Histopathology and Anatomical Pathology

Loyce Christianson

Quentin Blevins

First Edition, 2012

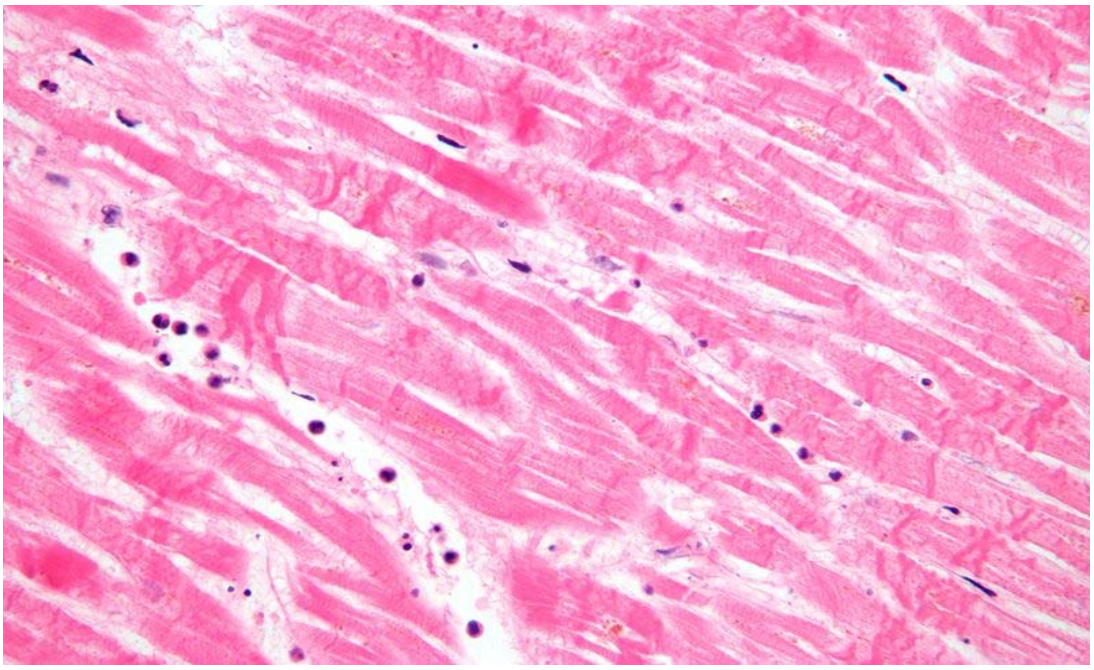
ISBN 978-81-323-1402-8

Published by:
College Publishing House
4735/22 Prakashdeep Bldg,
Ansari Road, Darya Ganj,
Delhi - 110002
Email: info@wtbooks.com

Table of Contents

Chapter 1

Histopathology



Micrograph showing contraction band necrosis, a **histopathologic** finding of myocardial infarction (heart attack).

Histopathology (compound of three Greek words: *ἱστός* histos "tissue", *πάθος* pathos "disease-suffering", and *-λογία* -logia) refers to the microscopic examination of tissue in order to study the manifestations of disease. Specifically, in clinical medicine, histopathology refers to the examination of a biopsy or surgical specimen by a pathologist, after the specimen has been processed and histological sections have been placed onto glass slides. In contrast, cytopathology examines free cells or tissue fragments.

Collection of tissues

Histopathological examination of tissues starts with surgery, biopsy, or autopsy. The tissue is removed from the body or plant, and then placed in a fixative which stabilizes

the tissues to prevent decay. The most common fixative is formalin (10% formaldehyde in water).

Preparation for histology

The tissue is then prepared for viewing under a microscope using either chemical fixation or frozen section.

Chemical fixation

In chemical fixation, the samples are transferred to a cassette, a container designed to allow reagents to freely act on the tissue inside. This cassette is immersed in multiple baths of progressively more concentrated ethanol, to dehydrate the tissue, followed by toluene or xylene, and finally extremely hot liquid (usually paraffin). During this 12 to 16 hour process, paraffin will replace the water in the tissue, turning soft, moist tissues into a sample miscible with paraffin, a type of wax. This process is known as **tissue processing**.

The processed tissue is then taken out of the cassette and set in a mold. Through this process of **embedding**, additional paraffin is added to create a paraffin block which is attached to the outside of the cassette.

The process of embedding then allows the sectioning of tissues into very thin (2 - 7 micrometer) sections using a microtome. The microtome slices the tissue ready for microscopic examination. The slices are thinner than the average cell, and are layered on a glass slide for staining.

Frozen section processing

The second method of histology processing is called frozen section processing. In this method, the tissue is frozen and sliced thinly using a microtome mounted in a below-freezing refrigeration device called the cryostat. The thin frozen sections are mounted on a glass slide, fixed immediately & briefly in liquid fixative, and stained using the similar staining techniques as traditional wax embedded sections. The advantages of this method is rapid processing time, less equipment requirement, and less need for ventilation in the laboratory. The disadvantage is the poor quality of the final slide. It is used in intra-operative pathology for determinations that might help in choosing the next step in surgery during that surgical session (for example, to preliminarily determine clearness of the resection margin of a tumor during surgery).

Staining of the Processed Histology Slides

This can be done to slides processed by the chemical fixation or frozen section slides. To see the tissue under a microscope, the sections are stained with one or more pigments. The aim of staining is to reveal cellular components; counterstains are used to provide contrast.

The most commonly used stain in histopathology is a combination of hematoxylin and eosin (often abbreviated H&E). Hematoxylin is used to stain nuclei **blue**, while eosin stains cytoplasm and the extracellular connective tissue **matrix pink**. There are hundreds of various other techniques which have been used to selectively stain cells. Other compounds used to color tissue sections include safranin, Oil Red O, congo red, silver salts and artificial dyes. **Histochemistry** refers to the science of using chemical reactions between laboratory chemicals and components within tissue. A commonly performed histochemical technique is the Perls Prussian Blue reaction, used to demonstrate iron deposits in diseases like Hemochromatosis.

Recently, antibodies have been used to stain particular proteins, lipids and carbohydrates. Called immunohistochemistry, this technique has greatly increased the ability to specifically identify categories of cells under a microscope. Other advanced techniques include **in situ hybridization** to identify specific DNA or RNA molecules. These antibody staining methods often require the use of frozen section histology. Digital cameras are increasingly used to capture histopathological images.

Interpretation

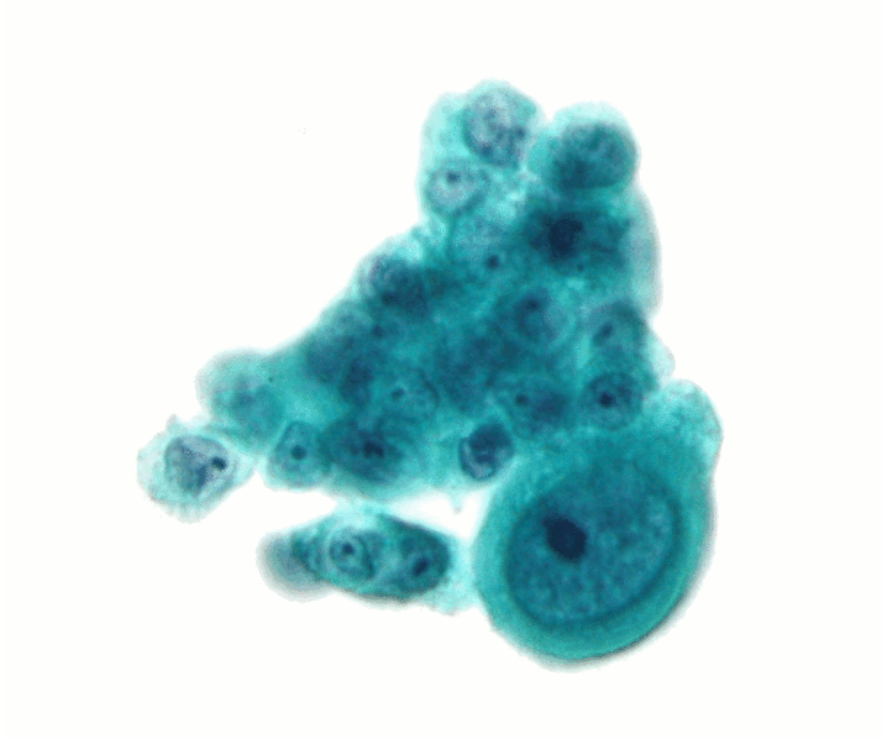
The histological slides are examined under a microscope by a pathologist, a medically qualified specialist. This medical diagnosis is formulated as a **pathology report** describing the histological findings and the opinion of the pathologist. In the case of cancer, this represents the **tissue diagnosis** required for most treatment protocols. In the removal of cancer, the pathologist will indicate whether the surgical margin is cleared, or is involved (residual cancer is left behind). This is done using either the bread loafing or CCPDMA method of processing.

In myocardial infarction

After a myocardial infarction, no histopathology is seen the first ~30 minutes. The only possible sign the first 4 hours is waviness of fibers at border. Later, however, a coagulation necrosis is initiated, with edema and hemorrhage. After 12 hours, there can be seen karyopyknosis and hypereosinophilia of myocytes with contraction band necrosis in margins, as well as beginning of neutrophil infiltration. At 1 – 3 days there is continued coagulation necrosis with loss of nuclei and striations and an increased infiltration of neutrophils to interstitium. Until the end of the first week after infarction there is beginning of disintegration of dead muscle fibers, necrosis of neutrophils and beginning of macrophage removal of dead cells at border, which increases the succeeding days. After a week there is also beginning of granulation tissue formation at margins, which matures during the following month, and gets increased collagen deposition and decreased cellularity until the myocardial scarring is fully mature at approximately 2 months after infarction.

Chapter 2

Cytopathology



A pair of micrographs of a cytopathology specimen showing a 3-dimensional cluster of cancerous cells (serous carcinoma)

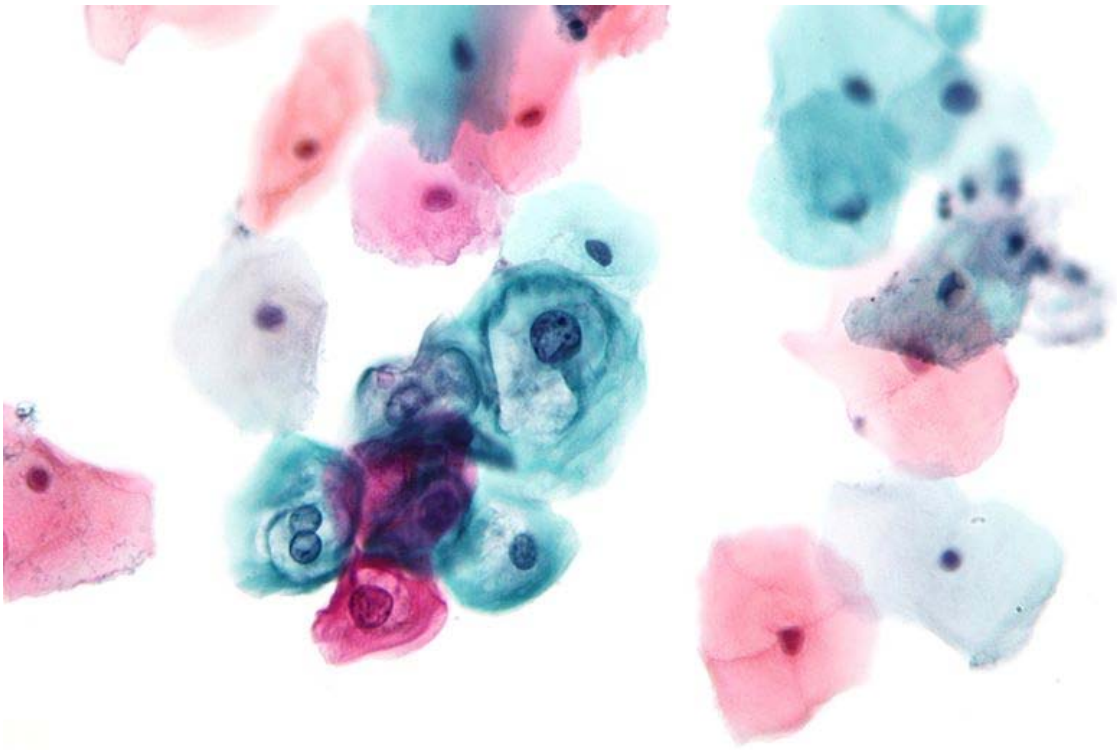
Cytopathology (from Greek κύτος, *kytos*, "a hollow"; πάθος, *pathos*, "fate, harm"; and -λογία, *-logia*) is a branch of pathology that studies and diagnoses diseases on the cellular level. The discipline was founded by Rudolf Virchow in 1858. A common application of cytopathology is the Pap smear, used as a screening tool, to detect precancerous cervical lesions and prevent cervical cancer. Cytopathogy is also commonly used to investigate thyroid lessions, diseases involving sterile body cavities (peritoneal, pleural, and cerebrospinal), and a wide range of other body sites. It is usually used to aid in the diagnosis of cancer, but also helps in the diagnosis of certain infectious diseases and other inflammatory conditions. Cytopathology is generally used on samples of free cells or tissue fragments, in contrast to histopathology, which studies whole tissues.

Cytopathologic tests are sometimes called **smear tests** because the samples may be smeared across a glass microscope slide for subsequent staining and microscopic examination. However, cytology samples may be prepared in other ways, including cytocentrifugation. Different types of smear tests may also be used for cancer diagnosis. In this sense, it is termed a *cytologic smear*.

Cytopathology is frequently, less precisely, called cytology, which means "the study of cells."

Cell collection

Two methods of collecting cells for cytopathologic analysis are:



A micrograph of an exfoliative cytopathology specimen (Pap test, Pap stain)

1. *Exfoliative Cytology* – In this method, cells are collected after they have been either spontaneously shed by the body ("spontaneous exfoliation") or manually scraped/brushed off of a surface in the body ("mechanical exfoliation"). An example of spontaneous exfoliation is when cells of the pleural cavity or peritoneal cavity are shed into the pleural or peritoneal fluid. This fluid can be collected via various methods for examination. Examples of mechanical exfoliation include Pap smears, where cells are scarped form the cervix with a cervical spatula, or bronchial brushings, where a bronchoscope is inserted into the trachea and used to evaluate a visible lesion by brushing cells from its surface and subjecting them to cytopathologic analysis.

2. *Fine Needle Aspiration Cytology* or *Needle aspiration biopsy* – A needle attached to a syringe is used to collect cells from lesions or masses in various body organs by microcoring, often with the application of negative pressure (suction) to increase yield. FNAC can be performed under palpation guidance (ie. the clinican can feel the lesion) on a mass in superficial regions like the neck, thyroid or breast; FNAC may also be assisted by ultrasound or CAT scan for sampling of deep-seated lesions within the body that cannot me localized via palpation. FNAC is is widely used in many countries, but success rate is dependent on the skill of the practitioner. If performed by a pathologist alone, or as team with pathologist-cytotechnologist, the success rate of proper diagnosis is superior than when performed by a non-pathologist. This may be due to the pathologist's ability to immediately evaluate specimens under a microscope and immediately repeat the procedure if sampling was inadequate.

Fine needles are 23 to 27 gauge. Because needles as small as 27 gauge can almost always yield diagnostic material, FNAC is often the least injurious way to obtain diagnostic tissue from a lesion. Sometime a syringe holder may be used to facilitate using one hand to perform the biopsy while the other hand is immobilizing the mass. Imaging equipment such as a CT scanner or ultrasound may be used to assist in locating the region to be biopsied.

Parameters

The nucleus of the cell is very important in evaluating the cellular sample. In cancerous cells, altered DNA activity can be seen as a physical change in the nuclear qualities. Since more DNA is unfolded and being expressed, the nucleus will be darker and less uniform, larger than in normal cells, and often show a bright red nucleolus.

While the cytologist's primary responsibility is to discern whether cancerous or precancerous pathology is present in the cellular sample analysed, other pathologies may be seen such as:

- microbial infections: parasitic, viral, and/or bacterial
- reactive changes
- immune reactions
- cell aging
- amyloidosis
- autoimmune diseases

Various normal functions of cell growth, metabolism, and division can fail or work in abnormal ways and lead to diseases.

Cytopathology is best used as one of three tools, the second and third being the physical exam and medical imaging. Cytology can be used to diagnose a condition and spare a patient from surgery to obtain a larger specimen. An example is thyroid FNA, many benign conditions can be diagnosed with a superficial biopsy and the patient can go back

to normal activities right away. If a malignant condition is diagnosed, the patient may be able to start radiation/chemotherapy, or may need to have surgery to remove and/or stage the cancer.

Some tumors may be difficult to biopsy, such as sarcomas. Other rare tumors my be dangerous to biopsy, such as pheochromocytoma. In general, a fine needle aspiration can be done anywhere it is safe to put a needle, including liver, lung, kidney, and superficial masses.

Many clinicians are not trained to perform fine needle aspiration biopsies properly, and then when they do not obtain diagnostic material, believe that cytology is not useful. Proper technique takes time to master. Cytotechnologists and cytopathologists can assist clinicians by going to procedures and assisting with collection techniques. A "quick read" is a peek under the microscope and can tell the clinician if they have obtained enough diagnostic material. Cytological specimens also need to be properly prepared so that the cells are not damaged.

Sometimes more information about the specimen is helpful. Immunohistochemical stains and molecular testing can be performed, especially if the sample is prepared using liquid based cytology. Often "reflex" testing is performed, such as HPV testing on an abnormal pap test or flow cytometry on a lymphoma specimen.

Body regions

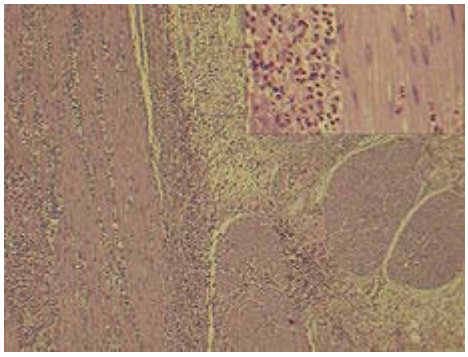
Cytopathologic techniques are used in the examination of virtually all body organs and tissues:

- Gynaecologic cytology - concerning the female reproductive tract
- Urinary tract cytology - concerning the ureters, urinary bladder and urethra
- Effusion cytology - concerning fluids collections, especially within the peritoneum, pleura and pericardium
- Breast cytology - principally concerning the female breast
- Thyroid cytology - concerning the thyroid gland
- Lymph node cytology - concerning lymph nodes
- Respiratory cytology - concerning the lungs and airways
- Gastrointestinal cytology - concerning the alimentary tract
- Soft tissue, bone and skin cytology
- Kidney and adrenal cytology
- Liver and pancreas cytology
- Central nervous system cytology
- Eye cytology
- Salivary gland cytology

Chapter 3

Eosinophilic Gastroenteritis

Eosinophilic gastroenteritis



H&E Stain: Dense Eosinophilic infiltration of gastro-duodenal wall

ICD-10	K52.8
ICD-9	558.3
DiseasesDB	32555
eMedicine	med/688

Eosinophilic gastroenteritis (EG) is a rare and heterogeneous condition characterized by patchy or diffuse eosinophilic infiltration of gastrointestinal (GI) tissue, first described by Kaijser in 1937. Presentation may vary depending on location as well as depth and extent of bowel wall involvement and usually runs a chronic relapsing course. It can be classified into mucosal, muscular and serosal types based on the depth of involvement. Any part of the GI tract can be affected, and isolated biliary tract involvement has also been reported. The stomach is the organ most commonly affected, followed by the small intestine and the colon.

Pathophysiology

Peripheral blood eosinophilia and elevated serum IgE are usual but not universal. The damage to the gastrointestinal tract wall is caused by eosinophilic infiltration and degranulation.

As a part of host defense mechanism, eosinophil is normally present in gastrointestinal mucosa, though finding in deeper tissue is almost always pathologic . What triggers such dense infiltration in EG is not clear. It is possible that different pathogenetic mechanisms of disease is involved in several subgroups of patients. Food allergy and variable IgE response to food substances has been observed in some patients which implies role of hypersensitive response in pathogenesis. Many patients indeed have history of other atopic conditions like eczema, asthma etc.

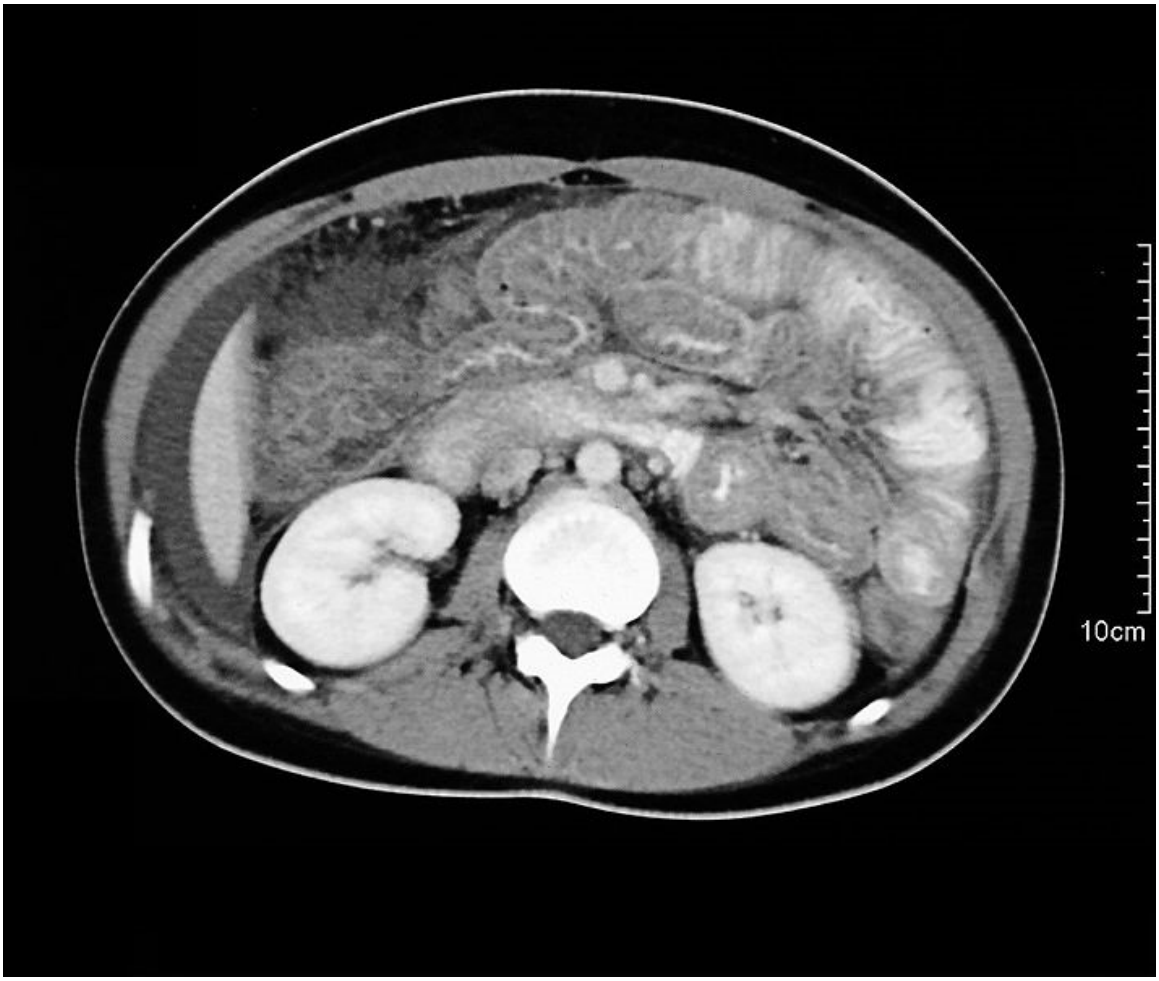
Eosinophil recruitment into inflammatory tissue is a complex process, regulated by a number of inflammatory cytokines. In EG cytokines IL-3, IL-5 and granulocyte macrophage colony stimulating factor (GM-CSF) may be behind the recruitement and activation. They have been observed immunohistochemically in diseased intestinal wall . In addition eotaxin has been shown to have an integral role in regulating the homing of eosinophils into the lamina propria of stomach and small intestine . In the allergic subtype of disease, it is thought that food allergens cross the intestinal mucosa and trigger an inflammatory response that includes mast cell degranulation and recruitment of eosinophils.

Symptoms and signs

EG typically presents with a combination of chronic nonspecific GI symptoms which include abdominal pain, nausea, vomiting, diarrhea, weight loss, and abdominal distension. Approximately 80% have symptoms for several years; a high degree of clinical suspicion is often required to establish the diagnosis, as the disease is extremely rare. Occasionally, the disease may manifest itself as an acute abdomen or bowel obstruction.

- **Mucosal EG** (25-100%)is the commonest variety, which presents with features of malabsorption and protein losing enteropathy. Failure to thrive and anaemia may also be present. Lower gastrointestinal bleeding may imply colonic involvement.
- **Muscular EG** (13-70%) present with obstruction of gastric outlet or small intestine; sometimes as an obstructing caecal mass or intussusception.
- **Subserosal EG** (4.5 % to 9 % in Japan and 13 % in the USA) presents with ascites which is usually exudative in nature, abundant peripheral eosinophilia, and has favourable responses to corticosteroids.
- Other documented features are Cholangitis, pancreatitis, eosinophilic splenitis, acute appendicitis and giant refractory duodenal ulcer.

Diagnosis



Spiral CT showing ascites and concentric thickening of colon and ileum in EG

Talley et al. suggested 3 diagnostic criteria which is still widely used:

1. the presence of gastrointestinal symptoms,
2. histological demonstration of eosinophilic infiltration in one or more areas of the gastrointestinal tract or presence of high eosinophil count in ascitic fluid (latter usually indicates subserosal variety),
3. no evidence of parasitic or extraintestinal disease.

Hypereosinophilia, the hallmark of allergic response, may be absent in up to 20% of patients, but hypoalbuminaemia and other abnormalities suggestive of malabsorption may be present.

CT scan may show nodular and irregular thickening of the folds in the distal stomach and proximal small bowel, but these findings can also be present in other conditions like Crohn's disease and lymphoma.

The endoscopic appearance in eosinophilic gastroenteritis is nonspecific; it includes erythematous, friable, nodular, and occasional ulcerative changes. Sometimes diffuse inflammation results in complete loss of villi, involvement of multiple layers, submucosal oedema and fibrosis.

Definitive diagnosis involves histological evidence of eosinophilic infiltration in biopsy slides. Microscopy reveals >20 eosinophils per high power field. Infiltration is often patchy , can be missed and laparoscopic full thickness biopsy may be required.

Radio isotope scan using technetium (^{99m}Tc) exametazime-labeled leukocyte SPECT may be useful in assessing the extent of disease and response to treatment but has little value in diagnosis, as the scan does not help differentiating EG from other causes of inflammation.

When eosinophilic gastroenteritis is observed in association with eosinophilic infiltration of other organ systems, the diagnosis of idiopathic hypereosinophilic syndrome should be considered.

Management

Corticosteroids are the mainstay of therapy with a 90% response rate in some studies. Appropriate duration of steroid treatment is unknown and relapse often necessitates long term treatment. Various steroid sparing agents e.g. sodium cromoglycate (a stabilizer of mast cell membranes), ketotifen (an antihistamine), and montelukast (a selective, competitive leukotriene receptor antagonist) have been proposed, centering around an allergic hypothesis, with mixed results. An elimination diet may be successful if a limited number of food allergies are identified.

Epidemiology

Epidemiology may differ between studies, as number of cases are small, with approximately 300 EG cases reported in published literature.

EG can present at any age and across all races, with a slightly higher incidence in males. Earlier studies showed higher incidence in the third to fifth decades of life.

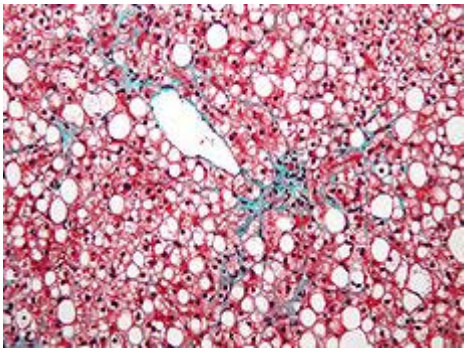
Other gastrointestinal conditions associated with allergy

- Eosinophilic esophagitis
- Eosinophilic ascites
- Coeliac disease
- Protein losing enteropathy from intolerance to cow's milk protein
- Infantile formula protein intolerance

Chapter 4

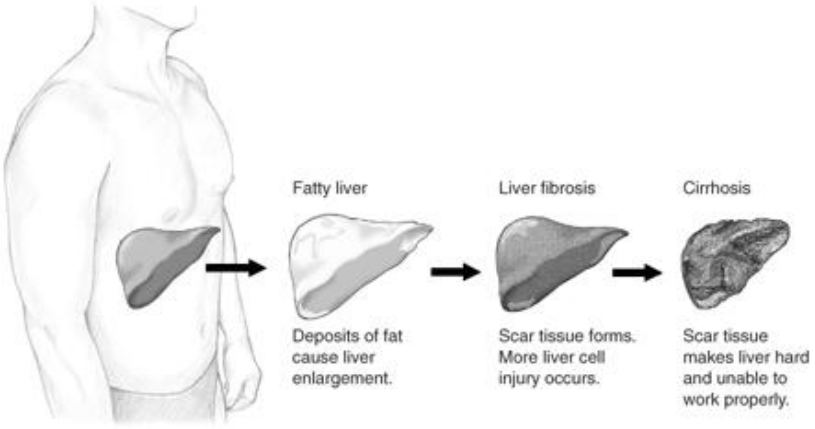
Fatty Liver

Fatty liver



Micrograph showing a **fatty liver** (macrovesicular steatosis), as seen in non-alcoholic fatty liver disease. Trichrome stain.

ICD-10	K70., K76.0
ICD-9	571.0, 571.8
DiseasesDB	18844
eMedicine	med/775 article/170409
MeSH	*C06.552.241*



Different stages of liver damage

Fatty liver, also known as **fatty liver disease** (**FLD**), is a reversible condition where large vacuoles of triglyceride fat accumulate in liver cells via the process of steatosis (i.e. abnormal retention of lipids within a cell). Despite having multiple causes, fatty liver can be considered a single disease that occurs worldwide in those with excessive alcohol intake and those who are obese (with or without effects of insulin resistance). The condition is also associated with other diseases that influence fat metabolism. Morphologically it is difficult to distinguish alcoholic FLD from non alcoholic FLD and both show micro-vesicular and macrovesicular fatty changes at different stages.

Accumulation of fat may also be accompanied by a progressive inflammation of the liver (hepatitis), called steatohepatitis. By considering the contribution by alcohol, fatty liver may be termed alcoholic steatosis or non-alcoholic fatty liver disease (NAFLD), and the more severe forms as alcoholic steatohepatitis (part of alcoholic liver disease) and non-alcoholic steatohepatitis (NASH).

Causes

Fatty liver is commonly associated with alcohol or metabolic syndrome (diabetes, hypertension, obesity and dyslipidemia) but can also be due to any one of many causes:

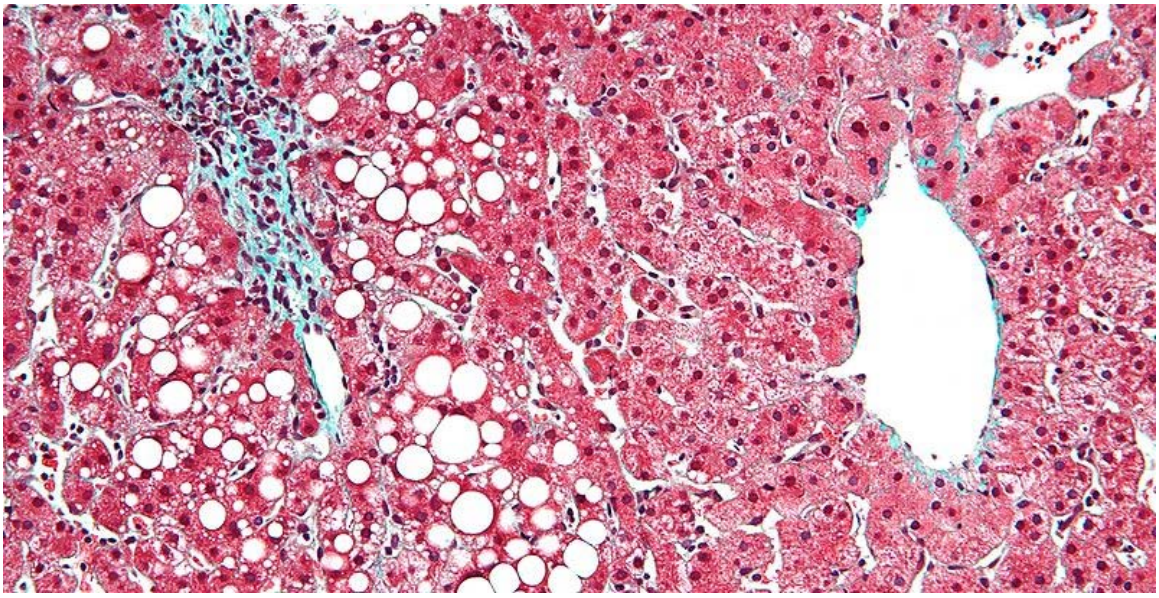
Metabolic
: Abetalipoproteinemia, glycogen storage diseases, Weber-Christian disease, acute fatty liver of pregnancy, lipodystrophy

Nutritional
: Malnutrition, total parenteral nutrition, severe weight loss, refeeding syndrome, jejuno-ileal bypass, gastric bypass, jejunal diverticulosis with bacterial overgrowth

Drugs and toxins
: Amiodarone, methotrexate, diltiazem, highly active antiretroviral therapy, glucocorticoids, tamoxifen, environmental hepatotoxins (e.g., phosphorus, mushroom poisoning)

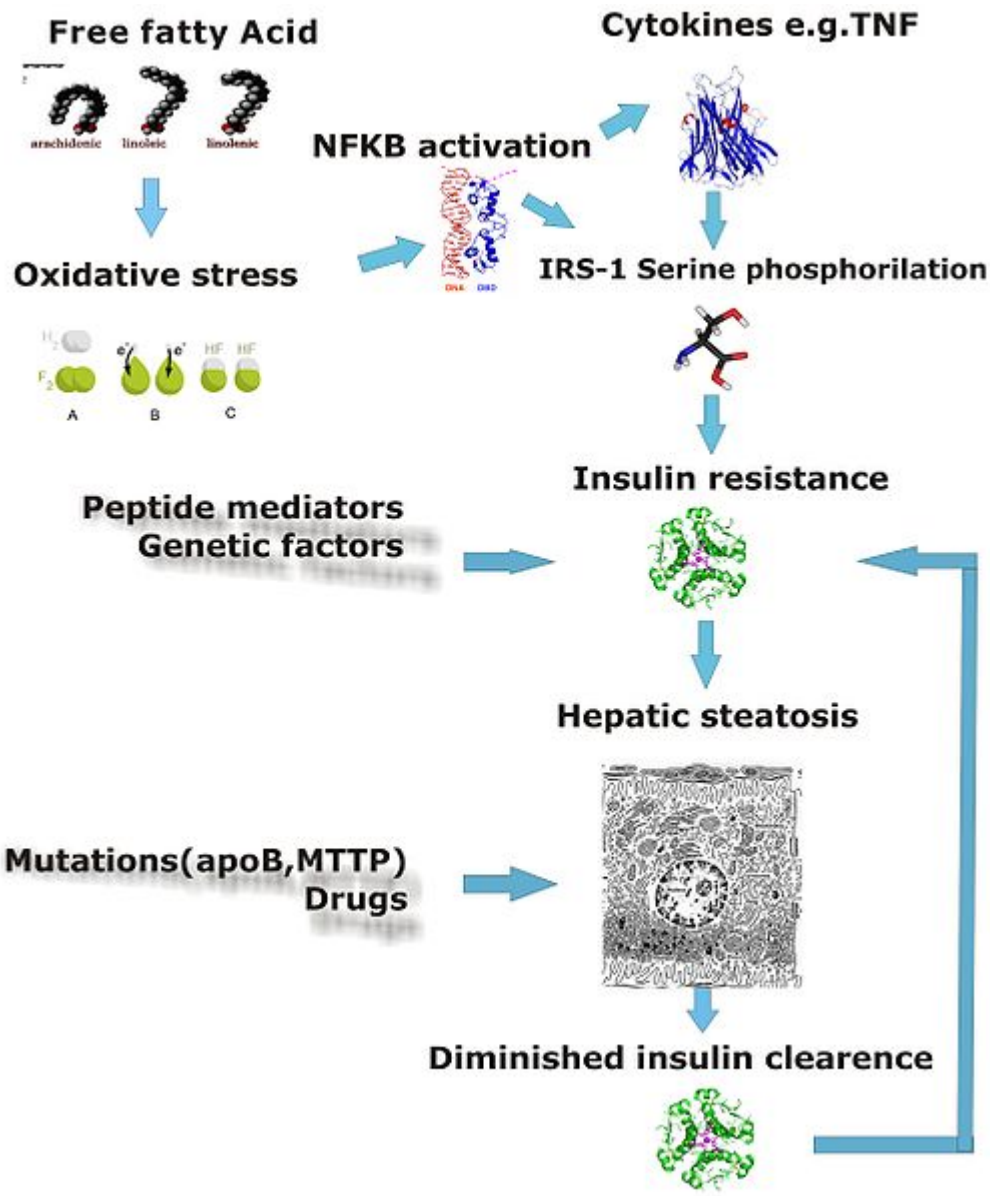
Other
: Inflammatory bowel disease, HIV, Hepatitis C especially genotype 3, and Alpha 1-antitrypsin deficiency

Pathology



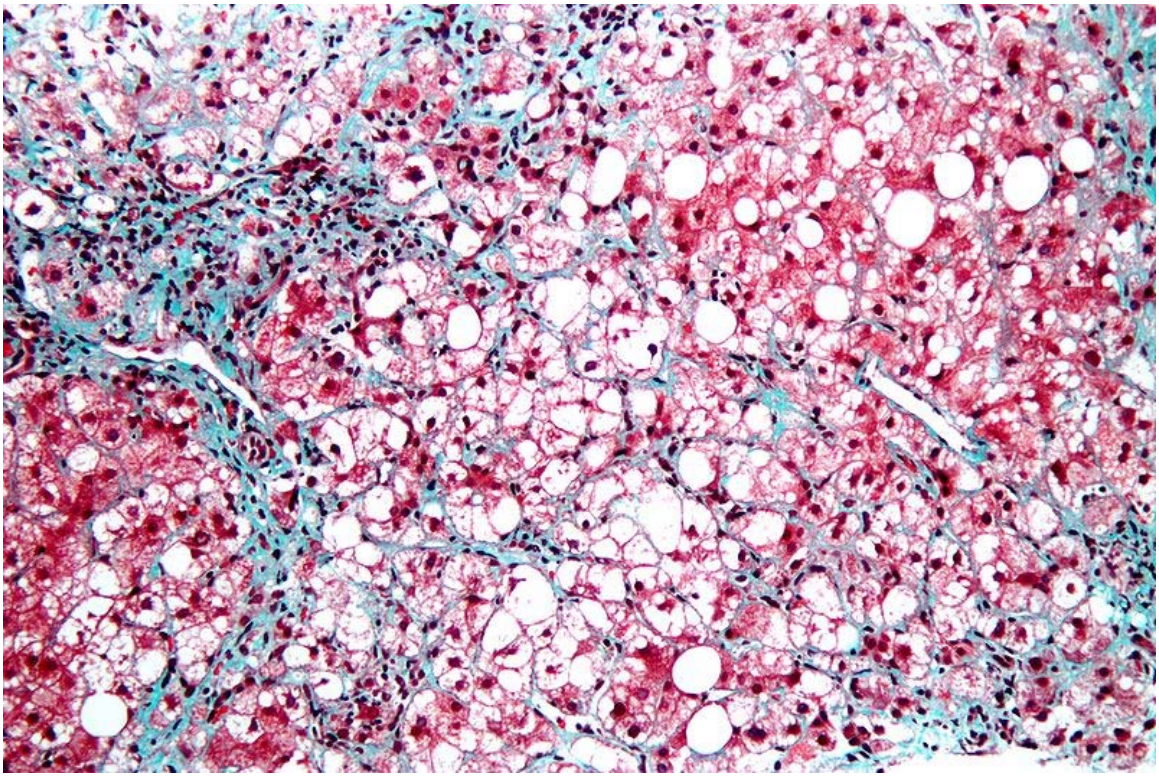
Micrograph of periportal hepatic steatosis, as may be seen due to steroid use. Trichrome stain.

