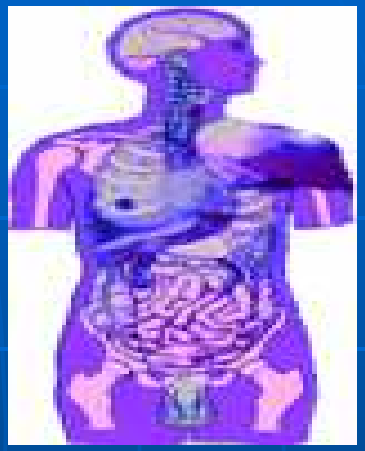
MEDICAL UNIVERSITY - PLEVEN
FACULTY OF MEDICINE
DEPARTMENT OF GENERAL AND CLINICAL PATHOLOGY

Lecture

Techniques for study of pathology & Pathological diagnosis of neoplasm

Prof. S. Popovska, DSc

Conventional diagnosis of tumor



- Signs and symptoms
  - Palpable lump, pain
  - Fever, Fatigue, Weight gain or loss
  - Altered metabolism
- Laboratory tests- PSA, CA 15-3, AFP
- Medical imaging: X-ray, CT,MRI,PET.
- Gold standard: diagnostic pathology methods

A) MORPHOLOGICAL METHODS

i. **FNAC**
ii. **Exfoliative cytology**
iii. **Abrasive cytology**
iv. **Washings and lavage Techniques**

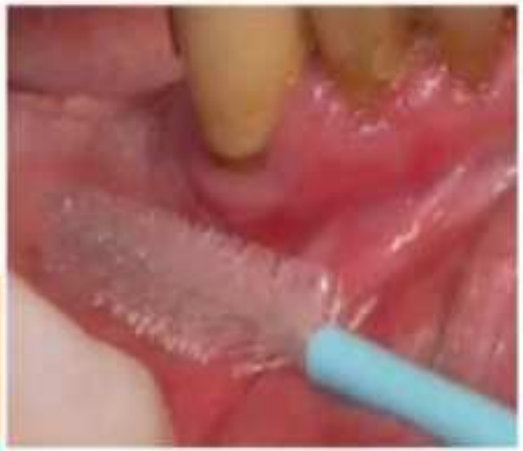
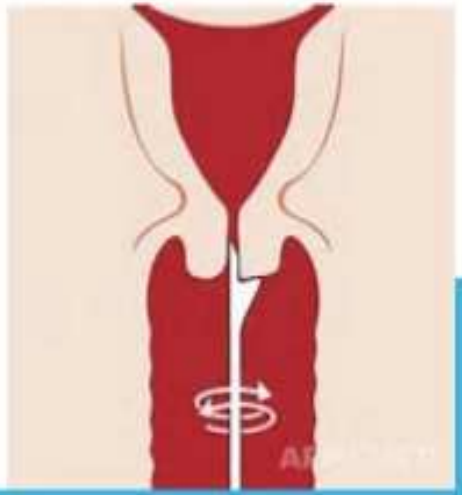


Fig. 1. Obtaining an exfoliative smear with the CytoBrush® (Andalc Barcelona, Spain).
Toma de una muestra mediante CytoBrush® (Andalc Barcelona, España).



Autopsy

„Hic locus est ubi mors gaudet succurrere vitae.“

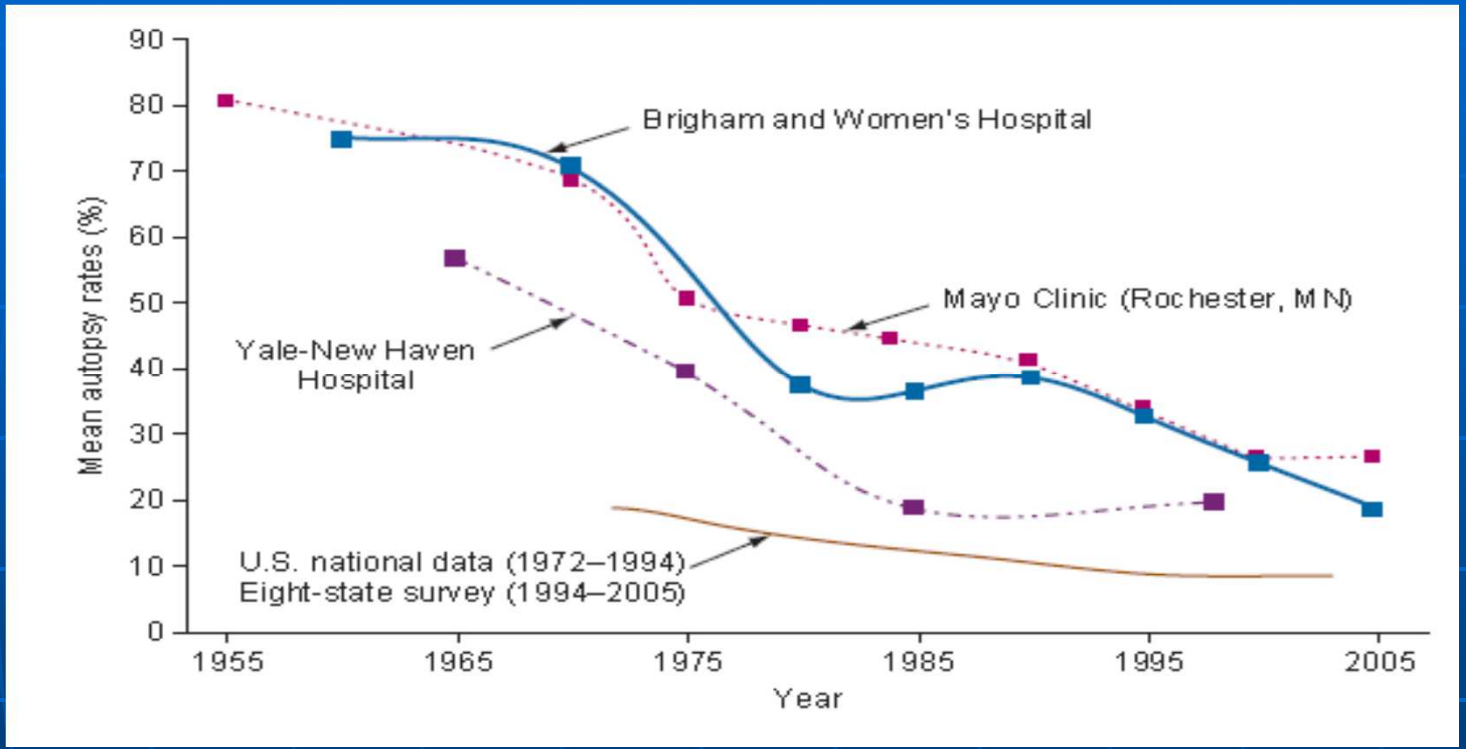
„This is the place where death rejoices to help those who live.“

- The main purposes of autopsy are as under:

1. Quality assurance of patient care by:
   - confirming the cause of death
   - establishing the final diagnosis
   - study of therapeutic response to treatment

2. Education of the entire team involved in patient care by:
   - making autopsy diagnosis of conditions which are often missed
   - discovery of newer diseases
   - study of demography and epidemiology of diseases
   - affords education to students and staff of pathology

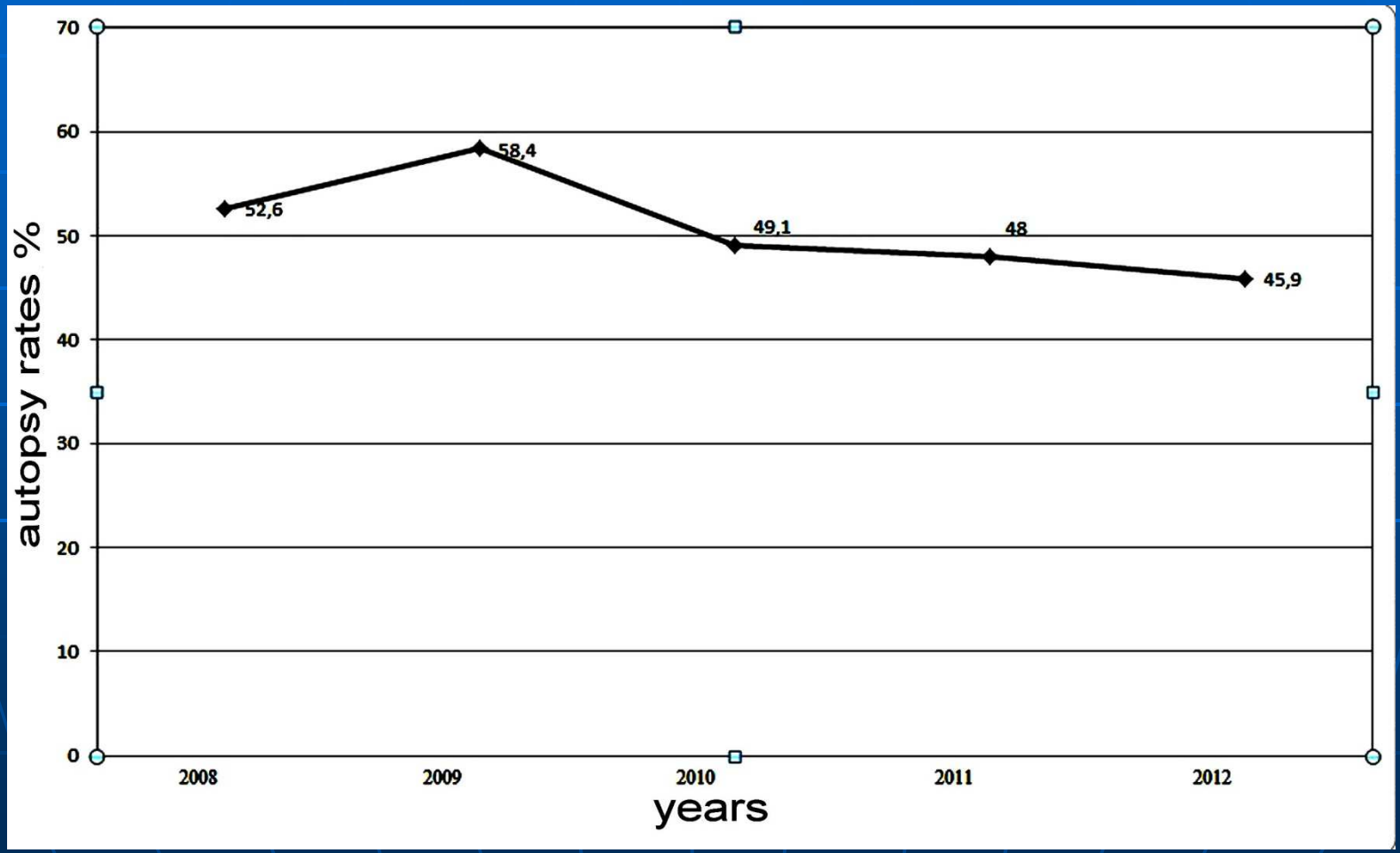
Declining autopsy rate



Declining autopsy rate throughout world in the recent times is owing to the following reasons:

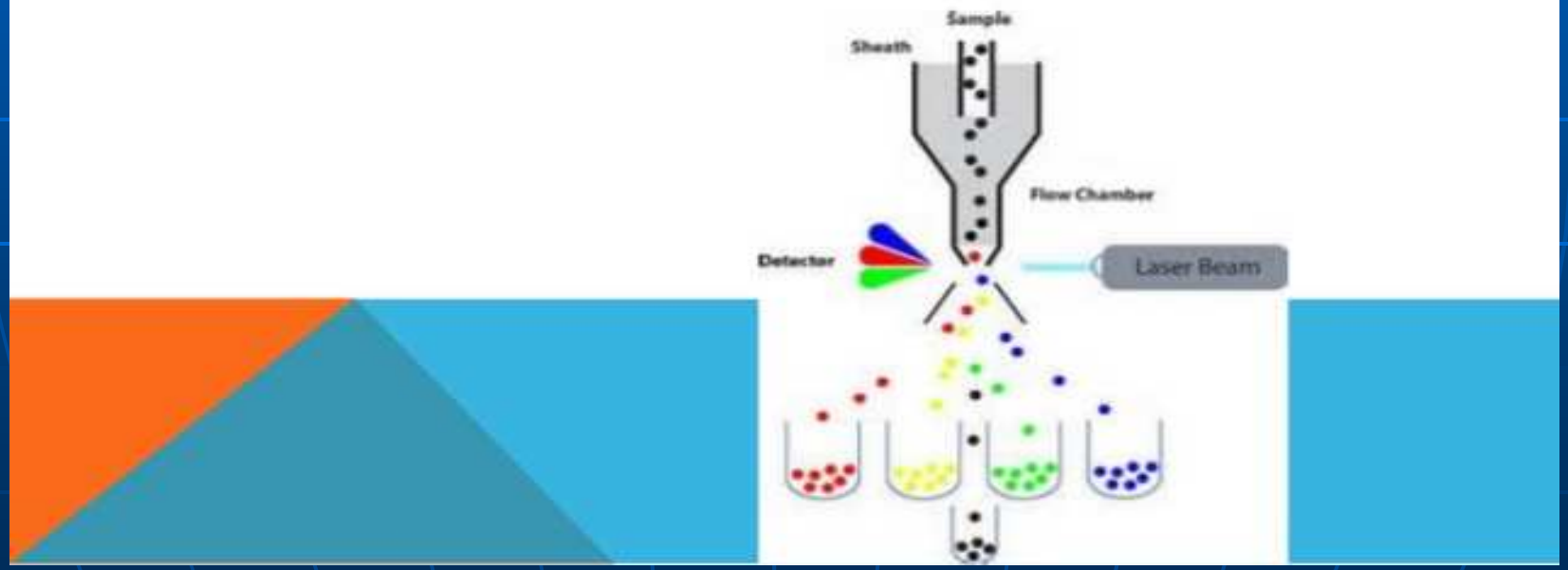
1. Higher diagnostic confidence made possible by advances in imaging techniques e.g. CT, MRI, angiography etc.
2. Physician's fear of legal liability on being wrong.

