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Comparison of contrast, sensitivity and efficiency of signal amplified and nonamplified immunohistochemical reactions suitable for videomicroscopy-based quantification and neuroimaging

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Abstract

In recent years, many different technical modifications of immunohistochemical methods have been developed. The selection of a suitable technique for quantitative purposes such as mapping studies can be quite difficult. Various features of a certain method must be considered such as the sensitivity, costs, duration and practicability with respect to serial sectioned specimens. Background and foreground difference or contrast and the influence of artifacts are major problems of quantitative immunohistochemistry. It is not known which of the different modifications of immunohistochemical signal amplifications and non-amplifications gives optimal results in respect to image analytical-based quantification. However, for image analysis, it is important to analyze sections which offer a sufficient contrast between foreground and background. The sensitivity of a system is crucial when quantitative immunohistochemistry should be applied to scarce material with longer postmortem and storage times which occur often by processing human brains. In addition, the enzyme–substrate reactions have an obvious influence on this criterion; therefore, different substrates were also tested. The contrast may be as well effected by the quality and specificity of the primary antibody, the type of tissue and naturally by preparative (fixation, postmortem delay, storage) and individual factors (age, circadian effects, diseases, sex). Because all of these factors may yield to different results by combining them with different neuronal structures, we used three different antigen expressions for a specific analysis: fibrillary, granulary and perikaryal antigen distributions in brains from Wistar rats.

Principally, the sensitivity of the modifications of immunohistochemical amplifications is revealed more strongly than without enhancement steps; however, the contrast between foreground and background structures does not necessary increase by applying a certain amplification technique. The lowest contrast (15%) was detected after applying the labelled streptavidin–biotin technique. All other methods offer comparable contrasts in between 30% and 40%. The *catalyzed signal amplification* reaction has been found to give optimal results (40% contrast) for image analysis. However, from the technical point of view and variability of protein expression, storage and postmortem delay, it was necessary to adapt the commercial CSA Kit from Dako (K1500). The modified technique, called C2 method, offers better results with respect to sensitivity, total costs, duration and contrast (60%) and variability of contrast.

Theme: Motor systems and sensorimotor integration
Topic: Cerebellum

Keywords: Immunohistochemistry; Amplification; Enhancement; PAP; ABC; LSAB®; CSA; TSA®; C2; Silver precipitation; EnVision+™; Biotinylated tyramine; Image analysis; Contrast measurement; Videomicroscopy; Rat brain; Human brain; Tyrosine hydroxylase; Fixation; Unmasking; HRP visualization

1. Introduction

Most authors *describe* their immunohistochemical results [22,33,49]; however, the disadvantage of doing so is that comparable and resembling results may be described differently. Quantification of antigen–antibody visualizations would gain importance with respect to objectivity and comparability [41,45]. At least different quantified antigen distributions could be mapped and integrated into models

Abbreviations: 4CN, 4-chloro-1-naphthol; ABC, avidin–biotin complex; AEC, 3-amino-9-ethylcarbazol; APAAP, alkaline phosphatase–anti-alkaline phosphatase; C2, optimized catalyzed signal amplification method; CSA, catalyzed signal amplification; DAB, diaminobenzidine; EnVision+™, enhanced polymer one-step; EPOS, enhanced polymer one-step; HRP, horseradish peroxidase; HYR, Hanker–Yates reagent; LSAB®, labelled streptavidin–biotin; PAP, peroxidase–antiperoxidase; PBS, phosphate-buffered saline; Tris, trishydroxymethylaminomethan; TSA®, tyramine signal amplification

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[40]. In many cases, it is unknown which specific method is most suitable for image analytical-based quantification because no measurements of contrasts are available. A decision of which protocol should be applied for quantitative immunohistochemistry is confusing this issue because they are quite similar. So far, the contrast, sensitivity and efficiency of different immunohistochemic techniques based on enzyme–substrate reactions have not been systematically and quantitatively compared with each other. Therefore, the objective of this study is to ascertain if there exists a convincing method which offers reliable results, optimal contrasts for image analysis and optimal conditions for applying on a section series as well as on very large sections (costs, duration) for neuroimaging with respect to neuromapping investigations.

The detection of antigenic structures by immunohistochemical techniques is a complex task with respect to parameters like substrate reactions [12] and blocking schemes (protein blocking, endogenous peroxidase blocking, endogenous avidin–biotin blocking) influencing the results strongly. Therefore, standardized protocols as published by suppliers of different immunoreaction visualization kits or protocols used in other laboratories were investigated (silver precipitation method, peroxidase–antiperoxidase method). Furthermore, these methodic standards were studied in normal rat brains that exhibit only small variations of the antigens at specified brain regions and cytologic structures. Many additional parameters influence immunoreactivity like diverse fixation techniques, variability of postmortem delays, different types of antibodies (quality, specificity) that should bind at the same antigen and storage of material. In order to determine an optimal immunohistochemical method for the quantitative evaluation of central nervous tissue, we concentrate on the properly immunohistologic techniques and not exploring numerous combinations of conditions mentioned before. Because quantitative immunocyto- and immunohistochemistry is a rather difficult issue (camera calibration, microscope adjustment, type of light source) and experimental setups must be realized prudent, the histo-biological models used here were investigated systematic under the same conditions. Moreover, it could be crucial not to disregard aspects of economy and time because a potential useful method should be suitable for antigen visualization up to several hundreds of serial sections and/or very large sections in order to study the chemoarchitecture of multiple transition regions of the human cerebral cortex. We must emphasize that economic and time considerations with respect to a certain methodic recommendation are important for the

