Bioscience Solutions

Creating RAFT™ 3D Cell Cultures with Different Thicknesses and Different Cell Types

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Introduction

The RAFT™ 3D Cell Culture System allows the culture of different cell types within a collagen type 1 matrix that mimics tissue-like environment more closely. Using the RAFT™ Standard Protocol, 3D Cell Cultures with a thickness of approximately 100 µm are obtained.

In this Technical Note, we show how RAFT™ 3D Cell Cultures with different thicknesses ranging from approximately 30 µm to 140 µm can be generated with only a few minor modifications to the standard protocol. RAFT™ Cultures with decreased thickness could be of interest when limited amounts of cells are available and there is a need to reduce number of cells used per RAFT™ Culture. When quick proliferating cells or high cell density models are used, better oxygen and nutrient supply can likely be achieved in thinner cultures resulting in higher cell viability. On the other hand, thicker RAFT™ Cultures could be of interest for example for tumor models. The increased thickness gives so-called tumoroids more room to grow into all three dimensions.

This Technical Note also explains how two different cell types – neonatal Normal Human Dermal Fibroblasts (NHDFneo) and the colon cancer cell line HCT 116 – can be cultured in two distinct RAFT™ Layers. This proof-of-principle experiment can be the starting point for the development of more complex tissue models. For example, larger blood vessels are surrounded by distinct layers of smooth muscle cells and fibroblasts.[1]

Materials and Methods

- NHDFneo (Lonza Cat. No.: CC-2509)
- FGM™-2 BulletKit™ Media (Lonza Cat No.: CC-3132)
- Reagent Pack™ Subculture Reagents (Lonza Cat. No.: CC-5034)
- HCT 116 cells (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)
- D-PBS (Lonza Cat. No.: BE17-512F)
- Trypsin (Lonza Cat. No: BE02-007E)
- RAFT™ Reagent Kit for 3D Culture (Lonza Cat. No.: 016-0R94)
- RAFT™ 96-well Absorber Kit (Lonza Cat. No.: 016-0R92)
- ViaLight™ Plus Cell Proliferation and Cytotoxicity BioAssay (Lonza Cat. No.: LT07-221)
- CalceinAM (Fisher Scientific Cat. No.: C3100MP)
- Propidium Iodide (Sigma/Aldrich Cat. No.: 70335)
- Total Collagen Assay (Quickzyme Biosciences Cat. No.: QZBTOTCOL2)
- CTS2 Device (Lein Applied Diagnostics)
- Accuracy balance (Denver Instrument SI-234)
- Zeiss Microscope Axio Observer Z.1 (Zeiss)
- Fluoroskan Ascent FL (Labsystems)

Set-up of RAFT™ 3D Cultures

NHDFneo and HCT 116 cells were cultured according to the instructions provided by the supplier, before being plated in RAFT™ 3D Cultures. The RAFT™ Collagen Mix was created following the protocol supplied with the RAFT™ 3D Cell Culture Kit.

For cultures with different thicknesses, cell-collagen mix volumes of 80 µL, 160 µL, 240 µL (standard volume as a control culture), or 320 µL per well were dispensed in triplicate into the wells of a standard 96-well cell culture plate. After the 15 minutes gelling step at 37° C / 5% CO_2, the cultures were compressed according to the protocol using the specialized RAFT™ Absorbers. Subsequently, 200 µL of corresponding medium was added to each well. RAFT™ Cultures were incubated for 48 hours at 37°C and 5% CO_2 in a standard cell culture incubator (Heraeus).

For NHDFneo final cell concentration was 100,000 cells/mL, which corresponds to 24,000 cells per standard 96-well RAFT™ Culture. For HCT 116 final cell concentration was 200,000 cells/mL, which corresponds to 48,000 cells per standard 96-well RAFT™ Culture. Please refer to the RAFT™ 3D Cell Culture Kit Protocol for more detailed information.

