Cryostat, cryotechniques

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The *cryostat* (greek *cryo* [cold] and *stat* [stable]) is a device by which low temperatures can be maintained at defined levels by special refrigeration methods. In biosciences, the cryostat is a key instrument to make frozen cut histological sections (cryosections). It is principally a microtome placed in a deep-freezer.

For many histological activities such as enzyme histochemistry, immunohistology and molecular ligand binding studies, classical techniques of tissue fixation, dehydration and embedments (paraffin, resin etc.) are often too harsh for a study goal. Thus, alternative methods are necessary from which cryotechniques and cryomicrotomy are a choice. This way, frozen cut sections are helpful for a number of histological approaches. Apart from the selective stainings, classical histological dyes can be also applied.

Frozen sections

The preparation of frozen cut sections has a long tradition, and important contributions came from W STIRLING (1870), W RUTHERFORD (1871, 1873), TS CULLEN (1895), H PLENGE (1896), L PICK (1897), CR BARDEEN (1901) and LB WILSON (1905). In the early days, frozen sections for histopathological purposes were cut with microtomes containing a box with a freezing mixture (crushed ice and salt) or, later, equipped with a freezing unit cooled by liquefied carbon dioxide. Yet, no fine-tuning was available at that time. Histological cryo-techniques received a general boost with the presentation of a *cryostat-microtome* by K LINDERSTROM-LANG and KR MORGENSON (1938). The cryostat-microtome consisted of a cryo-chamber and a rotary microtome with an innovative anti-roll plate for the collection of sections.

The unit (called *cryostat*) facilitates reliable cryosectioning because cryo-chamber and microtome are run at preselected temperatures being kept constant. With all new models, cryostat handling and cutting process can be controlled from outside. The cryostat technique is beneficial for histopathological diagnostics and for special histological applications as alternative to classical embedding techniques. Cryostat temperatures may range from 0º C to - 50º C The best cutting temperature depends on the tissue type, the majority of tissues are cut between - 20º C to - 30º C.

Cryostat sections proved to be useful in diagnostics and research, especially in enzyme cytochemical studies. Numerous techniques as well as theories and practical advices were published by PEARSE AGE (1953, 1980), LILLIE DR (1965), LILLIE RD and FULLMER HM (1976), LOJDA Z et al. (1979) and KIERNAN JA (1999, 2008). Cryomicrotomy is still an important tool in many areas of medical, biochemical and cell research. Apart fom its

usefulness in classical enzyme cytochemistry, cryostat techniques are suggested to be helpful in experimentation with the ever-expanding panel of histological labelling techniques.

Since the introduction of antibody labelling with fluorochromes by AH COONS and coworkers (COONS AH et al. [1942, 1950, 1951, 1955]), the method of histological immunofluorescence became very popular for biomedical studies in the following decades. Particularly, the success of immunofluorescence depended on continuous improvements of fluorochome labelling and tissue processing methods (NAIRN RC [1976]). Some early studies may be called to mind: HOLBOROW EJ et al. (1957), FRIOU GJ (1958), FRIOU GJ et al. (1958), PARONETTO F et al. (1962), SAINTE-MARIE G (1962), CRABBÉ PA et al. (1965), PARONETTO F and KOFFLER D (1965), TOMASI TB et al. (1965),CRABBÉ PA and HEREMANS JF (1966), GITLIN D et al., (1967), KUHLMANN WD (1968), LINDER E and SEPPÄLÄ M (1968) TOURVILLE DR et al. (1969), KUHLMANN WD et al. (1970), ENGELHARDT NV et al. (1971), CURRAN RC and GREGORY J (1978).

The localization of molecules in cells such as proteins, enzymes and other molecules in the course of cellular fluctuations, for monitoring cellular changes and dynamics of cell functions is of considerable interest in bioscience, see f.e. KUHLMANN WD et al. (1981), WOLF CR et al. (1984), BUCHMANN A et al. (1985). The tracing of biomolecular events during normal cell differentiation (evolution, phylogeny, ontogeny) or in pathological conditions such as carcinogenesis, toxicity, metabolic disorders, regeneration and other defined models is possible with appropriate marker systems. Amongst them, enzyme cytochemistry, immunological and other ligand labelling techniques are constantly further developed.

