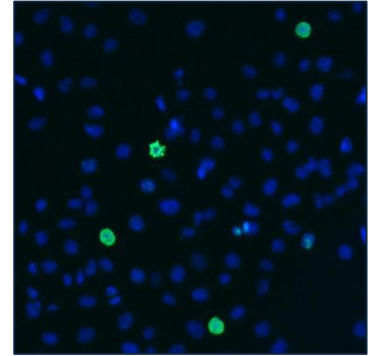


Measurement and Evaluation of Mitosis in Cell Culture

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Abstract

Identification of mitotic status of cells can be important e.g. in cell production processes as well as for cancer related research on proliferation. With the CELLAVISTA[®] System, mitotic cells can be automatically imaged and analyzed. Before imaging, cells are stained with the nuclei dye Hoechst 33342 and immunohistochemically with a fluorescence labeled mitotic marker, e.g. α -pHH3 antibody. Samples are imaged in two fluorescence channels, one for Hoechst to determine the total number of cells, and one for the subpopulation stained with the marker. Mitotic cells and Hoechst stained cells can be individually quantified and evaluated concerning fluorescence intensities, size and multiple other characteristics. In this Application Note we describe the staining procedure and how to measure cells with the CELLAVISTA[®] System.



Application Note
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Introduction

Mitosis is a critical and highly coordinated event in the cell cycle. Its correct orchestration is essential for accurate transmission of genetic information. Errors in mitosis can lead to uncontrolled proliferation as in cancer associated diseases or chromosome missegregation and aneuploidy.

After DNA-synthesis (S-phase) and after transition to G2-phase cells enter mitosis (M-phase). In the prophase of mitosis chromosomes undergo condensation. During prometaphase the nuclear envelope disintegrates and chromosomes are attached to microtubules. After the alignment of chromosomes in the meta-phaseplate each chromatid is pulled to opposite ends of the cell. Telophase is a reversal of prophase and prometaphase events: a nuclear envelope forms around each set of chromosomes while they begin to decondense back to chromatin.

As a final step after mitosis usually cytokinesis takes place and results in two individual daughter cells which differentiate and proliferate.

A central role in regulation of mitosis is fulfilled by Histone 3 (H3), one of the five main histone proteins involved in the structure of

chromatin in eukaryotic cells [1]. Phosphorylation of H3 (at serine 10 and 28, [2]) can first be detected starting from late G2-phase and accelerates as mitosis proceeds until metaphase [3]. Metaphase chromosomes are always found to be heavily phosphorylated [4] (Fig. 1).

Dephosphorylation of H3 begins in anaphase and is completed at early telophase. Likewise mitosis, phosphorylation of H3 can also be detected in condensed metaphase chromosomes during meiosis I and II.

Anti-phosphorylated H3 antibody (α -pHH3) is commonly used to detect mitotically dividing metaphase cells. Therefore it is a valuable marker for various approaches like the synchronization of cell cultures or to measure the effect of compounds on mitosis rates. Furthermore, pHH3 antibody can also be used to detect premature mitosis or senescence of cells.

With the help of the CELLAVISTA[®] Imaging System, pHH3 and other markers (e.g. Ki-67 [5] or MPM-2 [6]) can be used to gain quantitative and qualitative information on the cell cycle status of cells.

The CELLAVISTA® offers automated detection, quantification and detailed evaluation of your cells. Screening can be performed in many culture formats, on a small scale as well as in a

high throughput manner, according to the needs for the identification of therapeutic compounds involved in the inhibition of cell proliferation and therefore in cancer research.

