Video Article

Quantitative Analysis of Alternative Pre-mRNA Splicing in Mouse Brain Sections Using RNA *In Situ* Hybridization Assay

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Abstract

Alternative splicing (AS) occurs in more than 90% of human genes. The expression pattern of an alternatively spliced exon is often regulated in a cell type-specific fashion. AS expression patterns are typically analyzed by RT-PCR and RNA-seq using RNA samples isolated from a population of cells. *In situ* examination of AS expression patterns for a particular biological structure can be carried out by RNA *in situ* hybridization (ISH) using exon-specific probes. However, this particular use of ISH has been limited because alternative exons are generally too short to design exon-specific probes. In this report, the use of BaseScope, a recently developed technology that employs short antisense oligonucleotides in RNA ISH, is described to analyze AS expression patterns in mouse brain sections. Exon 23a of neurofibromatosis type 1 (*Nf1*) is used as an example to illustrate that short exon-exon junction probes exhibit robust hybridization signals with high specificity in RNA ISH analysis on mouse brain sections. More importantly, signals detected with exon inclusion- and skipping-specific probes can be used to reliably calculate the percent spliced in values of *Nf1* exon 23a expression in different anatomical areas of a mouse brain. The experimental protocol and calculation method for AS analysis are presented. The results indicate that BaseScope provides a powerful new tool to assess AS expression patterns *in situ*.

Video Link

The video component of this article can be found at https://www.jove.com/video/57889/

Introduction

Alternative splicing (AS) is a common process that occurs during pre-mRNA maturation. In this process, an exon can be differentially included in mature mRNA. Thus, through AS, one gene can generate many mRNAs that code for different protein products. It is estimated that 92–94% of human genes undergo alternative splicing[1,2]. Abnormal alternative splicing patterns resulted from genetic mutations have been linked to a large number of diseases, including amyotrophic lateral sclerosis, myotonic dystrophy, and cancer[3,4]. It is thus crucial to investigate and better understand alternative splicing regulatory mechanisms in an attempt to find new treatments of human diseases.

AS is often regulated in a cell type-specific fashion. It is important to determine the AS expression pattern of a specific gene in a given biological system. However, this becomes complicated when a complex organ that contains many different types of cells, such as the brain or heart, is studied. In this case, an ideal choice of assay system is RNA *in situ* hybridization (ISH) using tissue sections so the AS expression pattern of a specific gene can be detected in many cell types simultaneously. Indeed, exon-specific probes have been used to assess expression levels of an alternative exon[5,6,7]. However, this approach is not well suited for AS pattern analysis for the following reasons. First, conventional ISH methods usually use probes longer than 300 bp, while the average size of vertebrate internal exons (not first or last exon) is 170 nucleotides[8,9]. Second, when an exon-specific probe is used to examine the splicing pattern of an internal alternative exon, the only mRNA isoform detected by the probe is the one that contains the exon, while the mRNA isoform without the exon cannot be detected. Thus, calculation of the percent spliced in (PSI) value for the alternative exon is complicated. Furthermore, conventional fluorescent ISH often combines ISH with immunostaining, which reduces the detection efficiency and robustness. For example, in a study that investigated the stress-induced splicing isoform switching of the acetylcholinesterase (AChE) mRNA, digoxigenin was incorporated into the ISH probe and detected using anti-digoxigenin antibody. Alternatively, biotin-labeled probe was detected by an alkaline phosphatase/streptavidin conjugate and a substrate for alkaline phosphatase[10]. Neither method uses any amplification strategy to increase the sensitivity of detection. As a result, it is challenging to detect mRNA transcripts that are expressed at low levels. Thus, a simpler and more robust ISH assay system is needed to analyze AS expression patterns *in situ*.

BaseScope was recently developed based on the platform of RNAscope, a well-established and widely used ISH assay system. Both assay systems employ a target-specific amplification technology that increases the sensitivity of detection[11,12]. What distinguishes one from the other is the length of the target sequence, which is as short as 50 nucleotides for BaseScope, and 300–1,000 nucleotides for RNAscope. Thus, it is possible to design probes that target exon-exon junctions to detect specific alternative mRNA isoforms. In the current study, a procedure was

established to examine AS expression patterns of neurofibromatosis type 1 (*Nf1*) exon 23a, an alternative exon extensively studied in the same laboratory[13,14,15,16,17], in mouse brain sections. The results demonstrate that BaseScope is an ideal system to study expression patterns of *Nf1* exon 23a *in situ*. As this assay system can be adapted to analyze AS expression patterns of many alternative exons, it represents a powerful new tool in the studies of AS.

