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# Using intracellular SCGB1A1-sorted, formalin-fixed club cells for successful transcriptomic analysis

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## A B S T R A C T

As opposed to surface marker staining, certain cell types can only be recognized by intracellular markers. Intracellular staining for use in cell sorting remains challenging.

Fixation and permeabilization steps for intracellular staining and the presence of RNases notably affect preservation of high-quality mRNA. We report the work required for the optimization of a successful protocol for microarray analysis of intracellular target-sorted, formalin-fixed human bronchial club cells. Cells obtained from differentiated air-liquid interface cultures were stained with the most characteristic intracellular markers for club cell (SCGB1A1<sup>+</sup>) sorting. A benchmarked intracellular staining protocol was carried out before flow cytometry.

The primary outcome was the extraction of RNA sufficient quality for microarray analysis as assessed by Bioanalyzer System. Fixation with 4% paraformaldehyde coupled with 0.1% Triton/0.1% saponin permeabilization obtained optimal results for SCGB1A1 staining.

Addition of RNase inhibitors throughout the protocol and within the appropriate RNA extraction kit (Formalin-Fixed-Paraffin-Embedded) dramatically improved RNA quality, resulting in samples eligible for microarray analysis.

The protocol resulted in successful cell sorting according to specific club cell intracellular marker without using cell surface marker. The protocol also preserved RNA of sufficient quality for subsequent microarray transcriptomic analysis, and we were able to generate transcriptomic signature of club cells.

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### Keywords:

Club cells  
SCGB1A1 sorting  
RNA quality

Transcriptomic analysis

## 1. Introduction

Human bronchial epithelial cells (HBEC) maintained in Air-Liquid Interface (ALI) culture is a reliable model of the airway epithelium [1–3]. The structure and composition of the *in vivo* epithelium are relatively respected in this *ex vivo* model. The

different cell types can be found: ciliated, goblet, basal, neuroendocrine, tuft, and club cells, thus paving the way for studies on bronchial epithelial biology. However, the varying cell composition of the airway epithelium gives rise to the questioning of the respective roles of each cell type. ALI cultures offer a valuable opportunity to investigate this when cells can be sorted. Several studies have already focused on one cell type of the bronchial epithelium; basal cells with a known surface marker which facilitates sorting [4]. In contrast, club cells have not yet been investigated, and to do so appears challenging given their lack in

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identification of a specific surface marker.

Club cells, characterized by SCGB1A1 expression, are known to be non-ciliated secretory cells in the respiratory epithelium [5]. They are multifunctional cells with various pathophysiological roles; They can act as stem/progenitor cells [6,7] and participate in injured epithelial repair [8]. They secrete various proteins including Club Cell Secretory Protein (CCSP) that has been described as having anti-inflammatory properties [9,10]. Dysregulation of the club cells contributes to many respiratory diseases like asthma [11,12], IPF [13] and COPD [14]. Even if evidences highlight important role of club cells and CCSP in airway epithelium homeostasis, biological functions of club cells and CCSP have not been completely described and elucidated. Focus on these cells will help on the understanding.

Sorting cells that can only be identified by intracellular proteins, especially those lacking a known surface marker [15,16], is problematic. This is all the more so when RNA integrity must be preserved for subsequent microarray analysis. The multiple steps required to achieve reliable intracellular staining are well described [17], although subject to protocol personalization. To date, no specific surface marker for these cells has been identified, thus hindering flow cytometry cell sorting without intracellular staining. Additionally, SCGB1A1 is a highly biologically active protein in the airway and SCGB1A1 expression levels are subject to strong variation, creating thus further difficulty for single-cell RNA-seq techniques.

The goal of this study was to develop a robust protocol for the sorting of club cells based on their intracellular SCGB1A1 protein expression without compromising both RNA quantity and quality. We aimed to describe the specificities of this SCGB1A1 cell sorting protocol required for performing microarray analysis from the HBEC fixation step to the RNA sample quality assessment step.