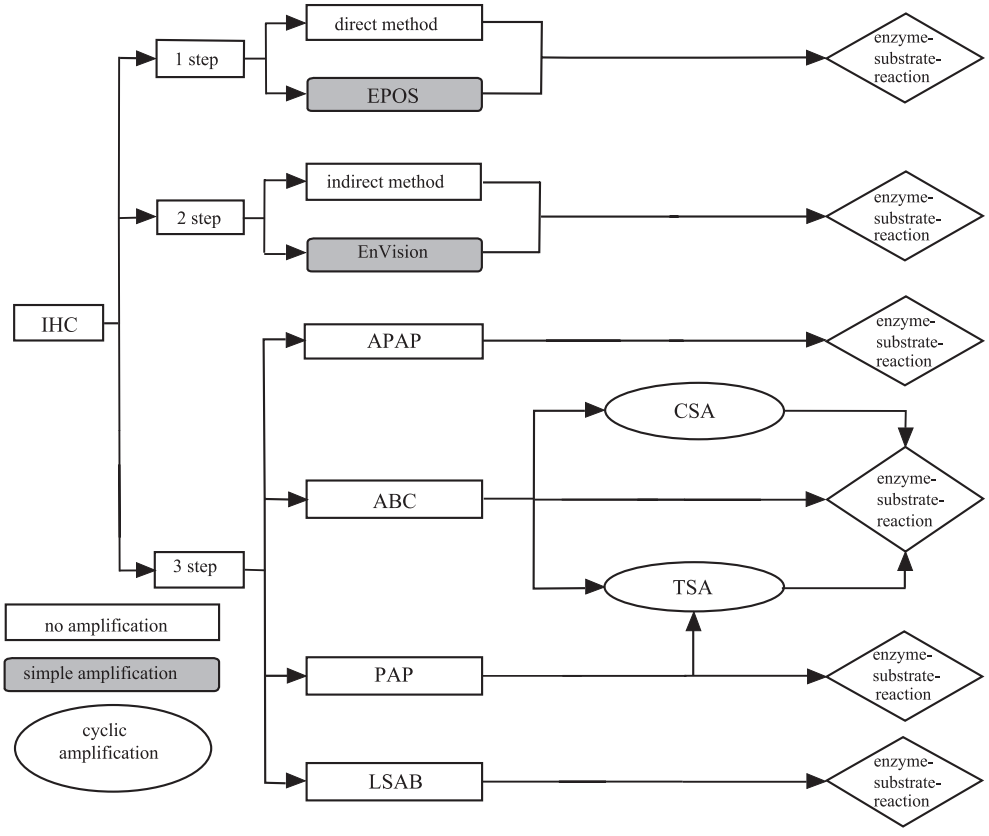

Fig. 1. Overview of the immunohistochemical (IHC) methods. The ABC method is integrated in the CSA procedure. Abbreviations: EPOS, enhanced polymer one-step; EnVision+™, enhanced polymer one-step; APAP, alkaline phosphatase–anti-alkaline phosphatase; CSA, catalyzed signal amplification; ABC, avidin–biotin complex; TSA®, tyramine signal amplification; PAP, peroxidase–antiperoxidase; LSAB®: labelled streptavidin–biotin.

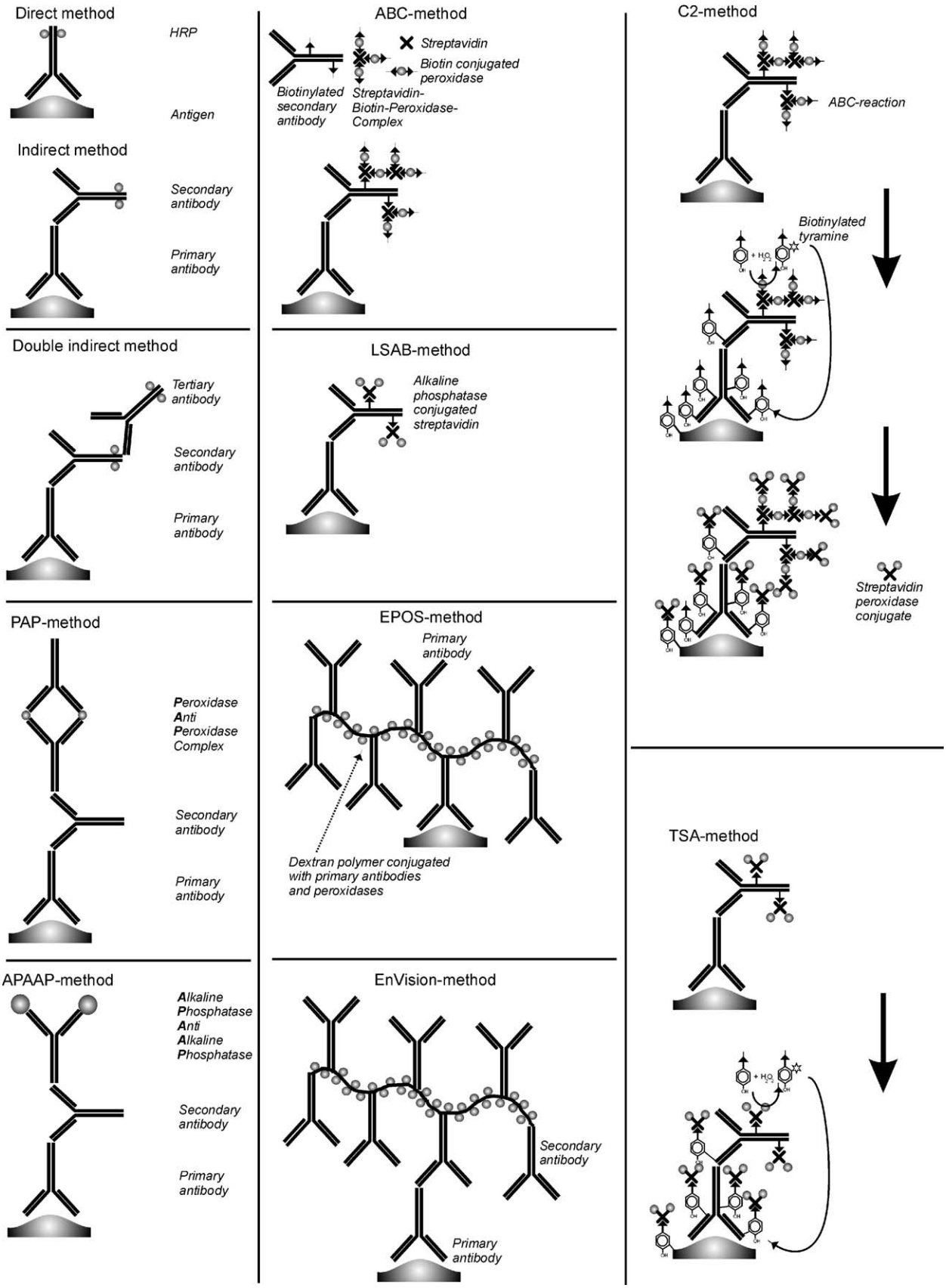

Fig. 2. Overview of the immunohistochemical methods which are used most often. The direct, indirect, APAAP and EPOS methods were not tested in this study.

feasibility of three-dimensional quantitative immunohistochemic studies of large sections of the cerebral cortex but they are considered as secondary issues. Primary aspects are contrast, homogeneity of the immunoreactions, the missing of artifacts and optical differentiation of small amounts of endproducts of the enzyme–subtrate reactions. Finally, the results should be of practical interest for investigations of *real* neuronal systems and not only for sophisticated model systems like neuronal cell cultures or a nitrocellulose model [31]. Quantitative results of the polymer-based enhancement method which was introduced by Bobrow et al. [5,6,7] and applied by Chilosi et al. [10] are presented here for the first time.

A prerequisite for an optimal immunoreactivity is optimal fixation by a coagulative procedure (alcohol or acetone) or a covalent method (formaldehyde, glutaraldehyde, paraformaldehyde). In order to receive comparable results a unique fixation procedure was applied in this study. Therefore, fluctuations in background staining obtained from less well-fixed tissue are negligible.

Additionally, immunoreactivity can be influenced by unmasking the fixed antigens and increase the sensitivity of the detection system [50]. Finally, the condition of the material and the reactivity of the primary antibody must also be taken into account. The subject of this study is a comparison of the contrast results—the most important factor for quantitative issues—of different immunohistochemical procedures. Thus, we aimed to hold as many conditions (fixation, material, material preparation) as possible constant in order to quantify the essential effects of the immunohistochemic techniques.

We followed the proposal of Bourne [8] in which a fixation is only a compromise and any new primary antibody or antigen requires additional testing with different fixatives to optimize results. Fig. 1 summarizes the compared methods in this study and Fig. 2 offers an overview of the immunohistochemical methods which are preferred in most laboratories.