Declining autopsy rate
Pleven University Hospital



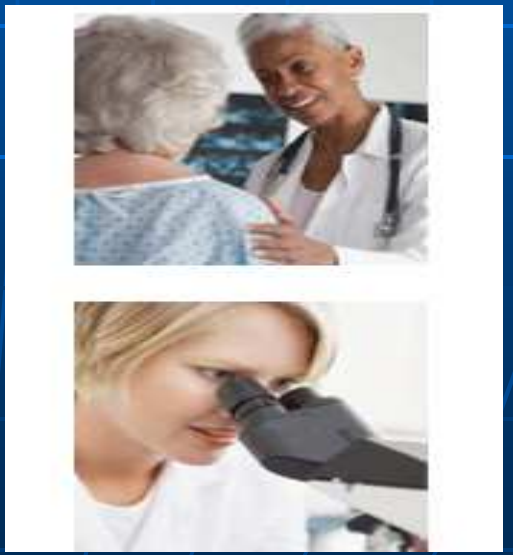
VARIOUS METHODS OF DIAGNOSIS

a. **Cytological and Histopathological techniques**
b. **Immunocytochemistry & immunohistochemistry**
c. **Molecular Diagnosis**
d. **Flow cytometry**
e. **Tumour markers**



Cytological diagnosis

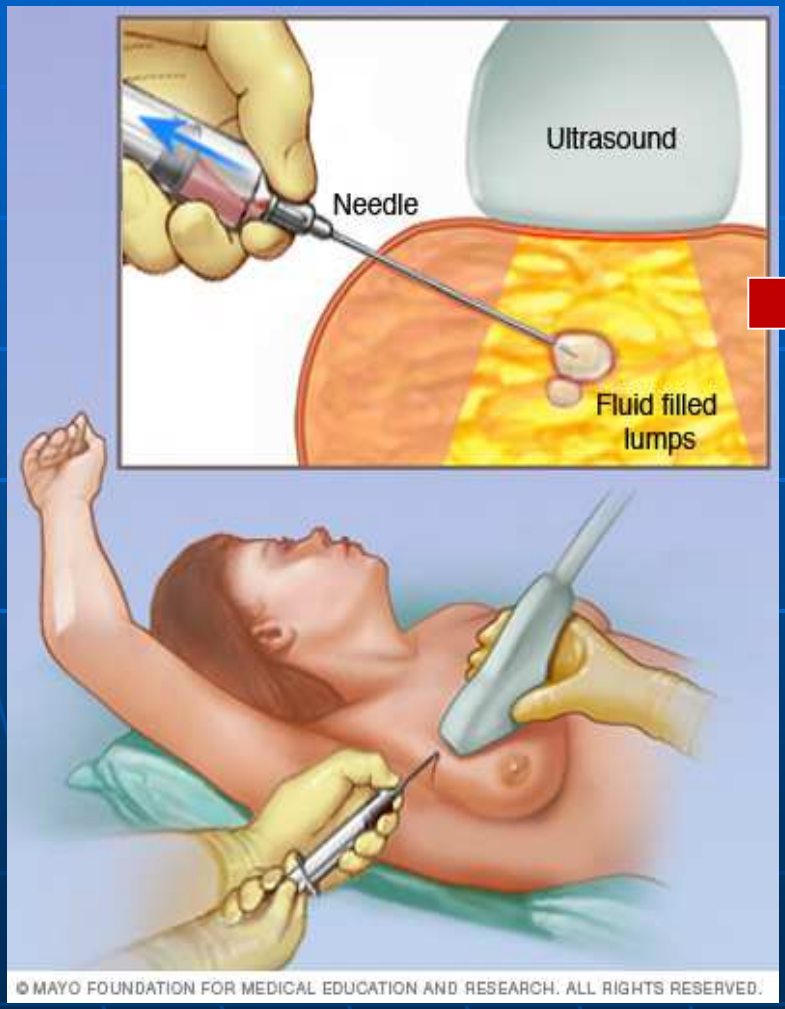
- *Cytopathology* refers to diagnostic techniques that are used to examine cells from various body sites to determine the *cause or nature of disease.*
- The first important decision for suspicious case is:
  inflammation vs neoplasia
- Second important decision is
  Benign vs malignant
- Cytological diagnosis methods
- Fine-needle aspiration
- Abrasive cytology
- Exfoliative cytology

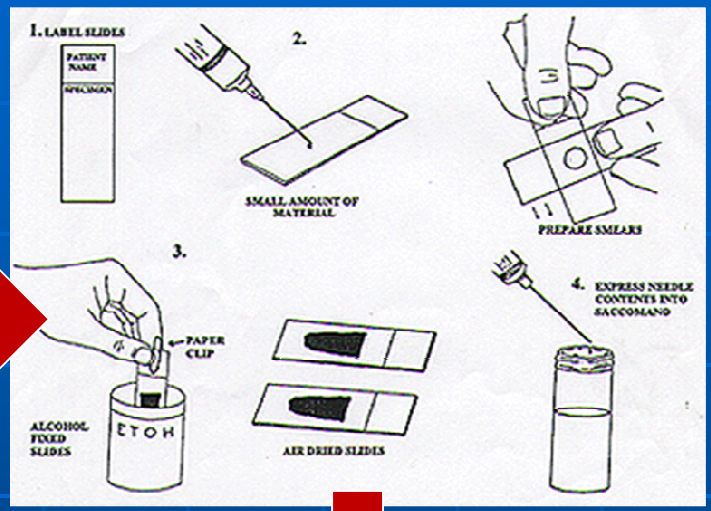


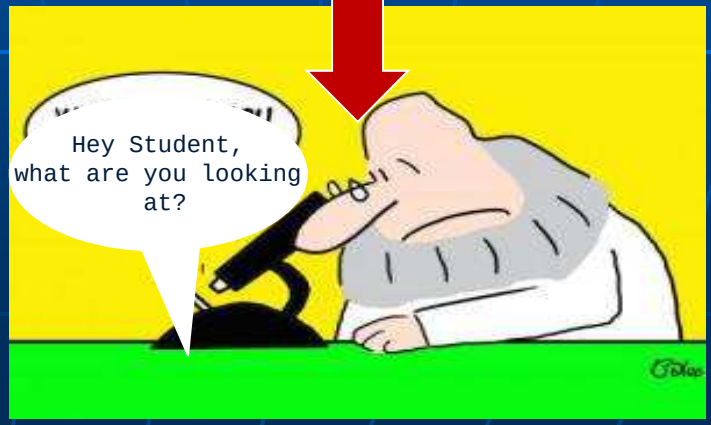
Fine-needle aspiration

- FNA cytology may be indicated in the following clinical situations:
  - investigation of palpable masses, regardless of whether they are considered benign or malignant
  - investigation of impalpable image-detected masses that are considered likely to be benign or with typically malignant features
  - investigation of suspected local recurrence of cancer, as suggested by the presence of palpable masses, impalpable image-detected masses, or lymph node involvement
  - evaluation of cystic lesions with atypical imaging features
  - confirmation of a diagnosis of cancer when core biopsy is not available, not possible or contraindicated.
- The use of FNA significantly decrease health care costs by decreasing the number of open surgical biopsies
- A diagnosis of malignancy allows preoperative discussion of available therapeutic options
- Patient-friendly procedure

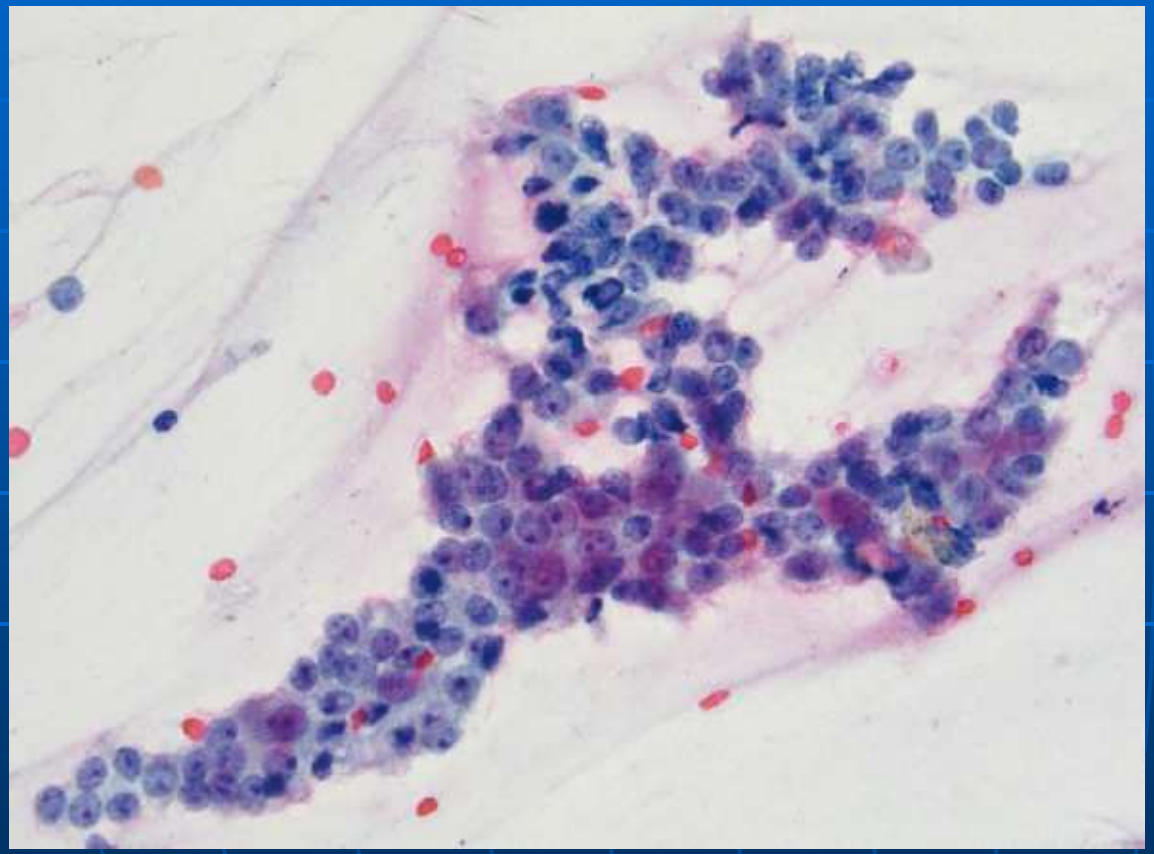
Fine-needle aspiration







Fine-needle aspiration



Fibroadenoma (FA). Note the prominent nuclear atypia with nucleoli. The tightness of the cluster and a background of single bipolar cells and stripped nuclei are important clues in avoiding an overdiagnosis of malignancy (Papanicolaou stain).

Abrasive cytology

- The purpose of this procedure is to enrich the sample with cells obtained directly from the surface of the target of interest. Hence, cell specimens are usually obtained through superficial scraping of the lesion (artificial mechanical desquamation) .
- Examples include:
- Cervical scraping or the so called Pap smear: the cervical scraper introduced by Ayre in 1947 (i.e., Ayre's spatula) allows a direct sampling of cells from the squamous epithelium of the cervix and the adjacent endocervical canal.
- Buccal mucosal smear
- Skin scraping of various lesions
- Brushing techniques through using rigid endoscopic & fibroptic instruments to collect cell samples from the gastrointestinal tract, bronchial tree, etc…

Abrasive cytology
Pap Smear Test

- Is used mainly to detect precancerous and cancerous lesions of the uterine cervix.
- It is based on the fact that cancer of the cervix is one of the most preventable cancers, because most of the cellular changes which may lead to carcinoma can be detected and accordingly treated at an early stage before progression.
- In most cases, cervical carcinoma develops slowly (over a period of up to 10 years), whereby it passes into different pre-neoplastic conditions before it reaches the cancer stage, termed dysplasia or Cervical Intraepithelial Neoplasia (CIN) or Squamous Intraepithelial Lesion (SIL):

III) ABRASIVE CYTOLOGY

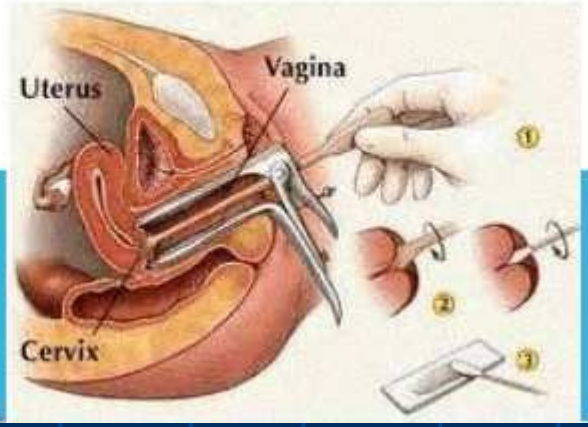
Surface scraped using Ayre's Spatula or brush.

With or without optic guidance

Examples: cervical smears (PAP smear)

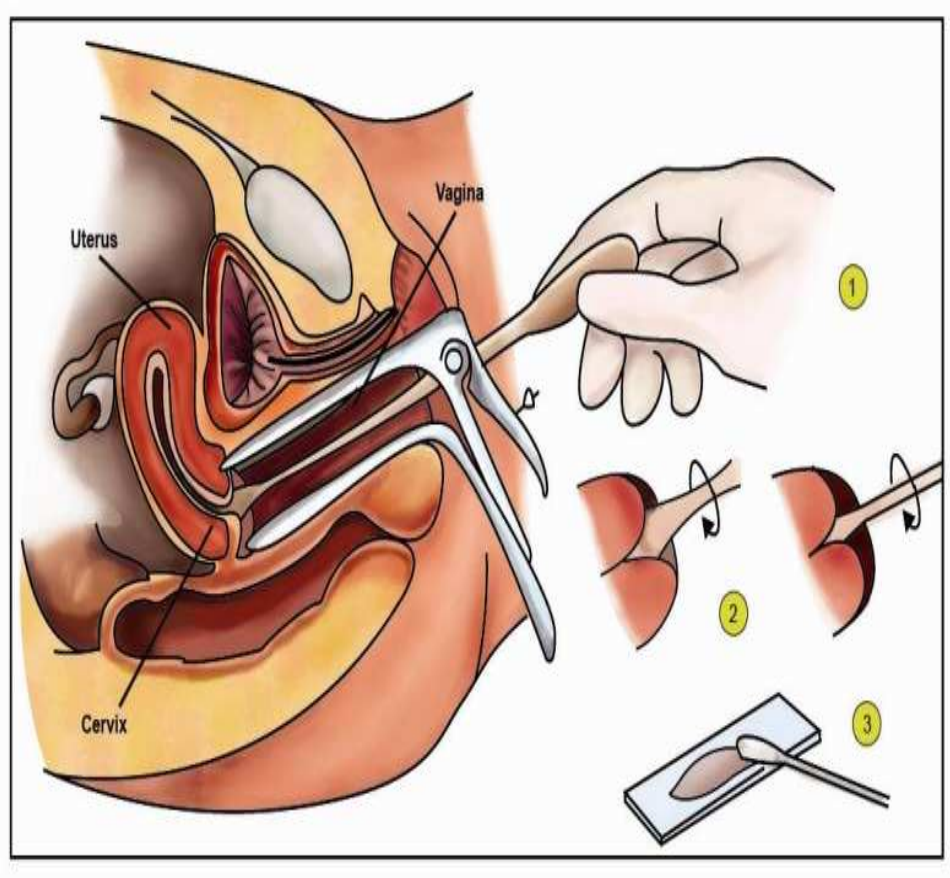
bronchoscopic brushings

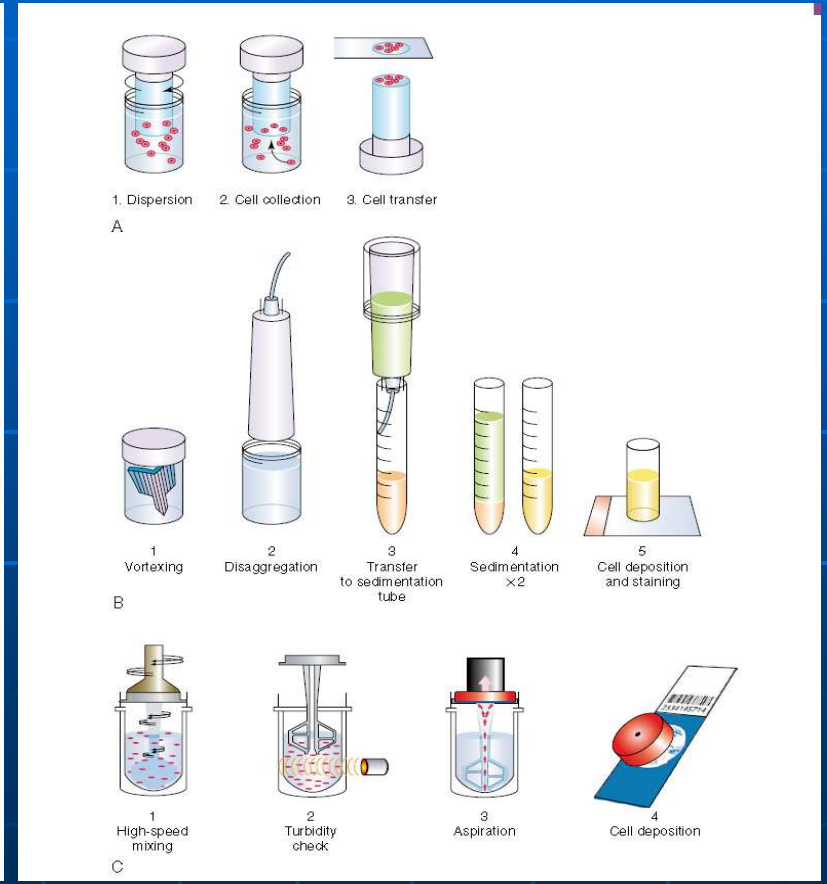
endoscopic brushings from lesions of GIT



