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Determination of RAFT™ 3D Cell Culture 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, due to higher cell density and the abundant presence of extracellular matrix molecules.

In this technical note, we show that the ViaLight™ Plus Cell Proliferation and Cytotoxicity BioAssay can be used to assess cell viability in 3D cultures developed with the RAFT™ 3D Cell Culture System in both the 96-well and 24-well format.

The ViaLight™ Assay is based on the bioluminescent detection of cellular ATP as a measure of cell viability (Figure 1). The two-step (Figure 2), non-radioactive assay protocol 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 30 minutes is optimal to determine cell viability in RAFT™ 3D Cell Cultures containing either the HCT 116 colon carcinoma cell line or normal human dermal fibroblasts (NHDF).

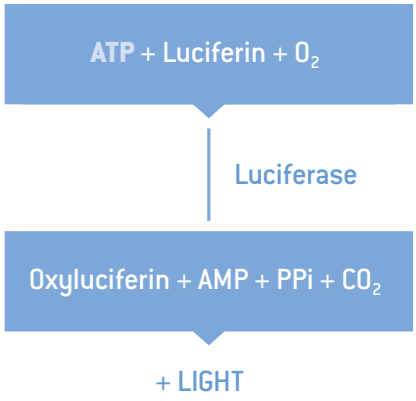


Figure 1
Principle of the ViaLight™ Plus BioAssay. Catalyzed by the luciferase enzyme contained in the ViaLight™ Plus Kit cellular ATP, luciferin and O_2 react to oxyluciferin, AMP, pyrophosphate and CO_2 paralleled by the emission of light that can be detected using suitable luminometers or scintillation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

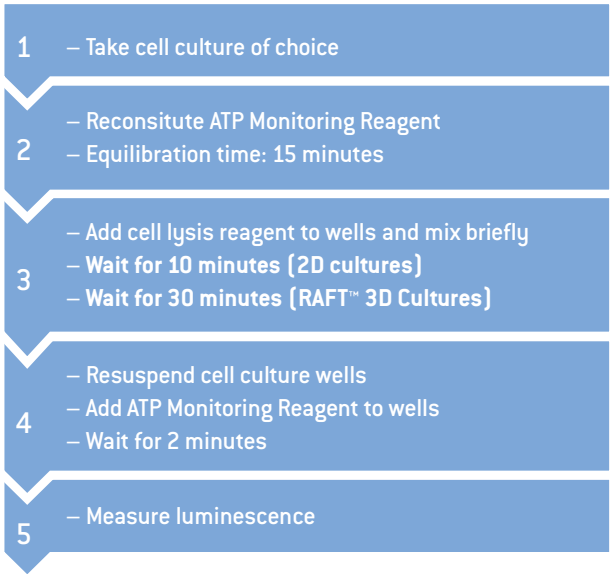


Figure 2
Overview of the ViaLight™ Plus BioAssay Procedure. Please refer to the ViaLight™ Plus Kit Protocol for detailed information. For RAFT™ 3D Cultures it is recommended to increase the lysis time (Step 3) from 10 minutes to 30 minutes to ensure efficient lysis of the approximately 100 – 120 µm thick cultures.

Materials and Methods

- Neonatal 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]
- RAFT™ Reagent Kit for 3D Culture [Lonza Cat. No.: 016-0R94]
- RAFT™ 96-well Absorber Kit [Lonza Cat. No.: 016-0R92]
- RAFT™ 24-well Absorber Kit [Lonza Cat. No.: 016-1R32]
- White-walled 96-well cell culture plates e.g. white walled/clear bottom 96-well plates [Greiner Bio-One Cat. No.: 655098]
- 24-well cell culture plates e.g., Corning® Costar® Cell Culture Plates [Corning Cat. No.: 3526]
- 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)

NHDFneo primary cells and HCT 116 cell line were cultured according to the instructions provided by the supplier before being plated in either 2D cultures or RAFT™ 3D Cultures for cell viability assessment. RAFT™ Cultures were made following the protocol supplied with the RAFT™ 3D Cell Culture Kit. Please refer to the RAFT™ 3D Cell Culture Kit Protocol for more detailed information.