2. Material and methods

2.1. Material

Five adult Wistar rats were anesthetized by an i.p. injection of 16 ml/kg body weight of 2 mg/ml Nembutal® (pentobarbital-sodium) (Sanofi Ceva, Hannover) and 1 mg/ml Rompun® (Bayer, Leverkusen) in 0.9% NaCl. The animals were perfused through the left ventricle with Ringer solution containing sodium heparinate (1000 IU/100 ml). This blood clearance was followed by fixation in Somogyi fixans [42]. The brain was dissected and fixated by immersion for further 12 h. Fixation was followed by cryoprotecting the brains in a 30% sucrose 0.01 M PBS solution for 12–24 h. The specimens were subsequently frozen in liquid nitrogen and 20 μm thick sections were sliced serial for reasons of comparability at a cryotome at −20 °C and dried on gelatinized glass slides.

2.2. Tissue processing

The sections were rinsed three times for 20 min in 0.01 M phosphate-buffered saline (PBS, pH 7.4; 4 × PBS solution: 45 g NaCl + 1.352 g $NaH_2PO_4 \cdot H_2O$ + 14.397 g $Na_2HPO_4 \cdot 12H_2O$ + 1000 ml H_2O dest.). After rinsing, the endogenous peroxidase was blocked by 3% H_2O_2 for 10 min followed by further rinsing in PBS. The endogenous avidin and biotin binding sites were blocked with biotin blocking solution provided by DAKO (X0590) containing 0.1% avidin and 0.01% biotin in 0.05 M Tris–HCl (pH 7.2–7.6). Nonspecific protein binding was blocked by a protein blocking solution (1 ml normal swine serum (Biochrome S0163) + 30 ml fetal bovine serum (Biochrome S0213) + 70 ml Tris buffer) (incubation for 15 min). In order to determine the contrast of different types of antigen distributions (filamentous, granulary, perikaryons), monoclonal antibodies against non-phosphorylated neurofilament (DAKO M0762), synaptophysin (Sigma S-5768) and parvalbumin (Sigma P-3088) were applied. After antibody incubations and enzyme–substrate reactions, all sections were mounted with Aquatex® (Merck 8562). Antibody concentrations were optimized for each method until their contrast results (distance between foreground and background) were maximized. Immediate-

Table 1
The following four substrates were used for the horseradish peroxidase reaction

Substrate	Abbreviation	Supplier	Composition
3,3′-diaminobenzidine 4HCl	DAB	Serva 18865	1. 2.5 mg DAB 2. 5 ml PBS 3. 50 μl 1 M imidazole 4. 100 μl 3% H_2O_2
3-amino-9-ethylcarbazol	AEC	Sigma A-5754	1. 3.125 ml 0.1 M acetate buffer (pH 5.2) 2. 125 μl AEC (4 mg AEC + 1 ml N.N-dimethylformamide + 14 ml 0.1 M acetate buffer (pH 5.2)) 3. 37.5 μl 3% H_2O_2
4-chloro-1-naphthol	4CN	Serva 16928	2 × 1 ml PBS (pH 7.4) 55 °C 10 μl 4CN (3 mg 4CN + 0.1 ml abs. ethanol + 10 ml 0.05 M Tris (pH 7.6)) 10 μl 4CN + 10 μl 3% H_2O_2
Hanker–Yates Reagent	HYR	Sigma H-7507	75 mg Hanker–Yates reagent 50 ml 0.1 M Tris 0.2 ml 1% H_2O_2

ly after polymerization of Aquatex®. the contrast measurement was performed. The four substrates as shown in Table 1 were tested again [12] because new immunoreaction techniques were applied.

2.3. Peroxidase–antiperoxidase method

The peroxidase–antiperoxidase method (PAP) was first developed by Avrameas and Uriel [2] and Sternberger et al. [43] and is a three-step technique. Alternatively horseradish peroxidase (HRP) or alkaline peroxidase can be used for visualization. In this study, HRP was used according to the protocol of Preuße [34].

Nonspecific protein binding was blocked with 10% normal goat serum for 30 min followed by incubation of the primary antibody (Table 2). The secondary antibody incubation lasted from 2 to 12 h followed by rinsing in PBS for 10 min (Table 2). The PAP complex was applied for 3 h by rinsing in PBS for 10 min followed by the enzyme–substrate reaction. The procedure was finished with mounting in Aquatex®.

2.4. Avidin–biotin complex methods

Additionally, the three-step methods with labeled streptavidin–biotin (LSAB®, see below) and avidin–biotin complex (ABC) [19] were tested. For evaluating the ABC method, the ABC Kit from Vectastain was used (Vectastain Universal Elite ABC Kit, PK 6200) and applied as proposed by the Kit instructions. The staining result of this method is shown in Fig. 7.

2.5. Labelled streptavidin–biotin method (LSAB®)

The labelled streptavidin–biotin method is similar to the ABC method. The difference consists in the conjugated

Table 2
Overview of the protocols which are adapted in this study

Peroxidase–antiperoxidase method (PAP)		Avidin–biotin complex method (ABC)		Silver precipitation method	
1 Fixation		1 Fixation		1 Follow steps 1–6.1 of the ABC method	
1.1 Drying of sections at 37 °C	3 min	1.1 Drying of sections at 37 °C	3 min		
1.2 Fixation of sections in Somogyi fixans	10 min	1.2 Fixation of sections in Somogyi fixans	10 min	2 Enzyme–substrate reaction: 3,3'-diaminobenzidine (DAB)	
1.3 Rinse in Tris	10 min	1.3 Rinse in Tris	10 min	2.1 Rinse in PBS	
1.4 Rinse in Tris	10 min	1.4 Rinse in Tris	10 min		
1.5 Rinsing for prefixed sections	30 min	1.5 Rinsing for prefixed sections	30 min	3 Silver precipitation	
				3.1 Rinse in distilled water I 56–60 °C	10 min
2 Blocking of endogenous peroxidase		2 Blocking of endogenous peroxidase		3.2 Rinse in distilled water II 56–60 °C	10 min
				3.3 Rinse in distilled water III 56–60 °C	10 min
2.1 3% H_2O_2	10 min	2.1 3% H_2O_2	10 min	3.4 Ammoniacal silver nitrate solution	10 min
2.2 Rinse in PBS	10 min	2.2 Rinse in PBS	10 min	56–60 °C (Prepare: Stock ammonium hydroxide (25–30%) is diluted 1:1	
3 Incubation with 10% normal goat serum (NGS)	30 min	3 Incubation with diluted normal blocking serum	20 min	with H_2O to facilitate titration of the dilute silver solution. Add ammonium	
3.1 Blot excess serum from sections		3.1 Blot excess serum from sections		hydroxide drop by drop; the 0.5% silver nitrate solution will become cloudy and then clear again. Heat to 56–60 °C)	
4 Incubation with primary antibody	over night	4 Incubation with primary antibody	over night	3.5 Rinse in distilled water	15 sec
				3.6 Rinse in 1% sodium thiosulfate	15 sec
				3.7 Rinse in distilled water	15 sec
4.1 Rinse in PBS	10 min	4.1 Rinse in PBS	5 min	3.8 Tone in 0.2% gold chloride	2 min
				3.9 Rinse in distilled water	15 sec
5 Incubation with 1% secondary antibody	12 h	5 Incubation with diluted biotinylated secondary antibody	30 min	3.10 Treat in 0.5 % oxalic acid	2 min
				3.11 Rinse in distilled water	15 sec
5.1 Rinse in PBS	10 min	solution		3.12 Treat in 5% sodium thiosulfate	5 min
		5.1 Rinse in PBS	5 min	3.13 Rinse thoroughly in tap water, dehydrate, mount and coverslip	
6 Incubation with 1% PAP complex	3 h				
		6 Incubation with Vectastain®	30 min		
6.1 Rinse in PBS	10 min	Elite ABC reagent			
		6.1 Rinse in PBS	5 min		
7 Enzyme–substrate reaction: 4-chloro-1-naphthol		7 Enzyme–substrate reaction: 4-Chloro-1-naphtol			
7.1 Rinse in PBS		7.1 Rinse in PBS			
8 Mounting in Aquatex®		8 Mounting in Aquatex®			

Some solutions of the ABC Kit (Vectastain PK6200) are ready to use. Their compositions are not published by the suppliers. The CSA and EnVision+ ™ protocols were not changed. They are used as recommended by the supplier. Therefore, it was to do without the repetition of these protocols.

