A modified protocol for the detection of three different mRNAs with a new-generation in situ hybridization chain reaction on frozen sections

Qian-qian Sui[1], Jiao Zhu[1], Xiangnan Li[2,3], Gillian E. Knight[4], Cheng He[1], Geoffrey Burnstock[4,5],Hongbin Yuan[2], Zhenghua Xiang[1]

[1]Department of Neurobiology, Key Laboratory of Molecular Neurobiology, Ministry of Education, Second Military Medical University, Shanghai 200433, PR China

[2]Department of Anesthesiology, Changzheng Hospital, Second Military Medical University, Shanghai, 200003, China

[3]Department of Anesthesiology, the Third People's Hospital of Yancheng, Yancheng, 224001, China

[4]Autonomic Neuroscience Centre, University College Medical School, Rowland Hill Street, London NW3 2PF, UK and

[5]Department of Pharmacology and Therapeutics, The University of Melbourne, Australia

Qian-qian Sui, Jiao Zhu and Xiangnan Li contributed equally to this work.

Corresponding author: Fax: +86 21 65492132. E-mail address: zhxiang@hotmail.com (Z Xiang). E-mail: jfjczyy@aliyun.com (H Yuan)

Abstract: A new multiple fluorescence in situ hybridization method based on hybridization chain reaction was recently reported, enabling simultaneous mapping of multiple target mRNAs within intact zebrafish and mouse embryos. With this approach, DNA probes complementary to target mRNAs trigger chain reactions in which metastable fluorophore-labeled DNA hairpins self-assemble into fluorescent amplification polymers. The formation of the specific polymers enhances greatly the sensitivity of multiple fluorescence in situ hybridization. In this study we describe the optimal parameters (hybridization chain reaction time and temperature, hairpin and salt concentration) for multiple fluorescence in situ hybridization via amplification of hybridization chain reaction for frozen tissue sections. The combined use of fluorescence in situ hybridization and immunofluorescence, together with other control experiments (sense probe, neutralization and competition, RNase treatment, and anti-sense probe without initiator) confirmed the high specificity of the fluorescence in situ hybridization used in this study. Two sets of three different mRNAs for oxytocin, vasopressin and somatostatin or oxytocin, vasopressin and thyrotropin releasing hormone were successfully visualized via this new method. We believe that this modified protocol for multiple fluorescence in situ hybridization via hybridization chain reaction would allow researchers to visualize multiple target nucleic acids in the future.

Kew words: multiple fluorescence in situ hybridization; hybridization chain reaction; brain section

Introduction

In situ hybridization (ISH) is a technique used for the detection of specific nucleic acid sequences in cells or tissue sections. The ISH technique is based on the complementary binding of a nucleotide probe to a specific target nucleic acid. The probes are labeled with either radioactive-labeled or non-radioactive-labeled bases, then visualized in situ by either autoradiography or immunohistochemistry, respectively. The ISH technique is used extensively in basic research as well as having clinical diagnostic applications.

In 1969, Gall and Pardue reported the localization of nucleic acids (ribosomal RNA) by ISH with a tritium-labeled RNA probe (Pardue and Gall 1969). Initially, radioactive nucleotide probes were used for ISH. As there are disadvantages to using radioactive nucleotide probes for ISH, non-radioactive nucleotide probes are now widely used. Non-radioactive probes can be used to visualize two or more mRNAs in the same specimen simultaneously. The advantages of this include a practical reason of keeping the tissue sections affixed to slides during hybridization and washing procedures, a particular problem with ISH on tissue samples (Wilcox, 2000; Brown, 1998), as well as the possibility of incompatibility of reagents or the use of shorter DNA oligonucleotides for amplification, which then requires an assay re-optimisation. ~~Generally, the sensitivity of the ISH technique using radioactive nucleotide probes is higher than that of using non-radioactive nucleotide probes (Forster et al. 1985; Dagerlind et al. 1992), but it is very difficult to detect two or more different mRNAs simultaneously.~~ Researchers have been working to design a highly sensitive, non-radioactive ISH technique able to detect two or more mRNAs in the same specimen simultaneously. A new ISH technique using hybridization chain reaction (HCR) would fulfill these requirements. Dirks and Pierce (2004) first introduced the concept

