Tech Note

Determination of Cell Confluence using Automated Cell Imaging for Verification of Cell Retention during Rigorous Cell Washing

Abstract

Here we show the use of automated cell imaging and automated analysis of cell confluence for verification of cell retention following rigorous cell washing. Minimal loss of cells was evident over three wash cycles for three different cell lines.

Introduction

Increasingly, automated workflows have gained in popularity with the ongoing adoption of cell-based assays performed in higher density microplate formats. Improved sample throughput and assay reproducibility are best achieved when processes such as media exchanges and cell washing are automated in conjunction with reagent additions. These automated workflows can be designed to offer walk-away solutions with increased performance when compared to manual methods.

Many cell-based assays rely on a combination of fluorescent reporter proteins and dyes spanning the light spectrum to achieve multi-parametric analysis on a single microplate well (Figure 1). Primary to any cell based assay is optimization of cell seeding density, while cell retention throughout the workflow is a key factor to assure optimal assay performance. This becomes more significant as workflows become increasingly complex as exemplified by experimental procedures that require a number of liquid handling steps including one or multiple cell washing and reagent dispensing steps. The importance of cell retention is compounded when analysis relies on data from several different time points when performing live cell assays. Here we describe the use of automated image capture, processing and analysis as a method to quantify cellular retention in the microplate and to aid in optimization of automated liquid handling parameters and normalization of data during analysis.

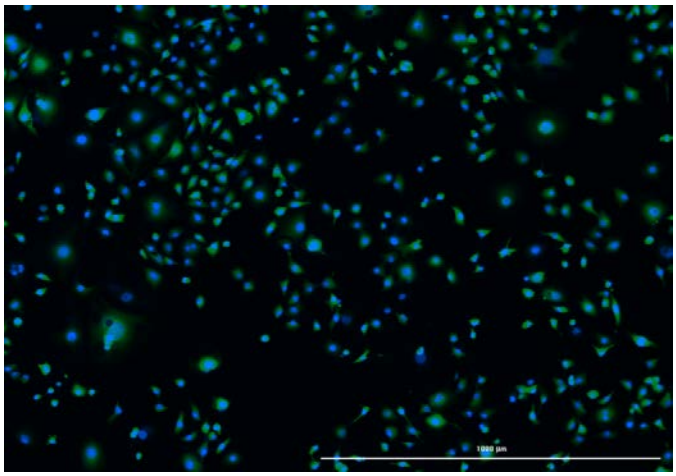


Figure 1. **MCF7 cells constitutively expressing GFP (green) and stained for the nucleus (blue) at 4x magnification.**

Materials and Methods

Materials

MCF7, HeLa and NIH3T3 cells were grown in Advanced DMEM (cat # 12491) from Life Technologies (Waltham, MA, USA) supplemented with 10% FBS, 2mM glutamine. Black sided, clear bottom 96-well (cat# 3904) were from Corning.

Instrumentation

Liquid Handling

All automated cell washes were carried out using the EL406 Combination Washer Dispenser. The EL406 provides full plate washing along with three reagent dispensers in one, compact instrument. Both peristaltic pump and syringe pump dispensers were used. In order to maintain sterility, the EL406 Washer Dispenser was placed in a biosafety cabinet and 5 µL cassettes for the peristaltic pump were sterilized by autoclaving prior to use.

Cell Culture and Microplate Seeding

MCF7, HeLa, and NIH3T3 cells were cultured in Advanced DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37 °C in 5% CO_2. Cultures were routinely trypsinized (0.05% Trypsin-EDTA) at 80% confluence. For experiments, cells were plated into Corning P/N3904 black sided clear bottom 96-well microplates at 5,000 cells per well using the peripump dispenser of the EL406. Prepared plates were kept at room temperature and protected from light prior to imaging.

Imaging

Cells were imaged using a Cytation 5 Cell Imaging Multi-Mode Reader (BioTek Instruments, Inc., Winooski, VT) configured with a GFP light cube. The microscope uses a combination of LED light sources in conjunction with band pass filters to provide the appropriate wavelength of light. The GFP light cube uses a 470/22 nm excitation filter and a 510/42 nm emission filter. Installed objectives included 4x and 10x magnifications.

Gen5 Analysis

Automated image capture, processing and analysis was performed using Gen5™ Microplate Reader and Imager Software (BioTek Instruments, Inc., Winooski, VT). Images were captured using both the blue and green channels prior to and following each cell wash step.

All collected images were preprocessed to flatten background prior to performing analytical methods. Image statistics were performed using the green channel to measure constitutively expressed cellular GFP for determination of cell confluence. Default parameters resulted in adequate computational data for further analysis.

Results

Automated image capture of several cell lines constitutively expressing GFP were used to evaluate the integrity of a cell monolayer for optimization of cell washing parameters for use with conventional workflows. Twelve representative wells of a 96-well microplate were imaged both before and after several rounds of automated cell washing using optimized wash parameters (Figure 2). An estimate of cell retention in a microplate well can be determined by visualization of a single 4x magnification image within a well. However, automated image analysis provides a fast, quantitative determination of cell confluence for validation of workflow processes. Analysis of GFP signal intensity (green channel) was used for determination of % confluence (Figure 2a and b).

