Microtomy of tissue specimens, collection of sections

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In the beginnings of histology, tissue sections from organs were prepared by hand with knives or razor blades. Sufficient quality depended on skill and experience, but the cutting method was far from being reproducible. The early histologists realized soon that apart from tissue sectioning appropriate preparation steps were needed for microscopical work with animal and human organs. The development of so-called histological microtechniques included stabilization of cell structures by chemicals such as solvents like ethanol, salt solutions, aldehydes or other measures followed by substitution of water (water replacement by dehydration or by inert substances), embedding media for example wax, polyethyleneglycol, cellodin or alternative ground mass. Thus, biological tissues will obtain a quite balanced strength for microtome sectioning.

Apart from the above and depending on the type of study, the use of a vibratome can be a useful alternative to dehydration and wax embedding when the processes of dehydration and embedding are too harsh procedurse for the biological material under study. In addition, the necessary strength is obtained by freezing the sample, when f.e. dehydration or embedding will lead to alteration of the specimen so that subsequent histochemical procedures (immunostaining, molecular ligand bindings) would be compromised. Until now, numerous papers have been dealt with histological microtechniques such as (a) organ dissection; (b) specimen stabilization; (c) tissue sectioning; (d) collection of sections; and (e) histological staining. Some of the many publications are suitable to follow the history of efforts, f.e. QUEKETT J (1848), KLEBS TAE (1869), APÁTHY S (1896), TELLYESNICZKY K (1898) MANN G (1902), GAGE SH (1911), SANNOMIYA N (1926), BAKER JR (1958), HUMASON GL (1962), ROMEIS B (1968), LILLIE RD and FULLMER HM (1976), BRACEGIRDLE B (1977, 1978, 1989), PEARSE AGE (1980), LANG G (2013), GRUNOW B et al. (2015). For more details, the following chapters are recommended:

General aspects in tissue preparation
[link: https://www.kuhlmann-biomed.de/wp-content/uploads/2020/11/IET_tissue_01.pdf],

Fixation of biological specimens
[link: https://www.kuhlmann-biomed.de/wp-content/uploads/2020/11/IET_tissue_02.pdf],

Fixatives
[link: https://www.kuhlmann-biomed.de/wp-content/uploads/2020/11/IET_reagents_01.pdf],

Tissue dehydration and embedment
[link: https://www.kuhlmann-biomed.de/wp-content/uploads/2020/11/IET_tissue_03.pdf].

Survey of microtomy

Milestones in microtechniques fairly paralleled the development of tissue cutting techniques with mechanical devices, the so-called microtomes. The origin of microtomes is largely obscure, probably because the first microtomes were just simple devices and not documented in classified order. The precursor of “cutting instruments” is thought to be the so-called

cutting engine invented by G ADAMS/A CUMMINGS (ADAMS G [1771], ADAMS G [1798]) being described by J HILL (1770) for the preparation of botanical specimens. The *cutting engine* was hand-operated: the sample held in a cylinder and sections cut from the top using a crank arm. The construction of substantial microtomes occurred much later when histologists strongly asked for tissue sections of reproducible thickness and quality.

During the nineteenth century the development of microtomy was particularly forced by the new concepts of cell biology and pathology and the demand for tissue sections of high quality. G VALENTIN (1840), B STILLING (1842) and W HIS (1870) were notably engaged in tissue sectioning and the development of microtomes. In addition, A OSCHATZ (1843), GF DE CAPANEMA (1848), H WELCKER (1856), P SCHIEFFERDECKER (1887a, 1887b) described several prototypes. The "nouveau microtome" presented by G RIVET (1868) on the occasion of the *Séance du 13 mars 1868* (Bull Soc Bot France15, 31-32, 1868) might be a new type of microtome, but neither details nor the category of instrument were specified in that paper. Much later J GRÖNLAND (1878) described RIVET's microtome as made of wood and as a sliding microtome (movement of knife by means of slides). Prototypes of rotary microtomes were independently developed by A PFEIFER (instrument maker at The Johns Hopkins University) and by CS MINOT (embryologist at The Johns Hopkins University) in the time between 1879 to 1886. From the PFEIFER microtome only a few items were made which were not put on the market. Finally, MINOT's rotary microtome around 1886 received much attention. It applied to all future modifications of rotary microtomes. The development of cutting is described in detail by NEEDHAM J (1873), KLEBS TAE (1877), LEE AB (1890), BECK A (1897), MINOT CS (1897), MINOT CS (1903), SMITH GM (1915), THRELFALL R (1930), COWLES RP and RICHARDS OW (1947), BRACEGIRDLE B (1989), GUILLERMO C (2000, 2001).

From the quantity of proposed microtomes, only few have prevailed. Three main types of microtomes are longstanding: (a) the automatic microtome (*rocking microtome*) designed by WH CALDWELL and R THRELFALL in 1883 and made in the university workshops of Cambridge (data from ENCYCLOPAEDIA BRITANNICA) which became further developed in conjunction with H DARWIN and introduced for the market by the Cambridge Scientific Instrument Company in 1885 (ANONYMUS [1885], THRELFALL R [1930]); (b) the *Thoma-Jung* sliding microtome elaborated since 1872) and ready for the market in 1881 (THOMA R [1881]); and (c) the MINOT rotation microtome from 1886 (MINOT CS [1897], MINOT CS [1903]).

