# Effective Cell Identification and Segmentation in Fluorescence Microscopy with New Fluorescent Whole Cell Stains

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## Introduction

High-content screening (HCS) and other quantitative cellular imaging assay methods that use fluorescence microscopy require effective fluorescent labeling and identification of the target cell(s) [(1)]. Fluorescent probes that stain cells as the primary objects are used to identify and count individual cells, as well as to define the region(s) of each cell to which target-specific image analysis is applied (1). For this purpose, the primary object can be a major component of the cell, such as the nucleus or a large organelle, or the whole cell itself. When the whole cell is the primary object, a high-quality cell stain is needed to delineate the intact cell from bordering cells without also interfering in target-specific detection and analysis. Thermo Scientific Cellomics® Whole Cell Stains (Thermo Fisher Scientific Inc., Rockford, Ill.), available in blue, green, orange and red, provides image staining and the ability to quantify the whole cell volume in HCS and fluorescence microscopy assays.

Cellomics Whole Cell Stains (WCS) were designed for ease-of-use: the WCS is added to fixed and permeabilized cells, incubated for a minimum of ten minutes and then rinsed. Cell staining is stable and bright for several weeks. Here we demonstrate the use and analysis of WCSs with several cell types in practical HCS applications. The images of fluorophore-labeled cellular targets in cells labeled with these WCSs were acquired with the Thermo Scientific Cellomics ArrayScan® HCS Reader and quantitatively analyzed using Cellomics automated image processing software modules, such as the Morphology Explorer BioApplication (1). We show that confluent touching cells can be segmented to identify individual cells by using a watershed algorithm in this BioApplication or by other segmentation routines in other image analysis software when the cells are properly labeled with a fluorescent volume marker such as the WCSs. Although WCSs are designed for high-content screening cell-based assays, these WCSs also work well with conventional fluorescence microscopic techniques.

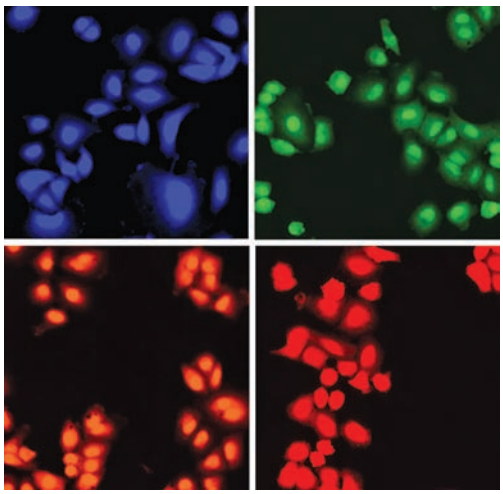

**Figure 1.** *A549 cells stained with the different WCSs: 1) WCS Blue, 2) WCS Green, 3) WCS Orange and 4) WCS Red*

## Materials and Methods

HMVEC, A549, NIH 3T3, MDCK or HT1080 cells were cultured, fixed and then permeabilized with 0.1% Triton X-100. Cells were stained directly with a WCS or blocked with 0.3% BSA in PBS, probed with primary and fluor-labeled secondary antibodies for 60 and 30 min respectively, and then stained with the WCS for the indicated times. Images were acquired automatically and analyzed using the Thermo Scientific Cellomics ArrayScan V$^{TI}$ HCS Reader. Automated image analysis and cell-to-cell separation were performed using the watershed algorithm in the Thermo Scientific Cellomics Morphology Explorer BioApplication.

