IMPRS NeuroCom

Neurohistology I

Methods of obtaining and processing CNS tissue
(Fixation, cryo, paraffin techniques)

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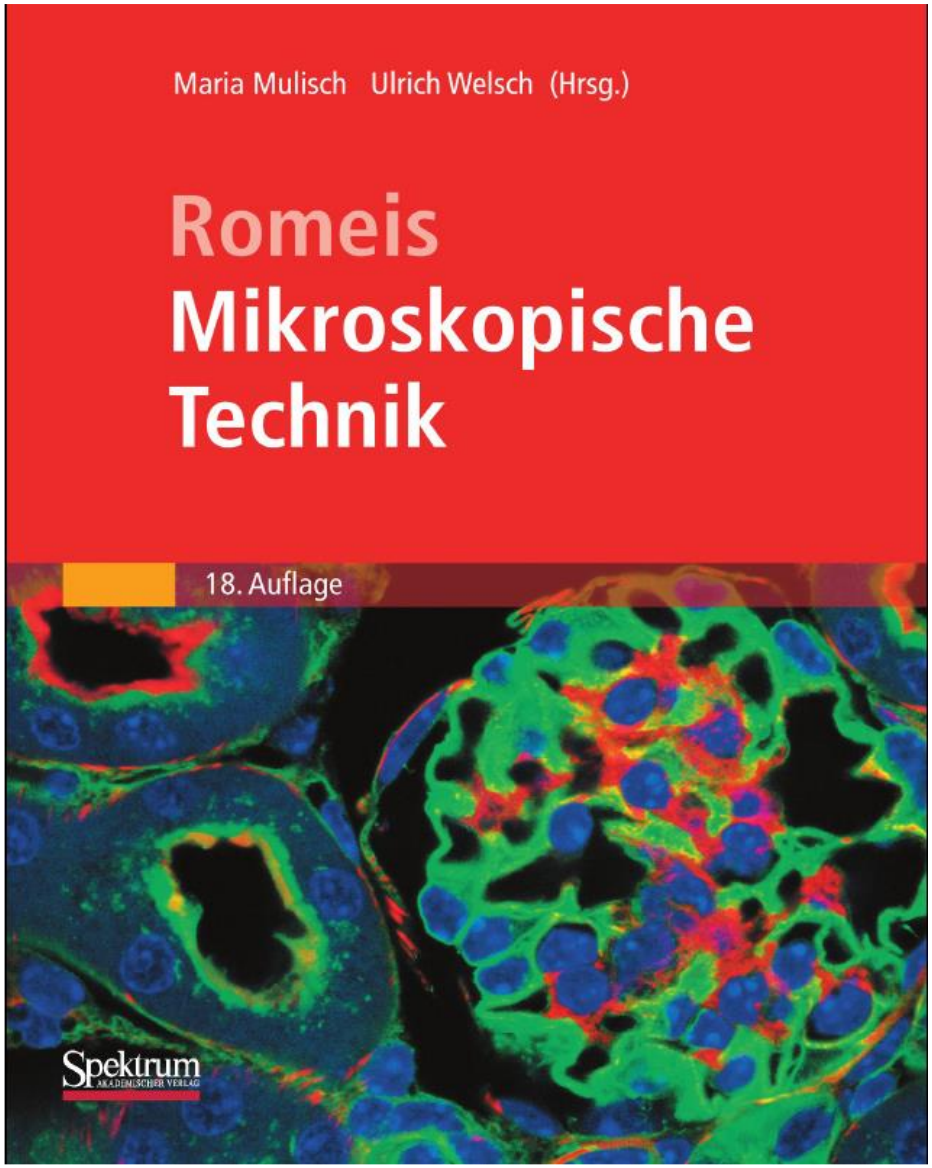
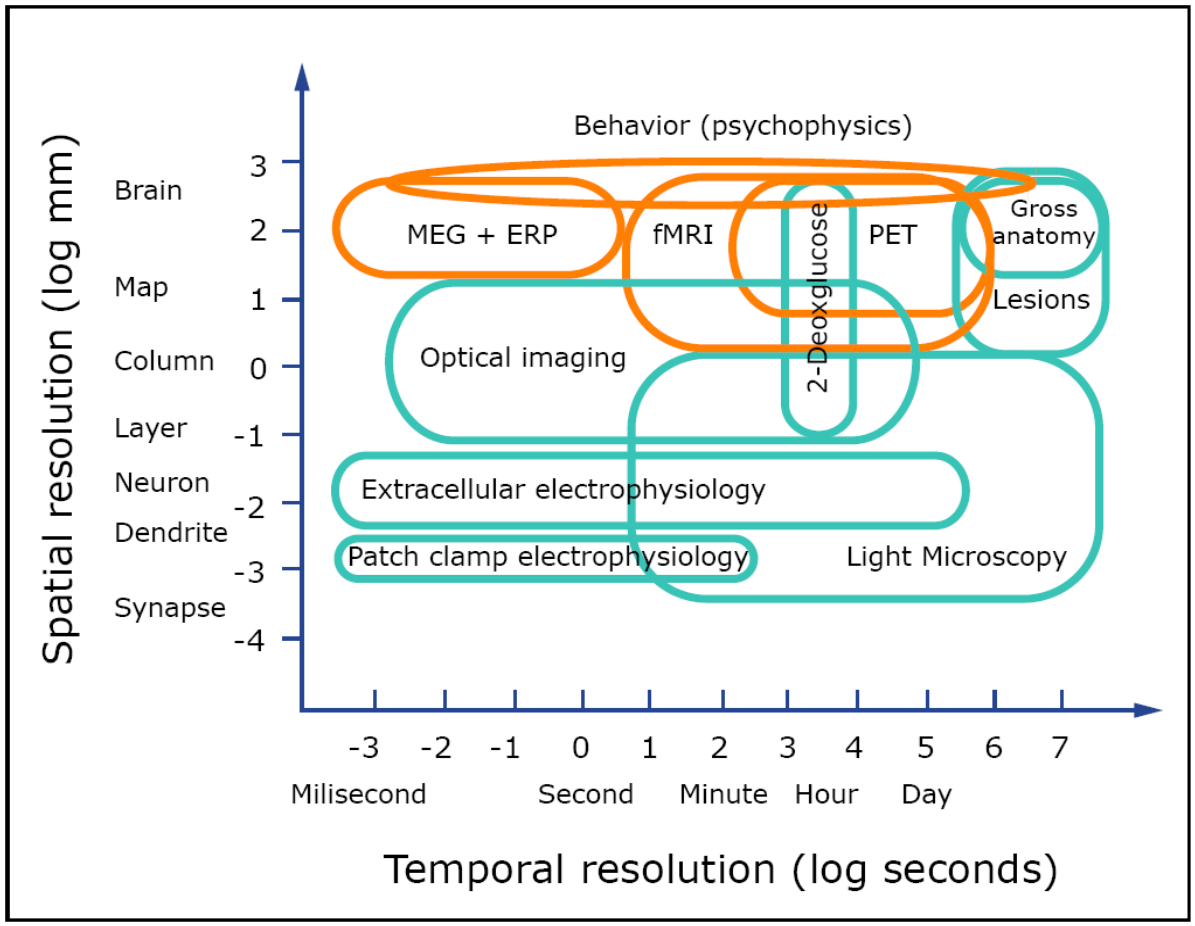


