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Histopathology is the science that studies disease pathology through morphological changes that can be seen microscopically. The word histopathology was first known to be introduced in a book entitled “On the Nature and Structure Characteristic of Cancer” by Johannes Muller in 1838. Before histopathology, doctors distinguished diseases based on macroscopically visible morphology organ changes (anatomy) in the operating room or during forensic autopsies. However, this expertise requires another diagnosis method to ensure that several differential diagnoses indicate the same anatomical morphological changes. Definitive diagnosis is obtained by taking a small organ tissue sample during surgery (biopsy), which is then viewed microscopically. This diagnosis method is called histopathological diagnosis. For example, a person with hearing loss has received a differential diagnosis of conduction or sensorineural hearing loss. An external macroscopic physical examination for this patient is as necessary as internal imaging. The condition’s pathological cause can be ascertained by additional histopathology biopsies. However, invasive histopathology procedures can be uncomfortable and cause harm. Histopathology for diagnostic or research purposes requires two fundamental things: the microscope as a tool and the development of histopathological techniques.

This handout made up of three chapters encompassing several titles and subtitles is intended for master 1 cell biology and pathology students. The first chapter deals with histopathological techniques starting with the organization of the pathological anatomy and cytology laboratory, the identification and control of laboratory risks and finally the stages and different techniques of the histocytopathological study. The second chapter is devoted to the different routine and special coloring techniques while the third chapter deals with the different immunohistochemical and immunocytochemical techniques.

Therefore, this handout is useful for students of cellular pathology as well as practicing biologists and laboratory technicians.

I. Histocytopathological study:

1. Pathology laboratory management:

Management of the histopathology laboratory in today's environment requires a balancing act of technical knowledge, business skills, fiscal responsibility, understanding of the workforce and a quality focus.

The objective of this chapter is not to be a comprehensive guide to the subject, but discuss and concentrate on specific areas which are unique and significant to the operation of the laboratory, namely:

- Regulation and Accreditation.
- Quality Management in the laboratory setting.
- Safety concerns specific to Pathology

The laboratory manager is accountable for the service provided by the laboratory and for the safety and well-being of their staff. It is imperative that the manager remains up-to-date on regulatory and technical changes as well as safety and quality requirements.

A. Regulation and accreditation:

Governmental and national standards have been set to assure that laboratories meet minimal standards in order to protect the public. In the USA, the Clinical Laboratory Improvement Amendments (CLIA), are federal regulatory standards which apply to all clinical laboratory testing performed on humans. The Centers for Medicare and Medicaid Services (CMS) have responsibility for the operation of the CLIA program with the objective of ensuring quality laboratory testing. CMS, through CLIA, inspects and certifies all laboratories, either directly or through voluntary organizations with deemed status such as the College of American Pathologists (CAP) or the Joint Commission on Accreditation of Healthcare Organizations (JCAHO). Failure to pass a CLIA, CAP or JCAHO inspection can result in revocation of a laboratory's license to practice.

On-site inspections are generally conducted every two years, with self-inspections done in the intermediary years. When deficiencies are discovered, the laboratory is given the opportunity to correct the problem before any disciplinary action is taken. A full catalog of all required standards for the CLIA and CAP can be obtained on line using the websites in further reading. These extensive standards cover all aspects of laboratory operations including the facility, personnel, test performance, safety, quality assurance and quality

control. The standards are updated regularly and it is wise to assure that you are working from the most recent set of regulations.

In the UK, the quality of the clinical laboratory service is certificated through accreditation to the laboratory standards pertaining to ISO15189 and previously to Clinical Pathology Accreditation (CPA) standards, rather than through licensing. If a laboratory performs any of the licensable activities regulated by the Human Tissue Authority (HTA), such as the processing and storage of post mortem tissue, then an HTA license under the relevant sector is required. The HTA was established under the Human Tissue Act and inspects establishments carrying out licensable activities to ensure the requirements of the Act are being met. The establishment must demonstrate at inspection how it meets the standards laid out by the HTA, including quality and governance systems, equipment and facilities in order to retain their licence.

B. Accreditation :

Accreditation in the American pathology laboratory is a voluntary process to confirm that the department meets specific standards for testing, staffing and quality services. Accrediting agencies often have more stringent requirements than the minimal governmental regulations, so laboratories may choose accreditation to identify themselves as a higher quality organization. CAP is a leading organization which serves patients, pathologists and the public by promoting excellence in the practice of pathology and laboratory medicine worldwide. It provides peer-conducted inspections on a bi-annual basis. Along with accreditation, CAP also offers educational and proficiency testing programs to promote quality practice.

The Joint Commission is an independent, not for-profit organization which accredits and certifies nearly 21,000 healthcare organizations and programs in the United States.

Joint Commission accreditation and certification is recognized as a symbol of quality which reflects an organization’s commitment to meet certain performance standards.

The International Organization for Standardization (ISO) standards are being adopted by many as the standards they wish to work to and be accredited by. ISO is the world’s largest developer and publisher of international standards which cover many areas of activity; the ones which affect medical laboratories are: ISO 15189 – Medical laboratories – Requirements for quality and competence. This is the main standard which affects medical laboratories and to which the majority will seek to become accredited.

ISO 17043 – Conformity assessment – General requirements for proficiency testing. This standard specifies general requirements for the competence of providers of proficiency testing schemes. This includes external quality assurance schemes.

ISO 17011 – Conformity assessment – General requirements for accreditation bodies accrediting conformity assessment bodies. To assess and accredit laboratories by ISO standards within their own country. National accreditation bodies such as the CPA in the UK must themselves be accredited under this standard.

ISO 27001 – Information Security Management Standard (ISMS). This provides a framework for information security standards. Laboratories may be expected to demonstrate how they conform to these standards when responding to tenders for external work. In the UK, clinical laboratories are accredited by the United Kingdom Accreditation Service (UKAS) to the ISO15189 standards which cover all aspects of the laboratory, from the management structure of the organization and quality management system to competence of personnel, suitability of equipment and the validity and quality assurance of test methods. Laboratories are assessed on an annual cycle, accreditation is not as a laboratory, but at test level.

Unaccredited clinical laboratories may struggle to remain viable if they are unable to demonstrate the quality and accuracy of the test results they provide. The National Health Service (NHS) England advise commissioners to prioritize accredited diagnostic services, stating that accreditation should be seen as the 'baseline standard for diagnostic services across the NHS in England'. Laboratories must also achieve accreditation by the Institute of Biomedical Science (IBMS) if they wish to train persons to become registered Biomedical Scientists. The IBMS inspects laboratories to ensure that they demonstrate the required standard of service and training provision before granting accreditation.

C. Quality management:

A robust quality management system is essential to provide the best possible service for the patient and clinicians. Quality is defined as a measure of how well a product or service does the job for which it is designed, i.e. conformity to specification. Internal quality control (QC) of work processes is an important part of quality management and has been the traditional way that bench work has been checked for many years. External quality assurance (EQA) schemes provide benchmarking against other laboratories and often provide access to best practice methods and expert advice on improving techniques/specific tests. However, a full quality management system should also encompass systems to ensure consistency, quality of service, confidence, standardization and continual improvement of all laboratory processes. Quality management of a laboratory should ensure that there are systems in place to monitor and improve areas such as organization and quality management systems. This will involve

liaison with users, human resources, premises, the local environment, equipment management, information systems and materials. It will address the pre-examination process, the examination process, and the post-examination phase as well as evaluation and quality assurance. Regular audit of the various components of the system will provide evidence of compliance with standards for accreditation. It should identify any trends and issues for concern, and confirm quality systems are working. Taken as a whole, all of these measures should identify areas for quality improvement and show whether any improvements are working. .

Quality control (QC):

These systems check that the work process is functioning properly. It includes processes utilized in the laboratory to recognize and eliminate errors ensuring that the quality of work produced by the laboratory conforms to specified requirements prior to its release for diagnosis. Errors and/or deviations from expected results must be documented and include the corrective action taken, if required. In the laboratory, quality control has long been a component of accreditation requirements and should be ingrained in scientists as a daily practice. Most laboratories have experienced scientists and support staff who have the responsibility of performing routine quality control checks prior to the release of slides for diagnosis.

This QC evaluation will include, but is not limited to, accurate patient identification, fixation, adequate processing, appropriate embedding techniques, acceptable microtomy, freedom from artifacts, and inspection of controls to determine the quality and specificity of special staining including immunohistochemistry methods.

Criteria should be established which would trigger a repeat if the QC findings were qualitatively or quantitatively unacceptable. Despite having a conscientious QC system in the laboratory, pathologists perform the final QC examination as they assess/report the slide. It is their responsibility to determine that this is adequate for diagnostic interpretation. However, all personnel are responsible and errors and incidents should be recorded and audited regularly to identify trends. This will highlight any training needs and gaps.

External quality assurance (EQA)

In addition to local data collection and monitoring for internal quality control, external mechanisms provide valuable information regarding quality and peer comparisons and also serve as an educational tool.

In the UK, quality assurance of laboratory techniques is organized on a national basis. It is a system of peer review and registration with appropriate approved schemes. The non-profit-making National External Quality Assurance Scheme (NEQAS) organizes programs for histochemistry and immunohistochemistry. The UK quality assurance schemes were started by members of the profession in order to establish quality standards within histopathology. Registration with the schemes is now a requirement for accreditation. The quality assurance process is based on peer review of the stained sections submitted by participating laboratories. There are also medical quality assurance schemes for pathologists which cover many of the sub-specialties of histopathology. The quality assurance schemes currently used in the UK are coordinated under the auspices of UK NEQAS. Within this organization there are two individual schemes for histopathology, NEQAS for immunohistochemistry and NEQAS for cellular pathology techniques.

The immunohistochemistry scheme gives participants the option to be assessed on general antibody panels or more specialist laboratories may choose to participate only in their specific areas. The cellular pathology scheme is subdivided into general, veterinary and neuropathology.

In the USA, the National Society of Histotechnology (NSH) in partnership with the College of American Pathologists (CAP) created the Histology Quality Improvement Program (HistoQIP). This quality assurance system scores each slide, assessing the fixation, processing, embedding, microtomy, staining and covers lipping for routine H&E slides, special stains and immunohistochemistry. Participants receive an evaluation specific to their laboratory, an educational critique and a participant summary report which includes peer comparison data, performance benchmarking data and information regarding best-performing procedures and techniques. Additionally, CAP establishes national surveys for immunohistochemistry. Accreditation standards require that action is taken by poor performers to improve the quality of their preparations. Most schemes offer expert assistance and advice to laboratories which fall below the defined acceptable score.

D. Process improvement:

This is the system which is used proactively to approach and identify opportunities to improve quality before problems occur. It operates through evaluation and audit of all systems and processes in the laboratory. The goal is to improve care and safety for patients and staff through recognition of potential problems and errors before they can occur.

Good managers realize that failures, errors and problems are often due to the system processes, and not necessarily the fault of the employee(s).

Regular and thorough auditing of the many components of the laboratory's quality management system and performance should be mapped against accreditation standards. This will help highlight any problem areas. Feedback from users provides useful information when evaluating the effectiveness and quality of the service.

Any criticism received may prompt an unscheduled audit of that part of the system. Continuous Quality Improvement (CQI) should include auditing of the laboratory's procedures against, not only accreditation standards, but also those of the host organization/other services. Any audit findings which show that the laboratory's processes are not adequate should result in corrective actions. These audit findings may also highlight opportunities for improvements in processes, documentation, staff training, or monitoring aspects of competence. Any corrective actions required should be completed as soon as possible so that services can be improved and brought up to the correct standard quickly.

CQI is a continuous cycle of audit and assessment of the service. If not monitored regularly, quality standards can slip as staff, equipment and reagents change. It is useful for the manager to establish an audit calendar to ensure that all areas are audited regularly, paying particular attention to 'problem areas'

E. Risk management:

Risk management is an essential and central part of all laboratory work. To comply with legislation and maintain accreditation a laboratory must have an effective risk management policy. Any chance of something going wrong should be either negated or minimized and therefore a laboratory's risk management process should have procedures in place for:

- Identifying all risks which exist within the environment.
- Assessing those risks for likelihood and severity.
- Eliminating those risks which can be removed.
- Reducing the effect of risks which cannot be eliminated.

The pathology laboratory should have close links with, and feed into, the host organization's risk management process. In most hospital laboratories, the laboratory manager will be

accountable for risk management and may be supported by a Risk Lead who will be responsible for the operational aspects of the system.

To function effectively and safely, all the laboratory procedures and activities must be subjected to the risk management process. Health and Safety, as well as quality assurance, incorporate a major aspect of risk management. All aspects of our working life incorporate a degree of risk, and the risk management process allows us to prioritize, evaluate and handle the risk appropriately. It is not possible to avoid or eliminate all risks and in reality, this may not be practical. It is important to identify and understand the risks which are involved in a laboratory's working practices.

A laboratory manager should be concerned with all the risks associated with the department they manage, and also how these may impact on other areas of the organization such as porters transporting samples or chemicals to the laboratory. The laboratory manager would also be required to alert the organization to the presence of risks which cannot be adequately controlled within or by the department. The laboratory management team will deal with any laboratory associated risk by ensuring that adequate resources are available to deliver a service which is safe for both staff and patients. Staffing levels and competence, timeliness and quality of results, budgetary management, consumable and equipment supplies, and maintenance are some of the areas of concern. The laboratory management team must also ensure that risk management procedures are in place for every aspect of a laboratory's processes and its environment. The laboratory manager must ensure that day to-day errors do not arise because of inadequacies in laboratory procedures and that quality control checks are in place to minimize the possibility of human errors, e.g. a transcription error or mislabeling. Standard operating procedures (SOPs) should include Control of Substances Hazardous to Health (COSHH) data, risk assessments or equivalent, and also other health and safety information relevant to the procedure. This should include national legislation and guidance where available.

It is important that where risks are identified, the risk management measures are regularly audited to assess whether they are being followed and that they remain appropriate and effective.

Risk identification:

The risks within each laboratory section are best identified by the section lead and members of that team working in conjunction with the laboratory's Health and Safety lead. This ensures that the broadest possible spectrum of viewpoints is considered.

During this process, it is useful to divide the risks into different categories. These include clinical, physical, chemical, infectious, and even organizational, financial and political, depending on the area being risk assessed. For example, a support worker unpacking the samples delivered to the laboratory may have noticed that samples have leaked, possibly putting both themselves and the porter at risk from infection and exposure to the fixative; if any of the contents have leaked beyond the specimen bag, there could be a risk to other health workers and patients/ visitors using the same route. This could just be a problem with one batch of specimen containers, but could also be a training issue for staff putting the samples into the containers. In raising the issue with their supervisor and giving them the opportunity to investigate the root cause, the support worker may have prevented harm to others and potential damage to the sample, which may impede its diagnosis.

Risk analysis and evaluation:

Analysis and evaluation of potential risks is an essential part of the process and one that is used to identify both the likelihood and severity of these risks. By scoring the risks for likelihood and severity, it is then possible to use a matrix such as the one described below as a tool which will put a value on specific risks. This will then help prioritize them for further action. The risk manager should put a system in place whereby all incidents and accidents are reported no matter how small. It is only by recording all the data that the full picture can be obtained and analyzed and areas possibly overlooked initially, be risk assessed and managed. There are numerous ways of controlling risk, but frequently there will be expert guidance or regulations issued by professional bodies or government agencies which the Risk Lead should ensure are implemented. Informal networking with professionals in similar laboratories can also provide valuable information and ideas as to how others have overcome the challenges of managing risks.

Risk assessment tool:

Severity and likelihood values The following is an example of a severity scoring scale for incidents:

1. Low:

• Minor injury or harm.

• Minor loss of non-critical service.

• Minor non-compliance with standards.

• Minor out-of-court settlement.

• Publicity mostly contained within organization.

Local press coverage of no more than one day.

2. Slight:

• Injury or harm requiring less than 3 days absence from work, or less than 2 days hospital stay.

• Loss of service for less than 2 hours in several non-critical areas, or less than 6 hours in one area.

• Single failure to meet internal standards.

• Civil action with or without defense, improvement notice.

• Regulatory concern.

• Local media coverage of 2 to 7 days.

3. Moderate:

• Medical treatment required and more than 3 days absence from work, or more than 2 days extended hospital stay.

• Loss of services in any critical area.

• Repeated failures to meet internal standards or follow protocols.

• Class action, criminal prosecution or prohibition notice served.

4. Severe:

• Fatality, permanent disability or multiple injuries.

• Extended loss of essential service in more than one critical area.

• Failure to meet national standards.

• Executive officer fined or imprisoned, criminal prosecution and no defense.

• Political concern, questions in parliament, national media coverage longer than 3 days. 5. Catastrophic

• Multiple fatalities.

• Loss of multiple essential services in critical areas.

• Failure to meet professional standards.

• Imprisonment of executive from organization.

• Full public enquiry.

Incidents may also be scored 1–5 for likelihood:

1. Incident unlikely to occur.

2. Incident likely to occur once in a 5 year period.

3. Incident likely to occur yearly.

4. Incident likely to occur once in a 6 month period.

5. Incident likely to occur once every 4 weeks or more frequently.

The Risk Factor is the severity, multiplied by the likelihood of occurrence:

Very Low Risk, 1–2 – The majority of control measures in place, or harm or severity small. Action may be long term.

Low Risk, 3–4 – Moderate probability of major harm or high probability of minor harm if control measures are not implemented. Action in the medium term.

Moderate Risk, 5–10 – Urgent action to remove or reduce the risk.

High Risk, 12–25 – Immediate action to remove/reduce the risk.

Audit:

This is an essential tool in risk management. Regular audits of the effectiveness of the risk management measures put in place, and the frequency and nature of incidents, will allow the laboratory's risk management team to assess them, and amend and improve if required.

Audit will also identify areas or tasks which may need additional or increased monitoring, and may highlight training gaps for individuals or groups of staff. In addition, regular and targeted audits will provide evidence to assist with driving change should the risk be due to the lack of funding for certain tasks or processes, or to identifying processes outside the control of the laboratory management, e.g. labeling of samples in the operating room.

Risk funding:

Risk management should also consider insurance, individual or laboratory, as an important option. All medical staff carry medical liability insurance which covers them in the event of any negligence claims. Similarly, professional indemnity insurance is commonly available today for non-medical laboratory staff who are much more at risk in today's litigation conscious society.

The decision regarding whether or not to insure should be based on the risk assessment and the severity and likelihood of the risk. Some risks will not be appropriate for insurance coverage and in these instances the risk must be accepted by the organization.

F. Safety :

The Pathology laboratory manager is responsible for the safety and well-being of all the staff and visitors to the department. Safety concerns include the common, e.g. slips and falls, and those more specific to histopathology. Pathology practice commonly includes a multitude of physical, biologic and chemical hazards which must be evaluated and eliminated or reduced. Elimination is possible, e.g. by looking for alternatives to high-risk, harmful chemicals used in the laboratory. Where hazards remain, efforts should be made to reduce the effect or possibility of the risk. Consideration must be given to altering work practices, making engineering changes and use of Personal Protective Equipment (PPE).

There are numerous ways of controlling hazards and frequently there is expert guidance or regulations issued by professional bodies or government agencies which the Safety Officer should ensure are implemented. Informal networking with professionals in similar laboratories can also provide valuable information and ideas as to how others have overcome the challenges of managing certain risks.

In the UK, organizations such as the Health and Safety Executive (HSE) and the Health Protection Agency (HPA) exist to ensure the safety of employees, patients and the general public. Regulations made under the Health and Safety at Work Act 1974 apply to all work situations, e.g. COSHH regulations and the Workplace (Health, Safety and Welfare) Regulations. The HSE enforces this act along with others, including the Health and Safety Offences Act 2008. The overall message is that employees are entitled to work in environments where risks to their health and safety are properly controlled, i.e. minimized. Under health and safety law, the primary responsibility is owed by employers, with employees expected to ensure their own safety and that of their colleagues and/or patient's by adhering to policies and procedures.

In the USA, the mission of the Occupational Safety and Health Administration (OSHA) is to prevent work-related injuries, illnesses and occupational fatality by issuing and enforcing standards for workplace safety and health. Most countries will have equivalent bodies and standards. OSHA guidelines require:

• Availability and maintenance of safety equipment.

• Measures to control the risk of exposure to chemical hazards or biological specimens by employees of the laboratory testing facility.
• Sanitary condition of the testing laboratory.
• Annual inspections.
• Waste management program.
• Procedures for infectious material response, ventilation failure, first aid, fires or emergencies.
• Documentation of all spills and exposure incidents. To comply with legislation and maintain accreditation, a laboratory must have an effective safety program or risk management policy.

Any chance of something going wrong should be either negated or minimized and therefore, a laboratory's safety management process should have procedures in place for:

• Identifying all risks which exist within the environment.
• Monitoring and assessing those risks for likelihood and severity.
• Eliminating those risks which can be removed.
• Reducing the effect of risks which cannot be eliminated.

The pathology laboratory should have close links with, and feed into, the host organization's safety program or risk management process. In most hospital laboratories, the laboratory manager will be accountable for the health and safety of the staff in their department and often will be supported by a Safety Officer who will be responsible for the operational aspects of the system.

In the USA, the 'Right to Know' law is the legal principle giving an individual the right to know the chemicals to which they may be exposed in their daily living and work life. This law requires that employees be trained on the hazards they work with and know which precautions should be used to prevent exposure. Safety Data Sheets are required to be supplied by all chemical manufacturers and they must be available for all the employees in the laboratory. These will provide comprehensive information about a substance or mixture for use in workplace chemical management.

Xylene and formalin exposures in pathology are one of the common concerns. Along with reducing exposure by altering work practices, engineering changes and the use of personal protective equipment, vapor monitoring must be done on a regular basis, usually annually, or when there has been a change within the laboratory. This is often done by employees wearing a monitoring badge during their work tasks which is then evaluated by an independent

laboratory. Results may be expressed as an 8-hour Time Weighted Average (TWA) or as Short Term Evaluation Limit (STEL).

G. Laboratory procedures:

In most laboratories, SOPs must be carefully established to comply with regulatory standards. A laboratory's testing procedures may be multiple and complex and it is essential that the methodology for all the procedures and tests are documented in SOPs allowing all staff to operate in a standardized and appropriate way. SOPs should cover all aspects of the testing process from delivery and storage of samples or reagents, to the issuing of the final laboratory report. The SOPs include not only the laboratory procedures but also those carried out by the pathologists and clerical staff. It is important that SOPs which impact on areas of staff outside of the laboratory, e.g. porters delivering samples from operating rooms, are shared with the other departments responsible for managing that part of the process.

Accreditation standards require that SOPs and other policies are within a document control system. This is usually a central database which holds authorized copies of documents, with controls on who can modify these data. The document control system must also ensure that only authorized and up-todate copies of SOPs and policies are being used by staff performing the tasks. Any changes to a procedure must be recorded within a further updated SOP. This must then be issued and any old SOPs removed from circulation.

H. Departmental organization:

An appropriate management structure for the department should exist ensuring that the main functions can be adequately delivered. Staff at all levels should be qualified and trained for the work they do, and hold appropriate registration, if required. Competencies for the tasks performed should be regularly assessed, checked and recorded.

Many departments publish a mission statement outlining their business and aims. Quality objectives need to be documented so that all have clear objectives outlining who is responsible for achieving them and when they should be achieved by.

A laboratory will have multiple users, including patients, clinicians and those purchasing its services. It is essential when planning and developing a laboratory service that all users are consulted. Likewise, when monitoring the effectiveness and quality of a service, user feedback should be sought so that the service can be properly evaluated. Any complaints/ praise should be recorded and followed up immediately. These should feed into the quality management system.

I. Workflow:

Managing workflow in the histopathology laboratory involves consideration of efficiency, quality and safety. The turnaround time for diagnosis continues to be a major concern and workflow must be established which provides the earliest possible diagnosis for patient treatment. Specimens must be moved from task to task in the most efficient manner possible. Laboratories have adopted continuous flow processing models which encourage small batch grouping and discourage bottlenecks and delays for specimens at any step.

