Spatial transcriptomics for respiratory research and medicine

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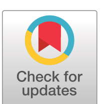

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In combination with scRNA-seq, spatial transcriptomics have the potential to lead to an unprecedented view of lung architecture at the single cell level, providing original information on lung physiology and physiopathology https://bit.ly/3uDYir0

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Introduction

Recent developments in single-cell technologies, and particularly single cell RNA sequencing (scRNA-seq), have provided invaluable tools to decipher complex biological systems like the lung. In the respiratory system, scRNA-seq analyses have led to the discovery of new cell types, such as ionocytes, as well as to a refined classification of the cells composing the lung [1–4]. Profiling of more than 300 000 cells from patients suffering lung pathologies, such as idiopathic pulmonary fibrosis (IPF), has allowed to identify new sub-populations of aberrant basal and endothelial cells that are specific to IPF [5]. Furthermore, collective efforts such as the Human Cell Atlas, aiming at characterising all cells in the human body at the molecular and spatial levels, have flagged the lung as a priority organ [6]. Ongoing efforts are now directed towards the development of spatial transcriptomic techniques allowing identification of cell localisation and description of prevailing cell–cell interactions in the organ in order to define a physiological cell atlas. Although technologies able to sequence *in situ* the transcriptome of individual cells are rapidly emerging, they still lack the resolution required to depict the extreme cell heterogeneity that characterises the anatomy of the lung. This article will present an overview of the different spatial transcriptomic methodologies that could be applied to the lung, as well as their potential impact for respiratory research and medicine.

One goal, different methodologies

The central goal of spatial transcriptomics is to depict the transcriptome landscape at the single cell level in native histological tissue sections. Two orthogonal techniques to study transcription at the cellular level have been firmly established during the last decade. First, scRNA-seq provides, from a cell suspension, access to an unbiased view of the transcriptome of each sequenced cell. Computational analysis permits the identification of different cell types present along with information on their respective transcriptome. Although scRNA-seq analysis provides key information to understand transcriptional alterations, it lacks sensitivity and, most importantly, loses spatial context due to the experimentally required cell dissociation. Second, conventional (single molecule) fluorescence *in situ* hybridisation (smFISH) detects RNA expression by direct hybridisation of fluorescently labelled probes on tissue sections. This allows the quantitative measurement of RNA expression with high sensitivity and specificity at the single cell level in their native spatial context, but not on the scale of the entire transcriptome [7, 8].

Recent developments further substantially improved these techniques, and innovative combinations of several approaches, such as multiplexing and amplification, provide an even deeper view. Here, we provide a brief overview of some of the recent milestone developments in spatial transcriptomics reached by either

improving approach or by implementing innovative combinations. Benefits and limitations of the different spatial transcriptomic methodologies are summarised in table 1.

scRNA-seq combined with hybridisation-based approaches

Computational analysis of scRNA-seq data allows the identification of distinct cell types present using published canonical markers. The analysis also allows identification of other cell-specific mRNA markers, usually 2–3, for each cell type, that could be used in hybridisation. Next, a set of cell type-specific probes is designed, labelled and hybridised on tissue sections using smFISH protocols (see below), and imaged by either wide-field or confocal microscopy to identify individual mRNAs [9].

The list of classic cell type-specific markers has been considerably enriched by information obtained in scRNA-seq, thus allowing extensive characterisation of cell types in some organs, such as the brain [10]. However, imaging the required number of markers in tissue remains particularly challenging and multiple factors (*i.e.* low expression level, high background due to off-target hybridisation or high tissue autofluorescence) can degrade signal quality. To circumvent these issues, different approaches, which may be combined, aim to: 1) amplify the actual RNA signal [11–15]; 2) increase the specificity of the probes and thus reduce off-target binding and background [16]; and/or 3) remove autofluorescence by tissue clearing [17, 18].

To reliably detect different cell types, several tens to hundreds of mRNA species have to be detected on the same histological sections. Nevertheless, the number of mRNA species that can be detected in conventional smFISH is limited by the number of spectrally resolved fluorophores, usually 3–4. To circumvent this limitation, sequential rounds of hybridisation are performed on microscopes equipped with microfluidic devices that allow, with adequate software, to fully automate imaging and buffer exchange.

TABLE 1 Comparison of the different spatial transcriptomic methodologies

	Hybridisation-based methods	High throughput strategies	*In situ* sequencing methods
Techniques	• seqFISH • osmFISH • HCR[#] • smHCR[#] • ClampFISH[#] • SABER[#] • PLISH[#] • SCRINSHOT[#] • RNAscope[#] (Bio-Techne)	• seqFISH+ • MERFISH	• *In situ* sequencing for RNA • FISSEQ • ExSeq • STARmap • Slide-seq/Slide-seqV2 • HDST • Visium (10XGenomics)
Imaging requirements	• Widefield (osmFISH, ClampFISH, SABER, SCRINSHOT, RNAscope) • Confocal (HCR, smHCR, PLISH) • Super-resolution (seqFISH)	• Widefield (MERFISH) • Confocal (seqFISH+)	• Widefield (Visium) • Confocal (FISSEQ, STARmap, Slide-seq/ Slide-seqV2, ExSeq)
Benefits	• High sensitivity • Cellular resolution • Low cost	• High throughput (up to 10 000 transcripts) • High sensitivity • Cellular resolution • Untargeted approach	• High throughput • Untargeted approach • *De novo* identification of spatial patterns
Limitations	• Low throughput (2–35 transcripts) • Targeted approach • Time consuming	• Technically challenging • High level of image analysis • Intermediate cost	• Low sensitivity • Low spatial resolution • High cost
Applications in the lung	• PLISH: comparison of AT2 distribution of idiopathic pulmonary fibrosis *versus* control samples, automated cell type identification • SCRINSHOT: spatial mapping of tracheal cell types	NA	• *In situ* sequencing for RNA: spatial mapping of 34 immune markers in granulomas present in *Mycobacterium tuberculosis*-infected lungs

[#]: with fluorescent signal amplification.

