Use of TAI-FISH to visualize neural ensembles activated by multiple stimuli

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Researchers in behavioral neuroscience have long sought imaging techniques that can identify and distinguish neural ensembles that are activated by sequentially applied stimuli at single-cell resolution across the whole brain. Taking advantage of the different kinetics of immediate–early genes' mRNA and protein expression, we addressed this problem by developing tyramide-amplified immunohistochemistry–fluorescence *in situ* hybridization (TAI-FISH), a dual-epoch neural-activity-dependent labeling protocol. Here we describe the step-by-step procedures for TAI-FISH on brain sections from mice that were sequentially stimulated by morphine (appetitive first stimulus) and foot shock (aversive second stimulus). We exemplify our approach by FISH-labeling the neural ensembles that were activated by the second stimulus for the mRNA expression of *c-fos*, a well-established marker of neural activation. We labeled neuronal ensembles activated by the first stimulus using fluorescence immunohistochemistry (IHC) for the c-fos protein. To further improve the temporal separation of the *c-fos* mRNA and protein signals, we provide instructions on how to enhance the protein signals using tyramide signal amplification (TSA). Compared with other dual-epoch labeling techniques, TAI-FISH provides better temporal separation of the activated neural ensembles and is better suited to investigation of whole-brain responses. TAI-FISH has been used to investigate neural activation patterns in response to appetitive and aversive stimuli, and we expect it to be more broadly utilized for visualizing brain responses to other types of stimuli, such as sensory stimuli or psychiatric drugs. From first stimulation to image analysis, TAI-FISH takes ~9 d to complete.

INTRODUCTION

Animal and human brains experience numerous stimuli on a daily basis. Understanding the neural coding mechanisms that establish connections between stimuli and stimuli-evoked neural activity patterns is essential to uncovering the neuronal circuit and the cellular mechanisms of the corresponding brain functions.

As part of the neural coding mechanism, neural ensembles activated by a stimulus have been identified by visualizing the upregulation of genes such as *c-fos*, *Arc* and *zif268* (refs. 1–4), which are immediately and transiently activated after stimulation, and are therefore named immediate–early genes (IEGs). Today, the mRNA or protein signals of these genes are routinely identified by either FISH or IHC, respectively, to visualize neuronal populations activated by a specific stimulus.

What remains challenging is to identify and distinguish neural activation patterns that are induced by multiple stimuli or experiences, especially for subpopulations of neurons within individual brain structures that are activated by different stimuli. For example, subpopulations of the nucleus accumbens (NAc) and basolateral amygdala (BLA) neurons can be activated either by appetitive stimuli such as morphine, sweet-tasting sucrose, appetitive odor (peanut oil, for example) or the presence of a female animal, or by aversive stimuli such as foot shocks, predator odor or bitter-tasting quinine[5–8]. BLA and NAc are, however, far from the only brain structures that are activated by both appetitive and aversive stimuli. For example, the cortex and ventral tegmental area have been demonstrated to be activated by stimuli or experiences of different valence[9–13]. It has been difficult to determine whether, and to what extent, these neuronal subpopulations have shared neurons by comparing different animals that have received different stimuli. So a technique that is capable of identifying and distinguishing activated neural activation patterns induced by different stimuli in the same animal brain is sorely needed.

Previous dual-epoch activity-dependent neural labeling techniques that utilize endogenous IEG signals

To this end, several different approaches have been developed. For instance, cellular compartment analysis of temporal activity by FISH (catFISH) was developed to investigate hippocampal neural activation in rats evoked by different novel environments[14,15]. It has also been used to identify neurons activated by fighting and mating behaviors[16] or by foot shock and nicotine, respectively[12]. This technique takes advantage of the kinetics of nucleus-to-cytoplasm transport of stimulation-induced *Arc* mRNA to discriminate neural activations induced by different stimuli. Two stimuli are applied sequentially with a temporal interval of 20–30 min, and when the animal is sacrificed after the second stimulation, *Arc* mRNA induced by the first stimulus has been completely transported from the nucleus to the cytoplasm, whereas *Arc* mRNA induced by the second stimuli is still exclusively in the nucleus. As such, neurons activated by either stimulus can be distinguished based on *Arc* mRNA's subcellular distribution.

However, catFISH requires high-magnification imaging (40- to 64-fold) in order to differentiate nuclear from cytoplasmic mRNA localization, which is challenging for whole-brain imaging. Also, the time interval (20–30 min) between the two stimuli may be too short if the first stimulus of behavioral state may affect the response to the second stimulus.

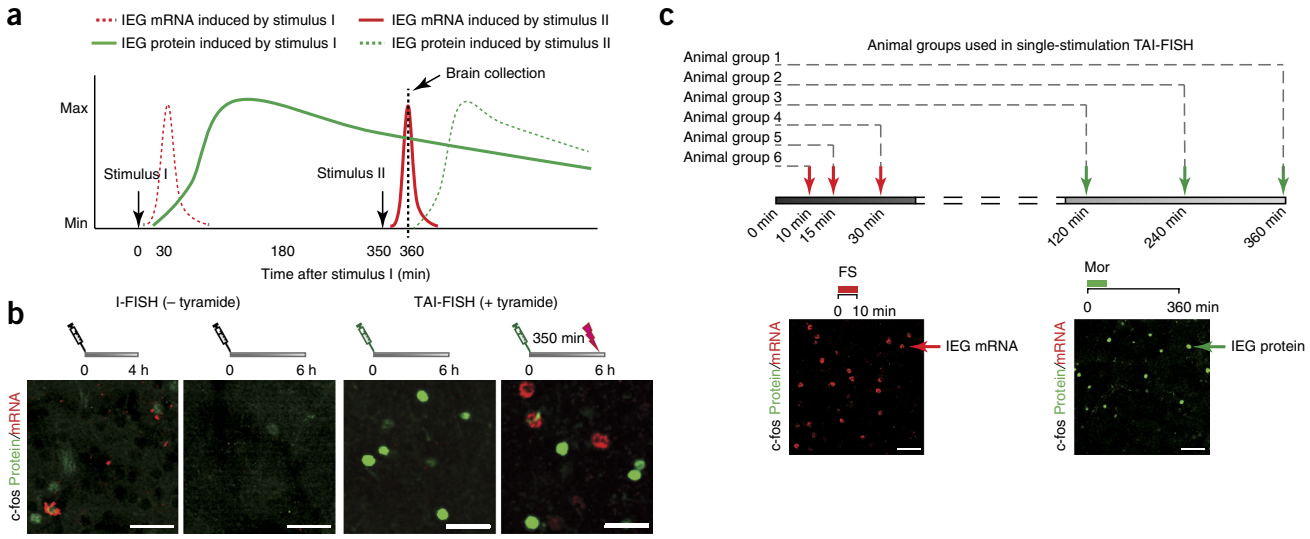

Figure 1 | TAI-FISH uses tyramide-based amplification to achieve robust temporal separation of IEG signals induced by two stimuli. (**a**) Schematic of the general principle of the dual-epoch activity-mapping techniques TAI-FISH and I-FISH. At the time of brain collection, neural activations induced by Stimulus I and Stimulus II are exclusively represented by IEG protein (solid green line) and mRNA (solid red line), respectively. IEG mRNA induced by Stimulus I (dashed red line) decays shortly after stimulation and will not interfere with that induced by the second stimulus, whereas IEG protein induced by the second stimulus (dashed green line) will not be captured because it peaks much later than when the brain is collected. This schematic is based on TAI-FISH using morphine as the first stimulus and foot shock as the second stimulus to identify activated neural ensembles in mouse NAc. Note that for different brain regions and/or stimuli, the order and temporal separation of the two stimuli must be tested for optimal results. (**b**) Tyramide amplification of IHC signals is essential for TAI-FISH. Without tyramide amplification, I-FISH was unable to reliably identify c-fos protein signal induced by morphine (needle) 6 h after stimulation in NAc (middle left). Note that after 4 h, *c-fos* mRNA (red) is still detectable by FISH (left), therefore it will interfere with that induced by foot shock at this time point. TAI-FISH utilizes tyramide to amplify c-fos protein signal induced by morphine (first stimulus) at 6 h (middle right), providing superior signal-to-noise ratio and enabling the separation of c-fos protein induced by morphine from *c-fos* mRNA induced by the foot shocks (right, second stimulus). The images were taken in the NAc. Scale bars, 30 µm. (**c**) In single-stimulation TAI-FISH, the accumulation and peaking of stimulus-induced IEG mRNA are typically monitored at multiple time points during a 30-min window after stimulation (red arrows). IEG proteins and the decay of IEG mRNA are monitored at later points because of IEG protein's slower kinetics as compared with IEG mRNA (green arrows). (Top) Schematic showing animal groups used in single-stimulation TAI-FISH. (Bottom left) Only *c-fos* mRNA signals are detected in NAc at 10 min after foot-shock stimulation. (Bottom right) Only c-fos protein signals are detected in NAc at 360 min after morphine stimulation (Mor). Scale bars, 50 µm. FS, foot shock. **b**,**c** adapted from ref. 5, Springer Nature. Arrows indicate positive c-fos signals. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Institute of Neuroscience, Chinese Academy of Sciences.

Chaudhuri *et al.* developed a dual-epoch activity-dependent neural labeling technique that combines regular *in situ* hybridization (ISH) with IHC (we termed it 'I-FISH') to simultaneously visualize the *zif268* mRNA and protein signals in the primate visual cortex after sequential application of two visual stimuli (**Fig. 1a**)[17]. It is known that *zif268* mRNA is induced within 30 min of the onset of stimulation and decays quickly afterward, whereas *zif268* protein accumulates more slowly and peaks ~2–3 h later[18,19]. So by separating the two stimulations by about 2 h and sacrificing test animals 30 min after the second stimulation, neural activations induced by the first stimulus can be visualized as zif268 protein by IHC and those induced by the second stimulus can be visualized as *zif268* mRNA by ISH. Furthermore, these signals can be detected by low-magnification imaging (for example, 10×), thus allowing whole-brain mapping.

