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High Contrast and Homogeneous Staining of Paraffin Sections of Whole Human Brains for Three Dimensional Ultrahigh Resolution Image Analysis

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ABSTRACT. The gallocyanin chromalum stain belongs to the classical DNA-RNA staining techniques in histochemistry. It has some important features for successful object orientated image analysis of whole sections of the human brain. To obtain reproducible staining results with these large sections, the method of Einarson was adapted to image analytical requirements. We discuss staining in a warm staining solution, pH adjustment, and optimal stain composition. The embedding procedure for whole human brains is considered as well.

Key words: CLSM, gallocyanin chromalum, human brain, image analysis, light microscopy, paraffin embedding, sections, three dimensional imaging

It is almost impossible to apply all analytical methods of morphological sciences on a single section or preparation without losing certain features that may be of interest for subsequent investigations. To overcome this methodological limit, different analytical methods including enzyme histochemistry, immunohistochemical detection of neurotransmitters and neuropeptides, in situ hybridization of gene expression, in situ polymerase chain reaction (PCR) of low gene expressions, channel and receptor characterization, neurophysiological features, synaptic connection patterns etc. can be applied to samples of brain areas and integrated the results into a real geometric model as a statistical feature distribution. To obtain a realistic geometric model, a human brain should be used as a model. This can be difficult, however, because analysis of the brain is complex with respect to histochemistry, section handling and investigation of large sections by image analysis. Successful image analysis requires optimal production of sections and their staining. Schmitt and Eggers (1997) have presented a staining procedure that possesses desirable features toward this goal. The gallocyanin chrome alum stain (GCA) introduced by Preoscher and Arkush (1927) and Einarson (1932) provides high contrast, homogeneous, stable and artefact-free staining that does not require subjective differentiation. GCA is a well investigated metal complex of the oxazine dye gallocyanin (Green 1991, Horobin and Murgatroyd 1968, Beswick 1958, Eimarson 1951 Gray et al. 1956, 1957) that specifically reacts with RNA and DNA (Stenram 1953, Boer and Sarnaker 1956, Dutt 1972, 1974, 1977, 1980, 1982).

The characteristics of GCA staining are important for analyzing a whole human brain. Approximately 8000 frontal sections (assuming 20 μm section

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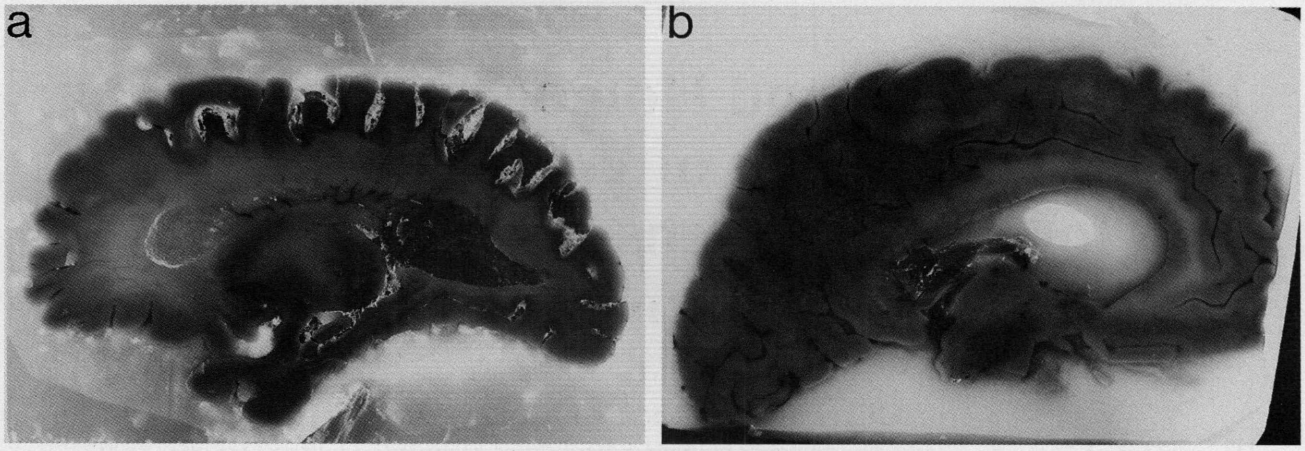

