A SYSTEMATIC REVIEW AND META-ANALYSES OF LABORATORY DIAGNOSTIC TECHNIQUES INMEDICAL PATHOLOGY

Esoh Rene Tanwieh[1*], Tata Elvis Fon[2], Solomon Gyampoh[3]

ABSTRACT

Systematic reviews and meta-analyses are generally accepted to represent the highest level of evidence, and are a corner stone in practicing evidence-based medicine. So far, these efforts have been largely confined to the evaluation of the efficacy and effectiveness of therapeutic andpreventive interventions. Systematic reviews in laboratory medicine are scarce and many of them do not meet quality criteria. [clin chem. Lab. Med. 38(2000) 577]. Most of these problemsare related to the poor design and heterogeneity of primary research, and that there are no agreedmethods or quality standards of making systematic reviews in laboratory medicine. Laboratory techniques in histopathology and cytology are the foundation of the diagnostic pathology. It isextremely essential to know all the basic and advanced techniques in laboratory. This article discusses the principles, steps, and troubleshooting areas of all the essential laboratory techniques in both histology and cytology laboratories. It contains multiple information's that explain the techniques. In addition to the various advanced techniques, microscopy in the laboratory have been discussed. Histological procedures aim to provide good quality sections that can be used for a light microscopic evaluation of human or animal tissue changes in either spontaneous or induced diseases. Routinely, tissues are fixed with neutral formalin 10%, embedded in paraffin, and then manually sectioned with a microtome to obtain 4–5 Mm-thick paraffin sections. Dewaxed sections are then stained with hematoxylin and eosin (H&E) or can be used for other purposes (special stains, immunohistochemistry, in situ hybridization, etc.). During this process, many steps and procedures are critical to ensure standard and interpretablesections. Key recommendations are given here to achieve this objective. I hope that the article will help all the students in pathology, practicing pathologists, and laboratory technologists.

KEY WORDS: Systematic Review, Clinical Pathology, Medical Laboratory Techniques/Technology, Histological/Chemical And Cytological/Chemical Techniques

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Systematic reviews The need for systematic reviews

Healthcare providers, consumers, researchers, and policy makers are inundated with unmanageable amounts of information, including evidence from healthcare research. It is unlikely that all will have the time, skills and resources to find, appraise and interpret this evidence and to incorporate it into healthcare decisions. Cochrane reviews respond to this challenge by identifying, appraising and synthesizing research-based evidence and presenting it in an accessible format (Mulrow 1994).

What is a systematic review?

A systematic review attempts to collate all empirical evidence that fits pre-specified eligibility criteria in order to answer a specific research question. It uses explicit, systematic methods that are selected with a view to minimizing bias, thus providing more reliable findings from which conclusions can be drawn and decisions made (Antman 1992, Oxman 1993). The key characteristics of a systematic review are:

- a clearly stated set of objectives with pre-defined eligibility criteria for studies;
- an explicit, reproducible methodology;
- a systematic search that attempts to identify all studies that would meet the eligibilitycriteria;
- an assessment of the validity of the findings of the included studies, for example through the assessment of risk of bias; and
- a systematic presentation, and synthesis, of the characteristics and findings of theincluded studies;

Many systematic reviews contain meta-analyses. Meta-analysis is the use of statistical methods to summarize the results of independent studies (Glass 1976). By combining information fromall relevant studies, meta-analyses can provide more precise estimates of the effects of health care than those derived from the individual studies included within a review. They also facilitateinvestigations of the consistency of evidence across studies, and the exploration of differencesacross studies.

Medical laboratory technology

Medical Laboratory Technology is fast developing along with growing population and technological advancement. It is the most sought job titles in the global Health Care System. Medical Laboratory Technology is a broad area comprising of different disciplines like Clinical Pathology, Hematology, Biochemistry, Bacteriology, Immunology, Virology, Mycology, Parasitology, Histopathology, Cytology, and Cytogenetics & Molecular biology. In a country like ours, where fast and tremendous technological advancement and population growth happens, the demand and supply of trained man power is not on par. Introduction of a certificate course in Medical Laboratory Technology at higher secondary level is the remedy to this majorskill gap in the country. Medical Laboratory Technology plays a crucial role in the diagnosis of diseases, prognosis and treatment. Apart from the laboratory diagnosis, application of medical laboratory technology extends to detection of genetic disorders, epidemiology of infection diseases, detection of metabolic disorders and even to answer unraveled questions in forensic medicine.

Clinical pathology

A change that takes place in the human body during the process of disease is always reflected in the chemical composition of body fluids. Clinical examination of these fluids reveals the presence of abnormal constituents, altered cellularity, microorganisms and other physical evidences. These evidences from a clinical pathology lab provide endless support to a physicianin reaching an early and accurate diagnosis. Apart from the common importance like that of any other laboratory investigation, its importance is paramount in the sense that it includes most of common clinical investigations that are routinely done in a clinical laboratory. Hence an adequate and appropriate understanding of the accurate procedure of these investigations is very essential for a technician. Lack of sufficient automation, decreased sensitivity in microscopy and less specific chemical reactions in the absence of enzyme chemistry are some of the inherent limitations of clinical pathology analysis. Even though the advances in fibro-optic technique enables a pinpoint observation of lower respiratory tract and gastro intestinal tract, the basic analysis of sputum and stool samples still remains in mainstay and so the case of other samples.Easy availability of samples, rapid results, and reasonable precision justifies the need of a clinical pathology lab in a hospital and in the curriculum too. Reporting of positive abnormal finding of the clinical pathology results are important equally to knowledge about the absence of abnormalities for correct diagnosis of a disease.

Histological/chemical and cytological/chemical techniques

Histopathological technique is that branch of biology concerned with the demonstration of minute tissue structures in disease. Since the differences between diseased and normal tissues

are often slight, it follows that the majority of the methods involved may be used for both. Before such structures can be demonstrated the tissue must be prepared in such a manner that it is sufficiently thin (one to two cells thick) to be examined microscopically, and that the many and complex structures which go to make up tissue may be differentiated. This differentiation is usually achieved by selective coloring, and, since it is impossible to demonstrate all these structures in one preparation, methods are employed which stain one or more in each section orslice of tissue. The term 'histochemistry' has come into prominence in recent years as the studyof the chemistry of tissue components by histological methods, and it is probable that the impetus given in the post-war years to this type of method, with its greater accuracy and control, is responsible for the impression that it is of recent origin. In fact, Raspali, in 1830, wrote an essay, Essai de Chimie Microscopique Applique a la Physiologie, which is generally accepted as the beginning of recorded histochemistry. Mann (1902) said 'the object of all staining is to recognize micro chemically the existence and distribution of substances which we have been made aware of macro chemically. It is not sufficient to content ourselves with using acid and basic dyes and speculating on the acid or basic nature of the tissues, or to apply color radicles with oxidizing or reducing properties. We should find staining reactions which will indicate thepresence of certain elements such as iron, phosphorus, carbohydrates, nucleus or protamines, and so on.' Mann's words are as cogent today as they were 55 years ago when they were written.There are special methods of preserving and preparing the tissue in mass, known as fixation, which precede the special staining methods employed. This process of fixation is used even when tissue or body fluid is smeared on glass slides. When blocks of tissue are to be examined,they must, after fixation, be cut into thin slices or sections. In order that such sections may be cut and manipulated they are normally impregnated and embedded in a firm medium, usually paraffin wax. The various methods of examination of tissue cells and structures are summarizedin this article.

Cytopathology is a discipline that involves the morphologic study of cells. It is broadly vided into exfoliative cytology and aspiration cytology.

Exfoliative cytology involves examination of specimens that contain cells exfoliated from body cavities and surface. It is further subdivided into gynecological cytology (Pap/cervical smears) and non-gynecological cytology (pleural fluid, peritoneal fluid, cerebrospinal fluid, urine, sputum, brushing, etc.).

Aspiration cytology involves examination of cells that are actively obtained by fine needle aspiration.

INTRODUCTION

Histopathology is the microscopic study of diseased tissue. It is an important investigative medical tool that is based on the study of human or animal histology (also called microscopic anatomy). It is performed by examining a thin tissue section under light microscopes. Histotechnique consists of a number of procedures that allow visualization of tissue and cell microscopic features and recognize specific microscopic structural changes of diseases. Observation of tissues under a light microscope is an old concern for science and medicine. The earliest evidence of magnifying glass forming a magnified image dates back to 1021 when the physicist Ibn Al-Haytham (965–1039) published the "Book of Optics." The name "microscope" was crafted by the German botanist Johann Faber (1574–1629). The light microscope used by Anton Van Leeuwenhoek's (1632–1723) was a small, single convex lens mounted on a plate.Nowadays, sophisticated light microscopes use multiple lenses and are widely used in researchand in diagnostic. Most histology techniques were described in the nineteenth century. They mostly use physicochemical reactions with tissue components that allow preservation, cutting or staining of tissues. It is only in the 1970s that the processing of tissue has become partiallyautomated, but some critical steps such as embedding and sectioning are still manual. Thesesteps are delicate and time and resource intensive. Histopathology evaluation basically compares diseased or experimentally altered tissues with matching sample from healthy or control counterparts. Therefore, it is very important to rigorously standardize every histology process (i.e., specimen sampling, trimming, embedding, sectioning, and staining). The purposeof this article is to describe some routine histological processing steps used for histopathological evaluation and, in particular, paraffin embedding, sectioning, and staining. Several othertechniques are available and could be performed on tissues for specific purposes (special stains, immunohistochemistry, in situ hybridization, etc.)

Tissues can also be frozen or embedded in plastic, but these latter techniques are beyond the scope of this article. Many/other websites can be consulted to get further protocols in histological techniques.

