

Video Article

Detection of Axonally Localized mRNAs in Brain Sections Using High-Resolution *In Situ* Hybridization

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Abstract

mRNAs are frequently localized to vertebrate axons and their local translation is required for axon pathfinding or branching during development and for maintenance, repair or neurodegeneration in postdevelopmental periods. High throughput analyses have recently revealed that axons have a more dynamic and complex transcriptome than previously expected. These analyses, however, have been mostly done in cultured neurons where axons can be isolated from the somato-dendritic compartments. It is virtually impossible to achieve such isolation in whole tissues *in vivo*. Thus, in order to verify the recruitment of mRNAs and their functional relevance in a whole animal, transcriptome analyses should ideally be combined with techniques that allow the visualization of mRNAs *in situ*. Recently, novel ISH technologies that detect RNAs at a single-molecule level have been developed. This is especially important when analyzing the subcellular localization of mRNA, since localized RNAs are typically found at low levels. Here we describe two protocols for the detection of axonally-localized mRNAs using a novel ultrasensitive RNA ISH technology. We have combined RNAscope ISH with axonal counterstain using fluorescence immunohistochemistry or histological dyes to verify the recruitment of *Atf4* mRNA to axons *in vivo* in the mature mouse and human brains.

Video Link

The video component of this article can be found at <https://www.jove.com/video/52799/>

Introduction

Axonal mRNA recruitment and local translation enable axons to respond to extracellular stimuli in a temporally and spatially acute manner¹. Intra-axonal protein synthesis is best understood in the context of neurodevelopment where it plays crucial roles in growth cone behavior²⁻⁸, axon pathfinding⁹⁻¹¹ and retrograde signaling^{12,13}. But far less is known about the functional significance of axonal protein synthesis in post-developmental neurons when axonal mRNA and ribosome levels are greatly reduced^{14,15}. Mature vertebrate axons have long been thought to be translationally inactive¹⁶. However, recent studies indicate that local translation is reactivated in mature axons under pathological conditions. For instance, a subset of mRNAs is recruited to regenerating axons following nerve injury and intra-axonal protein synthesis is required for the correct regeneration of these axons¹⁷. Additionally, our group has demonstrated that specific mRNAs are recruited to axons after local exposure to the Alzheimer's disease peptide A β ₁₋₄₂, and local translation of the transcription factor ATF4 is required to propagate the neurodegenerative effects of A β ₁₋₄₂ from axons to the neuronal soma¹⁸. Finally, high throughput analyses have revealed that mature axons have a more complex and dynamic transcriptome than expected¹⁸⁻²¹, especially under pathological conditions. In light of these studies, a highly sensitive and specific method to detect axonally localized mRNAs in the adult nervous system is needed.

Much of the work on mRNA recruitment and local translation in mature axons has been performed on cultured neurons. This is especially true for transcriptome analyses since specialized culturing methods exist that allow the isolation of axons from the somato-dendritic compartment¹⁸⁻²⁰. Although such studies have given valuable insight into the role of local translation in mature axons, the question whether cultured neurons faithfully represent the situation *in vivo* or if mRNA recruitment is an adaptive response of axons to culturing conditions is still open. Few studies have provided evidence of mRNA recruitment to mature axons *in vivo*. For example, the transcript coding for the olfactory marker protein has been detected in axons in adult sensory neurons²². A transgene containing the 3' UTR of β -actin mRNA is transported to axons in peripheral and central nervous system neurons in mice and is locally translated after developmental periods²³. Lamin b2 mRNA is localized to retinal axons in *Xenopus laevis* tadpoles and its depletion affects axon maintenance after axonal development²¹. Interference with the axonal transport of the mRNA encoding for cytochrome C oxidase IV alters mouse behavior²⁴. Finally, *Atf4* mRNA is found in adult axons of mice and human brains in the context of A β ₁₋₄₂-induced neurodegeneration¹⁸.

High throughput transcriptome analyses have proven to be useful to identify mRNA profiles in isolated axons *in vitro* but have limitations for *in vivo* studies since in whole tissues axons are never found in isolation but intermingled with neuronal cell bodies, glial cells and other cell types. Thus, such analyses have to be combined with imaging techniques that confirm the subcellular localization of mRNAs. RNA *in situ* hybridization (RNA ISH) allows the detection and visualization of specific RNA sequences in cells and tissues. However, original RNA ISH assays were suitable only for the identification of highly abundant RNAs²⁵, which is rarely the case for axonally localized mRNAs. For the last decades increasing efforts have been put into developing novel technologies that allow the detection of mRNAs at the single-molecule level^{25,26}. For instance, Singer

and colleagues have developed ISH probes to detect mRNAs in single cells, consisting in 5 non-overlapping fluorescently labelled 50-mers (for details refer to ²⁷). The main difference between the above mentioned techniques and the one here described is that the later uses 20 double Z-structure (not linear) probes that typically target ~ 1 kb region of the RNA of interest ensuring specificity and low background levels. Probes are then hybridized with preamplifier and amplifier sequences that are finally fluorescently labeled or conjugated with enzymes that allow chromogenic reactions. These amplification steps improve the signal-to-noise ratio compared to other ISH technologies²⁸. Here we describe two protocols using RNAscope combined either with fluorescence immunocytochemistry or with histological dyes allowing axonal counterstaining. Both protocols are suitable to visualize axonal localization of *Atf4* mRNA in adult mouse and human brains.

