High Content Cell Cycle Screening: Pairing FUCCI Technology with Thermo Scientific HCS Studio 2.0 Software and FCS Express Image Cytometry

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Introduction

Measuring disruptions in the cell cycle of proliferating cells and their repair is an important area in cancer biology and drug discovery research. It is also recognized that understanding cell cycle progression in tumor growth is important for new cancer therapies. We have demonstrated a high content screening (HCS) assay, which distinguishes between phases of the cell cycle enabling systematic investigation of cell proliferation. The assay was conducted using the Premo™ FUCCI cell cycle sensors, which allow detection of cells cycling through G1 (RFP), G2 (GFP), and S (GFP/RFP mix) phases of the cell cycle, simultaneously using a live cell assay based on immunofluorescence detection in cells grown on standard high-density microplates. The DNA intercalating dye, Hoechst, was used to determine the nuclear size and morphology while a cytoplasmic cell label was used to analyze the whole cell morphology. By using the Thermo Scientific™ ArrayScan™ XTI High Content Analysis (HCA) Reader and Premo FUCCI cell cycle sensor, compounds affecting the cell cycle can be readily analyzed and reported with the De Novo Software's FCS Express™ Image Cytometry Software.

Methods and Materials

Hoechst 33342 Dye, wash buffer (10X Dulbecco's PBS), Permeabilization Buffer (10X Dulbecco's PBS with 1% Triton™ X-100), Cytochalasin D (Fisher Scientific Product No BP606-5), FUCCI Cell Cycle reagents (cat# 36238), paraformaldehyde (16%) (Thermo Scientific Product No. 28908 or 28906), Thermo Scientific™ Nunc™ Edge clear-bottom 96-well microplates, U-2OS cells and medium.

Cells and Cell Culture

This protocol is optimized for live U-2OS bone carcinoma cells (American Type Culture Collection Product No. HTB-96) growing on standard high-density microwell plates. Using cell lines other than U-2OS might require protocol optimization.

For routine culture of U-2OS cells use F-12K Complete Media (Thermo Scientific™ HyClone™ Product No. SH) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100µg/ml streptomycin, 1X non-essential amino acids and 1mM sodium pyruvate. Split cells when they reach 70-80% confluence (2-3 times per week) at a ratio of 1:3-1:8. Use cells at a passage number ≤ 20. Harvest cells by trypsinization, dilute into F-12K Complete Medium and determine cell density. Calculate the appropriate volume of reagent A (Premo™ Cdt1-RFP) and reagent B (Premo™ geminin-GFP) such that the MOI=10 parts per cell. Dilute cells to 105 cells/ml in F-12K Complete Medium and add 100µl of the cell suspension per well of a 96-well microplate (recommended plating density = 8,000 cells/well).

Procedure (For use with an SBS-format 96-well plate)

1. Plate 8,000 U-2OS cells in 100µl F-12K complete media per well and incubate 16-24 hours at 37°C in 5% CO_2. Assign control wells in the microplate.

2. Prepare compounds to be tested in stock solution in sterile DMSO. Dilute test compounds in culture medium (e.g., 30µl of 1.6mM Cytochalasin D in 10ml F-12K complete media) or other test compound to appropriate concentration. Add 50µl of 4.8µM Cytochalasin D to each treatment well and add 50µl of culture medium to the control wells. Incubate plate for 24 hours at 37°C in 5% CO_2.

Imaging and Analysis using HCS Studio 2.0 Software

Images were acquired on an ArrayScan XTI HCA Reader using 10x magnification. For analysis, the Thermo Scientific™ CellCycleV4 BioApplication was used. Readout measurements included cell number, cell size, nuclear area and intensity. Images were exported in .tif format and values for output features were exported in .ICE format (Image Cytometry Experiment) using the Thermo Scientific™ HCS Studio™ 2.0 Image and Data Export Tool. FCS Express Image Cytometry was used to import data and images for single cell sub-population analysis.

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Results

The purpose of the assay is to quantify cell cycle features under different conditions and drug treatments. U-2OS cells were treated with Cytochalasin D (1.6mM), Hydroxyurea, Nocodazole, Na Butyrate, Aphidicolin and Vinblastine and image sets were acquired at the 1-, 24- and 48-hour time points. Cell cycle phases were detected using Premo FUCCI cell cycle sensors for geminin-GFP and Cdt1-RFP. Negative and positive controls for the FUCCI reagents are demonstrated and indicate a measurable model for GFP and RFP cell cycle analysis based on visual inspection (Figure 1).

