Tissue processing

Tissue and organ preservation (perfusion fixation), tissue processing for microscopy (dehydration, embedding, cutting, mounting, coverslipping)

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Morphology (anatomy)

• Science studying the structure of cells, tissues and organs
• morphology’s main research tool is the microscope
• to be observed under a microscope, tissues have to be transparent
• to make tissues transparent, tissues are cut in thin slices
• to be cut in thin slices, tissues have to be hard (and preserved from decay) « fixation »

Morphology is the art of cutting thin slices of tissues
Boiling an egg and pickling a cucumber represent examples of preservation, in which heat or vinegar stabilizes the organic materials.

**Chemical fixation** ↔ **Physical fixation**

**Chemical**
- Protein cross-linking
- Protein precipitation
- Oxydation
- Aldehydes
- Alcohol, acetic acid
- Osmium tetroxide

**Physical**
- Freezing: Liquid Nitrogen (-170°C), Dry ice (-40°C)
- Heating: Microwave ± protein denaturation

**Slow**

**Rapid**
Skills in anatomy

• Fixation
  – Decalcification
• Embedding
  – Dehydration
  – Clearing
  – Infiltration
• Sectioning
• Staining
• Mounting
Fixation † purpose

• Hardens the tissue (helps further handling)
• Halts enzyme autolysis
• Halts bacterial putrefaction
• Confers chemical stability on the tissue
• May enhance later staining techniques
• Introduces a 'consistent artefact'
Procedures for fixation

**Perfusion fixation**
- Animals are deeply anesthetized
- Animals are transcardially perfused with saline followed by fixative through a left ventricular catheter. The perfusion solution drains through an incision in the right atrium.
- Fixatives are usually infused under gravity or using a pump.
- This is a terminal procedure, tissues are collected for analysis.

**Immersion fixation**
- Pieces are dissected from organs and fixed by immersion (biopsies)
- Whole organs are fixed by immersion (brain in neuropathology)
Fixatives † Aldehydes † formaldehyde

• Formaldehyde is a gas (HCHO) and dissolves in water to form methylene hydrate (HOCH2OH)
• Methylene hydrate molecules have the tendency to react with one another, combining to form polymers
• Higher polymers of formaldehyde are insoluble and sold as white powder, paraformaldehyde
• To be useful as a fixative, paraformaldehyde has to be dissolved to monomeric formaldehyde by heating (60°C) and addition of hydroxide ions
Fixatives: formaldehyde mode of action

- Formaldehyde causes proteins to cross link in a meshwork, stabilising the protein mass and preserving morphology.
- Formaldehyde fixes by an oxidative reaction, forming methylene bridges (-CH2-) between the side amino groups of lysine and glutamine on different protein chains.
- Substances like sugars, lipids and nucleic acids are trapped in the meshwork of insolubilized and cross-linked protein molecules, but are not chemically changed by formaldehyde.
Formalin is the saturated (37-40%) solution with formaldehyde existing as low polymer. Formalin contains 1% methanol (to slow down polymerization) and formate ions.

Routinely, a 10% buffered formalin solution is used. This corresponds to a ~4% formaldehyde concentration.

Formalin is the main fixative in the dissection room.
Fixatives \( \equiv \) formaldehyde \( \equiv \) Practical considerations

- Formaldehyde penetrates tissue quickly but its reaction with proteins (cross-linking) is slow.
- Adequate fixation takes days, particularly if tissue has to withstand the stress of dehydration and paraffin embedding.
- Formaldehyde is toxic.
Fixatives: Glutaraldehyde

- Glutaraldehyde has two aldehyde groups, separated by a flexible chain of 3 methylene bridges (HCO-(CH2)3-CHO).
- Potential for cross-linking is higher than for formaldehyde because it can occur through both CHO groups and over variable distances.
- There are also many left-over aldehyde groups, that cannot be washed out of the tissue.
Fixatives † Glutaraldehyde practical considerations

