# IMPROVEMENT IN THE EVALUATION OF FOS-NEUROPEPTIDE COLOCALIZATION BY EMPLOYING FREE-FLOATING CRYO-SECTIONS OF DELICATE THICKNESS: DOUBLE AND TRIPLE COLORED IMMUNOHISTOCHEMISTRY

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**Objective.** The present study was aimed to select a methodical approach to optimize the thickness of cryo-processed free-floating sections for precise recognition between a single Fos signal and Fos/neuropeptide colocalizations in sequential double or triple colored immunohistochemical stainings. For this purpose brain sections of variable (5-20 μm) thickness were tested utilizing enzyme–substrate detection system employing oxytocin (OXY) and vasopressin (AVP) antisera.

**Methods.** The animals were perfused by fixative 90 min after i.p. administration of 5 ml of hypertonic saline (1.5 M NaCl) which was used to stimulate the hypothalamic osmosensitive neurons. The brains were removed, soaked with 30% sucrose in 0.1 M PBS, cryo-sectioned throughout the hypothalamus into 5, 10, 15, and 20 μm thick coronal sections, collected and washed in 0.2 M glycine buffer for 10-15 min, and finely stored in 0.1 M PBS. Single Fos and Fos/OXY and Fos/OXY/AVP colocalizations were processed employing avidin-biotin-peroxidase (ABC) complex and diaminobenzidine chromogen with or without adding Nickel chloride salt as a black and blue color inducer. Evaluation of the Fos-neuropeptide co-labeled perikarya manifestation was performed on a computerized Leica light microscopy.

**Results.** The present data demonstrate that cryoprocessing enables generate free- floating sections of 5, 10, 15, and 20 μm thickness. Except the 5 μm thickness, all the other sections sizes tested exhibited well preserved tissue stability and excellent immunohistochemical properties either for single Fos reaction or double Fos/OXY and triple Fos/OXY/AVP costainings.

**Conclusions.** We adapted and optimized Fos immunohistochemistry for use of fixed and cryocut processed free-floating brain sections. The present data indicate that except 5 μm thickness all the other sorts of cryosections tested were sufficiently resilient for performing a sequential double or triple colored immunohistochemical stainings. However, 10 μm thickness reached the borderline of the handling safety, therefore, 15 μm section thickness will be the thickness of the choice recommended, which gave relevant immunoreaction, retained good tissue preservation, and ensured an appropriate clarity for accurate recognition between a single and colocalized Fos signals.

**Key Words:** Fos, immunohistochemistry, cryostat sections, oxytocin, vasopressin, double labeling, triple labeling, hypertonic saline, rat

During the last three decades, an enormous progress in applicative light microscopic immunohistochemistry has been achieved (GIEPMANS et al. 2006; MORIYA et al. 2006). Throughout these years, various immunohistochemical methods have been developed which have became available and now may be used to localize a broad spectrum of

tissue-bound antigens. Immunohistochemistry has not only been applied as a single antigen technique, but also as a complex of sequential immunoprocedures, i.e. double (Murdoch et al. 1990; Smith and Day 1993; Wurden and Homberg 1993; Tao et al.1994; Mikkelsen et al. 1994; Hunyady et al. 1996; Shindler and Roth 1996; Tornehave et al. 2000; Ino 2004), triple (Van der Loos et al. 1987; Kiss et al. 1988; Staines et al. 1988; Brouns et al. 2002), quadruple (Ferri et al. 1997) or even multiple (Tsurui et al. 2000) immunostainings where antigen-antibody interactions have been visualized by different markers such as fluorescent dyes (FITC, rhodamine), enzymes (peroxidase, alkaline phosphatase), or radioactive markers. Moreover, multiple immunofluorescence labeling in combination with confocal laser scanning microscopy has been shown to be a powerful strategy to visualize the spatial relationship between different antigens in individual cells (Adriaensen et al. 1998). Therefore, immunohistochemistry has become a crucial technique which today is widely used in many basic and medical research laboratories as well as in clinical diagnostic facilities (Pargaonkar et al. 2003; Oliveira and French 2005).