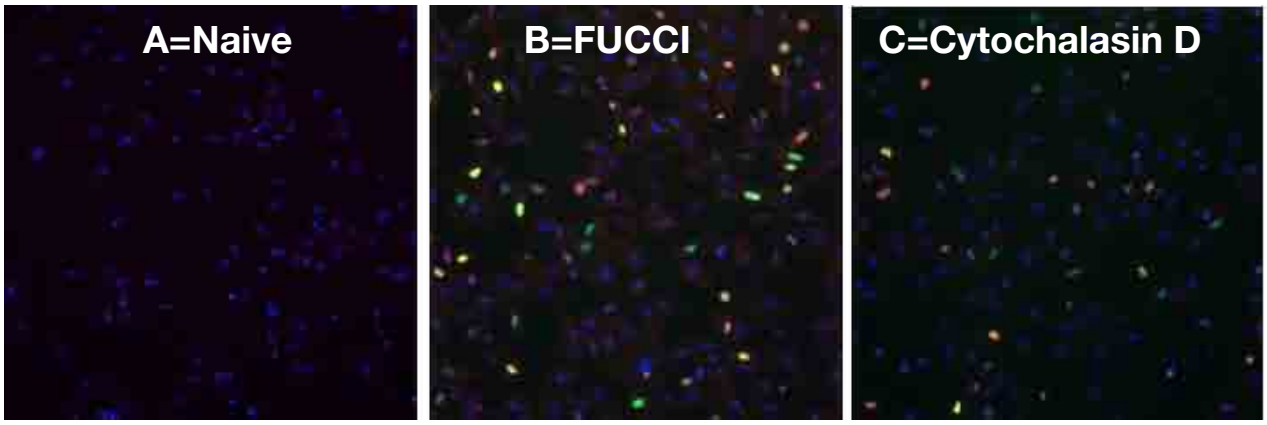
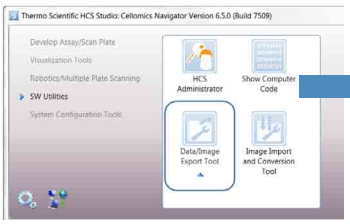


Figure 1: Images of U-2OS cells at 10x magnification showing untreated controls (A= naive, B = FUCCI) and treated control (C = Cytochalasin D) for phases G1 RFP, G2 GFP and S (RFP/GFP mix).

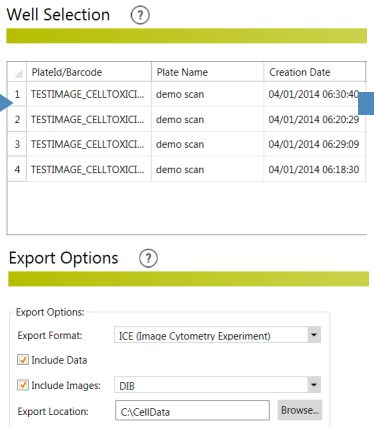
Cells were treated with the six aforementioned compounds while a kinetic time course was used for imaging over a 24-hour period. Multiwell plates of cells were fixed with paraformaldehyde and imaged along with Phalloidin 660. Data analysis begins with automated image acquisition and data generation via HCS Studio software. Using the Exporter Tool, data and images are then exported in a format compatible for import into the FCS Express software. (Figure 2).

Assay Image Aquisition and Data Handling Workflow

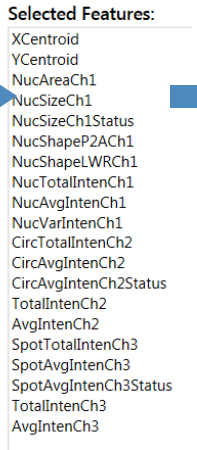
Step 1- Launch Data/Image Export Tool from Thermo Scientific HCS Studio



Step 2- Select Plates and the ICE (Image Cytometry Experiment) Format for Export



Step 3 – Select Cell Level Features to Export



Step 4 – Export and Open Data In FCS 4 Image Cytometry Software

Figure 2: Thermo Scientific HCS Studio 2.0 Software allows data and images from scanned plates to be quickly exported in the .ICE (Image Cytometry Experiment) file format standard for use in FCS Express Image Cytometry.

Using FCS Express 4 Image Cytometry for Cell Level Analysis

FCS Express 4 Image Cytometry was used to compare standard well level results to a single cell phenotypic-based approach to identify and quantitate subpopulations in a flow cytometry styled manner (Figure 3).

