QUANTIFICATION AND COMPARISON OF SIGNAL AMPLIFICATION AND NON-AMPLIFICATED IMMUNOHISTOCHEMICAL REACTIONS OF THE RAT BRAIN BY MEANS OF IMAGE ANALYSIS

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ABSTRACT

The study of the distribution of neurotransmitters, neuropeptides and metabolising enzymes of neurofunctional relevant substances in the CNS provides important information about structural and functional differences of the neurochemical architecture of the cortex cerebri. Immunohistochemical methods are essential tools for the investigation of such antigens. In order to quantify and visualize distribution patterns of these substances in situ by an image analysis system an optimal reporter system for incubations has to be determined. Therefore, the staining results i.e. contrasts obtained by application of eight different reporter methods at the brain of Wistar rats were evaluated by an image analysis system. After determination of the most efficient reporter system area 1 of the somatosensory cortex of a human brain was incubated with an antibody against tyrosine hydroxylase. Finally the variability of this antigen expression in consecutive sections was analyzed. It has been observed that a modification of a commercially available catalysed signal amplification system provides optimal results. Furthermore, we have determined for the first time the expression pattern of tyrosine hydroxylase in the somatosensory cortex in man and have shown that the variability of tyrosine hydroxylase expression in area 1 is relatively small.

KEYWORDS: Immunohistochemistry, enhancement, image analysis, brain

INTRODUCTION

The investigation of the expression and distribution of enzymes and structural proteins in the CNS in situ can be easily performed by immunohistochemical methods [2]. Since immunohistochemistry is working under relatively standardized conditions more work has been invested into the quantification and distribution of immunoreactivity. Quantification of immunoreactivity is an essential issue in neurohistology because cells in neuronal tissues are distributed in a highly structured manner like laminae and different kinds of columnae [9] and consequently functionally

relevant, immunopositive structures, too. The topographical quantification of immunoreactivity is considered here as a mapping of an in situ result into another visualization system like an image or coordinate system. Therefore, we will speak in the following of mapping of neurofunctionally relevant proteins. Image analytical quantification of immunoreactive sites in nervous tissue is a non trivial task because problems on the image analytical side and those on the immunohistochemical side have to be solved. In this study we concentrated on immunohistochemical problems in respect to quantification by an image analyzer. At first, different methods of immunohistochemical reactions were optimized in respect to standardized brain tissue of Wistar-rats. Then the staining results of the different immunohistochemical methods were compared. This was achieved by a semiautomatic procedure at the image analyzer. Here, we determined the local contrasts [27] of the foreground (immunoreactive region) and the background (non-immunoreactive region) of chromogens which were visualized by a HRP reaction. The different methods were compared in respect to their time of execution and material costs in order to decide which technique is most efficient. After the determination of the method which supplies the best contrasts in images captured by a videomicroscopic device this method was further optimized. In order to receive information about different kinds of patterns of antigen distribution, perikarya were visualized by an antibody against parvalbumin, nerve fibres were registered by an antibody against neurofilament and a pattern of small and scattered located antigens were quantified after incubation with an antibody against synaptophysin. At the end, the optimized method was applied to a specimen of human cerebral cortex (area 1). Here, consecutive cryosections were incubated with an antibody against tyrosine hydroxylase (TH) in order to determine the variability of TH immunoreactivity in different sections of the human area 1. At last we will present an adaption of the optimized immunohistochemical method to vibratom sections of the human somatosensory cortex.

