A quantitative approach to cytoarchitectonics: software and hardware aspects of a system for the evaluation and analysis of structural inhomogeneities in nervous tissue

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Cytoarchitectonic studies are based on the analysis of structural inhomogeneities in nervous tissue. Boundaries of brain regions are established where local structural properties such as numerical cell density, size, shape or orientation change. The measurement of these properties from histological sections with automatic devices is biased due to the thickness of the sections. In this study, the grey level index (GLI) is measured with a TV-based image analyzer from routine histological sections. This parameter is a biased estimate of the volume density of Nissl-positive structures. The histological section is digitized into GLI values by a computer-controlled scanning procedure. The result is stored in an image matrix which is processed by digital filtering in order to visualize the laminar pattern. GLI statistics of brain regions are evaluated from the pictorial data by delineating these regions with a cursor on a hard copy fixed to digitizer. Information from a series of sections is stored in a standardized data file and combined by specific application programs.

Introduction

Stereology has introduced a variety of methods for the quantitative analysis of nervous tissue (Weibel, 1979). With the aid of stereological parameters (e.g. volume density, surface density and numerical density), structural changes caused by experimental procedures or growth processes can be established and structural differences between tissues from different locations can be demonstrated.

Due to the statistical nature of this approach, only global properties of specific brain regions (e.g. the total number or the volume density of nerve cells) can be estimated.

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Cytoarchitectonics, in contrast to stereology, is concerned with the parcellation of the central nervous system into regions differing in structure. It is based on the analysis of local properties including cell size, shape and orientation. From the inspection of Nissl-stained histological sections, boundaries of brain regions are established where these local properties change. Since no quantitative methods were available to measure these properties from a large sample of brains and serial sections, the classic cytoarchitectonic studies are purely qualitative in nature (for reference see Zilles et al., 1982). Problems arose, when minor morphological differences were regarded as sufficient to establish new boundaries, and these could then not be confirmed by other investigators. Cytoarchitectonic studies were, consequently, criticized in general (Lashley and Clark, 1946; Bailey and v. Bonin, 1951). Although automatic, TV-based image analyzing devices had been available for 10 years (Fisher, 1967) no applications to cytoarchitectonics were found until 1978 (Schleicher et al., 1978; Zilles et al., 1978). This can be explained by the methodological difficulties which arise when structural properties are measured from histological sections. Due to the thickness of the sections, measurements of stereological quantities are subject to systematic errors mostly of unknown magnitude (Underwood, 1970). This problem was either ignored (Adhami, 1973), or confronted by developing semiautomatic methods, which, at least in part, overcame these shortcomings with the implementation of manual and, therefore, time-consuming interaction (e.g. Haug, 1972, 1976; Terry and Deteresa, 1982).

A fully automatic, quantitative approach was introduced by two of us (Schleicher et al., 1978; Zilles et al., 1978). In this method, there is no correction for section thickness. The measured grey level index (GLI) is, nevertheless, correlated to the volume density of Nissl-positive elements (Wree et al., 1982) and sensitively indicates changes in the cellular composition which are not obvious to visual inspection (Schleicher and Zilles, 1983).

Material and Methods

Specimen

The GLI values can be measured in routine histological sections (Nissl staining) of brains which have been embedded in paraffin, celloidin or resin, or cut in a cryostat. Routine preparations were analyzed, because the GLI method is not intended to replace visual inspection of the microscopic image, but rather to supplement it. Nissl-stained sections were used, because most cytoarchitectural studies are based on Nissl-stained paraffin sections. The method should also be able to process already existing serial sections of rare species, from which new series cannot be obtained. Most of our sections are 20 µm in thickness, but since sectioning is of special interest for the GLI method, it will be discussed in detail in the following section.

Measuring procedure

A quantitative approach to cytoarchitectonics requires the measurement of a quantity which describes the local properties of the tissue's structure in a large
sample. In order to keep time and effort within reasonable limits, sophisticated procedures, like shape analysis or the estimation of the numerical density of neurons, cannot be performed. It is, furthermore, important that the measured quantity should not be dependent on technical procedures such as fixation and staining. It should, on the other hand, sensitively indicate changes in the local cellular composition.