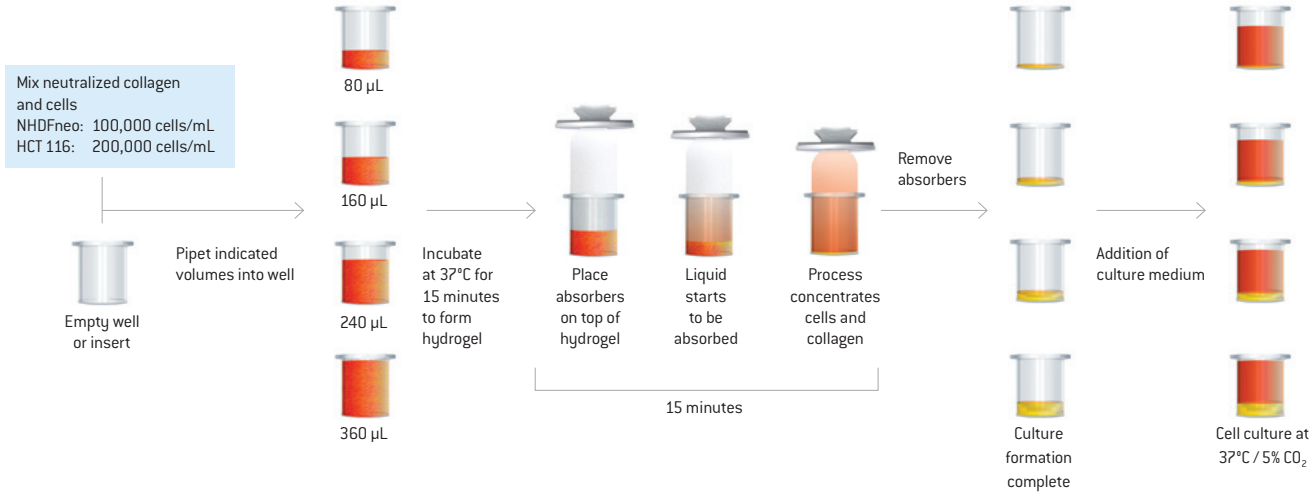

Figure 1
Creation of RAFT™ Cultures with different thicknesses. For details see Materials and Methods – Set-up of RAFT™ 3D Cultures.

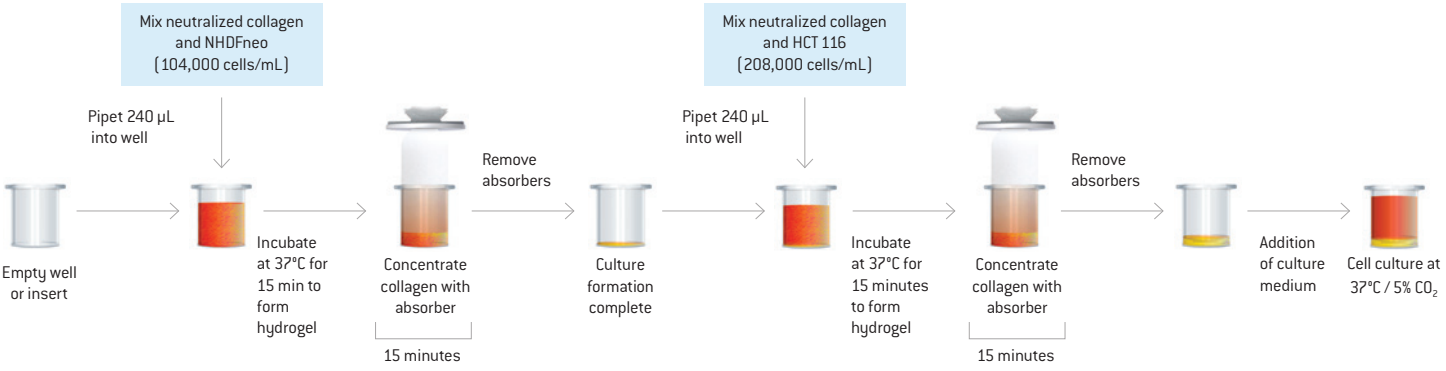

Figure 2
Creation of RAFT™ Cultures containing two different cell types in two distinct RAFT™ Culture Layers. For details see Materials and Methods – Set-up of RAFT™ 3D Cultures.

For RAFT™ Cultures containing two different cell types, a first RAFT™ Culture with NHDFneo cells (25,000 cells in 240 µL collagen mix/well) was created 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. Afterwards a second culture with HCT 116 cells (50,000 cells/well) was prepared on top of the first RAFT™ Culture following again the supplied protocol. 200 µL of FGM™-2 Media was added to each well. RAFT™ Cultures were incubated for 48 hours at 37°C and 5% CO_2 in a standard cell culture incubator (Heraeus).