Cryosections for light microscopy

The concept of cutting cryo-sections from fixed or not fixed biological samples is different from the classical paraffin procedure where fixation is the first step in a series of preparative steps with final embedding in paraffin wax. While problems can occur with cryostat sections inasmuch as soluble antigens may leak away during and after sectioning before sufficient tissue stabilization is attained, the paraffin technique has the disadvantage of possible denaturation of molecules due to the embedding sequence consisting of fixation, dehydration and hot wax. These objections have to be reconciled in the beginning of each project.

The need of fixation depends on the study material, the choice of a fixation protocol is linked with the analyses to be performed. Any treatment which will preserve cellular structures can be harmful to their biochemical composition. Obviously, there exists no ideal fixative for all study types. Tissue stabilisation can be done by organic solvents (e.g. acetone), aldehydes (f.e. formaldehyde freshly prepared from paraformaldehyde) or by other means.

Chemical fixatives which combine with proteins are called *additive fixatives*, and those which precipitate proteins are *coagulant or precipitant fixatives*. On the basis of this categorization, fixatives can be roughly classified into these groups:

- Additive fixatives, e.g. aldehydes, potassium dichromate, osmium tetroxide.
- Coagulant fixatives, e.g. acetone, ethanol, methanol, trochloroacetic acid.
- Additive coagulant fixatives, e.g. chromic acid, mercuric chloride, picric acid.
- Non-additive non-coagulant fixatives, e.g. acetic acid.

It can be expected that no matter what reagent is used the molecular structure may become altered: either chemically f.e. by cross-linking of proteins or physically by precipitation phenomena. Both actions may results in denaturation. The choice of fixatives and a defined fixation schedule (reagent, procedure, time, temperature, buffer salts) should be selected by experimentation. Even if many molecular structures can survive fixation, but the decision of fixation, freeze protection, sectioning, drying etc. will vary from tissue to tissue and is the result of trial and error; see *Fixation of biological specimens* [link: http://www.immunologie-labor.com/cellmarker_files/IET_tissue_02.pdf].

Tissue sections of high quality need standardized steps of tissue collection, freezing, cutting, and staining. All procedures should be as reproducible as possible. Specimens to be cut (either unfixed or fixed) are mounted on a metal "chuck" and frozen to cutting temperature. The use of an inert mounting media such as *Tissue-Tek*® (blend of water soluble glycols and resins) is a favorable supporting matrix. This ground mass enables regular and flat sectioning and improves the stability of the cut sections, even if the anti-roll plate is already a fairly good aid for flat sectioning. Frozen cut sections are directly mounted on a clean microscope slide and air-dried prior to further processing. Alternatively, sections are stored at - 80º C.

Frozen cut sections, especially those from unfixed specimens, are quite fragile, and they will not always adhere sufficiently strong to glass surfaces during the incubation steps or when harsh staining techniques are used. Several possibilities exist to prevent loss of sections, f.e. pretreatment of glass slides with adhesives such as egg albumin, polyvinyl acetate, gelatin or chrome alum gelatin. Poly-L-lysine, bovine serum albumin or silane are widely used and endorsed coatings. The type of slide treatment depend on subsequent histological reactions. Since tissue sections possess a net negative charge at physiological pH, adhesiveness of tissue sections can be enhanced when the slide surfaces are positively charged. This can be done by coating the slides with basic polymer or by chemical treatments which leave amino groups covalently bound to the glass slide. The introduction of positively charged glass surfaces by reaction of slides with 3-aminopropyltriethoxysilane (APES) according to M RENTROP et al. (1986) and PH MADDOX and D JENKINS (1987) proved to be a very useful and versatile method for enhanced adhesion of tissue sections onto microscopic glass slides.

Table 1: Cryostat sections for microscopy

Procedure	**Description**	**Remarks**
(1) Fixation	Buffered formaldehyde (e.g. 1-4%) prepared from paraformaldehyde	*Light microscopy*: optional step prior to cryomicrotomy
	Formaldehyde-glutaraldehyde mixture, other fixatives Alternatively: see (6)	*Electron microscopy*: important step for preembedding immuno-staining and in preparing ultrathin frozen sections
(2) Freeze protection	(a) Dimethylsulfoxide (DMSO), f.e. 10% DMSO in cacodylate buffer (b) 30% Sucrose in cacodylate buffer (c) 30% Glycerol in cacodylate buffer (d) Other type of freeze protection	*Light microscopy*: optional step *Electron microscopy*: customary in preembedding immuno-staining technique and in cryo-ultramicrotomy *Electron microscopy*: cryo-techniques for EM see text