In this example, cells were seeded in triplicates into 96-well cell culture plates or in duplicates into 24-well cell culture plates. Seeding densities of 1,500 – 96,000 cells or 30,000 – 480,000 cells were used in the 96-well format or the 24-well format, respectively. A triplicate of acellular cultures was included as a background control in the ViaLight™ Plus Cell Proliferation and Cytotoxicity BioAssay. The luminescence background in acellular RAFT™ 3D Cultures should be very low and similar to standard 2D cultures.

Standard 2D cultures were set up in either 100 µl (2-hour 96-well cultures), 200 µl (72-hour 96-well cultures) or 500 µl (2-hour 24-well cultures) of FGM™-2 Medium or HCT 116 medium. For RAFT™ 3D Cultures, the same amounts of medium were added to the cultures immediately after removing the RAFT™ Absorbers from the RAFT™ 3D Cultures (Step 5.5 in the RAFT™ 3D Cell Culture Kit Protocol). Subsequently, all cultures were incubated for either 2 hours or 72 hours at 37°C and 5% CO_2 in a standard cell culture incubator [Heracell or Heracell 240, Heraeus].

The ViaLight™ Plus Cell Proliferation and Cytotoxicity BioAssay Kit was used for 2D cultures according to the protocol provided by the supplier. Please refer to the ViaLight™ Plus Kit Protocol for detailed information. For RAFT™ 3D Cultures a slightly modified ViaLight™ Plus Protocol was used.

96-well RAFT™ 3D Cultures

2-hour 96-well RAFT™ 3D Cultures set up with 100 µl culture medium were used for the ViaLight™ Plus Assay as is. 72-hour RAFT™ 3D Cultures were set up with 200 µl of culture medium per well. Therefore, 100 µl of medium was 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, RAFT™ 3D Culture Plates were incubated at room temperature. After 30 minutes of incubation, 100 µl of ViaLight™ ATP Monitoring Reagent was added to each well. Plates were briefly shook again (30 seconds, 900 rpm, Fluoroskan Ascent FL) and incubated for 2 more 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 recommend 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 RAFT™ 3D 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.

24-well RAFT™ 3D Cultures

For 24-well RAFT™ 3D Cultures containing 500 µl of cell culture medium, 250 µl of Lysis Buffer were added to each well and plates were briefly shook (30 seconds, 900 rpm, Fluoroskan Ascent FL). Subsequently, RAFT™ 3D Culture Plates were incubated at room temperature. After 30 minutes of incubation, 100 µl of cell lysate were transferred in duplicate 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).

Results

Two different cell types were used in this study that show different growth behavior in RAFT™ 3D Cell Cultures. NHDFneo grow as individual cells interspersed in the RAFT™ 3D Collagen Scaffold (Figure 3A). This growth behavior resembles the growth of fibroblasts *in vivo*, as e.g. shown by Julie Daniels and colleagues who compared the central human cornea with a bio-engineered cornea based on plastically compressed collagen. In the bio-engineered cornea, human limbal fibroblasts were cultured within the plastically compressed collagen scaffold. Human limbal epithelial cells seeded on top of the collagen formed a stratified layer when airlifted.[1]

In contrast to NHDFneo, the colon cancer cell line HCT 116 forms aggregate-like structures (Figure 3B). The morphology of HCT 116 in RAFT™ 3D Cultures has been analyzed in more detail by Tarig Magdeldin and colleagues. They found a "grape-like" morphology of HCT 116 cell in RAFT™ Cell Cultures.[2] A similar morphology has been observed before in a panel of breast cancer cell lines cultured in Engelbreth-Holm-Swarm tumor extract.[3] Eight out of nine cell lines that formed a grape-like phenotype were isolated from tumor metastasis, indicating an acquired ability of these cells to metastasize over the course of their evolution.[3] Interestingly, HCT 116 cells are reported to show invasive behavior, too, for example in a Balb/c nude male mice model.[4]

