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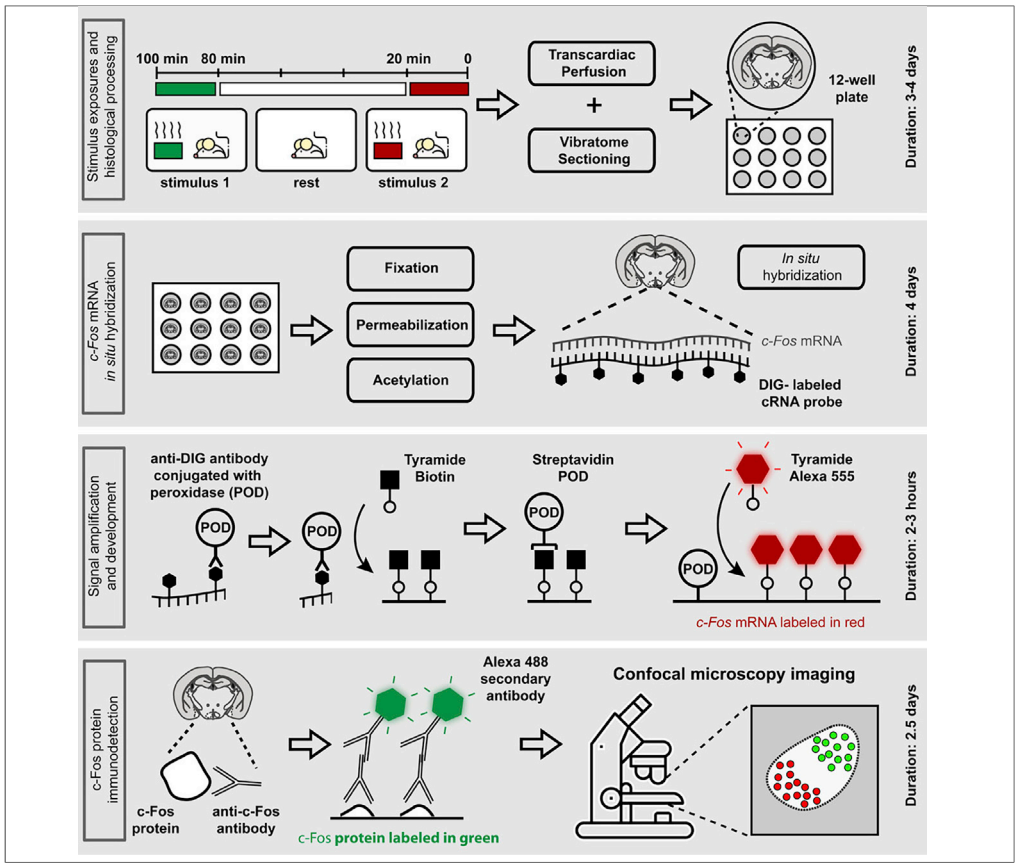
Protocol

Detection of Activated Mouse Neurons with Temporal Resolution via Dual c-Fos Staining



This protocol combines fluorescent *in situ* hybridization and immunostaining to simultaneously detect, in histological sections from the same animal, subpopulations of neurons activated after two episodes of sensory stimulation. It allows the identification of groups of cells singly activated by either stimulus or co-activated by both stimuli. Our method results in nuclear staining for *c-Fos* mRNA and c-Fos protein, allowing better spatial and temporal resolution than previously published protocols, although it requires quick brain fixation.

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HIGHLIGHTS

Dual staining identifies activated neurons after two episodes of sensory stimulation

Hybridization phase includes *c-Fos* mRNA hybridization and signal development

Immunostaining phase includes antibody detection of c-Fos protein

Cells activated by stimuli 1 and 2 are labeled in green and red, respectively

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SUMMARY

This protocol combines fluorescent *in situ* hybridization and immunostaining to simultaneously detect, in histological sections from the same animal, subpopulations of neurons activated after two episodes of sensory stimulation. It allows the identification of groups of cells singly activated by either stimulus or co-activated by both stimuli. Our method results in nuclear staining for *c-Fos* mRNA and c-Fos protein, allowing better spatial and temporal resolution than previously published protocols, although it requires quick brain fixation.

For complete details on the use and execution of this protocol, please refer to Carvalho et al. (2015, 2020).

BEFORE YOU BEGIN

This dual staining protocol was devised to detect activated neurons in mouse brain sections, allowing the distinction between cells activated after each one of two sequential episodes of sensory stimulation (Carvalho et al., 2015, 2020). Originally, our protocol was created to investigate, in the same animal, neurons in olfactory brain areas activated after stimulation with different chemosignals, but it is suited for the analysis of brain activity toward other types of sensory stimuli as well. Our method shares the same principles as the catFISH procedure (Guzowski et al., 1999, 2001; Lin et al., 2011) but we employ newly designed probes to simultaneously detect *c-Fos* mRNA and c-Fos protein (Carvalho et al., 2015). The *c-Fos* gene was chosen because it is a widely validated immediate early gene used as an indirect marker of neuronal activation in the brain, including in studies that focused on olfactory brain areas (Carvalho et al., 2015; Lin et al., 2011; Papes et al., 2010).

In our protocol, c-Fos protein is expressed in cells activated during the first window of sensory stimulation, while *c-Fos* mRNA is produced in cells activated during the second window of stimulation, allowing the identification of cells activated by one, the other, or both stimuli, with great temporal resolution (Figure 1 and Methods Video S1).

The protocol detailed below describes the stimulation procedure (with a focus on olfactory stimuli), brain dissection and sectioning, *in situ* hybridization to detect *c-Fos* mRNA, and immunostaining to detect c-Fos protein. The comments outline critical steps and provide a contrast to the previously published catFISH, underscoring the advantages of our method.

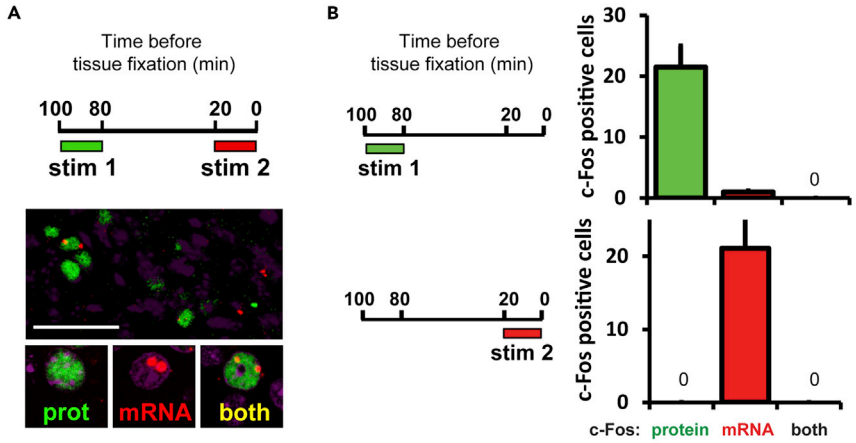

Figure 1. Example of Dual c-Fos Staining and Controls

(A) Top, time windows containing exposure to sensory stimuli, separated by 60 min of rest period. Bottom, example of microscopy image (maximum intensity projection in a z series of 20 confocal images). Green staining represents c-Fos protein labeling by immunostaining and red fluorescence indicates nuclear foci after *c-Fos* mRNA detection by *in situ* hybridization. Adapted from Carvalho et al. (2015), under the Creative Commons Attribution License (CC BY). Scale bar, 50 μm.

(B) Single stimulation controls, showing low *c-Fos* mRNA staining when stimulus is applied only in the first window and absence of c-Fos protein detection when stimulus is applied only in the second window. Data are represented as mean + SEM. Adapted from Carvalho et al. (2020).

Prepare Mouse Subjects, Stimuli, and the Procedure Room

Timing: 2 days

1. Most stimulation protocols require the mouse subject to be individually caged, either to prevent stimulation with conspecific signals or to prevent the generation of behaviors toward other co-housed individuals. We usually singly house each mouse subject in a clean cage with fresh bedding and leave it in its new home cage for at least 2 days before starting the habituation phase described in the protocol.
2. Clean cages may be obtained by washing with unscented soap, rinsing with running water for at least 10 min, followed by extra washing in distilled water. Optionally, add an additional step to remove odors from the cleaned cage by placing it in a vacuum desiccator container for 12 to 24 h.
3. Considering that each stimulation episode in this protocol lasts for only 20 min (and this should be strictly followed to allow the animal to rest without stimulation for 1 h prior to being stimulated a second time), it is important to have all stimuli fresh and ready when the exposure events are about to start. If using olfactory stimuli, do not prepare scented gauzes or cotton balls too far in advance, as the strength of each stimulus may decay, resulting in sub-optimal stimulation. This is especially true for the second window of stimulation, which starts 1 h and 20 min after the onset of the first stimulation. For a detailed description of the types, quantity, and modes of presentation of olfactory stimuli and preferred controls, please refer to previous publications (Carvalho et al., 2015, 2020; Isogai et al., 2011; Papes et al., 2010, 2018).
4. Experiments should be conducted in a clean and quiet procedure room that has been prepared in advance to remove unwanted odors. To avoid visual stimulation during exposure, it is also important to conduct all experiments in the dark, which can be better performed under red light illumination, as explained in detail in our previous publication (Papes et al., 2018). Additionally, the procedure room should be used for sensory stimulation and rest periods only, while perfusion and brain dissection should be performed in an adjoining surgery room.