MATERIAL AND METHODS

Five adult male Wistar rats were anaesthetized with an i.p. injection of 16 ml/kg body weight of 2 mg/ml Nembutal(r) (Sanofi Ceva, Hannover) and 1 mg/ml Rompun(r) (Bayer, Leverkusen) in 0.9% NaCl. For the subsequent histological studies the animals were perfused through the left ventricle with glucose Ringer solution containing sodium heparinate (1000 IU / 100ml). This wash out of blood was followed by a fixation with Somogyi fixans [29]. The brain was dissected and postfixed in the same fixans for further 12 hrs. Thereafter, the specimens were frozen in liquid nitrogen. At -20oC 20 um thick slices were sectioned and dried on gelatinized glass slides. In order to receive information about different kinds of patterns of antigen distribution perikarya were visualized by a monoclonal antibody against parvalbumin (Sigma P-3088) (type of cytoplasmatic pattern, TCP), nerve fibres were registered by a monoclonal antibody against neurofilament (DAKO M0762) (type of fibre pattern, TFP) and a pattern of small scattered located antigens (type of granular pattern, TGP) were quantified after incubation with a monoclonal antibody against synaptophysin (Sigma S-5768). For the second part of this study the brain of the corpse of a 75 year old male human without any neurological disorders was used. Six hours after death the brain was removed. The central sulcus was localized and the area 1 was prepared followed by a cryoprotection step (12 hrs in 30% sucrose 0.1M PBS solution at 4oC); finally the probes were frozen in liquid nitrogen. 20 um thick sections were produced and then fixed for 10 min in Somogyi fixans. All following steps were performed in the same kind as described above for the rats.

The sections were rinsed for three times a 10 min in 0.1M phosphate buffered saline (PBS, pH 7.4, 4X PBS: 45 g NaCl + 1.352 g NaH2PO4-H2O +

14.397 g Na2HPO4-12H2O + 1000 ml aqua dest.). This step was followed by a blocking of endogen HRP using 3% of H2O2 for 20 min. The inhibition of endogenous avidin and biotin sites was performed with the biotin blocking solution of DAKO (X0590) containing 0.1% avidin and 0.01% biotin in 0.05M Tris-HCl (pH 7.2-7.6, dilute 6.1 g Tris in 50 ml aqua dest. add 37 ml 1N HCl and fill up to 1000 ml with aqua dest.). Unspecific protein binding sites were efficiently blocked by the protein blocking solution of DAKO (X0909) after incubation for 15 min. After incubation with primary and secondary antibodies as well as ABC incubation, amplification and incubation with HRP labelled streptavidin, sections were rinsed in CSA buffer which contains 6.057 g Tris (pH 7.6), 17.532 g NaCl, 1 ml Tween-20 and 1000 ml aqua dest. 4-chloro-1-naphthol (4CN) was used as the substrate for the HRP reaction. This was followed by mounting the sections with Aquatex(r) (Merck 8562). Evaluation of sections by image analysis was performed immediately after mounting although these sections can be stored at least for 3 to 6 months at 4oC in the dark. In addition 3,3'-diaminobenzidine (DAB), 3-amino-9-ethylcarbazole (AEC) and Hanker-Yates reagent (HYR, Sigma H-7507) substrates were employed. In the following the differences of the immunohistochemical methods applied are described.

ABC METHOD (AVIDIN BIOTIN COMPLEX)

This method was introduced by Hsu et al. [8]. The secondary antibodies used in the ABC method are biotinylated. At these binding sites avidin, which possesses four binding sites, can be coupled. The HRP is labelled with biotin molecules. The avidin and biotin labelled HRPs are produced freshly before using the secondary antibody in order to build a complex of multiple streptavidin biotin molecules. Therefore, three HRPs with two biotin labels can bind to one avidin molecule which has a left binding site for the biotin labels at the secondary antibody. This technique provides a specific enhancement for the visualization of antibodies because more than four HRPs are accumulated at one secondary antibody. The ABC method was performed by using the Vectastain Elite Kit (Vector PK-6200).

LSAB(R) METHOD (LABELLED STREPTAVIDIN BIOTIN)

The secondary antibody of this method is labelled with biotin. However, the streptavidin molecule is coupled with two alkaline phosphatases (AP). Totally, four APs are provided at one secondary antibody. Neufuchsine was used as the appropriate substrate.