J. Personnel management:

One of the most important assets for a histopathology laboratory is its staff or personnel. Compared to any other laboratory specialty, the laboratory process in histopathology remains a manual procedure, from specimen receipt, through dissection (grossing), embedding, sectioning and staining. Many techniques are still reliant on skilled personnel rather than automation, and the laboratory manager must ensure that the department is staffed by an appropriate number of employees with the right level of skills to ensure that the process is robust, safe and cost-effective.

The laboratory manager is accountable for the service provided by the laboratory and should have the appropriate qualifications and experience to undertake this task. As well as being the lead technologist/ scientist for the department, laboratory managers are usually responsible for recruiting appropriately and managing the human resource needs and professional direction of their staff. All the staff should have comprehensive job descriptions so that they, their manager and supervisor know what is expected from them and to whom they are accountable. They should also have contracts which specify the terms and conditions of their employment.

The manager must ensure that there are appropriate numbers of staff with the required education, qualifications, training and competence to provide the service required. Managers must also ensure that the staff have access to further education as required to continue to keep up with the latest knowledge and techniques related to the service being provided.

The competency of staff to do the tasks within their job description needs to be assessed at regular intervals. This, together with regular formal appraisals should ensure that staff are supported and provided with the requirements to fulfill their roles. The manager must also address any issues with discipline or excessive absence from work ensuring that the workforce team functions optimally. Regular staff meetings should be held which involve all levels of staff to ensure that any new information is passed on, e.g. new procedures or

updates related to the risk and quality management systems. These meetings allow staff to feed back any information they have, or raise queries and gives them access to supervisors or managers who they may not see during their routine day.

K. Staffing the laboratory:

Ensuring the right number and level of staffing depends on the manager having a good understanding of the volume and complexity of the work received. Good information systems are essential for recording and analyzing the volume of work performed in a laboratory, and for understanding trends in workflow and complexity. Guidelines, e.g. those issued by the Royal College of Pathologists and the Institute of Biomedical Science in the UK, and by CLIA regulations in the US, advise what level of laboratory duties may be undertaken by which grade of staff. They have their own training and examination systems to enable consultant and postgraduate scientific staff to gain the qualifications they require.

Scientific staff working in accredited laboratories in the UK should be registered by the Health and Care Professions Council (HCPC), and in the US, they should be registered with the American Society for Clinical Pathology (ASCP). Some states in the US require laboratory personnel to be licensed. Licensing of technologists often requires a college degree and a specific exam or proof of ASCP certification, but requirements vary between state and specialty. Once the level and complexity of the workload is known the workforce can be profiled to match its requirements, remembering that to be cost-effective, tasks not requiring registered or licensed scientists should ideally be performed by support staff.

L. Premises, equipment and materials:

The laboratory environment and equipment must be fit for all the laboratory processes. Managers should ensure that there are adequate basic facilities for staff to do their jobs, e.g. rest and toilet facilities, adequate lighting, IT provision and space. There should also be enough space for equipment and storage. Equipment should be functional and regularly maintained for its safe use.

Staff must be trained and competent in their individual areas to use all of the equipment and materials in a safe and effective way. Display screen equipment (DSE) assessments must be carried out on all staff who regularly use computers.

Materials and equipment must be managed regarding stock control and servicing. Procurement policies should ensure that quality stock is purchased, is fit for purpose and value for money. Consideration should be given to whether to lease or purchase the

equipment, as a lease contract may enable upgrades of equipment or software as part of the contract.

2. Chemical safety in the laboratory:

Improper handling of hazardous chemicals can produce significant health and/or physical harm. For many years countries issued their own national regulatory standards to assure employees were informed of the hazards in the workplace. The regulations and descriptions of hazards varied between countries.

In 2003 the United Nations established the Globally Harmonized System (GHS) for the classification and labeling of chemicals. This GHS, adopted by the majority of the countries, has established a uniform system throughout the world for identifying chemicals and communicating their hazard information on labels and safety data sheets.

A. Classification of hazardous chemicals:

The United Nations GHS provides detailed criteria to assign the hazard classification to a chemical substance. Each classification is also assigned a category number. The category number indicates the severity of the hazard: Category 1 represents the greatest hazard risk; higher numbers have lower risk factor. Classifications are divided into three major groups (Table 1). The GHS assigns to each classification standardized text/symbols which includes hazard statements, a signal word, a pictogram, and precautionary statements.

A chemical can have multiple hazard classifications assigned. For example, the common clearing agent, xylene, is classified with multiple physical and health hazards. Table 2 shows the GHS information assigned to xylene.

- **Labeling of hazardous chemicals:**

In the USA, the Occupational Safety and Health Administration's (OSHA) Hazard Communication (29 CFR 1910.1200) requires the following elements to be placed on labels of hazardous chemicals:

1. Product name
2. Signal word (Danger or Warning)
3. GHS Hazard statement (classification)
4. GHS Pictograms
5. GHS Precautionary statements
6. Name, address and telephone number of the chemical manufacturer

Chemical manufacturers use the standardized GHS tests to classify a chemical and label it accordingly.

If a laboratory mixes its own reagents, they would need to follow the same GHS criteria to determine the hazard classification of the reagent in order to label it correctly. This would be time consuming and/or expensive.

For laboratory prepared reagents, searching the internet for a manufactured equivalent reagent can provide the GHS classifications.

Lot numbers, date of preparation (manufacturing), and person who prepared the reagent are other pieces of information that can be added to labels as required by a facility's protocols. In the pathology laboratory, fixatives, processing reagents and stains are considered medical devices and are thus exempt from the GHS labeling.

Table 1: GHS hazard classification.

Health hazards	**Physical hazards**	**Environmental hazards**
Acute toxicity	Explosives	Acute aquatic toxicity
Skin corrosion/irritation	Flammable gases	Chronic aquatic toxicity
Serious eye damage/eye irritation	Flammable aerosols	
Sensitization	Oxidizing gases	
Germ cell mutagenicity	Gases under pressure	
Carcinogenicity	Flammable liquids	
Reproductive toxicity	Flammable solids	
Specific target organ system toxicity-single exposure	Self-reactive substances	
Specific target organ system toxicity-repeated exposure	Pyrophoric liquids	
Aspiration toxicity	Pyrophoric solids	
	Self-heating substances	
	Substances which in contact with water emit flammable gases	
	Oxidizing solids	
	Oxidizing liquids	
	Organic peroxides	
	Substances corrosive to metal	

B. Working safety with hazardous chemicals:

Whenever possible, hazardous chemicals should be replaced with safer alternatives, or volume usage should be reduced. Mercury is an example of a hazardous material which has been banned in many U.S. hospitals; thermometers and sphygmomanometers are now using mercury replacements.

Pathology laboratories in turn have replaced the mercury-based fixatives (B-5, Zenkers) with zinc based fixatives.

It may not be possible to eliminate all hazards from a laboratory but the risk factor can at least be reduced. Formaldehyde is classified as a carcinogen. Formaldehyde-free fixatives using glyoxal have equivalent preservation characteristics without the carcinogenic properties of formaldehyde. Similarly, the clearing agent xylene requires solubility criteria for tissue processing and staining. The various xylene substitutes (aliphatic hydrocarbons, limonene) provide the solubility criteria without the physical flammability hazard of xylene or the high multiple health hazards.

- **Safety data sheets:**

Formerly known as Material Safety Data Sheets (MSDS), SDS are the primary source for identifying the hazards and proper handling requirements of a chemical. Prior to GHS there were multiple MSDS formats being used. Without a standardized format it was difficult to find information, especially during an emergency. The GHS established a standardized 16 section SDS format (Table 3). Sections 1-8 provide general information and also any information which may be needed quickly in an emergency (e.g. first-aid, spill, fire). Sections 9-12 have scientific data on which the classification is based and Sections 13-15 have regulatory information. Manufacturers are required to send SDS with the initial shipment of a chemical and whenever a change has been made in the SDS. Training programs should constantly be updated to assure dissemination of any new SDS information.

Table 2: GHS classification and information for xylene

Hazard classification	Flammable liquids, Category 3 Acute toxicity-dermal, Category 4 Acute toxicity-inhalation (vapors), Category 4 Skin irritation, Category 2 Serious eye damage/eye irritation, Category 2 Carcinogenicity, Category 2 Specific target organ toxicity-single exposure, Category 3

	Specific target organ toxicity-repeated exposure, Category 2 Aspiration toxicity, Category 1
Signal word	Danger
Hazard statement	Flammable liquid and vapor Harmful in contact with skin Causes skin irritation Causes serious eye irritation May cause respiratory irritation May cause drowsiness or dizziness Suspected of causing cancer May be harmful if swallowed and enters airways May cause damage to organs through prolonged or repeated exposure
Precautionary statements	Prevention, response, storage and disposal statements



Figure 1: Pictograms expressing toxicity by contact with the skin and by ingestion, carcinogenic effect or by inhalation.

Table 3: GHS safety data sheets format

Section 1	Identification of the substance or mixture and of the supplier
Section 2	Hazards identification
Section 3	Composition and information on ingredients
Section 4	First-aid measures
Section 5	Fire-fighting measures

Section 6	Accidental release measures
Section 7	Handling and storage
Section 8	Exposure controls and personal protection
Section 9	Physical and chemical properties
Section 10	Stability and reactivity
Section 11	Toxicological information
Section 12	Ecological information
Section 13	Disposal considerations
Section 14	Transport information
Section 15	Regulatory information
Section 16	Other information

- **Section 1: Identification of the substance or mixture and of the supplier:**

This section provides the name of the product and must match the name found on the label. The section may list other common chemical names. For example, formaldehyde gas is also known as methanal, methylene oxide and methyl aldehyde, the chemical formula may also be provided in Section 1. The recommended use of the chemical will also be provided. Generally, the latter will be generic with such listing as 'laboratory chemical'. The supplier's contact information and an emergency telephone number are also provided.

- **Section 2: Hazards identification :**

This section will provide the classification(s) of the chemical. In addition, all the label's elements will be found in this section of the SDS (signal word, pictogram(s), hazard statements, precautionary statements).

- **Section 3: Composition and information on ingredients :**

The chemical name as well as the common name will be listed for all components which contribute to the chemical hazard. Any hazardous impurities or stabilizers will also be included. An example of a hazardous stabilizer is found in 37% formaldehyde. Formaldehyde will polymerize to form solid paraformaldehyde and methanol, between 7-14%, is added to the aqueous solution to inhibit this reaction. Therefore, methanol, a hazardous chemical, is listed in the SDS as part of the composition of 37% formaldehyde.

- **Section 4: First-aid measures :**

This section contains universal/standard treatment measures for when an exposure has occurred (Table 4). The directions are intended for immediate medical care by untrained responders or those that have taken a basic first-aid course. An exposure to hydrochloric acid can have symptoms which vary from a simple irritation to severe burns. As indicated by the adage 'The dose makes the poison', this variability of symptoms results in the basic instructions ending with 'obtain medical attention'. Professional medical personnel are best able to determine the degree of injury and the correct way to remedy the specific exposure. GHS formatted SDS requires first-aid procedures to be given for the possible routes of exposure: inhalation, skin and eye contact, ingestion.

The symptoms for both acute and chronic exposure symptoms must be provided. Every work area should have a basic first-aid kit containing at least adhesive bandages, sterile gauze pads and adhesive tapes to dress a wound prior to obtaining advanced medical attention.

Eye washes can be plumbed or self-contained. The American National Standards Institute (ANSI) recommends that eyewashes deliver three gallons of tepid water per minute for 15 minutes. Plumbed eyewashes are connected to a source of tap water and have the advantage of delivering large volumes of water. The disadvantage of plumbed equipment is monitoring the temperature and possibly the quality of the tap water. Self-contained eyewashes store pH balanced rinse solutions which are more comfortable for rinsing the eyes.

Microorganisms can grow in eyewash stations (stagnant water) if the lines are not flushed on a regular basis. In addition, the self-contained eyewashes must be refilled/replaced according to manufacturer's recommendations. Improper maintenance of the eyewash can provide additional health hazards and therefore cause additional harm to the eyes beyond the chemical exposure. In the USA, the Formaldehyde Standard requires a laboratory to have eyewash stations within the immediate work area. In addition, workplaces which expose workers to corrosives are required to have eyewashes in the immediate area. Equivalent standards are required for shower equipment.

Table 4: Standard first-aid treatment.

Eye contact	Rinse immediately with plenty of water for at least 15 minutes. Obtain medical attention
Skin contact	Remove any contaminated clothing. Wash off immediately with plenty of water for at least 15 minutes. Obtain medical attention.

Inhalation	Move to fresh air. If breathing is difficult, give oxygen. If not breathing, give artificial respiration. Obtain medical attention
Ingestion	Do not induce vomiting. Obtain medical attention

- **Section 5: Fire-fighting measures :**

The specific type of extinguishing media to be used in case of a fire will be found in this section. Work areas generally have dry chemical multipurpose fire extinguishers for handling all types of fires. Laboratories should routinely inspect their areas for fire risks and have appropriate extinguishers readily available. The fire safety program of a facility should instruct the proper use of a fire extinguisher. Local fire authorities or hospital fire officers provide fire extinguishing training and in most institutions annual mandatory fire training programs occur. Besides the correct extinguisher to use, this section will also warn one of the inappropriate fire extinguishers. Special instructions are given for firefighters of any specific hazards resulting from a chemical fire and the required fire-fighting protective equipment which should be worn.

- **Section 6: Accidental release measures :**

One must know the hazard(s) of a chemical/mixture in order to know how to properly clean up any spillage. This section provides information on the specifics of the procedure for a spillage cleanup. It is required reading so that the laboratory is prepared with all the specific spill cleanup supplies (personal protective equipment [PPE], absorbents, neutralizers). Depending upon an institution's spill response program, either outside assistance and/or trained employees will perform the cleanup.

Most laboratory spillages involve small quantities and with the proper training, PPE and cleanup supplies they are easy to remedy. Evaluating the risk of a spill is an important element in the cleanup. It is more than the size of a spill that will determine the cleanup procedure. The characteristics of the chemical and ventilation play a major role. In a well ventilated area, a few hundred milliliters of spilled alcohol might be considered a small spill and only require absorbent materials to contain and remove it. However, a few hundred milliliters of formaldehyde in a poorly ventilated room could exceed exposure limits and produce a higher health risk requiring a different mode of cleanup involving respirators.

A spill response procedure must be developed to cover every chemical used in the laboratory. The written procedure must document evacuation routes and alarms in case they are needed. All spill cleanup materials and methods required for each chemical (group) must be included in the spill response document. General procedures for a spill cleanup include:

1. Evaluate the risk to determine if trained employees can neutralize the spill or if outside assistance is needed.

2. Evacuate people from the area if required.

3. All individuals performing any cleanup must wear appropriate PPE: goggles, gloves, shoe covers, aprons, and respirators. The PPE must be chemically resistant for the material being cleaned up.

4. Prevent the spread of any vapors by increasing ventilation. Turn off all ignition sources if cleaning up a flammable liquid.

5. Control the spread of a liquid by using absorbent materials. Absorbent socks, booms, pillows and pads are convenient and less messy than vermiculite or kitty litter. Special pads are available for formaldehyde which neutralizes the chemical and absorbs the formaldehyde vapors.

6. Absorb the liquid. Neutralize if possible. Acids are easily neutralized with sodium bicarbonate; bases with citric acid. The neutralizing procedure is exothermic and a bubbling reaction is normal.

7. Collect contaminated materials (booms, pads, neutralized residue, contaminated PPE). If cleaned up material is hazardous, properly label the contaminated items and dispose of as hazardous waste. Non-hazardous (neutralized material) may be disposed in regular trash.

8. Wash down the contaminated area with suitable cleaning solutions. Generally, soapy water is used. Non-aqueous spills will need cleanup solvents which are not water based. Continue ventilating the area to rid it of any vapors resulting from the spill.

9. Perform all notifications and required reports. Special cleanup procedures are required for metallic mercury.

Metallic mercury from a broken thermometer can disperse toxic vapors. Generally, the mercury can be seen and collected into larger droplets with a damp cloth and collected for disposal. If metallic mercury is used in the laboratory, special mercury absorbing sponges should be part of the spill kit. A special mercury vacuum is also an option for a metallic mercury spill cleanup. In addition to the implementation of a chemical spill cleanup program, the lab should also have an equivalent program for biohazards. Detailed information for

handling biohazard spills are beyond the scope of this chapter. However, the general protective steps listed for a chemical cleanup should be followed.

Decontamination procedures and disinfectants will vary. A freshly prepared 10% bleach solution is commonly used to disinfect solid surface areas.

- **Section 7: Handling and storage :**

This section will provide the environmental conditions for safe storage of a chemical whilst also maintaining the integrity of the chemical. In addition, the physical incompatibilities (e.g. heat, light, water) for improper storage are also provided.

Room temperature storage is recommended for most laboratory chemicals. Generally, chemicals should not be exposed to heat or direct sunlight. For example, hematoxylin stains will break down faster if stored in direct sunlight. Acetic acid should be stored above 61°F (16.6°C) to prevent it from freezing. Its characteristic of forming ice crystals at such high temperatures is where it acquired the common name, glacial acetic acid.

Chemicals will be identified as to whether they require storage in special cabinets due to flammability or corrosive characteristics. Flammables and corrosives should each be stored in approved storage cabinets. Ethyl alcohol, isopropyl alcohol and xylene are used in large volumes in histology and are all Class I flammables. Local fire departments often set their own limitations. Explosion-proof laboratory refrigerators should be used when flammables need to be refrigerated.

A household refrigerator/freezer should never be used to store flammable materials. Storage incompatibilities for chemicals are provided in Section 10 of the SDS. Chemicals should never be stored alphabetically except when grouped within the same hazard class.

Acids should be stored by themselves; segregation of the different acid groups from each other will also be noted in this section of the SDS. Chemicals should be stored in a location, e.g. a shelf, below head level. This prevents any chance of spillages from contaminating the head while reaching for chemicals. Storing chemicals on secondary trays allows the collections of miscellaneous drips or containment in case of a bottle breakage.

It is good practice when transporting a bottle of concentrated acid or base, even across the room, to place it in a secondary container such as plastic bucket with an appropriate lid.

- **Section 8: Exposure controls/personal protection :**

Exposure control limits are issued by governmental agencies or are recommended by the chemical manufacturer. The weighted average (TWA) represents the exposure limit averaged over an eight-hour time period. A TWA is appropriate in a manufacturing facility where

chemical exposure is constant during a work shift. A TWA is not practical in a laboratory environment where there could be a high exposure for a very short time period and none for the rest of the day.

The short term exposure limit (STEL) does take a laboratory environment into consideration. A STEL measures the exposure for a 15-minute time frame. The SDS will identify the source of the exposure limit with the name of regulatory agency, organization or manufacturer. As well as providing any exposure limits, Section 8 also provides engineering controls to control/ reduce exposure. General laboratory ventilation systems should have an adequate exchange rate to ensure vapors do not accumulate or recirculate through the building. Laboratory ventilation is recommended to be 6 to 12 room air changes per hour.

Chemical hoods must be used when general room ventilation is inadequate and the release of odoriferous vapors can occur if used out in the open. Styles of exhaust hood vary and the choice must take into considerations the chemicals used, how it will be used and available air exchange/engineering controls. An explosion-proof chemical hood must be used with flammable chemicals. Chemicals or equipment must never be stored in a hood because they could disrupt the air flow. When working with chemicals, they should be 6 inches (15 cm) inside the hood with the sash in the lowest possible position.

The face velocity of the chemical hood should be checked on a regular basis When engineering controls or work practices do not prevent the exposure to hazardous chemicals, personal protective equipment (PPE) is required. PPE should be readily available at all times and stored in a familiar location. The laboratory's safety hygiene plan should document which procedures (e.g. changing solvents on tissue processor, loading tissue cassettes into baskets) require which type of PPE. As with spill cleanups, PPE must be of a material and style appropriate for the type of chemical (e.g. acids, petroleum solvent, aqueous), nature of exposure (e.g. liquid, vapors) and level of exposure (above or below PEL).

- Eye protection

Safety glasses are not adequate protection when using chemicals. They are designed to protect eyes from flying objects. Splashes and dust can still reach the eyes when using safety glasses, even those with a wraparound style.

Goggles provide a secure shield on the face and are required especially when handling liquids. Non-vented goggles are the best to use because they prevent vapors and dust from reaching the eyes. Long term usage of goggles may result in fogging.

Anti-fogging coatings on the goggles are available as well as wipes to reduce fogging. Face shields can be used as secondary protection with safety glasses or goggles. Face shields should not be worn as the only source of PPE for the eyes.

- Skin protection

'One size fits all' does not apply when choosing a glove to prevent exposure to a chemical. Glove material and required dexterity for the required task must be taken into consideration when selecting a glove. The glove material will determine the compatibility with the chemical. Chemical compatibility charts for gloves are available on the internet to help choose the correct protective glove. Selection of a glove's material must take into consideration its characteristics of degradation time, breakthrough time and permeation rate of the chemical being handled. Manufacturers can vary glove thickness. Therefore, a glove made of the same material from different manufacturers will vary in the degree of protection. Whenever possible check with the manufacturer for their glove test data. Latex gloves which provide protection against pathogens provide minimal protection against chemical exposure, especially organic solvents like xylene. Chemical safety in the laboratory Latex gloves are also associated with allergy reactions in a significant part of the population, thereby limiting their value. Nitrile and neoprene gloves can be used as an alternative for protection from acids and organic solvents and are more regularly used now. Aprons, protective suits and footwear coverings should also be chosen based on the risk level encountered. Measuring out 1 ml of acetic acid needs minimal protection of gloves and a disposable apron, while cleaning up a large acid spill generally requires a protective suit and footwear/boots.

Laboratory coats can have protective finishes which are fluid resistant. These are generally acceptable for very small volumes and are designed more as a protective layer for stains and spills from reaching underlying clothing.

- Respiratory protection

Particle (dust) masks and respirators are the two major types of respiratory protection used in the laboratory. Particle masks can be disposable or reusable. The National Institute of Occupational Safety and Health (NIOSH) rates particle masks, assigning them a letter and a number to designate intended use and capacity respectively. The letter N indicates the mask cannot filter oil-based particles; R represents it is oil resistant up to 8 hours; P represents the mask is oil proof beyond 8 hours. The number represents the percentage of one-micrometer particles which can be filtered from the air. Most commonly used in the hospital is an N95 particle mask. It will filter 95% of 1 micrometer sized, non-oil-based airborne particles from the air. While an N100 provides more particle protection, all particle masks are not suitable

for use with chemical vapors. Respirators with chemical cartridges must be worn when handling chemicals releasing vapors above the PEL or when the user is susceptible to the gases/ vapors.

Respirators may be full face or half-mask. The half-mask covers only the mouth and nose so gas-proof goggles must be used with them to protect the eyes from exposure to the vapors. Cartridges are chosen based on the chemical being used (e.g. formaldehyde, organic vapors, ammonia, acid gases). Cartridges contain activated carbon treated to absorb the vapors and are color coded to indicate the contaminant it can filter, e.g. white for acid gases, black for organic vapors, and olive for formaldehyde. Combination cartridges are available which can filter multiple classes of gases, particles, and vapors (3M Personal Safety Division). When respirators are required to be used a respiratory fit testing program must be established. The respiratory fit testing assures the wearer is capable of wearing a respirator since breathing through a respirator is more difficult than breathing normal air. People with inadequate lung capacities or eyesight problems may not be able to work using a respirator. Also the respiratory fit testing program must show the user how to wear the respirator correctly.

- Ingestion protection

Laboratories which are accredited by the College of American Pathologists (CAP) are required to have a written policy which prohibits eating, drinking, smoking, application of cosmetics, and mouth pipetting in the laboratory. Such requirements are common in all laboratories and help prevent any contamination of these items which are ingested or applied to the mouth area.

