Using the RAFT™ System as a versatile tool to build *in vitro* models relevant for toxicity testing

Lonza
Pharma & Biotech

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Introduction

Conventional *in vitro* assays are based on cells grown on two-dimensional (2D) substrates, which are not representative for the true *in vivo* cell environment. In tissues, cells interact with neighboring cells and with the extracellular matrix (ECM). Three-dimensional (3D) cell culture methods mimic these interactions and allow cells to grow in structures resembling more the *in vivo* environment.
The RAFT™ 3D Culture System uses a collagen matrix at physiologically relevant concentrations. The resulting models provide valuable tools to investigate tissue function in an *in vivo*-like *in vitro* system. Here we illustrate how the three different physiological barriers skin, lung and mammary epithelium can be modeled using the RAFT™ 3D Culture System.

Materials and methods

Cell Culture: All cells were obtained from Lonza and cultured according to manufacturer's protocols before being transferred into RAFT™ Cultures. The following cell types and culture media were used: **Skin**: Clonetics™ Human Dermal Fibroblasts (NHDF) with Fibroblast Growth Medium (FGM™ 2) medium, Human Epidermal Keratinocytes (NHEK) with Keratinocyte Basal Medium (KBM™ Gold) and KGM™ CD SingleQuots™ (KGM™ Gold CD). **Lung**: Normal and Diseased (Asthma) Human Bronchial Epithelial (NHBE; DHBE) with Bronchial Epithelial Cell Growth Medium (BEGM™), Normal and Diseased (Asthma) Human Bronchial Smooth Muscle (BSMC; DBSMC-AS) with Smooth muscle Growth Medium (SmGM™ 2). **Liver**: For control cultures, 65,000 hepatocytes were plated on collagen coated 96-well plates (Corning, Cat. No. 354407) in complete Hepatocyte Plating Medium (Lonza, Cat. No. MP-100-1) or in complete Hepatocyte Culture Medium (HCM, Lonza, Cat. No. CC-3199 and CC-4182). Cells were overlayed with 0.3 mg/mL Matrigel™ (Corning, Cat. No. 354234).
RAFT™ 3D Cell Culture System: RAFT™ 3D Cell Culture Kits (Lonza) were used for the formation of cell seeded collagen gels as per the manufacturer's protocol. Briefly, cells and neutralized collagen were mixed and dispensed into wells of 96-well, 24-well, or 24-well insert cell culture plates, and subsequently incubated at 37°C to allow hydrogel formation. RAFT™ Absorbers were placed on top of the hydrogels. The absorbers gently remove abundant medium and compact the hydrogel to a layer approximately 100 µm thick (Figure 1). The cultures are then ready to use, but additional epithelial or endothelial cells may be added on top. For details about the individual models, please see individual tissue models sections.