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of HCR. Two stable species of DNA hairpins coexist in solution until the introduction of initiator strands trigger a cascade of hybridization events that form a nicked double helices, analogous to alternating copolymers (Dirks and Pierce 2004). HCR amplification has been applied to the detection of different targets, including nucleic acids (Dirks et al. 2004; Tang et al. 2012), proteins (Song et al. 2012; Zhao et al. 2012) and other small molecules (Han et al. 2013; Zhuang et al. 2013). HCR amplification cascades have been used to generate diverse output signals, including fluorescence (Choi et al. 2010; Zhu et al. 2013), chemiluminescence (Shimron et al. 2012; Wang et al. 2013), bioluminescence (Xu et al. 2013), and energy dissipation (Tang et al. 2012; Wang et al. 2012). Due to its high sensitivity, specificity and small molecule probes and hairpins of the HCR system, it is widely used for the detection of biomarkers. More recently HCR has been used together with other methods, such as with the northern blot approach to demonstrate multiple micro-RNAs simultaneously (Schwarzkopf and Pierce 2016), as well as in intact-tissue transcriptional analysis (Sylwestrak et al. 2016; Nguyen et al. 2016), the detection of protein interactions and post-translational modifications in microscopy and in flow cytometry (Koos et al. 2015). In 2014, Choi et al applied the HCR technique to multiple fluorescence ISH. This new ISH technique enabled simultaneous detecting of multiple target mRNAs. This new generation ISH protocol was designed to detect mRNAs in intact zebrafish embryos or mouse embryos and employed significantly milder conditions than previous HCR-amplified protocols. The length of the DNA oligonucleotide probe and hairpin set was 132bp and 72bp, respectively (Choi et al. 2014; Huss et al. 2015).~~of~~ ~~sare~~ The sensitivity of in situ HCR depends on the number of labeled molecules ~~number~~ within the copolymers that~~which~~ are formed by the hairpins. The greater the number of~~more~~ labeled molecules, the greater the ~~more~~ sensitivity~~e~~ of the in situ HCR.

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Generally each hairpin is labeled with a fluorescence molecule at 5' or 3'. If the same length of copolymer~~s~~ can form~~ed~~ either~~by~~ short~~er~~ or long~~er~~ hairpins, the number of labeling molecules ~~number~~ will be greater~~more~~ if the hairpins are shorter. That is to say, the sensitivity of in situ HCR with shorter hairpin amplification will be higher than that with longer hairpins. Generally speaking, the smaller ~~of~~ the oligonucleotide probe/hairpins, the higher the capacity~~ability of~~ for tissue penetration, since~~. That is to say, the~~ smaller oligonucleotide probe/hairpins ~~are easier to~~ penetrate into the tissue more easily, especially those thick section or whole-mount samples. In addition, the cost of shorter oligonucleotide probe/hairpin synthesis is much cheaper than that~~ose~~ of longer oligonucleotide probe/hairpin synthesis, especially over 100bp. For these reasons, in the present study, we investigated the use of a shorter oligonucleotide probe (89nt) and hairpin set (36nt) in the HCR amplification system. We found that brighter fluorescence was obtained when using the shorter (36-nt) hairpin set. The optimal parameters (HCR time and temperature, hairpin and salt concentration) for frozen tissue sections~~In the present study, we introduce a simple, sensitive and specific protocol for the detection of three different mRNAs in frozen sections using this new generation ISH technique.~~ under HCR amplification conditions of oligonucleotide probe (89nt) and hairpin set (36nt) are described in this study.

Materials and Methods

Probe Synthesis

The design of the oligonucleotide probes was reported in Choi et al (2014). DNA two initiator probes are 89-nt long (5' 18-nt initiator, 4-nt spacer, 45-nt mRNA recognition sequence, 4-nt spacer, 3' 18-nt initiator). Message RNAs are addressed by ~~probe~~ sets of three probes, each probe ~~containing one that~~ hybridizes at 45-nt binding sites. Probe sequences are displayed in Table 1. DNA probes were supplied by Sangon, Shanghai, China. DNAs probes were re-suspended in Tris-EDTA buffer (TE) at a concentrations of 1μM/L.