By now, the Cambridge rocking microtome is still used but not widespread. The so-called sledge microtomes are quite common, i.e. microtomes with (a) fixed object and moving knife; or (b) fixed knife and moving object. The "fixed object and moving knife" type such as the sliding microtome **JUNG Hn 40** according to R THOMA (1881) is very popular.

Microtomes essentially consist of three main parts:

- frame (main body)
- object holder (clamp for the embedded specimen)
- sledge (knife block, knife holder with knife)

With the JUNG HN40 sliding microtome, the knife is guided against the firmly clamped object. The knife in the knife carriage slides on the rails of the main body and cuts the required sections from the block according to the set cutting thickness. The cutting thickness can be varied between 0.5 and 50 µm. The movement is coupled to the feed mechanism so that the object holder is raised by the set cutting thickness after each cut. The block (Knife holder with knife) is fed by the carriage movement, which sets the fine gear in motion via a feed rod. This raises the object holder vertically by the set cutting thickness in a stable cyclinder guide. The

block holder with the mounted paraffin block is lifted upwards by the set cutting thickness after each cut. In this way, subsequent cuts are always made with the same set thickness.

Fig. 8413 JUNG HN40 microtome prepared for cutting sections

Fig. 8400 JUNG HN40 microtome ready for cutting sections from paraffin embedded tisuue

Here, the paraffin block is mounted in a clamp. The knife block (the “sledge”) containing the knife is sliding on three rails of the microtome body (frame). A dedicated feeding mechanism raises the tissue block by a calibrated toothed wheel.

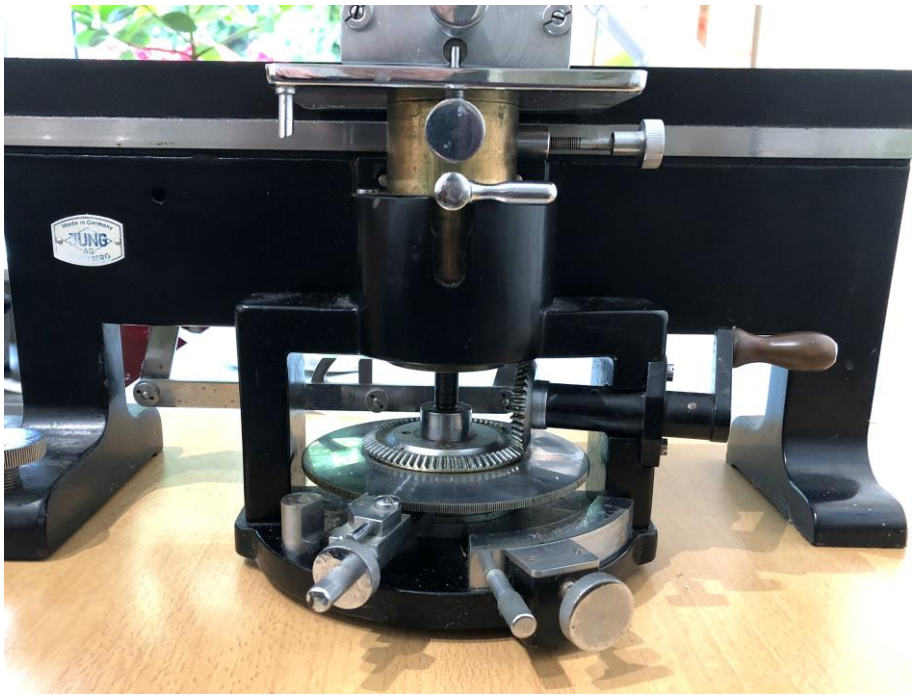

Fig. 8322 Vertical feed of the object by a screw spindle

The knife holder has five contact points for sliding on the microtome body. The so-called “fixed knife and moving object” type microtomes are specially designed heavy microtomes to cut large or hard objects.

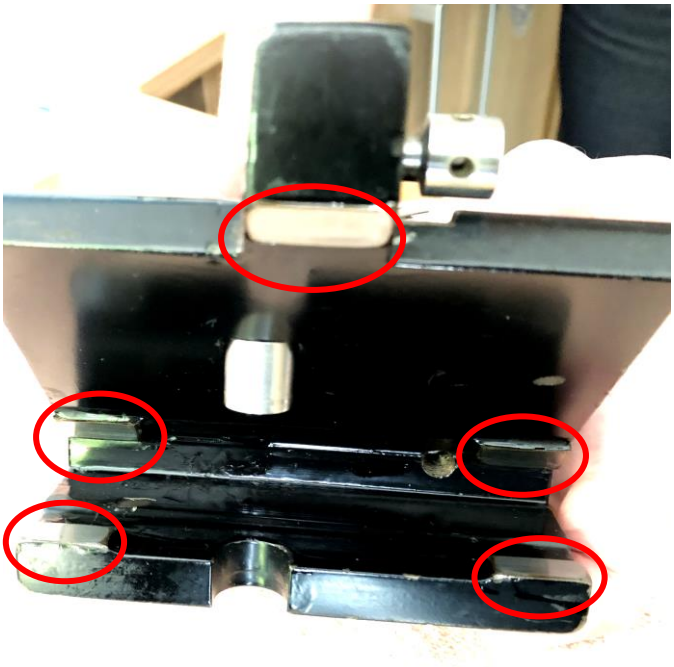

Fig. 8402 The sledge with its five contact points for stable contact with the rails of the microtome body and for stable sliding on the microtome body

The automatic MINOT rotation microtomes, preferentially the newer evolutions, are very common and much favoured for the cutting of serial sections from paraffin blocks. The microtome design yields good cutting properties enabling uniform series of thin sections. Other important types of microtomes are freezing microtomes, cryo-microtomes, vibratomes, ultramicrotomes, laser microtomes. The choice of a dedicated microtome type depends on the

material studied. As with all microtomes, it is to know that good cutting results are a matter of personal skill in handling the microtome.