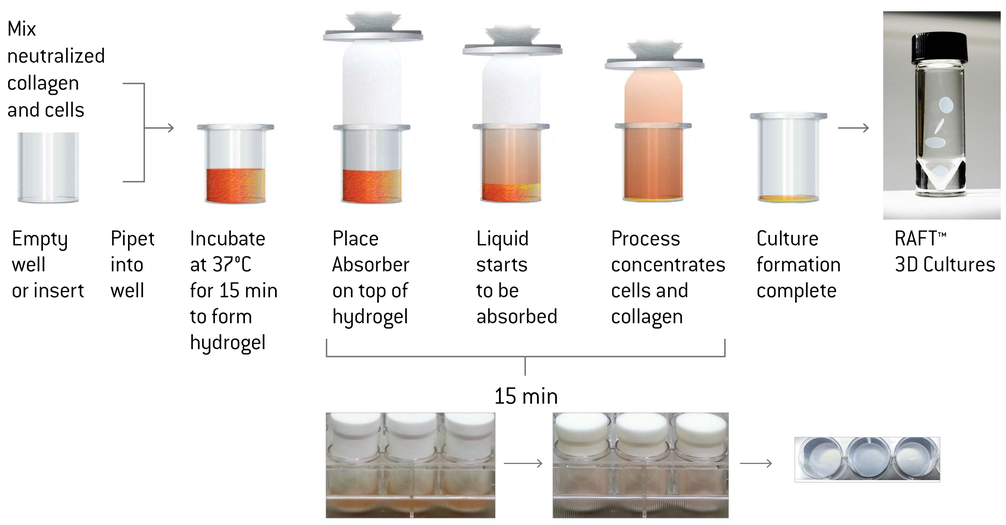

Figure 1. RAFT™ 3D Cell Culture Process Overview

Immunohistochemistry: Skin equivalent tissues were fixed in 4% paraformaldehyde and processed for histology. Sections were either Hematoxylin and Eosin stained or deparaffinized and labeled with the indicated antibodies. Other RAFT™ Cultures were fixed with methanol, washed with PBS and stained with the indicated antibodies (red) as well as DAPI (blue, nuclei).
ELISA: Spent media were collected for ELISA and analyzed using Raybiotech's Quantibody® Human Angiogenesis Array 3 testing service.

Bronchial epithelial model

A 3D lung co-culture model containing normal or asthmatic bronchial epithelial and smooth muscle cells was created by embedding BSMC within the RAFT™ Collagen, followed by an overlay with HBE. In comparison to standard 2D co-culture, no undesired overgrowth of HBE was observed in the 3D model. Differences in the secretion of CXCL16 (see Figure 2B) as well as *e.g.* Follistatin, uPAR and VEGF (data not shown) were observed between normal and asthmatic cultures as well as between different culture systems.

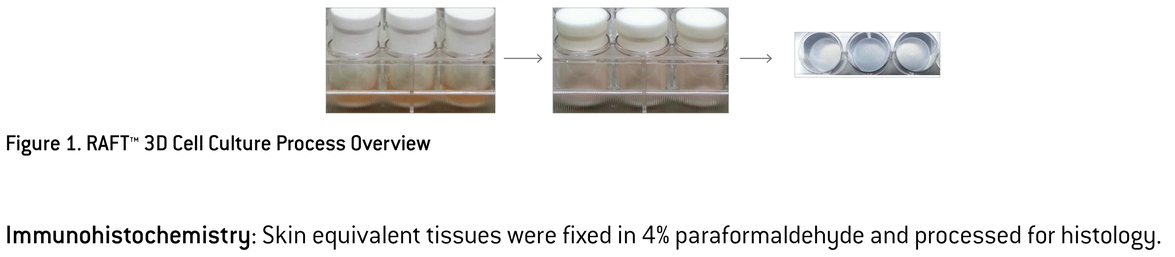

Figure 2. Bronchial Epithelial RAFT™ Model.
(A) Visual representation of the model. BSMC were embedded in collagen and overlaid with HBE. (B) ELISA analysis on spent culture medium from day 7. Differences in CXCL16 levels were observed between normal and asthmatic cells as well as between different culture systems. Immuno-fluorescent labeling of (C) BSMC for α smooth muscle actin (red) and (D) NHBE for pan cytokeratin (red) in RAFT™ 3D Cultures. Scale bars: 100 µm

Summary

- The RAFT™ 3D Cell Culture System allows the co-culture of different cell types in physiological orientation.
- In contrast to standard 2D cultures, undesired overgrowth of certain cell types is observed to a lesser extent in RAFT™ 3D Cultures.
- Phenotypic differences, for example in cell morphology or protein secretion, are detected when comparing cells cultivated in the RAFT™ 3D System to those in standard 2D culture.
- Primary hepatocytes survived and remained more metabolically active for up to 17 days in culture, which enables long-term drug metabolism studies and toxicity analysis.

Skin model

A full-thickness skin model was generated by embedding NHDF in 120 µL of RAFT™ Collagen in a 24-well transwell insert followed by a compression step according to RAFT™ Kit instructions. Subsequently, 60 µL of NHDF/collagen solution was added without further compression. The dermis layer was allowed to develop for 5 days, before NHEK were added. 48 hours after seeding, medium was exchanged to CnT-PR-FTAL (CellnTec). After another 24 hours, cultures were air-lifted allowing keratinocyte differentiation for 10-12 days. The resulting cultures resembled actual human skin (Figure 4).

