Automated Tissue Culture Cell Fixation and Staining in Microplates

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Abstract

High Content Screening (HCS) plays a significant role in the drug discovery process, including secondary and toxicity screening. Perhaps the most common HCS application is using antibodies labeled with fluorochromes to localize and quantify proteins within cells. This requires fixing and permeabilizing cells such that the antibodies can penetrate the plasma cell membrane. Often other fluorescent stains are used in addition to identify cells and the nucleus, such as DAPI or Hoechst dyes or cytoskeleton features such as F-actin using fluorescently labeled phalloidin. The workflow for performing these experiments is a laborious multi-step process that also requires multiple cell washing steps within the plate. BioTek's microplate washers have been employed for the washing steps in HCS fixed cell workflows for many years and have become an HCS industry standard. In this poster, we detail the use of a combination washer dispenser for automating not only cell washing in microplates, but also all dispense steps including fixative, detergent for permeabilization and fluorescent probes.

Methods

Liquid Handling

All cell washes and reagent additions for cell fixation, permeabilization and fluorescent staining 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. For expensive biological reagents, such as primary and secondary antibodies, the peristaltic pump dispenser is optimum as unused portions of the reagent can be easily retrieved; for inexpensive chemical reagents such as fixatives and detergents used to permeabalize cells, syringe pumps can be used. In order to maintain sterility, the EL406 was placed in a biosafety cabinet and 5 µL cassettes for the peristaltic pump were sterilized by autoclaving prior to use.

Imaging

Cells were imaged using an Cytation™3 Cell Imaging Multi-Mode Reader (BioTek Instruments, Winooski, VT) configured with DAPI, GFP and Texas Red light cubes. The reader uses a combination of LED light sources in conjunction with bandpass filters and dichroic mirrors to provide appropriate wavelength light. The DAPI light cube is configured with a 377/50 excitation filter and a 447/60 emission filter; the GFP light cube uses a 469/35 excitation filter and a 525/39 emission filter; while the Texas Red light cube uses a 586/15 excitation and 647/57 emission filters.

Assay Process

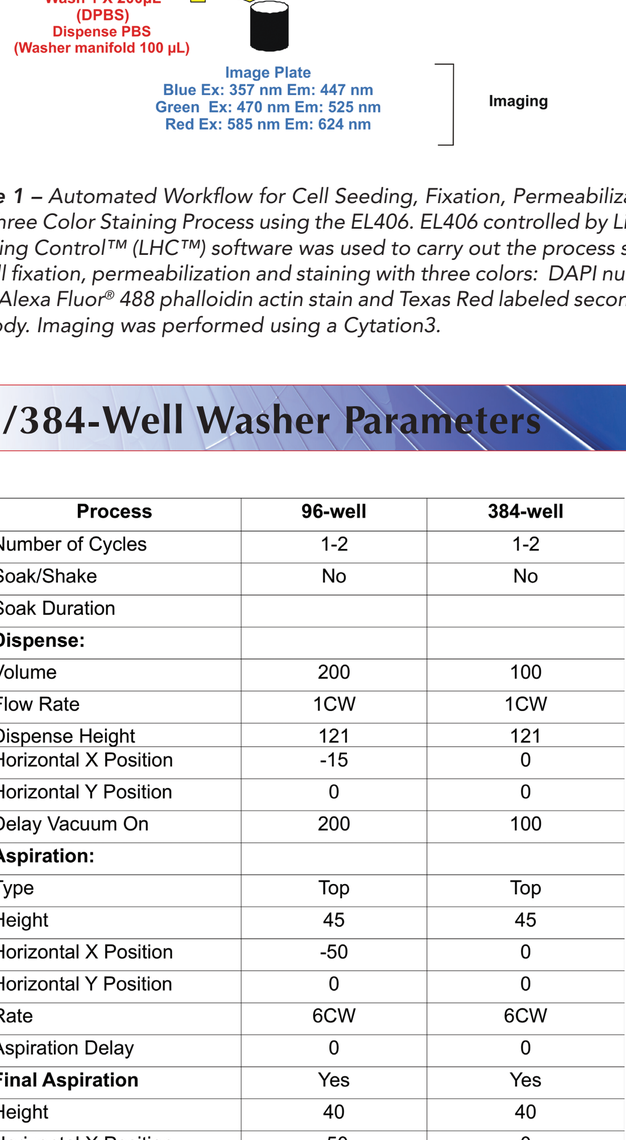

Figure 1 *– Automated Workflow for Cell Seeding, Fixation, Permeabilization and Three Color Staining Process using the EL406. EL406 controlled by Liquid Handling Control™ (LHC™) software was used to carry out the process steps for cell fixation, permeabilization and staining with three colors: DAPI nuclear stain, Alexa Fluor® 488 phalloidin actin stain and Texas Red labeled secondary antibody. Imaging was performed using a Cytation3.*