Analysis of RAFT™ 3D Cultures

The thickness of the resulting RAFT™ Cultures was determined with a CTS2 Device (Lein Applied Diagnostics), following the guidelines of the supplier. The CTS2 Device is a non-contact thickness measurement system designed to measure 3D-engineered tissues within standard tissue culture plates. For measuring the thickness of 24-hour old RAFT™ Cultures, cell culture medium was removed completely from the cultures. The cell culture plate was placed on the calibrated CTS2 Device and each sample was measured in triplicate.

To determine the weight of the RAFT™ Culture, each culture was removed carefully from the well with forceps and tapped on a paper towel to remove excessive cell culture medium. Each culture was transferred to an object slide and weighed using an accuracy balance.

Measuring Cell Distribution and Viability in RAFT™ 3D Cultures

To monitor cell viability and distribution in the RAFT™ Cultures, the cultures were stained with CalceinAM and Propidium Iodide (PI). The cultures were washed for 10 minutes with 100 µL D-PBS at room temperature. Subsequently, the cultures were stained with 100 µL of 0.4 µM CalceinAM / 10 µg/mL PI in D-PBS for 15 minutes at room temperature in the dark. Pictures were taken with the Zeiss Axio Observer Z.1 microscope equipped with appropriate fluorescence filters and a 10x objective.

The ViaLight™ Plus Cell Proliferation and Cytotoxicity BioAssay Kit was used with a slightly modified protocol to quantitatively determine cell viability in RAFT™ Cultures. Please refer to the RAFT™ and ViaLight White Paper for details.

Results

RAFT™ Cultures with Alternative Thicknesses

To obtain RAFT™ Cultures with alternative thicknesses different volumes of cell-collagen mix were pipetted into the wells of a 96-well plate and compressed using the standard RAFT™ Protocol. The 240 µL represent the standard volume for RAFT™ Cultures. The maximum volume of 320 µL was determined by the maximum filling volume for a 96-well plate well. As shown in Figure 3A, the average thickness of a cell containing RAFT™ Culture was approximately 100 µm when made out of 240 µL cell-collagen mix. This was independent of the cell type embedded. When the cell-collagen volume was increased to 320 µL, the culture thickness increased accordingly to approximately 140 µm. When the cell-collagen volume was reduced to 160 µL, the thickness of the RAFT™ Cultures decreased to a corresponding 60 µm. The thickness of the 80 µL RAFT™ Culture was determined to be approximately 50 µm by the CTS2 Device. This is likely a measuring artifact as the optimal measurement range of the CTS2 Device has been left.

The results of the CTS2 Device could be confirmed by determining the wet weight of the RAFT™ Cultures (Figure 3B). Standard 240 µL starting volume cultures had a weight of ~3 mg after compression, whereas 320 µL cultures had a wet weight of ~4.5 mg and 160 µL cultures of ~2 mg. The 80 µL starting volume cultures had a wet weight of ~1 mg. This indicates that a reduction of the collagen starting volume to a third decreased the weight of the final culture also to a third. Accordingly, the result of the CTS2 Device for such thin cultures seems to be indeed a measurement artifact.

While the final thickness of RAFT™ Cultures is dependent on the starting collagen-cell mix volume, the final collagen concentration was not altered and comparable in all cultures. This was confirmed with the Total Collagen Assay Kit (data not shown).

In order to evaluate whether a changed RAFT™ Culture thickness has an impact on cell viability, the RAFT™ Cultures were analyzed using the ViaLight™ Plus Cell Proliferation and Cytotoxicity BioAssay Kit. It could be shown that cell viability is comparable in all NHDFneo containing cell cultures, when normalized to 240 µL cell-collagen starting volume (set to 100 in Figure 4).

In HCT 116 cultures, relative cell viability or the amount of cells per collagen volume decreased with increasing culture thickness. This could indicate that for high cell numbers or quick proliferating cells, thinner cultures could be beneficial with respect to e.g. nutrient and oxygen supply.

As shown in Figure 5, good cell viability was confirmed in all cultures using CalceinAM/PI staining. Few PI positive, dead cells were observed in all cultures, while the majority of cells were viable and Calcein AM positive. Also comparable cell morphology and distribution was observed in all cultures.

In summary, these results indicate that RAFT™ Cultures with different thicknesses can be obtained, while maintaining the performance and composition of standard RAFT™ Cultures.