Activation of neurons by a variety of stimuli influences the level of expression of the immediate-early gene c-fos, and the protein of the gene product, Fos, regulates transcription of other genes via binding to other proteins in the AP-1 complex (Curran and Morgan 1995; Robertson et al. 1995). Therefore, Fos immunohistochemistry has become an approach successfully used for over a decade to visualize patterns of neuronal activity in the brain (Dragunow and Faull 1989; Smeyne et al. 1993; Laorden et al. 2000; Ueta et al. 2000; Hoffman and Lyo 2002; Pirnik et al. 2005) and spinal cord under different experimental conditions enabling to map neuronal activities throughout the whole brain or spinal cord (Herrera and Robertson 1996; Chaudhuri 1997; Lin et al. 1998) or to identify physiological or pharmacological impacts in a selected population of neurons (Pirnik and Kiss 2005). In addition, utilization of Fos in multiple immunohistochemistry increased its physiological significance since it made possible to reveal the physiological status of chemically defined groups of neurons in many areas of the brain and spinal cord. There exists a number of recommended immunohistochemical protocols dealing with the Fos visualization in context of its colocalization patterns (Mikkelsen et al. 1998; Sundquist and Nisenbaum 2005). Published protocols for immunohistochemistry are diverse, however, one of the most reproducible and convincing results have been shown employing free floating cryostat sections (Nitsch and Klauer 1989). However, in some densely packed neuronal conglomerations, e.g. in the hypothalamus or brain stem, since the frequent cell overlappings, it is often difficult to perform a clear differentiation between the single Fos and Fos-neuropeptide co-labelings on commonly used 40 μm thick free-floating cryocut sections. In this study an effort was done to reveal options to optimize the well established Fos immunohistochemistry methodology for its use in multiple as much as thinner free floating sections and to verify its handling capability during their processing and its usefulness in the processing double and triple Fos-neuropeptide immunohistochemistry.

## Materials and Methods

**Animals.** Adult male Wistar rats (Charles River Wiga, Silzfeld, Germany), weighing 260-280 g were used. The animals were housed, four per cage, in a temperature-controlled room with controlled light (14 h/day), humidity (55 %) and temperature (23 °C). They were kept under a regular rat chow (dry pellets) and tap water available *ad libitum.* Principles of laboratory animal care and the experimental procedures used have been approved by Animal Care Committee of the IEE SAS Bratislava, Slovak Republic. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

**Hyperosmolality.** Ninety min before sacrificing, the animals were injected i.p. by 5 ml of hypertonic (1.5 M) saline and kept in home cages without access to water. Then the animals were deeply anesthetized with sodium pentobarbital (50 mg/kg ip, bodyweight) and perfused with 50 ml of ice-cold isotonic saline containing 450 μl of heparin (5000 IU/l) followed by 150 ml of 0.1 M phosphate buffer (PB, pH 7.4) containing 4 % paraformaldehyde, 0.1 % glutaraldehyde and 10 % (w/w) picric acid at room temperature. The brains were removed from the skull and post-fixed in fresh fixative overnight. Thereafter, the brains were washed in 0.1 M PBS at 4 °C overnight, infiltrated with 30 % sucrose-PB, frozen in isopentane at – 35 °C, put into dry ice for 30 sec, and at the level of the hypothalami, sectioned into 5, 10, 15, and 20 mm thick coronal sections. During the cryoprocessing the tissue block temperature varied between -16 and -18 °C.

**Single Fos-immunohistochemistry.** The immunohistochemical procedure with 5, 10, 15, and 20 mm thick coronal sections was performed in glass vials (antibody incubations) or Netwell (ProSciTech) tissue processing baskets (washings). Glass sticks, instead of metal instruments, were used for handling of the delicate sections.

