Harvesting Cells from RAFT™ 3D Cell Cultures

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Introduction

The RAFT™ 3D Cell Culture System allows the culture of different cell types within a tissue-like environment that is based on collagen type I. Cells like fibroblasts, smooth muscle cells, astrocytes or cancer cells can be embedded within the RAFT™ Collagen. For the formation of barrier models, endothelial or epithelial cells can be seeded on top of RAFT™ Cultures.

RAFT™ Cultures can be analyzed directly using a variety of methods including immunocytochemistry, histology or viability assays. For some analysis methods like Fluorescence-activated Cell Scanning (FACS) it is required to retrieve the cells from RAFT™ Cultures.

In this Technical Note, we explain how Normal Human Lung Fibroblasts (NHLF) and the colon cancer cell line HCT 116 can be recovered from within RAFT™ Cultures [Figure 1A and 1B]. In addition, we show that Normal Human Bronchial Epithelial Cells (NHBE) cultured on top of RAFT™ Cultures [Figure 1C and 1D] can be harvested while leaving the collagen culture mostly intact. This allows for separation of two different cell populations within and on top of RAFT™ Cultures.

Materials and Methods

- NHLF [Lonza Cat. No.: CC-2512]
- FGM™-2 BulletKit™ Medium [Lonza Cat. No.: CC-3132]
- NHBE [Lonza Cat. No.: CC-2540]
- BEGM™ BulletKit™ Medium [Lonza Cat. No.: CC-3170]
- 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 (PAN Biotech Cat. No.: P30-3306) and 1% Penicillin/Streptomycin (Lonza Cat. No.: DE17-602E)
- D-PBS [Lonza Cat. No.: BE17-512F]
- HBSS [Lonza Cat. No.: BE10-527F]
- RAFT™ Reagent Kit for 3D Culture [Lonza Cat. No.: 016-0R94]
- RAFT™ 96-well Absorber Kit [Lonza Cat. No.: 016-0R32]
- RAFT™ 24-well Absorber Kit [Lonza Cat. No.: 016-1R32]
- Collagenase from Clostridium histolyticum [Sigma-Aldrich Cat. No.: C-9722]
- EDTA [Invitrogen Cat. No. 15575-038]
- Propidium Iodide [Sigma-Aldrich Cat. No.: 70335]
- CalceinAM [Fisher Scientific Cat. No.: C3100MP]
- CellTracker™ Orange CMRA Dye [ThermoFisherScientific Cat. No.: C34551]
- CellTracker™ Green CMFDA Dye [ThermoFisherScientific Cat. No.: C7025]
- 96-well U-bottom plates [Falcon Cat. No.: 353910]
- APC Mouse Anti-Human CD90 Clone 5E10 [BD Biosciences Cat. No.: 559869]
- APC Mouse IgG1, κ Isotype Control Clone MOPC-21 [BD Biosciences Cat. No.: 555751]
- Beriglobin [CSL Behring, 160 mg/mL]
- BSA [Sigma-Aldrich Cat. No.: A-7906]
- APC Mouse Anti-Human CD40 Clone 5E10 [BD Biosciences Cat. No.: 559869]
- APC Mouse IgG1, κ Isotype Control Clone MOPC-21 [BD Biosciences Cat. No.: 555751]
- FACSCalibur® (BD 343202 - FACSCALIBUR 4 CLR BASIC SENSOR UNIT) equipped with an HTS module
- FACSCalibur® (BD 343202 - FACSCALIBUR 4 CLR BASIC SENSOR UNIT) equipped with an HTS module
Bioscience Solutions – Harvesting Cells from RAFT™ 3D Cell Cultures

– Zeiss Microscope Axio Observer Z.1 (Zeiss) equipped with an AxioCam Mrm Camera (Zeiss Cat. No.: 426509-9901-000) and an Apotome (Zeiss Cat. No.: 1156-290)
– Olympus CX40 Microscope (Olympus)
– Standard cell culture incubator (Heraeus, HERAcell™ 240) set to 37°C and 5% CO2
– Centrifuge (Heraeus Megafuge 1.0R)

Set-up of RAFT™ 3D Cultures
NHLF, HCT 116 and NHBE cells were cultured according to the instructions provided by supplier, before being seeded within or on top of RAFT™ 3D Cultures. The RAFT™ Collagen Mix was prepared following the protocol supplied with the RAFT™ 3D Cell Culture Kit.

For up to 72 hours old RAFT™ Cultures, NHLF were used with a final cell concentration of 100,000 cells/mL of neutralized collagen, which corresponds to 24,000 cells per standard 96-well RAFT™ Culture. For HCT 116 cell concentration ranged from 85,000 cells/mL to 375,000 cells for up to 72 hours old RAFT™ Cultures, which corresponds to 20,000 to 90,000 cells per standard 96-well RAFT™ Culture. Please refer to the RAFT™ 3D Cell Culture Kit Protocol for more detailed information. For longer culture duration, cell numbers might need to be reduced to compensate for cell proliferation.

