ACTA STEREOL 1985; 4/2: 175—180
PROC ESS IV-1 GÖTEBORG 1985
ORIGINAL SCIENTIFIC PAPER

IMAGE ANALYSIS AND THE DETERMINATION OF THE ENTITY OF COEXISTENCE OF NEUROACTIVE SUBSTANCES IN NERVE CELL BODY POPULATIONS

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ABSTRACT

Automatic image analysis has been employed to use the occlusion method for the quantitative determination of coexistence. Also a method has been developed using the dilatation function of the IBAS image analyzer combined with the performance of a logical operation "and", to quantitatively evaluate the codistribution of profiles. Thus, this procedure allowed the determination of a dilated overlap area (FA^{D}_{AB}), which could be related to the mean dilated field area $(FA^{D}_{A}+FA^{D}_{B})/2$.

Keywords: Codistribution, coexistence, image analysis, neurons, transmitter.

INTRODUCTION

Stereological methods (Weibel 1979) and computer assisted evaluation of images have had a substantial impact in neurobiology. There exist two methods for evaluation of the entity of coexistence of neuroactive substances in nerve cell bodies using image analysis. The so called occlusion method allows an overall quantitative evaluation (Agnati et al. 1982a) and the overlap method, which allows a quantitative evaluation of coexistence cell by cell (Agnati et al., 1984).

Principles of the Experimental Procedures

A. The occlusion

This method is based on the analysis of three adjacent sections stained in a random way with antisera against A and B alone and with the two antisera together. The entity of coexistence is obtained by subtracting from the sum of the number of the immunoreactive nerve cell bodies demonstrated with antiserum A alone and antiserum B alone, the number of the immunoreactive nerve cell bodies demonstrated with the combined use of antisera A and B (Fig. 1). The procedure is based on the fact that the coexisting cells are demonstrated with both antiserum A alone and antiserum B alone as well as with the combined use of the two antisera. The second method is the s.c. overlap method (Agnati et al. 1984). In this analysis the image analyzer records the position and the perimeter of each

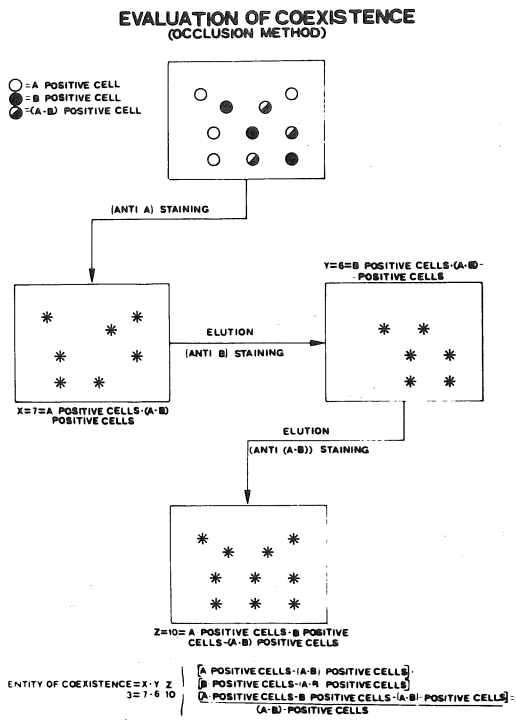


Fig. 1. Schematic representation of the morphometrical evaluation of coexistence by means of the occlusion method. For further details, see text.

nerve cell body demonstrated by an antiserum A in a Cartesian plane. In an adjacent section or in the same section following elution of the anti-A immunoreactivity staining for B immunoreactivity is performed using an antibody anti-B. The image analyzer records the position and the perimeter of the B immunoreactive nerve cell bodies in the same Cartesian plane (see Fig. 2).

The computer then considers coexistence to have taken place when the A and B immunoreactive areas overlap by at least 30 %. This method offers the possibility to assess coexistence of neuroactive cell bodies in each individual nerve cell body of a selected nerve cell group. In order to obtain absolute values of coexistence by means of the overlap procedure the same section must be used with electrophoretic elution. Previously we have used these methods in combination with semiautomatic image analysis on sections stained by means of the indirect immunoperoxidase procedure. We will now present a procedure involving automatic image analysis using the IBAS image analyzer (Zeiss-Kontron). In this way the methods can be performed much more rapidly.