Freezing microtome

A special procedure of tissue sectioning concerns the preparation of frozen sections. Early attempts were described in the publications of FV RASPAIL (1825a, 1825b, 1830), B STILLING (1842a, 1842b) and B STILLING and J WALLACH (1842); frozen sections were cut by hand. Freezing of tissue samples serves to prepare hardened specimens with preserved anatomical propeties for sectioning. Later, W STIRLING (1870) and W RUTHERFORD (1871, 1873) invented "section machines" for the preparation of frozen sections. The advanced model of W RUTHERFORD (1873) contained a box to hold a freezing mixture (powdered ice and salt). As soon as the freezing process begins, the specimen had to be kept in the hole of a brass plate (previously filled with a "neutral" medium) until it is frozen. Then, the specimen of frozen tissue and frozen fluid is cut with a razor blade. Freezing methods with freezing mixtures (ice, salt, water) were later replaced using volatile fluids like ether.

Freezing microtomes and freezing techniques became further refined. A significant advance was the introduction of freezing microtomes with carbon-dioxide tanks as described by CR BARDEEN (1901). Liquefied carbon dioxide as cooling agent replaced the conventional freezing mixture of ice/salt for a better and uniform freezing of the specimens. Later, the whole device was able to cool down the knife, too. Frozen sections became very popular for diagnostic purpose. The best way to freeze biological samples with their inherent high contents of water is rapid cooling by shock-freezing for example with liquid nitrogen/liquid nitrogen cooled isopentane etc. so that the water reaches an amorphous phase at its best without ice crystallization.

In order to obtain sections with solid quality, the tissue ought to be prefixed. TS CULLEN (1895), H PLENGE (1896) and L PICK (1897) independently proposed formaldehyde fixed specimens for the preparation of frozen sections. Later, LB WILSON (1905) described a rapid and reliable technique for the preparation of frozen sections of tissues taken from patients intraoperatively for rapid diagnostics while the patient was still on the operating table. With standardized steps of freezing, cutting, and staining, WILSON's procedure provided the surgeon with a diagnosis within few minutes. The significance is clear and even today frozen cut sections are important for rapid diagnosis in surgery.

Frozen sectioning received a certain boost when the newly developed cryostat-microtome was presented (K LINDERSTROM-LANG and KR MORGENSON [1938]). The microtome in a deep freeze cabinet proved useful for quantitative and qualitative cytochemical work. Sectioning is easier when both refrigerated microtome and tissue are kept at the same temperature. The innovation of an anti-roll plate was a technical progress for cutting flat sections and enabling the direct attachment of sections onto the microscope slide. The cryostat technique is beneficial for histopathological diagnostics, for special applications and often an alternative to paraffin histology. Since these early times of enzyme cytochemistry, cryostat sections are useful for all types of histological staining including histochemical, immunohistochemical and other ligand binding stainings. In any case, the best way for histological staining has to consider the possible deleterious effects of freezing, fixation, dehydration and embedment on cells for the studied aim.

The use of a cryo-chamber equipped with a rotary microtome is preferred for reliable cryo-sectioning. Refrigeration must be kept stable (maintaining selected temperature) and capable

of reaching at least -30° C. Best cutting of snap-frozen tissue blocks is done with the aid of an anti-roll plate. Tissue sections may be stored at -80º C. directly mounted on acetone cleaned or specially coated glass slides. Coating is done for better adhesion of sections, f.e. with poly-L-lysine, bovine serum albumin, silane or other molecules. For fixation, sections are immediately immersed into a fixative of choice. Organic solvents, formaldehyde freshly prepared from paraformaldehyde or other reagents may be used. Fixation depends on the studied material and on previously established schedules. For details see chapter *Cryostat technique* [link: https://www.kuhlmann-biomed.de/wp-content/uploads/2020/11/IET_tissue_04.pdf].

Tissue chopper, Vibratome

There exist several types of tissue chopper and vibrating microtomes. The aim of both devices is quite similar: tissue sectioning of fresh or fixed specimens without freezing or embedding and selection of a wide range of section thickness for cytochemical studies. In comparison to wax/plastic embedding, tissue sectioning does not need dehydration and embedding. Also, freezing of tissue samples for sectioning is not necessary. The section thickness can be in the range from 10 to 500 µm depending on the cutting device and the study type.

Tissue chopper operate by a chopping action to cut tissue specimens while Vibratomes cut sections by a more slicing action (principle of blade vibration). The principal feature is the lateral blade movement during penetration of the specimen. Lateral and advance speed are the key parameters in the sectioning performance of specimens. Features of both instruments for cytochemical studies were published by RE SMITH (1970).

The Sorvall TC-2 instrument was among the first developed non-frozen type tissue sectioners which were introduced in the 1960s (SMITH RE and FARQUHAR MG [1963], SMITH RE and FARQUHAR MG [1965] and became very popular for cytochemical studies; see for example NOVIKOFF AB et al. (1966), HIRANO H and OGAWA K (1967), KARNOVSKY MJ (1967), SELIGMAN AM et al. (1967), DOTY SB (1980), TAKANO K (1982). Moreover, the tissue chopper was used in ligand-binding studies such as immunolocalization of antigens and antibodies or lectin bindings (KUHLMANN WD and MILLER HRP [1971], WOOD JG et al. [1974], RANTALA I et al. [1980]).

