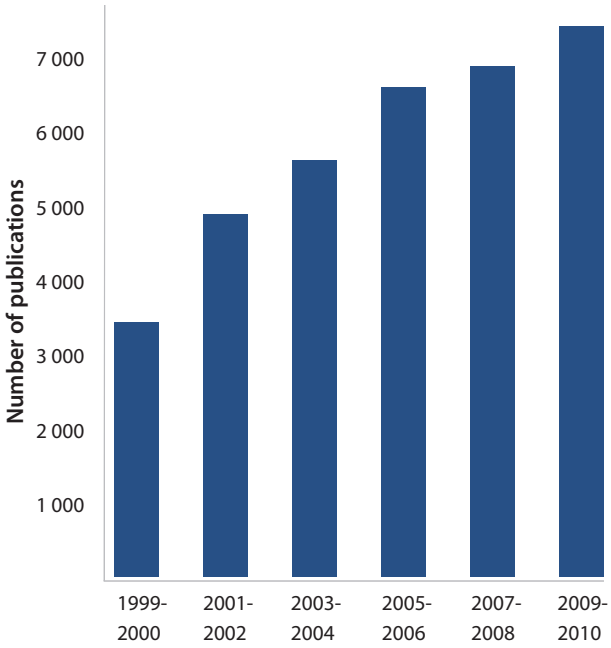
Multicellular tumor spheroids represent models for the three-dimensional microenvironment of tumors. Spheroids are superior to monolayer cultures for applications such as anti-cancer drug research. Apoptosis of tumor cells in multi-cellular spheroids can be quantified using the **M30 CytoDeath™ ELISA**.

APOPTOSIS *DETECTION IN* MULTICELLULAR SPHEROIDS

Publications containing "tumor spheroids"



The interest of the scientific community to use **multicellular spheroids** as *in vitro* models of tumors has increased in recent years. Multicellular spheroids are known to better mimic the 3D conditions of tumors compared to monolayer cultures. The inner spheroid cell layers contain non-proliferating, quiescent cell populations that respond poorly to radiation and chemotherapy.

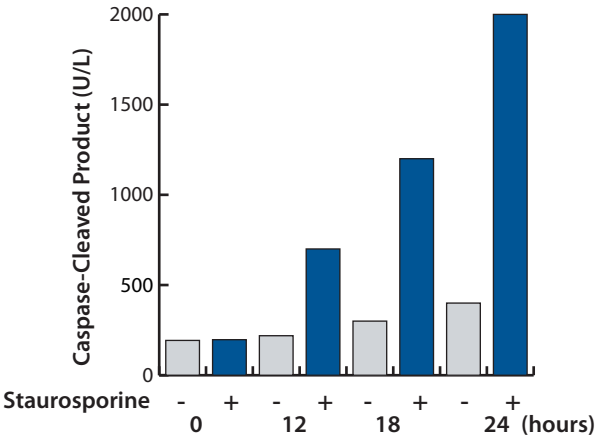
Simple procedures to determine cell proliferation and cell death in spheroids have been lacking. Spheroids are compact and difficult to dissociate into single cell suspensions. Exogenous substrates to measure cell viability may not penetrate into spheroids.

The M30 CytoDeath™ ELISA allows the quantitation of an endogenous apoptosis product (caspase-cleaved keratin 18) using spheroid lysates.

Multicellular Spheroids as Models for 3D Tumor Tissue

Two-dimensional (2D) monolayer cultures are universally used in experimental studies in biology. Monolayer cultures are convenient to use but do not provide accurate models for the microenvironment of tumors. Tumor cells exist in an organized 3D matrix where they are surrounded by other cells such as fibroblasts, macrophages and lymphocytes. Whereas nutrients and oxygen will be easily accessible to all tumor cells in 2D culture, this is not the situation in 3D tumor tissue. A large number of studies have reported differences in cell proliferation and phenotypic properties between cells grown in 2D or 3D culture. It is therefore advisable to use multicellular spheroids in various projects.