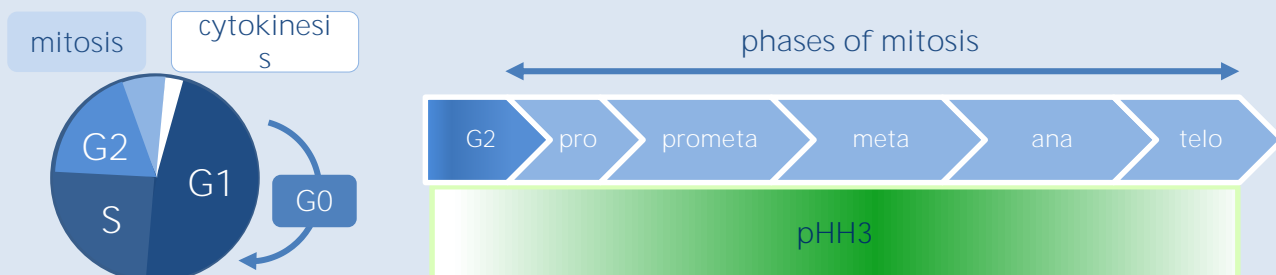


Fig. 1: Cell cycle and mitosis with pHH3 gradient

Cell cycle (left) showing mitosis and cytokinesis as well as interphase (G1-S-G2) and G0 resting phase. Mitosis (right) consists of pro-, prometa-, meta-, ana- and telophase. The mitosis marker pHH3 can be detected starting from late G2-phase with its maximum intensity at metaphase of mitosis. pHH3 signal starts to disappear with the beginning of anaphase.

Material and Methods

Material:

- Adherent monolayer forming cells, here CHO-K1 cells
- DMEM/Ham's F12 1:1 with 5 % fetal bovine serum and 1 % Penicillin/Streptomycin
- Culture dish, e.g. 96-well plate
- Phosphate buffered saline (PBS⁺⁺, with Ca²⁺ and Mg²⁺)
- Trypsin 0.25 %
- Mitotically active compound
- 2 % formaldehyd in PBS⁺⁺
- PBS⁺⁺ + 0.1 % Tween 20 (PBT)
- Hoechst 33342 (use at 2 µg/mL in PBS⁺⁺)
- α-pHH3 FITC-conjugated antibody or α-pHH3 antibody and fluorescent labeled secondary antibody against pHH3 host animal, use according to manufacturer's recommendations
- One of SYNENTEC's imaging systems (here: CELLAVISTA® 3.1)

Methods:

Cells were trypsinized to detach, and transferred to a 96-well plate. Dilution was set in a way to obtain a confluency of less than 70 % after incubation of cells at 37 °C for 10-24 hours. Subsequently, wells were incubated with mitotically active compound for 2-24 h depending on the compound and its concentration.

Cells were washed 2x with PBS⁺⁺ and then fixed with 2 % formaldehyd for 20 min at

room temperature. Excessive fixative was washed off with PBS⁺⁺ (3x). Solubilization of cells was done by incubation in PBT for 15 min. The primary antibody was diluted in PBS⁺⁺ according to the manufacturer's recommendations, and incubated with the cells at 4 °C over night in the dark.

From now on stained cells were protected from light. The next day unbound antibody was washed off with PBS⁺⁺ (6x).

If non-fluorescent labeled primary antibody has been used, secondary antibody should be applied under the same conditions as the primary antibody. If you expect strong background signal from unspecific binding, include a blocking step with 3 % BSA in PBS⁺⁺ previous to primary antibody incubation and enhance washing.

Measurement with the CELLAVISTA[®]

Insert your plate into the CELLAVISTA[®] System and choose the *Generic Measurement* of the SYNENTEC YT-software[®]. In the *Setup* dialogue create a *New Experiment* and define the *Experiment Name* and *Path*. Select your predefined *Plate Type* and *Nuclei 2F* as *Application Type*. Set the *Objective* to 10x and select focus each image and well.

In the *Well Definition* dialog mark the wells you want to be measured by dragging the mouse cursor (with left button pressed and then released) over the required area of the plate. For each group choose the focus option (set to *focus each well and image*) and the *Well Layout*. For a 96-well plate format we recommend to select only 9 central images inside the well to be imaged.