Protocol

All animal procedures were approved by the IACUC of Columbia University and applicable guidelines for the care and use of laboratory animals were followed. Note: Prepare all buffers used for the ISH procedures in RNase-free or DEPC-treated water. This recommendation is not strictly necessary after ISH has been completed but it is suggested that buffers are still prepared in autoclaved double-distilled water and/or sterilized by filtering.

1. Detection of *Atf4* mRNA Localized to Cholinergic Axons in the Adult Mouse Brain using Fluorescence *In Situ* Hybridization (FISH) Followed by Immunohistochemistry

1. Sample preparation for paraformaldehyde-fixed frozen brain slices
 1. For the following example, prepare slices from fixed frozen mouse brains.
 1. Briefly, sacrifice 9 month-old mice by anesthesia with ketamine (100 mg kg⁻¹ body weight) and xylazine (10 mg kg⁻¹ body weight) followed by transcardial infusion of 4% paraformaldehyde in PBS (pH 7.4).
 2. Remove brains and post-fix in 4% paraformaldehyde for 24 hrs at 4 °C.
 3. After fixation, wash samples three times in PBS and cryoprotect in 30% sucrose in PBS (pH 7.4) for 24 hrs at 4 °C.
 4. Embed brains in OCT freezing compound, serially section at 20 µm on a cryostat and mount on poly-D-lysine treated microscope slides. Keep brain slices at -20 °C until use.
 2. Remove paraformaldehyde-fixed brain slices from freezer and air-dry for 30 min at RT.
 3. Wash three times with 1x PBS.
 4. In a fume hood, re-fix brain slices 20 min with 4% paraformaldehyde at RT.
NOTE: CAUTION: 4% paraformaldehyde is toxic and must be handled and discarded appropriately following safety protocols.
 5. Wash three times with 1x PBS.
 6. Dehydrate brain slices.
 1. Prepare 50%, 75% and 100% ethanol solutions.
 2. Immerse slides in 50% ethanol for 5 min at RT.
 3. Immerse slides in 75% ethanol for 5 min at RT.
 4. Immerse slides in 100% ethanol for 5 min at RT.
 5. Immerse slides in fresh 100% ethanol for 5 min at RT. Note: Alternatively, slides can be stored in 100% ethanol at -20°C up to 1 month.
2. FISH assay followed by immunohistochemistry using heat-induced antigen/RNA unmasking
 1. Before starting prepare all required material:
 1. Heat the hybridization oven to 40 °C and place a tray containing distilled water in the oven to create a humidified environment.
 2. Take a slide box and place paper towels soaked in distilled water inside to humidify the slide box. Place the slide box in the hybridization oven.
 3. Remove probes (both negative and target probes) from fridge and heat them in the hybridization oven for 10 min. Let the probes cool down at RT.
 4. Equilibrate amplification reagents AMP 1-4 (included in the Fluorescent Multiplex Reagent Kit) at RT.
 5. Prepare antigen retrieval solution: 10 mM sodium citrate (pH 6), 0.05% Tween 20. Note: Solution can be stored at RT indefinitely and be used for future staining.
 6. Prepare 1x wash buffer diluting the 50x stock solution (included in the Fluorescent Multiplex Reagent Kit) in RNase-free or DEPC-treated water.
NOTE: 1x wash buffer can be stored at RT for 1 month and be used for future staining. 50x wash buffer might precipitate. If so, heat 50x wash buffer at 40 °C for 10 min before preparing the 1x solution.
 2. Remove slides from 100% ethanol and air-dry for 5 min at RT.
 3. Prepare a coplin jar containing 50 ml of antigen retrieval solution. Immerse slides in antigen retrieval solution and boil them for 10 min in a microwave oven.
NOTE: Alternatively place slides horizontally in a 100 mm diameter round glass dish containing 100 ml of retrieval solution.
 1. Fill up a 1 L beaker with distilled water and place it in the microwave.
NOTE: The water will "buffer" the heat when performing unmasking.
 2. Place the slides in antigen retrieval solution inside the microwave. Boil slides at high power for 5 min. Immediately after samples have stopped boiling, heat slides again at high power for extra 5 min.
 3. Cool down slides at RT.
NOTE: CRITICAL STEP: boiling duration and procedure should be determined by user, depending on the microwave characteristics. The faster the boiling starts the higher the chances of solution evaporation. In this case it is recommended that samples are boiled for shorter time periods more than twice to complete a total unmasking time of 10 min. On the contrary, if

heating is performed at medium or low power, unmasking can be performed once for 10 min. However, if samples do not boil continuously for a total of approximately 10 min, this might result in partial unmasking of both the target RNA and protein to be detected.