SILVER AMPLIFICATION METHOD [21]

At first the ABC method is employed followed by incubation in DAB as the substrate of the HRP. Then sections were incubated in a 0.5% ammoniacal silver nitrate solution, followed by 5% sodium thiosulfate and bleaching in a 0.2% gold chloride solution.

MULTIPLE HRP AND SECONDARY ANTIBODY LABELLED DEXTRAN-POLYMER METHOD (ENVISION(R), DAKO K4000) [22]

The technique of labelling a dextran-polymer was developed in the laboratories of DAKO. The technique makes use of the ability of dextran polymers to bind multiple HRP enzymes as well as IgGs. IgGs could be secondary antibodies or primary antibodies (Epos(r) system). The latter will act as a so-called one-step immunohistochemical method whereas the first one works as a two-step system. However, enhancement is provided by the dextran-polymer on which multiple HRPs and secondary antibodies are coupled. The HRPs on these macromolecules are reacting in the same manner as described above.

TYRAMINE SIGNAL AMPLIFICATION METHOD (TSA(R), NEN TM LIFE SCIENCE PRODUCTS, NEL700)

The secondary antibody of this method is labelled with biotin. HRP is coupled to streptavidin so that this link molecule can bind to the biotin labelled secondary antibody. Therefore, the accumulation reaction is not as strong as in the ABC method. Then biotinylated tyramine (BT), a phenol derivate, is oxidatively condensed in form of a dimerization of the phenolic compound by a free radical mechanism of the HRP. The reactive form binds covalently to a component of the protein (i.e. tyrosine) [5, 16] in the immediately adjacent surface. Thereafter, the precipitated biotins are detected by streptavidin which is coupled to HRP. At last, the multiple HRPs are transforming their substrate 4-chloronaphthol to reactive form which is able to bind at amino acids.

CATALYSED SIGNAL AMPLIFICATION METHOD (CSA(R), DAKO K1500)

The principle of this method was originally called catalysed reporter deposition [4, 5, 6] and modified by Adams [1], Chen et al. [7], Hunyady et al. [10] and Sanno et al. [24]. Modifications of this method are necessary in respect to differences in specimens, antibodies and further conditions according to von Wasielewski et al. [31].

The first steps of this method are the same as performed by the plain ABC technique (Vectastain Elite Kit, Vector PK-6200). Instead of providing the substrate to the coupled HRPs they are incubated with BT which is transformed in the same way as in the TSA system. After the fall out of reactive BT, streptavidin molecules which are double labelled with HRP react at the biotin sites of BT. The lacking of HRP labelled biotin is the main difference to the TSA method. Instead of the HRP-biotin molecule the TSA method makes use of a HRP labelled streptavidin. Visualization is performed as described above by precipitation of 4CN by HRPs. The peroxidase-antiperoxidase (PAP) method [2, 30] was used for comparing different substrate solutions. DAB solution contains 6 mg 3,3' diaminobenzidine tetrahydrochloride, 10 ml 0.05 M Tris (pH 7.6) and 0.1 ml 3% H2O-2. AEC was produced with 4 mg 3-amino-9-ethylcarbazole, 1 ml N,N-dimethylformamide, 14 ml 0.1M acetate buffer (pH 5.2) and 0.15 ml 3% H2O2. 4CN was made of 3 mg 4-chloro-1-naphthol, 0.1 ml absolute ethanol, 10 ml 0.05 M Tris (pH 7.6) and 0.1 ml 3% H2O2. HYR solution contains 5 mg of paraphenyldiaminedihydrochloride, 10 mg pyrocatechol 1,2-benzenediol, 10 ml 0.05 M Tris (pH 7.6) and 0.1 ml 3% H2O-2. All solutions were filtered prior to use.