## 2. Material and methods

### 2.1. Human bronchial epithelial cell cultures

ALI cultures of primary HBEC were derived from bronchial biopsy specimens obtained during fiberoptic bronchoscopy. All donors signed a consent form after being informed about the biomedical research on airway epithelium performed thanks to their donation. The protocol was approved by the institutional ethics commission of Sud Méditerranée III (CHRU Montpellier-AOI 9244–NCT02354677). All experiments and methods were performed in accordance with relevant guidelines and regulations. HBEC were then cultured as previously described [18]. Briefly, cells were mechanically dissociated from biopsies. After an expansion phase in monolayers, cells were plated on uncoated nucleopore membranes (24-mm dia., 0.4 µm pore size, Transwell Clear, Costar) in PneumaCult-Ex Plus medium (Stemcell Technologies) and grown to confluence. From here, ALI cultures were established by applying media (PneumaCult ALI maintenance (Stemcell Technologies)) only at the basal side (day 0). Cells were cultured for 21 days to obtain a polarized and differentiated cell population with a mucociliary phenotype.

### 2.2. Flow cytometry

HBEC were collected at D21 using enzymatic solutions (ACF enzymatic dissociation solution and ACF enzymatic inhibition solution, Stemcell Technologies). The cells were then fixed using 4% paraformaldehyde (PFA) and permeabilized with 0.1% Triton/0.1% saponin before intracellular staining. After a blocking step with 10% donkey serum (Sigma) in 1% BSA/PBS for 1 h at Room Temperature (RT), cells were labeled with anti-SCGB1A1 primary antibody (club

cell protein CC16 human, rabbit polyclonal antibody, Biovendor) overnight at 4 °C. Finally, the cells were incubated with secondary antibody (Alexa Fluor 488 donkey anti rabbit IgG (H + L), Invitrogen) for 1 h at RT. The primary antibody was omitted as a negative control.

During all these steps (fixation, permeabilization, and antibody staining), solutions were kept in sterile conditions and 1000U of RNasin Plus Ribonuclease Inhibitor (Promega) was added. Finally, cells were either analyzed by flow cytometry using the Beckman Coulter Gallios or sorted according to the presence/absence of the intracellular SCGB1A1 marker using the Aria IIIu Becton Dickinson flow cytometer. Sorted cells were recovered in PBS supplemented with 1000U of RNasin Plus Ribonuclease inhibitor (Promega).

### 2.3. RNA extraction

Total RNA was extracted from sorted HBEC cells using the Formalin-Fixed Paraffin-Embedded (FFPE) Recover All Total Nucleic Acid Isolation kit (ThermoFisher) according to the manufacturer's instructions.

The first step was sample digestion with protease digestion buffer at 50 °C for 1 h. This reaction was terminated at 80 °C for 15 min.

### 2.4. RNA integrity, microarray hybridization, and data processing

RNA integrity was examined using the Agilent Bioanalyzer 2100 System (Agilent RNA 6000 PICO kit, Agilent Technologies). The Bioanalyzer System profile demonstrates the extent of degradation and quantifies recovered RNA. The GeneChipT WT transcription Pico Amplification Kit (WT Pico 12rxs, Life Technologies SAS), specifically for amplification of degraded RNA, was used according to the manufacturer's instructions. Then, double stranded complementary DNA (ds cDNA) was measured using the NanoDrop and Bioanalyzer System. Briefly, biotinylated ds cDNA was prepared according to the WT Pico kit manual Rev1 (P/N: 703262 Rev1) from 1 to 4 ng of total RNA. Following fragmentation and terminal labeling, 5.5 µg of ds cDNA were hybridized for 16 h at 45 °C and 60 rpm to the GeneChip Human Gene 2.0 ST array (Affymetrix, Thermo Fisher Scientific, Inc.). Arrays were washed and stained in the Affymetrix fluidic station 450 with hybridization wash and stain kit, and finally scanned using the Affymetrix GeneChip Scanner 3000 7G. Overall, 6 chips (3 chips for SCGB1A1<sup>-</sup> cell samples and 3 chips for SCGB1A1<sup>+</sup> cell samples) were used to study the transcriptomes of sorted cells. Data processing (.CEL files containing intensity data were normalized with RMA algorithm at a gene and an exon level) and primary analyzes were performed using the Expression Console Software 1.4.1. Differentially expressed genes and volcano plots were determined using the Transcriptome Analysis Console version 4.0 (Affymetrix).