Washing your hands and face after handling chemicals and after removing gloves is essential in a medical laboratory. This simple measure will prevent ingestion of chemical vapors and microbes which could have settled on the skin.

- **Section 9: Physical and chemical properties**:

There are 18 physical/chemical properties which are required. If no data are available or are not applicable, then the SDS will state so. Many of these properties are used to determine the chemical classification(s). In the histology laboratory many of these properties can provide additional information on the handling of the chemical. When using an unfamiliar solvent for the first time, the appearance and odor data can confirm that the physical characteristics are correct.

Histological stains have pH ranges that produce optimal staining results. When the pH is out of the range, staining results will be abnormal (non-specific, too light, too dark). Knowing the correct pH value is helpful in troubleshooting. Multiple properties are listed to provide the specific temperatures to prevent melting or freezing of the chemical. Solubility data assist in cleanup information by informing whether the chemical is water or oil soluble.

- **Section 10: Stability and reactivity :**

Chemicals and mixtures not only have health hazards but also, if improperly stored or mixed, can produce physical hazards such as the release of toxic vapors or an explosion. For example, mixing acetic acid with bleach can generate toxic chorine gas. Some chemicals are self-reactive under heat or pressure. Picric acid, used in Bouin's fixative, is stable in liquid form (greater than 30% water), but the dry form is sensitive to friction, heat and shock, and is explosive. When storing a picric acid solution checks should be performed on a monthly basis to ensure that the quantity of water is maintained to prevent it from drying out.

When pouring liquid picric acid one should wipe the inside edges of the cap and the rim of the bottle free of any liquid to prevent any drying of picric acid solution. The cap should be kept loose to prevent any chance of even the smallest explosion due to the friction when turning the cap.

Hazardous decomposition is the term used to describe the breakdown of an unstable molecule into smaller molecules. This breakdown can result in an explosion, fire or release of vapors. Such chemicals are rarely used in the histology laboratory. This section of the SDS identifies such chemicals or mixtures. Section 7 also provides the safe handling and storage of such chemicals.

- **Section 11: Toxicological information :**

This section contains the supportive data used to classify the health hazards of the chemical. Numerical measures of toxicity such as LD50 and LC50 are provided.

LD50 is the lethal dose in which 50% of the tested population died; LC50 is the lethal concentration (used for vapors) in which 50% of the tested population died.

Specific symptoms observed for the inhalation, ingestion, and skin and eye contacts are provided.

Toxicology information is provided for both long-term and short-term exposure as well as the delayed and immediate effects. For example, skin exposure to acids produces an immediate effect from redness (short exposure) to severe burns (long-term exposure). By contrast, breathing in asbestos bodies over a long term has a delayed reaction of possible

severe lung disease. This section of the SDS can be used to assist medical professionals during medical treatment. The listed numerical data are usually based on animal studies, where experiments are carefully controlled so that the exposure is only to the one chemical. It has been difficult to assign inhalation dose and effects in human occupational studies since most worker are exposed to multiple chemicals in their work and private life.

- **Section 12: Ecological information :**

Whilst discussion is not warranted for this safety chapter, this section of the SDS has important information to help determine disposal options.

- **Section 13: Disposal considerations :**

Guidelines are given for disposal of a chemical. However only the applicable governmental agency has the authority to provide the disposal method. Each country has developed its own environmental laws and regulations. In the USA, the Environmental Protection Agency (EPA) oversees disposal controls. However, each of the state and local municipalities in the USA are able to issue more stringent rulings than the federal government. Therefore, each user must dispose of any chemical in accordance with their local governmental directives. Drain disposal is the easiest disposal option. One must get permission from their public-owned wastewater treatment plant to assure that drain disposal will not affect the functions of the facility. One step in the treatment process involves bacteria and it is important that the drain-disposed chemical is not toxic to the bacteria. It is beneficial when the chemical being disposed is water soluble, since the toxic effect is reduced through standard water dilution whilst traveling through the pipe systems. Wastewater treatment plants vary in their capabilities, and therefore the volume and classification of the chemical being drain disposed will determine whether permission is granted. Most hospital solid and liquid wastes are discarded with the services of a licensed waste hauler. Work with the waste hauler to properly segregate and label the waste material.

Recycling

Recycling is a viable option to reduce waste volumes and disposal costs in the histology laboratory. Distillation recycling systems for alcohol and xylene/xylene substitutes offer great cost-savings on these two high-volume solvents. The capital expense for the equipment will be offset by the reduced waste hauling expense. These systems are able to deliver the same purity of the original product purchased. Recycling units are programmed with the boiling point of the solvent. During the distillation process, the solvent is boiled and the vapors are collected for use. Whilst the recycled material for xylene can be used in the same capacity as

any unused clearant, recycled alcohols have limitations. The concentration of recycled alcohol is approximately 95% due to the azeotrope properties of alcohol and water. Therefore, distilled alcohol cannot be used in procedures which call for 100% alcohol. Formalin distillation recyclers use the same principle of the alcohol and xylene distillation units. However, the action of boiling can destroy formaldehyde. It is standard procedure to test the recycled formaldehyde to assure the concentration is 10%. Commercial assay test kits make this easy to determine. During formaldehyde distillation the buffering salts are not recycled. Therefore, it is necessary to re-buffer the recycled formalin before use.

Sometimes the recycled material may smell fishy. This is due to dissolved serum proteins in the waste solvents from the tissue processor. During exposure to the high temperatures required for distillation the serum proteins are broken down into odoriferous amines (producing the fishy smell). Keeping the distillation unit properly cleaned can easily prevent this fishy smell.

Manufacturers of distillation units have recommended cleaning procedures. Filtration recycling units do not use distillation but instead use absorbents to remove the color and solid materials from the waste solvent. Water from the tissues during processing, or carryover from the staining procedure, contaminates formalin and/or alcohols reducing the solvents' concentrations. The excess water is not removed by filtration and the concentration of the recycled material stays the same as the starting (waste) material. When using filtration recycling units, chemical testing of the formalin and an alcohol hydrometer needs to be used to determine the concentration of the recycled material and its applicable use.

- **Section 14: transport information :**

The classification procedure to determine the hazards classification of a chemical for transportation is based upon regulations written by the Department of Transportation (DOT) (USA) and the International Air Transportation Agency (IATA). The transportation classification text uses terminology such as proper shipping names, packing groups and packing classes. Both GHS and transportation standards use pictograms but with different criteria. Transportation regulations only identify a chemical as corrosive when they react with skin or metal; a chemical used in the workplace which is corrosive to the eyes will have a GHS corrosive pictogram on the bottle label but the shipping box containing the bottle may not have the same pictogram. Therefore, do not use any of the information found in the transportation section (e.g. proper shipping name, transportation diamonds) to identify the hazards which are encountered in the workplace.

- **Section 15: Regulatory information :**

The SDS is designed to provide information for occupational safety. This section will list all other national and state regulatory standards (environmental, transportation, consumer protection) which the chemical/mixture must comply with.

- **Section 16: Other information :**

This section will minimally have the SDS issue and/ or revision date. Many SDS will have the information for the NFPA hazard diamond (composed of 4 smaller diamonds). Each smaller diamond is uniquely colored for flammability (red), health (blue), instability (yellow) and special hazards (white). The hazard risk is indicated with a number in each smaller diamond ranging between zero (least hazardous) and four (most hazardous).

The GHS classification category numbers however, represent hazards inversely from the NFPA numerical system. GHS category 1 represents the most hazardous; category 4 indicates least hazardous. It is therefore important not to use the GHS category numbers to complete NFPA diamonds. The NFPA standards (National Fire Protection Association, 2012) identify the hazard risks which are experienced during a fire or spill emergency and are not to be used for occupational exposure. Despite the intended use and how the hazard risks have been assigned, many hospitals adhere NFPA diamonds onto their chemical bottles. The NFPA diamonds are actually to be attached to the outside of buildings or areas to warn emergency responders of the possible hazards they may encounter.

II. Methods of study in histology

A. Generalities and definitions:

Histology means, etymologically, the science of tissues. The concept of tissue, of dermis, was inaugurated at the end of the 17th / early 18th century by Xavier Bichat, without a microscope; this concept was developed thanks to his work of anatomical dissection.

Histology was formed around the mid-19th century from the conjunction of two events, thanks to the advent of cell theory (1838), established from the work of Theodore Schwann and Schleiden.This theory postulates that every organism is made up of cells, and is supplemented by Virchow in 1858, when the latter claims that every cell comes from another cell: the elementary unit of life is therefore the cell.

On the other hand, protozoa are made up of a single cell; other organisms, on the contrary, are made of multiple cells: they are methazoa.

From the beginning of the 19th century, and especially around 1840, the optical microscope was improved and the optics were greatly improved. Much more efficient lenses are actually being manufactured, which makes it possible to obtain images that are much closer to reality. It is at this time that histological techniques are put in place. A tissue is made up of cells and the substances they synthesize; these substances constitute the extracellular matrix or MEC. Although more complex, histological examination is now often likened to microscopic examination.

Histology quickly became interested in the morphology of cells (histochemistry), but also in their functioning and the design of tissues. (histophysiologie). In the early 1960s, the creation of the electronic microscope improved growth and introduced a new way of seeing cells and tissues. Today, histology aims to highlight in-cell, in situ (as opposed to biochemistry), proteins, DNA and mRNA.

There are four families of tissues, which are studied within the framework of general histology:

- Epithelia.
- Connective tissues.
- Muscle tissue.
- Nervous tissues.

These tissues are characterized by the molecular composition of their extracellular matrix, the nature of their cells and their ratio to the extracellular matrix.

Special histology is concerned with the study of the structure of organs by studying microscopic anatomy on the one hand and histophysiology on the other. The organs are grouped into systems or apparatus, each of which has one or more well-defined functions and ensures the functioning of the body.

Pathological anatomy, on the other hand, studies pathological tissues. Diagnosis of many pathologies is based on microscopic examination of small tissue samples called biopsies.

B. Tissue preparations:

1. Optical Microscopy:

The light microscope is an essential part of the histopathology laboratory as it is the device with which histological preparations are studied. The designs and specifications of modern microscopes vary widely, but the basic principle is the same as the original simple microscope which used sunlight as its light source. Electric bulbs or light emitting diodes (LEDs) are now used to produce a beam of light which is focused on the tissue section or sample, and then the transmitted light passes through a set of objectives, along the tube and through the eyepiece into the eye of the microscopist.

The lens system of the light microscope allows the eye to see an image of the target tissue at varying magnifications depending on the objectives used. The varying lenses seen in the modern microscope are present within the substage condenser below the slide, as well as above it. The additional objective lenses above the sample can be brought into position depending on the tissue magnification required. The objectives are usually mounted in a rotating disc and are brought into alignment with the main body tube of the microscope to select higher or lower magnifications. The different magnifications required are achieved by altering three variables; firstly, the angle at which the light strikes the lens, the angle of incidence; secondly, the curvature of the lens and finally, the density of the glass or refractive index (RI). Parallel light entering a lens from a small object is brought to a sharp focus at a point behind the lens, then the eyepiece allows a magnified real image to be formed below the eyepiece. This is the basic principle of light microscopy.

a) Sampling:

In human histology, the sampling can be done on a corpse (as soon as possible after death), from surgical specimens (quickly or the specimens must be fixedq uickly) or even globally, without recutting, in the case of biopsies.

A separate room is required for specimen reception which acts as the interface between non-laboratory hospital staff, other visitors and the pathological laboratory. The area must be

equipped with appropriate easily cleaned benching, adequate lighting, good ventilation, safety equipment, disinfectants, absorption granules and protective clothing.

In the event of specimen spillage, e.g. body fluids, fixative leakage or other mishap, the immediate response by staff in this area will limit any potential local health risk and prevent risk to other laboratory personnel. The key point of this room is to receive samples safely and securely. Any new specimen should have its identity confirmed and assigned a unique laboratory specimen identifier, usually a complex number. Mapping of the specimen identifier against the clinical request form is mandatory, along with checking of appropriate clinical details mentioned against the specimen. Corroborative data, in the form of the hospital number/registration index, national patient identifier number, the full name, date of birth and address are valid ways of verifying the identity of any specimen.

Multiple sources of cross reference are always advocated, and if there is any doubt with regard to the probity of a specimen then it should not be passed onwards until the clinician concerned has confirmed all the appropriate details. In many situations the two-person rule is best followed with two independent laboratory practitioners verifying the various details of the specimen at all the different stages of examination.

Confirmation of a minimum of three unique identifiers (as detailed above) is advisable. Once validated and identified, the case, usually labeled with a self-adhesive bar or quick response (QR) code label, can be passed to the dissection room for examination, specimen description and block sampling.

The usual numerical method of specimen identification is simply the year, expressed in two digits, with a sequential numbering system starting with one (1) and proceeding up to the final specimen of each year. There may be a check digit, often in the form of a letter applied, but this simple system allows surgical pathology samples to be processed with ease and to be correlated against paraffin blocks, photographs and other tests

Particular attention must be paid to cases with unusual or common names. Names which have a variety of different spellings and any specimens which have incomplete information should be carefully considered before being accepted. Those with imprecise verification data and poorly handwritten data should not be accepted. In some cases, multiple specimens from a single patient may be received on the same day for analysis. Some laboratories prefer to annotate each sample with a separate number. However, a single laboratory number may suffice, but with sub-parts of the specimen being separately designated, e.g. sample A, sample B, etc. Within this framework, if multiple blocks are taken from a sub-part of the

specimen then these can be designated with individual numbers or letters in a similar ascending fashion.

The departmental computer system can be set to track specimen movement through the laboratory from its receipt to the final histopathologist report authorization.

Sharp instruments such as the scalpel are used in order not to crush the tissues and thus to avoid the formation of artifacts. It isnecessary to cutthin slices takingintoaccount the general orientation of the organ.

There are different types of tissue samples:

- Smears: Medical sampling using a sterile swab, a small brush or a small spatula (for example rom the cervix), allowing cells to be collected (figure 2).

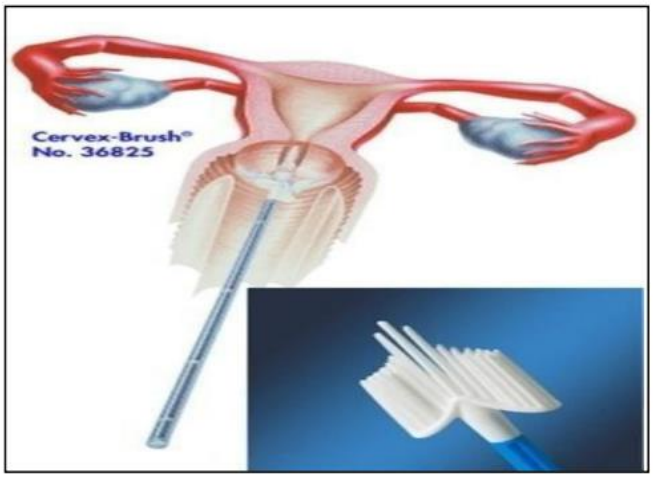


Figure 2: Brush for cervico-vaginal smear

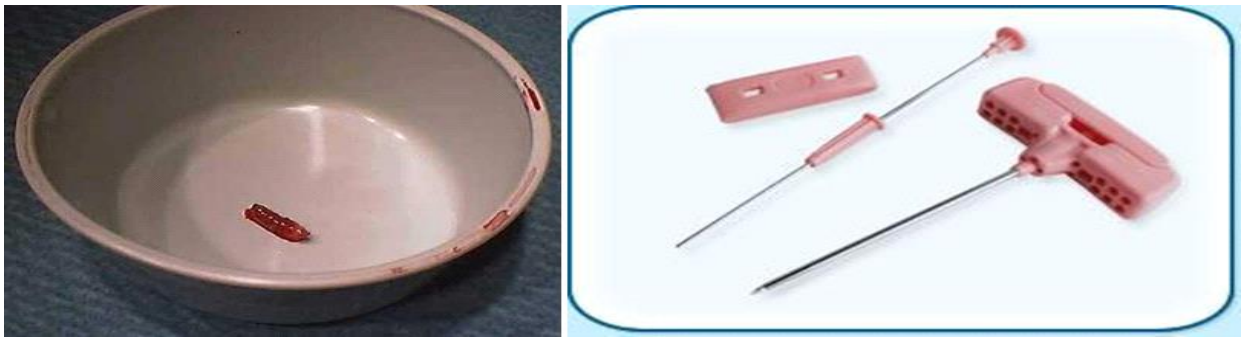


Figure3: A: Biopsies: very small size samples of a tissue. **B:** Trocar and biopsy forceps

- Partial or complete excision: Surgical removal of an organ or part, a lesion, a foreign body (tumor). It is performed in an operating block or for an autopsy (Figure 4).

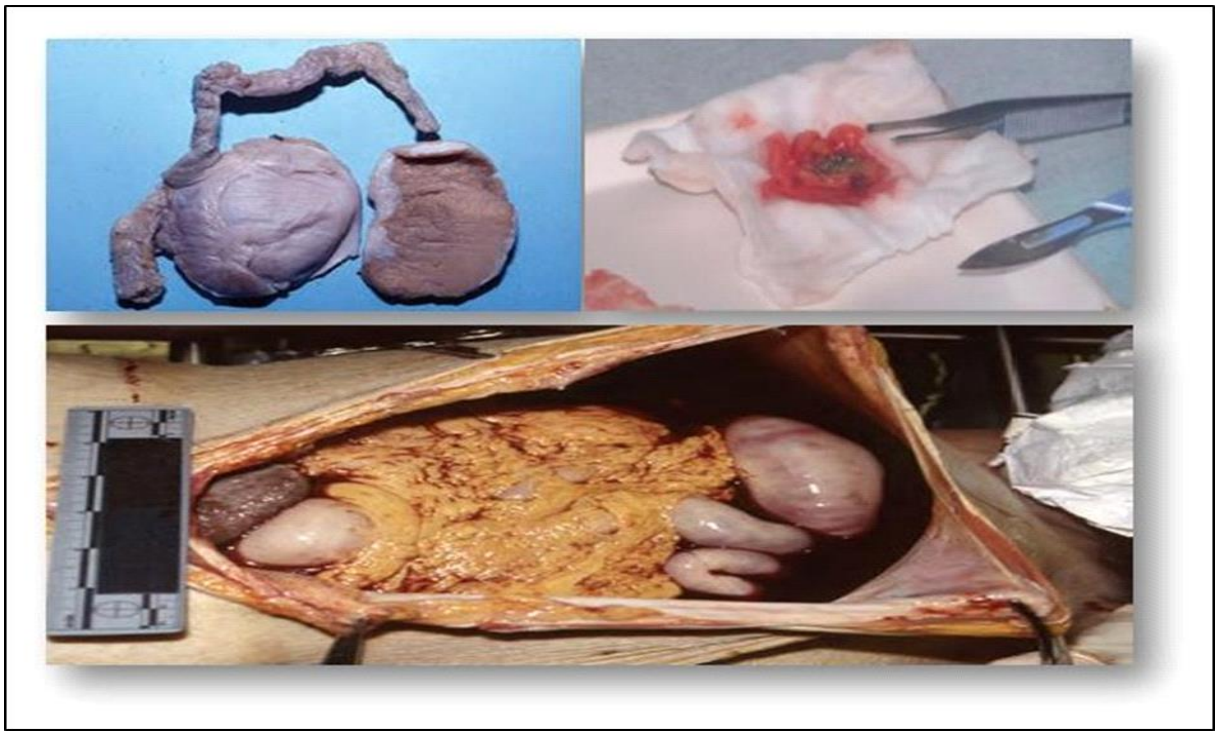


Figure 4 : A : orchidectomy. **B :** adenectomy. **C:** necropsy

Prior to fixation it may be relevant to reserve some fresh tissue from the specimens for microbiology assessment by placing into appropriate culture media and/or electron microscopy which requires glutaraldehyde fixation. Fresh tissue may be taken for frozen section immediate analysis. Unfixed tissue can also be taken for DNA extraction, cytogenetics and molecular pathology techniques.

The latter is becoming increasingly common and important in the arena of personalized therapy by reflecting the tumor genotype and characteristics. Other specialized tests, e.g. enzyme assay and mass spectroscopy may also require tissue retention before standard formalin fixation.

Some specimens are only examined macroscopically, possibly with photography and other physical techniques. Examples include various mechanical/ prosthetic implants, foreign bodies, bullets, gallstones and medical devices. These must be dealt with according to the needs of the specific request/ case. It should be noted that some specimens may require retention for a prolonged period of time, e.g. in forensic/criminal investigation cases. At this point, if no preliminary sampling is performed, the specimen is usually fixed, by immersion into formalin. This process fixes the sample, allowing storage for a prolonged period without

tissue degradation. Some samples need fixation and then decalcification in ethylene diamine tetra acetic acid (EDTA).

Photographing the macroscopic specimen, whole or during the dissection, is particularly important in cases of complex surgical excision, e.g. Wertheim's hysterectomy, pneumonectomy and localization samples. It may also be of use in later analysis/ case discussion. Digital photography with hand-held basic devices has been a major bonus to the laboratory superceding film-based photography. The opportunity to enhance the patient record has greatly improved all pathology disciplines and allowed retrospective case reviews. Specialist and professional photography may still be required however for cases which may be used in visual teaching presentation, journal/book publication or in a medico-legal situation. Consideration of the potential to recognize a patient's sample should be made, with some guidance existing on the subject.

b) Fixation:

.It is fair to say that the appropriate fixation of tissues for histological examination is central to all histology tests, as without this process all tissues would degrade and analysis would be useless. The last century has seen the development of a range of fixatives, with few recent modifications. The mechanisms and principles by which specific fixatives act fall into several broad groups. These include the covalent addition of reactive groups and cross-links, dehydration, the effects of acids, salt formation, and heat. Compound fixatives may function using several of these mechanisms.

When choosing a fixative there is a balance between the advantages and disadvantages which each fixative possesses. These include molecular changes or losses from 'fixed' tissues, swelling or shrinkage of tissues, variations in the quality of histochemical and immunohistochemical staining, the effect on biochemical analysis and the ability to maintain the structure of cellular organelles.

The major objective of fixation in pathology is to maintain clear and consistent morphological features.

The development of specific fixatives has usually been empirical, although much of the understanding of the mechanisms of fixation has been based upon information obtained from leather tanning and vaccine production. In order to visualize the microanatomy of stained tissue sections, the original microscopic relationships between cells, cellular components (e.g. the cytoplasm and nuclei) and the extracellular material must be maintained with little disruption to the organization of the tissue. The local chemical composition of the tissue must

also be maintained. Many tissue components are soluble in aqueous acid or other liquid environments and to reliably view the microanatomy and microenvironment of these tissues the soluble components must not be lost during fixation and tissue processing. Minimizing the loss of cellular components which include large proteins, small peptides, mRNA, DNA and lipids, prevents the destruction of macromolecular structures such as cytoplasmic membranes, smooth endoplasmic reticulum, rough endoplasmic reticulum, nuclear membranes, lysosomes and mitochondria.

Each fixative, combined with the tissue processing protocol, maintains some molecular and macromolecular aspects of the tissue better than other fixative/processing combinations. If soluble components are lost from the cytoplasm of cells, the color of the cytoplasm on hematoxylin and eosin (H&E) staining will be reduced or modified and aspects of the appearance of the microanatomy of the tissue, e.g. mitochondria, will be lost or damaged. Similarly, immunohistochemical evaluations of structure and function may be reduced or lost.

Almost any method of fixation induces shrinkage or swelling, hardening of tissues and color variations in various histochemical stains. Various methods of fixation always produce some artifacts in the appearance of tissue on staining. However, for diagnostic pathology it is important that such artifacts are consistent, predictable and understood. The chosen fixative acts by minimizing the loss or enzymatic destruction of cellular and extracellular molecules, maintaining macromolecular structures and protecting tissues from destruction by microorganisms. This results in one view of a dynamically changing, viable tissue.

The fixative should also prevent the subsequent breakdown of the tissue or molecular features by enzymatic activity and/or microorganisms during long term storage. These tissues removed from patients are an important resource which may at a later stage be subjected to further specialized tests, e.g. DNArelated or gene analysis.

