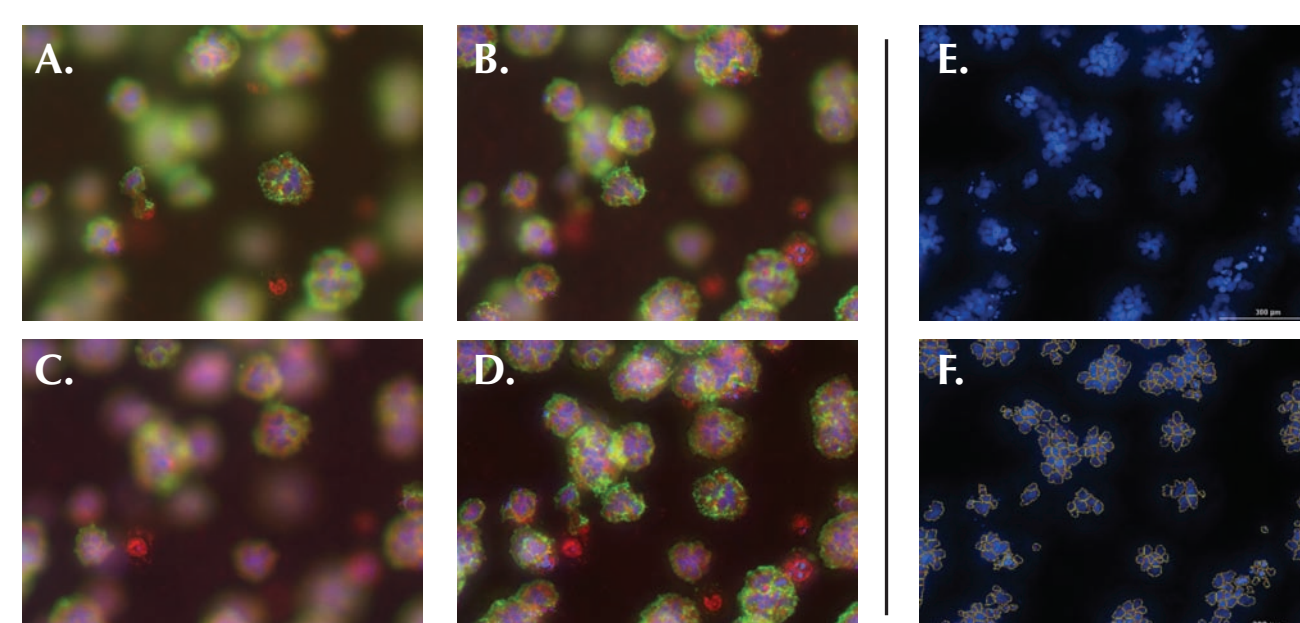


Figure 6 – Single plane and focus stacked tumoroid imaging and analysis. (A-C) Single plane 20x tumoroid images. (D) Focus stacked 20x image. (E) Focus stacked 20x image – DAPI channel only. (F) Cell count performed by Gen5 software on DAPI channel focus stacked image.

The image shown in Figure 6F demonstrates the ability to perform accurate analyses (such as cell counting) using the Gen5 software on focus stacked images (Figure 6D) of 3D tumoroids created using the RAFT system.

2D-3D Cell Culture Comparison

pelf4E Assay

The pelf4E assay was performed using traditional 2D cell culture methods, in addition to the RAFT system. HCT116 cells, at a concentration of 25,000 cells/well, were dispensed into 96-well clear bottom, TC treated plates (Catalog No. 3904) from Corning Life Sciences (Corning, NY) or the 96-well culture plates provided in the RAFT kit. Cercosporamide and PI 103 were once again used as the test pathway inhibitors. Serial titrations were created for each compound and added to the cells or tumoroids as previously described. The assay in each cell culture format was repeated twice to ensure repeatability and accuracy of results. IC₅₀ values were then compared to determine if differences in inhibitor pharmacology could be discerned that could be attributed to the different cell culture methods.