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Prepare Solutions and Reagents

Timing: 2 days

5. The following solutions must be prepared beforehand and may be stored as stock solutions (check the Materials and Equipment section for recipes on how to prepare some of these solutions):
   a. RNase-free 20% sucrose solution
   b. RNase-free 6 M HCl stock
   c. RNase-free 0.2 M HCl working solution
   d. RNase-free 10 mg/mL yeast tRNA
   e. 10% Tween-20
   f. 10% Triton X-100
   g. TNB buffer
   h. cRNA riboprobes (see section below for details)
6. The following solutions must be prepared right before the beginning of each experiment or at the beginning of each day of experimentation. These solutions should not be stored.
   a. RNase-free 4% paraformaldehyde fixative solution
   b. RNase-free 0.1 M triethanolamine-HCl pH 8.0 solution
   c. Pre-hybridization and hybridization solution
   d. 5× SSC, 2× SSC, 0.2× SSC, 0.1× SSC
   e. PTw solution
   f. TN buffer
   g. TNT buffer
   h. 0.1 M HCl/0.9% NaCl
   i. 0.3% Triton X-100/1× PBS permeabilization solution
   j. 0.1% Triton X-100/1× PBS wash solution
7. The following solutions must be prepared immediately before they are used:
   a. 0.1% H_2O_2 in 1× PBS-DEPC
   b. Acetylation solution (triethanolamine + acetic anhydride)
   c. Peroxidase-conjugated anti-digoxigenin antibody working solution
   d. Tyramide-biotin working solution
   e. Peroxidase-conjugated streptavidin solution
   f. Tyramide-Alexa 555 working solution
   g. 3% H_2O_2 in 1× PBS
   h. 0.3% Triton X-100/1% BSA/1× PBS
   i. Anti-c-Fos primary antibody working solution
   j. Alexa 488-conjugated anti-rabbit secondary antibody working solution
   k. Nuclear counterstaining solution

Prepare Critical Equipment

Timing: 2 h

8. The following instruments must be set up and tested beforehand:
   a. Perfusion device (peristaltic pump or 20 mL syringes) connected to appropriate tubing and a needle to puncture the heart's ventricle, which will be needed in step 6 of the Step-By-Step Method Details section.
   b. Humidified chamber containing pieces of Kimwipes wetted with 5× SSC (for hybridization steps) or 1× PBS (for antibody incubations), which will be needed in steps 26–28, 31–32, 37, and 42 of the protocol.
   c. Heated water bath (or hybridization oven) at 58°C, which will be needed in steps 26–28, 31–32, and 37 of the protocol.

d. Heated water bath or dry block at 85°C, which will be needed in step 29 of the protocol.

9. For all steps until the end of hybridization washes (step 32), glassware and plasticware that contact the specimen or tissue sections must be RNase-free.
   a. We recommend baking glassware at high temperature (more than 200°C) for at least 2 h (preferably 8 h or more).
   b. Plasticware should be new disposable RNase-free tubes (Eppendorf, conical tubes, pipette tips). If new plasticware is not available, such as for graduated cylinders, beakers, slide racks, etc., treat them with 3% H_2O_2 for 15–30 min, followed by extensive washes in RNase-free ultrapure water (such as DEPC-treated water).
   c. Metal instrumentation (such as dissecting tools, forceps, and slide jars) should be made of stainless steel and treated with 3% H_2O_2 for 15 min followed by extensive washes with RNase-free water.

Synthesize Riboprobes

Timing: 1 week

Our protocol uses cRNA riboprobes that are not commercially available, designed to recognize the *c-Fos* mRNA. We created and validated two 1 kb cRNA probes based on a region that spans the *c-Fos* gene coding sequence. We found that use of both probes increases the efficiency of hybridization with the corresponding mRNA and signal intensity after *in situ* hybridization (Carvalho et al., 2015). The experimenter may decide to order the probes as RNA oligos or amplify the corresponding DNA fragments, clone them into a suitable PCR cloning vector, followed by *in vitro* transcription to synthesize the digoxigenin-labeled cRNA probes. The experimenter is welcome to use the following protocol for this important preparatory step.

10. PCR amplify and clone the two fragments from the *c-Fos* gene
    a. Use the primer pairs listed in the Key Resources Table and the following PCR conditions to amplify two 1 kb fragments from the *c-Fos* gene, using mouse brain cDNA as template and the proofreading amplification enzyme Platinum *Taq* DNA polymerase High Fidelity.

Component	Final Concentration	Amount	Volume
Mouse brain cDNA	-	25 ng	1 µl
10× High Fidelity PCR Buffer	1×	-	2.5 µl
50 mM $MgSO_4$	2 mM	-	1 µl
10 mM dNTP Mix	0.2 mM each	-	0.5 µl
10 µM forward primer	0.2 µM	-	0.5 µl
10 µM reverse primer	0.2 µM	-	0.5 µl
Platinum® Taq DNA Polymerase High Fidelity (5 U/µL)	1 U/reaction	1 U	0.2 µl
PCR grade water	-	-	for 25 µl

Cycling Parameters		
1 x	94°C	5 min
35 x	94°C	1 min
	55°C	1 min
	68°C	1 min
1 x	68°C	5 min

Note: If using a different amplification enzyme, it should be noted that, since the resulting PCR amplicons will be cloned into a plasmid vector, it is mandatory to use a PCR enzyme that leaves A overhangs at the 3′ ends of each amplified DNA strand or treat the resulting amplicon with a suitable enzyme to add the A overhangs after the PCR reaction.

b. Run the fragments separately on a 0.8% agarose gel and isolate the gel pieces containing the appropriate bands (~ 1.0 kb) using a razor blade or scalpel.
c. Purify the DNA fragments from the gel pieces using a suitable mini-column-based purification kit (e.g., QIAquick gel extraction kit from Qiagen).
d. Quantify the purified DNA fragments by spectrophotometric methods and clone them into a suitable PCR cloning plasmid vector. We recommend using pGEM-T-Easy (Promega), as it contains easy-to-use RNA polymerase promoters flanking the cloned PCR product.
e. After the resulting recombinant plasmids containing each PCR product have been successfully produced, transform them into a competent, recombination-free bacterial strain, such as One Shot TOP10 (or Stbl3) competent cells, via chemical transformation or electroporation.
f. Seed isolated bacterial transformants into complete rich liquid medium (such as LB, TB, or 2xYT) and extract plasmid DNA from the cultures via miniprep or midiprep.
g. Restriction digest the resulting plasmid to verify the identity of the cloned DNA fragments and their orientation in the cloning vector. We use a combination of *Bgl*II and *Nco*I for PCR fragment amplified with the first primer pair in the Key Resources Table and *Bst*XI for PCR fragment amplified with the second primer pair.

11. It is recommended to sequence the plasmids to verify the identity of each cloned fragment, confirm its orientation relative to the RNA polymerase promoters flanking the multicloning site, and rule out the existence of mutations introduced as a consequence of amplification errors.
12. Choose one of the RNA polymerase promoters (T7 or SP6 promoters in pGEM-T-Easy, for example) based on the DNA insert orientation, considering the type of probe to be generated. For antisense *c-Fos* probes used in this protocol, choose the RNA pol promoter that sits downstream relative to the coding strand in the plasmid.
13. Digest 10 μg of DNA from each plasmid DNA with a suitable restriction enzyme to linearize the plasmid. The restriction enzyme should be chosen based on the following properties: (a) it should uniquely cut the plasmid DNA; (b) it should digest the DNA at the multicloning site on the side opposite to the chosen RNA pol promoter (for pGEM-T-Easy, if the SP6 promoter is chosen, select an enzyme located on the side containing the *Nco*I restriction site); and (c) it should digest the DNA leaving 5′ overhangs. For pGEM-T-Easy, we usually use *Nco*I or *Nde*I restriction enzymes.
14. Run the resulting digests on a 0.8% agarose gel, purify each linearized plasmid (e.g., with the QIAquick gel extraction kit from Qiagen), and quantify using spectrophotometric or fluorometric methods.

Note: Alternatively, the digested plasmids may be purified using a DNA clean-up kit rather than running the reaction on a gel and purifying the linearized plasmid with a gel extraction kit.

15. Using RNase-free tubes and reagents, set up the following *in vitro* transcription reaction:
    a. Add 1 μg of linearized plasmid to the tube.
    b. Add RNase-free water (DEPC-treated water or RNase-free ultrapure water) to a total of 13 μL.
    c. Add 2 μL of 10× transcription buffer, 2 μL of 10× DIG NTP labeling mix, and 1 μL of RNase inhibitor (from the DIG RNA labeling kit, Merck/Roche).
    d. Mix the reaction components and add 2 μL of T7 or SP6 RNA polymerase (Merck/Roche).
16. Incubate the reaction at 37°C for 3 h.