The use of vibratome sections or “microslicer” sections from a modified vibrating microtome (MAYAHARA H et al., 1981) in preembedding cytochemistry is an approach to avoid difficulties with staining from resin embedded tissue. Stained areas are selected with the light microcope that is useful when antigens are distributed within the tissue in a patchy fashion. The procedure of vibratome sectioning has proven useful for a number of physiological and histological studies. When some inert embedding is desirable, pieces of tissue can be embedded in a matrix such as agarose (low gelling temperature) prior to cutting. After solidification, excess agarose is trimmed away and blocks are attached to a metal chuck. Techniques and application of vibratome sections for studying organs of various origin have been published. The method is valuable for histochemistry, immunohistology, DNA/RNA sensing and other defined histological projects of both animal and plant tissues. Examples are described by HÖKFELT T and LJUNGDAHL A (1972a, 1972b), LINDVALL O et al. (1973), SALLEE CJ and RUSSELL DF (1993), SHIM K (2011), VIALL CA et al. (2014), GRUNOW B et al. (2015), VERHERTBRUGGEN Y et al. (2017), WONG TTW et al. (2017).

Laser microtome

Laser microtomy is an alternative to all other microtomes in the preparation of tissue sections. The laser microtome is a device for contact-free slicing of tissues with the help of photons instead of steel blades. Biological specimens are processed in their native states. Chemical fixation, embedding or freezing is not required. Depending on the properties of the sample, 10 to 100 µm thick sections are possible. The laser microtome is not for routine pathology, however, it has its advantage over standard prcedures for the preparation of tissue slices in its native state (similar to a vibratome, see above *Vibratome*).

The microtome operates by ultra short infrared laser pulses. Laser radiation is directed onto a scanning mirror-based optical system which allows three-dimensional positioning of the beam crossover and beam traversal to the desired area. Laser microdissection of internal areas in tissues and cellular structures is possible. The laser beam is sharply focused into the sample by a high-numerical objective. Due to the extreme intensity inside the focus, multiphoton absorption causes ionization of the tissue and separation occurs (photo-disruption). The laser pulse durations are limited (femtoseconds). The energy expended at the target area is controlled and limits the interaction zone of the cut to under 1 µm. Short beam applications introduce outside of this zone minimal or no thermal damage to the remainder of the sample (LUBATSCHOWSKI H, 2007).

Rotary microtome (paraffin)

The MINOT rotary microtome is a very common instrument for cutting paraffin embedded tissues in classical histology. Major aspects of paraffin embedding and sectioning are given in the following section *Sliding microtome (paraffin)*. The technique of sectioning is quite identical with that of sliding microtomes, merely the direction of movement is modified. Factors affecting section thickness are the microtome setting such as speed of rotation, clearance angle and the cutting edge of the knife. Furthermore, sectioning is improved when specimen and paraffin embedding matrix are well matched in hardness. Prior to microtomy, paraffin blocks are chilled on a cold plate or on melting ice.

The knife is fixed in a horizontal position, the device operates with a staged rotary action. Modern rotary microtomes are so-called “retracting” microtomes. This means that the specimen retracts away from the blade upon the up stroke. The tissue block is moved by rotation action horizontally towards the cutting level (automatic or handwheel), and cut by vertical action. At the highest point of the rotary motion (vertical object movement), the sample block is advanced by the selected thickness. The actual section remains at the knife. The following section pushes the preceding section getting caught by this one. The latter one again will remain at the knife. Thus, single sections or ribbons are easily obtained.

Rotary microtomes can be equipped with flywheels. Flywheels are of advantage for cutting clean sections because the large mass of the wheel prevents the sample from being stopped during the cutting process.

Sliding microtome (paraffin)

The paraffin procedure is the most applied embedding method in routine and suitable for both rotary and sliding microtomes. Objections involve the limitations due to the nature of paraffin itself and possible influences on the tissue from the dehydration process and the elevated

temperature during infiltration. Paraffin is a mixture of hydrocarbons which solidifies into types of crystals varying with the proportion of harder and softer hydrocarbons. Thus, different samples of paraffin have different plastic points; the plastic point being the lowest temperature at which permanent deformation may occur without fracture. Consequently, with varying plasticity and melting points, the melting point may be adapted to the temperature of the room in which the tissue sectioning is realised. In addition, paraffin can be conditioned f.e. by the addition of other waxes.

Dehydration, embedding and cutting procedures will cause some distorsions. Such phenomena must be expected with paraffin embedment. Paraffin changes in size with temperature (see above), and section thickness will depend on the temperature of the tissue block at the time of sectioning. In addition, sections are compressed towards cutting direction which, finally, results in shorter and thicker sections. All these effects are more or less prominent with different materials. Additionally, the way of separation of a cut section from a given block will vary from material to material. It is trivial to say that microtomy requires experimental skill.

Paraffin embedded tissue blocks are usually cut by steel knives, steel blades (disposable blades) or tungsten carbide knives. In the case of disposable blades, appropriate holders are needed. The knife must be made of a good grade of steel with proper hardness. The composition of the steel in the cutting edge, i.e. the right balance of the different phases of steel obtained by tempering the material, is very important. Size and shape of microtome knives have come developed over a long time. The nature of the edge is of special interest for cutting. The actual cutting facets are very small in relation to the knife.

