

Serial Thick, Frozen, Galloxyanin Stained Sections of Human Central Nervous System

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Abstract

A rapid method for macroscopic and microscopic investigation of human CNS is proposed. After formalin fixation, gelatin or agarose embedding, and cryoprotective treatment, frozen human spinal cords, brainstems, or hemispheres can be serially cut into 0.7 mm thick slices. Stained with galloxyanin-chromalum, these slices facilitate cytoarchitectonic, neuropathologic, and quantitative examination. Regions of interest from parallel formalin-stored unstained slices can be embedded into paraffin and stained by any immunocytologic and histologic stain compatible with formalin fixation and paraffin embedding. (*The J Histotechnol* 14:167, 1991)

Key words: CNS, comparative anatomy, cytoarchitectonics, neuroimaging, neuropathology, Nissl stain, quantitative anatomy

Introduction

The human brain is an organ of considerable size and immense complexity. These features render both the study of normal anatomy and the assessment of subtle pathological changes difficult. Economo and Koskinas proposed a "block method" in their study of the human cerebral cortex (1). At the expense of the destruction of the three-dimensional structure, more than 280 blocks per hemisphere guaranteed vertical sections through the major brain regions. From this mosaic of blocks the authors reconstructed and illustrated their well known brain map. This methodological approach has been heavily criticized by the Vogts and later by Sanides (2,3). The dissection procedure frequently destroys the boundaries of adjacent cytoarchitectonic fields, especially in the depth of the sulci, thus making an exact delineation of neighboring areas impossible. On the other hand, embedding of complete brains or hemispheres, as proposed by the Vogts and by Brodmann, into paraffin or celloidin with subsequent serial sectioning at section thickness from 20 to 40 μ m is time-consuming and expensive (4). Alternatively, neuropathologists perform macroscopic dissection of formalin fixed brains with slice thickness up to 20 mm before micro-

scopic examination. Although macroscopically visible processes or changes can be detected and diagnosed after histological examination with this procedure, discrete changes and minute tumors within functionally important brain regions may escape detection. Furthermore, the three-dimensional structure of the brain is destroyed by macroscopic slicing. We propose a different method that is rapid, easy to perform, and allows macroscopic inspection with unstained or stained slices at different levels of low and high power light microscopic enlargement, thus yielding complete serial sections.

Materials and Methods

We fixed human brains or brain slices in 4% formalin (1 part 40% formaldehyde + 9 parts tap water) for 2 wk to several yr. The fixation fluid, which should always be in excess (at least 5000 ml per brain), was changed weekly. For our quantitative investigations we left the brain in formalin for at least 3 mo because, according to our continuous volume and weight recordings, the brain volume initially increased, thereafter decreased, and reached its original weight and volume only after about 3 mo (5). At the end of the fixation procedure, vessels and meninges were carefully removed from the brain surface as arteriosclerotic plaques and fibrotic meninges make serial sectioning difficult by producing tears in the brain tissue.

Before serial freeze sectioning, the brain was pretreated with cryoprotective media to prevent ice crystal formation. Thereafter, it was embedded in either 15% gelatin or 3% agarose. Details of cryoprotective pretreatment, gelatin, or agarose embedding are listed below.

Pretreatment of complete human hemispheres with cryoprotective media according to Rosene et al (6)

- 1) Place in a solution with 4% formaldehyde, 2% dimethyl sulfoxide (DMSO) (Merck, Darmstadt, FRG), 10% glycerol for 8 days. Change solution after 4 days.
- 2) Place in a solution with 4% formaldehyde, 2% DMSO, and 20% glycerol for 8 days. Change solution after 4 days.

With smaller tissue blocks this protocol can be abbreviated to 1 wk. In solution 2, sinking of the tissue block indicates the degree of penetration of the cryoprotective medium into

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the tissue. (Gloves must always be worn when working with DMSO.)

Gelatin or agarose embedding

- 1) Take the hemisphere from the cryoprotective media, and remove excess fluid by carefully wrapping in the hemisphere in filter paper.
- 2) Prepare a 3% agarose solution on a boiling water bath with constant stirring or a 15% gelatin solution that can be directly prepared on a warming plate at 60°C with constant stirring. Put the hemisphere into either a warm (45–50°C) 15% gelatin or 3% agarose solution. Ensure that the warm embedding medium fills the sulci by carefully widening the latter with your thumbs.
- 3) Put the embedded tissue into a refrigerator (4°C) overnight. When the block has hardened, remove excess embedding medium. A mantle of embedding medium 5–10 mm wide should surround the brain tissue, and the base of the tissue block should be large enough to serve as a "pedestal" for freezing the block onto the microtome table.
- 4) Allow gelatin embedded tissue to harden in 4% formaldehyde for up to 3 days.