For instance, in the cyclic-ouroboros smFISH method, three sets of probes targeting three different mRNAs are hybridised on a tissue section. After imaging, probes are stripped using high formamide concentration. Next, three new probe sets are hybridised on the same section, imaged and stripped, allowing the number of targets to linearly increase with the number of hybridisation rounds [10]. Computational analysis then uses probabilistic models to infer the cell types in the images when compared to the expression levels measured in previously obtained scRNA-seq.

Multiplexing strategies for hybridisation-based approaches

To increase the number of transcripts that can be detected in RNA FISH, more complex strategies using specific encoding strategies have been developed in the recent years. Here, a given mRNA is not uniquely identified with a single hybridisation but with a unique barcode established over several hybridisation rounds. This permits several species to be targeted simultaneously in each round. The mRNA expression is then computationally inferred, often with error-correction strategies being included in the encoding schemes. Among these approaches are Multiplexed Error-Robust FISH (MERFISH) and seqFISH, which have been improved over several iterations [17, 19, 20]. For instance, with seqFISH+, 10 000 different mRNAs can be imaged in brain sections, allowing precise cell type identification and molecular characterisation in their spatial organisation [21].

In situ *sequencing strategies*

These approaches aim to perform sequencing directly on histological tissue sections. Methods such as FISSEQ (fluorescent *in situ* sequencing) and STARmap (spatially resolved transcript amplicon readout mapping) reverse transcribe the mRNAs within cells and amplify them before sequencing [22–24]. Other approaches, such as Slide-seq/Slide-seqV2 and HDST (high definition spatial transcriptomic) analysis, use coverslips or slides coated with barcoded oligo(dT) to capture mRNAs from tissue sections while recording their position at the same time [25–28]. After next generation sequencing, decoding of the barcodes allows visualisation of expression patterns of mRNAs present on the slide. A recent approach, ExSeq, combines spatial expansion of the biological sample with RNA sequencing, permitting highly multiplexed mapping of RNAs in tissue sections [29]. Compared to the hybridisation-based approach that targets a relatively limited group of markers with high sensitivity, the unbiased approach of sequencing-based methodologies should theoretically depict the whole transcriptome landscape of every cell in the tissue and thus it holds great promise. However, some limitations still need to be overcome, such as the lack of sensitivity and the fact that the spatial resolution does not classically reach the single cell level.

Altogether, spatial transcriptomic methodologies have the potential to lead to an unprecedented understanding of tissue architecture and physiology. However, these technologies remain challenging and researchers need to evaluate what fits best their needs in terms of resolution and throughput (table 1).

Spatial transcriptomics in the lung and future applications

Three spatially resolved transcriptomic studies have been performed in the lung so far. First, PLISH (proximity ligation *in situ* hybridisation) is a FISH-based approach relying on probes that act after a ligation step as a template for signal amplification. This allows to achieve high specificity, high sensitivity and high signal-to-noise ratio [30]. The PLISH protocol works on cryo-preserved as well as formalin-fixed paraffin-embedded human tissues and can be combined with classical immunostaining, making it suitable for clinical studies and diagnosis. As a proof of concept, PLISH was used to compare the spatial distribution of AT2 cells in control and IPF human lung tissue. In addition, the authors demonstrated that images from iterative staining for classical markers of epithelial cells (*e.g.* AT1, AT2, club cells) and macrophages fed into a dedicated analysis pipeline allowed an automated mapping of these cells onto the histological lung section.

Second, SCRINSHOT (single cell resolution *in situ* hybridisation on tissues) is validated in different organs, including the respiratory tract [31]. It uses padlock probes, which have complementary arms to the target mRNA sequence and a common backbone. After hybridisation, padlock probes are ligated using the SplintR ligase to create circular single-stranded DNA molecules that serve as a template for amplification. Staining and analysis of 29 markers by SCRINSHOT allowed mapping of the distinct epithelial cells present in the tracheal epithelium, including the recently discovered ionocytes [1, 2]. In the distal airways and alveoli, 15 markers were used to robustly identify macrophages and epithelial cells, such as AT1 and AT2 cells, as well as club and neuroendocrine cells.

Third, Carow *et al.* [32] used an *in situ* sequencing technique to localise 34 immune transcripts in lung tissue infected by *Mycobacterium tuberculosis*. Briefly, RNAs present in the lung section are reverse

transcribed and padlock DNA probes specific to the targeted transcripts are hybridised. A ligation step allows the circularisation of the probes required for the amplification and then, the amplified products are subjected to sequencing. Here, spatial transcriptomics allowed mapping of immune cells that characterise tuberculosis granulomas.

The spatial transcriptomics field has evolved very rapidly in recent years. With community-driven efforts such as *Tabula Muris* and The Human Cell Atlas that prioritise the respiratory system [6, 33, 34], lung cell classification is being refined and molecular markers for each cell type are now available from lung scRNA-seq datasets. Analysis of these cell type-specific markers with spatial transcriptomics will allow a better characterisation of the physiological interactions between cell types as well as their alterations in respiratory diseases, providing key insights in the understanding of their physiopathology.

In clinical practice, spatial transcriptomics, with an effort to standardise methods and create automated analysis pipelines, may eventually provide a useful complementary tool to analyse molecular markers or spatial patterns specific of lung pathologies on histological sections.

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