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Fast Fos: rapid protocols for single- and double-labeling c-Fos immunohistochemistry in fresh frozen brain sections

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Abstract

Immunohistochemical localization of c-Fos immunoreactivity has been used successfully for over a decade to visualize patterns of neuronal activity in the brain and spinal cord. These experiments are extremely useful in identifying physiological or pharmacological activation of specific populations of neurons. Unfortunately, conventional c-Fos immunohistochemical protocols are very time and resource intensive. We have adapted and optimized established c-Fos immunohistochemistry (IHC) methodologies for use with fresh frozen brain tissue mounted directly onto slides. The resultant rapid protocols, which we refer to as "Fast Fos", include applications for single- and double-labeling, utilizing either enzyme–substrate or fluorescent detection systems. These protocols provide increased assay throughput and reproducibility, which can be further enhanced by use of an automated slide stainer. Taken as a whole, the c-Fos IHC protocols described in this report provide a flexible system for the identification of neuronal activation that substantially reduces time and resource expenditure while increasing quality and reproducibility of data.

Keywords: Immunocytochemistry; Immunohistochemistry; Cryostat; c-Fos; Autostainer; Frozen; Peroxidase; Immunofluorescence; Double-labeling

1. Introduction

The rapid and transient induction of the immediate early gene c-Fos in response to diverse pharmacological and physiological stimuli can be utilized to produce high-resolution maps of cellular activation in the central nervous system (Dragunow and Faull, 1989; Herdegen and Leah, 1998; Herrera and Robertson, 1996; Morgan and Curran, 1991; Sheng and Greenberg, 1990). The immunohistochemical localization of c-Fos protein has been used successfully for over a decade to visualize patterns of neuronal activity in the brain and spinal cord (Chaudhuri, 1997; Chaudhuri et al., 2000; Hughes and Dragunow, 1995; Hyman et al., 1993; Sharp et al., 1993; Smith and Day, 1993). Double-labeling studies that combine c-Fos immunohistochemistry (IHC) with localization of a second antigen further expand the utility of c-Fos mapping by allowing examination of activated neurons within a neurochemically defined group of cells (Hoffman and Lyo, 2002; Kovacs, 1998; Mikkelsen et al., 1994). Review of previously published protocols for c-Fos IHC reveals the conventional methodologies to be quite time and resource intensive (Cohen et al., 2003; Conde et al., 1995; Deutch et al., 1991; D'Hondt et al., 1999; Hughes et al., 1992; Ishida et al., 2002; Leman et al., 2000; Lin et al., 1998; Sebens et al., 1998; Smith and Day, 1993).

In an effort to improve the ease and utility of c-Fos IHC, we undertook a step-wise optimization of each protocol stage from tissue harvest to coverslipping. This methodical approach allowed us to increase speed in some areas while improving reproducibility in others; resulting in an efficient, streamlined protocol for c-Fos IHC that increases throughput without sacrificing accuracy. The resulting rapid protocols, which we refer to as "Fast Fos", are described in the present report. These protocols can be performed with standard laboratory equipment or with an autostainer (DakoCytomation, Carpinteria, CA), which provides further advantages in assay throughput and reproducibility. Subsequent application of this methodology to a wide variety of studies required adaptations to allow for both enzyme–substrate and fluorescent visualization of both single- and double-labeled tissue.

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This report offers detailed instructions for each adaptation of the protocol for both manual and autostainer applications along with subsets of data from studies carried out using these protocols.

2. Materials and methods

The purpose of this report is to describe the "Fast Fos" protocols for immunohistochemical localization of c-Fos in fresh frozen tissue. A complete discussion of the details of individual experiments is therefore beyond the scope of this communication. For illustrative purposes, we present examples of staining from several assays in which these protocols were employed. Briefly, antipsychotic drugs (APD) have been shown to induce c-Fos in brain regions such as the prefrontal cortex, nucleus accumbens and dorsolateral striatum in a characteristic pattern or profile, and the differences in these profiles have been utilized as a means of distinguishing between classes of APDs (Ananth et al., 2001; Robertson and Fibiger, 1996; Robertson et al., 1994). Further, it has been shown that APDs associated with weight gain induce c-Fos in orexin-containing cells of the lateral hypothalamus (Fadel et al., 2002). We have used the APD olanzapine to illustrate the application of the protocols outlined in the present paper (see Section 2.2 for details of drug administration).