Fatty change represents the intra-cytoplasmic accumulation of triglyceride (neutral fats). At the beginning, the hepatocytes present small fat vacuoles (liposomes) around the nucleus (microvesicular fatty change). In this stage liver cells are filled with multiple fat droplets that do not displace the centrally located nucleus. In the late stages, the size of the vacuoles increase pushing the nucleus to the periphery of the cell giving characteristic signet ring appearance (macrovesicular fatty change). These vesicles are well delineated and optically "empty" because fats dissolve during tissue processing. Large vacuoles may coalesce and produce fatty cysts which are irreversible lesions. Macrovesicular steatosis is the most common form and is typically associated with alcohol, diabetes, obesity and corticosteroids. Acute fatty liver of pregnancy and Reye's syndrome are examples of severe liver disease caused by microvesicular fatty change. The diagnosis of steatosis is made when fat in the liver exceeds 5–10% by weight.



Mechanism leading to hepatic steatosis

Defects in fat metabolism are responsible for pathogenesis of FLD which may be due to imbalance in energy consumption and its combustion resulting in lipid storage or can be a consequence of peripheral resistance to insulin, whereby the transport of fatty acids from adipose tissue to the liver is increased. Impairment or inhibition of receptor molecules (PPAR-α, PPAR-γ and SREBP1) that control the enzymes responsible for the oxidation and synthesis of fatty acids appears to contribute towards fat accumulation. In addition, alcoholism is known to damage mitochondria and other cellular structure further impairing cellular energy mechanism. On the other hand non alcoholic FLD may begin as excess of unmetabolised energy in liver cells. Hepatic steatosis is considered reversible and to some extent nonprogressive if there is cessation or removal of underlying cause.



Micrograph of inflamed fatty liver (steatohepatitis)

Severe fatty liver is sometimes accompanied by inflammation, a situation that is referred to as *steatohepatitis*. Progression to alcoholic steatohepatitis (ASH) or non-alcoholic steatohepatitis (NASH) depend on persistence or severity of inciting cause. Pathological lesions in both conditions are similar. However, the extent of inflammatory response varies widely and does not always correlate with degree of fat accumulation. Steatosis (retention of lipid) and onset of steatohepatitis may represent successive stages in FLD progression.

Liver with extensive inflammation and high degree of steatosis often progresses to more severe forms of the disease. Hepatocyte ballooning and hepatocyte necrosis of varying degree are often present at this stage. Liver cell death and inflammatory responses lead to the activation of stellate cells which play a pivotal role in hepatic fibrosis. The extent of fibrosis varies widely. Perisinusoidal fibrosis is most common, especially in adults, and predominates in zone 3 around the terminal hepatic veins.

The progression to cirrhosis may be influenced by the amount of fat and degree of steatohepatitis and by a variety of other sensitizing factors. In alcoholic FLD the transition to cirrhosis related to continued alcohol consumption is well documented but the process involved in non-alcoholic FLD is less clear.

Diagnosis

Most individuals are asymptomatic and are usually discovered incidentally because of abnormal liver function tests or hepatomegaly noted in unrelated medical condition. Elevated liver biochemistry is found in 50% of patients with simple steatosis. The serum ALT level usually is greater than the AST level in non-alcoholic variant and the opposite in alcoholic FLD (AST:ALT more than 2:1).

Imaging studies are often obtained during evaluation process. Ultrasonography reveals a "bright" liver with increased echogenicity. Medical imaging can aid in diagnosis of fatty liver; fatty livers have lower density than spleen on computed tomography (CT) and fat appears bright in T1-weighted magnetic resonance images (MRIs). No medical imagery, however, is able to distinguish simple steatosis from advanced NASH. Histological diagnosis by liver biopsy is sought when assessment of severity is indicated.

Treatment

The treatment of fatty liver depends on what is causing it, and generally, treating the underlying cause will reverse the process of steatosis if implemented at early stage.

Complication

Up to 10% of cirrhotic alcoholic FLD will develop hepatocellular carcinoma. Overall incidence of liver cancer in non-alcoholic FLD has not yet been quantified, but the association is well established.

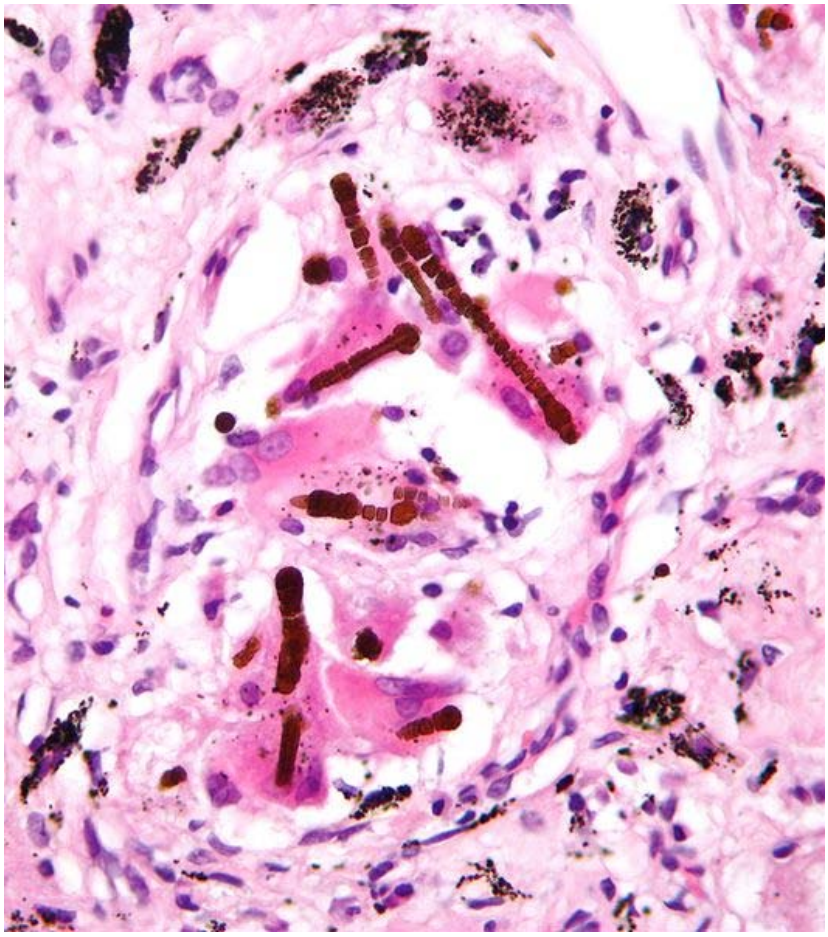
Epidemiology

The prevalence of FLD in the general population ranges from 10% to 24% in various countries. However, the condition is observed in up to 75% of obese people, 35% of whom will progress to non-alcoholic FLD, despite no evidence of excessive alcohol consumption. FLD is the most common cause of abnormal liver function test in the US. "Fatty livers occur in 33% of European-Americans, 45% of Hispanic-Americans, and 24% of African-Americans."

Chapter 5

Ferruginous Body, Fibrinoid Necrosis and Field Stain

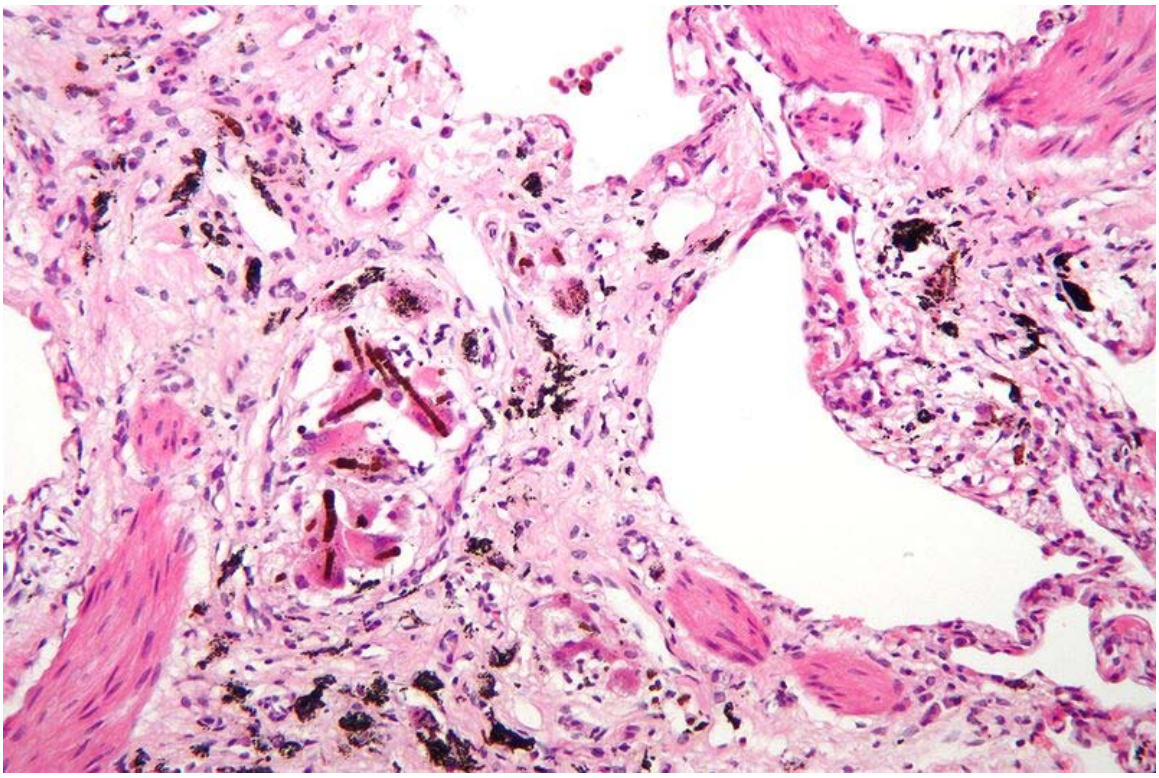
Ferruginous body



Ferruginous bodies. H&E stain.

A **ferruginous body** (pl. ferruginous bodies), is a histopathologic finding in interstitial lung disease suggestive of significant asbestos exposure (asbestosis).

They appear as small brown nodules in the septum of the alveolus. Ferruginous bodies are typically indicative of asbestos inhalation (when the presence of asbestos is verified they are called "asbestos bodies"). In this case they are fibers of asbestos coated with an iron-rich material derived from proteins such as ferritin and hemosiderin. Ferruginous bodies are believed to be formed by macrophages that have phagocytized and attempted to digest the fibers.



Micrograph of asbestosis with prominent **ferruginous bodies**. H&E

Fibrinoid necrosis

Fibrinoid necrosis is a form of necrosis, or tissue death, in which there is accumulation of amorphous, basic, proteinaceous material in the tissue matrix with a staining pattern reminiscent of fibrin. It is associated with conditions such as immune vasculitis (e.g. Henoch-Schonlein purpura), malignant hypertension, or hyperacute transplant rejection.

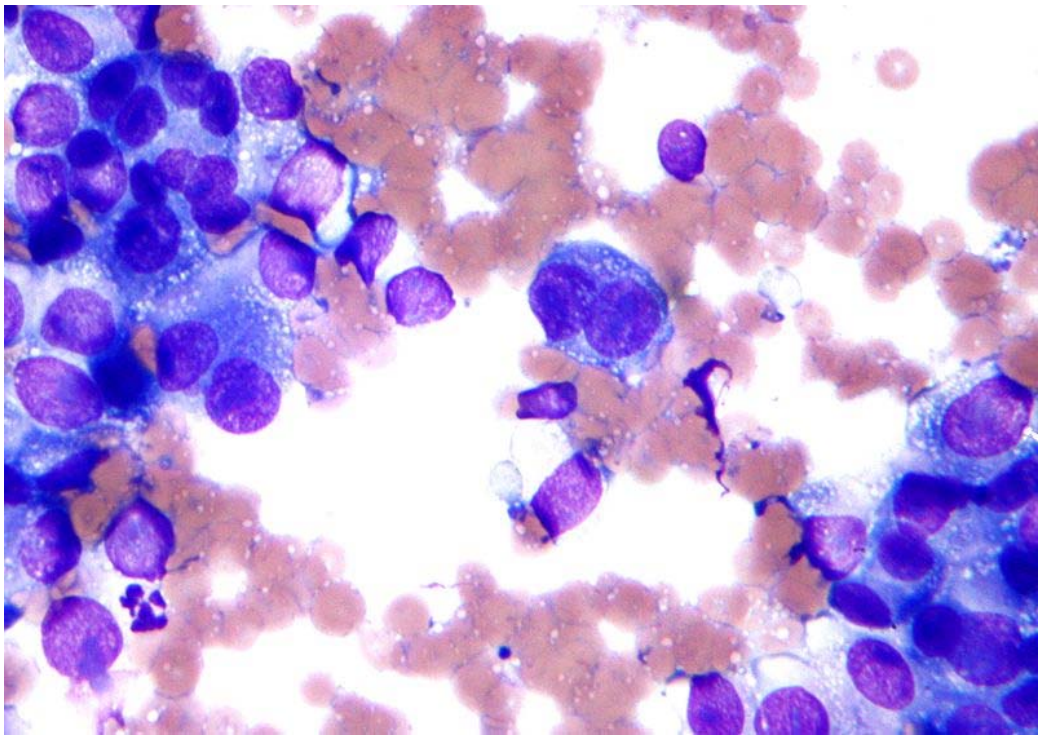
Fibrinoid material does usually contain fibrin and tends to be eosinophilic (staining red with the acidic dye eosin). However, in systemic lupus erythematosus the fibrinoid deposits may contain significant amounts of nuclear debris, including acidic DNA, and may be haematoxyphilic (staining purple or blue with the basic dye haematoxylin). Fibrinoid necrosis is distinguished from hyaline deposits, which are more homogeneous

and glassy, and caseous necrosis, which is associated with the tuberculosis. Fibrinoid is now a somewhat historic term, based on traditional haematoxylin and eosin staining. Its significance is different in different contexts, such as malignant hypertension and rheumatoid nodules. However, in context it remains a diagnostically useful term. The idea that autoimmune diseases such as systemic lupus and rheumatoid arthritis were based on a common lesion of 'fibrinoid necrosis of collagen' led to the popular term 'collagen vascular disease', which is now obsolete. It is cells that are dead, not collagen. The presence of fibrin indicates that nearby blood vessels have become highly permeable and often themselves destroyed but vasculitis is not necessarily present at sites of fibrinoid deposition.

Fibrinoid necrosis can be associated with immune-complex associated disease, as in the synovitis and nodules of rheumatoid arthritis and various forms of immune- based vasculitis and glomerular disease. It may be associated with deposition of immune complexes and complement components in vessel walls. In small vessel vasculitis fibrin plugs frequently occur in the vessel lumen, but the term fibrinoid is usually used to refer to material outside the lumen of a vessel.Fibrinoid necrosis also occurs in the walls of arterioles in malignant hypertension (blood pressure greater than 200/130 mmHg).

Fibrinoid necrosis involves the deposition of immune complexes in areas such as the glomerulus and capillaries. The immune complexes do not cause the damage, but activate the alternative complement pathway, specifically C5a, which attracts neutrophils. The neutrophils are then responsible for causing fibrinoid necrosis.

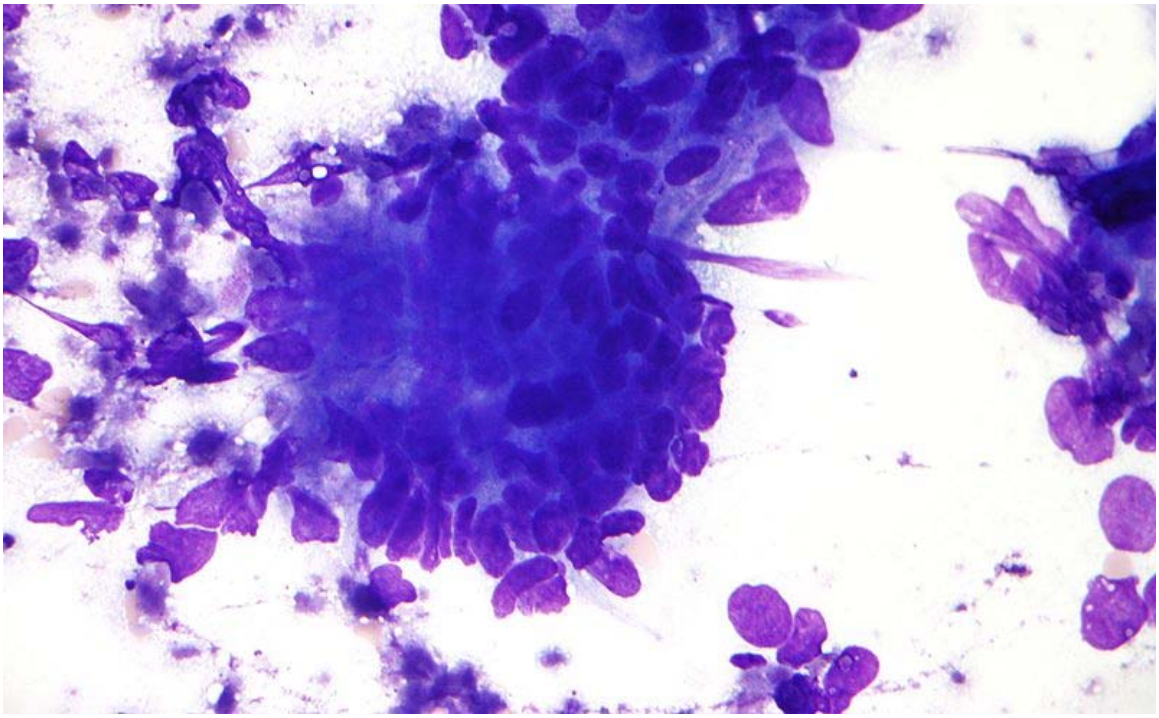
Field stain



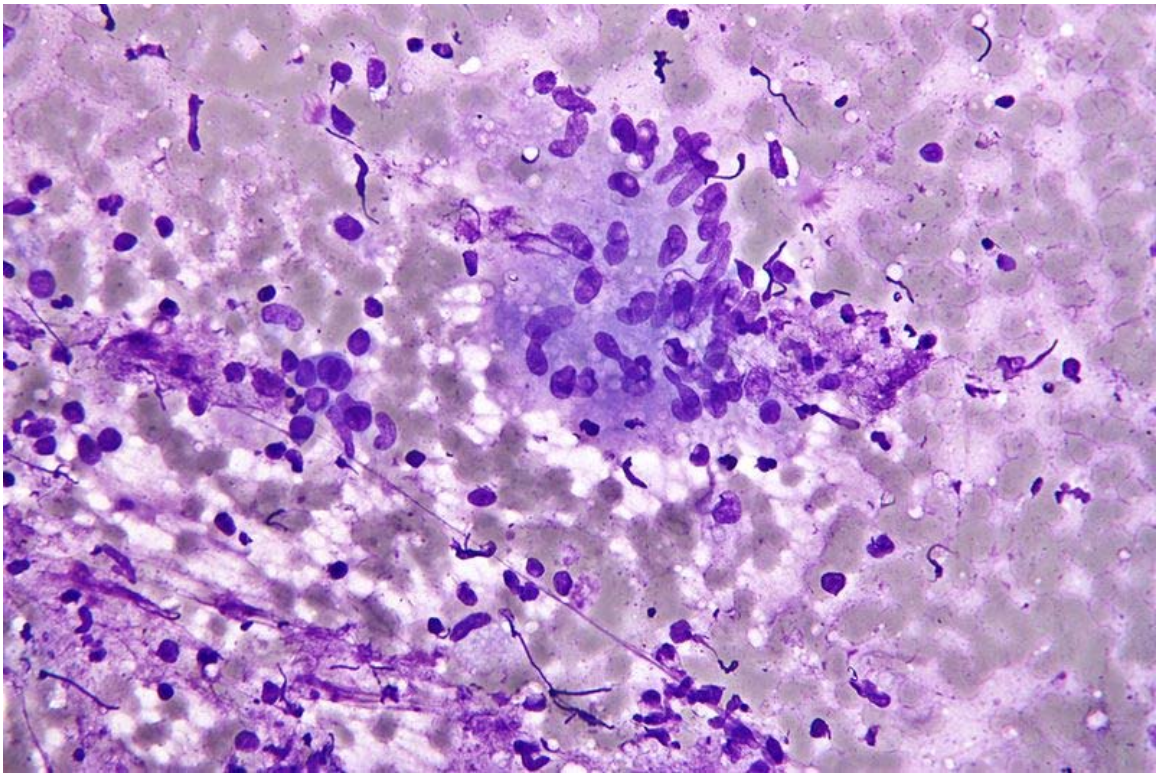
Micrograph of a Field stain showing malignant melanoma.

Field stain is a histological method for staining of blood smears. It is used for staining thin blood films in order to discover malarial parasites. Field's stain is a version of a Romanowsky stain, used for rapid processing of the specimens.

Field's stain uses methylene blue and Azure 1 dissolved in phosphate buffer solution, and Eosin Y in buffer solution.



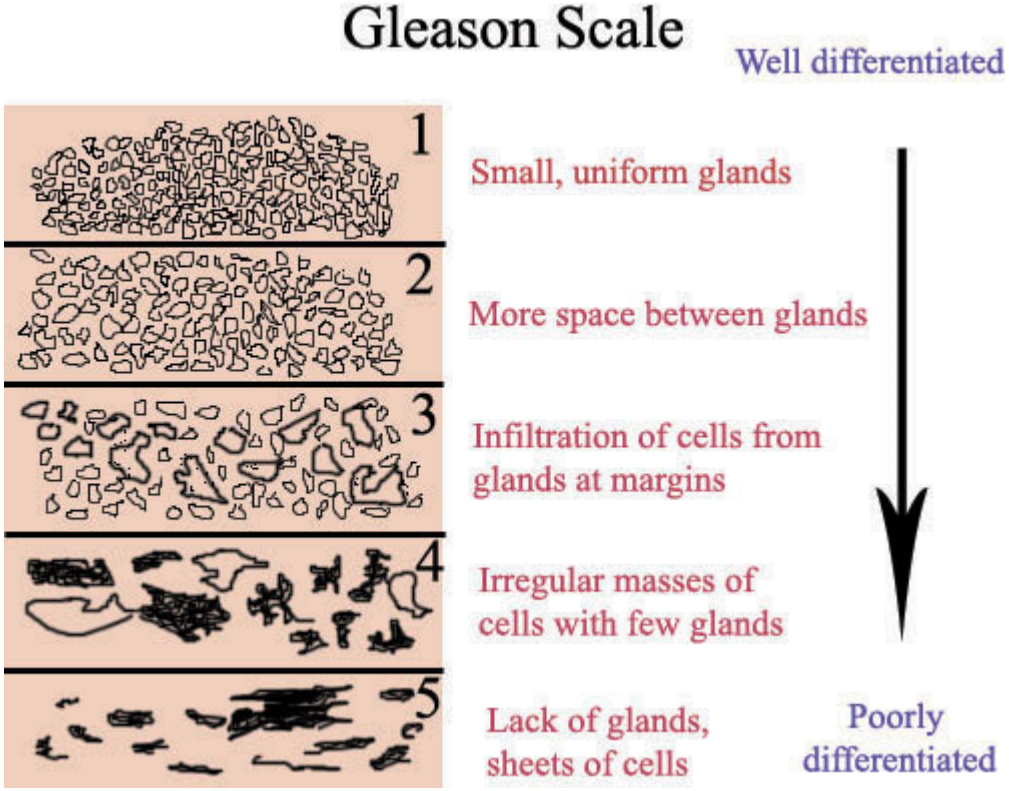
Colorectal adenocarcinoma. Field stain.



Granuloma. Field stain.

Chapter 6

Gleason Grading System



Gleason grade Lower grades are associated with small, closely packed glands. Cells spread out and lose glandular architecture as grade increases. Gleason score is calculated from grade as described in the text.

The **Gleason Grading system** is used to help evaluate the prognosis of men with prostate cancer. Together with other parameters, it is incorporated into a strategy of prostate cancer staging which predicts prognosis and helps guide therapy. A Gleason score is given to prostate cancer based upon its microscopic appearance. Cancers with a higher Gleason score are more aggressive and have a worse prognosis.

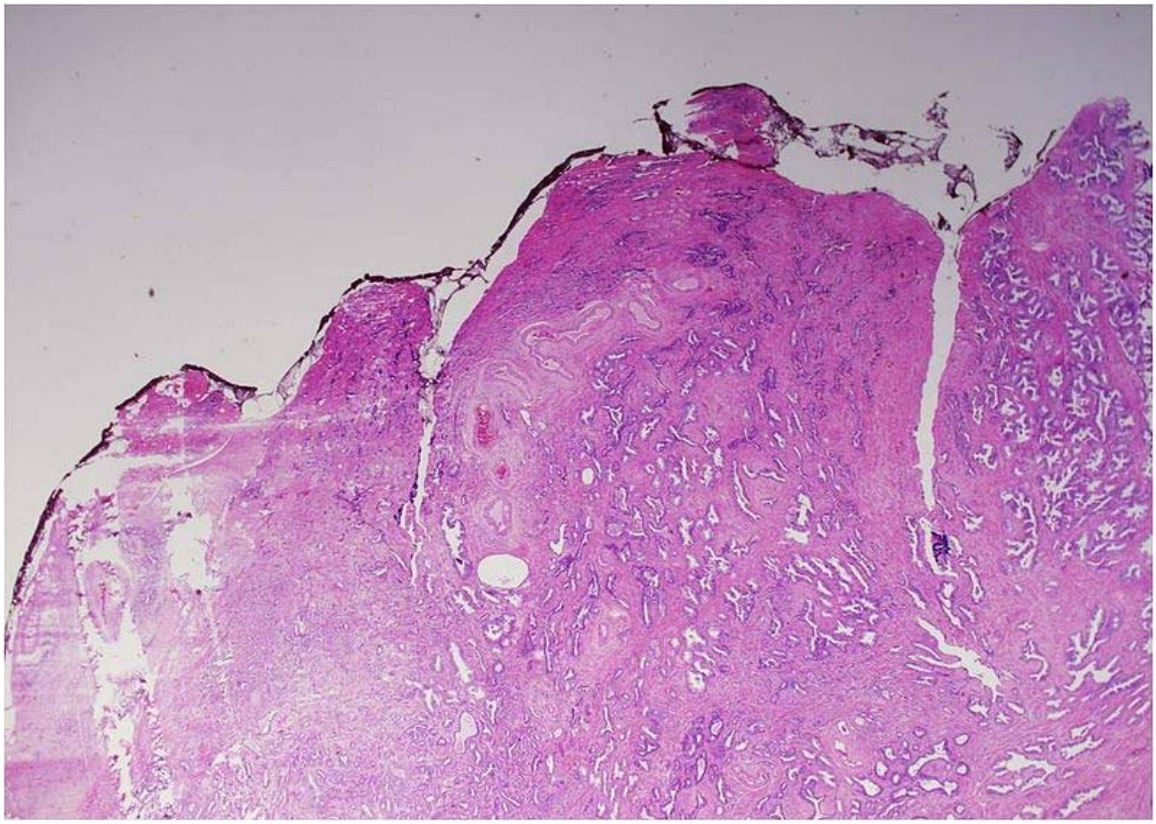
Process

Most often, a urologist or radiologist will remove a cylindrical sample (biopsy) of prostate tissue through the rectum, using hollow needles, and prepare microscope slides. After a prostate is removed in surgery, a pathologist will slice the prostate for a final examination.

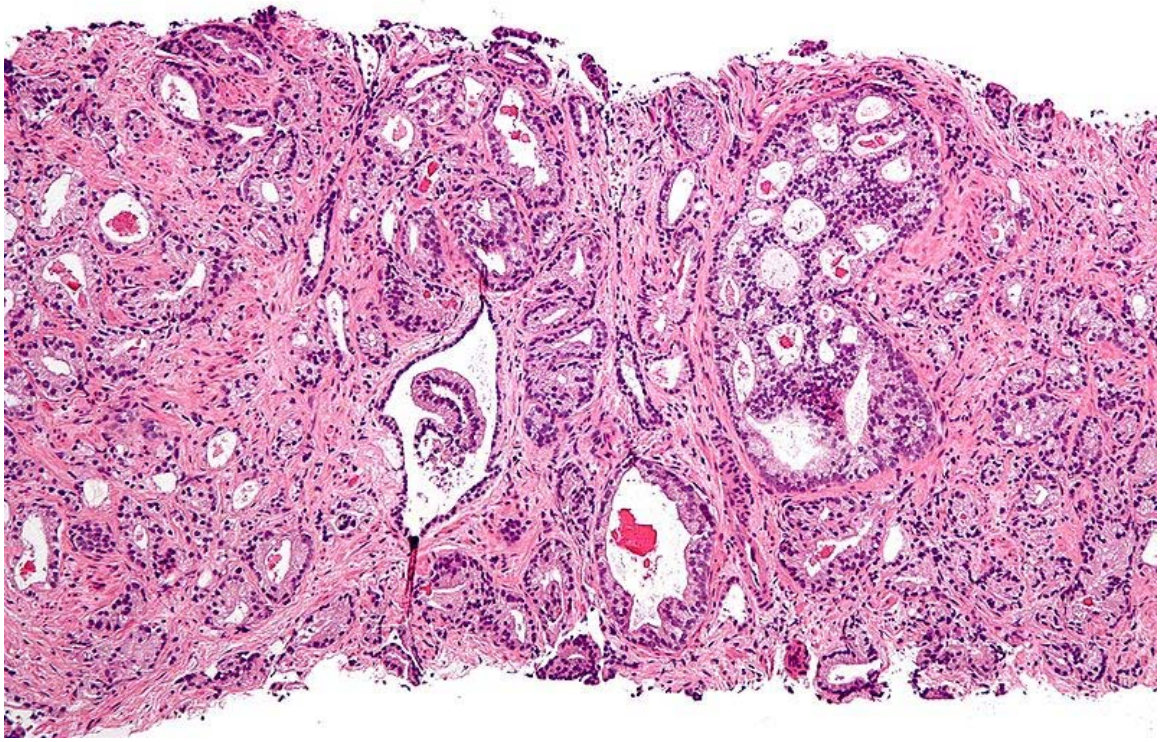
Grades and scores

The pathologist assigns a grade to the most common tumor pattern, and a second grade to the next most common tumor pattern. The two grades are added together to get a Gleason Score. For example, if the most common tumor pattern was grade 3, and the next most common tumor pattern was grade 4, the Gleason Score would be 3+4 = 7. The Gleason Grade is also known as the Gleason Pattern, and the Gleason Score is also known as the Gleason Sum. The Gleason Grade or Gleason Pattern ranges from 1 to 5, with 5 having the worst prognosis. The Gleason Score ranges from 2 to 10, with 10 having the worst prognosis. For Gleason Score 7, a Gleason 4+3 is a more aggressive cancer than a Gleason 3+4. Also, there is not really any difference between the aggressiveness of a Gleason Score 9 or 10 tumor.

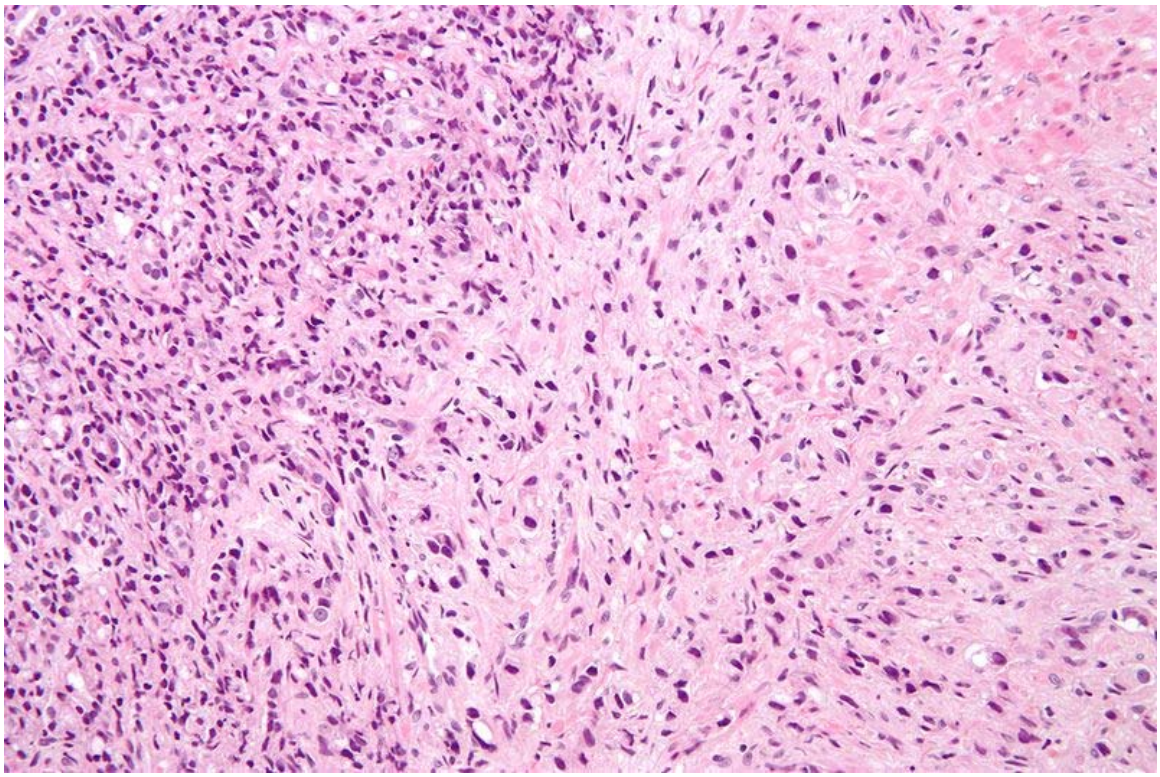
Patterns 1 through 5



Gleason Pattern 3. H&E stain.



Gleason pattern 4. H&E stain.



Gleason pattern 4 (left of image) and Gleason pattern 5 (right of image). H&E stain.

Gleason patterns are associated with the following features:

- Pattern 1 - The cancerous prostate closely resembles normal prostate tissue. The glands are small, well-formed, and closely packed.
- Pattern 2 - The tissue still has well-formed glands, but they are larger and have more tissue between them.
- Pattern 3 - The tissue still has recognizable glands, but the cells are darker. At high magnification, some of these cells have left the glands and are beginning to invade the surrounding tissue.
- Pattern 4 - The tissue has few recognizable glands. Many cells are invading the surrounding tissue
- Pattern 5 - The tissue does not have recognizable glands. There are often just sheets of cells throughout the surrounding tissue.

In the United Kingdom, prostate cancer of Gleason pattern 1 and 2 are almost never seen. Gleason pattern 3 is by far the most common.

Primary, secondary, and tertiary

A pathologist examines the biopsy specimen and attempts to give a score to the two patterns.

- First called the primary grade, represents the majority of tumor (has to be greater than 50% of the total pattern seen).
- Second - a secondary grade - relates to the minority of the tumor (has to be less than 50%, but at least 5%, of the pattern of the total cancer observed).

These scores are then added to obtain the final Gleason score.

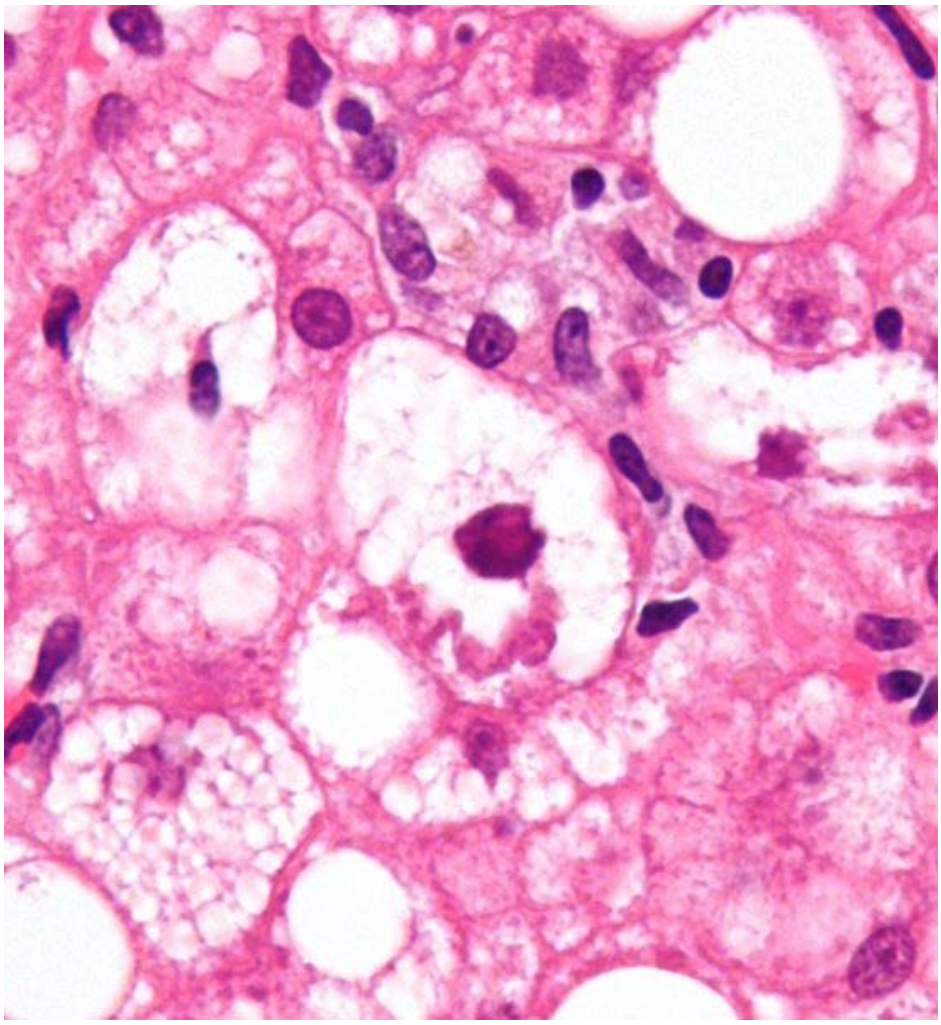
Increasingly, pathologists provide details of the "tertiary" component. This is where there is a small component of a third (generally more aggressive) pattern. So there could be a Gleason 3+4 with a tertiary component of pattern 5 - this would be considered to be more aggressive than a prostate cancer that was Gleason 3+4 with no tertiary pattern 5. Although it is debatable as to what the full extent the tertiary component has on the aggressiveness of a cancer.

History

The scoring system is named after Dr. Donald Gleason, a pathologist at the Minneapolis Veterans Affairs Hospital who developed it with other colleagues at that facility in the 1960s. It remains an important tool.

Chapter 7

Mallory body



Micrograph showing a **Mallory body** with the characteristic *twisted-rope* appearance (centre of image - within a ballooning hepatocyte). H&E stain.

In histopathology, a **Mallory body**, also known as **Drew Parlin bodies**, **Mallory-Denk body**, and **Mallory's hyaline**, is an inclusion found in the cytoplasm of liver cells.

Associated conditions

Mallory bodies are classically found in the livers of people suffering from alcoholic liver disease and were once thought to be specific for that.

They are most common in alcoholic hepatitis (prevalence of 65%) and alcoholic cirrhosis (prevalence of 51%).