Abrasive cytology

Pap Smear Test





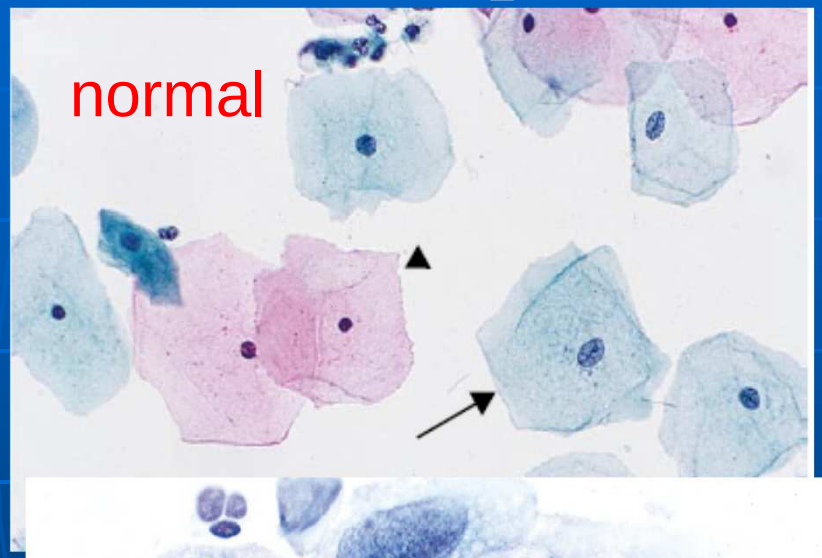
Abrasive cytology

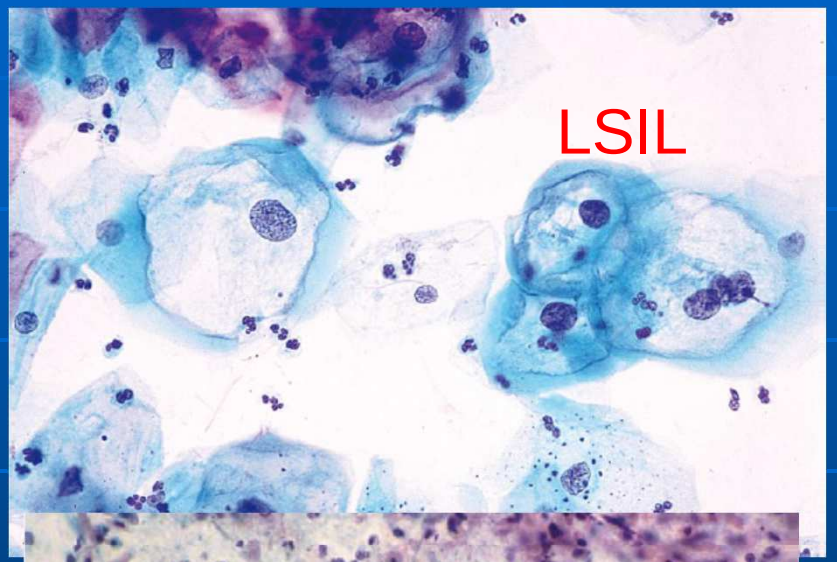
Pap Smear Test report

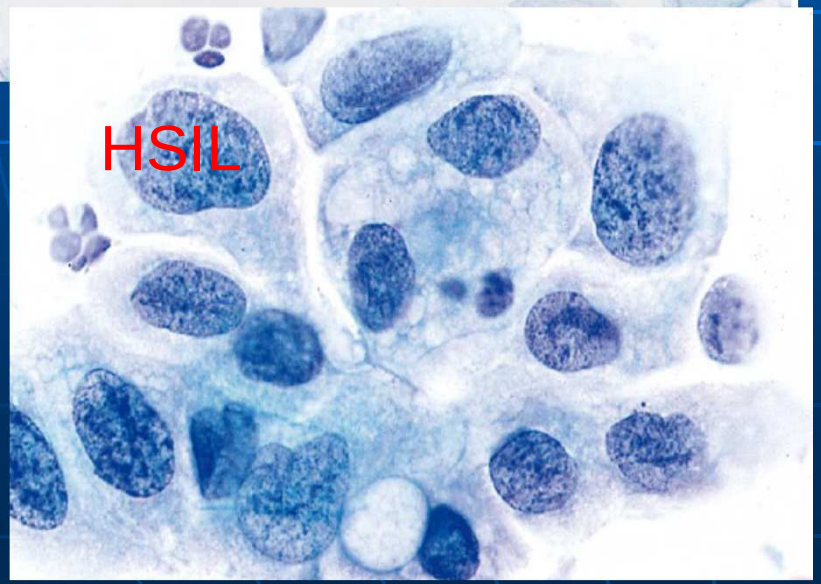
Cytological classification (used for screening)		Histological classification (used for diagnosis)	
Pap	Bethesda system	CIN	WHO descriptive classifications
Class I	Normal	Normal	Normal
Class II	ASC-US ASC-H *	Atypia	Atypia
Class II	LSIL	CIN 1 including flat condyloma	Koilocytosis
Class III	HSIL	CIN 2	Moderate dysplasia
Class III	HSIL	CIN 3	Severe dysplasia
Class IV	HSIL	CIN 3	Carcinoma in situ
Class V	Invasive carcinoma	Invasive carcinoma	Invasive carcinoma

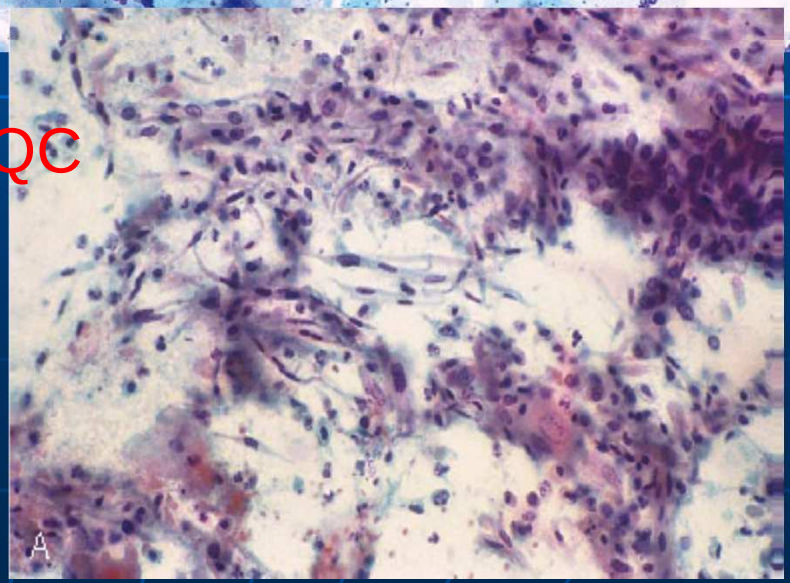
* ASC-US atypical squamous cells of undetermined significance ,ASC-H-atypical squamous cells HSIL

Abrasive cytology
Pap Smear Test report









Exfoliative cytology

- Exfoliative cytology– based on spontaneous shedding of cells derived from the lining of an organ into a cavity, where they can be removed by nonabrasive means
- Typical examples are:

  -Vaginal smear

  -Urine

  -Sputum

  -Nipple discharge

  -Effusions in body cavities (pleura, pericardium, peritoneum)

IV) WASHINGS AND LAVAGE

- **Normal saline is instilled into body cavity and reaspirated back, collecting shed cells**

Examples: gastric lavage, peritoneal lavage, bladder lavage

- **Fixative used: 95% ethylalcohol or cytospray**
- **Stains used: Papanicolaou stain or Giemsa stain**

Cytological diagnosis Advantages *vs* Disadvantages

- Advantages
  - Samples can be collected quickly and easily
  - Inexpensive
  - Little or no risk to the patient
  - Examine the cause or nature of disease
    - *Specific vs nonspecific inflammation*
    - *Inflammation vs neoplasia*
  - Direct therapy
  - Determinate next diagnostic procedures
- Disadvantages

  It is not always possible to:
  - Localize neoplastic lesion
  - Distinguish preinvasive of invasive cancer
  - Distinguish reactive of dysplastic and neoplastic changes
  - Determine tumor type

Histopathological diagnosis

i.e.

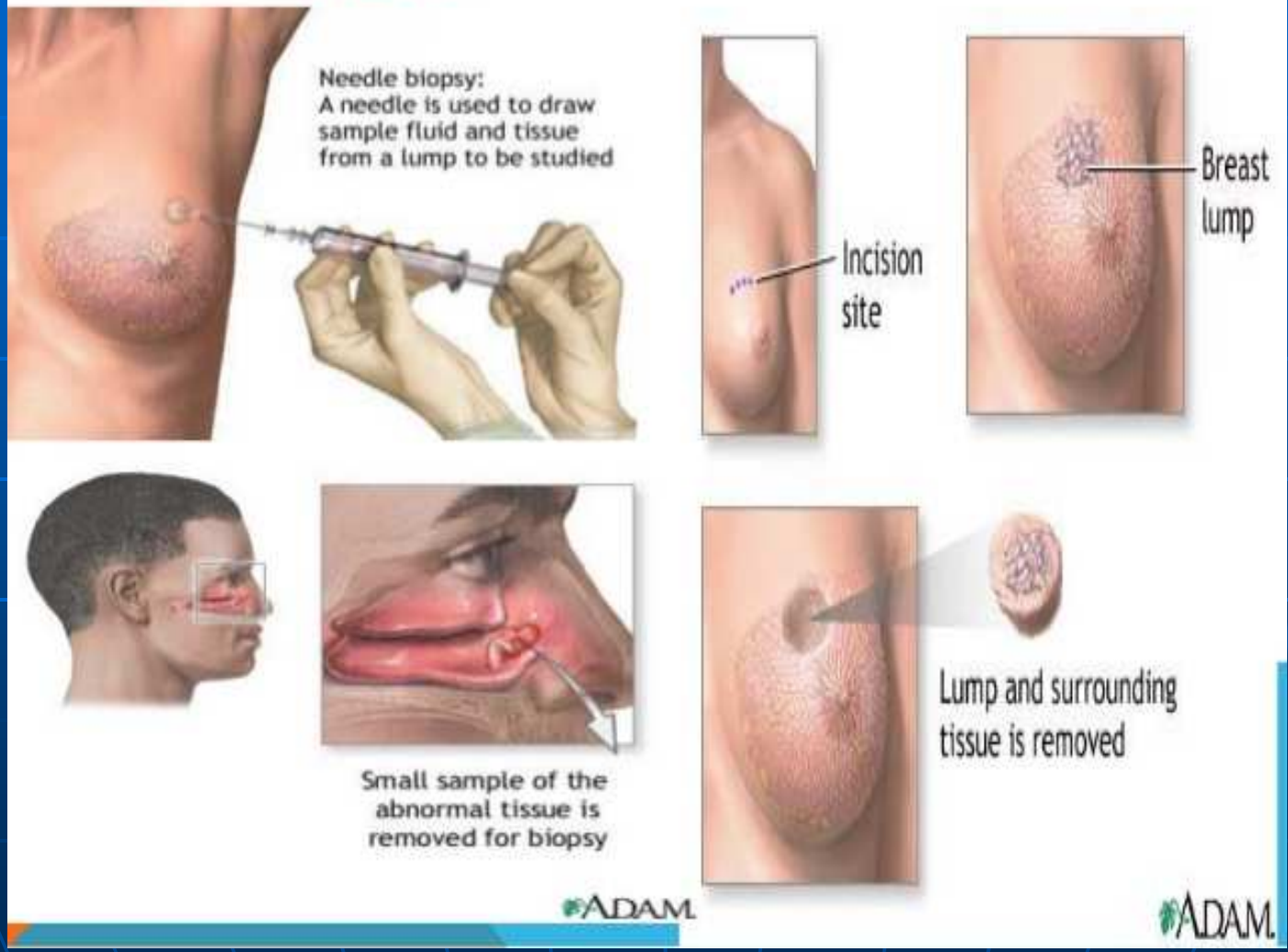
Surgical pathology

- **Surgical pathology is the classic method of tissue diagnosis made on gross and microscopic study of tissues.**
- **The surgical pathologist examines tissues and foreign objects removed from patients to identify disease processes, document surgical procedures, and release tissue for research.**
- **Specimen-comes from the Latin word specere, meaning "to look."This is a sample for medical testing.**

Specimens submitted for examination include:

- **Fluids, cells, and tissues.**
- **Products of conception.**

HISTOPATHOLOGY



Histopathological diagnosis

1.Collecting specimens:

-*Biopsy:*

core biopsy

incisional biopsy

excisional biopsy

-*Surgical excision:*

regional lymph nodes

organs or tissues with the tumors

2.Submitting pathology specimens

-Request form

Patient identification

Identification of the individual(s) requesting the examination

Procedure date&time

Adequate clinical history

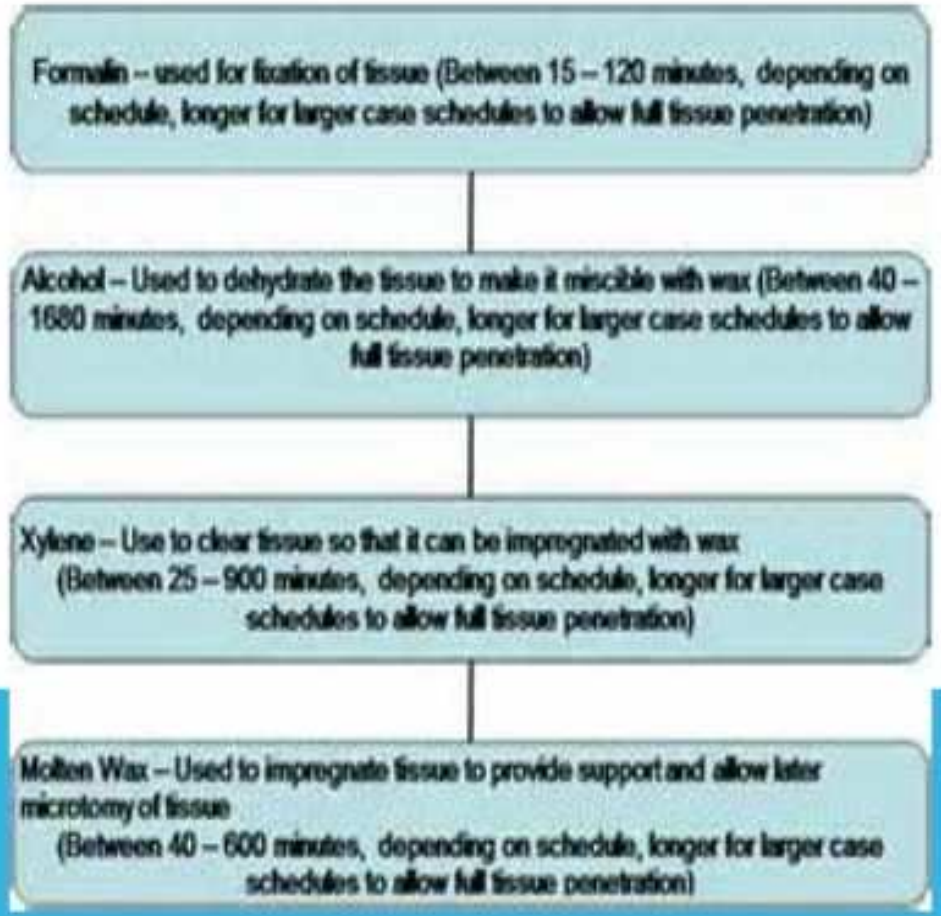
Specimen identification, including tests requested and any special handling required

Instructions for the disposition of gross specimens

Fixative used: 10% neutral Formalin

Various steps:

1. Fixation
2. Dehydration
3. Clearing
4. Impregnation
5. Staining
6. Examination of slide



Histopathological diagnosis

- EXAMPLES OF SPECIMENS REQUIRING SPECIAL PROCESSING

TYPE OF SPECIMEN OR REQUESTED STUDY	CONDITION OF SPECIMEN	COMMENTS
Gout	**Unfixed**	**Uric acid crystals dissolve in formalin. Tissue should be fixed in 100% ethanol for anaqueous processing.**
Liver: acute fatty liver	**Unfixed**	**Lipids are dissolved during routine processing. Demonstration of fat requires frozen section and special stains.**
Liver: copper	**Special**	**The specimen must not be touched with metal tools to avoid trace contamination.**

Histopathological diagnosis

3.Transportation - must be fast and secure

- Autolysis immediately begins and immunoreactivity is diminished for some markers (e.g., for receptors in breast cancers).In some cases, it is appropriate for clinicians to directly place specimens into fixatives at 15 to 20 times the volume of the tissue. The type of fixative must be identified on the container. The time of placing the specimen in the

fixative should be included when appropriate (e.g., for fixatives containin mercury such as Zenker's, if rush processing is requested, or if time in fixatio affects the results of requested immunohistochemical studies).

- Hazard - all tissues may be hazardous and must be transported in a safe fashion.

4.Tissue accession - The laboratory staff receiving the biopsy specimen must always match the ID of the patient on the request form with that on the specimen container.For routine tissue processing by paraffin-embedding technique, the tissue must be put in either appropriate fixative solution (most commonly 10% formol-saline or 10%buffered formalin) or received fresh-unfixed. For frozen section, the tissue is always transported fresh- unfixed.