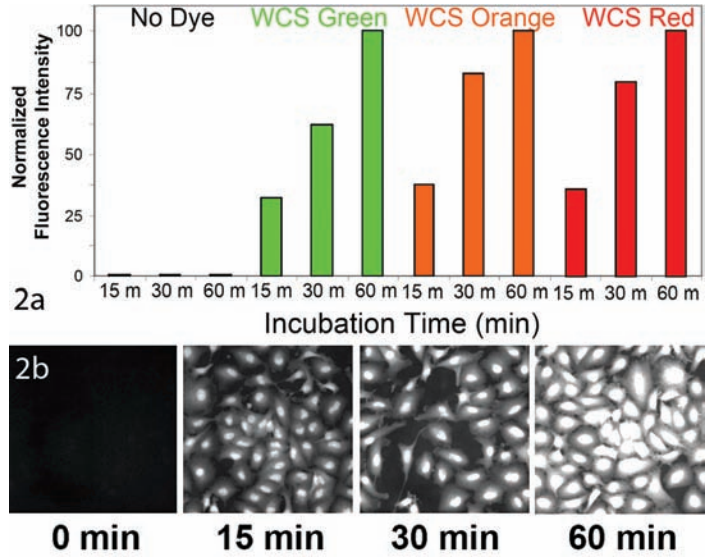


**Figure 2.** *A549 cells plated on 96-well microplates and stained with WCS Green, Orange and Red for 15 min, 30 min and 60 min. Normalized fluorescence intensity in the nuclear region increases with longer staining time (a and b).*

## Results and Discussion

We developed and evaluated new fluorescent whole cell stains that enable accurate cell identification and segmentation. Four stains have been developed with their fluorescence emissions in blue, green, orange and red wavelengths (λex/λem = 350/450, 493/518, 550/568, 654/673 nm, respectively). Cells labeled with these stains can be imaged using standard fluorescence filters and optics (Figure 1). These WCSs are effective with most cell types and are easy to optimize for proper fluorescence labeling of the cell because of their time-dependent

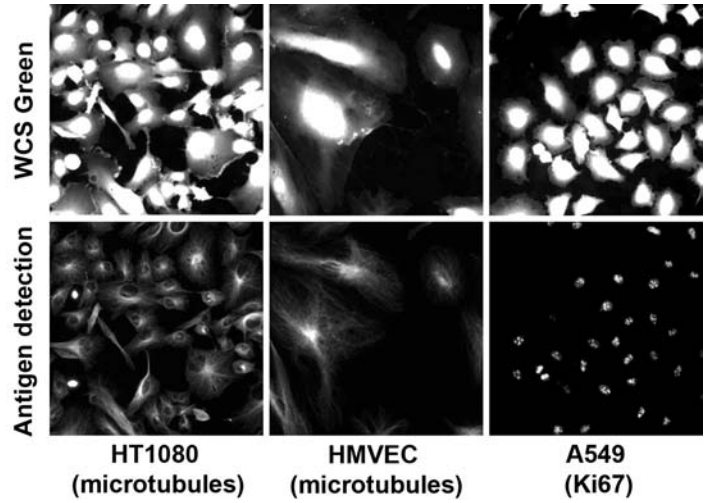


**Figure 3.** *HT1080, HMVEC and A549 cells stained with WCSs and different antibodies. WCS Green does not affect multiplexed cytoplasmic microtubule detection or nuclear Ki67 detection. Other antigens tested: BrdU, cyclins, F-actin and α-tubulin. Antibodies were from the Thermo Scientific Cellomics HCS Reagent Kits for Cyclin B1 (Cat. 8404402), Cytoskeleton Rearrangement (Cat. 8402402) and BrdU Ki67 Cell Proliferation (Cat. 8401102). Tests with WCS Blue, Orange and Red gave similar results.*

https://doi.org/10.1017/S1551929500054262 Published online by Cambridge University Press

