High-content screening-based phenotypic analysis of 3D organotypic bronchial tissues

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Introduction

Increasing requirements for more physiologically relevant *in vitro* systems have led to the development of more complex cellular models, which range from 2D co-cultures to 3D organotypic tissues with one or more cell types. To further evolve our High Content Screening (HCS) assay portfolio, we sought to establish HCS methods of 3D bronchial tissues to perform:

1. **Tissue quality control**: to verify differentiated phenotype and tissue homogeneity.
2. **Experimental endpoints**: to evaluate phenotypic changes upon tissue treatment.

Objectives

When grown to confluence on a Transwell™ system and cultured at the air-liquid interface (ALI), normal human bronchial epithelial (NHBE) cells form a polarized, pseudostratified epithelium composed of basal, ciliated, and goblet cells (Figure 1).

We investigated phenotypic markers that are known to characterize this 3D model. Cell type-specific markers, such as β-Tubulin 4, Muc5AC, and p63, were selected to identify ciliated, goblet, and basal cells, respectively. In addition, ZO-1, a member of the family of tight junction proteins, was included.

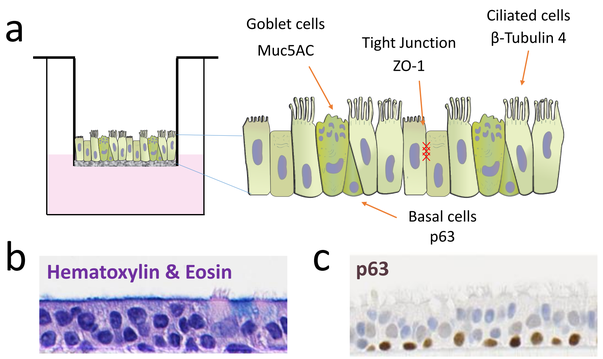

Figure 1. a) Representation of the bronchial pseudostratified epithelium. b) Tissue cross-section after staining with hematoxylin, eosin, and Alcian blue. c) Tissue cross-section after immunohistochemical detection of p63.

As a proof of concept, an *in vitro* model of IL13-driven goblet cell hyperplasia was evaluated. To induce goblet cell hyperplasia, 3D bronchial tissues were treated with IL13 (basolaterally) for two weeks. The resulting phenotype was examined by Muc5AC-specific antibody staining.

Methods

Immunostaining *Fixation:* 4% (w/v) paraformaldehyde for 20 min ⟶ *Blocking:* 0.5% Triton X-100 5% normal goat serum (NGS) + 2% bovine serum albumin (BSA) for 60' ⟶ *Staining:* PBS with 1% normal goat serum +1% BSA overnight.
Antibodies: β-Tubulin 4 AF647 (Abcam), Muc5AC AF555 (Abcam), ZO-1 AF594 (Invitrogen), p63 (Abcam) in combination with α-goat anti-mouse AF488 (Invitrogen). Hoechst 33342 was used at 1:2000 dilution. Images were acquired with the CellInsight™ CX7 platform (Thermo Fisher) and analyzed with Studio™ software (Thermo Fisher) .

Method Development

Autofocus and imaging strategy

- HCS-based methods of 2D cell cultures rely on the nuclear staining for focal plane and for cell identification (Figure 2a).
- Same approach proved to be challenging when applied to more complex 3D cultures due to their increased cellular complexity (Figure 2b).
- Spatial distribution of the selected markers along the z-axis appeared to be more consistent (Figure 1b).

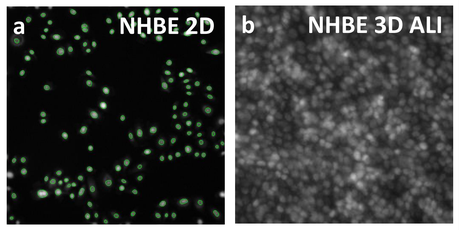

Figure 2. High-content images of Hoechst-stained a) NHBE 2D cells and b) NHBE 3D ALI culture.

→ Selected phenotypic markers were used to identify the optimal focal plane for their imaging.
→ Nuclear compartment was then imaged by applying a specific offset.

