

# Sample preparation for high-throughput live cell imaging using Thermo Scientific Wellwash Versa

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## Abstract

Cell-based multiwell assays using high-content analysis and high-throughput screening instruments are becoming increasingly common in biomedical research. However, analytical methods usually need multiple wash steps and incubation of reagents. These steps can be extremely laborious when performed manually, thus it is preferable to automate these steps with a microplate washer. While using a microplate washer on fixed cells is usually not difficult, it can be quite problematic when working with live cells, especially for some loosely adherent cells. In this application note, we evaluated the performance of the Wellwash® Versa, a microplate washer specifically designed for working with cells, for washing rat cerebellar granule neurons, a standard primary culture model widely used for studying neuronal function that is relatively loosely adherent and delicate, possessing a substantial amount of fine neuronal processes (Björkblom *et al.*, 2005). Some tips for optimizing the use of the microplate washer will also be covered here.

## Introduction

Cell-based assays in 96-, 384-well or higher density plates are not only restricted to simple colorimetric/fluorometric endpoint microplate reader assays, but also include high-content analysis and live cell imaging assays. Reducing well size reduces the consumption of experimental reagents and materials, and the generation of waste without decreasing the sample size, thereby permitting substantial increases in the range of conditions evaluated. This is particularly important in both cell-based high-content analysis (HCA) and high-throughput screening (HTS), which usually involve a large number, sometimes up to millions, of screening conditions.

In HCA/HTS, cells are usually cultivated in microplates and the assay also takes place within the well. However, the culture medium, in which the cells are maintained, is often not optimal for imaging assays. It may be necessary to transfer cells to an imaging buffer with low background fluorescence and defined chemical composition, or cells are loaded with a fluorescent probe, which may need removal before image acquisition. Many assays perform best in a specific buffer, and may involve multiple wash steps with different buffers. The manual pipetting steps required to wash cells, define well volumes and for assay setup are laborious and inefficient.

In such situations, a microplate washer can improve efficiency. The use of microplate washers for *in vitro* assays, such as ELISA is routine. However, the application to cell-based assays requires that they be sufficiently gentle. For example, primary cultured neurons have a complex and fragile structure. They may easily be detached by sudden movements, or be stressed by vigorous fluid flow inside the well. The Wellwash Versa provides adjustable aspiration and dispensing speeds and positions. The dispense head is angled and the solution can be dispensed to the well side walls and flow slowly to the well bottom. This offers gentle but efficient washing and maintains the cells in a good condition for experiments. In this application note, we describe the development and optimization of protocols for Wellwash Versa to accomplish specific needs and add some technical tips for using the unit for cell washing.

## Materials and Methods

### Cell culture and wash/imaging buffer

Rat cerebellar granule cells were isolated from cerebella of P7 Wistar rats essentially as described (Courtney *et al.*, 1990) and maintained in minimal essential medium (MEM) containing 10% FBS, 33 mM glucose, 2 mM Glutamine, 20 mM KCl, Pen/Strep (5 U / 5 µg/ml) and 10 µM AraC (applied 24 h after plating) for 6–10 days before using for all tests in this application note. The wash/imaging buffer used in this application note is Locke's buffer, which is composed of 154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO<sub>3</sub>, 5.6 mM Glc, 5 mM HEPES and 1.3 mM CaCl<sub>2</sub> at pH 7.4 (Cao *et al.*, 2004).

### Imaging system

The BD Pathway 855 automated imaging system, which is equipped with an integrated environmentally controlled imaging chamber for maintaining defined temperature and CO<sub>2</sub> levels, was used for performing live cell imaging. Cells were plated in 96-well clear bottom plates (Greiner 655180) and images were captured by 4X or 20X long working distance objectives (Olympus).

### Basic cell wash

Brightfield images were acquired when the cells were in culture medium before any wash step. Plates were then washed by the Wellwash Versa using Locke's buffer and the cells were kept in Locke's buffer during the second acquisition for the images after the wash.

To monitor depolarization-induced intracellular calcium increases in neurons, cells expressing the genetically encoded calcium reporter YC3.60 (Nagai et al., 2004, Cao et al., 2005) were washed with calcium-containing Locke's buffer either manually or using the Wellwash Versa, and kept in a 100  $\mu$ l volume before performing live cell imaging. Images through YFP and CFP emission filters (542nm/27 and 483nm/32 bandpass filters, AHF) under CFP excitation (438nm/20 filter) were captured over a period of 1 min to obtain a baseline. Then the cells were depolarized by addition of 30 mM KCl, using the dispensing device of the automated imaging system, and imaged for a further 5 min. The increase in the YFP/CFP emission ratio during the experiment indicated the increase in intracellular calcium level.