The Role of Histopathology Specimens

Histopathology specimens are a vital cornerstone in patient care. They not only establish a tissue

diagnosis but are crucial in clinical management decisions and provide important prognostic data. They are nodal events in a patient's illness shaping the choice of relevant medical and surgical therapies and determining follow-up strategy. The data they provide are used to assess the efficiency of current and new investigation and treatment regimes and to monitor the impact of population screening programmes. Clinical governance has recognized their key role in auditing not only individual clinicians but also the patterns and quality of overall health care provision. Biomedical research with advances in investigations and therapy would flounder without them. They are therefore a precious resource to be handled with great care by sufficient numbers of appropriately trained and experienced personnel. The data generated are of a confidential nature privy to the patient, consultant clinician or general practitioner, and the reporting pathologist. This information may be shared as appropriate with other directly involved health care professionals, for example, in the context of multidisciplinary team meetings, but laboratory practice (e.g., telephoned results and report authorization) must be geared to protect patient confidentiality at all times. The patient not only has a right to see and have explained the information in his/her specimen but must undergo a process of informed consent prior to the clinical procedure. Thus, the nature, purpose, extent, and side effects of the procedure are explained in understandable terms. This process extends to the laboratory as patients can express their wish for disposal and use of the tissue not only for diagnosis but also for educative, audit, and research purposes. Additionally, research projects should be verified by an appropriate research ethics committee. Patient denial of any of these uses must then be communicated to the laboratory and incorporated into the handling and disposal procedures. The histopathology specimen report forms a permanent part of the patient's medical record and as such may be used as medico-legal evidence in negligence and compensation cases. These various factors serve to emphasize the importance of the care that should be taken with these specimens by histopathology laboratory personnel.

The Handling of Histopathology Specimens

Specimen transportation, accession, clinical prioritization, dissection, audit, and reporting are considered

Specimen Transportation

There must be close liaison between pathology and clinical staff to ensure appropriate transportation of specimens between the outpatient department, operating theatre, and the laboratory, for example, prompt transport of fresh specimens or the provision of special fixatives. This must be reflected in shared protocols, a user information manual, and educationof the clinical and pottering staff.

Specimen Accession

Allocation of a unique laboratory number and accurate computer registration of patient details are fundamental to maintenance of a meaningful and practicable histopathology database. This is important not only to individual patient care (e.g., a sequence of biopsies) but also for provision of statistics, for example, download to cancer registries.

Specimen Prioritization

With ever increasing workload and limited staffing resources, pathologists may find it necessaryto put in place a specimen pull-through protocol related to clinical need to ensure that diagnostic results are available within an appropriate time frame. This can be based on various criteria such as specimen type and request form information. Suggested overall turnaround times for histopathology specimens are 80% and 90% of cases reported within 7 and 10 working days, respectively, subject to individual case needs and in agreement with local clinical teams.

Specimen Dissection

Traditionally the role of a medical pathologist specimen dissection is now also being performedby an increasing number of biomedical scientists (BMSs) as has been the situation for several decades in some laboratories in America (Pathologist Assistants) and the UK. BMSs, trainee, and consultant pathologists are all appropriate to the task provided that several principles are adhered to:

- The histopathology specimen and its report remain the overall responsibility of the reporting consultant pathologist.
- There is close proximity and ready availability of active consultant pathologist supervisionbefore, during, and after handling of the specimen.
- There is workforce stability and staff are prepared to work together as a team. The working unit comprises a variable combination of two people (junior/senior, medic/BMS) fulfillingthe roles of dissector/writer/supervisor with active overarching consultant pathologist supervision.
- Staff recognize that acquisition of dissection skills is an at-the-bench apprenticeship based on sufficient knowledge, time, experience, and supervision. This knowledge base requires insight

into normal anatomy, clinical presentation, and investigations relevant to request form information, common pathological conditions, and their effect on specimens, surgical considerations in production of the specimen, and core report data tailored to patient management and prognostic information. Consequently, the information in this article is structured accordingly under these headings. The cut-up supervisor plays a vital role in passing on verbal knowledge but this is supplemented by various means, for example, publications (inhouse protocols, ACP broadsheets, College datasets, and textbooks) or training courses. A structured training programme facilitates learning and progression. Staffmust also be familiar with the laboratory process of checking patient details, specimen labelling, and past history (cytology, biopsy, and treatment), the importance of specimen opening for adequate fixation, demonstration of resection margins, and use of macroscopic and microscopic digital photography. Knife etiquette and sampling blocks of appropriate thickness and fixation are crucial. The supervising pathologist must provide active feedbackas to the significance and adequacy of these blocks. Line diagrams are an invaluable communication tool between dissector and reporters. Specimens not infrequently need to be revisited prior to report authorization or following new information gained from the multidisciplinary team meeting. Retention of "wet" specimens must be sufficiently long (minimum 4 weeks) to allow this process to happen.

- Dissectors should only work to their individual level of experience and competence— this is determined by the structured training programme, audit process (see below), and categorization of specimens according to their complexity.
- Dissectors should actively seek supervisor input if a specimen is complex, novel, shows anunusual variation on a usual theme, or if they have any doubt.
- The principles and a working practice of surgical cut-up are referred to in Appendices.

Specimen Dissection Audit

The quality of specimen dissection must be meaningfully monitored, and the majority of this is done actively at the laboratory bench by the consultant pathologist/BMS supervisor team as part of the specimen dissection pre-/ peri-/post-view and reporting feedback procedures. In addition, this team should carry out formal periodic audit and assessment of dissectors' skills.This combination of approaches forms the basis for an individual dissector's continued practice and progression between specimen categories. It also identifies the areas of subspecialist expertise or in need of further training. It must be recognized that category progression cannot be proscribed by rigid time frames but rather related to the aptitude of the individual dissector and spectrum of workload that is encountered.

Specimen Reporting

Histopathology specimen reports remain the responsibility of an appropriately trained and experienced medical pathologist. Increasingly Royal College of Pathologist Cancer Datasets are mandating key audit data to assess the standards of specimen dissection and reporting, for example, colorectal cancer mean lymph node harvest and the reported percentages of serosal and extramural vascular involvement by tumor. Other key service quality indicators include pathologist participation in relevant interpretive histopathology external quality assurance (EQA) schemes and appropriate continuing professional development (CPD) activity. These issues are discussed at annual appraisal and are foundational to medical revalidation. The overall quality of a surgical pathology service depends on a number of key performance indicators summarized in a Royal College of Pathologists document (Key performance indicators—proposals for implementation. http://www.rcpath.org/). They include availability and timeliness of clinical advice, participation at multidisciplinary meetings, coding of histopathology reports, use of cancer resection report proformas, documentation of second opinions, results transmission, communication of critical and unexpected results, report turnaround times, monitoring of outstanding reports, appraisal, CPD, participation in appropriate EQA schemes, user satisfaction surveys, staff qualification, teaching, training, supervision, and succession planning. The principles and practice of surgical cut-up and sampleprotocols for general specimen handling and categorization are included. Other aspects of service quality and safety are also actively addressed by appropriate guidance documents available on the Royal College of Pathologists website.

The Core Data in Histopathology Specimens

Specimen dissection must be geared to provide information relevant to the clinician who is managing the patient. Reports must be timely, that is, prompt, but in the context of an adequate period of fixation so that acquisition of accurate data is not compromised. The report content must not only

come to an interpretationally accurate diagnosis but also be qualified byassessment of various evidence-based prognostic indicators. In the field of surgical cancer pathology, this is reflected by the trend towards set format reports or datasets for the common cancers. Thus, the core content should include gross specimen description, tumor histological type and grade, extent of local tumor spread, lymphovascular invasion, lymph node involvement, relationship to primary excision margins, and any associated pathology.

Gross Description

Clear distinction should be made between biopsy and resection specimens as they are handled differently and represent different nodal points in a patient's illness. This should be reflected in use of appropriate SNOMED T (topography) and P (procedure) codes—this also facilitates audit of biopsy and resection—proven cancer numbers and correlation with other techniques such as cytology, radiology, and serum markers. The site, distribution, size, edge, and appearance of a tumor within an organ greatly influence the specimen handling and creationof a diagnostic shortlist for microscopy. For example, a gastrointestinal malignant lymphoma may be multifocal, pale, and fleshy with prominent mesenteric lymphadenopathy, whereas a carcinoma is more usually ulcerated and annular, firm and irregular with more localized lymph node disease and vascular involvement.

Histological Tumor Type

There are marked prognostic and therapeutic differences between the diagnoses of carcinoma, sarcoma, germ cell tumor, and malignant lymphoma. This is further highlighted within a given anatomical site, for example, lung, where a diagnosis of carcinoma can be of various subtypes requiring either primary surgical (squamous cell carcinoma) or chemo/radiotherapeutic (small cell carcinoma) approaches and with very different biological outcomes.

Histological Tumor Differentiation or Grade

Tumor differentiation or grade reflects the similarity to the ancestral tissue of origin anddegree of cellular pleomorphism, mitoses, and necrosis. It too greatly influences choice of therapy and prognosis, for example, low-grade versus high- -grade gastric lymphoma (antibiotics versus chemotherapy/surgery) or grade I (surgery alone) versus grade III (surgery and chemotherapy) breast cancer. An accurate histological tumor type and grade cannot be ascertained unless there is appropriate specimen handling with adequate fixation.

Other Pathology

Predisposing, concurrent, and associated conditions should be noted, blocked, and documented, for example, colorectal carcinoma and adenomatous polyps, gastric carcinoma andgastric atrophy or synchronous malignant lymphoma (MALToma).

Ancillary Techniques in Histopathology Specimens

The vast majority of histopathology specimens can be adequately reported by close attention to careful gross description, dissection and block selection and microscopy of good quality formalin-fixed paraffin sections stained with hematoxylin and eosin. However, key ancillary techniques are required in a proportion of cases (see Chap. 46). Some examples are

Frozen sections: confirmation of parathyroidectomy, assessment of operative resection margins in cancer surgery, and cancer versus inflammatory lesions at laparotomy or thoracotomy.

Histochemical stains: demonstration of mucin in adenocarcinoma, congophilia in amyloid, iron in haemochromatosis, and organisms (pyogenic bacteria, tubercle, and fungus) ininfection.

Immunofluorescence: glomerular deposits in renal biopsies, deposition of immunoglobulin, and complement in blistering skin disorders.

Immunohistochemistry: the surgical pathologist's "second H and E" and invaluable in assessing tumor type, prognosis, and predictive factors in treatment, for example, carcinoma (cytokeratins) versus malignant lymphoma (CD45) and malignant melanoma (S100), or better prognostic and hormone responsive breast cancer (estrogen receptor positive). Tumor antigenic profile is often crucial in specifying the site of origin for a metastasis, for example, prostate carcinoma (PSA/PSAP positive).

Electron microscopy: valuable in medical renal biopsy diagnosis, and tumors where morphology and immunohistochemistry are inconclusive, for example, malignant melanoma (pre- /melanosomes) and neuroendocrine carcinoma (neurosecretory granules).