Fig. 1. a) Micrographs of brains embedded in paraffin a) without vacuum and b) with vacuum. The blocks are not stained.

thickness and an average length of the human brain from the frontal to the occipital pole of 160 mm) are required for whole brain analysis. When the GCA staining method is used in its original version, artefacts like dye precipitation, nonhomogeneous staining, lower contrast and irregular staining of dendrites and axons occur. We describe here a technique that overcomes these problems.

MATERIAL AND METHODS

After 12 hr post mortum a human brain without neuropathological changes was perfused with a 4% buffered PBS solution (pH 7.4). The pia mater and the superficial vessels were removed and the brain prepared for embedding 3 months later. The cerebellum was detached below the inferior colliculi. Dehydration was performed as suggested by Berg (1978), Wüstenfeld (1956), Kraus (1960) and Zilles (personal communication).

A detailed description does not exist in the literature for embedding whole human brains in paraffin. We placed the brain in a large piece of gauze for transport. The organ was placed in 80% isopropyl alcohol or ethanol for one week, two baths of 96% isopropyl alcohol or ethanol for 1 week each, three baths of 100% isopropyl alcohol or ethanol for one week each, four baths of chloroform for 3 days each, and immersed in four baths of chloroform for 3 days each, and immersed in four baths of degased paraffin at 60 C for three days each. The brain was positioned in a plastic box, the inner surfaces of which were covered with a protein-glycerin solution (10 ml egg albumin + 10 ml 85% glycerin). A final portion of degased paraffin then was poured into the box. Vacuum infiltration was carried out at 10 mbar for 1 hr at 60 C to avoid air filled holes over the sulci and in the ventricles (Fig. 1). The paraffin was allowed to cool slowly at room temperature and atmospheric pressure. Using the Polycut

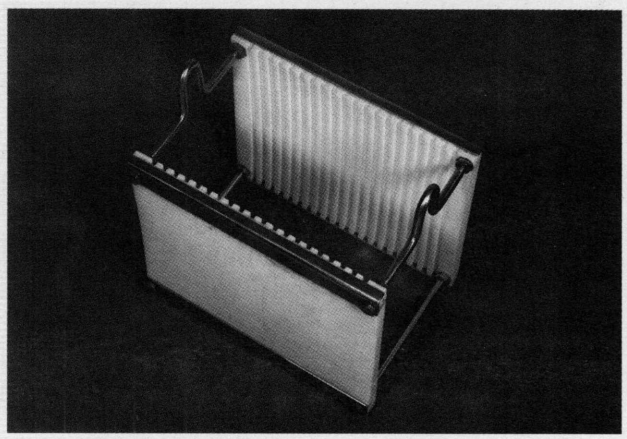

Fig. 2. The teflon transporter used gross sections.