17. Degrade the plasmid DNA by adding 2 µL of DNase I and incubate at 37°C for 15 min.
18. Stop the reaction with 2 µL of RNase-free 0.25 M EDTA pH 8.0.
19. Purify the cRNA probe from the reaction using a suitable mini-column-based RNA clean-up kit (e.g., RNeasy MinElute Clean-up kit from Qiagen or ProbeQuant G-50 micro columns from Cytiva Biosciences).
20. Run the eluate on a RNase-free agarose gel to check for probe integrity. Alternatively, use an automated RNA analysis method to check for probe integrity, such as running samples on a BioAnalyzer (Agilent BioAnalyzer High Sensitivity RNA Analysis kit) or tapestation (Agilent High Sensitivity RNA ScreenTape). If running on an agarose gel, it is normal to observe several bands forming a ladder pattern around the presumptive location of the expected band size.
21. Determine probe concentration using a sensitive method, such as Qubit fluorometric measurement (Troubleshooting Problem 1).
22. Long-term storage of cRNA probes should be done by freezing at −80°C. When stored at this temperature, cRNA probes are stable for up to 3 years. It is also possible to dilute each probe with formamide (final concentration: 50%) before freezing it, which increases cRNA stability for long-term storage up to 5 years.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Peroxidase-conjugated anti-digoxigenin (Fab fragments)	Merck (Roche)	11207733910; RRID: AB_514500
Anti-c-Fos (Ab-2) (4–17) rabbit polyclonal antibody	Merck (Sigma-Aldrich)	PC05L; RRID: AB_2106886
Alexa 488-conjugated goat anti-rabbit secondary antibody	Thermo Fisher Scientific	A-11070; RRID: AB_142134
Peroxidase-conjugated anti-rabbit secondary antibody (polyHRP-conjugated goat anti-rabbit reagent from Alexa Fluor 488 Tyramide SuperBoost kit)	Thermo Fisher Scientific	B40922
Chemicals, Peptides, and Recombinant Proteins		
Olfactory stimuli	n/a	Papes et al. (2010), Carvalho et al. (2015), Carvalho et al. (2020)
10× Phosphate-buffered saline (PBS)	Thermo Fisher Scientific	AM9625
Diethyl pyrocarbonate (DEPC)	Merck (Sigma-Aldrich)	40718
30% Hydrogen peroxide (H_2O_2)	Merck (Sigma-Aldrich)	H1009
Paraformaldehyde (PFA)	Merck (Sigma-Aldrich)	P6148
Agarose	Merck (Sigma-Aldrich)	A9539
DNase I	Thermo Fisher Scientific	18047019
Platinum® Taq DNA Polymerase High Fidelity	Thermo Fisher Scientific	11304011
RNase-free ultrapure water	Thermo Fisher Scientific	10977015
Triethanolamine	Merck (Sigma-Aldrich)	T58300
Acetic anhydride	Merck (Sigma-Aldrich)	320102
Deionized formamide	Merck (Sigma-Aldrich)	F9037
Yeast tRNA	Merck (Roche)	10109495001
Denhardt's solution (50×)	Merck (Sigma-Aldrich)	D9905
Dextran sulfate solution (50%)	Merck (Chemicon)	S4030

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
1 M Tris-Cl pH 8.0	Thermo Fisher Scientific	15568025
1 M Tris-Cl pH 7.5	Thermo Fisher Scientific	15567027
0.5 M EDTA pH 8.0	Thermo Fisher Scientific	AM9260G
20× SSC	Merck (Calbiochem)	8310-OP
Triton X-100	Merck (Sigma-Aldrich)	T8787
Tween-20	Merck (Sigma-Aldrich)	P9416
Blocking reagent (from TSA biotin kit)	Akoya Biosciences (Perkin Elmer)	SAT700001EA
Amplification diluent (from TSA biotin kit)	Akoya Biosciences (Perkin Elmer)	SAT700001EA
Tyramide-biotin conjugate (from TSA biotin kit)	Akoya Biosciences (Perkin Elmer)	SAT700001EA
Peroxidase-conjugated streptavidin (from TSA biotin kit)	Akoya Biosciences (Perkin Elmer)	SAT700001EA
20× amplification diluent (reaction buffer from Alexa Fluor 555 Tyramide SuperBoost kit)	Thermo Fisher Scientific	B40923 or B40922
Tyramide-Alexa 555 conjugate (from Alexa Fluor 555 Tyramide SuperBoost kit)	Thermo Fisher Scientific	B40923
1× Blocking buffer (from Alexa Fluor 488 Tyramide SuperBoost kit)	Thermo Fisher Scientific	B40923 or B40922
30% Bovine serum albumin (BSA) solution	Merck (Sigma-Aldrich)	A9576
Tyramide-Alexa 488 conjugate (from Alexa Fluor 488 Tyramide SuperBoost kit)	Thermo Fisher Scientific	B40922
TO-PRO-3 iodide (642/661) solution	Thermo Fisher Scientific	T3605
DAPI nuclear stain	Thermo Fisher Scientific	D1306
Hoechst 33342 nuclear stain	Thermo Fisher Scientific	H1399
ProLong Gold antifade mountant	Thermo Fisher Scientific	P36934
Critical Commercial Assays		
TSA biotin kit	Akoya Biosciences (Perkin Elmer)	SAT700001EA
Alexa Fluor 555 Tyramide SuperBoost kit	Thermo Fisher Scientific	B40923
Alexa Fluor 488 Tyramide SuperBoost kit	Thermo Fisher Scientific	B40922
DIG RNA Labeling Kit (SP6/T7)	Merck (Roche)	11175025910
QIAquick gel extraction kit	Qiagen	28506
RNeasy MinElute clean-up kit	Qiagen	74204
High Sensitivity RNA ScreenTape Analysis reagents (buffer, ladder, and tape)	Agilent	5067-5580, 5067-5581, and 5067-5579
ProbeQuant G-50 Micro Columns	Cytiva Biosciences	28903408
Experimental Models: Organisms/Strains		
Mouse: C57BL/6J inbred strain	Jackson Laboratories	Stock No: 000664; RRID: IMSR_JAX:000664
Bacterial and Virus Strains		
One Shot TOP10 Chemically Competent *E. coli*	Thermo Fisher Scientific	C404003
One Shot Stbl3 Chemically Competent *E. coli*	Thermo Fisher Scientific	C737303

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
c-Fos probe 1 - forward primer: 5′ CAGCGAGCAACTGAGAAGAC 3′	Integrated DNA Technologies	Carvalho et al. (2015)
c-Fos probe 1 - reverse primer: 5′ GCTGCATAGAAGGAACCGGAC 3′	Integrated DNA Technologies	Carvalho et al. (2015)
c-Fos probe 2 - forward primer: 5′ GGAGCCAGTCAAGAGCATCAG 3′	Integrated DNA Technologies	Carvalho et al. (2015)
c-Fos probe 2 - reverse primer: 5′ AATGAACATTGACGCTGAAGGAC 3′	Integrated DNA Technologies	Carvalho et al. (2015)
Recombinant DNA		
pGEM-T-Easy vector	Promega	A1360
Other		
Plastic histology embedding mold	Thermo Fisher Scientific	22-19
Vibrating blade microtome (vibratome)	Leica Biosystems	VT1000 S
Humidified chamber (if used at higher temperatures, it will need to be sealed inside a plastic Tupperware container)	Thermo Fisher Scientific	22-045-034
SuperFrost Plus microscope slides	Thermo Fisher Scientific	12-550-15
RNA tapestation (or BioAnalyzer)	Agilent	4200 TapeStation System (or 2100 BioAnalyzer Instrument)
Qubit 4 Fluorometer	Thermo Fisher Scientific	Q33238
Serological pipettes	Fisher Scientific	07-200-571, 07-200-574, 07-200-573, 07-200-575
1 mL syringes	Fisher Scientific	14-829-10F
Pipette p1000, p200, and p20 disposable tips	Fisher Scientific	02-707-408, 02-707-411, 02-707-438
Conical tubes (15 mL and 50 mL)	Fisher Scientific	14-432-22, 14-959-49B
RNase-free glass or plastic graduated cylinders and beakers	Fisher Scientific	10-462-833, 02-555-25B, 02-555-25D
6-well or 12-well tissue culture plates	Fisher Scientific	12-567-099, 07-200-81
PCR thermocycler	Thermo Fisher Scientific	4375786
Electrophoresis equipment	Fisher Scientific	09-528-110B
Microcentrifuge	Eppendorf	5401000013
Mouse cages	InnoVive	M-BTM, MVX1
Movie camera	Sony	2964179
Dissecting tools and forceps	Roboz	RS-6802, RS-8124, RS-7110, RS-5111
Peristaltic pump for trans-cardiac perfusion fixation	Harvard Apparatus	70-7000
Micropipettes	Gilson	FA10006M, FA10005M, FA10003M, FA10001M
Razor blades or scalpels	Fisher Scientific	12-640
Heated plate	Fisher Scientific	HP88850200
Fine point paintbrushes	Winsor & Newton	10269097
Stereomicroscope	Leica Microsystems	M80
Plate rocker	Fisher Scientific	2217765
Water bath	Thermo Fisher Scientific	TSCIR19
Confocal microscope equipped with filters for Alexa-488, Alexa-555, and TO-PRO-3 dyes (or similar filters)	Leica Microsystems	TCS SP5II

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MATERIALS AND EQUIPMENT

⚠ CRITICAL: Whenever possible, treat all solutions with diethyl pyrocarbonate (DEPC) to eliminate RNases. This can be performed by diluting DEPC (99% v/v) to a final concentration of 0.1% (v/v), followed by vigorous mixing, and incubation at 37°C for 12 to 16 h. After treatment, the solution must be autoclaved to destroy DEPC, after which procedure the solution acquires a slight fruity smell. For certain solutions, DEPC treatment is not possible, either because the solute reacts with DEPC (such as Tris) or because it cannot be autoclaved (SDS, for example). If DEPC treatment is not possible, prepare the corresponding solutions by using DEPC-treated water or RNase-free ultrapure water. Additionally, bake all glassware used in the experiment at 200°C for 8 to 12 h and use RNase-free disposable plasticware.

Alternatives: When baking at high temperature is not possible, treat surfaces, containers, or solid materials with 3% hydrogen peroxide followed by extensive washes in RNase-free water. Alternatively, treat surfaces and solid materials with an RNase decontamination solution (e.g., RNase AWAY or RNA Zap).

RNase-free 4% Paraformaldehyde Fixative Solution

Reagent	Final Concentration	Amount
Paraformaldehyde	4% (w/v)	4 g
PBS	1×	100 mL
NaOH 10 M	n/a (to adjust pH)	20 µL
Total		~ 100 mL

Store at 4°C for a maximum of 2 h.