HCR Hairpin Design and Synthesis

The sequences of the hairpins sets were modified from those reported in Choi et al (2014). DNA HCR hairpins are 36-nt long (6-nt toehold, 12-bp stem, 6-nt loop). DNA HCR hairpins were synthesized by Sangon, Shanghai. The standard DNA oligonucleotides were end-labeled with a fluorophore (3'-end for H1 and 5'-end for H2). To ensure that H1 and H2 form hairpin monomers, the strands were snap-cooled in 4×SSC (saline sodium citrate buffer) before use (heat at 95℃ for 90 s, cool to room temperature on the bench top for 30 min). All HCR hairpin sequences are shown in Table 2.

Gel Electrophoresis

DNA HCRs in Figure 1 were performed in 4×SSC for 1h reactions with each hairpin at 500nM at 25℃. DNA hairpins were snap cooled separately at 5μM in 4×SSC. The oligonucleotide probes with initiators were diluted to 1μM in ultrapure water. In the HCR gel, each lane was prepared by mixing 5μL 8x SSC, 3μL of oligonucleotide probe with initiator, and 1μL of each hairpin to obtain a reaction volume of 10μL. ~~each lane was prepared by mixing 10μL of 5μl 8×SSC, 3μL of oligonucleotide probe with initiator, and 1μL of each hairpin.~~ When an initiator was absent (lane no initiator), 3μL of ultrapure water was added to bring the reaction volume to 10μL. The reactions were incubated at 25℃ for 1 h. The samples were supplemented with 2μL 6×loading buffer (50% glycerol with bromophenol blue and xylene cyanol tracking dyes) and loaded into a 2% agarose gel. The gel was run at 100 V for 45 min at room temperature and imaged using a scanner (Tagon).

Section Preparations

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Second Military Medical University. Twenty five adult Wistar rats (200–300 g) were used. The rats were anesthetized with sodium pentobarbitone and perfused through the aorta with 0.9% NaCl solution and 4% paraformaldehyde in 0.1 mol/~~l~~L phosphate buffer pH 7.4. The brains were removed, and the hypothalamus was microdissected out immediately and immersed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.2) for 4-6 h. The hypothalamus blocks were then transferred to 25% sucrose in PBS and kept in the solution until they sank to the bottom. Thereafter, the hypothalamus blocks were rapidly frozen in a Leica cryostat. Coronal sections (25μm) of the hypothalamus were cut and floated in PBS.

In Situ Hybridization

The protocol for ISH was modified from a protocol used before (Xiang et al., 2001). Briefly, floating rat hypothalamic sections were washed 3×5 min in 0.01 mol/l L PBS pH7.2, incubated in 0.4% Triton X-100/PBS for 10 min. The sections were then incubated in protease K (5.0μg/ml) in PBS for 10 min at 37 °C. The activity of protease K was stopped by fixation in 4% paraformaldehyde for 5 min, followed by 2×3 min washes in PBS to remove fixative from the sections. The sections were washed in 0.6 mol/l L sodium chloride and 0.06 mol/l L sodium citrate (2×SSC) for 10 min. Cy3-labeled or FITC-labeled oxytocin (OT), AMCA-labeled vasopressin (VP), Cy3-labeled somatostatin (SST) or OT, thyrotropin releasing hormone (TRH) oligonucleotide probes were added to hybridization buffer containing 50% formamide, 10% dextran sulfate, 0.3 mol/l NaCl, 1×Denhardt's solution, 0.05 mol/l Tris–HCl (pH 8.0), 1 mmol/l L EDTA and 250 μg/ml E. coli tRNA (RNase-free). Hybridization was carried out for 16 h at 45°C in a hybridization oven. The sections were washed in 2×SSC, 1×SSC, 0.5×SSC, 0.25×SSC for 10 min at 37°C, respectively. Finally the sections were rinsed in 4×SSC for 10 min at room temperature before HCR.

