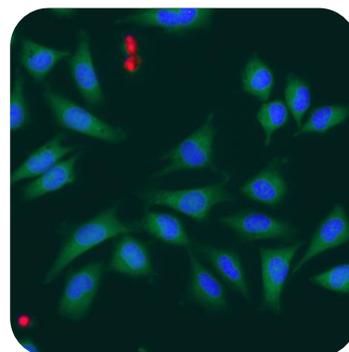


Versatile Cytotoxicity Assays for Animal Cells with the NyONE

Abstract

Cell-based *in vitro* models are helpful systems in wide areas of compound screening. This modern way in drug development and environmental sciences goes hand in hand with the development of cell culture viability assays. These assays are able to generate a large amount of data in short time, which takes up a lot of your valuable labor time to analyze. SynenTec has developed a completely automated fluorescence cell imager and an assay kit exactly for this purpose. This Cytotoxicity Assay Kit allows to determine the toxicity of compounds using live, dead and total cell staining.

This report demonstrates that the Cytotoxicity Assay Kit is able to determine EC₅₀ values for DMSO (dimethyl sulfoxide) for the animal cell line CHO with the NyONE system.



Application Note
AN-F340-XIV-04

Assay principle

The SynenTec Cytotoxicity Assay Kit is a three color fluorescence assay to determine simultaneously the numbers of live, dead and total cells. It is based on the enzymatic hydrolyzation of the cell permeant, non-fluorescent Calcein AM (Calcein acetoxymethyl ester) by intracellular esterases in viable cells. Calcein is an intensively green fluorescent dye and causes a uniform staining of the cell interior. The Calcein spectra show that optimal excitation is achieved at ~495 nm and emission at ~515 nm, respectively.

These fluorescent properties of Calcein allow a safe counting of living cells in your sample.

Just like esterase activity, membrane integrity is essential for the viability of cells. After cell death the membrane integrity is affected. Since Propidium iodide (PI) cannot pass the cell membrane of living cells, it is a good probe for dead cells. It can permeate through the plasma membrane of dead cells and reaches the nucleus where it intercalates into the DNA. The fluorescence is enhanced 20-30 fold combined with an excitation and an emission shift ^[1] upon intercalation.

The complex of Propidium iodide and DNA owns an excitation maximum at ~528 nm and an emission maximum at ~617 nm. The number of fluorescent nuclei is therefore a measure for the cytotoxicity.

We used the dye Hoechst 33342 (2,5'-Bi-1H-Benzimidazol) to compensate for variations in the number of cells seeded in the individual wells. In contrast to Propidium iodide Hoechst is a plasma membrane permeable agent and binds to the minor grooves of DNA ^[2].

Optimal excitation and emission of Hoechst 33342 bound to DNA takes place around 352 nm (ex) and 461 nm (em).