Additional cryoprotection after gelatin embedding

Before freezing, we soaked both the agarose and the gelatin blocks in cryoprotective media to prevent ice crystals from forming within the embedding medium and complicating sectioning.

- 1) Put agarose-embedded blocks directly into a solution with 4% formaldehyde, 2% DMSO, 10% glycerol (2 wk), but gelatin embedded tissue into the solution only after 3 days' hardening (see Section B, step 4).
- 2) Keep blocks or tissue in a solution with 4% formaldehyde, 2% DMSO, 20% glycerol for 2 wk. Time for smaller blocks can be abbreviated (see Pretreatment).

Freezing

To facilitate freezing, we divided the tissue block into a larger rostral part and a smaller caudal one with the plane of section caudal to the splenium corporis callosi.

The gelatin or agarose blocks were frozen in –60°C isopentane after the pretreatment in cryoprotective media. Isopentane was poured into a sufficiently large Dewar vessel and cooled by carefully adding small blocks of dry ice. At the beginning of the procedure, isopentane boiled up vehemently. Cooling was monitored by a thermometer. Within 15 min of recording the desired temperature, the gelatin or agarose blocks were carefully dipped into isopentane. Time of freezing depended on the size of the blocks. Small blocks could be immediately cooled down to –60°C. In our experience, rapid freezing of larger blocks and temperature below –60°C was sometimes related to cracking of the blocks within the embedding medium and to artifactual damage of brain tissue.

The blocks were left for additional 15 min in the –60°C isopentane; the temperature was constantly monitored and lowered by dry ice if necessary. Then the blocks were removed from the vessels, and excess isopentane was absorbed by filter paper. The blocks were sealed into plastic bags and transferred to a freezer at –80°C where they were left at least overnight or several days.

Sectioning and storage

The deeply frozen blocks were affixed with water onto the table of a freezing microtome. We use a Tetrandler, a special sliding microtome, produced by Jung in Nußloch. This microtome is supplied with a large freezing table that is cooled by an external aggregate. If no aggregate is available, the blocks can be frozen and kept cool by a wall of powdered dry ice surrounding them.

Because the cold tissue block is rather hard and sectioning of 700 micrometer thick slabs could either damage the microtome knife or fragment the block, we devised a trick: we warmed an aluminium block (12 × 18 cm in width, 2 cm in height, and supplied with a handle) on a warming plate at 80°C. When the hot metal was pressed onto the cold gelatin or agarose block, the most superficial layers, 0.5–1 mm, were warmed. It is a matter of experience what temperature is chosen on the warming plate and for how long and by what force the aluminium block is pressed onto the tissue. If the superficial layers of the block are too warm, the slice distorts and coils; if too cold, fissures on the surface will indicate inappropriate temperature. Furthermore, room temperature, size of the block, temperature of the block, and the speed of serial sectioning are parameters that are easily managed after some trials. With experience, one hemisphere could be serially sectioned within 1 hr.

We stored the 0.7 mm thick serial slices together with 4% formalin in plastic boxes. Filter paper circles (Schleicher & Schuell, Dassel, FRG) were inserted between every 5th slice to facilitate retrieval of certain parallel sections. During or after the serial sectioning, a macroscopic neuropathological inspection was made to reveal or exclude gross morphological changes.

Staining and mounting

The slices were then prepared for the staining procedure in gallocyenin-chromalum (7). According to Merker, pretreatment with performic acid enhances the selectivity of Nissl stains (8).

The sections were left overnight in petri dishes (185 mm diameter, 40 mm in height) with freshly prepared performic acid (1 part of 100% formic acid, 3 parts of 30% H₂O₂, and 6 parts of distilled water). Use double gloves to prevent accident burns by this aggressive mixture! The sections were constantly stirred on a swinging electromechanical shaker with filter paper on the surface of the performic acid to keep the sections immersed in the fluid.

The performic acid was removed the next morning by running water for 1 hr.

The sections were stained in gallocyenin-chromalum with constant slight stirring for 3 hr (7). Dissolve 1.5 g gallocyenin (Aldrich-Chemie, Steinheim, FRG) in 1000 ml of 5% potassium chromium(III) sulfate dodecahydrate (Merck). Boil the mixture for at least 10 min. (In some instances, the liquid may squirt from the vessel, so great care must be taken.)