(3) Snap-freezing	Liquid nitrogen cooled isopentane: immersion of tissue block mounted on a tissue holder with a drop of Tissue Tek® as supporting matrix [1)]	Frozen tissue blocks can be stored in liquid nitrogen
	Direct snap-freezing in liquid nitrogen	*Electron microscopy*: Cryo-techniques for EM see text
(4) Cryosectioning	*Light microscopy*: sections are cut at 4-8 µm thickness	*Light microscopy*: cryochamber at -30°C
	Electron microscopy: thick sections (10-40 µm thickness, preembedding immuno-staining)	*Electron microscopy*: cryochamber operating at -30°C to cut thick sections (preembedding immuno-staining)
	Electron microscopy: ultrathin frozen sections	*Electron microscopy*: cryochamber operating at -90°C to -120°C for ultrathin frozen sections
(5) Collection of sections	*Light microscopy*: frozen sections are mounted in the cryochamber on clean and conditioned glass slides, then transferred to room temperature; slides can be stored for several days at -20 to -80°C before staining	*Light microscopy*: sections may be air-dried (variable times) and/or fixed (in acetone or ethanol) at 4°C / -20°C for 5-15 min Alternatively: other type of fixation
	Electron microscopy: frozen cut sections are dropped straight as free-floating sections into vials with 10% DMSO	*Electron microscopy*: sections for preembedding immuno-staining are kept free-floating during all subsequent steps until final resin embedment
	Electron microscopy: ultrathin sections are cut at a thickness of about 50-70 nm; sections are picked up and transferred for direct imaging or immuno-staining by avariety of methods	*Electron microscopy*: ultrathin frozen sections are collected dry on nickel grids or spread on 50% DMSO and transferred with a wire loop onto a drop of water at room temperature; optionally: sections are picked up with a loop containing a drop of 2.3 mol/L sucrose or 1% methyl cellulose; the loop is then touched onto the surface of a grid to deposit the sections
(6) Fixation Postfixation	*Light microscopy*: air-dried sections may be fixed, f.e in acetone at 4°C or at -20°C for 5 to 15 min, then allowed to dry	*Light microscopy*: different methods to be tried depending on the study object
	Alternatively: other type of fixation	*Electron microscopy*: postfixation (OsO_4) of frozen sections is optional
(7) Immuno-staining	*Light microscopy*: see chapter (a) *Immuno-staining, other ligand detection* [2)] (b) *Selection of staining protocols* [3)]	*Light microscopy*: differences in mmuno-staining of paraffin and cryostat sections have to be reconciled, f.e. antigen retrieval, enzyme nhibition schedules
	Electron microscopy: see chapter (a) *Preembedding immuno-staining for electron microscopy* [4)]	*Electron microscopy*: treatment of ultrathin frozen sections is different from preembedding immuno-staining of thick frozen sections

[1)] Tissu Tek® is a formulation of water-soluble compounds (polyvinyl alcohol, polyethelene glycol and other non-reactive ingredients (Sakura Inc.)

[2] Link: http://www.immunologie-labor.com/ZM-CI/zmci_rubrik.php?lang=en&rubrik=26

[3] Link: http://www.immunologie-labor.com/ZM-CI/zmci_rubrik.php?lang=en&rubrik=27

[4] Link: http://www.immunologie-labor.com/ZM-CI/zmci_paragon.php?lang=en&content=79&rubrik=27

Usually, snap-frozen tissue blocks are cut by cryostat microtome with a clean blade, and sections are mounted on acetone cleaned or specially coated glass slides. After thawing on glass slides (room temperature), the latter are immersed into a fixative; unfixed sections may be stored at - 80ºC. For fixation purposes, a variety of solutions can be used, f.e. (a) cold acetone for 5 min; (b) cold methanol for 5 min; (c) 70% ethanol for 15-30 sec; (d) 4% formaldehyde freshly prepared from paraformaldehyde for 5 min; (d) mixtures from ethanol and formaldehyde or any other fixative (see above chemical fixatives). Be aware that acetone is not a real fixative like aldehydes. Acetone will solve fatty structures, and associated antigens may solve, too.

After fixation, the objects are rinsed in phosphate buffered saline at pH 7.4 and subjected to immuno-staining. Cryo-sections fixed with acetone alone are quite vulnerable with respect to surfactants (Tween etc.) and substances for blocking endogenous enzyme activities. The latter being often necessary in immuno-stainings with enzymes as markers; see *Immuno-staining, ligand detection* [link: http://www.immunologie-labor.com/ZM-CI/zmci_rubrik.php?lang=en&rubrik=26].