Our studies are based on the grey level index (GLI) as an estimate of the local volume density of cellular structures. This estimate is biased by the section thickness and by the TV-based image analyzer which measures the areal proportion as a numerical equivalent for the volume density.

The measuring system is designed around a Microvideomat 2 image analyzer (Carl Zeiss, Oberkochen, F.R.G.) which is used for image acquisition, and a 2200MVP laboratory computer (Wang, Lowell, U.S.A.; multiuser system, 2 × 64 kbyte R/W memory, Basic 2) for processing. This computer controls all functions of the image analyzer via an 8-bit parallel interface and is used as a host computer for data storage, analysis and presentation. A schematic representation of the system is given in Fig. 1.

The GLI is defined as the ratio of the area covered by image elements which are darker than a given grey value threshold, to the entire area of the measuring field, which is of fixed size. The measuring procedure is demonstrated in Fig. 2. Fig. 2a shows structures in lamina III of the monocular part of the primary visual cortex (Oc1M) in a coronal section of a rat brain (50 × magnification; Universal microscope, Carl Zeiss, Oberkochen, FRG; 100 W, monochromatic, 540 nm, stabilized power supply). The image is corrected for shading. The segmentation of the image is achieved by thresholding (Rosenfeld and Kak, 1970). The grey value threshold is set to the grey value of the boundary between the dark cellular image elements and the bright background by analyzing the grey value histogram of the image, according to Prewitt and Mendelssohn (1966) and Eins and Gallyas (1977). The plots in Fig. 3

Fig. 1. Schematic arrangement of the image analyzing and data processing system.
Fig. 2. Image analyzing procedure for the evaluation of GLI values. Since the procedure is comprised of elementary functions which are independent of the image analyzer type, we used an IBAS II system (Kontron, Eching, F.R.G.) with a model 4 videoprinter (Polaroid, Offenbach, F.R.G.) for documentation.

a: microscopic image of cellular elements in lamina III of the monocular part of the primary visual cortex (Oc1M) in the rat (section thickness 20 μm, Nissl staining) on the computer display. The image width is 160 μm. b: boundaries of the cellular elements which correspond to the grey value of 124 are indicated as black contours. c: binary image, thresholded by the grey value demonstrated in b. d: image of c. processed to fill the cell masks. The image is subdivided into 5×5 quadratic measuring fields, each measuring 32 μm in size.

represent the grey value histograms of 3 images from 3 different brain regions in the same section. The appropriate grey value threshold, in these histograms, is located at the local minimum between the left peak which is caused by the dark elements, and the right peak which is caused by the background (116, 121 and 121 in these examples). This demonstrates that the grey value threshold shows only minor variations, when it is determined at several locations within the same section. Table
I gives a set of grey value thresholds from locations which were selected to cover a wide range of GLI values. All images of the same histological section are, therefore, segmented with a constant grey value threshold, in order to increase the sampling rate. This global threshold is calculated as a mean value from several histograms in different regions. Fig. 2b shows this boundary superimposed upon the microscopic image in Fig. 2a.

The corresponding binary image (Fig. 2c) fails to show some cellular elements (e.g. nuclei), but if these are completely surrounded by cytoplasm, which is darker than the grey value threshold, they can be filled by processing this image with the image analyzer's closing function (Serra, 1972; Fig. 2d). The positions of 5 × 5 quadratic measuring fields, each having a length of L = 32 μm are shown for Fig. 2d. The size of a measuring field is adjusted according to the desired spatial resolution, which is determined by the size of the smallest details (e.g. the width of a cortical lamina) to be resolved. According to the sampling theorem, only details which are larger than twice the length of the measuring field can be resolved in full contrast (Prewitt, 1965). Measuring fields of up to 40 μm have shown to give good resolution for the analysis of areal patterns in the cortex, while keeping the amount of data within reasonable limits. Detailed analyses of laminar pattern, however, require very high resolutions, and measuring fields of as little as 10 μm.