DOI
https://doi.org/10.1007/978-3-642-55190-1

Neurohistology

Why do we do neurohistology ? (systems perspective)



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What is the right tool for the job?

Brain tissue

- **Where does the material come from**
- **Human tissue:**
- Body donations, autopsies, surgical / biopsies

- **Animal tissue:**
- MEZ (Medical Experimental Center of the Medical Faculty of the University of Leipzig (laboratory animal breeding / stable))

- **Animal species (most common):**
- Mice, rats, gerbils, guinea pigs, rabbits, pigs that are bred for biomedical research.

- The use of animals is subject to strict regulations, the use is subject to a so-called Animal killing notification (T) or an animal experiment application (TVV) beforehand, an approx. 20-page application that must be approved by an animal welfare / ethics committee and the state government

  (Duration of the approval process 3-12 months)

Why can't all research be done on human autopsy material?

- „Post mortem interval “human autopsy tissue min. 12 hours often up to 7 days
- strong tissue autolysis that makes many examinations impossible
- Mainly “old patients” 65+, often multiple clinical pictures, healthy “controls” are rare
- Genetic manipulation is not possible
- Availability severely restricted approx. 400 autopsies per year
- Biopsy material is extremely limited and only available in very small quantities.

Animal killing

To ensure a safe and “pain free" death, the following factors must always be met in their entirety:

Minimizing stress, fear and excitement before animals pass out

Rapid loss of consciousness

Respiratory and cardiac arrest after onset of deep anesthesia

Loss of brain function

Reliable determination of the occurrence of death and irreversibility

No endangerment of persons involved and present from the killing procedure

Animal killing

Adult laboratory animals (e.g. mice) are killed by different methods that has to be approved by government and a ethical commission.

CO2 inhalation until death.

- The CO_2 concentration is slowly increased, so even at relatively low concentrations of approx. 7.5% there is an analgesic effect, at approx. 30-40% (different depending on the species) there is a narcotic effect and if the concentration is increased further, breathing stops .
- Death occurs from hypoxia, which leads to an insufficient supply of oxygen to the brain and death.

Intravenous or intraperitoneal injection with pentobarbital

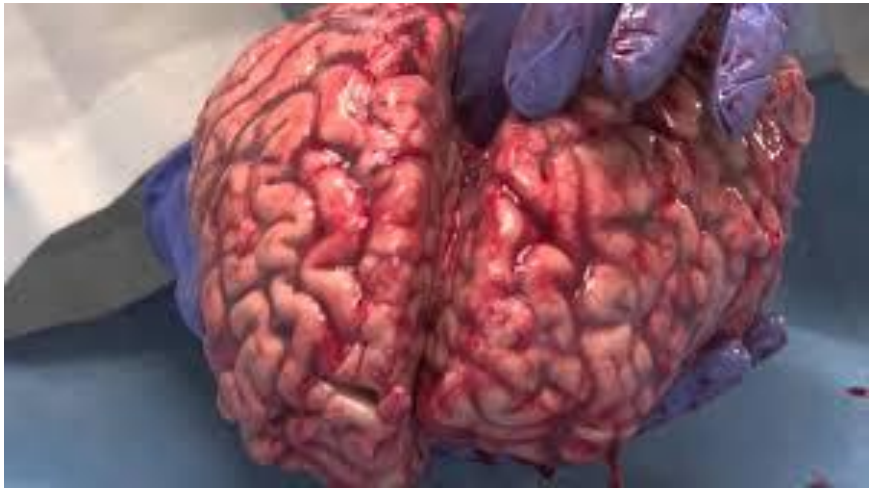
a medium-acting barbiturate (derivative of barbituric acid). In human medicine it has long been used as a sleep aid, in veterinary medicine and research it is used for euthanasia.

Decapitation (e.g. young rodents) P0-P13, since there is a relatively high resistance to hypoxia (CO_2 killing not practicable)

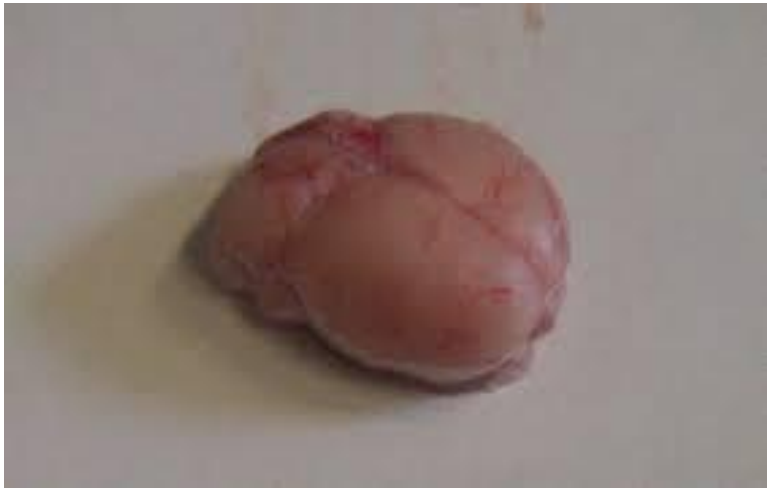
- Cervical dislocation (Fast without equipment, requires safe technology and handling)

- Rare methods: Electrical stunning and bleeding (Mainly in meat production)
  Microwaves (complex technology)
  Shock freezing (Fur loose small animals under 4g)
  Potassium chloride (Muscle cramps and pain)

Starting tissue unfixed



Human brain, native (unfixed)



Mouse brain, native (unfixed)

Fixation

Immersion

Perfusion

Goal of Fixation

The fixation

is intended to preserve cells and tissue in their natural, current state for examination.