Free floating sections were repeatedly washed in cold 0.1 M PB followed by a preincubation with 3 % $H_2O_2$ in 0.1 M PB (Fisher Scientific, Fair Lawn, NJ, USA) for 30 min at room temperature. Thereafter the sections were rinsed 3x 10 min in 0.1 M PB. Then the sections were incubated with a polyclonal Fos protein antiserum (1 : 2000, No 94012) diluted in 0.1 M PB containing 4 % normal goat serum (Gibco, Grand Island, NY, USA), 0.5 % Triton X-100 (Koch-Light Lab. Ltd.,Colnbrook Berks, England) and 0.1 % sodium azide (Sigma

Chemical Ltd. St. Louis MO, USA) for 48 h at 4 °C. After several rinsing in PB, the sections were incubated with biotinylated goat anti-rabbit IgG (1: 500, VectorStain Elite ABC Kit, Vector Lab., Burlingame, CA, USA) in PB for 2 h at room temperature. Next PB rinsings were followed by incubation with the avidin-biotin peroxidase complex (1: 250) for 2 h at room temperature. After several washings in 0.05 M sodium acetate buffer (SAB) (pH 6.0), Fos antigenic sites were visualized on the sections by 0.02 % 3,3´-diaminobenzidine tetrahydrochloride (DAB, Sigma), DAB-Nickel (0.0625 % DAB, 2.5 % nickel ammonium sulfate, Sigma), in SAB containing 0.03 % hydrogen peroxide, for 6-10 min. The heavy metal-intensification of DAB yielded to black staining in the Fos labeled nuclei. Finally, the sections were either mounted into 1% of gelatine dissolved in 0.0125 M SAB, air-dried, coverslipped with Permount (Sigma), and examined under computerised Leica DMLS light microscope or they were further processed in double immunohistochemistry.

**Double immunohistochemistry.** Sections containing Fos labeled black cell nuclei were used for Fos/OXY colocalizations. After washing in PB the sections were incubated with OXY antiserum (diluted 1:2000) for 48 h at 4 °C. Likewise in the above mentioned procedure, the Fos labeled sections were, after completing the avidin-biotin peroxidase incubations, washed in 0.1 M PB. The PB rinsings were followed by several washings in 0.05 M SAB (pH 6.0), OXY antigenic sites on the sections were visualized by 0.02 % 3,3´-diaminobenzidine tetrahydrochloride (DAB, Sigma), DAB-Nickel (0.0625 % DAB, between 0.25- 0.50 % nickel ammonium sulfate, Sigma), in SAB containing 0.03 % hydrogen peroxide, for 6-10 min. The lower concentration of nickel ammonium sulfate produced bluish color. Finally, the sections were either mounted into 1% of gelatine dissolved in 0.0125 M SAB, air-dried, coverslipped with Permount (Sigma), and examined under computerised Leica DMLS light microscope or they were processed in triple colored immunohistochemistry.

**Triple immunohistochemistry.** Sections containing Fos labeled black cell nuclei and blue colored OXY perikarya were, after washing in PB, exposed to AVP antiserum (diluted 1:2000) for 48 h at 4 °C (Kiss et al. 1988). Likewise in the previous two steps, the Fos/OXY labeled sections, after completing the avidin-biotin peroxidase incubations, were stained with a single DAB reaction in Tris buffer (pH 7.4) using the same approach as mentioned above. To reach the appropriate yellow-brown color for the third staining, the section DAB colorization was inspected under the light microscope. Immunostaining of negative control, which did not show any antiserum immunolabeling, included substitution of the primary antisera with normal rabbit serum, and sequential elimination of the primary or secondary antibody from the staining series.

**Antisera.** The polyclonal Fos antiserum (No 94012) was raised against the N-terminal peptide similar to 2-17 of the rat Fos protein according to the protocol described elsewhere (Woldbye et al, 1996; Mikkelsen et al, 1998). Vasopressin antiserum (VPC-2) was kindly provided by Dr. R.L. Eskay (NIH Bethesda, Maryland, USA) and OXY antiserum was purchased from Chemicon (Temicula, CA).

