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Focused REVIEW

RNA seqFISH: A High-Resolution Method for Spatial Transcriptomics

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ABSTRACT

By revealing the molecular processes and interactions within cells, omics technologies have transformed our comprehension of cellular functions and metabolism. RNA seqFISH is a spatial transcriptomics method that enables high-resolution imaging of thousands of genes in single cells within tissues, revealing the spatial organization and dynamics of the transcript One in situ. In this article, we review the method itself and the applications of this technology in various biological contexts, such as development, differentiation, and disease. We discuss the benefits of the RNA seqFISH technique to provide us new insights into the molecular mechanisms underlying cellular function and diversity, as well as the limitations of this technique. We conclude that RNA seqFISH is a powerful and promising tool for studying spatial transcriptomics, and that it will inspire more researchers to adopt and advance this technology.

Keywords

spatial omics, transcriptomics, in situ hybridization, RNA seqFISH.

Abbreviations

Expansion Microscopy (ExM); Fluorescence in situ hybridization (FISH); In situ hybridization (ISH); In situ sequencing (ISS); next generation sequencing (NGS); RNA sequential FISH (RNA seqFISH); single-molecule FISH (smFISH); single-molecule hybridization chain reaction (smHCR); transcription active site (TAS).

SUMMARY

1. Introduction

Omics technologies have revolutionized the way we understand cellular activities, function and metabolism. As a subdiscipline of omics, transcriptomics investigates the gene expression patterns in cells. Even more so, spatial transcriptomics has set the goal of maintaining locational information about gene expression by retrieving not only data about the RNAs present in a cell, but also the exact location of the RNAs inside the cell. Spatial transcriptomics methods can be divided into in situ sequencing (ISS), in situ hybridization (ISH) and next generation sequencing (NGS) based-sequencing with region capture

methods. While each of these classes of spatial transcriptomics methods have made significant advances throughout the recent years, ISH methods clearly stand out to yield the greatest number of genes studied in an experiment with the highest efficiency compared to the other two classes of methods.

ISH methods build on basic fluorescence in situ hybridization (FISH), which is a ubiquitously used technique to label specific sequences of nucleic acid for biological, diagnostic or research purposes. In FISH a complementary sequence is delivered to cells to bind to the target sequence, making it detectable and visible. Single-molecule FISH (smFISH) was developed to exploit FISH for spatial transcriptomics[1–3]. To increase the number of RNA that can be studied in a single experiment, researchers have created new methods building on the basic functioning principle introduced by smFISH.

One very promising ISS technology is RNA sequential FISH (seqFISH) which was first described by Lubeck et al. in 2014 and applied by Shah et al. in 2016 by demonstrating its applicability in molecular neuroscience by describing cellular clusters in the hippocampus[3,4]. In this review, we want to present the RNA seqFISH method as previously described and elaborate on how it has improved research in the field of transcriptomics.

2. The RNA seqFISH method

THE CONCEPT. RNA seqFISH relies on the allocation of specific barcodes to each RNA type targeted in the experiment. To do so a color-schemed barcode is used[5]. The order of colors in the code corresponds to the color of the fluorophore that will be observed in the respective seqFISH cycle at the specific RNA spot, e.g. a color code of green-blue-red will produce a green fluorescence in spot x in the first cycle, a blue fluorescence in spot x in the second cycle and a red fluorescence in spot x in the third cycle[4,5]. These colored fluorescences can be obtained by the addition and subsequent hybridization of readout probes to the primary FISH probe. The primary FISH probe is a FISH probe that is specifically designed for each targeted RNA type and bound to their target region before multiplexing cycles start[4,5]. It can be bound by specific readout probes and due to the intelligent design of FISH probe and readout probes, the barcode can be obtained during the cycles[5]. Each of the multiplexed seqFISH cycles contain the following steps[5]: (1) readout probes with differently colored fluorophores are used for every cycle which hybridize to the complementary regions on primary FISH probes, (2) Imaging is performed, (3) the hybridized FISH probes are removed with DNase I to prepare for the next cycle. Furthermore, Shah et al. have introduced a modification to the hybridization of the FISH probes to the RNAs, which they call single-molecule hybridization chain reaction (smHCR)[4]. HCR has been previously described as a method for biosensing nucleic acids and is based on two hairpins (H1 and H2), that overlappingly hybridize upon exposure to the initiator sequence to increase fluorescence signal strength[6].