- Glutaraldehyde fixative has to consist of monomers, with molecules small enough to penetrate quickly (EM-grade GA <-> technical grade for tanning)
- Penetration rapidity is only 2-3 mm / 12 hours
- Free aldehyde groups can lead to non-specific binding of antibodies. The free aldehyde has to be blocked or removed
- Strong cross-linking of tissue make penetration of paraffin difficult. Plastic monomer (Epon, Araldit) penetrates better
- Enzyme histochemistry and immunohistochemistry are severely impaired by glutaraldehyde
Glutaraldehyde fixation of the brain allows to differentiate between inhibitory (F: flat vesicles) and excitatory synapses (S: spherical vesicles).

D: dendrite
Electrical synapse † Mauthner

George Gray manuscript
Fixatives ‡ Karnovsky mixture of formaldehyde and glutaraldehyde

• Advantage of rapid penetration of formaldehyde ‡ structural stabilization
• Thorough cross-linking brought about by glutaraldehyde
Fixatives: Osmium tetroxide

- Osmium tetroxide exists as a pale yellow-brown crystalline solid with a characteristic acrid odor similar to ozone. In fact, the element name osmium is derived from osme, Greek for odor. OsO₄ is volatile: it sublimes at room temperature.
- OsO₄ is a staining agent widely used in transmission electron microscopy to preserve lipids and provide contrast to the image.
- Osmium tetroxide reacts with double bonds to form a very stable diester.
Fixatives ‡ OsO4 ‡ Myelin ‡ Mauthner axon
Fixation  †  Alternatives to chemical fixation

• Freezing
  – Fluid N₂
  – Dry ice

• Microwaves

Cutting frozen sections in a cryostate

Further processing like aldehydes treated tissue
Decalcification

Anorganic acid: nitric and hydrochlorid
Organic acid: formic acid and acetic acid
EDTA

After decalcification
ribs

Micro-CT’s
Skills in anatomy

- Fixation
- Decalcification
- **Embedding**
  - Dehydration
  - Clearing
  - Infiltration with embedding medium
- Sectioning
- Staining
- Mounting

LM: light microscopy
EM: electron microscopy
Araldite or Epon
Embedding medium

- Ideal qualities of embedding medium:
  - Easily available, cheap
  - Uniformity from one batch to another
  - Solubility in dehydrating agents
  - Low viscosity as monomer for penetration
  - Uniform polymerization
  - Little volume change during polymerization
  - Good preservation of fine structure
  - Good sectioning quality that includes homogeneity, hardness, plasticity and elasticity
  - Resistance to heat generated by sectioning
  - Adequate specimen stainability
  - Stability in electron beam
  - Electron lucent
Embedding ‡ Dehydration and clearing

- embedding media like paraffin (and araldite) are water insoluble
- water in the tissue has first to be exchanged with ascending concentrations of alcohol
- alcohol is exchanged with xylol, a clearing agent miscible with paraffin
- xylol is exchanged with paraffin

Propylenoxyd
Araldit or Epon for electronmicroscopy
Microtomes have the essential machinery of a baloney-slicer:
- a cutting edge (which may be a razor, a heavy knife, a piece of broken glass, or a finely sharpened diamond),
- a specimen holder,
- a screw to advance the specimen toward the blade (ultramicrotomes may use carefully-controlled thermal expansion in lieu of a screw),
- a crank, such that each turn of the crank raises the specimen, advances it, and then lowers it across the blade.

Sectioning

Microtom † Paraffin † LM

Ultramikrotron † Araldit † EM

« Je härter der Block, je dünner der Schnitt »
Hard blocks give thin sections

Section thickness: 3-5 µm
Section thickness: 60 nm
Embedding ‡ Paraffin for LM

Advantages:
1. Polymerize uniformly and rapidly
2. Thin sections (3 µm) can be cut
3. Cheap and therefore routine in pathology

Disadvantages:
1. Must be dissolved before staining of specimens can occur
2. Strong shrinkage of specimens (20-30%)
Embedding † Miscellanea

• In chemistry, **paraffin** is the common name for the alkane hydrocarbons with the general formula C\(_n\)H\(_{2n}+2\). **Paraffin wax** refers to the solids with \(n=20-40\).