Nuclear area identification and quantification

- Nuclear staining was analyzed using the spot detection method, a flexible image analysis algorithm that can be utilized to provide generic spot analysis (Figure 3a).
- Evaluation of the area of the field of view effectively covered by the tissue is enabled, allowing identification and quantification of fields where the tissue is damaged or detached as well as those where the camera reaches the border of the inserts (Figure 3b).

→ The obtained values are then used for normalizing the values obtained from the quantification of the specific markers.

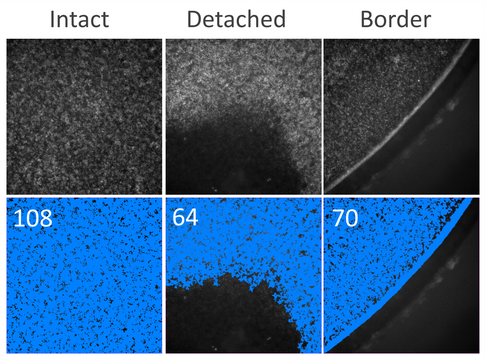

Figure 3. Representative images of different fields of Hoechst-stained 3D tissues. Lower panels show the spot detection mask applied for the analysis (in **blue**), and for each field, the spot total area (pixel number) is reported.

Phenotypic marker identification and quantification

- Similar to the nuclear signal detection, the expression of specific phenotypic markers was also investigated by applying a spot detection-based mask, which enables the evaluation of their expression and the distribution (Figure 4a).
- One of the advantages of high-content imaging, similar to other fluorescence-based methods, is the possibility to investigate several markers simultaneously (Figure 4b).

→ Such a combination enables gathering biological insights while decreasing cost, as more markers can be investigated simultaneously in the same tissue.

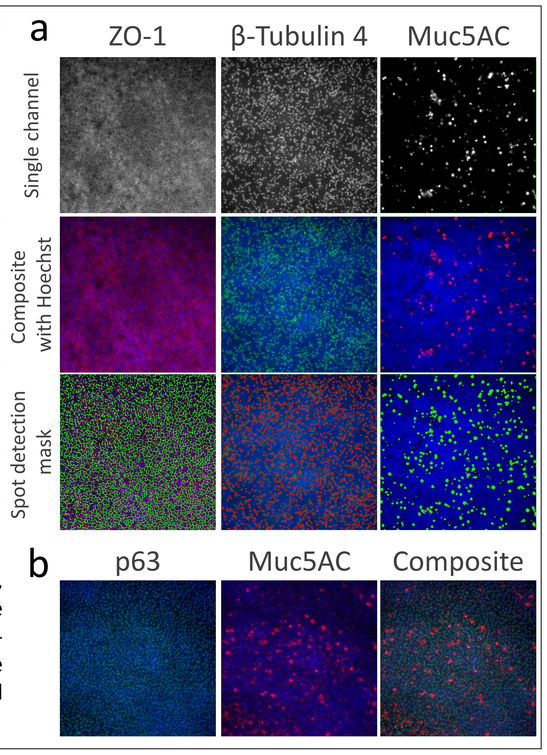

Figure 4. a) Raw single channel image, composite image of Hoechst-stained tissue and spot detection mask image of ZO-1, β-Tubulin 4 and Muc5AC staining. b) Single channel and composite images of p63 and Muc5AC multiplex staining.

Applications

Tissue quality control

- Single field of view images, taken in both fluorescence and bright-field mode, can also be mounted for whole tissue visualization (Figure 5a).
- Additional tools for quality check can include the evaluation of marker expression and distribution after maturation (Figure 5b).
- In the example reported here, tissue ciliation is quantified by staining with anti-β-Tubulin 4 (Figure 5c).

→ The visualization of this image has proven to be very valuable in the evaluation of the whole tissue structure. In fact, structural damage and abnormalities can be spotted easily. In addition, heterogeneous marker expression can be identified and quantified quickly.