Molecular and chromosomal studies: immunoglobulin heavy chain and T cell receptor gene rearrangements in the confirmation of malignant lymphoma and the characterization of various cancers (malignant lymphoma, sarcoma, and some carcinomas, e.g., renal) by specific chromosomal changes. Distinctive molecular findings in a wide range of solid tumors are being

increasingly used with regard to diagnosis, prognosis, and predicting response to specific targeted therapies. This trend towards personalized oncological medicine also requires consideration of the pre-analytical phase with regard to optimal tissue preservation, fixation, and processing.
Quantitative methods: prognostic indicators include the Breslow depth of invasion in malignant melanoma, muscle fibre typing and diameter in myopathies, and the mitotic activityindex in breast carcinoma.

Diagnostic Cytology
SThe direct smear/cytospin/liquid-based preparations are supplemented by formalinfixed paraffin processed cell blocks of cell sediments and needle core fragments (mini-biopsies) which can combine good morphology and robust immunohistochemistry. Correlation between the cytology and histopathological findings is pivotal to accurate diagnosis (e.g., lungcancer) and staging (e.g., pelvic washings in gynecological cancer). Cytology may also provide a diagnosis where biopsy fails due to sampling error, inaccessibility of the lesion, or biopsy crush artefact

Basic Laboratory Techniques in Histopathology Laboratory
Materials Fixation
1. Fixative solution (usually commercially available formalin).
2. Phosphate buffer (pH = 6.8).
3. Rubber or gloves.
4. Protective clothing.
5. Eyeglasses and mask.
6. Fume hood.
7. Containers with appropriate lids (volume is commensurate with sample size. Largeneck plastic containers are preferable and can be reused)
8. Labels and permanent ink.

Trimming
1. Fume hood.
2. Rubber or gloves.
3. Protective clothing.
4. Eyeglasses and mask.
5. Dissecting board (plastic boards are preferred as they can be easily cleaned andautoclaved)
6. Blunt ended forceps (serrated forceps may damage small animal tissues).
7. Scalpels blades and handle.
8. Plastic bags and paper towels.
9. Containers for histological specimens, cassettes and permnent labels. Containers and cassettes, should be correctly labeled before starting tissue trimming.

Pre-embedding
1. Disposable plastic cassettes for histology (with appropriate lids). For small samples, disposable plastic cassettes for histology with subdivision (Microsette®).
2. Foam pads (31 × 25 × 3 mm) can be used to immobilize tissue samples inside the cassettes.
3. Commercial absolute ethyl alcohol and 96% ethanol solution.
4. 90% and 70% ethanol solutions.
5. Paraffin solvent/clearing agent: xylene or substitute (e.g., Histosol®, Neoclear®).
6. Paraffin wax for histology, melting point 56–57°C (e.g., Paraplast® Tissue Embedding Media).
7. Automated Tissue Processor (vacuum or carousel type).

Embedding
1. Tissue embedding station (a machine that integrates melted paraffin dispensers, heated and cooled plates).
2. Paraffin wax for histology, melting point 56–57°C (e.g., Paraplast® Tissue Embedding Media).
3. Histology stainless steel embedding molds. These are available in different sizes (10 ×10 × 5 mm; 15 × 15 × 5 mm; 24 × 24 × 5 mm; 24 × 30 × 5 mm, etc.).
4. Small forceps.

Sectioning
1. Rotary microtome.
2. Tissue water bath with a thermometer. Alternatively, a thermostatic warm plate can beused.
3. Disposable microtome blades (for routine paraffin sections use wedge-shaped blades).
4. Sharps container to discard used blades.
5. Fine paint brushes to remove paraffin debris.
6. Forceps to handle the ribbons of paraffin sections.
7. Clean standard 75 × 25 mm microscope glass slides (other dimension microscope glass slides are commercially available).
8. Laboratory oven (set at 37°C).
9. Coated glass slides (e.g., Superfrost® or Superfrost Plus®). This is especially recommended when slides are used for immunohistochemistry.
10. 0.1% gelatin in water (1 g of gelatin in 1 L of distillated water). This should not be usedwith Superfrost® or Superfrost Plus® slides and should be reserved for immunohistology sections.

Staining and Cover Slipping

1. Harris hematoxylin (commercial solution, ready to use).
2. Eosin Y solution.
3. Hydrochloric acid 37%.
4. Absolute ethanol.
5. Ethanol 96%.
6. Clearing agent (xylene or substitute e.g. Histosol®, Neoclear®).
7. Staining dishes and Coplin jars suitable for staining.
8. Permanent mounting medium (e.g. Eukitt®).
9. Glass cover slips (25 × 60 mm).
10. Filter paper. 11. Ethanol solutions:

 Add 12.5 mL of water to 1 L of commercial 96% ethanol to obtain 95% ethanol.

Add 408 mL of water to 1 L of commercial 96% ethanol to obtain 70% ethanol.

Storage of Paraffin Blocks and Slides

1. Paraffin blocks storage cabinets.
2. Histological slides storage cabinets.

Methods Fixation

Autolysis is a combination of postmortem changes due to rupture of cell homeostasis that leads to uncontrolled water and electrolytes dynamics in and out of the cell and of alteration of enzymatic activity. These changes are favorable conditions for bacterial and fungal growth andultimately result in complete destruction of tissue structures. To halt autolysis, tissues should be preserved in an appropriate fixative that permanently cross-link its proteins and stabilize it.

The process of autolysis virtually begins immediately after death. Therefore, rapid and adequatefixation after sampling is essential. This can be achieved by immersion of the tissue sample in an adequate volume of fixative solution. There are several methods of fixation including aldehydes, mercurials, alcohols, oxidizing agents, and picric acid derivatives. Tissue immersion in aldehyde (formaldehyde or glutaraldehyde) is the most frequently used fixation method in biomedical research. Formalin (formaldehyde) is commercially available as 38–40% or 10% neutral phosphate buffered solutions. It is generally accepted that a volume ratio of tissue to fixative of 1:10 to 1:20 is necessary for optimal fixation. Small tissue samples are usually fixedat room temperature after 12–48 h. larger specimen may require more fixation time as formalinslowly penetrates tissues.

1. Place the fixative container under a fume hood.
2. Plunge tissue samples in the fixative solution.
3. Stir gently the fixative container for a few seconds to make sure the tissue sample does not stick to the container surface.
4. Replace cap over the container after each tissue (one container can be used for several tissue samples).
5. Add identification label on each container

Trimming

After fixation, tissue samples need to be properly trimmed to reach the adequate size and orientation of the tissue. This step is also important to reach a sample size that is compatible with subsequent histology procedures such as embedding and sectioning.

Hard tissues (such as bones and teeth) must be decalcified before trimming.

DECALCIFICATION

Definition: Decalcification is a process of complete removal of calcium salt from tissues like bone and teeth and other calcified tissues following fixation. Decalcification is done to assure that the specimen is soft enough to allow cutting with the microtome knife. Unless the tissues in completely decalcified the sections will be torn and ragged and may damage the cutting edgeof microtome knife.

The steps of decalcification

1. To ensure adequate fixation and complete removal of the calcium it is important that the slices are 4-5 mm thick. Calcified tissue needs 2-3 hours only, for complete decalcification to be achieved so it in necessary to check the decalcification after 2-3 hours.
2. Fixative of choice for bone or bone marrow is Zenker formal or Bouin's fluid. Unfixed tissue tends be damaged 4 times greater during decalcification than a properly fixed tissue.

Decalcification is affected by one of the following methods.

(a) Dissolution of calcium by a dilute mineral acid.
(b) Removal of calcium by used of dilute mineral and along with ion exchange resin to keep the decalcifying fluid free of calcium.
(c) Using Chelating agents EDTA.
(d) Electrolytic removal of calcium ions from tissue by use of electric current.The Criteria of a good decalcifying agents are.

1. Complete removal of calcium.
2. Absence of damage to tissue cells or fibres.
3. Subsequent staining not altered.
4. Short time required for decalcification.

Removal of calcium by mineral acids - Acid decalcifiers are subdivided into- Strong acid and weak acid.

Strong acid - eg.Nitric and hydrochloric acid. Nitric acid- 5-10% aqueous solution used.

They decalcify vary rapidly but if used for

longer than 24-48 hrs. causes deterioration of stainability especially of the nucleus.
Hydrochloric acid - 5-10% aqueous solution decalcification slower than nitric acid but still rapid. Fairly good nuclear staining.
Weak acid e.g., formic, acetic and picric acid of these formic acids is extensively used as acid decalcifier. 5-10% aqueous solution or with additives like formalin or buffer are used.

Formic acid
1. Brings out fairly rapid decalcification.
2. Nuclear staining is better.
3. But requires neutralization and thorough washing prior to dehydration.

Aqueous nitric acid Nitricacid 5-10 ml Distilled water to 100 ml.

Procedure
1. Place calcified specimen in large quantities of nitric acid solution until decalcification is complete (change solution daily for best results).
2. Washing running water for 30 minutes
3. Neutralize for a period of at least 5 hours in 10% formalin to which excess of calcium or magnesium carbonate has been added.
4. Wash in running water over night
5. Dehydrate, clear and impregnate in paraffin or process as desired.

Note: Overexposure to nitric acid impairs nuclear staining. Nitric acid is the solution of choicefor decalcifying temporal bones.
Perenyi's fluid 10% nitric acid 40.0ml Absolute alcohol 30.0 ml.
0.5% chromic acid. 30.0 ml.
Note all these ingredients may be kept in stock and should be mixed immediately before use. This solution may acquire of blue violet tinge after a short while but this will have no effect in the decalcifying property.
It is slow for decalcifying hard bone but excellent fluid for small deposits of calcium eg. calcified arteries, coin lesions and calcified glands. Also good for human globe which containscalcium due to pathological conditions. There is little hardening of tissue but excellent morphologic detail is preserved.
Formalin Nitric acid Formalin 10 ml Distilled water 80 mlNitric acid 10ml
Nitric acid causes serious deterioration of nuclear stainability which partially inhibited by formaldehyde. Old nitric acid also tends to develop yellow discoloration which may beprevented by stabilization with 1% urea.
Aqueous formic acid
90% formic acid 5-10 ml Distilledwater to 100 ml.
Gooding and Stelwart's fluid.90% formic acid 5-10ml.
Formalin 5ml
Distilled water to 100 ml.
Evans and Krajian fluid
20% aqueous trisodium citrate 65 ml
90% formic acid 35 ml
This solution has a pH of - 2-3
Formic acid sodium citrate methodProcedure
1. Place calcified specimen in large quantities of formic acid-sodium citrate solution until decalcification is complete (change solution daily for best results).
2. Wash in running water for 4-8 hours
3. Dehydrate, clear and impregnate with paraffin or process as desired.