Development and overview of TAI-FISH

We sought to identify and distinguish neural activation patterns induced by emotionally appetitive and aversive stimuli in the limbic forebrain at single-neuron resolution[5]. Initially we sequentially stimulated mice with morphine and foot shock and conducted *c-fos*-based I-FISH to identify and differentiate *c-fos* mRNA and protein signals induced by those two stimuli. We found that in about half of the brain regions of interest, I-FISH did not provide sufficient separation of *c-fos* mRNA and protein signals induced by foot shock and morphine, respectively[5]. This was because by the time the *c-fos* mRNA levels induced by morphine were low enough, the levels of c-fos protein translated from morphine-induced *c-fos* mRNA also had become too low for I-FISH to detect. Therefore, I-FISH in its current form was not suitable for our study.

To optimize I-FISH, we incorporated a tyramide-based IHC signal amplification step into the I-FISH protocol and therefore named our new technique TAI-FISH[5]. TSA has been used to improve the detection of IHC signals[20,21]. By incorporating it into I-FISH, we greatly improved the sensitivity and robustness of IHC and the separation between c-fos protein induced by morphine and *c-fos* mRNA induced by foot shock, thus making the modified version of I-FISH applicable to more brain structures and behavioral paradigms (**Fig. 1b**).

For TAI-FISH to succeed, it is critical to choose the proper time points for applying the two stimuli and for sacrificing the animal. At the time when the animal is sacrificed, (i) the mRNA signal of the first stimulus should have decayed, whereas its protein signal should still be high; and (ii) the protein in response to the second stimulus should not have been expressed yet, whereas its mRNA signal should still be high. Therefore, we devised a general guideline for conducting a 'single-stimulation' TAI-FISH to determine the proper sequential order and temporal interval for the two stimuli to be given to the test animals (**Fig. 1c**).

TAI-FISH consists of three main stages, of which an overview is provided in **Figure 2**. The first stage involves standard brain sample harvesting, which includes fixation, dehydration and sectioning (Steps 1–6). In the second stage, FISH is used to identify IEG mRNA signals to label neural ensembles activated by the second stimulus (Steps 7–26). In the third stage, IHC is used to identify IEG protein signals to label neural ensembles activated by the first stimulus (see Steps 27–36 in the 'PROCEDURE' for detail). The IHC portion of the protocol includes TSA to amplify the c-fos protein signals to help segregate the time course of *c-fos* mRNA and protein signals. This is a unique step in TAI-FISH and provides an advantage over other dual-epoch labeling techniques such as I-FISH.

In addition, we developed specific control experiments to minimize false-negative and false-positive results that can occur in TAI-FISH[5]. We proposed a positive TAI-FISH control to test the sensitivity and robustness of the experimental setup for TAI-FISH. We also introduced 'no-stimuli' controls to minimize false positives caused by the context in which stimuli are given (see 'Experimental design').

We have applied TAI-FISH to identify and distinguish the neural coding patterns evoked by two stimuli of different emotional valences in the limbic forebrain[5]. We chose *c-fos* as the neural activity marker because it is generally expressed at a low basal level across the limbic forebrain in resting animals and is readily induced by many stimuli, although IEGs such as *Arc*, *zif268* and *Homer1a* can also potentially be used[17,22]. We used morphine and foot shock as appetitive and aversive stimuli, respectively, in our study because both can evoke robust emotional behavioral reactions: morphine induces a significant ($P = 0.048$) conditioned place preference, and foot shock causes robust place avoidance[23,24]. We sequentially gave mice morphine and foot shocks, and then performed TAI-FISH to simultaneously visualize c-fos protein expression induced by morphine and *c-fos* mRNA evoked by foot shocks. Using this approach, we have successfully identified stable and stereotypic neural activation patterns induced by those stimuli within several limbic structures, e.g., the convergent pattern in the paraventricular nucleus of hypothalamus (PVN), the segregated pattern in the central amygdala (CEA) and the intermingled pattern in the NAc[5] (**Fig. 3a**,**b**). Our study demonstrated that TAI-FISH is a robust and reliable technique for visualization of neural activations induced by emotional stimuli of opposing valences at a single-cell resolution, and for capturing the valence map throughout the limbic forebrain.

We also determined the cellular identities of neuronal populations that are activated by morphine and foot shock by staining for a number of endogenous neuronal markers and determining the overlap between these markers and the TAI-FISH signals. For instance, we demonstrated that the mosaic activation patterns in the NAc correlate with the heterogeneous D1- and D2-receptor expression patterns[5]: we found that ~80% of morphine-activated neurons are D1-positive, whereas >60% of foot shock-activated neurons are D2 positive (**Fig. 3c**). It should be noted that here we used a variation of TAI-FISH in which FISH was used to identify D1- or D2-type dopamine receptor mRNA instead of *c-fos* (i.e., morphine- or foot-shock-activated NAc neurons were identified by TSA-enhanced c-fos IHC signals, whereas the identity of activated neurons was determined by FISH, which probed for D1- or D2-type dopamine receptor's mRNA).

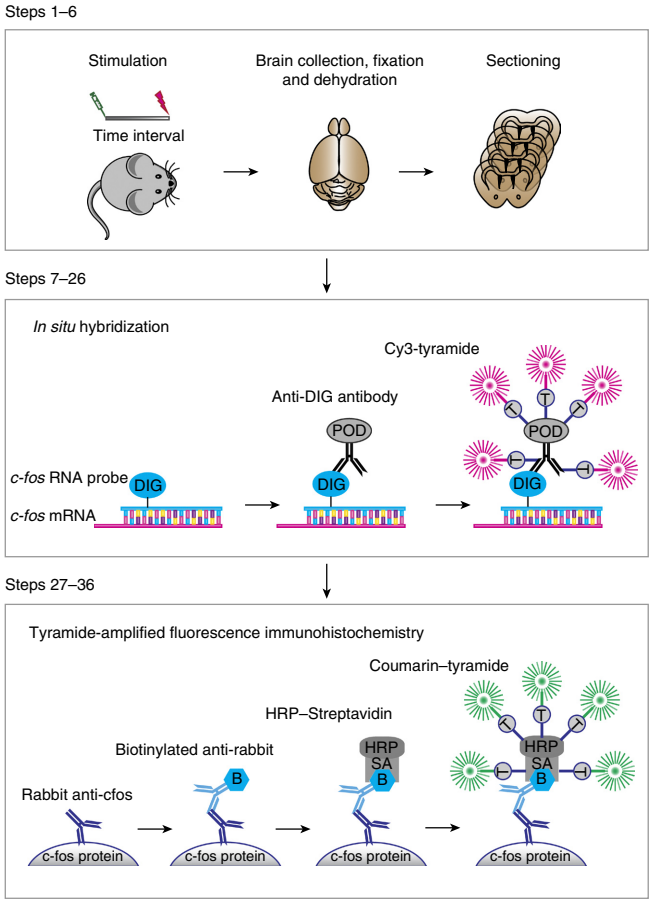

Figure 2 | Schematic overview of TAI-FISH protocol. First, test animals are given two sequentially applied stimuli and are sacrificed. Then their brains are collected, prepared and sectioned (Steps 1–6); Second, IEG mRNA signals activated by the second stimulus are identified by FISH after sample treatments (Steps 7–26); Finally, IEG protein signals activated by the first stimulus are identified by IHC, which can utilize tyramide to amplify protein signals (Steps 27–36). Thus, neural ensembles activated by the two stimuli are now labeled by different detection methods with different colors and are readily distinguished by imaging. Note that other IEGs can be used instead of *c-fos*. B, biotin; DIG, digoxin; POD, peroxidase; HRP, horseradish peroxidase; SA, streptavidin; T, tyramide.

Advantages of TAI-FISH

Based on our previous work, we believe TAI-FISH has several advantages over other available dual-epoch activity-dependent labeling techniques. First, it offers a more robust separation of neural ensembles activated by different stimuli. Comparing TAI-FISH with I-FISH[17,25], we found that in tested brain structures that are not applicable for I-FISH because of the low signal-to-noise ratio of IHC, TAI-FISH was able to produce robust separation of *c-fos* mRNA and protein signals and allowed us to differentiate neural activations induced by different stimuli. This is due to the incorporation of TSA for IHC signals in TAI-FISH.

Second, TAI-FISH is better than catFISH at minimizing interference between the two stimuli[14,16]. As described earlier, catFISH requires the two stimulations to be separated by ~30 min. In comparison, the two stimulations used in TAI-FISH can be separated by 2–6 h, depending on the brain structures and stimulations. Thus, TAI-FISH further minimizes the chance of the two

stimulations interfering with each other with regard to induced neural activation.

Third, TAI-FISH is more suitable than catFISH for whole-brain imaging. Unlike catFISH, which requires 40- to 64-fold magnification for subcellular-resolution imaging, a 10-fold magnification is typically sufficient for TAI-FISH imaging (**Fig. 3a**), making it easier to conduct investigation at the whole-brain scale.

Last but not least, TAI-FISH does not require transgenic animals such as TetTag mice[26] or TRAP mice[27]. Such mice use IEG promoters to express fluorescent proteins to label activated neural ensembles, and can also be used to capture and differentiate neural ensembles activated by different stimuli. TAI-FISH can be performed on wild-type animals, making it more approachable to researchers who may have limited access to such transgenic animals. More importantly, because of the often-observed discrepancies between neurons labeled by endogenous IEG and those labeled by transgenic IEG-promoter-driven fluorescent protein, it is likely that the activated neural ensembles captured by TAI-FISH are the more faithful representation of neural responses to stimuli.

Limitations of TAI-FISH

TAI-FISH has provided us with a robust technique to identify neural ensembles activated by multiple stimuli and their spatial distributions at single-cell resolution across the same animal brain. It has the potential to be widely used in many other neuroscience studies, either as a stand-alone technique or in combination with others.

