BioResearch

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Determination of Spheroid Viability and Proliferation Using the ViaLight™ Plus Cell Proliferation and Cytotoxicity BioAssay

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Introduction

It is a common requirement to assess the viability and proliferation of cells in culture. This requirement is the same for advanced 3-dimensional (3D) tissue-like culture systems as it is in planar 2D cultures. However, the assessment of cell viability in tissue-like structures can be more challenging than in cell monolayers, since the cells are less accessible for e.g. assay reagents due to the 3-dimensional structure.

In this Technical Note, we show that the ViaLight™ Plus Cell Proliferation and Cytotoxicity BioAssay can be easily used to assess cell viability in so-called spheroids. Spheroids are aggregates of cells that can be formed on non-adherent substrates, in hanging drop cell culture plates or in spinner flasks. Instead of attaching to a synthetic polystyrene cell culture surface, cells in spheroid culture attach to each other and thereby mimic natural tissues.

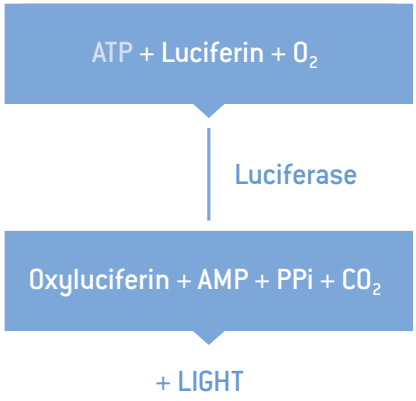

Figure 1
Principle of the ViaLight™ Plus BioAssay. Catalyzed by the luciferase enzyme contained in the ViaLight™ Plus Kit cellular ATP, luciferin and O_2 react to oxyluciferin, AMP, pyrophosphate and CO_2 paralleled by the emission of light that can be detected using suitable luminometers or scintillation counters.

The ViaLight™ Bioassay is based on the bioluminescent detection of cellular ATP as a measure of cell viability (Figure 1). The two-step, non-radioactive assay protocol (Figure 2) was designed to provide robust, quick and sensitive measurements of cell viability and cell proliferation. In addition, the two-step assay protocol allows users to adapt the length of the first lysis step according to the needs of their cell culture system. In this study, it could be shown that a lysis time of 60 minutes is optimal to determine cell viability in spheroid cultures made of either the HCT 116 colon carcinoma cell line or normal human dermal fibroblasts (NHDF).

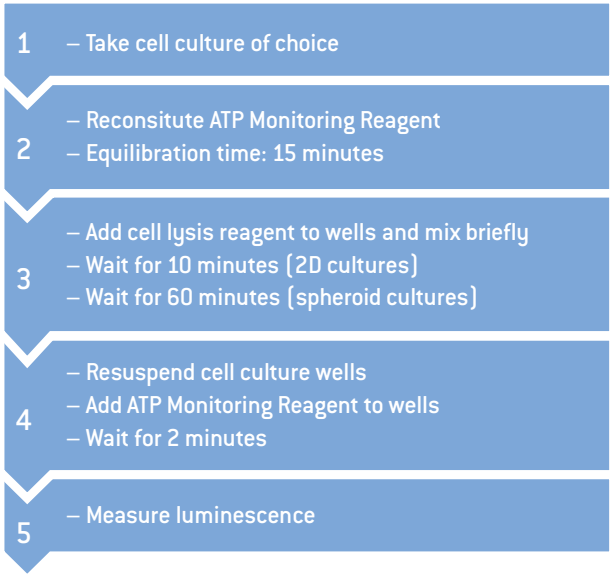

Figure 2
Overview of the ViaLight™ Plus BioAssay Procedure. Please refer to the ViaLight™ Plus Kit Protocol for detailed information. For spheroid cultures, it is recommended to increase the lysis time (Step 3) from 10 minutes to 60 minutes to ensure efficient lysis of spheroids with a diameter of up to 800 µm.