After cooling, the solution is filtered and enough distilled water is added to bring its volume up to 1000 ml. The resulting pH of the solution is 1.8. This solution is freshly prepared and can be used two times.

In most cases, a pH of the solution of 1.8 was optimal. If the stained tissue was only faintly blue, raising the pH to 2.0–2.2 yielded better results.

Remove excess gallocyanin by thorough rinsing in running water for 1 hr.

The sections were transferred from tap water into 50% ethanol and flattened by smoothing them between 2 sheets of filter paper circles. Thereafter, individual sections pressed between filter paper were stored in a stack of ceramic desiccator discs 150 mm in diameter (Hoechst CeramTec, Marktredwitz, FRG), which keeps them smooth and allows diffusion of increasing concentrations of alcohol. A stack consisting of 4 discs fits in petri dishes 185 mm in diameter and 40 mm in height.

Dehydrate in a graded series of alcohol:

80% ethanol, 1 hr; 100% previously used ethanol, 1 hr; 100% fresh ethanol, overnight, propananol 100%: xylene 1:1, 2 hr; xylene previously used, 2 hr; xylene (fresh) overnight. Use double gloves for the transfer of desiccator discs into solutions containing xylene.

We immersed the sections about 1 min in Permount (Fisher Scientific, Pittsburgh, PA) and mounted the sections from the resin onto microscopic slides. We kept led blocks on the coverslide for at least 1 wk to prevent formation of air bubbles.

Because of the relatively thick layer of resin, polymerization will take several months. However, we were able to view the slides carefully before complete hardening occurred.

Results

Routine section thickness of paraffin- or celloidin-embedded brain tissue is 10 to 20 μm . Although examination of 500 μm thick sections is uncommon and seems to be associated with considerable loss of details, compared with the brains of rodents, the nerve cell density of the human CNS is low. Consequently, after exclusive staining of glial cell nuclei and nerve cell nuclei and their perikarya with gallocyanin, neuronal arrangement in subcortical nuclear complexes and cortical laminar sheets became more clearly visible than in routine histological preparations.

Even at low magnification subcortical and cortical grey matter could be unequivocally distinguished (Figure 1). The individual nuclei of the thalamus (A pr, Do, M), especially the lateral nucleus (L po) with its complicated structure, were easily delineated. The same holds true for the nuclear complex of the amygdaloid body (C a), which is separated from the basal nucleus of Meynert (NbM) by the substantia innominata (S i). The border between these nuclei and the substantia innominata in routine sections is sometimes arbitrary, especially at more frontal levels.

Unlike other subcortical nuclear masses, the outlines of the globus pallidus (Gpi and Gpe) appear blurred in the photo. This was not the case after microscopic examination with a stereo microscope. The nerve cell density of the globus pallidus and the fundus striati (nucleus accumbens of other authors) is low; but these nuclei appeared rusty brown in our Nissl preparations, and this particular natural color stands out against the unstained fiber tracts surrounding these structures. This feature renders microscopical delineation of these structures simple and unequivocal. We photographed the Nissl stained section with a red filter. This procedure enhanced the contrast of the blue stained nerve cells in the majority of cortical and subcortical grisea at the expense of the delineation of the globus pallidus in this photo.

Several types of cortex could be recognized easily with the naked eye. The insular cortex (Figure 1, In) and the cortex of

