High Throughput Gene Expression Analysis of 3D Airway Organoids

Application Note

CORNING

Hilary Sherman[1], Nathan Elliott[2], Andrew White[2], and Ann Rossi[1], Ph.D.
[1]Corning Incorporated, Life Sciences, Kennebunk, ME USA;
[2]NanoString Technologies, Inc., Seattle, WA USA

Introduction

For over 30 years, researchers have been using permeable support systems for air-liquid interface culture to study the airway epithelium. Utilizing this traditional technique, primary airway cells are cultured on permeable supports and differentiated into ciliated, goblet, and basal cells to model lung tissue for research in toxicology and respiratory disorders, such as asthma and cystic fibrosis. There is continued need for research in these areas to identify treatments, necessitating an increase in throughput for airway models. Three-dimensional (3D) airway organoids pose a solution for modeling airway tissue. Airway organoids produced from primary airway cells have the same ability to differentiate into polarized structures consisting of ciliated, goblet, and basal cells without the need for a permeable support system[1]. This allows for increased throughput of airway studies beyond the currently available 96-well format to 384-well and potentially larger formats. Importantly, advances in characterization of these model airway cultures is also critical to understanding these pathologies and facilitates high throughput cell-based assays for therapy research and development. An essential aspect of studying respiratory disorders is comparison of gene expression of healthy versus diseased tissue, as alterations in the composition of the differentiated cells or cytokines produced are often associated with various pathologies. We use the nCounter® PlexSet™ assay to characterize the gene expression of airway organoids generated from healthy and asthmatic primary bronchial cells. The PlexSet assay provides a simple and cost-effective solution for multiplex sample analysis of up to 96 genes in 96 samples per run. The assay is compatible with whole cell lysate, without the need for RNA purification and was simple to incorporate into the airway organoid workflow. The result is high throughput gene expression data without amplification, cDNA conversion, or library prep.

Materials and Methods

Airway Organoid Culture

Normal and Asthmatic human bronchial epithelial cells (HBEC; Lonza Cat. Nos. CC-2540S and 00194911, respectively) were cultured in PneumaCult™-Ex Plus medium (STEMCELL Technologies Cat. No. 05040) per manufacturer's protocol. Cells were harvested with Accutase® (Corning Cat. No. 25-058-CI) and seeded onto 50 µL of undiluted, polymerized Corning® Matrigel® matrix for Organoid Culture (Corning Cat. No. 356255) per well of a 96-well microplate (Corning Cat. No. 353219). Cells were seeded at a density of 1.4 x 10^4 cells per well in a volume of 80 µL per well of assay medium. Assay medium consisted of complete PneumaCult-ALI medium (STEMCELL Technologies Cat. No. 05001) containing 0.45 mg/mL Matrigel matrix. Medium was exchanged 3 times per week for 20 to 23 days.

Airway Organoid Staining

Organoids were collected from Matrigel matrix by pipetting up and down with Axygen® wide bore tips (Corning Cat. No. TF-205-WB-R-S) and incubating with Corning Cell Recovery Solution (Corning Cat. No. 354253) at 4°C for 20 minutes. Organoids were washed several times with cold phosphate buffered saline (PBS) before fixing with cold 4% paraformaldehyde for 15 minutes at 4°C. Organoids were permeabilized with 0.5% Triton X™-100 for 20 minutes prior to washing with PBS and staining. One microliter of primary conjugated antibody was added to each well containing 49 µL of PBS for overnight incubation at 4°C. Airway organoids were immunolabeled with Abcam antibodies for beta IV tubulin, mucin 5AC, cytokeratin 5, and isotype control (Abcam Cat. Nos. ab204034, ab218714, ab193894, and ab199091, respectively). The next day, organoids were washed with PBS and nuclei were stained with 10 µg/mL of Hoechst 34580 (Thermo Fisher Cat. No. H21486). Images were captured with the Thermo Fisher Scientific CellInsight™ CX7 Hight-Content Screening Platform using a 40X objective.

