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HISTOCHEMISTRY - A TOOL FOR PRIMARY IMAGE FORMATION IN NEUROBIOLOGY

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ABSTRACT

Neurohistochemical techniques are presented with the aim to form an introduction to the neurobiology section of the IV-ESS proceedings. Detailed protocols with comments of the Falck-Hillarp formaldehyde fluorescence methodology and the indirect immunofluorescence technique of Coons and collaborators, respectively, are given to enable a clear understanding of the basis for image formation in certain types of neurobiological investigations.

Keywords: Distribution, Falck-Hillarp formaldehyde fluorescence method, histochemistry, immunofluorescence method, localization, neurotransmitters.

INTRODUCTION

Transmitter histochemistry can, together with biochemical and pharmacological techniques, provide knowledge on the cellular localization of a substance, and a basis for the study of transmitter systems and their interactions. For these purposes, many histochemical techniques have been developed during recent years. Among these, various autoradiographic approaches have been tested for different substances such as biogenic amines, amino acid transmitters, peptides and receptors. Also ultrastructural techniques have been developed utilizing certain cytochemical methods. Furthermore, recent innovations have led to very specific tracing techniques enabling the investigator not only to trace pathways but also to combine this with other techniques e.g. immunohistochemistry, thus elucidating the transmitter content of the very same fiber system (for more detailed information as well as reference material, the reader is referred to Björklund and Hökfelt (1983-84) and Johansson (this Volume)). The most recent development in the morphological sciences involves the use of computerized quantitative image analysis where nerve cells and fiber networks can be measured from morphometric and stereologic points of view. However, in the following we will concentrate on two major contributions, namely the formaldehyde fluorescence technique of Falck, Hillarp and their colleagues as well as the indirect immunofluorescence technique of Coons and his collaborators. For further aspects on the various methods, the reader is referred to the appropriate literature in the reference list.

STANDARD PROCEDURE FOR THE FORMALDEHYDE FLUORESCENCE TECHNIQUE

In the early 1960's, Falck, Hillarp and coworkers introduced the gas-phase formaldehyde condensation reaction for visualization of catecholamines and indolamines (Falck et al., 1962). In the original formaldehyde method, freeze-dried tissue pieces or air-dried thin tissue sheets are exposed to formaldehyde vapour. The formaldehyde will react with monoamines resulting in the formation of intensely fluorescent compounds which are easily traced in

an ordinary fluorescence microscope (cf. Fig. 1). Originally, it was considered of fundamental importance for the high sensitivity of this method that all steps in the histotechnical procedure should be performed under dry or nearly dry conditions. During the last few years, several fluorescence histochemical techniques based on application of the reagent in solution have, however, been worked out. In some cases, these methods have considerably higher sensitivity for catecholamine containing structures than the standard formaldehyde method. This has proved to be the case, for example, for the introduction of a new reagent, glyoxylic acid, and its use alone or in combination with formaldehyde (Lindvall et al., 1973; Lindvall and Björklund, 1974). In the following a brief description of the standard formaldehyde fluorescence method will be given (for further aspects of the methodology as well as references, see Björklund, 1983).

Preparation of Tissue

The animal is killed by decapitation and the organs dissected out as quickly as possible. The tissue pieces are rapidly frozen to a very low temperature. Freezing is performed by the use of various intermedia. Usually, a mixture containing liquid propane and propylene in proportion of roughly 9:1 is used as an intermedium. The specimens can be stored in liquid nitrogen for prolonged periods until they are freeze-dried.

Freeze-Drying

Freeze-drying implies the removal of water from rapidly frozen tissue by sublimation at a temperature below the freezing point. After freeze-drying the specimens should be handled carefully because they are very brittle and will easily break.

Formaldehyde Treatment

The freeze-dried specimens are transferred to a glass vessel containing an excess amount of paraformaldehyde and heated in an oven at +80°C. During heating, the paraformaldehyde is depolymerized to gaseous formaldehyde. Beside the specific condensation reaction with the monoamines that yields the intensely fluorescent derivatives, the formaldehyde treatment also gives a mild but sufficient fixation of the tissues. It should be noted that, various types of tissues, and even various parts of the same tissue, may behave differently toward different types of paraformaldehyde. It is therefore often adviseable to test several pieces of the tissue to be studied, obtained from the same dryer, in different paraformaldehydes and to choose the one that gives the optimum reaction.

Embedding and Sectioning

The specimens should be embedded in degassed paraffin as soon as possible after formaldehyde treatment. Embedding is preferentially performed *in vacuo* to ensure complete and rapid penetration of the dry tissue. After the specimen is blocked in paraffin, it is sectioned and mounted on dry slides. Mounting usually has a negligible immediate effect on the observable fluorescence intensity, but is often necessary to avoid light-scattering in the sections that might be misinterpreted as specific fluorescence. The fluorescence in deparaffinized sections shows notable fading within a few days with concomitant and disturbing increase in unspecific background fluorescence. Storage of paraffin blocks, non-deparaffinized sections and mounted sections at low temperatures (down to -20°C), at low humidity and in darkness postpones these changes.