This technique gives better staining results then nitric acid method, since formic acid and sodium citrate are less harsh on the cellular properties. Therefore, even with over exposure of tissue in this solution after decalcification has been complete, causes little loss of staining qualities. This method of choice for all orbital decalcification including the globe.

Surface decalcification- The surface of the block to be decalcified is trimmed with scalpel. Theblock is then placed in acid solution at 1% hydrochloric acid face downwards so that acid bathesthe cut surface for 15- 60 min. As penetration and decalcification is only sufficient for a few sections be cut the block shall be carefully oriented in microtome to avoid wastage ofdecalcified tissue.
Decalcification of Bone marrow biopsy.
Tissue after fixation in Bouin's or Zenker's fixative is decalcified for 2½ hours followed by an hour of washing. The tissue in then dehydrated beginning with alcohol.

Use of Ion exchange resins
Ion exchange resins in decalcifying fluids are used to remove calcium ion from the fluid. Therefore, ensuring a rapid rate of solubility of calcium from tissue and reduction in time of decalcification. The resins an ammoniated salt of sulfonated resin along with various concentrations of formic acid are used. The resin is layered on the bottom of a container to a depth of = ½ inch, the specimen is allowed to rest on it. After use, the resin may be regeneratedby washing twice with dilute N/10 HCL followed by three washes in distilled water. Use of Ionexchange resin has advantage of (ii) faster decalcification (ii) tissue preservation and (iii) cellular details better preserved.

Chelating agents
Chelating agents are organic compounds which have the power of binding certain metals. Ethylene-diamene-tetra-aceticacid, disodium salt called Versenate has the power of capturing metallic ions. This is a slow process but has little or no effect on other tissue elements. Some enzymes are still active after EDTA decalcification.
Versenate10 gm. Distilled water 100 ml(pH 5.5 to 6.5) Time
7-21 days.

Electrolytic method
This is based on the principle of attracting calcium ions to a negative electrode when in solution.

Decalcifying solution
HCL (Conc.) 80ml Formic acid 90% 100 ml Distilled
water 1000 ml.
Decalcify with electrophoresis apparatus with the above-mentioned decalcifying fluid. Thismethod has no added advantage over any other method.

Neutralization: It has been said that following immersion in mineral acids, tissues should be deacidified or neutralized, before washing by treatment with alkali. This may be affected by treatment overnight in 5% lithium or sodium sulphate.

Washing: washing of the tissue before processing is essential to remove acid (or alkali if neutralized has been carried out) which would otherwise interfere with staining.

Determination of end point of decalcification 1. Flexibility method
Bending, needling or by use of scalpel if it bends easily that means decalcification is complete. Unreliable, causes damage and distortion of tissue.

2. X-ray method
Best method for determining complete decalcification but very costly. Tissue fixed in mercuric chloride containing fixatives cannot be tested as they will be radio opaque.

3. Chemical Method
It is done to detect calcium in the decalcifying fluid when no further calcium is detected, decalcification is considered complete.

Procedure
Take 5 ml of decalcifying fluid from the bottom of container which has been in contact with thetissue for 6-12 hrs. Add 5 ml each of 5% ammonium oxalate and 5% ammonium hydroxide. Mix and let it stand for 15-30 min. A cloudy solution caused by calcium oxalate indicates that specimen is not thoroughly decalcified. Absence of turbidity indicates completeness of decalcification. This test is only suitable for use in decalcifying agents whose acid content is less than 10%.

Treatment of hard tissues
Keratin and chitin are softened by use of concentrated sulphuric and with the aid of heat keratin is completely dissolved from the tissue sections. But much tissue distortion will also occur. For softening of chitin full procedure gives a satisfactory result.
1. Fix the specimen in fixative of choice.
2. Place the specimen in following solution until complete dechitinized. Change the solution every two days for best results.

Mercuric chloride - 4 gm Chromic acid - 0.5gm Nitric acid (Conc.) - 10.0mlEthyl alcohol 95% - 50.0 mlDistilled water - 200.0ml
3. Wash in running water for 3 hours
4. Dehydrate, clear and impregnate with paraffin.

Prenyi's fluid
Immersing hard tissues in these solutions for 12-24 hours will make sectioning easier andexcellent preparation of calcified arteries, thyroid and calcified glands is possible.

Lendrum's technique
It is very useful for tissues which became hard at the time of fixation. Following washing outof the fixative, tissue is immersed in a 4% aqueous solution of phenol for 1-3 days.
Wax blocks - The treatment of wax embedded block of hard tissue may be done by soaking insoap water overnight.
Trimming includes;
1. Under a fume hood, remove tissue samples from the fixative container or jar.
2. Trim one or more small pieces of tissues and organs and fit them into cassettes.
3. Place a lid on the cassette.
4. Label each cassette with a permanent ink.
5. Store cassettes in a fixative container.

There are specific rules that should be followed for trimming of each tissue and organ. These depend on the goal of the histopathology evaluation. Standardized methods for toxicology studies in drug safety evaluation are available online. These are also described in the comprehensive papers from the RITA group (Registry of Industrial Toxicology Animal data). Specific methods have been proposed for many organs: gut, heart, male

reproductive system, female reproductive system, or muscle.

Pre-embedding

The goal of pre-embedding is to infiltrate tissue samples with paraffin and replace water contentof tissue by this wax material. Paraffin is used as a supporting material before sectioning. Histology grade paraffin wax has a melting point around 56 or 57°C, a temperature that does not alter the structures and key morphologic characteristics of tissues, thus allowing adequate microscopic evaluation by the pathologist. At room temperature, paraffin wax offers enough rigidity to allow very thin sections just a few micrometers thick (usually 4 or 5 Mm).Preembedding is a sequential process that consists of dehydration of tissues in increased concentrations of alcohol solutions, then gradual replacement of alcohol by a paraffin solvent. Xylene (or its substitutes; e.g., Histosol®, Neoclear®, and Histoclear®) has the advantage to be miscible in both alcohol and paraffin. As a result, the tissue sample is dehydrated and fully infiltrated by paraffin. This step is generally automated using a variety of vacuum or carousel type tissue processors.

When using a tissue processor, the following steps should be followed:

1. Check if the baskets and metal cassettes are clean and free of wax.
2. Do not pack the tissues too tightly to allow fluid exchange.
3. Check if the processor is free of spilt fluids and wax.
4. Check if the fluids levels are higher than the specimen containers.
5. Select the appropriate protocol and check the clock.
6. Prepare 95% and 70% ethanol solutions.
7. Dehydrate in a graded series of ethanol:

a. Wash in 70% ethanol for 1 h.
b. Wash in 95% ethanol for 1 h (two times).
c. Wash in 100% ethanol for 1 h (two times).

8. Clear with a paraffin solvent (xylene) for 1 h (two times).
9. Infiltrate with paraffin for 1 h (two times).
10. Tissue sampled are retrieved at the end of the processing program (automates are usuallyrun overnight to start the embedding process in the next morning).

The following is a list of rescue procedures that can be helpful to consider in case the preembedding procedure is not completed normally:

1. Recovery of tissues that have air-dried because of mechanical or electrical failure of theprocessor:

a. Rehydrate the tissue in Sandison's solution (absolute alcohol 30 mL, formaldehyde 37% 0.5 mL, and sodium carbonate 0.2 g water up to 100 mL) orVan Cleve and Ross' solution (trisodium phosphate 0.25 g in 100 mL of water).
b. Immerse the tissues in one of these two solutions for 24–72 h (actually most of tissues rehydrate and soften within 4–6 h).
c. Process to dehydration and pre-embedding as usual, starting in 70% ethanol.

2. Recovery of tissues accidentally returned to fixative following wax infiltration. Discard all contaminated fluids:

a. Rinse in 70% ethanol followed by 95% ethanol
b. Rinse in absolute ethanol (two to three times).
c. Rinse in xylene (or substitute).
d. Carry out the paraffin infiltration three times, 30–60 min each.

3. Recovery of tissues accidentally returned to ethanol 70% following wax infiltration:

a. Discard all contaminated fluids.
b. Rinse in ethanol 95%.
c. Rinse in absolute ethanol (two changes).
d. Rinse in xylene (or substitute).
e. Carry out the paraffin infiltration two to three times, 30–60 min each. The samesteps can be used for manual tissue processing. Melt the paraffin in an oven at 60°C in glass containers. Immerse the specimens into the melted paraffin.

Embedding

Once tissue samples are infiltrated by paraffin, they are removed from the cassettes and carefully positioned inside a metal base mold. This step is critical as correct orientation of the tissue is essential for accurate microscopic evaluation. The mold is filled with melted paraffin and then immediately placed on a cooling surface. To trace each tissue specimen, the cassette with permanent tissue and study identification is placed on top of the metal base mold and incorporated in the paraffin block before cooling. In this manner, the cassette will be used as abase of the paraffin block for microtome sectioning (once the metal base mold is removed).

i. Check that the different compartments of the station have the appropriate temperature. Paraffin should be liquid in the paraffin reservoir, work surface should be warm, and cool plate should be cold. Stainless steel molds should be kept warm.
ii. Remove the cassettes from the last tissue processor bath (normally melted paraffin) and transfer to the warm compartment of the embeddingstation.
iii. Transfer one cassette onto the hot plate. iv.

Snap off the cassette lid anddiscard it.

v. Select a preheated stainless-steel mold of the appropriate size.The specimen must not come into contact with the edge of themold.

vi. Transfer the mold onto the hot plate. vii. Pour melted paraffinfrom the paraffin dispenser.

viii. Transfer the paraffin-infiltrated tissues into the mold. ix. Using heated forceps, orientate the tissue inside the mold to obtain the desired position in relation with the cutting axis; the specimen surface in contact with the base of the mold being the one that will be on the slide after sectioning.

x. Center the specimen in the mold ensuring that paraffin entirely surrounds the edge of the tissue. 11. Carefully transfer the mold onto the cool plate. Allow a few seconds to paraffin to turn white (this means thatparaffin returned to solid phase). During cooling, the paraffin will shrink(up to 15% of its initial volume); this compression will be fully recovered later after sectioning.

xi. Make sure that the specimen does not move during this step and still keep its desired orientation. If not, put the mold back onto the warm work surface until the whole paraffin liquefies then start again from step

9. Immediately place the base of the original cassette on top of the mold.Incorporation of the cassette in the paraffin block before cooling allowstracing the specimen identification and uses the cassette as a holder during sectioning.

xii. Carefully fill the mold with paraffin to above the upper edge of the cassette.

xiii. Carefully transfer the mold and cassette onto the cool plate and allow time (at least 15 min) until the paraffin has hardened.

xiv. Snap off the mold. xv. Bring the paraffin blocks together. xvi. Store the paraffin blocks at room temperature until sectioning.

