Automation of HTRF® eIF4E Kinase Assay Using a 3D Tumoroid-Based Cell Model

Incorporation of the MultiFlo™ FX Microplate Dispenser to Create 3D Cell Cultures using the RAFT™ System and Perform the Steps of an HTRF® Cellular Kinase Assay

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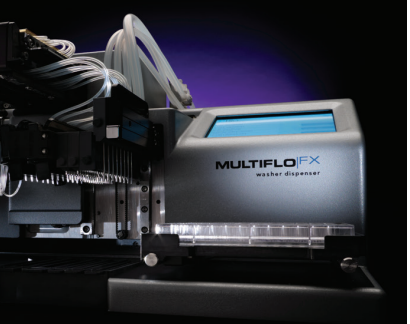

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Three-dimensional (3D) cell culture is poised to meet the need for a more *in vivo*-like cellular model with which to test large and small molecules. This is accomplished by providing a method that allows for the reorganization of cells into a format which re-establishes the necessary cellular architecture and communication networks seen in normal tissue. Recently a methodology has been put forth that incorporates a simplified procedure for the creation of a cell and collagen hydrogel mix. The inclusion of appropriate liquid handling instrumentation can further simplify the process and ensure the generation of accurate, robust results.

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. Biochemical assays used in screening campaigns are being replaced by cell-based, functional assays at ever increasing rates. Initially, these assays typically used the over-expression of drug targets in immortalized cell lines; but now more and more assays are conducted with human primary cells with endogenous expression of drug targets.

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 environment[1]. 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 apoptosis[2]. 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-extracellular matrix (ECM) interactions to develop in an *in vitro* environment. However, as with any assay procedure performed in a cell-based manner, it is imperative that correct instrumentation be incorporated for dispensing and cell washing to further ensure that proper conclusions can be made with these promising cell models. The robustness of the 3D cell culture structure to automated aspiration and dispensing of media, compounds and reagents will be assessed.

In this application note we demonstrate an *in vitro* microplate assay that can quantify total, as well as phosphorylated eIF4E. The assay was performed wherein cells were cultured using a novel 3D culture system called RAFT™ to create a cell/collagen hydrogel mix. The assay workflow involved a two plate protocol where cells are plated and compounds are added to inhibit basal activity of the eIF4E signaling pathway. Levels of phosphorylated and total eIF4E are then quantified by transferring the cell lysates to a second plate and detection reagent addition. All 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. Validation data generated using the automated assay procedure confirms the ability of the instrument to perform accurate, repeatable addition and removal steps throughout the entire process.

Materials and Methods

Materials

Cells

Colorectal carcinoma HCT116 cells (Catalog No. CCL-247) were obtained from ATCC (Manassas, VA). The cells were propagated in McCoy's 5A Medium (Catalog No. 16600) plus Fetal Bovine Serum, 10% (Catalog No. 10437) and Pen-Strep, 1X (Catalog No. 15140) from Life Technologies (Carlsbad, CA). The cells were plated at a final density of $2.5x10^5$ cells/mL for 72 hours prior to performing the assay.

Inhibitors

Cercosporamide (Catalog No. 4500), and PI 103 (Catalog No. 2930) were purchased from R&D Systems (Minneapolis, MN).

RAFT Reagents and Plates

96-Well RAFT Plate (Catalog No. A-0051) and 96-Well Culture Plate (Catalog No. A-9WE2) are part of the 4 x 96 RAFT Plate Kit (Catalog No. 016-0R93). Collagen Solution (Product Code A-0052), 10X Minimum Essential Medium (Product Code A-0053), and RAFT Neutralising Solution (Product Code A-0054) are part of the RAFT Reagent Kit. All RAFT components were supplied by TAP Biosystems (Hertfordshire, UK).

Instrumentation

MultiFlo™ FX Microplate Dispenser

MultiFlo FX is a modular, automated reagent dispenser for 6- to 1536-well plates. Up to four independent reagents are dispensed in parallel without potential carryover. The choice of peristaltic or syringe pumps allows reagent conservation and unattended operation down to 500 nL. A wash module is available for use with 6- to 384-well plates.

Cytation™3 Cell Imaging Multi-Mode Reader

Cytation3 combines automated digital widefield microscopy and conventional microplate detection. This patent pending design provides rich phenotypic cellular information with well-based quantitative data.