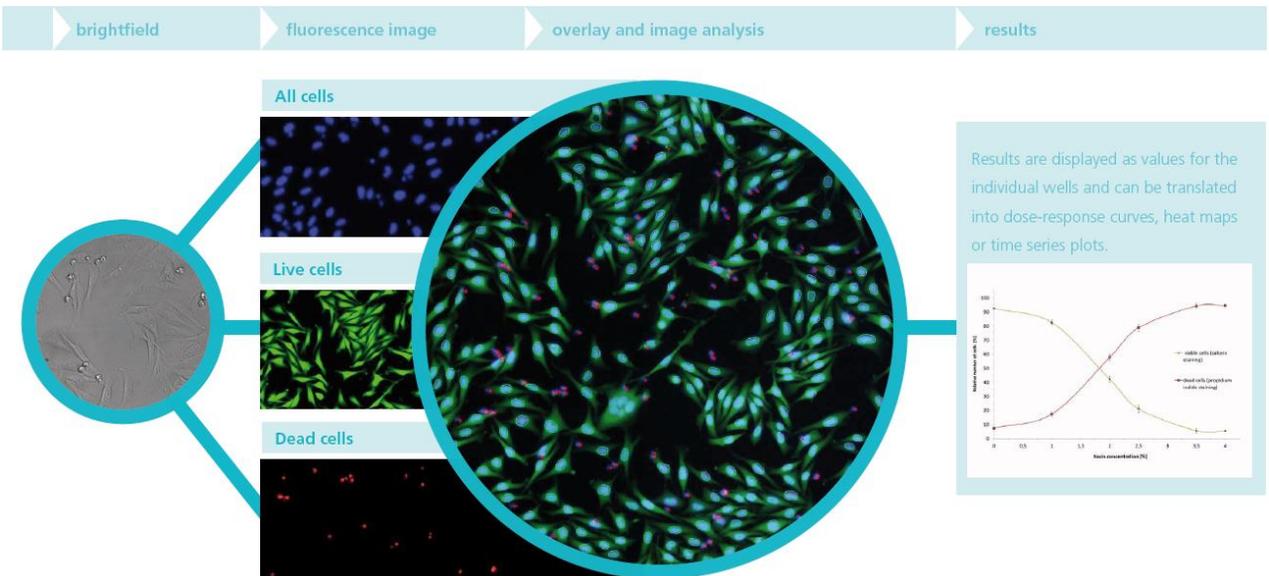
Materials

- Cytotoxicity Assay Kit
- CHO-K1 cells (ATCC)
- DMEM/Ham's F12 Growth Media (Biochrom)
- Fetal Bovine Serum (Biochrom)
- Phosphate buffered saline (with Ca²⁺ und Mg²⁺), pH 7.4 (Biochrom)
- Nunclon 96-well microplates (Nunc)

Methods

CHO cells were plated in 96-well polystyrene plates at a density of 5000 – 10000 cells/well (depending on the cell line) in 200 µL of growth medium. After cell seeding the microplates were gently placed under the laminar hood avoiding agitation.

The cells were allowed to settle down homogeneously on the well bottom for 30 minutes. Afterwards the cells were incubated 24 h at 37 °C in a 5 % CO₂ and 95 % humidity atmosphere.



After incubation the growth medium was removed carefully and the cells were exposed to various concentrations of DMSO in serum-free media. A volume of 100 µL/well was used so that each column of the microplate had four replicates and two concentrations of DMSO. The toxin was incubated for 24 h at 37 °C in a 5 % CO₂ and 95 % humidity incubator.

Following to the toxin treatment 15 mL of staining solution was prepared in PBS (with Ca²⁺ und Mg²⁺), according to the kit manual.

A volume of 100 µL staining solution was added to each well. Cells were incubated with the dyes for 30 min at 37 °C in the incubator and then analyzed using the NyONE.

Instrumentation

Measurements were performed with the NyONE **Live/Dead operator**. Nine pictures per well were taken. The read time for a whole 96-well plate was around 30 minutes for the given experimental parameter settings for CHO. They were set as followed:

The **Hoechst-channel** is preconfigured with the 390 nm excitation and the 452/45 nm emission filter.

Optical settings:

- 100 % intensity
- 300 ms exposure time
- 50 % gain

The **PI-channel** is preconfigured with the 485 nm excitation and the 628/32 nm emission filter.

Optical settings:

- 100 % intensity
- 100 ms exposure time
- 35 % gain

The **Calcein-channel** is preconfigured with the 485 nm excitation and the 530/43 nm emission filter.

Optical settings:

- 100 % intensity
- 50 ms exposure time
- 45 % gain

Data analysis

The NyONE image analysis module uses blob detection algorithms to detect the fluorescent cells in the three different fluorescence channels. The predefined image analysis parameters can be fine-tuned to account for differences in cell morphologies between the cell lines covered in this report.

To determine the total number of cells in each well, the Hoechst stained nuclei were counted.

Dead cells were counted when the software detected co-localized Propidium iodide and Hoechst stains. Determining of the viable cells was done using a combination of the signals in the Hoechst and the Calcein channel.

Fig. 1 shows a snapshot from an image overlay in the analysis software for visual control of cell counting.

Results

Figure 2 shows the results of CHO experiments. For the CHO cells that receive a 24 h DMSO cell treatment we obtained an EC_{50} (half maximal effective concentration) of 24h DMSO at a concentration of 1.8 % DMSO in growth media.

Furthermore we could observe, that there is a sigmoidal concentration dependent increase of

dead cells in combination with an antiproportional decrease of Calcein stained living cells with rising DMSO-concentration.

The relative standard deviations for all measurements (four replicates) were well below 5 %.