However, TAI-FISH does have some limitations. First, TAI-FISH depends on a strict time window between when the mRNA of the first stimulus is fully degraded and when the protein is still present. In some brain regions, the first stimulus-induced IEG mRNA is long-lasting and inseparable from its protein time course. For example, foot shock-induced *c-fos* mRNA in the NAc degrades slowly (**Fig. 4a**,**b**), thus foot shock is unsuitable as the first stimulus for TAI-FISH. Second, TAI-FISH relies on robust antibodies/FISH probes for the target of interest. In our experience, some antibodies suitable for regular IHC may not work on brain slices after FISH treatment in TAI-FISH. Indeed, we found that only 2 of the 3 c-fos antibodies that have proven to be robust in standard IHC worked for TAI-FISH (see 'PROCEDURE' for details). Therefore, TAI-FISH users are encouraged to use our recommended antibodies, or to thoroughly test their candidate antibodies before implementing TAI-FISH. Third, structures that have high basal-level IEG expression in the resting state or in no-stimuli controls, such as sensory cortexes for *c-fos* are not suitable. Finally, expression/stability patterns of mRNA/protein for different brain targets can be very different and will always have to be optimized. For example, 6 h is suitable to separate NAc activation patterns stimulated by morphine and foot shock (**Fig. 4b**), whereas 3 h is ideal for CEA stimulated by the same stimulus pair[5].

Applications of the method

TAI-FISH was designed to identify and distinguish neural activations induced by multiple stimuli. In addition to morphine and foot shock, we have also used TAI-FISH to capture and compare neural ensembles activated by chocolate, cocaine or restraint stress[5]. Xue *et al.* recently used TAI-FISH to map amygdala neural ensembles coding different forms of nicotine reward memories[28].

Indeed, in principle, TAI-FISH can be applied broadly to capture neural activations evoked in brain functions such as emotion, memory, sensory processing and drug responses. For example, it is possible to use TAI-FISH to identify odor maps in the olfactory bulb in response to different odors. Similarly, we suggest that neural activations induced by multiple chemicals, hormones or drugs are also ideal for investigation using TAI-FISH. This technique may be particularly useful in studying comorbid psychiatric

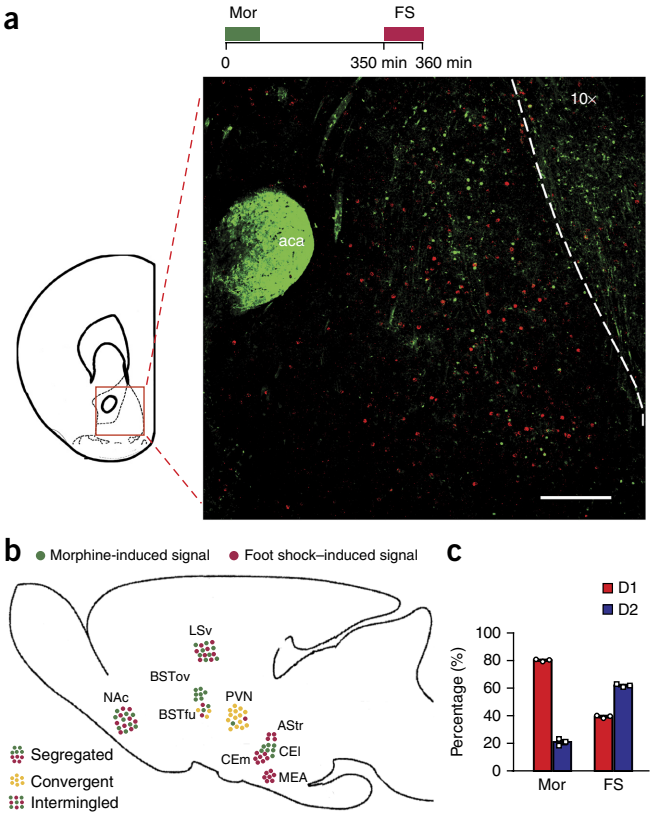

Figure 3 | TAI-FISH identifies and distinguishes activated neural patterns at single-neuron resolution across a whole brain. (**a**) Neural activity patterns identified by *c-fos*-based TAI-FISH in NAc stimulated by morphine (i.p. injection) and foot shock. (Left) Schematic of section of NAc imaged. (Right) A single microscopic field taken from a brain slice 1.70 mm anterior to the bregma with a 10× objective, showing that TAI-FISH can map large brain areas. Red: *c-fos* mRNA; green: c-fos protein. Scale bar, 200 μm. (**b**) TAI-FISH reveals different patterns of spatial distribution of neural representations of morphine and foot shock in different regions of the limbic forebrain (each dot represents 5 neurons counted from representative sections from each brain structure). (**c**) TAI-FISH identifies and quantifies the percentage of *c-fos*$^+$ neurons induced by morphine (left) and foot shock (right) that express D1-type (red) or D2-type (blue) dopamine receptors. Counting was performed for the whole NAc dorsomedial shell (n = 3 for both stimuli. Each dot represents one mouse. aca, anterior commissure, anterior part; AStr, the amygdalostriatal transition area; BST, bed nucleus of stria terminalis; BSTfu, the fusiform nucleus of the BST; BSTov, oval nucleus of the BST; CEA, central amygdala; CEl, the lateral division of the CEA; CEm, the medial division of the CEA; FS, foot shock; LSv, the posterior ventral part of the lateral septum; MEA, medial amygdala; Mor, morphine; PVN, paraventricular nucleus of hypothalamus; Image adapted from ref. 5, Springer Nature. All procedures were approved by the IACUC of the Institute of Neuroscience, Chinese Academy of Sciences.

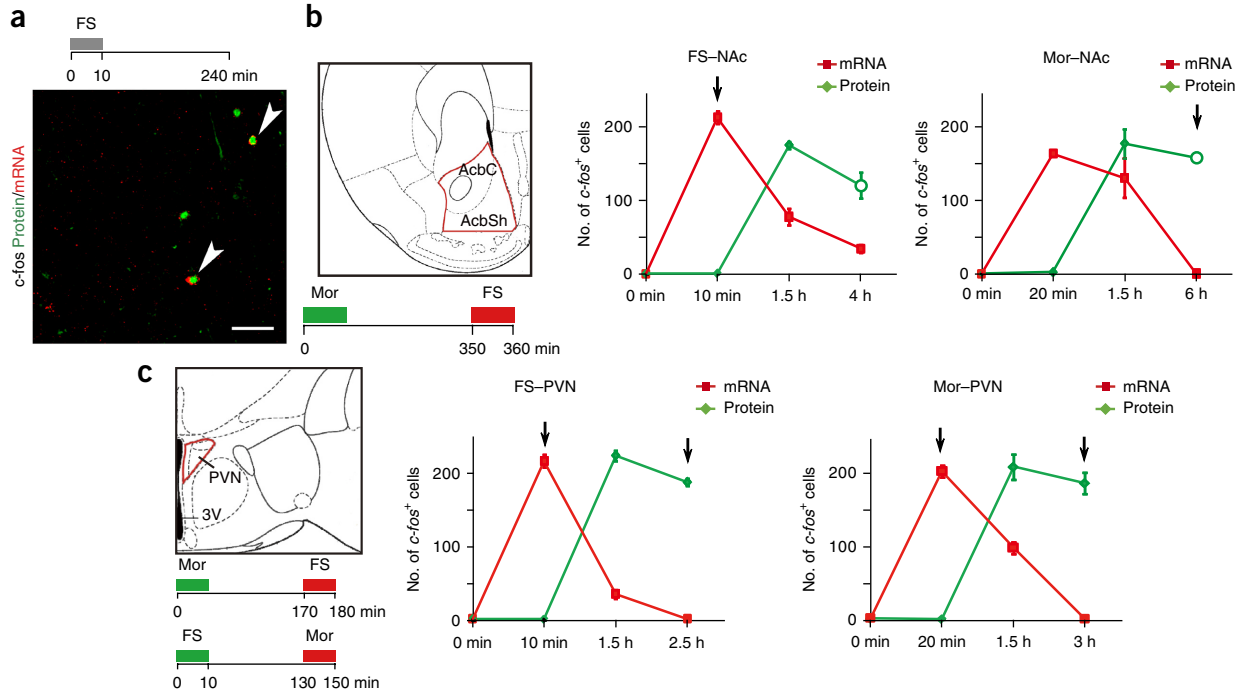

Figure 4 | Time course mapping for TAI-FISH and I-FISH. (**a**) 4 h after foot shock, mRNA signals of *c-fos* still exist in a substantial number of NAc neurons, whereas protein signals have decayed. Therefore, it is not ideal to choose foot shock as the first stimulus in NAc. Arrowheads indicate overlapped *c-fos* mRNA and protein signals. Scale bar, 50 μm. (**b**) Mapping of time course of foot shock and morphine for the dual labeling experiments in NAc. (Top left) Schematic of location of NAc shell imaged. (Bottom left) Optimal order and intervals chosen for the dual-labeling experiment in NAc. (Center and right) Time mapping of a single stimulus using TAI-FISH (open circles) or I-FISH (closed rhombus). Arrows indicate the time points chosen for the dual-labeling experiment in **Figure 3**. Data collected from brain slices at 1.70 mm anterior to the bregma. (**c**) Mapping of time course of foot shock and morphine for the dual-labeling experiments in the PVN. (Top left) Schematic of location of PVN imaged. (Bottom left) Optimal orders and intervals chosen for the dual-labeling experiment in the PVN. (Center and right) Time mapping of single stimulus using I-FISH in the PVN. Arrows indicate the time points that can be chosen for the dual-labeling experiment. Data collected from brain slices at 0.82 mm posterior to the bregma. AcbC, NAc core; AcbSh, NAc shell; 3V, third ventricle. **b**, **c** adapted from ref. 5, Springer Nature. All procedures were approved by the IACUC of the Institute of Neuroscience, Chinese Academy of Sciences.

disorders, for which multiple drugs are often administrated to one patient.

We also propose combining TAI-FISH with other techniques to expand its application. For example, TAI-FISH can be employed with a host of retrograde labeling techniques, such as retrograde tracer cholera toxin subunit B (CTB)-[29], canine adenovirus-2–(ref. 30), herpes simplex virus–[31], rabies virus–[32], rAAV2-retro-[33] or RG-EIAV lentivirus–based[34] tracing, to map functional connections at the neural circuit level: by injecting retrograde tracer or virus downstream of the brain region visualized by TAI-FISH and identifying IEG-positive neurons that also have retrograded reporter signals, we can map the functional projections from the activated neural ensembles in this brain region to their downstream targets and thus start to reveal the functional circuits underlying the behavioral responses toward the stimuli. TAI-FISH is also applicable to other hapten-labeled riboprobes such as biotin- or fluorescein-labeled probes, making it possible to introduce the double-FISH (dFISH) method with TAI-FISH (TAI-dFISH). As described earlier, non-IEG genes such as dopamine receptors D1 or D2 in conjunction with *c-fos* can be detected by TAI-FISH[5]. Therefore, it is possible to use TAI-dFISH to simultaneously identify neural ensembles activated by different stimuli and discover their cellular identity by probing for both activated IEG and specific cellular markers during dFISH.