### Sipping protocol

To quantify liquid volumes accurately and maintain surface-tension properties, 0.002% bromophenol blue was added into the medium for cerebellar granule cells and distributed to the wells of a 96-well plate. A single aspiration step with different aspiration heights were applied to different columns as indicated. The standard curve was prepared by manual pipetting. The absorbance of the wells was measured at 595 nm and the volume of the medium after aspiration was calculated according to the standard curve.

### Results and Discussion

The flexibility to optimize parameters is necessary for every microplate washer, especially when working with microplates containing live cells. The Wellwash Versa provides sufficient parameters for optimizing performance for different cell types and plate types, including the positioning of the aspiration and dispensing head, aspiration and dispensing speed and height. The most important criterion is to be gentle enough to the cells without compromising the washing efficiency. Primary neurons can be quite loosely attached on tissue culture plates. The neurites of differentiated neurons form a mesh of processes interconnecting individual soma. This can catch liquid flow and result in the entire cell sheet peeling off during wash steps. First, we aimed to find a basic wash protocol that can wash the well adequately but with minimal disturbance to the loosely attached cells.

A two-cycle procedure, using settings shown in Table 1, included an aspiration step down to 2–3 mm from the bottom, followed by an aspiration-dispensing step, i.e., simultaneous aspiration during the dispensing of 500  $\mu$ l wash buffer per well at a higher position. This was shown to be adequate to rinse the well, as evaluated by comparing the absorbance of a well containing dye-labeled buffer to a dye-free well (blank) before and after washing with different parameters (Table 2). Primary cultured neurons and their neurite network were therefore washed thoroughly without showing physical signs of disturbance (Figure 1).

**Table 1. Parameters for the optimized wash step:**

Buffer source	A
Wash volume ( $\mu$ l)	500
Wash cycles	1 / 2
Soak time (mm:ss)	00:00
Shake speed	Off
Wash mode	Plate
Strip over mode	No
Aspirate height	5 / 6 mm (2 / 3 mm from bottom)
Wash head speed	10
Aspirate speed	Medium
Aspirate time (s)	1
Dispense height start	7
Dispense height end	9
Dispense offset	1.2
Dispense tip touch	1.3
Final aspirate	No

	A595	Standard Deviation
No dye	0.041	0.003
Unwashed	0.570	0.028
2 mm from bottom, 1 cycle	0.055	0.004
2 mm from bottom, 2 cycles	0.041	0.003
3 mm from bottom, 1 cycle	0.070	0.009
3 mm from bottom, 2 cycles	0.042	0.003

Table 2. Absorbance at 595 nm of a 96-well plate with 0.002% bromophenol blue after performing wash steps with different aspiration height and cycles. 150  $\mu$ l of dye was distributed into all wells except those designated "no dye" (blank control), and the wells were washed by washing steps as indicated below. A final aspiration step, defining a remaining volume of 100  $\mu$ l, was applied to the whole plate to normalize the pathlength of all the wells for subsequent quantitation of washing efficiency. The numbers show the mean absorbance of 16 wells.

