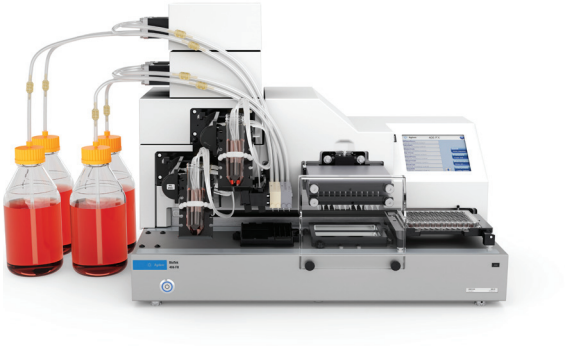
Application Note

Cell Analysis

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Automated Cell Fixing and Antibody Staining in Microplates



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Abstract

Fluorescence microscopy has traditionally been performed on microscope slides, but there is a growing trend towards the use of 96- and 384-well microplates, as this allows greater number of samples to be easily processed and automated, consistent with high-content analysis (HCA). This application note describes the use of the Agilent BioTek 406 FX washer dispenser to automate the fixing and staining processes typically used before fluorescence imaging.

Introduction

HCA is a method of identifying cellular phenotypic changes brought about by substances that interact with the cell. Such substances include small molecules, receptor ligands, and RNAi, which may induce: phenotypic changes that increase or decrease the production of specific proteins, internalization of G protein–coupled receptors (GPCRs), nuclear hormone receptor translocations, apoptosis, changes in post-translational modification of proteins, and changes in cell morphology. While some morphological changes can be investigated using transmitted-light observations (brightfield), many more require the use of physical stains (typically fluorescent) or inherent fluorescence (e.g., GFP proteins).

The hallmark of fluorescence microscopy is the use of specific antibodies to recognize and bind to cellular structures. While primary antibodies are occasionally directly labeled, more often a labeled secondary antibody that recognizes the species and type of the primary antibody is used to visualize the structure. In addition to antibody-based detection, the expression of genes can be accomplished using chimeric fluorescent protein constructs. These small genetic elements are inherently fluorescent and can be linked to endogenous proteins without loss of function in many cases. Fluorescent counterstains are also often used to identify cellular organelles, such as the nucleus and cytoskeleton, to provide the spatial context for the antibody staining (Figure 1).

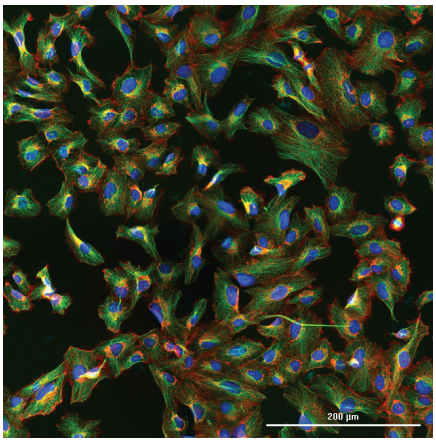


Figure 1. Fixed and stained HT-1080 cells. HT-1080 cells were fixed and stained for actin (red), tubulin (green), nuclei (blue), and mitochondria (orange) using the Agilent BioTek 406 FX washer dispenser to perform the liquid handling. Widefield montage (2 x 2) was captured with a 40x objective using the Agilent BioTek Cytation C10 confocal imaging reader.

Regardless of the combination of antibody and fluorescent dye used in high-content screening assays, a series of aqueous reagent additions and wash steps to remove unbound materials is required for specific staining with low background. This application note describes the use of the Agilent BioTek 406 FX washer dispenser to automate reagent addition and the necessary wash steps for the preparation of cell samples in 96-well plates before HCA.

The 406 FX is a modular system, fully programmable from either its built-in touch screen or from the Agilent BioTek Liquid Handling Control (LHC) software on an attached PC. The system is capable of rapidly washing either 96- or 384-well microplates without changing washer manifolds. The combination of up to four syringe pumps and two peristaltic pump dispensers allows for the addition of multiple reagents. The peristaltic pump dispenser, in conjunction with a 1 µL cassette, allows the user to minimize the amount of overage required for an adequate dispense. Unused reagent can also be recovered by reversing the peristaltic pump and purging the lines back into the reagent container. Less-precious reagents can be added using the syringe pump dispenser. Bulk dispensing of buffer can also be accomplished with the washer manifold.