Hybridization Chain Reaction

1μl of each fluorescently labeled hairpin (10μm/L) in 4×SSC was heated at 95 °C for 90 seconds and allowed to cool to room temperature on the bench top for 30min. The snap-cooled hairpins were added at room temperature to 500μl of 4x SSC to obtain a final concentration of 20nM/L~~The snap-cooled hairpins were added to 500μl (20nM/L) of 4×SSC at room temperature~~, which was called the amplification mixture. The 4x SSC solution was removed from the sections and replaced by the 500μl amplification mixture. HCR~~ybridization chain reaction~~ on the sections was preformed for 1-2 hours at 25°C.~~The sections incubated in 4×SSC were added to 500μl amplification mixture for 1-2 hours at 25°C.~~ The sections were then washed 3x5 min in 0.01 mol/l PBS.

Finally the sections were mounted on slides, dried and covered with 50% glycerin PBS.

Eight different times for HCR amplification (0min, 5min, 10min, 30min, 1h, 2h, 8h and 16h), eight different concentrations of hairpins (0.625, 1.25, 2.5, 5, 10, 20, 40, 80nM~~/L~~), eight different incubation temperatures (4, 15, 20, 25, 30, 37, 45, 50°C), and six different salt concentrations in amplification buffer (0.5, 1, 2, 4, 8, 16×SSC, 4×SSC+10% dextran sulphate, 8×SSC+10% dextran sulphate) were tried to obtain the optimal parameters for the HCR.

Combined Use of the New-generation In Situ Hybridization and Immunofluorescence

In order to evaluate the specificity of ISH, a combination of the use of a new generation ~~in situ hybridization~~ISH and immunofluorescence was carried out. The protocol was as follows: after amplification of HCR, the sections were washed 3-5min in PBS, and then preincubated in a blocking solution (10% normal bovine serum, 0.2% Triton X-100, 0.4% sodium azide in 0.01 mol/l PBS pH 7.2) for 30 min followed by incubation with the primary antibodies (VP (1:2,000, rabbit polyclonal antibody, Abcam, ab39363); OT (1:1,000, rabbit polyclonal antibody, Abcam, ab2078)), at room temperature overnight. Subsequently, the sections were incubated with Cy3-conjugated donkey anti-rabbit IgG (Jackson, 711-165-152) diluted 1:400. All incubations were separated by 5-10 min washes in PBS.

Control Experiments

In order to confirm the specificity of mRNA signals, a variety of control experiments for ISH were carried out. 1. Anti-sense probe without relative initiator control: some sections were hybridized with antisense probes (VP, OT, TRH and ST) without relative initiator sequences (the sequences are shown in Table 1). Other experimental

conditions were the same as mentioned above. 2. RNase-treated control: some sections were treated with 20μg/RNase A in PBS at 37°C for 30min before hybridization. Thereafter these sections were used to visualize mRNAs of VP, OT, TRH and SST. 3. Sense probes with relative initiators: some sections were hybridized with sense probes (VP, OT, TRH and SST) with relative initiator sequences (see Table 1). Other experimental conditions were the same as mentioned above. 4. Neutralization and competition controls: the neutralization and competition assays of OT, VP, TRH and SST probes were carried out as follows. Different combinations of sense and antisense probes, with relative initiator concentrations (μm), were used. Sense probe: antisense probe = 8:1; 4:1; 2:1; 1:1 and 0:1. Other experimental conditions were the same as mentioned above.

Photomicroscopy

Images were taken with a Nikon digital camera DXM1200 (Nikon, Japan) attached to a Nikon Eclipse E600 microscope (Nikon). Images were imported into a graphics package (Adobe Photoshop 5.0, USA).

Image Analysis

The value of the average area optical density (AAOD) for fluorescence images from the supraoptic nucleus of the hypothalamus was measured using an NIS-elements D3.1 system (Nikon, Japan). Five sections were used for each experiment and the mean number of these five sections was calculated. Five separate experiments were carried out.

Statistical analysis

Results are expressed as mean ± SEM (n=5). Values were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. $p<0.05$ was considered to be statistically significant.