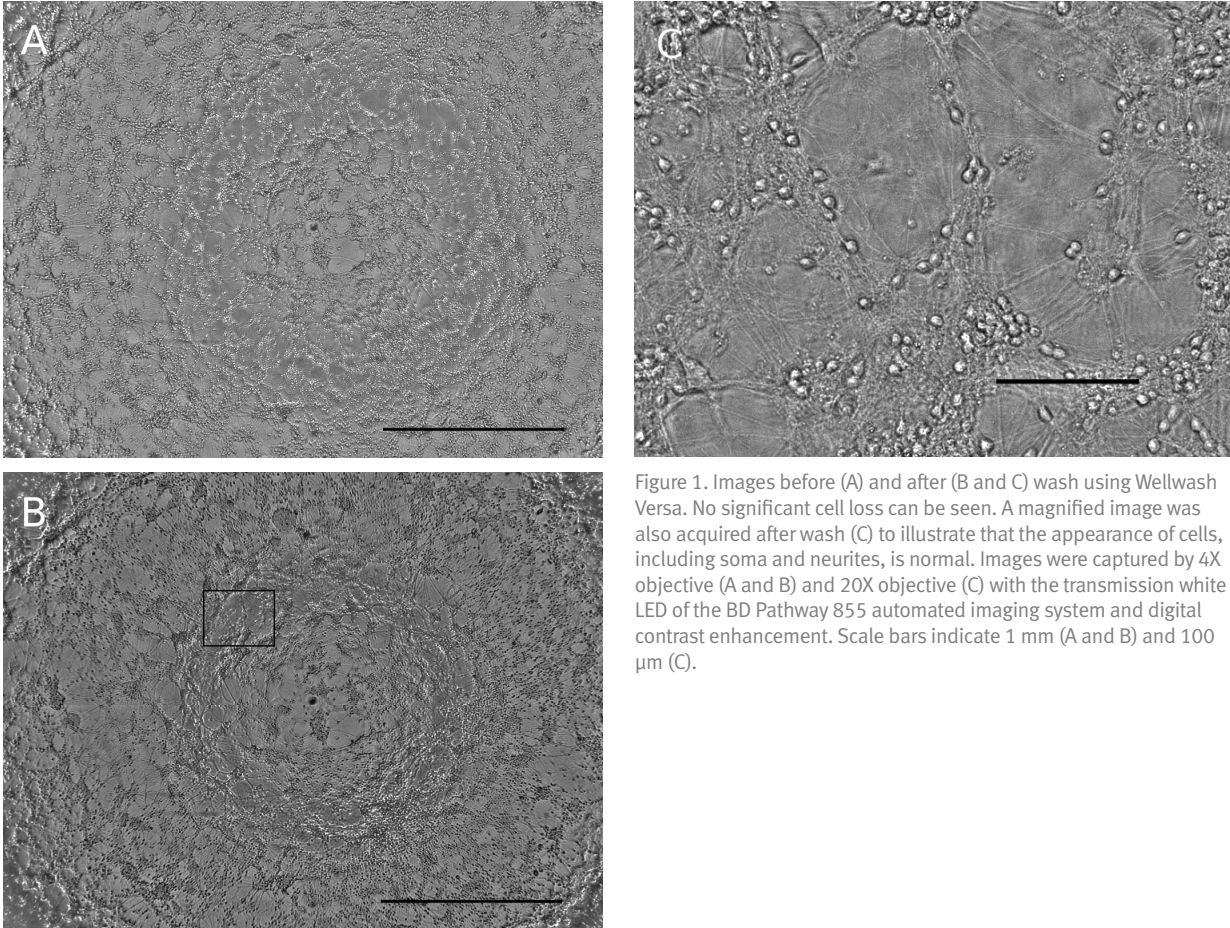


Figure 1. Images before (A) and after (B and C) wash using Wellwash Versa. No significant cell loss can be seen. A magnified image was also acquired after wash (C) to illustrate that the appearance of cells, including soma and neurites, is normal. Images were captured by 4X objective (A and B) and 20X objective (C) with the transmission white LED of the BD Pathway 855 automated imaging system and digital contrast enhancement. Scale bars indicate 1 mm (A and B) and 100  $\mu\text{m}$  (C).

One concern of using a microplate washer is that the processes of aspiration and mechanical dispensing may interfere with the functional behavior of cells, ultimately resulting in incorrect conclusions from misleading experimental results. It is well known that when cerebellar granule neurons are depolarized by extracellular potassium, extracellular calcium will enter through calcium channels and result in a sudden increase in intracellular calcium (Courtney et al., 1990). In order to demonstrate that cerebellar granule neurons give the same response after the washing steps by Wellwash Versa as after manual washing, the optimized washing process was used to prepare cells for measurement of intracellular calcium upon depolarization. Intracellular calcium levels were detected by a fluorescent protein-based FRET probe. The response of cerebellar granule cells upon depolarization is similar between those washed manually with a multichannel pipette or by Wellwash Versa (Figure 2).

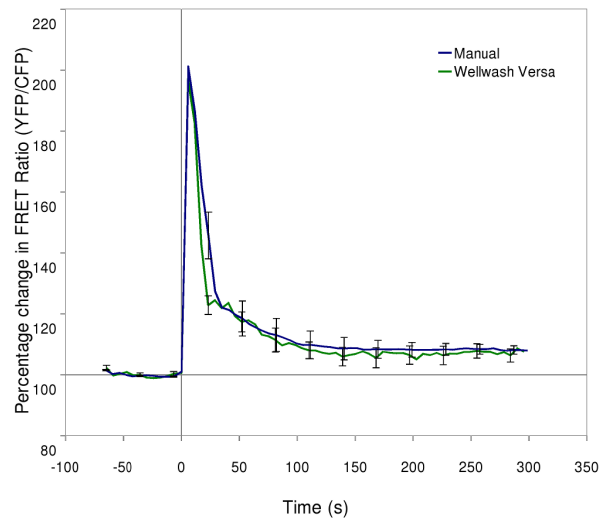


Figure 2. Intracellular calcium levels of cerebellar granule cells before and after depolarization. Blue: Cell washed by manual pipetting. Green: Cell washed by Wellwash Versa (error bars show the S.E.M. of average values from 6 wells).