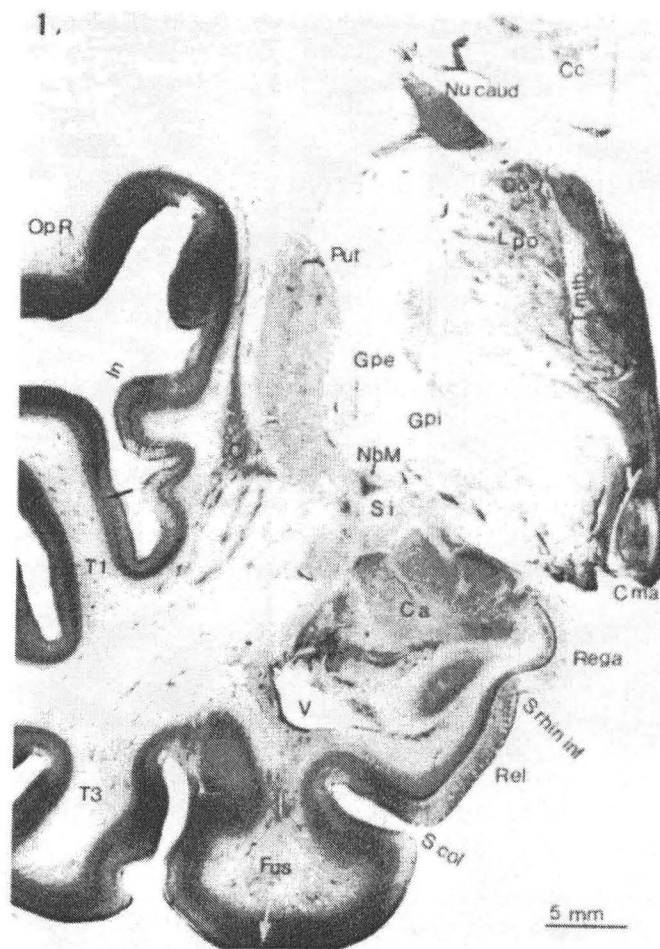


Figure 1. Diencephalon and left hemisphere with temporal and basal frontal lobe of a 46-year-old female, death by accidental CO-poisoning, post-mortem interval less than 24 hr. A pr = Ncl anteropolaris thalami, C a = nuclear complex of Corpus amygdaloideum, Cc = Corpus callosum, Cl = Claustrum, C ma = Corpus mamillare, Do = Ncl dorsalis thalami, Fus = Gyrus fusiformis (occipitotemporalis lateralis), Gpe = Globus pallidus externus, Gpi = Globus pallidus internus, In = Insula, L po = Ncl lateropolaris thalami, M = Ncl medialis thalami, NbM = Ncl basalis Meynert, Nu caud = Ncl caudatus, Op R = Operculum Rolando, Put = Putamen, Rega = Regio entorhinalis gyri ambientis, Rel = Regio entorhinalis lateralis, S col = Suclus collateralis, S i = Substantia innominata, S rhin inf = Suclus rhinencephali inferior (Retzius), T mth = Tractus mamillothalamicus, T1 = Gyrus temporalis superior, T3 = Gyrus temporalis inferior, V = Cornu inferius ventriculi lateralis.

the entorhinal region (Rel, Rega) are considered to represent allocortical (primitive) cortical types, whereas the six-layered isocortex of the temporal lobe is regarded as the product of a progressive evolutionary trend.

The cortex of the ventral allocortical insula was characterized by an intensely basophilic bandlike pyramidal layer (layer V) in the lower third of the cortex. It appeared to merge with the internal granular layer of the isocortex in T1 (Figure 1, arrow), but inspection with higher microscopic magnification showed that internal pyramids of T1 were far less basophilic than their insular counterparts and that layer V was now bordered by the intensely basophilic layer IV and the less basophilic layer VI. In our preparations, the isocortical temporal layer V appeared light and faintly stained.

Layer IV disappeared on the crest of the fusiform gyrus (Figure 1, Fus, arrow), thus marking the medial border of the temporal isocortex that coats most of the temporal gyri.

In contrast to the rather uniform six-layered composition of the temporal isocortex, the allocortex of the entorhinal region exhibited a complicated multilamellar structure with characteristic nerve cell glomeruli in the second layer. A nerve cell-poor, fiber-rich lamina dissecans separated outer components (lamina principalis externa) from inner layers (lamina principalis interna) of this progressive allocortex.

The brainstem contains a variety of nuclear complexes that represent either the origin or termination of axons of the cranial nerves or that are intercalated in long ascending or descending fiber tracts. The outline of some of these nuclei can be recognized in Figure 2. We labeled only the main nuclei, as at this low magnification there was not enough space to designate all visible nuclei. The complex architecture of the external cuneate nucleus (Cu 1) is noteworthy.

The diencephalic substantia nigra is an integral part of the so-called extrapyramidal motor loops. This nuclear complex extends from the caudal diencephalon to the rostral pons and is subdivided into a melanin-rich pars compacta (Figure 3, pc) and a melanin-poor pars reticulata (Figure 3, pr). The pars compacta consists of up to 21 subnuclei, some of which regularly degenerate in idiopathic Parkinson's disease (9,10). The arrangement of melanin-rich neurons in subnuclei of the pars compacta substantiae nigrae was readily visible in Figure 3. Oil immersion microscopic objectives with long working distances allowed direct scrutiny of cells in thick frozen, gallocyanin stained sections. Depending on the thickness of the cover slide (0.17 mm), the most superficial 50–100 μm of the thick sections could be examined (Figure 4). Because of the high section thickness contrast was less brilliant than with 10 μm thick sections.