Protocol

All of the experiments described here that involve mice have been approved by the Case Western Reserve University Institutional Animal Care and Use Committee. The title of the protocol 2016-0068 (PI: Hua Lou) is "Role of alternative pre-mRNA splicing in vertebrate development".

NOTE: Information of all of the equipment, reagents and supplies used in this protocol is included in **Table of Materials**.

1. Prepare Formalin Fixed Paraffin Embedded (FFPE) Sections

1. **Euthanize 1 CD1 mouse and 1 C57BL/6J mouse by cervical dislocation. Carefully cut open the skull with surgical scissors and forceps. Remove the brain with a scoop.**
   NOTE: 5-month-old CD1 male mice and 3-month-old C57BL/6J male mice were used and showed similar results.
   1. Wash the brain with PBS. Fix the whole brain with a solution containing 10% formalin at room temperature (RT) for 30 h by putting the whole brain in 50 mL of the formalin solution.
   2. Change the fixing solution to PBS and incubate the brain in PBS overnight at 4 °C. Dehydrate and embed the brain with xylene and paraffin using standard procedures[18].

2. **Use a microtome to cut embedded tissue into 5 µm sections. Put the paraffin ribbon in a RT water bath first, and then in a 45 °C water bath to spread the ribbon.**
   1. When the wrinkles and folds in the ribbon disappear, immediately mount sections on glass slides. Air dry slides overnight at RT. Bake slides for 1 h at 60 °C before use.

2. Sample Pretreatment

1. **Deparaffinize tissue sections. Carry out these steps in a fume hood.**
   1. Fill two washing dishes (**Figure 1A**) with 250 mL of fresh xylene and two with 250 mL of 100% ethanol. Insert tissue slides into a washing rack (**Figure 1A**) and place the washing rack in washing dishes following the order and incubation time indicated in **Table 1**.
   2. During each incubation, lift the washing rack up and down more than 3 times in the washing dish. After each incubation, quickly move the washing rack into the next washing dish to prevent the tissue sections from drying out.
   3. After completing the incubation steps, remove the washing rack with slides from the washing dish. Place the rack in a 60 °C incubator for 5 min or until the slides are completely dry.

2. Draw a 0.75'' x 0.75'' barrier surrounding the tissue section with a hydrophobic barrier pen. Air dry the barrier for 3 min at RT.
3. **Pretreat slices**
   1. Prepare reagents and equipment
      1. Turn on the hybridization oven (**Figure 1B**) and set temperature to 40 °C 30 min before use.
      2. Wet the humidifying paper (**Figure 1E**) with distilled water completely and put it in humidity control tray (**Figure 1D**, left). Keep the humidity control tray in the hybridization oven.
      3. Prepare 700 mL of fresh 1x Target Retrieval Reagents by adding 630 mL of dH_2O to 70 mL of 10x Target Retrieval Reagents.
   2. Apply hydrogen peroxide.
      1. Put the deparaffinized slides on the bench. Add 5–8 drops of hydrogen peroxide to a section to completely cover the tissue. Incubate for 10 min at RT.
      2. Tap the slides on a piece of absorbent paper to remove the hydrogen peroxide solution, one slide at a time. Immediately insert the slide into a washing rack and move the rack to a washing dish filled with dH_2O.
      3. Wash the slides by moving the rack up and down in distilled water for 3–5 times. Move the washing rack to a washing dish filled with fresh distilled water and wash slides one more time.
   3. Perform target retrieval
      1. Place a 1,000 mL glass beaker on a hot plate. Add 700 mL of fresh 1x Target Retrieval Reagents into the beaker and cover it with foil. Insert a thermometer in the beaker through the foil cover (**Figure 1C**). Turn on the hot plate and set on high for 10–15 min.
      2. When the temperature of retrieval reagents reaches 98 °C, switch the temperature control of the hot plate from high to low to maintain a mild boil. Do not boil more than 15 min.
      3. Meanwhile, carefully open the foil and put the washing rack with slices inside the retrieval regent. Cover the beaker again and incubate for 15 min.
      4. Use forceps to remove the rack from the beaker to a washing dish filled with 200 mL of dH_2O and rinse for 15 s.
      5. Move the rack to a washing dish containing 100% fresh ethanol and let sit for 3 min. Remove slides and dry them in a 60 °C incubator for 10 min.
   4. Apply Protease III
      1. Place the slides on the stain rack (**Figure 1D**, right). Add 5 drops of Protease III to cover the tissue. Carefully put the rack in the humidity control tray (**Figure 1D**, left) and insert the tray into the 40 °C hybridization oven. Incubate for 30 min.