Materials and Methods

- Neonatal human dermal fibroblasts (NHDFneo) [Lonza, Cat. No.: CC-2509]
- Fibroblast Growth Media, FGM™-2 BulletKit™ [Lonza, Cat. No.: CC-3132]
- Reagent Pack™ Subculture Reagents [Lonza, Cat. No.: CC-5034]
- Human colon carcinoma cell line (HCT 116) (ATCC®, Cat. No.: CCL-247™)
- HCT 116 medium: McCoy's 5A Medium [Lonza, Cat. No.: BE12-688F] supplemented with 10% Fetal Calf Serum and 1% Penicillin/Streptomycin [Lonza, Cat. No.: DE17-602E]
- DPBS w/o Ca and Mg [Lonza, Cat. No.: BE17-512F]
- Trypsin Versene® (EDTA) mixture (10x) [Lonza, Cat. No.: BE02-007E] diluted 1:10 in DPBS w/o Ca and Mg [Lonza, Cat. No.: BE17-512F]
- Corning® 96-well Clear Round Bottom Ultra Low Attachment Microplate [Corning, Cat. No.: 7007] or e.g. InSphero GravityTRAP™ ULA Plate 96-well [PerkinElmer, Cat. No.: ISP-09-001]
- White-walled 96-well cell culture plates, e.g. white walled/clear bottom 96-well plates [Greiner Bio-One, Cat. No.: 655098]
- ViaLight™ Plus Cell Proliferation and Cytotoxicity BioAssay [Lonza, Cat. No.: LT07-221]
- ATP [e.g. Lonza, Cat. No.: LT27-008 or Sigma-Aldrich, Cat. No.: A7699-1g]
- Fluoroskan Ascent FL [Labsystems]

Cell Culture

NHDFneo primary cells and HCT 116 cell line were cultured according to the instructions provided by the suppliers, before being plated in 96-well Round Bottom Ultra Low Attachment Microplates. 300 – 10,000 cells were seeded in quadruplicates in 200 µl of the appropriate cell culture medium per well. Microplates were transferred onto an orbital shaker (Edmund Bühler KM2) set to 90 rpm in a standard cell culture incubator (37° C, 5% CO_2). Subsequently, plates were cultured for the indicated number of days. A quadruplicate of cell culture medium alone was included as a background control. The luminescence background of the ViaLight™ Plus BioAssay should be very low.

ViaLight™ Plus BioAssay

For detailed information about the ViaLight™ Plus Cell Proliferation and Cytotoxicity BioAssay Kit please refer to the ViaLight™ Plus Protocol. The assay was used according to the protocol provided by the supplier with slight modifications.

100 µl of medium were removed carefully from each well using a standard multichannel pipet, so that 100 µl of cell culture medium remained. 50 µl of ViaLight™ Cell Lysis Reagent was added to each well and plates were briefly shook (30 seconds, 900 rpm, Fluoroskan Ascent FL). Subsequently, culture plates were incubated at room temperature within the Fluoroskan Ascent FL set to a soft shaking mode (360 rpm). After 60 minutes of incubation, samples were briefly resuspended and 100 µl of cell lysate were transferred into a white-walled 96-well cell culture plate. 100 µl of ViaLight™ Substrate were added to each well. Plates were briefly shook (30 seconds, 900 rpm, Fluoroskan Ascent FL) and incubated for another 2 minutes at room temperature in the dark. Luminescence was measured in the Fluoroskan Ascent FL Reader using a read time of 1 second (integrated).

For standard 2D cell cultures, it is recommended that the plates are not shaken as this can induce frothing. Bubbles produced may deflect the light signal away from the detection unit, reducing the number of RLUs observed and producing an artificially low result. However, for spheroid cultures careful shaking is recommended to ensure complete lysis as well as optimal mixing of the ViaLight™ ATP Monitoring Reagent. Shaking conditions were selected that resulted in minimal to no frothing. In case bubbles were observed despite careful handling, mixing and shaking, a standard hair dryer (Compact 1000, Philips) was used to remove them.

Microscopic Analysis

Spheroids were analyzed with a Zeiss AxioObserver Z.1 microscope equipped with a Zeiss N-Achroplan 10x Objective, appropriate fluorescence filters and an AxioCam MRm camera. To assess lysis efficiency, spheroids were stained for 15 minutes with 0.4 µM Calcein AM [LifeTechnologies, Cat. No.: C3100MP] and 1 µg/ml Propidium Iodide (PI) [Fluka, Cat. No.: 70335]. Subsequently, medium was exchanged to 100 µl of the appropriate cell culture medium containing 1 µg/ml PI. 50 µl of ViaLight™ Plus BioAssay Lysis Buffer were added and spheroids were monitored microscopically at the indicated time points.