All components should remain unchanged in size and shape and in their normal environment.

should not change molecular properties, colorability, antigenicity and enzyme activities.

should prepare for further preparation, i.e. strengthen the tissue and make it cutable and not brittle or softened.

Problem

- **There is no such thing as a fixation that meets all of the aforementioned criteria.**
- **The closest approximation is obtained through cryopreparation**
- For most uses, in particular

  light microscopic examination, a cryopreparation is not absolutely necessary.

- You should therefore be clear about the goal you are pursuing before starting the preparation in order to choose the easiest or fastest route with good results.

Fixation Methods

Name	Zusammensetzung	Anwendung	Eignung
BOUINsches Gemisch	• 15 ml gesättigte wässrige Pikrinsäure • 5 ml 40 % Formalin • 1 ml Eisessig	• direkt vor Gebrauch mischen • 2–24 h fixieren • in 70–80 % Ethanol mehrmals waschen • entwässern und einbetten	• Übersichtspräparate • Cytologische Präparate • Protozoen • Embryonen • *in situ*-Hybridisierung • Immunmarkierung
CARNOYsches Gemisch	• 600 ml 99,9 % Ethanol • 300 ml Chloroform • 100 ml Eisessig	• direkt vor Gebrauch mischen • je nach Größe 1–4 h fixieren	• Glykogennachweis • Darstellung von Kernstrukturen
Formol nach **LILLIE**	• 100 ml 36 % Formol • 4 g $NaH_2PO_4 \times H_2O$ • 6,5 g Na_2HPO_4 • 900 ml H_2O pH 7,0	• direkt vor Gebrauch mischen • je nach Größe 1–3 Tage bei 4 °C fixieren	• Histologische Färbungen
Formol-Calcium nach **BAKER**	• 10 ml 36 % Formol • 1 g $CaCl_2$ • 90 ml H_2O	• zur Neutralisation einige Stücke $CaCO_3$ hinzufügen • in dunkler Flasche aufbewahren	• Hartgewebe
Formol-Alkohol nach **BURKHARD**	• 324 ml 36 % Formol • 540 ml Ethanol oder Methanol (absolut) • 130 ml Barbital-Natrium-Puffer, pH 7,4 • 6 g Glucose	• Aufbewahrung in dunkler Flasche	• Hartgewebe
Ethanol-Essigsäure-Gemisch nach **WOLMAN** und **BEHER**	• 950 ml 99,9 % Ethanol • 50 ml Eisessig	• bis 1 cm Größe: 4 h fixieren bei −6–8 °C • Nachbehandlung: – über Nacht in 99,9 % Ethanol bei RT – 2× 20 min in reinem Benzol – Paraffineinbettung	• Nachweise: – alkalische Phosphatase – Lipase – Phosamidase – Cholinesterase
MAXIMOWsches Gemisch	• 100 ml Müllersche Flüssigkeit • 5 g $HgCl_2$ • 10 ml Formol • 10 ml 2 % OsO_4 in H_2O	• direkt vor Gebrauch mischen • 1–6 h fixieren • auswaschen in Leitungswasser	• Blut, Blutbildungsorgane • Fett
MÜLLERsche Flüssigkeit	• 2,5 g Kaliumdichromat • 1 g Natriumsulfat • 100 ml H_2O		

Fixation Methods

Pikrinsublimat nach **RABL**	• 100 ml gesättigte, wässrige Pikrinsäure • 100 ml gesättigte, wässrige Sublimatlösung ($HgCl_2$) • 200 ml H_2O	• direkt vor Gebrauch mischen • 12 h fixieren • Nachbehandlung: – Übertragen in niedrig konzentriertes Ethanol – aufsteigende Ethanol-Reihe – Zusatz von Iodtinktur und Lithiumcarbonat in 99,9 % Ethanol	• ältere Embryonen • Keimscheiben
ROSSMANNsche Lösung	• 90 ml gesättigte, ethanolische Pikrinsäure • 10 ml 40 % Formalin	• direkt vor Gebrauch mischen • 3–8 h fixieren • in 99,9 % Ethanol übertragen	• Kohlehydrate • Glykogennachweis
SCHAFFERsches Gemisch	• 100 ml 36 % Formalin (neutralisiert mit $CaCO_3$) • 200 ml 80 % Ethanol pH 7,2–7,4 (evtl. mit 1 N NaOH einstellen)	• 1–2 Tage fixieren • in 80 % Ethanol überführen	• Darstellung von Schleimen • bei rascher Weiterbehandlung: dotterreiche Embryonen • Hartgewebe • fluorochrommarkierte Gewebe