Histopathological diagnosis

5.Fixatives

- Choice of fixative may limit the opportunities for other special studies.Before fixing tissue, consideration should be given to cytogenetic (cell culture) studies and frozen tissue (RNA and DNA analysis), which require, or are best performed on unfixed tissue.

- Formalin -10% phosphate - buffered formalin. Standard fixative and has been used in many studies of special stains and immunohistochemistry. It fixes most tissues well and is compatible with most histologic stains. Tissue can be preserved in formalin for many months. Fixation occurs due to cross-linking of proteins. Formalin penetrates tissue at about 0.4 cm each 24 hours. Overfixation can diminish immunoreactivity. To some extent this is reversed by antigen retrieval methods. Modifications adding zinc may also preserve antigenicity.

Histopathological diagnosis

Decalcification –

Bone and other calcified tissues (blood vessels with calcified plaques, some teratomas, intervertebral discs, some meningiomas, some ovarian tumors, calcified infarcted epiploic appendages, etc.) must have the calcium removed in order to allow the specimen to be sectioned.

Some fixatives(e.g., Bouin's and Zenker's) will both fix and decalcify tissues. Other decalcifying agents are not fixatives and tissues must be fixed first before using such agents.

Decalcification will adversely affect histologic detail and preservation of some nuclear antigens, especially ER, PR, p53, and Ki-67.It may not be possible to perform FISH or other assays requiring intact DNA on decalcified tissue. Specimens of diagnostic importance (e.g., tumors) should be decalcified for the least amount of time necessary by checking the tissue every few hours.

Decalcification with 10% EDTA has the best results for molecular studies.

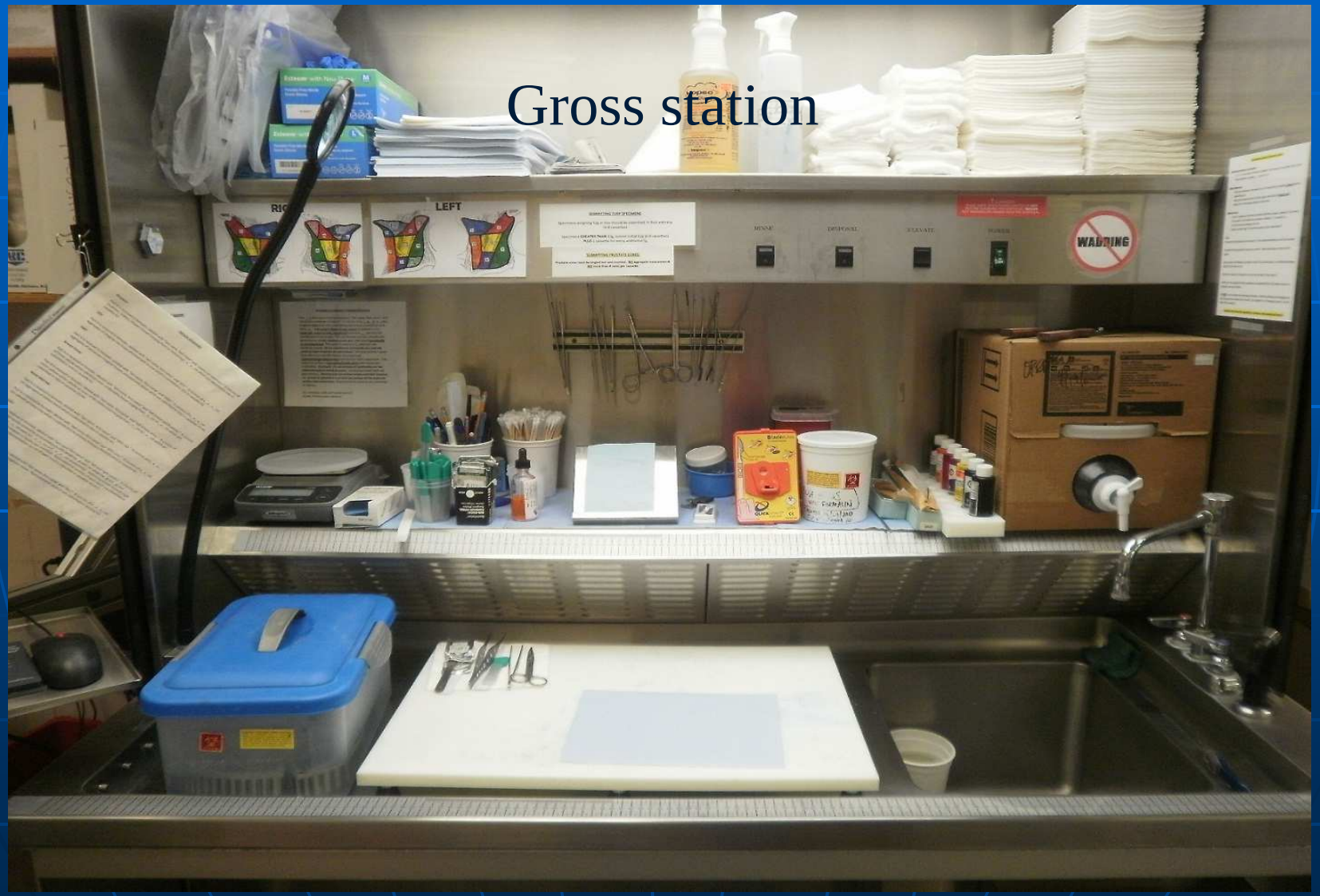
Histopathological diagnosis

6. Gross examination of the specimen received is the next most important step. Proper gross tissue cutting, gross description and selection of representative tissue sample in larger specimens is a crucial part of the pathologic examination of tissue submitted. Complacency at this step cannot be remedied at a later stage and might require taking the tissue pieces afresh if the specimen is large enough and that may delay the report, or if the biopsy is small and lost in processing the entire surgical procedure for biopsy may have to be done again.

Modern compact grossing stations have inbuilt system for recording gross description through dictaphone without the aid of an assistant to write it. Some laboratories have a protocol of doing gross specimen photography and specimen radiography, before and after tissue cutting for documentation.

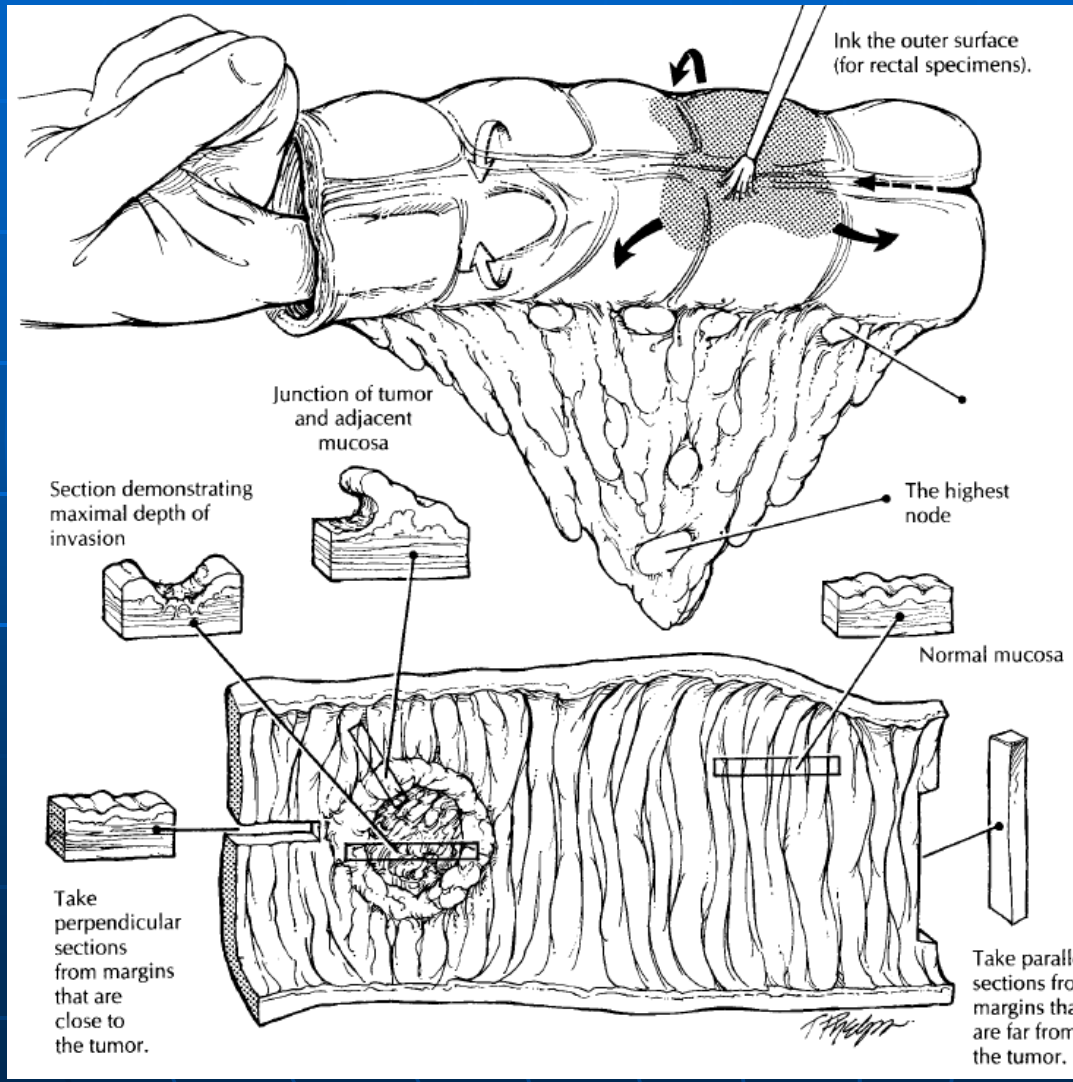
Macro imaging systems

Histopathological diagnosis



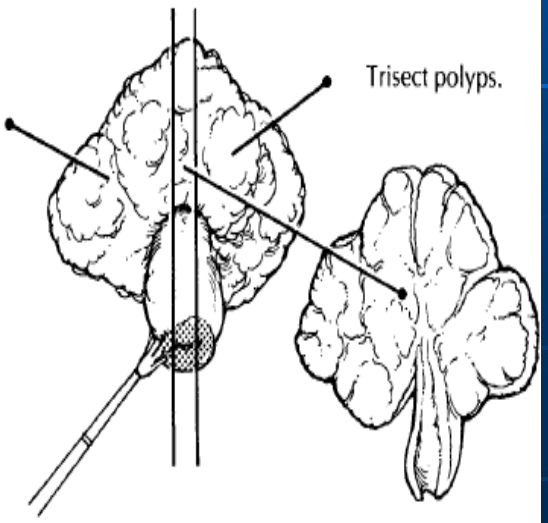
Gross station

Histopathological diagnosis



Resections of Intestinal Neoplasms

1. Orient the specimen. Record its length and diameter proximal and distal to the tumor. Describe the serosa.
2. Open the bowel on the side opposite the tumor. Record the size of the tumor and the distance to each margin. Section the tumor, and document its deepest gross penetration.
3. Remove the mesentery, and submit representative sections of each node by level. Look for vascular invasion.
4. Submit sections of tumor to demonstrate its deepest penetration and relationship to normal mucosa. Submit sections of the proximal and distal margins, other lesions, normal mucosa, all lymph nodes, and any attached structures or organs.



FROZEN SECTION

Cryostat used

Liquid N at -190 C or liquid carbon dioxide at -90 C used to freeze IC water into ice (embedding medium)

Used for:

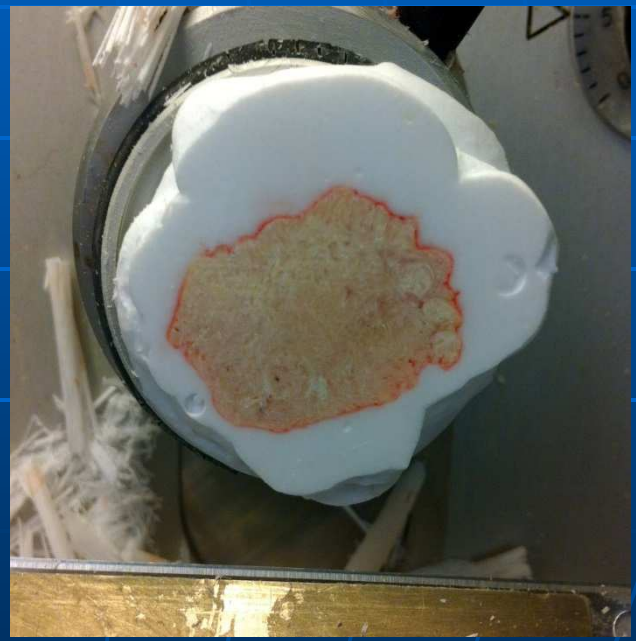
- ✓ **Rapid 'on-table' diagnosis**
- ✓ **Preservation of enzymes and labile substances like lipids and glycogen**
- ✓ **Determining nature of mass lesion**

Histopathological diagnosis

7. Frozen section-cryostat or frozen section eliminates all the steps of tissue processing and paraffin-embedding. Instead, the tissue is quickly frozen to ice at about – 25° C which acts as embedding medium and then sectioned. Sections are then ready for staining. Frozen section is a rapid intraoperative diagnostic procedure for tissues before proceeding to a major radical surgery. Frozen section is stained with rapid H & E or toluidine blue routinely. Special stains can be employed for either of the two methods according to need. The sections are mounted and submitted for microscopic study.

Histopathological diagnosis

Cryostat for cutting sections by freezing technique



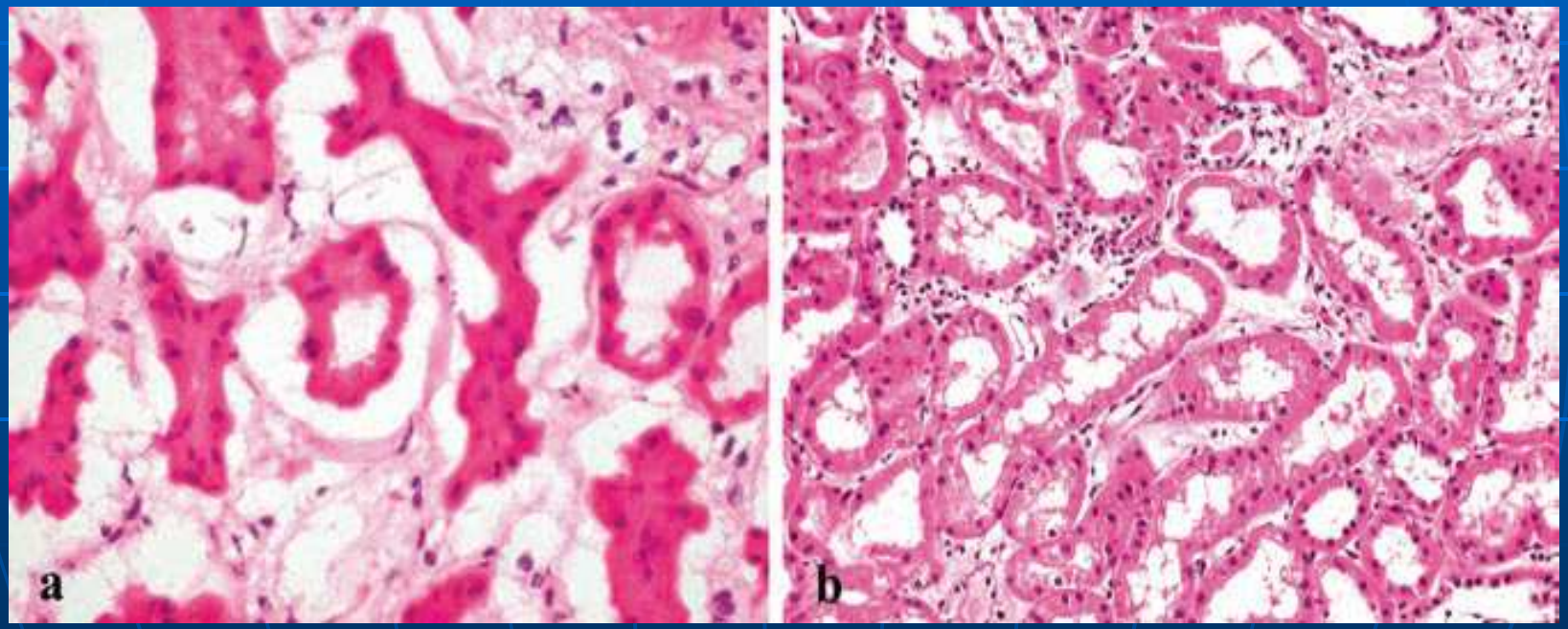
Histopathological diagnosis

Paraffin-embedded tissue vs Frozen section

	Paraffin-embedded tissue section	Frozen section
Specimens	Fixed tissues	Fresh tissues
Making time	24 - 48 hours	10 - 20 minutes
Saving time	Permanent	Months
Morphology under microscopy	Clarity	Opacity
Application	Pathological Diagnosis	Intraoperative consultation

Histopathological diagnosis

Paraffin-embedded tissue vs Frozen section



Freeze artifacts: Compression artifact. Figure (**a**) is frozen section micrograph of kidney tissue at 400× magnification. There is scalloping and compression of the renal tubules by the bubble like crystals forming in the surrounding watery stroma. Figure (**b**) is the paraffin embedded section from the tissue used to prepare the slide. The watery stroma is evident but compression is not present in this paraffin embedded control

Histopathological diagnosis

8. Paraffin-embedded tissue - Tissue cassettes along with unique number given in the gross room to the tissue sample is carried throughout laboratory procedures.

