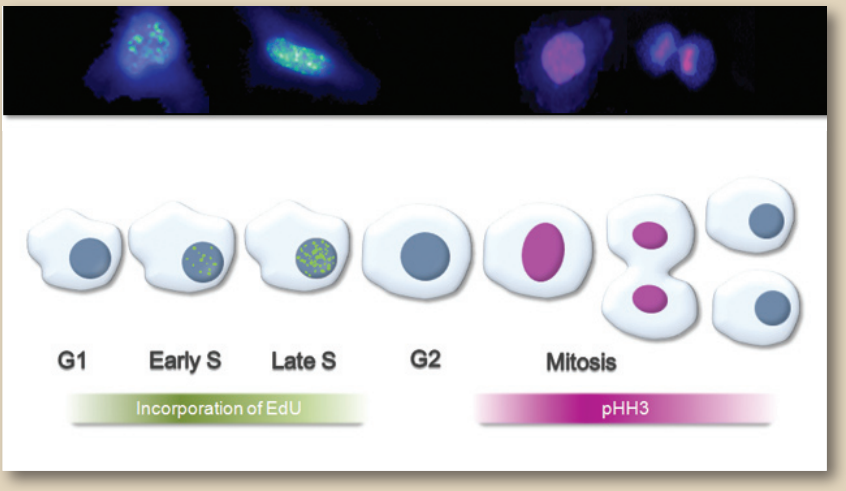


APPLICATION NOTE

# Determination of Cell Cycle Phase using the Operetta

## Key Features

- Automated Image acquisition using the Operetta™ High Content Screening System
- Data analysis using the versatile Harmony™ Software
- Image-based classification of cells in S-phase and M-phase

# Incorporation of EdU, pHH3

## Background

The cell cycle is a ubiquitous, complex process that underlines the growth and proliferation of cells, organismal development, regulation of DNA damage repair and tissue hyperplasia as a response to injury and diseases such as cancer. The characterization of pharmacological compounds that regulate cell proliferation and division is therefore particularly important for drug discovery research.

The cell cycle can be morphologically subdivided into interphase and the stages of the mitotic (M-) phase, which include prophase, metaphase, anaphase and telophase [Alberts B *et al.*, 1983]. The interphase encompasses G1-, S- and G2-phase. The G1- and G2-phases of the cycle represent the "pauses" in the cell cycle that occur between DNA synthesis and mitosis. In the first pause, G1-phase, the cell is preparing for DNA synthesis. In S-phase, cells are synthesizing DNA and therefore have an aneuploidic DNA content between 2N and 4N. The G2-phase is the second pause in the cell cycle during which the cell prepares for mitosis (M-phase).

Here we present a flexible High Content Analysis approach for determining the effects of compounds on cell cycle phases. We have multiplexed the measurement of two cell cycle markers, phospho-histone H3 (M-phase) and EdU (S-phase), with a nuclear stain (DNA content).

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## Application

In this application we present a simple, image-based, validated and ready-to-use strategy for the characterisation of compounds which modulate the cell cycle. HeLa (human cervix carcinoma) cells were seeded at a density of 2500 cells per well into a 384 CellCarrier microtiter plate, and were cultured over night. After an 18 h incubation with various concentrations of Thymidine[1] and Nocodazole[2] cells were fixed with 3.7 % formaldehyde. The DNA content was calculated by staining nuclei with Hoechst 33342. The compound induced inhibition of cell proliferation could be followed using the Click-iT™ EdU Alexa Fluor® 488 Kit. Furthermore, a specific M-phase marker, pHH3, was used in combination with the Alexa Fluor® 647 antibody. Images were recorded on the Operetta using the 20x LWD objective and were analyzed with the Harmony "Cell Cycle-1" module selected from the Ready-Made Solution collection.