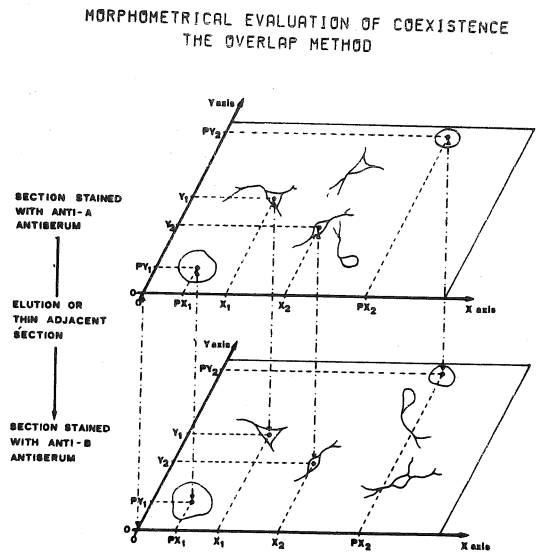
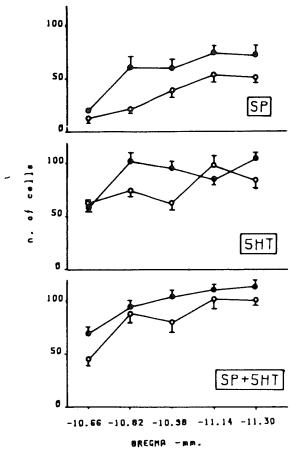


Fig. 2. Schematic representation of the morphometrical evaluation of coexistence by means of the overlap method. The neuroanatomical landmarks are indicated by the coordinates P (x_1, y_1). Only for the cells for which there is coexistence (i.e. they are positive in both sections) have the respective coordinates been given.

RESULTS AND DISCUSSION

On the use of the occlusion method by means of automatic image analysis

In these new procedures we employed the occlusion principle for studies on coexistence in thin adjacent sections. In this procedure the sections showing A and B immunoreactivity alone are adjacent to one another, so that in the same program also codistribution of A and B immunoreactive nerve cell bodies can be quantitated. In this procedure the section stained with both antisera is analyzed first. By means of the shading and discrimination functions a binary image is obtained, which only demonstrates A and B immunoreactive profiles. By means of the measurement and the identification programs the field area (FA_{AB}) of the immunoreactive cell bodies is determined. The "skip" function in the image analysis makes it possible to automatically remove too small and too large immunoreactive profiles. The A immunoreactive nerve cells and B immunoreactive nerve cells are subsequently analyzed in a similar way. Thus, field area A (FA_A) and field area B (FA_B) is determined as well as the mean area A ($\bar{A}\pm SD_A$) and mean area B ($\bar{B}\pm SD_B$). The mean areas and the standard deviations were obtained automatically from the histograms of the areas of the immunoreactive profiles. The entity of coexistence is by this program obtained by taking the sum of the field areas for A and B immunoreactive profiles (FA_A+FA_B) minus FA_{AB}. The entity of coexistence can then be expressed in per cent of FA_A or FA_B (see Agnati et al., 1985a,b). Using the occlusion principle it could e.g. be demonstrated that during the



X ± SEM

	YOUNG	OLD
SP	274±9.8	158±6.9
SHT	446±8.3	382±7.0
SP+SHT	495±8.2	422±10.3

THEORETICAL BASIS	OBSERVED VALUES YOUNG	OLD
$(N_{SP} + N_{SHT/SP}) +$	296 +	158 +
$(N_{SHT} + N_{SHT/SP}) +$	446 +	382 +
$-(N_{SP} + N_{SHT} + N_{SHT/SP})$	- 495	-422
$N_{SP} + N_{SHT} + 2N_{SHT/SP} -$ $N_{SP} + N_{SHT} + N_{SHT/SP}$		
$/ \quad / \quad N_{SHT/SP}$	247	118
% OF COEXISTENCE	55	31