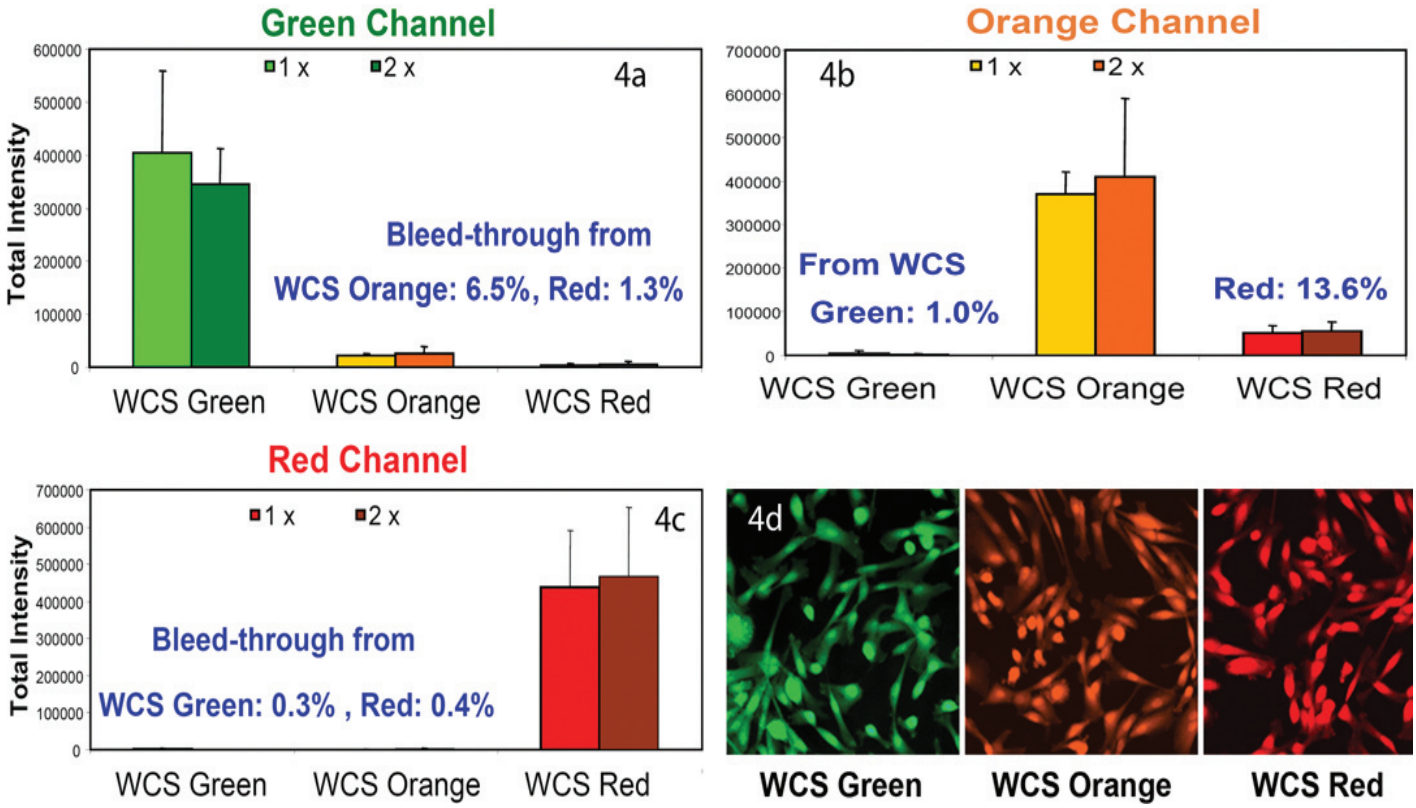


**Figure 4.** *MDCK cells stained with the WCS Green (a), Orange (b) and Red (c) and measured for bleed-through. Staining was for 30 min using normal (1x) and elevated (2x) stain concentrations. (d) images corresponding to data in (a)-(c).*

fluorescence labeling properties (Figure 2). The stains are bright and versatile to label a wide range of cell types with excellent fluorescence-stability (weeks). Because these stains have minimal bleed-through and do not hamper target-specific detection by antibodies or other standard fluorescent probes, they can be used in multiplexed target detection (Figures 3 and 4). WCSs are compatible with different coating materials, and common surfaces (polystyrene, poly-L-lysine or collagen coating) in cell culture plates (Figure 5).

Cells labeled with WCSs can be accurately identified and separated from contacting neighboring cells by using standard object segmentation image processing algorithms. In Figure 6, confluent U2OS cells labeled with WCS Green are effectively segmented using the watershed algorithm in the Cellomics Morphology Explorer BioApplication. The identified cell boundaries are shown in red.