240 µL of cell-collagen mix were dispensed in triplicate into the wells of a standard 96-well cell culture plate. 1,300 µL of cell-collagen mix were used for 24-well RAFT™ Cultures. After 15 minutes gelling at 37°C / 5% CO2, the cultures were compressed according to protocol using the specialized RAFT™ Absorbers. Subsequently, 200 µL (96-well plate) or 1,000 µL (24-well plate) of corresponding cell culture medium was added to each well. RAFT™ Cultures were incubated in a standard cell culture incubator until further use.

NHBE cells were re-suspended in their respective cell media and added on top of RAFT™ Cultures after compression (Figure 1C). 40,000 cells were applied in 200 µL of cell culture medium per 96-well RAFT™ Culture. For acellular RAFT™ Cultures, 100% BEGM™ BulletKit™ Medium was used (Figure 1C). For NHBE cells on NHLF containing RAFT™ Cultures, a 1:1 mixture of BEGM™ BulletKit™ Medium:FGM™-2 BulletKit™ Medium was used (Figure 1D). As a control, the same cell numbers were seeded into standard 2D 96-well cell culture plates.

Harvest of Cells from within RAFT™ 3D Cultures
RAFT™ Cultures were washed twice with HBSS for 10 minutes at room temperature. (Please find information about the required buffer volumes in Table 1.) After washing, Collagenase from Clostridium histolyticum was added at a concentration of 1 mg/mL (2.7 U/mL) in HBSS. Culture plates were incubated for approximately 30 minutes in a standard cell culture incubator. Dissociation of RAFT™ Cultures was controlled under a standard cell culture microscope. At the end of the dissociation process, the collagen scaffold wasn’t visible anymore and cells were seen as single cells or small cell clusters. Once RAFT™ Cultures were dissolved, appropriate cell culture medium was added. Samples were gently but thoroughly re-suspended and used for downstream analysis. In some cases, for example in long-term cultures with high cell density, it was required to agitate the RAFT™ Cultures every 5 – 10 minutes by pipetting during the collagenase incubation step.

<table>
<thead>
<tr>
<th>Washing step</th>
<th>96-well</th>
<th>24-well</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td>100 µL</td>
<td>1 mL</td>
</tr>
<tr>
<td>Repetitions</td>
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<td>2</td>
</tr>
<tr>
<td>Incubation time</td>
<td>10 minutes</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Incubation temperature</td>
<td>Room temperature</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Collagenase-I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume</td>
<td>100 µL</td>
<td>200 µL</td>
</tr>
<tr>
<td>Concentration</td>
<td>1 mg/mL</td>
<td>1 mg/mL</td>
</tr>
<tr>
<td>Incubation time</td>
<td>30 minutes</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Incubation temperature</td>
<td>37°C (incubator)</td>
<td>37°C (incubator)</td>
</tr>
<tr>
<td>Medium</td>
<td>100 µL</td>
<td>200 µL</td>
</tr>
</tbody>
</table>

Table 1
Harvest of cells from within RAFT™ 3D Cultures - Overview
Harvest of Cells from the Top of RAFT™ 3D Cultures

The Reagent Pack™ Subculture Kit was used to harvest NHBE cells from the top of RAFT™ Cultures. This kit has been designed specifically for the harvesting and passaging of primary cell types. RAFT™ Cultures were washed once with HEPES Buffered Saline and 75 µL of Trypsin/EDTA were applied to each 96-well RAFT™ Culture. Culture plates were incubated for 5 – 10 minutes in a standard cell culture incubator. Cell detachment was observed under a standard cell culture microscope. Once the majority of cells were detached from the RAFT™ Culture, the reaction was stopped by the addition of 75 µL appropriate Fetal Calf Serum (FCS)-containing cell culture medium or Trypsin Neutralizing Solution (TNS). Samples were re-suspended carefully. The supernatant containing the detached cells was transferred into another vessel for further analysis. The remaining RAFT™ Cultures can be used for further analysis. For example, cells can be harvested from within the RAFT™ Cultures using the protocol above.

Cell Counting

Cells were counted with the Nucleocounter® NC-250™. 20 µL of cell suspension were gently mixed with 1 µL Solution 18. Subsequently, 12 µL of cell suspension were transferred into a chamber of a NC-Slide A8. The slide was inserted into the Nucleocounter® NC-250™ and the number of viable cells per mL and the percentage of viable cells were measured according to the protocols provided by Chemometec. The number of cells per well or RAFT™ Culture were calculated to determine cell harvest efficiency or seeding efficiency.