Sectioning

The objective of this step is to cut 4–5 Mm-thick sections from paraffin blocks. This is achievedusing precision knives (microtomes). To obtain constant high quality and extremely thin tissue sections, disposable blades should be used and changed after a limited number of blocks. The paraffin block is mounted on the microtome holder. Sections are cut as a ribbon and are floated on a water bath maintained at 45°C to stretch the paraffin section. A standard microscope glassslide is placed under the selected tissue section and removed from the water bath. Tissue sections are then allowed to dry, preferably in a thermostatic laboratory oven at 37°C. Tissue sectioning and floating steps are delicate operations that should be performed by trained personnel.

i. Heat the tissue water bath to 45°C and fill it with water. To avoid microorganisms' growth, the bath should be carefully cleaned every day and the water flotation bath discarded.

ii. Put the paraffin blocks on a cold surface (e.g., refrigerated cold plate or ice) to harden the cut surface. Avoid prolonged cooling and very cold surfaces as they may lead to cracking in the block surface.

iii. Install a disposable blade in the microtome. iv. Set angle between the blade edge bevel and the block to 2–5 degrees (clearance angle). A correct angle should be set to avoid compression in cut sections and to reduce friction as the knife passes through the block. Angles in the above-mentioned range are recommended for paraffin sections, but the exact angle is generally found by trial and error.

v. Lock the blade in place.

vi. Lock the microtome hand-wheel. vii. Trim the edges of one block with a sharp razor blade so that the upper and lower edges of the block are parallel to the edges of the knife. Otherwise, a ribbon cannot be cut. Keep 2–3 mm of paraffin wax around the tissue.

viii. Fit the cassette paraffin block onto the cassette holder of the microtome. Orientate the block so that its greater axis is perpendicular to the edge of the knife, and also that the edge offers the least resistance (e.g., the smallest edge will be cut first).

ix. Unlock the hand-wheel.

x. Advance the block until it is in contact with the edge of the knife. Paraffin block edges must be parallel to the knife. If not, adjust the blockorientation.

xi. Set the section thickness around 15 microns. xii. Coarse cut the block at 15 microns until the whole surface of the embedded tissue can be cut.

xiii. Lock the microtome hand-wheel. xiv. Return the trimmed block to cold plate for 1–2 min. xv. Set the section thickness to 4–5 Pm. xvi. Remove wax debris from the knife with alcohol. Avoid use of xylene to clean the paraffin debris as it often leaves an oily remnant on the knife and following sections will stick (not mentioning the xylene hazards for the operator).

xvii. Move to an unused area on the blade or install a new disposable blade.

xviii. Install the cassette paraffin block onto the cassette holder again.

xix. Cut a series of paraffin sections. If sectioning is doing well, you willobtain a ribbon of serial sections.
xx. Gently breath upon the sections to eliminate static electricity, to flattenthe sections, and to facilitate the removal of the ribbon from the blade.
xxi. Separate the ribbon (including four to five sections) from the knife edge with a paint brush.
xxii. Transfer the piece of ribbon onto a glass slide coated with a drop of gelatin-water, or to the surface of the water bath.
xxiii. Gently separate the floating sections on the water bath with pressure from the tips of forceps.
xxiv. Collect sections on a clean glass slide. Hold the slide vertically beneaththe section and lift carefully the slide up to enable tissue adherence.
xxv. Label slides with a histo-pen or pencil. Avoid pens with non- alcoholresistant ink (ballpoint or felt-tipped pens).
xxvi. Allow the slides to dry horizontally on a warm plate for 10 min to ensure that the section firmly adheres to the glass slide. Alternatively, slides can be dried vertically in an oven for 20 min at 56°C.
xxvii. Transfer the slides (vertically placed) to a laboratory oven overnight at37°C.
xxviii. Store the slides in dry boxes at room temperature. For immunohistochemistry, slides should be stored at 4°C to minimize antigen loss.
xxix. Empty the water bath and wipe it with a damp cloth at the end of each day.

Staining and Mounting

Unstained paraffin sections offer very low contrast and therefore cannot be evaluated microscopically in routine histopathology. It is necessary to apply coloring reagents (mostly chemicals) to stain tissue structures. There are many histochemistry staining techniques that canbe applied to examine specific tissue or cell structures. As most of these dyes are water soluble,tissue sections should be rehydrated to remove paraffin (using xylene, alcohol solutions endingin water). Hematoxylin and Eosin (H&E) is the routine staining used to study histopathology changes in tissues and organs from animals in toxicity studies. Hematoxylin is a basic dye thathas affinity for acid structures of the cell (mostly nucleic acids of the cell nucleus), and eosin isan acidic dye that binds to cytoplasmic structures of the cell. As a result, H&E stains nuclei in blue and cytoplasms in orange-red. A variant to this staining method is the Hematoxylin–Eosin and Saffron (HE&S) stain. As compared to the H&E method, HE&S stains collagen in yellow-orange, allowing a better highlight of interstitial conjunctive tissue.

The following protocol describes manual H&E staining technique. It is suitable for small seriesof slides. This operation is usually automated to allow high-throughput staining of slides.

- Prepare the Harris hematoxylin working solution. Filter the commercial solution through filter paper to remove the metallic precipitant that forms in the solution upon standing.
- Prepare 0.1% aqueous eosin Y working solution. Dissolve 1 g of eosin Y in 1 L of deionized water. Add four drops of HCl to obtain a pH between 4 and 5.
- Add a thymol crystal to prevent molds growth.
- Label and date the solutions. Aqueous eosin solution is stable for at least 2 months at room temperature.
- Prepare the differentiation solution (acid alcohol). Add 1 mL of 37% HCl to 100 mL of 70% ethanol.
- Dewax the paraffin sections in xylene 2 × 5 min each.
- Rehydrate in 100% ethanol 2 × 5 min each.
- Rehydrate in 95% ethanol 2 × 5 min each. 9. Wash in running tap water for 3 min.
- Stain for 3–5 min in Harris hematoxylin.
- Wash in running tap water for 3 min.
- Decolorize briefly in acid alcohol for 2 s.
- Wash and blue the sections in running tap water for 3 min.
- Stain for 2–5 min in 0.1% aqueous eosin Y.
- Rinse in tap water for 30 s.
- Dehydrate in 95% ethanol two times for 2 min each.
- Dehydrate in 100% ethanol two times for 2 min each.
- Clear sections in clearing agent two times for 2 min each.
- The slides may remain in clean clearing agent until cover slipping.
- The nuclei will be stained in blue, the cytoplasms and other tissue components in pinkorange. Decreased staining intensity of the sections (after approximately 500 slides) indicates that the staining solutions should be renewed

After staining, a very thin glass should be placed over the tissue section to protect it and to enhance the optical evaluation of the tissue. This also allows tissue section storage for several years. Cover slipping process consists of gluing the cover slip glass over the tissue section on the microscope slide

glass. The mounting medium is usually insoluble in water. Therefore, thetissue should be dehydrated again using solutions of increasing concentrations of alcohol and xylene.

The following protocol describes manual cover slip mounting technique. It is suitable for smallseries of slides:

1. Wipe the surface under the slide while keeping the tissue section covered with the clearing agent.
2. Apply two or three drops of the mounting medium (e.g., Eukitt®).
3. Place a cover slip on the slide and avoid the formation of bubbles. Press gently with forceps to remove any bubble.

Dry the slides overnight at room temperature on a flat surface within the fume hood. To allowhigh-throughput slide preparation, this operation is usually automated. These automated machines are commercially available from many suppliers.

Storage of Paraffin Blocks and Slides

1. After sectioning, store paraffin block at room temperature.
2. Store-stained slides in appropriate boxes (avoid prolonged exposure to light).

Notes

1. Vinyl gloves are preferable as latex gloves may be allergenic.
2. Formaldehyde is an eye, nose, respiratory tract, and skin irritant. It is a strong skin sensitizer and can cause cancer in humans. Therefore, it should not be handled without gloves or outside a fume hood. As a result, a variety of alternative fixatives, mostly compound fixatives containing chemicals with differing fixation characteristics, have been investigated. However, as of today, there is no unique formalin successor.
3. The best fixation is achieved by intracardial perfusion of the body with formaldehyde or glutaraldehyde. However, this technique needs specific training and is time consuming and therefore only used on a case-by-case basis.
4. The following is a non-exhaustive list of hazards associated with some of the abovementioned chemicals used during pre-embedding.

a) Absolute ethanol is inflammable and irritant to the eye. It should never be handled closeto a naked flame or heat. The vapor is heavier than air and can travel a considerable distance to a source of ignition.

b) Paraffin is not hazardous for health when pure, but wax additives can be potential carcinogen as, e.g., dimethyl sulfoxide (DMSO). Molten wax should not be inhaled, asit produces small lipid droplets that can lead to lipid pneumonia. Prefer paraffin wax without DMSO.

c) Xylene is an aromatic compound that contains benzene. It is moderately inflammable, a mild eye and mucous membrane irritant, a primary skin irritant that may causes dermatitis. It might cause central nervous system depression. Overexposure can lead to respiratory failure. Avoid contact with skin. Xylene substitutes are preferred, but if hazards of xylene are well documented, its substitutes have not been so thoroughly evaluated.

5. Alternative techniques of paraffin embedding exist; they use plastic or resin polymers that should be considered when paraffin embedding is not appropriate (i.e., methyl methacrylate tosection non decalcified bone, Epon to obtain very thin sections in the range of 1 Mm or for electron microscopy, etc.). These embedding media require specific reagents and equipment. These techniques will not be described in this chapter as they are not used in routine histology in the drug safety evaluation environment.
6. The following is a non-exhaustive list of hazards associated with some of the chemicals used during staining/cover slip mounting:

(a) Harris hematoxylin: irritant to eyes, skin, and mucous membranes. Toxic by inhalation and ingestion. Handle with care.

(b) Eosin Y: bromofluorescein dye and other dyes of this group are highly toxic. They are skin and eye irritant and reported as being carcinogens.

(c) Mounting medium: this is usually xylene or a xylene-based solvent.

Basic Laboratory Techniques in Cytology Laboratory

The advantages of diagnostic cytology are that it is a non-invasive, simple procedure, helps in faster reporting, is relatively inexpensive, has high population acceptance and facilitates cancer screening in the field. Diagnostic cytology can be carried out by different methods, which includes collection and examination of exfoliated cells such as vaginal scrapes, sputum, urine,body fluids etc. Collection of cells by brushing, scraping or abrasive techniques is usually employed to confirm or exclude malignancy. Fibreoptic endoscopes and other procedures can be used for collecting samples directly from the internal

organs.