Table 3
Overview of antibodies, the tested concentration, the contrasts which were determined by interactive image analysis, costs (100% correspond to 664.68 Euro) and performance of the methods (σ_{FG}: standard deviation of foreground signal and σ_{BG}: background signal)

Methods	Contrast [%]	σ_{FG} [%]	σ_{BG} [%]	Antibody concentration	Costs/200 slides [%]	Time [h]
PAP neurofilament	49	16	4	1:40	0.08	4
PAP parvalbumin	47	14	5	1:100		
PAP synaptophysin	20	7	2	1:800		
ABC neurofilament	46	10	5	1:2000	9.23	2
ABC parvalbumin	47	11	6	1:1000		
ABC synaptophysin	32	9	5	1:1000		
LSAB® neurofilament	18	7	3	1:40	26.15	1
LSAB® parvalbumin	12	7	6	1:100		
LSAB® synaptophysin	18	4	5	1:800		
EnV neurofilament	36	13	2	1:2000	71.54	0.75
EnV parvalbumin	36	11	5	1:500		
EnV synaptophysin	25	10	2	1:500		
SILVER neurofilament	24	15	11	1:2000	13.08	3
SILVER parvalbumin	35	16	16	1:500		
SILVER synaptophysin	45	13	7	1:2000		
TSA® neurofilament	49	12	3	1:5000	100	3
TSA® parvalbumin	31	8	4	1:5000		
TSA® synaptophysin	37	11	2	1:5000		
CSA neurofilament	43	11	4	1:5000	64.62	2
CSA parvalbumin	34	7	3	1:10,000		
CSA synaptophysin	41	9	3	1:5000		
C2 neurofilament	59	11	2	1:5000	13.08	2
C2 parvalbumin	62	11	3	1:10,000		
C2 synaptophysin	53	9	3	1:5000		

enzyme: streptavidin is labelled with alkaline phosphatase. The alkaline phosphatase-labelled streptavidin is coupled directly to the secondary antibody. Reaction complexes do not emerge by this procedure; therefore, the ABC method produces a stronger signal enhancement. Because new fuchsin is used as a substrate for alkaline phosphatase, the pink to red reaction product can be differentiated clearly from dark red to brown DAB or AEC reaction products. This is of advantage for enzyme–substrate-based colocalizations.

The LSAB® Kit from DAKO (K676) was applied according to the Kit instructions. For antibody concentrations see Table 3.

2.6. EnVision+™ method

The EnVision+™ System of DAKO (rabbit K4002, mouse K4000) is a two-step technique. It is based on a dextran–polymer conjugated with about 20 secondary antibodies and about 100 HRP molecules. This method makes no use of cyclic enhancement: it is a one-time amplification procedure. Like the other techniques, the EnVision+™ System needs the same substrates: DAB (diaminobenzidine), AEC (3-amino-9-ethylcarbazol) or 4-chloro-1-naphthol (4-CN).

Because the primary antibodies were derived from mice, the EnVision+™ System against mice (DAKO K4000) was tested. The procedure was performed as proposed by the Kit instructions. The concentrations of antibodies are shown in Table 3 and the result of the staining is presented in Fig. 7.

2.7. Silver precipitation method

Previously, many amplification methods have been published that enhance the visible intensity of the endproduct of a DAB–enzyme–substrate reaction [15,16,28,30,35]. The method of Quinn and Graybiel [35] was tested with the result that unspecific precipitation of silver ions at the argyrophil components in the tissue produces a disturbing background. This background can be greatly suppressed by heeding differences in the kinetic behavior of the reaction. The period of development of the DAB endproduct is shorter than that of the silver precipitation at the argyrophil components; therefore, the DAB reactions should be sufficient and completed before the silver precipitation step begins [28].

The procedure starts with performing the ABC method as proposed by the instructions of the Vectastain Kit (PK-6200) followed by the silver enhancement step (Table 2). For antibody concentrations see Table 3, and the staining result for neurofilament and parvalbumin is shown in Fig. 7.

2.8. Catalysed signal amplification method (CSA)

This procedure starts with the ABC method. Before the enzyme–substrate reaction takes place the amplification with biotinylated tyramine occurs. The mechanism of enhancement by biotinylated tyramine is described as follows. Tyramine is the biogen amine of the aromatic amino acid tyrosine and is oxidized in the presence of HRP and H_2O_2.

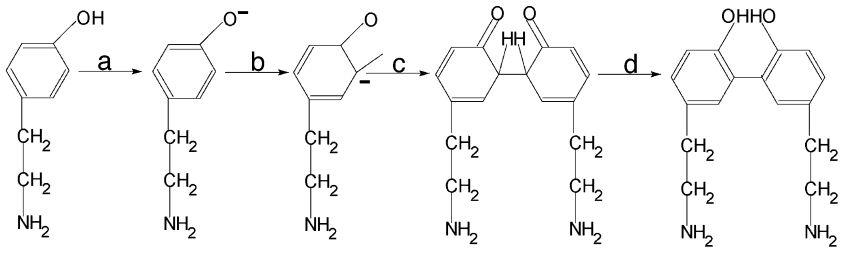

Fig. 3. The tyramine reaction in the presence of H_2O_2 and peroxidase.

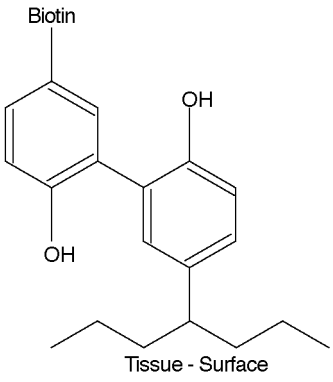

Fig. 4. Structure of biotinylated tyramine at phenol-rich tissue components.

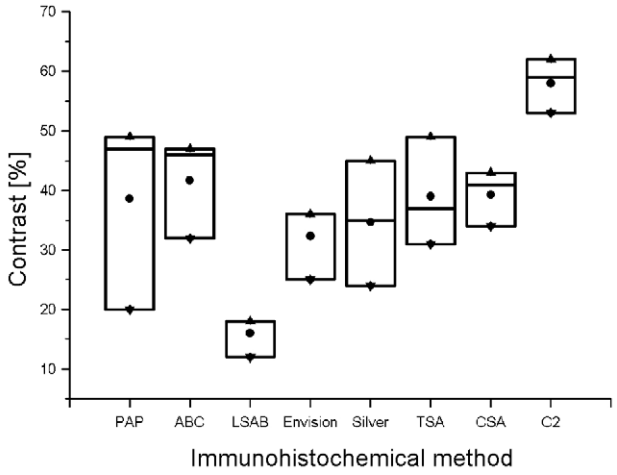

Fig. 6. Minima, maxima, mean and deviations of contrasts of all tested methods. The C2 method has a small standard deviation and the largest contrasts.