Modern microtome knives are either wedge-shaped with slightly hollow ground side or plano-concave (the latter is primarily used for celloidin sectioning). From the available knife types, the knife type C is most usual in routine work. The angle between the two cutting facets results from the sharpening method. Sharpening the knives by hand is a complex and elaborated task and is no longer practiced in routine. Microtome knives are preferentially resharpened by an outside firm. Alternatively, automatic knife sharpening devices may be used. When the knife is mounted on the microtome it must be tilted (lower side) so that there is clearance between the cutting facet next to the tissue block and its surface. The tilt is obtained by trial and error.

Microtomy of paraffin embedded tissue, collection of sections

The microtome is set up (knife removed) and the trimmed paraffin block (leave the specimen surrounded with some paraffin wax) is mounted in the specimen clamp. When all adjustments are done, the specimen clamp is locked. If necessary, final orientation is done when the knife is in its position. Declination of the knife is defined by the knife's cutting angle with respect to the cutting direction. For example, with a rotary microtome the knife has a straight through position (declination of 90º). With a sliding microtome the declination be widely varied (declination of 120-160º); the harder the material the more is the knife tilted. The "0" setting of the knife holder corresponds with a clearance angle of about 0º. The clearance angle prevents contact between the knife facet and the face of the paraffin block. The facet angle is the angle between the two facets and forms the cutting edge. Since this angle can vary with the knife type (and the manufacturer), the clearance angle must be optimally set for each blade type. Settings are often between 1º and 5º.

In the case of a JUNG-THOMA HN 40 microtome, the cutting angle (inclination) is set with the *cutting angle setting handle*. Inclination correlates with the slope of the knife towards the block plane, the clearance angle between the facet and the block surface. Important knife settings are cutting/clearance angle and declination angle. Finally, sectioning is started after a last check of the microtome. For historical development of microtomy and details in paraffin sectioning see SCHIEFFERDECKER P (1882), SCHIEFFERDECKER P and KOSSEL A (1891), KISSER J (1926), LÖW W (1931), DEMPSTER WT (1942), LEE AB (1950), GETTNER ME and ORNSTEIN L (1956), BAHR GF et al. (1957), STEEDMAN HF (1960), GRAY P (1964), MARENGO NP (1967), CLAYDEN EC (1971), WILLEY RL (1971) GALIGHER AE and KOZLOFF EN (1971), LANG G (2013).

Under almost all conditions for cutting, sections have to be flattened before attaching them to the slide. For example, the section is placed on the slide, a few drops of distilled water previously added. Then, the slide is warmed until the section spreads to full size. Floating out is done at about 30-40º C, at least significantly less than the melting point of the paraffin. Alternatively, sections are placed onto warm water and, after expansion, they are recovered by placing a slide underneath the sections. Standard microscopic glass slides are used for section mounting.

Table: Sectioning of paraffin embedded tissue

Material, equipment	**Procedure and collection of sections**
Microtome, knife Container with ice Tissue blocks Water bath 30-40 ºC, usually 5-9 ºC below the melting pint of paraffin wax Microscope glass slides Oven 37 ºC	• Tissue blocks: waste paraffin is cut away leaving the specimen surrounded only by some wax • chill tissue blocks on ice before sectioning • water bath is filled with pure water (distilled water) and heated to 30-40 ºC • prepare the microtome: knife in the holder, set the clearance angle etc. according to the microtome manufacturer's instructions • insert the paraffin block, orientate so that the blade will cut straight across the block • approach carefully the tissue block with the blade, cut a few thin sections to ensure the correct positioning, adjust if necessary • final trim of the block (thickness of 10-30 µm) to expose the tissue surface to a level where a section can be cut • cut sections of about 4-6 µm thickness • pick up the sections with a paint brush and float them on the surface of the water in order to flatten out (water bath) • use microscope glass slides (clean, silane or otherwise coated) to pick the sections out of the water bath, remove excess of water (drain vertically for a brief time) and put in a slide rack • place the slide rack in an oven and allow to dry for several hours or overnight at 37 ºC

For artefacts and troubleshooting in paraffin embedding and difficulties with tissue sectioning see website LEICA MICROSYSTEMS:

Scientia Leica Microsystems' Education Series
https://www.leicabiosystems.com/news-events/news-details/article/scientia-education-series-microtomy-and-paraffin-section-preparation/News/detail/

Scientia Leica Microsystems' Education Series, Difficult Blocks and Reprocessing
https://www.leica-microsystems.com/fileadmin/academy/2011/95.9890_Rev_A_Difficult_Blocks.pdf

Ultramicrotomy

Ultramicrotomy is the usual term to describe the technique for cutting sections from biological specimens thin enough for electron microscopic studies. Tissue sections are cut in the order of 50 to 100 nm thickness which is adequate for most fine structural studies. Section thickness is estimated by thin-film interference colors of reflected light (PEACHEY LD [1958], WILLIAMS MA and MEEK GA [1966]).

The concept of electron microscopy and the desire to study biological specimens (RUSKA H, 1939) called for new embedding media and special microtomes for the preparation of very thin sections which are thinner than the usually cut section from paraffin embedded tissue. The first attempts of thin sectioning for electron microscopy date back to the initiative of M VON ARDENNE (1939): a rotary microtome was modified to produce wedge-shapes sections from which the thinner edges could be penetrated by the electron beam. From here, a number of cutting theories and instrumentation designs have followed which are described in the papers of O'BRIEN HC and MCKINLEY GM (1943), FULLAM EF and GESSLER AE (1946), PEASE DC and BAKER (1948), HILLIER J and GETTNER ME (1950), GEREN BB and MCCULLOCH D (1951), BRETSCHNEIDER LH (1952), SJÖSTRAND FS (1953), PORTER KR and BLUM J (1953), SITTE H (1956), GETTNER ME and ORNSTEIN L (1956), HUXLEY AF (1957), PEASE DC and PORTER KR (1981), VILLIGER W and BREMER A (1990).