Drug Exposure

Twenty-four hours prior to lysing organoids, media was replaced with assay medium containing 1 µM Dexamethasone (Sigma Cat. No. D2915) or media.

Gene Expression Analysis

Samples were sent to NanoString Technologies for analysis via their nCounter platform (Figures 1 and 2). Prior to analysis, organoids were removed from Corning Matrigel matrix for Organoid

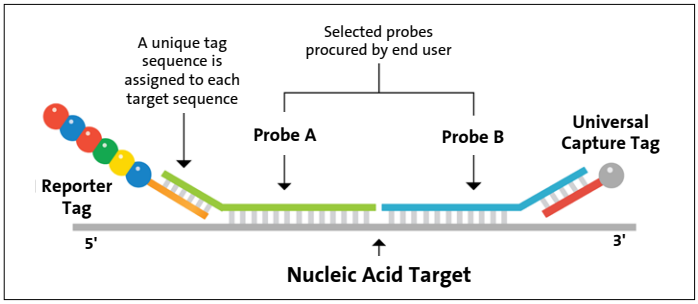

Figure 1. nCounter barcoding technology for target counting. nCounter barcoding technology uses color-coded molecular barcodes to hybridize and count nucleic acids, and is applied to analysis of RNA, DNA, and protein.

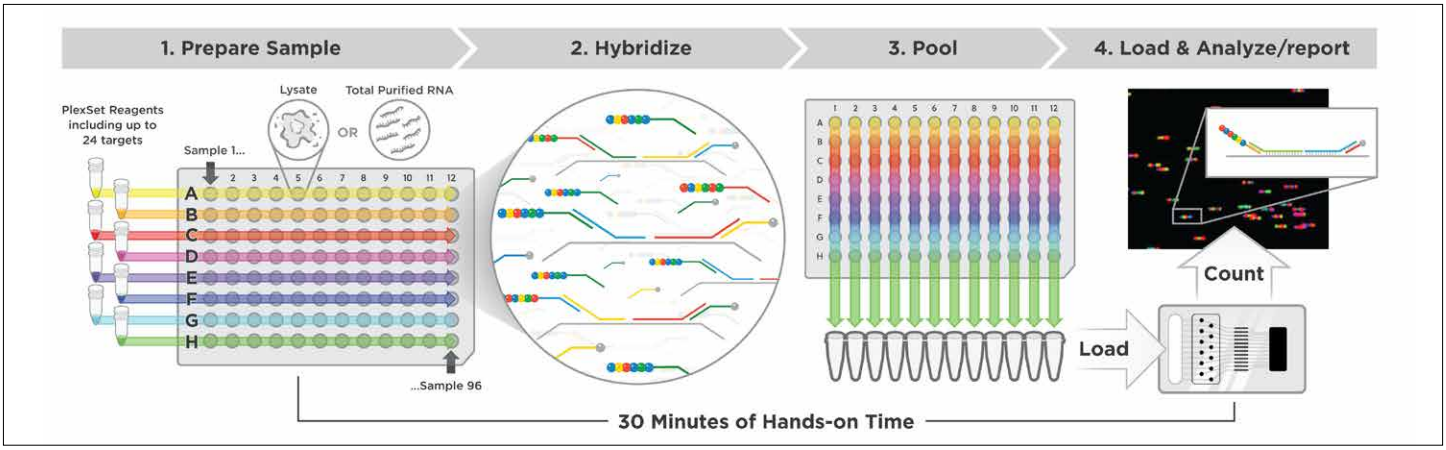

Figure 2. nCounter technology workflow. Schematic of workflow for high throughput gene expression analysis directly from cell lysates.

Culture as previously described. After organoids were separated from Matrigel matrix, they were lysed with iScript™ buffer (Bio-Rad Cat. No. 1708898) per manufacturer's protocol. Samples were immediately frozen at -80°C and shipped on dry ice for analysis. Five microliters of each lysate were hybridized with nCounter® PlexSet™ reagents for 16 hours at 67°C. Data was collected on the nCounter MAX instrument system. Digital counts were normalized to the geometric mean of internal positive control and reference gene counts, and log2 transformed. Reference genes ABCF1, GUSB, HPRT1, LDHA, POLR1B, and RPLP0 are commonly used by NanoString Technologies in many of their commercially available panels.