4. Wash slides three times with 1x PBS.
5. Add 2 - 3 drops (~100 μ l) of the *dapB* probe set to those brain slices used to assess background staining. Note: The *dapB* probe set targets a bacterial gene and should not recognize any mammalian RNA.
6. Add 2 - 3 drops of the targeting probe set to those brain slices used to detect the RNA of interest. Note: a probe set targeting residues 20 - 1,381 of the mouse *Atf4* mRNA is used in this example.
7. Cover slides with parafilm to avoid probe evaporation, place them in the humidified slide box inside the hybridization oven and incubate them at 40 °C for 2 hrs.
NOTE: OPTIONAL: additional brain slices can be hybridized with a probe set targeting mouse *Polr2A* and used as positive controls.
8. Wash slides twice with 1x wash buffer for 2 min at RT.
9. Add 2 - 3 drops of amplification reagent AMP 1-FL to each brain slice, cover slides with parafilm, place them in the slide box and incubate at 40 °C for 30 min in the hybridization oven.
10. Wash slides twice with 1x wash buffer for 2 min at RT.
11. Add 2 - 3 drops of amplification reagent AMP 2-FL to each brain slice, cover slides with parafilm, place them in the slide box and incubate at 40 °C for 15 min in the hybridization oven.
12. Wash slides twice with 1x wash buffer for 2 min at RT.
13. Add 2 - 3 drops of amplification reagent AMP 3-FL to each brain slice, cover slides with parafilm, place them in the slide box and incubate at 40 °C for 30 min in the hybridization oven.
14. Wash slides twice with 1x wash buffer for 2 min at RT.
15. Add 2 - 3 drops of amplification reagent AMP 4-FL to each brain slice, cover slides with parafilm, place them in the slide box and incubate at 40 °C for 15 min in the hybridization oven.
16. Wash slides twice with 1x wash buffer for 2 min at RT and twice with 1x PBS.
17. Add 100 - 200 μ l (or enough volume to completely cover slices) of a blocking solution containing 3 mg/ml BSA, 100 mM glycine and 0.25% Triton X-100 in PBS (pH 7.4) to the slides, cover them with parafilm and incubate for 30 min at RT.
18. Add 100 - 200 μ l of anti-ChAT antibody diluted in blocking solution (1/100) to the slides, cover them with parafilm and incubate for 2 days at 4 °C. Make sure that brain slices don't dry out. If needed, re-apply anti-ChAT antibody solution to brain slices 24 hrs after incubation.
19. Wash slides three times in 1x PBS for 5 min at RT.
20. Add 100 - 200 μ l of the appropriate Alexa-conjugated secondary antibody (e.g., Alexa-594 donkey anti-goat for this particular example) to the slides, cover with parafilm and incubate for 1 hr at RT.
21. Wash slides three times in 1x PBS for 5 min at RT.
22. Wash slides once with distilled water.
23. Mount slides with DAPI-containing mounting medium.
24. Visualize brain slices under a fluorescence microscope.

2. Detection of *Atf4* mRNA Localized to Axons in Human Brain Samples using Chromogenic *In Situ* Hybridization (CISH) Followed by Luxol Fast Blue and Cresyl Violet Counterstaining

1. Sample preparation for formalin-fixed paraffin-embedded human brain samples
 1. Bake slices in a dry oven at 60 °C for 1 hr.
 2. Deparaffinize brain slices in a fume hood.
NOTE: CAUTION: reagents are toxic and must be handled and discarded following the appropriate security guidelines
 1. Immerse slides in xylene alternative clearing agent twice for 10 min.
 2. Immerse slides in 100% ethanol twice for 1 min.
 3. Air-dry slices 5 min at RT. Note: Alternatively samples might be dried at RT O/N but must be used within 24 hrs.
2. Diaminobenzidine (DAB)-based chromogenic ISH assay followed by luxol fast blue and cresyl violet stain using heat-induced and protease-induced RNA unmasking
 1. Before starting prepare required materials:
 1. Heat the hybridization oven to 40 °C and place a tray containing distilled water to create a humidified environment.
 2. Take a slide box and place paper towels soaked in distilled water inside to humidify the slide box. Place the slide box in the hybridization oven.
 3. Prepare 1x Pretreat 2 by diluting the 10x stock solution (included in the CISH Reagent Kit) in RNase-free or DEPC-treated water. If pretreatment is performed on a hot plate, add 100 ml of pretreat solution to a 100 mm diameter glass dish to increase the contact surface between the dish and the hot plate (if pretreatment is performed in a microwave, a coplin jar can be used instead as specified in 1.2.3). Heat solution to 100 °C and keep it boiling no longer than 30 min before submerging samples.
 4. Heat negative and positive probes 10 min at 40 °C and cool down at RT.
 5. Equilibrate amplification reagents AMP 1-6 (included in the CISH Reagent Kit) at RT.
 6. Prepare 1x wash buffer diluting the 50x stock solution (included in the CISH Reagent Kit) in distilled water. Note: 1x wash buffer can be stored at RT for 1 month and be used for future staining.
 2. Add ~4 drops (~120 μ l) of Pretreat 1 to each brain slice, cover with parafilm and incubate at RT for 10 min.
 3. Wash 3 to 5 times in fresh distilled water.
 4. Perform heat-induced unmasking:

1. Transfer slides to hot Pretreat 2 (included in the CISH Reagent Kit) solution and boil for 15 min. Note: Boiling can be performed on a hot plate ensuring continuous boiling or in a microwave following critical steps specified in 1.2.3).
5. Immediately transfer slides to distilled water and wash 3 to 5 times.
6. Wash slides 3 to 5 times in fresh 100% ethanol.
7. Air-dry slides 5 min at RT. Note: Alternatively slides can be dried O/N.
8. Perform protease-induced unmasking:
 1. Add 4 drops of Pretreat 3 (included in the CISH Reagent Kit), cover with parafilm and incubate 30 min at 40 °C in the hybridization oven.
9. Wash slides 3 to 5 times in fresh distilled water.
10. Add 4 drops of the *dapB* probe set to those brain slices used to assess background staining.
11. Add 4 drops of the targeting probe set to those brain slices used to detect the RNA of interest.
NOTE: A probe set targeting residues 15 - 1,256 of the human *ATF4* mRNA is used in this example. OPTIONAL: additional brain slices can be hybridized with a probe set targeting human PPIB and used as positive controls.
12. Place slides in the slide box and incubate for 2 hrs. at 40 °C in the hybridization oven.
13. Wash slides twice with 1x wash buffer for 2 min at RT.
14. Add 4 drops of AMP 1 reagent to each brain slice, cover slides with parafilm, place them in the slide box and incubate at 40 °C for 30 min in the hybridization oven.
15. Wash slides twice with 1x wash buffer for 2 min at RT.
16. Add 4 drops of AMP 2 reagent to each brain slice, cover slides with parafilm, place them in the slide box and incubate at 40 °C for 15 min in the hybridization oven.
17. Wash slides twice with 1x wash buffer for 2 min at RT.
18. Add 4 drops of AMP 3 reagent to each brain slice, cover slides with parafilm, place them in the slide box and incubate at 40 °C for 30 min in the hybridization oven.
19. Wash slides twice with 1x wash buffer for 2 min at RT.
20. Add 4 drops of AMP 4 reagent to each brain slice, cover slides with parafilm, place them in the slide box and incubate at 40 °C for 15 min in the hybridization oven.
21. Wash slides twice with 1x wash buffer for 2 min at RT.
22. Add 4 drops of AMP 5 reagent to each brain slice, cover slides with parafilm and incubate 30 min at RT.
23. Wash slides twice with 1x wash buffer for 2 min at RT.
24. Add 4 drops of AMP 6 reagent to each brain slice, cover slides with parafilm and incubate 15 min at RT.
25. Wash slides twice with 1x wash buffer for 2 min at RT.
26. Mix equal volume of BROWN-1 and BROWN-2 reagents (included in the CISH Reagent Kit), add ~120 µl of solution to each brain slice, cover with parafilm and incubate 10 min at RT.
27. Wash slides twice with 1x wash buffer for 2 min at RT and rinse once in distilled water.
NOTE: CRITICAL STEPS: the following steps are critical to obtain an optimal axonal counterstain without masking the presence of RNA granules.
28. Before performing counterstain check the presence of mRNA of interest under a brightfield microscope. Define reference areas in the brain samples in which to monitor the presence of puncta throughout the counterstain procedure.
29. Preheat luxol fast blue solution at 60 °C.
30. Place slides in luxol fast blue and incubate 30 min at 60 °C.
31. Rinse several times in distilled water.
32. Dip slides several times in 0.05% lithium carbonate solution to start differentiation.
33. Dip slides twice in fresh 75% ethanol.
34. Rinse in distilled water.
35. Check brain slices under a brightfield microscope. Note that grey and white matter should start to be distinguishable and RNA granules still visible as dark blue/black puncta. Verify that axons start appearing as light blue fibers.
36. Repeat steps 2.2.28 to 2.2.32. Perform incubations with luxol fast blue solution in 10 - 20 min steps, carefully monitoring the counterstain and the presence of RNA granules. Note: Typically, optimal counterstain is acquired in 60 - 90 min (total incubation time) but time can be shortened if it is suspected that RNA granules might be masked by the luxol fast blue stain (see **Figure 2** for examples).
37. Incubate slides in cresyl violet solution for 10 min at RT.
38. Rinse slides in distilled water.
39. Dip slides 5 to 10 times in 70% ethanol.
40. Dehydrate brain slices through 3 quick changes of 100% ethanol.
41. In a fume hood, clear brain slices by incubating twice in xylene alternative clearing agent for 2 min and a third time for 5 min at RT.
42. Mount brain slices in xylene-based permanent mounting medium.
43. Analyze samples under a brightfield microscope.