The CSA system was optimized to our needs (Tab. 1). This modification is the so-called C2 method. BT was produced according to Adams [1]. NHS-LC-Biotin (sulfosuccinimidyl-6-(biotinimide) hexanoate was obtained from Toronto Research Chemicals (s69025). 100 mg NHS-LC-Biotin and 31.2 mg tyramine-HCL (Sigma T-2879) were mixed in 40 ml of 50mM borax buffer (pH 8.0). The reaction lasted 12 hrs at room temperature (20oC). The solution was filtered through a 0.45 um pore filter. The concentration of BT is 7 uM. This solution can be stored at 4oC for 3 months. Aliquots may be stored at -20oC and can be used for over a year. Before application 1 ul of BT solution was diluted in 1 ml 0.1M PBS and 1 ul 30% H2O2 in PBS. In the modified CSA procedure C2 the DAKO protein blocking solution was employed. The biotinylated secondary antibody against mouse or rabbit was made in goat which was delivered from DAKO (E0413, E0432), too. It was diluted 1:500 in 0.1% BSA (bovine serum albumine), 10% NGS (normal goat serum) and 0.05% thimerosal in PBS (pH 7.4). The streptavidin-biotin-complex conjugated with HRP was obtained from DAKO (K0377). 8 ul streptavidin and 8 ul biotin were diluted 30 min before use each in 1000 ul PBS. The HRP conjugated streptavidin was made by DIANOVA (016-030-084) and applied in a concentration of 1:200 in PBS.

FREE FLOATING SECTIONS

50 um thick vibratom sections of the area 1 of the human brain were used for the adaption to the C2 procedure. The sections were rinsed for 12 hrs in Tris buffer. Protein, endogenous streptavidin and biotin blocking was performed as described above. Incubation with the primary antibodies lasted 12 hrs at 4oC. The primary antibody was diluted in 0.01% BSA, 0.01% thimerosal and 0.01% sodium azide. The rinsing after ABC incubation lasted 30 min instead of a fast washing out of the ABC reagents. Here, 4CN was used as the substrate for HRP, too. The same reagents may be used at the first day for about 4-7 different sections without an observed decrease of staining intensity. For a further day the same solutions may be used but the ABC complex should be restored.

MEASUREMENT OF IMMUNOREACTIVITY

For the quantification of immunoreactions a videomicroscopic set-up was used [27]. Measurements took place at a resolution of 0.503 um/pixel. The selection of sections out of several probes incubated with different primary antibody concentrations was done by visual inspection. 50 measurements of immunoreactive regions (foreground (FG)) and non-immunoreactive regions (background (BG)) were performed of each section. Mean grey values of FG and BG in three different colour channels were calculated and subtracted. The results were normalized and expressed in percent as contrast values (Fig. 1, see 993-031.pcx).

MAPPING OF IMMUNOREACTIVITY

50 um thick vibratom sections of area 1 were incubated with anti-tyrosine hydroxylase and treated according to the C2 protocol. The slides were fixed on a scanning stage (Märzhäuser) under the light microscope (Zeiss Universal Forschungsmikroskop). A grey level ray tube camera (Siemens K30) was adapted to the system. The condensor was calibrated according to Köhlers illumination condition and a reference image which is necessary for shading correction within the imaging process was grabbed. Thereafter, the rectangular region of the tissue which should be scanned was determined by the investigator. Then the image analysis system (IBAS 2.5, Zeiss-Vision) moved the scanning stage meander-like through the predefined evaluation region and grabbed a 5122 image after each step with a dynamic range of 256 grey levels. These images were stored on hard disk. A generation of a grey level mosaic (Fig. 2, see 993-032.pcx) (very large scale digital image (VLSDI)) was produced after finishing the scan. At last, the images were transformed into GLI (grey level index) [26] and mean optical density MOD [23, 28] respectively. The GLI is defined as the percental portion of the area of the specific foreground signal to the whole area of an evaluation frame (32 x 32 pixel) which is generated by masking the whole of the GLI image systematically by marching meander-like through the 512 2 image area. The normalized GLI values are mapped into a new image which has been colour coded by a look-up-table. The MOD is defined as the mean grey value of the foreground signal. The MOD is normalised and gives information about the intensity of immunoreactivity at all regions of an VLSDI. After calculating GLI and MOD maps the pial surface and the border to the white matter were determined by the investigator. Along to these borderlines further orthogonal lines were generated by the software. On each pixel of these lines the GLI or MOD values were picked up and added into a vector. After finishing this evaluation the cumulated values were averaged and normalized. The curves resulting from this calculation are called profiles. These profiles present information about the average distribution of immunoreactive sites orthogonal to the pial surface of the cortex cerebri (Fig. 3, see 993-033.pcx).