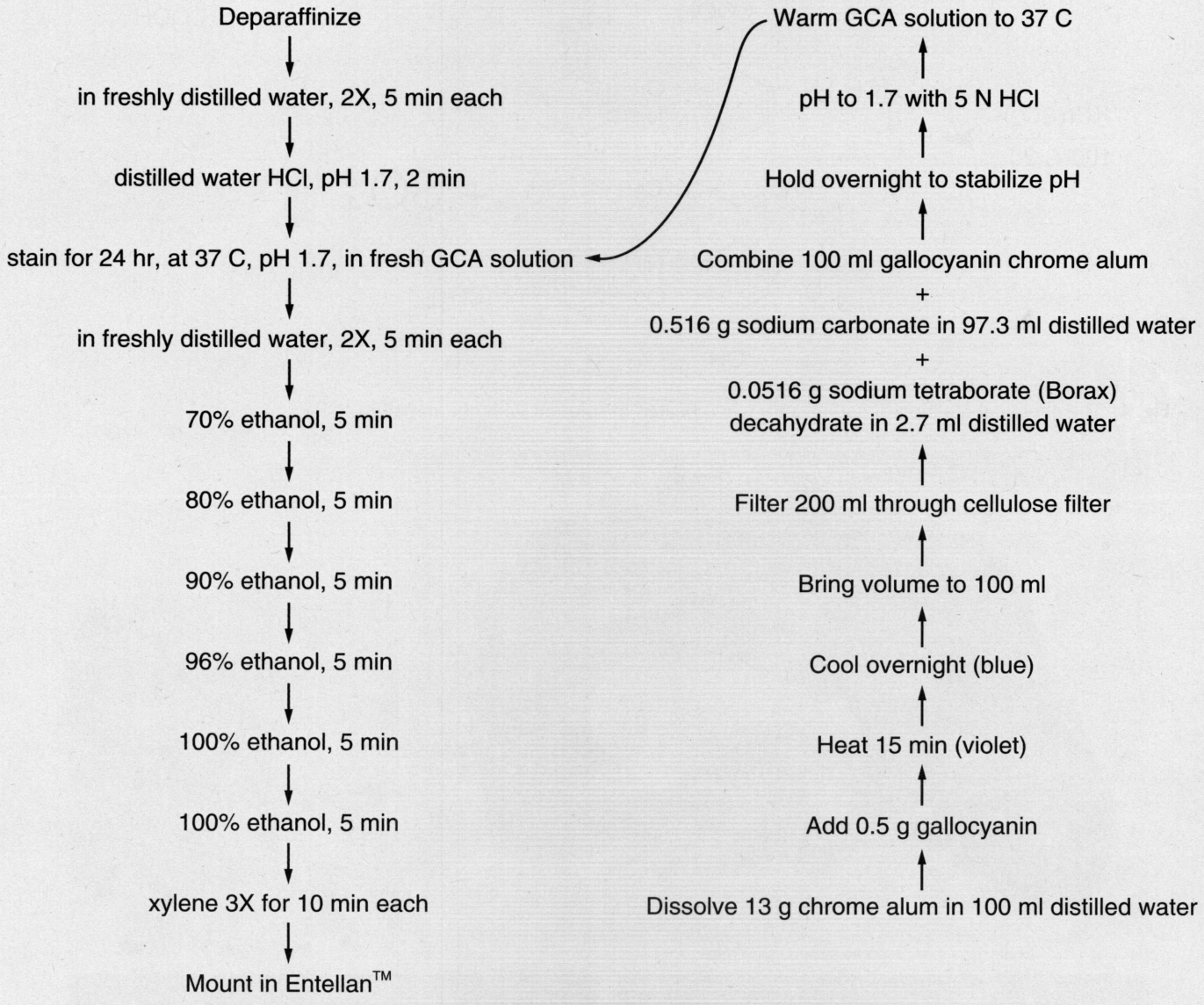

Fig. 3. Gallocyanin chrome alum staining protocol.