### 2.5. Real-time RT-qPCR

0.4 µg of RNA was reverse transcribed using a one-step RT-PCR kit (Verso cDNA synthesis kit, ThermoFisher Scientific) and real-time RT-qPCR was performed using SYBR Green (Light Cycler 480 SYBR Green I master, Roche Applied Science). The comparative Cycle Threshold (Ct) method for relative quantification of gene expression was used (2<sup>-ΔCt</sup>, where ΔCt represents the difference in the threshold cycle between the target and housekeeping genes). Data were normalized to GAPDH mRNA levels. Primer sequences were as follows:

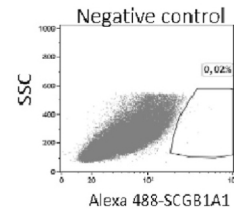
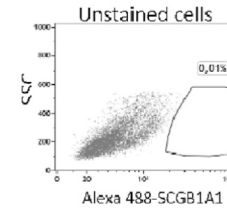
## 2.6. Microscopy

SCGB1A1<sup>-</sup> and SCGB1A1<sup>+</sup> sorted cells were mounted on slides and visualized using the Zeiss Axiomager microscope using the Axiovision software (Zeiss).

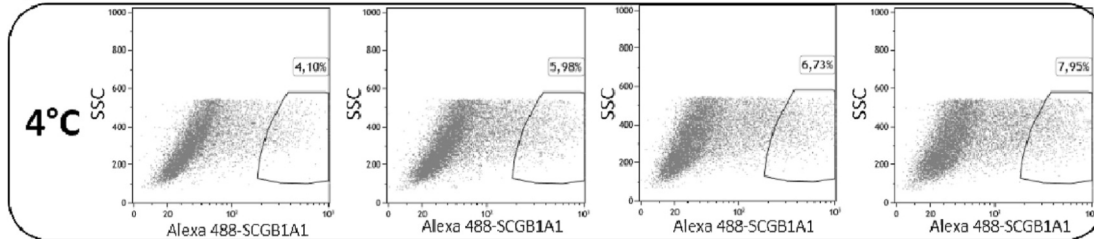
Primers	Sequences (5' to 3')
GAPDH	Forward: CCATCTTCAGGAGCGAG Reverse: CTTGAGGCTGTGTCATACT
SCGB1A1	Forward: CATGAAACTCGTGTCAACC Reverse: GATGACACGCTGAAAGCTCG

**A**

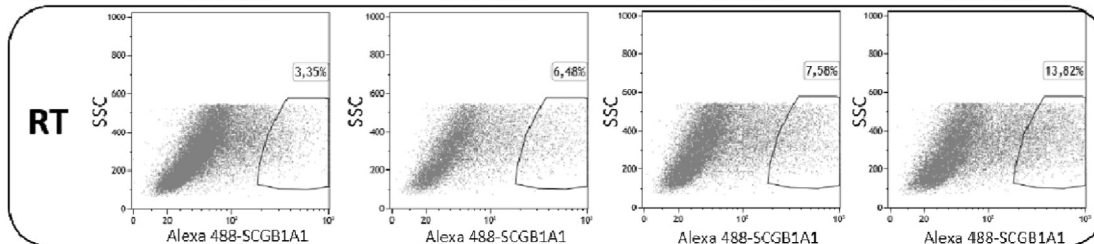
Fixation	Incubation time	Staining
70% ethanol	15 min at RT	-
1% PFA	15 min at RT or 4 °C	+
2% PFA	15 min at RT or 4 °C	++
3% PFA	15 min at RT or 4 °C	+++
4% PFA	15 min at RT or 4 °C	++++



**B**



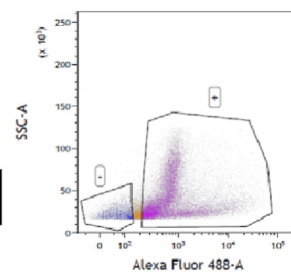
**C**



**D**

Permeabilization	Incubation time	Staining
0.1% Triton	10 min at RT	-
0.1% Saponin	10 min at RT	-
0.1% Triton-Saponin	10 min at RT	+

**E**



**Fig. 1.** Optimal fixation and permeabilization steps during SCGB1A1 staining for flow cytometry cell sorting.

Fixation with 70% ethanol or paraformaldehyde (PFA) (1–4%) was tested for 15 min at Room Temperature (RT) or 4 °C. Staining with SCGB1A1 antibody was performed and cells were injected into a flow cytometer to visualize SCGB1A1 staining. All fixation tests carried out for optimal SCGB1A1 staining are resumed in table A. "-" indicates poor staining; "+" corresponds to positive staining strength. The inset highlights optimal fixation for SCGB1A1 staining. B and C are flow cytometry profiles for different fixations with PFA at 4 °C or RT. D- Permeabilization tests for optimal SCGB1A1 staining. "-" indicates poor staining; "+" corresponds to positive staining strength. The inset highlights optimal permeabilization for SCGB1A1 staining. E-flow cytometry profiles for permeabilization with 0.1% Triton/0.1% saponin.