Furthermore, it is possible to combine TAI-FISH with other IEG-dependent tools or techniques to develop a triple-epoch neural activity–labeling protocol. For example, TAI-FISH can be combined with catFISH to map and differentiate neural activations induced by three distinct stimuli. This can also be achieved by combining TAI-FISH with activity-dependent ensemble-labeling tools such as *Arc*-CreER[35], E-SARE[36], TetTag[26,37,38], TRAP[27], RAM[39], Cal-Light[40] and FLARE[41]. In this strategy, activity-dependent viral or mouse tools are used to label neural ensembles activated by the first stimulus, whereas neural ensembles activated by the second and third stimuli are visualized by TAI-FISH. This more advanced version of triple-epoch mapping provides more flexibility in analyzing neural activation induced by multiple stimuli in a single animal.

Experimental design

Before TAI-FISH: considerations for the IEG to be used in TAI-FISH. The basic principle for choosing an appropriate IEG for TAI-FISH is that the IEG must have a high signal-to-noise ratio in the stimulated brain structure: it must have low basal-level expression without stimulation or with a stimulation control (e.g., saline injection as a control for morphine injection) and be robustly induced by both stimuli used in TAI-FISH. We calculated the signal-to-noise ratio of an IEG in a brain structure by comparing the number of neurons positively stained in stimulated animals and

in no-stimuli controls. We routinely use 3:1 (stimulated/no-stimuli) as a minimal cutoff for an acceptable signal-to-noise ratio. An IEG that is expressed at a substantial level without stimulation will introduce false-positive signals in TAI-FISH; alternatively, if it cannot be substantially induced by a stimulus, neural ensembles activated by this stimulus will not be captured by TAI-FISH, causing false negatives. Therefore, TAI-FISH users should determine the mRNA and protein levels of their choice of IEG in both resting animals and stimulated animals.

Basal levels of IEG expression can vary depending on IEGs, brain structures and stimulus types. For example, we found *c-fos* in general has a low basal-level expression in resting brains, except in the sensory cortex and anterior lateral septum (LS), and can be rapidly activated by many stimuli. So, broadly speaking, *c-fos* is very suitable for TAI-FISH.

Although we used *c-fos* in our published study, in principle, any molecules that faithfully capture neural activation can be used for TAI-FISH, such as *zif268*, *Arc* and others, as long as they meet the criteria mentioned above.

We also found that basal-level and stimulus-induced IEG expression can vary between animals, depending on a number of additional factors, such as their genetic background, sex, age, housing conditions and animal handling, all of which may influence the mental state (for example, stress or anxiety) of individual animals. So TAI-FISH users should thoroughly test their animals to determine basal-level IEG expression and its inducibility, and take precautions to minimize animal stress. For example, to reduce cross-influence on cagemates and minimize stress-caused *c-fos* signals, we usually divide mice into two per cage 4 d before the start of the experiment, apply treatments on two cagemates simultaneously, sacrifice them within 10 s of the treatment termination and perfuse them; this is accomplished by two people working simultaneously within ~10 min (ref. 5).

Before TAI-FISH: determination of the time course of stimulations. The key to the experimental setup is that when the stimulated animal is sacrificed, IEG signals induced by the first stimulus all exist as protein, whereas those induced by the second stimulus all exist as mRNA. To ensure this is the case, TAI-FISH users must conduct a series of single-stimulation TAI-FISH experiments to determine the kinetics of IEG signals induced separately by the two stimuli, i.e., monitoring the accumulation, peaking and degradation of both mRNA and protein signals.

Based on our experience and the literature, for each stimulus we recommend first-time that users perform a series of single-stimulation TAI-FISH experiments at 5–8 different time points after stimulation to conclusively determine the kinetics of induced IEG signals (**Fig. 1c**). These time points can be categorized into two groups. The first group is at 10, 15 and 30 min after the stimulation to capture the rapid accumulation and peaking of IEG mRNA. The second group of time points start at 90–120 min after stimulation and are separated hourly, the last of which occurs at 3 to 6 h after stimulation. This group serves two functions: (i) to monitor the accumulation and decay of IEG protein, which are usually slower than that those of mRNA; and (ii) to monitor the decay of induced mRNA signals, which may take hours to become sufficiently low as to not interfere with those induced by a second stimulus. As usual, multiple test animals should be used at each time point to determine the variation between test animals and experimental performances.

Stimuli that induce short-lived IEG mRNA, but long-lasting IEG protein, should be used as the first stimulus, whereas those inducing rapid IEG mRNA accumulation should be used as the second. For example, we found that NAc *c-fos* mRNA induced by morphine is completely degraded 6 h after stimulation, whereas c-fos protein remains near its peak level[5]. Meanwhile, mRNA induced by foot shock peaks 10 min after stimulation. So, for NAc activation, morphine and foot shock can be given as first and second stimuli, respectively.

However, it should be noted that the kinetics of mRNA and protein signals of IEGs vary markedly depending on the brain structures tested and the dose of the stimuli used. For instance, contrary to the NAc, stimulating with either morphine or 10 foot shocks induces persistent *c-fos* mRNA signals in the BLA hours after stimulation[5]. Therefore, *c-fos*-based-TAI-FISH is not suitable when morphine or foot shocks are applied at this dosage as the first stimulus when studying the BLA because *c-fos* mRNA induced by either stimulus will persist and interfere with those induced by a second stimulus. This highlights the necessity for TAI-FISH users to optimize the time points in single-stimulation tests for the brain regions and stimuli of interest, and to retest the time course when new IEGs, brain structures and/or behavioral paradigms are studied.

Controls to be performed with TAI-FISH. We recommend that TAI-FISH users perform two types of controls simultaneously with their TAI-FISH experiments to minimize false-negative and false-positive results.

To establish a positive control for the TAI-FISH protocol, we recommend that first-time TAI-FISH users repeat our published TAI-FISH treatment, in which morphine and foot shocks were used as the first and second stimulus for NAc activation, respectively, before starting TAI-FISH on their brain structure and/or stimulus of interest. By performing this control, TAI-FISH users can verify that their experimental setup is sufficiently sensitive and robust to detect IEG mRNA and protein signals, i.e., there is very little RNase contamination during FISH, and the antibody used to detect IEG protein has sufficiently high affinity.

We also recommend including a 'no-stimuli' control, which controls for the IEG activation induced by the experimental conditions under which the stimuli were applied, i.e., false-positive results. For example, in TAI-FISH experiments in which mice were stimulated with morphine and foot shocks, we injected saline (the solvent for morphine) into control mice to account for IEG signals induced by injection itself. Afterward, we performed sham foot shocks by placing control mice in the foot-shock chamber for the same duration as the foot-shocked group to control for IEG signals induced by the context of foot shocks and a novel environment. These 'no-stimuli' controls (saline injection as a control for morphine and foot-shock chamber as a control for foot shock) are essential to isolating neural activation induced solely by stimuli from those induced by experimental setups, and therefore must be performed for each TAI-FISH experiment.

The necessity of tyramide-based IHC signal amplification. The key improvement of TAI-FISH over I-FISH is that we used a TSA step to enhance IEG protein signals. This technique maximizes the separation of IEG mRNA and protein signals induced by two stimuli, and thus robustly increases the number of

TAI-FISH-compatible brain structures and behavior paradigms. For example, when we tested I-FISH, we found that c-fos protein signals in the NAc remain suitable for detection 4 h after morphine injection, when there is still substantial *c-fos* mRNA signal left, which will interfere with *c-fos* mRNA induced by a second stimulus (**Fig. 1b**). On the other hand, when morphine-induced *c-fos* mRNA almost completely decayed 6 h after stimulation, c-fos protein signals also become too weak to be detected by IHC. This makes it extremely difficult to use morphine as the first stimulus in I-FISH. Using tyramide-based IHC signal amplification, we solved this conundrum by robustly amplifying morphine-induced c-fos protein signals, which makes it possible to identify c-fos protein signals 6 h after stimulation when *c-fos* mRNA is gone and does not interfere with *c-fos* mRNA induced by a second stimulus (**Fig. 4b**).

In addition to the NAc, we found that TSA of the IHC signal is required for additional brain structures, such as the posterior ventral part of the LS and the bed nucleus of stria terminalis[5]. We suspect that this will probably be the case for many other IEGs, brain structures and behavior paradigms, especially for drug-induced neural activations, because chemicals may have long-lasting stimulating effects.

However, it should also be noted that some brain regions and/or stimuli may not need TSA for IHC signals. For example, we found that in the PVN (**Fig. 4c**) and CEA, standard I-FISH is sufficient to identify and distinguish neural ensembles activated by morphine or foot shock[5]. In our experience, brain regions whose first stimulus-induced IEG mRNA accumulates and decays rapidly are more likely to be suitable for I-FISH: the rapid decay of the first stimulus-induced mRNA (for example, in the PVN, *c-fos* mRNA decays to background levels 90 min after foot shock) allows the second stimulus to be given within a short time interval from the first stimulus. This in turn allows the level of first-stimulus-induced IEG protein to remain sufficiently robust for standard IHC detection, and therefore does not require TSA.

To determine whether or not TSA of the IHC signal is needed for a specific stimulus–brain region combination, we recommend performing a standard IHC without TSA, along with the single-stimulation TAI-FISH test, and comparing the signal-to-noise ratio of IHC signals with or without TSA.