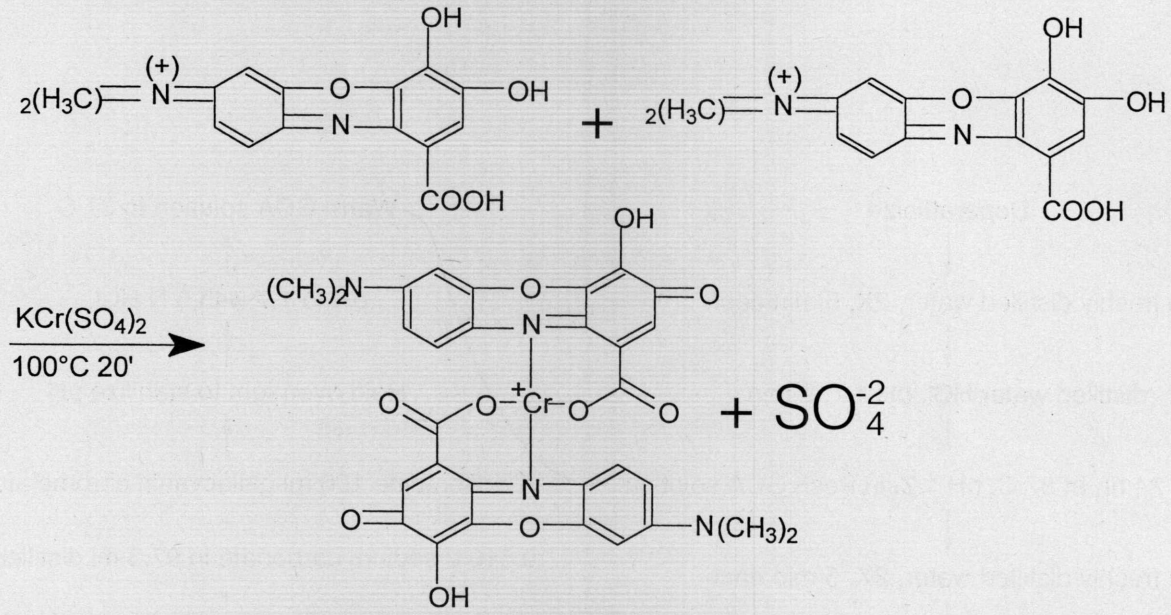

Fig. 4. The chemical reaction of gallocyanin when heated in solution with chrome alum.