-10% formalin for fixation

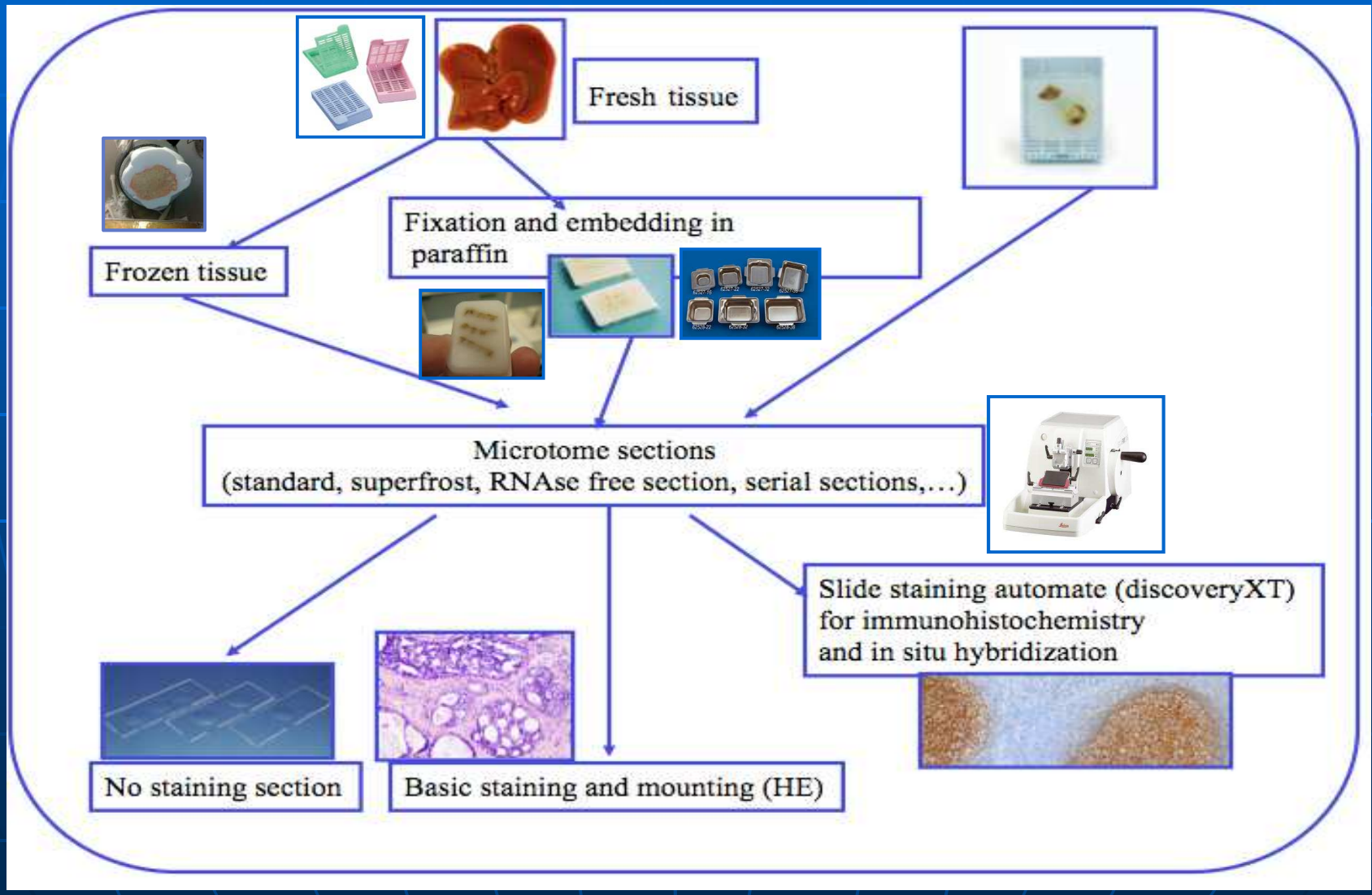
- ascending grades of alcohol (70%, 95% through 100%) for dehydration for about 5 hours in 6 -7 jars

- xylene/toluene/chloroform for clearing for 3 hours in two jars

- and paraffin impregnation for 6 hours in two thermostat - fitted waxbaths.

Embedding of tissue is done in molten wax, blocks of which are prepared using metallic L (Leuckhart's) moulds. The entire process of embedding of tissues and blocking can be temperature controlled for which tissue embedding centres are available .The blocks are then trimmed followed by sectioning by microtomy, most often by rotary microtome. Paraffin - embedded sections are routinely stained with haematoxylin and eosin (H & E).

Histopathological diagnosis



9. Staining

Staining	Presented structures	Examples from forensic practice
Alcian blue	Detection of acid mucopolysaccharides	Mucoid lakes, for example, in cases of idiopathic cystic Erdheim–Gsell medial necrosis and dissected aortic aneurysm
Azan staining (azo carmine and aniline blue)	Connective tissue staining (red): azo carmine stains cell nuclei, erythrocytes, fibrin, fibrinoid, acidophilic cytoplasm, epithelial hyalin; Aniline blue (blue): collagen fibers, fibrous hyalin, basophil cytoplasm, mucus	Differentiates basophilic and chromophobe cells in the hypophysis; loss of detectability, for example, in the case of Sheehan syndrome
Best's carmine stain	Classified as a glycogen stain, but is not specific; also stains mucus, fibrin, gastric glands, and mast cell granules	Glycogen detection in kidney distal tubular cells in the case of hyperglycemia (Armanni–Ebstein cells)
Elastin staining according to Weigert	Stains elastic fibers violet-black	For example, elastic fibers in the aortic media
Elastika van Gieson (EvG)	Combined staining of collagen fibers (red) and elastic fibers according to Weigert (black and brown); cytoplasm, musculature, amyloid, fibrin, and fibrinoid (yellow)	Fibrotic zones in the myocardium, fibrosis in other organs, liver cirrhosis, cystic medial necrosis
Iron stain (Prussian blue reaction)	Stains trivalent iron, in particular hemosiderin; detection of iron deposits	Siderosis of the lung, posttraumatically deposited siderophages, e.g., for wound age determination
Fibrin staining according to Weigert	Blue: fibrin and bacteria Red: cell nuclei; is not considered a specific fibrin stain	Detection of microfibrin in the placenta, hyaline membrane in the lung post shock event
Gomori's stain	Argyrophilic reticular fibers (silver)	Glomerular basal membranes in the case of a membrane-proliferative glomerulonephritis type I (MPGN) – so-called tram tracks; reticular fiber network in the case of hepatic peliosis
Grocott stain	Ideal fungal stain: fungal conidia, fungal fibers stain black	Fungal infection
Haematoxylin–eosin (H&E) staining	Acidophilic cytoplasm is red, basophil nuclei are blue, erythrocytes are red	Routine staining
Congo red stain	Amyloid stain	Amyloidoses of any type, in particular cardiovascular
Kossa stain	Calcified bone tissue stains black in a non-calcified specimen	Sediments in renal tubules and vascular walls following ethylene glycol intoxication
Luxol fast blue (LFB)	Evidence of myelin and phospholipids	Myelin sheath staining
Mallory's stain	Trichrome stain; collagen and reticular connective tissue is light-blue, nuclei are red, smooth musculature is violet, striated musculature orange-red, mucus is blue	Connective tissue stain, for example, in the case of liver cirrhosis
Masson–Goldner stain	Red-orange: parenchyma and fibrin Green: mesenchyme Black: cell nuclei	Hyaline fibrin thrombi in the case of shock
May-Grünwald–Giemsa stain (MGG)	Nuclei are purple-red, nucleoli are blue, cytoplasm is light blue-gray to red-violet, erythrocytes are pink to orange (except in the case of alkaline pH where they are green-blue)	Hematopoietic marrow, differentiation of cells of the myeloid and lymphatic line; eosinophil granula is red
Methylene blue	Nuclei are sharp blue, plasma cells are deep blue, erythrocytes are greenish	Suitable to detect agents, e.g., Helicobacter pylori
Naphthol AS-D chloroacetate esterase stain (Moloney et al. 1960) (enzyme-histochemical stain; abbreviated to ASD)	Neutrophil myeloid cells with all preliminary stages stain wine red	Mostly selective detection of neutrophil granulocytes in purulent inflammation of all kinds (phlegmons, abscesses)

Staining	Presented structures	Examples from forensic practice
Nissl stain	Detects cell nuclei and tigroid bodies in nerve cells; cell nuclei and Nissl substance violet, nerve cells light blue, the rest is colorless	Detection of nervous tissue
Orcein stain	Detection of elastic fibers, used to identify the Australia HBsAG	Hepatocellular single cell necrosis in the case of active hepatitis B – detection of hepatitis B surface antigen; result should be checked immunohistochemically
Papanicolaou stain	Cells are blue to black, nucleoli are black to red, cytoplasm is blue-green (cyanophil) to pink-red (eosinophil); erythrocytes are bright red	Standard stain for vaginal wet mount
PAS (periodic acid-Schiff's reagent)	Stains carbohydrates, in particular glycogen, purple-red (magenta) and epithelial mucin	Glycogen positive Armanni–Ebstein cells in the renal tubules in the case of diabetic coma
Periodic acid – silver	Stains basal membranes, Alzheimer's plaques, and fungi black	Detection of basal membranes, for example, in the kidney
PTAH	Phosphotungstic acid-hematoxylin according to Mallory	Used to differentiate between smooth and striated muscle fibers, detects fibrin; suitable in the case of muscle damage, also in the myocardium
Prussian blue	Blue: hemosiderin, Fe III	Siderosis of the lung, hemosiderin macrophages full of pigments
Reticulin stain	Silvering of fine (pre-) collagen reticulin fibers	Basal membranes, newly formed fibers
Sudan III	Fat stain; lipids stain yellowish-red; Sudan IV stains more orange-red	Fat embolisms, fatty liver
Toluidine blue	Detects striation of muscle fibers and metachromatic substances	Striated muscle tissue, mast cell granules
Silvering	Black: reticular fibers, nervous fibers Brown: collagen fibers	Hepatic peliosis, glomeruli
Ziehl–Neelsen stain	Acid-resistant rods, mycobacteria (also lepra bacteria) stain bright red	In particular tuberculosis; microscopy ×1000, oil immersion

There are numerous other simple and combined staining methods that are described in the relevant literature.

Histopathological diagnosis

10. Microscopy - Light microscope - Simple microscope
- Dark ground illumination (DGI)
- Polarising microscope
- Immunofluorescence
- Electron microscope (EM)

11. Pathology report -The final and the most important task of pathology laboratory is issuance of a prompt, accurate, brief, and prognostically significant report.

The ideal report must contain five aspects:

- History (as available to the pathologist including patient's identity).
- Precise gross description.
- Brief microscopic findings.
- Morphologic diagnosis which must include the organ for indexing purposes .
- Additional comments in some cases.

Histopathological diagnosis

Pathology reports for neoplasms are complex and they include:

1. Gross description
   Specimen: description
   Tumour: Site
   Size
   Appearance
   Edge
2. Histological type
3. Differentiation/grade
4. Extent of local tumour spread
5. Lymphovascular invasion
6. Lymph nodes
7. Excision margins
8. Other pathology
9. Other malignancy
10. Staging

Benign vs Malignant

Characteristics	Benign	Malignant
Morphology and Differentiation	Well-differentiated appearance Structure similar to tissue origin Little or no anaplasia	Usually some lack of differentiation Structure often atypical Variable degree of anaplasia
Rate and pattern of growth	Slow, progressive expansion Rare mitotic figures Normal-appearing mitotic figures	Slow to rapid growth; erratic growth rate Mitotic figures often numerous and sometimes abnormal
Local invasion	No Invasion Cohesive and expansile growth Capsule often present	Local Invasion Infiltrative growth Usually no capsule
Metastasis	No metastasis	Frequent metastasis (definitive criteria for malignancy)
Damage to human body	Relatively smaller	Relatively bigger
Prognosis	Good	Bad

Histopathological diagnosis

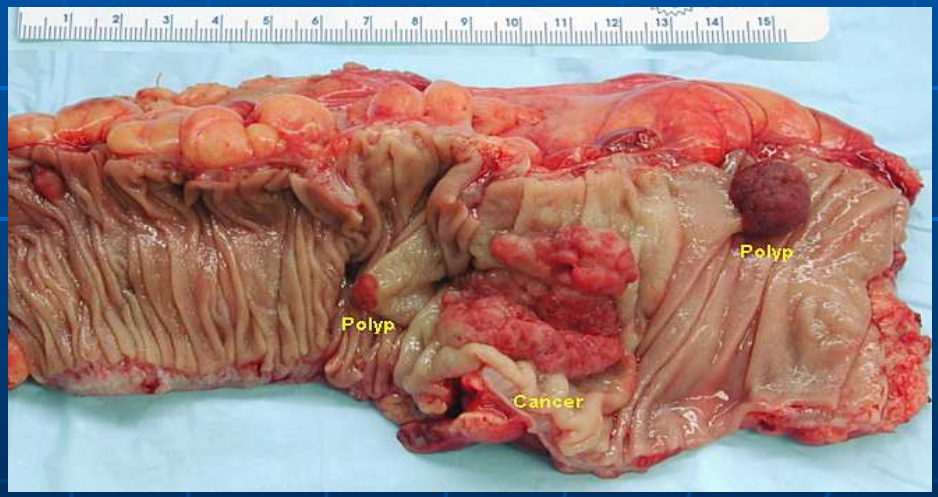
Example: Pathology report for colorectal carcinoma

1.Gross description

- rectal/sigmoidoscopic/colonoscopic biopsy,
- local resection
- right or left hemi -/transverse/sigmoid/ subtotal or total colectomy
- weight (g) and size/length (cm), number of fragments.

Tumour:

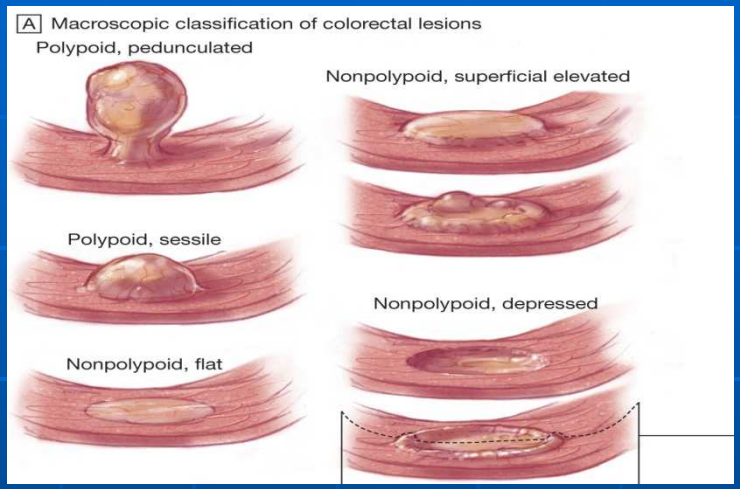
- site-caecum
- ascending colon
- hepatic flexure
- transverse colon
- splenic flexure
- descending or sigmoid
- rectum/multifocal



- size - length × width × depth (cm) or maximum luminal dimension (cm).