Results

HCR analysis showed that the three sets of hairpins for OT, VP and SST/TRH oligonucleotide probes were metastable in the absence of their oligonucleotide probes with initiators (lane no initiator) ~~and the formation of HCR polymers when mixed with their initiators (lane initiator)~~ and formed HCR polymers when mixed with their initiators (lane initiator) (Figure 1).

In order to obtain the optimal time of HCR amplification, we used the same experimental parameters: HCR amplification temperature was 25°C; hairpins concentration was 20nM/L; HCR amplification buffer was 4×SSC. No fluorescence hybridization signals for OT mRNAs were detected before amplification (Figure 2A). Five mins after amplification, weak signals were detected in some neurons of the supraoptic nucleus. After 10 mins, the number of OT mRNA positive neurons increased significantly, the signals were mainly detected in the cytoplasm~~a~~ and almost no signals were detected in the nucleus of the neurons. After 30 mins, the fluorescence intensity increased significantly. After 1 hour, the fluorescence intensity was strong. Extending the amplification for up to 16 hr did not significantly increase fluorescence intensity further (Figure 2). AAOD at the different experimental amplification times are summarized in Figure 2.

In order to obtain the optimal concentration of hairpins in HCR amplification, we used the same experimental parameters mentioned above and an amplification time of 1 hour. Nine different concentrations of hairpins (80nM, 40nM, 20nM, 10nM, 5nM, 2.5nM, 1.25nM, 0.625nM and 0nM) were used. The results clearly showed that the fluorescence intensity increased with increasing hairpins concentration from 0nM to 20nM. Thereafter, fluorescence intensity did not change significantly with increasing

hairpins concentration. AODD at the different experimental hairpins concentrations are summarized in Figure 3.

In order to obtain the optimal temperature of HCR amplification, we used the same experimental parameters mentioned above, an amplification time of 1 hour and hairpins concentration of 20nM. Eight different temperatures for the HCR amplification (4, 15, 20, 25, 30, 37, 45 and 50°C) were used. The results showed that the fluorescence intensity increased by increasing the HCR amplification temperature from 4 to 25°C. The fluorescence intensity did not change significantly between 25-45°C, but the intensity decreased at 50°C. AODD at the different experimental temperatures are summarized in Figure 4.

In order to obtain the optimal salt concentration for the HCR amplification buffer, we used the same experimental parameters mentioned above, the amplification time was 1 hour and the hairpins concentration was 20nM. Six different salt concentrations (0.5×SSC, 1×SSC, 2×SSC, 4×SSC, 8×SSC, and 16×SSC) were used. The results showed that there was no fluorescence signal when the salt concentration was 0.5×SSC, moderate fluorescence signals were obtained when the salt concentration was 1×SSC, and strong fluorescence signals were obtained when the salt concentration was 2×SSC. There was no difference in the fluorescence intensities obtained when the salt concentration increased from 2 up to 16×SSC. AODD at the different experimental concentrations of salt in the HCR amplification buffer are summarized in Figure 5.

Under the same optimal parameters (amplification temperature was 25°C; HCR amplification buffer was 4×SSC; hairpins concentration was 20nM/L; HCR amplification buffer was 4×SSC; amplification time was 1 hour). We compared the fluorescence intensity of OT mRNA positive neurons in the supraoptic nucleus, which

was visualized with differential lengths of hairpins (36-nt and 72-nt). The result showed that the higher fluorescence signals were obtained when using shorter hairpins (Figure 6).

After the main experimental parameters for this new generation ISH protocol were optimized, we carried out the combined use of this new ISH and immunofluorescence to visualize VP and OT mRNAs and their relative peptides within the same cell. The results showed that ISH fluorescence signals of OT mRNAs (green) and VP mRNAs (blue) were only detected in the neuronal cell bodies with OT (red) or VP (red) immunoreactivities, respectively (Figure ~~6~~7). We found that 100% OT- and VP-positive cells visualized by ISH fluorescence, were also immunoreactive for OT and VP, respectively. The reverse was also true, 100% immunoreactive cells were also positive for ISH fluorescence. This demonstrated that this new generation ISH technique displayed a high level of specificity.