FACS Analysis

For FACS analysis, 130 µL cell suspension was transferred into a 96-well U-bottom plate. An APC-coupled mouse-anti-human CD90 antibody (Clone 5E10; BD Biosciences Cat. No.: 559869) or an appropriate isotype control were diluted 1:110 in D-PBS supplemented with 0.5% Bovine Serum Albumin (BSA) and 1.6 mg/mL Beriglobin. Beriglobin is used to prevent unspecific antibody binding. 100 µL of antibody suspension was added to each sample and incubated for 30 minutes at room temperature in the dark. Subsequently, cells were pelleted by centrifugation for 10 minutes at room temperature and 130 g (800 rpm, Heraeus Megafuge 1.0R). 170 µL of supernatant were discarded and cells were re-suspend in 70 µL of D-PBS/0.5% BSA. 5 µL PI (5 µg/mL) and 20 µL APC beads (500/µL) were added to each sample. 100 µL of each sample were analyzed on a BD FACS Calibur equipped with an HTS-module at a flow rate of 3 µL per second. Data was analyzed with the Cell Quest Pro Software [BD, Version 5.2.1].

Results

Cells Can Be Efficiently Retrieved from RAFT™ 3D Cell Cultures

RAFT™ Cultures containing NHLF were created according to standard procedures in either 96-well cell culture plates or 24-well cell culture plates. Cells were harvested with Collagenase from Clostridium histolyticum (Sigma-Aldrich Cat. No.: C-9722) according to the protocol described above. As depicted in Figure 2A, approximately 80% of the seeded NHLF were recovered from the RAFT™ Cultures after 24 hours of culture. It can be speculated, that at least part of the remaining 20% of NHLF died during seeding, culture, harvest or were lost during washing and centrifugation steps. Viability of harvested cells was determined to be around 95% (Figure 2B).

Other classical cell harvest reagents like Trypsin/EDTA, Accutase, Dispase or TrypLE™ Select were not suitable for dissolving RAFT™ Cultures (data not shown). Other collagenase preparations than the one used in this study, might require a different concentration of the enzyme and optimization of incubation times.

Harvesting of NHLF from RAFT™ 3D Cultures. A) Approximately 80% of NHLF were recovered from 24-hour old 96-well (n = 3) or 24-well RAFT™ Cultures. (n = 3) B) Viability of harvested cells was determined to be around 95%, (n = 3)
For HCT 116 colon cancer cells comparable results as for NHLF were obtained. Approximately 110% of seeded HCT 116 cells could be retrieved after 24 hours of RAFT™ Culture. The recovery rate of more than 100% is very likely caused by cell proliferation (Figure 3A).

One difference that was observed between NHLF and HCT 116 was that NHLF were harvested mostly as single cells from RAFT™ Cultures (Figure 4A). In contrast, HCT 116 cells tend to form tumoroid-like cell aggregates in RAFT™ Cultures. These tumoroids could not be dissolved by collagenase treatment alone (Figure 4B). In this case, and dependent on the desired downstream analysis method, further steps like additional Trypsin/EDTA treatment might be required.

Overall, it needs to be considered that the given protocol has been optimized for 1 – 4-day old RAFT™ Cultures containing NHLF or HCT 116 cells. However, different cell types tend to modify the RAFT™ Collagen Matrix in different ways\(^1\) and tend to behave differently. Therefore, the harvest protocol presented here can only serve as a basic guideline on how to retrieve cells from RAFT™ Cultures. Other cell types, higher cell numbers, combinations of different cell types, longer culture periods or different culture conditions might require modifications of the presented protocol.

Cells Harvested from RAFT™ 3D Cell Cultures Can Be Re-plated into Standard 2D Culture

In order to get a basic understanding of the functionality of cells harvested from RAFT™ Cultures, we determined the seeding efficiency of those cells by plating them into standard 2D cell culture plates. Cell re-attachment is dependent on the absence of collagenase activity in the sample. In comparison to trypsin, collagenase is not inactivated by for example Fetal Calf Serum (FCS). Therefore, we tested alternative methods to remove remaining collagenase activity from the cell samples. As option 1, EDTA was added to a final concentration of 20 mM. As option 2, cells were pelleted by centrifugation for 8 minutes at room temperature and 130 g before being re-suspended in fresh cell culture medium. As option 3, both methods were combined. All options were compared to samples that were neither centrifuged nor treated with EDTA.

As shown in Figure 5, treatment with 20 mM EDTA followed by a centrifugation step is required to obtain good cell re-attachment.
Cells Can Be Efficiently Retrieved from the Top of RAFT™ 3D Cell Cultures

RAFT™ Cultures without cells were created according to standard procedures in 96-well cell culture plates. Cultures were overlaid with 40,000 NHBE. NHBE were harvested with Trypsin/EDTA according to the protocol described above.

