

Image-Based Quantification of Cyclin B1 and DNA content during Cell Cycle using the Opera™ system

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1 Introduction

One of the most important tasks in anti-cancer treatment is the inhibition of cell proliferation by interruption of the cell cycle. As the cell cycle is subdivided into the four phases G1-, S-, G2- and M-phase, there are diverse targets for arresting cells either during DNA replication in S-phase or during division in mitosis. Several cytoplasmic proteins like the cyclins have been identified to be proprietary in cell cycle control whereas Cyclin B1 in particular is essential for initiation of mitosis. With transition from G2 to M- phase, it is translocated from the cytoplasm to the nucleus where it coordinates the division of the chromosomes by promoting spindle formation and suppression of cytokinesis. Treatment with anti-mitotic drugs that arrest a cell in G2/M-phase will therefore increase the amount of Cyclin B1 which can be monitored in a sub-cellular resolution. We used the Opera™ system for confocal image capture in combination with the high content image analysis software Acapella™ to precisely quantify intracellular Cyclin B1 and DNA in order to evaluate the effects of the cell cycle inhibiting drugs Aphidicolin, Demecolcin and Nocodazole.

The antibiotic *Aphidicolin* specifically inhibits eukaryotic DNA-Polymerase and therefore arrests cells in S-Phase. The alkaloid *Demecolcin* as well as the anticancer drug *Nocodazole* has been used as antimitotic agents, arresting cells in M-Phase.

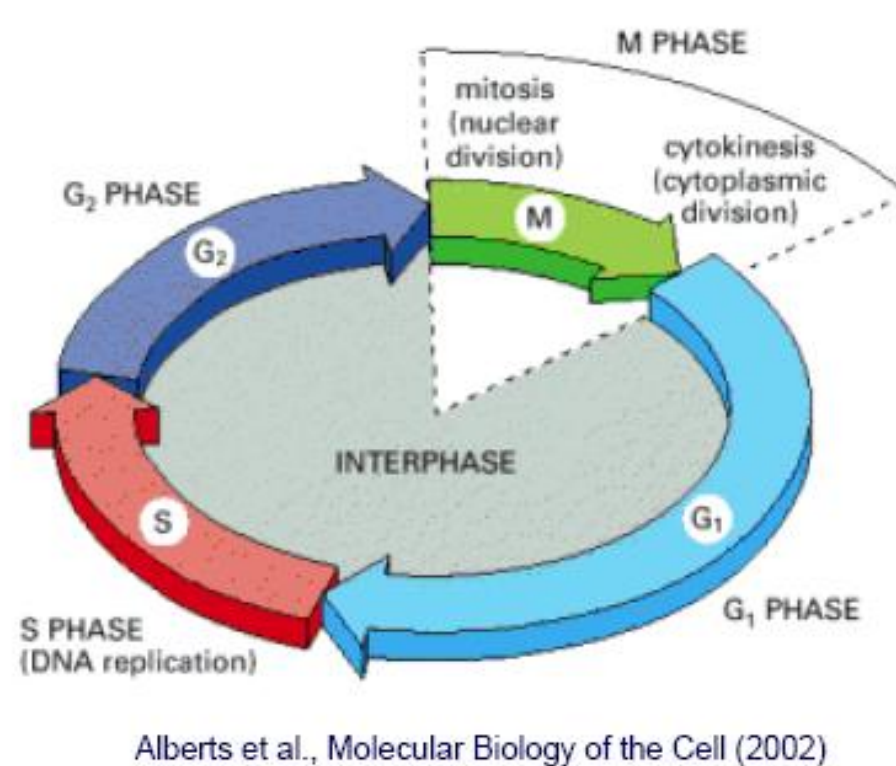


Figure 1: Schematic drawing of the cell cycle

2 Material & Methods

HeLa cells were grown in CellCarrier-384 tissue culture plates using RPMI-1640 medium with Glutamax supplemented with 10 % fetal bovine serum at 37°C, 5 % CO₂/ humidified air. After 18 h and at semi-confluence, medium was replaced by dilution series of Aphidicolin, Demecolcin or Nocodazole prepared in culture medium followed by incubation for 6 h at 37°C, 5 % CO₂ humidified air. Cells were formaldehyde fixed and permeabilised prior to immunostaining which was done at room temperature with 5 µg/ml of α-Cyclin B1 primary antibody and 10 µg/ml of AlexaFluor®488 goat α-rabbit IgG for 1 h each. Nuclei were stained at room temperature with DRAQ5™ at a final concentration of 5 µM.