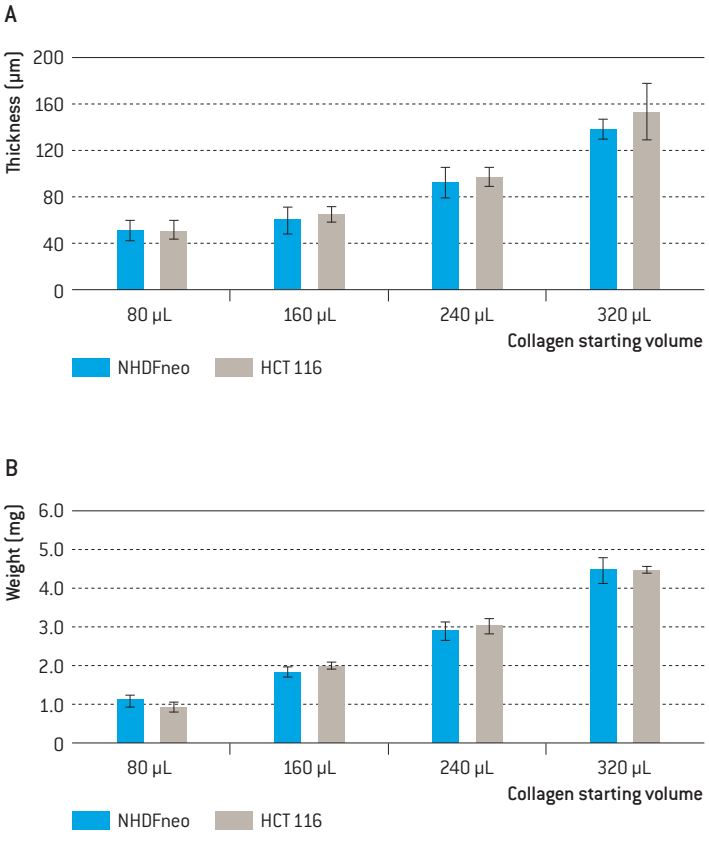

Figure 3

Dependence of thickness of RAFT™ 3D Cultures from collagen starting volume. **A)** Thickness of RAFT™ Cultures measured with the non-contact CTS2 Device according to the method described above. **B)** Wet weight of RAFT™ cultures. The thickness and the wet weight of RAFT™ Cultures correlate with the respective collagen starting volume. (n = 3)

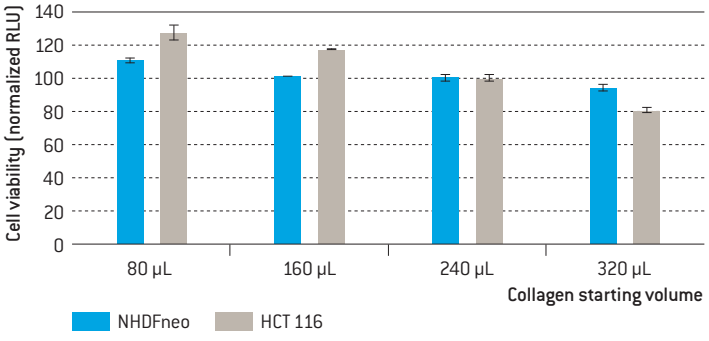

Figure 4

Impact of thickness of RAFT™ 3D Cultures on cell viability. RAFT™ Cultures containing either NHDFneo or HCT 116 were generated with the indicated starting volumes. 48 hours after culture set-up, cell viability was determined with the ViaLight™ Plus Cell Proliferation and Cytotoxicity BioAssay Kit. The raw data was normalized for each cell type to the standard collagen volume of 240 µL. The viability of the standard 240 µL RAFT™ Culture was set to 100 and all other cultures are represented accordingly. (n = 3)