The histological section, or a selected part of it is digitized by scanning it as a series of adjoining measuring fields, which are of length L. The computer-controlled scanning procedure combines the electronic scanning of the preprocessed image as M × M measuring fields of length L (Fig. 2d) with mechanical scanning in M × L μm increments, using a mechanical scanning stage (Märzhäuser, Wetzlar, F.R.G.; 2000 × 1 μm/s, scanning range 150 mm × 100 mm). This method considerably
TABLE I

STATISTICS ON THE SETTING OF GREY VALUE THRESHOLDS AT VARIOUS LOCATIONS (Loc.) IN THE SECTION SHOWN IN FIG. 5

1, OC2MM, lamina II; 2, OC2ML, lamina II; 3, OC2ML, lamina I; 4, OC1M, lower part of lamina VI; 5, OC1M, upper part of lamina VI (abbreviations of occipital cortical areas according to Zilles et al., 1985): n, number of fields analyzed at each location; GLI, arithmetic mean of n GLI values; thr, arithmetic mean of n grey value thresholds; thr, median value; S.D., standard deviation of n grey value thresholds; range, range of grey value thresholds.

<table>
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<tr>
<th>Loc.</th>
<th>n</th>
<th>GLI</th>
<th>thr</th>
<th>thr</th>
<th>S.D.</th>
<th>Range</th>
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<td>2.2</td>
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<td>17.4</td>
<td>122</td>
<td>122</td>
<td>6.3</td>
<td>116–131</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>23.4</td>
<td>124</td>
<td>124</td>
<td>6.1</td>
<td>119–127</td>
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<td>24.8</td>
<td>120</td>
<td>120</td>
<td>6.5</td>
<td>117–126</td>
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<tr>
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<td>8</td>
<td>29.9</td>
<td>125</td>
<td>126</td>
<td>3.8</td>
<td>119–129</td>
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<td>19.5</td>
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<td>126</td>
<td>5.0</td>
<td>116–132</td>
</tr>
</tbody>
</table>

reduces the time and effort expenditure, since the total length of the pathway of the scanning stage is reduced by a factor of approximately 1/M.

Depending upon the size of the measuring field, up to 10 measurements per second can be performed; this includes data transfer to the host computer and storage on the hard disk. Following initialization, the scanning process is fully controlled by the measuring program; it requires no manual interaction. An auto focus is engaged after a previously defined number of images have been scanned.

The GLI values are stored as an image matrix, on hard disk (2 x 5 Mbyte; model 2260BC; Wang, Lowell, U.S.A.). The pixel coordinates are given by the x, y-count of the measuring field; the pixel value is set to the GLI-value measured in that field. The size of the image matrix to be stored for further processing is limited by the 64 kbyte R/W memory, which is available for the associated analyzing programs, to a row length of 1024 elements.

GLI and volume density

Due to the thickness of the histological section, the image elements measured by the analyzer are projections of cellular elements within a rectangular solid whose cross-section is a square of constant area, which is identical with the area of the measuring field, and whose depth is constant but unknown. This depth depends upon the focal depth and the section thickness. The unbiased estimation of the volume fraction, however, requires that profiles be generated by a test plane. The GLI values are expected to be larger than the volume density, because the projections can only be larger than the corresponding profiles generated by a test plane (Holmes, 1927). This is shown in Fig. 4 (modified from Wree et al., 1982). GLI values were measured in lamina II of the regio praepiriformis from a guinea pig. The sections ranged from 2 to 37.3 μm in thickness (block staining with galloycyanin, Araldite embedding). The volume density was estimated from 1 μm-thick sections by point counting for comparison.

The GLI values show a monotonous, non-linear dependency upon section
thickness. The GLI underestimates the volume density, in sections thinner than 5.6 μm. This is due to the cellular compartments which have high grey values and are thus not detected by thresholding. As thickness and contrast increase, the Holmes effect leads to increasing overestimation of up to 78% for 20 μm-thick sections. The increase is not limited by the focal depth (about 3 μm for the optics used). As the section thickness increases, elements, of which only blurred images are detected, contribute to the measured areal fraction. This overestimation was found to be even higher in other cortical regions with higher cell packing densities.

Since GLI values are dependent on section thickness, standardization and control of the sectioning procedure are necessary when the GLI values from different sections are to be compared. The graph in Fig. 4 predicts a value of 62.5% at a section thickness of 20 μm. Variations of ±1 μm result in a grey value fluctuation between 60.8% and 63.9%. Depending on the local slope of the approximation curve, this effect will decrease with increasing section thickness.

In some applications, the GLI analyses compile data from a series of sections. In these cases, the effect of variations in section thickness will be eliminated to a large extent by averaging.