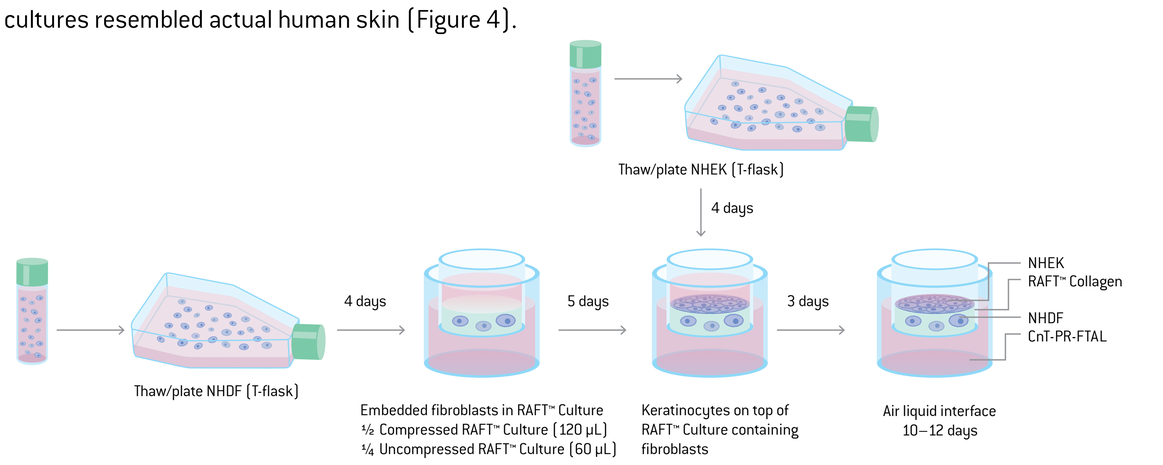

Figure 3. Creation of a RAFT™ 3D skin model

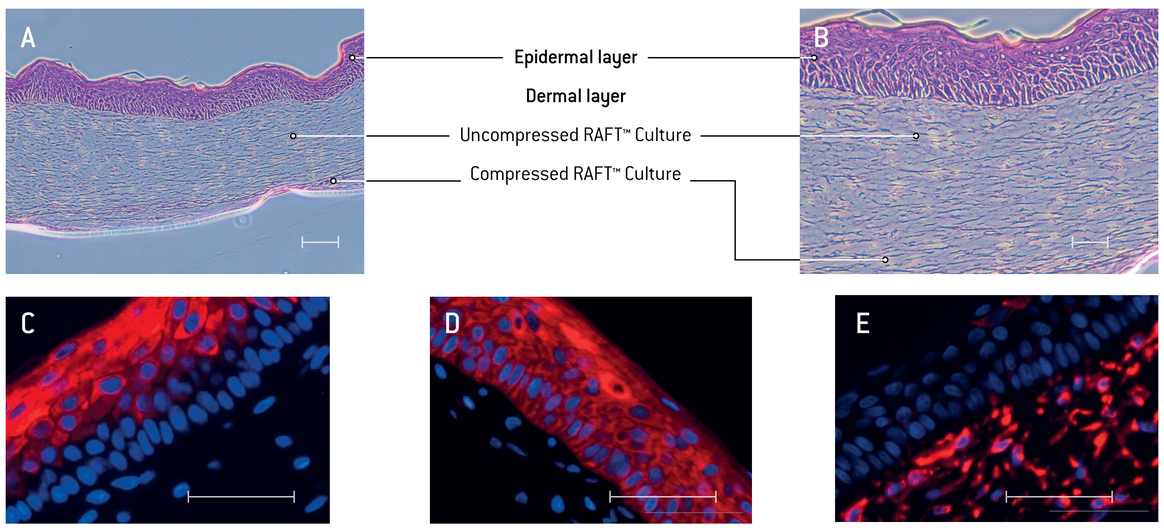

Figure 4. Histology of RAFT™ Skin model. (A, B) Hemotoxylin and eosin staining showed a distinct epidermal layer containing differentiated epidermal keratinocytes juxtaposed to the dermal layer containing dermal fibroblasts in RAFT™ Hydrogel. (C, D) Immunofluorescent labeling of cytokeratin 10 (red, C), cytokeratin 14 (red, D) and vimentin (red, E). Scale bars: 100 µm (A), 50 µm (B – E)

Liver model

A 3D liver model was created by embedding 65,000 cryopreserved primary human hepatocytes within the RAFT™ Collagen. Hepatocytes survived and remained more metabolically active for longer periods of time than in the traditional Sandwich Culture.