Experimental

Materials

Cell culture

HT-1080 cells (part number CCL 121; ATCC, Manassas, VA) and NIH3T3-expressing GFP (part number AKR 214; Cell Biolabs Inc., San Diego, CA) were grown in advanced DMEM (part number 12491; Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS, penicillin, streptomycin, and 2 mM glutamine. Black-sided, clear-bottom 96-well microplates (part number 204626-100) were from Agilent. TopSeal-A adhesive plate sealers (part number 204626-100) were from PerkinElmer (Waltham, MA). DAPI dihydrochloride stain (part number D1306), Alexa Fluor 488 phalloidin (part number A12379), Texas Red phalloidin (part number 7471), Alexa Fluor 488 anti-mouse IgG (part number A10680), Alexa Fluor 555 anti-rabbit IgG (part number A27039), and DPBS (part number 14190) were obtained from Thermo Fisher Scientific. Anti-Tom20 monoclonal antibody (part number 42406) was obtained from Cell Signaling Technology (Danvers, MA).

CF633 phalloidin conjugate (part number 00046) was from Biotuim (Fremont, CA). Paraformaldehyde (part number P6148) and Hoechst 33342 (part number B2261) powders were obtained from Sigma-Aldrich (St. Louis, MO), while Triton X-100 (part number X198-05) was from J.T.Baker.

Cell imaging

Cells were imaged using the Agilent BioTek Cytation C10 confocal imaging reader configured with confocal and widefield DAPI, GFP, TRITC, and CY5 detection filter cubes. For widefield imaging, the Cytation C10 uses a combination of LED light sources in conjunction with band-pass filters and dichroic mirrors to provide light of appropriate wavelength. For confocal imaging, the Cytation C10 uses multiple lasers in conjunction with a Nipkow spinning disk and filter cubes.

Liquid handling

All cell washes and reagent additions for cell fixing, permeabilization, and fluorescent staining were carried out using the 406 FX washer dispenser. The 406 FX provides full-plate washing along with six reagent dispensers in one, compact instrument. Both peristaltic and syringe pump dispensers were used. For expensive biological reagents, such as primary and secondary antibodies, the peristaltic pump dispenser is optimum as portions of the reagent can be easily retrieved. For inexpensive chemical reagents such as fixatives and detergents used to permeabilize cells, syringe pumps can be used.

Cell seeding

The 406 FX was used to seed tissue culture cells into microplates using autoclaved 5 µL peristaltic pump cassettes. To allow adequate time for cell recovery and attachment after cell dispersal, this process was carried out the preceding day. After dispersion with trypsin, cells were rinsed with complete media and resuspended in complete media at a concentration of 1 x 10^6 cells/mL. Using sterile peristaltic pump tubing sets, various volumes of cell suspension were dispensed into the wells to achieve the desired cell number in each well. The second peristaltic pump was then used to add complete media without cells such that all the wells in the microplate had a total of 200 µL media.

Cell fixing and staining

Depending on the experimental protocol, all or a subset of the process steps outlined in the "Fix and stain" workflow (Figure 2) may be used. For example, a four-color immunofluorescent stain process that uses two primary and (labeled) secondary antibody pairs along with two separate counterstains can require as many as seven different dispensers to completely automate the process (Table 1). Alternatively, if the cells need only to be fixed and counterstains added without requiring antibodies, the blocking step and antibody additions can easily be eliminated.

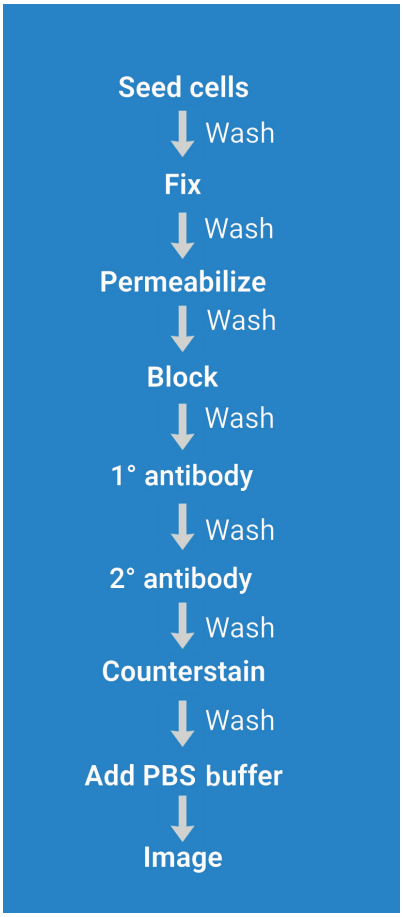


Figure 2. Fix and stain workflow.