• The name is derived from the Latin *parum* (= barely) + affinis with the meaning here of "lacking affinity", or "lacking reactivity". This is because alkanes, being non-polar and lacking in functional groups, are very unreactive.

• Paraffin wax (or simply "paraffin") is mostly found as a white, odorless, tasteless, waxy solid, with a typical melting point between about 47 to 64 °C.
Embedding † Araldite and Epon for EM

Classics
• Araldite: Glycerol based aromatic resin that has very little volume shrinkage on polymerization.
• Epon 812 (Shell) Glycerol based aliphatic epoxy resin
• Not reactive with alcohol, require use of transition solvent (acetone or propylene oxide)
• Require addition of curing agents to convert them to a tough, extremely adhesive and highly inert solid
• Polymerization accomplished by the addition of various bifunctional setting groups which link with the resin to produce a three-dimensional structure

Advantages:
1. Polymerize uniformly with little change in volume (as low as 2%)
2. Relatively stable in electron beam
3. Mixtures can be stored for several weeks at 4 C and many months at -20 C

Disadvantages:
1. Relatively high viscosity. Necessitates lengthy graded infiltration procedure
2. Some reduction in contrast between specimen and background
3. Can cause severe irritation to skin
Skills in anatomy

• Fixation
• Decalcification
• Embedding
  – Dehydration
  – Clearing
  – Paraffin infiltration

• Sectioning
• Staining
• Mounting
Sectioning + special equipment

Vibratome
Vibrating razor blade
For cutting fixed, non-embedded tissue

Cryostate
Microtome in a freezer
For cutting frozen tissue
Skills in anatomy

• Fixation
• Decalcification
• Embedding
  – Dehydration
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  • Staining
• Mounting
Stains

Same colorants used for textiles
Stains ± Staining versus impregnation

- Silver impregnation used in
  - histology to stain reticulin fibers (collagen III), DNA and nerve cells
  - Biochemistry to stain proteins after SDS-PAGE

Golgi impregnation: the 'black reaction' (la reazione nera)

- Nervous tissue hardening in potassium dichromate and impregnation with silver nitrate.
- Staining dependent upon the initial reduction by the oxidizing agent chromic acid of a small number of silver ions by proteins
- Staining impregnates a limited number of neurons at random
- Permits a clear visualization of a nerve cell body with all its processes in its entirety

« The gain in the brain is mainly in the stain »

Floyd Bloom, Path to discovery, MIT Press, 1975
Stains † how they work

• The components involved in histological staining are dyes and proteins. The fundamental process involved is the chemical bonding between the carboxyl groups of one and the amino groups of the other. The commonest bonds involved are ionic bonds, although there are exceptions especially in the case of nuclear staining of DNA.

• Dyes are coloured, ionising, aromatic organic compounds, based on the structure of benzene. Colour is not an objective phenomenon, it is the human detection and perception of electromagnetic radiation. Visible wavelengths 380-750.

• When some of the wavelengths found in white light are absorbed, then we see what is left over as coloured light. The colour that we see is referred to as the complementary colour of the colour that was removed. For instance, if the red rays are removed from white light, the colour we detect is blue-green.
Stains † Dyes

• Chromophores (NO2, quinoid rings): alter the energy in the electron cloud of the benzene ring and this results in an absorption in the visible range

• Our eyes detect that absorption, and respond to the lack of a complete range of wavelengths by seeing colour

• Auxochromes (-OH, -NH3, -COOH): enhance a color

• Modifiers: alter the color of dyes
Without any methyl groups the parent dye is called *pararosanilin* and is red.

When four methyl groups are added we get the reddish purple dye *methyl violet*.