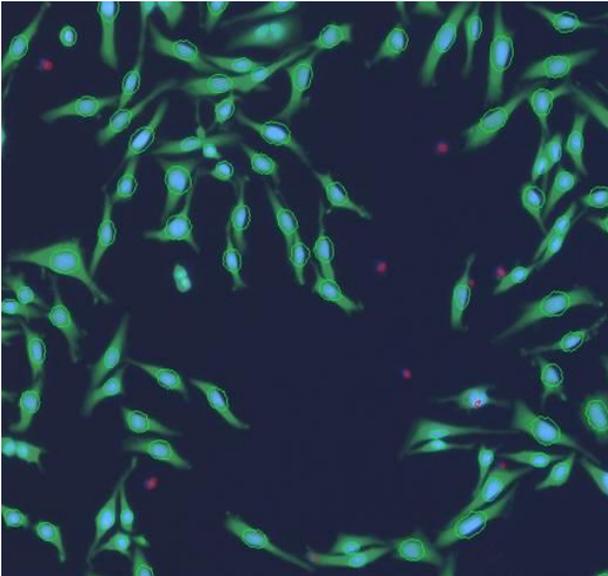


Fig. 1: This three-channel overlay illustrates the results of the image analysis process. Cells with a blue-stained nucleus and surrounded by a Calcein-stained cytosol are marked (circles) and counted as live cells. Cells with a nucleus which is stained by both Hoechst and PI, appear as purple and are counted as dead cells.

Conclusion

It has been demonstrated that the NyONE imaging system with its cell analysis software produces reliable data in combination with the Cytotoxicity Assay Kit. It is a simple and fast method to determine the cytotoxicity of toxins in multiwell formats.

Despite the fact that the CHO cells change morphology as a response to the toxin, the image analysis software was readily able to cope with this challenge.

We could show that there is a measurable effect of the organic solvent DMSO on animal cells. For further studies in which you will handle with DMSO as a solvent of your dyes or your compounds of interest, it is more than important to determine the individual EC_{50} of your specific cell culture. Thus you are able to minimize side effects of DMSO on measurement results.

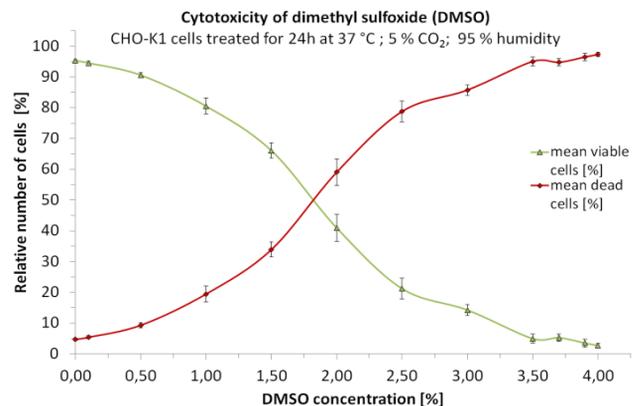


Fig. 2: In this dose response curve the results of the EC_{50} determination of CHO cells treated with different DMSO concentrations for 24 h are shown. The red line displays the averaged percentage of counted dead cells whereas the green line represents the viable cells. The data points are plotted with standard deviation of four replicates.

Our results agree with common experiences in the lab which shows an effect of DMSO on other animal cell lines like Caco-2 or HEK.

For example Da Violante and colleagues analyzed the DMSO concentration depended LDH (lactate dehydrogenase) activity on Caco-2 cells. They showed that the LDH activity decreases at DMSO concentrations exceeding 10 % [3].

In addition to this we were able to observe first morphological signs of toxic effects in CHO-cells at the lowest DMSO concentrations applied, demonstrating the sensitivity of the method. Further modules to exploit the potential of automated cell image analysis will be available soon.

References

- [1] Arndt-Jovin DJ, Jovin TM; Fluorescence labeling and microscopy of DNA; Methods Cell Biol. 1989;30:417-48.
- [2] Ellwart JW, Dörmer P; Vitality measurement using spectrum shift in Hoechst 33342 stained cells. Cytometry 1990;11:239-43.
- [3] Da Violante G et al.; Evaluation of the Cytotoxicity Effect of Dimethyl Sulfoxide (DMSO) on Caco2/TC7 Colon Tumor Cell Cultures. Biol. Pharm. Bull. 2002;25(12):1600-1603.



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