Fixation Methods

Name	Zusammensetzung	Anwendung	Eignung
STIEVEs Fixativ	• 76 ml gesättigte wässrige $HgCl_2$-Lösung • 20 ml Formol • 4 ml Eisessig	• vor Gebrauch mischen • 3–6 h fixieren • in 80 % Ethanol übertragen	• große Präparate
Sublimatalkohol nach **APATHY**	• 3–4 g $HgCl_2$ • 0,5 g NaCl • 100 ml 50 % Ethanol	• vor Gebrauch mischen • 12–24 h fixieren • in 70 % Ethanol übertragen	
Sublimat-Formol nach **HEIDEN-HAIN**	• 4,5 g $HgCl_2$ • 0,5 g NaCl • 80 ml H_2O • 20 ml 40 % Formalin	• vor Gebrauch mischen • 2–24 h fixieren • in 70 % Ethanol übertragen	• bindegewebsreiche Organe
Sublimat-Essig-säure nach **LANG**	• 100 ml gesättigte, wässrige Sublimatlösung ($HgCl_2$) • 5–10 ml Eisessig	• vor Gebrauch mischen • 0,5–6 h fixieren • in 70 % Ethanol übertragen	• Zellkernstruktur • embryonales Gewebe • bindegewebsarme Organe
SUSA-Gemisch nach **HAIDEN-HAIN**	• Lösung A: – 4,5 g $HgCl_2$ – 0,5 g NaCl – 70 ml H_2O • Lösung B: – 10 ml 20 % Trichloressig-säure • Lösung C: – 20 ml Formol • Lösung D: – 4 ml Eisessig	• Lösungen A, B, C, D vor Gebrauch mischen • 1–24 h fixieren • in 96 % Ethanol übertragen und mehrmals wechseln	• Muskelgewebe • kollagenes Bindegewebe
ZENKERsches Gemisch	• 100 ml Müllersche Flüssigkeit • 5 g Sublimat • 0,5–5 ml Eisessig • 5 ml 40 % Formalin	• vor Gebrauch mischen • 1–6 h fixieren • 24 h in fließendem Leitungs-wasser waschen	• hämatologische Unter-suchung • Übersichtspräparate

Alcoholic fixation

- **Ethanol penetrates tissue very quickly.**
- **Drainage hardens the material greatly.**
- **The preparations shrink very much.**
- Ethanol fixation for special questions that cannot be obtained with other means.
- This includes:
- Mucus, glycogen, uric acid, iron, calcium.
- The following are resolved:
- Fats and fatty substances, cholesterol compounds, chromaffin substances and many enzymes.
- 99.9% ethanol (absolute ethanol) is normally used for fixation. The fixation time is 15 minutes to 4 hours, depending on the size of the samples

Formaldehyde fixation

- Formalin or formol is a commercially available routine fixation as buffered 4% formalin.

- Formalin preserves the shape, color and structure of the preparations very well and also penetrates larger preparations. Fats and lipids are well preserved. In addition, formalin is very suitable for storing the fixed material without affecting the dyeability. It is therefore one of the most common fixatives and is included in many fixation mixtures