MATERIALS

REAGENTS

- Brain sections of test animals (we have successfully used mouse (C57BL/6; Shanghai SLAC Laboratory Animal Center) tissue) **! CAUTION** Any experiments involving mice must conform to relevant institutional and national regulations. Permission for the procedures described in this protocol were obtained from the Institutional Animal Care and Use Committee (IACUC) of the Institute of Neuroscience, Chinese Academy of Sciences.
- Diethylpyrocarbonate (DEPC; Sigma-Aldrich, cat. no. D5758) **! CAUTION** DEPC is a toxic hazard; work under a fume hood with gloves while using DEPC.
- 10 mg/ml Morphine hydrochloride (Shenyang No.1 Pharmaceutical, cat. no. 14-01) **▲ CRITICAL** Morphine hydrochloride is used in our example test, or serves as a positive control for new stimuli.
- RNaseZAP (Ambion, cat. no. AM9780 or AM9782) **! CAUTION** Always wear gloves while using RNaseZAP solution because prolonged contact with skin may cause irritation.
- Chloral hydrate ($C_2H_3Cl_3O_2$; Sinopharm Chemical Reagent, cat. no. 30037517)
- Paraformaldehyde (PFA; Sigma-Aldrich, cat. no. 158127) **! CAUTION** PFA is a toxic hazard; PFA is a cross-linking agent; wear personal protective equipment and work under a fume hood when using PFA.
- Sodium chloride (NaCl; Sinopharm Chemical Reagent, cat. no. 10019318)
- Potassium chloride (KCl; Sinopharm Chemical Reagent, cat. no. 10016318)
- Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$; Sinopharm Chemical Reagent, cat. no. 10020318)
- Potassium dihydrogen phosphate (KH_2PO_4; Sinopharm Chemical Reagent, cat. no. 10017618)
- Sodium hydroxide (NaOH; Sangon Biotech, cat. no. 0583) **! CAUTION** NaOH is corrosive. Wear gloves and protective goggles to avoid contact with eyes and skin.
- Sucrose (Sigma-Aldrich, cat. no. S1888-1KG)
- Tissue embedding matrix (OCT; Leica, cat. no. 020108926)
- Tris–sodium citrate dihydrate ($C_6H_5Na_3O_7{\cdot}2H_2O$; Sinopharm Chemical Reagent, cat. no. 10019418)
- 12 N Hydrochloric acid (HCl; Sinopharm Chemical Reagent, cat. no. 10011018) **! CAUTION** HCl is poisonous and corrosive. The acid's liquid and mist cause severe burns. The solution may be fatal if swallowed or inhaled.
- Deionized formamide (Sangon Biotech, cat. no. F0606) **! CAUTION** Deionized formamide is a toxic hazard.
- Ribonucleic acid, type X-SA, transfer from baker's yeast (yeast tRNA; Sigma-Aldrich, cat. no. R8759-10KU)
- Heparin sodium salt, from porcine intestinal mucosa (Sigma-Aldrich, cat. no. H3393)
- 100 × Denhardt's solution (Sangon Biotech, cat. no. EDE257)

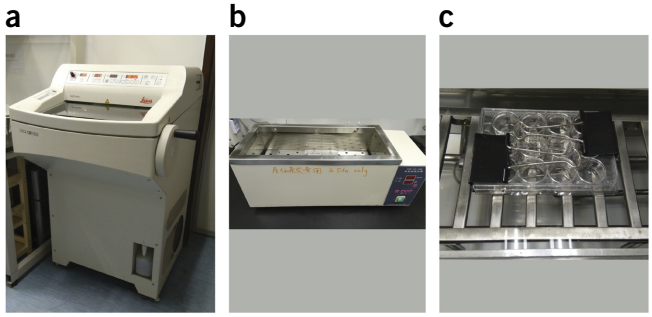

Figure 5 | Key equipment used in TAI-FISH. (**a**) Cryostat microtome (Leica, model no. CM1950) for sectioning animal brains. (**b**) Water bath (used with a cover) dedicated to hybridization in FISH. (**c**) During prehybridization and hybridization, tissue culture plate for incubation is clamped by binder clips to prevent potential contamination from bath water. This also keeps hybridization buffer from evaporating. The water level in the water bath is beneath the lid of the 12-well cell culture plate.

- Tween 20 (Sangon Biotech, cat. no. T0777)
- 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Sangon Biotech, cat. no. CD0110)
- Ethylene diamine tetraacetic acid, anhydrous, Sigma Grade, ~99% titration (EDTA; Sigma-Aldrich, cat. no. EDS-500G)
- 30% (vol/vol) Hydrogen peroxide (H_2O_2; Sangon, cat. no. 10011218) **! CAUTION** 30% (vol/vol) Hydrogen peroxide is a toxic hazard; be aware when opening containers, as they are often pressurized; hydrogen peroxide is corrosive; wear personal protective equipment.
- Triton X-100 (Sangon, cat. no. T0694-500ML)
- Triethanolamine (Sinopharm Chemical Reagent, cat. no. 10023118) **! CAUTION** Triethanolamine is flammable if heated.
- Acetic anhydride (Fluka, cat. no. 45830) **! CAUTION** Acetic anhydride is a toxic hazard; work under a fume hood while wearing gloves and a lab coat when working with acetic anhydride.
- RNase A (Roche, cat. no. 10109142001)
- Normal goat serum (NGS; Solarbio, cat. no. SL038)
- Sodium azide (NaN_3; Sangon Biotech, cat. no. DB0613) **! CAUTION** Sodium azide is a toxic hazard; work under a fume hood while wearing gloves and a lab coat when working with sodium azide.
- Streptavidin, HRP conjugate (Millipore, cat. no. 18-152)

- DMSO (Sigma-Aldrich, cat. no. D5879) ! CAUTION DMSO is a flammable and toxic hazard; work under a fume hood while wearing gloves and a lab coat when working with DMSO.
- Tyramide signal amplification (TSA) kits with tyramide-coupled fluorescein cyanine 3 or coumarin (PerkinElmer, cat. nos. NEL744B001KT and NEL703001KT, respectively)
- Glycerol ($C_3H_8O_3$; Sinopharm Chemical Reagent, cat. no. 10010618)
- Tris(hydroxymethyl)aminomethane (Tris base; Sigma, cat. no. 154563) ! CAUTION Tris base is an irritant; wear dust mask type N95 (US), eye-shields, and gloves when using Tris base.

Antibodies

- Anti-digoxigenin (DIG)-POD, Fab fragment (Roche, cat. no. 11207733910) ▲ CRITICAL Note that the anti-DIG-POD antibody is used in immunohistochemical detection of the DIG-labeled riboprobes used in this protocol. If researchers use other types of riboprobes (e.g., biotin-labeled probes or fluorescein-labeled probes), then the corresponding primary antibodies must be used.
- Biotinylated goat anti-rabbit IgG (H + L) (Vector Laboratories, cat. no. BA-1000)
- Anti-c-fos rabbit polyclonal antibody (Synaptic Systems, cat. no. 226003) ▲ CRITICAL The quality of c-fos antibody, i.e., signal-to-noise ratio for c-fos protein detection, is essential to TAI-FISH. c-fos antibodies that work for standard IHC do not necessarily work for TAI-FISH, presumably because of the RNA hybridization steps. We previously used Calbiochem (cat. no. PC38) c-fos antibody[5], but it was discontinued while the manuscript was being prepared. Xue *et al.*, successfully used c-fos antibody from Cell Signaling Technology (cat. no. 2250S; 1:500; ref. 28). We have tested several other c-fos antibodies and found that Abcam ab138471, ab190289 and ab87655 give no signal in standard IHC, whereas Santa Cruz sc-52 works for standard IHC, but gives inconsistent results in TAI-FISH.

EQUIPMENT

- Fear-conditioning apparatus (Coulbourn Instruments, cat. no. ACT-100A) ▲ CRITICAL Fear-conditioning apparatus is used in our example test and serves as a positive control for new stimuli.
- Cryostat microtome (Leica, model no. CM1950; **Fig. 5a**)
- 6-Well cell culture plate, tissue culture–treated, nonpyrogenic, polystyrene, sterile (Costar; Corning, cat. no. 3516)
- 12-Well cell culture plate, tissue culture–treated, nonpyrogenic, polystyrene, sterile (Costar; Corning, cat. no. 3513)
- Orbital shaker (Kylin-Bell Lab Instruments, cat. no. TS-200)
- Decoloring shaker (Kylin-Bell Lab Instruments, cat. no. TS-8)
- Orbital shaker (Kylin-Bell Lab Instruments, cat. no. TS-2)
- Binder clips (Deli Stationery)
- Water bath (Shanghai Jing Hong Laboratory Instrument, cat. no. DK-S22), required temperature = 60 °C (**Fig. 5b**)
- Water-jacket incubator (Shanghai Bluepard Instruments; Shanghai Yiheng Technical, cat. no. 9050)
- Epifluorescence microscope equipped with appropriate filters, or confocal microscope with proper lasers (e.g., Olympus, model no. Fluoview FV1000)
- Superfrost Plus slides (Fisher Scientific, cat. no. 12-550-15)
- 24×50-mm^2 glass coverslips (Citotest Labware Manufacturing, cat. no. 10212450C)
- Chinese brush (Transon Art & Brush, cat. no. 000)

REAGENT SETUP

10× PBS, pH 7.4 Dissolve 2 g of KCl, 80 g of NaCl, 35.8 g of $Na_2HPO_4{\cdot}12H_2O$ and 2.4 g of KH_2PO_4 in 800 ml of dH_2O. Adjust the pH to 7.4 with 1 M NaOH and bring the volume to 1 liter with dH_2O. Sterilize the solution by autoclaving. The solution can be stored at room temperature (20–25 °C) for more than 1 year.

1× PBS Add 100 ml of 10× PBS solution to 900 ml of dH_2O and mix. The solution can be stored at room temperature for 1 month.

DEPC-treated RNase-free PBS and water For DEPC-PBS, add 1 ml of DEPC to 1 liter of 1× PBS. For DEPC-H_2O, add 1 ml of DEPC to 1 liter of dH_2O. Stir the solution violently, then keep in the water-jacket incubator overnight at 37 °C. The solution should be autoclaved the following day to hydrolyze, and therefore inactivate, the DEPC. The solution can be stored at room temperature for 3 months. ! CAUTION DEPC is a toxic hazard; always work under a fume hood while wearing gloves when using DEPC.

30% (wt/vol) sucrose Add 30 g of sucrose to 50 ml of DEPC-PBS and mix thoroughly; bring the volume to 100 ml with DEPC-PBS. ▲ CRITICAL This solution should be freshly prepared.