Histopathological diagnosis

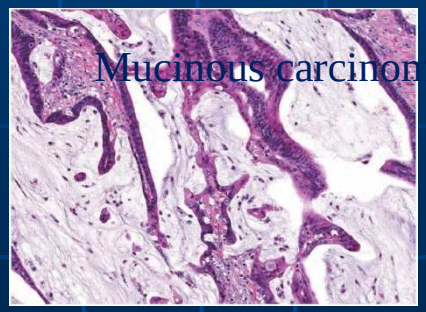
- appearance
- polypoid
- annular
- ulcerated
- mucoid
- linitis plastica
- stricture
- plaque



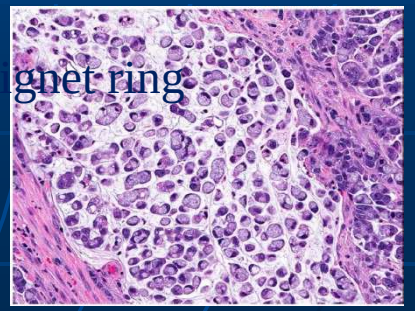
- edge - circumscribed/irregular (adverse prognostic indicator)
- perforation - present/absent

2. Histological type - adeno, mucinous , signet ring, neuroendocrine, others.

Adenocarcinoma



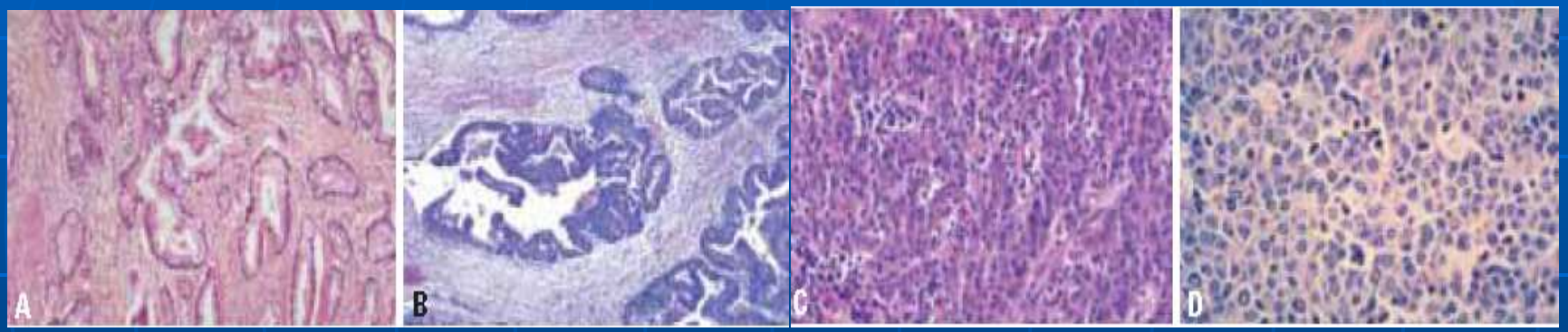
Mucinous carcinoma



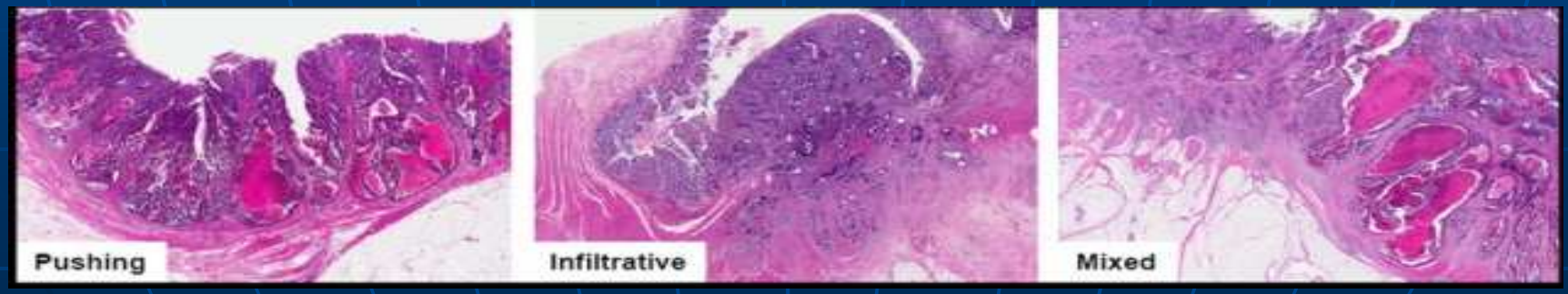
signet ring

Histopathological diagnosis

3. Differentiation/grade - Well/moderate/poor/undifferentiated, or, Grade 1/2/3/4 based on the percentage tumour gland formation(well/G1 > 95 %: moderate/G2 50 – 95 %: poor/G3 < 50 %). Undifferentiated carcinoma(grade 4) shows no gland formation.



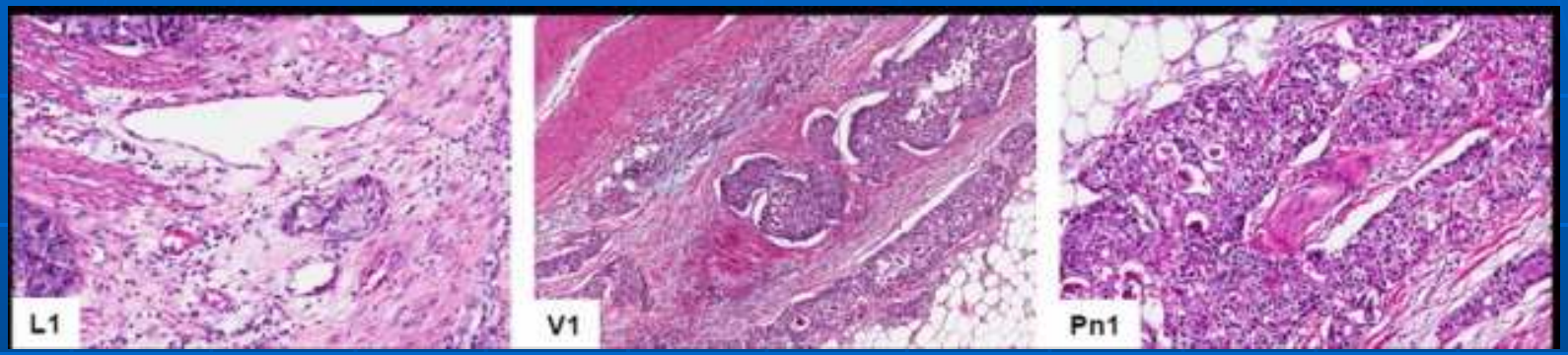
4. Extent of local tumour spread-Border: pushing/infiltrative.Lymphocytic reaction: prominent/sparse.



Histopathological diagnosis

5. Lymphovascular invasion - Present/absent. Intra - /extratumoural.

lymphovascular venous neural invasion



6. Lymph nodes - *Lymph nodes and liver are the commonest sites of metastases*. Other sites include *peritoneum* , *lung* , and *ovaries*, *vagina* and *bladder*, where the *metastases can mimic primary adenocarcinoma of those organs.*

7. Excision margins-Doughnuts/anastomotic rings/staple gun transections – involved/not involved.Distances to the nearest longitudinal resection limit (mm), mesorectal CRM (circumferential radial margin, mm), and mesocolic resection margin (mm).

Histopathological diagnosis

8. Other pathology- inflamatory, neoplastic.

9. Other malignancy.

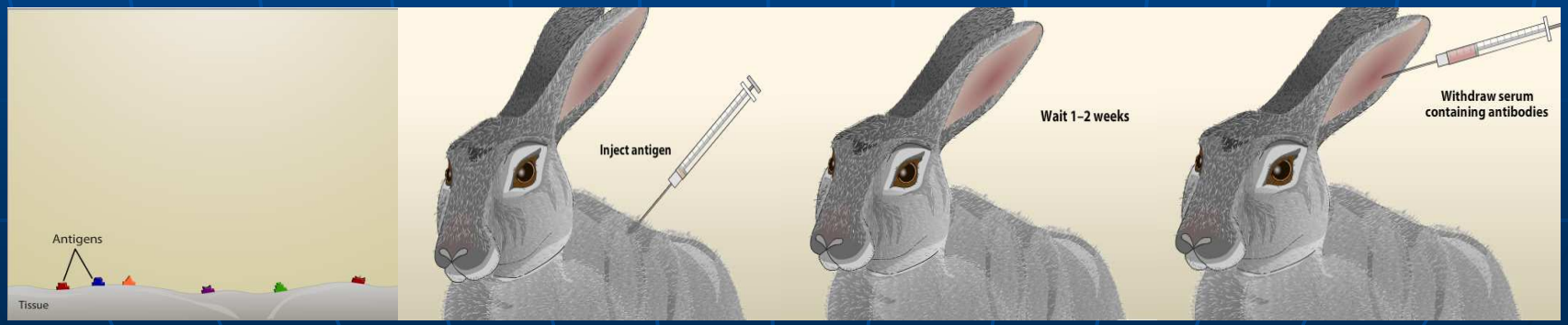
10. Staging.

Staging of Malignant Neoplasms	
Stage	**Definition**
Tis	In situ, non-invasive (confined to epithelium)
T1	Small, minimally invasive within primary organ site
T2	Larger, more invasive within the primary organ site
T3	Larger and/or invasive beyond margins of primary organ site
T4	Very large and/or very invasive, spread to adjacent organs
N0	No lymph node involvement
N1	Regional lymph node involvement
N2	Extensive regional lymph node involvement
N3	More distant lymph node involvement
M0	No distant metastases
M1	Distant metastases present

Histopathological diagnosis

12. Immunohistochemistry -This is a technique for identifying cellular or tissue constituents (antigens) by means of antigen - antibody interactions, the site of antibody binding being identified either by direct labeling of the antibody, or by use of a secondary labeling method.

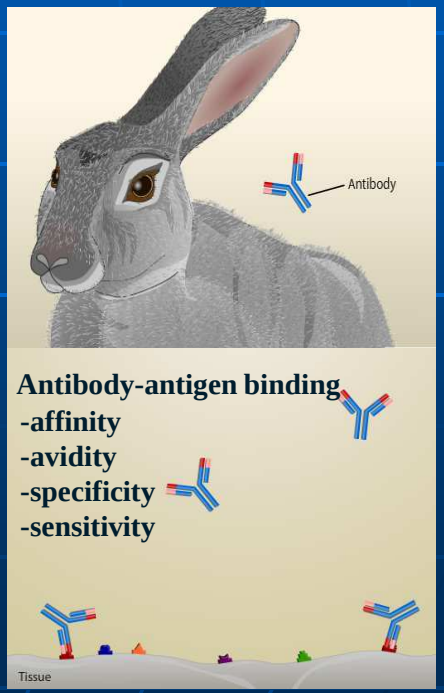
Antigens **-** An antigen is a molecule that induces the formation of an antibody and bears one or more antibody binding sites. These are highly specific topographical regions composed of a small number of amino acids or monosaccharide units, being known as antigenic determinant groups or epitopes.



Histopathological diagnosis

Antibody - Antibodies belong to the class of serum proteins known as immunoglobulins. The basic unit of each antibody is a monomer. They are formed in the humoral immune system by plasma cells, the end cell of B-lymphocyte transformation after recognition of a foreign antigen.

Antibody - antigen binding **-** The amino acid side - chains Of the variable domain of an antibody form a cavity which is geometrically and chemically complementary to a single type of antigen epitope .The analogy of a lock (antibody) and key (antigen) has been used, and the precise fit required explains the high degree of antibody-antigen specificity seen.



Histopathological diagnosis

Types antibodies:

Polyclonal antibodies - animal will produce numerous clones of activated plasma cells (polyclonal). Each clone will produce an antibody with a slightly different specificity to the variety of epitopes present on the immunogen.

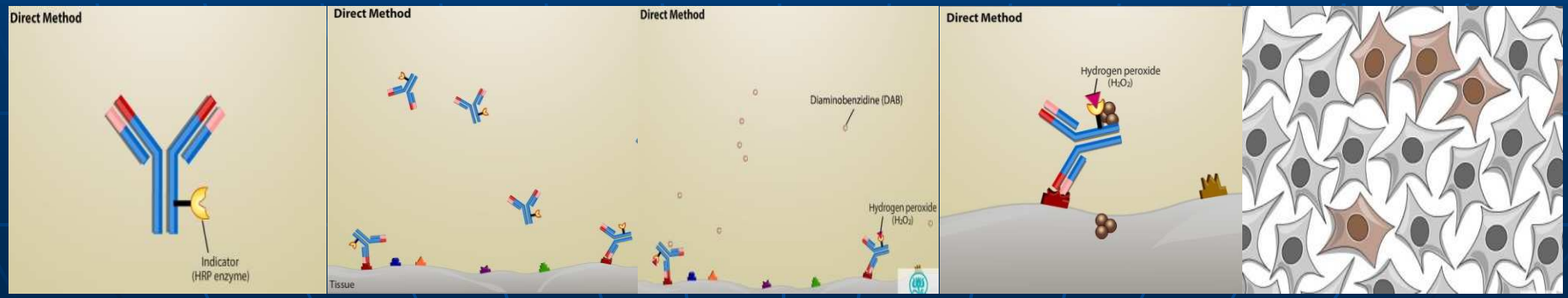
Monoclonal antibodies - hybridoma technique - produce monoclonal antibodies and revolutionized immunohistochemistry by increasing enormously the range, quality, and quantity of specific antisera. The method combines the ability of a plasma cell or transformed B lymphocyte to produce a specific antibody with the *in vitro* immortality of a neoplastic myeloma cell line; a hybrid with both properties can be produced. With the technique of cloning, this cell can be grown and multiplied in cell culture or ascitic fluid, theoretically to unlimited numbers.

Labels-enzymes, colloidal metal labels, fluorescent labels, radiolabels - produces a stable, colored reaction suitable for the microscope.

Histopathological diagnosis

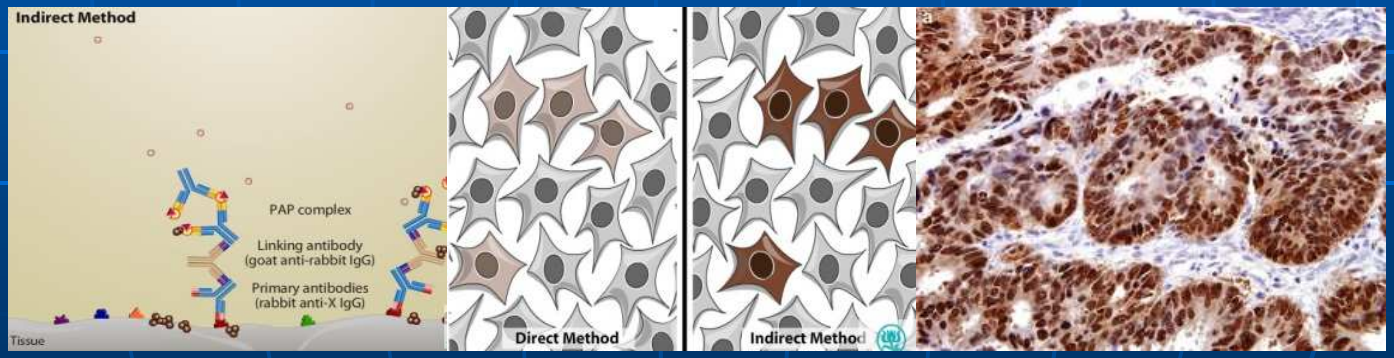
Immunohistochemical methods

1. Traditional direct technique - The primary antibody is conjugated directly to the label. The conjugate may be either a fluorochrome (more commonly) or an enzyme. The labeled antibody reacts directly with the antigen in the histological or cytological preparation. The technique is quick and easy to use. However, it provides little signal amplification and lacks the sensitivity achieved by other techniques.



Histopathological diagnosis

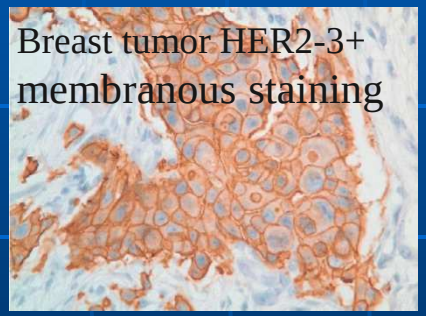
2. Indirect technique - A labeled secondary antibody directed against the immunoglobulin of the animal species in which the primary antibody has been raised visualizes an unlabeled primary antibody. Horseradish peroxidase labeling is most commonly used, together with an appropriate chromogen substrate. The method is more sensitive than the traditional direct technique because multiple secondary antibodies may react with different antigenic sites on the primary antibody, thereby increasing the signal amplification.