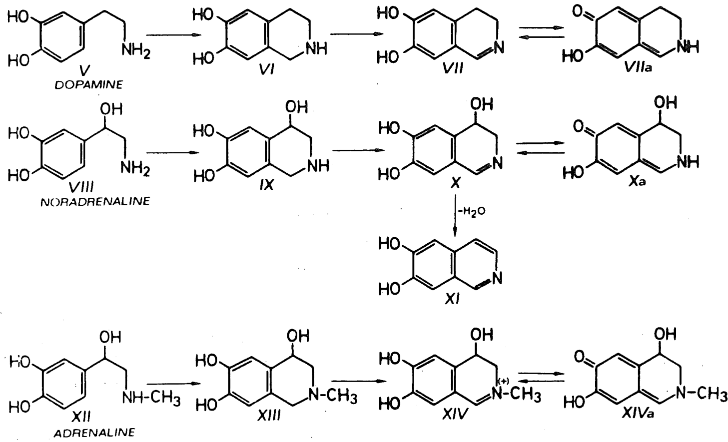

Fig. 1. The histochemical reaction between the biogenic catecholamines and formaldehyde. The catecholamine (V, VIII, XII) first reacts with formaldehyde forming a 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (VI, IX, XIII) which in a protein-promoted reaction is dehydrogenated to its corresponding 6,7-dihydroxy-3,4-dihydroisoquinoline (VII, X, XIV). This latter compound is in a pH-dependent equilibrium with its tautomeric quinoidal form (VIIa, Xa, XIVa), which exhibits a strong fluorescence with peak of emission at about 480 nm (Courtesy of Dr. G. Jonsson, Department of Histology, Karolinska Institutet, Stockholm, Sweden).

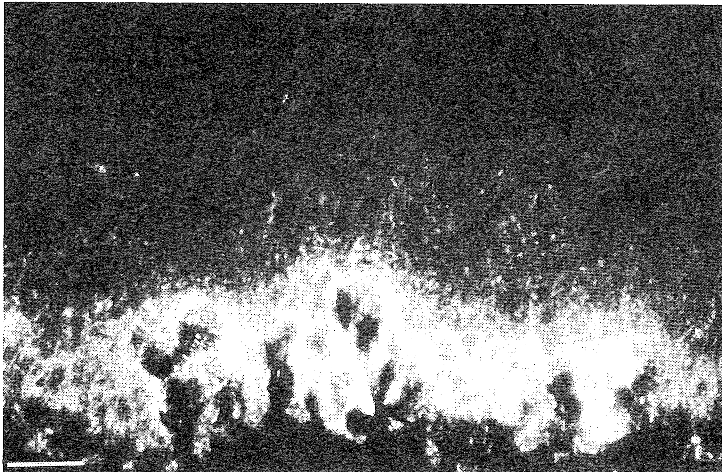

Fig. 2. Immunofluorescence micrograph showing TRH immunoreactive nerve fibers in the median eminence of rat. Corresponding ultrastructural immunocytochemistry is shown in Johansson (this Volume). Scale bar indicates 50 µm.

Microscopical Examination

The sections are examined in a standard fluorescence microscope equipped with a dark-field oil condenser. A Schott BG12 (3 or 4 mm), or alternatively a KP500 excitation filter, and a Zeiss 50 or LP520 barrier filter is used for the formaldehyde induced fluorescence.

STANDARD PROCEDURE FOR THE INDIRECT IMMUNOFLUORESCENCE TECHNIQUE

A very promising approach was initiated when Geffen, Livett and Rush (1969) purified dopamine-β-hydroxylase (DBH), raised antisera to this enzyme which converts dopamine to noradrenaline, and used the antiserum for immunohistochemical tracing of catecholamine cells. Their studies were based on the indirect immunofluorecence technique of Coons and collaborators (see Coons, 1958) and on the fact that DBH has antigenic properties, which had been discovered a few years earlier by Gibb et al. (1967). Although the Coons technique was about 25-year-old at that time, it had only been exploited to a limited extent in the field of neurobiology, probably due to lack of suitable antigens. In fact, the increased application of immunohistochemistry in neurobiology is a direct consequence of remarkable advancements in biochemistry which have led to the purification and structural characterization of peptides, polypeptides and proteins of various types. Immunohistochemical studies have revealed extensive networks (cf. Fig. 2) of neurons in the brain, spinal cord and periphery containing small peptides supporting the view that these types of substances may act as neurotransmitters or neuromodulators (cf. Hökfelt et al., 1984).

Preparation of Tissue

The animals (rats) are perfused via the ascending aorta with ice-cold 10 % formalin (or picric acid/formalin) for about 5-30 min. The formalin is prepared from paraformaldehyde powder in 0.1 M Sörensen's phosphate buffer. After perfusion, the tissues are rapidly dissected out and immersed in the same fixative for about 90 min. Thereafter they are rinsed in 0.1 M phosphate buffer, with 10 % sucrose, 0.01 % NaN_3 and 0.02 % Bacitracin (Sigma Chemical Co., St. Louis, MO., U.S.A.) added, for at least 24 h.