Cryotechniques for electron microscopy

Cryotechniques are a group of related procedures to stabilize biological specimens for microscopic observations. The preparative steps include cryoprotection of cell/tissue samples (fixed or not) by special measures and ultra-rapid freezing to maintain morphological structure and molecular composition prior to electron microscopic processing. Such techniques are important for a number of applications in cell biology. It is for example difficult or not possible to study under all conditions the dynamics in cellular distribution of macromolecules (enzymes, proteins, metabolites and other cell components) by methods of conventionally fixed, dehydrated and resin embedded tissue specimens because such procedures are often too harsh for molecular and cellular stuctures. Common cryo-techniques are cryo-fixation, cryo-substitution and cryo-ultramicrotomy:

- Cryo-fixation as initial step in tissue preparation provides excellent preservation of cellular ultrastructure. Cryo-fixation is a physical fixation for immobilization of biomolecules without the need of chemical reagents. Morphology remains near to the native state. Artefacts by denaturation are as much as possible avoided; cellular water becomes transformed into a vitreous state. Quick-freezing, high-pressure freezing, freeze substitution, freeze-drying are most suitable methods to give adequately frozen samples. High-pressure freezing, freeze substitution and low-temperature embedding can be combined. Adequate cryo-fixation is an important measure for cryo-electron microscopy of frozen hydrated specimens.
- Cryo-substitution is an alternative to classical resin embedment. The method is thought to avoid as much as possible the denaturing effects of room temperature, dehydration and resin embedment. Samples are frozen, dehydrated and embedded in a cryo-substitution unit operating with acetone at - 90° C. Water molecules replaced by acetone in a process like sublimation will minimize adverse effects on molecular structures. Finally, the sample is infiltrated with low viscosity resins. Resins of the Lowicryl® series are cured at low temperature with UV light. Sections are cut from tissue blocks with conventional ultramicrotomes and processed for subsequent staining.

– Cryo-ultramicrotomy covers the technical preparation of ultrathin frozen sections (50-70 nm thick). Cutting of ultrathin sections is achieved with an ultramicrotome in a cryochamber. Sections are placed on grids and stained for microscopy.

Hence, methods such as inert embedding and a variety of cryotechniques such as cryo-fixation, freeze-substitution, low-temperature embedding, cryo-electron microscopy are important alternatives to classical resin embedment either for fine structural studies or for studies on subscellular dynamics in bioscience. During the last decades, several cryo-techniques have been published with the aim to investigate biological samples in their native state as much as possible. For references see FERNANDEZ-MORAN H (1960), MOOR H (1964), PEASE DC (1966), BAUMEISTER W (1978), BAUMEISTER W (1982), HARVEY DM (1982), PLATTNER H and BACHMANN L (1982), EDELMANN L (1986), GILKEY JC and STAEHELIN AL (1986), MOOR H (1987), STEINBRECHT RA and ZIEROLD K (1987), DUBOCHET J et al. (1988), DAHL R and STAEHELIN LA (1989), HENDERSON R et al. (1990), HERMANN R and MÜLLER M (1992), DUBOCHET J (1995), MONAGHAN P et al. (1998), BAUMEISTER W et al. (1999), MCDONALD K (1999), STUDER D et al. (2001), EDELMANN L (2002), BIEL SS et al. (2003), BAUMEISTER W (2002), HSIEH CE et al. (2002), SAWAGUCHI A et al. (2004), MATSKO N and MUELLER M (2005), HSIEH CE et al. (2006), DUBOCHET J (2007), RICHTER T et al. (2007), STUDER D et al. (2008), TAKIZAWA T et al. (2015), BECK M and BAUMEISTER W (2016).