MEASUREMENTS OF VARIABILITY OF THE TYROSINE HYDROXYLASE

In a further experiment the variability of profile curves (Fig. 3, see 993-033.pcx) were studied because we did not know if expression of tyrosin hydroxylase in consecutive sections is regular or if the expression follows a random distribution. Therefore, GLI maps of four consecutive cryosections were prepared and the GLI profiles were calculated.

RESULTS

Comparing methodological efficiency in respect to costs, time and contrast results the modified CSA method called here C2 method represents a sophisticated and standardized solution for immunohistochemical staining of brain tissues. It provides the highest contrast values independent of the antigen distribution pattern (Fig. 2, see 993-032.pcx). The weakest contrasts were reached with the LSAB method. The PAP method and the ABC method are comparable to each other. TSA and CSA provide similiar results. For the TFP distribution pattern the TSA method leads to a slightly higher contrast whereas towards the other two patterns it provides lower contrast values. We have mentioned within the description of the TSA technique that HRP is coupled to streptavidin. This link molecule can bind to the biotin labelled secondary antibody. Therefore, the accumulation reaction is not as strong as in the ABC method. This effect can be observed within the parvalbumin contrast measurement (Fig. 1. see 993-031.pcx) but not for neurofilament and synaptophysin. The silver enhancement procedure showed a relatively high variability of contrast values among the different distribution patterns. The fastest of all methods is the EnVison method recently developed by DAKO. Tab. 1 shows the details of this evaluation. Finally different types of chromogens were compared. We observed that DAB produced the most intensive background signal followed by AEC and HYR. Best results were obtained by 4CN. The GLI map and MOD map of the TH immunoreactivity are presented in Figs. 2 (see 993-032.pcx). The molecular lamina does not show any TH immunoreactivity whereas the other laminas possess TH immunopositive regions. The distribution of GLI values of TH as shown in Fig. 2 (see 993-032.pcx) can better be analyzed in the corresponding profile (Fig. 3a, see 993-033.pcx).

A plot of four GLI profiles of the TH immunoreactivity of area 1 is shown in Fig. 3 (see 993-033.pcx). All four profiles possess four larger peaks with higher variability in their height and lower differences in their relative position to each other. This finding indicates a relative similarity of TH distribution in area 1 in different sections. The highest peaks of GLI profile peaks are located within lamina 2, 5 and 6 and to a lower extend in form of a plateau around lamina 3.

DISCUSSION

All methods were optimized before measurements were performed. Therefore, we have introduced the blocking of endogenous biotin and avidin by the blocking kit delivered by DAKO. Furthermore, it is necessary to perform a dilution of the primary antibody by the factor 50 to 100 in comparison to the PAP method. However, this can not be generalized because high antigen densities in a limited region of the tissue may lead to an increase of background staining by using an amplification method whereas the PAP method will produce a result with high contrast. Intensifying of immunoreactive sites by silver enhancement [21] produced sufficient contrast of the reporter reaction. However, nickel-DAB [17], ferric ferricyanide [20] and osmium tetroxide [12, 14] reactions among others were not investigated in this study. The fastest reporter method is the EnVison technique, however, it is also a rather expensive method and

provides only average contrast values in comparison to the other procedures. An advantage of the C2 method is its applicability to further methods like in situ hybridization [11]. Another amplification method was proposed by Linsenmeyer et al. [15] by using repeated incubation with primary and secondary antibodies called multiple-reaction cycling. However, positive results of this technique have been reported only after using monoclonal antibodies whereas the C2 method is applicable also to primary antibodies from rabbit.