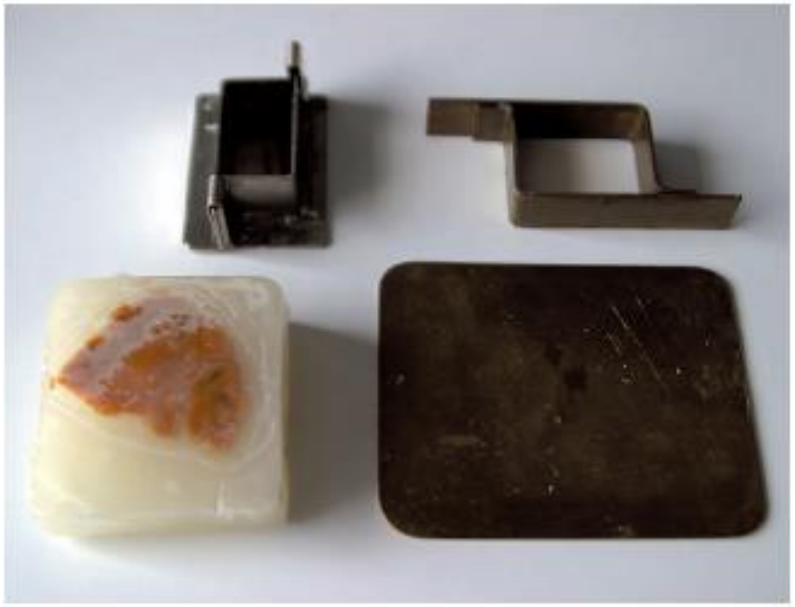
- Fixing properties are given primarily by denaturing the proteins and forming methyl bridges.

Native vs. fixed tissue

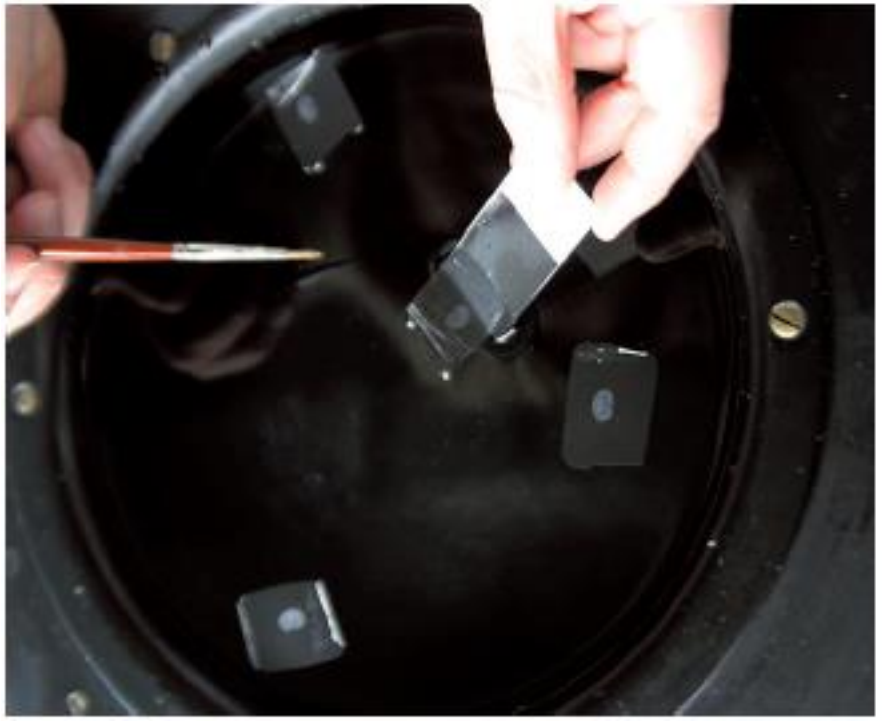
Embedding of tissue

- "Embedding" or preparation of the tissue to make it "cuttable".
- Paraffin embedding requires complete drainage of the tissue (ascending alcohol series 50-100%).
- Paraffin (histo wax) not soluble in alcohol,
- An intermediate is required, organic solvent: e.g. Methyl benzoate (but xylene, toluene or citrus oils are also possible)
- Ascending paraffin levels (at 50-65 ° C) up to complete penetration of the tissue
- Pouring out the tissue and cooling it in paraffin

Paraffin embedding



Paraffin cutting

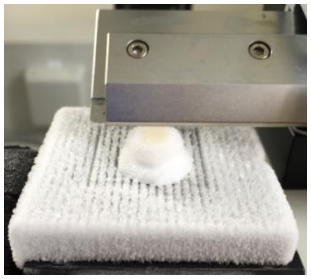


More cutting techniques

- Freeze cutting

Requirement, cryoprotection of the fixed tissue e.g. by incubation in an anti-freeze medium 30% sucrose