### 3. Results

Among the different steps required for sorting club cell based on their intracellular marker SCGB1A1, adjustments on critical steps are essential for successfully achieving the aim of this study: sorting club cells using an intracellular antibody without compromising future RNA-based analyzes.

#### 3.1. Fixation step

Different epithelial cell fixation protocols were tested (Fig. 1A) and validated by flow cytometry. 70% ethanol, used as previously described [19], failed to achieve adequate SCGB1A1<sup>+</sup> cell staining. Further tests were performed using different concentrations of PFA and incubation temperatures. The percentage of SCGB1A1-stained cells increased with increasing percentage of PFA at RT (Fig. 1B) or 4 °C (Fig. 1C). When comparing graphs for 4% PFA, increased cell staining rates were observed at RT. Overall, we put forward a 15min 4% PFA incubation at RT as the optimal fixation step.

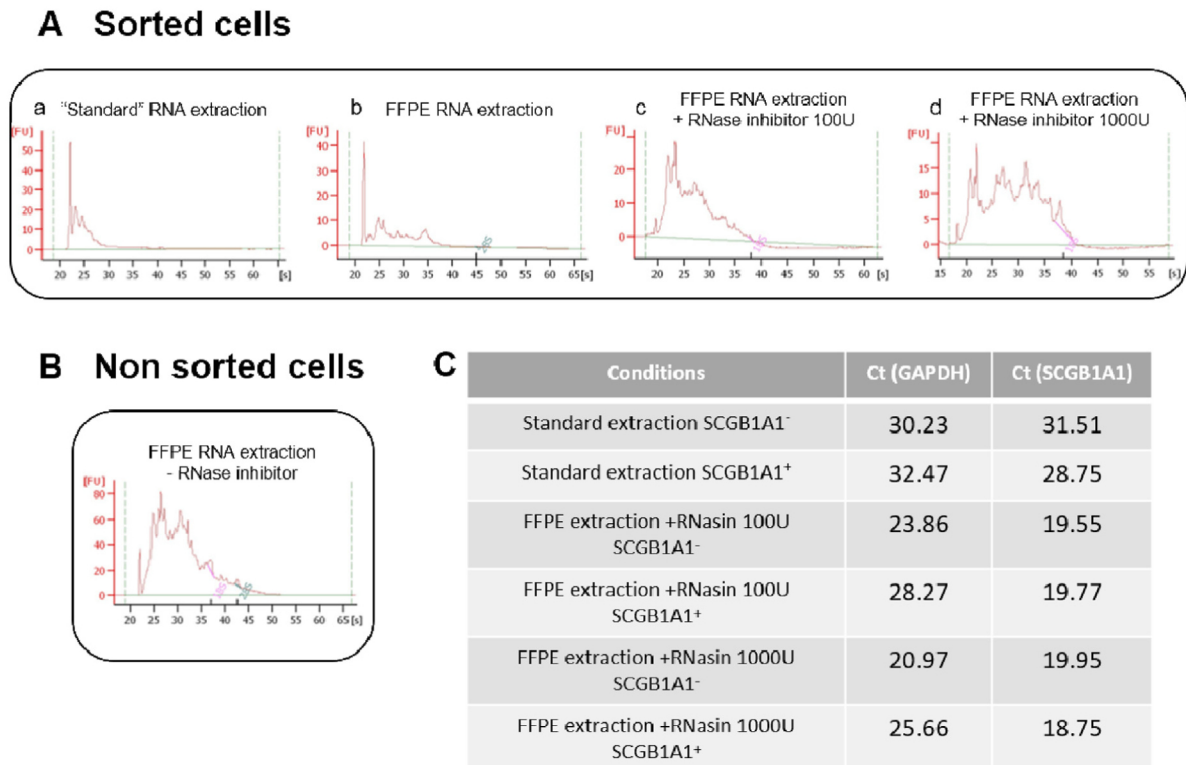
#### 3.2. Permeabilization step

Using the optimal 4% PFA fixation step, different permeabilization reagents were then tested (Fig. 1D); this step allows access of specific antibodies to the cellular cytoplasm [20]. First, 0.1% Triton or 0.1% saponin incubations were deemed inadequate, resulting in negative staining on the flow cytometry profile (data not shown). A combination of 0.1% Triton and 0.1% saponin was then tested, proving to be more successful as shown in Fig. 1E. In summary, the permeabilization step selected for further experiments was 0.1% Triton/0.1% saponin for 10min at RT.