Results

Both NHDFneo and HCT 116 cells formed compact spheroids after 72 hours of culture in ultra-low attachment microplates (Figure 3). Interestingly, spheroids formed by NHDFneo have a smaller diameter after several days of cell culture compared to HCT 116 spheroids. This can likely be explained by the slower cell proliferation rate of the primary cells NHDFneo compared to the quickly dividing cancer cell line HCT 116. In 2D cultures, average population doubling times of 15.0 ± 1.9 hours were observed for HCT 116 cells, whereas NHDFneo showed population doubling times of 27.8 ± 10.7 hours.

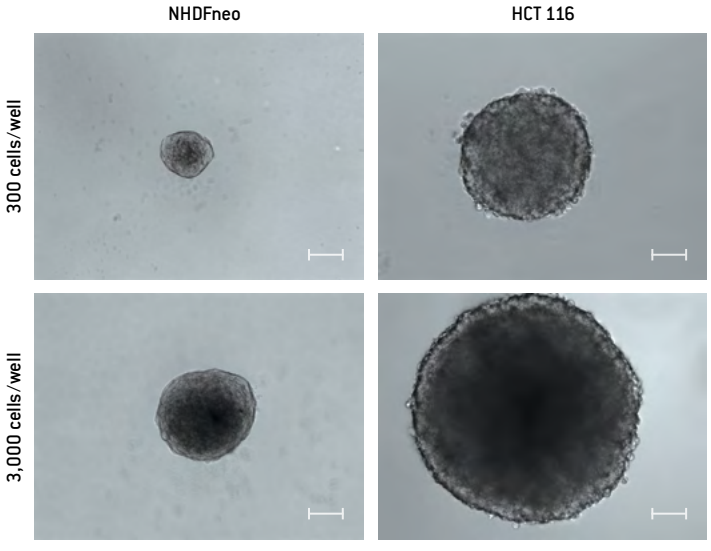

Figure 3
Spheroid formation of NHDFneo and HCT 116 cells. The indicated number of cells was seeded into 96-well ultra-low attachment plates and cultured for 96 hours (NHDFneo) or 72 hours (HCT 116). Scale bar: 100 µm.

In a first experiment an ATP-standard curve was generated to determine the linear range of the assay system used including the Fluoroskan Ascent FL Reader. Since different culture media may quench the light output from the bioluminescent reaction to differing degrees, the ATP standard was always diluted in the respective cell culture medium to obtain ATP concentrations of 0 – 15 µM and 100 µl of each dilution were added in triplicate to the assay plate. Examples of ATP-standard curves are shown in Figure 4. In the two different cell culture media different light output was observed, as measured by the Relative Luminescence Units (RLU). The HCT 116 medium seems to quench the bioluminescent reaction to a higher degree than the FGM™-2 Medium. However, a linear range of the ViaLight™ Plus BioAssay for ATP concentrations of up to 15 µM could be observed in both cell culture media with R^2 values of more than 0.99.

In addition to confirming the linear range of the ViaLight™ Plus BioAssay and allowing the researcher to compare results from cultures grown in different media, the usage of an ATP-standard curve can allow the comparison of experiments executed on different days. However, it needs to be considered that different cell types and cells in different assay conditions can possess different endogenous ATP levels. Therefore, it is advised to create an ATP-standard curve in each experiment in the same plate in which the cell calibration curve samples are generated.

One potential caveat of using cell culture assays established for 2D cultures in 3D cultures is the increased thickness and density of these 3D cultures. Reduced penetration of 3D cultures with assay reagents and insufficient lysis can be the result. Therefore, we tested whether the ViaLight™ Plus Lysis Buffer will permeabilize spheroid cultures.

Our results indicate that 30 minutes of incubation of spheroid cultures with the ViaLight™ Plus Lysis Buffer permeabilized spheroids efficiently as monitored by the entry of PI into the cells (Figures 5 and 6). In order to ensure complete lysis, we used a lysis time of 60 minutes in all subsequently presented experiments. Also, under these conditions, an efficient dissociation of the spheroids after resuspension could be observed (Figure 6).