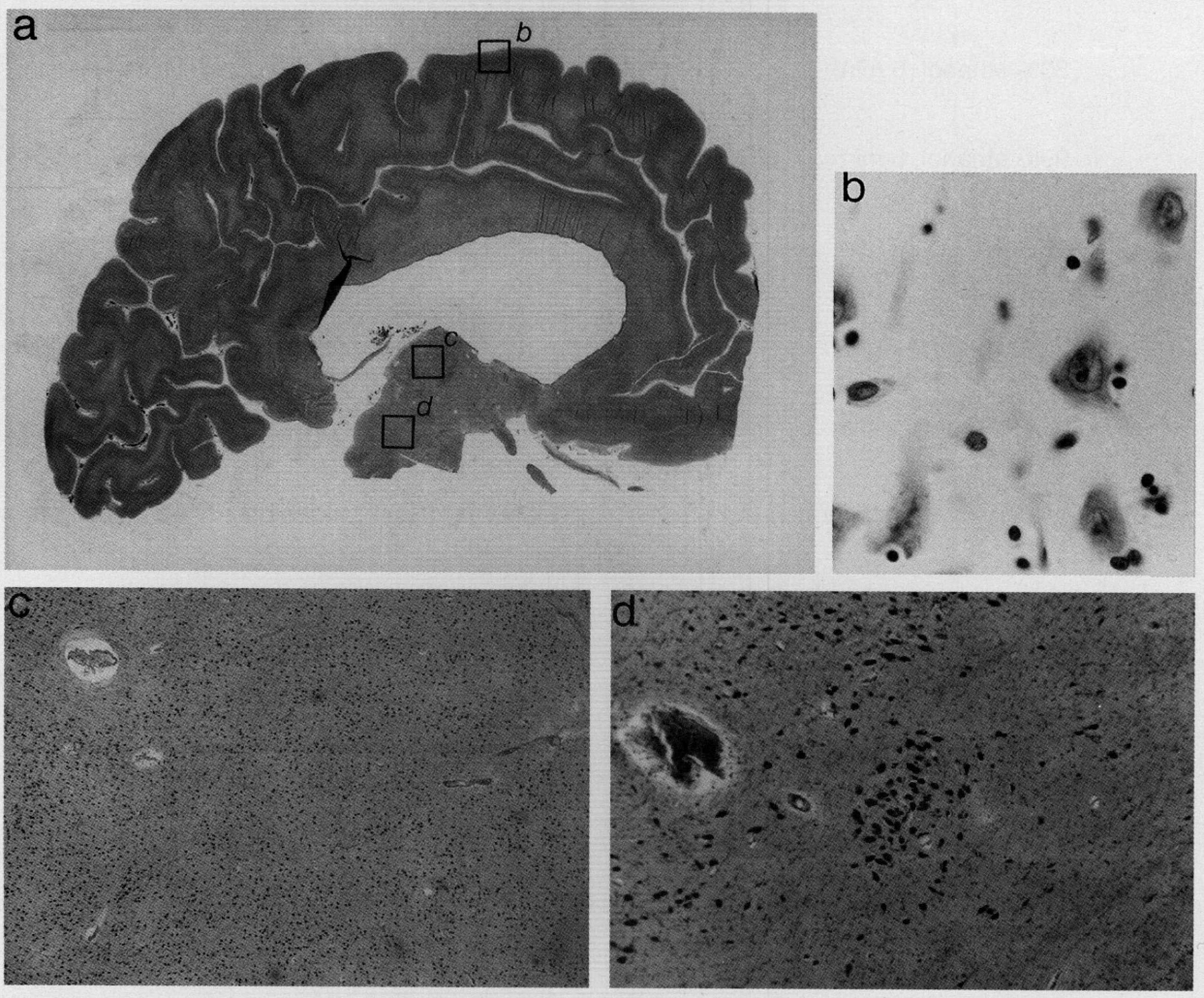

Fig. 5. a) Micrograph of a homogeneously stained gross section of the human brain. b) Cortex. c) Thalamus. d) Substantia nigra. Boxes indicate sources of samples shown in b–d.

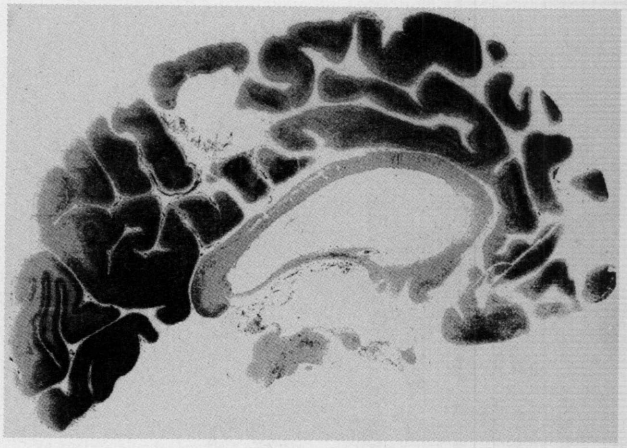

Fig. 6. A cresyl violet stained gross section. Note that the stained areas are not homogeneous compared to Fig. 4a.

gross section microtome from Reichert Jung, 20 μm thick gross sections of the paraffin brain block were made using a d-type knife and cutting angles of 0–5°. The sections were transferred manually onto a 55 C water bath for stretching, positioned on an extra large 0.2% gelatin coated slide, and dried overnight in an oven at 37 C. The sections were deparaffinized through xylene (3 x) and isopropyl alcohol to demineralized water. Because the contrast is influenced by pH, the large surfaces of the slides and transporter device were rinsed in acidified water (pH 1.7) and transferred into the gallocyanin (C.I. 51030, CAS 1562-85-2) chrome alum staining solution. Greater alkalinity results in co-staining of the cytoplasm, nucleoplasm and neuropil. After 12 hr at 37 C, the sections were rinsed two times in water. After optimizing the staining procedure, we have never observed overstaining. If overstaining should occur, however, the pH should be controlled in the staining solution and the sections can be cleared in acidified water (pH 1.0–1.7) immediately after staining. To transport the large slides, a teflon transporter (Fig. 2) was built. An aluminum transporter is not recommended because the dye-metal salt formation is disturbed by aluminium ions. The composition and preparation of the staining solution according to Sandritter et al. (1963) and Terner and Clark (1960a,b) is shown in (Fig. 3). The chemical mechanism consists of an inner-complex dye metal salt or dye-lake that reacts in the tissue to form a lake-tissue salt. The reaction of two gallocyanin molecules with one chrome alum is shown in Fig. 4.