Certain functions are common to all modern microtomes even if they are reached by different ways. In general, microtomes of the mechanical or thermal advance type are in use. The ultra-fine advance is achieved by mechanical or thermal expansion with an accurate linear and reproducible movement. An arm carrying the the specimen is moved downwards past a sharp cutting edge (the knife), and a very small movement of the specimen arm towards the knife between each cut gives a thin section being detached from the face of the tissue block. A small trough is attached to the knife to hold the liquid (water) on which the sections can float. Sections are picked up with special grids (copper, nickel etc.). Similar to paraffin sectioning, the selection of knife angle, cutting speed and other variables depend on the material under study and are part of technical experience.

The first knives used in ultramicrotomy were steel knives or razor blades. Then, H LATTA and JF HARTMANN (1950) found that freshly broken pieces of glass possessed qualified edges for cutting ultrathin sections. Several instruments for the preparation of glass knives (Knife-Makers) are available which are suitable for cutting thin sections from biological specimens (WEINER S [1959], ANDRÉ J [1962], SQUIER CA and RANDALL M [1965]). For a better cutting quality, H FERNÁNDEZ-MORÁN (1953) proposed knives from diamonds. The crystalline structure provides a very sharp and stable edge of unsurpassed hardness. Diamond knives, however, are very expensive. It is of advantage that so-called semi-thin sections in the order of 0.5 to 1 µm can be prepared from resin embedded tissues by ultramicrotomes. Such sections are very useful for classical, cytochemical and selective ligand-binding studies with the light microscope.

With the experience that conventional embedding and cutting techniques applied for light microscopy (e.g. paraffin wax) are not appropriate for the preparation of ultrathin sections for electron microscopy, new tissue preparations were developed. Major improvements covered tissue fixation and embedment procedures. Breakthroughs in biological specimen preparation were the introduction of buffered glutaraldehyde as fixative, glutaraldehyde and osmium tetroxide as double fixation, the development of new embedding media such as methacrylates, polyester resins, epoxy resins and alternative methods for the protection of cellular structures and their molecules. The various initiatives of tissue treatment are illustrated f.e. in the publications of PORTER KR et al. (1945), NEWMAN SB et al. (1949a, 1949b), PALADE GE (1952), GLAUERT AM et al. (1956), KELLENBERGER E et al. (1956), RYTER A and

KELLENBERGER E (1958), KUSHIDA H (1960), LUFT JH (1961), LOW FN and CLEVENGER MR (1962), SABATINI DD et al. (1963), MCLEAN JD and SINGER SJ (1964), PEASE DC (1966), SANDSTRÖM B and WESTMAN J (1969), MAZURKIEWICZ JE and NAKANE PK (1972), ROTH J et al. (1978), CARLEMALM E et al. (1980), WOLOSEWICK JJ (1980), CAPCO DG et al. (1984), NEWMAN GR and HOBOT JA (1987), NEWMAN GR (1989).

For special cytochemical applications, f.e. enzyme histochemistry or molecular ligand binding studies, it became obvious that dehydration and resin embedment are often too harsh for subsequent detection of molecular structures. Thus, methods were developed such as inert embedding, cryofixation, freeze-drying, freeze-substitution and cryo-ultramicrotomy. The preparation of ultrathin frozen sections from biological specimens appeared as a good alternative in special cases as compared to conventional resin embedding and sectioning. To this aim, shock-frozen specimens (prefixed or not) are cut with special cryo-ultramicrotomes which are run at deep temperatures by cooling the device down to a selected temperature with the aid of liquid nitrogen or by other deep-freezing designs. Different experimental set-ups were proposed, and the reader is referred to some them for a broader insight: FERNANDEZ-MORAN H (1952), SJOSTRAND FS and BAKER RF (1958), FERNANDEZ-MORAN H (1960), BERNHARD W and NANCY MT (1964), DOLLHOPF FL et al. (1969), HODSON S and MARSHALL J (1970), KOLEHMAINEN-SEVÉUS L (1970), BERNHARD W and VIRON A (1971), CHRISTENSEN AK (1971), KUHLMANN WD and MILLER HRP (1971), KUHLMANN WD and VIRON A (1972), PEASE DC (1973), MITRENGA D et al. (1974), TOKUYASU KT (1973), TOKUYASU KT and SINGER SJ (1976), SITTE H (1979), KUHLMANN WD and VIRON A (1981), DUDEK RW et al. (1982), PLATTNER H and BACHMANN L (1982), DUBOCHET J et al. (1988), BAUMEISTER W et al. (1999), TAKIZAWA T and ROBINSON JM (1994), SAWAGUCHI A et al. (2004), DURÁN I et al., (2011).