Fine-Needle Aspiration Cytology/ Biopsy (FNAC/FNAB) is now a widely accepted diagnostic procedure, which has largely replaced open biopsy. This method is applicable to lesions that are easily palpable, for example swellings in Thyroid, Breast, superficial Lymph node etc. Imaging techniques, mainly ultra-sonography and computed tomography, offer an opportunity for guided FNAC of deeper structures.

The practice of diagnostic cytology needs proper training of the laboratory personnel including cytopathologist, cytotechnologist and cytotechnician. The role of cytotechnician is very important in cancer control programmes where large numbers of asymptomatic population haveto be screened.

The accuracy of the cytologic examination from anybody site depends greatly on the quality of collection, preparation, staining and interpretation of the material. Inadequacy in any of these steps will adversely affect the quality of diagnostic cytology.

Diagnostic accuracy and reliability are major issues in cytology practice. Over the years many quality control measures have been introduced for ensuring high standards in cytology. Amongthem the most important are regular continuing education of medical and technical personnel, certification and accreditation of laboratory to national authorities.

Collection and Preparation of Material for Cytodiagnosis

Accurate interpretation of cellular material is dependent on the following factors:

- Methods of specimen collection.
- Fixation and fixatives.
- Preservation of fluid specimens prior to processing.
- Preparation of material for microscopic examination.
- Staining and mounting of the cell sample.

Methods of specimen collection

Individual cells may be studied in many ways.

A. Exfoliative Cytology: Exfoliative cytology is the study (microscopical and interpretation) of cells shed from membranes of various organs, cells from all organs communicate naturally or artificially with the exterior of the body. These cells may be recovered either from natural secretions such as sputum and vaginal or prostatic fluids. Normal epithelial cells are shed or thrown off spontaneously because of the continuous replacement of the individual warn out cells on the living process. The new cells which are formed by mitotic process of division underneath generate pressure which forces the cells to desquamate (exfoliate)

The rate of renewal varies greatly in different times and different locations

The exfoliative cells when collected and appropriately stained gives information on the state of living epithelium from which they have been derived. These are characteristics of cellular and nuclear appearances in cells drawn off from normal healthy epithelium which differs distinctly from those derives from inflamed or malignant time and this offers the basis of diagnostic importance of the method.

Cytology has become an established aid for the diagnosis of malignancy in various organs especially those of respiratory, urinary and female respiratory tracts. Studies from these organsis a means of detecting asymptomatic cancer. It also helps in accessing hormonal activities in women which is a value in some cases of sterility in certain endocrine disorders as well they offer a guide in the assessment of therapy taken to correct such hormonal abnormalities. Furthermore, information may be obtained concerning the nature and severity of inflammatory conditions in the vagina, cervix, uterine and occasionally also in the endometrial activity due to pathologic organisms such as *Trichomonas vaginalis* and monilia. Genetic sex is also determined using cytological observations of buccal smears.

Cytological diagnosis is based in some cases on the appearances of individual cells with respectto the cytoplasm. Nuclear abnormalities associated with cancer include the following.

1. Enlargement of nucleus without an increase in the overall size of the cells giving adecreased cytoplasm to nuclear ratio.
2. Irregularity of nuclear outline and variation in size and shape
3. Hyperchromesia due to increased amounts of DNA; the nuclei of malignant cells offerdeeply with basic dyes
4. Multi-nucleation resulting from abnormal cell division
5. Uneven distribution and variation in size of chromatin particles
6. Increase in size and number of nucleoli

B. Fine Needle Aspiration Cytology (FNAC): This is a technique used to obtain material from organs that do not shed cells spontaneously. It is valuable in of lesions of the breast, thyroid, lymph nodes, liver, lungs, skin, soft tissues and bones.

C. Body Fluids: Body fluids like Urine, Pleural fluid, Pericardial fluid, Cerebrospinal fluid, Synovial fluid and Ascitic fluid can be studied by cytology.

Collection and Preparation of Material for Cytodiagnosis

Female Genital Tract (FGT)

The cytological specimens collected from FGT include cervical smear, vaginal smear,aspiration from posterior fornix of vagina (vaginal pool smear) and endometrial smear.

Vaginal smear: Introduce an unlubricated speculum, scrape the lateral vaginal wall at the level of cervix with a spatula. The broad and flat end of Ayre's spatula is used for this purpose. The cellular material is rapidly but gently smeared on a clean glass slide and the smears are fixed immediately. If no spatula is available a cotton swab dipped innormal saline can be used.

Vaginal pool smear: The aspiration can be performed after the introduction of unlubricated speculum. The technique allows collection of cells under direct vision fromposterior fornix pool. When a speculum is not employed the pipette is gently introduced in to the vagina until resistance is encountered. It is important to compress the suction bulb during the introduction of the pipette to avoid collecting the cellular material of the lower vaginal origin. The cellular material is spread on a clean glass slide and fixed immediately.

Endometrial aspiration smear: After preliminary visualization and cleaning of cervixa sterile cannula is introduced into the uterine cavity and aspiration is then carried out with a syringe. The specimen is squirted on a clean glass slide, gently spread and rapidlyfixed.

Cervical smear:

Patient Preparation: Proper patient preparation is the beginning of good cervical cytology. The patient should be instructed before coming for smear collection, that she should not douche the vagina for at least a day before the examination. No intravaginal drugs or preparations should be used for at least one week before the examination and the patient should abstain from coitus for one day before the examination. Smear should not be taken during menstrual bleeding, because of contamination with blood, endometrial component, debris and histiocytes.

Collection: it is obtained by inserting a speculum into the vagina and passing an Ayres spatulaalong the vagina. A sample of the surface cells of the cervix is deposited on the edges of the spatula. The spatula then pulled along the microscope slide and the slide paced immediately into a fixative.

Advantages of Pap Smear:

1. It is painless and simple
2. Does not cause bleeding
3. Does not need anesthesia
4. Can detect cancer and precancer
5. Can identify non-specific and specific inflammations
6. Can be carried out as an outpatient procedure

Respiratory Tract

Sputum; specimens of sputum should be obtained from a patient before eating and thisslide should be sent fresh to the lab. Though immediate section is usually reliable, storage in the refrigerator until a convenient time is not detrimental. The specimens are collected by decanting the sputum into disposable Petri dishes placed in a black surface.The areas of smearing are those containing white flecks and blood-stained purulent areas. Particular care should be taken in order to obtain for examination any minute opalescent thread. These various parts should be smeared on to a microscope slide for each specimen

Smears From Fluid Specimens

It is very important that smears from these are fixed as soon as possible after collection. Refraction will arrest the destructive process only for a short time

On receipt, the specimens are distributed in centrifuge tubes and centrifuged at 2000RPM for 20 minutes. The supernatant is discarded and the sediment spreads evenly on slides with wire loops. The prepared slides are fixed immediately. Sediments of fluids containing little or no proteins e.g. urine and gastric washings tend to wash off the slides during fixation and staining.

Addition of these sediments is improved if the slides are lightly coated with meyer's egg albumin before spreading.

Other Sites

Oral lesions: Scrape the lesion with a tongue depressor, spread material on a clean slideand fix immediately.

Nasopharynx: Cotton tipped applicator is used to obtain material for cytological examination.

Larynx: A cotton swab smear of larynx may be a useful adjunct to clinical diagnosis ifbiopsy is not contemplated.

Oesophagus

Oesophageal washing and brushing are usually recommended for collecting cytology sample from oesophagus. To collect a good specimen for cytology one should first localize the suspicious lesion by oesophagoscopy .

Stomach

Cytology specimen can be collected from the surface of the lesion by scraping (abrasion)under direct vision of a flexible endoscope. The cells collected can be directlysmeared on a glass slide. Gastric lavage is also recommended for cytological

investigations.

- **Discharge from nipple of the breast**

Spontaneous nipple discharge and discharge produced by breast massage are collected by applying the slide directly to the nipple followed by immediate fixation.

- **Asceptic Precautions**

Care must be taken in the handling of specimens sent for cytology since some of the specimens sent for investigation may be highly infectious e.g., sputum All laboratory safety precautions must be observed. Specimens, glass wares and all materials used must be autoclaved or placed in aseptic solutions before cleaning or discarding.

- **Fixation**

Immediate fixation is vital for the fixation of cells subjected to cytological investigations. A thin layer of cells smear on the microscope slide rapidly dries in the air. In consequence, the cells tend to flatten and shrink. These artifacts are most noticeable in the nucleus where drying causes the nucleus to increase in size with a reduction of the density of nuclear staining. The cytoplasm suffers similar effects although it may not increase in size but rather shows alterations in its staining reactions.

The classical cytological fixative is ETHER or ALCOHOL. It relies on its action for theability of alcohol to precipitate and denature proteins. Acetone or 95% alcohol may be separately used as both denature proteins in the same way or similar manner. Althoughthese fixatives are ideal for the cytochemical aspect, they possess distinct practical disadvantages owing to their inflammable nature. The fluid and volatile nature of the fixative present additional hazards

Fluid fixatives may cause cells to float from positive smears and in other to prevent these, smears must be fixed in separate containers. New fixatives have recently been introduced that overcome the disadvantage of the older but otherwise excellent fixatives. Moreover, these new fixatives facilitate the postage of fixed slides. Alcohol is the main fixing agent incorporated with a protective coating usually water-soluble ware. This type of fixative may be obtained either in aerosol form or in simple and cheaper dropping services.

FIXATIVES ETHER/ALCOHOL

a) Ether…….1part, 95% alcohol……1part, fix for at least 10 minutes
b) 95% alcohol. fix for at least 10 minutes
c) Acetone fix for at least 10 minutes

a. SHUADIN'S FLUID

- Saturated aqueous mercuric chloride 2 vol
- 95$ alcohol1vol Fix for at least20 minutes

STAINING

The type of fixative employed detects the pre-staining treatment of the prepared slide. High alcoholic fixatives requires the preparation to be stained before preceding to aqueous solutions.