Representative Results

A brief summary of the procedures described above is shown in **Figure 1**.

Optimal detection of *Atf4* mRNA granules in cholinergic axons using heat-induced unmasking

When assessing axonal localization of mRNAs, it is critical to be able to identify the axons and to be able to visualize low abundant RNAs. The RNA ISH technology described here enables the detection of RNAs at a single-molecule resolution. Standard protocols using this technology suggest the combination of two unmasking procedures to efficiently detect target RNAs: protease-induced and heat-induced unmasking²⁸.

However, when ISH is combined with immunohistochemistry to identify axons, protease-induced unmasking might not result in optimal antigen detection²⁹.

In the first protocol described here, protease digestion was avoided, since the antibody used failed to recognize choline acetyltransferase (ChAT) typically found in axons of the dentate gyrus arising from the septohippocampal pathway (**Figure 2A** and **B**), although positive mRNA granules were detected. Heat-induced retrieval with 10 mM citrate buffer (pH 6), on the other hand, ensured the integrity of the epitope recognized by the anti-ChAT antibody and target mRNA was efficiently detected in ChAT-stained axons (**Figure 2C** and **D**). The results presented here (**Figure 2**) show the presence of *Atf4* in the dentate gyrus of adult mice where mRNA recruitment and local translation were induced by infusion of A β ₁₋₄₂¹⁸, oligomers into the hippocampus. Previous evidence suggests that *Atf4* mRNA is not detected in axons *in vitro* or *in vivo* under basal conditions¹⁸, and thus such an experimental paradigm is not shown.

Optimal detection of *ATF4* mRNA granules in axons using both heat-induced and protease-induced unmasking

Whereas the results described above show that antibody based protein detection is compatible with RNA ISH technology when performing heat induced unmasking it might not be useful if protease pre-treatment is required. Section 2 describes detection of *ATF4* mRNA within axons following previously published procedures^{28,30} combined with histological stains typically used for brain samples^{31,32}.

A critical step in this procedure is the optimal counterstain of myelinated fibers using luxol fast blue (LFB) without masking the presence of mRNA granules in axons stained by CISH. Reagents used for this purpose allow optimal counterstain with LFB following incubation times of 60 to 90 min. Counterstain might fail when both temperature and incubation times are decreased (**Figure 3A** and inset, and data not shown) and although mRNA granules will still be visible, their axonal localization cannot be verified. On the other hand, incubating brain samples for 60 min or longer at 60 °C without monitoring the presence of positive granules periodically might result in irreversible masking of the mRNA of interest by the dye (**Figure 3B** and inset). It is thus recommended that samples are incubated in LFB for 30 min and that further incubation is performed stepwise checking the samples every 10 to 20 min to obtain an optimal staining of both the axons and the RNA granules (**Figure 3D-F**). Following these guidelines *Atf4* positive granules can be clearly detected both in control (**Figure 3D**) and Alzheimer's disease (**Figure 3F**) brain samples.

