Optimization and image analysis of RNAscope® technology on 3D human organotypic ciliated respiratory epithelial culture

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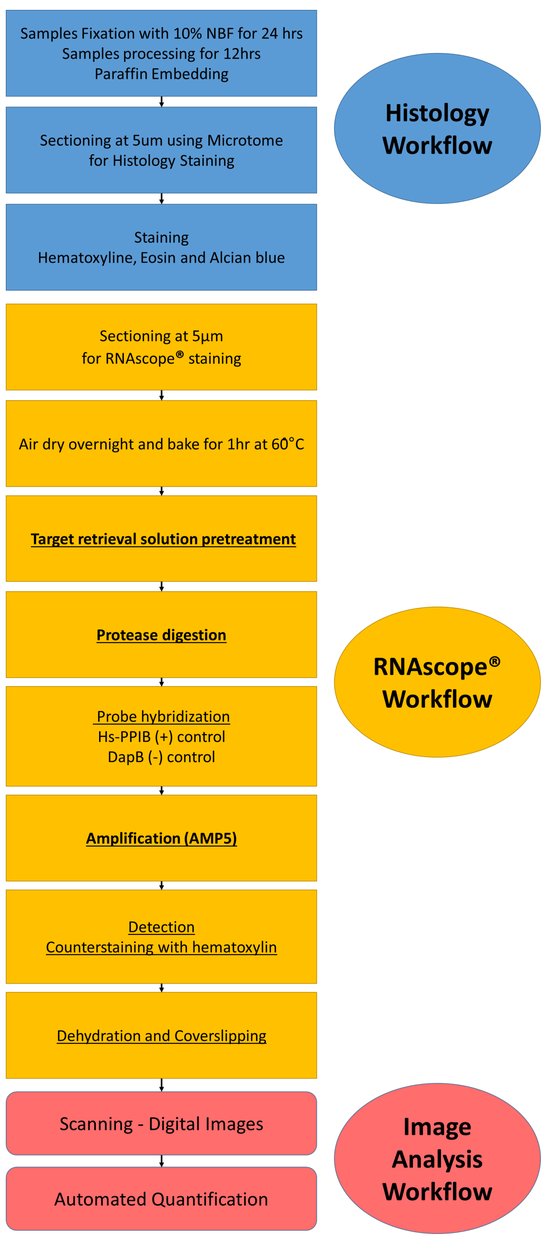
Introduction and Objectives

Introduction: RNAscope® is an *in situ* hybridization (ISH) technology, developed by Advanced Cell Diagnostics (ACD, Newark, NJ, USAD), used to visualize and detect RNA of interest in a cell-specific manner on formalin-fixed paraffin-embedded tissue sections. RNAscope® technology is a good alternative to immunohistochemistry, the success of which is strictly dependent on antibody availability. Human organotypic respiratory epithelial cultures derived from human primary cells comprise differentiated cells in a 3D organization of cells that better recapitulate the morphological, physiological, and molecular aspects of human tissues.

Objective: The objective of this work was to optimize the RNAscope® method for human organotypic nasal cultures and quantify the staining using Definiens software complemented by a fit-for-purpose, custom-built plug-in developed by Definiens.

Material and Methods

Workflow



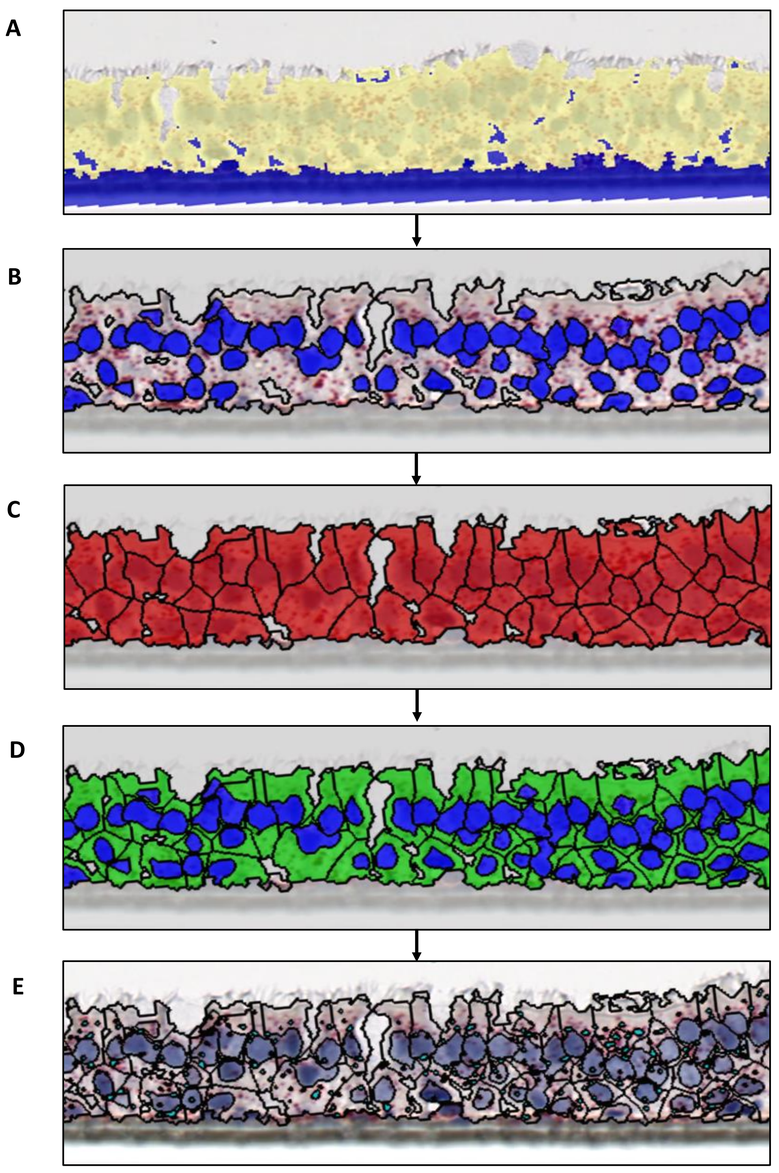
Histology workflow:
Five replicates of nasal cultures were fixed in 10% neutral-buffered formalin for 24 hours at room temperature and processed with the Leica ASP300S Tissue Processor on a 12-hour automated schedule. Slides with 5 µm-thick sections were stained with hematoxylin & eosin and Alcian blue to assess the morphology of received cultures.