#### 3.3. RNA extraction step

After staining and sorting club cells by flow cytometry, total RNA was extracted and the RNA integrity was verified using the Bioanalyzer System. First, sorted cells were recovered in RLT buffer (Qiagen) with 1% beta-mercaptoethanol to lyse the cells. Then, RNA was extracted using a "standard" kit (RNeasy mini kit, Qiagen) according to the manufacturer's instructions. Both the 18S and 28S ribosomal RNA peaks were absent from the Bioanalyzer System profile after this first extraction, suggesting that the RNA was degraded or absent from these RNA extracts (Fig.2Aa). This was further confirmed by very late RT-qPCR Ct values for both GAPDH and SCGB1A1 (Fig. 2C, samples in the "standard extraction SCGB1A1<sup>-</sup> and <sup>+</sup>" groups). We considered thus the "standard" extraction kit not appropriate and tested a second extraction technique dedicated to mRNA extraction from fixed tissues (RecoverAll Total Nucleic Acid Isolation kit, ThermoFisher). This extraction added a protease digestion step. This digestion suppresses protein-DNA crosslinking caused by the process of formalin fixation [21]. The resulting Bioanalyzer System profile showed not only the absence of peaks, but also a very minimal amount of RNA extracted (Fig.2Ab). The absence of a suitable Bioanalyzer System profile with both of the mentioned extraction tests cannot be entirely due to the formalin fixation process, thus suggesting that the RNA was degraded.

An RNase inhibitor (RNasin Plus RNase Inhibitor, 100–1000U, Promega) was then added to solutions throughout all the steps of the intracellular staining protocol to prevent RNA degradation. FFPE tissue extraction was carried out. A multitude of peaks indicating the presence of RNA was observed with the addition of both 100 or 1000U of RNasin (Fig.2Ac and d). Despite 1000U RNasin tending to



**Fig. 2.** Optimal RNA extraction protocol after cell sorting.

A- Bioanalyzer System profiles of RNA extracted from sorted cells. a- RNA extraction using the "standard" extraction kit. b- RNA extraction using the Formalin-Fixed-Paraffin-Embedded (FFPE) extraction kit. c and d- RNA extraction using the FFPE extraction kit with incubation with 100U or 1000U RNase inhibitor. B- Bioanalyzer System profile of RNA extracted from non-sorted cells using the FFPE kit. C- RT-qPCR Ct values of the housekeeper GAPDH and SCGB1A1 genes.

demonstrate the presence of more RNA (Fig.2Ad), the minimal RNA quantity still suggested some RNA degradation. Improved RT-qPCR Ct values for both GAPDH and SCGB1A1 further confirmed the presence of RNA in these samples (Fig. 2C, samples from "FFPE extraction + RNasin 100U or 1000U of SCGB1A1<sup>-</sup> and SCGB1A1<sup>+</sup>"). RNA extraction profiles assessed before and after cell sorting were similar (Fig. 2B), supporting the idea that RNA degradation cannot be attributed entirely to cell sorting. Accordingly, FFPE extraction with protease digestion was selected with RNasin (1000U) supplementation throughout the whole protocol.

### 3.4. Amplification step

Despite degraded Bioanalyzer System profiles, marked by the absence of distinct 18S and 28S ribosomal peaks, and a small amount of RNA (in the picogram order), we were able to proceed to chip hybridization using the WT Pico Amplification Kit (specific for low quantity and degraded RNA). After RNA amplification, NanoDrop readings (Fig. 3A) revealed sufficient RNA concentrations for ds cDNA synthesis (in the microgram order). Then, fragmentation and biotin labelling carried out on 5.5 µg of ds cDNA was validated by the Bioanalyzer (Fig. 3B). Adequate fragment sizes were detected (<50 bases). Finally, box-plot graphs of the transcript signal distributions per array after Robust Multiarray Averaging normalization was created using the Expression Console Software. These