⚠ CRITICAL: Paraformaldehyde powder is neurotoxic and can cause irritation of skin and mucous membranes. Weigh powder using gloves, mask, and chemical safety goggles. Paraformaldehyde solution is chemically active and should be handled with gloves and disposed of following institutional guidelines for hazardous chemical waste.

⚠ CRITICAL: 4% Paraformaldehyde fixative used in *in situ* hybridization steps of the protocol should be RNase-free. Either prepare the fixative with RNase-free PBS or dissolve a concentrated PBS stock (such as 10× PBS) using RNase-free ultrapure water and glassware.

RNase-free 20% Sucrose Solution

Reagent	Final Concentration	Amount
Sucrose powder	20% (w/v)	20 g
RNase-free 10× PBS	1×	10 mL
RNase-free ultrapure water	n/a	to make 100 mL
Total		~ 100 mL

Store at 23 to 26°C for a maximum of 2 months.

⚠ CRITICAL: This solution must be prepared in RNase-free conditions.

RNase-free 0.1 M Triethanolamine-HCl pH 8.0

Carefully pipet the viscous pure triethanolamine into a small volume of RNase-free ultrapure water using an RNase-free beaker. Insert an RNase-free magnetic stirrer and add enough volume of 6 M HCl to adjust the pH to 8.0. Make sure to wash the pH meter probe with RNase-free water after calibration and between each measurement cycle. After the pH is adjusted, complete the volume to

250 mL with RNase-free ultrapure water and store in an RNase-free glass bottle, in the dark or wrapped in aluminum foil.

Reagent	Final Concentration	Amount
Triethanolamine	0.1 M	3.33 mL
RNase-free ultrapure water	n/a	to make 250 mL
6 M HCl	to adjust pH	1.5 to 1.8 mL
Total		**250 mL**

Store at 23 to 26°C for a maximum of 2 h.

⚠ CRITICAL: This solution must be prepared in RNase-free conditions.

RNase-free Pre-hybridization and Hybridization Solution

50% dextran sulfate solution should be 500,000 molecular weight. This stock solution is really viscous and should be pipetted with care to ensure precision in volume measurements. If needed, yeast tRNA can be omitted from the pre-hyb solution.

Reagent	Final Concentration	Amount
deionized formamide	50% (v/v)	5 mL
5 M NaCl	600 mM	1.2 mL
10 mg/mL yeast tRNA	200 μg/mL	200 μL
10% SDS	0.25% (w/v)	250 μL
1 M Tris-Cl pH 8.0	10 mM	100 μL
50× Denhardt's solution	1×	200 μL
500 mM EDTA pH 8.0	1 mM	20 μL
50% dextran sulfate	10% (w/v)	2 mL
RNase-free ultrapure water	n/a	to 10 mL
Total		**10 mL**

Store at 4°C for a maximum of 2 h.

⚠ CRITICAL: This solution must be prepared in RNase-free conditions.

PTw Solution

Reagent	Final Concentration	Amount
10% Tween-20	0.1% (v/v)	1 mL
10× PBS	1×	10 mL
Ultrapure water	n/a	to 100 mL
Total		**100 mL**

Store at 23 to 26°C for a maximum of 2 h.

TN Buffer

Reagent	Final Concentration	Amount
1 M Tris-HCl pH 7.5	100 mM	10 mL
5 M NaCl	150 mM	3 mL
Ultrapure water	n/a	to 100 mL
Total		**100 mL**

Store at 23 to 26°C for a maximum of 2 h.

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TNB Buffer

Weigh the necessary amount of blocking reagent (Perkin Elmer), add to the adequate volume of TN buffer, and heat it up to 60°C until completely dissolved. Make 40 mL aliquots in conical tubes and freeze at −20°C, for up to 6 months.

Reagent	Final Concentration	Amount
Blocking reagent (Perkin Elmer)	0.5% (w/v)	1 g
TN buffer	n/a	200 mL
Total		**200 mL**

Alternatives: Blocking reagent from another brand or BSA can be used at the same concentrations.

TNT Buffer

Reagent	Final Concentration	Amount
10% Tween-20	0.05% (v/v)	5 mL
TN buffer	n/a	to 1 L
Total		**1 L**

Store at 23 to 26°C for a maximum of 2 h.

Plasticware and Glassware

The following is a list of plastic or glass materials needed to complete this protocol:

- Serological pipettes
- 1 mL syringes
- Pipette p1000, p200, and p20 disposable tips
- Conical tubes (15 mL and 50 mL)
- RNase-free glass or plastic graduated cylinders and beakers
- 6-well or 12-well tissue culture plates
- Plastic histology molds

Instruments

The following is a list of pieces of equipment needed to complete this protocol:

- PCR thermocycler
- Electrophoresis equipment
- Fluorometer or spectrophotometer for DNA quantitation
- Microcentrifuge
- Tapestation for assessment of riboprobe integrity
- Mouse cages
- Movie camera (if behavior scoring is needed)
- Dissecting tools and forceps
- Peristaltic pump for trans-cardiac perfusion fixation
- Micropipettes
- Razor blades or scalpels
- Heated plate
- Vibrating blade microtome (vibratome)
- Fine point paintbrushes
- Stereomicroscope

- Plate rocker
- Humidified chamber for histological staining
- Water bath
- Confocal microscope (for example, Leica model TCS SP5 II) equipped with filters for the fluorophores used. In this protocol, we use Alexa-488, Alexa-555, and TO-PRO-3 dyes, and therefore the confocal microscope must contain the corresponding filters.

Optional Instruments

- BioAnalyzer for assessment of riboprobe integrity
- Microwave oven
- Plate shaker
- Hybridization oven
- Heated block

STEP-BY-STEP METHOD DETAILS

Sensory Stimulation Procedure

Timing: 3 h

The procedure below describes how to expose mouse subjects to the appropriate olfactory stimuli. We usually use 8 to 12 weeks-old C57BL/6J mice, but other ages and strains may be used depending on the experimenter's interests.

Note: The two stimulation windows (both of which are 20 min long) are separated by a 60 min period. The long separation between both stimulation events was found to be of sufficient duration to allow the discrimination of cells which express *c-Fos* mRNA (activated during the second exposure window) and cells which express c-Fos protein (activated during the first exposure window), such that no mRNA deriving from the first exposure is still present when the animal is euthanized (80 min after the end of the first stimulation period) and little to no c-Fos protein has yet been synthesized as a result of the second exposure event (which is 20 min long and not of enough duration for c-Fos protein to be produced at high levels) (Figure 1B).

1. Preparation of exposure cages
   a. For each mouse to be exposed, prepare three exposure cages (numbered #1, #2, and #3), as follows:
      i. Cage #1 is prepared by transferring a third of the soiled bedding from the subject's home cage to a new clean deodorized cage (see Before You Begin section for steps on how to prepare deodorized cages). Do not overlay cage #1 with food compartment/grid or water bottle. Keep cage closed with a lid until ready to be used in step 2.
      ii. Cage #2 is prepared exactly like cage #1.
      iii. Cage #3 is the animal's home cage, with the remaining one-third of soiled bedding. This cage will contain the animal to be exposed at the beginning of the procedure. When ready to start the exposure sessions, remove the food compartment/grid and water bottle.
   b. Transfer cages #1 to #3 to a dark procedure room (preferably under red light illumination for ease of handling), without disturbing the animal with loud noises or sudden movements. The entire exposure session should be performed in the dark.
2. Exposure to first stimulus
   a. Transfer the animal to cage #1 and habituate it to the procedure room for 1 h.
   b. Slowly open the cage lid and insert the first olfactory stimulus source into the cage, on the side opposite to where the animal is located when the lid is opened.
      i. The type of olfactory stimulus obviously depends on the experimenter's interests. Suitable olfactory stimuli include medical gauze or cotton ball scented with liquid stimuli (urine,

saliva, purified odorants, or pheromones, as well as recombinant proteins diluted in buffer), gauze scented with bodily secretions (fur, skin, feces scents), bodily shedding (feathers, fur, shed skin pieces, etc.), scented bedding (from conspecifics or from another species' cage), or another animal (female or competitor male mouse, or an anesthetized predator).

ii. If using stimulus sources composed of several individual pieces (of scented gauze or bodily shedding, for example), attach them to a binder clip to prevent them from dispersing about the cage.

iii. If behaviors are to be scored later, the experimenter may decide to film the exposure session. More information can be found in our recent publication on behavioral assays in the study of olfaction (Papes et al., 2018).

c. Close back the lid and let the exposure proceed for 20 min, without disturbing the subject during this time window.

Note: If the olfactory stimulus is deposited on a solid substrate (cotton ball or medical gauze), control animals should be exposed to clean cotton ball or medical gauze. When the stimulus is liquid, control animals may be exposed to the solid substrate containing an equivalent volume of a behaviorally neutral odor diluent (e.g., PBS or mineral oil).

3. Rest period
   a. Transfer the animal back to cage #3 (home cage) at the end of the first exposure session.
   b. Let the animal stay undisturbed and unstimulated for 60 min.

⚠ CRITICAL: It is important not to insert scented material back into cage #3, because this is intended to be a rest period where the animal is kept without further olfactory stimulation (and preferably without visual and gustatory stimulation either). For example, avoid bringing scented bedding or pieces of bodily shedding together with the animal when it is transferred back to cage #3 in this step.

4. Exposure to second stimulus
   a. Transfer the animal to cage #2.
   b. Immediately insert the second olfactory stimulus source into the cage, on the side opposite to where the animal is located.
   c. Close with a lid and let the exposure proceed for 20 min, without disturbing the animal during this time window.