A fixative not only interacts initially with the tissue in its aqueous environment but it also has ongoing reactivity with any unreacted fixative and the chemically altered tissues. Fixation interacts with all phases of processing and staining from dehydration to staining of tissue sections using histochemical, enzymatic or immunohistochemical stains. It follows that any stained tissue section, produced after specific fixation combined with tissue processing, is a compromise of fixed tissue changes formed from the natural living tissue. To date, a universal or ideal fixative has not been identified.

Fixatives are therefore selected based on their ability to produce a final product needed to demonstrate a specific feature of a specific tissue. In diagnostic pathology, the fixative of choice for most pathologists has been 10% neutral buffered formalin.

An important constraint in using formaldehyde has been the loss of antigen immune recognition due to this type of fixation combined with processing the tissue to paraffin wax. However, from a clinical perspective the advent of heat-induced epitope retrieval methods, instigated in the early 1990s, have overcome many of these limitations. Similarly, the analysis of mRNA and DNA from formalin-fixed, paraffin embedded tissue has been problematic. All widely used fixatives are therefore selected by compromise, with their positive aspects balancing against their less desirable features. The most important characteristic of a fixative is to support high quality and consistent staining with H&E, both initially and after storage of the paraffin blocks for at least a decade, although new guidelines within the United Kingdom recommend that paraffin processed blocks are now kept for 30 years.

The fixative must have the ability to prevent short and long term destruction of the micro-architecture of the tissue by stopping the activity of catabolic enzymes and hence autolysis, minimizing the diffusion of soluble molecules from their original locations. Another important characteristic of a good fixative, which helps maintain tissue and cellular integrity, is the fixation and inactivation of infectious agents. It is also important to have good toxicological and flammability profiles which permit the safe use of the fixative. The advent of new biological methods, increased understanding of the human genome and the need to rapidly evaluate the biology of disease processes means that fixatives should also permit the recovery of macromolecules including proteins, mRNA, and DNA from fixed and paraffin-embedded tissues without extensive biochemical modifications. Other important characteristics of an ideal fixative include being useful for a wide variety of tissue types, including fat, lymphoid and neural tissues. It should preserve small and large specimens and support histochemical, immunohistochemical, in situ hybridization and other specialized procedures.

The fixative should penetrate and fix tissues rapidly, have a shelf life of at least one year and be compatible with modern automated tissue processors. It should be readily disposable or recyclable, support long term tissue storage to give excellent microtomy of paraffin blocks and should be cost effective.

Types of fixation:

Fixation of tissues can be accomplished by physical and/or chemical methods. Physical methods, e.g. heating, microwaving and freeze-drying are independent processes and not used commonly in the routine practice of medical or veterinary pathology, anatomy and histology. The exception is the use of dry heat fixation of microorganisms prior to Gram staining. Most methods of fixation used in the processing of tissue for histopathological diagnoses rely on chemical fixation carried out by liquid fixatives.

Reproducibility of the microscopic appearances of tissues after H&E staining is the prime requirement of the fixatives used for diagnostic pathology. Methods of fixation used in research protocols include the use of vapors and rarely, when fixation of a whole animal is needed, the perfusion of the animal's vascular system with a fixative. Several chemicals, or their combinations, can act as good fixatives and accomplish many of the stated goals of fixation. Some fixatives add covalent reactive groups which may induce cross-links between proteins, individual protein moieties within nucleic acids and between nucleic acids and proteins. The best examples of such 'cross-linking fixatives' are formaldehyde and glutaraldehyde. Another approach to fixation is to use agents which remove free water from tissues and precipitate and coagulate the proteins. Examples of these dehydrants include ethanol, methanol and acetone. These agents denature proteins by breaking the hydrophobic bonds responsible for maintaining the tertiary structure of proteins. Other fixatives, e.g. acetic acid, trichloroacetic acid, mercuric chloride and zinc acetate act by denaturing proteins and nucleic acids through changes in pH or via salt formation. Some fixatives are mixtures of reagents and are referred to as compound fixatives, e.g. alcoholic formalin fixes tissues in two ways: firstly, by adding covalent hydroxymethyl groups and crosslinking proteins and secondly, by coagulation and dehydration.

- **Physical methods of fixation :**

Heat fixation

This is the simplest form of fixation. Boiling or poaching an egg precipitates the proteins and, on cutting, the yolk and egg white can be identified separately. Each component is less soluble in water after heat fixation than the same component of a fresh egg. Picking up a frozen section on a warm microscope slide, both attaches the section to the slide and partially fixes it by heat and dehydration. Even though adequate morphology could be obtained by boiling tissue in normal saline, heat is primarily used to accelerate other forms of fixation as well as the other steps of tissue processing.

Microwave fixation:

Microwave heating can reduce times for fixation of some gross specimens and histological sections from more than 12 hours to less than 20 minutes. Microwaving tissue in formalin results in the production of large amounts of dangerous, potentially explosive vapors. In the absence of a hood for extraction or a microwave processing system designed to handle these vapors, this may cause safety problems. Commercial glyoxal-based fixatives which do not form vapors when heated at 55°C have been introduced as an efficient method of microwave fixation.

- **Chemical fixation :**

This utilizes organic or non-organic solutions to maintain adequate morphological preservation. Chemical fixatives can be considered as members of three major categories: coagulant, cross-linking, and compound.

Coagulant fixatives:

Both organic and non-organic solutions may coagulate proteins making them insoluble. Cellular architecture in vivo is maintained primarily by lipoproteins and fibrous proteins such as collagen. Coagulating these proteins maintains tissue histomorphology at the light microscope level. Unfortunately, because coagulant fixatives result in cytoplasmic flocculation and poor preservation of mitochondria and secretory granules, these fixatives are not useful in ultrastructural analysis.

Dehydrant coagulant fixatives:

The most commonly used in this group are alcohols (e.g. ethanol, methanol) and acetone. Methanol is closer to the structure of water than ethanol. Ethanol therefore competes more strongly than methanol in the interaction with hydrophobic areas of molecules and coagulant fixation begins at a concentration of 50–60% for ethanol but 80% or more for methanol.

The removal and replacement of free water from tissue by any of these agents has several potential effects on proteins within the tissue. Water molecules surround hydrophobic areas of proteins and, by repulsion, force hydrophobic chemical groups into closer contact with each other stabilizing hydrophobic bonding. By removing water, the opposite principle weakens hydrophobic bonding. Similarly, molecules of water participate in hydrogen bonding in hydrophilic areas of proteins, and therefore removal of water destabilizes this hydrogen bonding. Together, these changes act to disrupt the tertiary structure of proteins. Additionally, with the water removed the structure of the protein may become partially reversed, with hydrophobic groups moving to the outside surface of the protein. Once the tertiary structure of a soluble protein has been modified, the rate of reversal to a more ordered

soluble state is slow and most proteins after coagulation remain insoluble even if returned to an aqueous environment. Disruption of the tertiary structure of proteins (i.e. denaturation) changes their physical properties, potentially causing insolubility and the loss of function. Even though most proteins become less soluble in organic environments, up to 13% of protein may be lost, e.g. with acetone fixation.

Factors which influence the solubility of macromolecules include:

• Temperature, pressure, and pH.

• Ionic strength of the solute.

• The salting-in constant, which expresses the contribution of the electrostatic interactions.

• The salting-in and salting-out interactions.

• The types of denaturing reagents.

Alcohol denatures protein differently depending on the choice and concentration of alcohol, the presence of organic and non-organic substances and the pH and temperature of fixation. The protein denaturing effect of ethanol is > phenols > water and polyhydric alcohols > monocarboxylic acids > dicarboxylic acids.

Non-coagulant cross-linking fixatives:

Several chemicals were selected as fixatives secondary to their potential actions of forming cross-links both within and between proteins and nucleic acids.

Cross-linking may not be a major mechanism with short times of fixation and therefore 'covalent additive fixatives' may be a better name for this group. Examples include formaldehyde, glutaraldehyde and other aldehydes, e.g. chloral hydrate and glyoxal, as well as metal salts, e.g. mercuric and zinc chloride, and other metallic compounds, e.g. osmium tetroxide. Aldehyde groups are chemically and biologically reactive and are responsible for many histochemical reactions, e.g. the argentaffin reaction.

- **Formaldehyde fixation :.**

Formaldehyde, as 10% neutral buffered formalin (NBF) is the most common fixative used in diagnostic pathology. Pure formaldehyde is a vapor which, when completely dissolved in water forms a solution containing 37–40% formaldehyde and this aqueous solution is 'formalin'. The usual '10% formalin' used in the fixation of tissues is a 10% solution of formalin, which contains approximately 4% weight to volume of formaldehyde. The reactions of formaldehyde with macromolecules are numerous and complex. In an aqueous solution, formaldehyde forms methylene hydrate, a methylene glycol as the first step in fixation $H2C = O + H2O \rightarrow HOCH2OH$.

Methylene hydrate reacts with several side chains of proteins to form reactive hydroxymethyl side groups (–CH2–OH). When the current relatively short fixation times are used with 10% neutral buffered formalin (hours to days), the formation of hydroxymethyl side chains is the primary and characteristic reaction and the formation of actual cross-links may be rare. Formaldehyde also reacts with nuclear proteins and nucleic acids It penetrates between nucleic acids and proteins and stabilizes the nucleic acid-protein shell, also modifying nucleotides by reacting with the free amino groups as it does with proteins. In naked and free DNA, the cross-linking reactions are believed to start at adenine-thymidine rich regions and cross-linking increases with rising temperatures.

Formaldehyde reacts with C=C and –SH bonds in unsaturated lipids but does not interact with carbohydrates. The side chains of peptides or proteins which are most reactive with methylene hydrate have the highest affinity for formaldehyde; these include lysine, cysteine, histidine, arginine, tyrosine and the reactive hydroxyl groups of serine and threonine.

- **Reversibility of formaldehyde macromolecular reactions :**

The reactive groups may combine with hydrogen groups or with each other to form methylene bridges. If the formalin is washed away, reactive groups may rapidly return to their original states, but any bridging which has already occurred may remain. Washing for 24 hours removes approximately half of the reactive groups and after 4 weeks up to 90% are removed. This suggests that actual cross-linking is a relatively slow process and, most 'fixation' with formaldehyde prior to tissue processing in diagnostic pathology stops with the formation of reactive hydroxymethyl groups. During long term storage in formalin, the reactive groups may be oxidized to the more stable groups (e.g. acids –NH–COOH) which are not easily removed by washing in water or alcohol. Returning the specimen to water or alcohol following fixation therefore reduces the further fixation of the specimen because the reactive groups produced by the initial reaction with formalin may reverse and be removed. Although it was initially thought that cross-linking was the most important process in the fixation of tissue for biological uses (based on the limited number of cross-links over short periods of fixation), it is likely that the formation of these hydroxymethyl groups actually denatures macromolecules and renders them insoluble. As these washing experiments have not been reproduced, the actual mechanisms and their importance in fixation by formaldehyde remain uncertain. Over-fixation of tissue may also be partially corrected by soaking the tissue in concentrated ammonia plus 20% chloral hydrate.

The principal type of cross-link in short term fixation is thought to be between the hydroxymethyl group on a lysine side chain and arginine (through secondary amino groups),

asparagine and glutamine (through secondary amide groups) or tyrosine (through hydroxyl groups). For example, a lysine hydroxymethyl amine group can react with an arginine group to form a lysine–CH2–arginine cross-link. Similarly, a tyrosine hydroxymethyl amine group can bind with a cysteine group to form a tyrosine–CH2–cysteine cross-link. Each of these cross-links between macromolecules has a different degree of stability which can be modified by the temperature, pH and the type of environment surrounding and permeating the tissue. The time to saturation of human and animal tissues with active groups by formalin is approximately 24 hours, but cross-linking may continue for many weeks.

When formaldehyde dissolves in an unbuffered aqueous solution, it forms an acid solution (pH 5.0– 5.5) because 5–10% of commercially available formaldehyde is formic acid. Acid formalin may react more slowly with proteins than NBF because the amine groups become charged, e.g. –N+H3. In solution, this requires a much lower pH than 5.5. However, the requirement for a lower pH to produce –N+H3 groups may not be equivalent to that required in peptides. Acid formalin also preserves immunorecognition better than NBF. Indeed, the success of early immunocytochemistry methods to demonstrate immunoglobulins in paraffin-processed tissue sections probably relied on the fixation of the tissues in acid formalin.

The disadvantage of using acid formalin for fixation is the formation of a brown/black pigment with degraded hemoglobin. This heme-related pigment which forms in tissue, is not a problem unless patients have a blood abnormality, e.g. sickle cell disease or malaria. Formaldehyde primarily preserves peptide-protein bonds and the general structure of cellular organelles. It can interact with nucleic acids but has little effect on carbohydrates and preserves lipids if the solutions contain calcium.

- **Compound fixatives :**

Pathologists use formaldehyde-based fixatives to ensure reproducible histomorphometric patterns. Other agents may be added to formaldehyde to produce specific effects which are not possible with formaldehyde alone.

The dehydrant ethanol, for example, can be added to formaldehyde to produce alcoholic formalin. This combination preserves molecules such as glycogen and results in less shrinkage and hardening than pure dehydrants.

Compound fixatives are useful for specific tissues, e.g. alcoholic formalin for fixation of some fatty tissues, such as breast, in which the preservation of the lipid is not important. Additionally, the fixation of gross specimens in alcoholic formalin may aid the identification of lymph nodes embedded in fat. Some combined fixatives, including alcoholic formalin, are good at preserving antigen immunorecognition, but non-specific staining or background

staining in immunohistochemical procedures can be increased. Unreacted aldehyde groups in glutaraldehyde-formaldehyde fixation, for example, may increase background staining and alcoholic formalin may cause non-specific staining of myelinated nerves.

- **Factors affecting the quality of fixation :**

Buffers and pH :

The effect of pH on fixation with formaldehyde may be profound depending upon the applications to which the tissues will be exposed. In a strongly acidic environment, the primary amine target groups (–NH2) attract hydrogen ions and become unreactive (–NH+ 3) to the hydrated formaldehyde (methylene hydrate or methylene glycol), and carboxyl groups (–COO−) lose their charges (–COOH). This may affect the structure of proteins. Similarly, the hydroxyl groups of alcohols (–OH) including serine and threonine may become less reactive in a strongly acidic environment. The extent of formation of reactive hydroxymethyl groups and cross-linking is reduced in unbuffered 4% formaldehyde which is slightly acidic, because the major methylene cross links are between lysine and the free amino group on the side chains. The decrease in the effectiveness of formaldehyde fixation and hence cross-linking in the slightly acid environment has led some authors to suggest that unbuffered formalin is a better fixative than NBF for the immunorecognition of many antigens. This aided the detection of antigens prior to the advent of heat-induced epitope retrieval methods in immunocytochemistry.

However, minimal delay in effectively fixing labile antigens, such as the estrogen receptor, is vital in the immunohistochemical testing for a range of clinically important prognostic and predictive biomarkers. Whilst formaldehyde fixation remains the recommended method for optimal preservation of morphological features, proteins and nucleic acids in a clinical environment, the most reliable way of achieving optimal formalin fixation is through its buffering at pH 7.2–7.4, i.e. NBF. At the acidic pH of unbuffered formaldehyde, hemoglobin metabolic products are chemically modified to form a brown-black, insoluble, crystalline, birefringent pigment. This pigment forms at a pH less than 5.7 and the extent of its formation increases in the pH range 3.0 to 5.0. Formalin pigment is easily recognized and should not affect diagnoses except in patients with large amounts of hemoglobin breakdown products secondary to hematopoietic diseases. The pigment is removed easily with an alcoholic solution of picric acid. Using NBF avoids the formation of formalin pigment and it is used as the preferred formaldehydebased fixative. Factors affecting the quality of fixation 51 Acetic and other acids work mainly through lowering pH and disrupting the tertiary structure of proteins. Buffers are used to maintain the optimum pH. The choice of a specific buffer

depends on the type of fixative and analyte. Commonly used buffers are phosphate, cacodylate, bicarbonate, Tris and acetate. It is necessary to use low salt, buffered formalin in the new complex tissue processors in order to keep the machine 'clean' and reduce problems in its operation.

Duration of fixation and the size of specimens:

The time of fixation is approximately equal to the square of the depth to which the fixative must penetrate and most fixatives, such as NBF will penetrate tissue to the depth of approximately 1 mm in one hour, e.g. for a 10 mm sphere, the fixative will not penetrate to the center until (5)2, 25 hours of fixation. It is important to note that the components of a compound fixative will penetrate the tissue at different rates so it is better if these fixatives are used with thin specimens. Gross specimens should not rest on the bottom of a container of fixative, they should be separated from the bottom by wadded fixative-soaked paper or cloth allowing penetration of the fixative in all directions. Additionally, unfixed gross specimens which are to be cut and stored in fixative prior to processing, should not be thicker than 5 mm. When surgical specimens are to be processed to paraffin blocks the time of penetration by the fixative is more critical.

Fixation proceeds slowly – the period between the formation of reactive hydroxymethyl groups and the formation of a significant number of cross-links is unknown. Ninety percent of reactive groups can be removed by 4 weeks of washing, confirming that cross-linking is not a rapid process and may require weeks for the completion of potential bonds.

Proteins inactivate fixatives, especially in blood or bloody fluids, so these gross specimens should be washed with saline prior to being put into fixative. The fixative volume should be at least 10 times the volume of the tissue specimen for optimal, rapid fixation. Currently in some laboratories, thin specimens may be fixed in NBF for only 5–6 hours, including the short time of fixation in tissue processors. The extent of the formation of crosslinks during such rapid NBF fixation is uncertain. Consequently, the formation of hydroxymethyl groups may predominate as opposed to the more resilient cross-linking. It has been suggested that rapid fixation is acceptable as long as histochemical staining remains adequate, and that immunohistochemistry and other molecular techniques are probably enhanced by shorter times of fixation using an aldehyde-based fixative, e.g. formaldehyde. However, studies investigating the time taken to adequately fix clinical cases of breast cancer tissue for subsequent immunohistochemical detection of estrogen receptors illustrate that this practice can be detrimental to the optimal preservation of important antigens, and should be avoided. The guidelines for estrogen receptor and progesterone receptor testing produced by the

American Society of Clinical Oncology (ASCO) and College of American Pathologists (CAP) recommend a minimal fixation time of 6 hours in neutral buffered formalin for all clinical breast cancer specimens.

Temperature of fixation:

The diffusion of molecules increases with rising temperature due to their more rapid movement and vibration, i.e. the rate of penetration of a tissue by formaldehyde is faster at higher temperatures. Microwaves therefore have been used to speed formaldehyde fixation by both increasing the temperature and molecular movements. Increased vapor levels however, are a safety problem. Most chemical reactions occur more rapidly at higher temperatures and therefore formaldehyde reacts more rapidly with proteins in these conditions. Closed tissue processors have their processing retort directly above the paraffin holding stations which are held at 60–65°C, making the retort slightly warmer than room temperature.

Concentration of fixative:

Effectiveness and solubility primarily determine the appropriate concentration of fixatives. Concentrations of formalin above 10% tend to cause increased hardening and shrinkage. Additionally at higher concentrations formalin is present in its polymeric form which can be deposited as a white precipitate, as opposed to its monomeric form HO(H2CO)H, which at 4% is more soluble. Ethanol concentrations below 70% do not remove free water from tissues efficiently.

Osmolality of fixatives and ionic composition:

The osmolality of the buffer and fixative is important – hypertonic and hypotonic solutions lead to shrinkage and swelling respectively. The best morphological results are obtained with solutions which are slightly hypertonic (400–450 mOsm), although the osmolality for 10% NBF is about 1500 mOsm. Similarly, various ions (Na+, K+, Ca2+ and Mg2+) can affect cell shape and structure regardless of the osmotic effect. The ionic composition of fluids should be as isotonic as possible to the tissues being fixed.

Additives:

The addition of electrolytes and non-electrolytes to fixatives may improve the morphology of the fixed tissue. These additives include calcium chloride, potassium thiocyanate, ammonium sulfate and potassium dihydrogen phosphate. The electrolytes may react either directly with proteins causing denaturation, or independently with the fixatives and cellular constituents. The choice of electrolytes to be added to fixatives used on a tissue processor may vary. Fixatives buffered with electrolytes such as phosphates may cause problems with some

processors due to the precipitation of the salts. The addition of non-electrolyte substances such as sucrose, dextran and detergent has also been reported to improve fixation.

- **Selecting or avoiding specific fixatives :**

The choice of a fixative is a compromise, balancing their beneficial and detrimental effects.. The main problem with fixatives used in histological staining is the loss by solution or extraction of molecules which are the targets for specific histochemical methods. Typically, some molecules are soluble in aqueous fixatives, e.g. glycogen, whilst others are soluble in organic-based fixatives, e.g. lipids. Some fixatives may chemically modify the targets of histochemical staining and affect the quality of special stains, e.g. glutaraldehyde with silver stains. This includes the modification of staining secondary to changes in pH induced by fixation.

- **Fixation for selected individual tissues :**

Eyes:

The globe must be firmly fixed in order to cut good sections for embedding. Eyes may be fixed in NBF, usually for approximately 48 hours; to speed fixation one or two small windows can be cut into the globe, avoiding the retina and iris, after 24 hours.

After the gross description, the anterior iris and posterior optic nerve are removed with a new, sharp razor blade and the components of the globe are fixed for an additional 48 hours, or more, in buffered formaldehyde before being processed. Embedding may be in paraffin wax. Perfusion fixation of the eye is recommended for studies of the canal of Schlemm and/or the aqueous outflow pathways. Animal eyes may be fixed in Davidson's fixative. This fixative is rapid and gives no artifactual detachment of the retina. Fixation should not exceed 24 hours for rodents' eyes and 48 hours for larger species. Ocular globes are then transferred to 70% ethyl alcohol and processed to paraffin embedding.

Brain:

The problem of fixing a whole brain is to make it firm enough to investigate the neuroanatomy and to be able to produce sections for histopathology and possible immunochemistry. Conventionally this fixation takes 2-6 weeks.. Fixation may also be enhanced by the use of microwave technology. Alcoholic formalin should not be used for fixation if immunohistochemistry is to be performed using biotin-avidin (streptavidin) methods

Breast:

Clinical samples should be fixed in 10% NBF for a minimum of 6–8 hours, to a maximum of 72 hours and should be sliced at 5 mm intervals after appropriate gross inspection and margin designation. The time from the tissue acquisition to fixation should be as short as possible in order to prevent lysis of clinically important biomarkers such as estrogen receptors, progesterone receptors and the human epidermal growth factor receptor-2 (HER2). They should be placed in a sufficient volume of NBF to allow adequate tissue penetration. If the tumor specimen has come from a different geographical location to the laboratory, it should be bisected through the tumor on removal from the patient and sent to the laboratory immersed in a sufficient volume of NBF.

Lungs:

Lung biopsies are typically fixed in NBF. The lungs from lobectomy, pneumonectomy and autopsies may be inflated by, and fixed in NBF instilled under gentle pressure via the trachea or major bronchi. Such fixed lungs can be cut within 2-6 hours, and gross sections are fixed overnight, allowing sections to be processed and cut the next day.

Lymphoid tissue:

Special care should be taken with all lymphoid tissue as many organisms, e.g. Mycobacterium tuberculosis and viruses may be present in the lymphoreticular system. There is always a possible infection risk with such cases. The lymphoid tissue is usually sliced and a representative sample of fresh tissue taken for special studies, e.g. microbiology, flow cytometry or molecular analysis. The rest of the lymph node is fixed in NBF, although some laboratories fix part of the tissue in B5 or zinc.

Testis:

Biopsies of the testes are fixed routinely in NBF.

Muscle biopsies:

These are received fresh and a portion is separated for enzyme histochemistry. The tissue for routine histological assessment is fixed in NBF and embedded so the fibers of the specimen are viewed in cross section and longitudinally. After processing this is stained with H&E, a trichrome stain and Congo red if amyloid is suspected.