3 Image Acquisition on the Opera™ platform

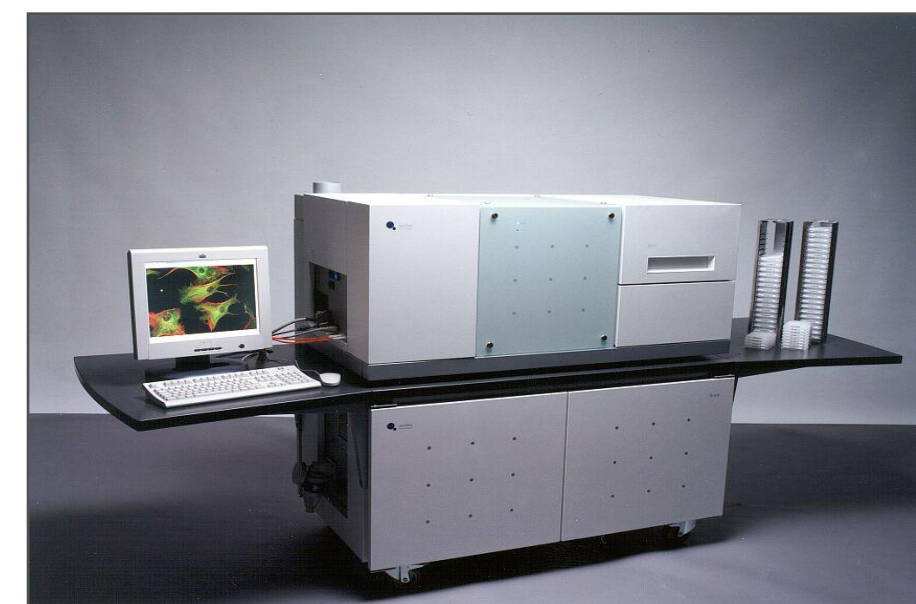


Figure 2: Opera™ screening platform

For high content analysis of Cyclin B1 and DNA, confocal images were acquired on an Opera™ QEHS using the 20x water immersion lens. AlexaFluor®488 was excited by the 488 nm laser and detected through a 540/75 bandpass filter. DRAQ5™ was excited by the 640 nm laser, and fluorescence was collected through 690/50 bandpass filter.

Ideally suited for high content analysis is the screening platform *Opera™ QEHS* which is equipped with up to five excitation sources (405, 488, 561, 640 nm laser lines and UV lamp) and four parallel recording detection channels rendering combination of high speed with high resolution. The additionally available *Columbus™ Gallery* is a database system that allows complex HCS multi-channel images to be stored and accessed by multiple users, providing a convenient and easy-to-use solution to high volume image management.