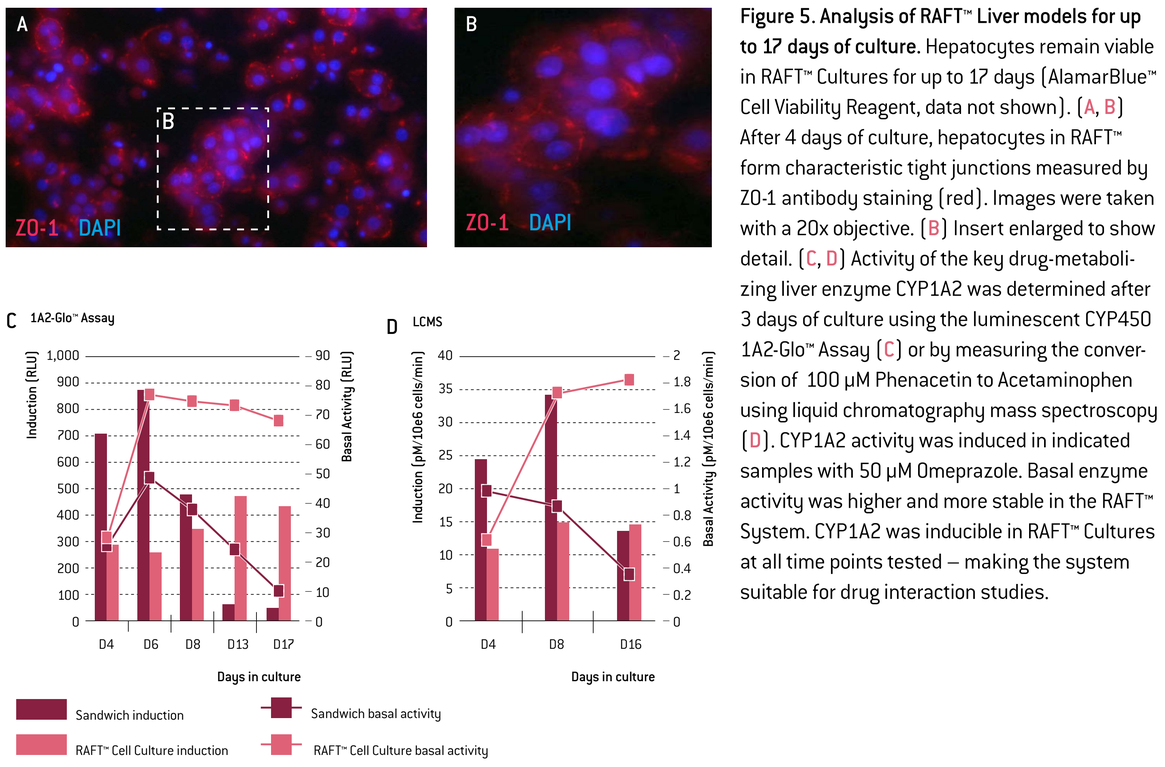

Figure 5. Analysis of RAFT™ Liver models for up to 17 days of culture. Hepatocytes remain viable in RAFT™ Cultures for up to 17 days (AlamarBlue™ Cell Viability Reagent, data not shown). (A, B) After 4 days of culture, hepatocytes in RAFT™ form characteristic tight junctions measured by ZO-1 antibody staining (red). Images were taken with a 20x objective. (B) Insert enlarged to show detail. (C, D) Activity of the key drug-metabolizing liver enzyme CYP1A2 was determined after 3 days of culture using the luminescent CYP450 1A2-Glo™ Assay (C) or by measuring the conversion of 100 µM Phenacetin to Acetaminophen using liquid chromatography mass spectroscopy (D). CYP1A2 activity was induced in indicated samples with 50 µM Omeprazole. Basal enzyme activity was higher and more stable in the RAFT™ System. CYP1A2 was inducible in RAFT™ Cultures at all time points tested – making the system suitable for drug interaction studies.

Conclusion

- The RAFT™ 3D Cell Culture System provides a valuable tool to investigate different cell types in single or co-culture in an *in vivo*-like collagen-based microenvironment.
- Standard analysis methods like immunocytochemistry, histology, ELISA, viability and metabolism assays can be applied to RAFT™ Cultures.
- The data presented here suggests that the RAFT™ 3D Cell Culture System can be a tool for potentially more relevant *in vitro* biomedical studies in the context of drug discovery or safety testing.