2. Remove the slide rack and put the tray back into the oven. Tap the slides to remove the excess liquid, one slide at a time. Insert the slide in a washing rack and place the rack in a washing dish filled with dH_2O. Move the rack up and down to wash the slides for 3-5 times.

3. ISH Assay

1. **Prepare materials**
   1. Pre-warm 50x wash buffer in a 40 °C incubator for 20 min. Mix 2.94 L of dH_2O and 60 mL of 50x wash buffer to make 1x wash buffer.
   2. Prepare 50% Hematoxylin staining solution by adding 100 mL of Gill's hematoxylin I to 100 mL of dH_2O. Prepare 0.02% (w/v) ammonia water by adding 1.43 mL of 1 N ammonium hydroxide to 250 mL of dH_2O. Mix well in a container.
   3. Put the probes and AMP 0–6 reagents at RT 30 min before use. Keep the humidity control tray in the 40 °C hybridization oven.

2. **ISH procedure**
   NOTE: To examine the expression of the *Nf1* splicing isoforms and calculate the percent spliced in (PSI) value for exon 23a, three different ISH probes that target exon 23a-included isoform, exon 23a-excluded isoform or both isoforms are used (**Figure 2**). The probes are used on adjacently cut tissue sections. To ensure the accuracy of the calculation, it is critical that the three tissue sections are treated exactly the same in the hybridization, amplification and signal detection steps.
   1. Probe hybridization.
      1. Remove the slides from the washing rack and tap the excess liquid from the slides. Place the slides in the stain rack.
      2. Use a pencil to label the slides with the probe name. Put the three adjacently cut brain tissue slides to be hybridized with three different Nf1 probes (**Figure 2**) together. Carry out the subsequent steps in the same order of slides.
      3. Add 4 drops of probe to cover the tissue. Do this one slide at a time to prevent the sections from drying out. Place the stain rack in the humidity control tray and insert it into the 40 °C oven for 2 h.
      4. Wash the slides twice with washing buffer. For each wash, fill the staining dish with 200 mL of 1x wash buffer and place a washing rack in it. Tap excess liquid on a paper towel one slide at a time and quickly insert it into the washing rack. Move the slide rack up and down in the dish for 3–5 times for 2 min at RT.
      5. For the second wash, use fresh wash buffer.

   2. Signal amplification.
      1. Tap the excess wash buffer from the slides and place them in the stain rack. Add 4 drops of AMP 0 to cover the tissue. Place the stain rack in the humidity control tray and insert it in the oven for 30 min at 40 °C.
      2. Wash the slides twice with washing buffer as described above.
      3. Repeat this step with AMP 1–6 following the order and condition of **Table 2**. Wash the slides twice after each hybridization step.

   3. Signal Detection
      1. Tap the excess wash buffer from the slides and place them in stain rack. Add 2 µL of Fast Red B to 120 µL of Fast Red A (1:60 ratio) in a microcentrifuge tube. Mix well and add to fully cover the tissue section.
         NOTE: The total volume of Red A/B mix is based on the number and size of tissue sections. For a 0.75'' x 0.75'' area, 120 µL is enough for one section. Use the mix within 5 min and avoid exposure of the mix to direct sunlight or UV light.
      2. Close the tray and incubate for 10 min at RT. Remove the solution to a waste container and immediately insert the slides into a washing rack and place the rack in a washing dish filled with tap water. Rinse again with fresh tap water.

3. **Counterstaining of the slides**
   1. Remove the washing rack from tap water, and quickly tap on absorbent paper to remove extra water. Put the rack into a 50% hematoxylin staining solution and immediately move the rack up and down several times. Incubate for 2 min at RT.
   2. Transfer the slide rack back into the tap water dish. Wash 3–5 times by moving the rack up and down and changing fresh tap water until the slides become clear, while the brain tissues remain purple.
   3. Move the rack to a dish containing 0.02% ammonia water. Move the rack up and down 2–3 times until the tissues turn blue. Wash the slides 3–5 times with fresh tap water in a staining dish.

4. **Mounting of the samples**
   1. Move the slide rack from the staining dish to a 60 °C incubator and incubate for at least 15 min until the slides are completely dry.
   2. Place 40 µL of mounting medium on the slide and carefully place a 24 mm x 50 mm coverslip over the tissue section. Air dry slides for 30 min at RT.