4 Typical images of Cyclin B1/ DNA staining

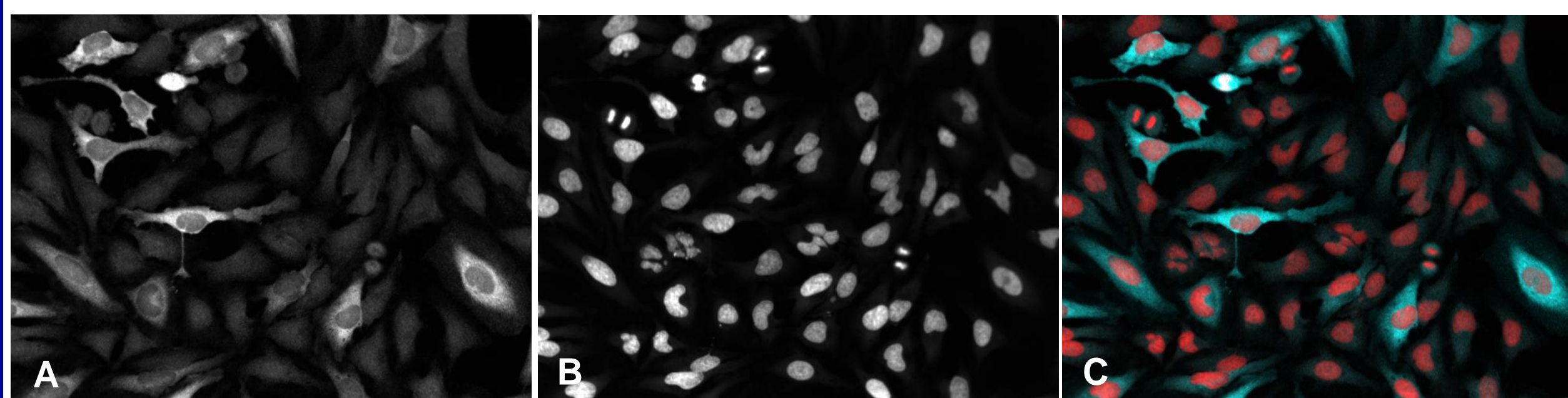


Figure 3: Confocal images of Cyclin B1 and DNA captured on Opera™ at 20x magnification. HeLa cells were formaldehyde fixed and immunostained against Cyclin B1. DNA was stained with DRAQ5™. A: Cyclin B1; B: Nuclei; C: Pseudo-coloured overlay of Cyclin B1 (turquoise) and DNA (red).

5 Acapella™ Image Analysis

The Acapella™ image analysis platform allows precise quantification of the location, shape, structure and amount of fluorescence on an individual cell level. A set of ready-made analysis algorithms referred to as scripts are available for a broad range of standard situations like counting, translocation, spot analysis, etc. Due to its high speed Acapella™ is ideally suited for on-the-fly analysis and analysis of large data sets.

For image analysis of the Cyclin B1/ DNA stained cells we used the Acapella™ *CellRegionAnalysis* script. Nucleus and cytoplasm were identified in the DRAQ5™ image as a basis for determination of the amount of Cyclin B1 in these both regions using the AlexaFluor®488 image. Via definition of certain thresholds, the number of Cyclin B1 positive cells was calculated.

We combined the quantification of Cyclin B1 with the determination of the DNA content using the *DNA Distribution Analysis* (DCD) script. This analysis is done on the DRAQ5™ image for displaying a DNA distribution in a histogram that shows a cell population's classification into G1-, S- and G2/M-phase via their DNA content.