The GLI measurement includes all Nissl-positive cell structures (i.e. the glial and endothelial cell nuclei contribute to the GLI value, in addition to the perikarya of neurons). Because the traditional cytoarchitectural studies are based on the analysis of neurons, Wree et al. (1980) studied the contribution of the different cell types to the GLI. Upon analyzing the visual cortex of Callithrix jacchus, they found a constant volume density of glial and endothelial cell nuclei throughout the cortex, from lamina I to lamina VI. This finding has been confirmed for other species and

![Graph showing dependency of GLI value on section thickness.](image)

Fig. 4. Dependency of the GLI value (ordinate) on section thickness (abscissa). Each point represents the mean of 50 measurements. Data approximation by a 5-parametric exponential function. The volume density was estimated by pointcounting from 1-μm sections and plotted together with the standard error as horizontal lines.
other cortical regions (Nurnberger, 1958; Tower and Young, 1973). Differences from one brain region to another which manifest themselves in the GLIs can, therefore, be interpreted as being correlative with differences in the volume density of neurons.

The sensitivity of the GLI values to the distribution and arrangement of cells in the brain tissue is discussed by Wree et al. (1982). The grey values may differ between brain regions which show the same volume density of Nissl-positive structures as established by point counting. This is of great importance in cytoarchitectonic investigations because the laminae can be separated on the basis of differences in the size or packing density of their perikarya.

**Data presentation**

In order to visualize the GLI distribution in a brain section, the stored image matrix is plotted with a graphic display (model 2282, Wang, Lowell, U.S.A.; 800 × 512 points) and copied with a matrix printer (model 2231-W3, Wang, Lowell, U.S.A.). Fig. 5b presents data for the brain section shown in Fig. 5a. This coronal section (rat, 20 μm, Nissl staining) was scanned by 20 μm measuring fields into an image matrix of 216 × 392 elements within 2.4 h.

The GLI image is plotted by subdividing the entire GLI range (0% ≤ GLI ≤ 100%) into 5 subranges by setting 4 GLI limits. Each range is plotted as a distinct density pattern. The limits are chosen to achieve the best visualization of the GLI distribution in the section.

No rules exist for the selection of the GLI limits, but an analysis of the cumulative frequency distribution (cfd) of the GLI values in the image matrix can be helpful in choosing these limits. This is shown in Fig. 6 for the data of the image in Fig. 5b. The GLI ranges from 0% (background; cumulative relative frequency \(H_0 = 34.7\)) to 95% (\(H_{95} = 100\%\)). A good visualization is produced, for most of the GLI images, when the GLI limits are set to values which meet the cfd at

\[H_1 = (100 - H_0) \cdot a_i,\]

where \(H_1\) is the cumulative relative frequency for a GLI of 1% (36.7% in this example) and the \(a_i\) are set to 0.4, 0.7 and 0.9. In this example, cfd values of 36.7%, 62%, 81.4% and 94.6% result in the GLI limits 1%, 15%, 21% and 28%, and the density patterns are defined as follows: GLI = 0%, 1% ≤ GLI < 15%, 15% ≤ GLI < 21%, 21% ≤ GLI < 28% and 28% ≤ GLI ≤ 100%. Small alterations of these limits may be necessary to satisfy specific demands. Attempts to increase the number of patterns representing various densities, and to use pseudocolor presentations did not result in a better visualization of the laminar pattern in cortical regions.

Fig. 5. GLI analysis of a coronal section (rat, 20 μm, Nissl staining). a: micrograph of the histological section including the primary visual cortex. The image width is 7800 μm. b: plot of the GLI matrix (216 × 392 elements) in 5 density patterns for the envisioning of the 5 GLI ranges (GLI = 0%, 1% ≤ GLI < 15%, 15% ≤ GLI < 21%, 21% ≤ GLI < 28%, 28% ≤ GLI ≤ 100%). c: data of b smoothed by 1 pass of a 3 × 3 median filter. d: data of b smoothed by 3 passes of a 3 × 3 median filter.
Fig. 6. Cumulative frequency distribution of the GLI matrix of Fig. 5d with the GLI limits used in the plots of Figs. 5b-d.