The last steps of dehydration were performed following routine histochemical methods and included isopropyl alcohol. Finally, the sections were cleared in three baths of xylene and mounted in Entellan. The Entellan was liquefied with xylene (1:1) to obtain a standardized composition. If this is not done, the distance between the large coverslides and slides can differ and

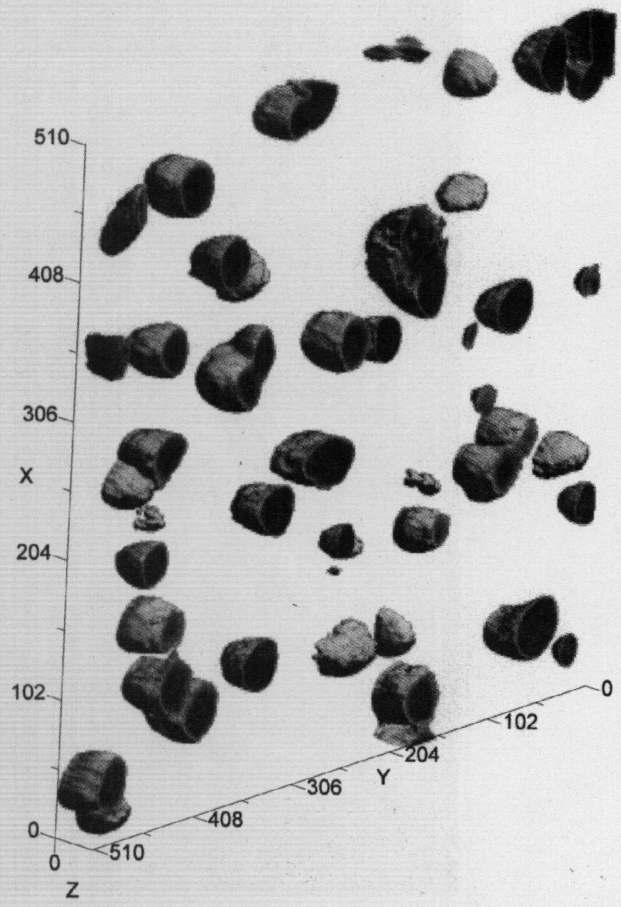

Fig. 7. A reconstruction of area 17 using conventional confocal microscopy. Simple dynamic thresholding was used to segregate the neuronal and glia elements.