Within this reaction, a hydrogen ion is split at the para-hydroxy group of the benzene ring of tyramine and produces two H_2O molecules from one H_2O_2. The oxygen ion has one free electron (Fig. 3a) which changes from the para to a meta position by isomerization (Fig. 3b). Two molecules can then bind covalent to the isomer form (Fig. 3c) leading to a dityramine. Finally, the enolstructure of the isomer is tautomerized to a ketostructure (Fig. 3d) [17]. An important prerequisite is the presence of many phenolic compounds in situ (for example, tyrosine or tryptophane) (Fig. 4).

After incubating with the biotinylated secondary antibody, the peroxidase-labelled streptavidin–biotin complex is bound and the ABC procedure is complete. At this time, many peroxidases have accumulated around the secondary antibodies. These peroxidases can transform the biotinylated tyramines as described above. The biotinylated tyramine can bind to phenolic compounds around the primary antibody binding sites. A further streptavidin–peroxidase complex incubation is then necessary. This complex binds at the free biotin sites of the tyramine (Fig. 2).

Theoretically, the chemical reaction may take place on the total surface area of the tissue but the reaction needs HRP as a catalyst. Therefore, the reaction is localized at the primary antibody binding site where HRP had previously

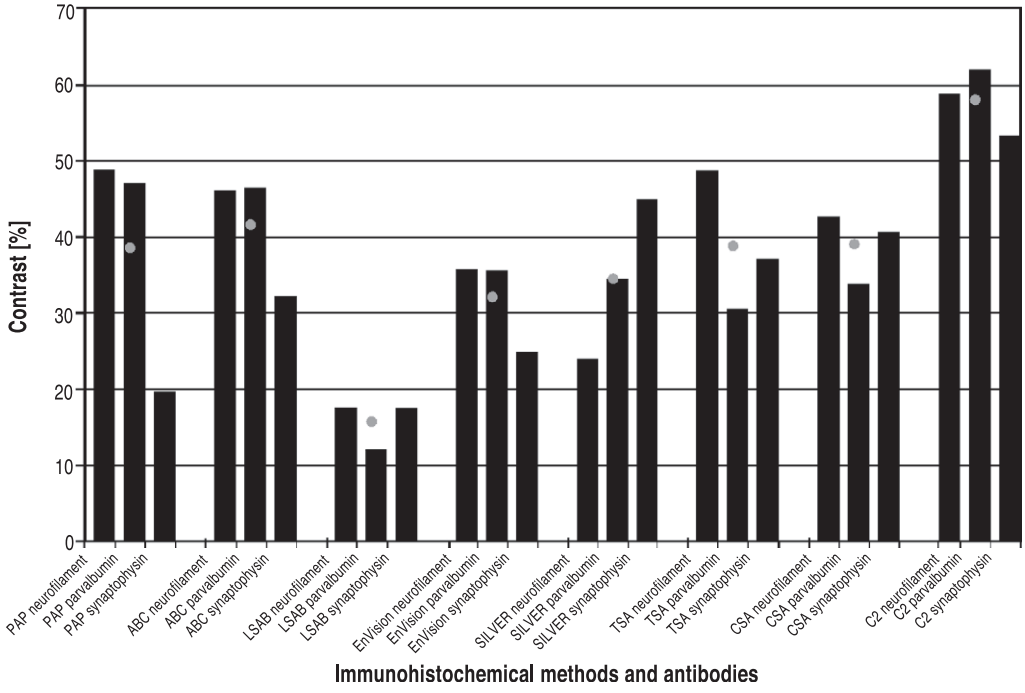

Fig. 5. Normalized contrast values [%] of the different immunohistochemical methods and antigen distributions of the analyzed rat brains. The gray points in the diagram indicate the mean contrasts of all three antigens in a certain method.

accumulated. The diffusion of biotinylated tyramine is limited to very short distances only because it has a short half-life [6]. Because streptavidin can be labeled with HRP as well as alkaline phosphatase or β-galactosidase, it provides significant flexibility [7].

This method was applied according to the instructions of the CSA Kit of DAKO (K1500). For antibody dilutions see Table 3. The staining result of this method is shown in Fig. 8.

2.9. Tyramine signal amplification method (TSA®)

The only difference between the TSA® and CSA method is that HRP-conjugated streptavidin is already bound to the secondary antibody. Then the biotinylated tyramine (Fig. 3) binds to electron-rich structures around the primary antibody (Fig. 4). This is followed by an incubation by HRP-conjugated streptavidin which binds at the biotin derivates of the biotinylated tyramine. The

