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Research Paper

Detection of single mRNAs in individual cells of the auditory system

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ABSTRACT

Gene expression analysis is essential for understanding the rich repertoire of cellular functions. With the development of sensitive molecular tools such as single-cell RNA sequencing, extensive gene expression data can be obtained and analyzed from various tissues. Single-molecule fluorescence *in situ* hybridization (smFISH) has emerged as a powerful complementary tool for single-cell genomics studies because of its ability to map and quantify the spatial distributions of single mRNAs at the subcellular level in their native tissue. Here, we present a detailed method to study the copy numbers and spatial localizations of single mRNAs in the cochlea and inferior colliculus. First, we demonstrate that smFISH can be performed successfully in adult cochlear tissue after decalcification. Second, we show that the smFISH signals can be detected with high specificity. Third, we adapt an automated transcript analysis pipeline to quantify and identify single mRNAs in a cell-specific manner. Lastly, we show that our method can be used to study possible correlations between transcriptional and translational activities of single genes. Thus, we have developed a detailed smFISH protocol that can be used to study the expression of single mRNAs in specific cell types of the peripheral and central auditory systems.

1. Introduction

Quantitative analysis of gene expression is a fundamental approach in studying cell functions and discovering biomarkers for various diseases. Spatiotemporal expression of mRNA molecules is commonly heterogeneous among different cells within the same tissue. Such variation in expression leads to differences in biological functions, both normal and pathological (Fox et al., 1995; Cao et al., 2017). Therefore, quantification of these individual mRNAs within single cells can be key to discovering their functions as well as their associations with diseases (Raj et al., 2008; Tsanov et al., 2016). *In situ* hybridization (ISH) is a technique that can determine where and when genes are expressed in specific cells and tissues. The principle behind ISH is that a labeled segment of DNA or RNA (a probe) hybridizes or binds with a complementary strand of DNA or RNA in a tissue sample (the target molecule). However, one problem with traditional ISH is that the precise locations of the target molecules cannot be determined. This is because the probe requires the use of an enzyme to catalyze chromogenic or fluorogenic reactions, and the products of these reactions can diffuse away from the target molecules. Fluorescence ISH (FISH) circumvents this problem by employing a probe directly linked with a fluorophore (Femino et al., 1998). The most recent FISH development is single-molecule FISH (smFISH), an emerging technique used to achieve quantitative visualization and localization of single mRNA transcripts within individual cells (Raj et al., 2008; Trcek et al., 2012; Hansen and van Oudenaarden, 2013; Kwon, 2013; Cabili et al., 2015; Stapel et al., 2016; Tsanov et al., 2016; Querido et al., 2017). This method has been tested in the developing auditory system, but signal detection has not been optimized for quantification (Pawlosky, 2014).

Here, we present a detailed smFISH method in tandem with immunohistochemistry for analysis of gene expression within single cells of the auditory system. We demonstrate the success of this method by focusing on well-established cell types, such as spiral ganglion neurons (SGNs), inner and outer hair cells (IHCs and

Abbreviations: IC, inferior colliculus; IHC, inner hair cell; ISH, *in situ* hybridization; Nrg1, Neuregulin-1; OHC, outer hair cell; SGN, spiral ganglion neuron; smFISH, single-molecule fluorescence *in situ* hybridization

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OHCs) of the cochlea, and neurons positive for γ-aminobutyric acid (GABA) in the inferior colliculus (IC). In addition, we modify an analysis pipeline for automated transcript detection and manual cell segmentation. To illustrate the power of our method, we combine smFISH method with immunohistochemistry to study the expression levels, subcellular localizations, and transcriptional and translational correlations of single mRNAs within individual cells of the auditory system.

2. Materials and methods

2.1. Probes

Probes for T-type calcium channels Cacna1h [*$Ca_v3.2$*, Cat. No. 459751-C3, Advanced Cell Diagnostics (ACD), USA] and Cacna1i (*$Ca_v3.3$*, Cat. No. 459781, ACD, USA), as well as Neuregulin-1 (*Nrg1*, Cat No. 422961-C3, ACD, USA), Myosin VI (*Myo6*, Cat. No. 313381, ACD, USA), glutamate decarboxylase 1 (*Gad1*, Cat. No. 400951, ACD, USA), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, Cat. No. 314091, ACD, USA) and a blank negative control (Cat. No. 300041, ACD, USA) were obtained from ACD, Newark, California. The technique we selected for our experiments, the RNAscope Multiplex Fluorescent Assay, achieves simultaneous single mRNA visualization in a sample with heightened specific signal amplification and minimal background noise (Wang et al., 2012). This is mainly due to the use of short Z-shaped oligonucleotide complementary DNA probes that selectively hybridize to the target mRNA in pairs (ZZ) with subsequent amplification steps that intensify the target-specific signals. Each of the target probes contains a region 18 to 25 bases in length that complements the target mRNA. In addition, there is a spacer sequence and a tail sequence 14 bases long. When a pair of target probes hybridizes contiguously to a target sequence, a 28-base hybridization site is formed for the preamplifier. The preamplifier contains 20 binding sites for the amplifiers, and each amplifier contains 20 binding sites for the fluorophores. Unlike previous smFISH probe designs, the ZZ design only allows the preamplifier to bind when both target probes bind simultaneously to the target mRNA, which ultimately reduces nonspecific hybridization and increases the validity of the signals (Pawlosky, 2014). The RNAscope probes are optimized specifically for the RNA targets as opposed to genomic DNA. Finally, the probes are screened against all validated reference sequence RNAs (e.g., non-coding RNA, long non-coding RNA, and ribosomal RNA) during design feasibility. Detailed information about the probe sequences can be obtained by signing a nondisclosure agreement provided by the manufacturer.