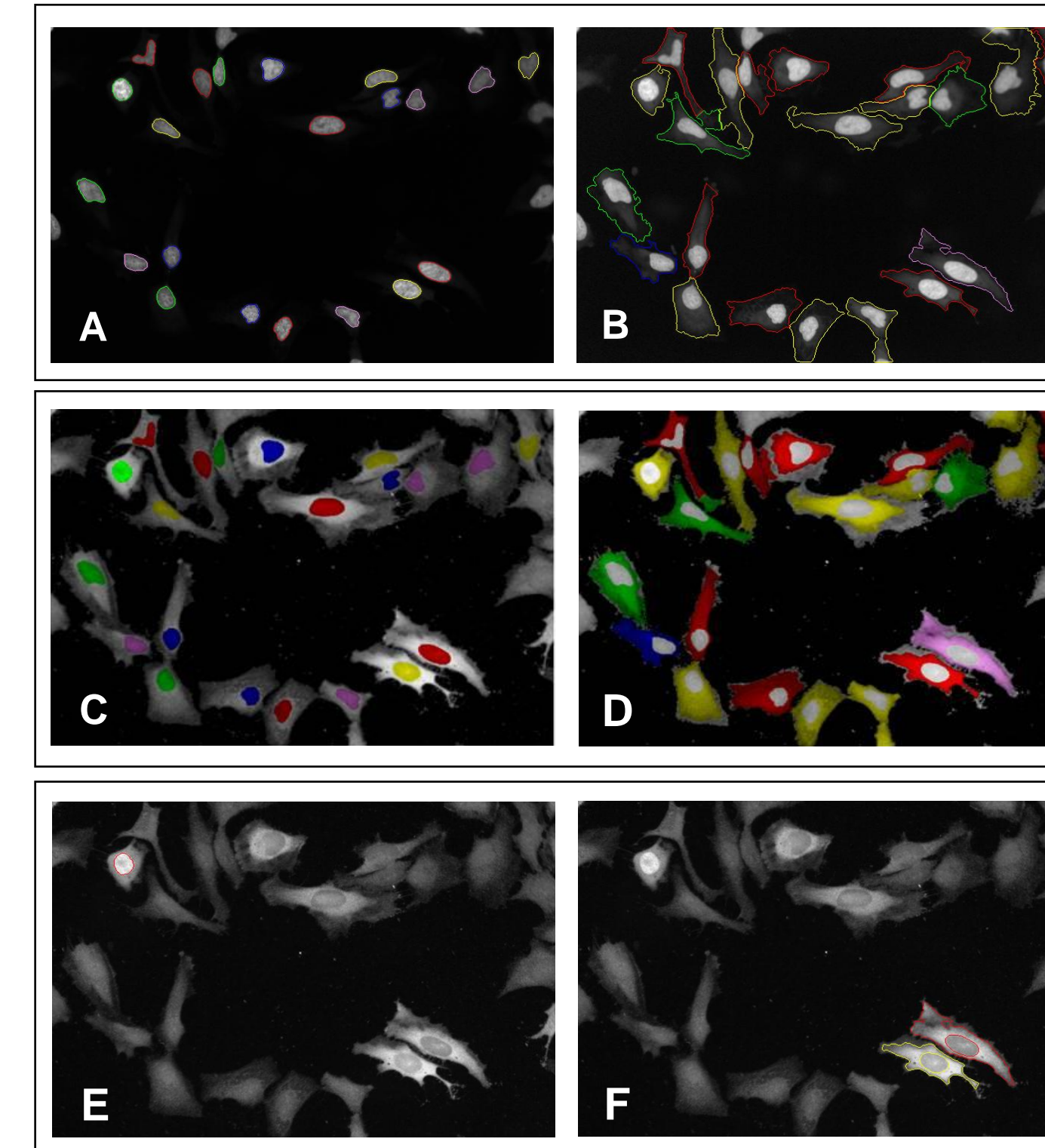


Figure 4: Image analysis strategy for quantification of Cyclin B1 using the Acapella™ *CellRegionAnalysis* script. Left: Nuclei detection (panel A) and cytoplasm detection (panel B) are used for subsequent definition of regions for detection of Cyclin B1. Middle: Detection of Cyclin B1 in the nuclear (C) and the cytoplasmic (D) cell region. Right: Based on distinct intensity thresholds for Cyclin B1 identified in each region, cells are marked as positive in either the nucleus (panel E) or the cytoplasm (panel F) region.