Figure 6A shows that comparable numbers of NHBE could be retrieved from RAFT™ Cultures and from standard 2D cultures. Viability of harvested NHBE was determined to be around 88% (Figure 6B), which is in alignment with standard 2D cultures. Similar results were obtained for primary Human Mammary Epithelial Cells (data not shown).

RAFT™ Cultures appeared to be unaffected by the removal of the NHBE from the top of the culture using Trypsin/EDTA. Therefore, it was evaluated whether NHLF embedded in remaining RAFT™ Culture can be retrieved using collagenase. In addition, the purity of the resulting cell populations was investigated using FACS analysis.

In order to distinguish between NHLF and NHBE, samples were stained against the fibroblast marker CD90. As shown in Figure 7, more than 95% of the NHLF express high levels of CD90, in both standard 2D cultures [Figure 7A] and in RAFT™ 3D Cultures [Figure 7B]. This indicates that cells isolated from RAFT™ Cultures using collagenase are suitable for FACS analysis. Also, general surface markers are not significantly impacted by the harvesting process. However, this needs to be specifically investigated for each marker as different markers can respond differently to the harvesting process. In addition, the results show that the typical fibroblast marker protein CD90 expression is well-maintained in RAFT™ 3D Cultures.
On the other hand, more than 95% of NHBE cells showed considerably less anti-CD90 fluorescence in FACS analysis compared to NHLF, and were therefore considered to be CD90 negative. This was observed for both standard 2D cultures (Figure 7D) as well as for NHBE seeded on top of RAFT™ Cultures (Figure 7E). Therefore, CD90 could be used as a marker to distinguish the two cell types.

For further analysis, RAFT™ Cultures were created containing NHLF within the collagen scaffold and NHBE on top of the scaffold. NHBE harvested from top of the RAFT™ Culture using Trypsin/EDTA were approximately 95% CD90 negative (Figure 7F). This confirms the macroscopic observation that the collagen scaffold remained rather intact during Trypsin/EDTA treatment and the resulting NHBE population is only minimally contaminated with NHLF. When the remaining RAFT™ Culture was dissolved using collagenase treatment, the resulting cell population was ~85% CD90 positive and ~15% CD90 negative (Figure 7G). This indicates that some NHBE were present in the fibroblast population.

One potential reason for the presence of NHBE in the NHLF population could be incomplete NHBE-detachment from the RAFT™ Culture. However, increasing the time used for Trypsin/EDTA treatment from 10 minutes to 30 minutes did not result in increased harvest efficiency (data not shown). In addition, about 100% of seeded NHBE could be retrieved from the top of the RAFT™ Cultures. Another explanation could be the invasion of epithelial cells into the collagen scaffold. In that case, the NHBE might not be accessible to Trypsin/EDTA harvest. However, this could not be proven microscopically. In a preliminary study, the two different cell populations were stained with CellTracker™ Green and CellTracker™ Orange and subsequently analyzed using fluorescence microscopy in combination with optical sectioning (Zeiss Microscope Axio Observer Z.1 equipped with an Apotome). In the resulting images, the NHBE were found in the upper layer of the RAFT™ Cultures, whereas the NHLF were embedded in the RAFT™ Cultures (data not shown).

In summary, we provide a protocol to harvest cells embedded within and from the top of RAFT™ Cultures. In the described harvesting process, the RAFT™ Culture can serve as kind of a boundary between the two different cell populations. This can lead to two separate cell populations after the harvesting process and facilitate the independent analysis of both cell populations in downstream assays. However, since the separation is not complete and dependent on the desired downstream analysis method, it might be required to further purify the cell populations using established cell culture tools like Fluorescence-activated Cell Sorting or Magnetic-activated Cell Sorting (MACS®).

Conclusions

In this Technical Note, we explain how cells like NHLF and the colon cancer cell line HCT 116 can be recovered from within RAFT™ Cultures. A similar approach has already been published by Sreekanthreddy et al. [2016]² and by Magdeldin et al. [2014].³

Harvesting efficiency in approximately 24-hour RAFT™ Cultures was determined to be 80 – 100% using the protocol provided in this Technical Note, while maintaining good cell viability. This supports analysis methods like FACS. In addition, cells from RAFT™ Cultures can be re-seeded in 2D cultures. For that, neutralization of collagenase activity with EDTA followed by centrifugation is recommended.

In addition, we show that NHBE cultured on top of RAFT™ Cultures can be harvested, while leaving the remaining collagen culture mostly intact. This allows the separation of two different cell populations within and on top of RAFT™ Cultures.

Overall, this protocol is intended to be a starting point for how to harvest cells from RAFT™ Cultures. Since other cell types, higher cell numbers, combinations of different cell types, longer culture periods or different culture conditions can lead to RAFT™ Cultures with different properties than the ones presented here, this may require modifications of the protocol for optimal results.

References

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