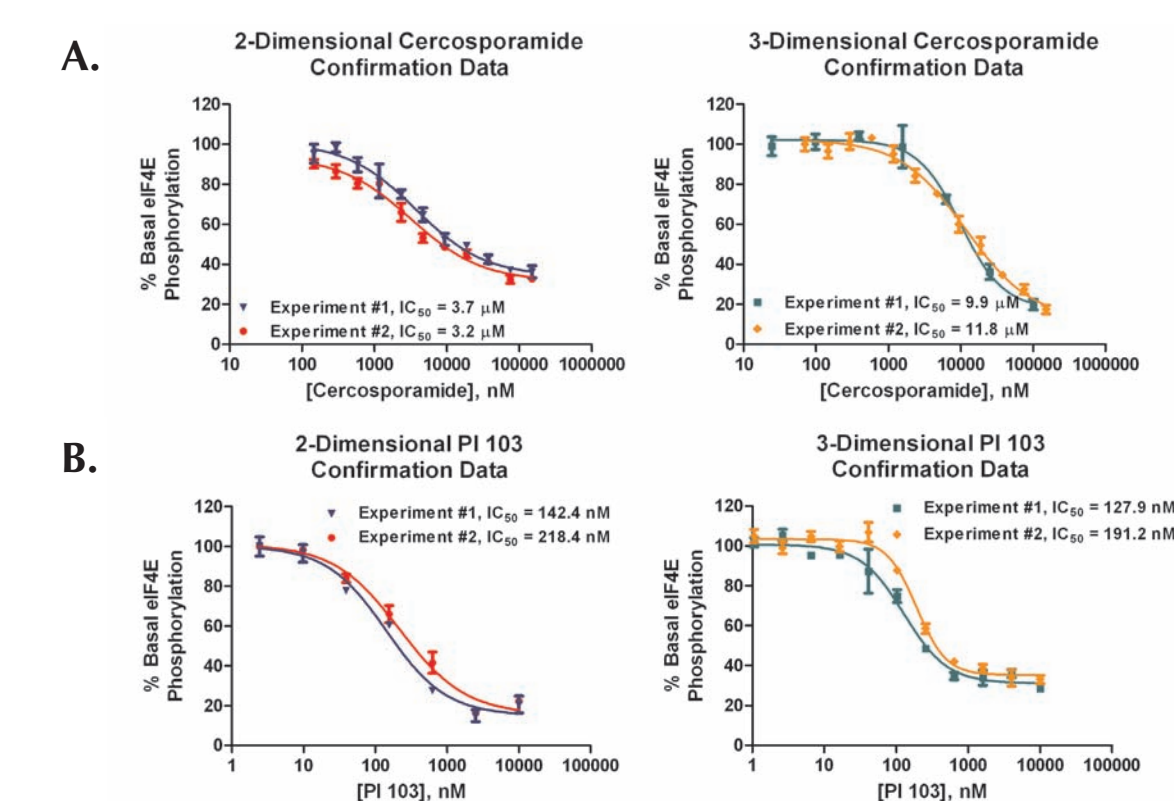


Figure 7 – 2D/3D pelf4E Assay Comparison Data. Cercosporamide and PI 103 inhibition of basal eIF4E phosphorylation results generated using either (A) 2D cell culture methods, or in 3D using the RAFT system (B). Dose response curve and IC₅₀ data from two separate experiments shown on each graph.

While no discernible difference was seen in IC₅₀ values between the two cell culture methods when using the upstream eIF4E pathway inhibitor, PI 103, it is clear that when cercosporamide, an inhibitor of direct eIF4E phosphorylation is incorporated, a disparity in pharmacology is observed. IC₅₀ values from dose response curves generated using 2D cell culture methods are greater than three times more potent than values calculated from dose response curves generated with assays performed using the RAFT system. This is most likely due to the fact that cells grown in a 2D monolayer are many times more easily effected by inhibitor compounds than cells cultured in more *in vivo*-like 3D tumoroid structures. This also serves to confirm the validity of the RAFT system for use in 3D cell signaling applications.

Total eIF4E Assay

The total eIF4E assay was also performed using the same cell culture methods and eIF4E pathway inhibitors. A unique HTRF antibody pair is incorporated to allow detection of both phosphorylated and unphosphorylated eIF4E protein.

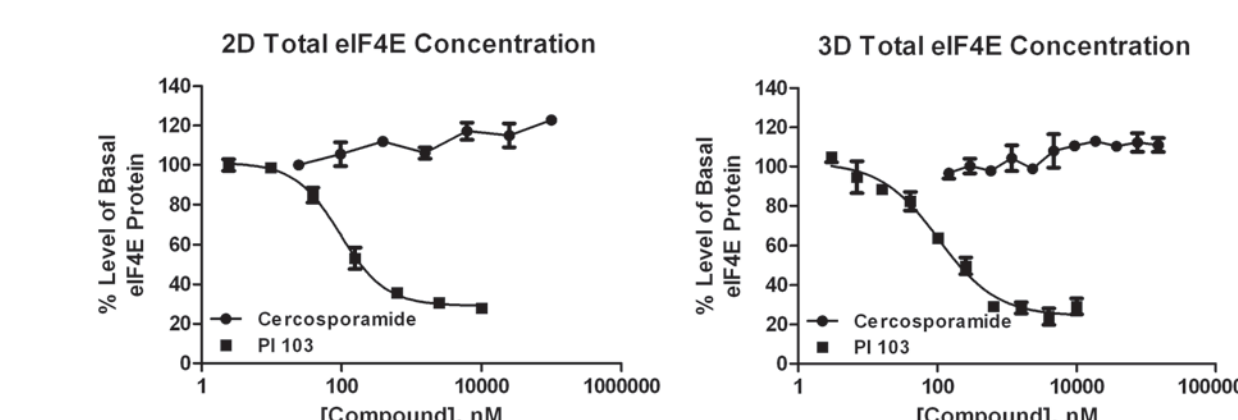


Figure 8 – 2D/3D total eIF4E Assay Comparison Data. Compound inhibition of signaling pathway leading to creation of eIF4E protein. Data generated using 2D cell culture or 3D RAFT system. Equivalent cercosporamide and PI 103 titrations incorporated as previously used in Figure 7.