Prior to staining with gallocyanin, we performed macroscopic inspection of every 15th section of our serially sectioned hemisphere to exclude macroscopically visible circulatory, neoplastic, or other changes. In one of our cases, we could detect cortical circulatory disturbances only after staining with gallocyanin. The cortical band of the superior and medial frontal gyrus appeared perforated by circular to

oval unstained patches (Figure 5). These focal vessel-associated nerve cell losses were confined mainly to the wall and depth of the superior frontal sulcus in a region extending about 40 mm in rostrocaudal direction of the prefrontal cortex. At higher magnification we could identify astrocytes and numerous endothelial cells in the periphery of these patches.

A disadvantage of thick frozen sections is the limited number of histological stains that can be applied with this method. Silver impregnations and other routine histological stains obscure most of the details. For this study, we stained only each 3rd section through the brainstem and hemisphere. Serial sections number 2, 5, 8, 11 (etc) and 3, 6, 9, 12 (etc) were stored in formalin filled plastic boxes in the order of the section procedure. If small lesions are detected in circumscribed regions of thick gallocyanin stained sections, these segments can be localized in the parallel preceding or ensuing unstained section, cut out, dehydrated, embedded in paraffin, and serially sectioned with a rotary microtome. We regularly obtained 20–30 serial paraffin sections at a section thickness of 15–20 μm . These sections could be stained with any histological, histochemical, or immunocytochemical stain that is compatible with formalin fixation and paraffin embedding. The removal of phagocytosed material and transport of the latter to the wall of a larger vessel (Figure 6) as well as the increase in number and density of processes of fibrous astrocytes (Figure 7) in the cortex and subcortical

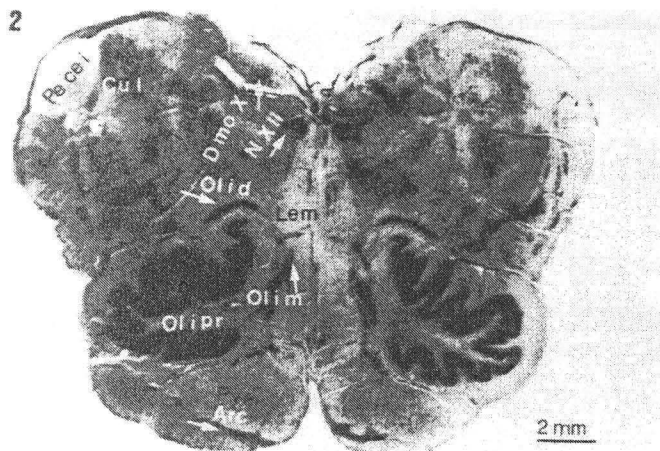


Figure 2. Frontal section through the medulla oblongata (level of section is through the center of the olives) of a 61-year-old female, death by bronchopneumonia, postmortem interval 72 hr. *Arc* = Ncl arcuatus, *Cu 1* = Ncl cuneatus lateralis, *Dmo X* = Ncl dorsalis motorius nervi vagi, *Lem* = Lemniscus medialis, *N XII* = Ncl nervi hypoglossi, *OIid* = Ncl olivaris inferior accessorius dorsalis, *OIim* = Ncl olivaris inferior accessorius medialis, *OIipr* = Ncl olivaris inferior principalis, *Pe cel* = Pedunculus cerebelli inferior, *Pyr* = Pyramis, *T sol* = Tractus solitarius.