Histological staining of tissue sections

Histological stainings by natural and synthetic dyes have a great tradition in microscopy. Dyes react more or less specifically with defined cell structures. A uniform theory of tissue staining does not exist because the mechanisms of dye binding with cell components are complex and heterogenous. In a particular case, both natural and synthetic dye stainings may be due to (a) chemical reaction, (b) physical adsorption or absorption, (c) other physico-chemical interactions; chemical and physical processes are running in parallel. For historical views, stains and descriptions see GIERKE H (1884), ROMEIS B (1968), BAMFORTH J and OSBORN GR (1958), GURR E (1971), CULLING CFA (1974), LILLIE RD and FULLMER HM (1976), BURCK HC (1988), TITFORD M (1993, 2001, 2005, 2009), COOK HC (1997), HOROBIN RW and KIERNAN JA (2002), KIERNAN JA (2008). Since the very beginning of histological microtechniques, a great number of procedures were developed for tissue sections either made from fixed or unfixed specimens and either cut by hand (razor blade) or by a microtome. Histological stainings exist for frozen sections as well for sections from paraffin or plastic embedded material. For details and further references, see the following chapters and internet data:

Natural and synthetic dyes in histology dehydration and embedment
[link: https://www.kuhlmann-biomed.de/wp-content/uploads/2020/11/IET_reagents_07.pdf],

Dyes, stains, and special probes in histology
[link: https://www.kuhlmann-biomed.de/wp-content/uploads/2020/11/IET_introduct_05.pdf],

Histological staining techniques
[link: https://www.kuhlmann-biomed.de/wp-content/uploads/2020/11/IET_introduct_04.pdf],

Mikroskopie, Farbstoffdaten (Eisner A)
[link: http://www.aeisner.de/daten/farbinh.html],

Internet resource for histotechnologists (Llewellyn BD)
[link: https://stainsfile.info/stain/stainindex.htm],

Selection of histological staining methods (Division Chroma®, Waldeck GmbH & Co. KG)
[link: https://www.waldeck-ms.de/wp-content/downloads/faerbemethoden_deutsch.pdf].

Paraffin embedded tissue blocks are sectioned by a conventional microtome (about 4-8 µm thick) as is usual in routine histopathology. Frozen tissues for light microscopy are preferably cut with a microtome (about 4-8 µm thick) at -25 °C to -35 °C housed in a cryochamber (cryostat-microtome).

In the case of resin embedded tissues (e.g. epoxy), semithin sections about 0.5-1.0 µm in thickness, and ultrathin sections (about 50 nm thick with silvery or gray-white appearance) are cut by an ultramicrotome equipped with glass or diamond knives. Sections for light microcopy are transferred with plastic rings (loop) onto glass slides; attachment is done by a hot plate. The partial removal of polymerized resin by defined etching methods is in certain cases necessary to achieve the desired staining effect.

Specimen	**Preparation of tissue sections**
Paraffin method	• Deparaffinize sections in xylene or xylene substitute • pass into absolute ethanol, rehydrate by decreasing series of ethanol, rinse in distilled water, alternatively in buffer • perform histological/immunohistological staining
Cryostat method	• Frozen cut sections are placed on microscopic glass slides, previously coated with silane, albumin or other adhesives • fixation is optional, f.e. with organic solvents, aldehydes, other fixatives • rinse in distilled water/buffer • perform histological/immunohistological staining
Semithin method	• semithin sections are placed with a drop of water on slides (f.e. silane coated glass slides) and dried on a hot plate plate (90 °C) for 20 min • sections can be conventionally stained or immunostained after resin etching (optional)
Ultrathin method	• Ultrathin sections are picked up with EM copper, nickel or other grids • Staining is done by classical methods (uranyl acetate, lead citrate, other contrasting methods, prior to immunostaining resin etching can be performed (optional)

Sections will not always adhere sufficiently strong even to clean glass surface during all incubation steps or when harsh staining techniques are applied. Several possibilities exist to prevent loss of sections, f.e. pretreatment of glass slides with adhesives such as egg albumin, polyvinyl acetate (white glue), gelatin or chrome alum gelatin. The most popular way to keep sections on slides, however, is to coat glass slides with polylysine (a basic polymer) or with APES (3-aminopropyltriethoxysilane). Moreover, epitope retrieval by special procedures is often necessary for immunostaining. Details are given in *Considerations prior to immuno-staining* [Link: https://www.kuhlmann-biomed.de/wp-content/uploads/2020/11/IET_immustain_02.pdf].

For sections from plastic embedded tissue it is often preferable to remove at least partially the resin matrix. This is f.e. done with sodium methoxide in methanol/benzene mixture (1:1) for 1-3 min, then passed stepwise through methanol/benzene mixture, absolute ethanol and

decreasing series of ethanol into distilled water (each solution 2 x 1 min). Prior to incubation, sections are washed in PBS.

Cryostat sections are allowed to air drying (either before or after a fixation step). Enhanced adherence of frozen sections can be achieved when sections are defrosted and dried in a microwave oven (LEONG AS and MILIOS J, 1986).

Prior to selective stainings in immunohistology, it is useful to examine the quality of tissue by a regular light microscope. To this aim, sections are histostained by an easily performed procedure such as toluidine blue (cryostat sections), hematoxylin-eosin or other conventional histological dyes (paraffin sections) or azur-methylene blue stains (Epon sections). In the case of resin embedded tissues, areas with the desired structures are easily identified. Then, tissue blocks are trimmed and the chosen areas accordingly cut by the microtome.