Table 1. Reagents and dispensers use for fluorescent staining.

Step	Reagent	Dispenser
1	Fixation	Syringe A
2	Permeabilization	Syringe B
3	Blocking	Syringe C
4	Primary antibodies	Primary peristaltic pump
5	Secondary antibodies	Secondary peristaltic pump
6	Counterstains	Syringe D
7	PBS	Washer manifold

Fixing (step 1)

Cells were washed three times with 200 µL DPBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 7.4 mM KH_2PO_4) before fixing using the 96-tube washer manifold of the 406 FX. Following this, 100 µL freshly made 4% paraformaldehyde (PFA) solution was added using one of the syringe pump dispenser manifolds of the 406 FX. Cells were fixed for 30 minutes at room temperature, followed by two washes using 200 µL DPBS.

Permeabilization (step 2)

Following the fixation process described above, cells were permeabilized for 30 minutes using 100 µL PBS containing 0.1% Triton X-100, which was added using a syringe pump dispenser manifold. Afterwards, the permeabilization was aspirated using the washer manifold.

Blocking (step 3)

A blocking solution of 100 µL, consisting of 3% bovine serum albumin (BSA) in permeabilization buffer, was added to each well using a syringe pump dispenser. The cells were blocked for a period of 30 minutes, after which the fluid was aspirated using the washer manifold.

Antibody staining (steps 4 and 5)

Primary antibodies were added using a peristaltic pump dispenser. Each well received 50 µL of both rabbit monoclonal anti-Tom20 and mouse monoclonal anti-tubulin antibodies, each diluted at 1:750 in a mixture of PBS, 30 mg/mL BSA, and 0.1% Triton X-100. The plate was incubated at room temperature for 60 minutes, then washed three times with PBS using the washer manifold. The second peristaltic pump dispenser was used to add secondary antibodies. Each well received 50 µL of both Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 555 goat ant-rabbit monoclonal antibodies, each diluted at 1:500 in a mixture of PBS, 30 mg/mL BSA, and 0.1% Triton X-100. The plate was allowed to incubate for 60 minutes at room temperature followed by three washes with PBS.

Counterstaining (step 6)

After washing to remove unbound secondary antibodies, cells were counterstained. Depending on the experiment, cells were counterstained with either a mixture of the nuclear stain Hoechst 33342 (10 µM) and the actin stain CF633-conjugated phalloidin, or Hoechst 33342 alone. Regardless of the mixture, a syringe pump dispenser was used to add 50 µL of the counterstains to all wells. The cells were stained for 30 minutes at room temperature followed by three washes.

Final buffer addition (step 7)

After completing of the staining process, the wells were filled with 275 µL PBS using the washer manifold and the plates sealed with an optically clear plate seal. Fixed and stained cells were refrigerated and protected from light before imaging.

Results and discussion

The initial use of the 406 FX washer dispenser involved the seeding of experimental cells. By using different volumes of a common cell suspension, the number of cells in the well can be varied. As demonstrated in Figure 3, when a linear volume titration of a cell suspension is dispensed into strips of a microplate, the subsequent cell count based on image analysis of fixed and Hoechst 33342–stained cells results in a linear response. In this experiment, the primary peristaltic pump was used to dispense various volumes of a common cell suspension of NIH3T3 cells. The secondary peristaltic pump dispenser was used to normalize all wells to a volume of 200 µL.The observed cell count, extrapolated to reflect the entire well area, correlates well with the expected cell number based on the cell concentration when seeding.