4. Data Collection and Analysis

NOTE: Use a slide scanner to scan the images at 40X magnification.

1. **Positive and negative controls**
   1. For each experiment, include positive and negative controls (**Figure 3**). As a good positive control, signal should be visible as punctate dots at 20-40X magnification.
   2. Use Mouse (Mm)-PPIB-1ZZ as a positive control. This probe targets a common housekeeping gene PPIB. For negative control, one dot to every 20 cells displaying background staining per 20X microscope field is acceptable.

3. Use the DapB-1ZZ probe as a negative control. This probe targets the bacterial gene dapB. These control probes have been used in many ISH assays[19].

2. **Signal detection and analysis of AS expression patterns of *Nf1* exon 23a**
    1. As the hybridization signals are indicated as punctate dots, count the dots manually. The number of dots is correlated with the level of the transcripts that is detected by a specific probe. Note that the number of dots can also be analyzed by ImageJ or SpotStudio software.
    2. Use three probes to hybridize to exon 23a-containing, exon 23a-lacking isoforms, or total *Nf1* transcripts (**Figure 2**). As the three probes cannot be used simultaneously, use three adjacent brain tissue sections to hybridize to each probe.
        1. To calculate percent spliced in (PSI) for exon 23a, count the number of cells and dots in several brain regions. For each region of interest, count more than 400 cells from several sub-regions as indicated in **Figure 4**.
        2. Collect data from the same sub-regions for each of the three probes. Calculate PSI as number of dots per cell with the exon 23a inclusion probe/(number of dots per cell with the exon 23a inclusion probe + number of dot per cell with the exon23a skipping probe) x 100%.

Representative Results

BaseScope ISH was carried out using three mouse strains: CD1 wild type mice, C57BL/6J wild type mice, and $Nf1^{23aIN/23aIN}$ mutant mice in the C57BL/6J background, in which exon 23a is included in all cell types as a result of engineered splice site mutations[14,15].

As a first step, the ISH assay system was tested using company provided reagents: slides that have 3T3 cells, and negative and positive ISH probes. As shown in **Figure 3**, no signal was detected when the negative control probe was used, while many punctate dots were detected when the positive control probe was used.

Next, ISH was carried out using mouse brain sections and *Nf1*-specific probes that are designed to detect *Nf1* transcripts that contain exon 23a, skip exon 23a or both isoforms (**Figure 2**). These probes target specific exon-exon junctions. Two brain regions were selected to examine the AS expression pattern of *Nf1* exon 23a: cortex and hippocampus CA3 (**Figure 4**). For each region, count three sub-regions, as indicated in **Figure 4**, recording both cell and dot numbers. For each region, approximately 400 cells were counted in total and used to calculate the dots/cell ratio. The AS expression pattern for *Nf1* exon 23a is calculated as PSI.

The ISH signals obtained with the three *Nf1* probes in the cortex and hippocampus of CD1 mice are shown in **Figure 5A-5F** and **Table 3**. The cortex region from C57BL/6J $Nf1^{+/+}$ and $Nf1^{23aIN/23aIN}$ mice was also analyzed (**Figure 6**).

The result of these experiments led to several conclusions. First, the sum of signals, shown as dots/cell, detected by exon 23a inclusion-specific and skipping-specific probes equals to that detected by the probe hybridizing to both isoforms (**Table 3**), which suggests that the three probes hybridized with the *Nf1* transcripts with similar efficiency. Second, the PSI value of exon 23a in cortex, 10%, is consistent with the previously reported RT-PCR result, in which cortex was dissected from adult C57BL/6J mouse brain followed by total RNA isolation and RT-PCR analysis[14,15]. This result suggests that the BaseScope ISH can be used to quantitatively analyze AS expression patterns in addition to transcript levels in tissue sections. Third, the results obtained with the $Nf1^{23aIN/23aIN}$ mutant brain tissues, in which all of the *Nf1* transcripts contain exon 23a[14,15], showed similar levels of signals when inclusion-specific and *Nf1* total probes were used and no signal when skipping-specific probe was used (**Figure 6**), indicating that the two probes targeting exon inclusion or skipping are highly specific.