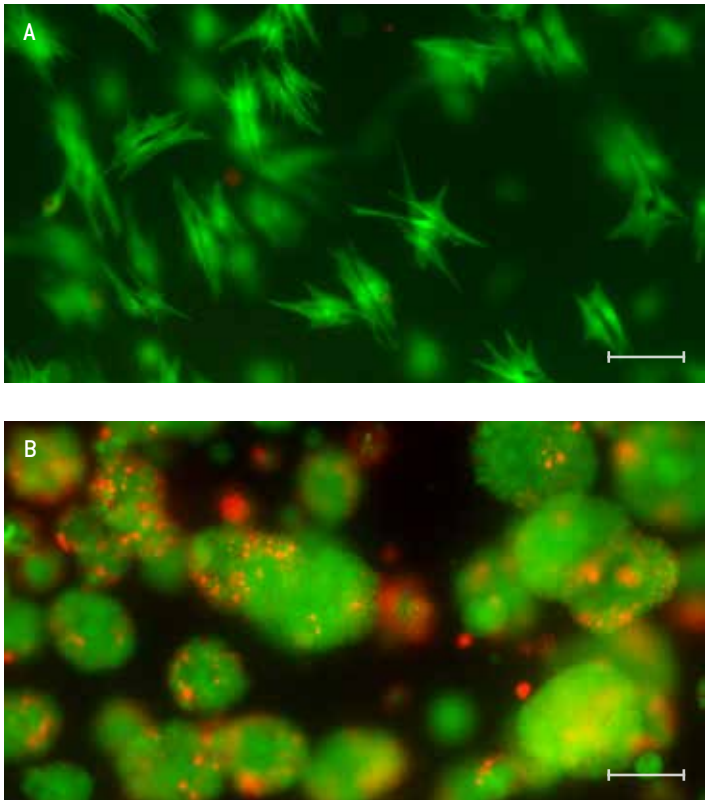


Figure 3

NHDFneo and HCT 116 cells in 24-well RAFT™ Cultures. At the indicated time points cells were stained for 30 minutes with 0.4 µM Calcein AM (LifeTechnologies, C3100MP) for viable cells (green) and 10 µg/ml Propidium Iodide (Fluka Cat. No. 70335) to detect dead cells (red). A) 30,000 NHDFneo cultured for 72 hours B) 50,000 HCT 116 cultured for 6 days. 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.

In addition, the ATP-standard curve was used to analyze whether the presence of RAFT™ 3D Cultures has an impact on the performance of the ViaLight™ Plus Assay. Therefore 100 µl of the ATP standard were applied to either standard white-walled 96-well cell culture plates or to the same type of plates containing 96-well acellular RAFT™ 3D Cultures.

Examples of ATP-standard curves are shown in Figure 4. Interestingly, different light output was observed in the two different cell culture media 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 Assay 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 for all conditions tested. What's more, no negative impact of the RAFT™ Collagen on the performance of the ViaLight™ Plus Assay could be observed.

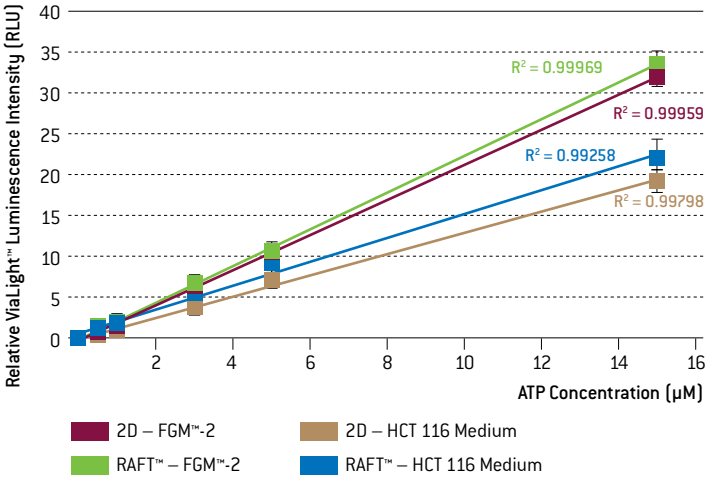


Figure 4

Linear Range of the ViaLight™ Plus BioAssay for an ATP standard in FGM™-2 and HCT 116 Medium in a standard white-walled 96-well cell culture plate with or without acellular RAFT™ 3D Cultures. The 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 indicated on the right hand side of the graph.