⚠ CRITICAL: Proceed to the following section immediately. After the second exposure window is finished, the animal must be perfused with fixative solution to stop the expression of *c-Fos* mRNA and protein. This is especially critical to prevent further c-Fos protein translation or *c-Fos* mRNA degradation in cells activated during the second exposure session.

Brain Dissection and Sectioning

Timing: 3–4 days

After the animal is exposed, it must be immediately anesthetized and subjected to cardiac perfusion with fixative solution, followed by dissection of the desired brain structures and sectioning under a vibratome. At the end, the experimenter will have produced thick sections suitable for the *in situ* hybridization and immunostaining steps to follow (staining as free-floating sections).

5. Remove the animal from cage #2 after the second stimulation session is ended and inject it intraperitoneally with an anesthetic combination composed of ketamine (100 mg/kg body weight) and xylazine (2–4 mg/kg body weight), dissolved in 50–100 μL of PBS, with the help of a 1 mL syringe.

6. After the animal is checked to be deeply anesthetized, dissect to open the rib cage and expose the cardiac chamber using appropriate dissection tools. Inject the ventricle with 20 mL of cold PBS and then with 40 mL of cold 4% paraformaldehyde fixative (prepared in PBS).

⚠ CRITICAL: This step must be conducted by an experienced experimenter with sufficient prior training in performing cardiac perfusion, to minimize the time between the end of exposures and the onset of fixation.

Optional: Alternatively, the animal could be decapitated with the aid of a mouse guillotine (provided it is permitted in the experimenter's IACUC-approved animal protocol), followed by brain dissection and fixation by immersion.

7. Dissect the brain out using appropriate dissection tools and technique, exposing either the entire brain or the experimenter's region of interest.
8. Immerse the dissected brain or brain region in enough volume of 4% paraformaldehyde fixative in a plastic 6- or 12-well tissue culture plate. Make sure the specimen is fully submerged in fixative solution. Cover the plate with a lid and place it at 4°C for fixation (12 to 16 h).

⚠ CRITICAL: All solutions used from this step until step 33 must be RNase-free. See notes at the beginning of the Materials and Equipment section.

9. On the following day, remove the fixative solution with the help of a p1000 pipetman or 5 mL serological pipette.

Note: Avoid the use of a vacuum line for steps requiring changes of solution, as they may cause loss of specimens or sections.

10. Add enough volume of 20% sucrose (in PBS) and incubate at 4°C for 2–3 days. It is normal for the specimen to float once it is initially incubated in this solution. Equilibration in sucrose solution hardens the neural tissue and improves sectioning at later stages.
11. Check that equilibration in sucrose solution has been successful by verifying that the specimen has sunk to the bottom of the well.
12. Remove sucrose solution and add enough volume of cold PBS.

⚠ CRITICAL: The specimen must be immediately embedded in agarose for vibratome sectioning. If short-term storage is absolutely needed, it should not be extended beyond a couple of days. Also, preservatives based on sodium azide must never be used at this step, as it chemically interferes with tyramide labeling in later stages of this protocol. Thimerosal preservatives are compatible.

13. Before embedding the specimen in agarose, trim it with a razor blade or scalpel and isolate the region of interest. Small specimens embed more efficiently and improve vibratome sectioning. We usually position the brain ventral side up and slice it coronally at two positions while keeping the brain stable with forceps. One cut is made at the level of the optic chiasma and another at a position immediately rostral to the cerebellum.

Optional: Alternatively, the brain may be cut using stainless steel or acrylic brain matrices (slicers).

14. Embed the specimen containing the region of interest in agarose:
    a. Prepare the agarose embedding solution by mixing 100 mL of 1× PBS, 3.5 g of agarose, and 8.0 g of sucrose pellets. Dissolve sucrose on a heated plate or in a microwave oven until the solution is homogenous and clear. Do not let the solution boil.

b. Let the clear solution cool down at 23°C–26°C until it is close to gelation.
c. Pour the embedding solution onto a plastic histology mold, enough to produce a 0.5–1.0 cm high block.
d. Quickly blot the desired specimen dry with Kimwipes and place it in the embedding mold while the agarose solution is still liquid, with the help of forceps. Push the specimen all the way down so it touches the bottom of the plastic mold.
e. The specimen orientation inside the embedding mold depends on the experimenter's interests but, usually, the side facing down will be the first to be sectioned at the vibratome.

⚠ CRITICAL: Drying the specimen is important because wet pieces will not adhere well to the agarose gel matrix, making vibratome sectioning difficult and producing sections of variable thickness.

15. Section the block on a vibratome:
a. Once the block has solidified, remove it from the plastic mold and trim it with a razor blade such that the agarose margins are approximately 3–5 mm from the specimen edges.
b. Glue the trimmed block to the vibratome stage with fast drying Super Glue.
c. Section through the block on a Leica VT 1000S vibratome (or similar instrument), under PBS, to produce 40 μm sections (see Troubleshooting Problem 2).

Note: This protocol has been optimized for 40 μm sections, which must be stained as free-floating sections in subsequent steps. Despite the difficulties inherent to dealing with staining of free-floating sections on a multi-well plate, this procedure has many advantages. First, it increases the number of cells for which the entire nucleus is stained, which is important because the two *c-Fos* mRNA nuclear foci are not always within the same cutting plane, and therefore the identification of *c-Fos*-positive cells after *in situ* hybridization requires that the entire nucleus be stained and imaged. Second, for brain areas that occupy a thick volume, staining of 40 μm sections is ideal since it allows the experimenter to visualize stained cells in the context of neighboring cells and structures inside the imaged area. Third, free-floating sections allow cRNA probes to penetrate the section from both surfaces, increasing staining efficiency, especially for deeply located cells. Lastly, free-floating section staining can be better achieved by using 40 μm section as opposed to thinner sections, which curl up more frequently and are harder to handle. Despite these advantages, it is possible to use this protocol to stain 12–20 μm cryostat sections collected onto glass slides, provided the necessary adaptations are included in the protocol. An extra advantage of using cryostat sections is that they could be stored at −80°C for up to 1 year without significant loss of RNA content. However, be aware that, due to low cRNA probe penetrability, use of cryostat sections may result in inefficient *in situ* staining on the side of the section facing the microscope slide. Moreover, thinner sections contain fewer layers of cells stained at their entirety. Finally, cryostat sections collected onto glass slides may trap air bubbles underneath the section, increasing *in situ* hybridization staining background.

d. Gently pick each section from the vibratome reservoir with a fine paintbrush and transfer to the wells of a multi-well plate containing PBS. To keep track of section order along the direction of cutting (rostral-caudal, lateral-medial, or dorsal-ventral), it is advisable to collect each section into an individual well of a multi-well plate. Alternatively, collect sets of sections into each well. Sections may be stored for up to 2 days in PBS at 4°C.

Pause Point: If short-term storage is needed, it should not be extended beyond a couple of days. Also, preservatives based on sodium azide must never be used in this step, as it chemically interferes with tyramide labeling at later stages. Thimerosal preservatives are compatible.

cRNA Probe Hybridization and Signal Development

Timing: 4 days

The first steps in our staining protocol include *in situ* hybridization to detect *c-Fos* mRNA, which is expressed in neurons activated during the second window of sensory stimulation (Figure 1). This phase includes pre-treatment of histological sections, probe hybridization, washes, probe immunodetection, and signal development.

16. **Pre-treatment of sections.** Fill the required number of wells in an RNase-free 12-well plate with RNase-free PBS. Under a stereomicroscope, choose suitable sections and transfer them to the plate with a paintbrush. Place a maximum of three sections per well.
17. Remove PBS and wash sections twice with 1× PBS, for 5 min each, under gentle agitation on a plate rocker.

Note: For all washes and incubations of thick vibratome sections on a 12-well plate, use 500 µL of liquid, unless specified otherwise in the protocol. If using well plates with other number of wells, adjust the volume accordingly. Using more than 500 µL of liquid for the 12-well plate may lead to leaking or poor agitation of sections. For all changes of solution, use a p1000 pipetman.

18. Remove PBS and add 4% paraformaldehyde (prepared in 1× PBS) and treat sections under agitation for 20 min.
19. Wash sections twice in 1× PBS under agitation, for 5 min each.
20. Permeabilize the sections with 0.2 M HCl (prepared in RNase-free ultrapure H_2O) under agitation, at 23°C–26°C for 10 min.
21. Wash sections twice in 1× PBS under agitation, for 5 min each.
22. Inactivate endogenous peroxidases by incubating sections in 0.1% H_2O_2 (prepared in RNase-free 1× PBS) for 30 min at 23°C–26°C under agitation, in the dark.

Note: During this incubation step, prepare hybridization solution for steps 28 and 29. For the pre-hyb phase (step 28), calculate the volume needed by multiplying 300 µL by the number of wells to be stained, and dispense the calculated volume in a conical tube, plus some additional extra to account for pipetting errors due to solution viscosity. Heat the tube at 58°C until ready to use. For step 29, aliquot hybridization solution as 300 µL fractions in 1.5 mL microfuge tubes and heat them up to 85°C for 15 min prior to use. Each fraction is enough for one well (probe/condition combination) in the hybridization step.

23. Wash sections twice in 1× PBS under agitation, for 5 min each.
24. Treat sections with acetylation solution:
    a. In a glass graduated cylinder, prepare acetylation solution by mixing 20 mL of 0.1 M triethanolamine-HCl pH 8.0 (freshly prepared right before the beginning of the protocol; see Materials and Equipment section for details) with 50 µL of acetic anhydride.
    b. Add 1 mL of acetylation solution per well.
    c. Pipet an additional 2 µL of concentrated acetic anhydride per well.
    d. Gently mix on a plate rocker for 10 min (Troubleshooting Problem 3).
25. Wash sections twice in 1× PBS under agitation, for 5 min each.
26. Transfer the plate to a humidified chamber and place it at 58°C. Water bath or hybridization oven may be used at this and following steps.