Cryoprotection of the tissue

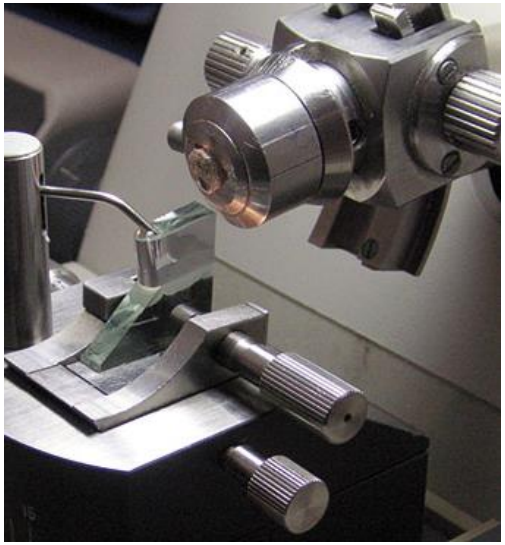


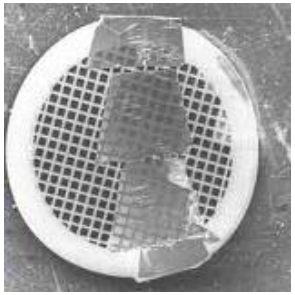
Preparation of tissue sections using the freeze-cut technique

30 µm coronal sections

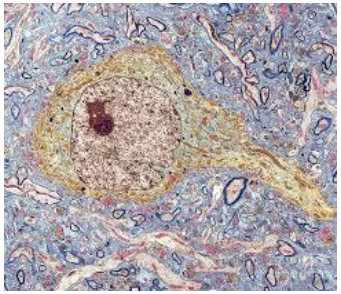
More cutting techniques

- Ultra- semi-thin cutting (30nm - 1µm)
- Embedding in "hard" media (resins)





3 mm

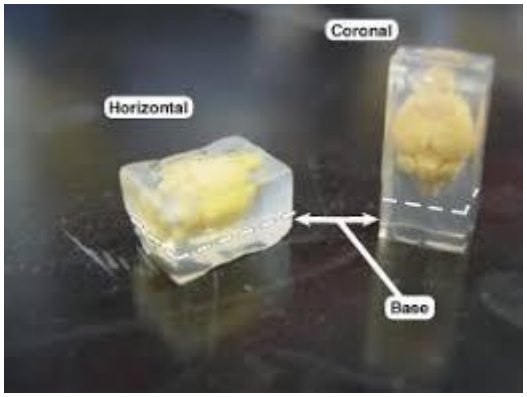


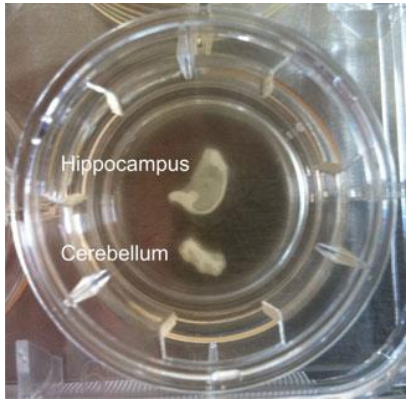
6 µm

- Electron microscopy (30-80nm)
- Light microscopy (semi-thin 0.5-1µm)
- Process from tissue to picture ~2-3 weeks

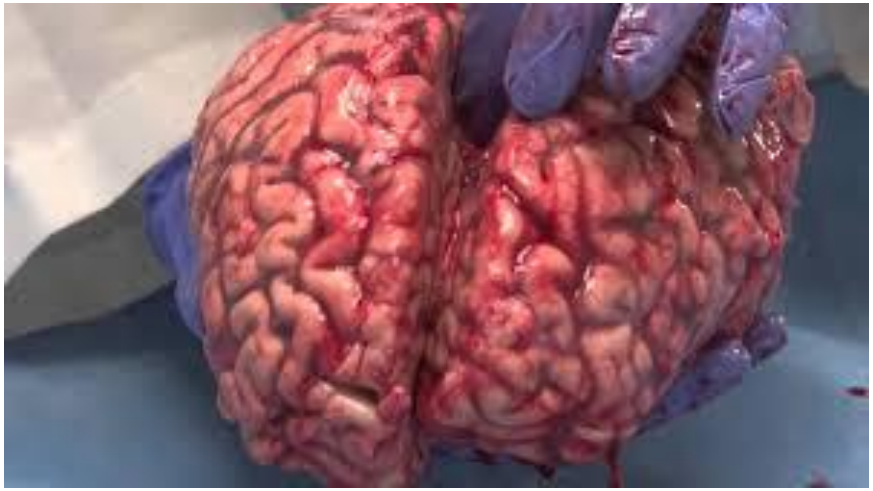
More cutting techniques

- Vibratome cutting (40-400µm) acute cuts for electrophysiology or for organotypic cultures