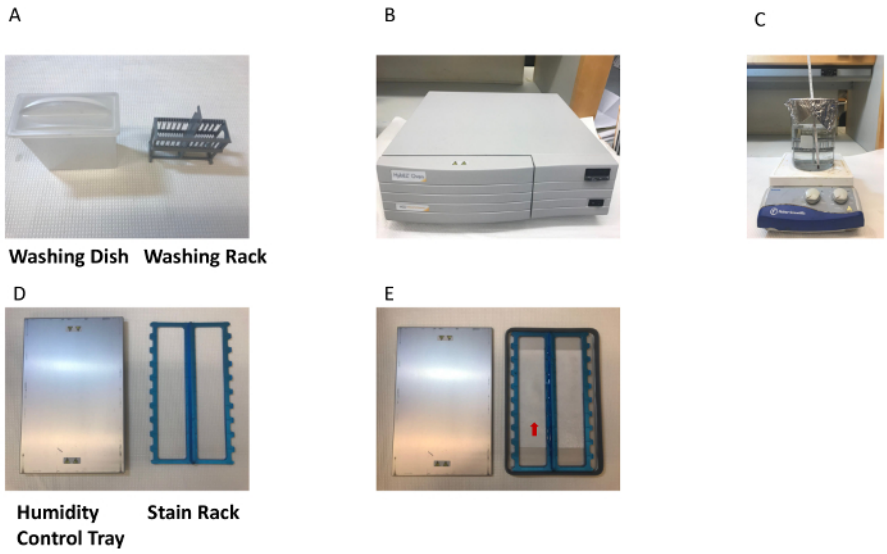

Figure 1: Equipment used in ISH. (**A**) Washing dish and washing rack. (**B**) Hybridization oven. (**C**) Target retrieval beaker and hot plate set. (**D**) Humidity control tray and stain rack. (**E**) Humidifying paper indicated by a red arrowhead inside the humidity control tray. Please click here to view a larger version of this figure.

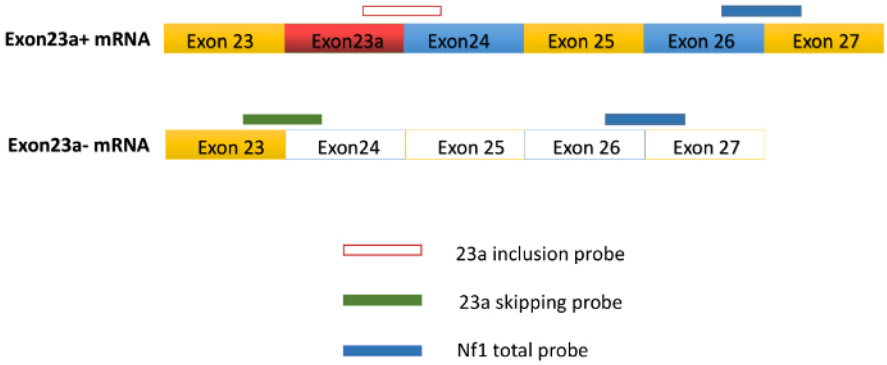

Figure 2: Probes used to detect *Nf1* exon 23a AS expression patterns. All of the probes contain one ZZ pair oligonucleotide. The inclusion-specific probe (red) targets the junction of exons 23a and 24, the skipping-specific probe (green) targets the junction of exons 23 and 24, and the *Nf1* total probe (blue) targets the junction of exons 26 and 27. Please click here to view a larger version of this figure.