## Results

Hypertonic saline injection activated a number of neurons in the brain. Owing to this experimental stimulus, a number of small cell clusters or larger cells aggregations exhibited black Fos immunoreactivity. Especially the hypothalamic magnocellular neurons of the paraventricular (PVN), supraoptic (SON), and accessory (ACC) nuclei were distinctly stimulated by hypertonic saline (Fig. 1A,B,C).

All four categories of sections, i.e. 5, 10, 15, and 20 μm thickness, displayed Fos signals, however, the handling difficulties forced us to exclude the sections of 5 μm thickness from double and triple immunoprocessings. In addition, the sections of 5 μm thickness were difficult to mount on the microscopic glasses regardless even of they infiltration with 0.05 % of gelatine.

The sections of 10 μm thickness exhibited excellent single Fos immunoreactivity with appropriate immunodensity and Fos signal delineation (Fig. 1D,E). Although the handling properties of this sort of sections was acceptable, even entire coronal plates could be obtainable, many of them tend to make large folders with a tendency to stick together. In spite of certain difficulties, this set of sections could be processed for double and triple immunostainings and they were well accept-

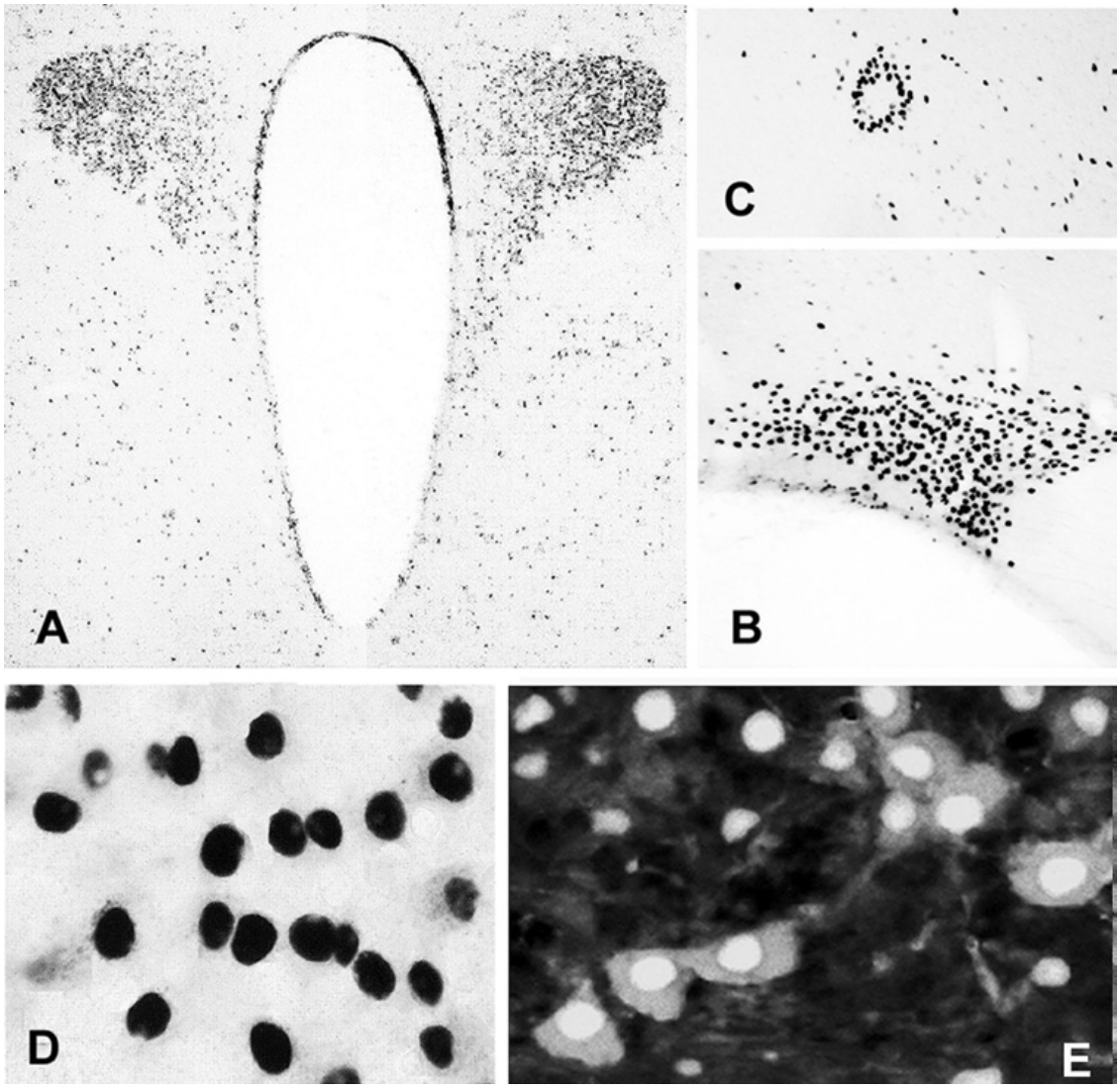