Additionally, we have observed that a very high dilution of the primary antibody (up to 1:500,000) will give valuable results, too. However, this extreme dilutions have to be tested for reliability for each antibody separately. High dilutions are important in respect to incubations of larger sections for the detection of chemoarchitectural areal differences in order to protect the experimental set-up against extreme increase of costs [13]. Costs can be reduced if the avidin biotin blocking and protein blocking will be optimized. However, we observed a strong increase of the background signal of unspecific protein binding after using normal goat serum, BSA or dry milk solutions in different mixtures and combinations. If protein blocking and avidin-biotin blocking will be performed by procedures developed in our laboratory the costs for 200 sections processed by the C2 method will be reduced. Hunyady et al. [10] and Adams [1] have reported that suboptimal fixation or formalin-resistance [32, 18] provide a strong decrease of antigenicity. Amplification reactions may overcome this problem. Considering antigen unmasking techniques like microwave irradiation [25] may enhance the C2 procedure to a further extend. Considering these different aspects we recommend the application of the C2 method for detection of neuronal antigens in rat and human tissue of the CNS. Furthermore, we are convinced that the C2 method can be used for immunohistochemical experiments in other organs, too. Tests of antibodies against different CD-antigens with antibodies against B-lymphocytes and T-lymphocytes in lymph nodes provided similiar results.

REFERENCES

1. Adams JC (1992) Biotin amplification of biotin and horseradish peroxidase signals in histochemical stains. J Histochem Cytochem 40: 1457-1463

2. Avrameas S, Uriel J (1966) Methode de marquage d'antigenes de proteines a l'aide d'immnoadsorbants et d'antigenes marques aux enzymes. C R Acad Sci Paris 272: 2705-2712

3. Avrameas S (1992) Amplification systems in immunoenzymatic techniques. J Immunol Meth 150: 23-32

4. Bobrow MN, Harris TD, Shaughnessy KJ, Litt GJ (1989) Catalysed reporter deposition, a novel method of signal amplification. J Immunol Meth 125: 279-285

5. Bobrow MN, Litt GJ, Shaughnessy KJ, Mayer PC, Conlon J (1992) The use of catalysed reporter deposition as a means of signal amplification in a variety of formats. J Immunol Meth 150: 145-149

6. Bobrow MN, Shaughnessy KJ, Litt GJ (1991) Catalysed reporter deposition, a novel method of signal amplification. J Immunol Meth 137: 103-112

7. Chen BX, Szabolcs MJ, Matsushima AY, Erlanger BF (1996) A strategy for Immunohistochemical signal enhancement by end-product amplification. J Histochem Cytochem 44: 819-824

8. Hsu S-M, Raine L, Fanger H (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 29: 577-580

9. Huang X, Chen S, Tietz EI (1996) Immunocytochemical detection of regional protein changes in rat brain sections using computer-assisted image analysis. J Histochem Cytochem 44: 981-987

10. Hunyady B, Krempels K, Harta G, Mezey E (1996) Immunohistochemical signal amplification by catalysed reporter deposition and its application in double immunostaining. J Histochem Cytochem 12: 1353-1362

11. Kerstens HMJ, Poddighe PJ, Hanselaar AGJM (1995) A novel in situ hybridization signal amplification method based on the deposition of biotinylated tyramine. J Histochem Cytochem 43: 347-352

12. Johansson O, Backman J (1983) Enhancement of immunoperoxidase staining using osmium tetroxide. J Neurosc Meth 7: 185-193

13. King G, Payne S, Walker F, Murray GI (1997) A highly sensitive detection method for immunohistochemistry using biotinylated tyramine. J Pathol 183: 237-241