Selected publications in chronological order

Hill J (1770)
Adams G (1771)
Adams G (1798)
Raspail FV (1825a, 1825b)
Raspail FV (1830)
Valentin G (1840)
Stilling B (1842a, 1842b)
Stilling B and Wallach J (1842)
Oschatz A (1843)
de Capanema GF (1848)
Quekett J (1848)
Welcker (1856)
Rivet G (1868)
Klebs TAE (1869)
His W (1870)
Stirling W (1870)
Rutherford W (1871)
Needham J (1873)
Rutherford W (1873)
Klebs TAE (1877)
Grönland J (1878)
Thoma R (1881)
Schiefferdecker P (1882)
Gierke H (1884a, 1884b, 1884c)
Anonymous (1885)
Schiefferdecker P (1887a, 1887b)
Lee AB (1890)
Schiefferdecker P and Kossel A (1891)
Cullen TS (1895a, 1895b)
Apáthy S (1896)
Plenge H (1896)
Beck A (1897)
Minot CS (1897)
Pick L (1897)
Tellyesniczky K (1898)

Bardeen CR (1901)
Mann G (1902)
Minot CS (1903)
Wilson LB (1905)
Gage SH (1911)
Smith GM (1915)
Kisser J (1926)
Sannomiya N (1926)
Threlfall R (1930)
Löw W (1931)
Linderstrom-Lang and Morgenson KR (1938)
von Ardenne M (1939a)
Ruska H (1939)
Dempster WT (1942)
O'Brien HC and McKinley GM (1943)
Porter KR et l. (1945)
Fullam EF and Gessler AE (1946)
Cowles RP and Richards OW (1947)
Pease DC and Baker RF (1948)
Newman SB et al. (1949a, 1949b)
Hillier J and Gettner ME (1950a, 1950b)
Latta H and Hartmann JF (1950)
Lee AB (1950)
Geren BB and McCulloch (1951)
Bretschneider LH (1952)
Fernandez-Moran H (1952)
Palade GE (1952)
Fernández-Morán H (1953)
Porter KR and Blum J (1953)
Sjöstrand FS (1953)
Gettner ME and Ornstein L (1956)
Glauert AM et al. (1956)
Kellenberger E et al. (1956)
Sitte H (1956)
Bahr GF *et al.* (1957)
Huxley AF (1957)
Baker JR (1958)
Bamforth J and Osborn GR (1958)
Peachey LD (1958)
Ryter A and Kellenberger E (1958)
Sjostrand FS and Baker RF (1958)
Weiner S (1959)
Fernandez-Moran H (1960)
Kushida H (1960)
Steedman HF (1960)
Luft JH (1961)
André J (1962)
Humason GL (1962)
Low FN and Clevenger MR (1962)
Sabatini DD et al. (1963)
Smith RE and Farquhar MG (1963)
Bernhard W and Nancy MT (1964)
Gray P (1964)

McLean JD and Singer SJ (1964)
Smith RE and Farquhar MG (1965)
Squier CA and Randall M (1965)
Novikoff AB et al. (1966)
Pease DC (1966)
Williams MA and Meek GA (1966)
Hirano H and Ogawa K (1967)
Karnovsky MJ (1967)
Marengo NO (1967)
Seligman AM et al. (1967)
Romeis B (1968)
Dollhopf FL et al. (1969)
Sandström B and Westman J (1969)
Hodson S and Marshall J (1970)
Kolehmainen-Sevéus L (1970)
Smith RE (1970)
Bernhard W and Viron A (1971)
Christensen AK (1971)
Clayden EC (1971)
Galigher AE and Kozloff EN (1971)
Gurr E (1971)
Kuhlmann WD and Miller HRP (1971)
Willey RL (1971)
Hökfelt T and Ljungdahl A (1972a, 1972b)
Kuhlmann WD and Viron A (1972)
Mazurkiewicz JE and Nakane PK (1972)
Lindvall O et al. (1973)
Pease DC (1973)
Tokuyasu KT (1973)
Culling CFA (1974)
Mitrenga D et al. (1974)
Wood JG et al. (1974)
Lillie RD and Fullmer HM (1976)
Tokuyasu KT and Singer SJ (1976)
Bracegirdle B (1977)
Bracegirdle B (1978)
Roth J et al. (1978)
Sitte H (1979)
Carlemalm E et al. (1980)
Doty SB (1980)
Pearse AGE (1980)
Rantala I et al. (1980)
Wolosewick JJ (1980)
Kuhlmann WD and Viron A (1981)
Mayahara H et al. (1981)
Pease DC and Porter KR (1981)
Dudek RW et al. (1982)
Plattner H and Bachmann L (1982)
Takano K (1982)
Capco DG et al. (1984)
Leong AS and Milios J (1986)
Newman GR and Hobot JA (1987)
Burck HC (1988)

Dubochet J et al. (1988)
Bracegirdle B (1989)
Newman GR (1989)
Villiger W and Bremer A (1990)
Sallee CJ and Russell DF (1993)
Titford M (1993)
Takizawa T and Robinson JM (1994)
Cook HC (1997)
Baumeister W et al. (1999)
Guillermo C (2000)
Guillermo C (2001)
Titford M (2001)
Horobin RW and Kiernan JA (2002)
Sawaguchi A et al. (2004)
Titford M (2005)
Lubatschowski H (2007)
Kiernan JA (2008)
Titford M (2009)
Durán I et al. (2011)
Shim K (2011)
Lang G (2013)
Viall CA et al. (2014)
Grunow B et al. (2015)
Wong TTW et al. (2017)
Verhertbruggen Y et al. (2017)

Full citation of publications is given in chapter *References*
link: https://www.kuhlmann-biomed.de/wp-content/uploads/2020/12/References.pdf

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