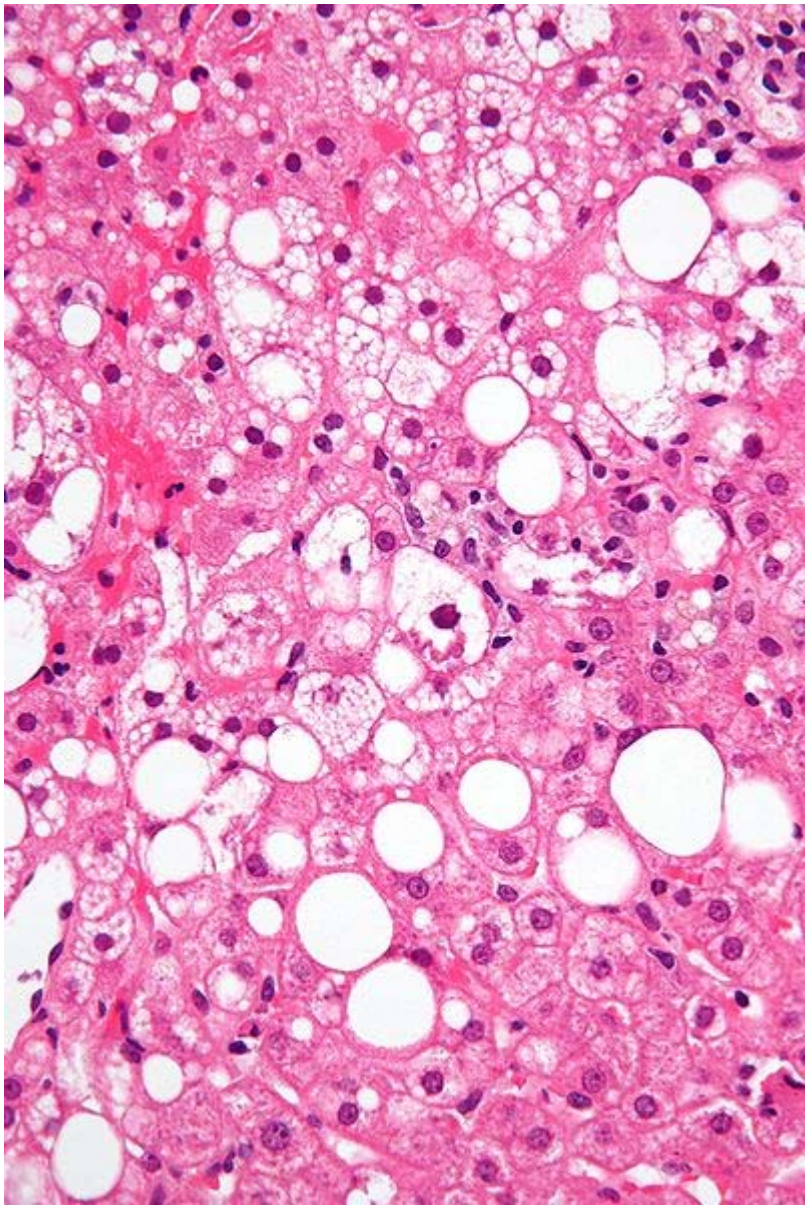
They are a recognized feature of Wilson's disease (25%), primary biliary cirrhosis (24%), non-alcoholic cirrhosis (24%), hepatocellular carcinoma (23%) and morbid obesity (8%), among other conditions.

Appearance

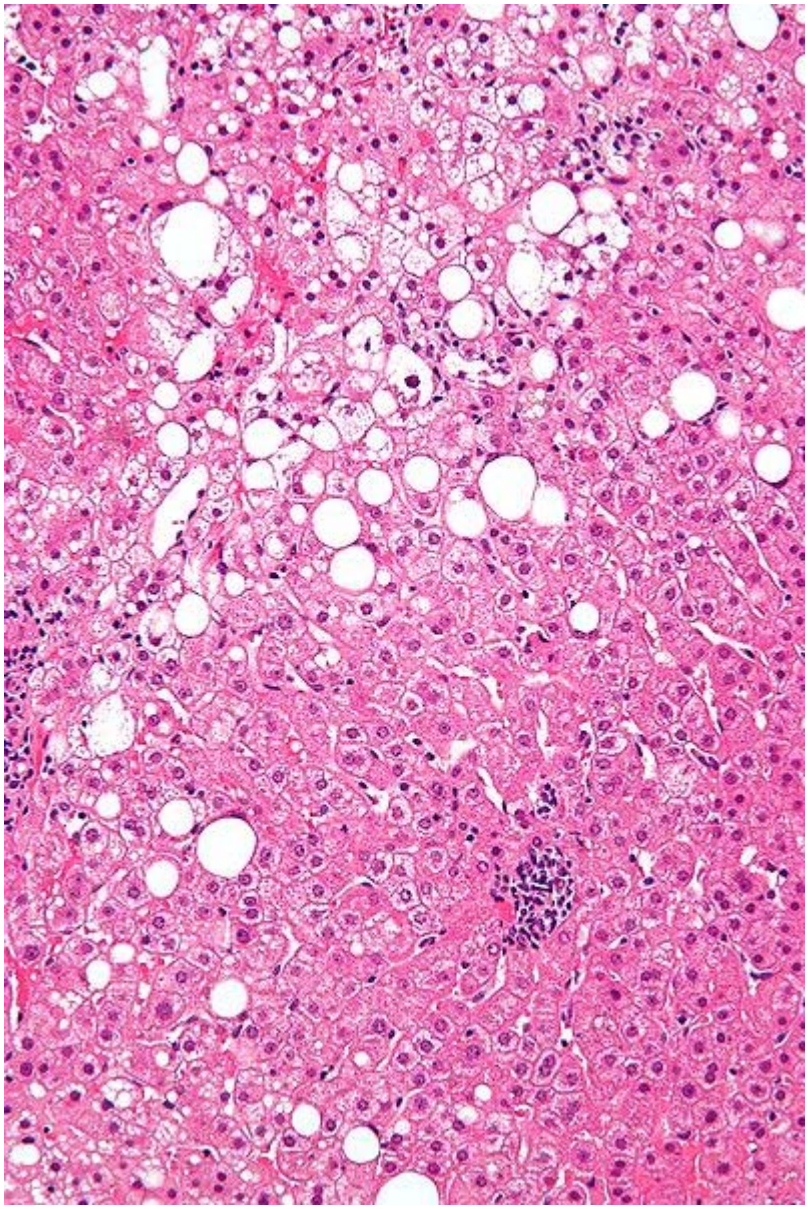
Mallory bodies are highly eosinophilic and thus appear pink on H&E stain. The bodies themselves are made up of intermediate keratin filament proteins that have been ubiquinated, or bound by other proteins such as heat shock proteins, or p62.

Eponym

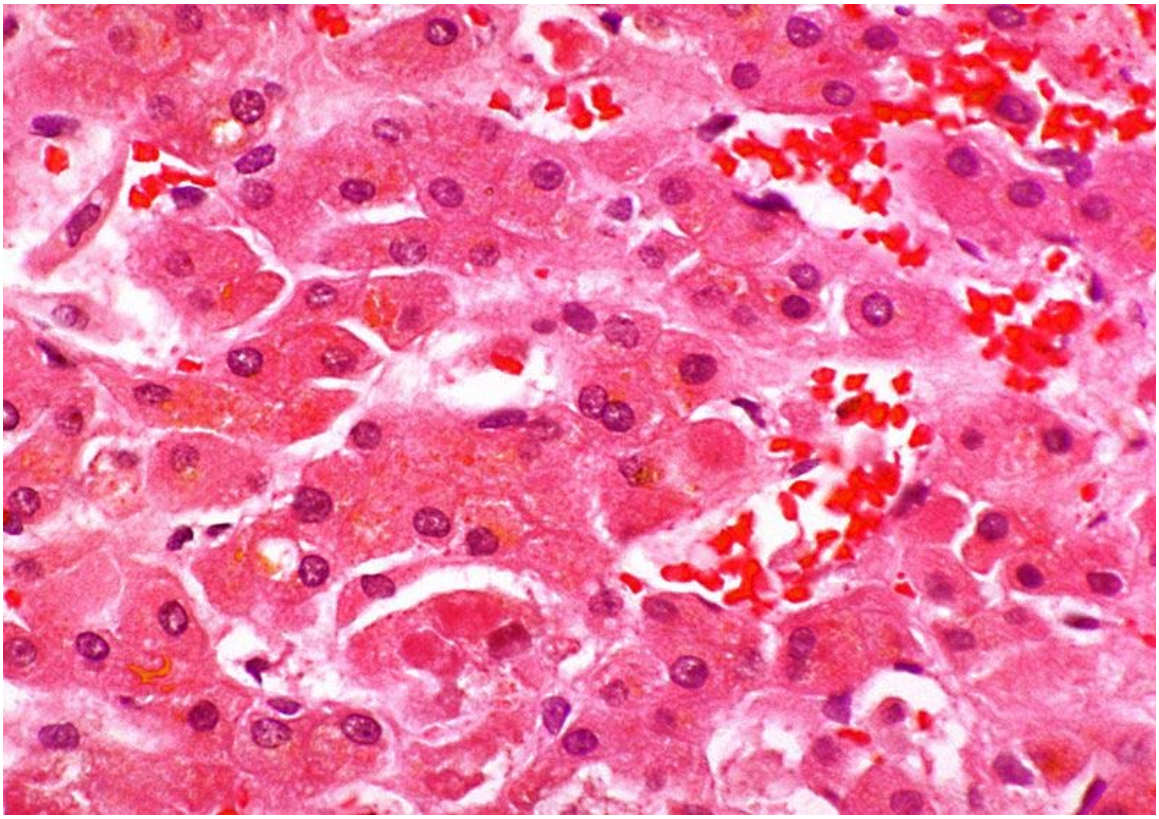
It is named for Frank Burr Mallory. It is was also described serendipitously at the same time by Dr. Drew Parlin.



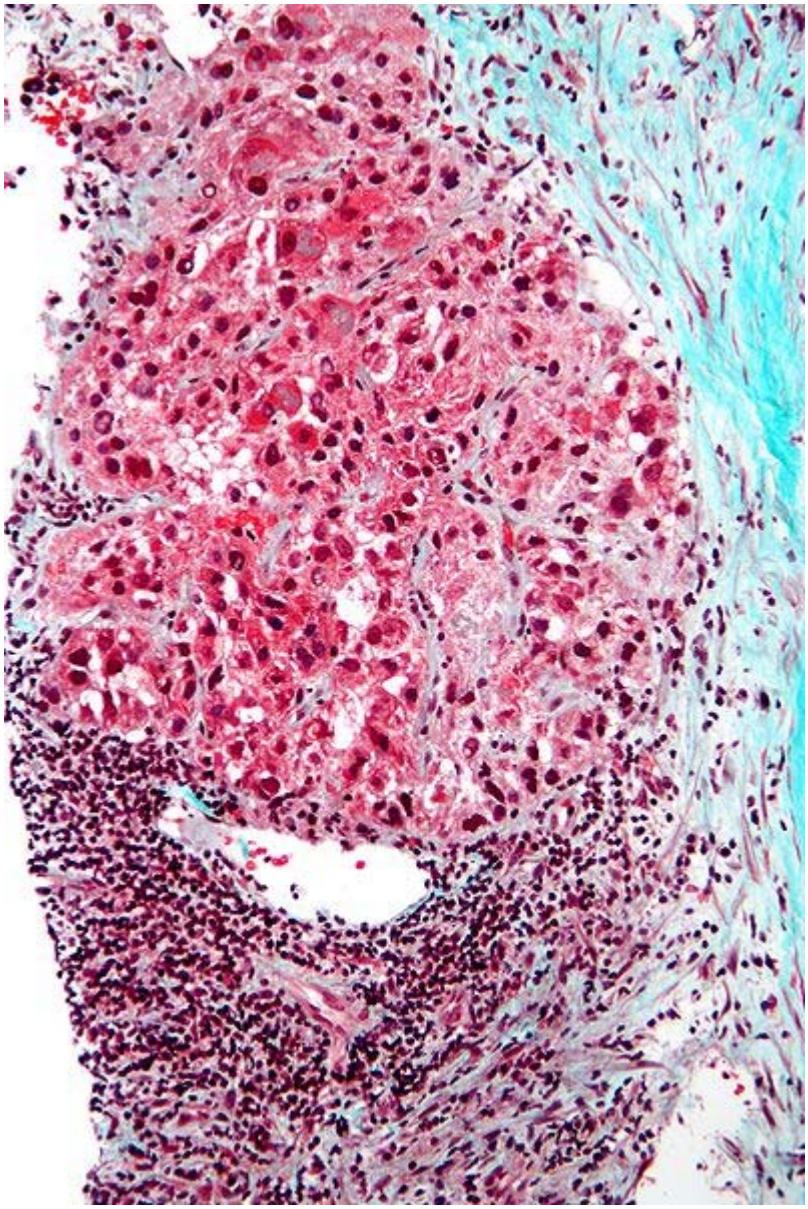
Micrograph showing a **Mallory body**. Original magnification



Micrograph showing a **Mallory body**. Original magnification.



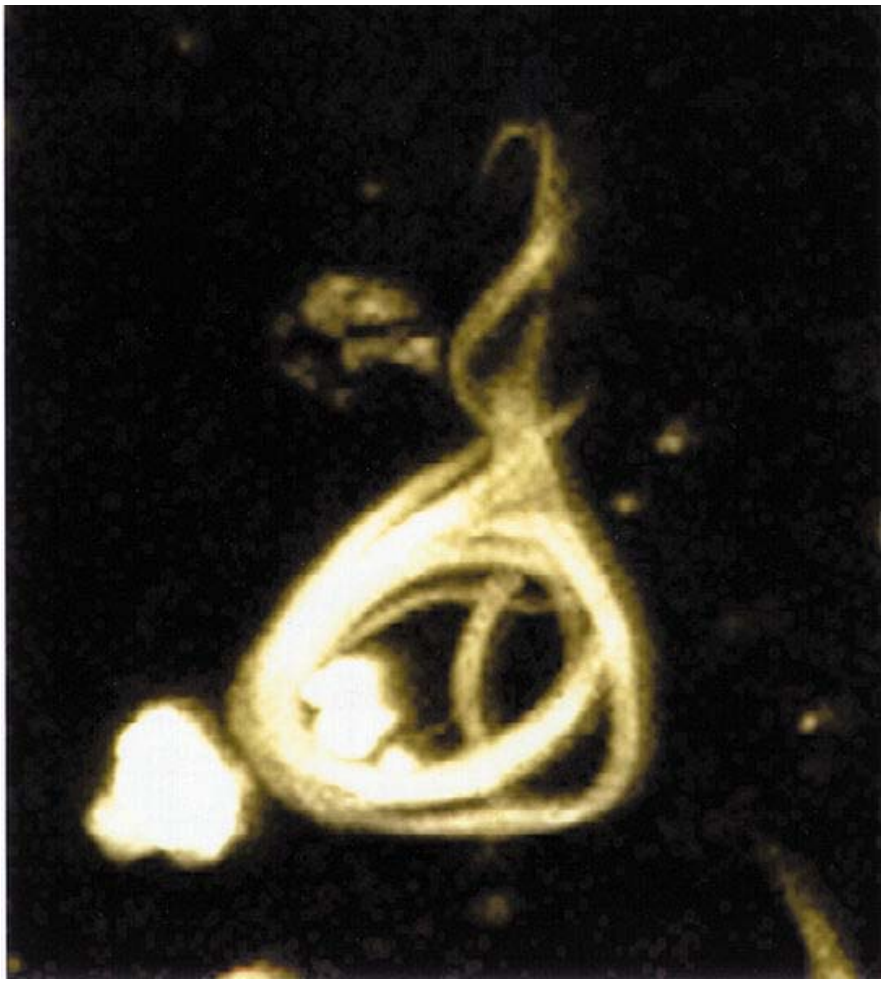
Liver micrograph showing abundant **Mallory bodies**, as seen in alcohol abuse



Mallory bodies in hepatocellular carcinoma. Trichrome stain.

Chapter 8

Neurofibrillary Tangle



Microscopy image of a neurofibrillary tangle, conformed by hyperphosphorylated tau protein

Neurofibrillary Tangles (NFTs) are aggregates of hyperphosphorylation tau that are most commonly known as a primary marker of Alzheimer's Disease. Their presence is also found in numerous other diseases known as Tauopathies. Little is known about their exact relationship to the different pathologies.

Formation

Neurofibrillary Tangles are formed by hyperphosphorylation of a microtubule-associated protein known as tau, causing it to aggregate, or group, in an insoluble form. (These aggregations of hyperphosphorylated tau protein are also referred to as PHF, or "paired helical filaments"). The precise mechanism of tangle formation is not completely understood, and it is still controversial whether tangles are a primary causative factor in disease or play a more peripheral role.

Cytoskeletal changes

Three different maturation states of NFT have been defined using anti-tau and anti-ubiquitin immunostaining. At stage 0 there are morphologically normal pyramidal cells showing diffuse or fine granular cytoplasmic staining with anti-tau. In other words cells are healthy with minimal tau presence; at stage 1 some delicate elongate inclusions are stained by tau antibodies (these are early tangles); stage 2 is represented by the classic NFT demonstration with anti-tau staining ; stage 3 is exemplified by ghost tangles (tangles outside of cells where the host neuron has died), which are characterized by a reduced anti-tau but marked anti-ubiquitin immunostaining.

Causes

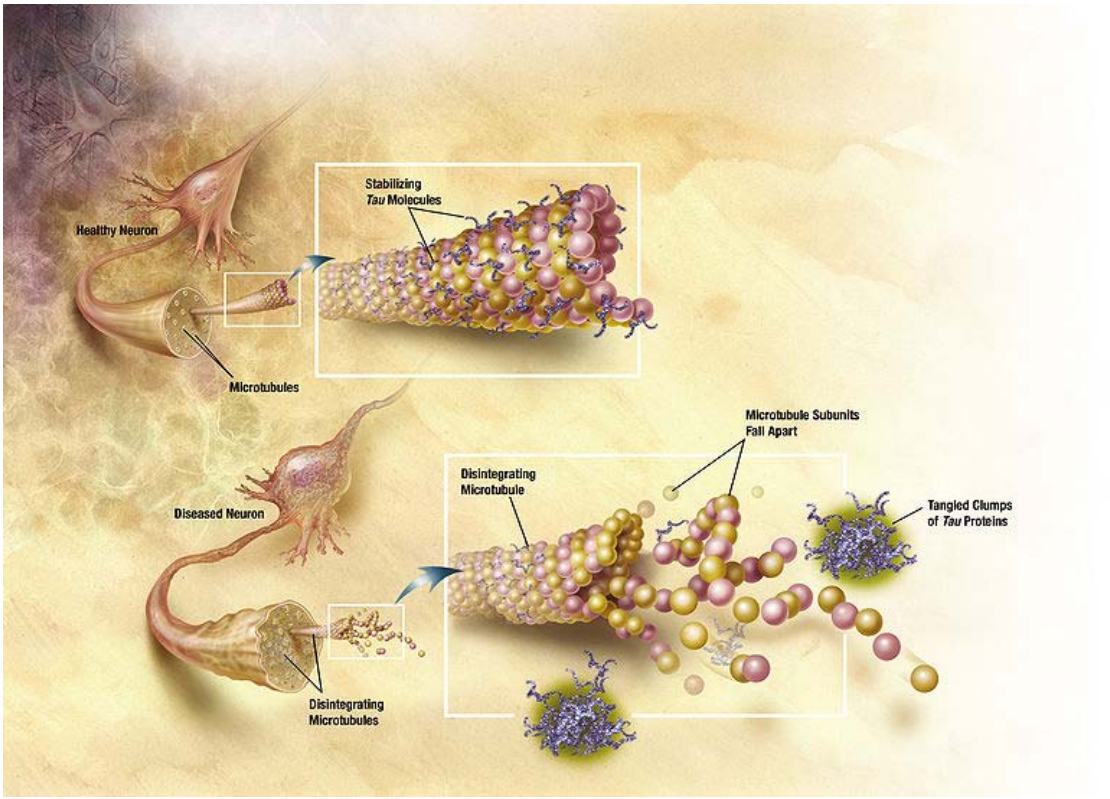


Diagram of how microtubules desintegrate with Alzheimer's disease

Mutated Tau

The traditional understanding is that tau binds to microtubules and assists with their formation and stabilization. However when tau is hyperphosphorylated, it is unable to bind and the microtubules become unstable and begin disintegrating. The unbound tau clumps together in formations called neurofibrillary tangles. More explicitly, intracellular lesions known as pretangles develop when tau is phosphorylated excessively and on improper amino acid residues. These lesions, over time, develop into filamentous neurofibrilary tangles (NFTs) which interfere with numerous intracellular functions. Seeking a reliable animal model for tau-related pathologies, researchers expressed the human mutant P301L tau gene in adult mice. This experiment resulted in the formation of neurofibrillary tangles and pretangle formations. The human mutant P301 tau gene is associated with frontotemporal dementia with parkinsonism, another tauopathy associated with NFTs. It was found that the degree of tau pathology was dependent on time and the level of gene expression. Groups receiving a combination of a promoter and enhancer in the vector saw increased tau expression, as early as 3 weeks after vector injection, which was measured using a Western blot.These groups also showed a greater pathology compared to those with less expression of the mutant tau. Additionally, NFTs were clearly detected by immunoelectron microscopy at 4 months but not at 2 months. However, at both 2 and 4 months, pretangle-like structures were observed suggesting the NFT formation is not complete by 4 months and will continue to progress with time.

Traumatic Brain Injury

Preliminary research indicates that iron deposits due to hemorrhaging, following traumatic brain injury (TBI), may increase tau pathology. While TBI does not routinely lead to accelerated NFT formation, further work may determine if other blood components or factors unrelated to hemorrhages are involved in this TBI-induced augmentation of tau pathology. NFTs are most commonly seen associated with repetitive mild TBI as opposed to one instance of severe traumatic brain injury. For example the clinical syndrome of dementia pugilistica, otherwise known as punch-drunk syndrome found in boxers, is highly associated with NFTs and neuropil threads.

Aluminum

The idea that there is a link between Aluminum exposure and the formation of neurofibrillary tangles has floated around the scientific community for sometime without having been definitively proven or disregarded. Recently a study examining the hippocampal CA1 cells from individuals with and with out Alzheimer’s disease showed a small portion of the pyramidal cells contain cytoplasmic pools within their somas containing early NFTs. These cytoplasmic pools are aggregates of an aluminum/hyperphosphorylated tau complex similar to mature NFTs. (Walton) While a connection between aluminum and NFTs and AD is maintained, there is evidence that Aluminum does not directly cause the formation of NFTs or AD.

Pathology

It has been shown that the degree of cognitive impairment in diseases such as AD is significantly correlated with the presence of neurofibrillary tangles.

Harmful or Protective?

There has been some suggestion that the formation of NFTs is not a causal relationship with disease. Rather that NFTs may be produced in response to a variety of conditions and may in fact be a compensatory response against oxidative stress and serves a protective function. Several points are made to argue the position that NFTs are perhaps protective instead of harmful. First there appears to be a dispute as to the impact of neurofibrillary tangles on neuronal viability because some neurons containing NFTs survive for decades. Furthermore, NFTs have been found in apparently healthy individuals, indicating that NFTs are not directly related to neural degeneration. It has been proposed that the formation of NFTs is part of a multifaced compensatory response where oxidative insult activates several kinases, which are then capable of phosphorylating tau. This then prompts the early formation of NFTs, which reduce oxidative damage and prolong the function of the neuron. While an intriguing theory, scientists have not come to a firm conclusion as to what role NFTs play in neurodegenerative diseases.

Neuron loss

Traditionally believed to play a major role in neuron loss, NFTs are an early event in pathologies such as Alzheimer's disease, and as more NFTs form, there is substantially more neuron loss. However, it has been shown that there is significant neuron loss before the formation of neurofibrillary tangles, and that NFTs account for only a small proportion (around 8.1%) of this neuron loss. Coupled with the longevity of neurons containing NFTs, it is likely that some other factor is primarily responsible for the bulk of neuron loss in these diseases, not the formation of neurofibrillary tangles.

NFT-predominant dementia vs. Classical Alzheimer's

It is currently unclear as to whether or not Neurofibrillary tangle-predominant dementia (NFTPD) (a.k.a. tangle-only dementia) is a variant of the traditional Alzheimer's disease, or a genetically distinct entity. Characterized by later onset and milder cognitive impairment, the distribution of NFT pathology is more closely related to that found in centenarians showing no or limited cognitive impairment. NFTs are generally limited to allocortical/limbic regions of the brain with limited progression to the neocortex but a greater density in the allocortical/hippocampal region. Plaques are generally absent.

Alzheimer disease with concomitant dementia with Lewy bodies (AD+DLB)

The degree of NFT involvement in AD is defined by Braak stages. Braak stages I and II are used when NFT involvent is confined mainly to the transentorhinal region of the brain. Stages III and IV indicated involvement of limbic regions such as the hippocampus, and V and VI when there's extensive neocortical involvement. This should not be confused with the degree of senile plaque involvement, which progresses differently.

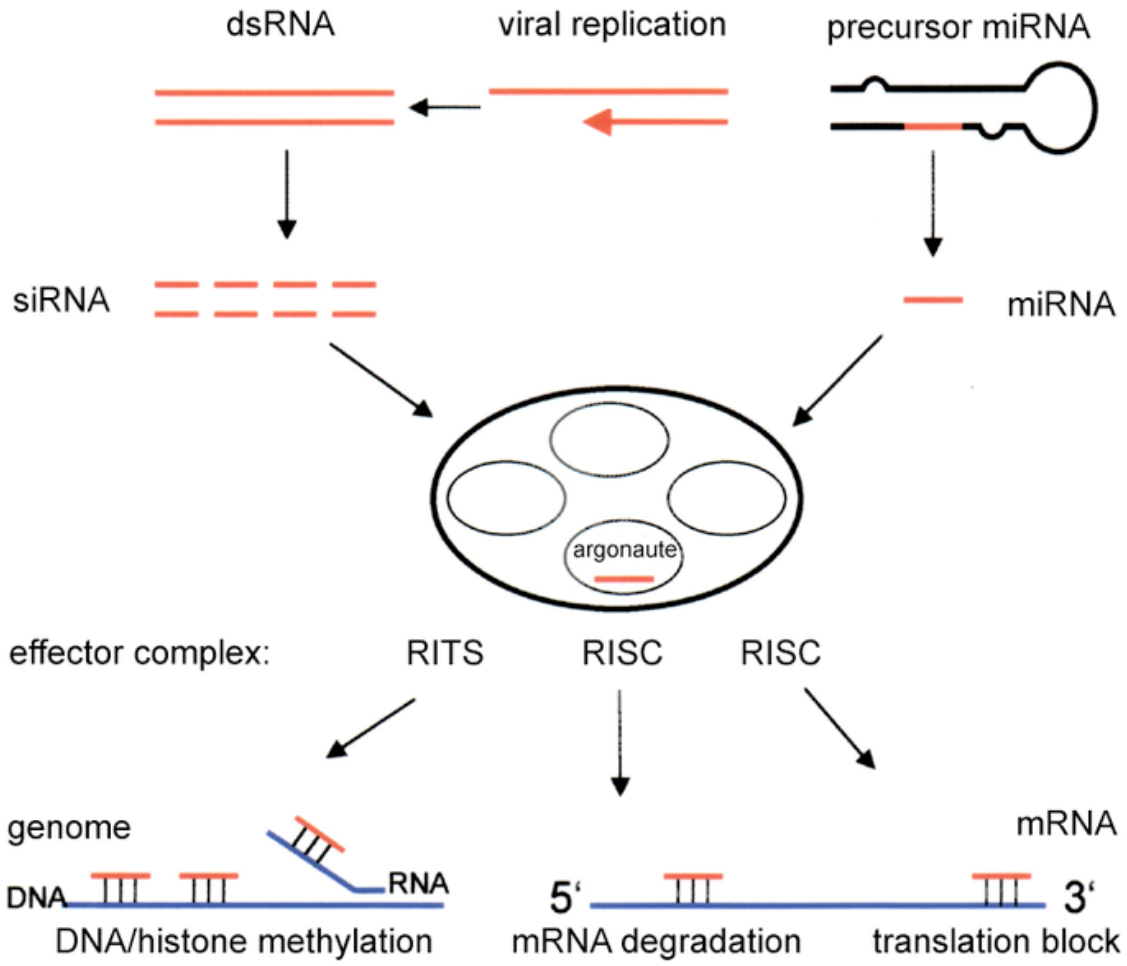
Neurofibrillary tangle and modified Braak scores were lower in AD+DLB, however, neocortical NFT scores show markedly different patterns between AD+DLB and Classical Alzheimers. In pure AD, NFT are predominately found at a high frequency: In AD+DLB, the distribution of NFT frequency was found to be bimodal: NFTs were either frequent or few to absent. Additionally, neocortical NFT frequency in the AD+DLB group tended to parallel the severity of other types of tau cytopathology.

Link to Aggression and Depression In Alzheimer's Patients

A recent study looked for correlation between the quantitative aspects of Alzheimer's disease (neuron loss, neuritic plaque and neurofibrillary tangle load) and aggression frequently found in Alzheimer's patients. It was found that only an increase in neurofibrillary tangle load was associated with severity of aggression and chronic aggression in Alzheimer's patients.While this study does indicate a correlation between NFT load and severity of aggression, it does not provide a causative argument.

Research has also indicated that patients with AD and comorbid depression show higher levels of neurofibrillary tangle formation than individuals with AD but no depression. Comorbid depression increased the odds for advanced neuropathologic disease stage even when controlling for age, gender, education and cognitive function.

Treatment



Overview of RNA interference

Statins

Statins have been shown to reduce the neurofibrillary tangle burden in mouse models, likely due to their anti-inflammatory capacities.

Cyclin-dependent kinase 5

Cyclin-dependent kinase 5 (CDK5) is a kinase that has been previously hypothesized to contribute to tau pathologies. RNA interference (RNAi) mediated silencing of the CDK5 gene has been proposed as a novel therapeutic strategy again tau pathology, such as neurofibrillary tangles. Knockdown of CDK5 has been shown to reduce the phosphorylation of tau in primary neuronal cultures and in mouse models. Furthermore, this silencing showed a dramatic reduction in the number of neurofibrillary tangles.

Lithium

Lithium has been shown to decrease the phosphorylation of tau.Lithium treatment has been shown to reduce the density of neurofibrillary tangles in transgenic models in the hippocampus and spinal cord. Despite the decrease in density of NFTs, motor and memory deficits were not seen to improve following treatment. Additionally, no preventative effects have been see using lithium treatment.

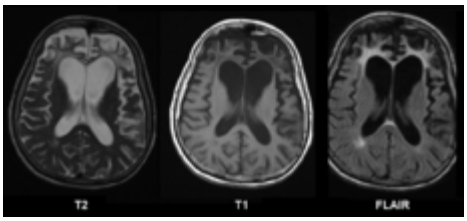
Other conditions

- Progressive supranuclear palsy although with straight filament rather than PHF tau
- Dementia pugilistica (chronic traumatic encephalopathy)
- Frontotemporal dementia and parkinsonism linked to chromosome 17 however without detectable β-amyloid plaques.
- Lytico-Bodig disease (Parkinson-dementia complex of Guam)
- Ganglioglioma and gangliocytoma
- Meningioangiomatosis
- Subacute sclerosing panencephalitis
- As well as lead encephalopathy, tuberous sclerosis, Hallervorden-Spatz disease, and lipofuscinosis

Chapter 9

Pick's Disease

Pick's disease



Brain MRI in Pick's disease

ICD-10	G31.0, F02.0
ICD-9	331.11
OMIM	172700
DiseasesDB	10034
eMedicine	neuro/311
MeSH	D020774

Pick's disease, also known as **Pick disease** and **PiD** (not to be confused with Pelvic Inflammatory Disease, also known as PID), is a rare neurodegenerative disease. While the term Pick's disease was once used to represent a class of clinical syndromes with symptoms attributable to frontal and temporal lobe dysfunction, it is now used (at least among professionals in the field) to mean a specific pathology that is just one of the causes of the clinical syndrome now known as frontotemporal lobar degeneration. Some people still use the term Pick's disease to mean the more general clinical syndrome of frontotemporal lobar degeneration, but this has previously led to confusion among both professionals and patients and so its use should be restricted to the specific pathological subtype described below.

Pick's disease (the pathology) causes progressive destruction of nerve cells in the brain and causes tau proteins in neurons to accumulate into silver-staining, spherical aggregations known as "**Pick bodies**" that are a defining characteristic of the disease.

History

Pick's disease is named after Arnold Pick, a professor of psychiatry from the University of Prague who first discovered and described the disease in 1892 by examining the brain tissue of several deceased patients with histories of dementia. As a result, the characteristic histological feature of this disease - a protein tangle that appears as a large body in neuronal tissue - is named a Pick body. In 1911, Alois Alzheimer also noted the complete absence of senile plaques and neurofilbrillary tangles as well as the presence of Pick Bodies and occasional ballooned neurons.

Symptoms

Pick's disease is one of the causes of the clinical syndrome of frontotemporal lobar degeneration which has three subtypes. Pick's disease pathology is associated more with the frontotemporal dementia and progressive nonfluent aphasia subtypes than the semantic dementia subtype.

Causes

While other pathologies causing frontotemporal lobar degeneration are associated with a genetic cause, there is no evidence in the modern literature that classical Pick's disease pathology can run in families or has a genetic cause.

Pathology and biochemistry

PiD was first recognized as a distinct disease separate from other neurodegenerative diseases because of the presence of large, dark-staining aggregates of proteins in neurological tissue as well as the aforementioned ballooned cells, which are known as Pick cells. Pick bodies are almost universally present in patients with PiD, but some new cases of atypical Pick’s disease have come to light that lack noticeable Pick bodies. A variety of stains can aid in the visualization of Pick bodies and Pick cells, but immunohistochemical staining using anti-tau and anti-ubiquitin antibodies have proven the most efficient and specific. Hematoxylin and eosin staining allows visualization of another population of Pick cells, which are both tau and ubiquitin protein negative. Several different silver impregnation stains have been used, including the Bielschowsky, Bodian, and Gallyas methods. The latter two techniques are sensitive enough to allow PiD to be distinguished from Alzheimer's disease as the Bodian will bind preferentially to cells with PiD as compared to the Gallyas method, which preferentially binds to the cells with Alzheimer's.

Numerous different areas of the brain are affected by PiD, but the specific areas that are affected allow for differentiation between PiD and Alzheimer’s disease. Pick bodies are almost always found in several different places in the brain, including the dentate gyrus, the pyramidial cells of the CA1 sector and subiculum of the hippocampus, and the neocortex as well as a plurality of other nuclei. Interestingly, it is the location within the different layers of the brain as well as the anatomical location that demonstrates some of

the unique features of PiD. A striking feature is that in the neocortex the Pick bodies are located in the II and IV layers of the cortex, which send neurons within the cortex and to thalamic synapses, respectively. While layers III and V have very few if any Pick bodies they show extreme neuronal loss that can, in some cases, be so severe as to leave a void in the brain altogether. Other regions that are involved include the caudate, which is severely affected, the dorsomedial region of the putamen, the globus pallidus, and locus cerulus. The hypothalamic lateral tuberal nucleus is also very severely affected. The cerebellar elements that are important in receiving input, including the mossy fibers as well as the monodendritic brush cells in the granule cell layer, and generating output signals, most notably the dentate nucleus, are stricken with lots of tau protein inclusions. Strangely, the substantia nigra is most often uninvolved or only mildly involved, but cases of extreme degeneration do exist.

PiD has several unique biochemical characteristics that allow for unique identification of Pick's disease as opposed to other pathological subtypes of frontotemporal lobar degeneration. The most striking of these is that this disease, which has tau protein tangles present in many affected neurons, contains only one or as many as two of the six different isoforms of the tau protein. All of these isoforms result from alternative splicing of the same gene. Pick bodies typically have the 3R isoform of tau proteins as not only the most abundant form but the only form of this protein, but a recent study has shown that a much greater number of different tau isoforms including 4R and mixed 3R/4R can be present in the Pick bodies. Not only do these tangles have the 3R tau protein predominately but they are also characteristically shaped with a round body and there is often an indentation in the area that faces the nucleus of the cell. The Pick bodies are also able to be labeled by N-terminal amyloid precursor protein segment, hyperphosphorylated tau, ubiquitin, Alz-50, neurofiliment proteins, clathrin, synaptophysin and neuronal surface glycoside (A2B5) specific stains. Moreover βII tubulin proteins are also suspected in playing a role in the formation of phosphor-tau aggregates that are seen in PiD as well as AD.

Differences from Alzheimer's disease

In Alzheimer's disease, all six isoforms of tau proteins are expressed. In addition, the presence of neurofibrillary tangles that are a hallmark of Alzheimer's can be stained with antibodies to basic fibroblast growth factor, amyloid P, and heparan sulfate glycosaminoglycan. Another difference is that in Pick's disease, a personality change occurs prior to any form of memory loss, unlike Alzheimer's, where memory loss typically presents first. This is used clinically to determine whether patient is suffering from Alzheimer's or Pick's.

Sample Presentation

(From biopsy "histological" verified, after death, case presentation)

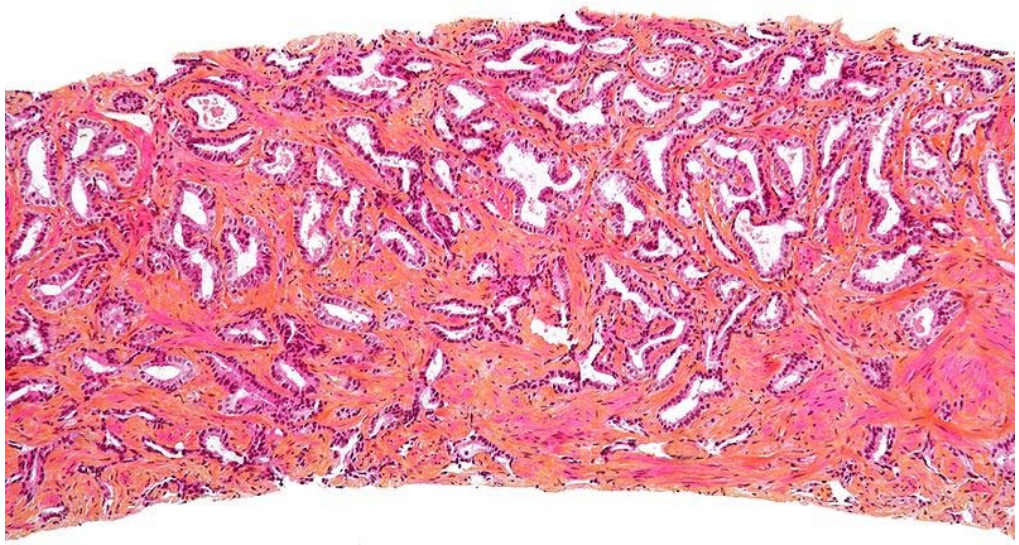
64 y/o retired professional male with progressive strange behavior according to his wife and children for several months without memory loss at start but mild problems with memory now (peoples names, and dates).

Additional signs/symptoms: Uncharacteristic impulsive behavior, easily distractible (again uncharacteristic for this former professional), lack of personal hygiene (stops bathing, brushing teeth, combing hair, etc without prompting from wife), appears unkempt/disheveled, uncharacteristically rude to friends and family, eats with fingers occasionally. No focal neurological signs/symptoms. Prominent grasp and snout reflex.

Chapter 10

Prostate Biopsy and Reed-Sternberg Cell

Prostate biopsy



Micrograph of a **prostate biopsy** showing prostate adenocarcinoma, acinar type, the most common type of prostate cancer. HPS stain.

Prostate biopsy is a procedure in which small samples are removed from a man's prostate gland to be tested for the presence of cancer. It is typically performed when the scores from a PSA blood test rise to a level that is associated with the possible presence of prostate cancer.

The procedure, usually an outpatient procedure, requires a local anesthetic, with fifty-five percent of men reporting discomfort during the biopsy. The most frequent complication of the procedure is bleeding in the urine for several days, some bleeding in the stool for several days, and blood in the ejaculate for several weeks afterwards.

The procedure may be performed transrectally, through the urethra or through the perineum. The most common procedure is transrectal, and may be done with tactile finger guidance, or, more commonly and precisely, with ultrasound guidance.

About a dozen samples are taken from the prostate gland through a thin needle - about six from each side. If the procedure is performed transrectally, antibiotics are prescribed to prevent infection. An enema may also be prescribed for the morning of the procedure. In both the transrectal and the transperineal procedure, the doctor inserts an ultrasound probe into the rectum to help guide the biopsy needles. A local anesthetic is then administered into the tissue around the prostate, similar to the local anesthetic administered for a dental procedure. A spring-loaded prostate tissue collection needle is then inserted into the prostate, through the rectum (or more rarely through the perineum), about a dozen times. It makes a clicking sound, and there may be considerable discomfort.

Prostate mapping

Prostate Mapping is a new biopsy method that uses a combination of multi-sequence MRI scans and template-guided multiple biopsies. It involves taking 30-50 biopsies through the skin that lies in front of the back passage instead of the rectum. The procedure is carried out under general anaesthetic. This method can determine with high accuracy the location and aggressiveness of cancer.