14. Lascano EF, Berria MI (1988) PAP labeling enhancement by osmium tetroxide-potassium ferrocyanide treatment. J Histochem Cytochem 36: 697-699

15. Linsenmayer TF, Fitch JM (1988) Multiple-reaction cycling: a method for enhancement of the immunochemical signal of monoclonal antibodies. J Histochem Cytochem 36: 1075-1078

16. Litt GJ (1996) A high-sensitivity signal enhancement method. Am Biotech Lab 12/98: 16

17. Merchenthaler I, Stankovics J, Gallyas F (1989) A highly sensitive one-step method for silver intensification of the nickel-diaminobenzidine endproduct of peroxidase reaction. J Histochem Cytochem 37: 1563-1565

18. Merz H, Malisius R, Mannweiler S, Zhou R, Hartmann W, Orscheschek K, Moubayed P, Feller AC (1995) Methods of laboratory investigation. ImmunoMax. Lab Invest 73: 149-156

19. Morrell JI, Greenberger LM, Pfaff DW (1981) Comparison of horseradish peroxidase visualization methods: quantitative results and further technical specifics. J Histochem Cytochem 29: 903-916

20. Nemes Z (1987) Intensification of 3,3'-diaminobenzidine precipitation using the ferric ferricyanide reaction, and its application in the double-immunoperoxidase technique. Histochem 86: 415-419

21. Quinn B, Graybiel AM (1996) A differentiated silver intensification procedure for the peroxidase-diaminobenzidine reaction. J Histochem Cytochem 44: 71-74

22. Sabatini E, Bisgaard K Ascani S, Poggi S, Piccioli M, Ceccarelli C, Pieri F, Fraternali-Orcioni G, Pileri SA (1989) The EnVisionTM+system: a new immunohistochemical method for diagnostics and research. Critical comparison with the APAAP, ChemMateTM,CSA, LABC, and SABC techniques. J Clin Path 51: 506-511

23. Salmon I, Camby I, Gras T, Rombuat K, Pasteels J-L, Kiss R (1994) The use of digital cell image-analysis of Feulgen-stained nuclei to

quantitatively describe morphonuclear features in a series of 174 meningiomas. Mod Pathol 7: 570-577

24. Sanno N, Teramoto A, Sugiyama M, Ito Y, Osamura RY (1996) Amplification of catalysed signal amplification in immunodetection of gonadotropin subunits in clinically nonfunctioning pituitary adenomas. Anat Path 106: 16-21

25. Shiurba RA, Spooner ET, Ishiguro K, Takahashi M, Yoshida R, Wheelock TR, Imahori K, Cataldo AM, Nixon RA (1998) Immunocytochemistry of formalin-fixed human brain tissues: microwave irradiation of free-floating sections. Brain Res Prot 2: 109-119

26. Schleicher A, Zilles K (1990) A quantitative approach to cytoarchitectonics: analysis of structural inhomogeneities in nervous tissue using an image analyser. J Micros: 157, 367-381

27. Schmitt O, Eggers R (1997) Systematic investigations of the contrast results of histochemical stainings of neurons and glial cells in the human brain by means of image analysis. Micron 28: 197-215

28. Schmitt O, Almert U, Mecke E, Eggers R (1998) Die quantitative Zytoarchitektonik der Areale nach Brodmann des menschlichen Cortex cerebri. Focus MUL 15: 144-155

29. Somogyi P, Hodgson AJ, Smith AD (1979) An approach to tracing neuron networks in the cerebral cortex and basal ganglia. Combination of Golgi staining, retrograde transport of horseradish peroxidase and anterograde degeneration of synaptic boutons in the same material. Neuroscience 4: 1805-1852

30. Sternberger LA (1979) Immunohistochemistry. Wiley and Son, New York, 104-169

31. Wasielewski R von, Mengel M, Gignac S, Wilkens L, Werner M, Georgii A (1997) Tyramine amplification technique in routine immunohistochemistry. J Histochem Cytochem 45: 1455-1459

32. Werner M, von Wasielewski R, Komminoth P (1996) Antigen retrieval, signal amplification and intensification in immunohistochemistry. Biochem Cell Biol 105: 253-260

LEGENDS

Fig. 1: Histogram of all measured contrasts of the different methods and antigen distribution patterns. The used primary antibody concentrations are shown in Tab.1.