Under the optimal experimental parameters identified in this study (a HCR amplification temperature of 25°C; hairpins concentration of 20nM~~/L~~; HCR amplification buffer 4×SSC; HCR time of 1-2 hours) we successfully and clearly visualized three different neuropeptide mRNAs from the same section simultaneously (Figure ~~7~~ 8 and ~~8~~9). We used two sets of oligonucleotide probes (VP, OT, somatostain or VP, OT, TRH) to detect VP, OT and SST or TRH mRNAs. The results showed that although the hybridization signals for VP (blue), OT (green) and ~~somatostatin~~ SST (red) or TRH (red) mRNAs could be detected in the same sections of rat hypothalamus, none of them could be detected in the same neuron (Figure ~~7~~ 8 and ~~8~~9). Occasionally VP and OT mRNAs could be detected in the same neuron of the supraoptic nucleus (SON), especially in the dorsal part of this nucleus (Figure ~~7d~~8d).

In control experiments, no ISH signals were detected in the sections hybridized with antisense probes without relative initiator sequences and sense probes with relative initiators. Similarly, no ISH signals were detected in RNase-treated sections. In neutralization and competition experiments of OT, VP, TRH and SST probes, no signals were detected when the ratio of sense and antisense probe concentrations was 8:1 or 4:1, while weak or moderate ISH signals were detected when the ratio was 2:1 or 1:1, respectively. Strong ISH signals were detected when the ratio was 0:1 (supplementary Figure 1-4).

Discussion

Double or multiple hybridizations have been successfully carried out by many researchers (Bresser et al. 1987; Cremer et al. 1986; Haase et al. 1985; Hopman et al. 1986, 1988; Nederlof et al. 1989, 1990; Wiegant et al. 1993). This is important in the fields of cytogenetics, oncology, and cell biology because of the increasing interest in the demonstration of multiple DNA or RNA targets in the same cell. In order to achieve this goal, researchers usually used different probes labeled with different haptens to hybridize, and finally the combined use of three different immunohistochemical affinity systems to visualize the hybrids (Nederlof et al. 1989, 1990). Even nucleotide probes have been directly labeled with fluorescents (Wiegant et al. 1993; Kosman et al. 2004; Chan et al. 2005; Raj et al. 2008). The sensitivity of previously used multiple hybridization protocols was low and their applications were limited. With different haptens labeled RNA probes, different antibodies for haptens and tyramide signal amplification (TSA) system, two- and three-color fluorescent ISH were successfully carried out in whole-mount zebrafish (Lauter et al. 2011a, 2011b). The drawback of this system is that two or three colors could not be demonstrated simultaneously. A step was needed to inactivate the perioxidase activity before the next detection round could be carried out. Recently, several highly sensitive FISH approaches have been ~~were~~ reported, including as branched DNA ISH (Player et al. 2001; Kenny et al. 2002), RNAscope (Wang et al. 2012, 2014; Grabinski et al. 2015), and ISH chain reaction (Choi et al. 2010, 2014). ISH chain reaction was used with the highly sensitive multiple fluorescence ISH technique, which enabled the simultaneous mapping of multiple target mRNAs within intact zebrafish embryos (Choi et al. 2014), and within intact mouse embryos (Huss et al. 2015) simultaneously. With this approach, DNA/RNA probes complementary to target mRNAs trigger chain reactions

in which metastable fluorophore-labeled DNA/RNA hairpins self-assemble into fluorescent amplification polymers. The formation of the specific, high polymers enhances greatly the sensitivity of multiple fluorescence ISH. The authors claimed that this new protocol addresses a longstanding challenge to scientists. In the present study, a shorter oligonucleotide probe (89nt) and hairpin set (36nt) was used in the HCR amplification system and we found that a brighter fluorescence was obtained when using the shorter (36-nt) hairpin set compared to the longer (72-nt) hairpin set. Using these conditions,~~In these two reports, intact zebrafish or mouse embryos were used. For this technique to be used for frozen tissue sections, the protocol would need to be modified. With this idea~~, we modified this new multiple fluorescence ISH protocol.