96-/384-Well Washer Parameters

Process	96-well	384-well
Number of Cycles	1-2	1-2
Soak/Shake	No	No
Soak Duration		
Dispense:		
Volume	200	100
Flow Rate	1CW	1CW
Dispense Height	121	121
Horizontal X Position	-15	0
Horizontal Y Position	0	0
Delay Vacuum On	200	100
Aspiration:		
Type	Top	Top
Height	45	45
Horizontal X Position	-50	0
Horizontal Y Position	0	0
Rate	6CW	6CW
Aspiration Delay	0	0
Final Aspiration	Yes	Yes
Height	40	40
Horizontal X Position	-50	0
Horizontal Y Position	0	0
Rate	6CW	6CW
Delay	0	0

Table 1 *– EL406 Combination Washer Dispenser Parameters.*

Brightfield

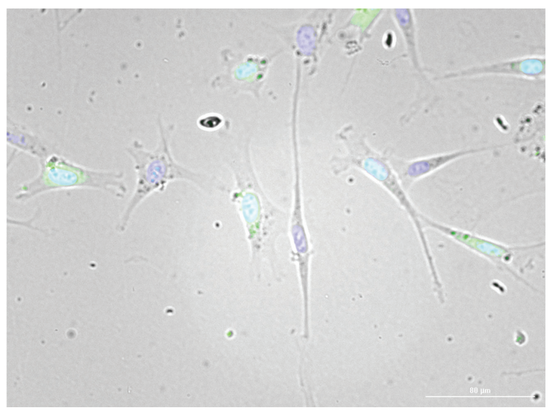

Figure 2 *– GFP expressing NIH 3T3 cells fixed with 4% Para-formaldehyde and stained with DAPI. Image represents an overlay composite of brightfield, and both blue and green fluorescence using 20x objective. Scale bar indicates the size of 80 µm.*

One-color Stain

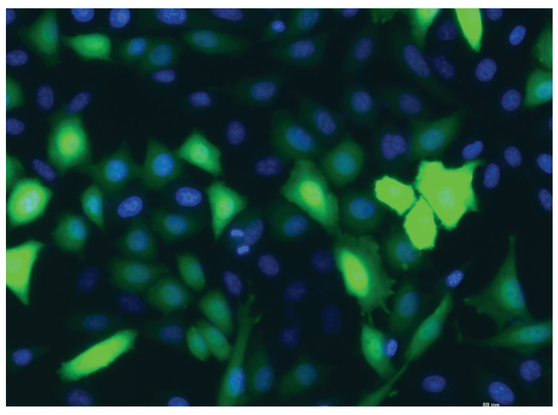

Figure 3 *– NIH 3T3 cells transfected with GFP. Cells in 96-well plates were fixed with PFA and stained with DAPI. Image represents an overlay composite of green and blue fluorescence using 20x objective. Scale bar is 80 µm in length.*

Two-color Stain

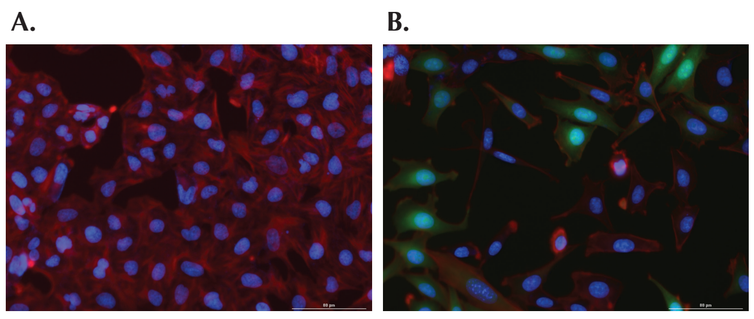

Figure 4 *– Two color staining. **(A)** Fixed U-2 OS cells in 96-well microplates stained for F-actin and DNA. F-actin was stained with Texas Red phalloidin, nuclear DNA was identified with DAPI. Image represents the overlay of separately captured blue and green fluorescent signals. **(B)** NIH 3T3 cells expressing GFP. F-actin was stained with Texas Red-phalloidin and nuclear DNA was identified with DAPI. Image represents the overlay of separately captured red, blue, and green fluorescent signals. Both images were obtained using using 20x objective. Scale bars represent 80 µm.*