In addition to confirming the linear range of the ViaLight™ Plus Assay 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 cells in RAFT™ 3D Cultures. Our results indicate that 10 minutes of incubation of RAFT™ 3D Cultures with the ViaLight™ Plus Lysis Buffer permeabilized NHDFneo in these cultures efficiently as monitored by the entry of Propidium Iodide into the cells (Figure 5). In order to ensure complete lysis also of cellular aggregates in RAFT™ 3D Cultures – like HCT 116 clusters – we used a lysis time of 30 minutes in all subsequently presented RAFT™ 3D Culture ViaLight™ Plus Assays. Under these conditions also an efficient dissociation of the RAFT™ Collagen Scaffold itself could be observed.

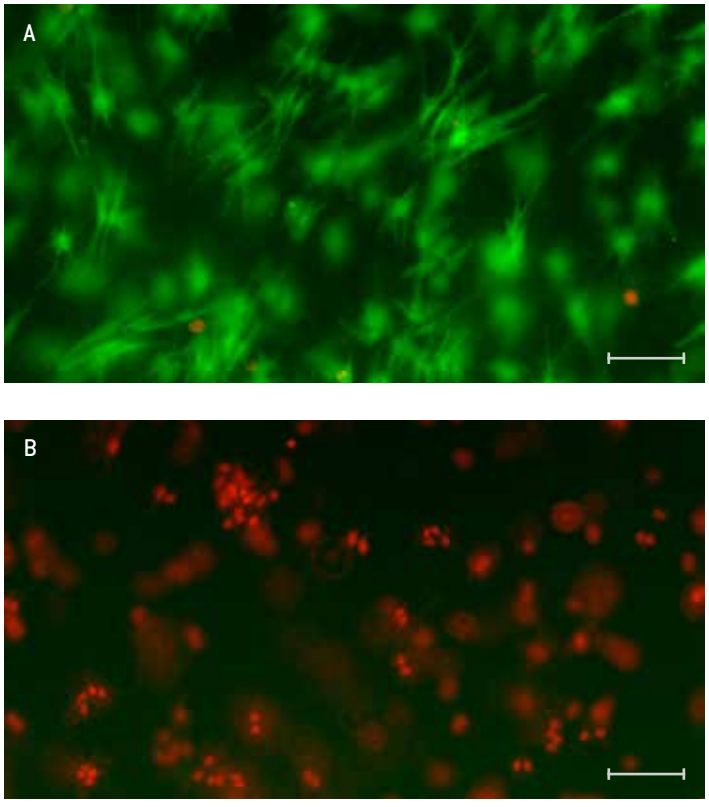


Figure 5

NHDFneo are efficiently permeabilized by the ViaLight™ Plus BioAssay Lysis Buffer. A) 60,000 NHDFneo were cultured for 72 hours in 24-well RAFT™ 3D Cultures before being stained for 30 minutes with 0.4 µM Calcein AM (LifeTechnologies, C3100MP) for viable cells (green) and 10 µg/ml Propidium Iodide (Fluka Cat. No. 70335) to detect dead cells (red). B) Subsequently the cells were exposed to the ViaLight™ Plus BioAssay Lysis Buffer while being monitored microscopically. After 10 minutes efficient lysis of cells was observed, indicated by the loss of Calcein AM fluorescence from the cells, while Propidium Iodide stained nuclei became visible. Scale bar: 100 µm.

In order to ensure that the extended lysis time has no impact on the performance of the ViaLight™ Plus Assay, we seeded different numbers of NHDFneo into standard white-walled 96-well cell culture plates and cultured them 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 6, no clear impact of varying the lysis time between 10 minutes and 60 minutes could be observed. 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.