Cryo-ultramicrotomy appears attractive and as a good alternative in certain cases when compared with resin embedded specimens. The advantage of cryo-ultramicrotomy of nearby naturally left tissue over resin embedded specimens is self-understanding. To this aim, shock frozen tissue is 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. More than 20 years of development were necessary to reach the point until this technique has become appropriate for ultrastructural studies. For a broader insight, the reader is referred to publications of FERNANDEZ-MORAN H (1952), SJOSTRAND FS and BAKER RF (1958), FERNANDEZ-MORAN H (1960), BERNHARD W and NANCY MT (1964), LEDUC EH et al. (1967), 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), TOKUYASU KT (1973), TOKUYASU KT and SINGER SJ (1976), SITTE H (1979), KUHLMANN WD and VIRON A (1981), GEUZE HJ et al. (1981), DUDEK RW et al. (1982), MCDOWALL AW et al. (1983), KELLER GA et al. (1984), STEINBRECHT RA and ZIEROLD K (1984), TAKIZAWA T and ROBINSON JM (1994), LIOU W et al. (1996), TAKIZAWA T and ROBINSON JM (2003), RICHTER T et al. (2007), BOS E et al. (2011), WEBSTER P and WEBSTER A (2014), TAKIZAWA T et al. (2015).

Selected publications in chronological order

Stirling W (1870)
Rutherford W (1871)
Rutherford W (1973)
Cullen TS (1895)
Plenge H (1896)
Pick L (1897)
Bardeen CR (1901)
Wilson LB (1905)
Linderstrom-Lang K and Morgenson KR (1938)
Coons AH et al. (1942)

Coons AH and Kaplan MH (1950)
Coons AH et al. (1951)
Fernández-Morán H (1952)
Pearse AGE (1953)
Coons AH et al. (1955)
Holborow EJ et al. (1957)
Friou GJ (1958)
Friou GJ et al. (1958)
Sjostrand FS and Baker RF (1958)
Fernández-Moran H (1960)
Paronetto F et al. (1962)
Sainte-Marie G (1962)
Bernhard W and Nancy MT (1964)
Moor H (1964)
Crabbé PA et al. (1965)
Lillie DR (1965)
Paronetto F and Koffler D (1965)
Tomasi TB et al. (1965)
Crabbé PA and Heremans JF (1966)
Pease DC (1966)
Gitlin D et al. (1967)
Leduc EH et al. (1967)
Kuhlmann WD (1968)
Linder E and Seppälä M (1968)
Dollhopf FL and Sitte H (1969)
Tourville DR et al. 1969)
Hodson S and Marshall J (1970)
Kolehmainen-Sevéus L (1970)
Kuhlmann WD et al. (1970)
Bernhard W and Viron A (1971)
Christensen AK (1971)
Engelhardt NV et al. (1971)
Kuhlmann WD and Miller HRP (1971)
Kuhlmann WD and Viron A (1972)
Tokuyasu KT (1973)
Lillie RD and Fullmer HM (1976)
Nairn RC (1976)
Tokuyasu KT and Singer SJ (1976)
Baumeister W (1978)
Curran RC and Gregory J (1978)
Lojda Z et al. (1979)
Sitte H (1979)
Pearse AGE (1980)
Geuze HJ et al. (1981)
Kuhlmann WD and Viron A (1981)
Kuhlmann WD et al. (1981)
Baumeister W (1982)
Dudek RW et al. (1982)
Harvey DM (1982)
Plattner H and Bachmann L (1982)
McDowall AW et al. (1983)
Keller GA et al. (1984)

Steinbrecht RA and Zierold K (1984)
Wolf CR et al. (1984)
Buchmann A et al. (1985)
Edelmann L (1986)
Gilkey JC and Staehelin AL (1986)
Rentrop M et al. (1986)
Maddox PH and Jenkins D (1987)
Moor H (1987)
Steinbrecht RA and Zierold K (1987)
Dubochet J et al. (1988)
Dahl R and Staehelin LA (1989)
Henderson R et al. (1990)
Hermann R and Müller M (1992)
Takizawa T and Robinson JM (1994)
Dubochet J (1995)
Liou W et al. (1996)
Monaghan P et al. (1998)
Baumeister W et al. (1999)
Kiernan JA (1999)
McDonald K (1999)
Studer D et al. (2001)
Baumeister W (2002)
Edelmann L (2002)
Hsieh CE et al. (2002)
Takizawa T and Robinson JM (2003)
Biel SS et al. (2003)
Sawaguchi A et al. (2004)
Matsko N and Mueller M (2005)
Hsieh CE et al. (2006)
Dubochet J (2007)
Richter T et al. (2007)
Kiernan JA (2008)
Studer D et al. (2008)
Bos E et al. (2011)
Webster P and Webster A (2014)
Takizawa T et al. (2015)
Beck M and Baumeister W (2016)

For full citation of publications see chapter *References*
link: http://www.immunologie-labor.com/cellmarker_files/References.pdf

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