Renal biopsies:

Renal core biopsies should be subdivided into three and each piece should contain an adequate number of glomeruli, e.g. 6-10 for light microscopy, 1-2 for electron microscopy and 3-6 for immunofluorescence. Each portion is then preserved depending upon the method to be used for subsequent analysis:

• NBF for routine histology.

• Buffered glutaraldehyde at pH 7.3 for ultrastructural analysis.

• Snap frozen in isopentane and liquid nitrogen for immunofluorescence examination.

c) Dehydration:

Dehydration involves removing the tissue from the water it contains. It is done by passing into ethanol baths of increasing concentration to the absolute ethanol (70°, 80°, 90°, 95°, 99°, and finally 100°). The tissues should only stay in the baths for the time necessary to balance the relative proportions of water and ethanol. Fibrous and dense tissues are more difficult to dehydrate than others. Poor dehydration can be the result of too long or too short stays in ethanol. A prolonged stay excessively hardens the tissue. Inadequate dehydration prevents the paraffin from fully penetrating the tissue; so areas where the paraffine has not penetrates do not become rigid enough to withstand the section.

The substitution is to replace ethanol that is not miscible to paraffin with a solvent: toluene or xylene. These two solvents are mixable to both the dehydratant and the inclusion agent, they are referred to as “clearing agents” (because they have the property of making the tissues they permeate translucent). Toluene (very toxic and flammable) is slower and more tolerant than xylene.

The main effects of improper replacement are the production of blocks of heterogeneous or inadequate consistency, excessive tissue retraction or distortion if the bath is too prolonged.

Impregnation is the replacement of the solvent with paraffin. This terminal stage is relatively aggressive as the paraffin is liquid only at 58°C and at this temperature the proteins are altered. Too long impregnation can cause significant retraction of all structures and excessive hardening of parts. At the end of the cycle, the baskets containing the cassettes are in a hot paraffin bath (liquid). They are drained before being transferred to the waiting basket at the inclusion post. There are different impregnation devices. Vacuum impregnation machines reduce the emission of solvent vapors into the working atmosphere. They reduce the risk of human error and ensure the homogeneous handling of all parts. They thus allow for better

paraffin impregnation and can contain a large number of cassettes arranged in specially designed baskets. They are programmable, most have a delay mechanism that allows you to adjust the chosen program or cycle in advance. This stage isusuallyperformed at night.



Figure 5: Dehydration machine

d) Paraffin embedding:

In order for light to pass through the tissue to be examined, it must be very thin. But the tissues are soft, so you need to give them a solid consistency.

The principle of the inclusion consists of a coating of the sample with liquid paraffin which is hardened thus preserving the architectural relations of the structures to each other and providing it with an external support both during and after the microtomy. It is necessary that the paraffin used for coating the parts has the same characteristics as the one used for impregnation. The inclusion is made satisfactorily only if the sample contains neither water nor solvent (ethanol) after impregnation.

The inclusion can also be made in resin for samples intended for electronic microscopic observation. For frozen samples the embedding is relatively simple. The sample is coated in a polymer that hardens when placed in isopentan cooled either by liquid nitrogen or by dry ice.

Cassetes placed in the waiting tank of the basket containing paraffin kept in liquid state (≈ 60 °C) are included manually when the temperature of the working plate has reached 70 °C and that of the cooling plate -15 °C.

- Output only one cassette at a time to avoid any risk of error.
- Carefully open the lid and remove it while checking that there is no sampling left hanging on the lid or between the foams
- Take a stainless steel mold suitable for the size of the sample (stored in the heated compartment of the workstation) and pour some paraffin into it.
- Grab a single fragment at a time using a preheated metal plier so that the paraffin or the fragment does not adhere to it. Place it gently at the center of the mold conserving the initial position and eventually reorient it properly. That is, the section plane must be positioned controlled bottom of the mold and some on the slice so that the section allows to observe simultaneously all structures, for example for the skin: the epidermis, the dermis and the hypoderma.
- Place the mold on the cooling disc so that the paraffin at the bottom of the mold is fixed and gently flatten the fragment(s) with preheated clamps without crushing or perforating them.
- Before the paraffin becomes massive, adjust the identification cassette on the mold (enabling to ensure its traceability). Cover with paraffin and remove any air bubbles.
- Immediately put the mold on the cooling plate and let the paraffin fig in order to seal the cassette with the mold.
- After total hardening of the paraffin and when the block is very cold, it is unmold.

The excess paraffin all around the cassette is removed by scraping using a knife (non-sharp) to facilitate the insertion of the block between the jaws of the microtome object holder when cutting..

A block of homogeneous fabric in consistency and elasticity is thus obtained, this character is of great importance for the making of the cuts.

The blocks are classified on cooling plates by registration number (following alphabetical or numerical order) and the series concerning the same patient are grouped together. The blocks awaiting microtomy are stored in a freezer at −20 °C.Besttissuessections are obatained when thoroughly cooled.

Note: Embedding can be made in Leuckhardt bars, that is to say between metal bars of variable size. This process is used in neuropathy for the embedding of large parts, such as the brain.



Figure 6: Operating mode of embedding

a. Embedding post.

b. Tissue fragments in cassette to include

c. Embedding of tissue fragments.

d. Embedding of a tissue sample.

e. Cooling and unmolding of paraffin blocks.

- **Embedding problems**

- If If the specimens are incorrectly embedded, the block can be remelted and the sample recoated.

- If the specimen is placed in a mold without having first filled it with paraffin or if its thickness is not sufficient, the surface of the part adheres to it and prevents the paraffin from coming into contact with the bottom of the mold. Once the block has hardened, the tissue can separate from the paraffin surrounding it.

- If the clamps are too hot, they can burn the tissue.

- If the block is cooled too slowly, the paraffin may have an irregular crystallization that can damage the making of the microtomy.

- If the block is cooled too quickly, the paraffin located between the part and the bottom of the mold shrinks and produces a block with a concave surface.

- If the action is not quick enough before placing the specimen in the mold containing the paraffin, a thick layer of paraffin may form which, after hardening, requires more planing at the time of cutting.

e) Microtomy:

Microtomy is the means by which tissue is sectioned and attached to the surface of a glass slide for further microscopic examination. The basic principles are applicable to both paraffin and frozen sections although most microtomy is performed on paraffin wax-embedded tissue blocks. The instrument used to cut sections is the microtome. This has an advancing mechanism which moves the object, the paraffin block, for a predetermined distance until it is in contact with the cutting tool, the knife or blade. The specimen moves vertically past this cutting surface and a tissue section is produced.

The microtomy is intended to obtain very thin quality sections of 2 to 4 μm (micron) and without vibration phenomena for all types of tissuesembedded in paraffin. This thickness allows the light rays of the microscope to pass through the preparation and avoid tissue superpositions. They are prepared at room temperature and require a microtome with a razor blade. The realization of thin sections depends on the specimen to be cut (hardness, elasticity), the performance of the microtomeand the manual skill of the technician.

Cryotomy is used during a surgical procedure or for some studies that require working on unfixed fresh tissue(lipids staining). The principle of the freezing section is the hardening of the tissue through the freezing of the water it contains. The results obtained depend on several factors such as the temperature of the refrigerated compartment, the type of tissue, the

maintenance of the cryostat and the experience of the technician. The sections are less thin (4–6 μm) and more fragile than those made after embedding. The tissue morphology is not of as good quality as after a fixation andparaffin embedding due to the freezing which alters cell morphologies.

Operating mode of a freezing section:

The fresh tissue is deposited on a plot or object carrying a coating resin (transparent at room temperature) that has the ability to harden at low temperatures (whitening).In the refrigerated compartment of a cryostat at -20 °C, freezing is facilitated by the use of a piston that accelerates cooling (in a few seconds) while promoting the achievement of a flat surface. After a slight degrossing, the microtome cut is collected directly from the shaver with a blade and it adheres to it spontaneously. The blade is equipped with an anti-drizzle plate which prevents it from rolling on itself while making it.After the cut, the remaining tissue fragment is recovered. According to the pathologist's instructions, this fragment is either placed in the tumor library in the state, or after depolymerization of the resin (at room temperature) placed on cassette, identified as an extemporaneous sample to be fixed in a formaline solution with the rest of the surgical sample.

Note: To avoid contamination betweentwosamples, change the shaver blade, decontaminate the plot and clean the cryostat compartment. Close the cryostat glass to keep the temperature at -20 °C.

Operating mode of a paraffin microtomy:

The operator rotates a motor wheel with the help of a handle. The shaver blade is fixed and the block moves vertically during the cuts. It undergoes two movements, one horizontal which determines the cutting thickness, the other vertical which makes it come into contact with the microtome’s blade. After changing the block or the position of the blade, it is necessary to make a new approximation between the block and the blade or even some adjustments.

Note: Risk of hands injury . Moving parts as well as the blade are a potential danger. Always put the drive wheel stop lever in a locked position when positioning or removing the blade from the block. Position the finger guard when handling the block to reduce the risk of injury . Be vigilant when wearing clothes. The microtome’s blade should never remain on the microtome in the absence of the manipulator. Stretch properly all the screws of the strapping lever.



Figure 7: Cryostat tissue cutting.

a. Fresh tissue sample deposited on a carrier.

b. Cold hardened tissue for cutting in a cryostat.

c. Manufacture of a microtome freezing cut (cryostat).

d. Blade recovery of the freezing cut

The expertise which must be gained to become a competent microtomist cannot be achieved from textbooks. Practical experience under the guidance of a skilled tutor is the best way to gain the confidence and coordination necessary to manipulate the microtome and the sections produced. Techniques will be described providing information and helpful hints for use during microscopy.

- **Setup of the microtome :**

Maintenance of the microtome is important to the production of quality slides for diagnosis. The manufacturer's recommendation regarding the proper care of the instrument should be closely followed. A departmental policy should be implemented outlining daily, weekly, quarterly and yearly preventive maintenance procedures. The water bath and the microtome should be ergonomically positioned to reduce stress and tension on the employee's neck and shoulders. The water bath may be filled with distilled or tap water and adjusted to the correct temperature for the paraffin wax. Care should be taken to reduce air bubbles which may distort the tissue section. The temperature of the water bath should be recorded and monitored throughout the day for quality purposes. The blade should be sharp and defect free. The blade or knife holder should be adjusted to optimize the clearance angle, the distance between the lower facet angle and the surface of the block face. The recommended angle varies from 2–4° for paraffin and 5–7° for frozen sections. The correct angle reduces friction as the blade passes through the block, preventing compression of the section. Determining the exact angle is largely a matter of trial and error. Clamps and screws must be firmly tightened. If a disposable blade is to be used, care should be taken to ensure enough pressure is being exerted on the blade to provide support, but it should not be over-tightened, as this causes thick and thin sectioning.

- **Sectioning**

Trimming the tissue blocks

The paraffin block may be faced or "rough cut" by setting the micrometer at 15–30 μm or by advancing the block using the coarse feed mechanism. Aggressive trimming will cause "moth hole" artifacts. Care must be taken to ensure that the block clamped in the chuck has been retracted so that there is no contact with the blade on the initial downstroke. It is possible to damage the tissue by gouging or scoring when trimming the block.

Cutting sections

Blocks should be arranged in numerical order on an ice tray or cooling mechanism, cooling both the tissue and the paraffin wax to a consistent temperature. A small amount of water is absorbed into the tissue causing slight swelling and making sectioning easier. Over-soaking may cause expansion and distortion of the tissue section. Proper processing greatly reduces or eliminates the need to pre-soak blocks.

Routine surgical material should be cut at 3–4 μm. The micrometer setting does not guarantee that each section will be that exact thickness. Thickness depends on many factors including temperature, knife angle and cutting speed. Experience will determine the speed of the stroke but in general, one should use a smooth, slow stroke. If there is difficulty cutting a smooth flat section, warming the block face with warm water, or gently exhaling breath onto the block surface during sectioning may help. This has the effect of expanding the block, giving a slightly thicker section. Ideally, successive sections will stick edge to edge, forming a ribbon. If the entire block is to be sectioned and retained, the ribbons are stored in a receptacle for future use.

Ribbons are the most convenient way of handling sections. When a ribbon of several sections has been cut, the first section is held by forceps or teasing needle and the last section eased from the knife edge with a small brush.

Floating out sections

The floating out of the ribbon must be smooth, the trailing end of the ribbon making contact with the water first. The slight drag produced when the rest of the ribbon is laid on the water is sufficient to remove most, if not all of the folds which occur. Sections are floated on the water bath shiny side down. Folds in the section may be removed by simply teasing with the forceps. Approximately 30 seconds should be long enough for a ribbon to flatten, longer on the water causes excessive expansion distorting the tissue. Individual sections or ribbons may be floated onto the slide. Circular structures such as eyes may be difficult to flatten. Various techniques are useful in these situations, e.g. placing the section on a slide which has been pre-flooded with 50% alcohol. The slide is gently immersed in the water bath and the section of eye will float on the surface. The presence of the alcohol will set up diffusion currents which help flatten the tissue section. The water bath should be cleaned after each block is cut, removing debris and tissue fragments by dragging tissue paper across the surface. Cleanlines cannot be overemphasized as debris from different sections (carryover) is a serious problem.

Drying sections

The small amount of water held under the section will allow further flattening to occur when heat is applied to dry the section. The temperature should be at the melting point of the paraffin wax. Automated stainers have integral drying ovens. Slides may be attached to the stainer with individual slide holders or in racks which are designed for the instrument. It is important to eliminate over-heating during the drying stage as cellular details may be compromised. Hot plates may cause localized overheating of the drying delicate tissues; less distortion will occur if the temperature is reduced and the time prolonged. Overnight drying at 37°C or room temperature is recommended for many tissues.

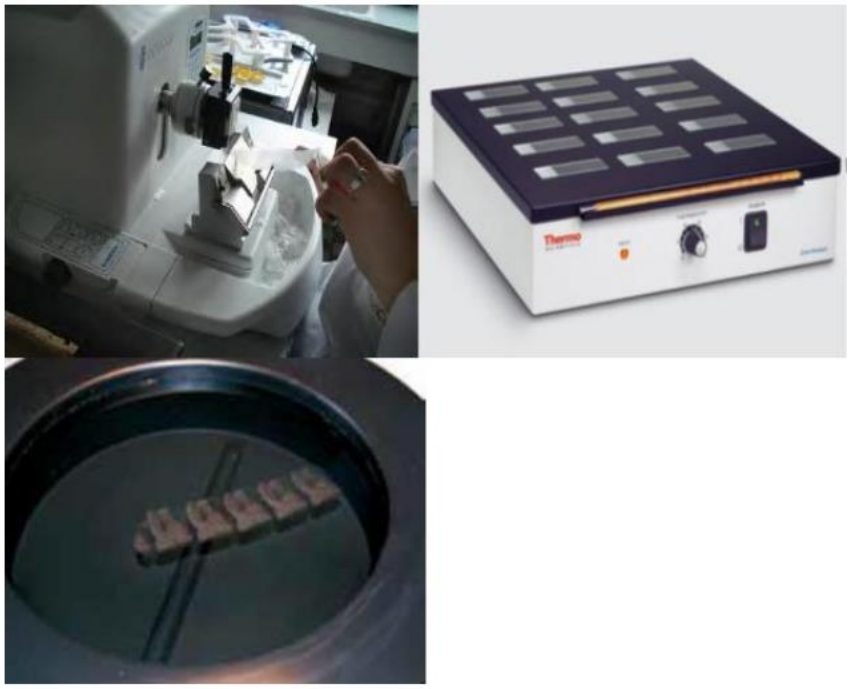


Figure 8: Making of ribbon with a microtome, drying of slides on a heating plate, spreading of ribbons in a water bath.

f) Staining:

The physicochemical mechanisms of most histological stains are now understood Histological staining methods from acid dyes to silver impregnation, involve broadly similar physicochemical principles.

Stain uptake is often due to dye-tissue or reagent tissue affinities. In the physicochemical literature, to say a tissue component has a high affinity for a dye merely means there is a tendency for a stain to transfer from solution onto a section and this concept is used here. The

magnitude of the affinity depends on every factor favoring or hindering this movement. The familiar stain-tissue attractions, including stain solvent and stain-stain interactions, can be influential, as can solvent-solvent interactions. This account initially assumes staining reaches equilibrium, and the consequences of this not being reached are discussed later. Moreover, uptake of dyes and reagents is often multistep in both space and time. Thus, a reagent may initially enter tissues due to coulombic attractions. Once inside it may form covalent bonds with some tissue grouping. The intensity of staining may also be influenced by stain solubility in solvent and tissue environments. Practical staining processes commonly involve several such factors. However, as histologists and histochemists often emphasize reagent-tissue attractions as affinity sources, these interactions are discussed first. Reagent-tissue interact

Body tissues are not spontaneously colored, making observations difficult. The stains used in histology are more or less selective; most are acid or basic components in aqueous media that form salts with ionized radicals from tissues.

Acidic components are used for basophilic tissue areas, and basic components are used for acidophilic tissue areas. The most commonly used coloring is HES: hematein/eosin/saffron. Hematine is a rather basic substance, which colours nuclei in purple thus colouring nucleic acids. Eosine is a rather acidic substance, which colours rather the cytoplasms in pink so colours the proteins. Finally, the saffron colored the collagen fibers in yellow.

However, However, in order to perform histological staining, some preparatory steps for staining are required

- **Slides drying**

To facilitate adhesion of the sections to the glass slide before the deparaffinization step, the slides must be dried.This drying eliminates (by evaporation) the film of water which is between the section and the slide. It is carried out in an oven whose temperature must be slightly lower than the melting point of the paraffin used .The slides racks are placed in a ventilated oven at 58°C for 1 hour.

- **Deparaffinization**

The first step of any histological sections coloring is to remove the paraffin needed so that the dyes can penetrate it. Exiting the oven, the slides undergo (in an automatic machine for good reproducibility) deparaffinization in order to eliminate the paraffin using a solvent (toluen,

xylen), then rehydration which consists of gradually replacing the tissue solvent with decreasing ethanol baths.

Since most of the dyes are usedin aqueous solution, their penetration can be ensured only if the sections are impregnated with water.The purpose of hydration is to remove the toluen and replace it with water. The slides must be completely submerged in clean toluen and ethanol baths (not contaminated with paraffin).The most common problems related to hydration are separation of sections and incomplete removal of toluene.

At the end of the deparaffinization cycle, the section appears transparent whereas impregnation with ethanol normally makes them opaque.

It is quickly observed whether the hydration has not been done properly because the tissue does not opacify in places where toluen remains.

- **Staining**

Hematoxylin and eosin (H&E) is the most widely used histological stain. It is simple to use, easy to automate and demonstrates different tissue structures clearly. Hematoxylin stains the cell nuclei blue-black, showing clear intranuclear detail, whilst eosin stains cell cytoplasm and most connective tissue fibers in varying shades and intensities of pink, orange and red. Automated staining machines and commercially prepared hematoxylin and eosin solutions are commonly used in today's laboratories for routine staining. Many laboratories now have to adhere to strict ISO standards to gain accreditation from regulating authorities. These standards cover validation of chemicals and stains used within the laboratory and variability between batches of 'inhouse' stains. These regulations and the underpinning paperwork have resulted in many laboratories using commercially produced reagents where validation is performed by the supplier. Students of histological techniques should have a basic knowledge of the dyes and their preparation techniques in order to troubleshoot and/or modify procedures for specialized use. Hematoxylin may also be used as a stain without eosin to demonstrate connective tissues such as elastic fibers, muscle striations and mucins.

Coloring the sections makes it possible to visualize the three main morphologicalconstituents of the tissues.T he most commonly used coloring is the hematoxyline coloring eosine saffron staining– HES

It provides information about the distribution, architecture, and structure of cells. The result of this trichrome staining is to combine a nuclear dye (blue nuclei), a cytoplasmic dye, and a connective tissue dye. (orange yellow).

Its main advantageis the orange-yellowcoloringgiven to collagen (major fibrous constituent of connective tissue) by saffron. Given the non-negligible cost of saffron, a bicromic method – the hematoxylineeosine(HE) staining – is sometimes used. The eosine tinted in rosenon only the cytoplasms but also the pale pink collagen and the elastic fibers in bright pink.HES is usually sufficient to carry a diagnosis but can be supplemented by special colorations.

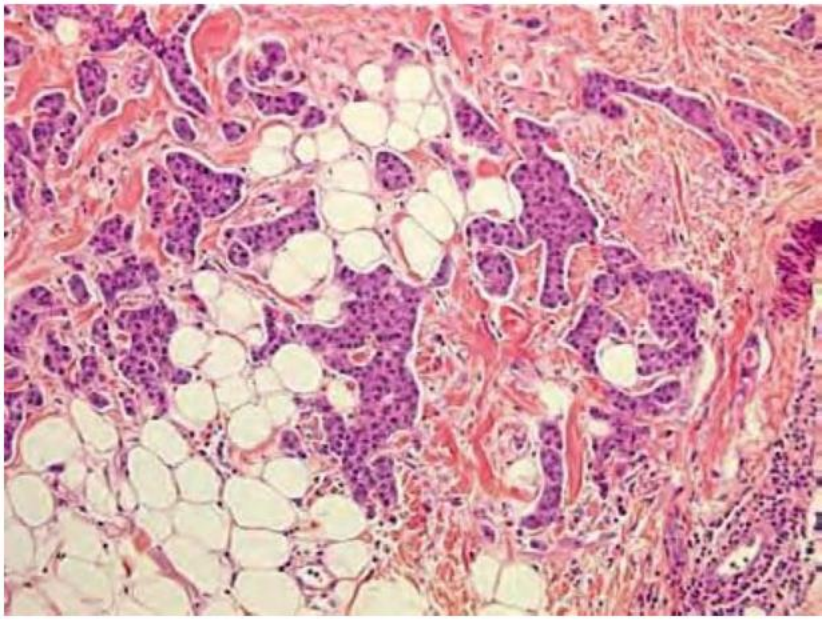


Figure 9: Microscopic appearance of hematoxylineeosine saffron HES. (breast, infiltrating ductal carcinoma of the breast).

- **Mounting:**

The slides are then mounted to preserve the stains, for this preparatory steps are necessary:

- Dehydration: This step prepares the slides after staining to make a resin mounting. The preparations undergo a dehydration that consists of removing the water from the sections

by successive baths of absolute ethanol and then a clarification in toluene baths, this being miscible with the mounting medium. This step must be quick because ethanol (especially in low concentrations) is likely to extract several dyes from the sectionts. The absolute ethanol of the last bath can be replaced by the isopropyl alcohol (isopropanol C3H8O, flammable liquid) which has less tendency to dissolve dyes, and which has the same properties as toluene, but is less harmful.

- Mounting:

This operation (automatic or manual) consists of fixing a slat on the slide with a synthetic resin to protect it from the chemical degradation of dyes which oxidize in the air and from mechanical breakage.

- **Microscopic observation:**

The pathologist judges whether the interpretation of the stained sections by HES is sufficient to establish a diagnosis or whether it needs to be supplemented by other techniques (histochemical and/or immunohistochemical). In some cases, the first section levels of a particular block or series of blocks are not informative to highlight lesional aspects. Therefore, a cross-section of the block or a macroscopy on the residues kept in reserve (which are identified as a second start) are requested.

- **Special tissue constraints:**

- Decalcification: In the study of bone tissue, its hardness requires treatment with chemical agents that allow calcium to be chelated. Decalcification facilitates the production of thin sections that can be exploited from a microscopic point of view.

The diversity of agents, so-called decalcifiers, allows to find an ideal compromise between the objective of the study and the time allocated to the decalcification process.

Example of decalcifier: E.D.T.A. 4.13% buffered at pH=7.4, nitric acid at 8%.



Figure 10: Kos Decalcification Microwave Automatic

- Fixation:

Fixation of certain organs takes much more time, for example, fixation of the encephalon is a long step, which requires 6 to 8 weeks of fixation.

