Multiplexing of DELFIA® Cell Proliferation and DNA Fragmentation Assays with Conventional Fluorometric Cell-Based Assays for Identification of Toxic Compounds

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1 Introduction

We have developed sensitive assays for Apoptosis, cell metabolism and cell proliferation monitoring these events. These non-radioactive assays can be used in secondary screening in the drug discovery process, for early cell toxicity testing, to identify compounds with toxic properties. Combining different assays provide more information regarding the cytotoxic properties of compounds. The DNA Fragmentation assay is a simple, cell-based TUNEL assay for quantitative detection of apoptosis, performed in microplate format. The DELFIA® Cell Proliferation assay measures the effect of growth regulatory substances. These assays can also be combined with one another, or combined with other conventional fluorometric assays or applications. We have set up cell-based assays where the DELFIA® detection technology is combined with conventional fluorometry, and where both assay technologies are performed on the same sample.

2 Overview of the ToxSuite Products

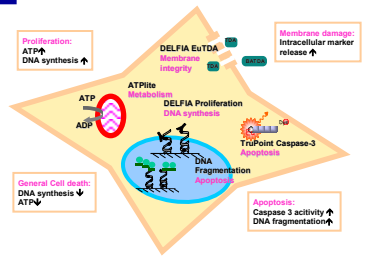
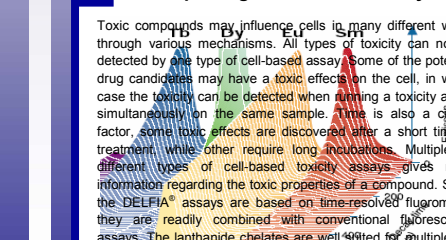


Figure 1. Non-radioactive cytotoxicity assays are used primarily in secondary screening in the drug discovery process. There is a trend, for early cell toxicity testing to identify compounds with toxic properties before lead optimization. These kinds of assays are also used in basic research, to study cell proliferation, apoptosis, etc. in for instance cancer research and immunology. The cell-based assays of the ToxSuite provide extensive information about the cytotoxic effects of compounds.

3 Multiplexing of cell-based assays

Toxic compounds may influence cells in many different ways, through various mechanisms. All types of toxicity can not be detected by one type of cell-based assay. Some of the potential drug candidates may have a toxic effects on the cell, in which case the toxicity can be detected when running a toxicity assay simultaneously on the same sample. Time is also a critical factor, some toxic effects are discovered after a short time of treatment, while other require long incubations. Multiplexing different types of cell-based toxicity assays gives more information regarding the toxic properties of a compound. Since the DELFIA® assays are based on time-resolved fluorometry, they are readily combined with conventional fluorescence assays. The lanthanide chelates are well suited for multiplexing assays. The use of the lanthanide chelates will maximize sensitivity and minimize signal spillover for the assays.



4 Assay Flow Chart

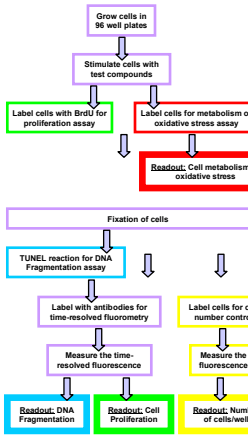


Figure 2. Assay flow chart for multiplexing.

All the assays are performed simultaneously on the same samples, and in the same wells. In addition to increasing the amount of data gained form each sample, this also saves reagents, plates, and test compounds.

The labeling of the cells for the proliferation assay, for the oxidative stress assay, and for the metabolism assay is done with live cells, without removing the culture media or the test compound.

After performing the measurement for the cell metabolism assay, the culture media is removed, and the cells are fixed.

The TUNEL reaction for quantification of DNA fragmentation is performed directly in the wells on the fixed cells.

Detection of incorporated nucleotides is done using components labeled with different lanthanide chelates.

A label for total number of cells in each well (SYTO24®) is also included in the detection mix.

All the assays are measured using the multilabel plate readers VICTOR™ and EnVision™.

5 Cell Proliferation & DNA fragmentation assays

The DELFIA® Cell Proliferation assay is based on the incorporation of BrdU into newly synthesized DNA strands of proliferating cells. Incorporated BrdU is detected using a labeled antibody. The DNA Fragmentation assay is a cell-based assay performed in 96-well micro plate format for quantitative detection of apoptosis. The labeling of the 3'-hydroxyl ends of DNA fragments provides a measure of cells undergoing apoptosis. Incorporated, labeled nucleotides can be detected by labeled streptavidin. We have combined this two assays, Europium-labeled anti-BrdU antibody for the proliferation assay, and Samarium-labeled streptavidin for the DNA fragmentation assay.