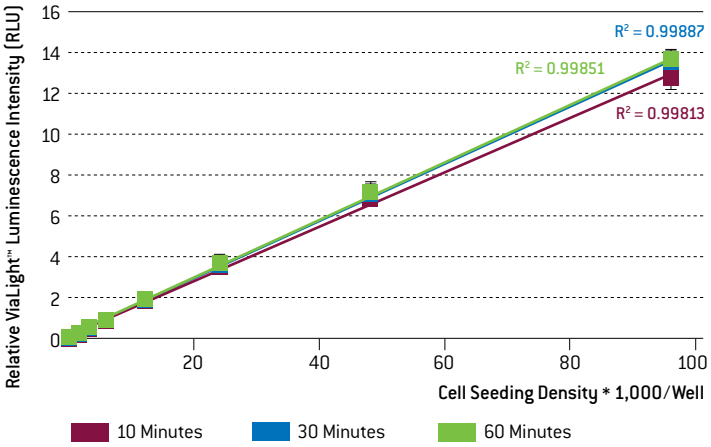
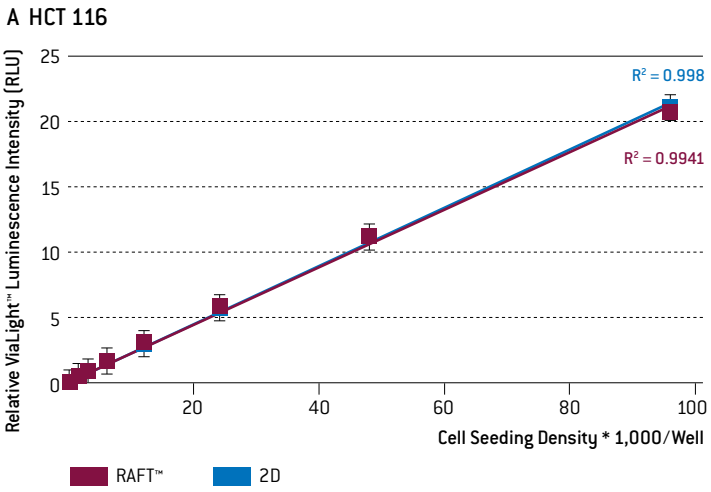


Figure 6

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.

In a next step, we analyzed whether the optimized ViaLight™ Plus BioAssay Protocol with the extended lysis time of 30 minutes can actually be applied to cell-containing RAFT™ 3D Cultures. In Figure 7, we show that for the range of selected cell densities (0 – 96,000 cells) the response of the ViaLight™ Plus BioAssay is linear for both 96-well 2D and RAFT™ 3D Cultures and for both HCT 116 cells and NHDFneo 2 hours after seeding of cells. The R^2 value for the linear regression is high in 2D and in RAFT™ 3D Cultures with R^2 values of >0.95. In addition, low background luminescence of 0.002 RLU was confirmed for acellular RAFT™ 3D Cultures, which is comparable to the value recorded for acellular 2D cultures.

Interestingly, HCT 116 cells show comparable RLU and therefore ATP-levels in both the RAFT™ 3D Cultures and in 2D cultures. On the other hand, lower ATP concentrations were detected in NHDFneo cultured in RAFT™ System as compared to standard 2D cultures. It can be speculated, that the metabolism of at least certain cell types is altered when exposed to a different, more *in vivo*-like cell culture environment. Actual differences in cell number due to enhanced cell proliferation in 2D cultures are not expected under the selected experimental conditions with a time span of only 2 hours between cell culture set-up and viability assessment. Higher mortality of cells within RAFT™ 3D Cultures compared to 2D cultures can also be excluded as a cause for the detected lower RLU, because the Calcein AM staining as shown in Figure 3A or Figure 5A indicates that almost all NHDFneo in the RAFT™ 3D Cultures are Calcein AM-positive and therefore viable, while almost no Propidium Iodide positive, dead cells can be detected.