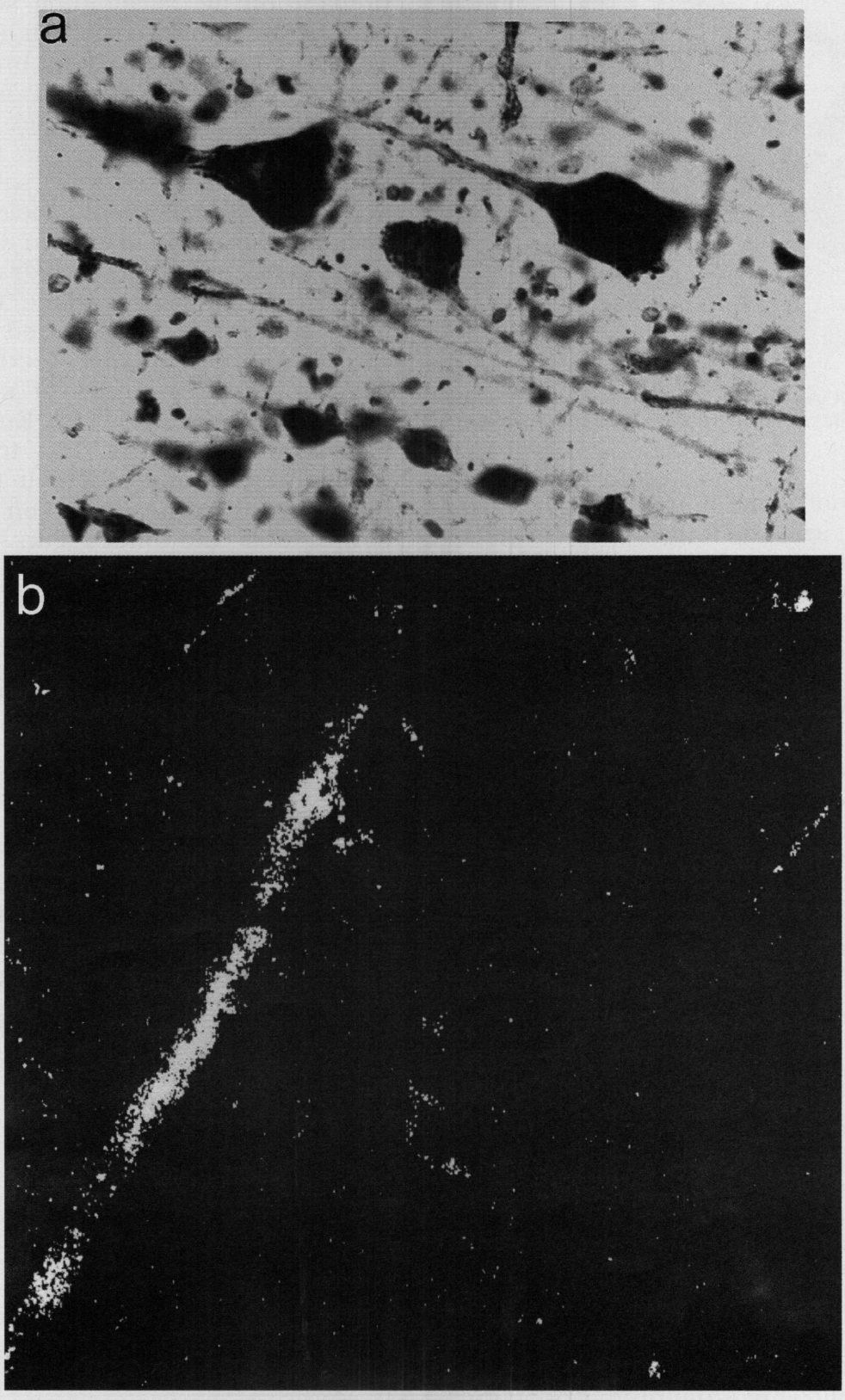

Fig. 8. a) Bright field image of neuronal and glial processes using the complex reaction. b) CLSM image using the reflection mode of the GCA stain for processes.

microscopic analysis will be disturbed. The sections should be stored in a horizontal position, for about 3 months until the Entellan is completely dried under the coverslide.

RESULTS

The micrographs (Fig. 5, b–d) of the gross sections show great contrast between glial and neuronal elements and the neuropil of the cortex, thalamus and substantia nigra. Furthermore, GCA staining of the CNS is homogeneous (Fig. 5a) over the entire section. In comparison, a gross section stained with cresyl violet is shown in Fig. 6. The staining pattern is not homogeneous owing to either the staining step or the differentiation. This phenomenon has been objectively analyzed by quantifying the staining contrast using techniques of color image analysis (Schmitt and Eggers 1997). The contrast is sufficient to permit the application of dynamic gray level thresholding by an image analytical procedure to delineate nuclei of neurons and glial cells. A confocal light microscopic three-dimensional visualization of a sample generated by 40 focal planes and using an automated stepping device is shown in Fig. 7. Using a CLSM in the reflection mode and modifying the staining solution by adding aluminium ions and adjusting the pH to 2.5 (complex reaction), the neuron processes can be visualized as shown in Fig. 8, a and b.