Cytation3's design places special emphasis on live-cell assays: features include temperature control to 45°C, CO_2/O_2 gas control, orbital shaking and full support for kinetic studies with BioTek's Gen5™ Data Analysis Software.

The filter-based system was used to detect the 665 nm and 620 nm fluorescent emissions from the HTRF® phospho and total eIF4E assay chemistries with the following settings: Delay after plate movement: 0 msec; Delay after excitation: 150 µsec; Integration time: 500 µsec; Read height: 10.5 mm. Imaging was also performed to analyze the tumoroid structure.

3D Assay Components

RAFT™ 3D Cell Culture System

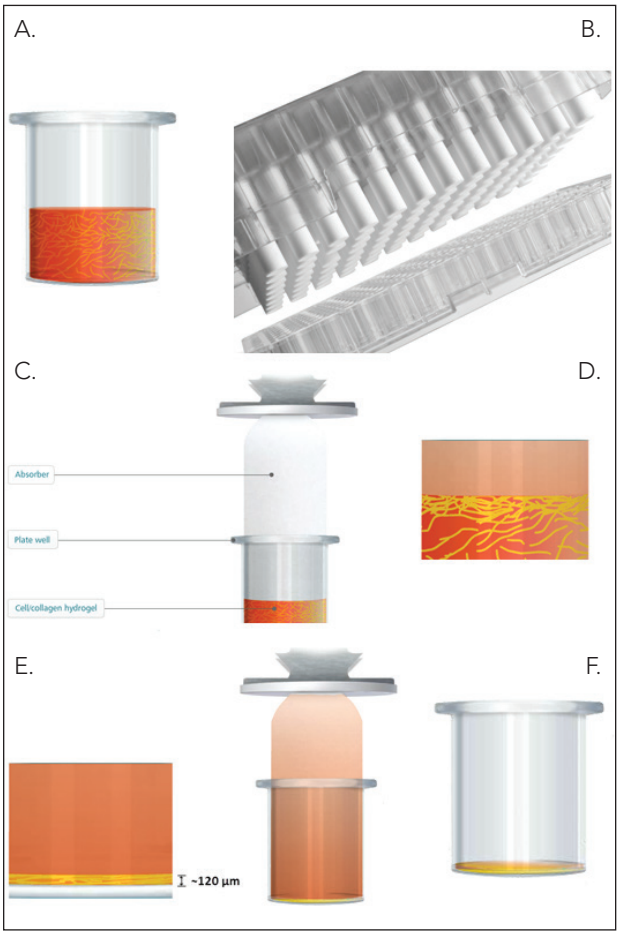

Figure 1. **Creation of 3-Dimensional Cell/Collagen Hydrogel using RAFT™ System. (A)** Cell/collagen mix dispensed to wells of 96-well plate. **(B)** 96-well RAFT plate containing individual sterile absorbers. **(C)** Absorber insertion into plate well. **(D)** Absorption of medium, concentrating collagen 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.

The RAFT (Real Architecture for 3D Tissue) cell culture technique developed by TAP Biosystems allows researchers to culture cell type(s) of their choice in an *in vivo*-like collagen environment. The technology uses the most abundant matrix protein in the body, type I collagen. The RAFT process raises the collagen concentration to physiological levels quickly and reproducibly. It takes less than 1 hour to generate cell cultures which are ~120 µm thick, biomimetic, dimensionally stable and transparent with high cell viability.

HTRF® Phospho- and Total eIF4E Assays

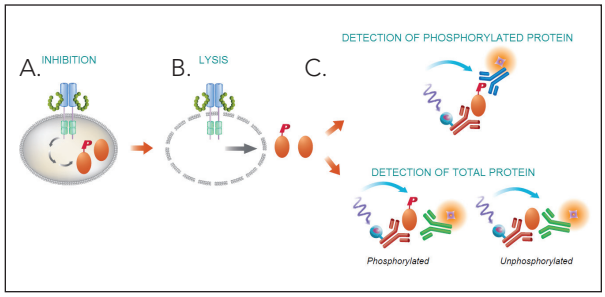

Figure 2. Two-Plate HTRF Human eIF4E Assay.

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 protein molecules. **(C)** In the detection step lysate is then transferred to a second plate, followed by antibody addition.