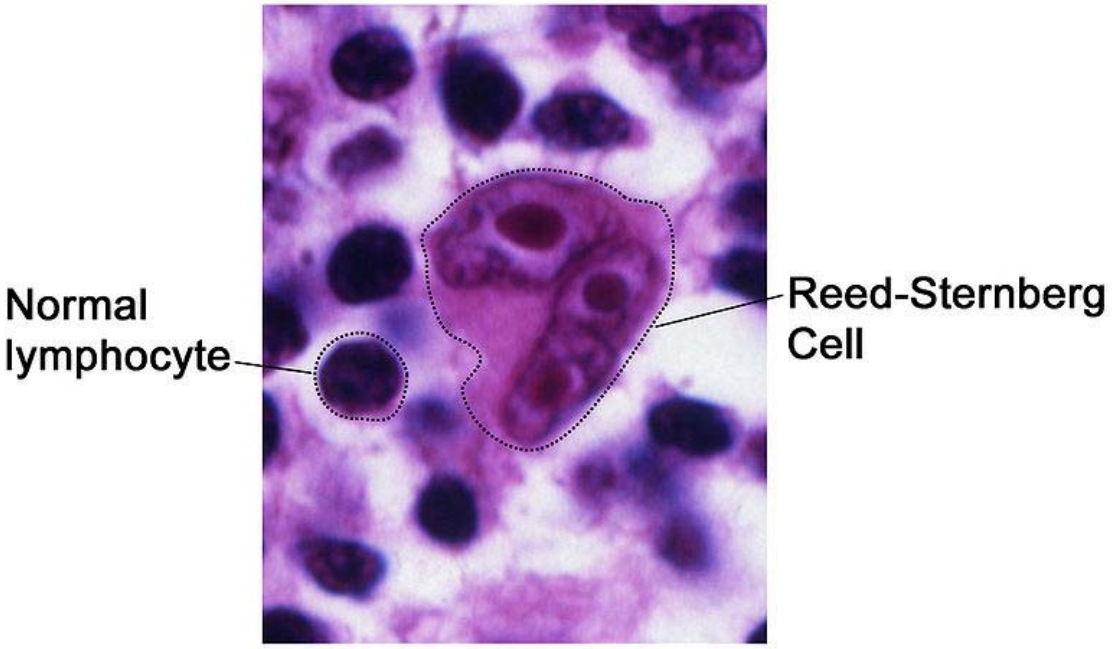
Gleason score

The tissue samples are then examined under a microscope to determine whether cancer cells are present, and to evaluate the microscopic features (or Gleason score) of any cancer found.

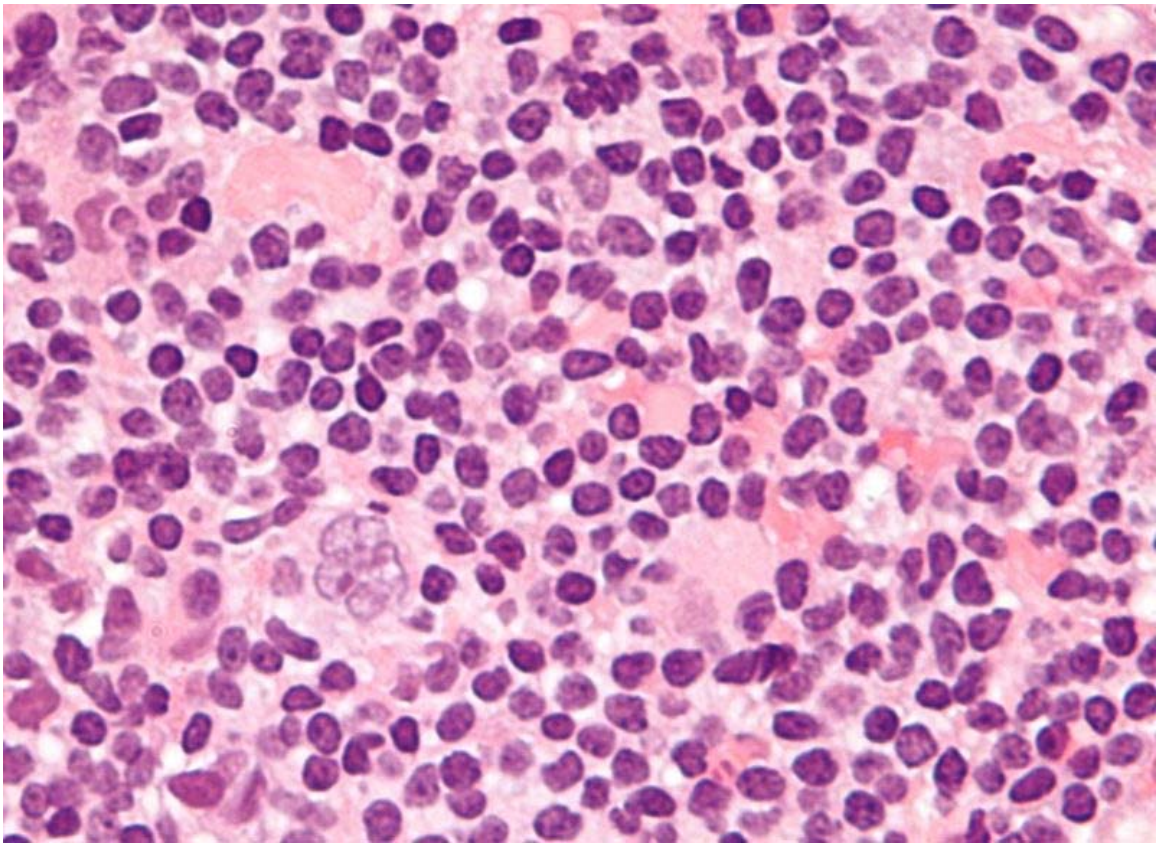
Tumor markers

Tissue samples can be stained for the presence of PSA and other tumor markers in order to determine the origin of maligant cells that have metastasized.

Reed-Sternberg cell



Micrograph showing a classic Reed-Sternberg cell



Micrograph showing a "popcorn cell", the Reed-Sternberg cell variant seen in nodular lymphocyte predominant Hodgkin lymphoma.H&E stain.

Reed-Sternberg cells (also known as **lacunar histiocytes** for certain types) are different giant cells found with light microscopy in biopsies from individuals with Hodgkin's lymphoma (aka Hodgkin's disease; a type of lymphoma) primarily due to EBV, and certain other disorders. They are usually derived from B lymphocytes.

They are named after Dorothy Reed Mendenhall (1874-1964) and Carl Sternberg (1872-1935), who provided the first definitive microscopic descriptions of Hodgkin's disease.

Reed-Sternberg cells are large and are either multinucleated or have a bilobed nucleus (thus resembling an "owl's eye" appearance) with prominent eosinophilic inclusion-like nucleoli. Reed-Sternberg cells are CD30 and CD15 positive, usually negative for CD20 and CD45. The presence of these cells is necessary in the diagnosis of Hodgkin's lymphoma - absence of Reed-Sternberg cells has very high negative predictive value. They can also be found in reactive lymphadenopathy (such as infectious mononucleosis, carbamazepine associated lymphadenopathy) and very rarely non-Hodgkin lymphomas.

A special type of Reed-Sternberg cells are *lacunar histiocytes*, whose cytoplasm retracts when fixed in formalin, so the nuclei give the appearance of cells that lie with empty spaces (called *lacunae*) between them. These are characteristic of the nodular sclerosis subtype of Hodgkin's lymphoma.

Chapter 11

Rheumatoid Nodule and Tauopathy

Rheumatoid nodule

Rheumatoid nodule

MeSH D012218

A **rheumatoid nodule** is a local swelling or tissue lump, usually rather firm to touch, like an unripe fruit, which occurs almost exclusively in association with rheumatoid arthritis. Very rarely rheumatoid nodules occur as 'rheumatoid nodulosis' in the absence of arthritis. They are usually subcutaneous especially over bony prominences such as the tip of the elbow or olecranon or over the finger knuckles. Less commonly they occur in the lining of the lung and other internal organs. The occurrence of nodules in the lung of miners exposed to silica dust was known as Caplan's syndrome. Nodules vary in size from that of a lentil or pea to that of a mandarin orange. Quite often they are associated with synovial pockets or bursae. About 5% of rheumatoid arthritis patients have such nodules within 2 years of disease onset, and the cumulative prevalence is about 25%. In the great majority of cases nodules are not painful or disabling in any way, being more of an unsightly nuisance, but in some cases they can be painful, especially if the overlying skin breaks down. Rarely, the nodules occur at diverse sites on body (e.g. upper eyelid, distal region of soles, vulva and internally in the gallbladder, lung, heart valves, larynx and spine).

Diagnosis

Histological examination of nodules shows that they consist of a shell of fibrous tissue surrounding a center of fibrinoid necrosis. Pea sized nodules have one centre. Larger nodules tend to be multilocular, with many separate shells or with connections between the necrotic centres. Individual necrotic centres may contain a cleft or several centres of necrosis may all open on to a large bursal pocket containing synovial fluid. The boundary between the necrotic centre and the outer fibrous shell is made up of the characteristic feature of the nodule, which is known as a cellular palisade. The palisade is a densely packed layer of macrophages and fibroblasts which tend to be arranged radially, like the seeds of a kiwi fruit or fig. Further out into the fibrous shell there is a zone that contains T lymphocytes and plasma cells in association with blood vessels. The overall

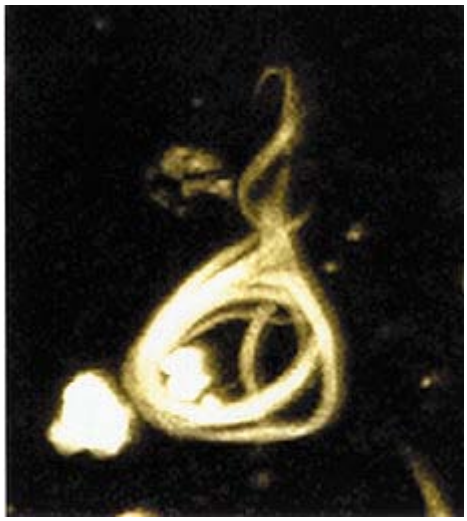
histological picture is essentially identical to that of rheumatoid synovitis with the main differences being that the palisade replaces the synovial intima (they may blend imperciptibly in bursae) and an almost total absence of B lymphocytes.

Treatment

Treatment of rheumatoid nodules is rarely a priority for people with rheumatoid arthritis. However, surgical removal is often successful, even if there is a tendency for nodules to regrow. Of the drug therapies commonly used in rheumatoid arthritis, methotrexate has the disadvantage of tending to make nodules worse. TNF inhibitors do not have a very reliable effect on nodules. B cell depletion with rituximab often leads to disappearance of nodules but this is not guaranteed.

Tauopathy

Tauopathy



Microscopy image of a neurofibrillary tangle, conformed by hyperphosphorylated tau protein

MeSH D024801

Tauopathies are a class of neurodegenerative diseases resulting from the pathological aggregation of tau protein in the human brain.

The best known of these illnesses is Alzheimer's disease (AD), where tau protein is deposited within neurons in the form of neurofibrillary tangles (NFTs). They were first described by the eponymous Alois Alzheimer in one of his patients suffering from the disorder. Tangles are formed by hyperphosphorylation of a microtubule-associated

protein known as tau, causing it to aggregate in an insoluble form. (These aggregations of hyperphosphorylated tau protein are also referred to as PHF, or "paired helical filaments"). The precise mechanism of tangle formation is not completely understood, and it is still controversial whether tangles are a primary causative factor in the disease or play a more peripheral role. AD is also classified as an amyloidosis because of the presence of senile plaques.

The degree of NFT involvement in AD is defined by **Braak stages.** Braak stages I and II are used when NFT involvent is confined mainly to the transentorhinal region of the brain, stages III and IV when there's also involvement of limbic regions such as the hippocampus, and V and VI when there's extensive neocortical involvement. This should not be confused with the degree of senile plaque involvement, which progresses differently.

Other conditions in which neurofibrillary tangles are commonly observed include:

- Progressive supranuclear palsy although with straight filament rather than PHF tau
- Dementia pugilistica (chronic traumatic encephalopathy)
- Frontotemporal dementia and parkinsonism linked to chromosome 17 however without detectable β-amyloid plaques.
- Lytico-Bodig disease (Parkinson-dementia complex of Guam)
- Tangle-predominant dementia, with NFTs similar to AD, but without plaques. Tends to appear in the very old.
- Ganglioglioma and gangliocytoma
- Meningioangiomatosis
- Subacute sclerosing panencephalitis
- As well as lead encephalopathy, tuberous sclerosis, Hallervorden-Spatz disease, and lipofuscinosis

In Pick's disease and corticobasal degeneration tau proteins are deposited in the form of inclusion bodies within swollen or "ballooned" neurons. Argyrophilic grain disease (AGD), another type of dementia, is marked by the presence of abundant argyrophilic grains and coiled bodies on microscopic examination of brain tissue. Some consider it to be a type of Alzheimer disease. It may co-exist with other tauopathies such as progressive supranuclear palsy and corticobasal degeneration.

Some other tauopathies include:

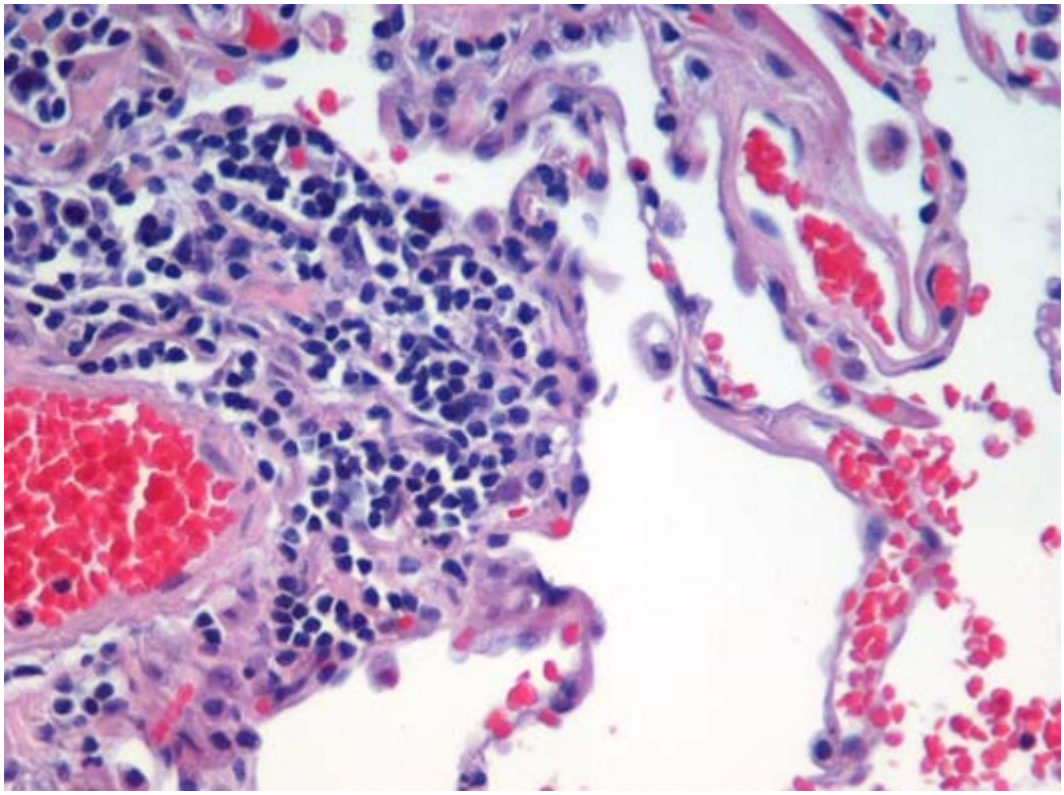
- Frontotemporal dementia
- Frontotemporal lobar degeneration

The non-Alzheimer's tauopathies are sometimes grouped together as "**Pick's complex**".

Chapter 12

Histology

A stained histologic specimen, sandwiched between a glass microscope slide and coverslip, mounted on the stage of a light microscope.



Microscopic view of a histologic specimen of human lung tissue stained with hematoxylin and eosin.

Histology (compound of the Greek words: *ἱστός* "tissue", and *-λογία* -logia) is the study of the microscopic anatomy of cells and tissues of plants and animals. It is performed by examining a thin slice (section) of tissue under a light microscope or electron microscope. The ability to visualize or differentially identify microscopic structures is frequently enhanced through the use of histological stains. Histology is an essential tool of biology and medicine.

Histopathology, the microscopic study of diseased tissue, is an important tool in anatomical pathology, since accurate diagnosis of cancer and other diseases usually requires histopathological examination of samples. Trained medical doctors, frequently board-certified as pathologists, are the personnel who perform histopathological examination and provide diagnostic information based on their observations.

The trained scientists who perform the preparation of histological sections are *histotechnicians,* histology technicians (HT), histology technologists (HTL), medical scientists, medical laboratory technicians, or biomedical scientists. Their field of study is called *histotechnology*.

Histology

Fixing

Chemical fixation with formaldehyde or other chemicals

Chemical fixatives are used to preserve tissue from degradation, and to maintain the structure of the cell and of sub-cellular components such as cell organelles (e.g., nucleus, endoplasmic reticulum, mitochondria). The most common fixative for light microscopy is 10% neutral buffered formalin (4% formaldehyde in phosphate buffered saline). For electron microscopy, the most commonly used fixative is glutaraldehyde, usually as a 2.5% solution in phosphate buffered saline. These fixatives preserve tissues or cells mainly by irreversibly cross-linking proteins. The main action of these aldehyde fixatives is to cross-link amino groups in proteins through the formation of CH_2 (methylene) linkage, in the case of formaldehyde, or by a C5H10 cross-links in the case of glutaraldehyde. This process, while preserving the structural integrity of the cells and tissue can damage the biological functionality of proteins, particularly enzymes, and can also denature them to a certain extent. This can be detrimental to certain histological techniques. Further fixatives are often used for electron microscopy such as osmium tetroxide or uranyl acetate

Formalin fixation leads to degradation of mRNA, miRNA and DNA in tissues. However, extraction, amplification and analysis of these nucleic acids from formalin-fixed, paraffin-embedded tissues is possible using appropriate protocols.

Frozen section fixation

Frozen section is a rapid way to fix and mount histology sections. It is used in surgical removal of tumors, and allow rapid determination of margin (that the tumor has been completely removed). It is done using a refrigeration device called a cryostat. The frozen tissue is sliced using a microtome, and the frozen slices are mounted on a glass slide and stained the same way as other methods. It is a necessary way to fix tissue for certain stain such as antibody linked immunofluorescence staining. It can also be used to determine if a tumour is malignant when it is found incidentally during surgery on a patient.

Processing - dehydration, clearing, and infiltration

The aim of Tissue Processing is to remove water from tissues and replace with a medium that solidifies to allow thin sections to be cut. Biological tissue must be supported in a hard matrix to allow sufficiently thin sections to be cut, typically 5 μm (micrometres; 1000 micrometres = 1 mm) thick for light microscopy and 80-100 nm (nanometre; 1,000,000 nanometres = 1 mm) thick for electron microscopy. For light microscopy, paraffin wax is most frequently used. Since it is immiscible with water, the main constituent of biological tissue, water must first be removed in the process of dehydration. Samples are transferred through baths of progressively more concentrated ethanol to remove the water. This is followed by a hydrophobic clearing agent (such as

xylene) to remove the alcohol, and finally molten paraffin wax, the infiltration agent, which replaces the xylene. Paraffin wax does not provide a sufficiently hard matrix for cutting very thin sections for electron microscopy. Instead, resins are used. Epoxy resins are the most commonly employed embedding media, but acrylic resins are also used, particularly where immunohistochemistry is required. Thicker sections (0.35µm to 5µm) of resin-embedded tissue can also be cut for light microscopy. Again, the immiscibility of most epoxy and acrylic resins with water necessitates the use of dehydration, usually with ethanol.

Embedding

After the tissues have been dehydrated, cleared, and infiltrated with the embedding material, they are ready for external embedding. During this process the tissue samples are placed into molds along with liquid embedding material (such as agar, gelatine, or wax) which is then hardened. This is achieved by cooling in the case of paraffin wax and heating (curing) in the case of the epoxy resins. The acrylic resins are polymerised by heat, ultraviolet light, or chemical catalysts. The hardened blocks containing the tissue samples are then ready to be sectioned.

Because Formalin-fixed, paraffin-embedded (FFPE) tissues may be stored indefinitely at room temperature, and nucleic acids (both DNA and RNA) may be recovered from them decades after fixation, FFPE tissues are an important resource for historical studies in medicine.

Embedding can also be accomplished using frozen, non-fixed tissue in a water-based medium. Pre-frozen tissues are placed into molds with the liquid embedding material, usually a water-based glycol, OCT, TBS, Cryogel, or resin, which is then frozen to form hardened blocks.

Sectioning

Sectioning can be done in limited ways. Vertical sectioning perpendicular to the surface of the tissue is the usual method. Horizontal sectioning is often done in the evaluation of the hair follicles and pilosebaceous units. Tangential to horizontal sectioning is done in Mohs surgery and in methods of CCPDMA.

For light microscopy, a steel knife mounted in a microtome is used to cut 10-micrometer-thick tissue sections which are mounted on a glass microscope slide. For transmission electron microscopy, a diamond knife mounted in an ultramicrotome is used to cut 50-nanometer-thick tissue sections which are mounted on a 3-millimeter-diameter copper grid. Then the mounted sections are treated with the appropriate stain.

Frozen tissue embedded in a freezing medium is cut on a microtome in a cooled machine called a cryostat.

Staining

Biological tissue has little inherent contrast in either the light or electron microscope. Staining is employed to give both contrast to the tissue as well as highlighting particular features of interest. Where the underlying mechanistic chemistry of staining is understood, the term histochemistry is used. Hematoxylin and eosin (H&E stain) is the most commonly used light microscopical stain in histology and histopathology. Hematoxylin, a basic dye, stains nuclei blue due to an affinity to nucleic acids in the cell nucleus; eosin, an acidic dye, stains the cytoplasm pink. Uranyl acetate and lead citrate are commonly used to impart contrast to tissue in the electron microscope.

Special staining: There are hundreds of various other techniques that have been used to selectively stain cells and cellular components. Other compounds used to color tissue sections include safranin, oil red o, Congo red, fast green FCF, silver salts, and numerous natural and artificial dyes that were usually originated from the development dyes for the textile industry.

Histochemistry refers to the science of using chemical reactions between laboratory chemicals and components within tissue. A commonly performed histochemical technique is the Perls Prussian blue reaction, used to demonstrate iron deposits in diseases like hemochromatosis.

Histology samples have often been examined by radioactive techniques. In historadiography, a slide (sometimes stained histochemically) is X-rayed. More commonly, autoradiography is used to visualize the locations to which a radioactive substance has been transported within the body, such as cells in S phase (undergoing DNA replication) which incorporate tritiated thymidine, or sites to which radiolabeled nucleic acid probes bind in in situ hybridization. For autoradiography on a microscopic level, the slide is typically dipped into liquid nuclear tract emulsion, which dries to form the exposure film. Individual silver grains in the film are visualized with dark field microscopy.

Recently, antibodies have been used to specifically visualize proteins, carbohydrates, and lipids. This process is called immunohistochemistry, or when the stain is a fluorescent molecule, immunofluorescence. This technique has greatly increased the ability to identify categories of cells under a microscope. Other advanced techniques, such as nonradioactive *in situ* hybridization, can be combined with immunochemistry to identify specific DNA or RNA molecules with fluorescent probes or tags that can be used for immunofluorescence and enzyme-linked fluorescence amplification (especially alkaline phosphatase and tyramide signal amplification). Fluorescence microscopy and confocal microscopy are used to detect fluorescent signals with good intracellular detail. Digital cameras are increasingly used to capture histological and histopathological image

Common laboratory stains

Stain	Common use	Nucleus	Cytoplasm	Red blood cell (RBC)	Collagen fibers	Specifically stains
Haematoxylin	General staining when paired with eosin (i.e. H&E)	Blue	N/A	N/A	N/A	Nucleic acids—blue ER (endoplasmic reticulum)—blue
Eosin	General staining when paired with haematoxylin (i.e. H&E)	N/A	Pink	Orange/red	Pink	Elastic fibers—pink Collagen fibers—pink Reticular fibers—pink
Toluidine blue	General staining	Blue	Blue	Blue	Blue	Mast cells granules—purple
Masson's trichrome stain	Connective tissue	Black	Red/pink	Red	Blue/green	Cartilage—blue/green Muscle fibers—red Keratin—orange
Mallory's trichrome stain	Connective tissue	Red	Pale red	Orange	Deep blue	Cartilage—blue Bone matrix—deep blue Muscle fibers—red
Weigert's elastic stain	Elastic fibers	Blue/black	N/A	N/A	N/A	Elastic fibers—blue/black
Heidenhain's AZAN trichrome stain	Distinguishing cells from extracellular components	Red/purple	Pink	Red	Blue	Muscle fibers—red Cartilage—blue Bone matrix—blue
Silver stain	Reticular fibers, nerve	N/A	N/A	N/A	N/A	Reticular fibers—

	fibers, fungi					brown/black
Wright's stain	Blood cells	Bluish/purple	Bluish/gray	Red/pink	N/A	Nerve fibers—brown/black Neutrophil granules—purple/pink Eosinophil granules—bright red/orange Basophil granules—deep purple/violet Platelet granules—red/purple Elastic fibres—dark brown
Orcein stain	Elastic fibres	Deep blue [or crazy red]	N/A	Bright red	Pink	Mast cells granules—purple Smooth muscle—light blue
Periodic acid-Schiff stain (PAS)	Basement membrane, localizing carbohydrates	Blue	N/A	N/A	Pink	Glycogen and other carbohydrates—magenta

Table sourced from Michael H. Ross, Wojciech Pawlina, (2006). *Histology: A Text and Atlas*. Hagerstown, MD: Lippincott Williams & Wilkins. ISBN 0-7817-5056-3.

The Nissl method and Golgi's method are useful in identifying neurons.

Alternative techniques

Alternative techniques include cryosection. The tissue is frozen using a cryostat, and cut. Tissue staining methods are similar to those of wax sections. Plastic embedding is commonly used in the preparation of material for electron microscopy. Tissues are

embedded in epoxy resin. Very thin sections (less than 0.1 micrometer) are cut using diamond or glass knives. The sections are stained with electron dense stains (uranium and lead) so that they can possibly be seen with the electron microscope.

History

In the 19th century, histology was an academic discipline in its own right. The 1906 Nobel Prize in Physiology or Medicine was awarded to histologists Camillo Golgi and Santiago Ramon y Cajal. They had dueling interpretations of the neural structure of the brain based in differing interpretations of the same images. Cajal won the prize for his correct theory and Golgi for the staining technique he invented to make it possible.

Histological classification of animal tissues

There are four basic types of tissues: muscle tissue, nervous tissue, connective tissue, and epithelial tissue. All tissue types are subtypes of these four basic tissue types (for example, blood cells are classified as connective tissue, since they generally originate inside bone marrow).

- Epithelium: the lining of glands, bowel, skin, and some organs like the liver, lung, and kidney
- Endothelium: the lining of blood and lymphatic vessels
- Mesothelium: the lining of pleural and pericardial spaces
- Mesenchyme: the cells filling the spaces between the organs, including fat, muscle, bone, cartilage, and tendon cells
- Blood cells: the red and white blood cells, including those found in lymph nodes and spleen
- Neurons: any of the conducting cells of the nervous system
- Germ cells: reproductive cells (spermatozoa in men, oocytes in women)
- Placenta: an organ characteristic of true mammals during pregnancy, joining mother and offspring, providing endocrine secretion and selective exchange of soluble, but not particulate, blood-borne substances through an apposition of uterine and trophoblastic vascularised parts
- Stem cells: cells able to turn into one or several of the above types

Note that tissues from plants, fungi, and microorganisms can also be examined histologically. Their structure is very different from animal tissues.

Related sciences

- Cell biology is the study of living cells, their DNA and RNA and the proteins they express.
- Anatomy is the study of organs visible by the naked eye.
- Morphology studies entire organisms.

Artifacts

Artifacts are structures or features in tissue that interfere with normal histological examination. These are not always present in normal tissue and can come from outside sources. Artifacts interfere with histology by changing the tissues appearance and hiding structures. These can be divided into two categories:

Pre-histology

These are features and structures that have being introduced prior to the collection of the tissues. A common example of these include: ink from tattoos and freckles (melanin) in skin samples.

Post-histology

Artifacts can result from tissue processing. Processing commonly leads to changes like shrinkage, washing out of particular cellular components, color changes in different tissues types and alterations of the structures in the tissue. Because these are caused in a laboratory the majority of post histology artifacts can be avoided or removed after being discovered. A common example is mercury pigment left behind after using Zenker's fixative to fix a section.

Chapter 13

Staining

A stained histologic specimen, sandwiched between a glass microscope slide and coverslip, mounted on the stage of a light microscope.

Staining is an auxiliary technique used in microscopy to enhance contrast in the microscopic image. Stains and dyes are frequently used in biology and medicine to highlight structures in biological tissues for viewing, often with the aid of different microscopes. Stains may be used to define and examine bulk tissues (highlighting, for example, muscle fibers or connective tissue), cell populations (classifying different blood cells, for instance), or organelles within individual cells.

In biochemistry it involves adding a class-specific (DNA, proteins, lipids, carbohydrates) dye to a substrate to qualify or quantify the presence of a specific compound. Staining and fluorescent tagging can serve similar purposes. Biological staining is also used to mark cells in flow cytometry, and to flag proteins or nucleic acids in gel electrophoresis.

Staining is not limited to biological materials, it can also be used to study the morphology of other materials for example the lamellar structures of semi-crystalline polymers or the domain structures of block copolymers.

In vivo *vs* In vitro

In vivo staining is the process of dyeing living tissues—*in vivo* means "in life" (compare with *in vitro* staining). By causing certain cells or structures to take on contrasting colour(s), their form (morphology) or position within a cell or tissue can be readily seen and studied. The usual purpose is to reveal cytological details that might otherwise not be apparent; however, staining can also reveal where certain chemicals or specific chemical reactions are taking place within cells or tissues.

In vitro staining involves colouring cells or structures that are no longer living. Certain stains are often combined to reveal more details and features than a single stain alone. Combined with specific protocols for fixation and sample preparation, scientists and physicians can use these standard techniques as consistent, repeatable diagnostic tools. A counterstain is stain that makes cells or structures more visible, when not completely visible with the principal stain.

- For example, crystal violet stains only Gram-positive bacteria in Gram staining. A safranin counterstain is applied which stains all cells, allowing the identification of Gram-negative bacteria as well.

Often these stains are called vital stains. They are introduced to the organism while the cells are still living. However, these stains are eventually toxic to the organism, some more so than others. To achieve desired effects, the stains are used in very dilute solutions ranging from 1:5000 to 1:500000 (Howey, 2000). Note that many stains may be used in both living and fixed cells.

In vitro methods

Preparation

The preparatory steps involved depend on the type of analysis planned; some or all of the following procedures may be required.

Fixation–which may itself consist of several steps–aims to preserve the shape of the cells or tissue involved as much as possible. Sometimes heat fixation is used to kill, adhere, and alter the specimen so it will accept stains. Most chemical fixatives (chemicals causing fixation) generate chemical bonds between proteins and other substances within

the sample, increasing their rigidity. Common fixatives include formaldehyde, ethanol, methanol, and/or picric acid. Pieces of tissue may be embedded in paraffin wax to increase their mechanical strength and stability and to make them easier to cut into thin slices.

Permeabilization involves treatment of cells with (usually) a mild surfactant. This treatment will dissolve the cell membranes, and allow larger dye molecules access to the cell's interior.

Mounting usually involves attaching the samples to a glass microscope slide for observation and analysis. In some cases, cells may be grown directly on a slide. For samples of loose cells (as with a blood smear or a pap smear) the sample can be directly applied to a slide. For larger pieces of tissue, thin sections (slices) are made using a microtome; these slices can then be mounted and inspected.

Staining proper

At its simplest, the actual staining process may involve immersing the sample (before or after fixation and mounting) in dye solution, followed by rinsing and observation. Many dyes, however, require the use of a mordant: a chemical compound which reacts with the stain to form an insoluble, coloured precipitate. When excess dye solution is washed away, the mordanted stain remains.

Most of the dyes commonly used in microscopy are available as **certified stains**. This means that samples of the manufacturer's batch have been tested by an independent body, the Biological Stain Commission and found to be meet or exceed certain standards of purity, dye content and performance in staining techniques. These standards are published in detail in the journal Biotechnic & Histochemistry Many dyes are inconsistent in composition from one supplier to another. The use of certified stains eliminates a source of unexpected results.

Negative staining

A simple staining method for bacteria which is usually successful even when the "positive staining" methods detailed below fail, is to employ a negative stain. This can be achieved simply by smearing the sample on to the slide, followed by an application of nigrosin (a black synthetic dye) or Indian ink (an aqueous suspension of carbon particles). After drying, the microorganisms may be viewed in bright field microscopy as lighter inclusions well-contrasted against the dark environment surrounding them. Note: negative staining is a mild technique which may not destroy the microorganisms therefore it is unsuitable for studying pathogens.

Specific techniques

Gram staining

Gram staining is used to determine gram status to classify bacteria broadly. It is based on the composition of their cell wall. Gram staining uses crystal violet to stain cell walls, iodine as a mordant, and a fuchsin or safranin counterstain to mark all bacteria. Gram status is important in medicine; the presence or absence of a cell wall will change the bacterium's susceptibility to some antibiotics.

Gram-positive bacteria stain dark blue or violet. Their cell wall is typically rich with peptidoglycan and lacks the secondary membrane and lipopolysaccharide layer found in Gram-negative bacteria.

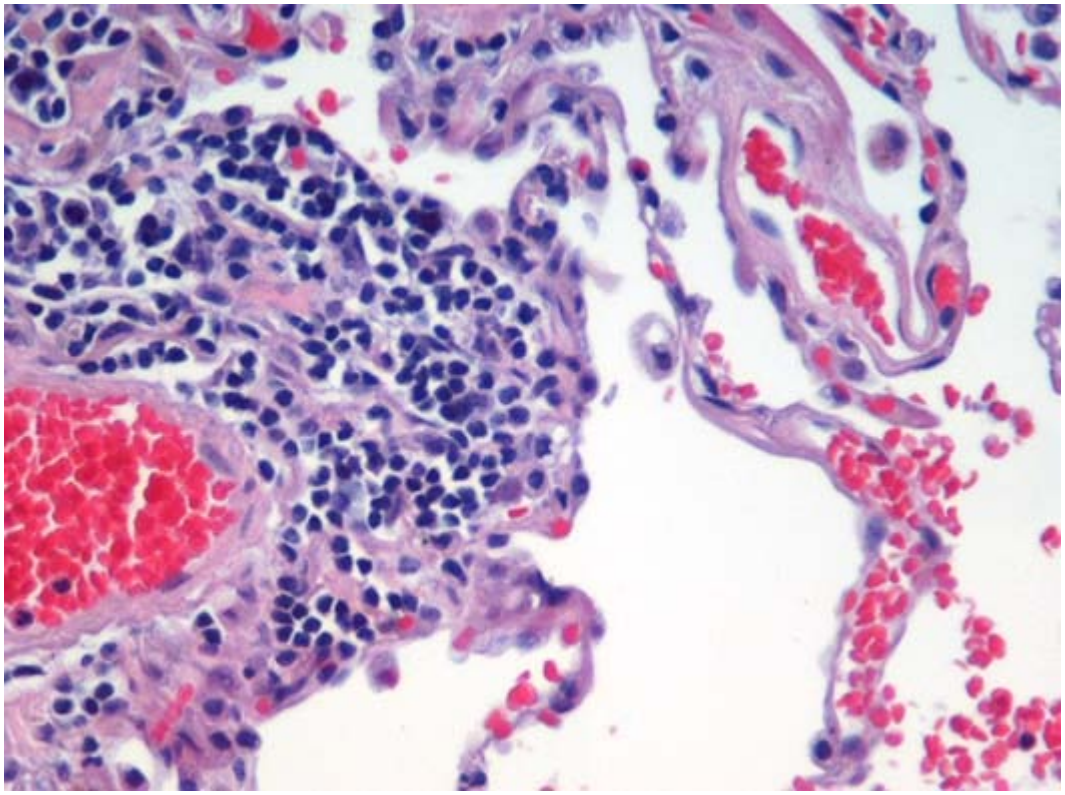
On most Gram-stained preparations, Gram-negative organisms will appear red or pink because they are counterstained. Due to presence of higher lipid content, after alcohol-treatment, the porosity of the cell wall increases, hence the CVI complex (Crystal violet - Iodine) can pass through. Thus, the primary stain is not retained. Also, in contrast to most Gram-positive bacteria, Gram-negative bacteria have only a few layers of peptidoglycan and a secondary cell membrane made primarily of lipopolysaccharide.

Ziehl-Neelsen stain

Ziehl-Neelsen staining is used to stain species of *Mycobacterium tuberculosis* that do not stain with the standard laboratory staining procedures like Gram staining.

The stains used are the red coloured Carbol fuchsin that stains the bacteria and a counter stain like Methylene blue or Malachite green.

Haematoxylin and eosin (H&E) staining



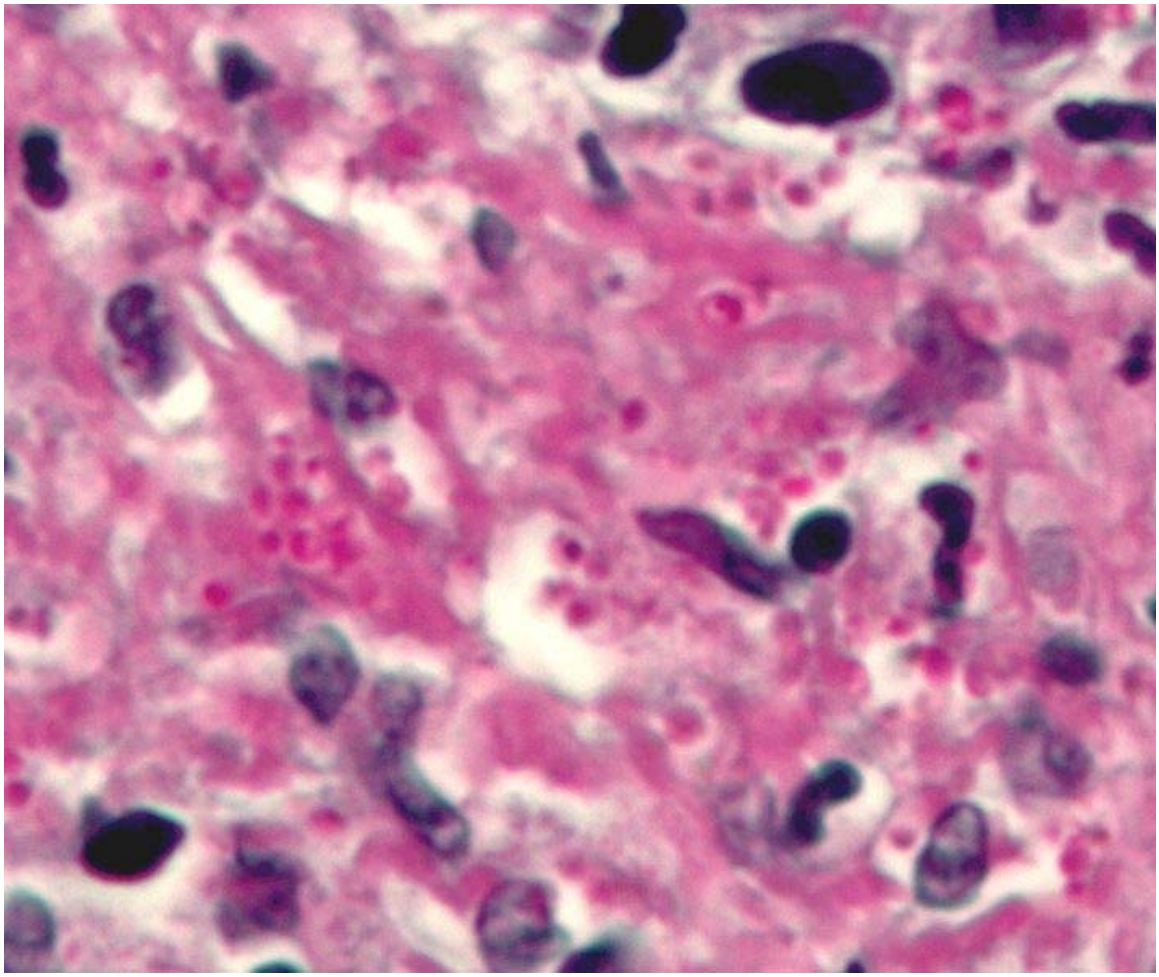
Microscopic view of a histologic specimen of human lung tissue stained with hematoxylin and eosin.

Haematoxylin and eosin staining protocol is used frequently in histology to examine thin sections of tissue. Haematoxylin stains cell nuclei blue, while eosin stains cytoplasm, connective tissue and other extracellular substances pink or red. Eosin is strongly absorbed by red blood cells, colouring them bright red. In a skilfully made H & E preparation the red blood cells are almost orange, and collagen and cytoplasm (especially muscle) acquire different shades of pink. When the staining is done by a machine, the subtle differences in eosinophilia are often lost.