Hematoxylin provides an ideal nuclear stain and is eminently suitable for the demonstration ofnucleus in cytological preparations. Unfortunately, the classical haematoxylin solution used inhistology lab requires relatively long staining times and could result in cells becoming detachedfrom the slide. For these reasons, Harris Haematoxylin solution is generally used as it allows excellent nuclear staining

PAPANICOLAOU'S STAIN

This method is to cytology as H & E is to histology; Nearly all cytological are stained by this method for it provides excellent staining with differential staining of cytoplasm. Moreover, the alcohol solvents used in the preparation of the dye solution assist in raising the refractive indexand thus the assessment of cell morphology. Cell relationship and multinucleation is more readily observed.

PREPARATION OF STAIN

EOSIN AZURE (E.A : 36 OR E.A : 50)

- 0.5% light green SF (yellowish) in 95% alcohol…45ml
- 0.5% Bismark brown (yellowish) in 95%alcohol…

10mls

- 0/5% eosin in 95% alcohol…45mls
- Phosphotungstic acid…0.2g
- Saturared aqueous lithium carbonate… 1drop

ORANGE GREEN

1. 0.5% orange green in 95% alcohol… 100mls
2. Phosphotungstic acid…0.015g

The EA number indicates the amount of light green in the solution. The higher the number, thelower the percentage of light green in solution. The amount of lithium carbonate added is critical for a slight variation causes the stain to precipitate. For this reason, it is preferably to obtain themadeup solution from a manufacturer who provides a constant standard preparation manufactured in bulk and evaluated by a cytological laboratory

To prepare EA 65 from the above solution, reduce the light green to 0.25% in 95% alcohol

METHOD

1. Bring slide to water
2. Stain in Harris or Cold's haematoxylin (4mins)
3. Rinse in water
4. Differentiate in 1% aced alcohol
5. Wash in water and blue it
6. Rinse in 70% alcohol and 25% alcohol
7. Stain in orange green(0.66) for 30 minutes
8. Rinse in 95% alcohol
9. Stain E.A 50 for 3 mins
10. Rinse in 95% alcohol
11. Dehydrate in absolute alcohol
12. Clear in xylene and mount

RESULTS

- Nuclear…..blue
- Malignant nuclei….Blue or hypochromia
- Acidophilic cells….red
- Basophilic cells… .....blues green
- RBCs…

Orange-Red

Other staining methods are ; -

1. **SHORR'S stain** for differential staining of vaginal smears for hormonal evaluation
2. **Methylene blue** that may be used for the staining of sputum but of disadvantage owingto poor refractive index. This method is used for screening and positive specimens maybe selected for more detached examination

METHOD

1. Mix equal parts of specimens and the reagent

2. Warm gently over a flame and cover with a coverslip (1% methylene blue)

RESULTS

- Nuclei…..dark blue
- Cytoplasm……shades of light blue

H & E; - same as applied in histology but the hematoxylin employed must be the shortstaining types because of cells detaching from the slide. It Is employed for general architecture of cells.

3. LEISHMAN STAIN

APPLICATION; - exfoliative cells when collected appeared well stained give the informationon the state of the epithelium from which they have been derived. They are cellular and nuclearappearance in cells exfoliate from normal epithelium that differs distinctly from the structures of those derived from the diagnostic importance of the method. In the exfoliative cytology of the vagina and genital tract features of cells provides evidence for hormonal activity. From sputum, one may be able to diagnose malignant lesions and diagnosis of cancer has been carriedout from smears in pathology laboratories

Advanced Techniques In Histology And Cytology Laboratories

A. Immunocytochemistry in Histology and Cytology.

Immunohistochemistry (IHC)/immunocytochemistry (ICC) is the technique to visualize recognition of antigen present in the tissue with the help of corresponding antibody. Coons et al. first-time applied immunofluorescence technique on the frozen section by using fluorescence labelled antibodies. The antibody conjugated with enzyme acid phosphatase and horseradish peroxidase was used first time by Nakane and Pierce in 1967. IHC technique was successfully introduced in routine formalin-fixed paraffin embedded (FFPE) section by Taylor and Burns in 1974. Subsequently the development of monoclonal antibody introduced a new era in the immunohistochemistry. However, it took another 10–15 years to have regular routine use of IHC in pathology diagnostic laboratory. Presently IHC is an essential technique in every pathology laboratory.

Basic Principles

The basic principle of immunocytochemistry is to demonstrate the specific antigen in the cell by applying the corresponding antibody to have antigen-antibody reaction. The antigen containsan epitope or antigenic determinant site that evokes specific immunologic response to develop antibody. The antigen epitope site and antibody binding site have complementary geometrical and chemical features. This is responsible for the antigen-antibody reaction. This antigenantibody reaction is further visualized by attaching certain label to the primary or secondary antibody.

The Sample of Tissues for Immunocytochemistry Histopathology

- Formalin-fixed paraffin-embedded tissue
- Frozen section

Cytology

- Cell block tissue
- Smear from liquid-based cytology
- Direct cytology smear: imprint or FNAC smear
- Cytospin smear

B. Flow Cytometry: Basic Principles, Procedure and Applications in Pathology

Flow cytometry (FCM) is the method by which the various characteristics of individual particleor cells are studied. FCM provides us very quick assessment of cell surface antigens, DNA content and intracellular proteins. The instrument is now used not only for research but also for routine clinical activities. It is now a well-established technique for the diagnosis and classification of lymphoid neoplasms, identification of malignant cells in effusion cytology and other body fluids. Modern flow cytometer is now much more user-friendly and smaller in size.

Principle of Flow Cytometry

Single dissociated cells in liquid medium are essential for flow cytometry. The specific component of the cell is identified by the antibody tagged with a fluorescent dye. Similarly, DNA can also be stained by a DNA stoichiometric dye. The single cells rapidly pass in front ofa laser beam, and the laser beam of particular wave length hits the cell. The individual cells absorb the light and emit light of different wavelength. The emitted light is detected by the photomultiplier tube and is converted into a digital pulse. The intensity of the digital signal is stored in the computer and expressed in a relative scale known as channel.

Dye Used Fluorochrome Dyes for FCM

What is Fluorescence: When a fluorochrome compound absorbs quantum of light, the electron moves from lower orbit to the higher orbit, and compound remains in excited state. When the compound comes back to its ground state, the electron returns to its original orbit, and the compound emits the quantum of light of lower wavelength with a different color. The whole phenomenon is known as fluorescence. As the excited light wave and emitted light waves are of different wavelength and color, the emitted light is easily visible and can be recorded. Each fluorochrome dye compound has certain properties:

- Specific excitation spectrum of light
- Specific emission spectrum of light
- The quantum efficiency the difference between the wavelength of excitation or absorption spectrum and emission spectrum is known as Stokes shift. The higher the value of the Stokes shift, the greater the separation of the excitation and emission spectrum.

Fluorochrome Dye for Nucleic Acid

The common dyes in DNA FCM include propidium iodide, ethidium bromide, Hoechst 33342and diamidino phenylindole.

Fluorochrome Dye for Antibody and Protein Various fluorochrome dyes are used for conjugating antibodies and protein. The most commonly used fluorochrome dye is fluorescein is othiocyanate (FITC). highlights the excitation and emission spectrum of different commonly used fluorescein dyes.

Dye for RNA Content Measurement RNA content is measured with acridine orange, pyronin Yand oxazine 1.

Samples for Flow Cytometry Cytology Samples

The various types of cytology specimens for FCM include:

1. Fine needle aspiration cytology materials: Lymph node, breast, lung, prostate, etc.
2. Exfoliative samples: Effusion fluid, CSF, bladder wash

Histology Samples

- Frozen section tissue
- Paraffin-embedded tissue Histopathology sample needs thorough disaggregation for flow cytometry. In fact, paraffin embedded tissue does not give good result on FCM.

C. Digital Image Analysis and Virtual Microscopy in Pathology

In the last few decades, there is massive development of the computer technology followed by marked advancement in the field of high-resolution image digitization, image storage, extraction of features and analysis. Virtual microscopy has also significantly advanced in the last few years. Previously the pathologists used to give opinion on holistic viewing of the imageof the tissue/cells. Presently there is increasing demand to provide semi-quantitative features along with the quantitative information of various biomarkers on the tissue section or cytologysmear. Digital image analysis (DIA) provides more objective and consistent information of theimages and also helps in the diagnosis, grading and classification of the disease. It provides various prognostic information and guidance to therapy. Nowadays it is essential to have a good knowledge on digital analysis and virtual microscopy for the effective improvement of reportingtissue specimen.

Applications of Digital Pathology

The digital pathology has the following applications in the field of pathology:

1. Education: Presently the entire slides can be scanned and stored in the computer system. The slides can be available in the web page or in the parent computer. The classical cases can be used for teaching purposes.

2. Online opinion (telepathology): With the help of digital imaging system, it is possible to send the scanned image of the whole slide to the distant centre. The experts can examine the virtual slide and give their opinion. Thereby the physical transfer of the slides can be avoided, and rapid opinion may be available in a particular case.
3. Detection of malignancy: DIA is useful in the detection of malignant cells in cytology smears and in images of histopathology sections. It helps in the detection of false negative cervical cytology smears. Large number of slides can be re-screened after initial manual screening. In case of any suspicious cells, the computer gives alarm signal, and the slide is againscreened in that particular area to avoid any false negative report.
4. Detailed morphologic study of DNA and chromosomes: DNA and structure of chromosome have been studied with the help of digital image analysis.
5. Pattern recognition and grading of carcinoma: Image analysis has been applied to diagnoseand grading of carcinomas. Accurate grading of prostate and breast carcinoma were done by different workers on the basis of image analysis.
6. Quantitation of immunohistochemistry: With the help of digital image analyzer and appropriate software, it is now possible to quantify the HER 2/ER/PR immunostaining on the histopathology section. Automated quantification of fluorescent in situ hybridization (FISH) is also possible on histology tissue.
7. Assessment of aggressiveness of a tumor for personalized medicine: With the help of digital image analysis, it is now possible to find out the aggressiveness of individual tumor in individual persons. It is often seen that the same tumor with same type of treatment has differentoutcome in two different patients. Digital image analysis can provide useful information on (a) tumor morphology, (b) tumor classification, (c) tumor grading, (d) tumor stroma reaction, (e) quantification of biomarkers and (f) molecular phenotypes. The combined data of all those features may be helpful to plan for personalize treatment.