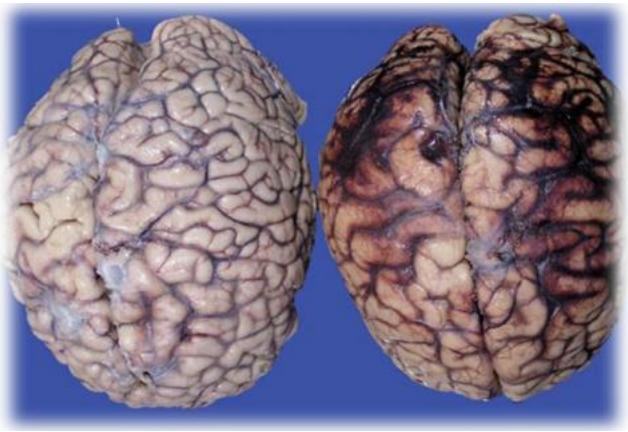


Figure 11 : A : Fixed brain. **B** : Non fixed brain

- Cryoprotection:

In the context of the study of the tissue that we wish to freeze, it may be necessary in some cases to practice cryoprotection. In fact, it will allow, through various substances, to prevent or delay the appearance of ice crystals: sucrose, dextran, glycerol, DMSO23 etc.

However, they can cause osmotic disturbances with their artifacts, if the tissues are little or not fixed.

Example of cryoprotector: Sucrose dissolved in P.B.S. pH=7.4 in progressive gradient.

2. Electron Microscopy:

Electron microscopy (ME) is based on exactly the same principles as optical microscope, but an electron beam replaces the photon beam and the samples are taken under vacuum; on the other hand, it uses magnetic lenses.

Tissue preparation involves the same major steps as in optical microscopy, but with adaptations due to the constraint of speed which is even more important for fixation.

A. Sampling:

It is imperative that the tissue be fixed as soon as it is taken. The doctor who wishes to request an electron microscopy study must ensure that there is fixative in his department.

. The tissue fragment must be quickly immersed in the fixative (glutaraldehyde at 2% stored at +4°C) to avoid osmotic shocks and contact with air that alter the cellular ultrastructure and sent directly to the pathological anatomy and cytology laboratory.

B. Fixation:

The fixative used is glutaraldehyde (several baths, 1 hour in total), and a post-fixation is usually performed with osmic acid (osmium tetroxide) in order to obtain better observations.

- First fixation in glutaraldehyde at 2%

Work under a hood. Using forceps, remove the sample from the pill bottle containing the fixative and place it on the Teflon plate. Cover the tissue with a few drops of fixative so that it does not dry out.

With a razor blade degreased with alcohol, cut into small fragments of 1 to 3 mm maximum. Using a plastic pipette, suck up the fixative and around twenty fragments to put them back in the bottle.

Note: for a tumor tissue, give preference to fragments concerning dense and whitish areas. Remove the hemorrhagic areas. Remove the rest of the sample in the eco-box. The razor blade is thrown into the sharp object boxes. Clean the teflon plate with the alcohol piss. Place the bottle containing the sample in the refrigerator for one hour.

There is no over-fixation with glutaraldehyde, the sample can possibly spend the night in the fixative if we cannot go further in the technique.

- Lung:

The air contained in the lung slows down the penetration of the fixative. The fixation's time must therefore be increased to two hours. If the sample is large enough, keep it submerged using a pin stuck in dental wax.

Once the fixation is complete, remove the tissue damaged by the pin.

- Brain:

Use a 3% glutaraldehyde solution and extend fixation up to four hours.

- Muscle:

- A muscle fragment is pinned to dental wax and dipped in fixative
- Put in the refrigerator for 20 minutes.
- On a teflon plate, remove the pins and remove dental wax.
- Cover with a few drops of fixative.
- With the help of a razor blade cut the two ends of the muscle fragment altered by the pins.

- Cut into five rod-shaped fragments of a maximum of 1 mm thickness
- Cut each fragment to a third of its length.
- We obtain five short rods for the longitudinal orientation of the fibers and five long rods for the transverse orientation.
- Place the bottle containing the muscle fragments to be fixed for one hour in the refrigerator

- Skin:

- Place the biopsy on the chopper blades, with the epidermis facing upwards.
- Slide the teeth of the comb between the blades.
- Press the biopsy.
- Collect the slices obtained using a pick
- Dip in Fixative
- Place the bottle containing the skin samples to be fixed for one hour in the refrigerator.

- Frozen sample:

- Immerse the frozen fragment in the fixative.
- Wait 30 minutes before cutting it into small pieces.
- Place the bottle containing the samples to be fixed for one hour in the refrigerator
- This technique gives an ultra-structural architecture of poor quality.

- Formalizedsample

:

- Cut the sample into small fragments
- Remove the fixative with a pasteur pipette.
- Fill the pill container with approximately 3 ml of rinsing liquid (0.1 M cacodylate buffer).
- Make 3 baths of 10 minutes each.
- The sample can stay 48 hours at +4°C in the rinsing liquid if we cannot go further in the technique.
- This technique gives an ultra-structural architecture of poor quality.

- paraffinembeddedsample :

- This technique gives an ultra-structural architecture of poor quality.

- Prefer the formalized reserve if a sample has not been taken for electron microscopy in the fresh state.
- Using a razorblade, take a portion of tissue from the histology block. Cut into $1mm^3$ fragments
- Make the following bath changes:

Deparaffinize in xylen for 1 hour

. Deparaffinize in xylen for 1 hour

Xylen/ethanol: 30 minutes.

Ethanol: 3x 20 minutes.

Ethanol+ 25% rinsing liquid: 20 minutes.

Ethanol+ 50% rinsing liquid: 20 minutes.

Ethanol+ 75% rinsing liquid: 20 minutes.

Fill the pill container with approximately 3 ml of rinsing liquid (cacodylate 0.1 M buffer).

Make 3 baths of 10 minutes each.

The sample can stay 48 hours at +4°C in the rinsing liquid if we cannot go further in the technique.

- Calcifiedsample:

- Place the sample for two hours in the refrigerator .

- Remove the fixative using a pasteur pipette.
- Fill the pill with approximately 3 ml of rinsing liquid (cacodylate buffer 0.1M+ sucrose 0.16M – PH 7.3 – 360 mOsm).
- Make 3 baths of 10 minutes each.
- Replace the last rinsing bath with 3 or 4 ml of decalcifier medium (EDTA 0.1M in a 0,1M cacodylate buffer containing 0.83% glutaraldehyde) and let the tissue demineralize for ten days in the refrigerator.

- Rinses in the cacodylate buffer 0.1M

- Remove the fixative with a pasteur pipette.
- Fill the pill container with approximately 3 ml of rinsing liquid.
- Make 3 baths of 10 minutes each.
- The sample can stay 48 hours at +4°C in the rinsing liquid if we cannot go further in the technique.

- Post-fixationin 2% osmium tetroxide

- Fix 1 hour at +4°C (do not exceed 1h30: risk of over-fixation)
- □ Remove the fixative with a pasteur pipette
- Rinse 5 minutes in distilled water
- □ Immerse the fragment in uranyl acetate at 0.5% If we cannot go further, a 72 hour storage is possible at 4°C.

C. Dehydration:

The dehydration is carried out in ethanol according to the following protocol:

- Ethanol to 70% in distilled water: 3x10 min at room temperature.
- Ethanol at 95%: 3x10 min.
- Pure ethanol (on silica gel): 3x20 min.
- Acetone/ethanol (volume/volume): 30mn.
- Pure acetone: 1 hour

Epoxy resin replacement (remove resin syringes from the freezer 1 hour before use):

- Standard impregnation:

- 1st bath: resin diluted to 50% in acetone = 1h (under stirring),
- 2nd bath: pure resin = 1hr (under stirring),
- 3rd bath: clean resin= 1h(under stirring) in a clean pill,
- 4th bath: fresh resin, not frozen = 1 night in clean pill and in the vacuum pump.

- Long-term impregnation for "hard" samples (epidermis, nerve, fibrous sample):

- 1 bath: 25 % resin diluted in acetone = 4 hours
- 2nd bath: 50 % resinediluted = 1 night
- 3rd bath: 75 % resindiluted = 4 hours
- 4th bath: pure resin = 1 night in the vacuum pump.

D. Embedding:

For the embedding, synthetic epoxy resins are used. These have the following advantages:

- stable to electronic bombardment,
- little shrinkage,
- uniform hardening.

The disadvantages are that these resins are very viscous and have limited compatibility with ethanol.

Operating mode:

- Bring the resin from the freezer 1h before use.
- Place the capsules on the racks.
- Pour 1 drop of resin at the bottom of each capsule.
- Place a sample fragment inside using a mounted tip.
- Fill the capsule up to 2mm from the edge.
- Insert on a strip of paper approximately 4mm wide over 2 cm long the Examination Number and a sub-number for the capsule.
- Generallymaketen capsules.
- Put the porters in the 60°C bowl for 48 hours for the polymerization of the resin.

- Let the capsules cool for at least two hours at room temperature before freezing.

Special cases:

- kidney biopsy: embed the entire biopsies.
- Muscle: embed 5 fragments (long sticks) in cross-section in special moulds.
- Skin: embed 20 fragments.

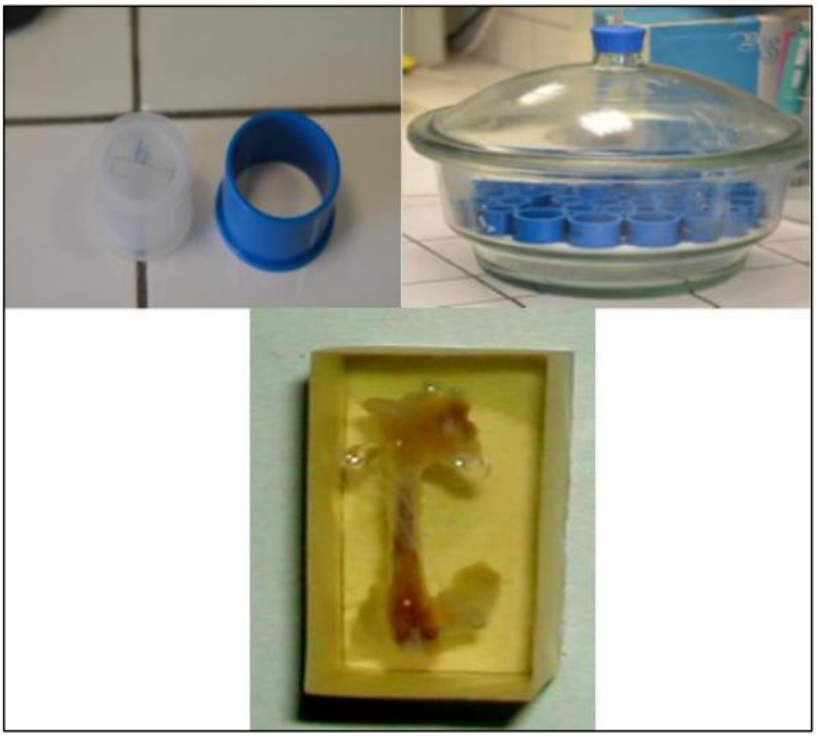


Figure 12: Resin embedding Epoxy

E. Ultra-microtomy:

The sections are made on an ultra-microtome with a glass or diamond blades: half-fine sections of approximately 0.5 to 1 mm thickness are obtained, as well as thin sections about 60 to 100 μm thick.

These sections are then placed on small copper grids measuring a few centimeters in diameter, and observation is made between the bars.



Figure 13:Ultra-microtome

F. Contrast:

There are no coloring techniques in electron microscopy, but tissue contrast techniques.

Uranyl acetate is frequently used to contrast nucleols and lead citrate to contrast membranes.

Note: : uranyl acetat and lead cytrate are salts of heavy metals.

G. Microscopic observation:

An electron microscope uses electron propagation which is associated with a much shorter wavelength than that of light. A shower of electrons is condensed on a sample part (from a few nanometers to a tenth of a millimeter).

A magnetic lens allows to form an image of the object with the electrons that interact strongly with the matter through.

The resolution power of the electron microscope (which can reach a magnification of × 5 000 000) allows a detailed study of the cellular and tissue ultrastructure. There are two variants of electron microscopy:

- The transmission microscope is a device that bombards an object with electrons that form a very enlarged image of the object in black and white on a fluorescent screen or a photographic plate. The emission of electrons is produced by heating a tungsten filament or a lanthanum hexaboride crystal

- A scanning microscope is a device that scans a sample of a beam of electrons to give a pseudo-3D image.

III. Different types of coloring:

There are two types of coloring, routine or topographic coloring and special or histochemical coloring.

Standard coloring

Standard dyes color a type of filler. They allow you to see the morphology of the cell, the position of the nucleus and its shape, the structure of a tissue. Thus, we can determine the number of cell types in the tissue. Depending on the specific conditions of each laboratory, the same method may have variations of technical processes, dye formulae, solution concentrations, time, etc.

A. Hematoxylin eeosine saffron (HES)

Signal coloring that highlights nuclei, cytoplasms and collagen fibers by capturing colorants selectively to get an idea of the overall topography of a tissue. Time of completion ≈ 3 minutes for rapid HES, 7 minutes for direct HES; 35 minutes with deparaffination for standard HES and 40 minutes for bone HES.

A trichrome method in which three colorants act in succession. an aqueous solution of haematoxyline (nuclear), an aquatic solution of eosine (cytoplasmic) and an alcoholic solution of saffron (collagen). Nuclear coloration is progressive. After hematoxyline (Mayer), a rapid differentiation in a saturated lithium carbonate solution (lithinated water) or simply with running water is carried out in order to blue the nuclei and facilitate the fixation of the dyes.

. The cytoplasmic coloration is regressive. After eosine, the excess is rinsed with running water, differentiation is carried out in successive baths of ethanol up to the absolute ethanol. Ethanol passages prepare the action of saffron which diffuses throughout the tissue through a phenomenon of absorption. At the end of coloring, the blades must be immersed in a solvent bath (toluene, xylene) for mounting. Under no circumstances should they dry in the open air, as the stains may precipitate into the tissue.

Results: The intensity of the coloring depends on many factors, including the relative proportion of stains, but above all the thickness of the tissue section. The coloration is more intense on thick sections:

• cytoplasm: pink (violet if it contains large amounts of RNA);

• nucleus: purple blue;

• collagen: orange yellow;

• muscle fiber: bright red;

• elastic fibre: pink;

•red blood cell: brightened red;

•mucus, basic substance of cartilage (chondroid) or bone (osteoid): oranged yellow.

Note: Eosine can be replaced by erythrosine, which gives a more vivid and stronger coloring than eosine.

Daily check the quality of the first coloring at the microscope. Filter the hematoxyline.Regularly change the alcohol baths (saturated with stains), the hematoxyline bath 2 times a week, the eosine bath 1 time a week and the saffron bath every 2 months, otherwise complete the level.

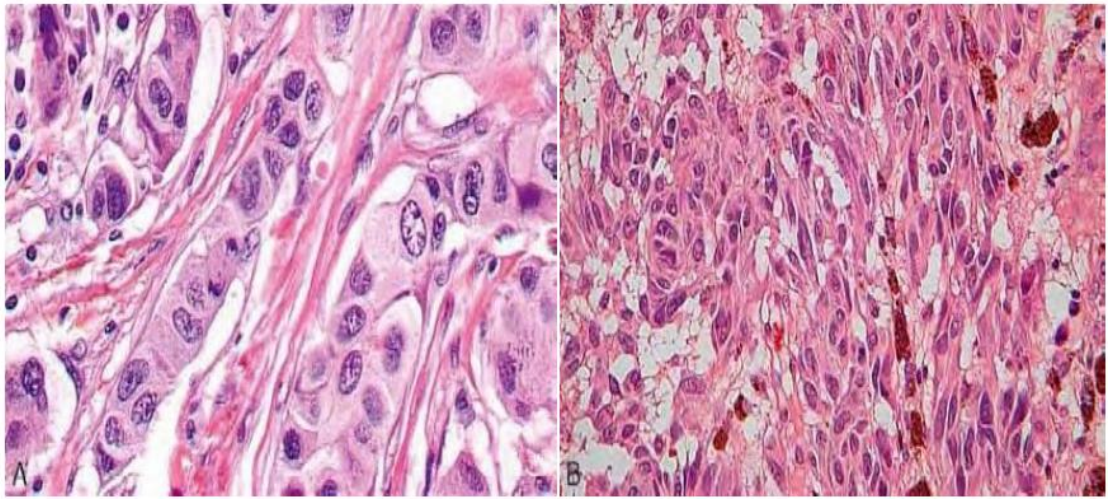


Figure 14:Hematoxyline Coloring eosine saffron HES, A: enlargement X40, B: presence of melanine

B. May-Grünwald-Giemsa (MGG)

Coloring that uses the different tinctorial affinities of May-Grunwald (Methylene Blue Eosinate) and Giemsa (Metylene Azure Eosinat) to color nuclei, cytoplasms and granulations in a highly nuanced way. These two stains are dissolved in methyl alcohol and are therefore inactive, it is the contact of water that gives them a coloring power. The salts then dissociate by dying red acid (eosine) and blue base (methylene blue azure). Methylene blue

eosinatecolours the cytoplasm. Eosinecolours the basic elements (acidophiles and eosinophiles) in pink, and the methylene blue colour the acidic elements (basophils) in blue.
Methylene azure eosinatestains the nuclei, azurophilic and basophilic granulations. For diluting stains, water must be buffered.
An acidic pH favors red hues, while a basic pH favors blue hues.
Time of completion ≈ 30 minutes.
Method that allows after fixation in methyl alcohol (solvent of dyes) the successive action of two neutral dyes. Each of them contains colored acidic (eosine) and basic (methylene blue and azure) ions that attach to the complementary cellular elements. At the end of coloring, the blades are rinsed with running water until the excess colorant is removed. The back of the shackle and the contours of the scrub are thoroughly wiped with absorbent paper. Let them dry in a vertical position on a door at room temperature. After complete drying, put them in a solvent bath (toluene) for mounting.
Results:
• cytoplasm:
– acidophilic: pink or light red,
– basophile: sky blue to dark blue;
• core:
– basychromatin: purple,
– oxychromatine: pink;
• nucleol: blue,
• red blood cell: greyish,
• keratin: intense Blue,
• mucus: pale blue.

NOTE: The use of urban water is strongly not recommended, because unpredictable and uncontrollable variations may occur compromising the result of the coloring, hence the interest of using a buffered water. Prepare the Giemsa solution daily. Regularly adjust the level of pure and diluted May-Grunwald baths and change them 1 time/month. Store these solutions in the refrigerator at +4 °C every evening.
Degrease the slides (except thin layer spreads) in toluene (under a fume hood in operation) before staining.

C. Papanicolaou staining

Reference pentachrome coloring in gynecological cytology and in epithelial cell examinations. It has the advantage of very finely drawing the detail of nuclear structures and highlighting cytoplasmic differentiation. Time of completion ≈ 30 minutes.

This method involves a polychromatic action resulting from the intervention of a nuclear stain in aqueous phase, the Harris hematoxyline, and two cytoplasmic stains in an alcoholic phase: the G orange and the polychrome solution (a mixture of green light, Bismarck brown and eosine).The coloration is based on the tinctorial affinity of the cells.

Harris's hematoxylinestains the nuclei in blue gray or purple according to DNA density, G orange stains glycogen in light brown and keratin in bright orange, and polychrome (EA 50) stains eosinophil cells' cytoplasm in pink and cyanophilic cells in blue.

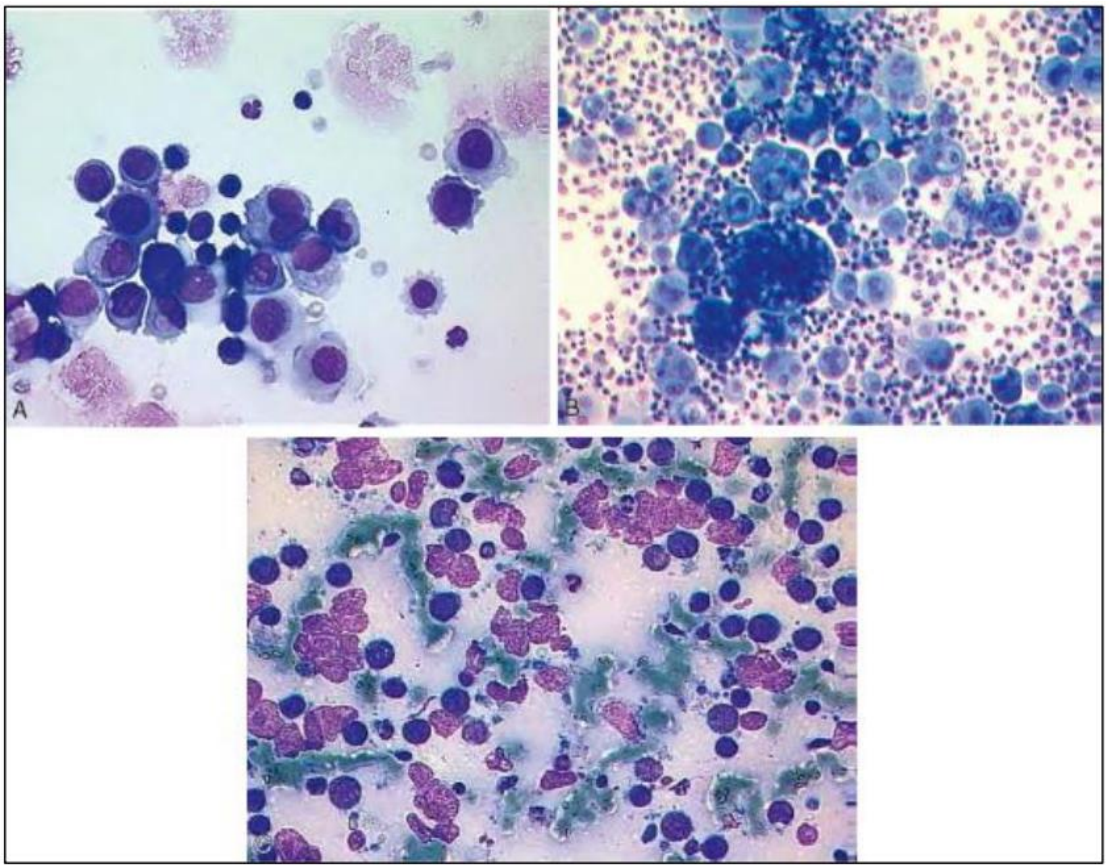


Figure 15: May-Grünwald-Giemsa (MGG).

a. Cytocentrifugation (Cytospin®) of a cerebrospinal fluid (carcinomatous meningitis).

b. Manual spreading after centrifugation of a pleural fluid (malignant mesothelioma).

c. Manual spreading of a lymph node aspiration (Burkitt’s lymphoma).

Rehydration is obligatory because it prepares the action of the nuclear stain. 37% hydrochloric water (a mixture of HCl and demineralized water) is used for nuclear differentiation. Ammonia alcohol (mixture of 3% ammonia concentrated in ethanol 70°) fixes the nuclear coloration and prepares the action of stains in alcoholic solution. At the end of coloring, the slides must be immersed in a solvent bath (toluene) for mounting.

Results:

• cytoplasm of cells:

– superficial (acidophile): pink to orange red,

– intermediate: blue-green,

– deep (basophile): darker green;

• nucleus: dark blue or purple;

• bacterium, Trichomonas: darkened blue;

• red bood cell: red orange;

• spore: slightly rosy;

• nucleolus: dark Blue;

• keratin: orange to bright orange.

Note: The aging of stains depends on the number of colored slides in the same solution baths. It is announced by the progressive disappearance of the tinctorial affinities of cytoplasms. If all the stains are changed, initially overly colored smears are obtained.

Filter hematoxyline daily. Regularly change alcohol and toluene baths. Complete the level of G orange and polychrome. Change the hydrochloride water and ammonia alcohol 1 time/month, otherwise adjust the levels.