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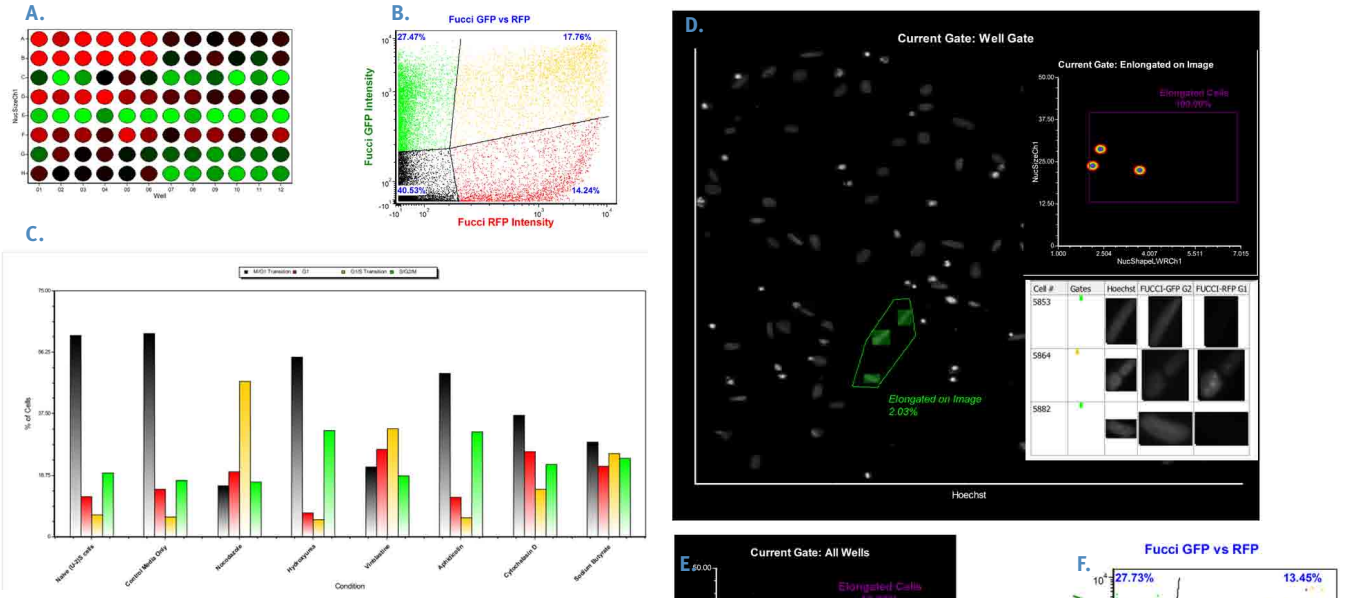

Figure 3: Well-level vs. single cell-based results.

A heat map representing the average nuclear size (green high, red low) of wells in the experiment (A). All cells were displayed in a 2D plot of FUCCI GFP vs FUCCI RFP signal (B). Quadrants created on the 2D plot (B) were converted to gates and applied to each condition resulting in a bar plot displaying the percentage of cells in each phase of the cell cycle (C) in each condition. A single cell approach was then taken by visual inspection of images to identify a phenotype of cells exhibiting elongated nuclei as shown in D (green cells). The elongated phenotype would normally have been grouped with all cells in a well-based analysis while a single cell approach allowed further interrogation. Cells identified in the green gate on the image were displayed on a 2D plot (inset D) and further evaluated in a cell gallery (inset D). A gate (purple) representing the elongated cell phenotype was set on the 2D plots (inset D – representative plot for one well) based on visual confirmation at the single cell level (D). The elongated cells gate was then visualized on a 2D plot displaying all events in the plate (E) and applied to a new FUCCI GFP vs. FUCCI RFP signal 2D plot (F) thus evaluating only cells of the elongated phenotype. The elongated cell phenotype was then assessed for percentage of cells in each phase of the cell cycle (G) to compare to well level results (C).

By comparing the results of bar plots in Figure 3C and Figure 3G it is clear that a single cell approach reveals new information about phenotypic subsets. The elongated cell phenotype exhibits substantial differences in each condition and for each phase of the cell cycle when compared to standard well level-based analysis. Notable changes include a lack of G1 and S phase cells in Nocodazole and Hydroyurea conditions, respectively, with the elongated phenotype. In a standard well-based approach, the elongated cells phenotype would not be identified and individually evaluated thus showing the benefit of an image cytometry approach. FCS Express Image Cytometry provides a means to identify cell subsets using raw images, objectively create gates based on control or interesting subsets in images, and use of gates to further analyze an entire screen of data. Such methods build upon well level-based screens by allowing a refinement of existing screening data at the single cell level.

Conclusions

This application note presents an image-based assay, which allows the simultaneous monitoring of cell proliferation and morphometric analysis in cells to provide insight on specific mechanisms involving cell cycle arrest and toxicity. Measuring phases of the cell cycle with Premo FUCCI sensors may be simultaneously performed in an automated manner using the ArrayScan XTI HCA Reader. Export of data and images from the HCS Studio 2.0 Software is seamlessly formatted for import into the FCS Express Image Cytometry.

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