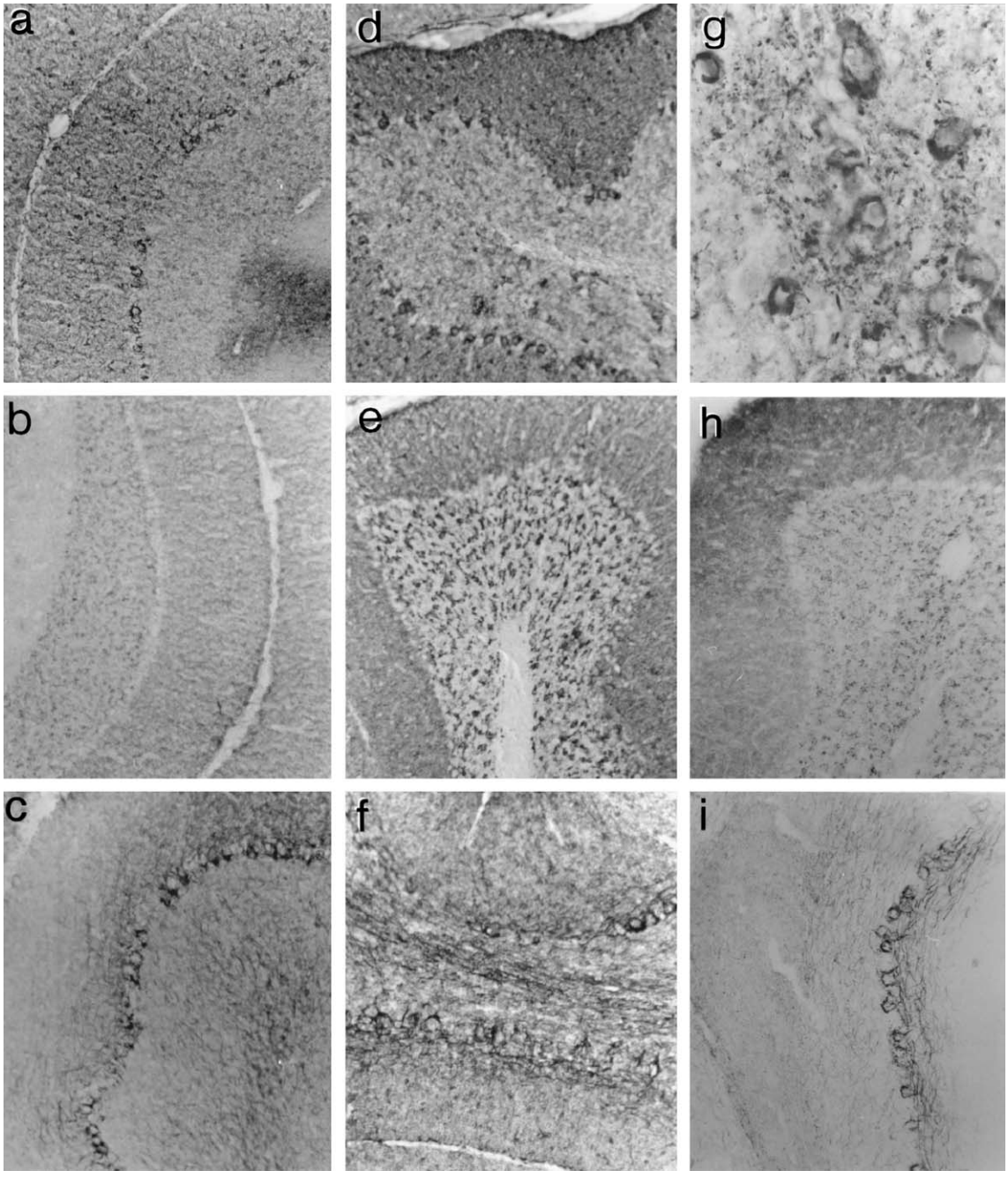

Fig. 7. Methods with simple amplification. (a) ABC method: Parvalbumin 36×. (b) ABC method: Synaptophysin 36×. (c) ABC method: Neurofilament 36×. (d) Silver enhancement: Parvalbumin 36×. (e) Silver enhancement: Synaptophysin 36×. (f) Silver enhancement: Neurofilament 72×. (g) EnVision+™: Parvalbumin 144×. (h) EnVision+™: Synaptophysin 36×. (i) EnVision+™: Neurofilament 36×.

bounded streptavidin–peroxidase is developed directly without using an ABC complex; therefore, the enhancement is not as strong as in the CSA method.

For the TSA® method, the TSA® indirect renaissance (NEL 700A) kit from NEN Life Science Products was used and performed as suggested by the Kit instructions. The primary antibody concentrations are documented in Table 3. The staining result is documented in Fig. 8.

2.10. C2 method

The CSA Kit was adapted and optimized with the resulting procedure being referred as C2 method. The mean incubation costs are considerably less than those of the CSA method. Biotinylated tyramine is produced according to Adams [1], that is, 100 mg HNS-LC-Biotin (sulfosuccinimidyl-6-biotinimide hexanoate) from Toronto Research

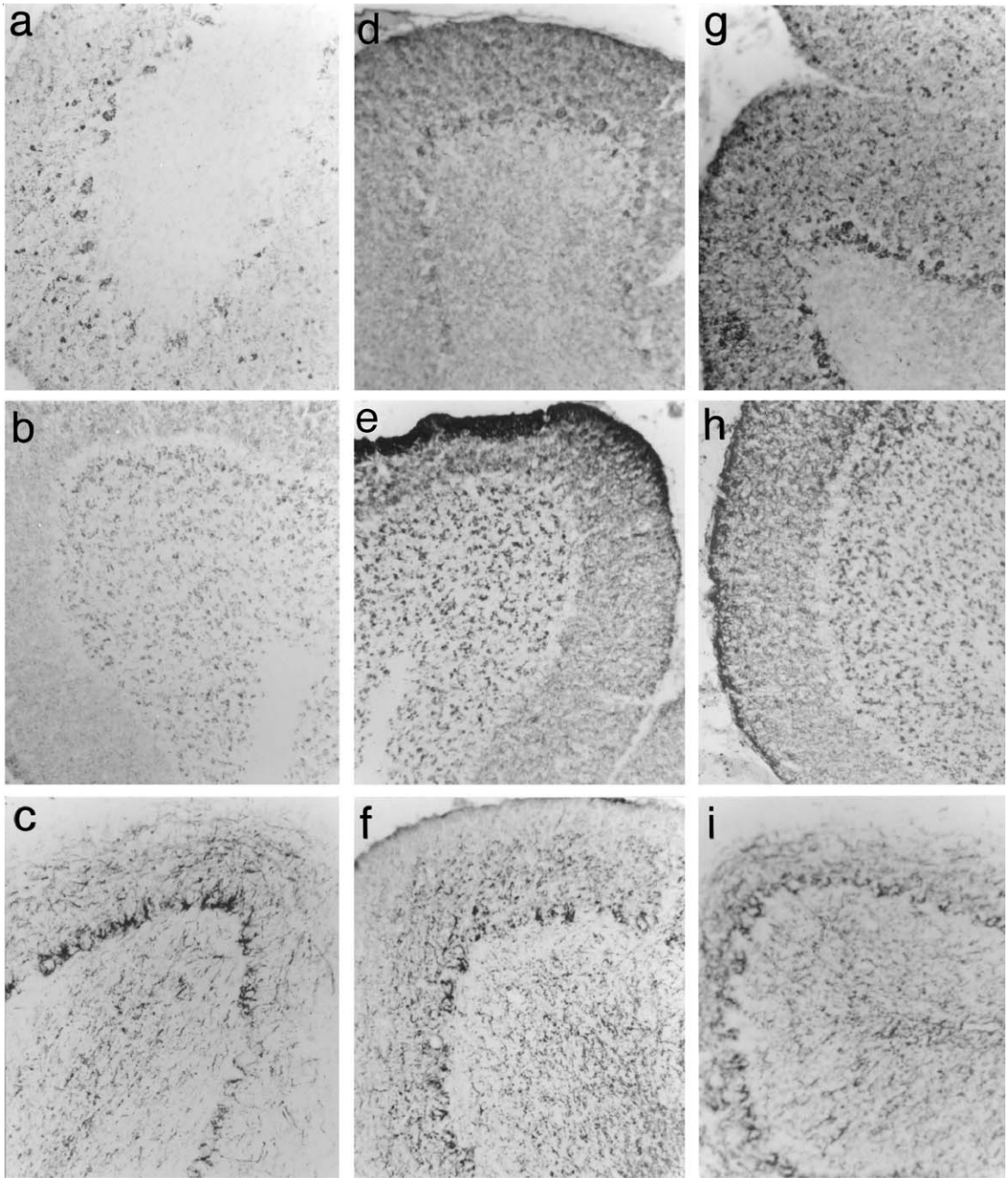

Fig. 8. Methods with cyclic amplification. (a) TSA® method: Parvalbumin 36 × . (b) TSA® method: Synaptophysin 36 × . (c) TSA® method: Neurofilament 36 × . (d) CSA: Parvalbumin 36 × . (e) CSA: Synaptophysin 36 × . (f) CSA: Neurofilament 36 × . (g) C2: Parvalbumin 36 × . (h) C2: Synaptophysin 36 × . (i) C2: Neurofilament 36 × .

Chemicals (s69025) and 31.2 mg tyramine–HCl (Sigma T-2879) mixed in 40 ml of 50 mM borax buffer (pH 8). After 12 h at room temperature (20 °C) the solution was filtered through a 0.45-μm pore filter. The resulting concentration of biotinylated tyramine (BT) is about 7 μM. This solution can be used for up to 6 months when stored at 4 °C or for long-term storage at −20 °C for 2 years (tested here). Thawing is also possible. Before use, 1 μl of the 7 μM solution and 1 μl 30% H_2O_2 was diluted in 1 ml 0.01 M PBS.