To determine quantitatively the performance of WCSs in cell separation and cell identification of adjacent and touching cells, a metric called the Cell Separation Index was formulated. The Cell Separation Index is defined the number of individual nuclei detected in an image divided by the number of individual cells identified with the watershed algorithm in the Morphology Explorer BioApplication. The ideal is a Cell Separation Index value of 1.0, indicating that all the non-dividing cells are separated correctly. We calculated the Cell Separation Index for cells that were labeled with the different WCSs and nuclear stains (*i.e.*, DAPI stain for cells labeled with WCS Green, Orange, and Red, and 7-AAD stain for cells labeled with WCS Blue). All four WCSs enabled excellent cell separation, providing for Cell Separation Indices near 1.0 (Figure 7).

The advantages of WCSs over conventional cell staining are: (1) the ability to label entire cell volume with four different fluorescent color options (Blue, Green, Orange and Red), (2) a quick and easy staining procedure, (3) bright and stable fluorescence with low background (4) low cost, (5) minimal bleed-through with good cell separation and (6) compatibility with HCS automation. In summary, WCSs are an effective solution for easy whole cell labeling and identification in multiplexed quantitative fluorescence microscopy assays. ■

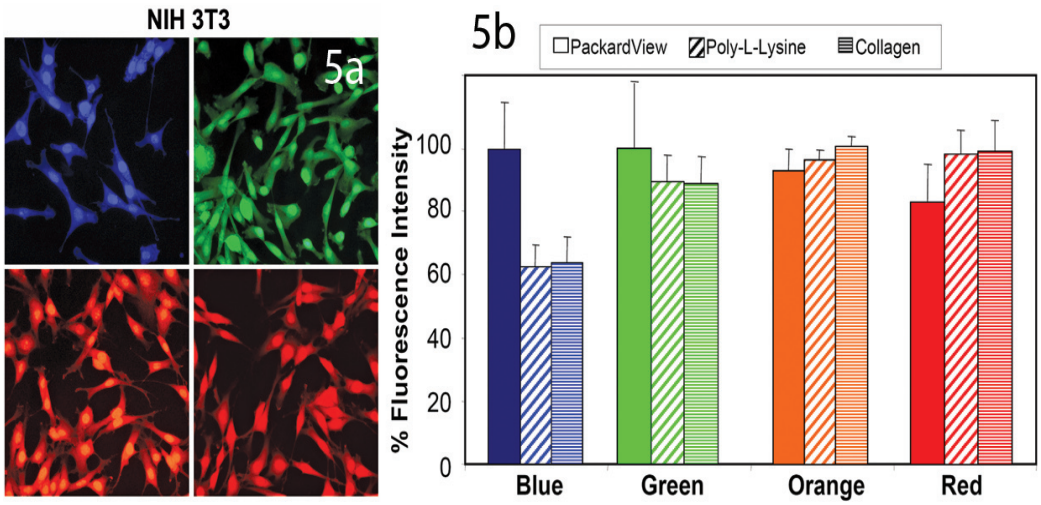


**Figure 5.** *WCSs tested for compatibility with coating materials on cell culture plates. (a) NIH 3T3 cells on PackardView®, BD BioCoat™ Poly-L-Lysine, and BD BioCoat Collagen plates stained with the WCS Blue, Green, Orange and Red for 15 min. (b) Cell images of NIH 3T3 cells on a PackardView Uncoated Plate stained with WCS Blue, Green, Orange and Red, respectively.*