No laminar pattern can be recognized in the presentation of the original data (Fig. 5b), because the GLI values show high local variations caused by inhomogeneities like cell clusters or small blood vessels. This type of local variation can be interpreted as noise, which an image can be cleansed of by median filtering (Pratt, 1978). Filtering an image by several passes of an $N \times N$ median filter will eliminate image details with a signal period lower than $(N + 1)/2$.

Fig. 5c demonstrates the result of one pass of a $3 \times 3$ median filter, while Fig. 5d demonstrates the result of 3 passes. Three passes of this filter are not sufficient to reach the final stage of the filtering process (Heygster, 1978), but it has proven to clarify the laminar pattern as demonstrated in Fig. 5d.

Median filters have the advantage over low-pass noise reduction filters with weighted or unweighted averages, that they will not blur edges and sharp transitions between regions having different GLI values.

Areal boundaries are expected to run along the interfaces between differing GLI patterns. Adjacent sections must be compared, to establish such boundaries. It is, furthermore, necessary to view the microscopic image, for the purpose of distinguishing true areal boundaries from non-significant changes in the GLI pattern, which are introduced by blood vessels and other irrelevant structural inhomogeneities.

Cortical maps for several species were produced, using this method (see Zilles et al., 1980; Wree et al., 1983).

**Data selection**

The second part of the GLI analysis consists of extracting statistical properties for selected brain regions from the pictorial data. In order to compare structural...
properties of different regions, and to determine the effects of experimental procedures and growth processes, the mean GLI value is calculated for a brain region whose boundaries (= closed contour) can be drawn on a series of consecutive sections. Another approach consists of analyzing the GLI distribution along a freely defined measuring line (= open contour) on the GLI image. This type of distribution is referred to as the GLI profile.

This analysis commonly requires a provision for user interaction for the purpose of extracting data from the image matrix. In our system, the contours are drawn on hard copies of the GLI images, as demonstrated in Fig. 7.

This procedure has several advantages over a system where the interaction is performed by tracing lines with a cursor on a computer display. The first advantage is that in most cases, information from one image is not sufficient to outline a brain region. Our procedure provides an opportunity to add information from adjacent sections, which have been processed with other methods (e.g. myelin staining, autoradiography, histochemistry). This is achieved by superimposing the microscopic image of these preparations on the hard copy of the GLI image with the help of a drawing apparatus attached to a microscope (Universal, Carl Zeiss, Oberkochen, F.R.G.). The images are aligned in two steps. The scaling adjustment of the drawing apparatus is calibrated to equalize the length of a reference line (e.g. a feret diameter of the hemisphere) in the two images. The print is then shifted and rotated to align corresponding structures (e.g. boundaries). Another advantage of our system over one which implements a cursor, comes from the unpredictable magnitude of the error which is introduced by the poor controllability of the cursor from a terminal. The unpredictability of this error becomes particularly acute, when the examined regions are small or narrow (e.g. cortical laminae). The third advantage of our system is that the exact positions of the drawn contours are documented and can be controlled or revised as desired.
To enter the contours into the computer, the hard copy is first affixed to the digitizer tablet (model Bit Pad One, Summagraphics, Fairfield, U.S.A.). Three reference points of the image matrix (Fig. 7) are marked on the hard copy with the cursor in the point mode of the digitizer. These reference points are used to calculate the matrix for the transformation of the digitizer coordinates to pixel coordinates. Contours on the hard copy are traced over with the cursor. At the same time, the digitizer coordinates are transferred to the CPU at a rate of 120 coordinate pairs per second. The program for transformation into pixel coordinates and the special routines for error recognition and end of contour recognition slow this rate to 20 pixel coordinates per second. This has shown to be fast enough, even for contours of complex shape. If the cursor moves too quickly for a continuous sequence of pixel coordinates, gaps are interpolated linearly. This feature is particularly helpful, when straight lines or polygons are to be entered, in which case, only distinct points (e.g. the endpoints of a straight line) are marked by the cursor.

For the purpose of calculating GLI statistics of a region outlined by a closed contour, special algorithms were implemented to define the interior of such a region and to extract the corresponding pixel values for statistical analysis (Pavlidis, 1982).

GLI profiles are generated as vectors with up to 1024 elements. Each of these elements is 6 bytes in length. The pixel coordinates are stored in 4 bytes; one byte is set to the pixel value, and the last byte is reserved for special purposes, like labelling of reference points or other markers in the profile.