RNAscope® optimization:
Unstained slides with 5 µm-thick sections were used for optimization of RNAscope®. The optimization was performed on the Leica BOND RX system using 2.5 LS Reagent Kit-RED by mainly focusing on three crucial parameters: pretreatment with Epitope Retrieval Solution 2 (ER 2), protease digestion to unmask the target RNA, and amplification with AMP5 to increase the sensitivity of the assay. For optimization, human peptidylprolyl isomerase B (Hs-PPIB) was used as a positive control, while dihydrodipicolinate reductase (dapB), a bacterial gene, was used as a negative control. The stained slides were checked under microscope.

RNAscope® quantification:
To quantify the average number of red spots per cells for each sample, the stained slides were scanned using the Hamamatsu NanoZoomer 2.0 slide scanner at 40x magnification. Automated quantification was then carried out with a custom-built solution designed by Definiens (described in Figure 2). The quantification scoring for positive and negative control (see Table 1) will determine whether the RNAscope® technique is optimized.

Figure 1. Workflow for the histology and RNAscope® processing. The experimental process is divided into three steps (indicated by different colored boxes).

Quantification



(A) Customized solution detecting region of interest (ROI) : The customized plug-in automatically detected the ROI (yellow) and separated the noncellular part (insert membrane, in blue) in all three culture types.

(B) Nucleus detection: Nuclei detection was carried out using color intensity of the hematoxyline stain threshold (0.20) and an average size of nuclei (36 µm²).

(C) Cell simulation: The area of each cell was simulated by the software by extending the area from each nucleus to a maximum distance of 12 µm.

(D) Cells and nuclei visualization: Visualization of both nuclei and cells after nucleus detection and cell simulation allowed for a manual check that each nucleus belonged to one cell.

(E) Spot detection: Finally, the stained spots were detected by adjusting the red color intensity threshold (0.27) and spot size (0.67 µm²).

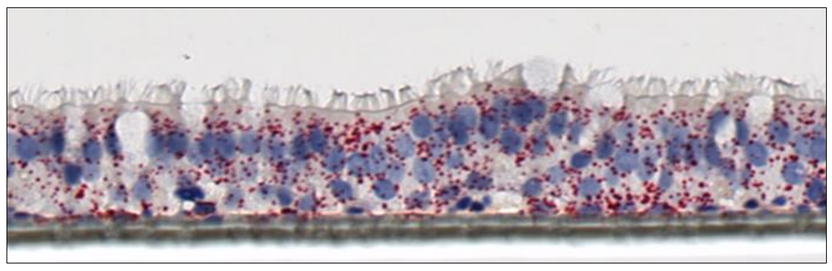
Score	Criteria
0	No staining or less than 1 spot per 10 cells
1	1 to 3 spots per cell
2	4 to 9 spots per cell
3	10 to 15 spots per cell
4	More than 15 spots per cell

Table 1: Scoring criteria for RNAscope® staining as per ACD recommendations. A scoring based on the average number of dots per cell is used to evaluate the staining results.

Figure 2. RNAscope® image analysis steps: Representative images of RNAscope® staining in nasal epithelial cultures **(A)** after ROI detection by the customized plug-in, **(B)** nuclei detection (blue), **(C)** cell simulation (red), **(D)** visualization of nuclei (blue) and cells (green), and **(E)** Hs-PPIB (positive control) and dapB (negative control) RNA detection in cells. The nuclei were stained with hematoxylin (blue). All shown images were scanned and quantified at 40x magnification.