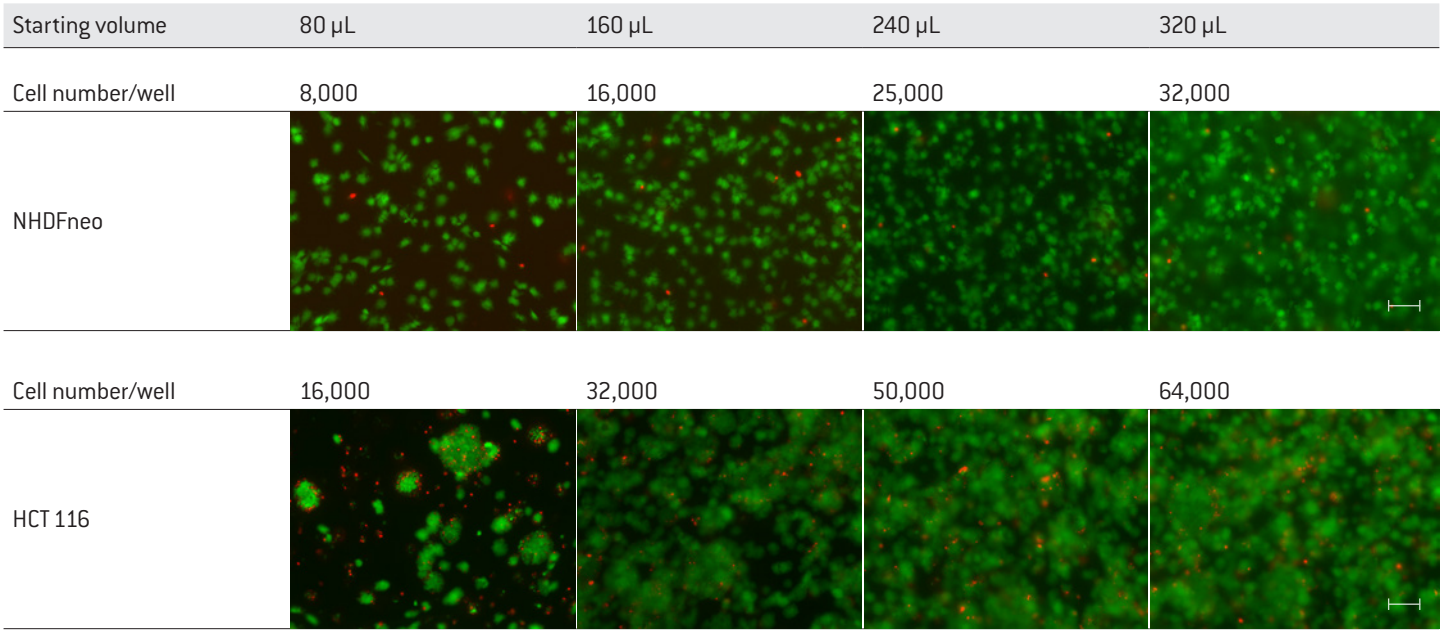

Figure 5

Viability and morphology of NHDFneo and HCT 116 cells in RAFT™ Cultures with alternative thicknesses. RAFT™ Collagen-mix was prepared with a cell concentration of 100,000 cells/mL for the NHDFneo cells and 200,000 cells/mL for the HCT 116 cells. The indicated volumes were dispensed in a 96-well plate and compressed following the standard RAFT™ Protocol. Cells were cultivated for 48 hours at 37°C / 5% CO_2 before being stained with CalceinAM to detect viable cells (green) and PI to detect dead cells (red). In all cultures, the majority of cells were CalceinAM positive and only few PI positive cells could be detected, indicating good cell viability in all cultures. Scale bar: 100 µm

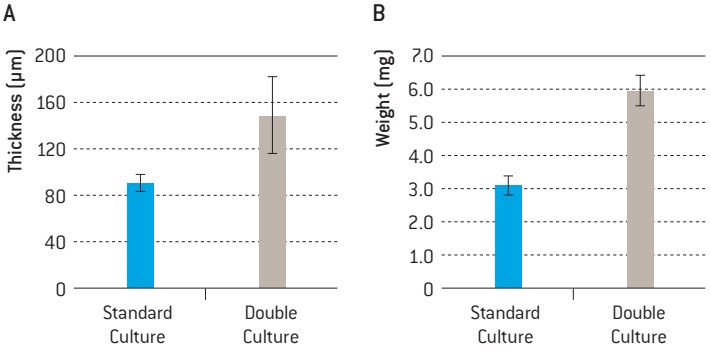

Figure 6

Generation of two-layered RAFT™ Cultures. The lower layer contained NHDFneo while the upper layer contained HCT 116 cells. 24 hours after creation of the RAFT™ Cultures **A)** the thickness was measured with the non-contact CTS2 Device and **B)** the wet weight was determined. The two-layered RAFT™ Culture was approximately twice as thick as a standard one-layer RAFT™ Culture.

RAFT™ Cultures with Two Layers

Many tissues are composed of different cell types in a specific spatial orientation. For example larger blood vessels are surrounded by distinct layers of smooth muscle cells and fibroblasts.[1] Therefore, it was evaluated whether a RAFT™ Culture containing NHDFneo could be overlaid with a second RAFT™ Culture containing HCT 116 cells.