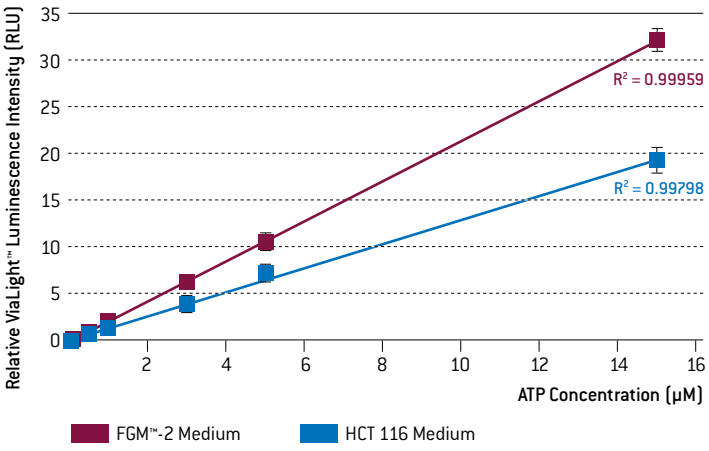

Figure 4

Linear Range of the ViaLight™ Plus BioAssay for an ATP standard in FGM™-2 Medium and HCT 116 medium in a standard white-walled 96-well cell culture plate. Mean luminescence recorded for each triplicate is plotted against the ATP concentration. The error bars represent the standard errors over each triplicate. A linear fit was generated for all ATP concentrations of 0 – 15 µM. The R^2 value for the linear regression is also indicated on the right hand side of the graph.

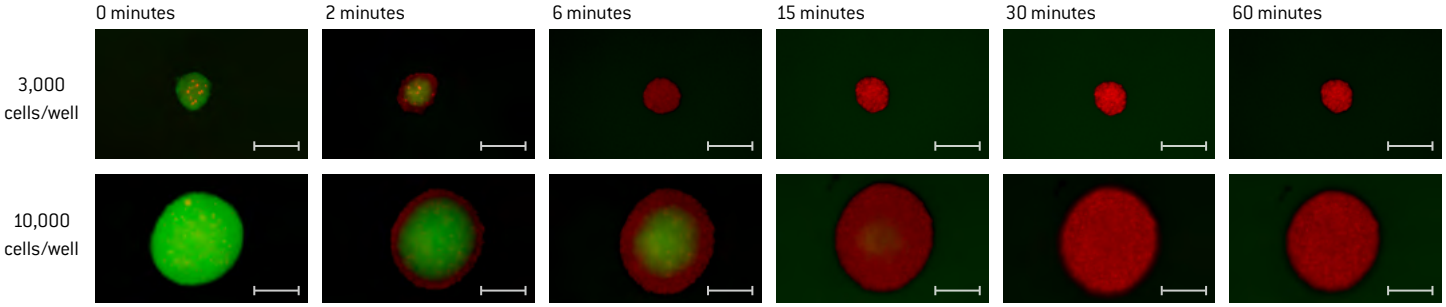

Figure 5

NHDFneo spheroids are efficiently permeabilized by the ViaLight™ Plus BioAssay Lysis Buffer. 3,000 or 10,000 NHDFneo were cultured for 72 hours in 96-well ultra-low attachment plates before being stained Calcein AM for viable cells (green) and PI to detect dead cells (red). Subsequently, 50 µl of ViaLight™ Plus BioAssay Lysis Buffer were added to each well. Dependent on the spheroid size, efficient lysis of the spheroids was observed after 15 or 30 minutes. This is indicated by the loss of Calcein AM fluorescence, while PI-stained nuclei became visible. Scale bar: 200 µm.