Before staining, immerse the slides fixed with a spray in a bath of distilled water to remove the protective film.

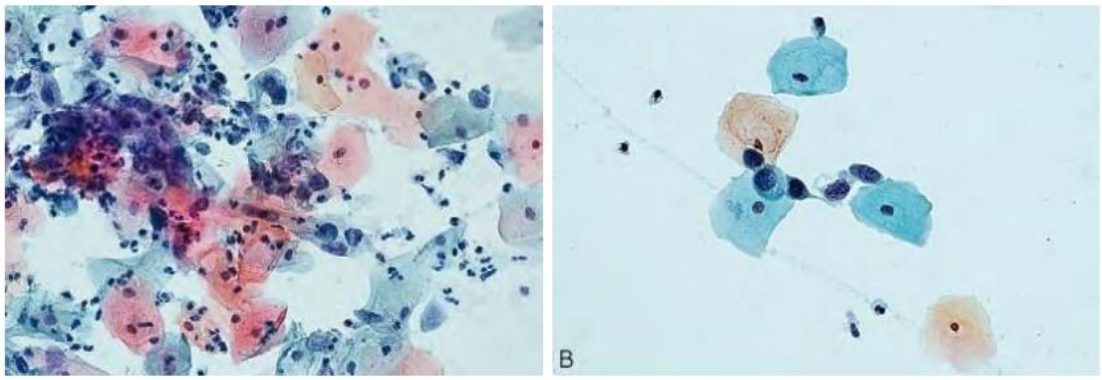


Figure 16: Cervical smear (high grade lesion) coloured by the Papanicolaou method.

a. Microscopic image of a conventional spreading.

b. Microscopic image of a thin layer spreading

D. Other commonly used stainings:

Histology has a considerable number of special staining and the variants possible techniques are almost endless.

- Alcian blue (BA)

This coloring allows for the highlighting of mucosubstances with an acidic character. A mucosubstance is considered a sugar-rich constituent.

• Use: mucosecreting adenocarcinoma, liposarcoma, myxoid tumor (myxoidchondrosarcoma).

• control : digestive (colon).

• Time of completion ≈ 40 minutes.

BA at a pH close to 1 colors sulfated carbohydrates (intestinal mucusa).

The BA at a pH between 2.5 and 2.6 colours carbohydrates with acidic carboxyl groups (extracellular matrix).

Counter stain with nuclear red, dehydrate and mount in resinous medium

Results:

• reactive substance: turquoise blue;

• bottom: pink;

• nucleus: red.

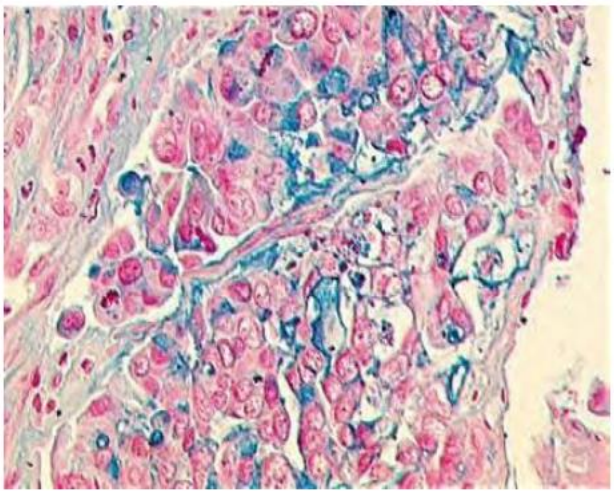


Figure 17: Alcian blue. Mucus secretions in turquoise blue (tissue section of an ovary).

- Giemsa Helicobacter staining

The Giemsa Helicobacter stain is a poorly differentiated Giemsa stain that allows spiral bacteria (Helicobacter) to be better seen.

Mount in a resin medium.

•utility: gastric cancers and lymphomas.

• Time of completion ≈ 35 minutes.

• Results: Helicobacter dark blue.

- Gram Coloring

This coloring allows the identification of several bacteria. Some germs remain insensitive to this staining, such as mycobacteria (the family to which the agents of tuberculosis and leprosy belong).

It is rarely used in tumor pathology.

• Gram (positive): Streptococcus, Staphylococcus.

• Gram (negative): pneumococcus.

• control: appendix, intestine.

•Time of completion ≈ 45 minutes.

Method based on the ability of certain bacteria to retain a violet crystal/ iodine complex (blue color) when differentiated by acetone or alcohol and dyed with a basic dye (fuchsine).

Mount in a resin medium. Iodine is a colour retention agent.

Results:

• Gram (positive) bacteria: blue;

• Gram (negative) bacteria: red;

• nucleus: Red;

• bottom: yellow.

- Grocott Coloring

Grocott is a method based on the high carbohydrate content of fungi walls and the identification of other micro-organisms.

• Use: bacteria (Actinomyces, Nocardia), mycosis (Candida), parasite

(Pneumocystis carinii).

• control: lung (fungi).

•Time of completion ≈ 2 hours.

The reactive groups of the carbohydrate component of fungi are oxidized by chromic acid and the released aldehydes are highlighted by reduction of a silver-methanamine complex (hexamethylenetetramine). Color the bottom with a light green solution and mount in resine medium.

Results

• fungi contour: black;

• internal part: grey;

• bottom: pale green;

• mycelial filament and yeast: dark;

• mucin: dark gray.

Note: Do not use metal objects.

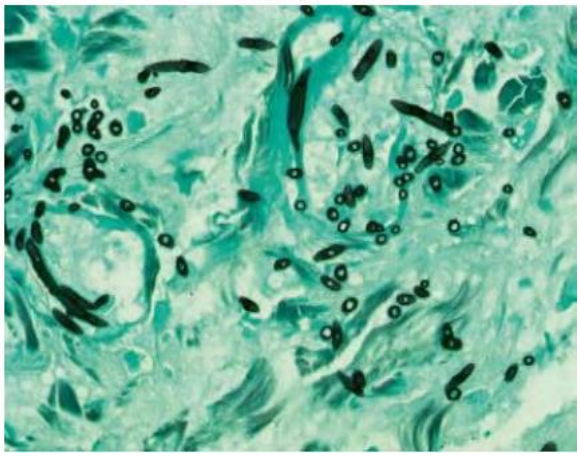


Figure 18: Grocott. Aspergillosis in black (tissue section of the lung).

- Periodic acid Schiff (PAS)

This coloring enables the identification of glycogen (storage form of carbohydrates in cells, such as hepatocytes and muscle cells), mucins (epithelial mucosubstances), neutral mucopolysaccharides (conjunctive muco-substances) as well as mycelial filaments and spores.

• Utility:

- chondrosarcoma, rhabdomyosarcoma, clear-cell sarcoma, alveolar soft tissue sarcoma,Ewingsarcoma, yolktumors.

Variable positivity incarcinomaswhich point towards a glandularorigin (adenocarcinoma).

- Mycosis (aspergillosis, candidiasis).

• control: digestive (colon), but this coloration has an internal control (polynuclear cells).

• Time of completion ≈ 1 hour. An oxidizing agent, periodic acid, is used to transform glycol groups into aldehydes. The Schiff reagent (basic fuchsin solution), decolorated by the sulfuric acid (or a running water wash) that reacts with the free aldehyde, forms a red-coloured condensation product. Counter-colour with hematoxyline, dehydrate and mount in a resin medium.

Results:

• reactive substance: pink to purplishred more or less intense

• nucleus: blue.

Note: Periodicacid (HIO4) is a selective oxidant that breaks bonds between two carbons of a number of chemical groups such as 1,2-glycol, 1,2-hydroxyl, amine groups. Too prolonged contact with periodic acid as well as a solution whose pH is too acidic results in false positives, because there is overoxidation of aldehydes to carboxyls.

Schiff's reagent must be perfectlyclear and colorless, otherwise throw it away. This solution is best stored in the refrigerator at + 4°C in a bottle that is opaque to light and tightly stoppered.

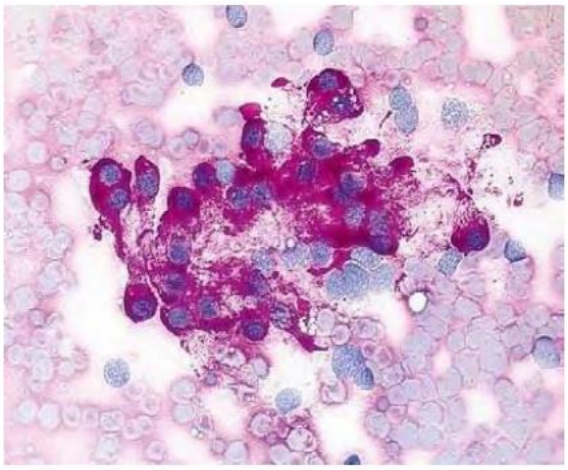


Figure 19: PAS positive. Intracytoplasmic glycogen in purplish pink

- *Periodic acid Schiff* /blue alcian (PAS/BA)

The PAS/BA coloring is a combined coloring in which the PAS highlights the neutral carbohydrates and the BA, the acidic carbohydrates.

• Utility: colon injury.

• control: digestive (colon).

• Time of completion ≈ 1 hour.

The differentiation is made at the level of the weakly sulfated acidic mucopolysaccharides colored by BA, and the neutral mucopolysaccharides colored by PAS.

Counter-colour with hematoxyline, dehydrate and mount in a resin medium.

Results:

• acid polysaccharide PAS (negative): blue;

• neutral polysackcharide: red;

• nucleus: dark blue.

- Perls staining

This staining allows the highlighting of ferric deposits (Fe+++) in erythroblasts and macrophages (siderophages)

• Utility: pulmonary embolism, resolving hematoma, differential from other pigments.

• control: resolving hematoma, spleen.

•Time of completion ≈ 1 hour.

In an acidic environment, iron ions react with potassium ferrocyanide to form an insoluble precipitation.

This precipitation of ferric ferrocyanide, of blue colour (Prussian blue), highlights a pigment, hemosiderine.

Counter-colour with nuclear red, dehydrate and mount in resin medium.

Results:

• Ferric iron and hemosiderin: blue to blue-green;

• nucleus: red.

Note: Potassium ferrocyanide and hydrochloric acid mixtures must be prepared in advance and have a yellow coloration. Throw away any solution with a slightly greenish tint. Do not use metal objects.

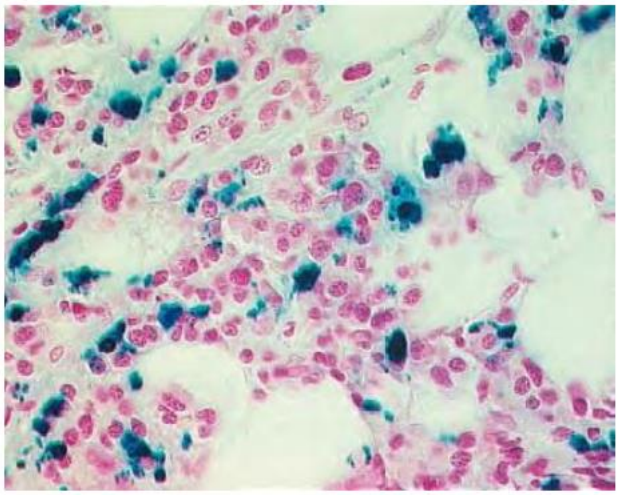


Figure 20: Perls. Ferric overload in blue (macrophages).

Osteomedullary tissue section.

- Reticulin

Reticulin allows the demonstration of reticulin fibers in the stroma (based on the argyrophilia of the fibers) and clarifies the architecture of tumors.

Reticulin fibers appear as very thin, highly branched, anastomosing fibrils (lattice fibers) under a light microscope

They can be more or less tight mesh networks.

• Utility: hemangiopericytoma, synovialosarcoma.

•The samples have an internal control.

• Time of completion ≈ 1 hour.

The oxidation of reticulin fibers means that they become capable of reducing the silver salts that precipitate on contact. The precipitate is stabilized and in solubilized (like a photograph) by a gold salt and sodium thiosulfate.

Counter-colour with nuclear red, dehydrate and mount in resin medium.

Results:

• reticulin fiber (collagen type III): intense black;

• collagen fiber I: purple brown;

• nucleus: pink red.

NOTE: Do not use a solution of ammonia silver nitrate that is more than 10 days old and metal object.

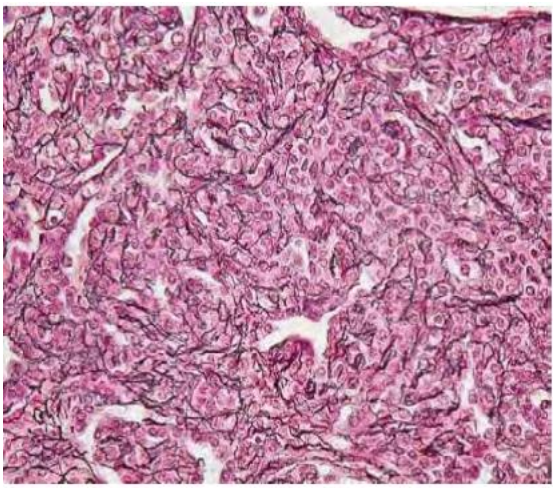


Figure 21: Reticulin. Black reticulin fibers. Pulmonary tissue section

- Trichrome of Masson staining

Masson's Trichrome allows the demonstration of collagen fibers (these fiber sappear as wavy, non-anastomosed bands under the optical microscope). This Trichromic staining is used to obtain brighter contrasts between cytoplasms and collagen

• Utility: leomyosarcoma (longitudinal striations), rhabdomyosarcome(transversal striations).

• This coloring does not require a control.

• Time of completion ≈ 50 minutes.

The successive combination of a nuclearlake (hematoxylin), a cytoplasmicdye (weakacidfuchsin/xylidine ponceau) and a connective tissue dye (aniline blue or light green), both strong acids, gives a good contrast.

Dehydrate and mount in a resin medium.

Aniline blue often gives a slightly more pasty coloring but allows better visualization of the fine collagen fibrils; it is generally used for neuropathy. Light green is more transparent and is more suitable for kidney and digestive biopsies.

.

Results:

• collagen: blue or green (depending on the colorant used);

• cytoplasm: pink to red (pink muscle and pink-green liver);

• nucleus: dark blue or brown;

• red blood cell and eosinophilic granulation: red;

• mucus: blue to green (depending on the colourant used);

• keratin: bright red.

• elastic fiber: pink

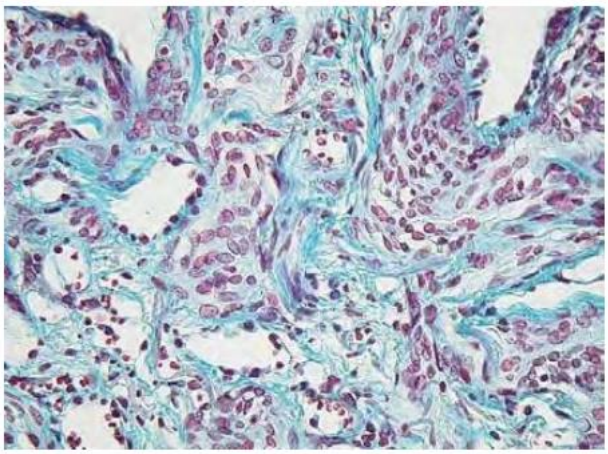


Figure 22: Masson's trichrome . Collagen and mucus in blue.Pulmonary tissue section

- Von Kossa staining

Von Kossa staining allows calcium salts to be highlighted. The specificity of this method is focused more on calcium phosphates and carbonates, the other anions not being highlighted. Abnormal accumulations of calcium are found in degenerating elastic fibers, as well as in certain specific lesions (caseatingl esions of tuberculosis).

Time of completion ≈ 50 minutes.

Revelation of calcium salts (carbonate, oxalate, phosphate, sulfate) by substituting a heavy metallic cation (silver nitrate) which is visualizedafterreductioninto black metallic silver.

Counter-colour with nuclear red, dehydrate and mount in resin medium.

Results:

• Calcium salts deposit: black;

• bottom: pink-red.

- Ziehl-Neelsen staining

The Ziehl-Neelsen stain allows the demonstration of certain microorganisms (in Particularly mycobacteria) on their acid-alcohol-fast characteristics.

• Utility: detection of bacteria (nocardiosis, tuberculosis), mycosis (aspergillosis).

• control: granuloma (tuberculous follicle).

•Time of completion ≈ 30 minutes.

Coloring of certain germs with a mixture of phenol and basic fuchsin followed by the action of ethanol with hydrochloric acid. Certain germs (Koch and Hansen's bacillus) resist the

bleaching action of hydrochloric alcohol (they are called acid-alcohol-fast), the other germs being bleached.

Counter-colour with methylene blue, dehydrate and mount in a resin medium.

Results:

• acid-fast (tuberculosis) bacilli: red;

• nucleus: dark blue to blue-green;

• bottom: pale blue.

Note: The alcohol/acid differentiation is mainly used to highlight Mycobacterium tuberculosis.

III. IHC immunohistochemistry and ICC immunocytochemistry:

Immunohistochemistry (IHC) staining revolves around sequential, well-defined steps, time intervals and prescribed temperatures so the whole process is ideal for automation. Indeed, in most laboratories this now happens in one form or another. Many different systems exist which can perform some, or all of the multistep process. The instruments are a combination delivery system with or without heat, controlled by computer software. Slides may be stationary or mobile and either in a linear or rotary fashion, whilst reagents are delivered via pipettes from reagent containers/ pre-packed cartridges. Some platforms also include deparaffinization, multiple antigen retrieval options and counterstain. All of these enable a wide range of immunohistochemical stains to be produced.

Automation of immunohistochemical techniques has been a major step in improving quality efficiency and reproducibility of results. Most IHC automated systems include flexibility for continuous flow and user defined steps. Some are capable of performing all the steps within the process, making them a true ‘walk-away’ system requiring far fewer man hours than previous manual or partly automated systems. Workflow features such as independent slide draws and reagent racks are essential if a ‘first-in-first-out’ workflow system is desired, and work can be continuously added and sent out. Integration of the platform’s software with the LIMS system can allow a full patient tracking package and remote requesting options whilst also removing the need for constant re-labelling of slides. More advanced platforms are available with an expanded range of functions, such as in situ hybridization and direct immunofluorescence.

Immunochemical techniques are powerful techniques for determining protein expression in cancer cells. They complement the standardized methods in pathology. Immunohistochemistry (or “IHC”) is the name of a method of localizing proteins in the cells

of a tissue section, by the detection of antigens using antibodies. Immunohistochemistry exploits the fact that an antibody binds specifically to antigens in biological tissues. Antibodies can be of polyclonal or monoclonal origin.

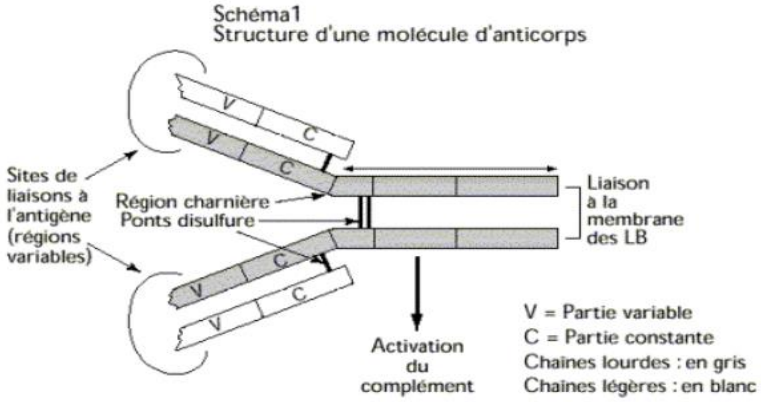
Immunohistochemistry is now a routine and invaluable procedure in the histo- pathology laboratory for the detection of many microorganisms. There are many commercially available antibodies for viral, bacterial and para- sitic organisms. Most methods today utilize (strept) avidin-biotin technologies. These are based on the high affinity that (strept)avidin (Streptomyces avidi- nii) and avidin (chicken egg) have for biotin. Both possess four binding sites for biotin, but due to the molecular orientation of the binding sites fewer than four molecules of biotin will actually bind. The basic sequence of reagent application consists of primary antibody, biotinylated secondary anti- body, followed by either the preformed (strept) avidin-biotin enzyme complex or the avidin-biotin complex (ABC) technique or by the enzyme-labeled streptavidin. Both conclude with the substrate solution. Horseradish peroxidase and alkaline phosphatases are the most commonly used enzyme labels.

A. Interest and definitions:

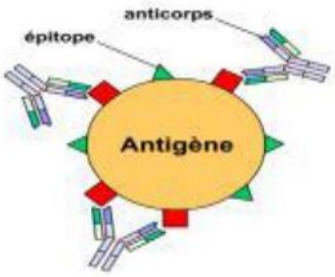
The advantage of this technique is to identify antigens (most often proteins) of interest at the cellular or extracellular level using specific antibodies.

An antigen is a molecule capable of activating the immune system and leading to the production of antibodies.

An antibodyis a glycoprotein, secreted by plasma cells, capable of specifically binding to the antigen.



On appelle épitope le site de l'antigène où se fixe l'anticorps.



Liaison anticorps-antigène

Figure 23: Antibody-antigen

B. Principles of immunohistochemistry and review of definitions:

• To exploit the immune response of various species to produce specific antibodies.

• achieve antigen-antibody-specific binding.

• Detect (visualize) the antigen-antibody complex.

An antigen usually has several epitopes.

Depending on the configuration of the antigen, an epitope may not be accessible to the antibody.

Several antibodies can bind to the same epitope with different affinities.

The same epitope can sometimes be present on several antigens.

The main difficulty of IHC is to have a good specific antibody capable of reaching its epitope on section.

Immunohistochemistry (IHC)

A technique for identifying cellular or tissue con- stituents, antigens, by means of antigen-antibody interactions. The site of antibody binding is identi- fied either by direct labeling of the antibody or by use of a secondary labeling method.

Antigen

An antigen is a molecule which induces the forma- tion of an antibody and bears one or more antibody- binding sites. These are highly specific topographical regions composed of a small number of amino acids or monosaccharide units known as antigenic deter-

minant groups or epitopes.

Antibody

Antibodies belong to the class of serum proteins known as immunoglobulins. The terms antibody and immunoglobulin are often used interchangeably. They are found in blood and tissue fluids, as well as many secretions. The basic unit of each antibody is a monomer; antibodies can be monomeric, dimeric, trimeric, tetrameric, or pentameric in format. The monomer is composed of two heavy and two light chains. If it is cleaved with enzymes such as papain and pepsin, two fragment binding antigens (Fab) fragments and a crystallizable fragment (Fc) are produced. They are formed in the humoral immune system by plasma cells, the end cell of B-lymphocyte transformation, after recognition of a foreign antigen. There are five types of antibody found in the blood of higher vertebrates: IgA, IgD, IgE, IgG, and IgM.

IgG is the most common and frequently used anti- body for IHC. The IgG molecule is composed of two pairs of light and heavy polypeptide chains linked by disulfide bonds to form a Y-shaped structure. The terminal regions of each arm vary in amino acid

sequence and are known as ‘variable domains’. This variability in amino acid content provides specificity for a particular epitope and enables the antibody to bind specifically to the antigen against which it was raised.

There are two types of antibodies: polyclonal antibodies and monoclonal antibodies.

Specific antibodies can be made by repeatedly injecting a sample of the antigen (protein to be detected) into an animal (most often a rabbit or goat), and then collecting the antibody-rich serum (antiserum). This antiserum contains different so-called polyclonal antibodies, produced by different plasma cells, recognizing various antigens of the protein of interest.

• Advantages: simple, cheap.

• Disadvantages:

- risk of cross-reactions (non-specific signal);

- limited quantities of serum.