Fig. 2: a) Photomicrograph of TH immunoreactivity of area 1 at a magnification of 100x. b) VLSDI of 6 x 18 grey level images. c) GLI map of the grey level image mosaic b). d) MOD map of the VLSI shown in Fig.2b).

Fig. 3: a) GLI profile of GLI map presented in 2c. b) Histogram which shows the variabilities of GLI profiles obtained of four consecutive sections incubated with an antibody against TH. Values of the x axes are normalized pixel values (min=1, max=64).

Tab. 1: Overview of the concentrations of primary antibodies, reporter

systems and duration of experiments (from incubation of primary antibody until mounting) (NF: neurofilament, PA: parvalbumin, SP: synaptophysin).

Reporter system	Costs / 200 sections [DM]	Antibody dilution	Duration/section
ABC	118.00	NF 1:2000 PA 1:1000 SP 1:1000	1.5 h
LSAB	340.00	NF 1:40 PA 1:100 SP 1:800	1.5 h
PAP	0.83	NF 1:40 PA 1:100 SP 1:800	3-4 h
Silver amplification	168.00	NF 1:2000 PA 1:500 SP 1:2000	2 h
EnVision	928.00	NF 1:2000 PA 1:500 SP 1:500	45 min
CSA	831.00	NF 1:5000 PA 1:10000 SP 1:5000	2.5 h
TSA	1286.00	NF 1:500 PA 1:5000 SP 1:5000	2.5 h
	168.00	NF 1:5000 PA 1:10000 SP 1:5000	2.5 h

Tab. 2: The protocol of the C2 method.

The C2 method	
1 Fixation	
1.1 Drying of section at 37oC	3 min
1.2 (Fixation of unfixed sections in Somogyi fixans)	10 min
1.3 Rinse in Tris	10 min
1.4 Rinse in Tris	10 min
1.5 Rinsing for prefixed sections	30 min
2 Blocking of endogenous peroxidase	
2.1 3% H_2O_2 (if eruption of blisters is to strong place bath on an electromagnetic stirrer)	20-30 min
2.2 Rinse in PBS	5 min
3 Blocking of endogenous avidin and biotin binding sites	
3.1 One drop of avidin	10 min
3.2 Rinse in PBS	5 min

3.3 One drop of biotin 10 min
3.4 Rinse in PBS 10 min

4 Blocking of unspecific protein binding sites
4.1 One drop of protein blocking solution 15 min

5 Incubation with primary antibody
5.1 Dilute antibody and add it directly withoutremoving the protein block
5.2 Incubation >1.5 or <12 hrs
5.3 Rinse in CSA buffer 10 min

6 Incubation with secondary antibody
6.1 Prepare ABC reagent (1 ml PBS + 8 ul streptavidin + 8 ul biotin)
6.2 One drop of species specific secondary antibody from goat (1:500) 15-30 min
6.3 Rinse in CSA buffer

7 Incubation with ABC reagent
7.1 50 ul ABC reagent / cm2 section 30 min
7.2 Rinse in CSA buffer 30 min

8 Amplification
8.1 Prepare amplification solution: 1 ml PBS + 1ul BT + 1ul 30% H_2O_2
8.2 50ul amplification solution / cm2 section 10 min
8.3 Rinse in CSA buffer 10 min

9 Marking of BT fall out
9.1 HRP labelled streptavidin 1:200 in PBS
9.2 50 ul HRP streptavidin / cm2 section 15 min
9.3 Rinse in CSA buffer 15 min

10 Chromogen reaction with 4-chloronaphthol
10.1 Rinse in PBS
10.2 Mounting in Aquatex