Papanicolaou staining

Papanicolaou staining, or Pap staining, is a frequently used method for examining cell samples from various bodily secretions. It is frequently used to stain Pap smear specimens. It uses a combination of haematoxylin, Orange G, eosin Y, Light Green SF yellowish, and sometimes Bismarck Brown Y.

PAS staining



PAS diastase showing the fungus Histoplasma

Periodic acid-Schiff staining is used to mark carbohydrates (glycogen, glycoprotein, proteoglycans). It is used to distinguish different types of glycogen storage diseases.

Masson's trichrome

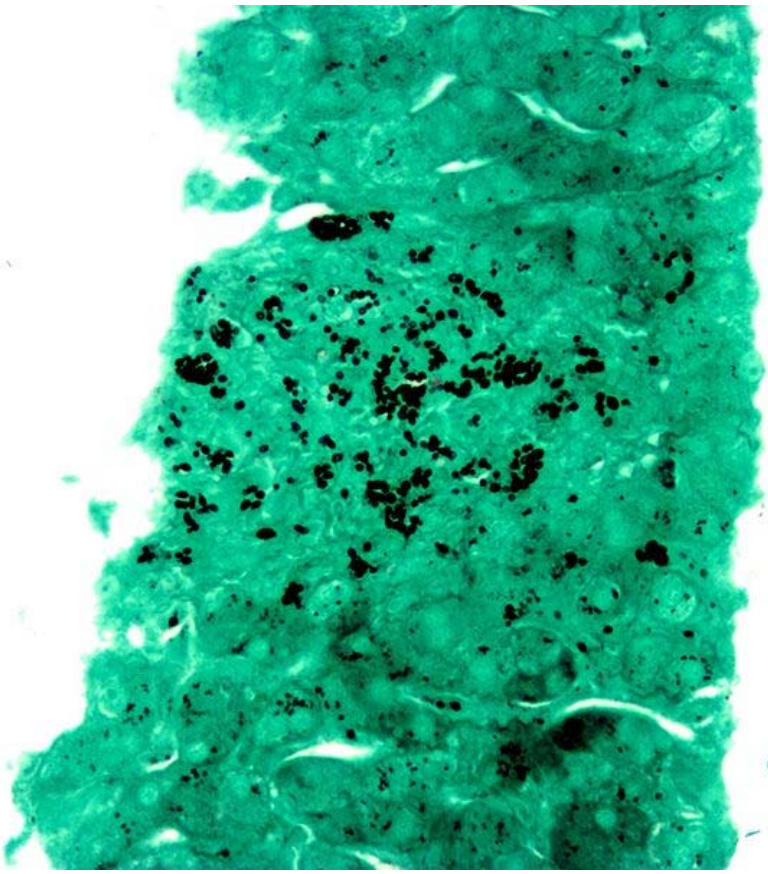
Masson's trichrome is (as the name implies) a three-colour staining protocol. The recipe has evolved from Masson's original technique for different specific applications, but all are well-suited to distinguish cells from surrounding connective tissue. Most recipes will produce red keratin and muscle fibers, blue or green staining of collagen and bone, light red or pink staining of cytoplasm, and black cell nuclei.

Romanowsky stains

The Romanowsky stains are all based on a combination of eosinate (chemically reduced eosin) and methylene blue (sometimes with its oxidation products azure A and azure B). Common variants include Wright's stain, Jenner's stain, Leishman stain and Giemsa stain.

All are used to examine blood or bone marrow samples. They are preferred over H&E for inspection of blood cells because different types of leukocytes (white blood cells) can be readily distinguished. All are also suited to examination of blood to detect blood-borne parasites like malaria.

Silver staining



Gömöri methenamine silver stain demonstrating histoplasma (black round balls).

Silver staining is the use of silver to stain histologic sections. This kind of staining is important especially to show proteins (for example type III collagen) and DNA. It is used to show both substances inside and outside cells. Silver staining is also used in temperature gradient gel electrophoresis.

Some cells are *argentaffin*. These reduce silver solution to metallic silver after formalin fixation. This method was discovered by Italian Camillo Golgi, by using a reaction

between silver nitrate and potassium dichromate, thus precipitating silver chromate in some cells. Other cells are *argyrophilic*. These reduce silver solution to metallic silver after being exposed to the stain that contains a reductant, for example hydroquinone or formalin.

Sudan staining

Sudan staining is the use of Sudan dyes to stain sudanophilic substances, usually lipids. Sudan III, Sudan IV, Oil Red O, and Sudan Black B are often used. Sudan staining is often used to determine the level of fecal fat to diagnose steatorrhea.

Conklin's staining

Special technique designed for staining true endospores with the use of malachite green dye, once stained, they do not decolourize.

Common biological stains

Different stains react or concentrate in different parts of a cell or tissue, and these properties are used to advantage to reveal specific parts or areas. Some of the most common biological stains are listed below. Unless otherwise marked, all of these dyes may be used with fixed cells and tissues; vital dyes (suitable for use with living organisms) are noted.

Acridine orange

Acridine orange (AO) is a nucleic acid selective fluorescent cationic dye useful for cell cycle determination. It is cell-permeable, and interacts with DNA and RNA by intercalation or electrostatic attractions. When bound to DNA, it is very similar spectrally to fluorescein.

Bismarck brown

Bismarck brown (also Bismarck brown Y or Manchester brown) imparts a yellow colour to acid mucins. Bismarck brown may be used with live cells.

Carmine

Carmine is an intensely red dye which may be used to stain glycogen, while Carmine alum is a nuclear stain. Carmine stains require the use of a mordant, usually aluminum.

Coomassie blue

Coomassie blue (also brilliant blue) nonspecifically stains proteins a strong blue colour. It is often used in gel electrophoresis.

Crystal violet

Crystal violet, when combined with a suitable mordant, stains cell walls purple. Crystal violet is an important component in Gram staining.

DAPI

DAPI is a fluorescent nuclear stain, excited by ultraviolet light and showing strong blue fluorescence when bound to DNA. DAPI binds with A=T rich repeats of chromosomes.DAPI also not visible with regular transmission microscopy. It may be used in living or fixed cells.

Eosin

Eosin is most often used as a counterstain to haematoxylin, imparting a pink or red colour to cytoplasmic material, cell membranes, and some extracellular structures. It also imparts a strong red colour to red blood cells. Eosin may also be used as a counterstain in some variants of Gram staining, and in many other protocols. There are actually two very closely related compounds commonly referred to as eosin. Most often used is eosin Y (also known as eosin Y ws or eosin yellowish); it has a very slightly yellowish cast. The other eosin compound is eosin B (eosin bluish or imperial red); it has a very faint bluish cast. The two dyes are interchangeable, and the use of one or the other is more a matter of preference and tradition.

Ethidium bromide

Ethidium bromide intercalates and stains DNA, providing a fluorescent red-orange stain. Although it will not stain healthy cells, it can be used to identify cells that are in the final stages of apoptosis - such cells have much more permeable membranes. Consequently, ethidium bromide is often used as a marker for apoptosis in cells populations and to locate bands of DNA in gel electrophoresis. The stain may also be used in conjunction with acridine orange (AO) in viable cell counting. This EB/AO combined stain causes live cells to fluoresce green whilst apoptotic cells retain the distinctive red-orange fluorescence.

Acid fuchsine

Acid fuchsine may be used to stain collagen, smooth muscle, or mitochondria. Acid fuchsine is used as the nuclear and cytoplasmic stain in Mallory's trichrome method. Acid fuchsine stains cytoplasm in some variants of Masson's trichrome. In Van Gieson's picro-fuchsine, acid fuchsine imparts its red colour to collagen fibres. Acid fuchsine is also a traditional stain for mitochondria (Altmann's method).

Haematoxylin

Haematoxylin (hematoxylin in North America) is a nuclear stain. Used with a mordant, haematoxylin stains nuclei blue-violet or brown. It is most often used with eosin in H&E (haematoxylin and eosin) staining—one of the most common procedures in histology.

Hoechst stains

Hoechst is a *bis*-benzimidazole derivative compound which binds to the *minor groove* of DNA. Often used in fluorescence microscopy for DNA staining, Hoechst stains appear yellow when dissolved in aqueous solutions and emit blue light under UV excitation. There are two major types of Hoechst: *Hoechst 33258* and *Hoechst 33342*. The two compounds are functionally similar, but with a little difference in structure. Hoechst 33258 contains a terminal hydroxyl group and is thus more soluble in aqueous solution, however this characteristics reduces its ability to penetrate the plasma membrane. Hoechst 33342 contains a ethyl substitution on the terminal hydroxyl group (i.e. an ethylether group) making it more hydrophobic for easier plasma membrane passage

Iodine

Iodine is used in chemistry as an indicator for starch. When starch is mixed with iodine in solution, an intensely dark blue colour develops, representing a starch/iodine complex. Starch is a substance common to most plant cells and so a weak iodine solution will stain starch present in the cells. Iodine is one component in the staining technique known as Gram staining, used in microbiology. Lugol's solution or Lugol's iodine (IKI) is a brown solution that turns black in the presence of starches and can be used as a cell stain, making the cell nuclei more visible. Iodine is also used as a mordant in Gram's staining, it enhances dye to enter through the pore present in the cell wall/membrane.

Malachite green

Malachite green (also known as diamond green B or victoria green B) can be used as a blue-green counterstain to safranin in the Gimenez staining technique for bacteria. It also can be used to directly stain spores.

Methyl green

Methyl green is used commonly with bright-field microscopes to dye the chromatin of cells so that they are more easily viewed.

Methylene blue

Methylene blue is used to stain animal cells, such as human cheek cells, to make their nuclei more observable. Also used to staining the blood film and used in cytology.

Neutral red

Neutral red (or toluylene red) stains Nissl substance red. It is usually used as a counterstain in combination with other dyes.

Nile blue

Nile blue (or Nile blue A) stains nuclei blue. It may be used with living cells.

Nile red

Nile red (also known as Nile blue oxazone) is formed by boiling Nile blue with sulfuric acid. This produces a mix of Nile red and Nile blue. Nile red is a lipophilic stain; it will accumulate in lipid globules inside cells, staining them red. Nile red can be used with living cells. It fluoresces strongly when partitioned into lipids, but practically not at all in aqueous solution.

Osmium tetroxide (formal name: osmium tetraoxide)

Osmium tetraoxide is used in optical microscopy to stain lipids. It dissolves in fats, and is reduced by organic materials to elemental osmium, an easily visible black substance.

Rhodamine

Rhodamine is a protein specific fluorescent stain commonly used in fluorescence microscopy.

Safranin

Safranin (or Safranin O) is a nuclear stain. It produces red nuclei, and is used primarily as a counterstain. Safranin may also be used to give a yellow colour to collagen.

Stainability of tissues

Positive affinity for a specific stain may be designated by the suffix *-philic*. For example, tissues that stain with an azure dye may be referred to as azurophilic. This may also be used for more generalized staining properties, such as acidophilic for tissues that stain by acidic stains (most notably eosin), basophilic when staining in basic dyes and *amphophilic* when staining with either acid or basic dyes. In contrast, Chromophobic tissues do not take up coloured dye readily.

Electron microscopy

As in light microscopy, stains can be used to enhance contrast in transmission electron microscopy. Electron-dense compounds of heavy metals are typically used.

Phosphotungstic acid

Phosphotungstic acid is a common negative stain for viruses, nerves, polysaccharides, and other biological tissue materials.

Osmium tetroxide

Osmium tetroxide is used in optical microscopy to stain lipids. It dissolves in fats, and is reduced by organic materials to elemental osmium, an easily visible black substance. Because it is a heavy metal that absorbs electrons, it is perhaps the most common stain used for morphology in biological electron microscopy. It is also used for the staining of various polymers for the study of their morphology by TEM. OsO_4 is very volatile and extremely toxic. It is a strong oxidizing agent as the osmium has an oxidation number of +8. It aggressively oxidizes many materials, leaving behind a deposit of non-volatile osmium in a lower oxidation state.

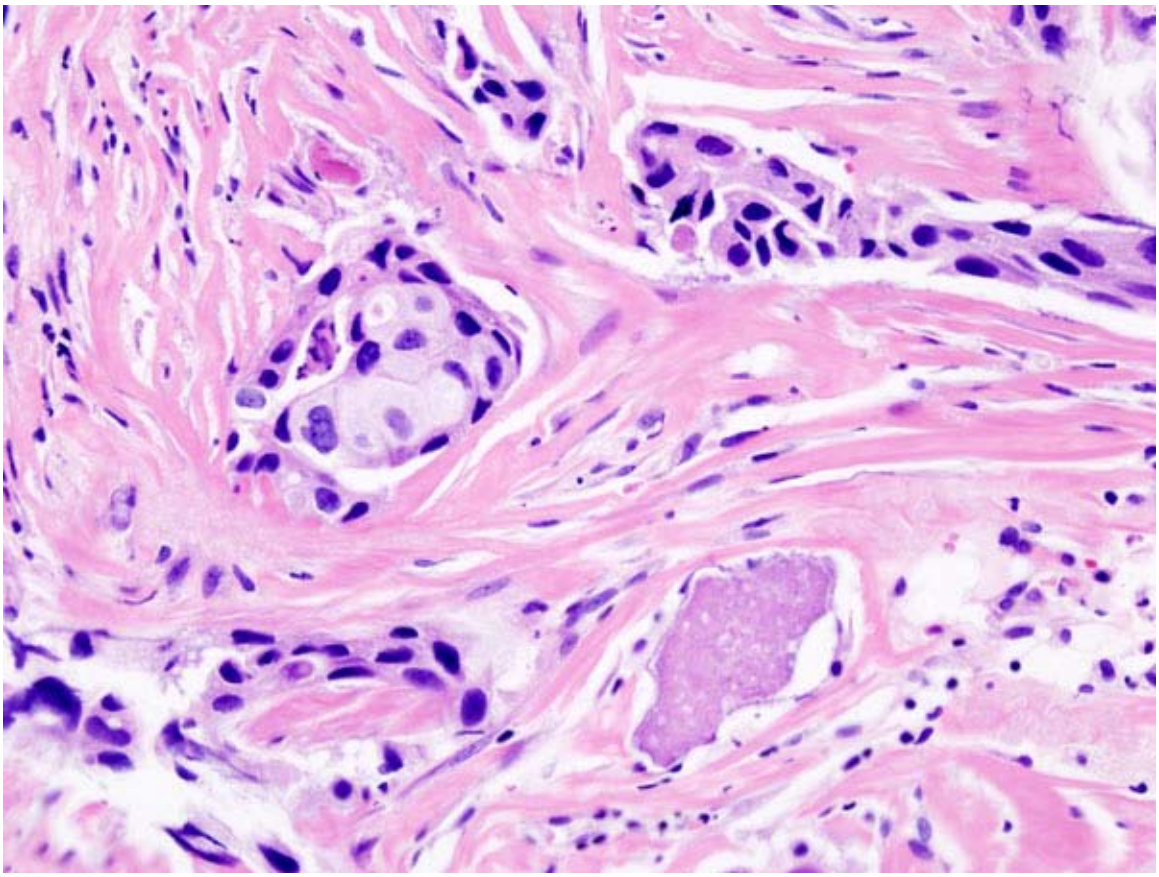
Ruthenium tetroxide

Ruthenium tetroxide is equally volatile and even more aggressive than osmium tetraoxide and able to stain even materials that resist the osmium stain, e.g. polyethylene.

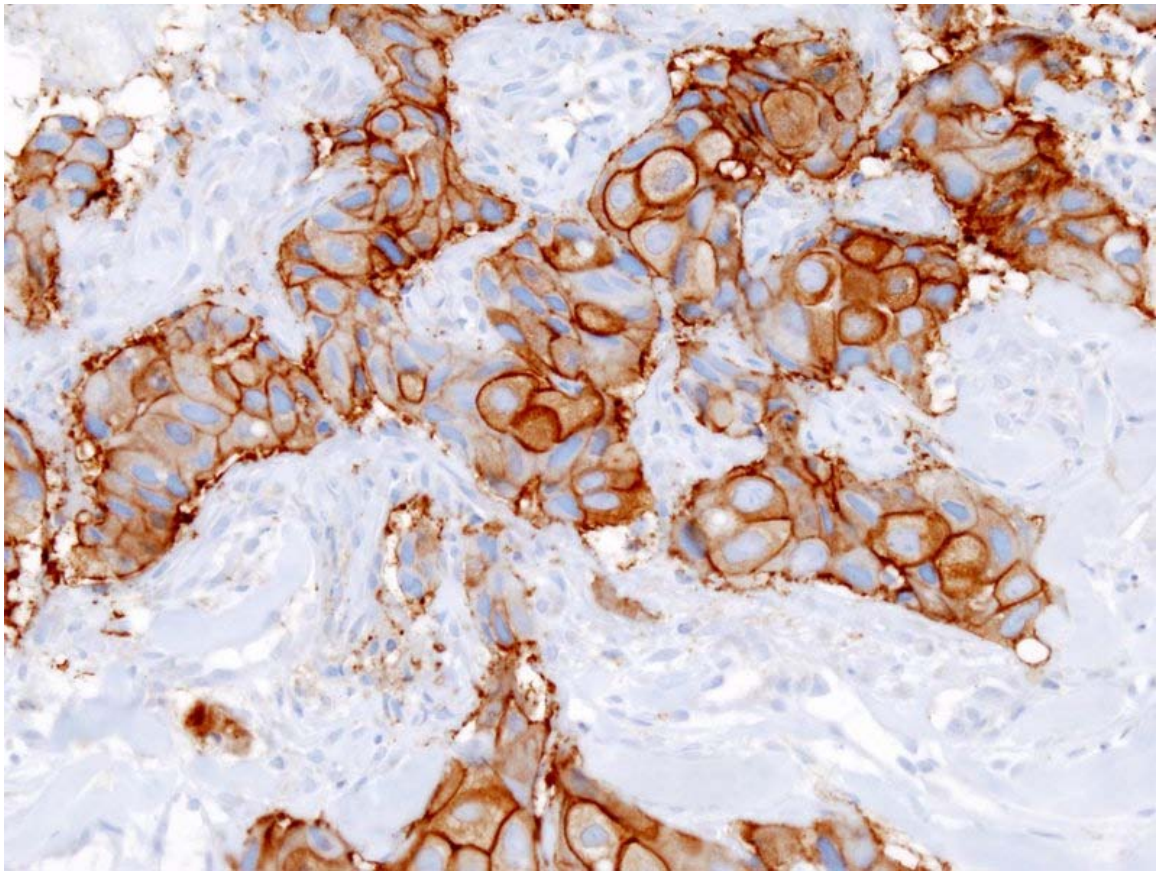
Other chemicals used in electron microscopy staining include: ammonium molybdate, cadmium iodide, carbohydrazide, ferric chloride, hexamine, indium trichloride, lanthanum nitrate, lead acetate, lead citrate, lead(II) nitrate, periodic acid, phosphomolybdic acid, potassium ferricyanide, potassium ferrocyanide, ruthenium red, silver nitrate, silver proteinate, sodium chloroaurate, thallium nitrate, thiosemicarbazide, uranyl acetate, uranyl nitrate, and vanadyl sulfate.

Chapter 14

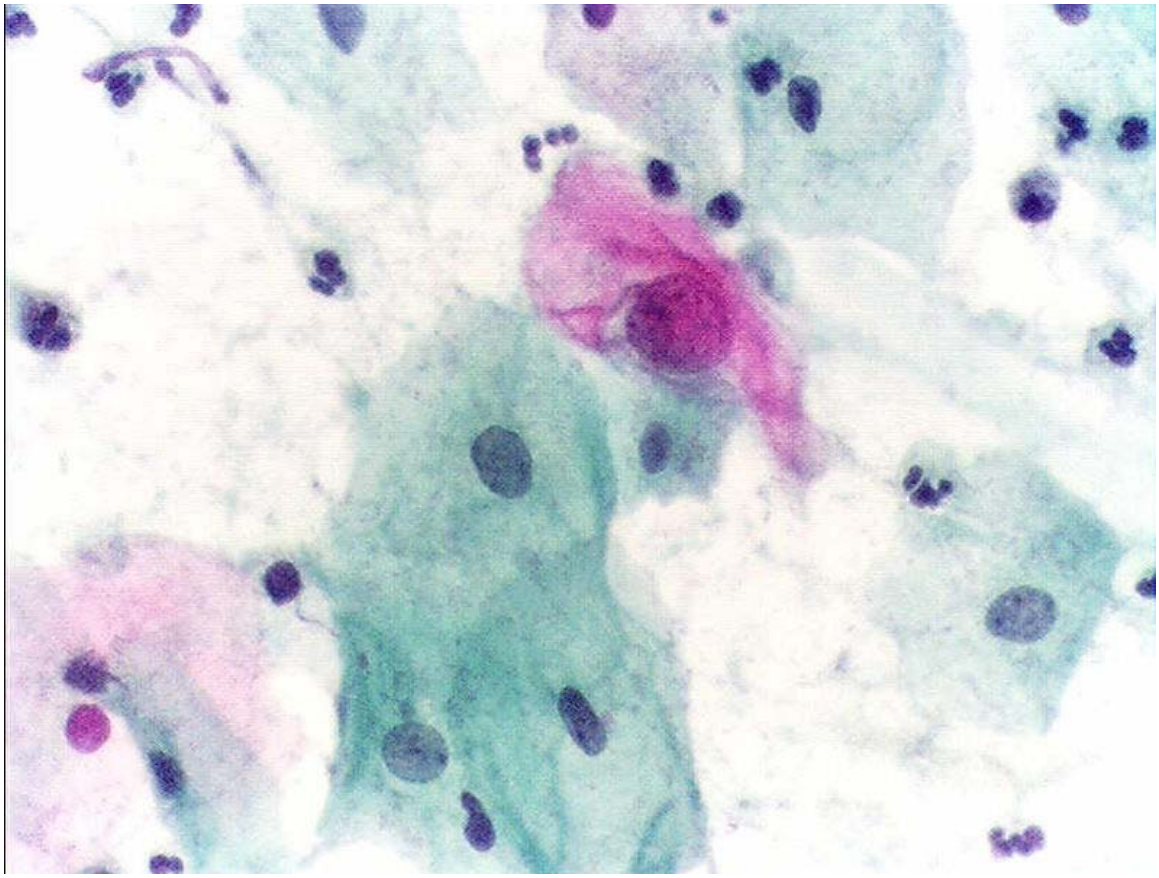
Anatomical Pathology



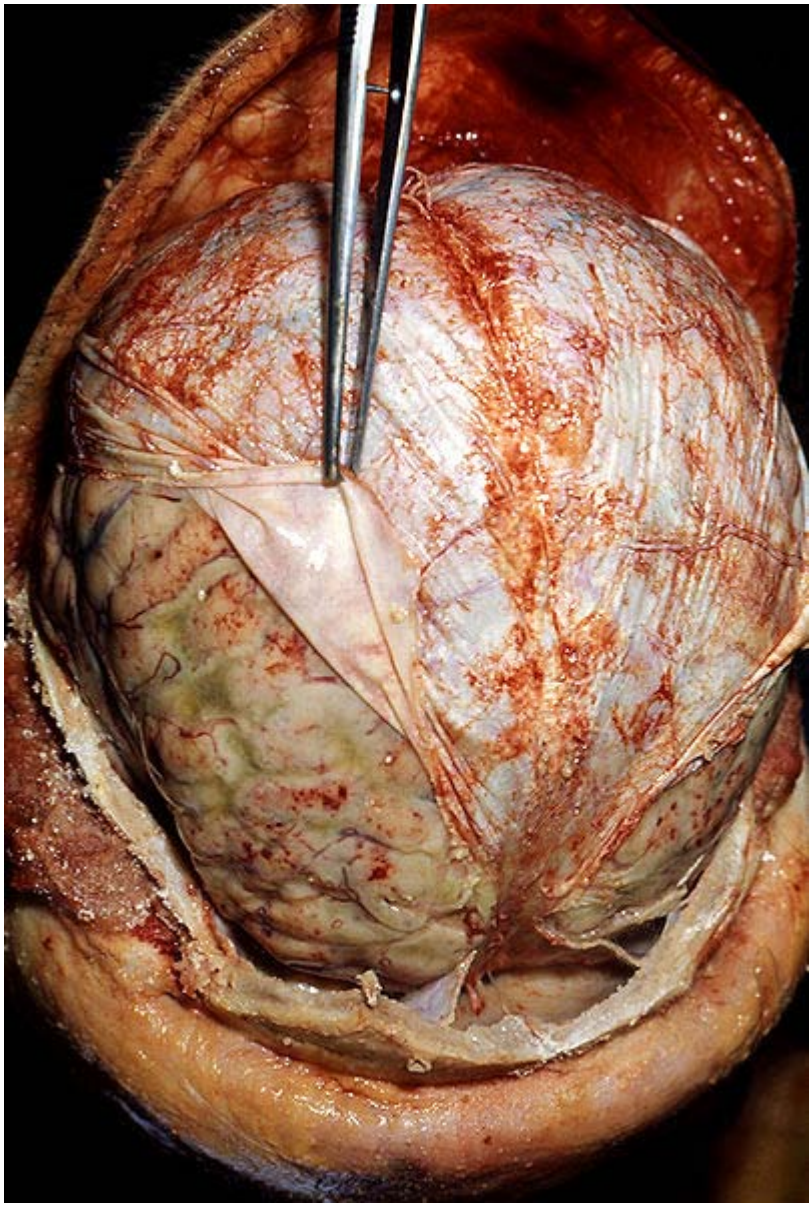
Histopathology: microscopic appearance of invasive ductal carcinoma of the breast. The slide is stained with Haematoxylin & Eosin.



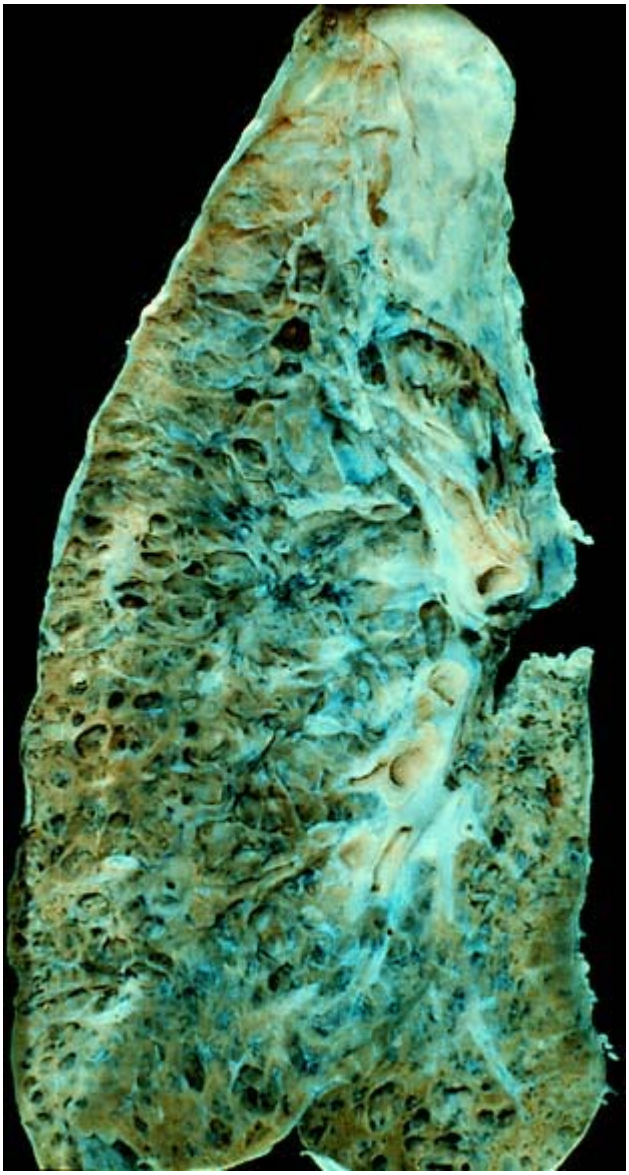
Histopathology: microscopic appearance of invasive ductal carcinoma of the breast. The slide is stained with an antibody (immunohistochemistry) against the ongene Her2neu. The dark brown reaction indicates that this tumor over-expresses this gene.



Cytopathology: microscopic appearance of a Pap test. The pink cell at the center with a large nucleus is abnormal, compatible with low grade dysplasia.



Autopsy: a brain surrounded by pus (the yellow-greyish coat around the brain, under the dura lifted by the forceps), the result of bacterial meningitis.



Gross examination: appearance of the cut surface of a lung showing the honeycomb pattern of end-stage pulmonary fibrosis.

Gross examination: appearance of a colorectal polyp (the cauliflower-shaped tumor) attached to the colon mucosa (the horizontal line at the bottom).

Anatomical pathology (*Commonwealth*) or **Anatomic pathology** (*U.S.*) is a medical specialty that is concerned with the diagnosis of disease based on the gross, microscopic, chemical, immunologic and molecular examination of organs, tissues, and whole bodies (autopsy).

Anatomical pathology is itself divided in subspecialties, the main ones being surgical pathology, cytopathology and forensic pathology. To be licensed to practice pathology, one has to complete medical school and secure a license to practice medicine. An approved residency program and certification (in the U.S., the American board of Pathology or the American Osteopathic Board of Pathology) is usually required to obtain employment or hospital privileges.

Anatomical pathology is one of two branches of pathology, the other being clinical pathology, the diagnosis of disease through the laboratory analysis of bodily fluids and/or tissues. Often, pathologists practice both anatomical and clinical pathology, a combination known as general pathology. The distinction between anatomic and clinical pathology is increasingly blurred by the introduction of technologies that require new expertise and the need to provide patients and referring physicians with integrated diagnostic reports. Similar specialties exist in veterinary pathology.

Skills and procedures

The procedures used in anatomic pathology include:

- Gross examination - the examination of diseased tissues with the naked eye. This is important especially for large tissue fragments, because the disease can often be

visually identified. It is also at this step that the pathologist selects areas that will be processed for histopathology. The eye can sometimes be aided with a magnifying glass or a stereo microscope, especially when examining parasitic organisms.

- Histopathology - the microscopic examination of stained tissue sections using histological techniques. The standard stains are haematoxylin and eosin, but many others exist. The use of haematoxylin and eosin-stained slides to provide specific diagnoses based on morphology is considered to be the core skill of anatomic pathology. The science of staining tissues sections is called histochemistry.
- Immunohistochemistry - the use of antibodies to detect the presence, abundance, and localization of specific proteins. This technique is critical to distinguishing between disorders with similar morphology, as well as characterizing the molecular properties of certain cancers.
- In situ hybridization - Specific DNA and RNA molecules can be identified on sections using this technique. When the probe is labeled with fluorescent dye, the technique is called FISH.
- Cytopathology - the examination of loose cells spread and stained on glass slides using cytology techniques.
- Electron microscopy - the examination of tissue with an electron microscope, which allows much greater magnification, enabling the visualization of organelles within the cells. Its use has been largely supplanted by immunohistochemistry, but it is still in common use for certain tasks, including the diagnosis of kidney disease and the identification of immotile cilia syndrome among many others.
- Tissue cytogenetics - the visualization of chromosomes to identify genetics defects such as chromosomal translocation.
- Flow immunophenotyping - the determination of the immunophenotype of cells using flow cytometry techniques. It is very useful to diagnose the different types of leukemia and lymphoma.

Subspecialties

Surgical pathology

Surgical pathology is the most significant and time-consuming area of practice for most anatomical pathologists. Surgical pathology involves the gross and microscopic examination of surgical specimens, as well as biopsies submitted by non-surgeons such as general internists, medical subspecialists, dermatologists, and interventional radiologists. Surgical pathology increasingly requires technologies and skills traditionally associated with clinical pathology such as molecular diagnostics.

Renal pathology

Renal pathology is a sub-discipline of anatomical pathology concerned with the diagnosis of medical (non-tumor) kidney diseases, including both native kidneys and transplants. Renal pathologists routinely employ techniques of immunofluorescence and electron microscopy, in addition to light microscopy, for kidney biopsy and nephrectomy

specimens. The renal pathologist integrates the microscopic findings from these three techniques along with the clinical presentation of the patient and the patient's clinical laboratory results to arrive at a diagnosis.

Oral and maxillofacial pathology

In the United States, subspecialty-trained doctors of dentistry, rather than medical doctors, can be certified by a professional board to practice Oral and Maxillofacial Pathology.

Cytopathology

Cytopathology is a sub-discipline of anatomical pathology concerned with the microscopic examination of whole, individual cells obtained from smears or fine needle aspirates. Cytopathologists are trained to perform fine-needle aspirates of superficially located organs, masses, or cysts, and are often able to render an immediate diagnosis in the presence of the patient and consulting physician. In the case of screening tests such as the Papanicolaou smear, non-physician cytotechnologists are often employed to perform initial reviews, with only positive or uncertain cases examined by the pathologist. Cytopathology is a board-certifiable subspecialty in the U.S.

Molecular pathology

Molecular pathology is an emerging discipline within anatomical and clinical pathology which is focused on the use of nucleic acid-based techniques such as in-situ hybridization, reverse-transcriptase polymerase chain reaction, and nucleic acid microarrays for specialized studies of disease in tissues and cells. Molecular pathology shares some aspects of practice with both anatomic and clinical pathology, and is sometimes considered a "crossover" discipline.

Autopsy pathology

General anatomical pathologists are trained in performing autopsies, which are used to determine the disease factors contributing to a person's death. Autopsies are important in the ongoing medical education of clinicians, and in efforts to improve and verify the quality of medical care. Dieners are non-physicians who assist pathologists in the gross dissection portion of the autopsy. The recently established field of Pathologists Assistant has taken over the role of Diener and contributes a great deal to the pre-diagnosis of disease within the pathology laboratory. Autopsies represent less than 10% of the workload of typical pathologists in the United States. However, the autopsy is central to public perceptions of the field, in part due to portrayals of pathologists on television programs such as *Quincy, M.E.* and *Silent Witness*.

Forensic pathology

Forensic pathologists receive specialized training in determining the cause of death and other legally relevant information from the bodies of persons who died suddenly with no known medical condition, those who die from non-natural causes, as well as those dying as a result of homicide, or other criminally suspicious deaths. A majority of the forensic pathologists cases are due to natural causes, often of a complex pathological nature. Often, additional tests such as toxicology, histology, and genetic testing will be used to help the pathologist determine the cause of death. Because of this, it is necessary for forensic pathologists to have a strong background in anatomical pathology before becoming forensic pathologists. Forensic pathologists will often testify in courts regarding their findings in cases of homicide and suspicious death. They also play a large role in public health, such as investigating deaths in the workplace, deaths in custody, as well as sudden and unexpected deaths in children. Forensic pathologists often have special areas of interest within their practice, such as sudden death due to cardiac pathology, deaths due to drugs, or Sudden Infant Death (SIDS), and various others.

Training and certification of Anatomical Pathologists

Australia

- **(Also New Zealand, Hong Kong, Singapore, Malaysia, and Saudi Arabia)**

Anatomical Pathology one of the specialty training programs offered by the Royal College of Pathologists of Australasia (RCPA). The RCPA. To qualify as a Fellow of the RCPA in Anatomical Pathology, the candidate must complete a recognised undergraduate or postgraduate medical qualification, then complete a minimum of 1 year of clinical medical experience (internship)as a prerequisite to selection as a training registrar. The training program is a minimum of 5 years, served in at least two laboratories, and candidates must pass a Basic Pathological Sciences examination (usually in first year), the Part 1 examination (not before 3rd year) and the Part 2 examination (not before 5th year). Fellows may then continue into subspecialty training.

Canada

Anatomical Pathology (AP) is one of the specialist certificates granted by the Royal College of Physicians and Surgeons of Canada. Other certificates related to pathology include general pathology (GP), forensic pathology, hematopathology, and neuropathology. Candidates for any of these must have completed four years of medical school and five years of residency training. After becoming certified in either AP or GP, it is common for pathologists to seek further fellowship training in a subspecialty of AP.

USA

Anatomic Pathology (AP) is one of the two primary certifications offered by the American Board of Pathology. The other is Clinical Pathology (CP). To be certified in

anatomic pathology, the trainee must complete four years of medical school followed by three years of residency training. Many US pathologists are certified in both AP and CP, which requires a total of four years of residency. After completing residency, many pathologists enroll in further years of fellowship training to gain expertise in a subspecialty of AP.

Anatomical pathology practice settings

- *Academic anatomical pathology* is practiced at university medical centers by pathologists who are also university faculty. As such, they often have diverse responsibilities that may include training pathology residents, teaching medical students, conducting basic, clinical, or translational research, and/or performing administrative duties, all in addition to the practice of diagnostic anatomical pathology. Pathologists in academic settings often sub-specialize in a particular area of anatomic pathology and may serve as consultants to other pathologists regarding cases in their specific area of expertise.

- *Group practice* is the most traditional private practice model. In this arrangement, a group of senior pathologists will control a partnership that employs junior pathologists and contracts independently with hospitals to provide diagnostic services, as well as attracting referral business from local clinicians who practice in the outpatient setting. The group often owns a laboratory for histology and ancillary testing of tissue, and may hold contracts to run hospital-owned labs. Many pathologists who practice in this setting are trained and certified in both anatomical pathology and clinical pathology, which allows them to supervise blood banks, clinical chemistry laboratories, and medical microbiology laboratories as well.

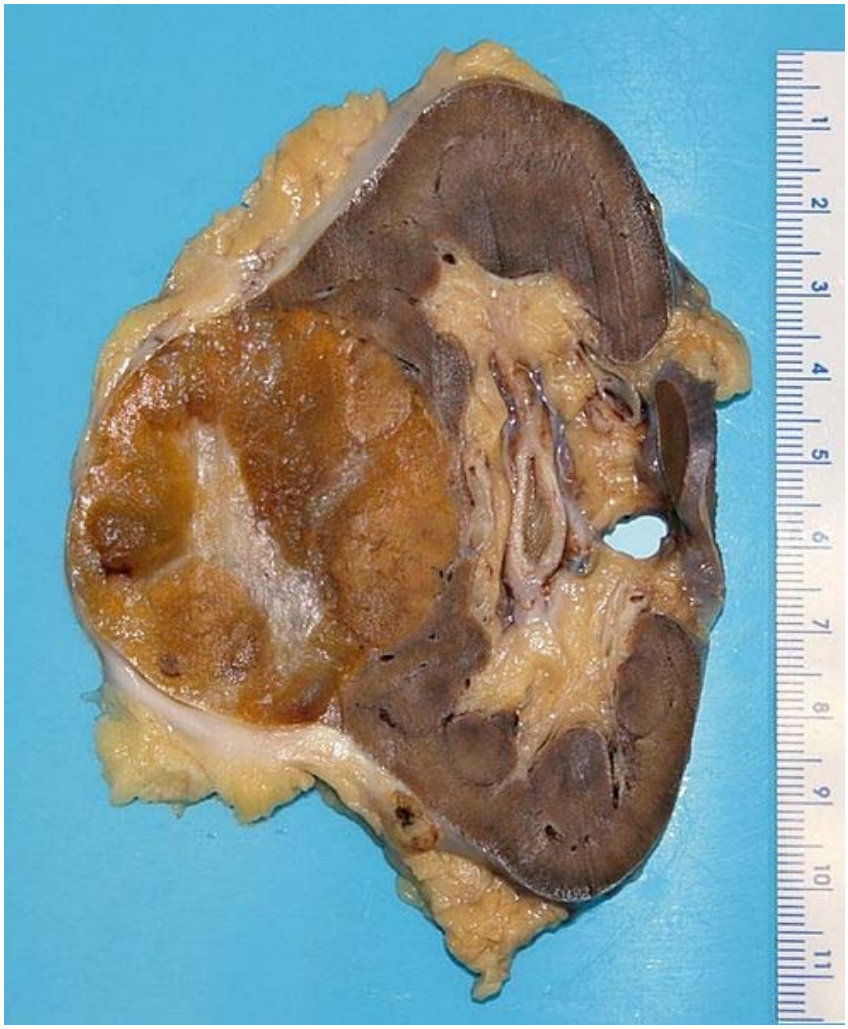
- Large *corporate providers of anatomical pathology services* have emerged in recent years, most notably *AmeriPath* in the United States. In this model, pathologists are employees, rather than independent partners. This model has been criticized for reducing physician independence, but defenders claim that the larger size of these practices allow for economies of scale and greater specialization, as well a sufficient volume to support more specialized testing methods.

- *Multispecialty groups*, composed of physicians from clinical specialties as well as radiology and pathology, are another practice model. In some case, these may be large groups controlled by an HMO or other large health care organization. In others, they are essentially clinician group practices that employ pathologists to provide diagnostic services for the group. These groups may own their own laboratories, or, in some cases may make controversial arrangements with "pod labs" that allow clinician groups to lease space, with the clinican groups receiving direct insurance payments for pathology services. Proposed changes to Medicare regulations may essentially eliminate these arrangements in the United States.