Note: Humidified chambers are commercially available and should be completely sealed to avoid evaporation during the long pre-hyb and hybridization steps. It is also possible to construct a humidified chamber by attaching railings made of serological plastic pipettes to

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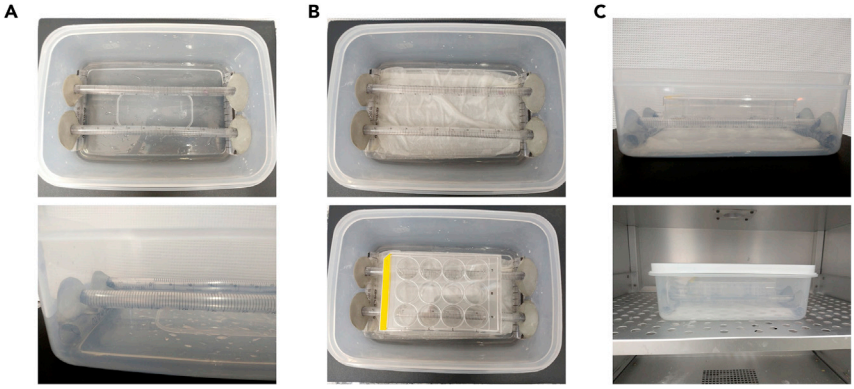

Figure 2. Critical Equipment and Setup during Dual Staining Protocol

(A) Home-made humidified chamber (top), showing railings constructed with serological pipettes attached to the bottom of a plastic container (bottom).

(B) Wetted Kimwipes placed under the railings (top) and positioning of 12-well plate inside humidified chamber (bottom).

(C) Lateral view of 12-well plate inside humidified chamber (top) and heated oven for high temperature incubations during the *in situ* hybridization phase of the dual protocol (bottom).

the bottom of a Tupperware plastic container (Figure 2A). Lay pieces of Kimwipes wetted with 5× SSC solution at the bottom of the container to create a humidified atmosphere with similar vapor pressure as the hybridization solution.

27. Equilibrate sections to the pre-hyb temperature by incubating in 1× PBS at 58°C for 5 min.
28. **Pre-hybridization.** Pre-hybridize sections with at least 300 µL of pre-warmed pre-hyb solution at 58°C for 1 h, in a humidified chamber. Place the plate on the railings and close the plate lid before sealing the chamber (Figures 2B and C) (Troubleshooting Problem 3).

Note: For all changes of solution at 58°C, remove the plate from the humidified chamber and place it on top of a heated block, returning it to the sealed chamber immediately after the change has been made.

29. Ten minutes prior to the end of the previous incubation step, mix pre-warmed hybridization solution with the two 1 kb digoxigenin-labeled cRNA riboprobes to detect *c-Fos* mRNA (see Before You Begin section for details). The concentration of each probe in the working hybridization solution should be 400 ng/mL. Quickly mix by inversion and place the tube containing probes and hyb solution at 85°C and denature for 5–10 min.
30. **Hybridization.** Remove pre-hyb solution from each well and add probe-containing working hybridization solution (300 µL per well). It is best to perform solution changes by removing and adding liquid to one well at a time.

⚠ CRITICAL: Make sure the sections lay flat at the bottom of each well for the hybridization step. Curled or folded sections result in artifactual staining. If necessary, unfold the sections with a paintbrush.

31. Return the plate to the humidified chamber, close the plate lid, seal the chamber, and place it back at 58°C. Perform hybridization for 16 h (Troubleshooting Problem 3).
32. Washes with solutions of increasing stringency:
    a. Pre-warm 2× SSC, 0.2× SSC, and 0.1× SSC to 55°C (see Materials and Equipment section for details on how to prepare wash solutions).
    b. Lower the temperature of the humidified chamber to 55°C by transferring it to another bath or oven for 15 min.

c. Remove hybridization solution from each well at a time and add 500 μL of pre-warmed 2× SSC wash solution.
d. Incubate for 30 min with occasional manual agitation.
e. Perform sequential washes in 0.2× SSC and 0.1× SSC at 55°C, for 20 min each.

33. Remove the humidified chamber from the bath or oven. Replace solution in each well with 0.1× SSC at 23°C–26°C. Incubate for 3–5 min to equilibrate sections to 23°C–26°C.
34. Permeabilize in PTw solution at 23°C–26°C for 10 min. Perform these and subsequent washes and incubations under gentle agitation.
35. Equilibrate sections in TN buffer by performing two washes at 23°C–26°C for 5 min each.
36. Block sections in TNB buffer at 23°C–26°C for 3 h.
37. **Probe immunodetection.** Incubate sections in TNB buffer containing horseradish-peroxidase-conjugated anti-digoxigenin antibody (1:400; Merck/Roche) at 4°C for 2 days in a sealed humidified chamber.

Note: The immunodetection step can be performed with 300 μL of antibody solution. If using limited volumes, make sure the agitation process does not lead to curling up of sections inside the well. The humidified chamber must contain Kimwipes wetted with PBS or water. Make sure the solution inside each well does not evaporate during this long incubation step.

38. Signal development:
    a. Wash each well six times in TNT buffer, for 5 min each, under gentle agitation at 23°C–26°C. During washes, prepare enough volume of tyramide-biotin solution, by mixing amplification diluent (Perkin Elmer) with H_2O_2 (to reach a final concentration of 0.0015%) and tyramide-biotin (1:50; Perkin Elmer). If using 30% H_2O_2 as a concentrated stock, it is advisable to generate intermediate dilution steps to reach the desired 0.0015% concentration with great precision.

⚠ CRITICAL: The tyramide-biotin solution must be prepared fresh immediately before the following step.

    b. Incubate sections in tyramide-biotin solution for 15 min at 23°C–26°C.

Note: If using a small volume of solution at this or subsequent signal development steps (less than 150 μL), overlay a round piece of Parafilm, cut to the size of the well, onto the sections to ensure that they are submerged in liquid (Methods Video S2).

    c. Wash 6 times in TNT buffer, for 5 min each, under gentle agitation at 23°C–26°C.
    d. Incubate sections in TNB buffer containing peroxidase-conjugated streptavidin (1:100; Perkin Elmer) at 23°C–26°C for 1 h.
    e. Wash 6 times in TNT buffer, for 5 min each, under gentle agitation at 23°C–26°C.
    f. During washes in the previous step, prepare enough volume of tyramide-Alexa 555 fluorescent development reagent, by mixing 1× amplification diluent (Thermo) with H_2O_2 (to reach a final concentration of 0.0015%) and tyramide-Alexa 555 (1:100; Thermo).
    g. Incubate sections in tyramide-Alexa 555 solution for 15 min at 23°C–26°C in the dark.

Note: From this point on, perform all steps without exposure to direct light (for example, in a dimly lit room) and cover the plate with aluminum foil during the incubations and washes.

    h. Wash 6 times in TNT buffer, for 5 min each, under gentle agitation at 23°C–26°C.

Pause Point: It is recommended to proceed to the next step (c-Fos protein immunostaining) immediately after *c-Fos* mRNA signal development. However, if necessary, a brief pause (up to 2 days at 4°C) may be introduced at this step. Staining resulting from tyramide labeling is chemically stable and concerns about mRNA integrity are no longer necessary.

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c-Fos Immunostaining

Timing: 2.5 days

After fluorescent detection of *c-Fos* mRNA by *in situ* hybridization with DIG-labeled cRNA riboprobes, sections are subjected to regular immunostaining to detect c-Fos protein, indicative of neuron activation relative to the first window of sensory stimulation (Figure 1B). This phase in the protocol includes blocking, incubation with primary anti-c-Fos antibody, incubation with fluorescently labeled secondary antibody, nuclear counterstaining, and section mounting onto glass microscope slides.

39. Permeabilize sections in 0.3% Triton X-100/1× PBS for 10 min.
40. **Blocking.** Perform a first blocking step in 1% blocking reagent (Thermo) for 1 h at 23°C–26°C under gentle agitation.
41. Perform a second blocking step in 0.3% Triton X-100/1% BSA/1× PBS for 30 min at 23°C–26°C under gentle agitation.
42. **Primary antibody incubation.** Incubate in anti-c-Fos antibody solution [0.3% Triton X-100/1% BSA/1× PBS containing anti-c-Fos antibody (1:1500; Ab2; Millipore)] for 2 days at 4°C, under gentle agitation.
43. **Washes.** Wash sections three times in 0.1% Triton X-100/1× PBS, for 15 min each.
44. **Secondary antibody incubation.** Incubate in anti-rabbit secondary antibody solution [0.3% Triton X-100/1% BSA/1× PBS containing Alexa 488-conjugated anti-rabbit secondary antibody (1:500; Thermo)] for 3 h at 23°C–26°C, under gentle agitation.