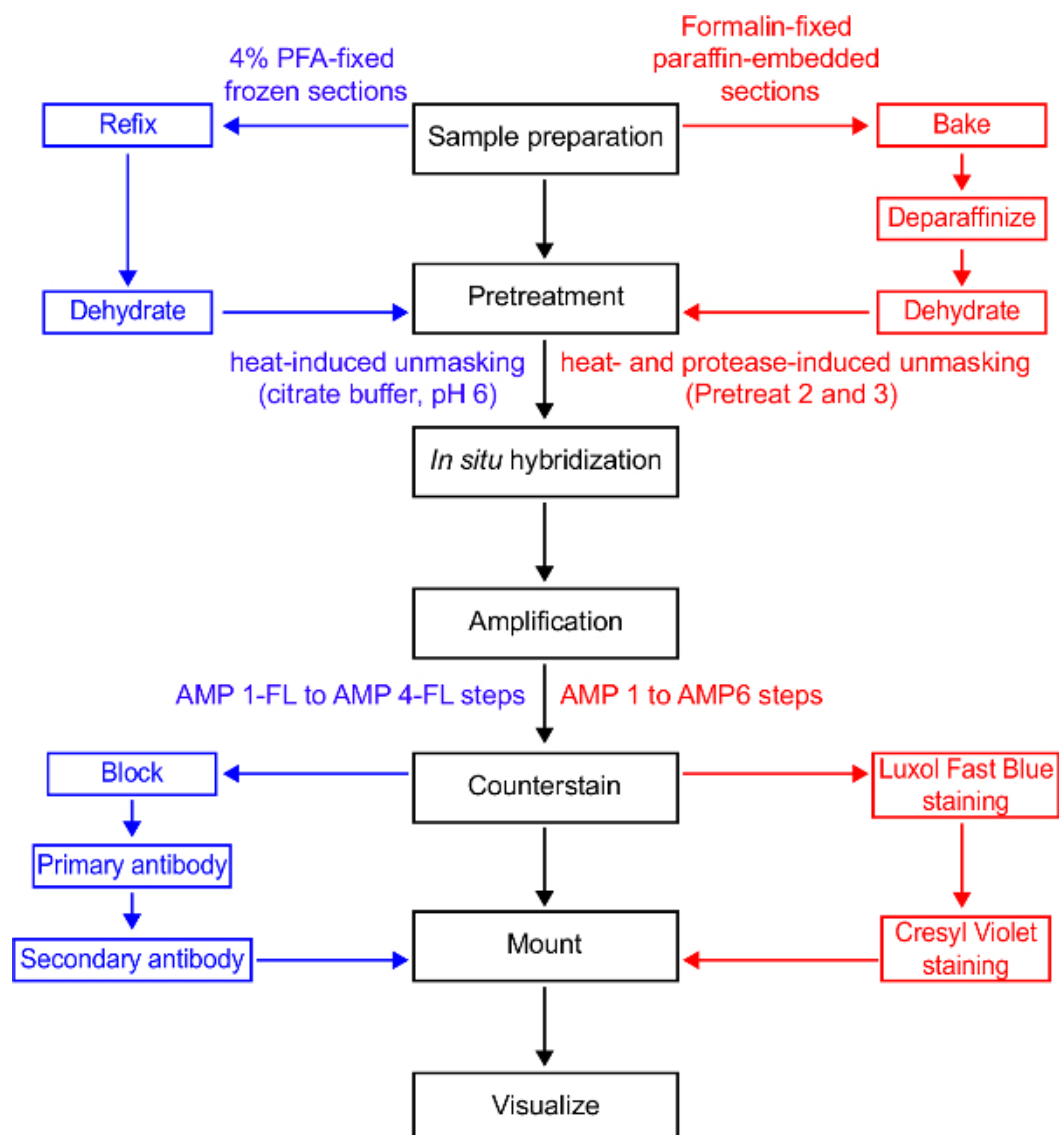


Figure 1. Summarized workflow of RNA ISH followed by axonal counterstain. Steps depicted in black are common to the two procedures exemplified. Steps highlighted in blue are specific to paraformaldehyde-fixed samples stained by fluorescent ISH whereas those highlighted in red are specific to formalin-fixed paraffin-embedded samples stained by chromogenic ISH. [Please click here to view a larger version of this figure.](#)