4% (wt/vol) PFA Dissolve 40 g of PFA in 1 liter of DEPC-PBS and heat it to 60 °C while stirring. After the PFA has completely dissolved, adjust the pH to 7.4 with NaOH. The solution can be frozen at −20 °C for 6 months or can be stored at 4 °C for 1 week. ! CAUTION PFA is a toxic hazard and should be used while working in a fume hood. ▲ CRITICAL Do not allow higher temperatures than 60 °C during heating.

10% (wt/vol) chloral hydrate Dissolve 5 g of chloral hydrate in saline to a final volume of 50 ml. The solution can be stored at room temperature for 1 year.

0.1 M Tris-HCl, pH 7.5 Dissolve 1.2114 g of Tris base in 80 ml of dH_2O while stirring. After the Tris base has dissolved, adjust the pH to 7.5 with HCl and bring the volume to 100 ml with dH_2O. The solution can be stored at room temperature for 6 months.

15 mg/ml (wt/vol) morphine hydrochloride Dissolve 150 µl of 10 mg/ml morphine hydrochloride in saline to a final volume of 1 ml. ! CAUTION Morphine hydrochloride is used in our example test or serves as a positive control for new stimuli. ▲ CRITICAL This solution should be freshly prepared.

20× Saline–sodium citrate buffer To make saline–sodium citrate buffer (SSC), dissolve 175.3 g of NaCl and 88.2 g of tris–sodium citrate dihydrate in 800 ml of DEPC-H_2O. Adjust the pH to 7.0 with HCl and bring the final volume to 1 liter with DEPC-H_2O. Sterilize the solution by autoclaving. The solution can be stored at room temperature for more than 1 year.

2× SSC Add 50 ml of 20× SSC to deionized water to a final volume of 500 ml and mix. 2× SSC may be stable at room temperature for more than 1 month; however, for our experiments, we recommend freshly preparing this solution before use.

0.2× SSC Add 50 ml of 2× SSC to deionized water to a final volume of 500 ml and mix. 0.2× SSC may be stable at room temperature for more than 1 month; however, for our experiments, we recommend freshly preparing this solution before use.

Prehybridization solution Mix the components shown below to prepare 50 ml of prehybridization solution. Prehybridization solution is used for stock solutions. ▲ CRITICAL All components should be RNase free and prepared in DEPC-treated water.

Component	Amount (ml)	Final concentration
Deionized formamide	25	50% (vol/vol)
20× SSC	12.5	5×
50-mg/ml Yeast tRNA	0.3	0.3 mg/ml
100-mg/ml Heparin sodium salt	0.05	100 mg/ml
100× Denhardt's solution	0.5	1×
10% (vol/vol) Tween 20	0.5	0.1% (vol/vol)
10% (wt/vol) CHAPS	0.5	0.1% (wt/vol)
0.5 M EDTA	0.5	5 mM
DEPC-H_2O	10.15	

Prehybridization solution can be stored at −80 °C for >1 year.

Hybridization solution Add riboprobes of interest to the prehybridization solution to a concentration of 1 µg/ml. ▲ CRITICAL Hybridization solution should be stored at −80 °C and used within 3 months.

2% (vol/vol) hydrogen peroxide Dissolve 3.33 ml of 30% (vol/vol) hydrogen peroxide in DEPC-PBS to a final volume of 50 ml. ▲ CRITICAL This solution should be freshly prepared to maximize the activity of hydrogen peroxide, which is used to inactivate endogenous peroxidase to reduce background and minimize false-positive signals.

0.3% (vol/vol) Triton X-100 Add 150 µl of Triton X-100 to DEPC-PBS to a final volume of 50 ml. Vortex to make sure the Triton X-100 is completely dissolved. 0.3% (vol/vol) Triton X-100 may be stable at room temperature for more than 1 month; however, for our experiments, this solution was freshly prepared before use.

1 M Triethanolamine (pH 8.0) Mix 6.8 ml of triethanolamine with DEPC-H_2O_2 to a final volume of 50 ml. Adjust the pH to 8.0 with HCl. The solution can be stored at room temperature for more than 1 year.

Acetylation solution Add 5 ml of 1 M triethanolamine and 0.125 ml of acetic anhydride to DEPC-treated water to a final volume of 50 ml. ▲ CRITICAL The acetylation solution is stable only for a few minutes after the addition of acetic anhydride; therefore, the solution should be freshly prepared before use. The acetylation step is aimed at decreasing static charges in the tissue, thereby reducing nonspecific binding of labeling probes and minimizing background signals.

10 µg/ml RNase A solution Prepare 10 mg/ml RNase A stocks by dissolving RNase A in DEPC-H_2O. Store the solution at −20 °C, for up to 2 years. Dissolve 50 µl of 10 mg/ml RNase A stock in 2× SSC to a final volume of 50 ml, yielding 10 µg/ml RNase A solution. ▲ CRITICAL RNase A solution should be freshly prepared and prewarmed in the incubator at 37 °C to retain the activity of the RNase A. The working concentration of RNase A solution can be varied, but we recommend 10 µg/ml. The RNase A step is aimed at digesting the residual probes that do not bind the corresponding mRNA.

0.05% (vol/vol) PBST Add 500 µl of Tween 20 to 1 liter of DEPC-PBS and mix. 0.05% (vol/vol) PBST may be stable at room temperature for more than 1 month; however, for our experiments we recommend freshly preparing this solution before use.

10% (vol/vol) NGS blocking buffer Add 3 ml of inactivated NGS to 0.05% (vol/vol) PBST to a final volume of 30 ml. ▲ CRITICAL The NGS must be inactivated by being incubated in a water bath at 60 °C for 15 min; otherwise, the complement components in the serum may affect the binding of antibodies to their antigens. 10% (vol/vol) NGS should be freshly made before use.

10% (wt/vol) NaN_3 Dissolve 5 g of NaN_3 in 1× PBS to a final volume of 50 ml. 10% (wt/vol) NaN_3 can be stored at room temperature for a few years. ! CAUTION NaN_3 is a toxic hazard and should be used in a fume hood while wearing protective equipment.

2% (wt/vol) NaN_3 Mix 2 ml of 10% (wt/vol) NaN_3 with 8 ml of 1× PBS. ▲ CRITICAL 2% (wt/vol) NaN_3 should be freshly made before use. The NaN_3 step is aimed at inactivating the peroxidase activity induced by the previous mRNA detection steps, thereby eliminating false-positive protein signals.

TNB blocking buffer Dissolve 0.25 g of blocking reagent (available in the TSA kits) in 0.1 M Tris-HCl (pH 7.5) to a final volume of 50 ml. Add NaCl to a final concentration of 0.15 M. It can be stored at −20 °C for more than 1 year. ▲ CRITICAL The blocking reagent should be added slowly to the buffer while the buffer is gradually heated to 55 °C.

TNT wash buffer Dissolve NaCl in 0.1 M Tris-HCl (pH 7.5) to a final concentration of 0.15 M. Add 500 µl of Tween 20 to 1 liter of the buffer and mix. The solution can be stored at room temperature for more than 1 year.

Cyanine 3 (Cy3)-Tyramide staining buffer Dissolve one tube of Cy3-tyramide powder in 150 µl of DMSO and store as stock solution at 4 °C. Before use, prewarm Cy3 stock solution and amplification diluents (provided in the TSA kit) at 37 °C and at room temperature, respectively, for 10 min. Dilute the Cy3-tyramide using amplification diluents to 1% (vol/vol). ! CAUTION Work under a fume hood with DMSO. ▲ CRITICAL The final solution should be freshly prepared. All the components should be pre-warmed because the freezing point of DMSO is 18.5 °C.

Coumarin–tyramide staining buffer Dissolve one tube of coumarin–tyramide powder in 300 µl of DMSO and store as a stock solution at 4 °C for up to 3 months. Prewarm the stock and amplification diluents (provided in the TSA kits) in the same manner as Cy3 before use. Dilute the coumarin–tyramide solution by 150-fold (vol/vol) using amplification diluents. ! CAUTION Work under a fume hood with DMSO. ▲ CRITICAL The final solution should be freshly prepared. All the components should be prewarmed because the freezing point of DMSO is 18.5 °C.

EQUIPMENT SETUP

Water bath for hybridization Clean the inside of the water bath carefully with RNaseZAP before use. Refill the water bath with dH_2O and set the temperature to 60 °C (**Fig. 5b**). ▲ CRITICAL The water bath should be cleaned each time before hybridization; the inside of the water bath should be carefully cleaned with RNaseZAP to minimize RNase contamination. Wash out the RNaseZAP with dH_2O several times before refilling the water bath.

Shakers The orbital shaker used at room temperature should be set to 80 r.p.m. The orbital shaker used at 4 °C should be set to 40 r.p.m. The decoloring shaker used at room temperature should be set to 10 r.p.m.

Water-jacket incubator Set the temperature of the water-jacket incubator to 37 °C.

PROCEDURE

Mouse stimulation and tissue preparation ● TIMING at least 3 d

▲ CRITICAL This section of the protocol takes at least 3 d, depending on experimental design and the volume of the sample. The example given here takes 3d.

1| Divide the mice into two per cage 4 d before stimulation to ensure they can be processed and sacrificed simultaneously, to avoid cross-influence on cagemates.

! CAUTION Any experiments involving live mice must conform to relevant institutional and national regulations. All procedures discussed here were approved by the IACUC of the Institute of Neuroscience, Chinese Academy of Sciences.

2| Apply the first stimulus to the mice. For illustration, we use our previously published TAI-FISH paper as an example[5] in which morphine and foot shocks were used as first and second stimuli, respectively. Inject the mice with 15 mg/kg morphine i.p. In addition, perform a 'no-stimuli' control on a parallel group of mice, in which mice are injected with an equal volume of saline. Return the injected mice to their cages and wait for 350 min, when foot shocks are to be applied.

▲ CRITICAL STEP The 'no-stimuli' control is to control for c-fos protein induced by the injection procedure. See 'Controls to be performed with TAI-FISH' section in 'Experimental design' for detail.

▲ CRITICAL STEP We strongly encourage first-time TAI-FISH users to repeat this morphine–foot shock TAI-FISH application to the NAc as a positive control to validate their experimental setup. See 'Controls to be performed with TAI-FISH' section in 'Experimental design' for detail.