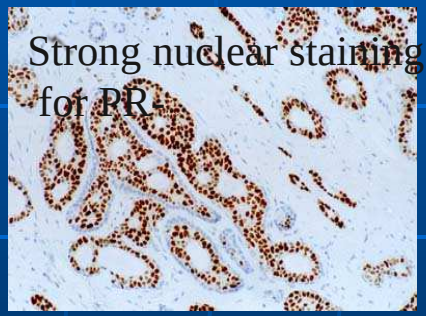


Strong and diffuse nuclear staining (with some cytoplasmic positivity) for CDX2 seen in a colorectal adenocarcinoma

Histopathological diagnosis

For interpretation of results of IHC stains, it is important to remember that *different antigens are localised at different sites in cells* (membrane, cytoplasm or nucleus) and accordingly positive staining is seen and interpreted at those sites e.g. membranous staining for leucocyte common antigen (LCA), nuclear staining for oestrogen - progesterone receptors (ERPR), cytoplasmic staining for smooth muscle actin (SMA) etc.


Breast tumor HER2-3+ membranous staining


Strong nuclear staining for PR


a-SMA cytoplasmic staining of blood vessels

IHC stains are used for the following purposes:

1. Tumours of uncertain histogenesis.
2. Prognostic markers in cancer.
3. Prediction of response to therapy.
4. Infections.

Molecular pathology

Molecular pathology seeks to apply gene expression against morphology and use gene expression analysis to validate large numbers of targets. Nucleic acid–based testing is becoming a crucial diagnostic tool not only in the setting of inherited genetic disease but in a wide variety of neoplastic and infectious processes. Following diagnosis, molecular testing can help guide appropriate therapy by identifying specific therapeutic targets of several newly tailored drugs.

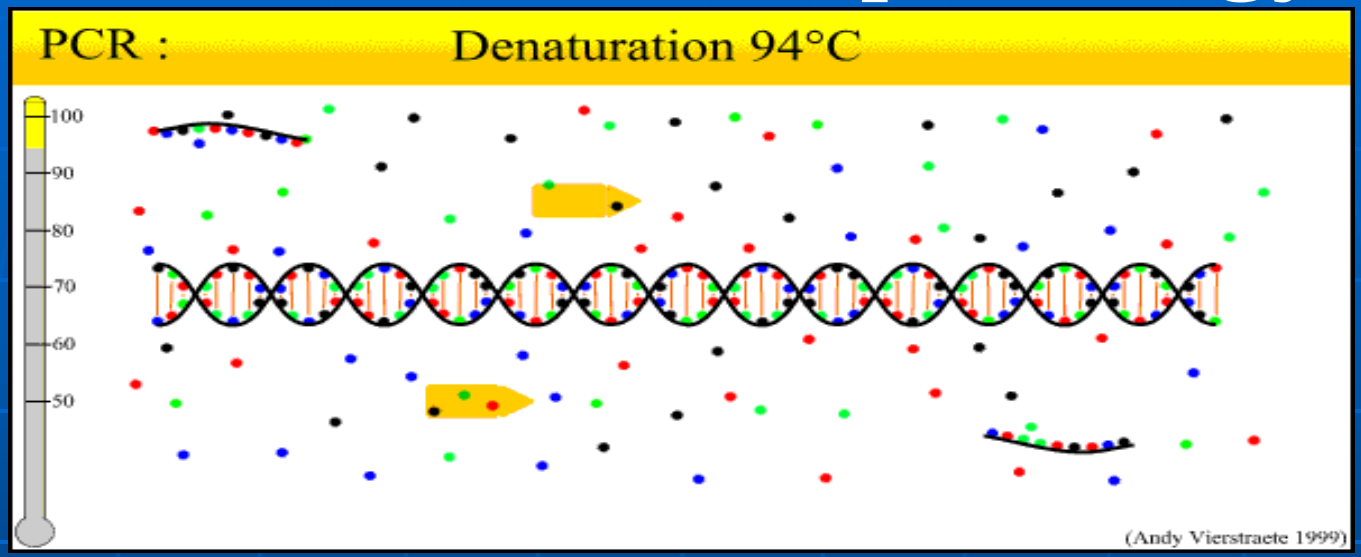
MOLECULAR DIAGNOSTIC TECHNIQUES

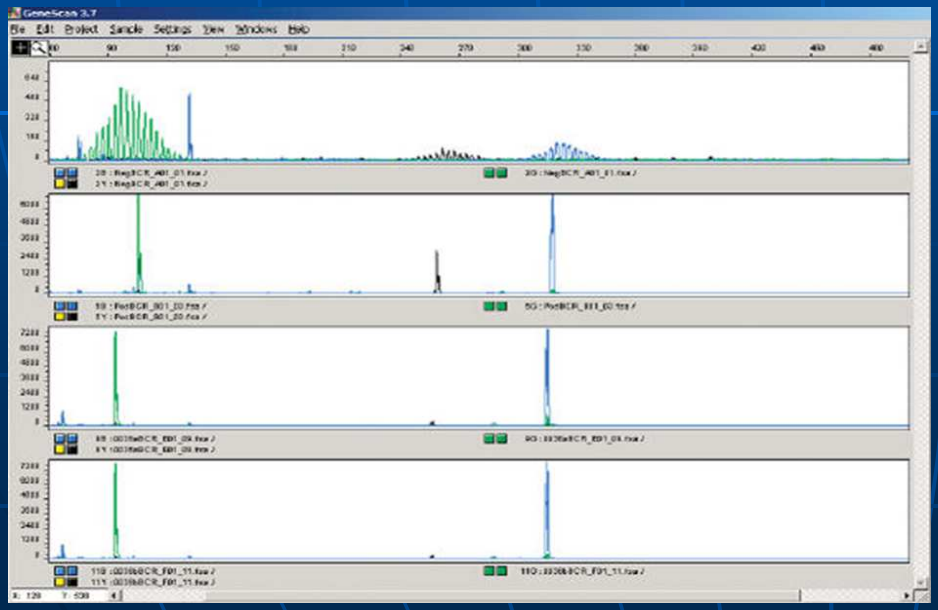
1. *Polymerase chain reaction* - Polymerase chain reaction (PCR) is a technique for molecular genetic purpose with widespread applications in diagnostics and research. The technique is based on the principle that a single strand of DNA has limitless capacity to duplicate itself to form millions of copies. In PCR, a single strand of DNA generates another by DNA polymerase using a short complementary DNA fragment; this is done using a primer which acts as an initiating template.

Molecular pathology

Each PCR cycle involves 3 basic steps: denaturing, annealing, and polymerization. The process is repeated 30 to 40 times, each cycle doubling the amount of the targeted genetic material. At the end of the PCR procedure, millions of identical copies of the original specific DNA sequence have been generated. Since these copies are identical in electrical charge as well as molecular weight, they are expected to migrate simultaneously, forming a single band, when applied to an electrophoretic gel. If oligonucleotide primers used during the PCR cycles are labeled with fluorescent dye, the PCR product can then be analyzed in a capillary electrophoresis instrument, which tracks the fluorescence of the identical PCR sequences as they migrate. The computerized instrument then generates a graph depicting a peak of fluorescence at the migration location of the PCR product .

Molecular pathology





Capillary electrophoresis analysis of a PCR product using a sequencer for fragment analysis . A distinct peak indicates a positive amplification.

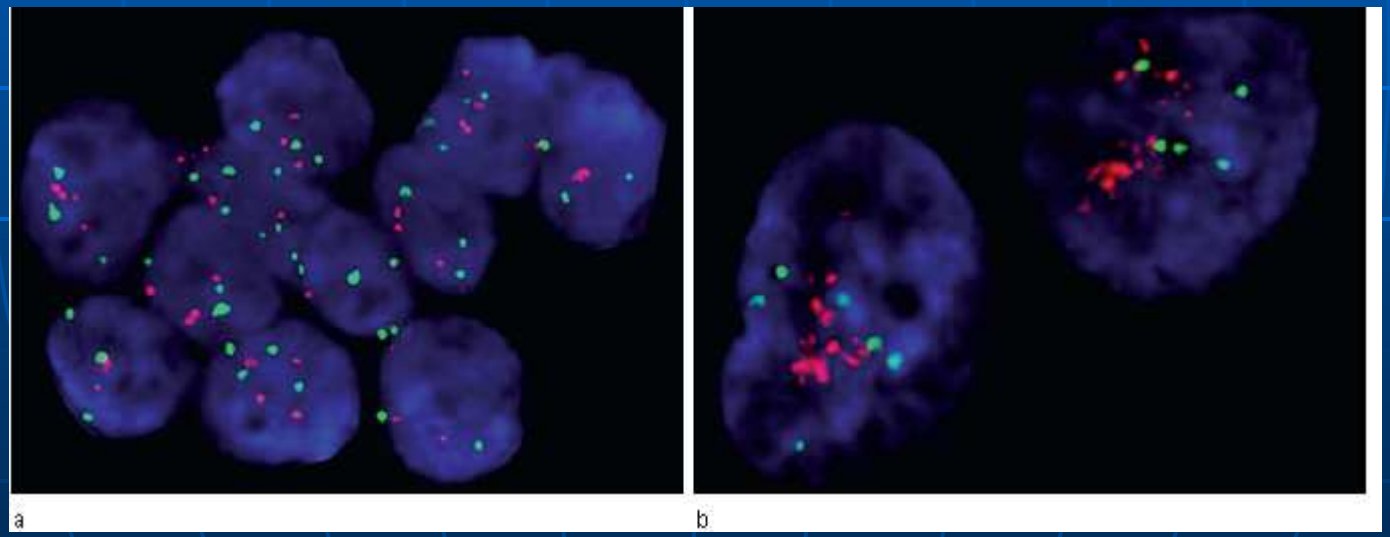
Molecular pathology

2. ***In situ hybridization (ISH)*** is a powerful technique for localizing specific nucleic acid targets within fixed tissues and cells, allowing to obtain temporal and spatial information about gene expression and genetic loci. While the basic workflow of ISH is similar to that of blot hybridizations - the nucleic acid probe is synthesized, labeled, purified, and annealed with the specific target - the difference is the greater amount of information gained by visualizing the results within the tissue. Today there are two basic ways to visualize your RNA and DNA targets in situ - fluorescence (FISH) and chromogenic (CISH) detection. Characteristics inherent in each method of detection have made FISH and CISH useful for very distinct applications.

Fluorescent in situ hybridization- *FISH* is based on the use of fluorescence-labeled oligonucleotide probes that specifically attach to their complementary DNA sequence target on the genome and label that region with fluorescence color . The labeled region can then be easily visualized under a fluorescence microscope. Currently, FISH is often used in evaluation of *HER2/neu* oncogene amplification in breast carcinoma and for detection of different translocations in chronic myelogenous leukemia and acute myelogenous leukemia.

Molecular pathology

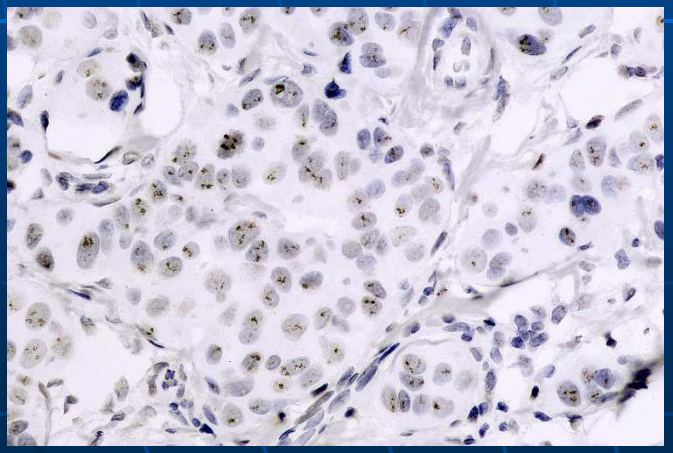
A normal cell should have 2 copies of the *HER2/neu* oncogene, 1 on each of its 2 copies of chromosome 17. Using a centromeric chromosome 17 probe (green signal) in combination with an allele-specific probe for the *HER2/neu* oncogene (orange signal), visualization of 2 green and 2 orange signals in each cell is expected (a ratio of 1). In a breast cancer cell undergoing *HER2/neu* oncogene amplification, 4 or more orange signals are visualized in nuclei showing only 2 green signals (a ratio of 2 or more).



Representative FISH (a) The signal ratio of the two probes was determined to be 1.13 in this sample and, therefore, no amplification of the HER2 gene was seen. (b) The signal ratio of the two probes was determined to be 5.48 in this sample and, therefore, amplification of the HER2 gene was determined to have occurred.

Molecular pathology

CISH, or chromogenic in situ hybridization, is a process in which a labeled complementary DNA or RNA strand is used to localize a specific DNA or RNA sequence in a tissue specimen. CISH methodology may be used to evaluate gene amplification, gene deletion, chromosome translocation, and chromosome number. CISH utilizes conventional peroxidase or alkaline phosphatase reactions visualized under a standard bright-field microscope, and is applicable to formalin-fixed, paraffin-embedded (FFPE) tissues, blood or bone marrow smears, metaphase chromosome spreads, and fixed cells.



HER2 CISH in a breast carcinoma with HER2 amplification

Molecular pathology

3. ***Flow citometry*** - Flow cytometry is a modern tool used for the study of properties of cells suspended in a single moving stream. Flow cytometric analysis finds uses in clinical practice in the following ways:

- Immunophenotyping haematopoietic neoplasias e.g. acute and chronic leukaemias, lymphomas (Hodgkin's and non-Hodgkin's), and plasmacytic neoplasms.
- Measurement of proliferation-associated antigens e.g. Ki67, PCNA.
- Measurement of nucleic acid content e.g. measuring RNA content of reticulocytes, quantifying DNA content and DNA ploidy counts in various types of cancers.
- Diagnosis and prognostication of immunodeficiency .
- To diagnose the cause of allograft rejection in renal transplantation.
- Diagnosis of autoantibodies in ITP, autoimmune neutropenia.

Molecular pathology

4. ***Cell proliferation analysis*** - Besides flow cytometry, the degree of proliferation of cells in tumours can be determined by various other methods. These include the following:

- Mitotic count.
- Radioautography.
- Microspectrophotometric analysis.
- Immunohistochemistry.
- Nucleolar organiser region.