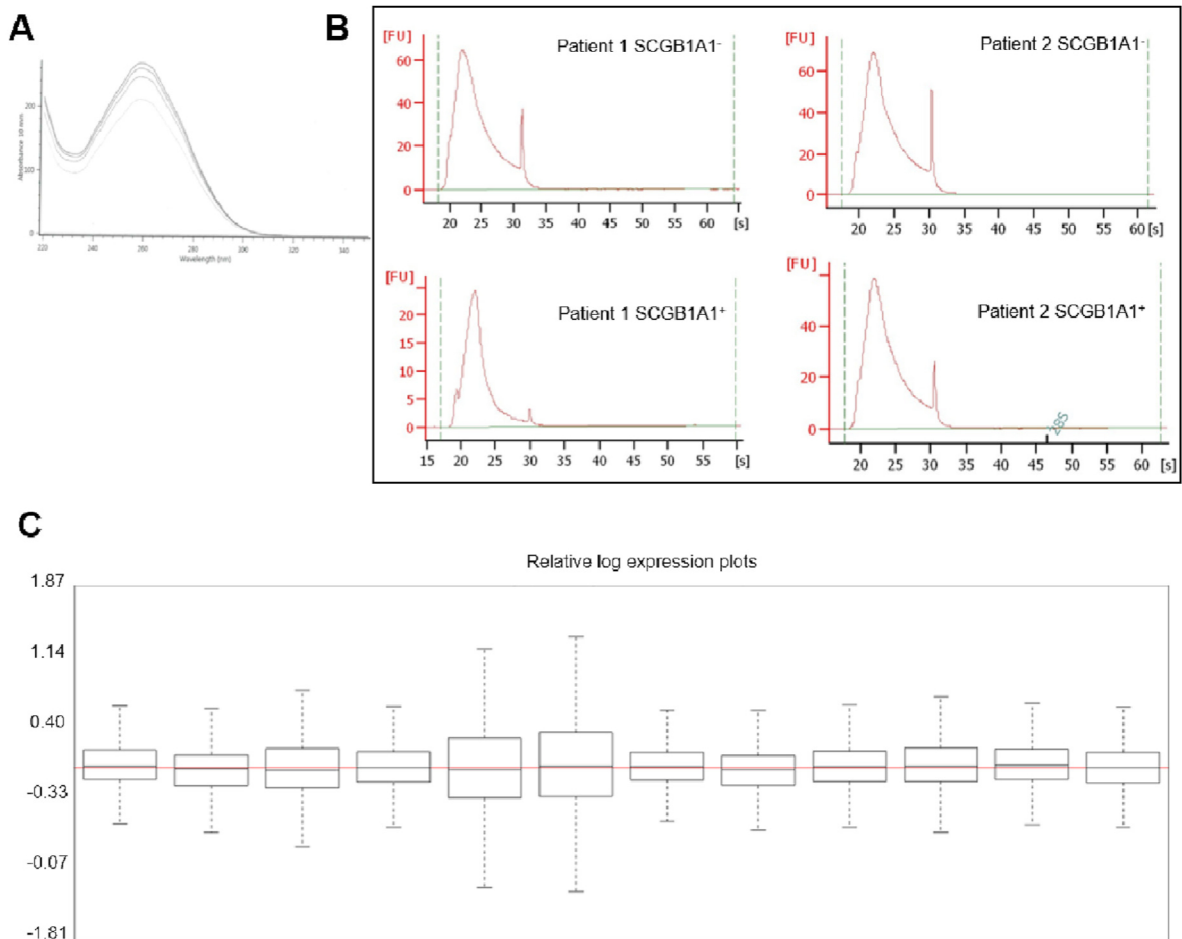
graphs show that the CHP file data are similarly distributed (Fig. 3C), confirming successful RNA amplification and microarray hybridization.

### 3.5. Sorting validation

SCGB1A1<sup>+</sup> and SCGB1A1<sup>-</sup> cell populations were recovered after flow cytometry cell sorting (Fig. 4A). Then, RNA was extracted with the above protocol and reverse-transcribed for further RT-qPCR experiments (Fig. 4B). As expected, the mRNA levels of SCGB1A1 were higher in the SCGB1A1<sup>+</sup> population among the three repeat experiments (Fig. 4B). Moreover, sorted cells mounted on slides and visualized by fluorescent microscopy confirmed sorting efficiency (Fig. 4C), displaying a higher level of fluorescence for SCGB1A1<sup>+</sup> versus SCGB1A1<sup>-</sup> cells.