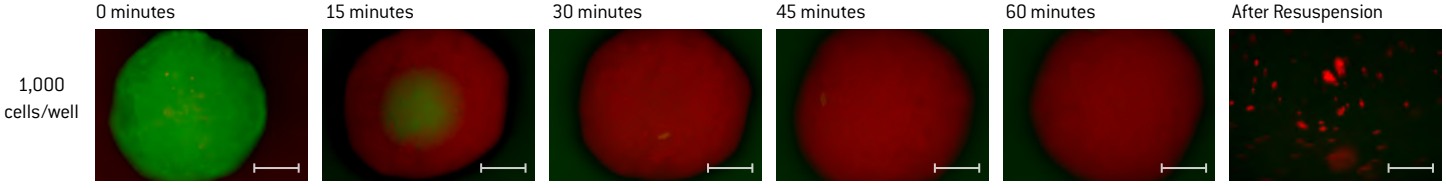

Figure 6

HCT 116 spheroids are efficiently permeabilized by the ViaLight™ Plus BioAssay Lysis Buffer. 1,000 HCT 116 cells were cultured for 5 days in 96-well ultra-low attachment plates before being stained with Calcein AM for viable cells (green) and PI to detect dead cells (red). Subsequently, 50 µl of ViaLight™ Plus BioAssay Lysis Buffer were added. Efficient lysis of the spheroids was observed after 30 minutes. This is indicated by the loss of Calcein AM fluorescence, while PI-stained nuclei became visible. After resuspension, the HCT 116 spheroids dissolved into smaller, PI-positive cell clusters. Scale bar: 200 µm.

In order to ensure that the extended lysis time has no impact on the performance of the ViaLight™ Plus BioAssay, we seeded different numbers of NHDFneo into standard tissue-culture-treated white-walled 96-well cell culture plates and cultured them in 2D for 2 hours in a standard cell culture incubator. Subsequently, we added 50 µl of ViaLight™ Plus Lysis Buffer to each well and incubated the plates for 10 minutes, 30 minutes or 60 minutes at room temperature. After addition of the ViaLight™ Plus ATP Monitoring Reagent, luminescence was measured as described above.

As shown in Figure 7, no clear impact of varying the lysis time between 10 minutes and 60 minutes could be observed. This indicates that a lysis of up to 60 minutes does not interfere with the performance of the ViaLight™ Plus BioAssay. Interestingly, for the highest cell number tested (96,000 cells) slightly higher RLU were observed with lysis times of 30 minutes and 60 minutes compared to 10 minutes. This indicates that for very high cell densities a prolonged lysis time can be beneficial.

In a next step, we analyzed whether the optimized ViaLight™ Plus BioAssay Protocol with the extended lysis time of 60 minutes can actually be applied to spheroid cultures. In Figure 8, we show that for the range of selected seeding densities (0 – 3,000 cells) the response of the ViaLight™ Plus BioAssay is linear for NHDFneo spheroids 72 hours after seeding. The R^2 value for the linear regression is high with an R^2 value of >0.99. In addition, low background luminescence of 0.001 RLU was confirmed in the medium only control.

In Figure 9, we analyzed HCT 116 spheroids with an initial cell seeding density of up to 10,000 cells after 72 hours of culture. Two independent assays showed a similar performance of the assay. A linear assay performance can be observed for initial cell seeding densities of up to 1,000 cells. With higher initial cell seeding densities lower RLU are observed. This is probably caused by slower cell proliferation rates and higher cell mortality at higher cell numbers. With initial cell seeding densities of 3,000 cells, spheroid diameters of more than 500 µm are reached after 72 hours of culture (Figure 3). Under these conditions the core of the HCT 116 spheroids is likely anoxic and nutrient supply is reduced, which limits cell proliferation. An incomplete lysis of the spheroids and insufficient accessibility of the ATP detection reagent can be excluded as a cause of the non-linear assay performance at higher cell seeding densities, as efficient lysis of spheroids could be shown for spheroids with diameters of up to 800 µm (Figure 6).

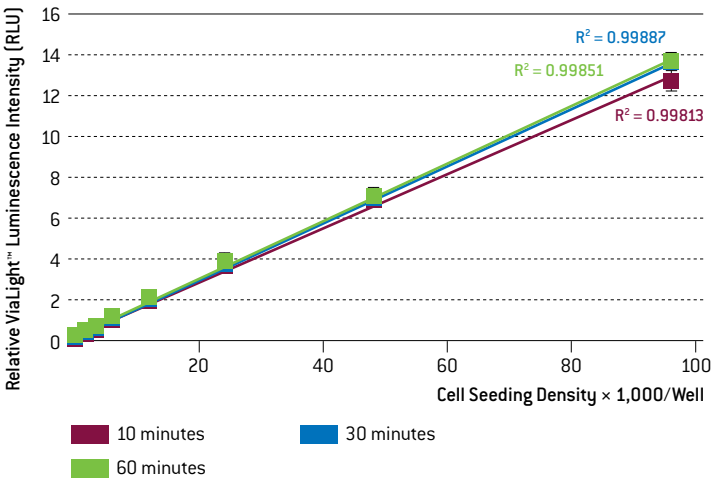

Figure 7

Impact of different lysis time on the ViaLight™ Plus BioAssay performance for NHDFneo in standard 96-well 2D cultures. NHDFneo were seeded in the indicated concentrations in standard white-walled 96-well cell culture plates and cultured for 2 hours at 37°C and 5% CO_2. The mean luminescence recorded for each triplicate is plotted against the cell seeding density. The error bars represent the standard errors over each triplicate. A linear fit was generated for cell densities of 0 – 96,000. The R^2 value for the linear regression is indicated on the right hand side of the graph.