In order to classify cells according to their DNA content, we calculated the integrated fluorescent intensity of the nuclear stain. Cells passing a defined intensity threshold, for example 2 x $10^6$ represent cells in mid S-phase and G2/M-phase (yellow labeled cells, Figure 1 A). Using a similar approach, we sorted cells, treated with different compounds, into three subpopulations: 2N (G1-phase), 2N - 4N (S-phase), and 4N (G2-/M-phase) (Figure 1 B).

For further verification of two cell populations, the mitotic cells and the S-phase cells, we introduced cell cycle specific markers into the assay. For fine discrimination of compound effects on the cell cycle, the use of EdU and pHH3 labeling allowed us to evaluate the proliferation and mitotic indices, respectively (Figure 2 A).

Furthermore, we calculated $EC_{50}$ values for Thymidine (G1/S arrest) and Nocodazole (G2/M arrest) treatments using numerical data from the Harmony software (Figure 2 B).

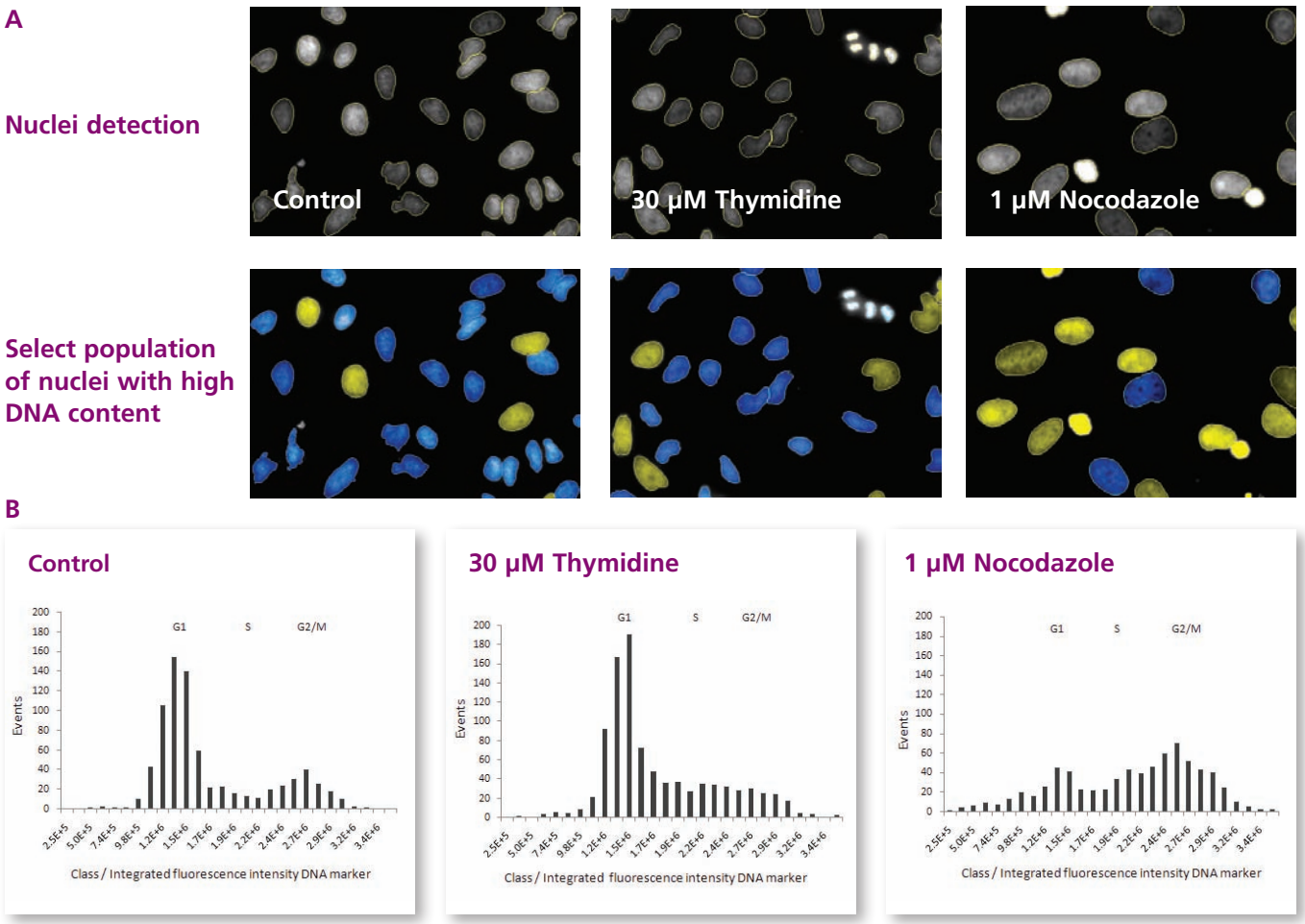


*Figure 1.* Image analysis strategy used to classify cells according their DNA content, using a nuclear stain only.
A | Top | Hoechst stained nuclei after an 18 h incubation with either Thymidine or Nocodazole. Bottom | Cells with a defined threshold of 2 x $10^6$ integrated DNA fluorescence intensity are highlighted in yellow. All other cells are highlighted in blue.
B | Histograms. Left | Non-treated cells (control). Middle | The increased number of cells in G1-phase after Thymidine treatment compared to untreated cells indicates a compound induced arrest in G1-phase. Right | After treatment with Nocodazole, the number of G1 cells decreases whereas the number of M-phase cells increases. This indicates an inhibition of cell division after Nocadazole treatment. To generate the histograms, the numerical data corresponding to single cells, from the 1000 cells analyzed per compound, were transferred into Excel®.