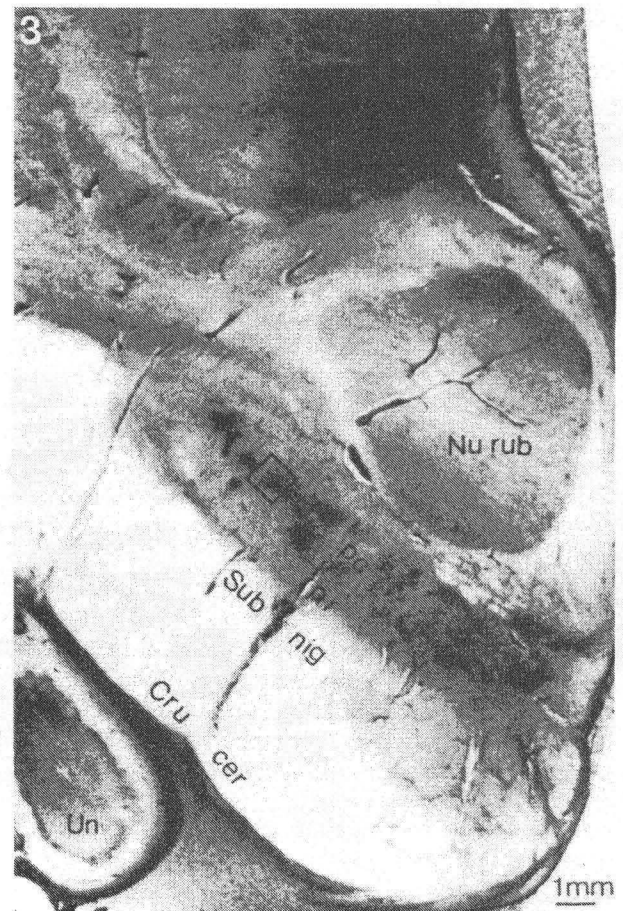


Figure 3. Crus cerebri of a 22-year-old male, death by accident, postmortem interval less than 24 hr. The gelatin is not retouched in this photograph and is visible in the upper right and the lower left corner of the photo. *Nu rub* = Ncl ruber, *pc* = pars compacta sub nig, *pr* = pars reticulata sub nig, *Sub nig* = Substantia nigra, *Un* = Uncus gyri parahippocampalis.

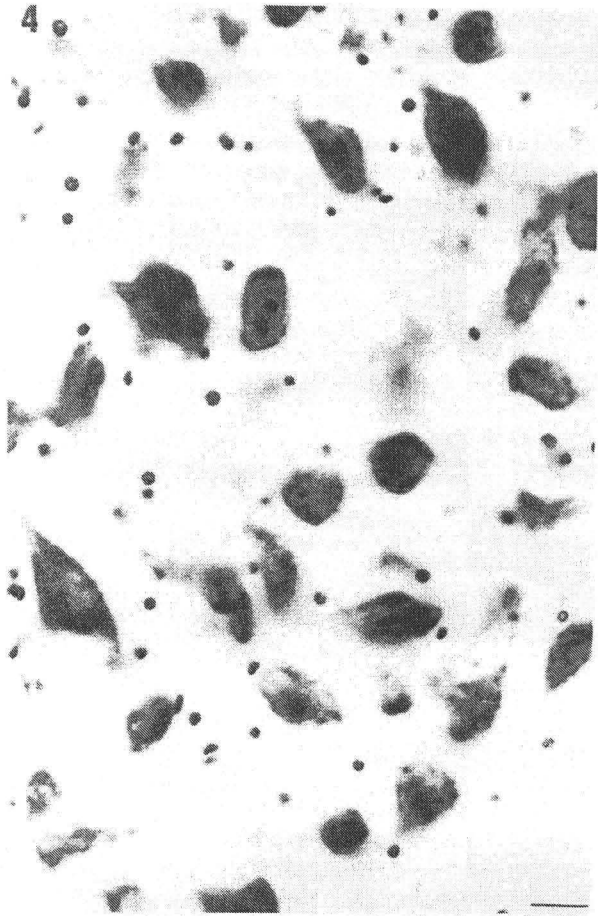


Figure 4. High power view from the area enclosed in the rectangle in Figure 3. The large oval- to spindle-shaped neurons of the substantia nigra with their large basophilic nucleolus and intermingled nuclei of mainly astroglial cells are readily visible in the superficial parts of 500 μm thick sections by the use of oil immersion microscopic objectives. Bar corresponds to 20 μm .



Figure 5. Frontal section through the left middle (above) and superior (below) frontal gyrus separated by the superior frontal sulcus at the level of the temporal pole of a 83-year-old female, death by myocardial infarct with only minimal arteriosclerosis in larger somatic and cerebral arteries. Post-mortem interval less than 24 hr.

white matter could be seen in reembedded tissue of parallel sections.

Discussion

The use of serial, thick, frozen gallocyanin stained sections through the human central nervous system offers some advantages compared with paraffin or celloidin embedded tissue.