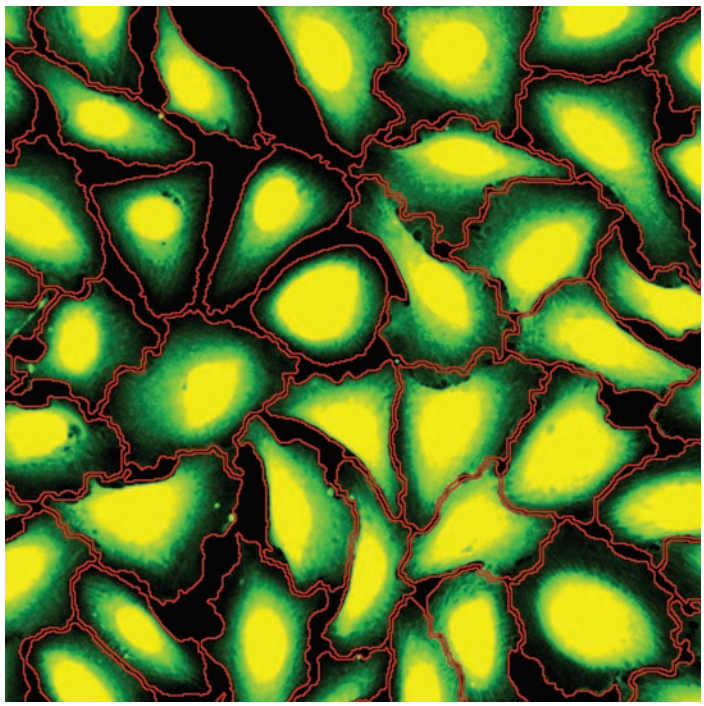

**Figure 6.** *Cell segmentation of confluent U2OS cells after labeling the cell volume with WCS Green. The cell boundaries identified by the watershed algorithm in the Thermo Scientific Cellomics Morphology Explorer BioApplication are shown as red traces.*

## References

1. R.N. Ghosh, O. Lapets, J.R. Haskins. (2007). Characteristics and value of directed algorithms in high content screening. In "High Content Screening: A Powerful Approach to Systems Cell Biology and Drug Discovery", Humana Press, eds. D.L. Taylor, K. Giuliano, J.R. Haskins. *Methods in Molecular Biology*. **356**:63-81.

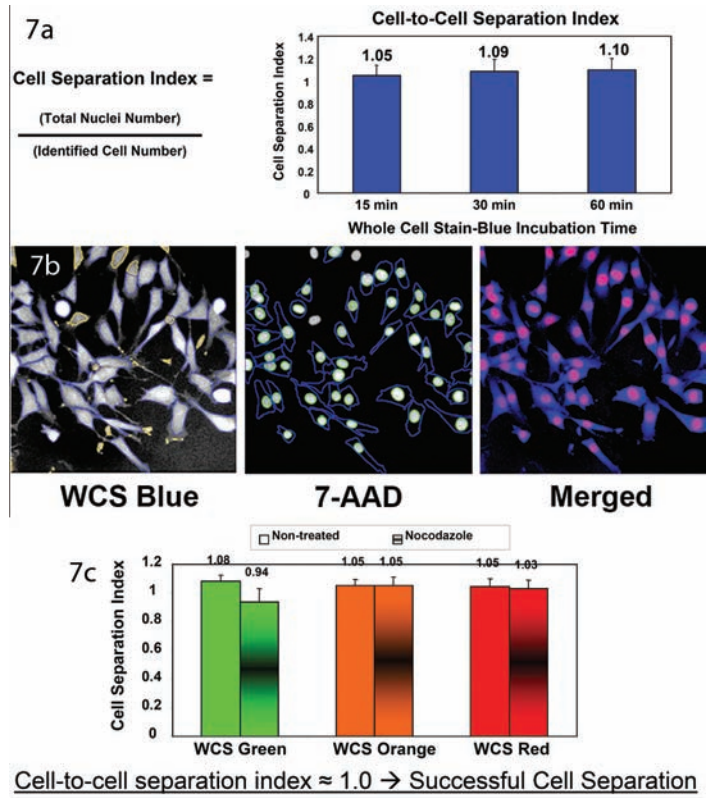


**Figure 7.** *Cell Separation Index values for sub-confluent NIH 3T3 cells stained with WCSs and 7-AAD (nuclear stain with WCS Blue). (a) Cells stained with WCS Blue for 15-60 min. (b) Individual cells in the image were identified using the Thermo Scientific Cellomics Morphology Explorer BioApplication. (c) Non-treated and Nocodazole-treated cells stained with WCS Green, Orange or Red. The values were close to 1.0, indicative of good cell separation.*