The speed of the polymer formation through HCR was quick, such that 1 to 2 hours of HCR was sufficient for HCR amplification. We found that above 20nM of hairpins was sufficient for ideal fluorescence signals. The optional temperature for HCR was found to be between 25 and 45℃. The optional salt concentration in the amplification buffer proved to be above 2×SSC. As there were no significant differences in fluorescence signal intensity when no dextran sulfate or 10% dextran sulfate was used, dextran sulfate was not used in our HCR amplification buffer.

The length of hairpins is an important parameter of the HCR. Usually the ends (3' or 5') of the hairpin oligonucleotides are~~were~~ labeled with a fluorophore. Thus, the longer the hairpin oligonucleotide, the fewer the labeling fluorophores of the HCR

polymer. The number of fluorophores in the HCR polymers determines the sensitivity of the fluorescence ISH. Shorter hairpin oligonucleotides result in a greater number of fluorophores in a certain length of polymer. In order to obtain the maximum number of fluorophores in a certain length polymer, 36-nt length hairpins sets were designed. In this study these 36-nt length hairpins sets were confirmed to be metastable without initiators and these hairpins assembled into HCR polymers after being mixed with the initiators linked with oligonucleotide probes (Figure 1). These hairpins were much shorter than those used previously (Choi et al. 2014).

In this study the combined use of fluorescence ISH and immunofluorescence was carried out in order to confirm the specificity of the fluorescence ISH. The results showed that 100% of the VP or OT immunoreactive neurons were also labeled with their relative mRNA signals, and the reverse was also true, 100% of OT and VP positive cells visualized by ISH fluorescence were also positive for immunoreactivity to OT and VP. Thus, no false positive cells were detected in this study. Very low background signals were obtained. These data show that the specificity of fluorescence ISH used in this study is high.

No ISH signals ISH were detected in any of the control experiments, including anti-sense probe without relative initiator, RNase-treated, sense probes with relative initiators, which confirmed the specificity of the VP, OT, TRH and SST probes.

Neutralization and competition assays further confirmed that the probes for VP, OT, TRH and SST were specific for their target mRNAs.

In summary, a set of optimal parameters for multiple fluorescence ISH via amplification using HCR in frozen tissue sections are described. The combined use of fluorescence ISH and immunofluorescence was carried out to confirm that the specificity of fluorescence ISH used in this study was high. Two sets of three different mRNAs for VP, OT, SST or VP, OT, TRH were successfully visualized via this new fluorescence ISH technique. We believe that this modified multiple fluorescence ISH via HCR protocol will allow researchers to visualize multiple target nucleic acids in frozen tissue sections in the future.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

This work was supported by the National Natural Science Foundation of the People's Republic of China (81471260 to Z. Xiang).

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Figure legends

Figure 1 Hybridization chain reactions were run with 500nM of each hairpin in 4×SSC at 25°C for 1 hour. In the lanes with no initiator, the hairpins were metastable, as no visible polymers were detected. In the lanes with initiator, almost all the hairpins (length=36bp) assembled into hybridization chain reaction polymers (most of them were 500 to 1000bp) as no visible remaining hairpins were detected. M is DNA marker. H1/2 for OT (length=36bp) was hairpin 1 and hairpin 2 for OT oligonucleotide probe with the initiator. H1/2 for VP (length=36bp) was hairpin 1 and hairpin 2 for VP oligonucleotide probe with the initiator. H1/2 for SST (length=36bp) was hairpin 1 and hairpin 2 for SST/TRH oligonucleotide probes with the initiator. H1/2 for OT (length=72bp) was 72-nt hairpin 1 and 72-nt hairpin 2 for OT oligonucleotide probe with the longer initiator (36-nt), DNA ladders were observed in the lane with initiator.

Figure 2 Fluorescence intensity of the supraoptic nucleus (SON) of the hypothalamus after different times of hybridization chain reaction amplification. No fluorescence hybridization signals of OT mRNAs were detected before amplification (A). Weak signals was detected in A after 5 min (B); the number of positive neurons increased significantly after 10 min (C); the fluorescence intensity increased significantly after 30min (D), and strong fluorescence signals were detected after 1 hour (E). F, G, H showed positive neurons after 2 h, 8h and 16h, respectively. The inserted image is a high magnification picture taken from the region indicated by a star in A to H. AAOD at the different experimental times are summarized in I. All scale bar =160μm.