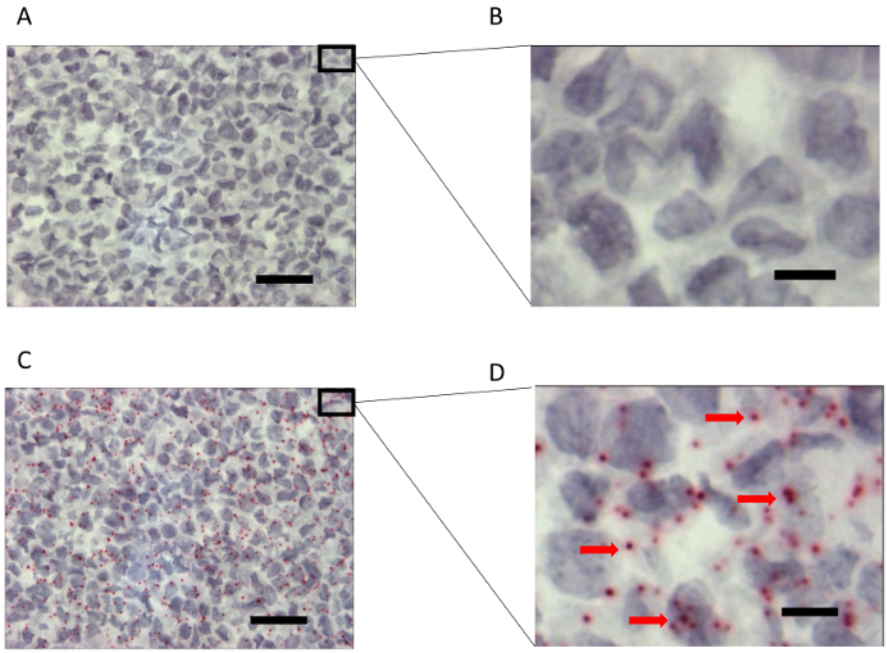

Figure 3: ISH using negative (**A-B**) and positive (**C-D**) controls on 3T3 cells. A control slide containing 3T3 cells was processed through steps 2 (sample pretreatment) and 3 (ISH procedure) in the protocol. Positive signals are shown as red punctate dots indicated by red arrows. B and D are zoomed in images of A and C, respectively. Scale bar = 20 μm (A and C); 5 μm (B and D). Please click here to view a larger version of this figure.

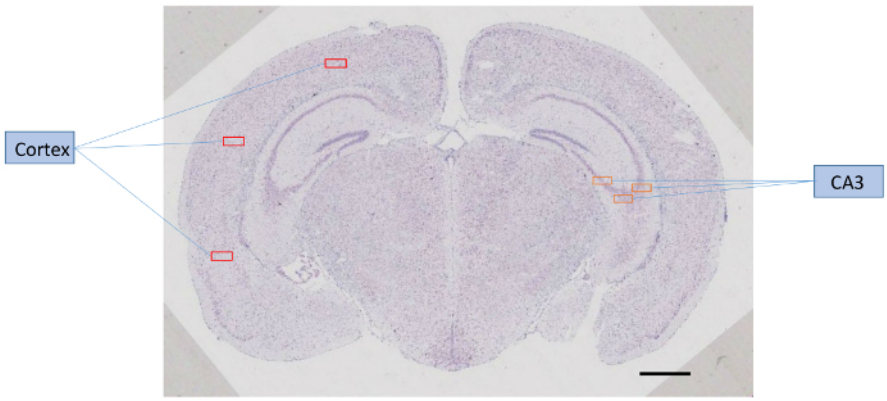

Figure 4: Picture of a mouse brain coronal section. The red boxes indicate the areas selected for counting of cell and signal numbers in cortex and hippocampus CA3 regions. Scale bar = 1 mm. Please click here to view a larger version of this figure.

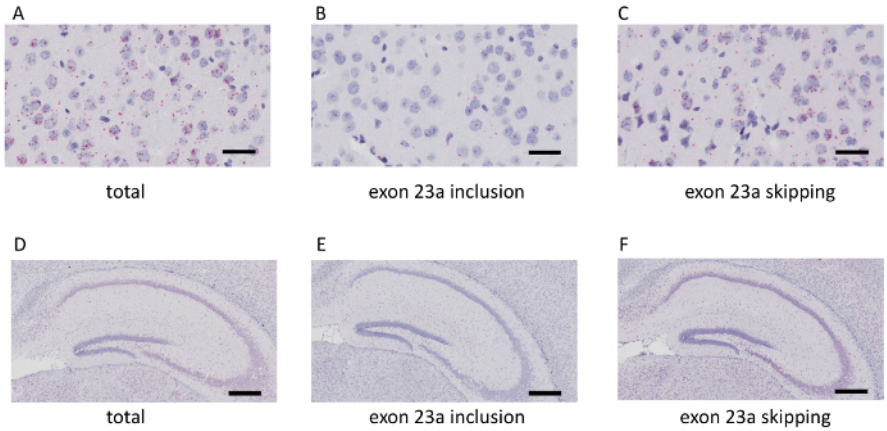

Figure 5: RNA ISH signals detected using *Nf1*-specific probes and mouse brain sections. CD1 brains were processed through steps 1-4 in the protocol. Red punctate dots indicate positive signals when the three *Nf1*-specific probes (shown in **Figure 3**) are used. Cortex (**A-C**) and hippocampus (**D-F**) are shown. *Nf*1 total probe was used in A and D, inclusion-specific probe in B and E, and skipping-specific probe in C and F. Scale bar = 40 µm (A-C); 400 µm (D-F). Please click here to view a larger version of this figure.