**Fig. 1 Single Fos protein immunostaining using Ni-DAB color inducers in different hypothalamic structures including PVN (A), SON (B), and the accessory circular nucleus (C) 90 min after injection of hypertonic saline. Detailed view on a single Fos (D) and Fos/OXY collabelings (E) in the SON of hypertonic saline treated rats. Thickness of the sections: A, B, C = 35 µm, D and E = 10 µm.**

able mainly in conditions when sections of lower size were used, for example, from the caudal brainstem or spinal cord tissue. The last two section thicknesses (15 and 20 µm) showed up as an excellent histological material for further double and triple immunoprocessings.

With respect to the goal of the study, i.e. to concentrate our attention to brain areas exhibiting densely packed cell perikarya with frequent overlappings and high background staining eventualities, the PVN and SON were selected. These structures showed up as an appropriate brain areas to test the handling strategies and staining capabilities of 10, 15, and 20 thick sections in the processing of double and triple Fos-neuropeptide immunohistochemistry.

For the double- and triple-immunohistochemical stainings, OXY and OXY/AVP synthesizing cells were selected because of their high sensitivity to hyperosmotic challenges (Pirnik and Kiss 2005). OXY immunoreactivity emerged in all the selected structures and in each of the three sort of the selected section thickness. The oxytocinergic perikarya were densely packed and well distinguishable one from another. Those perikarya which showed parallel Fos immunoreactive signal with OXY