Results and Discussion

Organoid Confirmation

Within 3 days of culture, 3D structures began to appear in the wells followed by lumen formation in the center of the structures by day 17 (Figure 3). After 23 days, many of the structures had moving cilia facing the lumen that were visible via bright-field imaging at 200X. Immunofluorescence staining revealed the presence of basal cells, ciliated cells, and mucus producing cells in both normal and asthmatic organoids (Figure 4). There was more positive mucin 5AC staining with the asthmatic organoids (Figure 4), which is consistent with primary literature reports[2].

Gene Expression Analysis

Reproducibility of nCounter data was demonstrated by generating a correlation plot of counts from two different wells of the same condition (Figure 5). The resulting R^2 value was 0.977 which indicates strong correlation[3], and thus, highly reproducible data from independent samples. The nCounter expression data was then utilized to compare expression of cell type specific genes for differentiated airway organoids relative to expression in the source HBECs prior to differentiation. Figure 6 shows downregulation of genes TP63 and IGA6 – the transcription factor tumor protein p63 and its downstream target integrin alpha 6, respectively – that are widely expressed in epithelial cells. Concurrently, there was an upregulation of genes associated with differentiated bronchial epithelial cells[4]. Specifically, expression of axonemal dynein intermediate chain 2 (DNAI2), which encodes for a protein that is part of the dynein complex in respiratory cilia, and forkhead box protein J1 (FOXJ1) transcription factor which is involved in ciliogenesis, were increased in the airway organoids relative to the source HBECs. MUC5AC and MUC5B that encode mucin proteins 5AC and 5B secreted by goblet cells were similarly upregulated in the organoids.

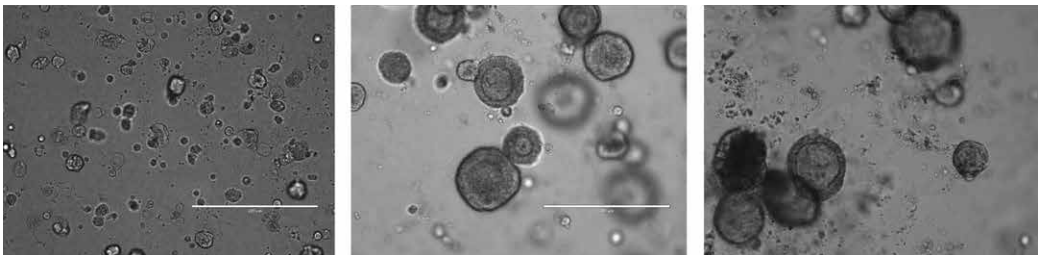

Figure 3. Airway Organoids. Representative photomicrographs of 3- (left), 17- (middle), and 29-day-old (right) airway organoids on Corning Matrigel matrix for organoid culture. Images were capture at 100X magnification with an EVOS® FL microscope. Scale bars are 400 µm.

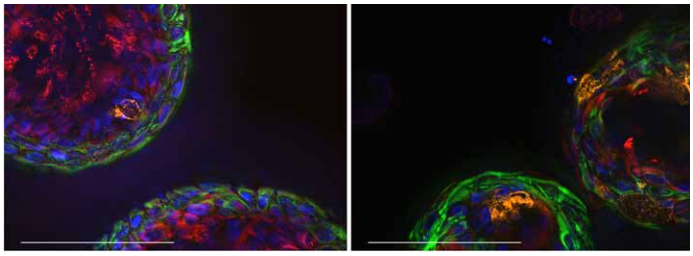

Figure 4. Normal versus asthmatic airway organoids. Representative photomicrographs of 23-day-old airway organoids from normal (left) and asthmatic (right) donors. Multicolor fluorescent labels indicate specific cells types: basal cells (green), ciliated cells (red), mucus production from goblet cells (orange), nuclei (blue). Images were captured at 40X magnification. Scale bars are 100 µm.