Chapter 15

Gross Examination and Immunohistochemistry

Gross examination



Gross examination of a kidney (right of image) with a renal oncocytoma (left of image).

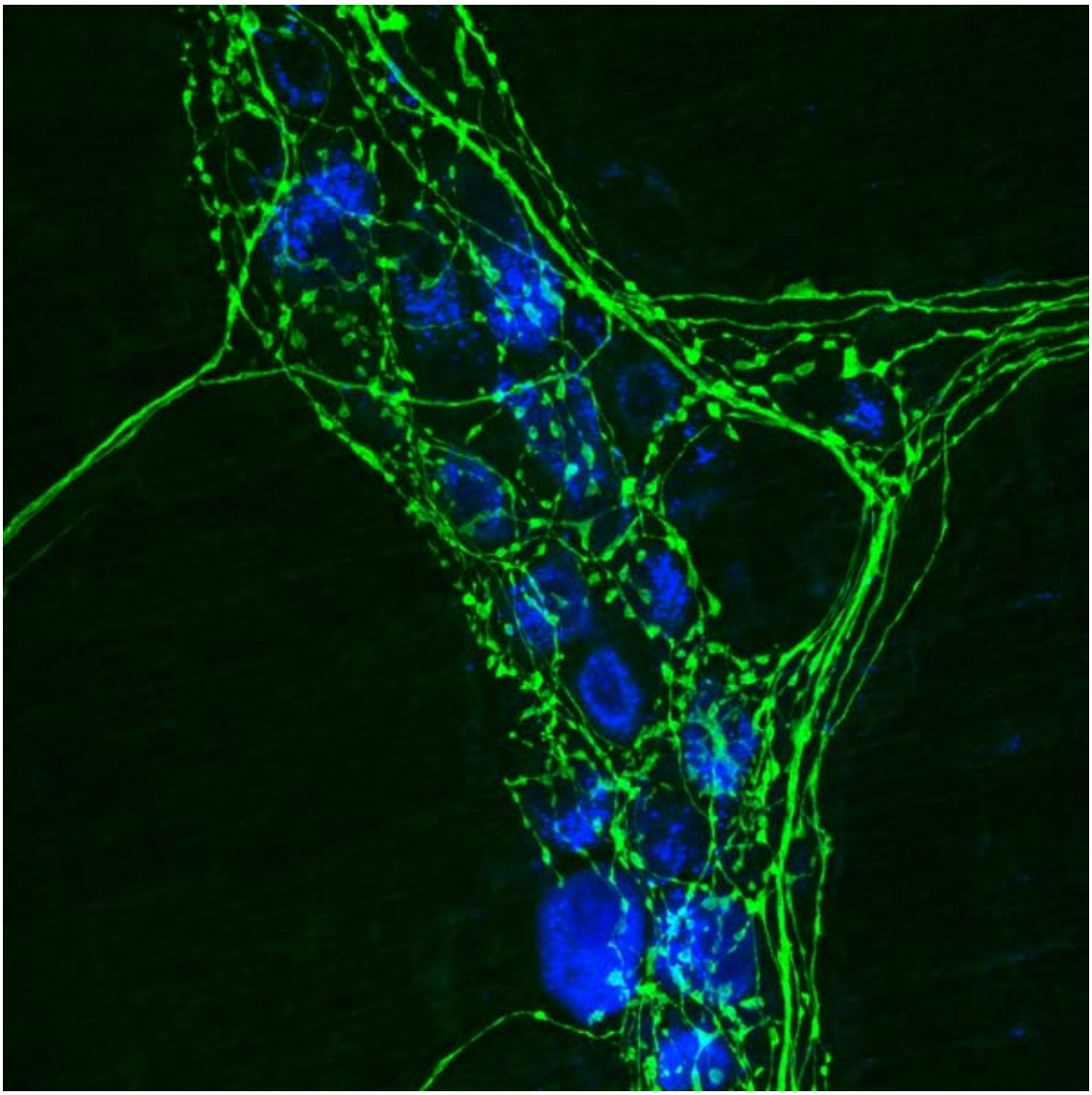
Gross examination or "grossing" is the process by which pathology specimens are inspected with the bare eye to obtain diagnostic information, while being processed for further microscopic *examination.*

Gross examination of surgical specimens is typically performed by a pathologist, or by a pathologists' assistant working within a pathology practice. Individuals trained in these fields are often able to gather diagnostically critical information in this stage of processing, including the stage and margin status of surgically removed tumors.

The initial step in any examination of a clinical specimen is confirmation of the identity of the patient and the anatomical site from which the specimen was obtained. Sufficient clinical data should be communicated by the clinical team to the pathology team in order to guide the appropriate diagnostic examination and interpretation of the specimen - if such information is not provided, it must be obtained by the examiner prior to processing the specimen.

There are usually two end products of the gross examination of a surgical specimen. The first is the gross description, a document which serves as the written record of the examiner's findings, and is included in the final pathology report. The second product is a set of tissue blocks, typically postage stamp-sized portions of tissue sealed in plastic cassettes, which will be processed into slides for microscopic examination. Since only a minority of the tissue from a large specimen can reasonably be subject to microscopic examination, the success of the final histological diagnosis is highly dependent on the skill of the professional performing the gross examination. The gross examiner may sample portions of the specimen for other types of ancillary tests as diagnostically indicated; these include microbiological culture, flow cytometry, cytogenetics, or electron microscopy.

Immunohistochemistry



Immunohistochemistry labels individual proteins, such as TH (green) in the axons of sympathetic autonomic neurons.

Immunohistochemistry or **IHC** refers to the process of detecting antigens (e.g., proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. IHC takes its name from the roots "immuno," in reference to antibodies used in the procedure, and "histo," meaning tissue (compare to immunocytochemistry). Immunohistochemical staining is widely used in the diagnosis of abnormal cells such as those found in cancerous tumors. Specific molecular markers are characteristic of particular cellular events such as proliferation or cell death (apoptosis). IHC is also widely used in basic research to understand the distribution and

localization of biomarkers and differentially expressed proteins in different parts of a biological tissue. Visualising an antibody-antigen interaction can be accomplished in a number of ways. In the most common instance, an antibody is conjugated to an enzyme, such as peroxidase, that can catalyse a colour-producing reaction. Alternatively, the antibody can also be tagged to a fluorophore, such as fluorescein or rhodamine.

Sample preparation

While using the right antibodies to target the correct antigens and amplify the signal is important for visualization, complete preparation of the sample is critical to maintain cell morphology, tissue architecture and the antigenicity of target epitopes. This requires proper tissue collection, fixation and sectioning. Depending on the purpose and the thickness of the experimental sample, either thin (about 4-40 μm) sections are sliced from the tissue of interest, or if the tissue is not very thick and is penetrable it is used whole. The slicing is usually accomplished through the use of a microtome, and slices are mounted on slides. "Free-floating IHC" uses slices that are not mounted, these slices are normally produced using a vibrating microtome.

Because of the method of fixation and tissue preservation, the sample may require additional steps to make the epitopes available for antibody binding, including deparaffinization and antigen retrieval; these steps often makes the difference between staining and no staining. Additionally, depending on the tissue type and the method of antigen detection, endogenous biotin or enzymes may need to be blocked or quenched, respectively, prior to antibody staining. Unlike immunocytochemistry, the tissue does not need to be permeabilized because this has already been accomplished by the microtome blade during sample preparation. Detergents like Triton X-100 are generally used in immunohistochemistry to reduce surface tension, allowing less reagent to be used to achieve better and more even coverage of the sample. Although antibodies show preferential avidity for specific epitopes, they may partially or weakly bind to sites on nonspecific proteins (also called reactive sites) that are similar to the cognate binding sites on the target antigen. In the context of antibody-mediated antigen detection, nonspecific binding causes high background staining that can mask the detection of the target antigen. To reduce background staining in IHC, ICC and any other immunostaining application, the samples are incubated with a buffer that blocks the reactive sites to which the primary or secondary antibodies may otherwise bind. Common blocking buffers include normal serum, non-fat dry milk, BSA or gelatin, and commercial blocking buffers with proprietary formulations are available for greater efficiency.

Sample Labeling

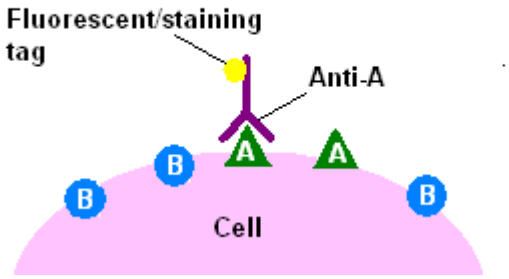
Antibody types

The antibodies used for specific detection can be polyclonal or monoclonal. Polyclonal antibodies are made by injecting animals with peptide Ag and, after a secondary immune response is stimulated, isolating antibodies from whole serum. Thus, polyclonal antibodies are a heterogeneous mix of antibodies that recognize several epitopes.

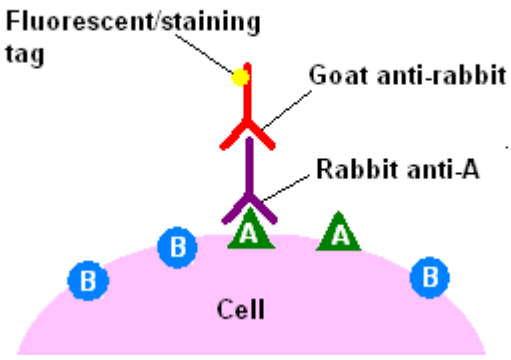
Monoclonal antibodies show specificity for a single epitope and are therefore considered more specific to the target antigen than polyclonal antibodies. For IHC detection strategies, antibodies are classified as primary or secondary reagents. Primary antibodies are raised against an antigen of interest and are typically unconjugated (unlabelled), while secondary antibodies are raised against immunoglobulins of the primary antibody species. The secondary antibody is usually conjugated to a linker molecule, such as biotin, that then recruits reporter molecules, or the secondary antibody is directly bound to the reporter molecule itself.

IHC reporters

Reporter molecules vary based on the nature of the detection method, and the most popular methods of detection are with enzyme- and fluorophore-mediated chromogenic and fluorescentdetection, respectively. With chromogenic reporters, an enzyme label is reacted with a substrate to yield an intensely colored product that can be analyzed with an ordinary light microscope. While the list of enzyme substrates is extensive, Alkaline phosphatase (AP) and horseradish peroxidase (HRP) are the two enzymes used most extensively as labels for protein detection. An array of chromogenic, fluorogenic and chemiluminescent substrates is available for use with either enzyme, including DAB or BCIP/NBT, which produce a brown or purple staining, respectively, wherever the enzymes are bound. Reaction with DAB can be enhanced using nickel, producing a deep purple/black staining. Fluorescent reporters are small, organic molecules used for IHC detection and traditionally include FITC, TRITC and AMCA, while commercial derivatives, including the Alexa Fluors and Dylight Fluors, show similar enhanced performance but vary in price. For chromogenic and fluorescent detection methods, densitometric analysis of the signal can provide semi- and fully-quantitative data, respectively, to correlate the level of reporter signal to the level of protein expression or localization.



The direct method of immunohistochemical staining uses one labelled antibody, which binds directly to the antigen being stained for.



The *indirect method* of immunohistochemical staining uses one antibody against the antigen being probed for, and a second, labelled, antibody against the first.

Target antigen detection methods

The *direct method* is a one-step staining method and involves a labeled antibody (e.g. FITC-conjugated antiserum) reacting directly with the antigen in tissue sections. While this technique utilizes only one antibody and therefore is simple and rapid, the sensitivity is lower due to little signal amplification, such as with indirect methods, and is less commonly used than indirect methods.

The *indirect method* involves an unlabeled primary antibody (first layer) that binds to the target antigen antigen in the tissue and a labeled secondary antibody (second layer) that reacts with the primary antibody. As mentioned above, the secondary antibody must be raised against the IgG of the animal species in which the primary antibody has been raised. This method is more sensitive than direct detection strategies because of signal amplification due to the binding of several secondary antibodies to each primary antibody if the secondary antibody is conjugated to the fluorescent or enzyme reporter. Further amplification can be achieved if the secondary antibody is conjugated to several biotin molecules, which can recruit complexes of avidin-, streptavidin or NeutrAvidin proteinbound-enzyme. The difference between these three biotin-binding proteins is their individual binding affinity to endogenous tissue targets leading to nonspecific binding and high background; the ranking of these proteins based on their nonspecific binding affinities, from highest to lowest, is: 1) avidin, 2) streptavidin and 3) Neutravidin protein.

The indirect method, aside from its greater sensitivity, also has the advantage that only a relatively small number of standard conjugated (labeled) secondary antibodies needs to be generated. For example, a labeled secondary antibody raised against rabbit IgG, which can be purchased "off the shelf," is useful with any primary antibody raised in rabbit. With the direct method, it would be necessary to label each primary antibody for every antigen of interest.

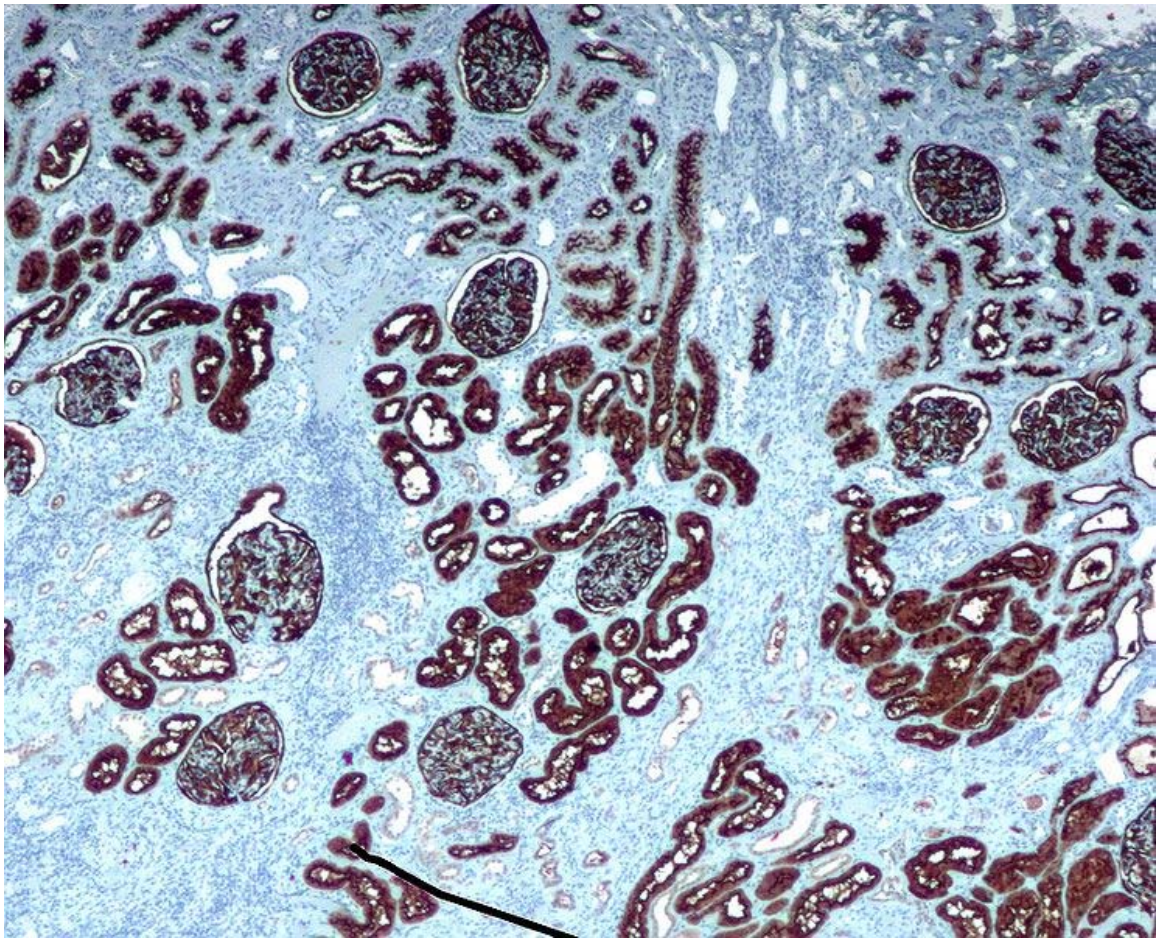
Counterstains

After immunohistochemical staining of the target antigen, a second stain is often applied to provide contrast that helps the primary stain stand out. Many of these stains show specificity for discrete cellular compartments or antigens, while others will stain the whole cell. Both chromogenic and fluorescent dyes are available for IHC to provide a vast array of reagents to fit every experimental design, and include: hematoxylin, Hoechst stain and DAPI are commonly used.

IHC Troubleshooting

In immunohistochemical techniques, there are several steps prior to the final staining of the tissue antigen, and many potential problems affect the outcome of the procedure. The major problem areas in IHC staining include strong background staining, weak target antigen staining and autofluorescence. Endogenous biotin or reporter enzymes or primary/secondary antibody cross-reactivity are common causes of strong background staining, while weak staining may be caused by poor enzyme activity or primary antibody potency. Furthermore, autofluorescence may be due to the nature of the tissue or the fixation method. These aspects of IHC tissue prep and antibody staining must be systematically addressed to identify and overcome staining issues.

Diagnostic IHC markers



Immunohistochemical staining of normal kidney with CD10

IHC is an excellent detection technique and has the tremendous advantage of being able to show exactly where a given protein is located within the tissue examined. It is also an effective way to examine the tissues .This has made it a widely-used technique in the neurosciences, enabling researchers to examine protein expression within specific brain structures. Its major disadvantage is that, unlike immunoblotting techniques where staining is checked against a molecular weight ladder, it is impossible to show in IHC that the staining corresponds with the protein of interest. For this reason, primary antibodies must be well-validated in a Western Blot or similar procedure. The technique is even more widely used in diagnostic surgical pathology for typing tumors (e.g. immunostaining for e-cadherin to differentiate between DCIS (ductal carcinoma in situ: stains positive) and LCIS (lobular carcinoma in situ: does not stain positive)).

- Carcinoembryonic antigen (CEA): used for identification of adenocarcinomas. Not specific for site.
- Cytokeratins: used for identification of carcinomas but may also be expressed in some sarcomas.
- CD15 and CD30 : used for Hodgkin's disease

- Alpha fetoprotein: for yolk sac tumors and hepatocellular carcinoma
- CD117 (KIT): for gastrointestinal stromal tumors (GIST)
- CD10 (CALLA): for renal cell carcinoma and acute lymphoblastic leukemia
- Prostate specific antigen (PSA): for prostate cancer
- estrogens and progesterone staining for tumour identification
- Identification of B-cell lymphomas using CD20
- Identification of T-cell lymphomas using CD3

Directing therapy

A variety of molecular pathways are altered in cancer and some of the alterations can be targeted in cancer therapy. Immunohistochemistry can be used to assess which tumors are likely to respond to therapy, by detecting the presence or elevated levels of the molecular target.

Chemical inhibitors

Tumor biology allows for a number of potential intracellular targets. Many tumors are hormone dependent. The presence of hormone receptors can be used to determine if a tumor is potentially responsive to antihormonal therapy. One of the first therapies was the antiestrogen, tamoxifen, used to treat breast cancer. Such hormone receptors can be detected by immunohistochemistry. Imatinib, an intracellualar tyrosine kinase inhibitor, was developed to treat chronic myelogenous leukemia, a disease characterized by the formation of a specific abnormal tyrosine kinase. Imitanib has proven effective in tumors, that express other tyrosine kinases, most notably KIT. Most gastrointestinal stromal tumors express KIT, which can be detected by immunohistochemistry.

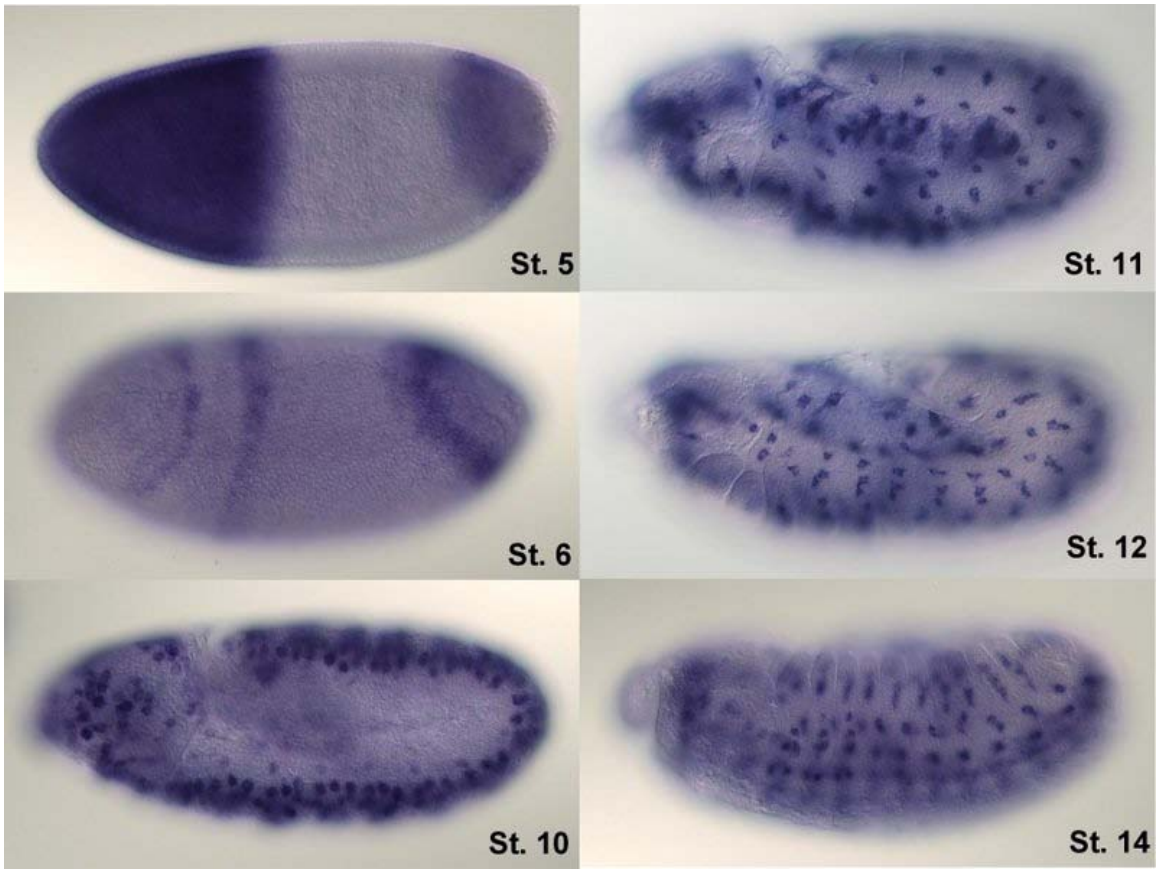
Monoclonal antibodies

Many proteins shown to be highly upregulated in pathological states by immunohistochemistry are potential targets for therapies utilising monoclonal antibodies. Monoclonal antibodies, due to their size, are utilized against cell surface targets. Among the overexpressed targets, the members of the epidermal growth factor receptor (EGFR) family, transmembrane proteins with an extracellular receptor domain regulating an intracellular tyrosine kinase, Of these, HER2/neu (also known as Erb-B2) was the first to be developed. The molecule is highly expressed in a variety of cancer cell types, most notably breast cancer. As such, antibodies against HER2/neu have been FDA approved for clinical treatment of cancer under the drug name *Herceptin*. There are commercially available immunohistochemical tests, Dako HercepTest and Ventana Pathway. Similarly, EGFR (HER-1) is overexpressed in a variety of cancers including head and neck and colon. Immunohistochemistry is used to determine patients who may benefit from therapeutic antibodies such as Erbitux (cetuximab). Commercial systems to detect EGFR by immunohistochemistry include the Dako pharmDx

Chapter 16

In Situ Hybridization and Electron Microscope

In situ hybridization



In situ hybridization of wild type *Drosophila* embryos at different developmental stages for the RNA from a gene called hunchback.

In situ hybridization (ISH) is a type of hybridization that uses a labeled complementary DNA or RNA strand (i.e., probe) to localize a specific DNA or RNA sequence in a portion or section of tissue (*in situ*), or, if the tissue is small enough (e.g. plant seeds,

Drosophila embryos), in the entire tissue (whole mount ISH). This is distinct from immunohistochemistry, which usually localizes proteins in tissue sections. DNA ISH can be used to determine the structure of chromosomes. Fluorescent DNA ISH (FISH) can, for example, be used in medical diagnostics to assess chromosomal integrity. RNA ISH (hybridization histochemistry) is used to measure and localize mRNAs and other transcripts within tissue sections or whole mounts.

Process

For hybridization histochemistry, sample cells and tissues are usually treated to fix the target transcripts in place and to increase access of the probe. As noted above, the probe is either a labeled complementary DNA or, now most commonly, a complementary RNA (riboprobe). The probe hybridizes to the target sequence at elevated temperature, and then the excess probe is washed away (after prior hydrolysis using RNase in the case of unhybridized, excess RNA probe). Solution parameters such as temperature, salt and/or detergent concentration can be manipulated to remove any non-identical interactions (i.e. only exact sequence matches will remain bound). Then, the probe that was labeled with either radio-, fluorescent- or antigen-labeled bases (e.g., digoxigenin) is localized and quantitated in the tissue using either autoradiography, fluorescence microscopy or immunohistochemistry, respectively. ISH can also use two or more probes, labeled with radioactivity or the other non-radioactive labels, to simultaneously detect two or more transcripts.

Basic Steps for Digoxigenin-labeled probes

1. permeabilisation of cells with proteinase K to open cell membranes (around 25 minutes, not needed for tissue sections or some early-stage embryos)
2. binding of mRNAs to marked RNA probe (usually overnight)
3. antibody-phosphatase binding to RNA-probe (some hours)
4. staining of antibody (e.g. with alkaline phosphatase)

The protocol takes around 2-3 days and takes some time to set up. Some companies sell robots to automate the process. As a result, large-scale screenings have been conducted in laboratories on thousands of genes. The results can usually be accessed via websites.

Electron microscope

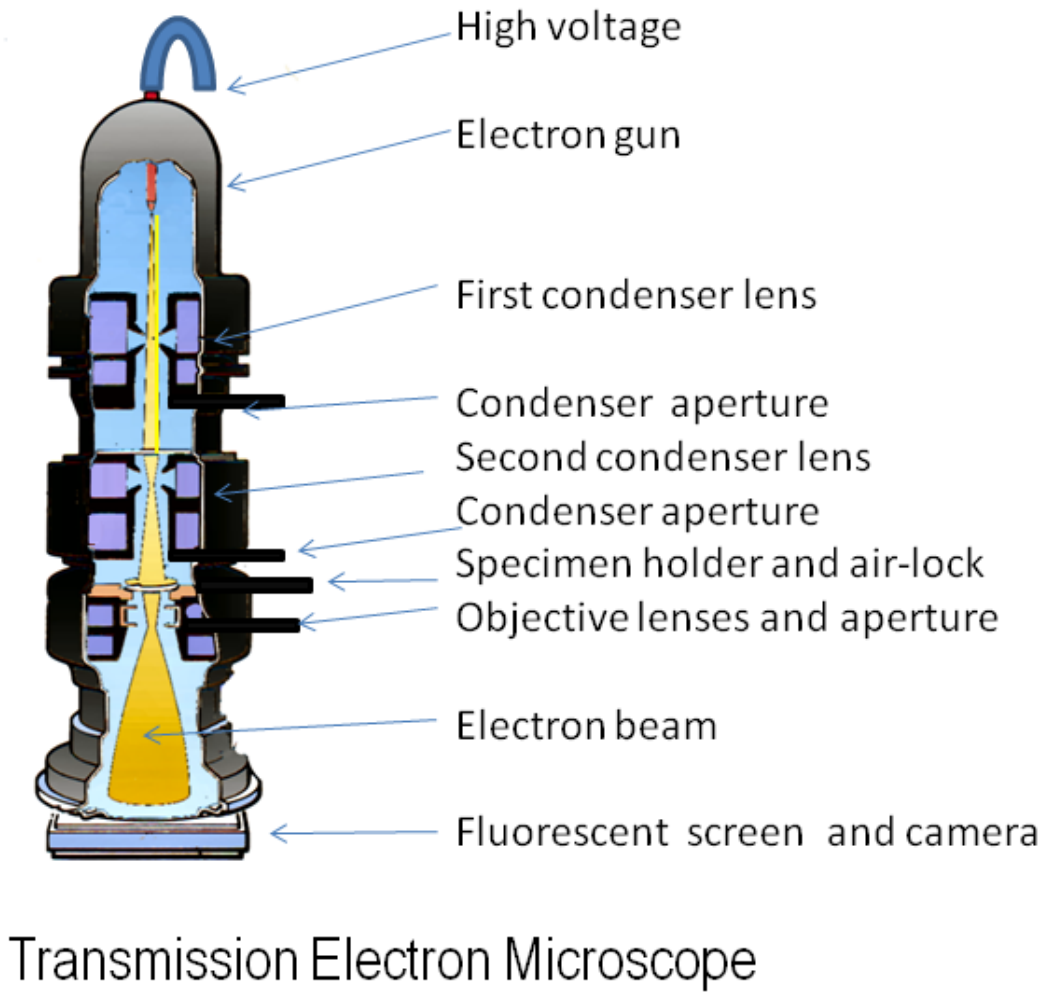


Diagram of a transmission electron microscope

A 1973 Siemens electron microscope, Musée des Arts et Métiers, Paris

An **electron microscope** is a type of microscope that uses a particle beam of electrons to illuminate the specimen and produce a magnified image. Electron microscopes (EM) have a greater resolving power than a light-powered optical microscope, because electrons have wavelengths about 100,000 times shorter than visible light (photons), and can achieve better than 0.2 nm resolution and magnifications of up to 2,000,000x, whereas ordinary, non-confocal light microscopes are limited by diffraction to about 200 nm resolution and useful magnifications below 2000x.

The electron microscope uses electrostatic and electromagnetic "lenses" to control the electron beam and focus it to form an image. These lenses are analogous to, but different from the glass lenses of an optical microscope that form a magnified image by focusing

light on or through the specimen. In transmission, the electron beam is first diffracted by the specimen, and then, the electron microscope “lenses" re-focus the beam into a Fourier-transformed image of the diffraction pattern for the selected area of investigation. The real image thus formed is magnified by a factor ranging from a few hundred to many hundred thousand times, and can be viewed on a detecting screen or recorded using photographic film or plates or with a digital camera. Electron microscopes are used to observe a wide range of biological and inorganic specimens including microorganisms, cells, large molecules, biopsy samples, metals, and crystals. Industrially, the electron microscope is primarily used for quality control and failure analysis in semiconductor device fabrication.

The advantages of electron microscopy over X-ray crystallography are that the specimen need not be a single crystal or even a polycrystalline powder, and also that the Fourier transform reconstruction of the object's magnified structure occurs physically and thus avoids the need for solving the phase problem faced by the X-ray crystallographers after obtaining their X-ray diffraction patterns of a single crystal or polycrystalline powder. The transmission electron microscope's major `disadvantage' is the need for extremely thin sections of the specimens, typically less than 100 nanometers. Biological specimens typically require to be chemically fixed, dehydrated and embedded in a polymer resin to stabilize them sufficiently to allow ultrathin sectioning. Sections of biological specimens, organic polymers and similar materials may require special `staining' with heavy atom labels in order to achieve the required image contrast.

History

Electron microscope constructed by Ernst Ruska in 1933

In 1931, the German physicist Ernst Ruska and German electrical engineer Max Knoll constructed the prototype electron microscope, capable of four-hundred-power magnification; the apparatus was a practical application of the principles of electron microscopy. Two years later, in 1933, Ruska built an electron microscope that exceeded the resolution attainable with an optical (lens) microscope. Moreover, Reinhold Rudenberg, the scientific director of Siemens-Schuckertwerke, obtained the patent for the electron microscope in May 1931. Family illness compelled the electrical engineer to devise an electrostatic microscope, because he wanted to make visible the poliomyelitis virus.

In 1937, the Siemens company financed the development work of Ernst Ruska and Bodo von Borries, and employed Helmut Ruska (Ernst's brother) to develop applications for the microscope, especially with biologic specimens. Also in 1937, Manfred von Ardenne pioneered the scanning electron microscope. The first *practical* electron microscope was constructed in 1938, at the University of Toronto, by Eli Franklin Burton and students Cecil Hall, James Hillier, and Albert Prebus; and Siemens produced the first *commercial* Transmission Electron Microscope (TEM) in 1939. Although contemporary electron microscopes are capable of two million-power magnification, as scientific instruments, they remain based upon Ruska's prototype.

Types

Transmission electron microscope (TEM)

The original form of electron microscope, the transmission electron microscope (TEM) uses a high voltage electron beam to create an image. The electrons are emitted by an electron gun, commonly fitted with a tungsten filament cathode as the electron source. The electron beam is accelerated by an anode typically at +100 keV (40 to 400 keV) with respect to the cathode, focused by electrostatic and electromagnetic lenses, and transmitted through the specimen that is in part transparent to electrons and in part scatters them out of the beam. When it emerges from the specimen, the electron beam carries information about the structure of the specimen that is magnified by the objective lens system of the microscope. The spatial variation in this information (the "image") is viewed by projecting the magnified electron image onto a fluorescent viewing screen coated with a phosphor or scintillator material such as zinc sulfide. The image can be photographically recorded by exposing a photographic film or plate directly to the electron beam, or a high-resolution phosphor may be coupled by means of a lens optical system or a fibre optic light-guide to the sensor of a CCD (charge-coupled device) camera. The image detected by the CCD may be displayed on a monitor or computer.

Resolution of the TEM is limited primarily by spherical aberration, but a new generation of aberration correctors have been able to partially overcome spherical aberration to increase resolution. Hardware correction of spherical aberration for the High Resolution TEM (HRTEM) has allowed the production of images with resolution below 0.5 Ångström (50 picometres) at magnifications above 50 million times. The ability to determine the positions of atoms within materials has made the HRTEM an important tool for nano-technologies research and development.

Scanning electron microscope (SEM)

An image of an ant in a scanning electron microscope

Unlike the TEM, where electrons of the high voltage beam carry the image of the specimen, the electron beam of the Scanning Electron Microscope (SEM) does not at any time carry a complete image of the specimen. The SEM produces images by probing the specimen with a focused electron beam that is scanned across a rectangular area of the specimen (raster scanning). At each point on the specimen the incident electron beam loses some energy, and that lost energy is converted into other forms, such as heat, emission of low-energy secondary electrons, light emission (cathodoluminescence) or x-ray emission. The display of the SEM maps the varying intensity of any of these signals into the image in a position corresponding to the position of the beam on the specimen when the signal was generated. In the SEM image of an ant shown at right, the image was constructed from signals produced by a secondary electron detector, the normal or conventional imaging mode in most SEMs.

Generally, the image resolution of an SEM is about an order of magnitude poorer than that of a TEM. However, because the SEM image relies on surface processes rather than transmission, it is able to image bulk samples up to many centimetres in size and

(depending on instrument design and settings) has a great depth of field, and so can produce images that are good representations of the three-dimensional shape of the sample.

Reflection electron microscope (REM)

In the **Reflection Electron Microscope** (REM) as in the TEM, an electron beam is incident on a surface, but instead of using the transmission (TEM) or secondary electrons (SEM), the reflected beam of elastically scattered electrons is detected. This technique is typically coupled with Reflection High Energy Electron Diffraction (RHEED) and *Reflection high-energy loss spectrum (RHELS)*. Another variation is Spin-Polarized Low-Energy Electron Microscopy (SPLEEM), which is used for looking at the microstructure of magnetic domains.

Scanning transmission electron microscope (STEM)

The STEM rasters a focused incident probe across a specimen that (as with the TEM) has been thinned to facilitate detection of electrons scattered *through* the specimen. The high resolution of the TEM is thus possible in STEM. The focusing action (and aberrations) occur before the electrons hit the specimen in the STEM, but afterward in the TEM. The STEMs use of SEM-like beam rastering simplifies annular dark-field imaging, and other analytical techniques, but also means that image data is acquired in serial rather than in parallel fashion.

Low-voltage electron microscope (LVEM)

The low-voltage electron microscope (LVEM) is a combination of SEM, TEM and STEM in one instrument, which operates at relatively low electron accelerating voltage of 5 kV. Low voltage increases image contrast which is especially important for biological specimens. This increase in contrast significantly reduces, or even eliminates the need to stain. Sectioned samples generally need to be thinner than they would be for conventional TEM (20-65 nm). Resolutions of a few nm are possible in TEM, SEM and STEM modes.

Sample preparation

An insect coated in gold for viewing with a scanning electron microscope

Materials to be viewed under an electron microscope may require processing to produce a suitable sample. The technique required varies depending on the specimen and the analysis required:

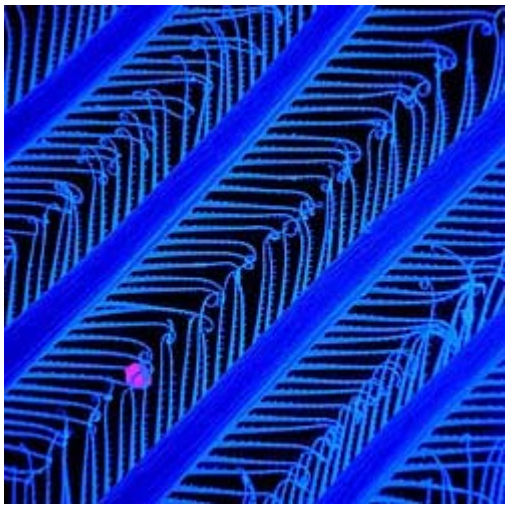
- *Chemical fixation* - for biological specimens aims to stabilize the specimen's mobile macromolecular structure by chemical crosslinking of proteins with aldehydes such as formaldehyde and glutaraldehyde, and lipids with osmium tetroxide.

- *Negative stain* - suspensions containing fine biological material (such as viruses and bacteria) are briefly mixed with a dilute solution of an electron-opaque solution such as ammonium molybdate, uranyl acetate (or formate), or phosphotungstic acid. This mixture is applied to a suitably-coated EM grid, blotted, then allowed to dry. Viewing of this preparation in the TEM should be carried out without delay for best results. The method is important in microbiology for fast but crude morphological identification, but can also be used as the basis for high resolution 3D reconstruction using EM tomography methodology when carbon films are used for support.
- *Cryofixation* – freezing a specimen so rapidly, to liquid nitrogen or even liquid helium temperatures, that the water forms vitreous (non-crystalline) ice. This preserves the specimen in a snapshot of its solution state. An entire field called cryo-electron microscopy has branched from this technique. With the development of cryo-electron microscopy of vitreous sections (CEMOVIS), it is now possible to observe samples from virtually any biological specimen close to its native state.
- *Dehydration* – freeze drying, or replacement of water with organic solvents such as ethanol or acetone, followed by critical point drying or infiltration with embedding resins.
- *Embedding, biological specimens* – after dehydration, tissue for observation in the transmission electron microscope is embedded so it can be sectioned ready for viewing. To do this the tissue is passed through a 'transition solvent' such as epoxy propane and then infiltrated with a resin such as Araldite epoxy resin; tissues may also be embedded directly in water-miscible acrylic resin. After the resin has been polymerised (hardened) the sample is thin sectioned (ultrathin sections) and stained - it is then ready for viewing.
- *Embedding, materials* - after embedding in resin, the specimen is usually ground and polished to a mirror-like finish using ultra-fine abrasives. The polishing process must be performed carefully to minimize scratches and other polishing artifacts that reduce image quality.
- *Sectioning* – produces thin slices of specimen, semitransparent to electrons. These can be cut on an ultramicrotome with a diamond knife to produce ultrathin slices about 60-90 nm thick. Disposable glass knives are also used because they can be made in the lab and are much cheaper.
- *Staining* – uses heavy metals such as lead, uranium or tungsten to scatter imaging electrons and thus give contrast between different structures, since many (especially biological) materials are nearly "transparent" to electrons (weak phase objects). In biology, specimens are can be stained "en bloc" before embedding and also later after sectioning. Typically thin sections are stained for several minutes with an aqueous or alcoholic solution of uranyl acetate followed by aqueous lead citrate.
- *Freeze-fracture or freeze-etch* – a preparation method particularly useful for examining lipid membranes and their incorporated proteins in "face on" view. The fresh tissue or cell suspension is frozen rapidly (cryofixation), then fractured by simply breaking or by using a microtome while maintained at liquid nitrogen temperature. The cold fractured surface (sometimes "etched" by increasing the

temperature to about –100 °C for several minutes to let some ice sublime) is then shadowed with evaporated platinum or gold at an average angle of 45° in a high vacuum evaporator. A second coat of carbon, evaporated perpendicular to the average surface plane is often performed to improve stability of the replica coating. The specimen is returned to room temperature and pressure, then the extremely fragile "pre-shadowed" metal replica of the fracture surface is released from the underlying biological material by careful chemical digestion with acids, hypochlorite solution or SDS detergent. The still-floating replica is thoroughly washed free from residual chemicals, carefully fished up on fine grids, dried then viewed in the TEM.