In the context of seqFISH, the readout probes do not just hybridize to their complementary region, but they are additionally amplified in situ to increase the strength of the fluorescent signal. In particular, this is of relevance concerning tissue with high autofluorescence levels such as the brain. Theoretically, seqFISH enables the study of F^n RNA types, where F stands for the number of fluorescent dyes used and n is the number of seqFISH cycles[5]. To correct errors caused by missing hybridization, Shah et al. introduced a supplementary hybridization round[4]. This enables the correct RNA type identification despite a mishybridization in one of the previous cycles.

THE PROCEDURE. In the first step, the targeted RNA types need to be selected from a database and each RNA is assigned a color-schemed barcode (Figure 5A)[4]. This is followed by design and synthesis of FISH probes that can specifically hybridize to their corresponding RNA type (Figure 5A)[4]. The FISH probes additionally contain an HCR adaptor as the initiator sequence for smHCR[4]. To enable multiplexing, multiple sets of readout probes are synthesized, but with different fluorescent dyes. For instance, Shah et al used 24 probes per RNA type and 5 fluorophores in 4 seqFISH cycles to analyze 125 genes[4].

Tissue sections are fixed and permeabilized. Then, seqFISH cycles consisting of hybridization, imaging and readout probe stripping can start (Figure 5B)[4]. Thus, the sample is put in a hybridization chamber overnight, where readout probes hybridize to their corresponding RNAs. This step is followed by HCR amplification. Therefore, hairpins are diluted in an amplification buffer and added to the sample[4]. After imaged under the fluorescence microscope for the