▲ CRITICAL STEP Choosing the optimal temporal interval, i.e., the waiting period, between the two stimuli is essential to the success of TAI-FISH. The appropriate time interval varies depending on stimuli and brain region examined, and should be

carefully tested by a 'single-stimulation' TAI-FISH before the double-stimulation TAI-FISH. The experimenter should sacrifice single-stimulated animals at a series of time points (for instance, start at 90–120 min after the stimulation and continue for 3–6 h) and perform TAI-FISH to visualize the IEG protein and mRNA signals induced by the stimulus. The time point at which the IEG's protein level induced by the first stimulus remains high, but its mRNA has decayed, is optimal for application of the second stimulus. See 'Before TAI-FISH: determine the time course of stimulations' section in 'Experimental design' for detail.
▲ **CRITICAL STEP** The incubation time between two stimuli should be followed exactly.

3| Apply the second stimulus to mice. For example, place the morphine-injected mice into a fear conditioning cage and give ten random foot shocks in a window of 10 min. Each foot shock should last for 2 seconds, with the shock intensity set to 1 mA.

For no-stimuli control, place the saline-injected mice in the fear conditioning cage for 10 min without foot shocks.
▲ **CRITICAL STEP** The 'no-stimuli' controls are placed in the fear conditioning cage to control for *c-fos* mRNA induced by experiencing the cage as a novel environment. See 'Controls to be performed with TAI-FISH' section in 'Experimental design' for detail.

4| Return the mice to their cage and wait till sacrifice. In this example, the mice are anesthetized immediately after being taken out from the foot-shock chamber. Anesthetize mice rapidly with 10% (wt/vol) chloral hydrate (i.p. injection) and perfuse them transcardially with ice-cold DEPC-PBS and 4% (wt/vol) PFA sequentially[42]. Dissect the brains and postfix them for 48 h in the same fixative solution at 4 °C.
▲ **CRITICAL STEP** For the second stimulus, it is essential to use 'single-stimulation' TAI-FISH experiments to determine the time point to sacrifice animal after stimulus is given: at a series of time points (usually 10, 15 and 30 min) after the single stimulation, the experimenter should sacrifice the stimulated animals and perform TAI-FISH. The time point when the second stimulus-induced IEG mRNA level is high, but the IEG protein has not emerged, is optimal for sacrifice. See 'Before TAI-FISH: determination of the time course of stimulations' section in 'Experimental design' for detail.
▲ **CRITICAL STEP** The waiting time between the second stimulus and sacrifice may vary depending on the stimuli and the brain structures, but it should be optimized and strictly enforced. This is because IEG protein induced by the second stimulus may emerge minutes after the optimal time point of sacrifice. Also, IEG mRNA induced by the second stimulus may not be sufficiently high minutes before the optimal time point. Both situations will complicate TAI-FISH results.
▲ **CRITICAL STEP** To minimize mRNA degradation and variation between samples, the mice should be sacrificed within 10 seconds after the termination of experiment. Two cagemates should be treated in parallel and perfused rapidly by two people side by side. Perfusion should be completed within 10 min.

5| Cryoprotect the brains by incubating them in 10 ml of 30% (wt/vol) sucrose in DEPC-PBS in a 6-well cell culture plate. After the brains sink to the bottom of the container (it usually takes 1 d at room temperature or 2 d at 4 °C), embed them in OCT and freeze them at −80 °C for future use (see 'The Art of Frozen Tissue Sectioning' for details (http://www.leicabiosystems.com/pathologyleaders/the-art-of-embedding-tissue-for-frozen-section/)).
▲ **CRITICAL STEP** Maintain an RNase-free environment by using RNase-free 6-well cell culture plates and cleaning the surface of the bench with RNaseZAP.
■ **PAUSE POINT** The brain samples can be stored at −80 °C for several months.

6| Cut the fresh frozen brain sections at a thickness of 40 µm on a cryostat at −20 °C. (see 'The Art of Frozen Tissue Sectioning' for details (http://www.leicabiosystems.com/pathologyleaders/the-art-of-embedding-tissue-for-frozen-section/)). Collect the brain sections in 6- or 12-well cell culture plates containing 10 ml (for 6-well cell culture plates) or 4 ml (for 12-well cell culture plates) of DEPC-PBS.
▲ **CRITICAL STEP** Maintain an RNase-free environment when carrying out all of the sectioning process. Clean all surfaces and equipment with RNaseZAP, wear a mask, gloves and a cap.
■ **PAUSE POINT** Free-floating brain sections can be left at 4 °C for no more than 1 week before being processed for TAI-FISH.

Treatments before prehybridization ● TIMING ~1.5 h
▲ **CRITICAL** Maintain the sections in the 12-well cell culture plate during all subsequent steps and transfer them using a small Chinese brush during the prehybridization (Step 13), hybridization (Steps 14), blocking (Steps 22 and 27), antibody incubation (Steps 23, 28 and 31), HRP–streptavidin incubation (Step 33), NaN_3 incubation (Step 29) and Cy3 or coumarin incubation (Steps 25 and 35) steps. In all other steps, the sections are kept in the 6-well cell culture plate. Manipulations before the end of the hybridization process must be performed using RNase-free buffers and in RNase-free conditions (clean the surface of bench, gloves, pipette and tip of the Chinese brush with RNaseZAP; wear mask, gloves and cap), in order to prevent RNase-mediated degradation of riboprobes and mRNA.

7| Wash the brain sections in 10 ml of DEPC-PBS for 10 min at room temperature on an orbital shaker at 80 r.p.m.

8| Incubate the sections with 6 ml of 2% (vol/vol) H_2O_2 (see Reagent Setup) for 10 min at room temperature on an orbital shaker at 80 r.p.m.
▲ **CRITICAL STEP** 2% (vol/vol) H_2O_2 solution must be freshly prepared.

9| Wash the sections in 10 ml of DEPC-PBS for 10 min at room temperature on an orbital shaker at 80 r.p.m.

10| Permeabilize the sections in 6 ml of 0.3% (vol/vol) Triton X-100 (see Reagent Setup) for 20 min at room temperature on an orbital shaker at 80 r.p.m.

11| Incubate the sections with 6 ml of acetylation solution (see Reagent Setup) for 10 min at room temperature on an orbital shaker at 80 r.p.m.
▲ **CRITICAL STEP** Acetylation solution should be added immediately before section treatment.

12| Wash the sections twice in 10 ml of DEPC-PBS, for 10 min each, at room temperature on an orbital shaker at 80 r.p.m.

Prehybridization ● TIMING ~1 h
13| Incubate the sections in 1–1.5 ml of prehybridization solution (see Reagent Setup) for 1 h in a water bath set to 60 °C.
▲ **CRITICAL STEP** The volume of prehybridization solution used in each well is dependent on the number of sections; ensure that all sections are well immersed in the prehybridization solution: use 1.5 ml of prehybridization solution for up to 15 normal-size mouse brain sections (e.g., coronal sections containing the NAc and striatum). If there are more than 15 brain sections, use multiple wells in a 12-well cell culture plate, or switch to a 6-well cell culture plate and add at least 2.5 ml per well instead.
▲ **CRITICAL STEP** The water level of the water bath should be kept below the cover of the 12-well cell culture plate to prevent contamination by water from the bath (**Fig. 5c**).
▲ **CRITICAL STEP** The 12-well cell culture plate should be carefully clamped with binder clips, one on each side, to avoid contamination and solution evaporation (**Fig. 5c**).

Hybridization ● TIMING ~16–20 h
14| Incubate the sections in 1–1.5 ml of hybridization solution containing the corresponding probe for 16–20 h at 60 °C in a water bath.
▲ **CRITICAL STEP** The volume of prehybridization solution used in each well is dependent on the number of sections; ensure that all sections are well immersed in the prehybridization solution: use 1.5 ml of prehybridization solution for up to 15 normal-size mouse brain sections. If there are more than 15 brain sections, use multiple wells in a 12-well cell culture plate, or switch to a 6-well cell culture plate and add at least 2.5 ml per well instead.
▲ **CRITICAL STEP** The water level of the water bath should be kept below the cover of the 12-well cell culture plate to prevent contamination by water from the bath (**Fig. 5c**).
▲ **CRITICAL STEP** The 12-well cell culture plate should be clamped with binder clips. Manipulate with great care to avoid contamination.

Post-hybridization washes ● TIMING ~2 h
15| Rinse the sections in 10 ml of 2× SSC solution (see Reagent Setup).
▲ **CRITICAL STEP** The 6-well cell culture plate containing 2× SSC solution should be pre-heated in a water bath for ~20 min before use.

16| Wash the sections twice in 10 ml of 2× SSC solution, for 15 min each, at 60 °C in a water bath.

17| Incubate the sections in 6 ml of 10 µg/ml RNase A solution (see Reagent Setup) for 30 min at 37 °C in an incubator.
▲ **CRITICAL STEP** RNase A solution should be freshly prepared and prewarmed in an incubator at 37 °C to maximize its activity.

18| Rinse the sections in 10 ml of 0.2× SSC solution (see Reagent Setup).
▲ **CRITICAL STEP** The 6-well cell culture plate containing 0.2× SSC solution should be preheated in a water bath for ~20 min before use.

19| Wash the sections twice in 10 ml of 0.2× SSC solution, for 30 min each, at 60 °C in a water bath.

Riboprobe detection ● TIMING ~1 d

20| Rinse the sections in 10 ml of 0.05% (vol/vol) PBST solution (see Reagent Setup).

21| Wash the sections twice in 10 ml of 0.05% (vol/vol) PBST solution, for 10 min each, at room temperature on an orbital shaker set to 80 r.p.m.

22| Incubate the sections in 10% (vol/vol) NGS blocking buffer (see Reagent Setup) for 1 h at room temperature on a decoloring shaker set to 10 r.p.m.

23| Incubate the sections in 1.5 ml of anti-DIG-POD antibody solution (1:500 dilution in 10% (vol/vol) NGS blocking buffer) overnight at 4 °C on an orbital shaker set to 40 r.p.m.

24| The next morning, wash the sections three times in 10 ml of 0.05% (vol/vol) PBST, for 10 min each, at room temperature on an orbital shaker set to 80 r.p.m.