Optional **(instead of steps 39–44).** Alternatively, instead of using a fluorescently labeled secondary antibody, it is possible to perform tyramide signal development for c-Fos immunodetection. Tyramide signal amplification boosts up staining after c-Fos detection. This is rarely necessary, even though it may be used if the experimenter has performed the protocol as indicated and concluded that the resulting c-Fos immunostaining is weak. This outcome is usually due to sub-optimal c-Fos antibody or failure to comply with the strict timing guidelines described in our protocol. Should tyramide amplification be applied, use the following procedure after step 38:

a. Incubate sections in 3% H_2O_2/1× PBS for 1 h to inactivate peroxidases from previous steps.
b. Incubate sections in 0.1 M HCl/0.9% NaCl for 15 min.
c. Wash 6 times in TNT buffer, for 5 min each.
d. Permeabilize sections in 0.3% Triton X-100/1× PBS for 10 min.
e. Perform a first blocking step in 1% blocking reagent (Thermo) for 1 h at 23°C–26°C under gentle agitation.
f. Perform a second blocking step in 0.3% Triton X-100/1% BSA/1× PBS for 30 min at 23°C–26°C under gentle agitation.
g. Incubate in anti-c-Fos antibody solution [0.3% Triton X-100/1% BSA/1× PBS containing anti-c-Fos antibody (1:1500; Ab2; Millipore)] for 2 days at 4°C, under gentle agitation.
h. Wash sections three times in 0.1% Triton X-100/1× PBS, for 15 min each.
i. Wash sections for an additional 15 min with 1× PBS to remove Triton X-100.
j. Incubate with peroxidase-conjugated anti-rabbit secondary antibody in 1% blocking reagent (1:100; Thermo) for 1 h at 23°C–26°C.
k. Wash three times with 1× PBS, for 15 min each.
l. During washes in the previous step, prepare enough volume of tyramide-Alexa 488 fluorescent development reagent, by mixing amplification diluent (Thermo) with H_2O_2 (to reach a final concentration of 0.0015%) with tyramide-Alexa 488 (1:100; Thermo).
m. Incubate sections in tyramide-Alexa 488 solution for 5–10 min at 23°C–26°C.

Note: If using a small volume of solution at this step (less than 150 μL), overlay a round piece of Parafilm, cut to the size of the well, onto the sections to ensure that they are submerged in liquid (Methods Video S2).

45. Wash three times with 1× PBS, for 15 min each.
46. Counterstain with TO-PRO-3 iodide solution (1:1,000; Thermo) in 1× PBS for 15 min.

Optional: If the confocal microscope used for imaging the sections contains a UV laser, sections may be alternatively counterstained with DAPI or Hoechst 33342.

47. Wash twice in 1× PBS, for 5 min each.
48. Section mounting:
    a. Pipet 1 mL of 1× PBS onto a glass microscope slide (SuperFrost; Thermo) and use a fine paintbrush to transfer each stained section to the pool formed by PBS. Unfold the sections and apply pressure to make them sink to the bottom of the pool and adhere to the glass slide.
    b. Carefully remove PBS from the microscope slide using a p1000 pipetman, making sure the sections are not disturbed and not made to overlap with each other.
    c. Remove the excess of liquid by tilting the slide and gently touching a piece of Kimwipes.
    d. Pipet 100–150 μL of ProLong Gold antifade mounting reagent (Thermo) and carefully and slowly overlay with a 24 x 60 mm glass coverslip, without introducing air bubbles.
    e. Maintain the slides at 23°C–26°C or at 4°C for at least 12 h in a dry container protected from light. This step will allow ProLong to harden before subjecting the slides to microscopy imaging.

Microscopy Imaging

After sections are stained and mounted onto a glass microscope slide, they may be imaged using a suitable instrument. Considering that the sections in this protocol are thick (40 μm), a confocal microscope or equivalent imaging technique is required. We usually perform collection of images as a z-series with either a 40× or a 63× planar objective, under oil, on a confocal microscope. Each optical section should be very thin (less than 0.5 μm thick). Individual optical sections are informative because they can reveal fine details of the location of *c-Fos* mRNA staining inside the nucleus. Such staining usually appears as two foci of red fluorescence (Methods Video S1). However, because these foci are rarely on the same optical plane, it may be required to produce maximum intensity projection images across the z-series to visualize both foci and the eventual co-localization with c-Fos protein derived from immunostaining, which is expressed across the entire nucleus of the activated neuron (Methods Video S1, Figure 1A) (Troubleshooting Problems 4, 5, and 6).

EXPECTED OUTCOMES

As a result of the staining method described here, the experimenter will obtain fluorescence microscopy images collected on a confocal microscope, usually as z-series. The detected *c-Fos* mRNA will appear as two fluorescent foci inside the nucleus of each activated cell relative to the second window of stimulation, while the detected c-Fos protein will be visualized as green fluorescent staining throughout the nucleus of each activated cell relative to the first window of stimulation (Figure 1A and Methods Video S1).

Both stimulation events in our protocol are 20 min long and are separated by a 60 min rest period without stimulation. This intermediate period is long enough to allow complete clearance of *c-Fos* mRNA in cells activated during the first stimulation window, meaning that any *c-Fos* mRNA detected should be related to the second stimulation period (Figure 1). Likewise, the second stimulation lasts for 20 min and the brain is fixed immediately after, preventing c-Fos protein from being synthesized in cells activated during such second stimulation period. Therefore, c-Fos protein is indicative of the

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first exposure (Figure 1). The separation time between stimulation windows was empirically determined to be ideal, allowing excellent temporal resolution in detecting c-Fos protein and mRNA (Carvalho et al., 2015). Moreover, this long separation between stimulation events gives an advantage to our method, because it allows better temporal resolution than methods that use cRNA probes for immature and mature immediate early gene mRNAs, in which the no-stimulation period is shorter (Lin et al., 2011).

Another advantage is that the signal related to the first stimulation window (c-Fos protein immunodetection) is localized in the same subcellular compartment as the signal related to the second stimulation window (c-Fos mRNA *in situ* hybridization detection) (Methods Video S1). As such, it is easier to identify green and red staining co-localization in a cell activated in both instances of sensory stimulation, an improvement over previously published protocols, which employ two *in situ* hybridization probes to detect immature and mature mRNA for an immediate early gene, such as *Arc* or *c-Fos* (Guzowski et al., 1999, 2001; Lin et al., 2011). In these studies, immature mRNA probe detects a nuclear signal, but the mature mRNA probe detects both nuclear and cytosolic signals, making it difficult to identify which cytosolic staining overlaps with which nuclear staining, especially when the nucleus position or disposition of cells in the tissue prevent a clear visualization of cell bodies and nuclei, such as when the experiment focuses on a densely populated brain region or on a neuroepithelium.

It should be noted that, in our protocol, fewer cells are activated by a certain stimulus compared to regular c-Fos immunostaining, because: (a) the c-Fos protein is detected 100 min after the onset of the first stimulation, which is longer than the period after which exposed animals are analyzed (60–90 min) in regular c-Fos immunostaining; (b) each stimulation window in our protocol is only 20 min long (to allow good and ideal temporal resolution), which is shorter than the usual 30 min long exposure events applied in regular c-Fos immunostaining protocols (Carvalho et al., 2015; Papes et al., 2010).

QUANTIFICATION AND STATISTICAL ANALYSIS

Microscopy images obtained as a result of this protocol are usually quantified to count the number of *c-Fos* mRNA-positive nuclei (containing red fluorescent foci), the number of c-Fos protein-positive cells (green fluorescence), and the number of co-stained cells. An important output measurement is the percentage of co-stained cells relative to the total number of red-labeled cells (activated by the second stimulus) or relative to the total number of green-labeled cells (activated by the first stimulus), as a way to determine the fraction of brain cells activated by one stimulus that are also activated by the other.

Mind that the staining intensity is less important in these experiments, as it may vary depending on probe concentration, temporal dynamics of *c-Fos* mRNA and protein synthesis and degradation, or position of the stained cell along the z-axis (cells located close to the surface of the section are usually more strongly stained).

LIMITATIONS

A technical limitation of this protocol is that the brain must be immediately fixed via cardiac perfusion (or decapitation) to prevent *c-Fos* mRNA from being degraded or translated into c-Fos protein. Another limitation is that each stimulation window is 20 min long, which leads to a population of activated cells that may not be as numerous as when the animals are stimulated for a longer period during a single exposure session. This may be a problem if the stimulus is weak or if the number of cells in the experimenter's region of interest is small. If the experiment is intended to compare the results from this method with regular c-Fos immunostaining, exposure durations in both types of experiments should match.

Another potential problem is that the harsh treatment conditions to which the histological sections are subjected during the *in situ* hybridization steps may degrade the c-Fos protein, decreasing the green fluorescent signal. To prevent this, the conditions in our protocol should be followed strictly, especially in regard to the duration of each treatment and concentration of reagents, as these have been successfully validated in our laboratory.

TROUBLESHOOTING

Problem 1: Low Riboprobe Yield

The expected concentration of riboprobes produced via *in vitro* transcription with the protocol described here is > 100 ng/μL. Lower probe concentrations may result from (a) low plasmid DNA template concentration, (b) cRNA degradation, (c) sub-optimal cRNA synthesis, or (d) loss during cRNA clean-up (step 21 in Before You Begin section).

Potential Solutions

- Low plasmid DNA template concentration
  - Digest 20 μg of plasmid DNA.
  - Do not leave the restriction digestion for more than 3 h at the appropriate temperature.
  - Purify using a reaction clean-up kit rather than a gel extraction kit.
  - Use more sensitive DNA quantification methods.
- cRNA degradation
  - Riboprobe that shows as a smear when run on an agarose gel or tapestation usually means it has been degraded (Figure 3A). Ensure that the transcription reaction is conducted in RNase-free conditions.
  - Store the purified cRNA at −80°C.
  - Follow the manufacturer's recommendations when purifying the cRNA probe using commercially available kits.
- Sub-optimal cRNA synthesis
  - Maintain *in vitro* transcription reaction components on ice while setting the reaction up.
  - Make sure to vortex the transcription buffer to dissolve DTT (if present).
  - Flick the DIG NTP labeling mix (Merck/Roche) several times before use to ensure homogenous thawing of the tube's contents.
  - If necessary, the volume of RNA polymerase enzyme or the mass of linearized plasmid template may be increased (to 2 μg, for example).
- Loss during cRNA clean-up
  - It may be necessary to test several purification kits before landing on one that works ideally. In our hands, silica-based mini-columns (ion exchange) tend to work less efficiently than gel exclusion chromatography-based mini-columns.
  - Also, make sure to use strict RNase-free technique during handling of synthesized probe, maintaining it on ice before and after purification and at the recommended temperature during the clean-up process.