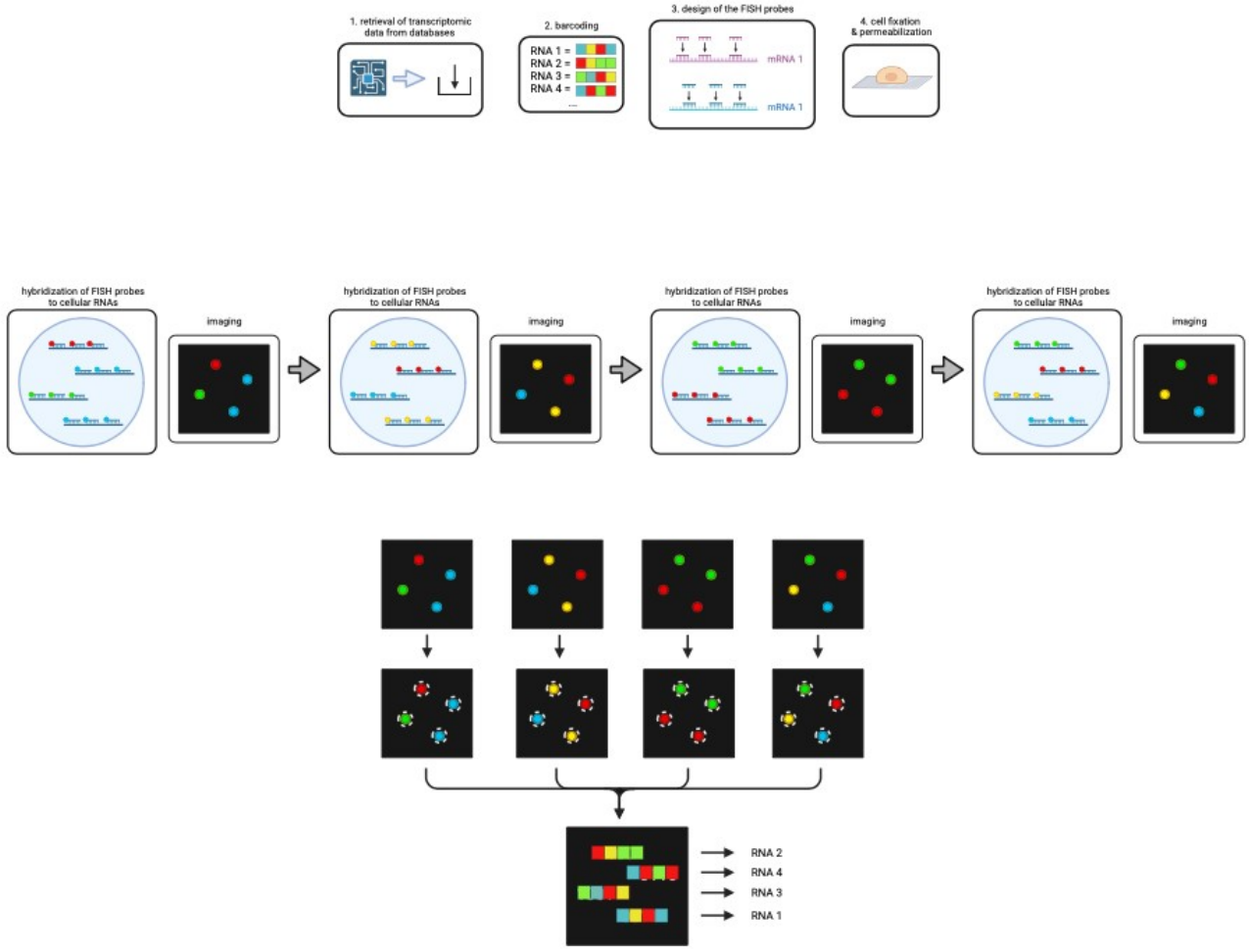

Figure 1. *Overview on the RNA SeqFISH method.* (A) planning and preparation. RNA data is retrieved and RNA types that are to be targeted in the seqFISH are determined. Each RNA type is assigned to a specific color-schemed barcode and FISH probes that are RNA-specific are designed and synthesized accordingly. The FISH probes also contain a HCR adaptor.(B) the seqFISH process. Cells are fixed and permeabilized. FISH probes of the first cycles are added and HCR amplification is performed. This is followed by imaging and DNase I stripping of FISH probes. (C) exemplary seqFISH experiment. To simplify the process, we depict the seqFISH procedure executed on four RNAs of yet unknown identity. Hybridization and imaging is depicted for each cycle, whereby DNase I stripping lies in between. (D) Data analysis. Analysis of the obtained images to obtain the color-schemed barcodes which indicate the RNA type present in the respective spot. (Created with Biorenders.com)

respective color channels of the fluorescent dyes of washing and nuclear staining with DAPI, samples are the experiment. As the last step of the seqFISH cycle, DNase I is used to remove the fluorescent labeling of RNAs[4]. This prepares for hybridization of the next set of readout probes in the next cycle. After all the cycles have been completed, image data is processed and the images of a cycle, each displaying only one color, are overlaid to obtain one image per seqFISH cycle displaying all fluorescent color emissions in the

tissue[4]. Images of different cycles are aligned and cellular borders can be determined using DAPI staining patterns and density of RNA signals[4]. Next, the fluorescent spots in the images are identified and the color barcode is read out (Figure 5C)[4]. The obtained data can then be used to characterize cell types in a cluster.