The creation of "double" RAFT™ Cultures turned out to be technically feasible. Interestingly, no distinct collagen layers were visible by eye and it was not possible to separate the two RAFT™ Layers using e.g. forceps. In comparison to a standard culture, the thickness of the two-layered RAFT™ Culture increased from 90 ± 8 µm to 149 ± 33 µm (Figure 6A). Accordingly, the wet weight of the cultures doubled from 3.1 ± 0.3 mg to 6.0 ± 0.5 mg (Figure 6B).

Microscopic observation of the two-layered RAFT™ Culture after CalceinAM/PI staining confirmed good cell viability (Figure 7). 24 hours after creation of the culture, only few PI positive, dead cells were observed, while the majority of cells were viable and Calcein AM positive. Using CalceinAM/PI staining it was not possible to differentiate between the upper and the lower RAFT™ Layer and between NHDFneo and HCT 116 cells.

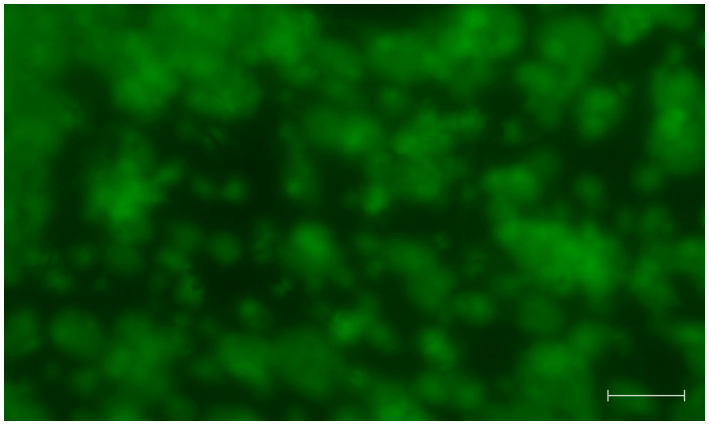

Figure 7
Viability of NHDFneo and HCT 116 cells in double-layered RAFT™ Cultures. Two-layered RAFT™ Cultures were generated with the lower layer containing NHDFneo and the upper layer containing HCT 116 cells. 24 hours after creation, the RAFT™ Cultures were stained with CalceinAM to detect viable cells (green) and PI to detect dead cells (red). It was not possible to distinguish between NHDFneo and HCT 116 cells. However, it was observed that the majority of cells were CalceinAM positive and only few PI positive cells could be detected, indicating overall good cell viability. Scale bar: 100 µm

Conclusions

We show in this Technical Note that it is feasible to generate RAFT™ Cultures with alternative thicknesses by varying the amount of collagen-cell mix used for initiating the RAFT™ Cultures. For 96-well plate RAFT™ Cultures, decreasing the starting volume to 80 µL reduces the thickness of the resulting RAFT™ Culture to approximately 30 µm. This could be of benefit when limited amounts of cells are available or for long-term, high cell density models, where optimal oxygen and nutrient supply is required. Increasing the starting volume to 320 µL increases the thickness to approximately 140 µm. A further increase in starting volume is not possible due to the maximum filling volume of a 96-well plate. In addition, with increasing thickness a slightly negative impact on cell viability was observed – in particularly for the quick proliferating HCT 116 colon cancer cell line. However, thicker RAFT™ Cultures could be of interest for example for larger, 3-dimensional tumor models.

We could also show that it is technically feasible to create RAFT™ Cultures consisting of two layers while maintaining overall good cell viability. With the methods applied in this Technical Note it was not possible to distinguish between the different layers of the resulting RAFT™ Culture. For this it would be required to stain the two different cell populations using different dyes prior to embedding the cells in the RAFT™ Culture. Alternatively, an immunocytochemistry approach with antibodies that are specific for markers characteristic for the two different cell types could be taken. However, this Technical Note gives first evidence that it could be possible to generate more complex tissue models containing different cell types in different parts of a RAFT™ Culture. This can be the starting point for the development of more complex tissue models like ones mimicking larger blood vessels that are surrounded by distinct layers of smooth muscle cells and fibroblasts.[1]

References

1. RT Schermuly et al. (2011) Mechanisms of disease: pulmonary arterial hypertension. *Nature Reviews Cardiology,* 8, 443–455

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