**Data storage**

To maintain flexibility in data interpretation for a variety of specific analyzing programs, the data extracted from each serial contour are stored in a data file with a standardized format, similar to the format proposed by Perkins and Green (1982).

The first two records of each data file are header records. The first of these stores general information, such as the name of the investigator, date, species, brain number, type of histological processing, section thickness, distance between measured sections, and data on the measuring procedure, such as spatial resolution, grey value threshold and illumination wavelength. The second header record contains a numbered list of the brain regions or structures being investigated. Up to 100 items can be entered, stored and edited. These two records are followed by any number of data blocks. Each data block has its own header record, in which the information concerning a single contour (closed or open) is stored. The relevant information includes section number, region number, number of GLI values or length of the vector in the following data block, number of sectors (each sector = 256 bytes) occupied by that data and the data mode (closed or open). Depending on the data mode, the data block either comprises the histogram of the GLI values within the closed contour or the GLI profile along the course of an open contour.

This data base is stored on the hard disk for quick access by a variety of analyzing programs.

**Data analysis and applications**

Statistics on the GLI values are calculated from a series of sections, for individ-
Fig. 8. GLI profiles from Oc2ML and Oc1M. The courses of the profiles from the pial surface (left) to the cortex/white matter boundary are indicated in Fig. 7. The sequence of peaks and depressions in the profile from left to right is associated with a sequence of cortical laminae in the following manner: Oc2ML: pial surface, I, I, II, III, IV, V, VI, VI; Oc1M: pial surface, I, I, II, III, IV, V, V, VI, VI; Oc1M: pial surface, I, I, II, III, IV, Va, Vb, VI, VI, VI, VI.

ual brain regions and for combinations of brain regions with closed contours (Schwientek et al., in press; Zilles and Wree, 1985).

Any combination of brain regions and section numbers can be analyzed with the associated program. Brain regions and section numbers which are to be included in the analysis are entered as ranges, groups or single items. One or more data files are searched for data blocks which meet these conditions, and the selected histograms are added. The number of GLI values, the arithmetic mean, the standard deviation, the minimum, the maximum and the range are calculated from the final histogram.

The laminar pattern of cortical regions was analyzed using GLI profiles as shown in Fig. 8. This plot compares two profiles from the areas Oc2ML and Oc1M of the rat (paraffin section, 20 μm, Nissl staining, which run from the pial surface to the white matter. The profiles were taken from the GLI image shown in Fig. 5d. Since the profiles may be taken from cortices of different thickness, they were standardized to the same relative width of 100% (Hudspeth et al., 1976). Standardized profiles from the same cortical area can be integrated in order to eliminate distortions caused by individual structural inhomogeneities, and to construct type profiles of an area's characteristic appearance. Parameters like the mean GLI value or the number of extreme values in the GLI profile were calculated from the profiles in order to describe changes which were caused by growth processes (Zilles et al., 1981).

The GLI profiles of a series of coronal sections were used to reconstruct a flattened map of lamina IV in the barrel field of the somatosensory cortex in the rat (Schleicher and Zilles, 1983). With the help of a reference system the series of
profiles which had been taken along the course of lamina IV, could be adjusted to make rows of a new image matrix.

Periodic alterations in the cell density of lamina IV of the striate area in various species were found by processing GLI profiles along this lamina with autocorrelation analysis (Schleicher and Zilles, 1985; Zilles et al., 1985). Depending upon the species, the signal period of these elements was found to range from 300 to 500 μm.

Summarizing our results, we can state, that the GLI method represents a powerful tool for the analysis of the structural inhomogeneities in nervous tissues. Although only one of many possible parameters which describe the structural composition of nervous tissue is measured, quantification introduces the decisive step towards a new approach to cytoarchitectonics. Visual inspection may reveal small variations in parameters like cell shape or mutual arrangement, but these differences can often not be perceived by all investigators. The GLI analysis, on the other hand, results in numbers which are not dependent on the investigator's degree of experience and can, therefore, be reproduced, provided that the measurements are carried out using the procedure described above. Since the human visual sense cannot perceive minute changes in volume density, the GLI analyses extend the knowledge on the structural composition of nervous tissue. This type of analysis is not limited to nervous tissues alone, but can be applied wherever particles can be detected by an image analyzer.

References