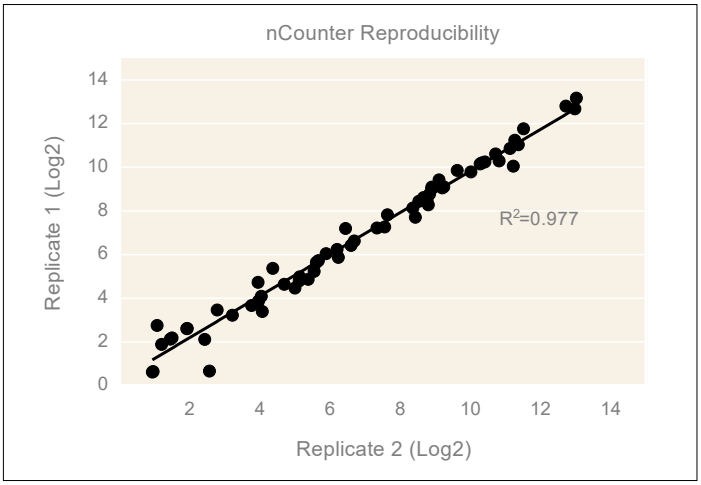

Figure 5. Reproducibility of gene expression data from replicates. Representative correlation plot demonstrating reproducibility of counts from two different wells of the same condition.

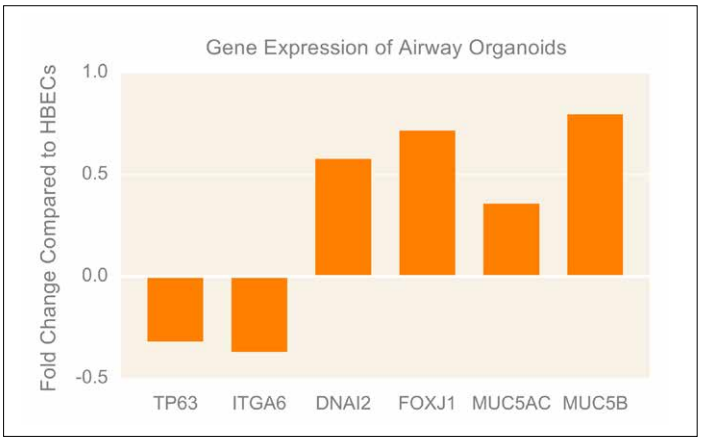

Figure 6. Changes in expression of genes associated with airway organoid generation. Fold change of gene expression of airway organoids compared to normal HBECs. TP63 and IGA6 expression are associated with basal cells. DNAI2 and FOXJ1 expression are associated with ciliated cells. MUC5AC and MUC5B expression are associated with goblet cells.