Sectioning and Incubation Procedure

The tissues are cut on a cryostat at 5-20 µm and the sections are thawed onto object slides. The sections are incubated at +4°C for 12-24 h with the primary antiserum, thoroughly rinsed, after which the fluorophore (fluorescein-isothiocyanate (FITC) or tetramethylrhodamine-isothiocyanate isomer R (TRITC)) conjugated antibody is applied. As controls, all antisera are routinely absorbed with the respective substance in excess amounts (=blocked antisera). Only immunofluorescence which is abolished by such absorbtion is regarded as specific. Preimmune serum or normal rabbit serum are used as controls for e.g. catecholamine synthesizing enzyme antisera. All antisera contain 0.3 % Triton X-100 (Hartman et al., 1972).

Mounting and Storage

After further rinsing in phosphate buffer, the sections are mounted in a mixture of glycerol and buffer (3:1) and stored in a deep freezer (-20°C).

Microscopical Examination

The sections are examined in a standard fluorescence microscope equipped with a dark-field oil condenser. A Schott BG12 (3 or 4 mm), or

alternatively a KP500 excitation filter, and a Zeiss 50 or LP520 barrier filter is used for FITC induced fluorescence. For TRITC fluorescence a BP546 excitation and a LP590 barrier filter is used. For further details and references regarding the methodology, see Hökfelt et al. (1973), Johansson (1983) and Johansson et al. (1984).

COMMENTS ON THE IMMUNOHISTOCHEMICAL TECHNIQUE

Our experience is based mainly on young adult male albino rats (Sprague-Dawley). In fact, very young rats (2-3 weeks) often seem to have a higher amount of peptide and transmitter related substance in their cell bodies than older rats. It is, however, clear that species differences exist and that other species may be better, at least when studied with some antisera. Immunofluorescence studies can also be carried out on tissues obtained from surgery, biopsy or autopsy in humans, but require good antibodies and comparatively rapid handling of the tissue.

Traditionally very mild fixatives have been used for tissues, which were to be processed for immunohistochemistry. They include various types of alcohol, acetone, etc., with the purpose of destroying antigenicity as little as possible. More recent developments have shown that, even if stronger fixatives are used, sufficient antigenicity is retained to allow immunohistochemical visualization. In our experience simple formalin or picric acid/formalin fixation gives acceptable results with most antisera.

A general rule seems to be that the quality of the immunofluorescence staining obtained is partly dependent on section thickness. Thus, it is easier to obtain good results with thick sections, whereas the morphology of thin sections often is inferior. The problem with thicker sections (up to 40 µm) is the increase in background fluorescence. When using cryostat sectioning, the cutting procedure is essential for good results. Generally, the sections should be cut at a low speed. Humidity and temperature inside the cryostat may influence the quality of the sections.

The microscopical analysis and photography of fluorescence slides is made difficult by the fact that the fluorescence rapidly fades under ultraviolet light exposure. However, this problem can now be avoided by addition of certain chemicals to the mounting medium, e.g. n-propyl gallate or paraphenylenediamine. We routinely use transmission illumination. This gives a more diffuse illuminated area and allows photography of montages without disturbing 'fading borders'. Furthermore, since the introduction of more advanced technical solutions, such as the 'condenser-scanner' method (Mårtensson and Björklund, 1984), this problem has become even smaller.

It is our experience that hardly any improvement in the histochemical procedure <u>per se</u> can compensate for a 'bad' antiserum. One should test as many antisera for a certain antigen as possible before initiating extensive immunohistochemical studies. High affinity antisera are not always the best for immunohistochemistry and antisera which are 'good' in radioimmunoassay are sometimes not 'good' enough for immunohistochemistry. The dilution of the antisera used must be tested individually for each antiserum and for each particular tissue and species. The dilution of the second antiserum must also be tested, and the ratio of specific against background fluorescence must be evaluated. It is our opinion that, at least in studies on neuronal tissues and particularly the central nervous system, it is an advantage to have a background high enough to allow recognition of land-marks and nuclei in the tissue both when examining the tissue in the microscope as well as when examining micrographs. This can be obtained by using the fluorophore conjugated antiserum in slightly higher concentrations. Furthermore, the higher concentrations may also be a positive factor, when trying to 'minimize' the fading effect (see above).

Several other problems are inherent to immunohistochemical techniques, particularly that of specificity. Apart from various technical problems such

as unspecific absorption of antibodies to sections, etc., which can be revealed by different control procedures, the main problem is the possible occurrence of cross-reactions. Thus, antisera may react with substances, which are structurally related to the immunogen, and it therefore seems appropriate to use expressions such as "somatostatin-like immunoreactivity", "somatostatin-immunoreactive", etc.

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