A monoclonal antibody corresponds to a population of identical ntibodies directed against the same antigenic site of a protein. These antibodies are produced in large quantities in culture by a clone of B lymphocytes using the hybridoma technique. After immunizing a mouse against a given antigen, B lymphocytes are taken from its spleen. These are fused with immortalized tumor plasma cells. After selection, the hybridomas thus obtained are a permanent and stable source of a single type of monoclonal antibody. The specificity of monoclonal antibodies is higher than that of polyclonal sera, but their sensitivity may be lower.

• Advantages: only one antibody per clone (limits cross-reactions);

• Disadvantages: long, expensive, uncertain results

Antibody-antigen binding

The amino acid side-chains of the variable domain of an antibody form a cavity-like site which is geometrically and chemically complementary to an antigen epitope molecule, as described by Capra and Edmundson in 1977. The analogy of a lock (antibody) and key (antigen) has been used, and the precise fit required explains the high degree of antibody-antigen specificity seen. The associated antibody and antigen are held together by a combination of hydrogen bonds, electrostatic interactions, and van der Waals' forces.

Affinity

Affinity is the three-dimensional fit of the antibody to its specific antigen, and is a measure of the binding strength between the antigenic epitope and its specific antibody-combining site

Avidity

Avidity is a related property referring to the heterogeneity of the antiserum which will contain various antibodies reacting with different epitopes of the antigen molecule. A specific but multivalent anti- body is less likely to be removed by the washing process than a monovalent antibody. Avidity there- fore is the functional combining strength of an antibody with its antigen.

Antibody specificity

This is the characteristics of an antibody to bind selectively to a single epitope on an antigen.

Sensitivity This is the relative amount of antigen which an IHC technique is able to detect. A technique with high sen- sitivity is able to detect smaller amounts of antigen than a technique with low sensitivity. If used to detect the same amount of antigen, the technique with high sensitivity would produce a larger signal than a method with low sensitivity.

Principle of an immunochemical technique: the ABC method

The technique uses a primaryantibodydirectedagainst the targetantigen to identify the proteinssought in the cell. Wethen use a biotinylatedsecondaryantibodydirectedspecificallyagainst the primaryantibody. An avidin–biotin complexcoupled to peroxidaseallows amplification of the signal duringdetection by diaminobenzidine (DAB).

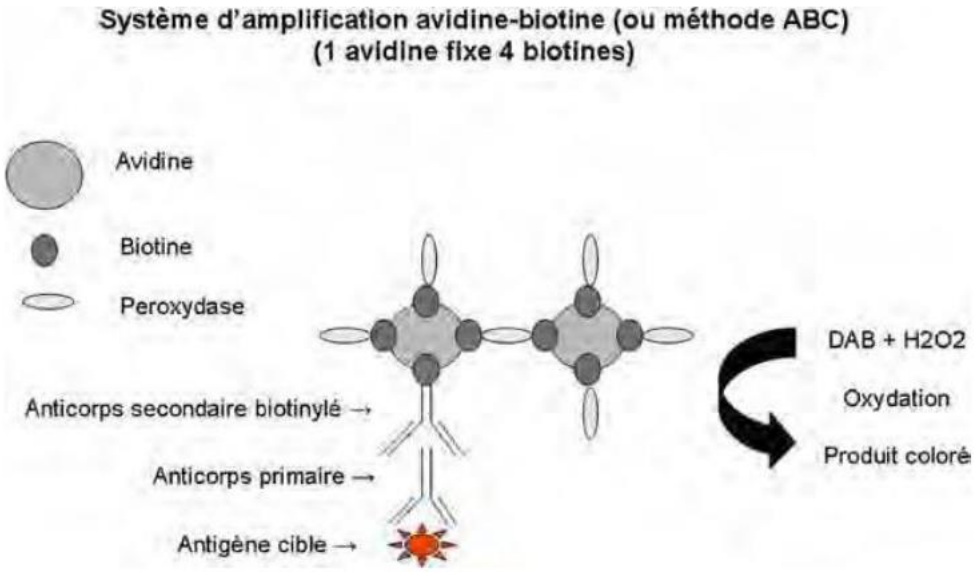


Figure 24 : ABC amplification method

Rinse solution:

Throughout the handling, washing and rinsing steps are necessary either in demineralized water or in buffered solutions of different molarity and pH, suitable for automated or manual marking.

Detergents, such as PBS and TBS, minimize nonspecific absorption of antibodies. Detergents are amphiphilic molecules, that is to say they have a hydrophilic part (charged or not) and a hydrophobic part.

Non-ionic detergents are particularly low denaturing and are used when we want to preserve protein activity.

Detergents containing a wetting agent, Tween 20 or triton, such as PBT which is PBS Tween 20, allow gentle permeabilization of the antigenic sites (creation of pores in the membrane phospholipid layer) because the antibodies are not capable of penetrating significantly. significantly inside or on the surface of cells, and reduce non-specific bindings

Technical recommendations

• For better adhesion of the section on the glass slide, preferably use special blades (positively charged). Then, fix the sections on the slides by spending one hour in a ventilated oven at 58°C, followed overnight at 37°C before deparaffination.

• All reagents must be balanced at room temperature (20–25 °C) before immuno-marking.

• The pH of the solutions must be titrated with a pH meter (previously calibrated).

• All incubations must take place at room temperature unless otherwise specified.

• Do not allow preparations to dry during the marking procedure (increased risk of non-specific marking). The samples can be surrounded by a ring of hydrophobic material to prevent dispersion of the reagents.

• For manual handling, place the slides in a humid environment (room humid) in a horizontal position during prolonged incubations.

• After blocking non-specific sites, do not rinse, remove excess reagent from the slides.

For proteolytic enzymatic treatment, the pH of the solution must correspond to the pH-action of the enzyme used.

• For heat unmasking in the microwave eoven

– Place the blades in a thermo-resistant microwave oven(s) containing the buffer solution;

– distribute the tanks in the enclosure of the calibrated microwave oven, wait for 97–98°C without touching the boiling point and maintain this level

– let the slides cool in the buffer outside the microwave at room temperature for 20 minutes ;

–Rinse the slides in rinsing buffer solution before continuing with the technique.

Determining the origin of technical problems

Immunochemical techniques are generally automated and computer-controlled. But the preparation of cellular or tissue samples and solutions is carried out manually, therefore operator/dependent. When there is a problem, you have to know how to identify it.

Note: Sodium azide (NaN3) is a highly toxic chemical agent in its pure state. It is an inhibitor of peroxydases. Do not use it in buffers revealing these enzymes.

Main Antibody Families

The main antibody families are defined by their target (the corresponding antigen).

• Intermediate filament

The intermediate filaments (IFs) form with the tubulin microtubules (24 nm in diameter) and the microfilaments or actin filaments (5 to 8 nm in diameter) the different architectures of the cytoskeleton (complex network of protein filaments contained in the cytoplasm of all cells). They group together five classes of proteins.

Actin is an important protein for cellular architecture and movement. It is present in all cells of the body and especially in muscle cells.

Vimentin and keratins are important proteins that are manufactured by cells with variable but often narrow specificity. Vimentin is responsible for maintaining cell shape, cytoplasm integrity, and stabilizes cytoskeletal interactions. It ispresent in most mesenchymal cells (fibroblasts, chondrocytes, vascular endothelial cells).

CK cytokeratins (8–10 nm in diameter) correspond to intracellular fibrous polypeptides present in virtually all epithelial cells.

There are around twenty different ones in humans. They are classified and numbered according to their molecular weight (pm) and their isoelectric point, and can be divided between an acidic subfamily (type I) numbered from 9 to 20 and a basic or neutral subfamily (type II) numbered from 1 to 8.

Each epithelium and each tumor corresponds to a particular cytokeratin profile.

Note: co-expression of keratinis possible with vimentin in glandular epithelia.

Vimentinis positive in most connective tumors, melanomas, mesotheliomas, certain non-Hodgkin's lymphomas (NHL) and carcinomas (endometrium, salivary glands, kidney, breast, thyroid). The detection of cytokeratins of different molecular weights is of great interest in the typing of certain tumors and/or their metastases.

Pancytokeratin KL1 (broad-spectrum antibody) is positive in many malignant tumors

: carcinoma (embryonalcarcinoma, neuroendocrinalcarcinoma), undifferentiated carcinoma of the nasopharynx (UCNT), choriocarcinoma, mesothelioma, epithelioid sarcoma, synovialosarcoma, teratoma, Merkel celltumor, desmoplastic tumor, germinal tumor, yolktumor).

Pancitokeratine AE1–AE3 (a cocktail of two monoclonal antibodies: Ac AE1 recognizes acid keratins and AcAE3, basic keratines) is used to identify epithelial differentiation in an undifferentiated tumor.

Carcinomas are almost always positive for anti-cytoceratin antibody cocktails (pancytoceratin: KL1 or AE1–AE3). Some cytoceratin subtypes may be better for diagnosis and in particular the CK 7/CK 20 profile.

• Surface Antigen

Most leukocyte typing CDs (cluster of differentiation = class of differentiation) allow the identification of the phenotype of a cell population and the recognition of the lymphoid cells involved.

CD molecules are divided into three main groups: markers expressed throughout the life of the cell, markers expressed transiently during a differentiation phase, and markers expressed when the cell is activated.

This is a complex area; more than 2000 CDs exist, some being made up of several proteins. Each class of differentiation antigens is followed by a number whose classification is based on an international nomenclature.

• Immunoglobulin (Ig)

Non-clustered antibodies for the heavy chains of immunoglobulins (anti-Ig A, G, M, D) and the light chains of immunoglobulins (anti-Kappa, -lambda) are used to detect the monoclonality of B lymphoid proliferation.

• Adhesion molecule

These are glycoproteins of the cell membrane which ensure the adhesion of cells to each other or to the extracellular matrix. They include cadherins, integrins and selectins.

• Tumor antigen (oncofoetal or neoantigen)

They highlight cellular constituents that are characteristic or relatively characteristic of one or more types of cancer.

• Others

PS 100 is a protein 100% soluble in ammonium sulfate, hence its name. It marks very diverse cells but can be useful to confirm the nervous or melanocytic origin of a malignant spindle cell tumor.

• Enzyme

- NSE (neuroenolase specific enzyme) to detect small cell lung carcinomas, Neuroblastoma, Feochromocytoma, Retinoblastoma, Merkel cell tumor, desmoplastic tumor, neuro-ectodermal tumor, neuroendocrine tumor.

- PAP (prostatic acid phosphatase) for Prostate carcinoma, germinal tumor

- PLAP (placental alkaline phosphate) for Embryo carcinoma, Ovary Dysgerminoma, Seminoma or germ cell tumor

• Cell differentiation marker

Calretininbelongs to the calcium-related superfamily. It is useful for the diagnosis of mesotheliomas. GCDFP 15 (grosscystic disease fluid protein 15) is useful for the diagnosis of apocrine carcinoma of the breast. Nuclear positivity of TTF 1 (thyroid transcription factor 1) points towards pulmonaryadenocarcinoma (primary or small cell neuroendocrine carcinoma of the lung) or thyroid (medullary, papillary and vesicular).

The positivity of thyroglobulin in thyroid carcinomas may allow secondarily to separate these two origins.

• Oncoprotein

Oncoproteins are proteins translating genes, some of which are expressed during cancers (oncogenes) while others are depressed or absent (anti-oncogenes):

• Bcl 2 is an oncogene involved in apoptosis pathways. It has the characteristic of being a powerful inhibitor of programmed cell death.

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• Bcl 6 (proto-oncogene) codes for a transcriptional repressor involved in the formation of germinal centers, B and T lymphocyte maturation, the cell cycle and apoptosis. Expression of Bcl 6 is observed with variable frequency in certain lymphomas of the B phenotype (lymphoma follicular, Burkitt lymphoma)

• CD 117 (c-kit oncogene) is a transmembrane protein with tyrosine kinase activity. Mutations are observed in digestive stromal tumors (GIST);

• EGFR (epidermal growth factor receptor) is highly expressed in squamous cell cancers of the head and neck, upper aerodigestive tract (AVDS), colon, pancreas, ovary and bladder;

• HER-2 is a transmembrane glycoprotein with tyrosine kynase activity. This protein is expressed in breast but also ovarian, colon and pancreatic cancers;

p53 is a protein involved in DNA repair or the induction of apoptosis after cellular stress. It is found in small quantities in normal cells but in large abundance in malignant tumors. Deletions or mutations of p53 are observed in almost 50% of cancers;

• WT 1 is a gene that codes for a transcription factor. It plays a critical role in the normal development of the kidney and gonads. Mutations in this gene cause kidney cancer in children (Wilms tumor).

Cell proliferation markers

They mark cells engaged in the cell cycle

• Ki 67 is expressed throughout the cell cycle, making it possible to evaluate the cell fraction in the growth phase. It may be useful to help determine the degree of malignancy of certain tumors such as digestive tract stromal tumors. A high Ki 67 index indicates that cancer cells are dividing rapidly

• alteration of p53 expression can be found in virtually all cancer types.

- Forecast marker

These are factors whose value helps to determine the evolutionary potential of cancer:

• the expression of ALK (anaplastic lymphoma kinase) is correlated with a good prognosis of anaplasmic T lymphocytes;

• Bcl 2 expression is corred with a poor prognose of large-cell diffuse B lymfoids;

• the over expression of HER-2 (Cerb B2) is cornered with a bad prognosi of invasive breast cancers.

- Predictive marker

They are used to determine the potential status of response to targeted antitumor therapy:

• the determination of hormonal receptors (estrogen and progesterone receptors) is carried out mainly on breast lesions in order to evaluate hormonal sensitivity with a view to specific treatment. The nuclear expression of the hormonal receptors is carried out by a semi-quantitative analysis.

The positivity of the receptors to estrogens and/or progesterone receptors (positivity threshold> 10% of labeled tumorcells) is associated with a better response to treatment with hormonal therapy.

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• The absence of Cerb B2 (HER-2) amplification (by FISH technique) is correlated with a lack of response to HerceptinR treatment in breast cancers.

C. Immunohistochemistry technique:

- Direct immunofluorescence on freezing section is a very specific method that avoids all non-specific reactions due to antibody attachment to immunoglobulin Fc receptor, provided the antibody is used at optimal dilution. But it is a non-sensitive method (no signal amplification) and requires the use of antibodies coupled to a fluorescent chromogen (Fluorescein, Rhodamine, Red Texas....).

Interest: Enables easy double marking and avoids endogenous peroxidase problems.

The marking is easy in time: special mounting environments eliminate the deletion of the marking.

- For the revelation of indirect immunohistochemistry, the enzymes used are peroxydase or alkaline phosphatase and the chromogens are most often diaminobenzidine (brown reaction) or aminoethylcarbazole (red reaction).

In the indirect immunoenzymatic method there is a risk of non-specific marking by attaching the secondary antibody to the immunoglobulin Fc receptor, so these non-spectical fixation sites must be blocked during the technique.

The intensity of the signal obtained after marking an antigen-antibody reaction depends on the number of visible colored molecules

Several amplification mechanisms are possible, including three-layer methods, especially since the divalent intermediate antibody can also be used to attach a reactive complex: peroxidase-antiperoxydase, avidine-biotin peroxydase (alkaline ophosphatase) and streptavidin-biotin-peroxidase

The number of reactive molecules can be multiplied by attaching them to a polymer, or amplification can be generated by creating a complex that multiplies the enzyme molecule attached non-covalently to the antibody. (tyramide).

Amplification can be achieved by increasing the incubation time and also by pre-treating the deparaffinated sections by heat or enzymes.

The use of frozen tissues or tissues fixed and included in paraffin has advantages and disadvantages:

• freezing:

need to have fresh tissues, a storage system (at least -80 °C), cryostatic sections; morphology less well preserved than on sections fixed and included in paraffin.

The main interest is to be able to visualize epitopes destroyed by fixation and inclusion processes. Nevertheless, immunohistochemistry indications on freezing sections tend to decrease, on the one hand due to the manufacture of new antibodies recognizing fixation-resistant epitopes, and on the other hand thanks to antigen detection techniques (microwave, , water bath) and ultra-sensitive detection systems.

• fixed tissues included in paraffin:

the advantages are the use a posteriori when the tissue has been fixed and the possibility of retrospective studies (on blocks or on slides) as well as a well preserved morphology.

The choice of the fixative is important (as a rule prefer the buffered formalin), because some epitopes are destroyed by certain fixatives (alcoholic mainly) and the possible decalcification.

Avoid excessive temperatures during the inclusion and drying of slides. Antigen unmasking (pre-treatment that breaks the molecular bonds created by the fixative and alters the spatial configuration of the epitopes and their accessibility to the antigens): Enzymatic digestion or more often heating of sections in a microwave oven , pressure cooker, or water bath.

IHC operating model:

The following procedure can be used as a guide (tissue sectionts fixed and included in paraffin):

1. sampling;
2. fixing (buffered formalin);
3. paraffin embedding;
4. sections making;
5. fixation of sections in oven;
6. deparaffination;
7. rehydration;
8. Pre-treatment: combined antigen restoration if necessary (enzymatic digestion followed by heat treatment);
9. Inhibition of endogenous peroxydases by H2O2 at 3%
10. Incubation of a normal horse serum that saturates the non-specific epitopes of the sought antigen;

11. incubation of the primary antibody that is fixed on the specific epitope of the sought antigen;

12. incubation of the biotinylated secondary antibody that is attached to the constant part of the primary antibodies;

13. Incubation of the complex consisting of avidine, biotin and peroxidase. The free sites of avidine attach the biotins present on the surface of the secondary antibody;

14. Incubation of the chromogenic/substrate DAB solution to reveal the Ag/Ac reaction site created;

15. slight hematoxyline counter-colouring that makes topographic determination of markings possible;

16. dehydration in increasing alcohols to reach toluene (or xylene);

17. mounting;

18. observation

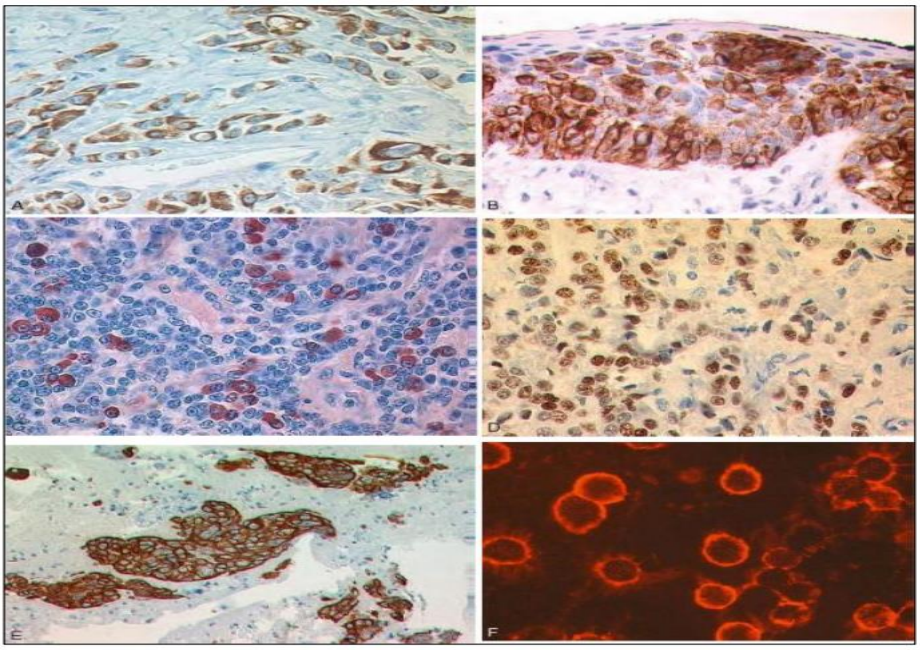


Figure 25: IHC. a.Cytoplasmic anti-cytoceratin KL1 marking(malignant mesothelioma), peroxydase/DAB. b. Anti-HMB 45 cytoplasmicmarking (melanoma), peroxydase/DAB. c. Anti-IgM cytoplasmic marking (lymphoma), peroxydase/AEC. d. Intranuclear anti-estrogen receptor marking RO (Matt Carcinoma), peroxydase/DAB. e. CB: cytoplasmic marking of anti-cytoceratin AE1–AE3 (epidermoid carcinoma metastasis), peroxydase/DAB. f. Fluorescent red membrane mark (quantum dot 605 nm) anti-HER-2/neu, no counter-colouring.

For immunocytochemistry, the operation mode is significantly identical. Except:

• the post-histological fixation stages;

• the antibody concentration adapted according to the fixative used;

• thin-layer spreading require for some nuclear epitopes (before the technique) additional fixation extemporaneously in a mixture (glacial acetic acid/absolute ethanol).

The fixed cell preparations are rehydrated in PBS. Depending on the antigen sought, they undergo antigenic restoration by heat and follow the same technical process. Enzymatic digestion should be prohibited as it leads to significant cellular decomposition and alterations.

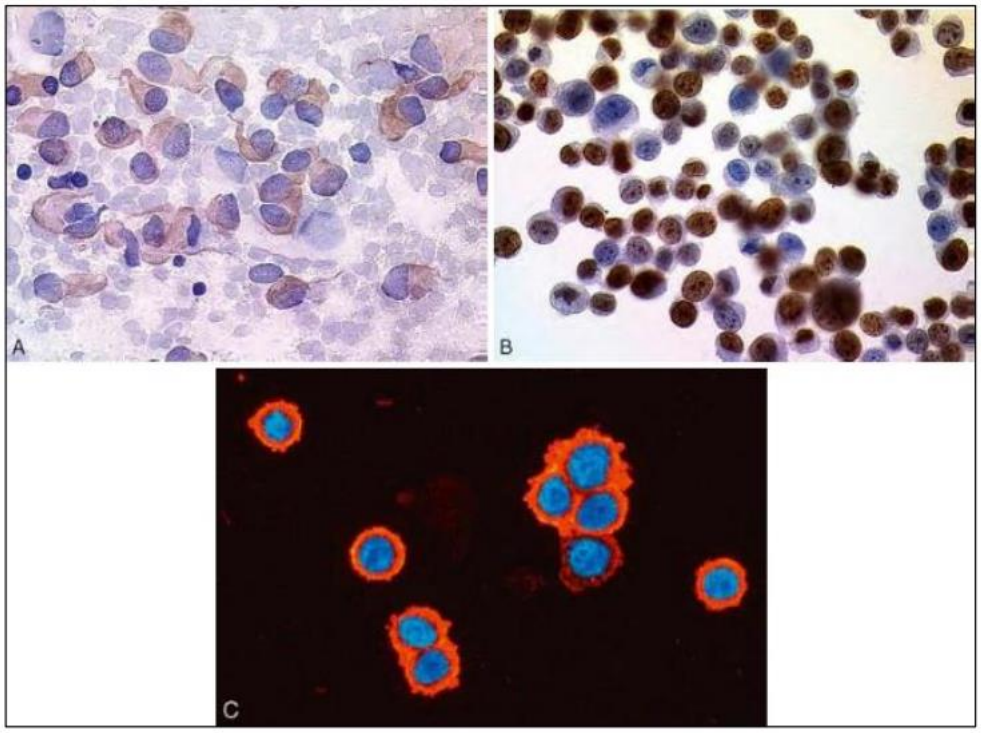


Figure 26: ICC. a.: cytoplasmic anti-desmine (rhabdomyosarcoma), peroxydase/DAB marking. b. BCL: intranuclear anti-Ki 67 (mammary carcinoma), peroxydase/DAB marking. c. Fluorescent red membrane marking (quantum dot605 nm) anti-HER-2/new, nuclear counter-colouring by the DAPI.

The value of IHC on cytological preparations has been established. Acetone-fixed smears or cytospins are often preferred by the immunohistochemist, as this allows a wide range of primary antibodies to be employed without destroying the target epitopes. Many cytology laboratories still insist on fixing cytological preparations in alcohol as opposed to acetone, and consequently the number of demonstrable antigens may be limited, but the morphology

may be superior. Where cytological samples are cellular enough, there is also the option of producing a cell block which can then be processed into paraf- fin wax. This gives greater flexibility for the number and range of tests which can then be performed on the sample and also provides archive material.

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