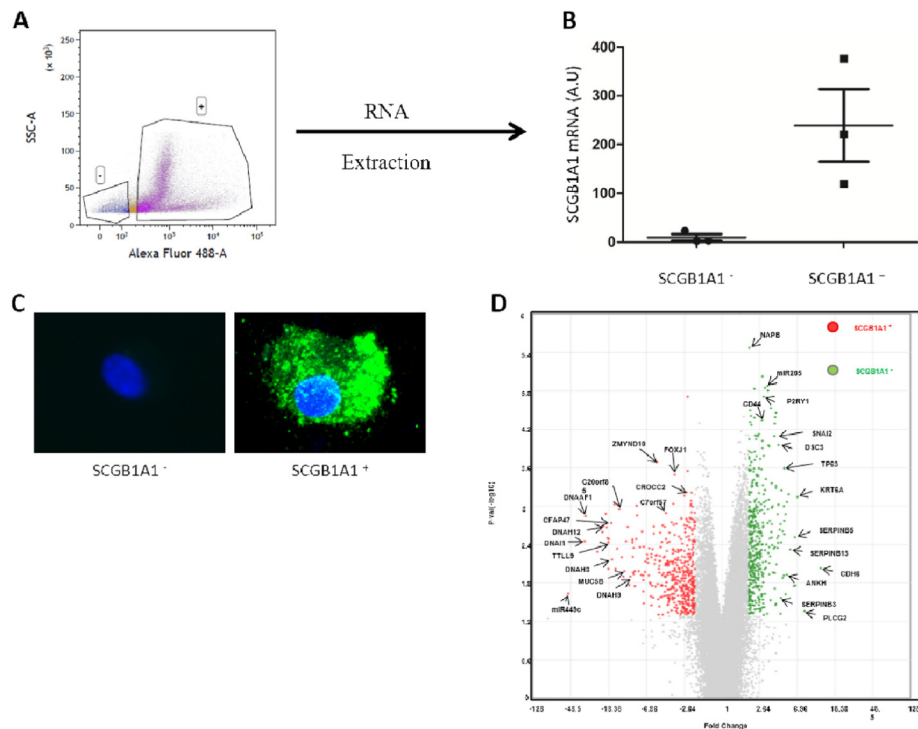
### 3.6. The transcriptomic signature of club cells

A transcriptomic signature was generated for the three repeat experiments using the hereby described protocol. The volcano plots (Fig. 4D) provide a clear overview of the genes differentially expressed according to club cell sorting. This confirms the robustness of our protocol for performing microarray analysis. Remarkably, SCGB1A1 was not found among the top upregulated genes. Cilia formation and ciliogenesis-associated genes were identified in



**Fig. 3.** Amplification and microarray controls.

After RNA extraction, an amplification step was required to improve RNA quantity before array hybridization. A- NanoDrop absorbance profile at 260 nm of 10 ng of RNA after amplification (GeneChip™ *in vitro* transcription WT Pico Amplification Kit). One curve per sample (n = 4). B- Bioanalyzer System profiles indicating adequate cDNA fragment sizes after amplification. C- Box-plot graphs of transcript distribution per array using the Expression Console Software. Data are grouped according to the sorted cell populations.



**Fig. 4.** Sorting efficiency.

**A-** Flow cytometry profile of sorted SCGB1A1 cells. The appropriate protocol for sorting club cells without compromising RNA integrity was performed on human bronchial epithelial cells: fixation with 4% paraformaldehyde (15min at room temperature), permeabilization with 0.1% Triton/0.1% saponin (10min at room temperature), staining with SCGB1A1 antibody with 1000U RNase inhibitor addition throughout the entire protocol. Cells were injected into a flow cytometer to visualize staining and sorting. Two cell populations (SCGB1A1<sup>-</sup> and SCGB1A1<sup>+</sup>) were obtained according to their fluorescence. **B-** After cell sorting, RNA from SCGB1A1<sup>-</sup> and SCGB1A1<sup>+</sup> cells was extracted with the Formalin-Fixed-Paraffin-Embedded extraction kit. RT-qPCR was conducted to validate the expression of SCGB1A1 in the different cell populations. Data were normalized to GAPDH mRNA levels (n = 3). **C-** Microscopy of sorted cells. After cell sorting, SCGB1A1<sup>+</sup> (green staining) and SCGB1A1<sup>-</sup> cells were mounted on slides for microscopy. **D-** Volcano plot of top up-regulated genes in SCGB1A1<sup>+</sup> (in red) and SCGB1A1<sup>-</sup> (in green) cells according to Affymetrix transcriptomic data analysis (three repeat experiments). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the SCGB1A1<sup>+</sup> population, including *FOXJ1*, *DNAH9*, *DNAH12*, *DNAH1*, and *miR449c*, but also some genes associated with mucus production, with *MUC5B* being the least unexpected. Top up-regulated genes from SCGB1A1<sup>-</sup> cells mostly enriched with classical basal-cell signatures, such as *KRT6A*, *TP63*, or *SERPINB3* genes.