Three-color Stain

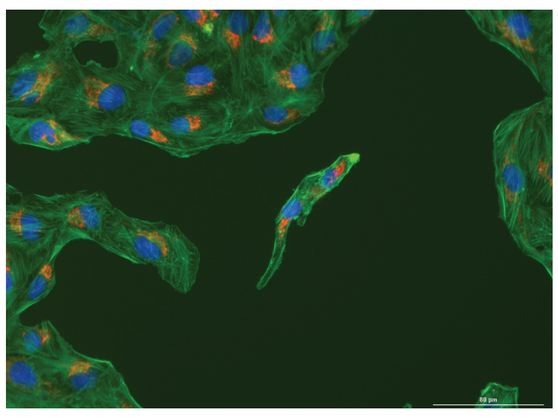

Figure 5 *– Three color staining of U-2 OS cells. Cells in 96-well plates were fixed and stained for mitochondria, F-actin, and DNA. Mouse monoclonal 1° anti-mitofilin antibody followed with a Texas Red labeled 2° anti-mouse IgG identified mitochondria, while (AlexaFluor 488 phalloidin identified cytoskeleton actin and DAPI stained nuclei. Images were obtained using using 20x objective. Scale bar indicates 80 µm.*

384-well Processing

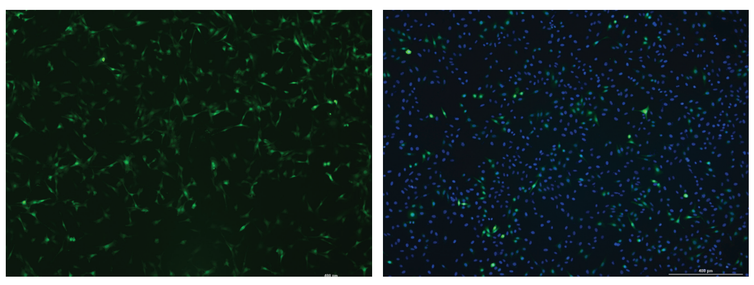

Figure 6 *– NIH 3T3 and HeLa cells expressing GFP in 384-well microplates. **(A)** HeLa cells expressing GFP were fixed with 4% PFA and stained with DAPI. Scale bar indicates 1000 µm. **(B)** NIH 3T3 cells expressing GFP were fixed with 4% PFA and stained with DAPI. Images were obtained using using 4x objective. Scale bar indicates 400 µm.*

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Figure 7 *– EL406 Combination Washer Dispenser.*

Figure 8 *– Cytation3 Cell Imaging Multi-Mode Reader.*

Results

Tissue culture cells in 96- or 384-well microplates can be fixed and stained using the EL406 Combination Washer Dispenser. After fixation with 4% paraformaldehyde, GFP expressing cells can be quantitated and transfection efficiency determined by the addition of DAPI or Hoechst 33342 dyes to identify nuclei (Figures 2-3). Likewise, fluorescently labeled phalloidin can be added in conjunction with nuclear dyes to identify F-actin cytoskeleton proteins and nuclei concurrently (Figure 4). Antibody based detection of cellular structures can also be performed using the automated system. Fixed cells were permeabilized using Triton X-100 followed by treatment with a mouse monoclonal antibody against the mitochondrial protein mitofilin. The 1° antibody was then visualized by the addition of a fluorescently labeled 2° antibody directed against Mouse IgG. With the addition of DAPI and AlexaFluor 488 phalloidin, three color staining of U-2 OS cells was demonstrated.

The EL406 Combination Washer Dispenser has a number of features that make it amenable for the process steps of fixing and staining tissue culture cells prior to fluorescence microscopy. Typically cells are washed with PBS or similar isotonic buffer between reagent additions to remove unwanted media or to eliminate unbound antibodies or dyes. The washer manifold has a full 96 head that is capable of rapidly washing either 96- or 384-well microplates without changing washer manifolds. In addition, the combination of syringe pump and peripump dispensers allows for the addition of different reagents. Use of the peripump dispenser (Table 2) in conjunction with a 1 µL cassette allows the user to minimize the amount of overage required for an adequate dispense. Unused precious reagent can also be recovered by reversing the peripump and purging the lines back into the reagent container. Additionally, the multiple reagent paths of the peripump can be used to add different antibodies or stains to different wells on the same microplate; thus allowing for side by side comparisons without the need for subsequent reagent additions. The syringe pumps allow for different reagents (e.g. PFA fixative and permeabilization buffer) to be added using separate fluid paths without any manual intervention.

These data also demonstrate the ability of the Cytation3 Cell Imaging Multi-Mode Reader to make 4X and 20X images from 96- and 384-well microplates.

Conclusions

1. Cytation3 provides high resolution images in 96- and 384-well microplates.
2. EL406 is capable of automating the fixation and staining of tissue culture cells in microplates
   - No loss of adherent cells.
   - Eliminates technician bias.
   - Process compatible with both 96- and 384-well microplates .
   - Wash steps accomplished using 96-head washer manifold.
   - Fixative and PBS bulk reagents added with syringe pumps.
   - Antibodies and stains added with peripump allowing recovery of unused amounts.