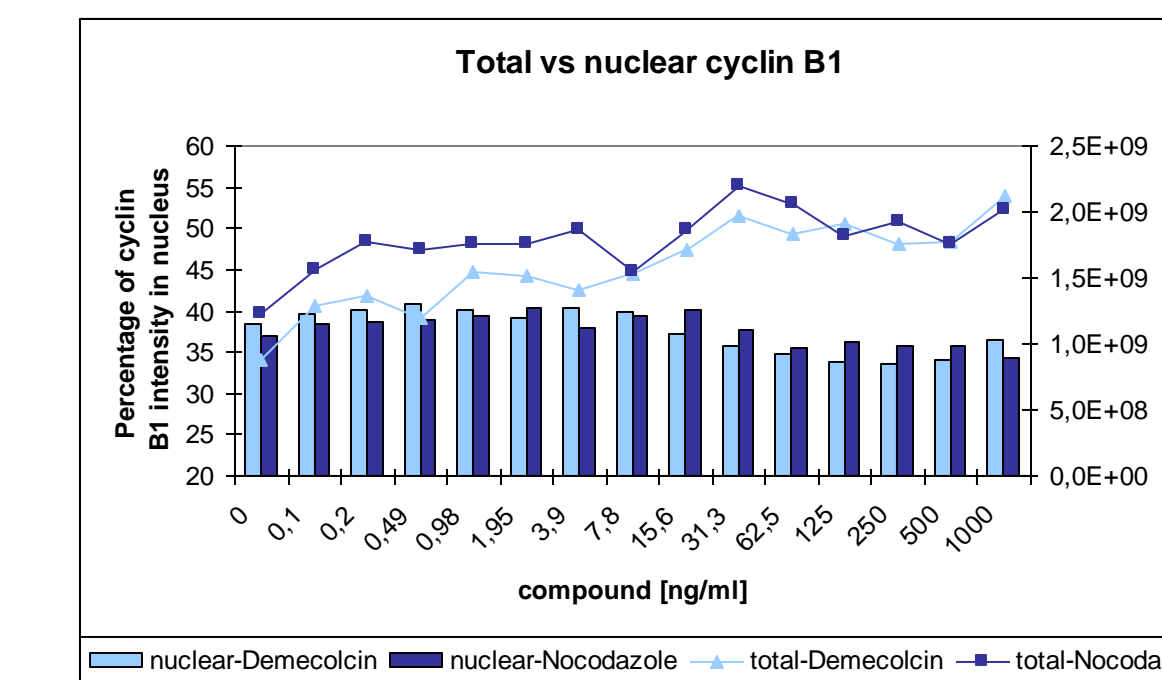


Figure 5: Image analysis results for Cyclin B1 quantity. The G2/M-cell population is considered to include all cells with an increased Cyclin B1 intensity in either the nucleus and/or the cytoplasm. All other cells are classified as G1/S cells. With increasing concentrations of anti-mitotic compound, the amount of total Cyclin B1 respectively the number of cells in G2/M-phase increases and is subsequently used for calculation of an EC₅₀ value.

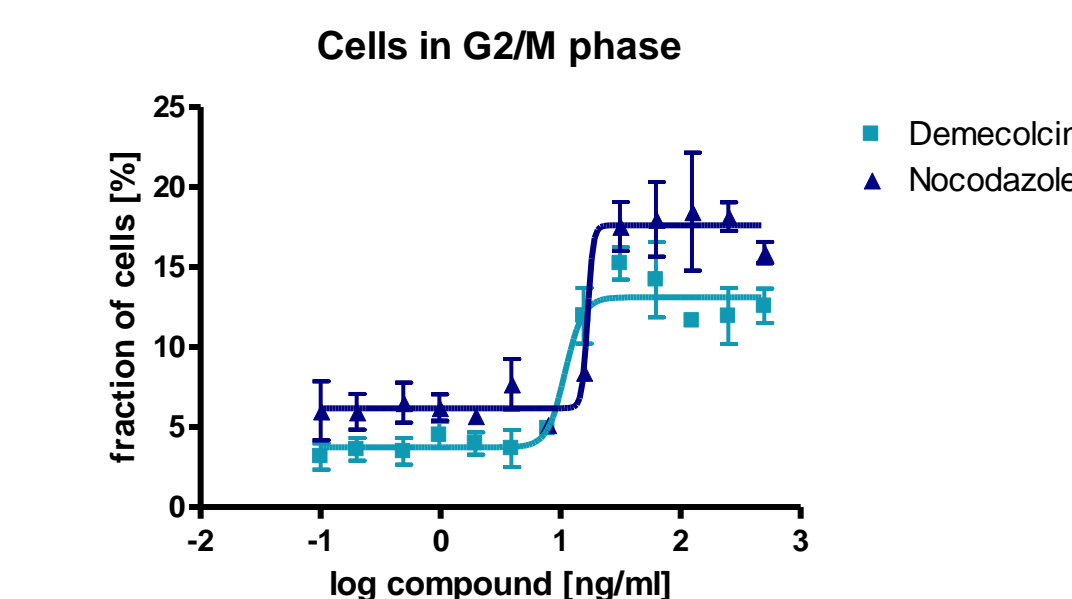


Figure 6: Numerical results for G2/M-phase based on Cyclin B1 quantity. Responses of cell count in G2/M-phase are shown as a function of Demecolcin and Nocodazole concentrations. EC₅₀ values calculated from the corresponding dose-response curves were at 16.8 ng/ml for Nocodazole and at 10.95 ng/ml for Demecolcin (Graph Pad Prism®).