- *Ion Beam Milling* – thins samples until they are transparent to electrons by firing ions (typically argon) at the surface from an angle and sputtering material from the surface. A subclass of this is Focused ion beam milling, where gallium ions are used to produce an electron transparent membrane in a specific region of the sample, for example through a device within a microprocessor. Ion beam milling may also be used for cross-section polishing prior to SEM analysis of materials that are difficult to prepare using mechanical polishing.
- *Conductive Coating* – an ultrathin coating of electrically-conducting material, deposited either by high vacuum evaporation or by low vacuum sputter coating of the sample. This is done to prevent the accumulation of static electric fields at the specimen due to the electron irradiation required during imaging. Such coatings include gold, gold/palladium, platinum, tungsten, graphite etc. and are especially important for the study of specimens with the scanning electron microscope. Another reason for coating, even when there is more than enough conductivity, is to improve contrast, a situation more common with the operation of a FESEM (field emission SEM).

Disadvantages



False-color SEM image of the filter setae of an Antarctic krill. (Raw electron microscope images carry no color information.)
Pictured: First degree filter setae with V-shaped second degree setae pointing towards the inside of the feeding basket. The purple ball is 1 μm in diameter.

Electron microscopes are expensive to build and maintain, but the capital and running costs of confocal light microscope systems now overlaps with those of basic electron microscopes. They are dynamic rather than static in their operation, requiring extremely stable high-voltage supplies, extremely stable currents to each electromagnetic coil/lens, continuously-pumped high- or ultra-high-vacuum systems, and a cooling water supply circulation through the lenses and pumps. As they are very sensitive to vibration and external magnetic fields, microscopes designed to achieve high resolutions must be housed in stable buildings (sometimes underground) with special services such as magnetic field cancelling systems. Some desktop low-voltage electron microscopes have TEM capabilities at very low voltages (around 5 kV) without stringent voltage supply, lens coil current, cooling water or vibration isolation requirements and as such are much less expensive to buy and far easier to install and maintain, but do not have the same ultra-high (atomic scale) resolution capabilities as the larger instruments.

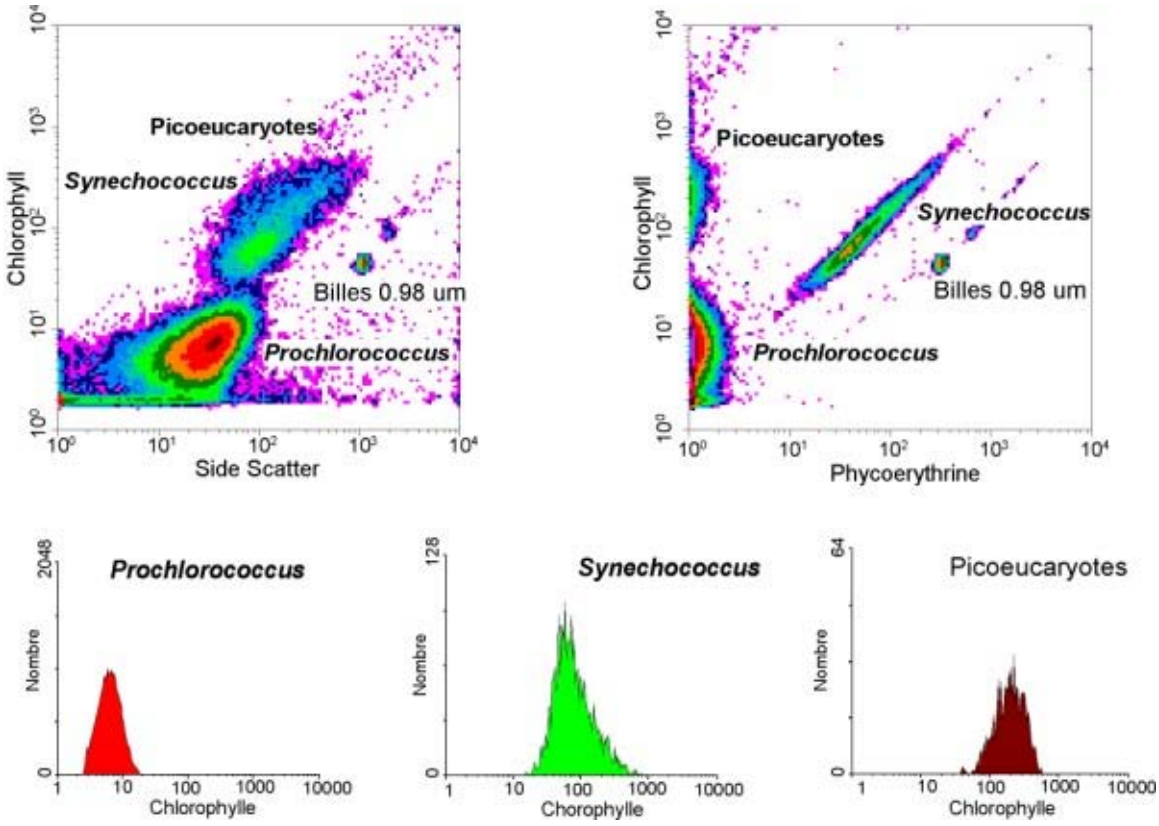
The samples largely have to be viewed in vacuum, as the molecules that make up air would scatter the electrons. One exception is the environmental scanning electron microscope, which allows hydrated samples to be viewed in a low-pressure (up to 20 Torr/2.7 kPa), wet environment.

Scanning electron microscopes usually image conductive or semi-conductive materials best. Non-conductive materials can be imaged by an environmental scanning electron microscope. A common preparation technique is to coat the sample with a several-nanometer layer of conductive material, such as gold, from a sputtering machine; however, this process has the potential to disturb delicate samples.

Small, stable specimens such as carbon nanotubes, diatom frustules and small mineral crystals (asbestos fibres, for example) require no special treatment before being examined in the electron microscope. Samples of hydrated materials, including almost all biological specimens have to be prepared in various ways to stabilize them, reduce their thickness (ultrathin sectioning) and increase their electron optical contrast (staining). These processes may result in *artifacts*, but these can usually be identified by comparing the results obtained by using radically different specimen preparation methods. It is generally believed by scientists working in the field that as results from various preparation techniques have been compared and that there is no reason that they should all produce similar artifacts, it is reasonable to believe that electron microscopy features correspond with those of living cells. In addition, higher-resolution work has been directly compared to results from X-ray crystallography, providing independent confirmation of the validity of this technique. Since the 1980s, analysis of cryofixed, vitrified specimens has also become increasingly used by scientists, further confirming the validity of this technique.

Chapter 17

Flow Cytometry



Analysis of a marine sample of photosynthetic picoplankton by flow cytometry showing three different populations (*Prochlorococcus*, *Synechococcus*, and picoeukaryotes)

Flow cytometry (abbreviated: **FCM**) is a technique for counting and examining microscopic particles, such as cells and chromosomes, by suspending them in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of up to thousands of particles per second. Flow cytometry is routinely used in the diagnosis of health disorders, especially blood cancers, but has many other applications in both research and clinical practice. A common variation is to physically sort particles based on their properties, so as to purify populations of interest.

History

The first impedance-based flow cytometry device, using the Coulter principle, was disclosed in U.S. Patent 2,656,508, issued in 1953, to Wallace H. Coulter. The first fluorescence-based flow cytometry device (ICP 11) was developed in 1968 by Wolfgang Göhde from the University of Münster and first commercialized in 1968/69 by German developer and manufacturer Partec through Phywe AG in Göttingen. At that time, absorption methods were still widely favored by other scientists over fluorescence methods . Soon after, flow cytometry instruments were developed, including the Cytofluorograph (1971) from Bio/Physics Systems Inc. (later: Ortho Diagnostics), the PAS 8000 (1973) from Partec, the first FACS instrument from Becton Dickinson (1974), the ICP 22 (1975) from Partec/Phywe and the Epics from Coulter (1977/78).

Name of the technology

The original name of the flow cytometry technology was "pulse cytophotometry" (German: *Impulszytophotometrie*). Only 20 years later in 1988, at the Conference of the American Engineering Foundation in Pensacola, Florida, the name was changed to "flow cytometry", a term that quickly became popular.

Principle of flow cytometry

A beam of light (usually laser light) of a **single wavelength** is directed onto a hydrodynamically-focused stream of fluid. A number of detectors are aimed at the point where the stream passes through the light beam: one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter (SSC) and one or more fluorescent detectors). Each suspended particle from 0.2 to 150 micrometers passing through the beam scatters the ray, and fluorescent chemicals found in the particle or attached to the particle may be excited into emitting light at a longer wavelength than the light source. This combination of scattered and fluorescent light is picked up by the detectors, and, by analysing fluctuations in brightness at each detector (one for each fluorescent emission peak), it is then possible to derive various types of information about the physical and chemical structure of each individual particle. FSC correlates with the cell volume and SSC depends on the inner complexity of the particle (i.e., shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness). Some flow cytometers on the market have eliminated the need for fluorescence and use only light scatter for measurement. Other flow cytometers form images of each cell's fluorescence, scattered light and transmitted light.

Front of desktop flow cytometer - the Becton-Dickinson FACSCalibur.

Flow cytometers

Modern flow cytometers are able to analyze several thousand particles every second, in "real time," and can actively separate and isolate particles having specified properties. A flow cytometer is similar to a microscope, except that, instead of producing an image of the cell, flow cytometry offers "high-throughput" (for a large number of cells) automated quantification of set parameters. To analyze solid tissues, a single-cell suspension must first be prepared.

A flow cytometer has 5 main components:

- a flow cell - liquid stream (sheath fluid), which carries and aligns the cells so that they pass single file through the light beam for sensing
- a measuring system - commonly used are measurement of impedance (or conductivity) and optical systems - lamps (mercury, xenon); high-power water-cooled lasers (argon, krypton, dye laser); low-power air-cooled lasers (argon (488 nm), red-HeNe (633 nm), green-HeNe, HeCd (UV)); diode lasers (blue, green, red, violet) resulting in light signals

- a detector and Analogue-to-Digital Conversion (ADC) system - which generates FSC and SSC as well as fluorescence signals from light into electrical signals that can be processed by a computer
- an amplification system - linear or logarithmic
- a computer for analysis of the signals.

The process of collecting data from samples using the flow cytometer is termed 'Acquisition'. Acquisition is mediated by a computer physically connected to the flow cytometer, and the software which handles the digital interface with the cytometer. The software is capable of adjusting parameters (i.e. voltage, compensation, etc.) for the sample being tested, and also assists in displaying initial sample information while acquiring sample data to insure that parameters are set correctly. Early flow cytometers were, in general, experimental devices, but technological advances have enabled widespread applications for use in a variety of both clinical and research purposes. Due to these developments, a considerable market for instrumentation, analysis software, as well as the reagents used in acquisition such as fluorescently-labeled antibodies has developed.

Modern instruments usually have multiple lasers and fluorescence detectors (the current record for a commercial instrument is **4 lasers and 18 fluorescence detectors**). Increasing the number of lasers and detectors allows for multiple antibody labeling, and can more precisely identify a target population by their phenotypic markers. Certain instruments can even take digital images of individual cells, allowing for the analysis of fluorescent signal location within or on the surface of cell.

Data analysis

Gating

The data generated by flow-cytometers can be plotted in a single dimension, to produce a histogram, or in two-dimensional dot plots or even in three dimensions. The regions on these plots can be sequentially separated, based on fluorescence intensity, by creating a series of subset extractions, termed "gates." Specific gating protocols exist for diagnostic and clinical purposes especially in relation to hematology.

The plots are often made on logarithmic scales. Because different fluorescent dyes' emission spectra overlap, signals at the detectors have to be compensated electronically as well as computationally. Data accumulated using the flow cytometer can be analyzed using software, e.g., WinMDI(deprecated), Flowjo, FCS Express, VenturiOne or CellQuest Pro. Once the data is collected, there is no need to stay connected to the flow cytometer. For this reason, analysis is most often done on a separate computer. This is especially necessary in core facilities where usage of these machines is in high demand.

Computational analysis

Recent progress on automated population identification using computational methods has offered an alternative to traditional gating strategies. Automated identification systems could potentially help findings of rare and hidden populations. Representative automated methods include FLOCK in Immunology Database and Analysis Portal (ImmPort) , FLAME in GenePattern and flowClust ,, in Bioconductor. Collaborative efforts have resulted in an open project called FlowCAP (Flow Cytometry: Critical Assessment of Population Identification Methods,) to provide an objective way to compare and evaluate the flow cytometry data clustering methods, and also to establish guidance about appropriate use and application of these methods.

Fluorescence-activated cell sorting

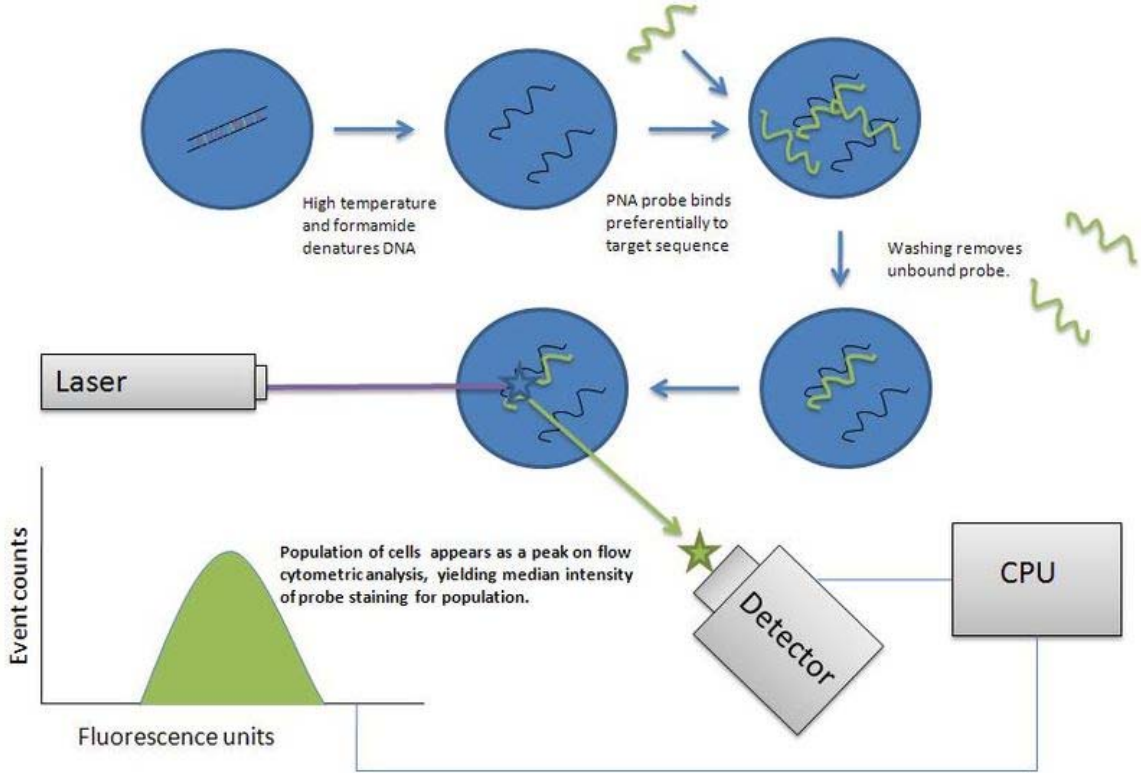
Fluorescence-activated cell sorting is a specialized type of flow cytometry. It provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. It is a useful scientific instrument, as it provides fast, objective and quantitative recording of fluorescent signals from individual cells as well as physical separation of cells of particular interest. The acronym FACS is trademarked and owned by Becton, Dickinson and Company. While many immunologists use this term frequently for all types of sorting and non-sorting applications, it is not a generic term for flow cytometry. The first cell sorter was invented by Mack Fulwyler in 1965, using the Coulter principle, a relatively difficult technique and one no longer used in modern instruments. The technique was expanded by Len Herzenberg who was responsible for coining the term FACS. Herzenberg won the Kyoto Prize in 2006 for his work in flow cytometry.

The cell suspension is entrained in the center of a narrow, rapidly flowing stream of liquid. The flow is arranged so that there is a large separation between cells relative to their diameter. A vibrating mechanism causes the stream of cells to break into individual droplets. The system is adjusted so that there is a low probability of more than one cell per droplet. Just before the stream breaks into droplets, the flow passes through a fluorescence measuring station where the fluorescent character of interest of each cell is measured. An electrical charging ring is placed just at the point where the stream breaks into droplets. A charge is placed on the ring based on the immediately-prior fluorescence intensity measurement, and the opposite charge is trapped on the droplet as it breaks from the stream. The charged droplets then fall through an electrostatic deflection system that diverts droplets into containers based upon their charge. In some systems, the charge is applied directly to the stream, and the droplet breaking off retains charge of the same sign as the stream. The stream is then returned to neutral after the droplet breaks off.

Fluorescent labels

A wide range of fluorophores can be used as labels in flow cytometry. These each have a characteristic peak excitation and emission wavelength. Also, the emission spectra of the

labels often overlap. Consequently, the combination of labels which can be used depends on the wavelength of the lamp(s) or laser(s) used to excite the fluorochromes and on the detectors available



Use of flow cytometry to measure copy number variation of a specific DNA sequence (Flow-FISH)

Measurable parameters

This list is very long and constantly expanding.

- volume and morphological complexity of cells
- cell pigments such as chlorophyll or phycoerythrin
- total DNA content (cell cycle analysis, cell kinetics, proliferation, etc.)
- total RNA content
- DNA copy number variation (by Flow-FISH)
- chromosome analysis and sorting (library construction, chromosome paint)
- protein expression and localization
- Protein modifications, phospho-proteins
- transgenic products *in vivo*, particularly the Green fluorescent protein or related fluorescent * cell surface antigens (Cluster of differentiation (CD) markers)
- intracellular antigens (various cytokines, secondary mediators, etc.)
- nuclear antigens
- enzymatic activity

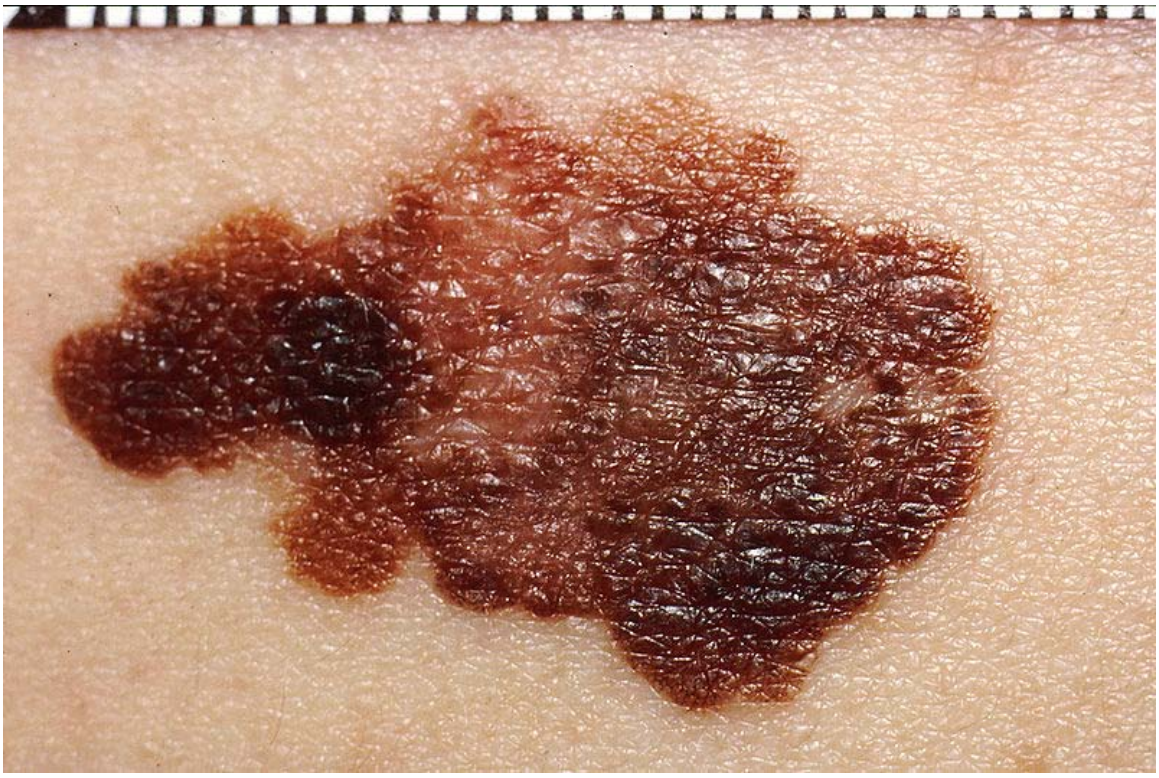
- pH, intracellular ionized calcium, magnesium, membrane potential
- membrane fluidity
- apoptosis (quantification, measurement of DNA degradation, mitochondrial membrane potential, permeability changes, caspase activity)
- cell viability
- monitoring electropermeabilization of cells
- oxidative burst
- characterising multidrug resistance (MDR) in cancer cells
- glutathione
- various combinations (DNA/surface antigens, etc.)
- cell adherence (for instance pathogen-host cell adherence)

Applications

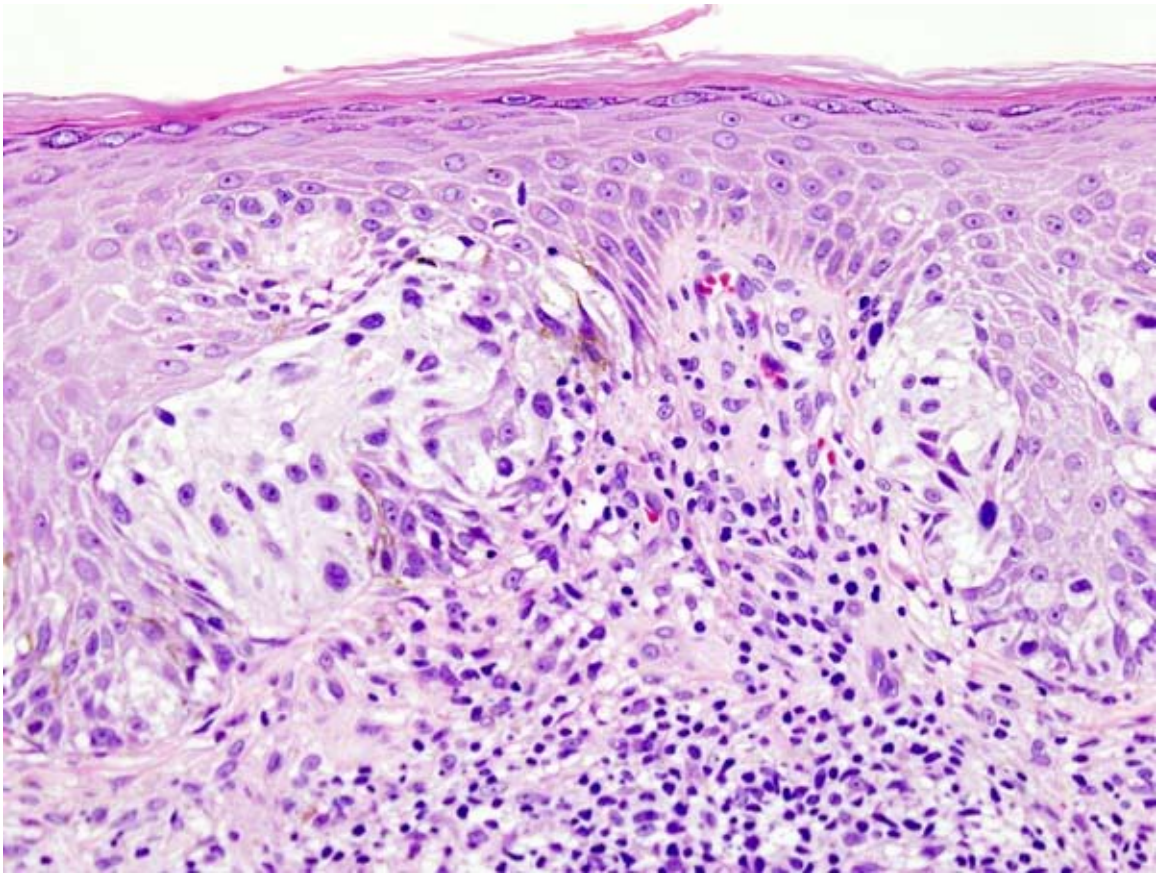
The technology has applications in a number of fields, including molecular biology, pathology, immunology, plant biology and marine biology. It has broad application in medicine (especially in transplantation, hematology, tumor immunology and chemotherapy, genetics and sperm sorting for sex preselection). In marine biology, the auto-fluorescent properties of photosynthetic plankton can be exploited by flow cytometry in order to characterise abundance and community structure. In protein engineering, flow cytometry is used in conjunction with yeast display and bacterial display to identify cell surface-displayed protein variants with desired properties. It is also used to determine ploidy of grass carp fry.

Chapter 18

Surgical Pathology



Malignant melanoma of the skin. This is as it would appear on the patient.



Malignant melanoma of the skin. This is a section of tissue, stained with hematoxylin & eosin, and viewed on a microscope slide

Surgical pathology is the most significant and time-consuming area of practice for most anatomical pathologists. Surgical pathology involves the gross and microscopic examination of surgical specimens, as well as biopsies submitted by non-surgeons such as general internists, medical subspecialists, dermatologists, and interventional radiologists.

The practice of surgical pathology allows for definitive diagnosis of disease (or lack thereof) in any case where tissue is surgically removed from a patient. This is usually performed by a combination of gross (ie. macroscopic) and histologic (ie. microscopic) examination of the tissue, and may involve evaluations of molecular properties of the tissue by immunohistochemistry or other laboratory tests.

Specimens

There are two major types of specimens submitted for surgical pathology analysis: *biopsies* and *surgical resections.*

A *biopsy* is a small piece of tissue removed primarily for the purposes of surgical pathology analysis, most often in order to render a definitive diagnosis. Types of biopsies

include *core biopsies* which are obtained through the use of large-bore needles, sometimes under the guidance of radiological techniques such as ultrasound, CT scan, or magnetic resonance imaging. Core biopsies, which preserve tissue architecture, should not be confused with fine needle aspiration specimens, which are analyzed using cytopathology techniques. *Incisional biopsies* are obtained through diagnostic surgical procedures which remove part of a suspicious lesion, while *excisional biopsies* remove the entire lesion, and are similar to therapeutic *surgical resections.* Excisional biopsies of skin lesions and gastrointestinal polyps are very common. The pathologist's interpretation of a biopsy is critical to establishing the diagnosis of a benign or malignant tumor, and can differentiate between different types and grades of cancer, as well as determining the activity of specific molecular pathways in the tumor. This information is important for estimating the patient's prognosis and for choosing the best treatment to administer. Biopsies are also used to diagnose diseases other than cancer, including inflammatory, infectious, or idiopathic diseases of the skin and gastrointestinal tract, to name only a few.

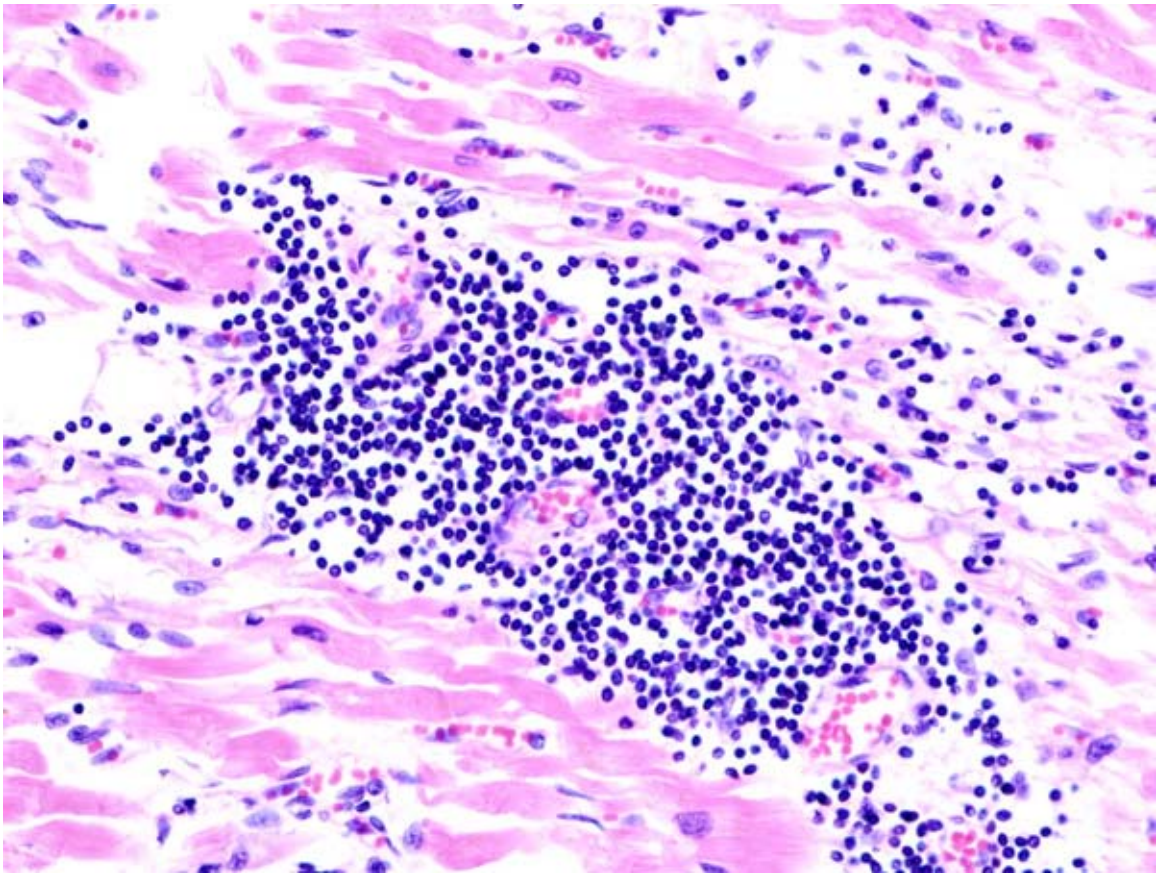
Surgical resection specimens are obtained by the therapeutic surgical removal of an entire diseased area or organ (and occasionally multiple organs). These procedures are often intended as definitive surgical treatment of a disease in which the diagnosis is already known or strongly suspected. However, pathological analysis of these specimens is critically important in confirming the previous diagnosis, staging the extent of malignant disease, establishing whether or not the entire diseased area was removed (a process called "determination of the surgical margin often using frozen section), identifying the presence of unsuspected concurrent diseases, and providing information for postoperative treatment, such as adjuvant chemotherapy in the case of cancer.

In the determination of surgical margin of a surgical resection, one can use the bread loafing technique, or CCPDMA. A special type of CCPDMA is named after a general surgeon, or the Mohs surgery method.

Surgical pathology workflow

- Gross examination
- Frozen section
- Fixation & Embedding
- Histopathologic examination
- Ancillary testing
- The surgical pathology report
- Direct consultation

Subspecialties



Histologic slide demonstrating viral myocarditis, an infection of the heart muscle

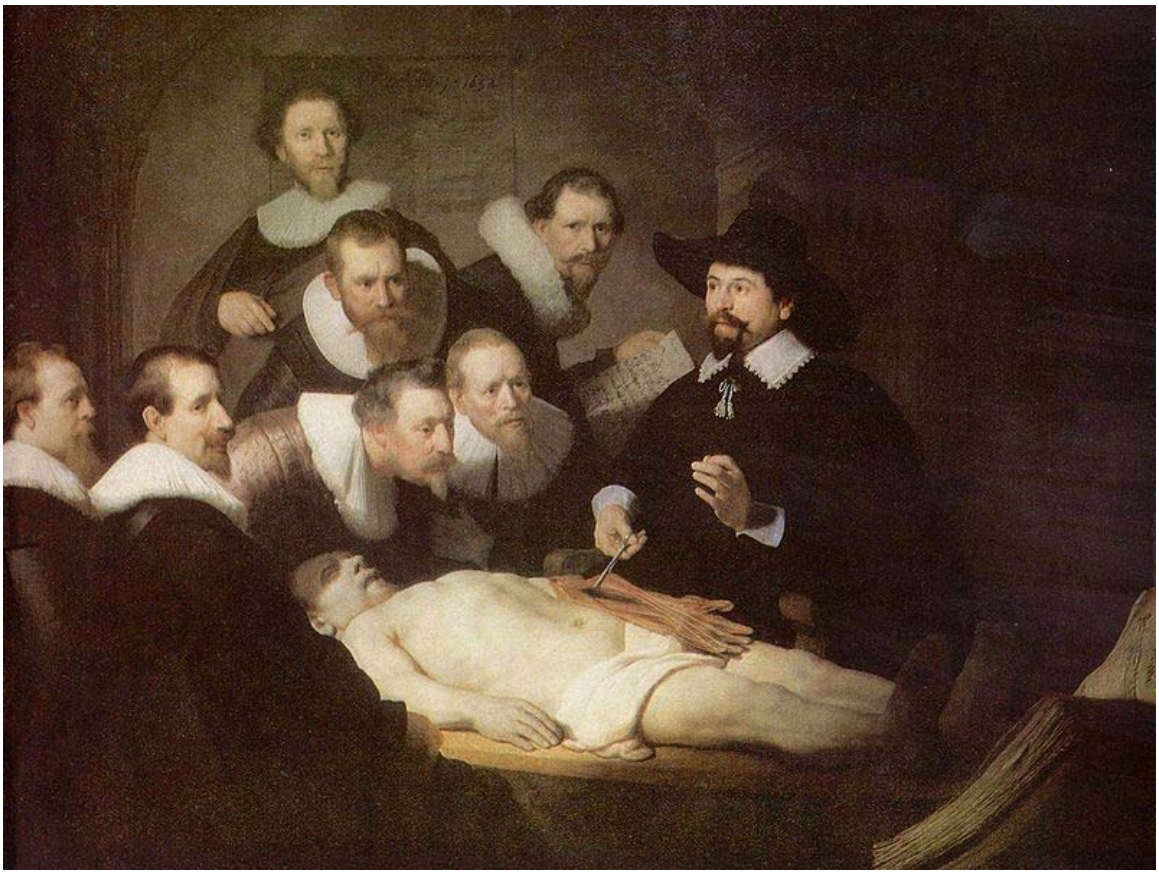
Many pathologists seek fellowship-level training, or otherwise pursue expertise in a focused area of surgical pathology. Subspecialization is particularly prevalent in the academic setting, where pathologists may specialise in an area of diagnostic surgical pathology which is relevant to their research, but is becoming increasingly prevalent in private practice as well. Subspecialization has a number of benefits, such as allowing for increased exerience and skill at interpreting challenging cases, as well as development of a closer working relationship between the pathologist and clinicians within a subspecialty area. Commonly recognized subspecialties of surgical pathology include the following:

- Bone pathology
- Cardiac pathology
- Cytopathology (A board-certifiable subspecialty in the U.S.)
- Dermatopathology (A board-certifiable subspecialty in the U.S.)
- Endocrine pathology
- Gastrointestinal pathology
- Genitourinary pathology
- Gynecologic pathology
- Head and Neck Pathology

- Hematopathology (A board-certifiable subspecialty in the U.S.)
- Neuropathology (A board-certifiable subspecialty in the U.S. and a recognised specialty in the U.K.)
- Ophthalmic pathology
- Pediatric pathology (A board-certifiable subspecialty in the U.S. and a recognised specialty in the U.K.)
- Pulmonary pathology
- Renal pathology
- Soft tissue pathology

Chapter 19

Autopsy



The *Anatomy Lesson of Dr. Nicolaes Tulp*, by Rembrandt, depicts an autopsy.

An **autopsy**—also known as a **post-mortem examination**, **necropsy** (particularly as to non-human bodies), **autopsia cadaverum**, or **obduction**—is a highly specialized surgical procedure that consists of a thorough examination of a corpse to determine the cause and manner of death and to evaluate any disease or injury that may be present. It is usually performed by a specialized medical doctor called a pathologist.

Autopsies are either performed for legal or medical purposes. For example, a forensic autopsy is carried out when the cause of death may be a criminal matter, while a clinical

or academic autopsy is performed to find the medical cause of death and is used in cases of unknown or uncertain death, or for research purposes. Autopsies can be further classified into cases where external examination suffices, and those where the body is dissected and internal examination is conducted. Permission from next of kin may be required for internal autopsy in some cases. Once an internal autopsy is complete the body is reconstituted by sewing it back together.

History

The term "autopsy" derives from the Ancient Greek *autopsia*, "to see for oneself", derived from αυτος (*autos*, "oneself") and όψις (*opsis*, "eye"). Around 3000 BC, the ancient Egyptians were one of the first civilizations to practice the removal and examination of the internal organs of humans in the religious practice of mummification.

Autopsies that opened the body to determine the cause of death are attested at least in the early third millennium BC, although they were opposed in many ancient societies where it was believed that the outward disfigurement of dead persons prevented them from entering the afterlife (as with the Egyptians, who removed the organs through tiny slits in the body). Notable Greek autopsists were Erasistratus and Herophilus of Chalcedon, who lived in 3rd century BC Alexandria, but in general, autopsies were rare in ancient Greece. In 44 BC, Julius Caesar was the subject of an official autopsy after his murder by rival senators, and the physician's report noted that the second stab wound Caesar received was the fatal one. By around 150 BC, ancient Roman legal practice had established clear parameters for autopsies.

The dissection of human remains for medical reasons continued to be practiced irregularly after the Romans, for instance by the Arab physicians Avenzoar and Ibn al-Nafis, but the modern autopsy process derives from the anatomists of the Renaissance. Giovanni Morgagni (1682–1771), celebrated as the father of anatomical pathology, wrote the first exhaustive work on pathology, *De Sedibus et Causis Morborum per Anatomen Indagatis* (The Seats and Causes of Diseases Investigated by Anatomy, 1769).

The great nineteenth-century medical researcher Rudolf Virchow, in response to a lack of standardization of autopsy procedures, established and published specific autopsy protocols (one such protocol still bears his name).

Purpose

The principal aim of an autopsy is to determine the cause of death, the state of health of the person before he or she died, and whether any medical diagnosis and treatment before death was appropriate.

In most Western countries the number of autopsies performed in hospitals has been decreasing every year since 1955. Critics, including pathologist and former *JAMA* editor George Lundberg, have charged that the reduction in autopsies is negatively affecting the

care delivered in hospitals, because when mistakes result in death, they are often not investigated and lessons therefore remain unlearned.

When a person has given permission in advance of their death, autopsies may also be carried out for the purposes of teaching or medical research.

An autopsy is frequently performed in cases of sudden death, where a doctor is not able to write a death certificate, or when death is believed to result from an unnatural cause. These examinations are performed under a legal authority (Medical Examiner or Coroner or Procurator Fiscal) and do not require the consent of relatives of the deceased. The most extreme example is the examination of murder victims, especially when medical examiners are looking for signs of death or the murder method, such as bullet wounds and exit points, signs of strangulation, or traces of poison. Many religions such as Judaism and Islam usually discourage the performing of autopsies on their adherents. Organizations such as Zaka in Israel and Misaskim in the USA generally guide families how to ensure that an unnecessary autopsy is not made.

In medicine

Autopsies are important in clinical medicine as they can identify medical error and assist continuous improvement.

A study that focused on myocardial infarction (heart attack) as a cause of death found significant errors of omission and commission, i.e. a sizable number cases ascribed to myocardial infarctions (MIs) were not MIs and a significant number of non-MIs were actually MIs.

A systematic review of studies of the autopsy calculated that in about 25% of autopsies a major diagnostic error will be revealed. However, this rate has decreased over time and the study projects that in a contemporary US institution, 8.4% to 24.4% of autopsies will detect major diagnostic errors.

A large meta-analysis suggested that approximately one third of death certificates are incorrect and that half of the autopsies performed produced findings that were not suspected before the person died. Also, it is thought that over one fifth of unexpected findings can only be diagnosed histologically, i.e. by biopsy or autopsy, and that approximately one quarter of unexpected findings, or 5% of all findings, are major and can similarly only be diagnosed from tissue.

One study found that "Autopsies revealed 171 missed diagnoses, including 21 cancers, 12 strokes, 11 myocardial infarctions, 10 pulmonary emboli, and 9 endocarditis, among others".