2.2. Sample preparation

Animal procedures were performed at the Northeast Ohio Medical University in accordance with the guidelines of its Institutional Care and Use Committee (IACUC). The sample preparation protocol was as follows:

1. Adult mice at 8 to 10 weeks of age were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and were transcardially perfused (highly recommended) with diethyl pyrocarbonate (DEPC)–treated phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer.
2. Tissue samples from cochleae (perforated at the apex) and the IC were harvested and kept in a 4% paraformaldehyde solution (DEPC-treated) overnight on a shaker at 4 °C.
3. Samples were washed with PBS, and cochleae were decalcified in 0.35 M ethylenediaminetetraacetic acid (EDTA) for 5 days on a shaker at 4 °C and washed with PBS.
4. Samples were sequentially dehydrated in 10%, 20%, and 30% sucrose solutions at 4 °C for 1 hr, 2 hrs, and overnight, respectively.
5. Samples were transferred into optimal cutting temperature (OCT) compound and kept there for a minimum of 1 hr at 4 °C, and then snap frozen using a dry ice–ethanol mixture.
6. Samples were cryosectioned to a thickness of 12 μm, and the cryosections were placed onto Superfrost slides, which were stored at −80 °C until further use.

2.3. smFISH protocol

To detect different single mRNAs in the same ISH process, each target probe was labeled in a different color channel. In the RNAscope Multiplex Fluorescent Assay, a Channel 1 (C1) probe was always present in the mixed probe solution to provide the target probe dilution buffer (Table 1). A blank C1 probe was used if only a C2 or C3 probe was needed in the experiments. The detailed smFISH protocol that we performed is described here:

1. Plastic slide mailers (Cat. No. 01253-MB, SPI Supplies, USA) were autoclaved.
2. Cryosectioned slides of cochlear or IC tissue were taken out of a −80 °C freezer and immersed in pre-chilled 4% PFA for 15 min at 4 °C.
3. Slides were dehydrated through the following series at room temperature (RT), 5 min/step: (a) 50% ethanol; (b) 70% ethanol; (c) 100% ethanol; (d) 100% ethanol; and (e) dried for 1–2 min.
4. Tissue sections were traced 2–4 times with an ImmEdge Hydrophobic Barrier Pen (Cat. No. H-4000, Vector Laboratories, USA), and the traces were dried for 1–2 min at RT.
5. One drop of protease 4 (Cat. No. 320842, ACD, USA) was added to each section, and slides were incubated at RT for 30 min.
6. Probes were warmed to 40 °C in an incubation oven for 10 min.
7. C1, C2, and C3 probes were combined in a tube at a respective volume ratio of 50:1:1 to create the probe master mix, which was mixed by inversion several times.
8. Slides were tapped to remove excess liquid, washed 5 times in PBS, and then fully immersed in PBS for 1 min. This step was repeated once.
9. The probe master mix (30 μl) was added to cover each section. Slides were incubated at 40 °C for 2 hrs.
10. The RNAscope Wash Buffer (50×, Cat. No. 310091, ACD, USA) was warmed to 40 °C for 20 min and diluted with distilled DEPC-treated water to prepare the 1 × wash buffer.
11. Slides were washed 5 times in 1 × wash buffer and then fully immersed in 1 × wash buffer for 1 min.
12. For the detection of four different mRNAs at the same time, the AMP 1-FL, AMP 2-FL, AMP 3-FL (Cat. No. 320851, ACD, USA) and AMP 4 Alt A-FL (Cat. No. 320855, ACD, USA) solutions were warmed to RT. One drop of each amplifier solution (AMP 1-FL, AMP 2-FL, AMP 3-FL, and AMP 4) was added to cover each section, and the section was incubated at 40 °C for 30 min, 15 min, 30 min, and 15 min, respectively. Step 11 was repeated after each incubation.
13. Slides were washed 5 times in PBS.