2.1. General guidelines

All steps should be carried out at room temperature (18–22 °C) unless otherwise specified. Antibody dilutions are provided as a guideline; optimal dilution of individual antibodies should be determined by the protocol user and re-evaluated periodically as antibody efficacy changes with age and lot number. These protocols were developed using conventional bench-top IHC techniques. All washes, fixation, peroxidase blocking and dehydration steps were carried out using metal slide racks in glass staining dishes (Fisher Scientific, Pittsburgh, PA). All washes were carried out under gentle agitation for 5 min and repeated three times in either buffer or de-ionized water as specified (when using an autostainer, this was replaced with two "wash" steps). Wash buffer consists of 50 mM Tris–HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.6, available as a concentrate from DakoCytomation and referred to in this report as TBS-T. This buffer was also used in diluting all reagents, except when using commercial kits, which were used according to the manufacturer's instructions. All antibodies were diluted in "antibody diluent", which consisted of TBS-T with 1% (weight by volume) fatty acid-free bovine serum albumin Fraction V (Calbiochem, San Diego, CA) as a general protein block. Incubations were carried out on flat slides in a humid chamber (Thermo Shandon, Pittsburgh, PA) and required 150–500 μl of reagent per slide to adequately cover the sections, depending upon the surface area of the tissue. After the protein blocking steps, excess reagent was carefully suctioned off by tipping the slide and applying gentle suction to a tissue-free area.

For convenience and repeatability, an autostainer can be used to perform c-Fos IHC. As automated staining is not an option for all researchers, protocols presented herein are suited for conventional bench-top IHC with asides for autostainer applications. The flowcharts shown in Fig. 1 provide schematics for each protocol and can be used as a guide for autostainer programming.

2.2. Tissue preparation

Male Sprague–Dawley rats weighing 155–175 g (Harlan Sprague Dawley, Inc., Indianapolis, IN) were housed in groups of four in a 22 °C room with lights on from 07:00 to 19:00 h for 1 week prior to experimentation to allow them to acclimate to their new environment. Food and water were freely available. Olanzapine (Eli Lilly and Co., Indianapolis, IN) was prepared at a concentration of 5 mg/ml in a vehicle solution of 0.4% lactic acid and a dose of 5 mg/kg was injected subcutaneously. Control rats received vehicle injections. Animals ($n = 4$–6 per group) were sacrificed by decapitation 2 h following vehicle or drug administration. Whole brains were rapidly removed and immediately immersed in isopentane (2-methyl butane, Fisher Scientific) over dry ice for approximately 25 s then placed in vials buried in dry ice, and stored at −80 °C until sectioned (see complete discussion of freezing technique in Section 2.6.3). Coronal sections through the nucleus accumbens and lateral hypothalamus were cut at 16 μm and coronal sections of the striatum were cut at 16–50 μm in a cryostat (HM 500 OM; Microm, Walldorf, Germany) and thaw-mounted onto Superfrost Plus slides (Fisher Scientific). Sections were allowed to air-dry at room temperature until completely dry (several hours for 16 μm sections up to 12 h for thicker sections) and were stored at −20 °C until processed (if long-term storage is required, they can be stored at −80 °C).

All data presented in the present report is from rat tissue immunolabeled for c-Fos using the "Fast Fos" protocols. However, we have also successfully utilized these protocols to immunolabel c-Fos in sections of mouse brain (results not shown).

2.3. Fixation

Slides were brought to room temperature and a hydrophobic marking pen (Immedge Pen; Vector Laboratories, Burlingame, CA) was used to encircle the tissue to prevent reagent run-off (unnecessary when using autostainer). Slides were immersed in a freshly-prepared solution of 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in phosphate-buffered saline (pH 7.4; Invitrogen, Carlsbad, CA) for 10 min then washed several times in TBS-T. At this point, slides were treated according to individual protocols as described below.