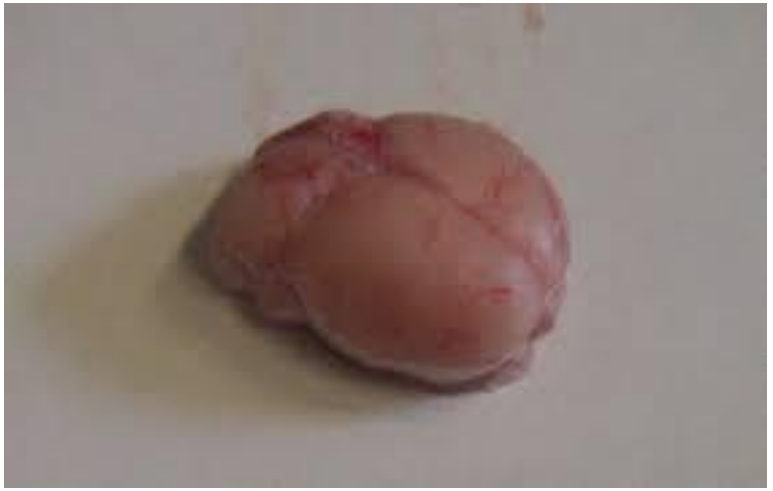




Starting tissue unfixed



Human brain, native (unfixed tissue)



Mouse brain, native (unfixed tissue)

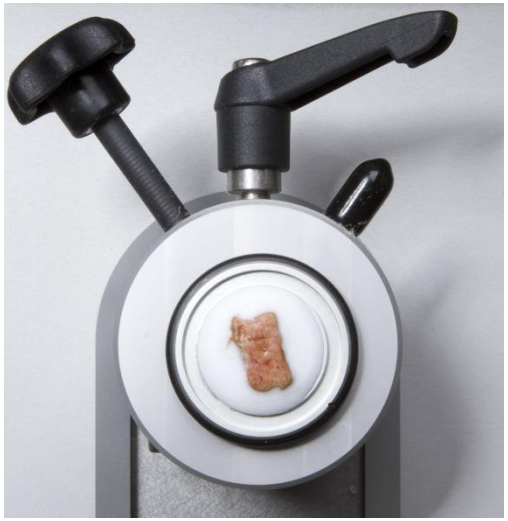
Native investigation

Live examination
Organotypic cell cultures

Protein biochemical, enzymatic, histologically

More cutting techniques

- Cryostat cutting (nativ tissue)



- Problem what do you do with the native cut? It must be "fixed" for further use!

Advantages / disadvantages of cutting techniques

	Paraffin cutting	Cryo cutting	Vibratome cutting	Ultrathin cutting
Principle	Drained/shrunken tissue, intermedium necessary, sections are drawn on OT, the section must be dewaxed for staining	Aqueous fixed tissue, cryoprotection (e.g. 30% sucrose) necessary, sections can be transferred	Aqueous native tissue, sections can be transferred, live measurements, cultivation possible	Drained tissue, intermedium necessary, contrasting, coloring necessary, cuts are drawn on tiny grids 2-3mm.
Application	Routine pathology, Research	Research	Research	Research
Pros	Thin cuts from approx. 1 µm to approx. 15 µm, extreme durability (over decades)	Quick easy method, free-floating staining	Fast, simple method, free-floating staining, also possible on native ice-cold tissue	Very thin sections from 40nm to 1µm, extreme durability (over decades), ultramicroscopy TEM possible
Cons	Staining on OS, time-consuming preparatory work, due to high temperature. Loss of reactivity, only on fixed tissue	Section thickness 20-60 µm, cryoprotection necessary, only on fixed tissue, limited shelf life	Cutting thickness 40 - 400 µm, limited shelf life	Elaborate embedding in resins (Epon, Durcupan), strong limitation of the sample size below 5mm, elaborate cutting technique, contrasting, coloring hardly possible