The DAKO protein blocking solution reduces the background more than any other tested blocking solution (normal goat serum, dry milk solution, bovine albumin solution). The biotinylated secondary antibody against mouse or rabbit was produced in goat (DAKO E0433, K1498) and diluted 1:500 in 0.1% bovine serum albumine, 10% normal goat serum and 0.05% thimerosal in PBS (pH 7.4). The streptavidin–biotin complex conjugated with HRP DAKO (K0377) is produced 30 min before incubation by mixing 8 μl streptavidin and 8 μl HRP-conjugated biotin each in 1000 μl PBS. The HRP-conjugated streptavidin (Dianova 016-030-084) was used in a concentration of 1:200 in PBS. The staining result of this method is presented in Fig. 7 and a precise protocol is presented in the Appendix A.

2.11. Contrast measurement

In order to quantify the contrasting results of the different immunohistochemical methods, a videomicroscopic setup was used [38]. The measurement was done with a high resolution (12 bit, 2996 × 3060 pixel) scanning camera (ProgRes 3012) from Zeiss Vision on the Zeiss Universal bright field microscope (objective 20×, secondary lens 1.25×) with IBAS 2.5 image analysis software. The resolution of a pixel is 0.503 μm. Those sections which show the smallest artifacts and homogeneous reactions were selected by visual inspection. Measurements were done at the same brain regions for reasons of comparability. On each section 50 measurements of the immunoreactive regions (foreground) and non-immunoreactive regions (background) were performed. The mean gray values of foreground and background in the three different color channels were calculated and subtracted. The mean gray values were normalized (percent) providing a measure for contrast of immunopositive foreground and immunonegative background.

3. Results

The DAB reaction with HRP leads to an intense response in immunoreactive regions; however, the background signal, in comparison to 4CN, is obviously stronger. Therefore, the contrast of DAB increases in relation to 4CN. The AEC gives a slightly lower background intensity than DAB; however, immunopositive sites are not as strongly visualized as in the DAB reaction. Therefore, the contrast resembles that of DAB. The Hanker–Yates reagent produces an intense foreground signal. The background is stained non-homogeneously and controlling of the reaction is difficult which can lead to sometimes a patch-like appearance in the reactive tissue. The best results were obtained by 4CN where the contrast is strong, the reaction homogeneous and specific and the reaction time optimal (5–10 min). The sections, however, must be stored at 4 °C in the dark; otherwise, the reaction product becomes blurred within 7–14 days. Within 3 months, 4CN reactions should be measured due to limited product stability.

The results of the contrast measurements are shown in Figs. 5 and 6. The differences are obvious in the diagrams whereas in the micrographs (Figs. 7 and 8) recognizing smaller contrast differences is not possible. The LSAB® method provides the lowest contrast, is quite fast (1 h) but is relatively expensive (Table 3). It leads always to a significant ($p < 0.05$) smaller contrast in comparison to all other methods. The fastest method is the EnVision+™-System (Fig. 7) but the contrasts lie only between 25% and 30% and costs are relatively high. The silver enhancement (Fig. 7) produced contrasts that lie between 25% and 45%. This method requires about 3 h and is relatively inexpensive. The TSA® and CSA methods yield comparable contrast results (Fig. 8), but because CSA works with a stronger enhancement, the antibody dilution of parvalbumin must be increased to 1:10,000 in comparison to the TSA® method where 1:5000 gives optimal results. The PAP and ABC methods (Fig. 7) have similar contrast values (in between 20% and 48%). The PAP method is more inexpensive. However, this procedure requires 4 h whereas the ABC method involves only 2 h. Furthermore, the ABC method produces significant ($p < 0.05$) stronger contrast in comparison to the EnVision+™ method. The optimized C2 method leads to the highest contrast values ranging between 50% and 60% that are close to the silver enhancement and ABC method. The C2 method produced significant ($p < 0.05$) stronger contrasts as compared to the rest of the methods (Table 4).

Table 4
The mean contrast values of the different epitope locations for each method were compared by the *t*-test

	PAP	ABC	LSAB®	EnVision+™	Silver	TSA®	CSA	C2
PAP			+					–
ABC			+	+				–
LSAB®	–	–		–	–	–	–	–
EnVision+™		–	+					–
Silver			+					–
TSA®			+					–
CSA			+					–
C2	+	+	+	+	+	+	+	

The significance level is $p < 0.05$ and the sample size is five. The methods within rows (left column) are compared with each other. We found that the PAP method in the first row gives a significant stronger (+) contrast than the LSAB® method. PAP in comparison with C2 leads to significant less (–) contrast.

4. Discussion

In order to quantify the results of different immunohistological methods using a unique enzyme–substrate technique, the AEC, DAB, HYR and 4CN chromogens were compared. It was found that 4CN gives reliable results with respect to the foreground and background signal intensity. Because the aim is to quantify immunohistological methods and not enzyme–substrate reactions, these were not quantified. With respect to immunohistological quantification and image analytic requirements, 4CN presents sufficient contrast without stronger artifacts. However, 4CN preparations have the disadvantage of an instable labeling. If the sections are stored at 4 °C in the dark, the labeling can be detected for at least 1 year. Nevertheless, quantification should be done as soon as possible after the enzyme–substrate reaction. If the measurement of the immunohistochemic reaction has been performed and micrographs are generated, the most important information of the experiment is available, independent of the original preparation.

Gray values show a nonlinear dependency on the local concentration not only of enzyme–substrate reaction products [13,21]. The effect of this nonlinearity on contrast measurements cannot be eliminated by calculating differences or proportions [21,32]. According to the Beer–Lambert absorption law, the concentration of a light absorbing substance is linearly correlated with the optical density (OD), a measure commonly used in videomicroscopy-based microdensitometry. The formal relation of transmission T and absorption or extinction E which can be calculated applying the Beer–Lambert law [14] is summarized as follows:

The transmission is determined pointwise (individual pixel i) by dividing the image (B) containing the transmission values of the dark field which depends on the camera offset and the image (W) grabbed at white field conditions [32]. The white field image contains the transmission values of the background. If the relation of the dark field image and the white field image is taken into account nonlinear effects are avoided. The mean extinction of a region of interest (ROI) (consisting of n pixels), for example, a perikaryon, is called the optical density (OD).

$$I_o = W - B$$

$$I_i = \bar{I}_i - B$$

$$T_i = \frac{I_i}{I_o}$$

$$E_i = -\log T_i = \log \frac{1}{T_i}$$

$$\mathrm{OD} = \frac{\sum_{i=1}^{n} E_i}{n}$$

$$\mathrm{IOD} = \sum_{i=1}^{n} E_i$$

Summing up the extinction values over a ROI leads to the integrated optical density (IOD). OD and IOD are determined in cytophotometry of Feulgen stains [18,36] in order to quantify changes of DNA contents in cell nuclei. Here we do not make use of the densitometric measurement method because illumination adjustments are reproducible and the black and white field images of the light–microscope–camera–system show negligible small changes within different measurement sessions. Slight fluctuations of transmission values mapped by pixels do have similar variations when contrasts of different methods and epitopes were compared with each other. The aim of this study is to find an optimal method which can be used for neuromapping purposes of histologic sections. In such mappings of immunoreactive sites, transformations for densitometric measurements are not necessary because topologic changes of the distribution of structural entities should be detected. An important approach, the gray level index (GLI: a measure of the area fraction of image analytical segmented cells), was introduced by Schleicher and Zilles [37] and is used in our studies, too. Within the GLI technique foreground informations are used in terms of structural information distributed over a certain region and not as optical density that is an indispensible measure for obtaining absolute values of staining intensities for cytologic-based quantifications. Measuring and mapping contrasts of immunopositive structures represent only one indicator for immunoreactivity. However, this indicator is a general measure that can be easily applied to any detectable signal obtained by a enzyme–substrate visualization of an immunhistochemic reaction. Therefore, we tested perikaryal, granular and fibrillar structures and no higher level or stereologic parameters like number of immunopositive perikarya, volume density of immunopositive nerve fibres.