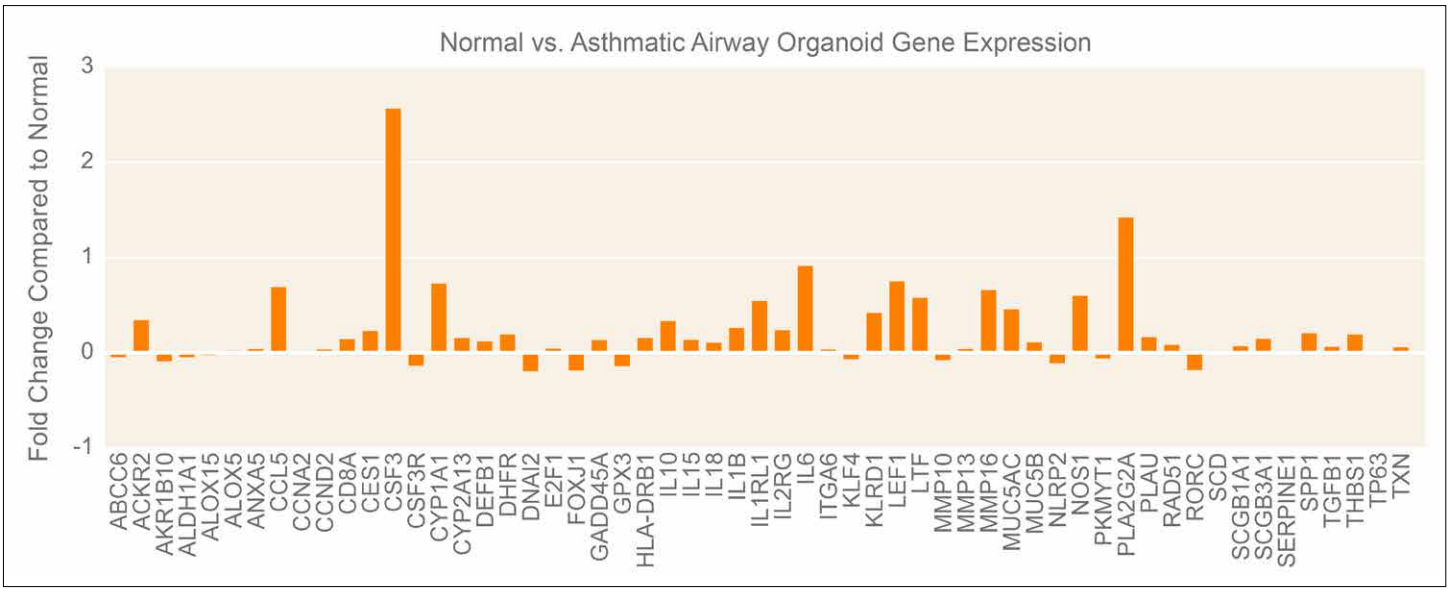

Figure 7. Fold change of gene expression in asthmatic organoids. Fold change of asthmatic organoid gene expression relative to expression in normal organoids. N = 24 from 2 independent studies.

To demonstrate high throughput gene expression analysis of diseased organoids, we compared normal and asthmatic airway organoids (Figure 7). Of note, we observed increased expression of chemokine ligand 5 (CCL5), colony-stimulating factor 3 (CSF3), interleukin 6 (IL6), interleukin 10 (IL10), interleukin receptor-like 1 (IL1RL1), mucin 5AC (MUC5AC), and phospholipase A2 (PLA2G2A) in the asthmatic organoids. These genes encode for chemokines, receptors, and other proteins associated with inflammatory processes in asthmatic airway cells[5-11]. Finally, we were able to detect differential gene expression with the nCounter® technology between normal and asthmatic organoids (Figure 8). Generally, the subset of genes upregulated in the normal organoids are the same as those genes downregulated in asthmatic airway organoids and vice-versa. Exposure to the anti-inflammatory dexamethasone changed expression (up- and downregulation) of a greater subset of genes in the asthmatic organoids relative to the normal organoids. Taken together, the data demonstrates the feasibility of airway organoid culture with accompanying high throughput gene expression analysis for characterization and 3D screening assays.

Conclusions

- Corning® Matrigel® matrix for organoid culture provides an ideal environment for the differentiation of bronchial epithelial cells to airway organoids.
- Corning Matrigel Matrix for organoid culture enables 3D cell culture of primary, patient-derived bronchial epithelial cells for high throughput 3D organoid research.
- Sample processing for nCounter analysis requires only cell lysis, eliminating the conventional steps for gene expression analysis (i.e., gene amplification, cDNA conversion, or library prep).
- The nCounter platform enables high throughput gene expression analysis of 3D organoid cultures for healthy and disease-model characterization and screening.

Figure 8. Fold change of gene expression of normal and asthmatic organoids in response to drug exposure. Heat map demonstrating relative gene expression of normal (left side) and asthmatic (right side) organoids, following normalization to reference genes ABCF1, GUSB, HPRT1, LDHA, POLR1B, and RPLP0 (not shown) after 24-hour exposure to DMSO control or dexamethasone. N = 24 from 2 independent studies.

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Corning Incorporated
Life Sciences
836 North St.
Building 300, Suite 3401
Tewksbury, MA 01876
t 800.492.1110
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