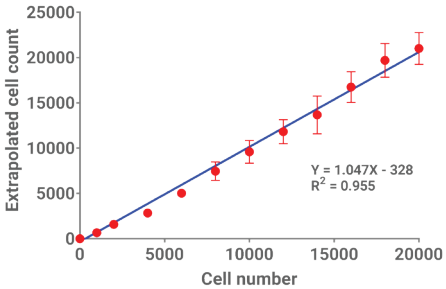


Figure 3. Variable cell number seeding. Various volumes of a NIH3T3 cell suspension were seeded into 96-well microplates using the Agilent BioTek 406 FX washer dispenser. Media without cells was also dispensed using the second peristaltic pump dispenser such that all wells received 200 µL media. The cells were fixed and stained with Hoechst 33342. All wells of the plate were imaged in a 2 x 2 montage using the DAPI channel and a 10x objective. Image analysis detected cell nuclei and the extrapolated results plotted against the number of cells originally seeded.

The 406 FX washer dispenser is completely programmable regarding dispenser usage. If a truncated process is desired, only the necessary dispensers need to be used. Figure 4 depicts images of fixed NIH3T3 cells expressing GFP. These cells were fixed and counterstained with DAPI and Texas Red phalloidin only. This process used the washer manifold, one peristaltic pump, and two syringe pump dispensers.

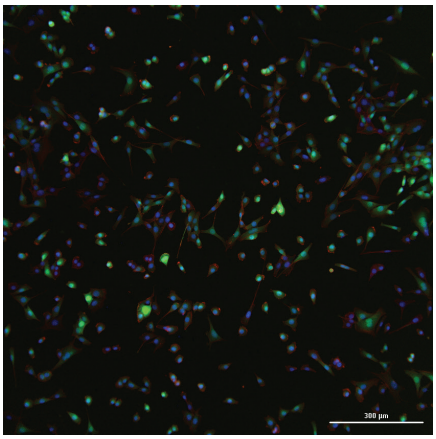


Figure 4. Three-color widefield 10x image after fixing and counterstaining with the Agilent BioTek 406 FX washer dispenser. NIH 3T3 cells expressing GFP were stained with Texas Red phalloidin (actin), and nuclear DNA was identified with DAPI. The image represents the overlay of separately captured red, blue, and green fluorescent signals using a 10x objective. Scale bar represents 300 µm.

The individual tubes of the peristaltic pump cassettes can be used to selectively add different reagents to the wells of a microplate. Figure 5 depicts images of HT-1080 cells counterstained with different combinations of Hoechst 33342 and fluorescent conjugates of phalloidin. HT-1080 cells were fixed and permeabilized using syringe pump dispensers to add reagents. Nuclear and cytoskeletal counterstains were then added using the peristaltic pump dispenser. In this experiment, different rows of the same microplate were stained with either Hoechst 33342 only or a combination of Hoechst 33342 and one of three different fluorescent phalloidin conjugates in parallel.

This process can also be used with antibody mixtures. It is often desirable to use antibody-negative controls to test the specificity of different antibodies. As shown in Figure 6, negative control wells for two different primary and two different secondary antibodies were generated by using different antibody mixtures in the separate tubes of both the primary and secondary peristaltic pump dispensers of the 406 FX. Wells that lack the primary rabbit anti-Tom20 antibody or the corresponding Alexa Fluor 555–labeled anti-rabbit IgG do not exhibit red fluorescence.

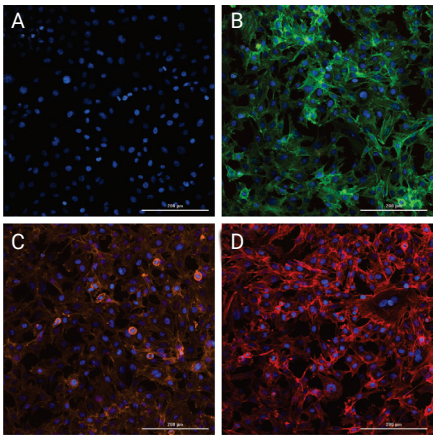
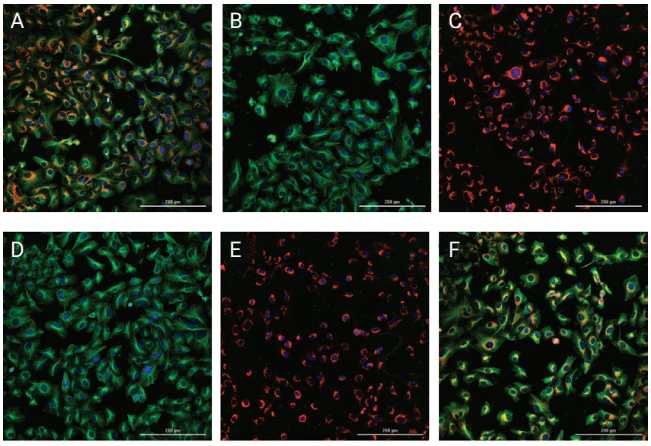