Basic Principles of Image Analysis

The basic principles and steps of image cytometry includes below;

- Image digitalization: Pixel is the basic unit of any image. Each pixel is located in particular X and Y axis. On the basis of the grey intensity of the pixel, the individual pixels are given a particular grey value. In a colored image in a particular pixel, three values are given based on the intensity of red, green and blue color. Therefore, the pixels are stored in the computer as the representationof a particular grey value. This is known as digitization of the image. From the digitized stored image, the original image can be easily retrieved.
- Image detection and segmentation: By adjusting grey threshold value, one can detect the cells or image of the interest.
- Image editing: With the help of editing, the images are processed, and the unwanted objects are eliminated from the background.
- Feature extraction: Next important step of image analysis is the measurement of various geometric data from the object of interest that include diameter, perimeter, area, roundness and texture. The other components of the features are the margin or periphery of the duct, gland or cell-stromal interface detection

D. Sanger Sequencing And Next Generation Gene Sequencing: Basic Principles And Techniques

DNA sequencing means the determination of the order of sequence of the base pair in DNA

Sanger Sequencing

Sanger sequencing is named under Sanger F. This is the widely used commercial DNA sequencing.

Basic Principle

In Sanger sequencing technique, 2′,3′-dideoxynucleotides are used for DNA synthesis. In the absence of 3′-hydroxyl group in 2′,3′-dideoxynucleotides, DNA cannot be synthesized further as no phosphodiester bond can be formed with the next dNTP, and the chain terminates. The four DDNTPs (dideoxynucleotides phosphates such as ddATP, ddTTP, ddCTP, and ddGTP) are labelled by different fluorochrome dyes so that they are recognized by laser beam. Each fluorescent-labelled terminated fragment of DNA is recorded and from this data DNA sequenceis assessed.

Reagents needed

- Primers: Small piece of single-stranded DNA is used.
- DNA template: This chain is sequenced.
- DNA polymerase enzyme.
- dNTPs (dATP, dTTP, dCTP, dGTP).
- ddNTP (fluorescent labelled): All four ddNTPs such as ddATP, ddTTP, ddCTP and ddGTP are labelled with different fluorochrome dyes. These are chain-terminating nucleotides.
- Mix the above reagents, and denature the complementary strands of DNA template by

heatingat 96 °C. • Lower the temperature to 50 °C for annealing the primer with DNA template.

- Raise temperature to 60 °C for the elongation of DNA with the help of DNA polymerase. The DNA chain elongates till it incorporates fluorescent-labelled ddNTP. At that point the DNA chain elongation is terminated. The DNA chain is terminated with various lengths bearing the terminal-labelled ddNTP (fluorochrome labelled A, T, C and G).
- The PCR cycle of DNA is repeated several times to have all the base position of the DNA template. At the end, variable lengths of DNA chains with terminal unique fluorescence labelled A, T, C and G are obtained.
- DNA chains undergone capillary gel electrophoresis, and then the colored emission spectrum of all these fluorochrome-labelled DNAs is recorded by capillary gel electrophoresis. With the help of a suitable software from the data of the terminal bases, the exact sequence of the bases in the DNA is generated.

E. Tissue Microarray in Pathology: Principal, Technique and Application

Tissue microarray (TMA) is a novel technology that helps to study a large number of tissue samples in a single section. In this technique few hundreds to thousands of tissue samples fromthe different paraffin block can be taken and are precisely put into a recipient paraffin block. TMA helps to perform immunocytochemistry (ICC), fluorescent in situ hybridization (FISH) or RNA analysis in the single section from the recipient block. Therefore, the technique is faster and cheaper than the conventional method

Tissue Microarray Technique

At first, hematoxylin- and eosin-stained section from the donor block is studied, and the representative area of the donor block is marked. With the help of tissue microarray instrument,core tissue biopsy (0.6–2 mm diameter) is taken from the donor paraffin block and is placed inan empty paraffin block (the recipient block) in precise manner. The recipient block can accommodate few hundreds to thousand specimens. The co-ordinates of the core biopsies in the recipient block are recorded typically in a spreadsheet (preferably in Microsoft Excel file). Now with the help of a microtome, 5 μ sections are cut from the TMA block to produce TMA slide.The section now can be used for IHC, FISH or other molecular studies.

TMA Construction and Generation of Grid

The construction of the TMA and the generation of the grid depend mainly on the type of the study.

1. At first make a list of suitable cases.
2. Study all the section of these cases.
3. Review the cases and reclassify if needed. Then mark the representative area on the stained section on glass slide. It is preferable to use different color for different diagnostic area such as green for normal area, red for tumor and blackfor in situ carcinoma.
4. Collect the representative block of the slides and identify the area

Designing theGrid

1. Capital letters indicate the quadrant, and small letters indicate the coordinateof the tissues in the recipient's block.
2. The primary data are entered for each tissue core such as biopsy number, location, diagnosis, grade of tumor, stage, etc. The data is usually entered ina Microsoft Excel file.
3. Protection wall: The peripheral boundary walls of the TMA are made of single row of core tissue. These core tissues may consist of any tissue and they are not analyzed
4. Control tissue: Positive and negative control tissues are placed in asymmetriclocation.
5. Tumor tissue: The tumor tissues are placed in small groups.
6. Normal heathy tissue: In between the groups of tumor tissue, the matched normal healthy tissues are placed in one or two rows. The presence of thesenormal tissue rows may help in the better orientation of the tumor tissues.
7. Empty cores in small row: Intentionally few cores in a row should be kept empty to immediately identify the orientation of TME grossly.
8. The different disease processes can be grouped together such as carcinoma cases; in situ carcinoma and normal cases are clubbed in different groups.

Diameter of the Core

The diameter of the punches of the core biopsy may vary from 0.6 to 2 mm. In fact, most of thepeople take 0.6 mm diameter core that includes 0.14 square mm tissue.

Number of Cores in TMA

In a 2.5 × 4.5 cm block, at least 1000 cores can be adjusted. However, it is preferable not to take more than 500 cores in a single block.

Recipient Block

The recipient block is made of fresh molten paraffin. The average size of the block is 2.5 × 4.5 cm. Adequate precautions should be taken to avoid any bubbles within the block.

Automated TMA

Presently automated tissue microarray machine is available that bypasses the tedious manual punching procedure. It helps to design the array design or layout, and the machine automatically creates holes in the paraffin wax and places the donor tissue in the hole of the recipient paraffinwax.

Advantages of TMA

There are several advantages of TMA that include:

1. Amplification of the resource: Ordinarily from a standard 5 mm thick section of tissue, we can get maximum 100 sections for study. Whereas depending on the size of the tissue in the original block, at least 200 corebiopsies can be taken to make TMA block. After the construction of the TMA, we can cut at least 1000 sections of 3 μ thickness from the 5 mm thick array block. Therefore, we get a total 1000 × 200 means 200,000 sections from the limited resource.
2. Uniformity in staining conditions: At the time of conventional staining, the different tissue sections are stained at different time (such as10 batches of 20 slides each), and the slide-to slide variation may occur dueto several variables such as antigen retrieval, concentration of different reagents, incubation period, washing time, etc. However, in case of TMA, each tissue section consists of 100–1000 core biopsies from the different patients, and the single section is stained that avoids all the slide-to slide variability.
3. Faster, cheaper and reduction of manpower: In TMA a single slide requires less reagents, labor and time. Therefore, TMA saves cost, time and total work force.
4. Original block can be preserved: Only a few core biopsies from the original block are sufficient to make TMA. The original block can be preserved and can be used for further sectioning.
5. Effective in quality assurance program: Due to significant amplification of the laboratory material, TMA can be used for external and internal quality control program. The TMA section can also be used for standardization of reagents for positive control.
6. The whole analysis of TMA is now automated and computer can keep track of the huge data.

F. Polymerase Chain Reaction: Principle and Technique

Polymerase chain reaction (PCR) is one of the most important techniques in molecular pathology. With the help of PCR, the single or the pieces of target DNA can be amplified manyfolds. This technique is now an integral part in every modern laboratory for both the diagnosisand research use.

What Is PCR and How It Works?

PCR acts like a "molecular photocopying" machine and amplifies the specific target DNA. Thebasic principles of PCR are:

- Double-stranded target DNA is made into single-stranded DNA by applying heat.
- Two oligonucleotide strands or primers are added. The oligonucleotide strand binds with its complimentary DNA strand to the 3′ ends.
- The DNA strand is now extended with the help of DNA polymerase (Taq polymerase). This polymerase enzyme incorporates the nucleotides in the DNAto make it elongated.
- The cycle is repeated.

Steps of PCR

The basic steps of PCR are described as:

- Denaturation step, 94 °C: DNA is heated to 94 °C to make it single stranded. Only 1–2 min is given to this heating process in each cycle.
- Annealing, 54 °C: The temperature is rapidly cooled. In this lowered temperature, the primer quickly anneals with the respective site of DNA. With the help of Taq polymerase, the reaction starts at the primer-DNA template site.
- Extension, 72 °C.
- The complementary nucleotides are attached from the 3′ to 5′ end of DNA. There is an exponential increment in the number of genes in each cycle. At least30 cycles of denaturation-annealing-extension is done in each PCR

Essential Ingredients of PCR

a. Primers: The primers are the small pieces of artificially made DNA strands thatare actually the complementary strands of the 3′ end of each strand of target DNA. Primers usually consist of 20–30 nucleotides.

b. DNA polymerase (Taq polymerase): Taq polymerase is a type of DNA polymerase enzyme that extends the new DNA strand. It combines at the end of the primer and then sequentially adds new nucleotides to the DNA strand at 3′ end complementary to the target DNA. High temperature (94 °C) is needed to separate the double-stranded DNA. Ordinary DNA polymerase breaks down in this temperature. However, the Taq polymerase has the unique characteristic towork efficiently in higher temperature. This Taq DNA polymerase is extracted from the bacteria Thermus aquaticus. These bacteria live in hot spring and can survive there

c. Deoxynucleotide triphosphates (dNTPs): Deoxynucleotide triphosphates (dNTPs) are

dATP, dCTP, dGTP, and dTTP. These are the raw material or the basic building blocks of the new DNA strands. The Taq polymerase captures these dNTPs from the working solution and attach them to the terminal part of the primers to extend the DNA chain.

d. The target DNA from the sample: The target DNA is extracted from the sample.
e. Buffer solution: It provides the optimum chemical environment for the reactionto occur.
f. Magnesium chloride (MgCl2): Magnesium chloride works as a cofactor of the Taq polymerase enzyme.

CONCLUSION

Systematic reviews of diagnostic interventions support clinical and policy decisions, the development of practical guidelines, clinical audit, technological assessment, economic evaluations, education and training, and identifying gaps in our knowledge for future research. systematic reviewing of laboratory data is expected to result in better, bigger and more reliable primary studies, which hopefully support the diffusion of new diagnostic technologies with scientifically proven efficacy and effectiveness in the future.

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