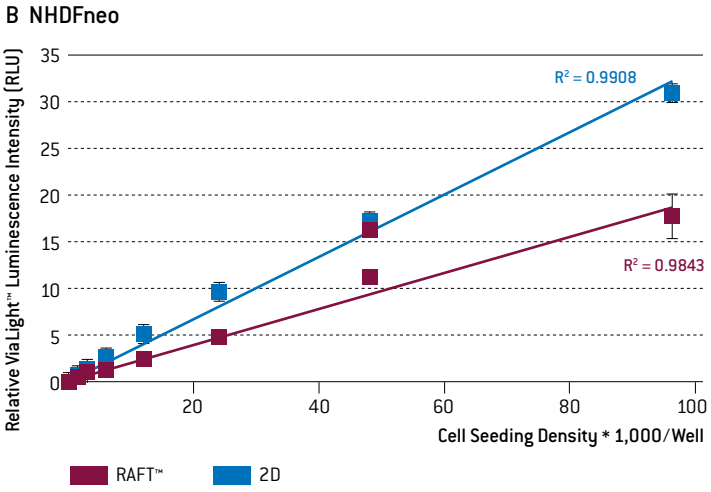
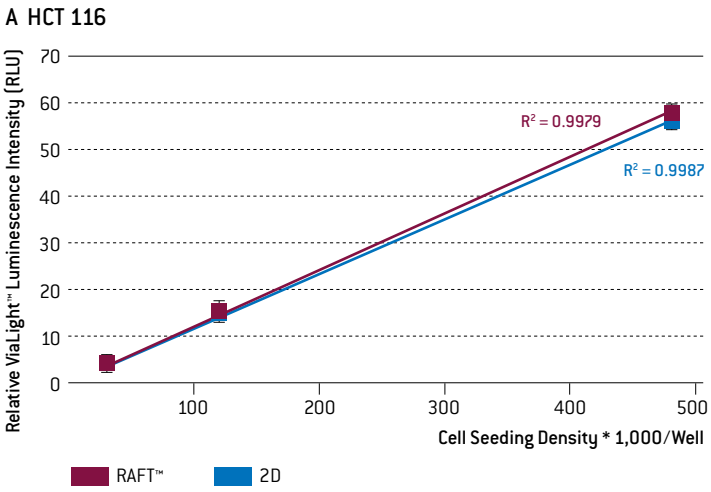


Figure 7

Linear Range of the ViaLight™ Plus BioAssay for A) HCT 116 cells and B) NHDFneo in 96-well 2D and RAFT™ 3D Cultures. Both cell types were seeded in the indicated concentrations in either 2D or RAFT™ 3D Cultures 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.



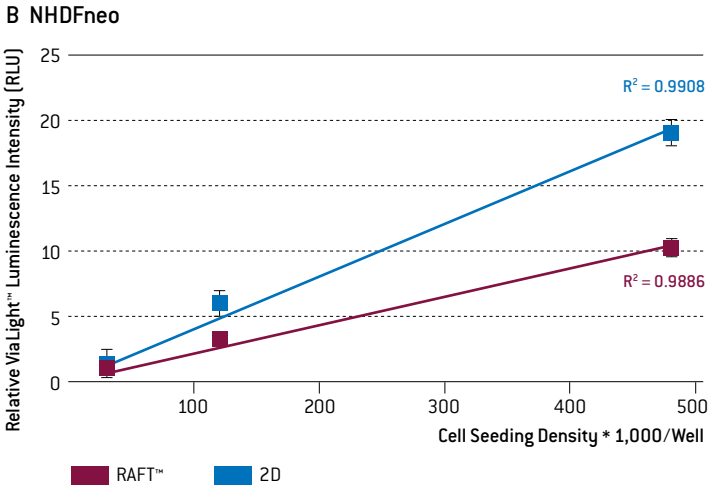


Figure 8

Linear Range of the ViaLight™ Plus BioAssay for A) HCT 116 cells and B) NHDFneo in 24-well 2D and RAFT™ 3D Cultures. Both cell types were seeded in triplicate in the indicated concentrations in either 2D or RAFT™ 3D Cultures in standard 24-well cell culture plates and cultured for 2 hours at 37°C and 5% CO_2. After lysis 100 µl cell lysate was transferred in duplicate into standard white-walled 96-well cell culture plates. After addition of 100 µl ViaLight™ Plus BioAssay Reagent, the mean luminescence recorded is plotted against the cell seeding density. The error bars represent the standard errors. A linear fit was generated for cell densities of up to 480,000. The R^2 value for the linear regression is indicated on the right hand side of the graph.