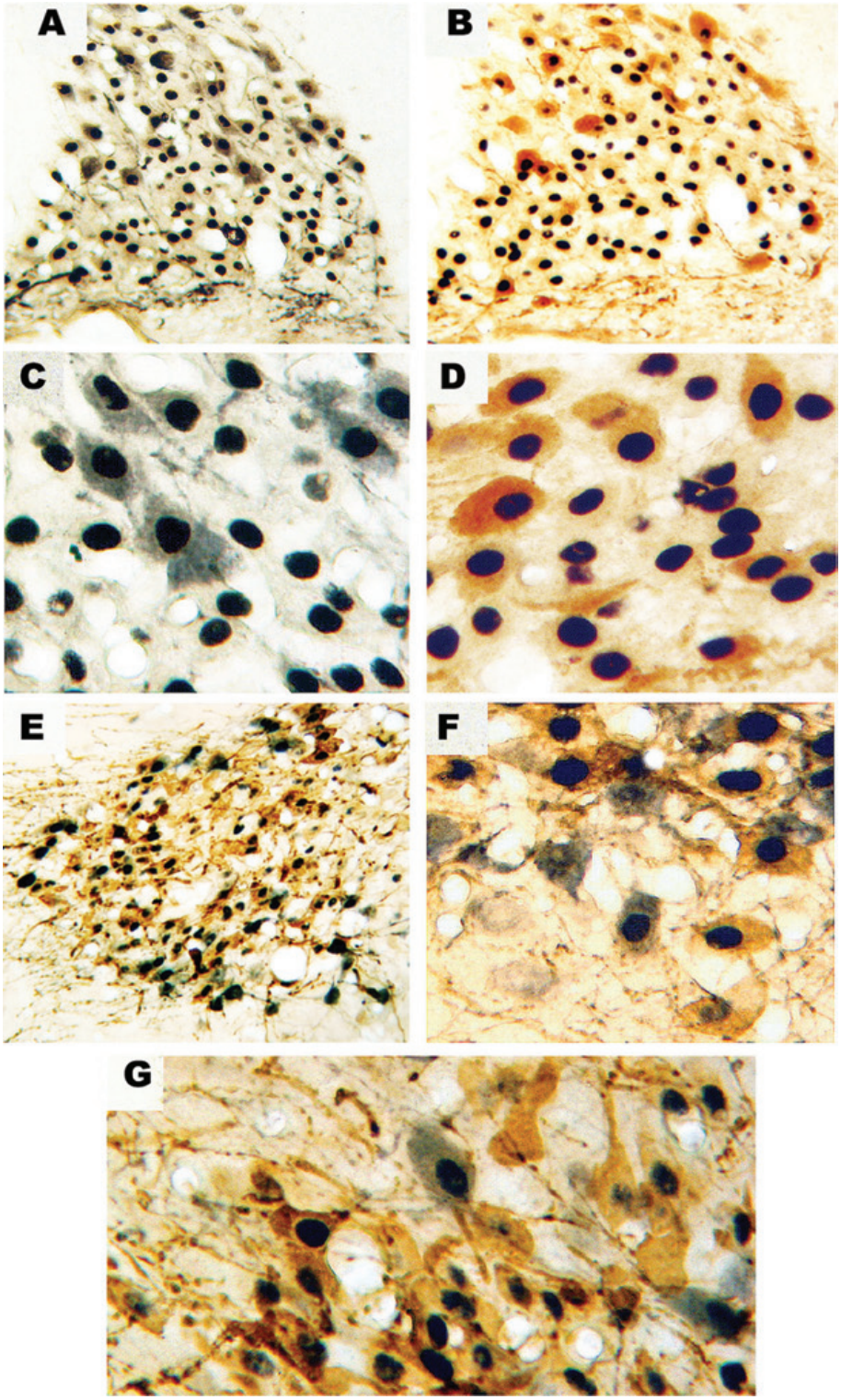


**Fig. 2 Fos evaluation in double (A, B, C, D) and triple (E, F, G) immunolabeled cells activated by hypertonic saline. Double Fos-OXY immunostaining of SON magnocellular neurons by using Ni-DAB combination (A, C) or a single DAB chromogen (B, D) application. Triple Fos/OXY/AVP immunostaining of PVN magnocellular neurons by using Ni-DAB (high Ni concentration) for black Fos protein labeling, Ni-DAB (low Ni concentration) for blue-gray OXY cells labeling, and single DAB for yellow-brown AVP cells labeling (E, F, G). Thickness of the sections: A, B, C, D = 10 µm, E, F, G = 15 µm.**

were well delineated and the Fos signal was well preserved. Microscopically, it was very easy to distinguish between the single Fos labelings and Fos-immunostaining within the OXY perikarya (Fig. 2 A,B,C,D).