Methods

Automated 3D HTRF eIF4E Assay Protocol
Day 1:
HCT116 cells were added manually to the prepared collagen solution. The peristaltic pump of the MultiFlo FX was then used to dispense the mixture to the 96-well plate in a volume of 240 µL per well. The final cell concentration equaled 25,000 cells/well. The cell plate was then incubated at 37°C/5% CO_2 for 15 minutes, followed by manual addition of the absorbers in the RAFT plate, and an additional 15 minute incubation at 37°C/5% CO_2 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 by the MultiFlo FX to the concentrated cell/collagen hydrogel. The plate was once again incubated at 37°C/5% CO_2 for three days to allow the tumoroid to form.

Automated 3D HTRF eIF4E Assay Protocol
Day 4:
Following the incubation period, the aspirate pins of the plate washing module removed the spent medium, and the peristaltic pump dispensed 100 µL of small molecule inhibitor back to the plate. The cells and compounds were incubated together for 60 minutes at 37°C / 5% CO_2. The aspirate and dispense procedure was repeated to remove each inhibitor concentration and add 75 µL of lysis reagent. This was followed by a 60 minute incubation at room temperature with shaking. 16 µL lystate 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 aliquots using the peristaltic pump, and the plate incubated for 4 hours before reading.

Delta F(%) Calculation
The Delta F(%), or assay window, was calculated by comparing results from wells containing compound to negative control wells containing no compound using the following formula:

$$((\text{HTRF Value}_{(\text{Test Well})} - \text{HTRF Value}_{(\text{Neg Ctl})})/\text{HTRF Value}_{(\text{Neg Ctl})})*100.$$

Automated Assay Z'-factor Validation
The 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. Forty replicates of each compound concentration were included. The process was also performed using the EL406™ Microplate Washer Dispenser, as well as manually, to compare the results from different methods.

eIF4E Pathway Inhibitor Dose Response Analysis
Small molecule inhibitors were then tested for their ability to inhibit the eIF4E signaling pathway. Compounds included cercosporamide, a known blocker of eIF4E phosphorylation, and the mTOR inhibitor PI 103. 8-point titrations were created for each compound with concentrations ranging from 100-0 µM for cercosporamide, and 10-0 µM for PI 103 using a serial 1:4 dilution scheme. Lysate aliquots were tested with the phospho eIF4E and total eIF4E assays to confirm at what level of the signaling pathway the inhibition takes place. The assay procedure was once again performed manually, as well as using the MultiFlo FX.

HCT116 Tumoroid Image Analysis
Images were captured of the HCT116 tumoroids following the three day post-dispense incubation period using 20x magnification. The cells were stained using DAPI, Alexa Fluor® 488 phalloidin, and CellMask™ Orange plasma membrane fluorescent probes.

Results and Discussion

Automated Assay Robustness Assessment

The MultiFlo FX automated assay procedure described previously was validated in a Z'-factor[3] experiment. The 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 replicates. A scale of 0-1 is used, with values greater than or equal to 0.5 indicative of an excellent assay. The same experiment was also executed using the EL406 automated procedure, as well as manually to properly assess the performance of the MultiFlo FX.

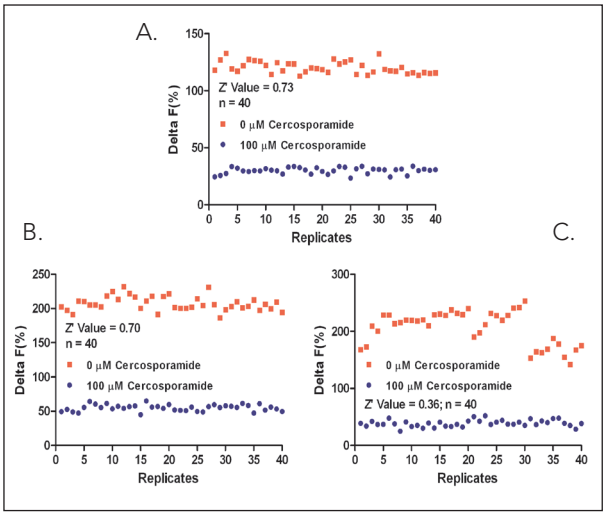

Figure 3. Z'-factor results using **(A)** MultiFlo FX automated; **(B)** EL406 automated; and **(C)** manual procedure to perform phospho eIF4E assay.

Per figure 3 data, the MultiFlo FX automated procedure provides equivalent results to the EL406. The Z' value for both, being >0.7, is indicative of a robust process. The values are also well above those achieved manually, demonstrating that repeatable aspiration and dispensing steps can improve accuracy and decrease variability within the final assay data.