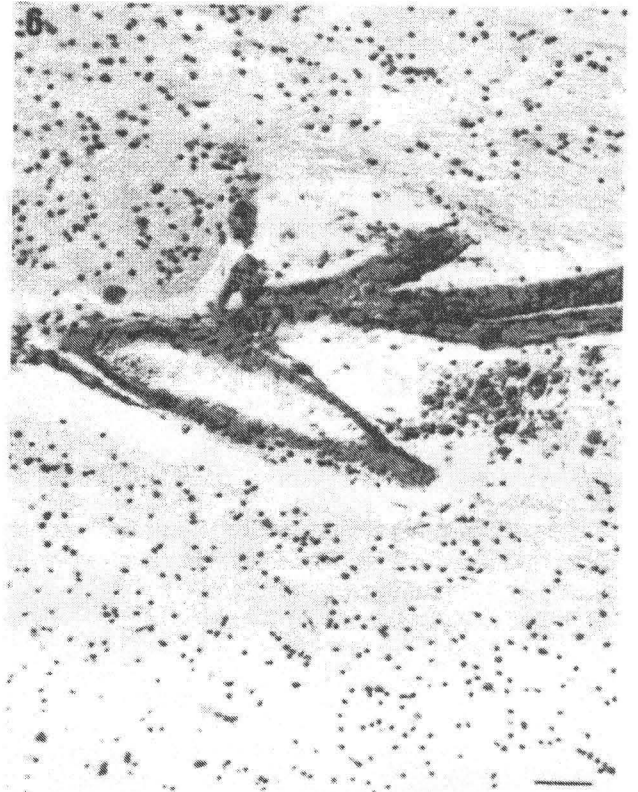


Figure 6. Fifteen- μm thick section through an ensuing 700 μm thick frozen section parallel to that in Figure 5 after embedding of a small piece of tissue in paraffin. The rectangle in Figure 5 marks the topography of Figure 6 in the preceding section. Gallocyanin-chromalum. Bar in Figure 6 corresponds to 50 μm .

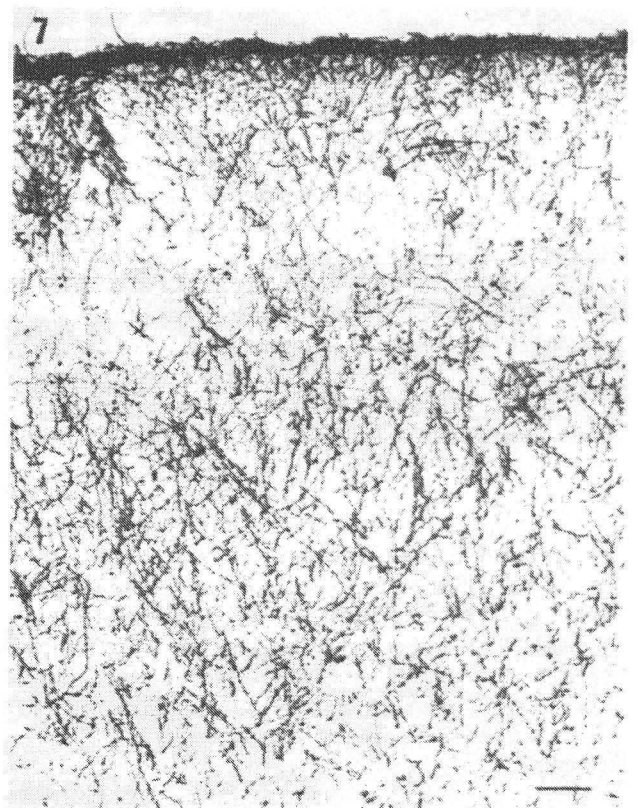


Figure 7. Serial 15- μm thick section, same technical procedure as in Figure 6. Staining of fibrous astroglia according to Gallyas (19). Bar in Figure 7 corresponds to 50 μm .

Embedding of complete human hemispheres into either paraffin or celloidin is a time-consuming procedure as the tissue must be carefully dehydrated and subsequently embedded in paraffin or celloidin. These steps together take several months. With our method, gelatin embedding and cryoprotection is done before serial sectioning to avoid breaking and freezing artifacts of the tissue. In our experience, gelatin embedding and pretreatment with cryoprotective fluids takes maximally 60 days or 2 mos. Furthermore, unlike with paraffin embedding, the use of toxic or environmentally hazardous intermedia like benzol or carbon tetrachloride can be avoided.

For fixation we prefer 4% formalin since, in our experience, formalin at this concentration penetrates complete brains better than mixtures of higher concentrations. Formalin at this concentration should be regularly changed, especially in the first weeks.