Results

Hs-PPIB (positive control) – Score of 3



dapB (negative control) – Score of 0

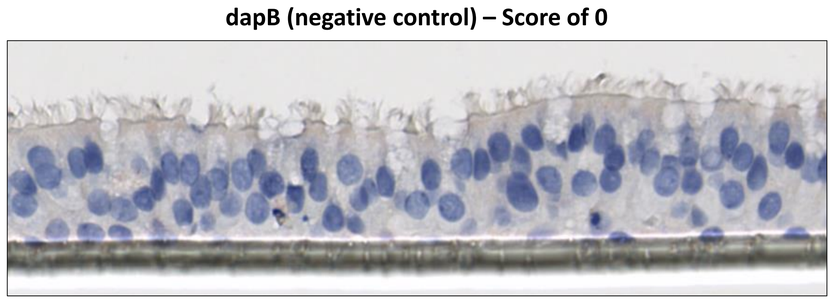


Figure 3. RNAscope® staining. From the initial microscopic evaluation, strong expression level of the Hs-PPIB positive control probe was observed with absence of background noise. No expression of the dapB negative control probe was observed.

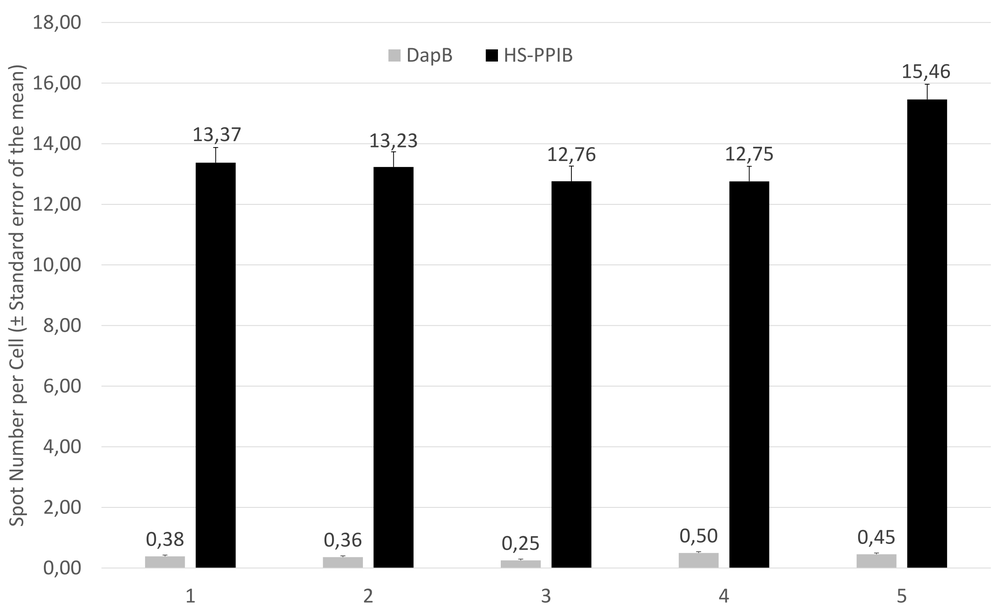


Figure 4. Quantification of the RNAscope® for the Hs-PPIB and dapB probes. Number of spots per cell in nasal organotypic epithelial cultures. Error bars indicate standard deviation (n=5).

The quantification showed that the five nasal culture samples stained with Hs-PPIB probes had 12.75 to 15.46 spots per cell and 0.25 to 0.50 with dapB.

Using the Hs-PPIB control probe, samples had an average number of spots per cell well within expected ACD guidelines (Table 1): for all replicates, the Hs-PPIB score was ≥ 2, and the dapB score was 0 (Table 2).

Table 2. Scores of the five replicates.

Sample	1	2	3	4	5
Score Hs-PPIB	3	3	3	3	4
Score DapB	0	0	0	0	0

Low values of the standard deviation suggested that there was no variability between the five replicates.

The image analysis confirmed that the RNAscope® technique for organotypic nasal cultures was optimized.

Discussion - Conclusion

In conclusion, it is important to note that tissue fixation and tissue processing have a critical role in maintaining **tissue morphology** and **RNA preservation** within tissues. We optimized RNAscope® protocols on nasal organotypic cell cultures based on three crucial parameters as **pretreatment**, incubation with **protease**, and various **amplification steps.** We succeeded in developing an **automated image analysis** with Definiens Tissue studio to count the **number of red dots per cell** and assign a **score** according to ACD recommendations. The quantification results demonstrated an accurate measurement of the optimized conditions for ISH using the RNAscope® technology on human organotypic respiratory epithelial culture samples.

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