Increase of Caspase-Cleaved K18 in Spheroids



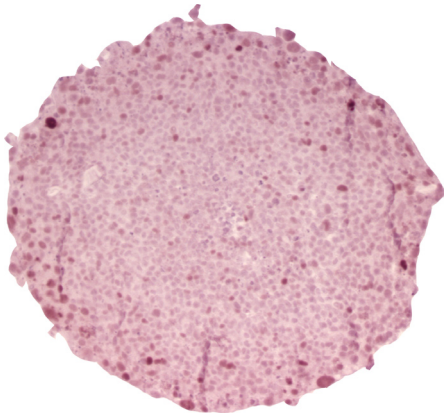
Measurement of apoptosis using the M30 CytoDeath™ ELISA

The spheroids were grown by the hanging drop method as described below and treated at the 7th day of growth; size 500 µm in diameter. Apoptosis was induced by treatment with 0.5 µM staurosporine in spheroid supernatants. M30 CytoDeath™ ELISA levels were measured 12, 18 and 24 hours after treatment. Comparison with untreated control spheroids.

In vitro Culture of Multicellular Spheroids

It is possible to generate spheroids of homogeneous size using the "**hanging drop method**" (Kelm *et al*., 2003). A spheroid diameter of 500 µm is reached after 7 days of incubation. Spheroids will contain proliferating cells in the outer layers, and quiescent, necrotic and apoptotic cells in the central portions. The possibility to generate one 500 µm spheroid per well in 96-well microtiter plate is very attractive in various biological studies, particularly in studies of the response of tumor cells to drugs.

It is possible to use spheroids for **drug screening**. Herrmann *et al*. (2008) have demonstrated excellent performance characteristics of a screening assay based on colon carcinoma spheroids and measurement of caspase-cleaved keratin 18 (Z' values of > 0.5). The M30 CytoDeath™ ELISA method provides the opportunity to restrict the number of primary hits in cell-based screening campaigns to compounds with good tumor penetration. Therefore, it is an ideal assay for primary screening of new anticancer compounds as well as for a secondary analysis of drug candidates identified in monolayer screenings.



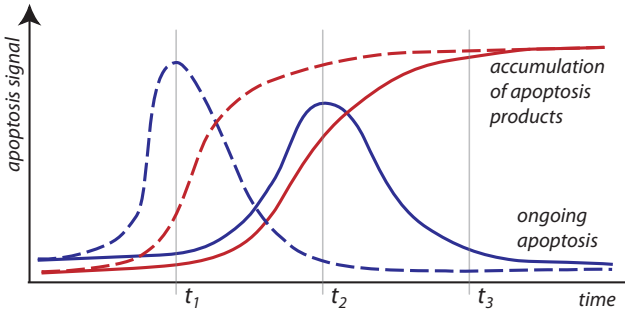
A HCT-116 colon cancer spheroid after 7 days of incubation.

Only One Measurement Time Point is Needed!

A major advantage of the M30 CytoDeath™ ELISA is that it measures a product of the apoptotic process. This means that the signal from cells that have undergone apoptosis remains in the medium, also after the apoptotic process is completed. When choosing a late time point for the sample analysis, all apoptosis that has occurred will be measured. This late time point (t_3 in the chart to the right) is sufficient to quantify the intensity of the apoptotic stimulus regardless of its kinetic.

In contrast, other methods for quantification of apoptosis, such as Annexin V, TUNEL or active caspase-3, reflect the ongoing apoptotic process *at a specific time point*. Given fast apoptosis induction (dotted lines in the chart), the measurement at time point 2 will return a misleading value with these methods. Multiple measurements are therefore required.

The M30 CytoDeath™ ELISA facilitates the determination of apoptosis and saves samples, work load, time and material by minimizing the number of required measurements.