Gelatin penetrates for about 50 μm into the brain tissue. After formalin induces hardening, the gelatin provides a shell that is insoluble in water, alcohol and xylene, as well as in acid and alkaline fluids. Tangentially cut gyri will not be lost from the slice. Therefore, gelatin embedding is well suited for quantitative investigations and three-dimensional reconstructions. Reembedding into paraffin of smaller tissue samples from a thick frozen section is possible, but the gelatin shell has to be carefully removed, as gelatin becomes brittle after dehydration in alcohol, and transferred via xylene into paraffin. Otherwise artifactual damage of 10 μm reembedded paraffin sections is a frequent consequence.

On the other hand, agarose does not penetrate brain tissue. Thick frozen sections, which are floated in 4% formalin solution after the section procedure, immediately lose their agarose mantle and their coherence is lost if not held together by deep fiber tracts of white matter. We prefer agarose embedding for serial sections through the brainstem or spinal cord.

In addition to time-saving in embedding procedures, serial sectioning of frozen human tissue is simple and rapid. After some experience a technologist can process a complete hemisphere within 1 hr, yielding up to 290 0.7 mm thick sections.

Presently, we stain every third section of a complete series through the human prosencephalon. As a result we obtain parallel serial sections 2.1 mm apart. The staining and mounting procedures take an additional week. This is comparable with modern NMR brain imaging techniques of human CNS, and the findings of both methods could yield complementary results (11).

It is generally agreed that frozen sections immediately attached to gelatin coated microscopic slides do not shrink in the absence of dehydration procedures. We have performed morphometry of thick frozen sections before staining and found minimal shrinkage. But the staining and mounting procedures of frozen sections cause considerable shrinkage. In general, section thickness linearly decreased from 700 to 550 μm after staining, dehydration, and mounting. Several factors, including age and a variety of antemortem conditions, have an unpredictable quantitative influence on frozen sections. Therefore, we correct each case with the individual shrinkage factor with point-counting morphometry and application of Cavalieri's principle (12,13).

In addition to rapid and precise quantitative investigation

of human CNS, thick frozen, gallocyenin stained sections facilitate architectonic studies of cortical and subcortical grisea with a stereo microscope and low power magnification. This instrument gives a three-dimensional aspect of nerve cells arranged in laminae or nuclear masses. Subtle changes in nerve cell density, shape, or arrangement that can be hardly detected in 10 or 20 μm thick sections are immediately perceived.

Tangential cuts are frequently encountered in frontal serial sections through the richly gyrated human cerebral hemispheres. Tangential planes of section make architectonic assessment of relative laminar width of cortex difficult. But tangential sections through cortical gyri can be, to a certain degree, optically corrected by tilting the thick sections under the view of a stereo microscope, thus compensating for the unfavorable plane of section. The use of thick frozen, gallocyenin stained sections appears especially advantageous in facilitating architectonic studies of large mammalian brains including herbivores like elephants or aquatic mammals like whales or dolphins. In these species, nerve cell density is as low as in human CNS. By optical superposition of nerve cells in thick section, the outlines of laminae or nuclei appear well demarcated in contrast to conventional section thickness of 20 or even 40 μm where laminar or nuclear boundaries are sometimes arbitrarily drawn. In this respect our method resembles Braak's pigment stain of thick frozen sections (2). In contrast to aldehydfuchsin, which selectively stains lipofuscin, gallocyenin allows assessment of all nerve cell types at low as well as high-power light microscopic magnification. Both methods are, therefore, complementary, and the results of both can be compared with parallel sections in serially cut CNS.

Larger myelinated fiber tracts (Figure 1 T mth, Figure 2 Pe ce i, Lem, T sol) remain nearly unstained as gallocyenin is stoichiometrically bound to nuclear DNA and Nissl bodies of neuronal perikarya (14). This feature facilitates architectonic study and gives a limited insight into myeloarchitectonics.

In addition to the study of normal architectonics, the assessment of either diffuse or localized nerve cell loss and gliosis in a variety of diseases is facilitated. Some preliminary results have been recently reported by us (15,16).

The obvious disadvantage of thick frozen sections is the limited number of routine histological stains that can be applied. Recently, we have modified the Gallyas method for demonstration of senile changes in Alzheimer's dementia, but other methods result in completely opaque thick sections (17,18). Therefore, reembedding of parallel serial gelatin sections in paraffin is proposed. Because our frozen sections are 700 μm thick, reembedded smaller tissue segments can be serially cut into 20 to 30 sections depending on the section thickness selected. This modification allows a variety of histological, histochemical, and immunocytochemical stains compatible with paraffin embedding to be applied on regions of interest.

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