Figure 3 Neurons positive for OT mRNA hybridization chain reaction signals and AAOD of SON after 1 hour amplification at 25ºC with different hairpin concentrations of 0.625nM (A), 1.25nM (B), 2.5nM (C), 5nM (D), 10nM(E), 20nM(F), 40nM (G) and 80nM (H). The results showed that the fluorescence intensity increased with increased hairpins concentrations from 0nM to 20nM. Thereafter, fluorescence intensity did not change significantly with increased hairpins concentrations. The inserted image is a high magnification picture taken from the region indicated by a star in A to H. I shows AAOD of SON at different hairpins concentration. All scale bars =160μm.

Figure 4 Neurons positive for OT mRNA hybridization chain reaction signals and AAOD of SON after 1 hour amplification at different incubation temperatures with a hairpin concentration of 20nM. The results showed that fluorescence intensity increased with increasing incubation temperatures from 4 to 25ºC (A, B, C, D). Fluorescence intensity did not change significantly from 25 to 45ºC (E, F, G). The intensity decreased when the incubation temperature was 50°C (H). The inserted image is a high magnification picture taken from the region indicated by a star in A to H. I shows AAOD of SON at different hairpins concentrations. All scale bars = 160μm.

Figure 5 Neurons positive for OT mRNA hybridization chain reaction signals and AAOD of SON after 1 hour amplification at 25ºC with a hairpin concentration of 20nM in different salt concentrations of SSC. The results showed that the fluorescence intensity increased with increasing salt concentrations from 0.5×SSC to 4×SSC (A, B, C, D). Thereafter, the fluorescence intensity did not change

significantly with increased salt concentrations from 4×SSC to 16×SSC (D, E, F). G and H show the fluorescence intensity of SON in 4×SSC and 8×SSC with added 10% dextran sulphate, respectively. The inserted image is a high magnification picture taken from the region indicated by a star in A to H. I shows AAOD of SON under the conditions above. All scale bars =160μm.

Figure 6 Neurons positive for OT mRNA hybridization chain reaction signals and AAOD of SON under the same optimal amplification parameters with different lengths of hairpins (36-nt and 72-nt) (A and B). The inserted image is a high magnification picture taken from the region indicated by a star in A and B. C shows AAOD of SON under the conditions above. The results show that higher fluorescence signals were obtained when using shorter hairpins. All scale bars =160μm.

Figure ~~6~~ 7 Co-existence of OT mRNA (green) and peptide immunoreactivity (red) (A, B, C) and VP mRNA (blue) and peptide immunoreactivity (red) (D, E, F) detected by the combined used of hybridization chain reaction and immunofluorescence. A is OT mRNA hybridization chain reaction signals; B is immunoreactivity to OT; C is the merged image of A and B. D is VP mRNA signals; E is immunoreactivity to VP; F is the merged image of D and E. All scale bars =160μm.

Figure ~~7~~ 8 Detection of OT (green), SST (red) and VP (blue) mRNAs in SON (A, B, C, D) and paraventricular nucleus (PVN) (E, F, G, H) with three color fluorescence in situ hybridization via hybridization chain reaction amplification. A, B, C show OT, VP, and SST mRNA hybridization signals of SON, respectively; D is the merged image of A, B and C (arrows indicate double-labeled cells). E, F, G shows OT, VP,

and SST mRNA hybridization signals of PVN, respectively; H is the merged image of E, F and G. Note that no double or triple labeling was detected in ~~D and~~ H. All scale bars =80μm.

Figure ~~8~~ 9 Detection of OT (green), TRH (red) and VP (blue) mRNAs in SON (A, B, C, D) and PVN (E, F, G, H) with three color fluorescence in situ hybridization via hybridization chain reaction amplification. A, B, C show OT, TRH, and VP mRNA hybridization signals of SON, respectively; D is the merged image of A, B and C. E, F, G show OT, TRH, and VP mRNA hybridization signals of PVN, respectively; H is the merged image of E, F and G. Note that no double or triple labell~~e~~d neurons were detected. All scale bars =80μm.