The rapid development of PAP, ABC, APAAP and LSAB® methods was further enhanced by the progress made in amplifying the signals at immunopositive sites. The amplification of immunopositive reactions was first described by Linsenmayer et al. [26] in which an initial primary and secondary antibody incubation was simply repeated using the two F_{ab} arms of the secondary antibody as a bridge for binding the second primary antibody. A further step was achieved by Lascano and Berria [25] who increased the visible endproduct of the enzyme–substrate reaction employing metals such as osmium or silver. The contrast values can be greatly increased by applying this method; however, the back-

ground increases as well. Therefore, differentiation steps like sodium thiosulfate, gold and a blocking step with copper hydrogen peroxide [15,16,28,30,35] have been introduced.

Tyramine-based amplification has proven to dramatically increase of sensitivity (by a factor of 10 to 1000) by preserving the strong contrast [11,20,23,24,27,44,47,48]. One advantage is to investigate specimens that were not optimally fixed [9]. The costs of primary antibodies are reduced because they are more diluted by maintaining and/or enhancing the contrast of immunopositive sites. The latest development consists of introducing the coupling of multiple secondary antibodies and enzymes on a single molecule such as dextran (EnVision+™ System). This method leads to sufficient contrast, easy handling and a fast visualisation after only 45 min. However, this method is the most expensive one which means that methodic duration seems to be negatively correlated with costs. Furthermore, it is unclear how strong the accessibility of antigens determines the result of the immunoreaction. If antigens are detected which are anchored just beneath the surface of the sections—especially thicker vibratome sections—because the huge dextran–enzyme complex cannot diffuse to deeper targets, then we get an amplification of a part of all antigens only. This may lead to wrong quantitative results.

All immunohistochemical methods have been investigated by quantitative image analysis. Here, quantitative results are presented for the first time. Features of primary interest are the contrast and the sensitivity. Those of secondary interest are costs, handling and duration. The features of secondary interest become important with respect to routine immunohistochemistry and incubation of very large sections or extensive serial sections, especially of the human brain for detection of changes in the chemoarchitecture.

The LSAB® method delivers the lowest contrast. The contrasts of the other methods (PAP, ABC, TSA®, CSA) are obviously stronger; however, their differences only vary slightly. Therefore, the contrast alone is not a sufficient feature in order to decide which method is optimal.

The second feature of primary interest is the sensitivity which depends on the concentration of the primary antibody by visualizing the same structures with the same intensity in comparison to other methods. This means that a high concentration of the primary antibody as applied by the PAP method produces a similar result in comparison to a 10- to 100-fold lower concentration of the primary antibody as applied by the CSA method. The CSA and TSA® methods make use of an amplification step, therefore it is not surprising that the primary antibody concentrations are relatively low in comparison to the PAP, ABC and LSAB® methods. Because the CSA method is using the ABC complex, it is slightly more sensitive than the TSA® method.

Considering the secondary features the CSA and TSA® methods are faster than the PAP and LSAB® methods. Furthermore, the CSA method gives optimal results with respect to image analytical-based quantification. However, the costs of the commercial kit are relative large and the practical usage of the kid components for larger free floating sections is not satisfactory. Therefore, it is necessary to modify and optimize the CSA method to the vulnerable neuronal tissue, especially that of the human brain. This leads to a further increase of contrast and sensitivity. The modification of the CSA method (the modification is called C2 method) leads to a reduction of the kit-cost by a factor of about 5.

The PAP method produces even quite strong contrasts and this kind of immunohistochemical standard technique is relative cheap. However, its sensitivity is extremely low. In addition, this cannot always be controlled by increasing the antibody concentration because the background staining increases too. Therefore, the PAP method can lead to problems by applying it to material with a reduced antigenicity. This point is an important problem within human brains because there postmortem time can only be standardized by extensive sampling which may result in unrealistic long phases of collecting material leading to further problem of storage and changes of the material. Adams [1] and Hunyady et al. [20] have reported that suboptimal fixation or formalin resistance [29,50] can be handled by applying a biotinylated tyramine-based amplification. A further advantage of the C2 method with respect to costs consists in the ability to apply the method to gross sections such as through whole brains, as it has been done on the human brain and large brains of mammalian species [3,39,40,46]. For some antigens–antibody combinations, it could be necessary to use an unmasking method (enzymatic digestion, cyclic freezing, microwave), before incubation of the primary antibody.

The C2 method is an optimized procedure for efficient and economic immunohistochemistry although it is not perfect solving each immunohistochemical visualization problem. Sometimes the sensitivity, the blocking of endogeneous peroxidase or biotin and streptavidin binding sites present themselves and must be dealt with [4,20,23,29,48]. The primary antibody concentrations must be adapted and tested by visualizing them as well as adjusting the primary antibody dilutions within the C2 method. However, by using a scheme of 1:1000, 1:2000 and/or 1:4000 dilution steps, an impression of the trend can determine the optimal dilution for further testing.

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Appendix A.

The C2-method		
1	Fixation	
1.1	Drying of section at 37°C	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
1.4	Rinsing for prefixed sections	30 min
2	Blocking of endogenous peroxidase	
2.1	3 % H_2O_2 (if eruption of blisters is too strong, place bath on a electromagnetic stirrer)	20-30 min
2.2	Rinse in PBS	5 min
3	Blocking of endogenous avidin and biotin sites of binding	
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 sites of protein binding	
4.1	One drop of protein blocking solution	15 min
5	Incubation with primary antibody	
5.1	Dilute antibody and add it directly without removing the protein block	
5.2	Incubation	1.5-12 hrs
5.3	Rinse in CSA-buffer	10 min
6	Incubation with secondary antibody	
6.1	Prepare ABC reagent (1 ml PBS+8 •l streptavidin+8 •l biotin)	
6.2	One drop of species specific secondary antibody from goat (1:500)	15-30 min
6.3	Rinse in CSA-buffer	10 min
7	Incubation with ABC-reagent	
7.1	50 • l ABC reagent/ cm5 section	30 min
7.2	Rinse in CSA-buffer	10 min
8	Amplification	
8.1	Prepare amplification solution (1 ml PBS+1•l BT+1•l 30% H_2O_2)	
8.2	50•l amplification solution/ cm5 section	10 min
8.3	Rinse in CSA-buffer	10 min
9	Labelling of tyramine with biotin (biotinylated tyramine: BT)	
9.1	HRP labelled streptavidin (1:200 in PBS)	
9.2	50•l HRP streptavidin/ cm5 section	15 min
9.3	Rinse in CSA-buffer	15 min
10	Enzyme-substrate-reaction with 4-chloro-1-naphthol	
10.1	Rinse in PBS	
11	Mounting in Aquatex	

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