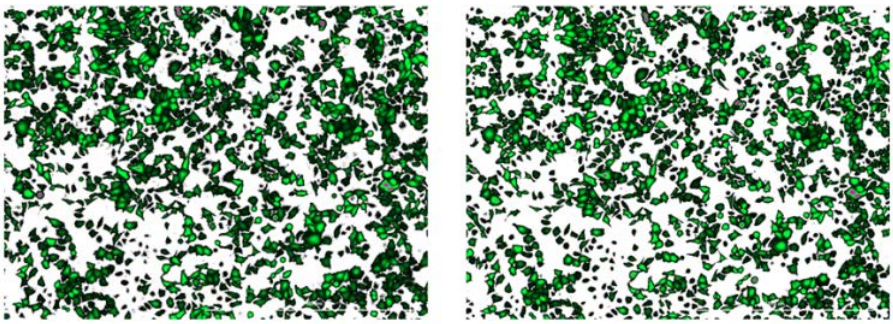
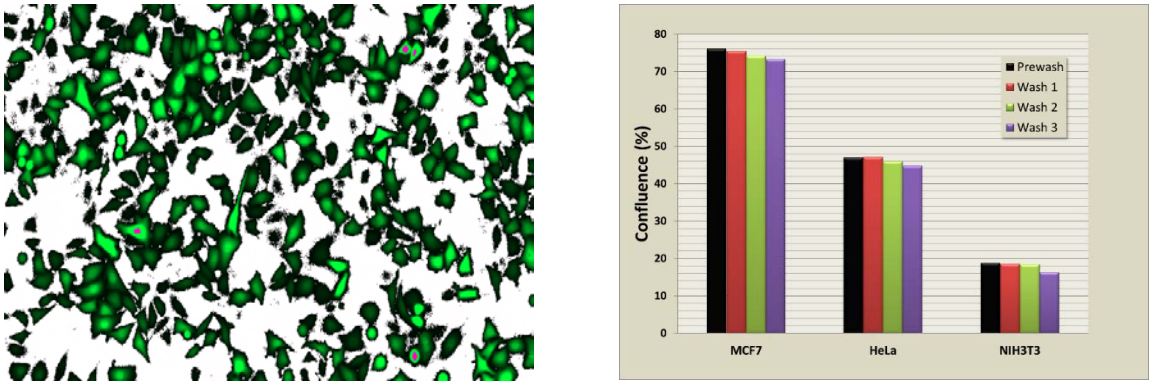


Figure 2. **Cell confluence analysis both pre- and post-automated cell washing.** MCF7 cells expressing GFP were seeded in a 96-well microplate for automated cell washing. The cells were imaged in 12 representative wells using a 4x objective before and after 3 cycles of automated cell washing with a primary mask applied to determine cell confluence. Images of GFP expressing MCF7 cells (A) pre-washing, (B) post-washing.

Cell confluence provides a method to determine cell density by identifying pixel intensity above a defined threshold representative of the object of interest (Figure 3a). Confluence is then calculated by dividing all pixels meeting the criteria by total image pixels multiplied by 100 (Figure 3b).



A B

Figure 3. Cell confluence analysis. Cell confluence is performed by identifying all pixels meeting the defined criteria in Gen5 software. In this case, the green channel is used for: (a) identification of the cell density based on pixel signal intensity; (b) percent (%) cell confluence is calculated by determining pixels above the defined threshold, green channel, divided by total pixels in the image multiplied by 100.

The entire well can also be imaged as a montage and stitched for determination of cell retention in the entire microplate well. Default settings in the Gen5 software for montage imaging align images such that there is sufficient overlap for appropriate stitching of the image tiles to provide the increased field of view. This ensures accurate downstream analysis such as cell counting or assessment of confluence measurements.

Figure 4 demonstrates GFP-expressing MCF-7 cells after vigorous washing. The image is composed of a 5x4 montage which has been stitched together to provide a field of view comprising a whole well of a 96-well microplate. It is apparent from the image that excellent retention of cells is maintained.

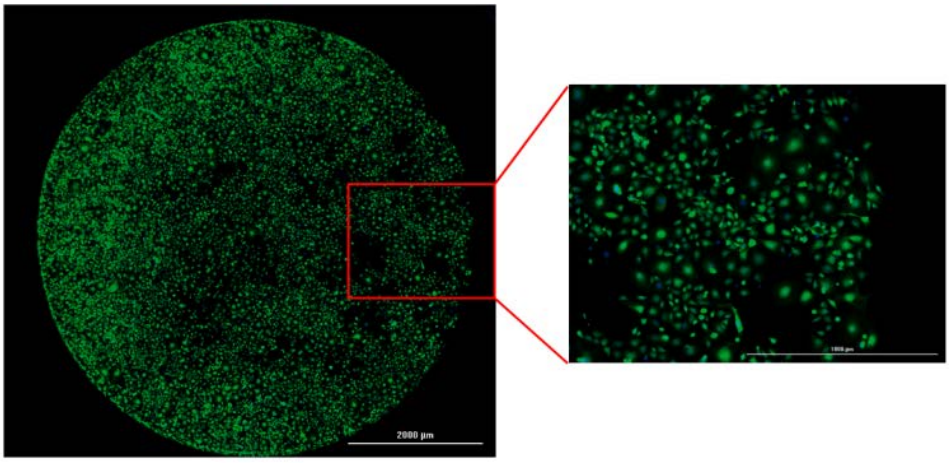


Figure 4. **Whole well imaging.** A 5x4 montage of images were captured using a 4x objective and stitched using Gen5 to allow the entire well surface to be viewed for analysis. A close-up detailing a section of the stitched image is shown (right image).

Conclusion

The use of automated processes to improve workflow can dramatically reduce variability and labor. The use of higher density microplates for cell based assays can further improve throughput and help reduce costs associated with reagent use. Optimization of automated processes such as routine media exchanges, cell washing and reagent additions help improve assay repeatability. With appropriate instrument settings to minimize cell loss, but still provide efficient washing, assay performance can be maintained.

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