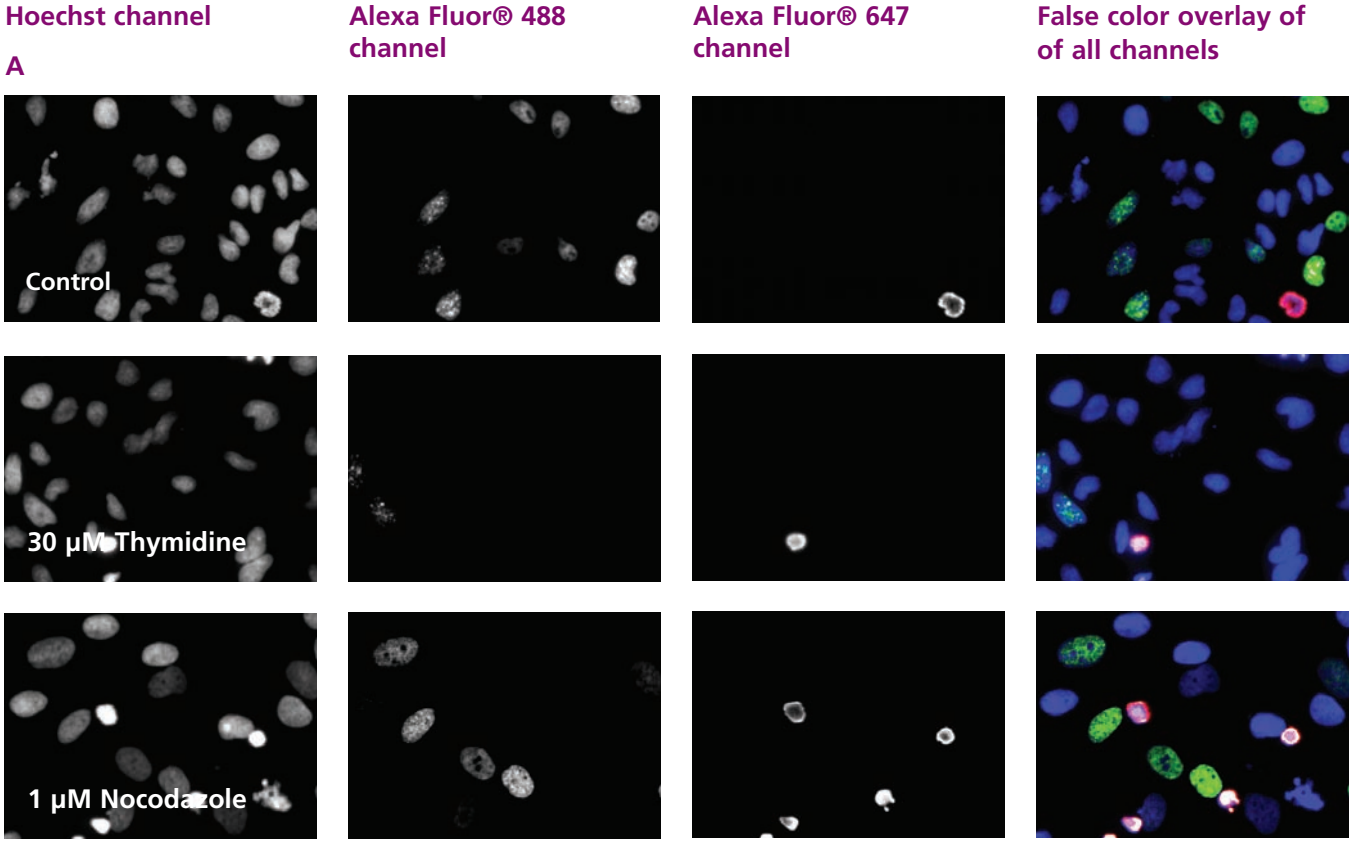


*Figure 2.* Image analysis strategy for quantifying the fluorescence signal of EdU (Alexa Fluor® 488) and pHH3 (Alexa Fluor®647) in cell nuclei.
A | Images of Hoechst stained DNA (Hoechst channel), Alexa Fluor® 488 labeled EdU (Alexa Fluor® 488 channel), Alexa Fluor® 647 labeled pHH3 (Alexa Fluor® 647 channel) and a false color overlay of all 3 channels (DNA: blue, EdU: green and pHH3: red) after an 18 h incubation with either Thymidine or Nocodazole. In order to classify cells as being positive for EdU (S-phase) or pHH3 (M-phase), mean intensity thresholds were defined; In this particular example these were 2000 and 200 respectively. The thresholds were adapted from a significant increase in the fluorescence intensity of the respective marker within a cell population.

B | Compound-generated dose-response curves for Thymidine (Top) and Nocodazole (Bottom). The percentage of cells positive for EdU (representing S-phase cells) is shown in gray, and the percentage of cells positive for pHH3 (representing M-phase cells) in red. When cells were treated with Thymidine, a significant decrease in the EdU signal was observed. Treatment of cells with Nocodazole however, led to a clear increase in the pHH3 signal due to its inhibitory effect during cell division (M-phase). $EC_{50}$ values for Thymidine (37.57 µM) and Nocodazole (0.11 µM) were calculated using the Prism software. N = 3 wells.

## Conclusions

We have presented a robust cell-based application that can be used in a High Content Screening approach to investigate the effects of anti-proliferative drugs. We show that by multiplexing the analysis of DNA content with an S- and M-phase specific marker, a precise classification of cells into the three main sub-populations (G1, S and G2/M) can be achieved.

*1 Thymidine (deoxythymidine) is a nucleoside composed of deoxyribose (a pentose sugar), joined to the base thymine. An excess of Thymidine leads to allosteric inhibition of the ribonucleotide reductase, which consequently prevents DNA synthesis (G1/S blocker).*

*2 Nocodazole is an anti-mitotic agent that blocks microtubule formation by binding to ß-tubulin, thus preventing nuclear and cell division (G2/M blocker).*

## References

Alberts BR, Bray DE, Lewis JU, Raff MA, Roberts KE, Watson JA (1983), Molecular Biology of the Cell, Garland, New York, NY, Alderborn.

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