Molecular pathology

5. ***DNA microarray*** **is the newer application of silicon chip technology for simultaneous analysis of large volume of data pertaining to human genes such as detection and quantification of point mutation and single nuceotide pleomorphism. The method eliminates use of DNA probes. Instead fluorescent labelling of an array of DNA fragment(complimentary or cDNA) is used to hybridise with target from test sample. High resolution laser scanners are used for detecting fluorescent signals emitted, while the level of gene expression and genotyping of the biologic samples is measured by application of bioinformatics.DNA microarrays is used for molecular profiling of tumours which aids in arriving at specific histogenetic diagnosis and predicting prognosis.**

DNA microarray

Purpose: multiple simultaneous measurements by hybridization of labeled probe

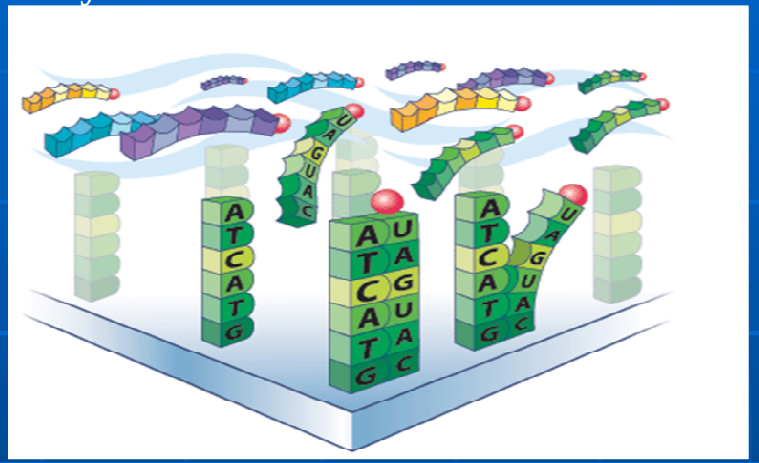
DNA elements may be:

- **Oligonucleotides**
- **cDNA's**
- **Large insert genomic clones**

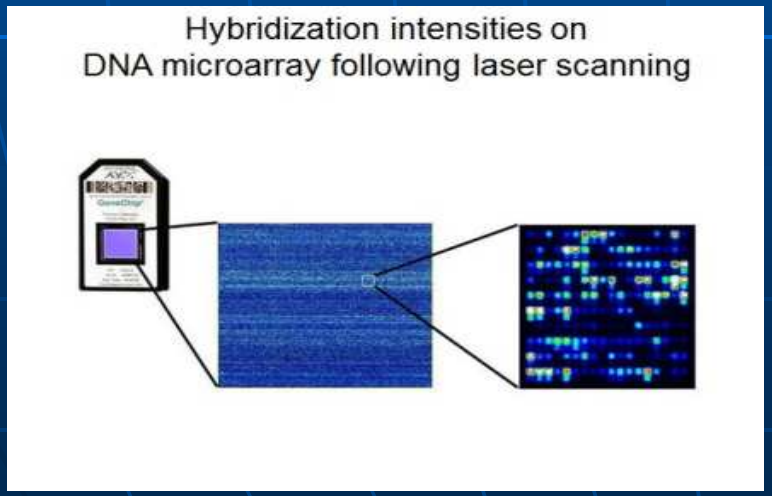
Microarray is generated by:

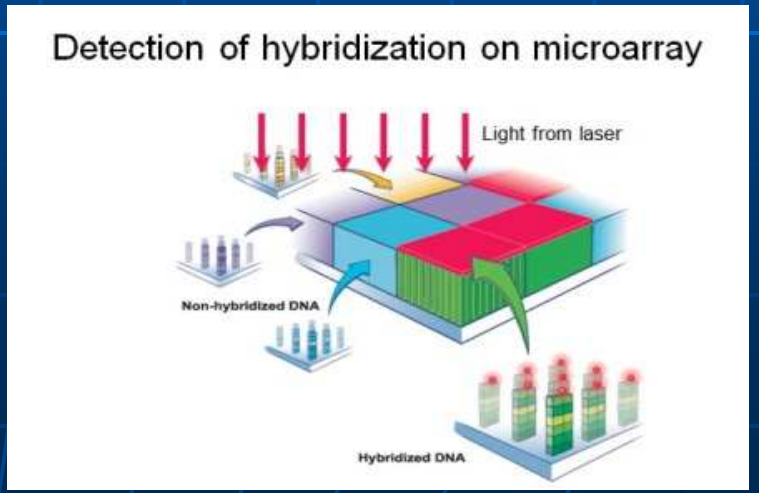
- **Printing**
- **Synthesis**

Hybridization of a labeled probe to the microarray



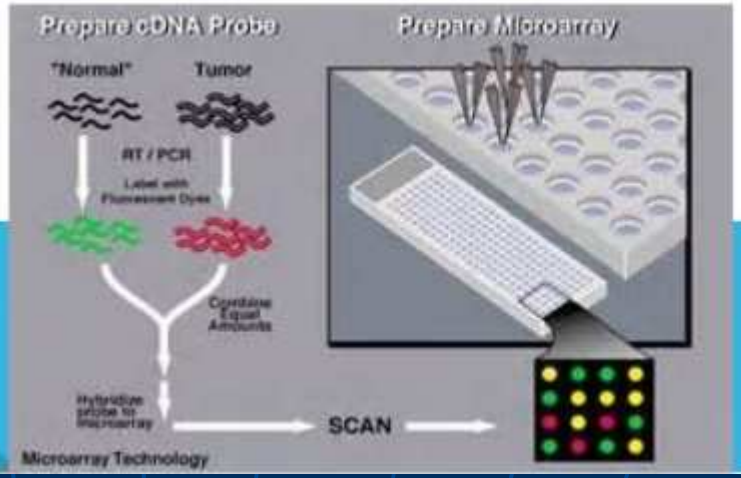
Detection of hybridization on microarray





- GENE CHIP METHOD

They are gene scanning techniques, based on oligonucleotide arrays called DNA chips, that provide a rapid method to analyze thousands of genes simultaneously



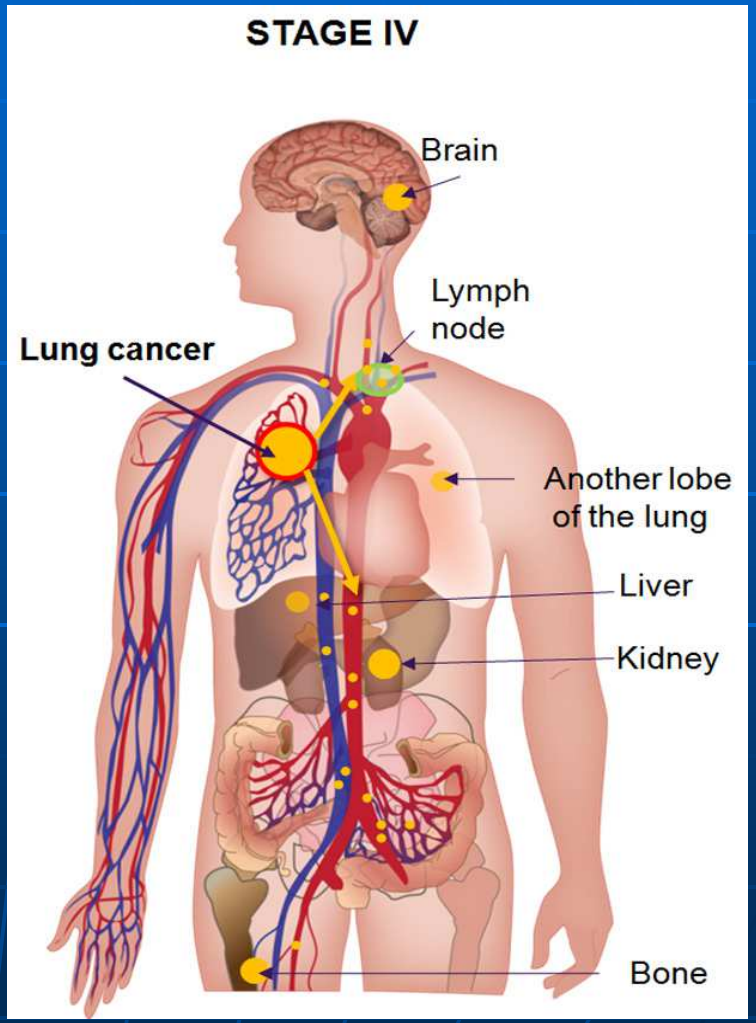
Circulating Tumor Cells (CTCs)
for Cancer Diagnosis and Prognosis

As individual cells present in low numbers, such disseminated cells can be hidden to standard methods of investigation such as microscopic examination of routinely stained cyto/histology slides. However, these cells are understood to be a source of eventual lethal metastases, the major cause of treatment failure in cancer patients. The cells that escape from the primary tumor settle down at a secondary site to cause metastasis are called Circulating Tumor Cells (CTCs). Metastatic disease is the most common cause of cancer-related death in patients with solid tumors. A considerable body of evidence indicates that tumor cells are shed from a primary tumor mass at the earliest stages of malignant progression. Some of these cells will travel via the peripheral blood to sites anatomically distant from the primary tumor and form metastases.

Detection and Characterization of these Cells is a Promising Method for Both Diagnosis and Clinical Management of Cancer Patients as well as Monitoring Treatment.

CTCs

There are a series of steps that a tumor cell must go through before metastasis can occur. After cells grow into a benign tumor in the epithelium, genetic alterations allow them to break out through the basement membrane, traverse through the extra cellular matrix and invade the capillary . Once tumor cells enter the circulation they can travel through the bloodstream to a distant site. CTCs must adhere the Vessel wall made up of endothelial cells,before they can extravasate.If the microenvironment is established, the tumor cell(s)may proliferate at this distant site and eventually form lethal metastases.



CTCs

Circulating Tumor Cells are the Messenger and the Message of Metastasis

Metastatic Cascade:

1. Cells grow as an in situ lesion.

2. Cells break through the basement membrane.

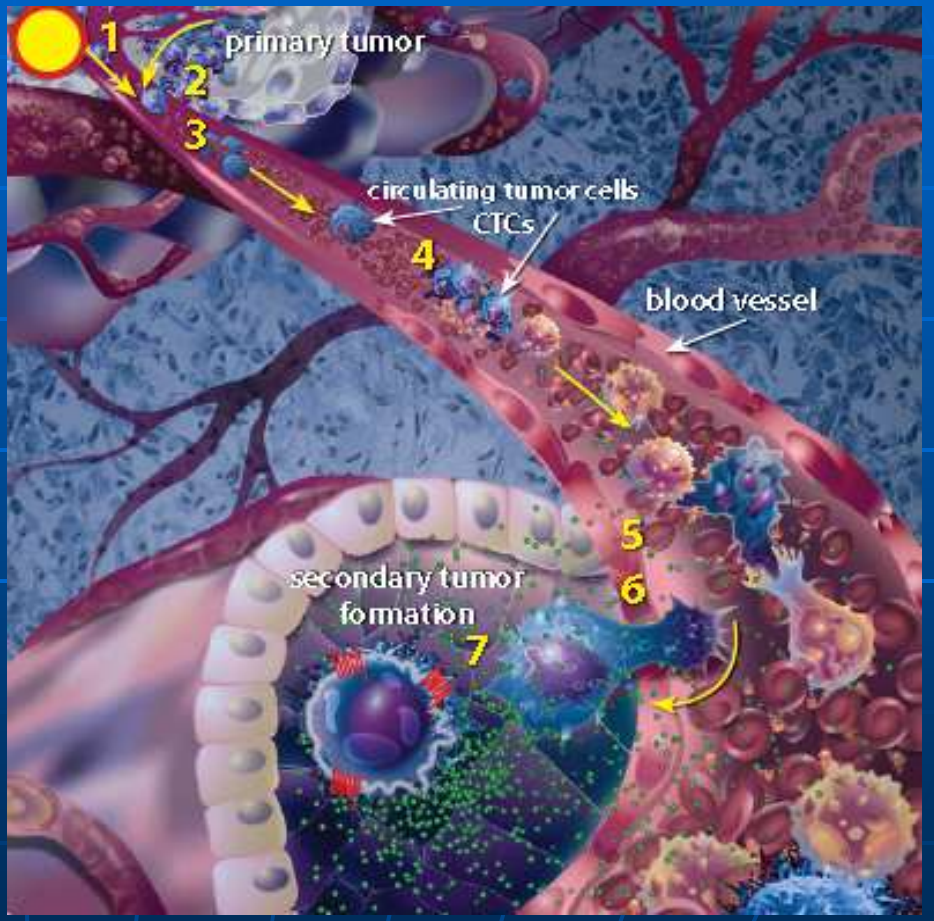
3. Invasion.

4. Travel through the blood stream.

5. Adhere to capillary wall.

6. Escape from blood vessel (Extravasation).

7. Proliferate to form metastases.

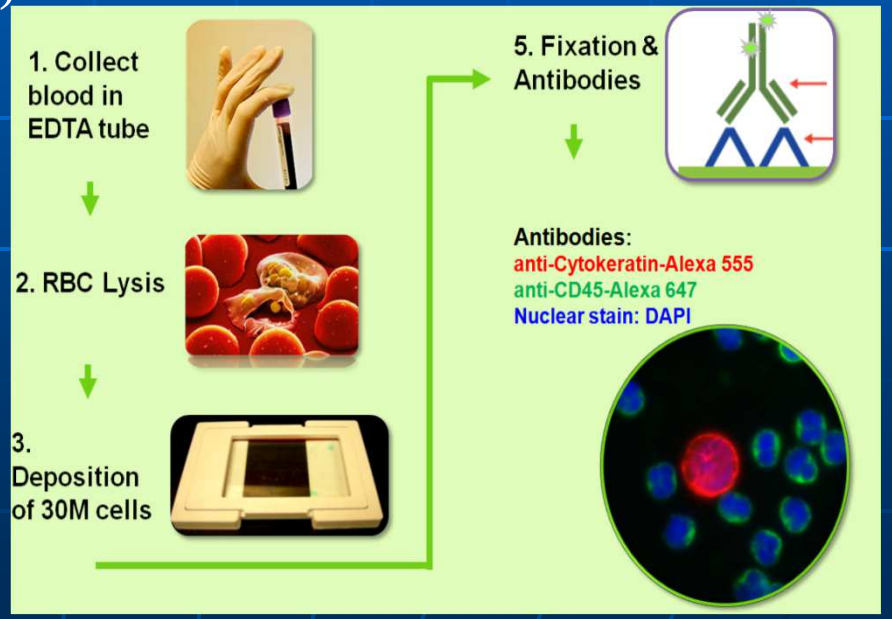


CTCs

The detection of CTCs may have important prognostic and therapeutic implications but because their numbers can be very small, these cells are not easily detected.

The approach that we use for the detection of occult micrometastases is measuring CTCs in peripheral blood (PB).

The reliable measurement of CTCs depends on both sample preparation protocol and detection technique, and hence research in this area must take both into account simultaneously in order to develop a reliable and clinically useful approach. There is good reason to be optimistic about the value of CTC detection.



CTCs

CTC Pleomorphism - Cytologically, the primary and metastatic tumor deposits show pleomorphism among the individual cells of the tumor tissue. Circulating cells from these same patients retain this cytomorphology, showing overall pleomorphism with variations in size, shape , and N/C as well. It appears that CTCs retain primary tumor cytologic characteristics, and that CTCs represent a random sampling of many phenotypic cell types present in the primary and metastatic tumor deposits.

CTCs

A potential application of CTC detection from peripheral blood (PB) is the early diagnosis of cancer.

At some ill-understood point during primary tumor growth, invasive sub-populations of the primary tumor cells gain access to the circulation. While the literature supports the concept that many tumor cells shed from a primary tumor are immediately destroyed in the circulation and most shed cells do not in fact result in metastasis, detection of such shedding may provide an early clue as to the presence of malignancy. With ancillary use of appropriate tissue and/or organ specific markers, CTC screening could be an early indicator of metastasis-capable malignancy.

Circulating Tumor Cells as a Real Time Fluid Biopsy

Enable Detection, Prognosis, and Individualized Therapy Management for Cancer Patients:

- Detect non-useful treatment even before scans/tumor markers show no response
- Tailor treatment/ monitor disease recurrence
- Therapy in the adjuvant setting
- Detect early cancer in "healthy" people

Next Generation Sequencing

DNA Sequencing

DNA sequencing = determining the nucleotide sequence (A, T, G, and C) of the DNA of a gene.

Next generation sequencing (NGS) is often referred to as massively parallel sequencing, which means that millions of small fragments of DNA can be sequenced at the same time, creating a massive pool of data. This pool of data can reach gigabites in size, which is the equivalent of 1 billion (1,000,000,000) base pairs of DNA. In comparison, previous methods could sequence one DNA fragment at a time, perhaps generating 500 to 1000 base pairs of DNA in a single reaction.

This is an incredibly powerful development, and whilst offering the potential to identify exactly where a gene alteration might be present in a patient, it does also provide the potential to identify some unexpected findings as well.

Looking at the molecular profile of the tumour sample using NGS might lead to the identification of an alteration that could suggest the best drug treatment option for a patient or perhaps a clinical trial that the patient could be entered into in the development of new and effective cancer treatments.

Telepathology

Telepathology **is defined as the practice of diagnostic pathology by a remote pathologist utilising images of tissue specimens transmitted over a telecommunications network.**

The main *components* of a telepathology system are :

- Conventional light microscope.

- Method of image capture.

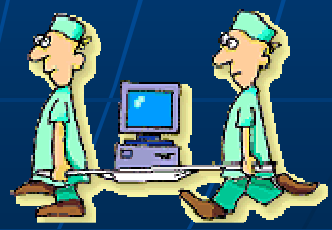
- Telecommunications link between sending and receiving side.

- Workstation at receiving end with a high quality monitor.

Telepathology system is of two *types*:

- *Static* selected images are captured, stored and then transmitted over the internet via e-mail attachment, file transfer protocol, web page or CD-ROM.

- *Dynamic* -images are transmitted in real-time .



Relax !

It's not THE END.