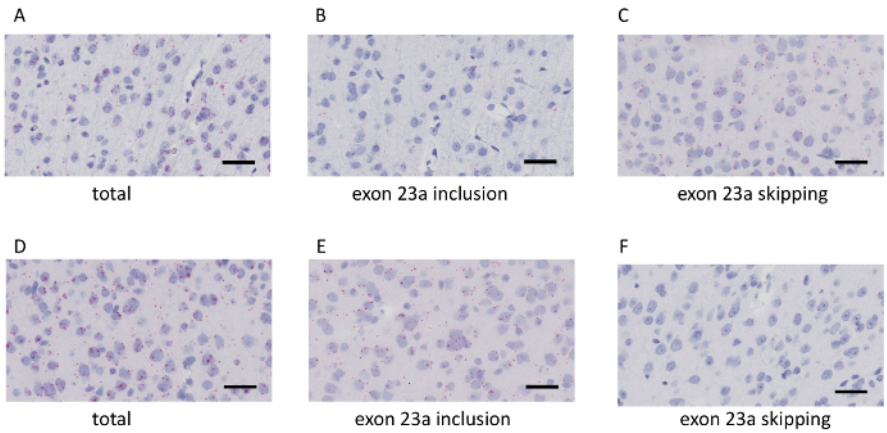

Figure 6: RNA ISH signals detected in *Nf1*$^{+/+}$ and *Nf1*$^{23aIN/23aIN}$ brain sections. Both mouse strains are in the C57BL/6J background. The brains were processed through steps 1-4 in the protocol. A-C. *Nf1*$^{+/+}$ cortex. D-F. *Nf1*$^{23aIN/23aIN}$ cortex. Nf1 total probe was used in A and D, inclusion-specific probe in B and E, and skipping-specific probe in C and F. Scale bar = 40 µm. Please click here to view a larger version of this figure.

Order of incubation	Chemicals in TT Dish	Time
1	250 mL of xylene	5 min
2	250 mL of xylene	5 min
3	250 mL of 100% ethanol	2 min
4	250 mL of 100% ethanol	2 min

Table 1: Incubation procedure of step 2.1.1.

AMP Solution	Incubation Time	Incubation Temperature
AMP 1	15 min	40 °C
AMP 2	30 min	40 °C
AMP 3	30 min	40 °C
AMP 4	15 min	40 °C
AMP 5-RED	30 min	Room Temperature
AMP 6-RED	15 min	Room Temperature

Table 2: Signal amplification procedure of step 3.2.2.

Region	skipping	inclusion	skipping+inclusion	total	PSI
cortex	2.9±0.1	0.32±0.03	3.21	3.22	10
CA3	5.37±0.2	0.2±0.04	5.56	5.65	3.71

Table 3: RNA ISH results calculated from the image shown in Figure 5. The numbers, shown in dots/cell, were calculated using more than 400 cells that are included in the three sub-regions in cortex and CA3 (**Figure 4**). Standard errors are included.

Discussion

This communication reports the use of BaseScope RNA ISH to examine AS expression patterns in mouse brain sections. It is demonstrated that anti-sense exon-exon junction probes shorter than 50 nucleotides can target exon inclusion and skipping isoforms robustly and specifically. Furthermore, the resulting signals can be used to calculate PSI of an alternative exon.

A few variations were tested in the procedure. For example, frozen tissue sections generated by cryostat sectioning were tested and shown to be successful for use in this ISH protocol. In this case, however, the deparaffinization step in 2.1 was changed to washing of OCT with PBS for 5 min. In addition, although it is very convenient to use the hybridization oven provided by ACD, use of a simple incubator set at 40 °C combined with a slide staining tray used for immunohistochemistry gives comparable results. The important thing is to keep the moisture in the box by including wet filter papers throughout the procedure.

There is a limitation in this procedure. Currently, it is not feasible to perform multi-color labeling on the same tissue slide. Thus, different probes can only be used on adjacently cut, different sections. To obtain accurate PSI values, it is critical to ensure that the incubation time of probe hybridization, signal amplification and detection is the same for every tissue section. In the future, implementing the use of multi-color probes on the same tissue slide, as used in the RNAscope procedures, will be highly desirable.

The RNA ISH described in this report provides a powerful new tool to quantitatively analyze AS expression patterns *in situ*. This technology can be applied to study many alternative exons. In a recent report, it was successfully used to examine the differential expression patterns of the four ErbB4 splicing isoforms in neurons and oligodendrocytes at cellular resolution[20]. As shown in this and several other reports, combination of BaseScope RNA ISH with immunochemistry will be a powerful approach to study the localization and function of the differentially expressed splicing isoforms[6,20].