In the last sequence of the immunolabelings, the Fos/OXY immunolabeled sections were immunoreacted with a vasopressin antiserum. Single DAB chromogen application elicited a third color in the triple immunosequence process and induced an yellow/brownish staining of vasopressinergic population of cells in all of the studied hypothalamic structures. After finishing the triple immunohistochemical procedure the presence of Fos signal was well distinguishable in both oxytocinergic and vasopressinergic perikarya in each of the 10, 15, and 20 μm sections (Fig. 2E,F,G).

## Discussion

The data of the present study indicate that free floating sections of 10 μm but not 5 μm thickness cut on a cryocut and fixed by our fixation solution are sufficiently resilient for performing a sequential double or triple colored immunohistochemical stainings, however, at the same time, they also indicate that benefit of the usefulness of 10 μm section thickness reach the borderline of the handling safety. To avoid the immunohistochemical processing failures and to reach more comfortableness in the immunohistochemical processing, the sections of 15 μm thickness are advisable to use since they allow a good Fos-neuropeptide colocalization performance and high quality of free Fos and Fos-neuropeptide colocalization differentiations.

Two types of cryocut sections, i.e. unfixed (fresh) and fixed (by immersion or perfusion), are generally used in biological studies: (1) quickly frozen unfixed sections, where the tissue block is first frozen and then cut and either air-dried or fixed prior to staining and (2) fixed frozen tissue, where the tissue is first fixed then cryoprotected with sucrose or other tissue stabilizers of cell structure prior to freezing and sectioning. The advantages of frozen sections are that they allow excellent antigen preservation, they are fast to perform, and they offer flexibility in optimization of fixative for each antigen. However, on the other hand, frozen sections may reveal less morphological details and structural resolution and dropout of some antigenicity.

A broad palette of tissues processed by vibratome (Hisano et al. 1994; Romijn et al. 1996; Horvath et al. 1997) or tissues soaked with sucrose and manufactured by cryocut (Newton et al. 2002; Regulier et al. 2003; Svensson et al., 2003) have widely been used in immunohistochemistry. Fixed cryocut sections are usually used in 35-50 μm thickness (Okere et al. 1999). Our experience indicated that although the immunoreactivity properties of cryocut sections are excellent there are also some difficulties in the evaluation of multiple immunostainings combined with Fos labeling: (1) it is difficult, mainly in the densely packed cell populations such as e.g. hypothalamic or brainstem nuclei, to clearly distinguish between the single Fos and colocalized Fos immunoreactivities due to the partial cells overlappings; (2) there is a high background staining streaming from the thickness of the 40-50 μm sections, i.e. lighting crossing the thicker tissue layer makes the whole tissue less transparent for microscopic evaluation. To avoid the above mentioned difficulties, the usefulness of thinner, i.e. of 5 - 20 μm thick, cryocut frozen sections was tested in the present study.

Our results clearly indicate that cryocut sections thicker than 10 μm are quite sufficient for performing a sequential double or triple colored immunohistochemical stainings, however, the best handling properties with free-floating sections were obtained using sections of 15 μm thickness. However, processing the single Fos signal and Fos/neuropeptide colocalization studies, to use the above mentioned delicate sized sections is a skill that is mastered over short time, and although it is not difficult to perform it, certain problems can occur and complicate the whole sectioning and staining procedures. To avoid of standard difficulties, the following steps in the tissue processing should be fulfilled: (1) the cryostat temperature should be stabilized between -16 °C and -18 °C; (2) the microtome knife should be well edge-sharped and during cutting regularly cleaned out of the tissue scraps to avoid of craks/splittings/scratches and other cutting spoilages; (3) buffer for the collection of cold sections should be osmotically well balanced to avoid tissue creasing and swelling; and (4) the sections should be avoided of frequent handlings. A special attention must be taken to the section transferring procedures which should be carried out by only glass sticks having a small ball-like edge to avoid of physical damages and damagaes caused by adhesion of sections induced by static electricity, typical, for example, for sodium acetate buffer. Handling and transferring the sections during the whole immunoprocessing should be carried out in special, i.e. tissue specimen baskets.

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