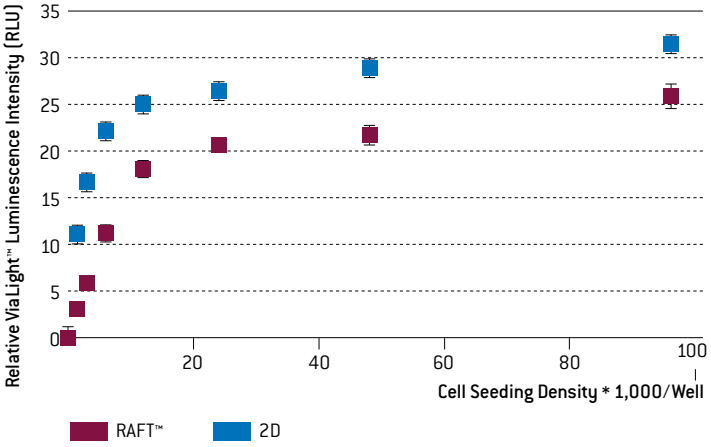
In Figure 8, we show that the ViaLight™ Plus BioAssay can also be applied to RAFT™ 3D Cultures in the 24-well format. A linear performance of the assay could be shown for both HCT 116 cells and NHDFneo with R^2 value of >0.95. In alignment with the 96-well culture results, in the 24-well format HCT 116 cells also showed comparable RLU and therefore ATP-levels in 2D and RAFT™ 3D Cultures, whereas lower ATP concentrations were detected for NHDFneo in RAFT™ 3D Cultures.

Finally, we analyzed the 2D and RAFT™ 3D Cultures 72 hours after seeding with the ViaLight™ Plus BioAssay (Figure 9). Confirming our previous results, also 72 hours after seeding detected ATP-levels were lower in RAFT™ 3D Cultures compared to 2D cultures with the same cell seeding densities. Moreover, this effect was not only observed for NHDFneo, but also for HCT 116 cells.

This observation is in alignment with microscopic observations (data not shown) and results of fluorescence-activated cell scanning (FACS) experiments (data not shown either). Calcein AM staining in combination with Propidium Iodide staining confirmed that reduced cell numbers after 72 hours in RAFT™ 3D Culture are due to reduced cell proliferation and are not caused by increased cell death (see e.g., Figure 3A).

This reduced cell proliferation might be seen as a drawback of 3D culture systems like the RAFT™ System. However, this reduced cell proliferation rate can reflect the *in vivo* growth properties of different cell types. For example fibroblasts usually slowly divide *in vivo* and only when a tissue is injured, the fibroblasts nearby proliferate and migrate into the wound, which helps to isolate and repair the damaged tissue.[5] Similarly, many cancer cell lines have population doubling times of hours to a few days in standard 2D cultures, whereas *in vivo* tumor growth often takes multiple weeks.[6]

A HCT 116



B NHDFneo

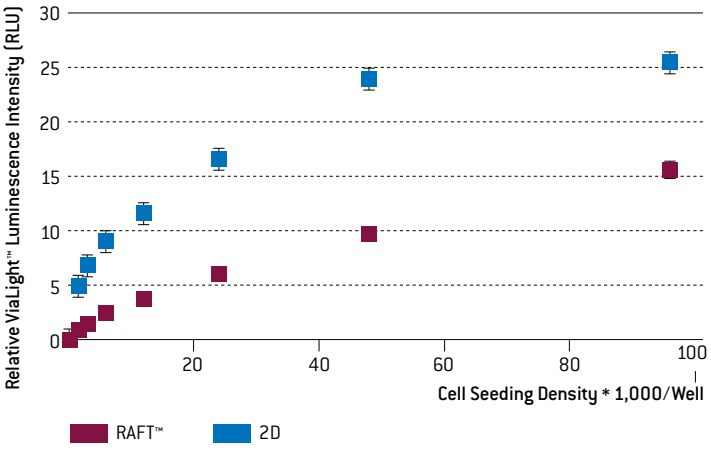


Figure 9

ViaLight™ Plus BioAssay Results for A) HCT 116 cells and B) NHDFneo cultured for 72 hours in 96-well 2D and RAFT™ 3D Cultures. Both cell types were seeded in the indicated concentrations in either 2D or RAFT™ 3D Cultures in standard white-walled 96-well cell culture plates and cultured for 72 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.

Conclusions

In this technical note, we show that ViaLight™ Plus Luminescent Cell Viability Assay can be used with RAFT™ 3D Cell Cultures with only slight modifications of the protocol. By extending the lysis time from 10 minutes to 30 minutes, efficient permeabilization of all cells within the RAFT™ 3D Cultures and complete dissolution of the RAFT™ 3D Collagen Scaffold could be achieved. For the cell types assessed here, a linear range of the assay is observed between 0 and 96,000 cells per 96-well and for up to 480,000 cells per 24-well.

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