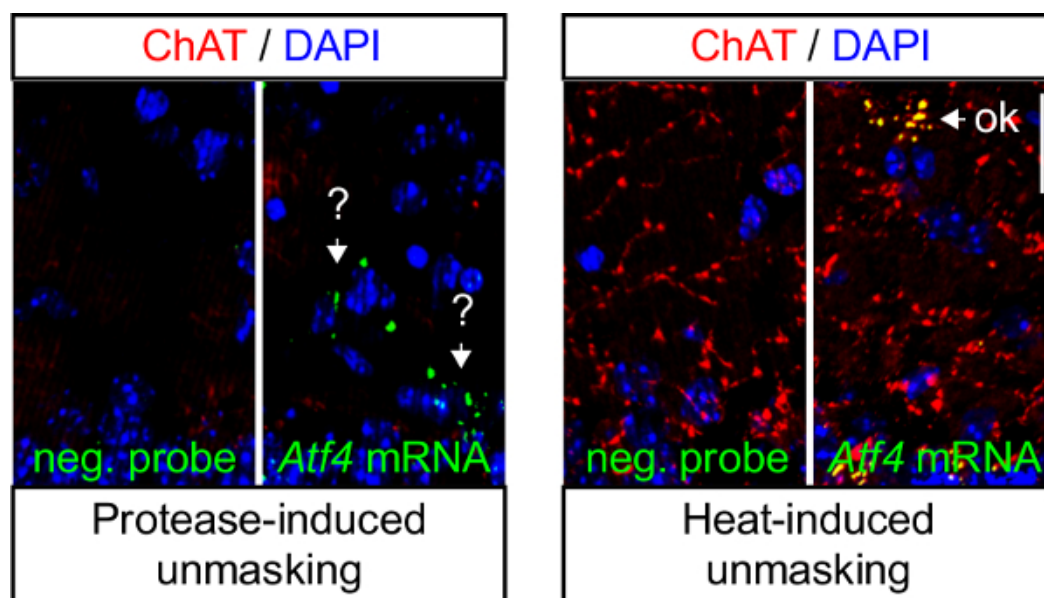


Figure 2. FISH for *Atf4* mRNA localized to cholinergic axons in the dentate gyrus of adult mice. FISH was performed using protease-induced (left panel) or heat-induced (right panel) unmasking followed by antibody detection of choline acetyltransferase (ChAT). The antibody failed to recognize ChAT when protease treatment was performed. Examples of results obtained with a non-targeting probe (*dapB*) and the *Atf4*-targeting probe using the same microscope settings and image adjustments are shown. *Atf4*-positive granules with unclear axonal localization are indicated with question marks while localized granules are marked as "ok". Scale bar 20µm. [Please click here to view a larger version of this figure.](#)

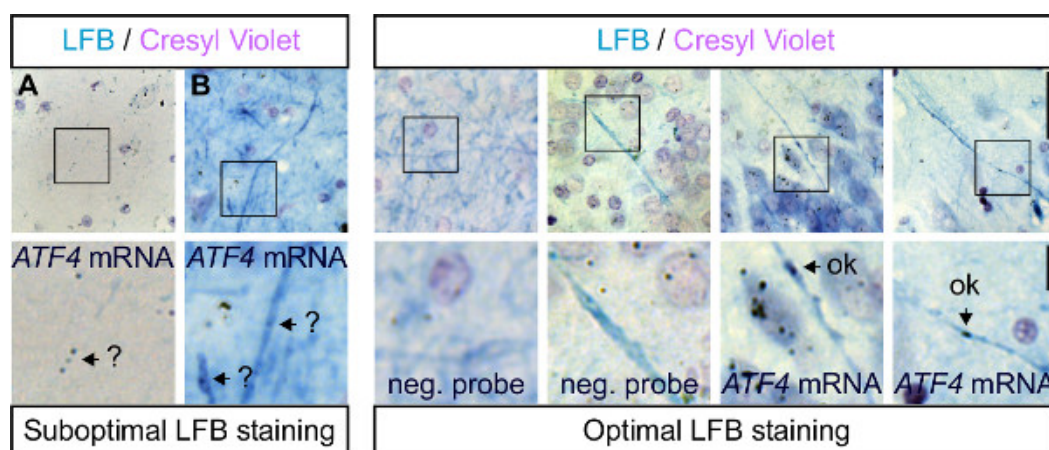


Figure 3. CISH for *ATF4* mRNA localized to myelinated axons in the human hippocampal formation. Following ISH, axons were counterstained with luxol fast blue (LFB) and neuronal somata were counterstained with cresyl violet. Suboptimal LFB staining might result if both temperature (**A** and inset represent samples stained with LFB at 40 °C for 4 hrs.) and incubation time (not shown) are decreased, or when counterstain is not checked after short incubation periods (**B** and inset). Examples of optimal LFB staining combined with ISH using a non-targeting probe (*dapB*) or the *ATF4*-targeting probe are shown (**C-F** and insets). Image acquisition was automatically adjusted for the best signal-to-noise ratio. *ATF4*-positive granules with unclear axonal localization are indicated with question marks while localized granules are marked as "ok". Scale bar 50 µm, insets 10 µm. [Please click here to view a larger version of this figure.](#)

Discussion

In this report we describe the use of a high-resolution ISH technology in the detection of axonally localized *Atf4* mRNA. These and previous published studies show that this technology is compatible with antibody-based protein detection in tissues or even whole embryos³³. Importantly, it has been recently used for the detection of *Arc* mRNA within dendrites of hippocampal neurons³⁴. It can also be combined with histological dyes for tissue staining. Finally, it is suitable for the simultaneous detection of multiple target RNAs^{28,30,33}. These findings exemplify the versatility of high-resolution RNA ISH technologies, and minor modifications to the original protocols do not decrease the sensitivity of this method.

Original protocols describing the use of Z-structure probes to detect mRNAs of interest in tissues at a single-molecule resolution suggest two steps for mRNA unmasking involving protease digestion and sample boiling^{30,35}. Here we show how protease-induced unmasking negatively affects the successful antibody-based detection of ChAT in cholinergic axons arising from the septo-hippocampal pathway (**Figure 2A and B**). Thus, we decided to exclude this step and perform only heat-induced unmasking if axonal counterstaining involved the use of antibodies. As shown (**Figure 2C and D**), cholinergic axons could be visualized with an anti-ChAT antibody and *Atf4* mRNA granules were detectable avoiding

protease digestion. Note that the positive detection of *Atf4* mRNA is based on the background fluorescence obtained from the negative probe. An extra control can be included at this point by treating brain samples with RNases and probing them with the mouse *Atf4* probes in order to test their specificity. This step is however not included in the procedures discussed here since previous evidence show, using this same technology, a complete depletion of *Atf4* mRNA in axons following siRNA injection in the mouse hippocampus¹⁸. Such results demonstrate the specificity of the ISH probes used here. The use of controls, other than the negative probes should be evaluated based on particular scientific questions. Finally, heat-induced retrieval might be substituted by or combined with protease-induced unmasking depending on the requirements of the antibody used for axon counterstaining. The choice of using one or other procedure or the combination of both should be empirically determined by the user.