25| Incubate the sections in 1–1.5 ml of Cy3-tyramide staining buffer (see Reagent Setup) for 20min at room temperature.
▲ **CRITICAL STEP** Cy3-tyramide staining buffer should be freshly prepared and kept from bright light.
▲ **CRITICAL STEP** The volume of prehybridization solution used in each well is dependent on the number of sections; use 1.5 ml of prehybridization solution for up to 15 normal-size mouse brain sections; if there are more than 15 brain sections, use multiple wells in a 12-well cell culture plate, or switch to a 6-well cell culture plate and add at least 2.5 ml per well instead.

26| Wash the sections three times in 10 ml of 1× PBS, for 10 min each, at room temperature on an orbital shaker set to 80 r.p.m.

IHC and tyramide-based signal amplification for protein detection ● TIMING ~3 d

27| Incubate the sections in 1.5 ml of TNB blocking buffer (see Reagent Setup) for 1 h at room temperature on a decoloring shaker set to 10 r.p.m.

28| Incubate the sections in 1.5 ml of c-fos antibody solution (1:10,000 dilution in TNB blocking buffer; see Reagent Setup) overnight at 4 °C on an orbital shaker set to 40 r.p.m.

29| Incubate the sections in 1.5 ml of 2% (wt/vol) NaN_3 (see Reagent Setup) for 15 min at room temperature.
▲ **CRITICAL STEP** This step is critical to inactivating residual peroxidase activity introduced during the detection of mRNA. 2% (wt/vol) NaN_3 must be freshly prepared.

30| Wash the sections three times in 10 ml of TNT wash buffer, for 10 min each, at room temperature on an orbital shaker set to 80 r.p.m.

31| Incubate the sections in 1.5 ml of biotinylated goat anti-rabbit antibody solution (1:400 dilution in TNB blocking buffer) overnight at 4 °C on an orbital shaker set to 40 r.p.m.
▲ **CRITICAL STEP** Biotinylated goat anti-rabbit antibody solution should be freshly prepared.

32| The next morning, wash the sections three times in 10 ml of TNT wash buffer, for 10 min each, at room temperature on an orbital shaker set to 80 r.p.m.

33| Incubate the sections in 1.5 ml of HRP–streptavidin solution (1:800 dilution in TNT) for 1.5 h at room temperature on a decoloring shaker set to 10 r.p.m.
▲ **CRITICAL STEP** HRP–streptavidin solution should be freshly prepared.

34| Wash the sections three times in 10 ml of TNT, for 10 min each, at room temperature on an orbital shaker set to 80 r.p.m.

35| Incubate the sections in 1–1.5 ml of coumarin–tyramide staining buffer (see Reagent Setup) for 20 min at room temperature.

▲ **CRITICAL STEP** The volume of staining buffer used in each well is dependent on the number of sections; ensure all sections are well immersed in the prehybridization solution: use 1.5 ml of prehybridization solution for up to 15 normal-size mouse brain sections. Use multiple wells in a 12-well cell culture plate, or switch to a 6-well cell culture plate and add at least 2.5 ml per well instead.
▲ **CRITICAL STEP** Coumarin–tyramide staining buffer should be freshly prepared and kept away from bright light.

36| Wash the sections three times in 10 ml of TNT, for 10 min each, at room temperature on an orbital shaker set to 80 r.p.m.

Slide mounting and imaging ● TIMING variable depending on volume of sample
37| Transfer the sections to Superfrost Plus slides (Fisher Scientific) using a small Chinese brush. Place drops of glycerol on the slide and seal it with a coverslip.
▲ **CRITICAL STEP** Fluorescent signals deteriorate over time. Image the samples as soon as possible after mounting.
■ **PAUSE POINT** Brain sample slides can be stored in the dark at 4 °C for 1 week.

38| Image the slides using a confocal microscope (e.g., Olympus Fluoview FV1000) with a 10× objective lens. Cy3 (reporting *c-fos* mRNA) and coumarin (reporting c-fos protein) fluorescent signals can be visualized by using 546-nm and 405-nm laser wavelengths, respectively. Take single-layer images with a 2,048 × 2,048-pixel size for data analysis.
▲ **CRITICAL STEP** Use the same image-capture parameters for parallel experimental groups.

39| Process the images and analyze the data blindly by coding images with random numbers generated by Excel. Process the control and experimental groups in parallel. Adjust the brightness and the contrast of the whole image with image processing software (e.g., Olympus Fluoview FV1000 v2.1b). Display processed images with the same amplification magnitude for manual signal counting. The c-fos protein signals should appear in the nucleus, be solidly round- or oval-shaped, and 6–10 μm in diameter. Typical *c-fos* mRNA signals appear in the cytoplasm as particle clusters of 8–14 μm in diameter. Determine the background signal by the mean gray value of an unlabeled brain region within the microscopic field. The intensities of the mRNA and protein signals are usually at least 80-fold greater than that of the background value. The c-fos protein and mRNA signals are counted in their respective single channels, and colabeling is determined by overlap in the merged channel.
? TROUBLESHOOTING

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
39	Few or no mRNA signal detected	The sample mRNA has degraded	Clean the water bath with RNaseZAP before use, seal the 12-well cell culture plate to prevent bath-water from leaking in. Keep the water level in the water bath below the cover of the cell culture plate during prehybridization and hybridization
		An unsuitable hybridization temperature was used	The hybridization temperature may vary for different riboprobes because of their various denaturation temperatures[43]. Thus, the hybridization temperature should be calculated and optimized if a different riboprobe is to be used Check the temperature of the water bath before sample incubation Fill the water bath with an appropriate volume of water to keep the temperature stable
		The detection reagents have degraded	Ensure that the anti-DIG-POD and/or tyramide reagents are active
		The signal deteriorates over time	After mounting, image the samples immediately because fluorescent signals deteriorate over time
	Low or no protein signals are being detected	Overfixation of the brain sections	Limit fixation to 48 h, as overfixation may decrease the binding of the primary antibody to its antigen

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
39		The sections have not been permeabilized properly	Use 0.3% (vol/vol) Triton X-100 detergent in the blocking buffer to allow full permeabilization of antibody into tissue sections
		Inappropriate primary and/or secondary antibodies were selected	Confirm that the primary and secondary antibodies are compatible by checking the species reactivity Confirm that the antibodies are effective in a positive control, such as morphine–foot shock-stimulated NAc. IHC antibodies and DIG antibody can be tested by single IHC and standard FISH, respectively. IEG antibodies should be further tested by performing a standard TAI-FISH procedure, because some *c-fos* antibodies suitable for standard IHC may not work after FISH treatment
		The antibody concentration was not optimal	Determine the optimal working concentration for primary antibody by titration
		Antibody incubation time was insufficient	Increase incubation time
		The IHC signal deteriorated over time	After mounting, image the samples immediately
	Non-specific and/or high background staining was observed	The acetylation solution was not fresh	The acetylation solution should be freshly prepared before use, as it will be stable only for a few minutes after acetic anhydride is added
		The hybridization and/or post-hybridization conditions were not optimized	Increase the hybridization temperature Increase the post-hybridization wash temperature Decrease the SSC concentration in the washing steps Ensure that the RNase A is active by running a digestion test on standard RNA samples; ensure sections are incubated for a sufficient time with RNase A at 37 °C
		Insufficient blocking time	Ensure that the samples are incubated in blocking buffer for at least 1 h before adding antibodies
		Immunohistochemical detection of digoxigenin (DIG)-labeled probes is not optimal	Ensure that the anti-DIG-POD is used in the optimal concentrations Incubate all primary antibodies overnight at 4 °C, as room temperature incubation increases unspecific binding and causes higher background
		Insufficient washing	Increase the washing time to sufficiently remove non-specifically bound antibodies
	False-positive protein signals are observed	Insufficient inactivation of peroxidase from previous mRNA detection steps	Increase the incubation time in H_2O_2 or NaN_3 solution during peroxidase inactivation steps Ensure that the H_2O_2 or NaN_3 solution is freshly made

● TIMING

Steps 1–6, mouse stimulation and tissue preparation: at least 3 d, depending on experimental design and the volume of sample (3 d for the example given here)
Steps 7–12, treatments before prehybridization: ~1.5 h
Step 13, prehybridization step: ~1 h
Step 14, hybridization step: ~16–20 h
Steps 15–19, post-hybridization washes: ~2 h
Steps 20–26, riboprobe detection: ~1 d

Steps 27–36, IHC and tyramide-based signal amplification for protein detection: ~3 d
Step 37–39, slide mounting and imaging: variable, depending on volume of sample

ANTICIPATED RESULTS

In this protocol, *c-fos* mRNA and protein are detected by Cy3-tyramide and coumarin–tyramide, respectively. Therefore, fluorescent signals can be visualized under a confocal microscope (e.g., Olympus Fluoview FV1000) by using 546-nm (for Cy3) and 405-nm (for coumarin) laser wavelengths. The c-fos protein signals showed a nuclear staining pattern, being solidly round or oval-shaped and having a diameter of 6–10 μm (**Fig. 1b**). *c-fos* mRNA signals appear mostly in the cytoplasm, being typically particle clusters of 8~14 μm in diameter. Individual neurons labeled using TAI-FISH are illustrated in **Figures 1b** and **3a**.

TAI-FISH will likely identify neural ensembles activated by two different stimuli to be organized in three distinct patterns (**Fig. 3b**) depending on the brain structure: convergent, in which a substantial portion of TAI-FISH-labeled neurons are labeled by both FISH and IHC, demonstrating that these neurons are activated by both stimuli; segregated, in which the two ensembles are located in two different subregions of a brain structure and share little common area; and intermingled, in which the two ensembles are located within the same region of a brain structure, but, despite the spatial proximity, few of these neurons are activated by both stimuli. A convergent pattern indicates that the two stimuli share at least part of the circuits they activate individually, whereas a segregated or an intermingled pattern suggests that the neural ensembles activated by the two stimuli most likely belong to two distinct neural circuits. Furthermore, a segregated pattern implies that a brain structure with this pattern may have functionally distinct subregions, whereas an intermingled pattern may indicate intricate interactions between those functionally different, but spatially mixed, neurons.

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