The ability of the RAFT system to generate accurate inhibitor pharmacology was further validated by the results illustrated in Figure 8. A decrease in endogenous total eIF4E is seen with increasing concentrations of PI 103, which is consistent with previously published data; that PI 103 inhibits expression of PI3K/Akt/mTOR downstream proteins⁴. In contrast cercosporamide, which blocks direct phosphorylation of eIF4E through suppression of Mnk kinase activity⁵, does not demonstrate a noticeable effect on total eIF4E protein levels. These findings suggest that the inhibition seen in Figure 7B for PI 103 is actually driven by a reduction of eIF4E protein expression; while the inhibition seen in Figure 7A is attributable to an inhibition in kinase activity.

Cytotoxicity

Cytotoxicity measurements were also performed in order to ensure that decreases seen in eIF4E phosphorylation were due to inhibitory effects on the signaling pathway and not due to toxicity from the compound concentrations being tested. The CellTox Green assay was incorporated due to the fact that it could be added to the medium used for compound dilution. This created an easy and efficient multiplexed assay procedure, did not interfere with the emission spectra of the HTRF detection antibodies, and allowed for the use of the "Hit Picking" capabilities in the Gen5 software. Following the compound incubation period, the plate was read using the filter-based system on the Cytation 3. Any wells generating a fluorescent value above a pre-determined threshold when compared to negative control wells trigger live-cell imaging of that well (also performed by the Cytation 3). Following the completion of the procedure, the remaining portion of the assay process is completed as previously described.

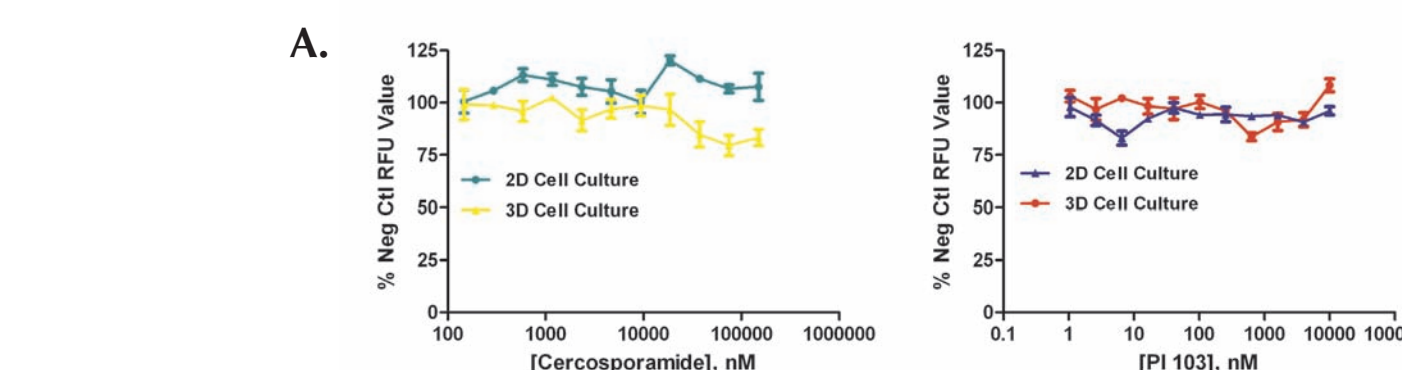


Figure 9 – Cytotoxicity Assessment Results. (A) % RFU values, when compared to those generated by negative control wells containing no compound, for all cercosporamide and PI 103 concentrations tested. 4x images also shown for (B) 100 µM cercosporamide and (D) 10 µM PI 103 using 2D cell culture; and (C) 100 µM cercosporamide and (E) 10 µM PI 103 using the 3D RAFT system.

Conclusions

1. The RAFT Cell Culture System represents a robust and accurate method to generate 3D cell-based assay data.
2. The HTRF phospho and total eIF4E assays provide easy-to-use procedures to measure cell signaling activity, and provide reliable results when combined with the RAFT system.
3. The filter-based detection system and fluorescence microscopy capabilities of the Cytation 3 present a unique and ideal way to capture the microplate reader and image-based results from the multiplexed assay procedure.
4. Incorporation of the MultiFlo FX serves to further enhance assay robustness by providing a repeatable, automated procedure.
5. The incorporation of a proper cell culture method, assay chemistry, and liquid handling and detection instrumentation, create an ideal combination to generate appropriate pharmacology and phenotypic results from test compounds.

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