Problem 2: Histological Sections of Variable Thickness

Sometimes, vibratome sectioning of large specimens or brain tissue poorly equilibrated in sucrose results in histological sections of variable thickness. This may result in inefficient staining due to low penetrability of cRNA probes, antibodies, and signal development reagents (step 15 in the Step-By-Step protocol).

Potential Solutions

- Make sure the specimen is equilibrated in sucrose by checking that it is hardened and have sunk to the bottom of the tube/well after sucrose treatment.

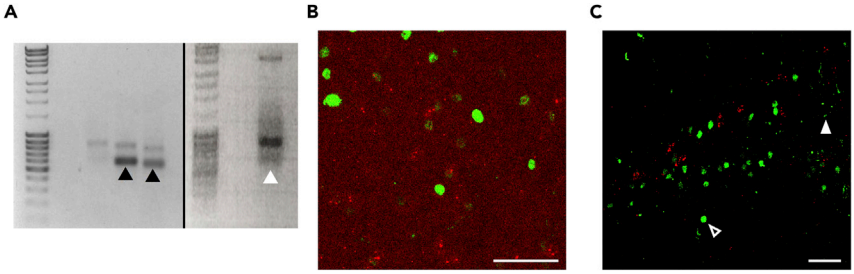

Figure 3. Selected Potential Problems during Dual Staining Protocol

(A) Left gel, examples of intact cRNA probes, displaying a ladder-like appearance after electrophoresis on an agarose gel, with clearly defined bands (black arrowheads). Right, degraded cRNA probe, presenting as a smear across the lane and a fainter lower band (white arrowhead). Additionally, note the undesirable presence of contaminating linearized plasmid near the top of the lane.

(B) Fluorescence microscopy image exhibiting high background after dual staining, particularly in the red channel, making it difficult to discern the *c-Fos* mRNA nuclear foci.

(C) Fluorescence microscopy image showing dirty specs (stained precipitates) after dual staining (filled triangle), making it difficult to discern real c-Fos protein staining (open triangle). In this case, c-Fos immunostaining was conducted with the alternative protocol to steps 39–43, including tyramide signal amplification (green).

Scale bars, 50 μm.

- Adjust the vibration speed on the vibratome for optimal sectioning results. Fast vibration speed usually results in irregular section thickness.
- Do not stop the sectioning process for too long, as the surface of the specimen facing the vibratome blade is constantly softening due to loss of sucrose to the PBS in the vibratome receptacle.
- Our protocol has been developed to produce 40 μm sections for mouse brain tissue. If a different thickness is required, it may be necessary to optimize the time for proper sucrose equilibration and the vibratome sectioning speed.

Problem 3: Folded Sections during Staining

Sometimes histological sections subjected to a free-floating method fold during staining due to constant agitation inside the well. If sections fold in steps prior to hybridization, they may remain distorted, creating problems during hybridization steps, during which sections tend to fold up solely because of the higher incubation temperatures (steps 24, 28, and 31 in the Step-By-Step protocol).

Potential Solutions

- Make sure the sections lay flat at the bottom of the well in each step during pre-treatment. If necessary, unfold them with a fine paintbrush.
- Slightly increase the volume of solution inside the well. However, mind that higher volumes may cause sections not to agitate well enough inside the well.
- For incubations with small volumes, such as during tyramide labeling steps, use round pieces of Parafilm cut to the size of the well and lay them on top of the sections after the staining solution has been added (Methods Video S2). This will compress the sections and maintain them flat against the bottom of the well.
- Sections that have been produced on a vibratome several days before the staining experiment are more likely to curl up or fold up than freshly prepared sections, because they lose sucrose and become softer.

Problem 4: Weak Fluorescent Signal

Weak fluorescent signal after staining may result from (a) low cRNA probe concentration, (b) poor section fixation leading to mRNA degradation, (c) loss of staining signal due to delayed observation

under a microscope, photobleaching, or sub-optimal *in situ* hybridization incubations (see Microscopy Imaging section).

Potential Solutions

- Low cRNA probe concentration
  - Double-check probe concentration and dilution.
  - Increase concentration of each *c-Fos* probe during *in situ* hybridization.
  - Make sure the pre-hybridization solution is completely removed from the well prior to adding hyb solution containing cRNA probe.
- Poor section fixation
  - Make sure fixative is freshly prepared.
  - Make sure perfusion is performed shortly after the end of the second stimulation window.
  - Section post-fixation at the start of the *in situ* hybridization phase should not be performed for less than 20 min.
- Loss of staining signal
  - Image sections as soon as possible after staining has been finished.
  - Avoid exposing microscope slides to direct light.
  - Do not overexpose a field of view to excitation light under the confocal microscope.
  - Make sure the duration of each step during the *in situ* hybridization phase is performed as indicated in the protocol, as extended incubation in the harsh fixation, permeabilization, or acetylation steps may decrease c-Fos antigenicity for the subsequent immunostaining phase.

Problem 5: Strong Background

Higher than normal background is expected when staining thick histological sections. However, in our protocol, the signal-to-noise is ideal, allowing clear identification of labeled cells. If higher background is experienced (Figure 3B), it may be due to (a) high probe concentration, (b) section over-fixation, (c) antibody solutions that are too concentrated, (d) tyramide staining for extended duration or with working solutions of inappropriate concentration, or (e) drying up of sections during staining (see Microscopy Imaging section).

Potential Solutions

- High cRNA probe concentration
  - Double-check probe concentration and dilution.
  - Decrease concentration of each *c-Fos* probe during *in situ* hybridization.
  - Make sure the hybridization or wash solutions are not evaporating during incubation steps.
- Section over-fixation
  - Do not extend fixation by either trans-cardiac perfusion or during the post-sectioning fixation step at the beginning of the *in situ* hybridization phase longer than indicated. Over-fixation creates cross-linked protein pockets that may trap the cRNA probes or antibodies.
- Over-concentrated antibody solutions
  - Make sure to spin down antibody stock tubes or aliquots to collect droplets retained in the tube's cap or wall prior to pipetting.
  - Do not use frost-free freezers to store antibody aliquots, as this may result in evaporation of liquid over time.
  - Mix antibody working solutions well before adding them to the sections.
- Extended or sub-optimal tyramide labeling
  - Tyramide labeling is one of the most critical steps in this protocol. Longer incubation times may result in strong background (sometimes accompanied by desirable stronger specific staining), so these steps may require optimization.

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- H_2O_2 concentration during these steps is critical, as higher than optimal concentrations may result in increased non-specific peroxidase activity, leading up to higher background. We recommend using an intermediate hydrogen peroxide dilution if the stock is too concentrated.
- When conducting several staining conditions (different exposure regimens, different stimuli, different probe concentrations), tyramide time-sensitive steps should be performed in a tiered fashion by washing each well (or small group of wells) at a time.

- Dry sections during staining
  - Sections must not dry at any step during this protocol, as this will result in increased background.
  - If performing staining over multiple wells, wash each well (or small group of wells) at a time.
  - Limit the time during which sections remain without liquid between washes, as they may dry up resulting in irreversible higher background.

Problem 6: Dirty Staining

Occasionally, specific staining is good but accompanied by dirty specs (Figure 3C). This phenomenon may be caused by (a) non-homogenous antibody solutions, (b) improperly dissolved tyramide, or (c) tyramide over-staining (see Microscopy Imaging section).

Potential Solutions

- Non-homogenous antibody solutions
  - Make sure each solution containing antibodies is dissolved and mixed well prior to use.
  - Keep each antibody working solution on ice until use but mix it right before adding to the sections if it sits on ice longer than 5 min.
  - Avoid pipetting antibody from the bottom of the stock tubes, as precipitates decant at the bottom over time.
  - Alternatively, flick the antibody stock tubes several times for 3 min, centrifuge them to collect the volume at the bottom, and pipet the desired amount from the surface of the liquid.
- Improperly dissolved tyramide-biotin or tyramide-Alexa.
  - In our protocol, tyramide is dissolved in DMSO so the tube must be equilibrated to 23°C–26°C for at least 30 min prior to use, followed by visual inspection that the solution is completely thawed and homogenous.
  - The amplification diluent where tyramide is dissolved should also be prepared and equilibrated to 23°C–26°C prior to use. Cold solutions lead to tyramide agglomerates that precipitate onto the tissue sections.
- Tyramide over-staining
  - As outlined above, tyramide labeling steps are time sensitive. Longer incubation times may result in dirty fluorescent precipitates (specs). Make sure to time staining for each well (or small group of wells) independently.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Fabio Papes (papesf@unicamp.br), upon reasonable request.

Materials Availability

The plasmids used to generate the c-Fos riboprobes are available through a Materials Transfer Agreement. No other specific materials were generated in this protocol.

Data and Code Availability

No datasets were generated nor analyzed during this study.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.xpro.2020.100153.

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AUTHOR CONTRIBUTIONS

Conceptualization, T.S.N., V.M.A.C., and F.P.; Methodology, T.S.N., V.M.A.C., M.A.A.S., G.Z.T., and F.P.; Investigation, T.S.N., V.M.A.C., M.A.A.S., G.Z.T., and F.P.; Writing – Original Draft, T.S.N. and F.P.; Writing – Review & Editing, T.S.N., V.M.A.C., M.A.A.S., and F.P.; Funding Acquisition, F.P.; Resources, F.P.; Supervision, F.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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