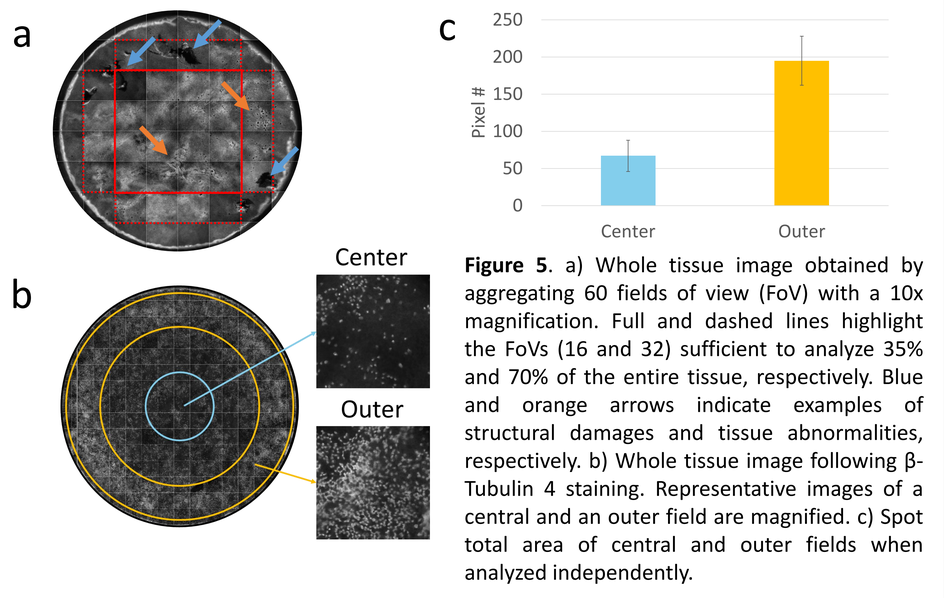

Figure 5. a) Whole tissue image obtained by aggregating 60 fields of view (FoV) with a 10x magnification. Full and dashed lines highlight the FoVs (16 and 32) sufficient to analyze 35% and 70% of the entire tissue, respectively. Blue and orange arrows indicate examples of structural damages and tissue abnormalities, respectively. b) Whole tissue image following β-Tubulin 4 staining. Representative images of a central and an outer field are magnified. c) Spot total area of central and outer fields when analyzed independently.

Experimental endpoint

- IL13 was applied repeatedly to the basolateral side of 3D bronchial tissues to induce goblet cell hyperplasia. Phenotype evaluation was performed by staining goblet cells with a Muc5AC-specific antibody.

→ Using our developed method, we demonstrated that IL13 induced an almost 4-fold increase in goblet cell density, as shown by the Spot total area detection. In addition, looking at the average intensity, we also noted a nearly 40% increase in Muc5AC production at the individual cell level.

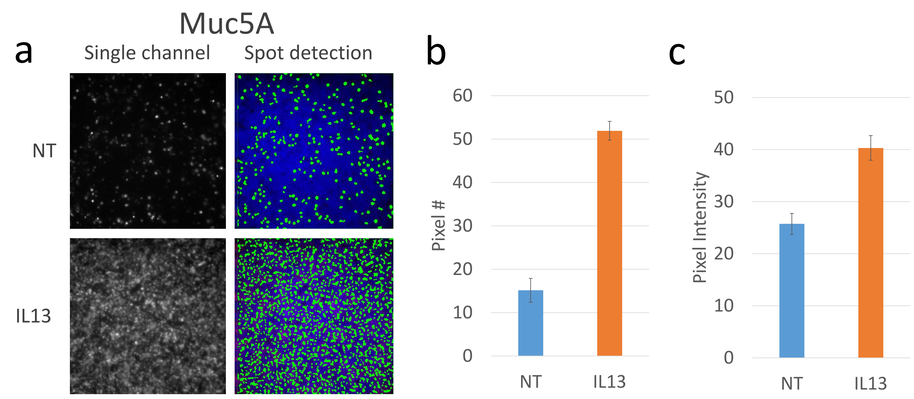

Figure 6. a) Muc5AC staining of untreated and IL13-treated tissues. b) Spot total area. c) Spot average intensity analysis of Muc5AC specific signal.

Conclusions

This approach demonstrates that high-content imaging can be used to evaluate more complex 3D *in vitro* models. In fact, it can be exploited either as a tool, for fast and accurate quality control of the tissue, evaluating both the expression and the distribution of tissue differentiation or maturation markers, or as an experimental endpoint in studies looking at specific phenotypic changes upon treatment.

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Declaration of Interest

Philip Morris International R&D is the sole source of funding and sponsor of this project.