Fig. 3. In the left part of the figure the number of 5-HT immunoreactive, Substance P (SP) immunoreactive and 5-HT plus SP immunoreactive profiles are given at various rostrocaudal levels of the rat medulla oblongata in the 3 month and 24 month old male rat. In the central part of the figure the total number of cells found after incubation with the SP antiserum alone, 5-HT antiserum alone and the two SP and 5-HT antisera are reported (n= 3; means ± s.e.m.). In the right part of the figure the occlusion method has been applied on the number of 5-HT, SP and 5-HT plus SP immunoreactive nerve cell bodies has been applied. In this way an overall evaluation of coexistence of Substance P and 5-HT has been obtained in the 24 month and 3 month male rat. The number of SP and 5-HT costoring cells has been expressed in per cent of the number of 5-HT immunoreactive nerve cell bodies. n. of cells= number of cell profiles. Mean SP profile area (Young) is 307.49 μm^2 and mean 5-HT profile area (Young) is 264.61 μm^2. Section thickness: 30 µm.

aging process the entity of coexistence of Substance P and 5-hydroxydopamine in nerve cells of the medulla oblongata of the rat was reduced from 55% to 31% (Fig. 3) (see Agnati et al., 1985c). It has previously been shown that there occurs a reduction in neuron size with age (Hung et al. 1981). Furthermore, the present area measurements are a measure of volume density (V_V) rather than number, since the neurons are selected according to a bias directly proportional to size. Therefore, it is possible that the reduction in SP/5-HT costoring profiles seen in aging may be related at least in part to a preferential reduction in the size of such profiles during aging.

A method to quantitate codistribution of profiles by automatic image analysis

In this automatic image analysis we also determined the codistribution of the A and B immunoreactive profiles as well as the eveness of their distribution in the selected field. To this purpose we used the medianization and dilatation functions in the IBAS image analyzer, by which small objects are removed and nerve cell bodies close to one another merge to form large area profiles ($\bar{A}^D \pm SD^D_A$ and $\bar{A}^D \pm SD^D_B$) (Agnati et al.,

Table I

IMAGE ANALYSIS OF CA FLUORESCENCE IN PERICYTES OF THE BRAIN CAPILLARIES VISUALIZED BY MEANS OF PARGYLINE L-DOPA TREATMENT IN CORONAL SECTIONS IN THE MALE RAT

CAs were trapped in the pericytes by treatment with the monoamine oxidase inhibitor pargyline (100 mg/kg, i.p., 4 h before killing) L-dopa (60 mg/kg, i.p., 30 min before killing). The pericytes in the ventromedial hypothalamic nucleus and the posterior cingulate cortex (layer II-IV) have been analized. Means ± s.e.m. are shown (n= 4-5 observations). $\bar{A}$= Mean value; N= Number of counts; N_D= Number of counts after dilatation; I%= $(N-N_D)/(N-1) \cdot 100$. Index of uniformity and/or density of pericytes; FA= Field area ($N \times \bar{A}$).

Brain area	$\bar{A}$ μm^2	N	FA μm^2	N_D	I%
Ventromedial hypothalamic nucleus	55±5.6	25±2	1419±220	7±2	73.5±2.6
Posterior cingulate cortex	63.3±3.8	46±5	2680±551	7±1	86.7±3.6

1985b). An octagonal shape was used in the dilatation procedure, and each time it was performed the size of the profiles was increased by one pixel at the boundary. The higher the density of immunoreactive profiles in the nerve cell body population the higher was the degree of formation of large area profiles with a reduction in the number of profiles. Furthermore, by comparing the ratios of the respective field area before and after dilatation (FA^D_A/FA_A and FA^D_B/FA_B) it was possible to obtain an evaluation of the eveness of distribution of the respective immunoreactive profiles A and B. As illustrated in Table 1, an index of uniformity and/or density of profiles can be developed by the dilatation function I%= $(N-N_D)/(N-1)x100$, where N= number of counts, N_D= number of counts after dilatation. Using semiautomatic image analysis we have also analyzed density maps by means of Lorenz curves and the Gini index to evaluate the uniformity of density distribution of profiles (Agnati et al., 1982b,c).