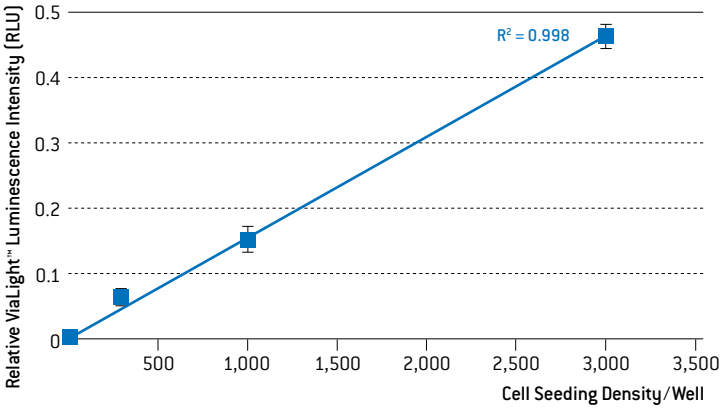

Figure 8

Linear range of the ViaLight™ Plus BioAssay for NHDFneo spheroids. NHDFneo were seeded in the indicated concentrations in 96-well ultra-low attachment plates and cultured for 3 days at 37°C and 5% CO_2. Subsequently, cell viability was assessed using the ViaLight™ Plus BioAssay with a lysis time of 60 minutes. The mean luminescence recorded for each quadruplicate is plotted against the cell seeding density. The error bars represent the standard errors over each quadruplicate. A linear fit was generated for cell seeding densities of 0 – 3,000. The R^2 value for the linear regression is indicated on the right hand side of the graph.

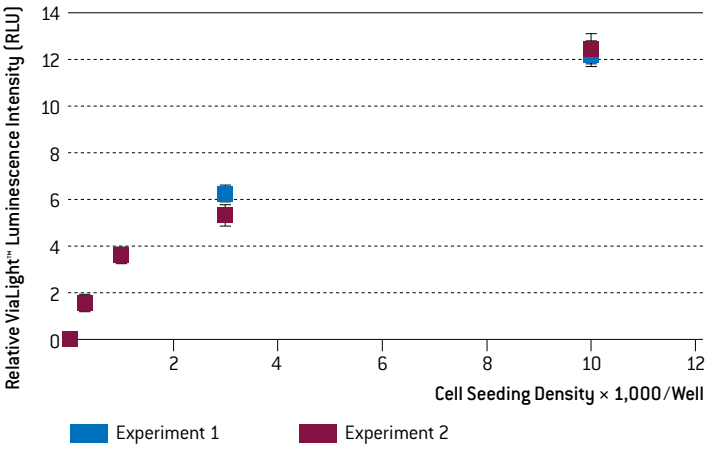

Figure 9

Performance of the ViaLight™ Plus BioAssay for HCT 116 spheroids. In two independent experiments, HCT 116 were seeded in the indicated concentrations in 96-well ultra-low attachment plates and cultured for 3 days at 37°C and 5% CO_2. Subsequently, cell viability was assessed using the ViaLight™ Plus BioAssay with a lysis time of 60 minutes. The mean luminescence recorded for each quadruplicate is plotted against the cell seeding density. The error bars represent the standard errors over each quadruplicate.

Conclusions

In this Technical Note, we show that the ViaLight™ Plus Luminescent Cell Viability BioAssay can be used with spheroid 3D cell cultures with only slight modifications of the protocol. By extending the lysis time from 10 minutes to 60 minutes efficient permeabilization of all cells within spheroids with a diameter of up to 800 µm and complete dissolution of the spheroids could be achieved. For the cell types assessed here, a cell-type dependent linear range of the assay is observed for cell seeding densities of up to 3,000 cells and spheroid diameters of up to 500 µm.

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