As more methyl groups are added we get the purple blue dye *crystal violet* which has six such groups.

If a seventh methyl group is added, the resulting dye is *methyl green*.
Stains ǂ  Fading versus fluorescence

• When light illuminates a dye, some of it is absorbed as energy. Since energy is not destroyed, something must happen
  – Some chemical changes occur which disrupt the dye's structure and cause it to lose colour ǂ  fading
  – When the return to the ground state is not gradual, but sudden ǂ  fluorescence or phosphorescence are emitted
Stains † acid and basic dyes

• Most histologic dyes are classified either as acid or as basic dyes. An acid dye exists as an anion (negatively charged) in solution, while a basic dye exists as a cation (positive charge). For instance, in the hematoxylin-eosin stain (H&E), the hematoxylin-metal complex acts as a basic dye. The eosin acts as an acid dye.

• Any substance that is stained by the basic dye is considered to be basophilic; it carries acid groups which bind the basic dye through salt linkages. When using hematoxylin, basophilic structures in the tissue appear blue. A substance that is stained by an acid dye is referred to as acidophilic; it carries basic groups which bind the acid dye. With eosin, acidophilic structures appear in various shades of pink. Since eosin is a widely used acid dye, acidophilic substances are frequently referred to as eosinophilic.
Stains ♦ Haematoxylin-Eosin

• At its minimum, there are three items needed to produce an effective nuclear staining alum hematoxylin.
  - Hematoxylin or hematein, as the dye
  - An aluminum salt, as the mordant
  - A solvent, the simplest being water
Stains † Haematoxylin

Haematoxyylon campechianum
Stains ‡ Haematoxylin

Hämatoxylin

Hämatein

Co-ordination complex between hematein and aluminum
Stains † Eosin

• Eosin
• (synthetic product)
• pH indicator
• Bacteriostatic (=mercurochrom)
• derives from fluorescein
Stain applications † Loss of photoreceptors

Wild type

Knock-out
Stains‡ Example‡ Heart fibrosis
Stains † Examples † Achalasia (megaoesophagus)

Wild-type

Knock-out
Stains ♦ Examples ♦ Hydrocephalus

Mutant: Hydrocephalus

Control: Normal

Nissl’ stain + Luxol fast blue
Stains † trichrome stain

- Staining methods which use two or more acid dyes of contrasting colours to selectively colour different basic tissue components. Most commonly they are used to demonstrate connective tissue, often in contrast to smooth muscle, but may also be used to emphasise fibrin in contrast to erythrocytes.

- In the trichrome stains, use is made of dye competition. For instance, acid fuchsin and picric acid are used in Van Gieson's trichrome stain. In the picric acid-fuchsin mixture, the small picric acid molecule reaches and stains the available sites in muscle before the larger fuchsin molecules can enter. Used by itself, acid fuchsin has no difficulty in staining muscle.
Stains ‡ lysochromes

- **Lysochrome** is the technical name for what are more plainly called **fat stains**, dyes such as sudan III, oil red O, and sudan black.
- The basis for these dyes colouring fats is that they dissolve into it. From another perspective, the fat is a solvent for the dye. The **lyso** part of lysochrome has the meaning of solution, and the **chrome** part refers to colour. The word therefore means *dissolve colourer*
Stains ♦ Accentuator

- An **accentuator** is any chemical which facilitates the staining process.

Accentuators fall into three groups: pH, salts and phenol.
A mordant is a metal with a valency of at least two. The two commonest metals used in histotechnology are aluminum and ferric iron, both with valencies of three.
Stains † Trapping agents

• Trapping agents are chemicals which inhibit removal of dyes from tissues
• By far the commonest example of a trapping agent is iodine in a Gram stain
Stains † Differentiation

- Differentiation is the process of removing excess dye from tissues in order to accentuate a structure which retains the dye while all about are losing theirs. It is similar to decolorising, but infers a high degree of selectivity.
Skills in anatomy

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