Figure 5. Different phalloidin counterstains. HT-1080 cells were fixed and counterstained with Hoechst 33342 and three different phalloidin conjugates on the same microplate using different peristaltic pump tubes to supply stain. Single-slice confocal images were captured in the DAPI, GFP, TRITC, and CY5 channels using a 20x objective. Shown are cells stained with: (A) Hoechst 33342 only; (B) Hoechst 33342 and AlexaFluor 488 phalloidin; (C) Hoechst 33342 and AlexaFluor 555 phalloidin; and (D) Hoechst 33342 and CF633 phalloidin.

Similarly, wells that lack either the primary mouse anti-tubulin primary antibody or the Alexa Fluor 488–labeled anti-mouse IgG antibody do not exhibit green fluorescence.

The 406 FX washer dispenser has several features that make it amenable for the steps of fixing and staining tissue culture cells before HCA. 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 is capable of rapidly washing either 96- or 384-well microplates without changing washer manifolds. In addition, the combination of multiple syringe pump and peristaltic pump dispensers allows for the addition of different reagents. Use of the peristaltic pump dispenser (Table 1) 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 peristaltic pump and purging the lines back into the reagent container. The multiple reagent paths of the peristaltic pump can be used to add different antibodies or stains to different wells on the same microplate, allowing for side by side comparisons without the need for subsequent reagent additions. The syringe pumps allow for different reagents (e.g., PFA fixative, permeabilization or blocking buffers, and counterstains) to be added using separate fluid paths without any manual intervention. In the process described, seven different reagents were added in sequence using seven different fluid paths.



	A	B	C	D	E	F
Rabbit anti-Tom20	+	—	+	+	+	+
Mouse anti-tubulin	+	+	—	+	+	+
Anti-rabbit IgG Alexa Fluor 555	+	+	+	—	+	+
Anti-mouse IgG Alexa Fluor 488	+	+	+	+	—	+
Hoechst 33342	+	+	+	+	+	+

Figure 6. Primary and secondary antibody controls. HT-1080 cells are fixed and stained. Individual reagent tubes from the primary and secondary peristaltic pump dispenser were used to dispense different antibody mixtures to separate rows of a microplate. As indicated by the matrix, Figures 6A and 6F had complete mixtures for both primary and secondary antibodies, while Figures 6B to 6E lacked one each of the antibodies. Widefield images in the DAPI, GFP, TRITC, and CY5 channels using a 20x objective.

The size of the 406 FX allows for the device to be placed in a sterile biosafety cabinet. Using fully autoclavable peristaltic pump fluid cassettes, solutions such as cell suspension of tissue culture media can be added under completely sterile conditions.

Automation of the staining process steps enables the ability to gently add and remove fluids from microplate wells without physically damaging cells in the wells. Physical trauma to cells can result from using a vigorous dispense rate. This is particularly true of the wash buffer being dispensed from 96 tubes simultaneously. Leaving a fluid residual serves to minimize the impact of incoming washer buffer. The residual used needs to be balanced with the dilution with any subsequent reagent added. In addition to the wash process previously described, most fix and stain procedures require the sequential addition of several different reagents.

The 406 FX can be programmed and run directly from the touch screen on the device. For complex workflows, the washer dispenser can be controlled using LHC software. Both methods allow for users to optimize assay performance through offsets of the wash and dispense manifolds, relative to the center of the microplate well. These offsets can be made in all three movement planes (X, Y, and Z).

Towards that end, physical offsets were used to move the dispense tubes for the washer manifold to the edge of the wells. Likewise, having the aspiration tubes in close contact with the cell layer for an extended period is harmful to the cell monolayer. A small yet strong vortex occurs near the aspiration tube that can damage fixed or live cells. The use of rapid aspiration dive rates minimizes the exposure of cells to this deleterious force.

Conclusion

HCA currently plays a significant role in the drug discovery process. Microscopic image-based analysis has historically required a significant amount of manual sample processing, which can be tedious and subject to process errors that can result in artifacts. The use of automation for these steps increases efficiency and walk-away time while reducing technician bias.

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