DISCUSSION

Gallocyanin chrome alum is a suitable stain for analyzing gross sections of human brain under the light microscope and digital image processing. It can be modified easily to visualize certain aspects of morphological features within the sections (Fig. 8). The staining results are optimal for image analysis and three dimensional imaging. These properties are basic features of an automated system that is suitable for mapping cytoarchitecture in gross sections of the human brain. Our method is a stable and reproducible standardized staining system for analyzing gross sections of whole human brains.

REFERENCES

Berg, G. 1978. Laborative Erfahrungen mit der Großflächenschnitt-Technik des Herzens. Zeitschr. DVTA 24: 1–9.

Beswick, T. S. L. 1958. The Einarson gallocyanin-lake staining technique. J. Pathol. Bacteriol. 76: 598–600.

Boer, J. de and Sarnaker, R. 1956. The gallocyanin stain for Nissl bodies. Med. Proc. 2: 218–221.

Dutt, M. K. 1972. Celestine blue B as a specific stain for cytochemical detection of nucleic acids. Acta Histochem. 44: 1–8.

Dutt, M. K. 1974. Cytochemical localization of nucleic acids with gallocyanin. Acta Histochem. 48: 149–151.

Dutt, M. K. 1977. Inorganic acids—their use in the differential extraction of RNA from fixed mammalian tissues. Acta Histochem. Cytochem. 10: 478–482.

Dutt, M. K. 1980. Absorption spectra of nuclei stained with two different basic dyes after selective extraction of RNA. Indian J. Exp. Biol. 18: 57–58.

Dutt, M. K. 1982. Specific staining of nuclei with aqueous solutions of celestine blue B and gallocyanine. Microsc. Acta 86: 201–205.

Einarson, L. 1932. A method for progressive selective staining of Nissl and nuclear substance in nerve cells. Am. J. Pathol. 8: 295–307.

Einarson, L. 1951. On the theory of gallocyanin-chromalum staining and its application for quantitative estimation of basophilia. A selective staining of exquisite progressivity. Acta Pathol. Microbiol. Scand. 28: 82–102.

Gray, P., Pickle, F. M., Maser, M. D. and Hayweiser, L. J. 1956. Oxazine dyes. I. Celestine blue B with iron as a nuclear stain. Stain Technol. 31: 141–150.

Gray, P., Day, W. M., Hayweiser, L. J. and Nevsimal, C. 1957. Oxazine dyes. 2. Celestine blue B, gallocyanin and gallamin blue with mordants other than ferric alum. Stain Technol. 32: 161–165.

Green, F. J. 1991. *The Sigma-Aldrich Handbook of Stains, Dyes and Indicators.* Milwaukee. pp. 386–387.

Horobin, R. W. and Murgatroyd, L. B. 1968. The composition and properties of gallocyanin-chrome alum stains. Histochem. J. 1: 36–54.

Kraus, C. 1960. Die Paraffineinbettung des Gehirns. Mikrotom. Nachrichten 6/1960: 1–19.

Proescher, F. and Arkrush, A. S. 1927. Metallic lakes of the oxazines (gallamin blue, gallocyanin and coelestin blue) as nuclear stain substitutes for hematoxylin. Stain Technol. 2: 1–3.

Sandritter, W., Kiefer, G. and Rick, W. 1963. Über die Stöchiometrie von Gallocyaninchromalaun mit Desoxyribonucleinsäure. Histochemie 3: 315–340.

Schmitt, O. and 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 (in press).

Stenram, U. 1953. The specifity of the gallocyaninchromalum stain for nucleic acids as studied by the ribonuclease technique. Exp. Cell Res. 4: 383–389.

Terner, J. Y. and Clark, G. 1960a. Gallocyaninchrome alum. I. Technique and specifity. Stain Technol. 35: 167–177.

Terner, J. Y. and Clark G. 1960b. Gallocyaninchrome alum. II. Histochemistry and specifity. Stain Technol. 35: 305–311.

Wüstenfeld, E. 1956. Experimentelle Beiträge zur Frage der Volumenänderungen und Eindringtiefe in der histologischen Technik. Z. Wiss. Mikrosk. 63: 86–102.