Create in the Channel list 2 channels. One for Hoechst (UV LED and Blue emission filter) and one for pHH3 (Blue LED and Green emission filter). Your Hoechst nuclear channel should be first, followed by your pHH3 fluorophore. In general: set *Excitation Source* and *Filter* according to the excitation/emission of your fluorophore if they vary to our dye selection.

In the *Preparation* dialog choose a well to adjust your optical settings (Navigator) and then press *Autofocus* and *Snapshot*. Decrease or increase exposure time and gain if signal is too low, high, respectively.

Stain nuclei with Hoechst 33342 for 30 min in the dark and wash with PBS⁺⁺ (6x).

For imaging of 96-well plate, add 100 µL PBS⁺⁺ per well and insert plate into the CELLAVISTA[®] Imaging System. The type of plate should be defined in advance.

Make sure that the background in both channels is kept to a minimum. Object intensities of the brightest >object in the image should be kept below 255, and the object to background brightness (O/BG) ratio should be at least 1.5 for a fluorescent object to ensure recognition by image analysis.

Standard Hoechst settings:

Intensity: 100 %
Exposure Time: 70 ms;
Gain: 20;

Standard pHH3 fluorophore settings:

Intensity: 100 %
Exposure Time: 100 ms;
Gain: 20;

Adjust the *Focus Offset* for each channel, so that nuclei and cells marked for mitosis are focused.

In the *Image Processing* dialog to the left of the *snapshot* you can adjust all *Image Processing* parameters per channel.

Start image acquisition by clicking on the upper green *Measurement* with the left mouse button. At the end of the measurement you can inspect and evaluate your data immediately or at a later date.