The two new images formed by the dilatation functions of the A and B immunoreactive nerve cell bodies were compared with each other using the "Boolop" function. In this way the dilated overlap area (FA^D_{AB}) between the A and B immunoreactive nerve cell bodies could be determined. The degree of codistribution of the A and B immunoreactive profiles was determined by calculating the ratio of the dilated field area for the overlap region (FA^D_{AB}) and the mean dilated field area of the A and B immunoreactive profiles $(FA^D_A+FA^D_B)/2$. Thus, in the same program it becomes possible to analyze in a quantitative way both coexistence using the occlusion procedure and codistribution of the two immunoreactive nerve cell body populations.

ACKNOWLEDGEMENTS

This work has been supported by a Grant (MH25504) from the NIH. We are grateful for the excellent secretarial assistance of Ms Anne Edgren.

REFERENCES

Agnati LF, Fuxe K, Locatelli V, Benfenati F, Zini I, Panerai AE, El Etreby MF, Hökfelt T. Neuroanatomical methods for the quantitative evaluation of coexistence of transmitters in nerve cells. Analysis of the ACTH- and β-endorphin immunoreactive nerve cell bodies of the mediobasal hypothalamus of the rat. J Neurosci Methods 1982a; 5: 203-214.

Agnati LF, Fuxe K, Zini I, Calza L, Benfenati F, Zoli M, Hökfelt T, Goldstein M. A new approach to quantitate the density and antigen contents of high densities of transmitter-identified terminals, immunocytochemical studies on different types of tyrosine hydroxylase immunoreactive nerve terminals in nucleus caudatus putamen of the rat. Neurosci Lett 1982b; 32: 253-258.

Agnati LF, Fuxe K, Calza L, Hökfelt T, Johansson O, Benfenati F, Goldstein M. A morphometric analysis of transmitter identified dendrites and nerve terminals. Brain Res Bull 1982c; 9: 53-60.

Agnati LF, Fuxe K, Benfenati F, Zini I, Zoli M, Fabbri L, Härfstrand A. Computer assisted morphometry and microdensitometry of transmitter identified neurons with special reference to the mesostriatal dopamine pathway. I. Methodological aspects. Acta Physiol Scand, Suppl 532, 1984: 5-36.

Agnati LF, Fuxe K, Janson AM, Zoli M, Härfsrand A. On the use of computer assisted morphometry and microdensitometry in the quantitative analysis of immunocytochemically stained neurons: a new powerful tool in the morphofunctional analysis of transmitter-identified neurons. In: Polak J, Van Noorden S, eds. Immunocytochemistry: modern methods and applications. 2nd edition. Bristol: John Wright & Sons Publ, 1985a: in press.

Agnati LF, Fuxe K, Janson AM, Zoli M, Härfstrand A, Zini I, Goldstein M. Morphofunctional studies on the central nervous system by means of image analysis. Eric K. Fernström Symposium on Neural Regulation of Brain Circulation: Effects of Neurotransmitters and Neuromodulators, Lund, Sweden. Amsterdam: Elsevier Biomedical Press, 1985b: in press.

Agnati LF, Fuxe K, Calza L, Giardino L, Zini I, Toffano G, Goldstein M, Marraa P, Gustafsson J-Å, Yu Z-Y, Cuello AC, Terenius L, Lang R, Ganten D. Morphometrical and microdensitometrical studies on monoaminergic and peptidergi neurons in the aging brain. In: Fuxe K, Agnati LF, Hökfelt T, eds. Wenner-Gren Symposium on "Quantitative Neuroanatomy in Transmitter Research", England: MacMillan Press, 1985c: in press.

Hung H, Knebel G, Mecke E, Orün C, Sass NL. The aging of cortical cytoarchitectonics in the light of stereological investigations. Prog Clin Biol Res 1981; 59B: 193-197.

Weibel ER. Stereological methods. Acad Press, London, 1979.