Disclosures

The authors have nothing to disclose.

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References

1. Wang, E.T. *et al.* Alternative isoform regulation in human tissue transcriptomes. *Nature.* **456**, 470-6 (2008).
2. Pan, Q., Shai, O., Lee, L.J., Frey, B.J., & Blencowe, B.J. Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nature Genetics.* **40**, 1413-5 (2008).
3. Cieply, B., & Carstens, R.P. Functional roles of alternative splicing factors in human disease. *Wiley Interdisciplinary Reviews: RNA.* **6**, 311-26 (2015).
4. Xiong, H.Y. *et al.* RNA splicing. The human splicing code reveals new insights into the genetic determinants of disease. *Science.* **347**, 1254806 (2015).
5. Shirai, Y., Watanabe, M., Sakagami, H., & Suzuki, T. Novel splice variants in the 5'UTR of Gtf2i expressed in the rat brain: alternative 5'UTRs and differential expression in the neuronal dendrites. *Journal of Neurochemistry.* **134**, 578-89 (2015).
6. Cui, Y., Liu, J., & Irudayaraj, J. Beyond quantification: *in situ* analysis of transcriptome and pre-mRNA alternative splicing at the nanoscale. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology.* **9** (2017).
7. Trifonov, S., Yamashita, Y., Kase, M., Maruyama, M., & Sugimoto, T. Glutamic acid decarboxylase 1 alternative splicing isoforms: characterization, expression and quantification in the mouse brain. *BMC Neuroscience.* **15**, 114 (2014).
8. Hollander, D., Naftelberg, S., Lev-Maor, G., Kornblihtt, A.R., & Ast, G. How Are Short Exons Flanked by Long Introns Defined and Committed to Splicing? *Trends in Genetics.* **32**, 596-606 (2016).
9. Cassidy, A., & Jones, J. Developments in *in situ* hybridisation. *Methods.* **70**, 39-45 (2014).
10. Meshorer, E. *et al.* Alternative splicing and neuritic mRNA translocation under long-term neuronal hypersensitivity. *Science.* **295**, 508-12 (2002).
11. Wang, F. *et al.* RNAscope: a novel *in situ* RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *Journal of Molecular Diagnostics.* **14**, 22-9 (2012).
12. Wang, H. *et al.* RNAscope for *in situ* detection of transcriptionally active human papillomavirus in head and neck squamous cell carcinoma. *Journal of Visualized Experiments.* (2014).

13. Zhu, H., Hinman, M.N., Hasman, R.A., Mehta, P., & Lou, H. Regulation of neuron-specific alternative splicing of neurofibromatosis type 1 pre-mRNA. *Molecular and Cellular Biology.* **28**, 1240-51 (2008).
14. Hinman, M.N., Sharma, A., Luo, G., & Lou, H. Neurofibromatosis type 1 alternative splicing is a key regulator of Ras signaling in neurons. *Molecular and Cellular Biology.* **34**, 2188-97 (2014).
15. Nguyen, H.T. *et al.* Neurofibromatosis type 1 alternative splicing is a key regulator of Ras/ERK signaling and learning behaviors in mice. *Human Molecular Genetics.* **26**, 3797-3807 (2017).
16. Zhou, H.L. *et al.* Hu proteins regulate alternative splicing by inducing localized histone hyperacetylation in an RNA-dependent manner. *Proceedings of the National Academy of Sciences of the United States of America.* **108**, E627-35 (2011).
17. Sharma, A. *et al.* Calcium-mediated histone modifications regulate alternative splicing in cardiomyocytes. *Proceedings of the National Academy of Sciences of the United States of America.* **111**, E4920-8 (2014).
18. Adzemovic, M.Z. *et al.* Immunohistochemical Analysis in the Rat Central Nervous System and Peripheral Lymph Node Tissue Sections. *Journal of Visualized Experiments.* (2016).
19. Shokry, I.M., Callanan, J.J., Sousa, J., & Tao, R. Rapid In Situ Hybridization using Oligonucleotide Probes on Paraformaldehyde-prefixed Brain of Rats with Serotonin Syndrome. *Journal of Visualized Experiments.* (2015).
20. Erben, L., He, M.X., Laeremans, A., Park, E., & Buonanno, A. A Novel Ultrasensitive In Situ Hybridization Approach to Detect Short Sequences and Splice Variants with Cellular Resolution. *Molecular Neurobiology.* (2017).