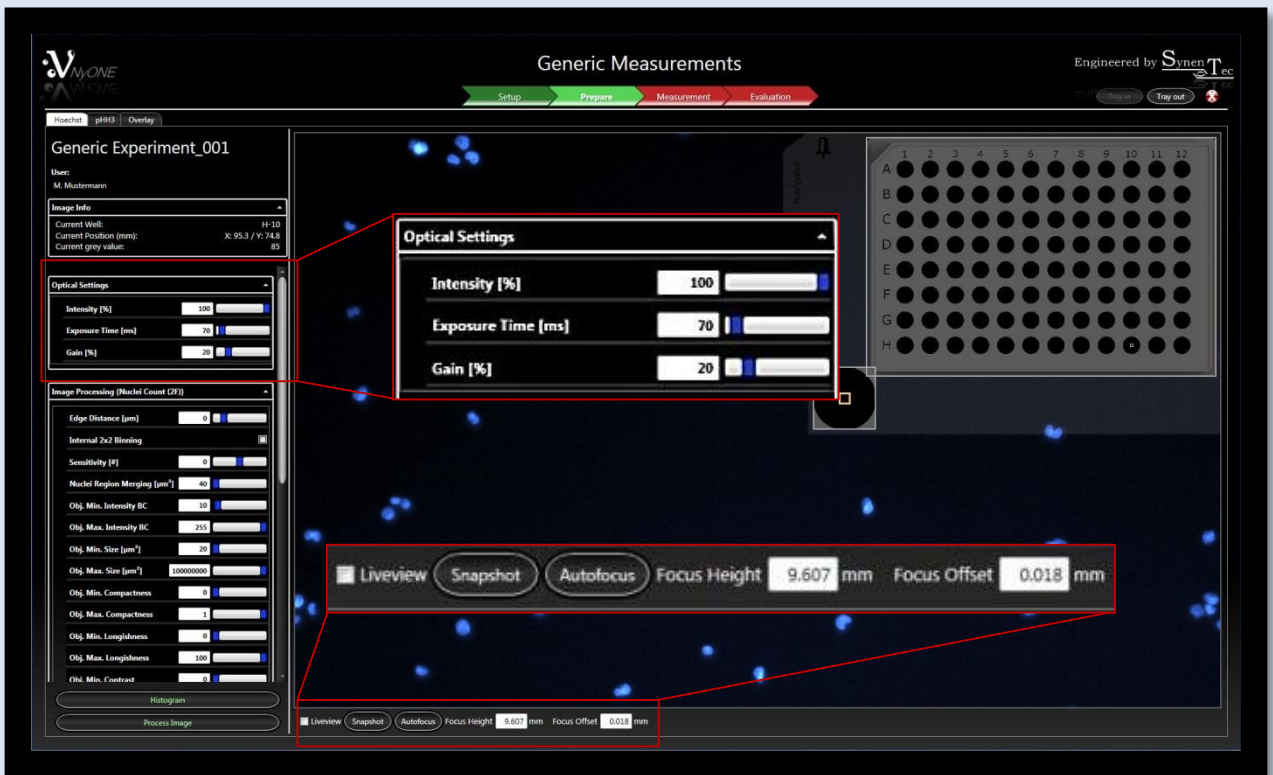


Fig. 2: Overview of *Experiment Preparation* and the *Optical Settings*

After pressing the *Prepare* button the *shown Dialog* appears. In this window the fluorescence channels are set up, one for Hoechst (shown) and one for the fluorophore of the mitotic marker. *Exposure Time* and *Gain* should be adjusted for each channel. If you press *Snapshot*, an image is taken with the current settings. If the fluorescence signal is too low enhance *Exposure Time* and *Gain*. In the *Experiment Preparation Dialog* adjust the *Focus Offset* for each channel. In this dialog you also find the dialogs for *the Image Processing*.

CELLAVISTA® Results

To inspect your experiment data you have to open your experiment and press the *Evaluation* button in the CELLAVISTA® main window.

If you have already processed the data automatically during measurement the table on the left hand will already contain some information about your cells, e.g.:

- Total Cell Count: All cell nuclei detected (nuclei dye positive)
- pHH3 (=M-Phase) Cell Count: Number of nuclei dye positive cells that are also pHH3 positive

- pHH3 (=M-Phase) M-Phase Cells [%]: Percentage of "pHH3 Cell Count" with respect to "Total Cell Count"
- Evaluated Area [mm²]: Total area analyzed by the evaluation program (operator) [mm²]

You can also look through your image data and processing results to check if processed data corresponds with your visual evaluation of cells (Fig. 3). To define an optimized processing parameter set for this and your future pHH3 mitosis assays, adjust the *Image Processing* sliders in the left panel per channel.

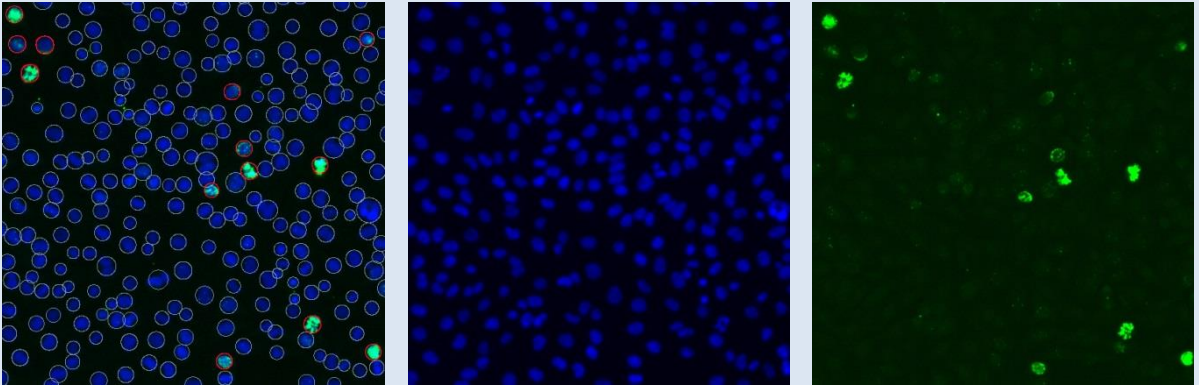


Fig. 3: CHO-K1 cells stained with Hoechst (blue) and α -pHH3 antibody (green)

CHO-K1 cells stained with Hoechst (blue) and α -pHH3 antibody (green). pHH3 signal was detected by a FITC- conjugated secondary antibody. Cell nuclei and pHH3 positive cells are automatically detected by the operator according to the user's settings. Nuclei are counted and marked by a white circle, while pHH3 cells are marked by a red circle.