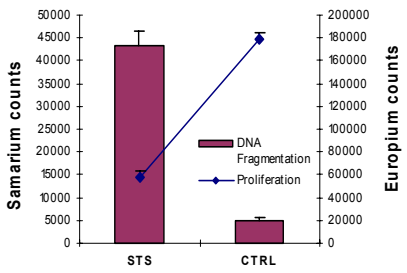


Figure 3. The data shows proliferation and DNA fragmentation in CHO cells, 10000 cells/well, grown overnight. The cells were treated with 10µM staurosporine (STS), for 6 hours to induce apoptosis. Apoptotic cells were compared to untreated cells. The data is expressed both as Samarium counts for the DNA fragmentation assay, and as Europium counts for the proliferation assay.

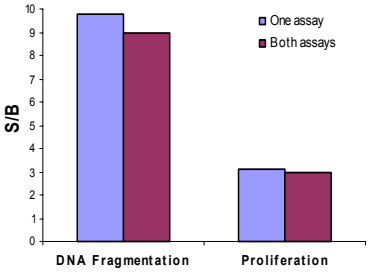


Figure 4. Proliferation and DNA fragmentation in CHO cells treated with STS. The two assays do not significantly affect each other when performed simultaneously in the same sample wells. The data is expressed as signal to background for both the proliferation- and the DNA fragmentation assays when performed either alone or together.

6 Oxidative stress & DNA fragmentation

The cells are loaded with CM-H2-DCFDA, treated with test compounds, and the oxidative stress is monitored in live-cells. CM-H2-DCFDA is a cell-permeable indicator of reactive oxygen species. The indicator remains non-fluorescent until removal of the acetate groups by intracellular esterases and until oxidation occurs within the cell. After live-cell, kinetic measurement of oxidative stress the cells can be fixed and the cell-based assay for DNA fragmentation performed.

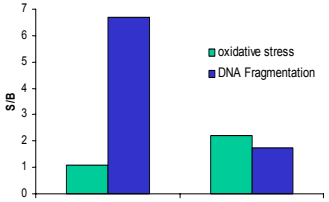


Figure 5. Oxidative stress and DNA fragmentation in CHO cells. The cells were loaded with CM-H2-DCFDA and treated with different compounds inducing oxidative stress or apcptosis. The data is expressed as signal from treated- compared to untreated cells.

7 Cell Proliferation & alamarBlue assays

Cell proliferation by incorporation of BrdU, and detection of metabolic activity in cells using the growth indicator alamarBlue. The BrdU- and the alamarBlue labeling reagents were added simultaneously, The homogenous alamarBlue assay is performed first and then the cells are fixed for the end-point DELFIA® Cell Proliferation assay.

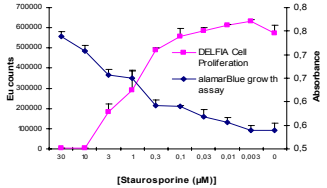


Figure 6. CHO cells were cultured over night at 10000 cells/well in a 96-well plate. Staurosporine, 0 – 30µM, was added and the cells were incubated for 6 hours before addition of the BrdU- and the alamarBlue labeling reagents. The cells were incubated in the presence of alamarBlue and BrdU overnight. The absorbance was measured, and the cells were fixed before detection of incorporated BrdU according to the DELFIA® Cell Proliferation kit insert.

8 Cell Proliferation, cell metabolism, and DNA fragmentation assays

Four different assays are performed in the same well. The cell metabolism assay is an absorbance measurement, the DELFIA® cell proliferation and the DNA fragmentation assays are time-resolved fluorescence assays and the label to correct for unequal number of cells/well is a conventional fluorescence label.

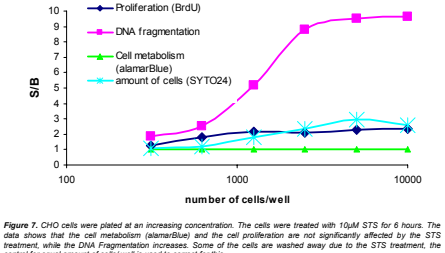


Figure 7. CHO cells were plated at an increasing concentration. The cells were treated with 10µM STS for 6 hours. The data shows that the cell metabolism (alamarBlue) and the cell proliferation are not significantly affected by the STS treatment, while the DNA Fragmentation increases. Some of the cells are washed away due to the STS treatment, the control for equal amount of cells/ well is used to correct for this.

9 Conclusions

Cell-based DELFIA® assays can be multiplexed and combined with assays and applications using conventional fluorescence measurements.

Multiplexing of DELFIA® assays saves time as well as reagents and more data is gained from one sample.

All assays can be performed on the same plate and read on the same time-resolved fluorescence reader.

When performing cell-based assays where cells are going through various treatments, it is important to include a control for equal number of cells in each well. We have used a nucleic acid stain for this. The label is not interfering with the time-resolved fluorescence.