Automated vs. Manual Assay Procedure Compound Pharmacology Comparison

Inhibition curves were plotted and IC_{50} values calculated for both compounds tested from the Delta F(%) values calculated by the Gen5™ Data Analysis Software using the original 620 and 665 nm emission signals.

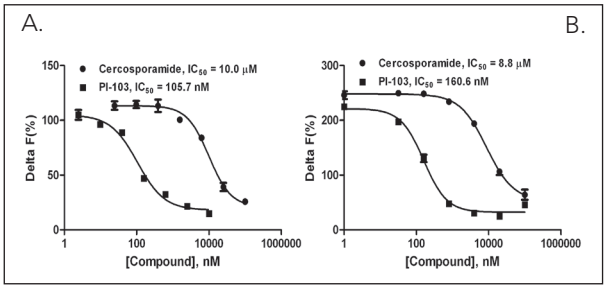

Figure 4. Dose response curves demonstrating inhibition of eIF4E phosphorylation. Results shown for the **(A)** MultiFlo FX and **(B)** manual assay procedure.

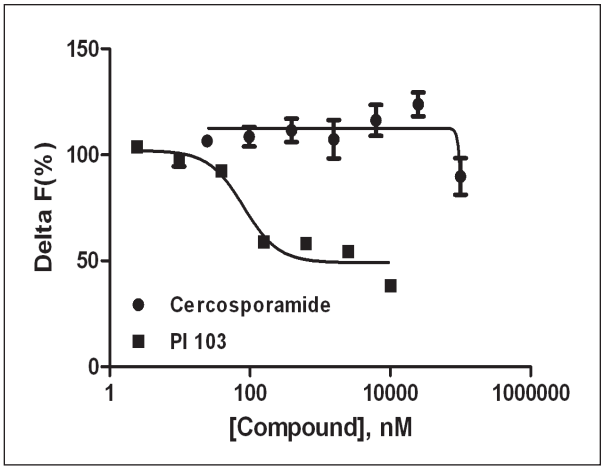

Figure 5. Compound inhibition of total eIF4E protein levels.

The ability to generate accurate inhibitor pharmacology is further validated by the results illustrated in figure 5. A decrease in endogenous total eIF4E is seen with increasing concentrations of PI 103, which is consistent with what has been previously published in the literature; that PI 103 inhibits expression of PI3K/Akt/mTOR downstream proteins[4]. In contrast cercosporamide, which blocks direct phosphorylation of eIF4E through suppression of Mnk kinase activity[5], does not demonstrate a noticeable effect on total eIF4E protein levels.

Cytation3 Imaging of 3D Tumoroid Structures

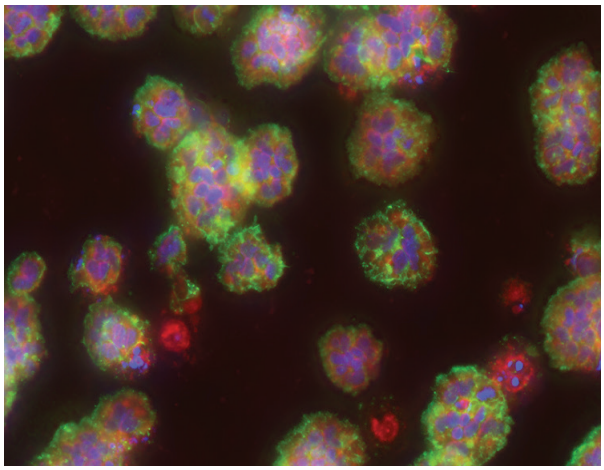

Figure 6. 20x z-stacked image of HCT116 tumoroids.

Multiple images were captured at different focal planes through the 120 µm hydrogel. A final z-stacked image (figure 6) was then created using the CombineZP software program.

Conclusions

The results shown here illustrate the ability of the MultiFlo FX to properly perform the necessary dispense and aspiration steps to complete the HTRF eIF4E assay using the RAFT 3D cell culture system. Z' validation data demonstrates that medium removal and addition can be completed without damaging the cell/collagen hydrogel layer, while volumes dispensed are repeatable across all replicates. Finally, pharmacology data confirms that accurate inhibition values can be generated when using the instrument to perform the multi-step process.

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