Adjustment of Parameters

The basic principle of the *Phospho Histone H3 Operator* is that it searches for distinct fluorescence objects in the first channel (Hoechst). Subsequent to nuclei detection the region around each nuclei is scanned for the presence of fluorescence signal (pHH3) in the second channel.

The *Image Processing* consist of different Parameters (Fig. 4). The *Hoechst Parameter Settings* work upstream of the *pHH3 Parameters*. That means if the *Hoechst parameters* are set too narrowly, only little objects can be found.

In the first step we recommend to change only the value for *Ch1 Hoechst Sensitivity* and test its effect on the evaluation of your selected well.

A high value means high sensitivity for the detection of Hoechst stained cell nuclei, so that also weakly stained nuclei are detected. This can also result in undesired artefact detection.

For the pHH3 *Parameter Settings* we recommend to adjust only the values for *Intensity to BG (background)*. This value defines in which range of minimal and maximal fluorescence values your pHH3 signal is counted as a pHH3 positive object. In the default settings a minimum value of 10 is defined, so that a signal 10 intensity value above background is considered as pHH3 positive.

You can test all your changes in parameter settings by pressing *Process Visible* (Fig. 4). If your settings are adequate to your needs you can name and save your settings with the *Save* button as a Template for following experiments. You can now use your optimized parameter settings to reprocess your experiment data. If the recommended adjustments of parameters are not sufficient to detect your cells properly please contact a member of the SYNENTEC team for further help.

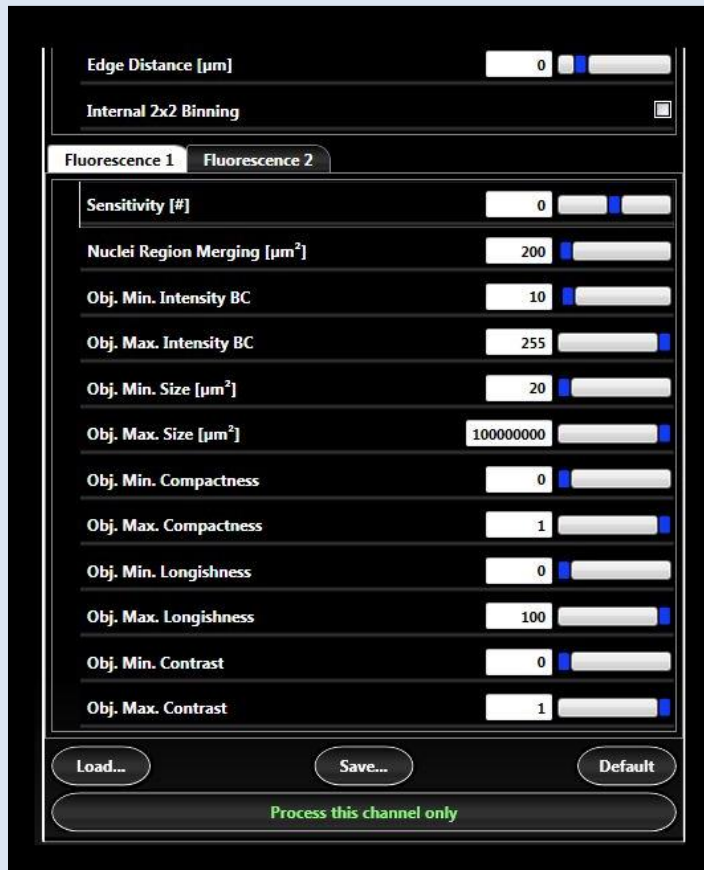


Fig. 4: Parameterlist of *Phospho Histone H3 Operator*

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