These results indicate Wellwash Versa can replace manual pipetting even when working on loosely attached primary cultured neurons. This makes high-throughput work much easier and less laborious. It also saves considerable time, especially for some cell-based assays which involve multiple changes of assay reagents and buffers before data acquisition.

### Sipping protocol

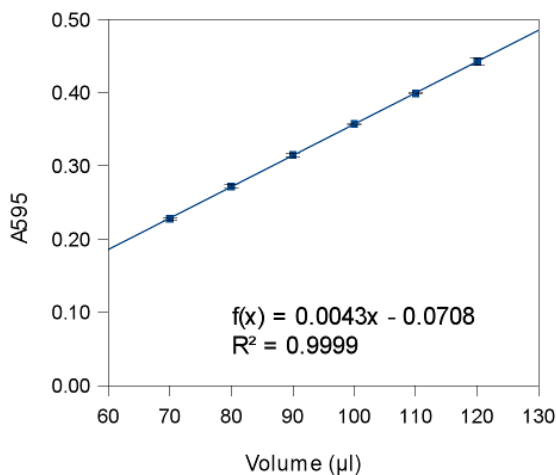
Cells are often cultured for a relatively long time, for example, for dividing cells to reach a particular level of confluence or differentiating cells to reach a defined level of maturity, compared with the duration of the cell-based assay. Therefore, it is necessary to culture cells in a larger volume than is necessary for the actual assay. However, during the treatment of cells with screening targets, or when cells are stained by synthetic fluorescent probes for image acquisition, a decrease in the medium volume is usually preferred for minimising reagent usage and conserving valuable large-scale libraries. It is laborious and time consuming to remove or set by manual pipetting a defined amount of medium in multiwell plates before assay.

For assays or assay steps that can be performed directly in cell culture medium, we made use of the aspiration function of the microplate washer and developed a protocol which can sip medium from the wells to leave the desired volume in each well. Figure 3, right panel, shows the volume remaining in the well versus the aspiration height. Manual pipetting is used for the standard and the volume is estimated by absorbance of each well with medium supplemented with 0.002% bromophenol blue as explained above. For example, according to the aspiration height to remaining volume conversion chart, when the aspiration step uses an aspiration height set at 6.1 mm, the volume in the wells is 99.6  $\mu\text{l}$  ( $\pm 3 \mu\text{l}$ ). Aspiration from center and the aspiration head goes down without stop-over time ("Aspirating from the centre of the well and the moving the aspiration head down without stop-over time" ("Aspiration time = 0")) give the highest reproducibility (data not shown). Note that adjustments have to be made for different plate types and different vendors because the dimensions of the plates may vary. The conversion table shown was obtained for Greiner 655180, and similar results were obtained with Greiner 655090.

Volume ( $\mu\text{l}$ )	A595	Standard Deviation
70	0.228	0.002
80	0.272	0.003
90	0.315	0.003
100	0.358	0.001
110	0.399	0.001
120	0.443	0.005

Aspiration height (mm)	Calculated Vol ( $\mu\text{l}$ )	StDev ( $\mu\text{l}$ )
5.4	71.78	3.63
5.6	78.67	3.36
5.7	82.51	2.64
5.8	86.66	2.37
6.0	95.34	2.58
6.2	104.43	3.07

Standard Curve



Volume in wells vs Aspiration height

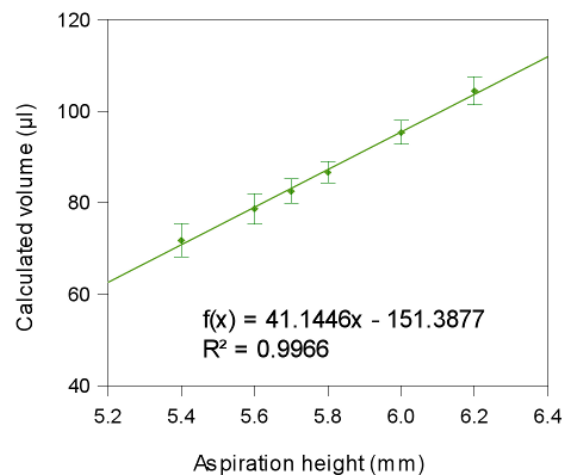


Figure 3. Tables and charts demonstrating the development of the sipping protocol for a defined medium and plate type. Left: Standard curve. Right: The relationship between aspiration height and remaining volume is shown.



## Conclusion

The Wellwash Versa is easy to use and to set up protocols for washing plates seeded with cells. It can suit different cell types as numerous parameters defining the behavior of the washer heads can be adjusted to optimize the required task. It can be integrated into an automated imaging pipeline via serial port commands as well as used as a standalone device without computer connection. The user interface is clear and easy to follow. In practice, the Wellwash Versa is gentle enough for loosely attached cells without compromising washing efficiency.

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