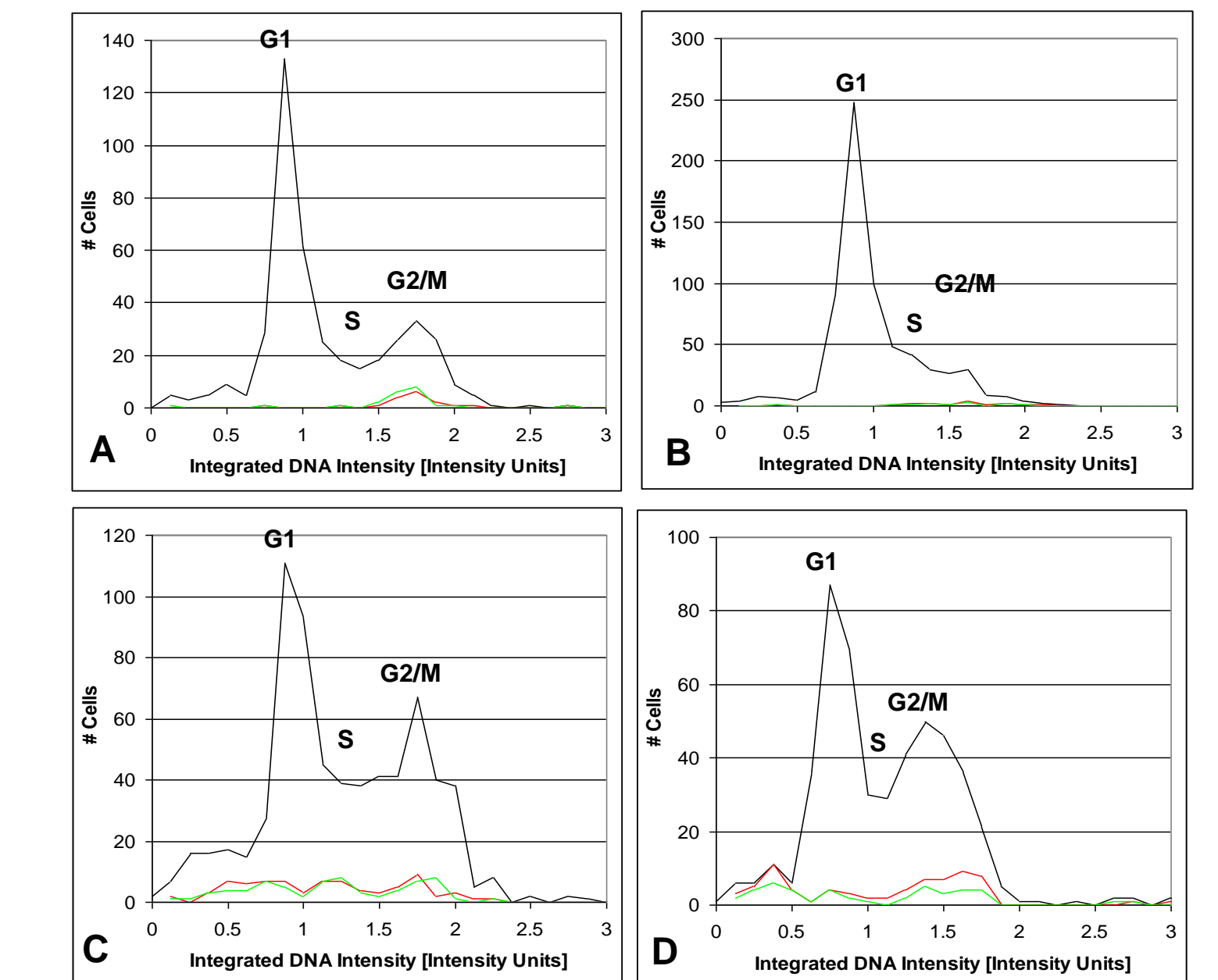


Figure 7: DCD subpopulation histograms derived from the Acapella DCD script. Subpopulations of cells in G1-, S- and G2/M-phase are differentiated by the integrated intensity of the DRAQ5™ stained DNA fluorescence and represented by the black line. The red line stands for number of cells positive for nuclear Cyclin B1, the green line for cell count of cytoplasmic Cyclin B1. A: Normal distribution of cells without treatment B: Distribution after treatment with 500 ng/ml Aphidicolin C: Distribution after treatment with 500 ng/ml Demecolcin D: Distribution of cells after treatment with 500 ng/ml Nocodazole Histograms of DCD analysis confirms that Aphidicolin arrests cells in S-phase as the respective cell count compared to the nontreated sample increases. However, the antimitotic compounds Demecolcin and Nocodazole clearly amplify the G2/M-population. Cyclin B1 distribution reconfirms DCD analysis as the number of positive cells has increased with compound addition.

6 Conclusions

This easy-to-use HCA approach for cell-based analysis by automated microscopy uses the outstanding Opera™ platform for simultaneous quantification of Cyclin B1 expression and DNA content. It is an ideal application for monitoring the effects of cytostatic drugs on cell cycle progression and can ideally be implemented as part of the drug development process during secondary screening. As the assay only uses two fluorescence emission channels and the *CellRegionAnalysis* Acapella™ script allows an even more extended analysis, multiplexing with any additional cell cycle marker as EdU or pRb can easily be implemented for even more detailed information on the cell populations' drug response.