#### 4. Discussion

The present protocol development study demonstrates the feasibility of isolating RNA from intracellular-SCGB1A1-target-sorted, formalin-fixed HBEC in order to perform microarray analysis and successfully generate a transcriptomic profile.

Similar results have been previously achieved using slightly different protocols, for example, the MARIS method [22]. However, this method was performed on a different cell type (human embryonic stem cells) and required adaptation to the bronchial epithelial cells used in the present study. In another study on HBEC cultures, a similar protocol was used but some differences appeared [23]. Even if the changes in the protocols seem minimal, they are essential to sort the club cells and obtain RNA with sufficient quality.

Our protocol was validated by both confocal microscopy and RT-qPCR. It was highly reproducible and was also used to sort different bronchial epithelial cells using different intracellular markers (Tubulin IV and Mucin5AC, data not shown). This protocol also obtained RNA of sufficient quality for the microarray analysis of fixed-permeabilized cells. However, despite all the precautions taken, a weakness lies in this protocol regarding the minimal RNA quantity and quality. Indeed, an amplification step using a

specialized kit was required prior to microarray preparation.

The lessons learned from this study allow us to highlight certain key points that require adjustments when designing an intracellular staining protocol against SCGB1A1 in airway epithelial cells. The first is adequate cell fixation, without which intracellular contents may leak and result in staining errors. Fixation can also affect RNA extraction, making it a determinant step for subsequent transcriptomic analyzes. When PFA is nonetheless required, the subsequent RNA extraction [21] can require adjustment with an adequate RNA extraction kit (FFPE extraction kit). Protease digestion should be considered and can require optimization according to cell type, percentage of PFA, and incubation time.

Permeabilization is a second crucial step requiring consideration when designing an intracellular staining protocol. Permeabilization must vary in “aggressiveness” according to the location of the molecule targeted by the staining. The more concealed the target within the intracellular space (i.e. within an organelle with a double membrane), the more permeabilization is required. Triton was not sufficient and add of saponin was necessary to better permeabilize. However, once the cell is permeabilized, the nucleic acids present becomes more vulnerable to the environment, and in particular to nucleases (enzymes that degrade nucleic acids), such as DNases and RNases. In this case, the use of enzymatic inhibitors can help preserve nucleic acids. If Bioanalyzer System RNA integrity numbers (RIN) are too low, a specific amplification kit (WT pico) is required to generate adequate sample-chip hybridization for microarray analysis.

Finally, three different samples successfully underwent Affymetrix microarray analysis in our study, leading to the generation

of transcriptomic signatures of SCGB1A1<sup>+</sup> and SCGB1A1<sup>-</sup> cells grown at the air-liquid interface. These signatures show consistency with the club cell (SCGB1A1<sup>+</sup>) and basal-cell (SCGB1A1<sup>-</sup>) signatures reported in the literature [4,15,16,24]. Our results also reveal some rather unexpected, upregulated pathways, such as pathways underlying both mucus production and ciliogenesis in SCGB1A1<sup>+</sup> cells. This reinforces the view that club cells have varying functions and are likely not terminally differentiated, but within a state that can orientate the airway epithelium phenotype by ruling the goblet-to-cilia ratio. The top differentially expressed genes in SCGB1A1<sup>+</sup> cells highly enriched match with the recently described deuterosomal cell signature [25]. The effect the culture medium used for growth and maintenance of epithelial cells in ALI cultures has on promoting orientation towards ciliated cells is therefore worthy of investigation. Although contamination by ciliated cells may be seen as a potential source of bias in these findings, it is likely that actually different Club Cell subtypes coexist more or less engaged toward a goblet or a ciliated fate indifferently captured by the presently performed gating strategy; more subtle and accurate strategies were developed by other teams and other markers are currently explored for sorting secretory cells [26–28].

Starting with this current tedious protocol optimization for microarray experiments, what additional protocol requirements can we expect in light of the more recent and seductive single-cell RNA-seq analyzes? We hope to elaborate some answers to this question ourselves as the team plans the performance of such complementary techniques.

Even if strategy for the characterization of common subsets of airway epithelial cells from HBEC using a sorting flow panel have been published [27], the specific Club Cells was not addressed. In conclusion, our work allowed successfully achievement of microarray analysis of intracellular SCGB1A1-sorted, formalin-fixed HBEC cultured at ALI.

## Declaration of competing interest

The authors declare no competing interests.

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