3. Applications, benefits and limitations

The RNA seqFISH method has been used and adapted in a variety of studies and research fields. While the original article by Shah et al. from 2016 deploys RNA seqFISH to create cell clusters based on the transcriptional profile of cells in the hippocampus[4], RNA seqFISH has been also used to study mouse organogenesis[7], the development of T-cells in the thymus[8], and the classification of cells in the ventrolateral subdivision of the ventromedial hypothalamus into cell types[9]. Other research groups further developed the seqFISH approach either to optimize its performance or to make it more suitable to the study of specific research questions. For instance, Eng et al. introduced optimizations to the RNA seqFISH method in 2019, calling the enhanced version RNA seqFISH+[10]. The major improvement made is the increase of colors used in the experiment by dividing each of the three color channels into 20 pseudocolors[10]. Pseudocoloring is the process of assigning pseudocolors to color or grayscale intensities, thus creating a pseudo-colored image. So in total, 20 * 3 = 60 pseudocolors can be analyzed per RNA seqFISH+ cycle[10]. The RNA seqFISH+ method has been tested on 10.000 genes in tissue of the mouse cortex, subventricular zone and olfactory bulb, allowing for the detection of on average ca. 35.000 genes per cell[10]. On the other hand, the versatility of the seqFISH approach has been proved by numerous researchers reporting on seqFISH based technologies that each tackle a specific problem. For example, Shah et al. presented intron seqFISH in 2019 which allows the detection of nascent RNA that is being transcribed at transcription active sites (TAS) in the nucleus[11]. This can be done by targeting transcribed intron sequences, which are removed shortly after by RNA processing[11]. Moreover, par-seqFISH has been introduced by Dar et al. in 2022, rendering the seqFISH compatible for the study of bacterial transcriptomes[12]. Table 1 explores benefits and limitations of the RNA seqFISH technology.

Table 1. *Benefits and Limitation of RNA seqFISH technology*[4,10,13,14]

Benefits	Limitations
• high RNA detection efficiency including spatial information (efficiency of 84% compared to smFISH) • single cell resolution • RNA seqFISH enables accurate cell type identification, classification and the creation of spatial cellular clusters based on transcriptional profiles • easy upscaling to allow for an increase in the amount of RNA types / genes studied by the increase of fluorophores used or of the cycles performed • signal amplification by HCR enables the application of RNA seqFISH on tissue with high autofluorescence • error detection and correction by a supplementary hybridization round ensures accurate RNA identification • the spatial compartmentalization of RNAs can be explored by antibody-labeling of subcellular structures (e.g. ER, nucleus) in the cells	• no untargeted approach is possible because RNA type sequences have to be known for the design of primary FISH and readout probes • compared to RNA-Seq only a low number of genes can be studied by RNA seqFISH (249 genes in the hippocampus by Shah et al.) • limited resolution because of optical crowding / high RNA density during imaging. This has been significantly improved by RNA seqFISH+. Other approaches for improving the resolution limit are the application of super-resolution microscopy or Expansion Microscopy (ExM) protocols on RNA seqFISH. • complex probe design and data analysis • multiplexing can damage the tissue structure, possibly leading to errors • costly, time-consuming, specialized equipment needed

4. Conclusion

In this article, we have discussed the transcriptomics method RNA seqFISH, which allows for high-resolution imaging of thousands of genes in single cells within tissues. We have reviewed the applications of this method in various biological contexts, such as development, differentiation, and disease and the great potential the seqFISH concept has to be adapted to address various research questions. We have also highlighted the benefits and limitations of this method, such as its ability to efficiently detect low copy number genes and its

requirement for complex probe design and data analysis. RNA seqFISH is a powerful tool that can reveal the spatial organization and dynamics of the transcriptome in situ, and provide new insights into the molecular mechanisms underlying cellular function and diversity. However, there are still challenges and opportunities for improvement, such as increasing the throughput, multiplexing, and compatibility of the method with other modalities. We hope that this article will inspire more researchers to adopt and advance this technology, and to explore the fascinating world of spatial transcriptomics.

Conflict of Interest

The authors declare no conflicts of interest.

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