When performing protease- and heat-induced unmasking in human brain samples, histological dyes can substitute antibody-based axonal counterstaining. Although the original protocol here followed suggests the use of hematoxylin for tissue counterstaining³⁰, such dye is not suitable for axon staining. Thus, luxol fast blue was used to stain myelinated axons and cresyl violet was used to visualize neuronal cell bodies. LFB, developed by Kluever and Barrera³¹, was chosen over other stains because it can be completed in less than 2 hr and if differentiation is optimized (**Figure 3C-F**) the light blue stain does not interfere with the mRNA granules that appear as dark brown-black dots. As shown (**Figure 3**), optimal LFB counterstaining can be accomplished when incubating samples at 60 °C for 60 - 90 min. It is however recommended that incubation is performed stepwise so that differentiation can be carefully monitored and mRNA granules are always visible. Other histological techniques such as Bielchowsky's or Bodian's silver staining yield grey-black axon staining³⁶ that might not be compatible with the DAB-based chromogenic reaction chosen in this report. Likely, such staining techniques should be combined with RNA CISH assays that allow the use of alternative dyes such as fast red or HRP green³⁰. Choosing the appropriate combination of RNA ISH assays and axonal stains should be empirically determined by user.

One limitation of the first procedure described in this report is the unsuccessful protein detection by immunohistochemistry if protease digestion is performed. This limitation can be overcome by avoiding protease-induced unmasking. In the particular case of axonally-localized *Atf4* performing heat-induced unmasking was sufficient to detect mRNA granules above background levels in cholinergic axons. This however might not be the case for other mRNAs of interest and protease digestion might be required. If so, axonal counterstaining should be performed using antibodies other than the anti-ChAT antibody here described. Alternatively, antibody-based counterstaining might be substituted by histological dyes as described in section 2 of the protocol.

LFB staining of fibers is not suitable for visualization of unmyelinated axons. Alternative staining techniques, such as Bielchowsky's or Bodian's silver staining, allow the visualization of both myelinated and unmyelinated axons. Both methods result in grey-black staining of axons that is not compatible with DAB CISH since the mRNA granules appear as brown-black puncta. However, there are other dyes available for the detection of mRNAs of interest under a brightfield microscope, such as fast red or HRP green³⁰.

As stated in the protocol section, one of the critical steps of both procedures is the heat-induced unmasking. If boiling is performed in a microwave, there are chances of solution evaporation depending on the characteristics of the device. Follow the steps specified in 1.2.3 and 2.2.4 to avoid solution evaporation. For fixed frozen tissue 10 min of unmasking should suffice, whereas for paraffin embedded tissues boiling for 15 min is recommended. If samples do not boil continuously for the recommended time this might result in partial unmasking. However times could slightly vary if other devices such as a rice cooker or a hot plate are chosen instead of a microwave. Boiling duration should be determined by user, depending on the method.

Another critical step is the LFB counterstaining. It is important that the intensity of the blue dye does not interfere with the visualization of the mRNA granules. Some modifications to the original protocol³¹ were performed in order to reduce the intensity of the dye, such as decreasing the temperature (**Figure 3A**) or the incubation time (data not shown), however we failed to clearly distinguish myelinated axons in human brain samples. On the other hand, incubating the samples in LFB solution at the suggested temperature (60 °C) resulted in optimal staining of myelinated axons. It is however recommended that counterstaining is performed stepwise carefully monitoring the presence of RNA granules at all times to ensure that LFB does not mask the mRNA of interest as specified in steps 2.2.28-2.2.36.

Finally, samples should never dry out. The methods described in this report involve multiple incubation steps at 40 °C, which increases the chances of reagent evaporation. As stated in the protocols, samples should be covered with parafilm in all steps to avoid evaporation.

In summary, the development of high-resolution RNA ISH and other ISH methods are enabling the visualization of low-abundant transcripts, including those localized to adult axons *in vivo*. This is especially important since for many years mRNA localization to and translation in adult axons was greatly overlooked as they were considered translationally inactive.

In conclusion we present a novel and promising technology for the detection of *Atf4* and potentially many other mRNAs in adult axons of the mammalian brain. RNAscope will facilitate future studies on mRNA localization and will help to unravel the biological significance of their local translation *in vivo*.

Disclosures

The authors have nothing to disclose.

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