Red lines: *signal in the M30 CytoDeath™ ELISA*

Blue lines: *signal of ongoing apoptosis (e. g. Annexin V)*

Dotted lines: *example of fast induction of apoptosis*

Solid lines: *example of slow induction of apoptosis*

t_1: *peak of the apoptotic rate for a quickly acting stimulus, but still very low signal upon treatment with slower induction.*

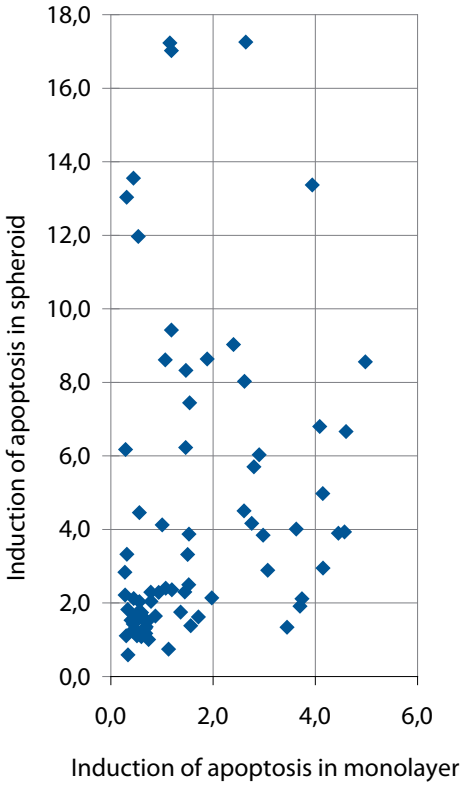
t_2: *peak of the apoptotic rate at slower apoptosis induction, but in the fast acting example, the apoptotic process in almost completed and leads to low signal.*

t_3: *late time point, where the apoptotic process is completed for both treatments; the signals of ongoing apoptosis is very low, but the concentrations of the accumulated apoptotic product measured by the M30 CytoDeath™ ELISA is at its plateau.*

3D and 2D Cultures Differ in Sensitivity to Treatment

The conditions inside a spheroid are different from monolayer cultures, for example by gene expression and levels of oxygen, nutrients and waste. Spheroids can mimic the different cell stages, from proliferating cells in the outer layer over quiescent cells to the necrotic core. With these differences in conditions, cells react differently to apoptotic stimuli, such as anti-cancer substances.

In the chart (to the right), the sensitivity to drug candidates in spheroids was compared to monolayer cultures. Not surprisingly, some substances showed anti-cancer potency in monolayer, but not in spheroids, which may be due to inability to penetrate the spheroids. More astonishing is the finding that some drug candidates were found that could effectively kill cancer cells in 3D, but not in monolayers.



Technical Notes

- The M30 CytoDeath™ ELISA can only be used for human, monkey or cow epithelial cell lines expressing keratin 18. Fibroblasts can be added to mimic *in vivo* conditions. Fibroblasts do not express keratins and will not contribute to the apoptosis signal.
- A cell suspension (50 000 cells/ml) is pipetted in the rings of the lid of a 96 well plate (each drop consisting of 20 µl). The lid is placed upside down and stored in a cell culture incubator with the drops hanging from the lid.
- At the third day, the spheroids can be centrifuged down to the wells of the 96 well plate (3 minutes at 1 000 rpm)
- After 5 – 7 days of incubation, the spheroids in the wells have grown to 500 µm in diameter and can be used for experiments (e. g. treated with drugs, cytokines or other agents).
- After treatment, spheroids are lysed by adding non-ionic detergent to the medium (1 % NP-40).
- Apoptosis is measured using the M30 CytoDeath™ ELISA.

6-Step Apoptosis Quantification

1. Transfer 25 µl sample to the microtiter plate
2. Add 75 µl diluted conjugate and incubate for 4 hours
3. Wash
4. Add 200 µl TMB and incubate for 20 minutes
5. Add 50 µl Stop solution
6. Read absorbance at 450 nm

Determination of Apoptosis by the M30 CytoDeath™ ELISA

The M30 CytoDeath™ ELISA quantifies apoptosis by measuring a neo-epitope generated by caspase cleavage of keratin 18.

The caspase-cleaved apoptosis products will be present in apoptotic cells and will also be released from dying cells into the cell culture. The total content of the apoptosis product in spheroid cultures is measured after addition of non-ionic detergent (NP-40) to the culture medium. The M30 CytoDeath™ ELISA method therefore provides an **integrative measure of apoptosis in spheroid cultures.**

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