Focusing intubated patients, one study found "abdominal pathologic conditions--abscesses, bowel perforations, or infarction--were as frequent as pulmonary emboli as a cause of class I errors. While patients with abdominal pathologic conditions generally

complained of abdominal pain, results of examination of the abdomen were considered unremarkable in most patients, and the symptom was not pursued".

In veterinary medicine

A field post-mortem exam of a ewe

Post-mortem examination is far more common in veterinary medicine than in human medicine. For many species that exhibit few external symptoms (sheep), or that are not suited to detailed clinical examination (poultry, cage birds, zoo animals), it is a common method used by veterinarians to come to a diagnosis.

Types

There are three main types of autopsies:

- *Medico-Legal Autopsy or Forensic* or *coroner's autopsies* seek to find the cause and manner of death and to identify the decedent. They are generally performed, as prescribed by applicable law, in cases of violent, suspicious or sudden deaths, deaths without medical assistance or during surgical procedures.

- *Clinical* or *Pathological autopsies* are performed to diagnose a particular disease or for research purposes. They aim to determine, clarify, or confirm medical diagnoses that remained unknown or unclear prior to the patient's death.

- *Anatomical* or *academic autopsies* are performed by students of anatomy for study purpose only.

Forensic autopsy

Autopsy room of the Charité Berlin

A forensic autopsy is used to determine the cause of death. Forensic science involves the application of the sciences to answer questions of interest to the legal system. In United States law, deaths are placed in one of five manners:

- Natural
- Accident
- Homicide
- Suicide
- Undetermined

In some jurisdictions, the Undetermined category may include deaths in absentia, such as deaths at sea and missing persons declared dead in a court of law; in others, such deaths are classified under "Other". But, medical examiners also attempt to determine the time of death, the exact cause of death, and what, if anything, preceded the death, such as a struggle. A forensic autopsy may include obtaining biological specimens from the deceased for toxicological testing, including stomach contents. Toxicology tests may reveal the presence of one or more chemical "poisons" (all chemicals, in sufficient quantities, can be classified as a poison) and, the quantity of those chemicals. Because post-mortem deterioration of the body, together with the gravitational pooling of bodily fluids, will necessarily alter the bodily environment, toxicology tests may overestimate, rather than underestimate, the quantity of the suspected chemical.

Most states require the State medical examiner to complete an autopsy report and many mandate that the autopsy be videotaped.

Following an in-depth examination of all the evidence, a medical examiner or coroner will assign a manner of death as one of the five listed above, and detail the evidence on the mechanism of the death.

Clinical autopsy

Clinical autopsies serve two major purposes. They are performed to gain more insight into pathological processes and determine what factors contributed to a patient's death. Autopsies are also performed to ensure the standard of care at hospitals. Autopsies can yield insight into how patient deaths can be prevented in the future.

Within the United Kingdom, clinical autopsies can only be carried out with the consent of the family of the deceased person as opposed to a medico-legal autopsy instructed by a Coroner (England & Wales) or Procurator Fiscal (Scotland) to which the family cannot object.

Prevalence

In 2004 in England and Wales, there were 514,000 deaths of which 225,500 were referred to the coroner. Of those, 115,800 (22.5%) resulted in post-mortem examinations and there were 28,300 inquests, 570 with a jury.

In the United States, autopsy rates fell from 17% in 1980 to 14% in 1985 and 11.5% in 1989, although the figures vary notably from county to county.

Process

Cadaver dissection table. Similar to those used in medical or forensic autopsies.

The body is received at a medical examiner's office or hospital in a body bag or evidence sheet. A brand new body bag is used for each body to ensure that only evidence from that body is contained within the bag. Evidence sheets are an alternate way to transport the body. An evidence sheet is a sterile sheet that the body is covered in when it is moved. If it is believed there may be any significant residue on the hands, for instance gunpowder, a separate paper sack is put around each hand and taped shut around the wrist.

There are two parts to the physical examination of the body: the external and internal examination. Toxicology, biochemical tests and/or genetic testing often supplement these and frequently assist the pathologist in assigning the cause or causes of death.

External examination

At many institutions the person responsible for handling, cleaning, and moving the body is often called a diener, the German word for *servant*. In the UK this role is performed by an Anatomical Pathology Technologist who will also assist the pathologist in eviscerating the deceased and reconstruction after the autopsy. After the body is received, it is first photographed. The examiner then notes the kind of clothes and their position on the body before they are removed. Next, any evidence such as residue, flakes of paint or other material is collected from the external surfaces of the body. Ultraviolet light may also be

used to search body surfaces for any evidence not easily visible to the naked eye. Samples of hair, nails and the like are taken, and the body may also be radiographically imaged.

Once the external evidence is collected, the body is removed from the bag, undressed, and any wounds present are examined. The body is then cleaned, weighed, and measured in preparation for the internal examination. The scale used to weigh the body is often designed to accommodate the cart that the body is transported on; its weight is then deducted from the total weight shown to give the weight of the body.

If not already within an autopsy room, the body is transported to one and placed on a table. A general description of the body as regards ethnicity, sex, age, hair color and length, eye color and other distinguishing features (birthmarks, old scar tissue, moles, etc.) is then made. A handheld voice recorder or a standard examination form is normally used to record this information. In some countries e.g. France, Germany, and Canada, an autopsy may comprise an external examination only. This concept is sometimes termed a "view and grant". The principles behind this being that the medical records, history of the deceased and circumstances of death have all indicated as to the cause and manner of death without the need for an internal examination.

Internal examination

If not already in place, a plastic or rubber brick called a "body block" is placed under the back of the body, causing the arms and neck to fall backward whilst stretching and pushing the chest upward to make it easier to cut open. This gives the prosector, a pathologist or assistant, maximum exposure to the trunk. After this is done, the internal examination begins. The internal examination consists of inspecting the internal organs of the body for evidence of trauma or other indications of the cause of death. For the internal examination there are a number of different approaches available:

- a large and deep Y-shaped incision can be made starting at the top of each shoulder and running down the front of the chest, meeting at the lower point of the sternum. This is the approach most often used.

- a T-shaped incision made from the tips of both shoulder, in a horizontal line across the region of the collar bones to meet at the sternum (breastbone) in the middle.

- a single vertical cut is made from the middle of the neck (in the region of the 'adam's apple' on a male body)

In all of the above cases the cut then extends all the way down to the pubic bone (making a deviation to the left side of the navel).

Bleeding from the cuts is minimal, or non-existent, because the pull of gravity is producing the only blood pressure at this point, related directly to the complete lack of

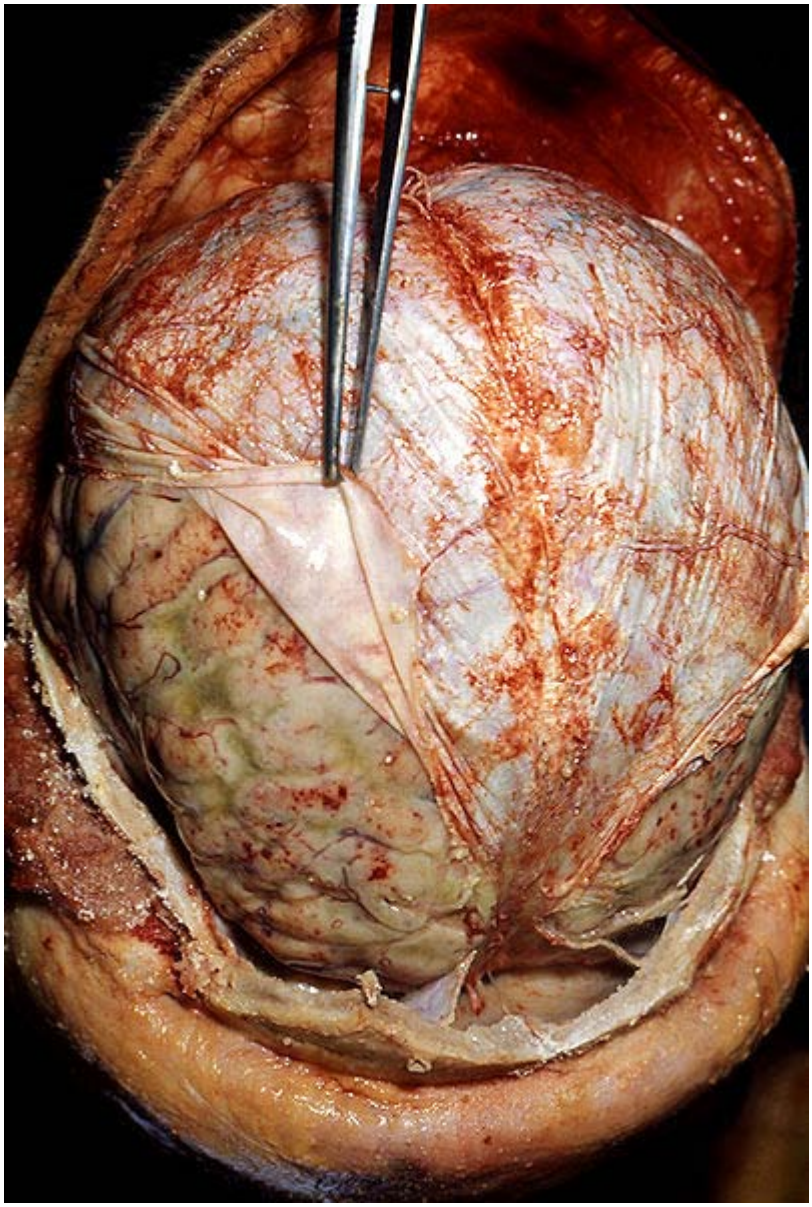
cardiac functionality. However, in certain cases there is anecdotal evidence to prove that bleeding can be quite profuse, especially in cases of drowning.

At this point shears are used to open the chest cavity. It is also possible to utilise a simple scalpel blade. The prosector uses the tool to saw through the ribs on the lateral sides of the chest cavity to allow the sternum and attached ribs to be lifted as one chest plate; this is done so that the heart and lungs can be seen in situ and that the heart, in particular the pericardial sac is not damaged or disturbed from opening. A scalpel is used to remove any soft tissue that is still attached to the posterior side of the chest plate. Now the lungs and the heart are exposed. The chest plate is set aside and will be eventually replaced at the end of the autopsy.

At this stage the organs are exposed. Usually, the organs are removed in a systematic fashion. Making a decision as to what order the organs are to be removed will depend highly on the case in question. Organs can be removed in several ways: The first is the en masse technique of letulle whereby all the organs are removed as one large mass. The second is the en bloc method of Ghon. The most popular in the UK is a modified version of this method which is divided into four groups of organs. Although these are the two predominant evisceration techniques in the UK variations on these are widespread.

One method is described here: The pericardial sac is opened to view the heart. Blood for chemical analysis may be removed from the inferior vena cava or the pulmonary veins. Before removing the heart, the pulmonary artery is opened in order to search for a blood clot. The heart can then be removed by cutting the inferior vena cava, the pulmonary veins, the aorta and pulmonary artery, and the superior vena cava. This method leaves the aortic arch intact, which will make things easier for the embalmer. The left lung is then easily accessible and can be removed by cutting the bronchus, artery, and vein at the hilum. The right lung can then be similarly removed. The abdominal organs can be removed one by one after first examining their relationships and vessels.

Some pathologists, however, prefer to remove the organs all in one "block". Then a series of cuts, along the vertebral column, are made so that the organs can be detached and pulled out in one piece for further inspection and sampling. During autopsies of infants, this method is used almost all of the time. The various organs are examined, weighed and tissue samples in the form of slices are taken. Even major blood vessels are cut open and inspected at this stage. Next the stomach and intestinal contents are examined and weighed. This could be useful to find the cause and time of death, due to the natural passage of food through the bowel during digestion. The more area empty, the longer the deceased had gone without a meal before death.



A brain autopsy demonstrating signs of meningitis. The forceps (center) are retracting the dura mater (white). Underneath the dura mater are the leptomeninges, which appear to be edematous and have multiple small hemorrhagic foci.

The body block that was used earlier to elevate the chest cavity is now used to elevate the head. To examine the brain, an incision is made from behind one ear, over the crown of the head, to a point behind the other ear. When the autopsy is completed, the incision can be neatly sewn up and is not noticed when the head is resting on a pillow in an open casket funeral. The scalp is pulled away from the skull in two flaps with the front flap going over the face and the rear flap over the back of the neck. The skull is then cut with what is called a Stryker saw, named for its manufacture, to create a "cap" that can be pulled off, exposing the brain. The brain is then observed in situ. Then the brain's connection to the cranial nerves and spinal cord are severed, and the brain is then lifted

out of the skull for further examination. If the brain needs to be preserved before being inspected, it is contained in a large container of formalin (15 percent solution of formaldehyde gas in buffered water) for at least two but preferably four weeks. This not only preserves the brain, but also makes it firmer allowing easier handling without corrupting the tissue.

Reconstitution of the body

An important component of the autopsy is the reconstitution of the body such that it can be viewed, if desired, by relatives of the deceased following the procedure. After the examination, the body has an open and empty chest cavity with chest flaps open on both sides, the top of the skull is missing, and the skull flaps are pulled over the face and neck. It is unusual to examine the face, arms, hands or legs internally. In the UK, following the Human Tissue Act 2004 all organs and tissue must be returned to the body unless permission is given by the family to retain any tissue for further investigation. Normally the internal body cavity is lined with cotton wool or an appropriate material, the organs are then placed into a plastic bag to prevent leakage and returned to the body cavity. The chest flaps are then closed and sewn back together and the skull cap is sewed back in place. Then the body may be wrapped in a shroud and it is common for relatives of the deceased to not be able to tell the procedure has been done when the deceased is viewed in a funeral parlor after embalming.

Chapter 20

Gastrointestinal Pathology

Gastrointestinal pathology is the subspecialty of surgical pathology which deals with the diagnosis and characterization of neoplastic and non-neoplastic diseases of the digestive tract and accessory organs, such as the pancreas and liver.

Sub-specialty recognition and Board Certification

Gastrointestinal pathology (including liver, gallbladder and pancreas) is a recognized sub-specialty discipline of surgical pathology. Recognition of a sub-specialty is generally related to dedicated fellowship training offered within the subspecialty or, alternatively, to surgical pathologists with a special interest and extensive experience in GI pathology. There are approximately 30 gastrointestinal pathology fellowship offered within the United States (predominantly academic, and more recently three "corporate" fellowships). This translates to fewer than 40 fellowship trained gastrointestinal pathologists being trained annually in the United States each year.

Fellowship in gastrointestinal pathology involves:
(1) diagnostic evaluation of surgical (whole organ) and biopsy pathology of gastrointestinal tissue, [with the exception of at least one corporate fellowship]
(2) consistent interaction with clinical colleagues (gastroenterologists, colorectal surgeons and gastrointestinal radiologists) to ensure understanding of the clinical aspects of gastrointestinal disease, treatment modalities and other diagnostic findings;
(3) research in gastrointestinal physiology, disease mechanisms and histomorphology and
(4) education of general pathologists and clinical colleagues. During the course of a one year gastrointestinal pathology fellowship, the GI-liver pathology fellow will review between 8,000 and 15,000 gastrointestinal and liver biopsy and surgical specimens with all clinical history, laboratory data and frequently, knowledge of response to treatment. This volume of cases is similar to approximately five years of case experience for general surgical pathologists in private practice.
Board certification in the United States requires approval by the certifying body: The American Board of Pathology. There has been considerable debate among academic and private practice gastrointestinal pathologists regarding the necessity, advantages and disadvantages of Board Certification in this subspecialty. The debate was last formally approached in 2001, during Dr. Joel K. Greenson's tenure as President of the Rodger C. Haggitt Gastrointestinal Pathology Society. The decision to seek Board Certification was declined by the membership of the Society.

History of the Rodger C. Haggitt Gastrointestinal Pathology Society

At the 1976 annual meeting of the organization then called the International Academy of Pathology (IAP), now the United States and Canadian Academy of Pathology (USCAP), the long course was devoted to gastrointestinal pathology. Due to its success, the first evening subspecialty conference devoted to gastrointestinal pathology was presented the following year at the IAP annual meeting in March 1977. That first evening session was organized by Jack Yardley from Johns Hopkins University, and included Henry Appelman (The University of Michigan), Harvey Goldman (Beth Israel Hospital/Harvard Medical School), Bill Hawk (The Cleveland Clinic), Tom Kent (The University of Iowa), Si-Chun Ming (Temple University), Tom Norris (The University of Washington), and Robert Riddell (The University of Chicago). This group, headed by Henry Appelman, organized a group for gastrointestinal pathologists. Every gastrointestinal pathologist in the United States and Canada received an invitation to attend the first organizational meeting, held the 1979 IAP meeting. At that meeting, the establishment of a society was approved, and four subcommittees were formed to deal with membership, finances, bylaws, and educational activities. During the next year, all organizational functions were completed, and at the 1980 IAP meeting, the organization was officially established, by-laws were approved, the first officers elected, the first dues collected, and the first educational program was offered. The original name for the organization was the Gastrointestinal Pathology Club. Only later did it achieve "Society" status. In 2001, after the tragic and untimely death of Dr. Rodger Haggitt, the name of the society was officially changed to Rodger C. Haggitt Gastrointestinal Pathology Society. The organization was developed as a mechanism:

1. for propagating interest in gastrointestinal pathology
2. for emphasizing the importance of gastrointestinal pathology as a sophisticated and complex area of pathology
3. for sharing information among members
4. for developing joint investigative efforts among its members
5. for establishing referral centers or referral mechanisms for specific types of case problems
6. for devising a registry for unusual cases.

The first three of these initial goals were accomplished early on. The Club was recognized as a companion society of the IAP and its first scientific session was presented about a year later. The fourth goal took longer, but it, too, has been fulfilled as exemplified by a published study on adenomas with invasive carcinoma that was shepherded by Harry Cooper. It took e-mail technology to really allow the fifth goal, the referral network, to operate. The development of an e-mail group from the Society, which became an opportunity for members to consult a wide group of GI colleagues regarding problem cases, has been functional sine 1995. The sixth goal, the registry issue, has yet to get off the ground. Currently, the Society has also awards prizes for resident presentations at the USCAP annual meeting and organizes educational symposia at national meetings, including the IAP, the American Society of Clinical Pathologists, Digestive Disease Week and the international congresses of the International Academy of Pathology.

Current Impact of Gastrointestinal Pathology as a Sub-Specialty

Digestive diseases affect 1 in 5 Americans annually – at least 60 million people in the United States each year. Digestive diseases account for approximately 50 million physician visits, nearly 25 million endoscopic diagnostic procedures and over 20 million gastrointestinal specimens directed to pathologists – representing $87 billion in direct medical costs attributable to gastrointestinal disease. The demand for gastroenterology care and diagnostic procedures continue to increase, driven by an increasing incidence of gastrointestinal disease and the need for colorectal cancer screening in the aging U.S. population. By 2010, gastroenterologists will perform at least 40 million endoscopic procedures annually, translating into $40 billion endoscopy and $12 billion related gastrointestinal pathology expenses annually in the U.S.

With less than 400 fellowship-trained gastrointestinal pathologists in practice in the United States, approximately 8% of the 20+ million gastrointestinal biopsies currently performed each year are reviewed by pathologists with fellowship training in digestive health and disease. The remaining 90 to 92% of biopsies are reviewed by highly skilled general surgical pathologists including some who have special interest in gastrointestinal pathology.

General surgical pathologists who practice with a focus in gastrointestinal pathology may attend continuing medical education courses offered by fellowship-trained academic and private practice gastrointestinal pathologists, may attend intensive "mini-fellowships" that are offered by select institutions (i.e. AFIP) to further hone their diagnostic and develop close working relationships with gastroenterologists to ensure a broad knowledge base with regards to clinical correlation of the findings under the microscope.

Whether a fellowship trained gastrointestinal pathologist, a general surgical pathologist with subspecialty interest in GI or an adept general surgical pathologist is reviewing a particular biopsy/surgical specimen, it is important to keep in mind that the training for the specialty of surgical pathology is rigorous. Following completion of both college and medical school, the surgical pathologist must have also completed an accredited residency in pathology and is board certified in Anatomic Pathology by the American Board of Pathology. Residency in pathology is one of the longest postgraduate training programs, encompassing 4–5 years. (In comparison, internal medicine and pediatrics are only 3 years.)

In most instances, gastrointestinal biopsies and surgical specimens represent common diseases that display common histomorphologic patterns, and are accurately evaluated and classified by adept general pathologists. Similar to many aspects of medicine, not all gastrointestinal tissue specimens require sub-specialist review for the accurate diagnosis to be rendered. Internal medicine physicians treat many gastrointestinal conditions without referral to a gastroenterologist specialist. Similarly, many gastrointestinal tissue biopsies and surgical specimens are accurately diagnosed without referral to a gastrointestinal pathologist. Certain diseases are histologically subtle or the differential diagnosis is complicated or complex. In such cases a close working relationship between

the pathologist and gastroenterologist with correlation of clinical, endoscopic, and biopsy findings may be of great benefit to the submitting physician and to the patient.

Appropriate biopsy classification of GI biopsies is critical. Current literature and other non-published reviews estimate that at least 10% of the diagnoses of Barrett's esophagus, a precancerous condition, are rendered in error. At least 15 – 20% of stomach biopsies have misclassified disease characteristics, 15% of colon polyps are misdiagnosed, and up to 50% of all cases of chronic inflammatory bowel disease are misdiagnosed or misclassified.

Though pathology diagnosis is a critical determinant of future cancer risk and screening interval, many of these diagnoses do not result in significant morbidity or mortality for patients; thus, errors may be "masked" by the limited effect to the patient in the present context.

The economic cost of inaccurate pathology diagnoses can be measured from the perspective of the healthcare system, the gastroenterologist practice, and the patient. Within the healthcare system, gastroenterology pathology diagnostic inaccuracy of 10% is equivalent to 2 million misdiagnoses each year. The vast majority of these patients will incur additional medical expenses from repeat clinician office visits; repeat diagnostic procedures, unnecessary enrollment in surveillance programs, and treatment with incorrect or completely unnecessary medications. The cost savings of the correct gastrointestinal pathology diagnosis being rendered at first opportunity by expert board certified surgical pathologists is conservatively estimated within the range of $200M – $1B.

Born out of increasing awareness of subspecialty gastrointestinal pathology services, increased marketing efforts by gastrointestinal pathology companies and increased patient awareness of laboratory errors, there is an increasing trend toward referral of tissue specimens to surgical pathologists with special interest in gastrointestinal pathology.

Subspecialty gastrointestinal pathology and Financial Relationships

Over the last decade, physician reimbursements have been declining steadily, both for clinical physicians (i.e. gastroenterologists) and for pathologists. To replace revenue lost from declining reimbursements, many physicians replace revenue by providing more services (seeing more patients) or explore capturing revenue from additional services (contract research or "ancillary services").

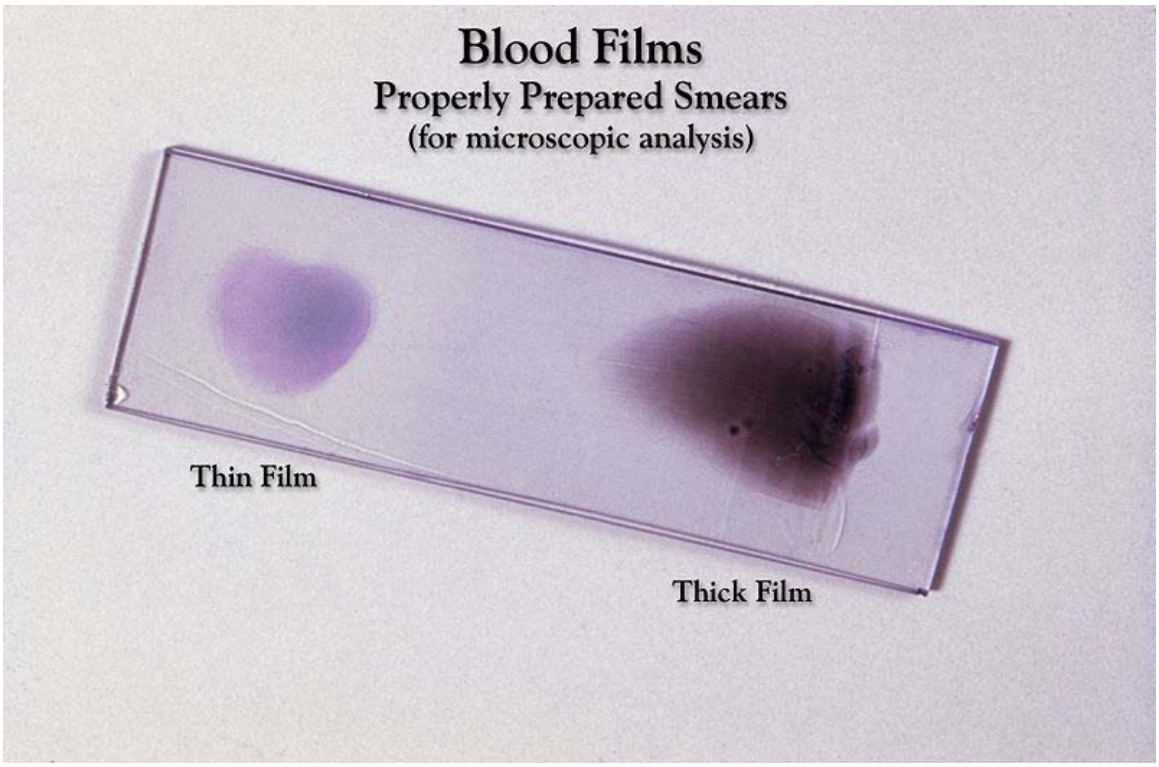
To ensure that physician's referral for additional services are motivated solely by the best interest of the patient, Federal Law (Stark) and many State laws prohibit physicians from referring patients to organizations or facilities in which they hold a financial interest - and therefore would profit from the referral.

Several pathology companies that offer "subspecialty" pathology services also offer to clinical physicians mechanisms by which the referral of their patient's tissue biopsies to a specific laboratory may result in additional revenue for the physician's practice. These mechanisms are generally designed to be practiced within the boundaries of certain loopholes in Stark laws and state regulations.

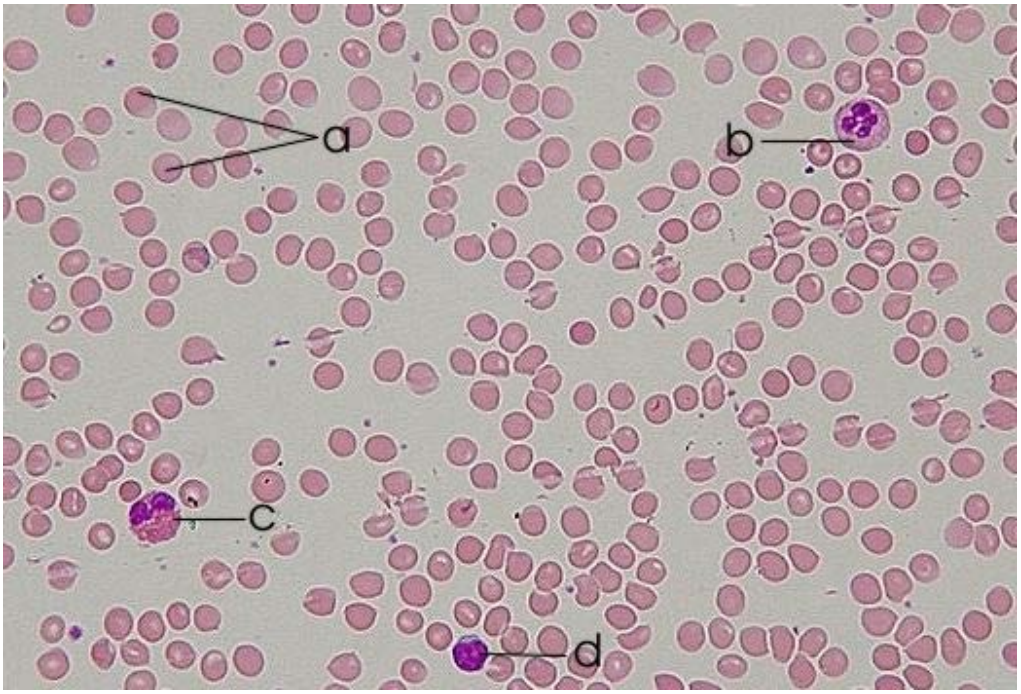
These financial relationships include:
1) Discounted client billing.
Client billing refers to a purchased services agreement between the physician caring for the patient and taking the biopsy and the pathology laboratory preparing the histologic slides and reviewing the biopsy material. The clinician and pathology company enter into a contract in which the clinican group buys pathology services from the pathologist at a rate substantially less than what the pathologist would be paid by insurance companies for the same service. The pathologist accepts payment from the clinician for the services as full compensation for pathology services. The clinician then bills and collection from the patient's insurance company for the full amount of the pathology service. In most instances, the clinician realizes a profit ($10 – 100) for each biopsy referred to the laboratory.
2) Condominium (pod) laboratories. 3) Technical component/professional component splitting.
4) Clinician owned histology laboratories.
5) Reverse TC/PC splitting.

Chapter 21

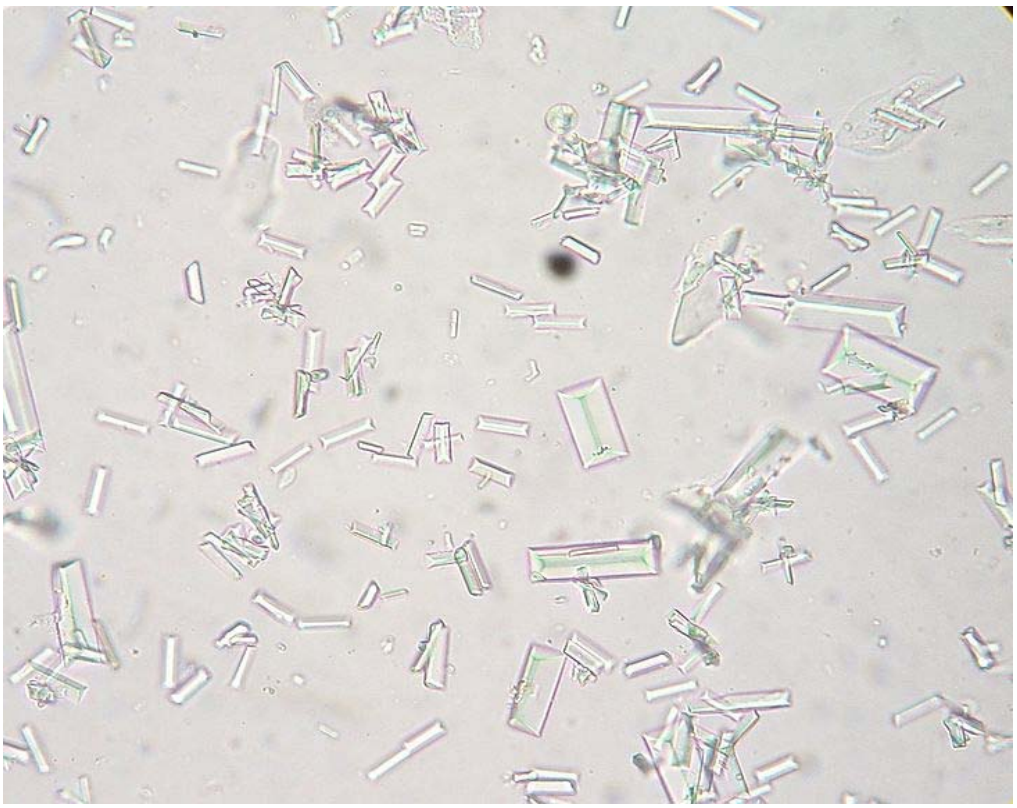
Clinical Pathology



Hematology: Blood smears on a glass slide, stained and ready to be examined under the microscope.

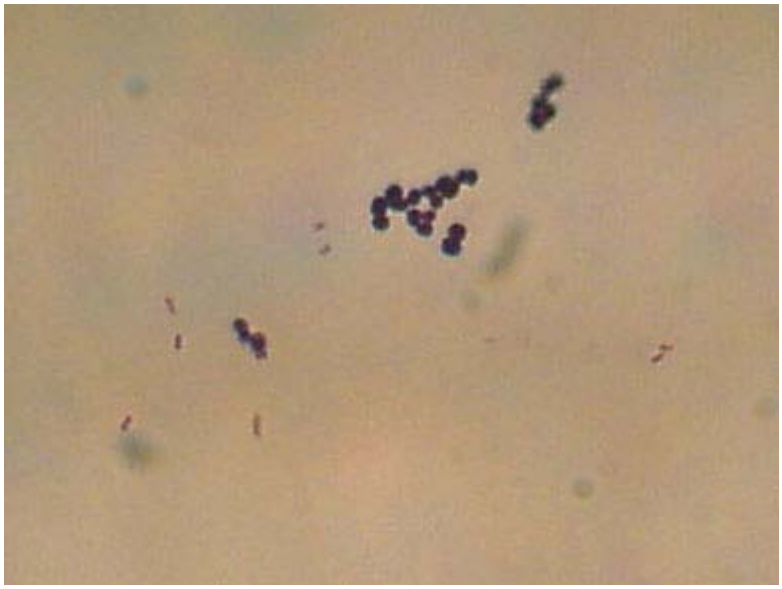


Hematology: microscopic image of a normal blood smear. a:erythrocytes, b:neutrophil, c:eosinophil, d:lymphocyte.



Clinical chemistry: microscopic image of crystals in urine

Bacteriology: Agar plate with bacterial colonies.



Bacteriology: microscopic image of a mixture of two types of bacteria stained with the Gram stain.

Clinical chemistry: an automated blood chemistry analyser.

Clinical pathology (US, UK, Commonwealth, Portugal, Italy), Laboratory Medicine (Germany, Romania, Poland, Eastern Europe), Clinical analysis (Spain) or Clinical/Medical Biology (France, Belgium, Netherlands, Austria, North and West Africa...), is a **medical specialty** that is concerned with the diagnosis of disease based on the laboratory analysis of bodily fluids, such as blood, urine, and tissues using the tools of chemistry, microbiology, hematology and molecular pathology. This specialty requires a medical residency and should not be confused with Biomedical science which is not necessarily related to medicine.

Licensing and subspecialties

The American Board of Pathology certifies clinical pathologists, and recognizes the following secondary specialties of clinical pathology:

- Chemical pathology, also called clinical chemistry
- Hematopathology
- Blood banking - Transfusion medicine
- Clinical microbiology
- Cytogenetics
- Molecular genetics pathology.

In some countries other subspecialties fall under certified Clinical Biologists responsibility :

- Assisted reproductive technology, Sperm bank and Semen analysis
- Immunopathology

Work organization

In United States of America

Clinical pathologists work in close collaboration with clinical scientists (clinical biochemists, clinical microbiologists, etc.), medical technologists, hospital administrators, and referring physicians to ensure the accuracy and optimal utilization of laboratory testing.

Clinical pathology is one of the two major divisions of pathology, the other being anatomical pathology. Often, pathologists practice both anatomical and clinical pathology, a combination sometimes known as general pathology. Similar specialties exist in veterinary pathology.

Clinical pathology is itself divided in to subspecialties, the main ones being clinical chemistry, clinical hematology/blood banking, hematopathology and clinical microbiology and emerging subspecialities such as molecular diagnostics and proteomics. Many areas of clinical pathology overlap with anatomic pathology. Both can serve as medical directors of CLIA certified laboratories. This overlap includes immunoassays,

flow cytometry, microbiology and cytogenetics and any assay done on tissue. Overlap between anatomic and clinical pathology is expanding to molecular diagnostics and proteomics as we move towards making the best use of new technologies for personalized medicine.

Studies and diplomas

Clinical pathologists are often medical doctors. In some countries in South-America, Europe, Africa or Asia, this specialty can be practiced by non-physicians, such as Pharm.D after a variable number of years of residency. For example, in France, Clinical Pathology is called Medical Biology ("Biologie médicale") and is both practiced by M.D.s and Pharm.D and this residency lasts four years. Specialists in this discipline are not called "Clinical pathologist" but "Clinical Biologist".

Place of work

Tools of Clinical Pathology

Microscopes, analysers, strips, centrifugal machines...

Macroscopic examination

The visual examination of the taken liquid is a first main indication for the pathologist or the physician. The aspect of the liquid in addition conditions the analytical assumption of responsibility which follow and the validity of the end results.

Microscopic examination

The microscopic analysis is an important activity of the pathologist and the laboratory assistant. They have for that recourse to many different colourings (GRAM, MGG, Grocott, Ziehl-Neelsen…). The immunofluorescence, cytochemistry, the immunocytochimy and the FISH are also used in order to look further into the diagnosis.

This stage makes it possible to affirm the character “normal”, tumoral, inflammatory even infectious of the liquid. Indeed, the microscopic examination often makes it possible to identify a causal infectious agent, generally a bacterium, a mould, a yeast, or a parasite, more rarely a virus.

Analyzers

The analysers, by the association of robotics and spectrophotometry, allowed these last decades a better reproducibility of the results of proportionings, in particular in medical biochemistry and hematology.

The companies of in vitro diagnosis henceforth try to sell chains of automats, i.e. a system allowing the automatic transfer of the tubes towards the various types of automats

of the same mark. These systems can include the computer-assisted management of a serum library.

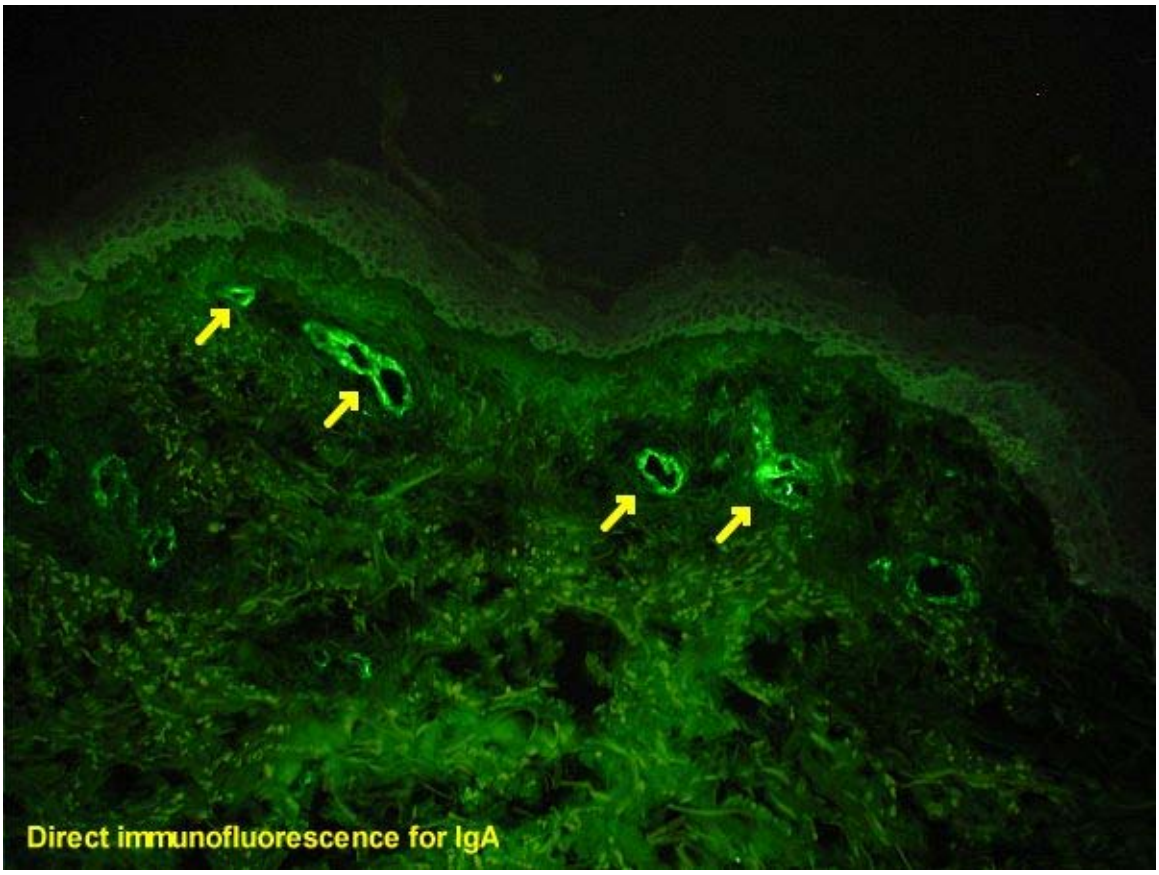
These analysers must undergo daily controls to guarantee a result just possible, one speaks about quality control. These analysers must also undergo daily, weekly and monthly maintenances.

Cultures

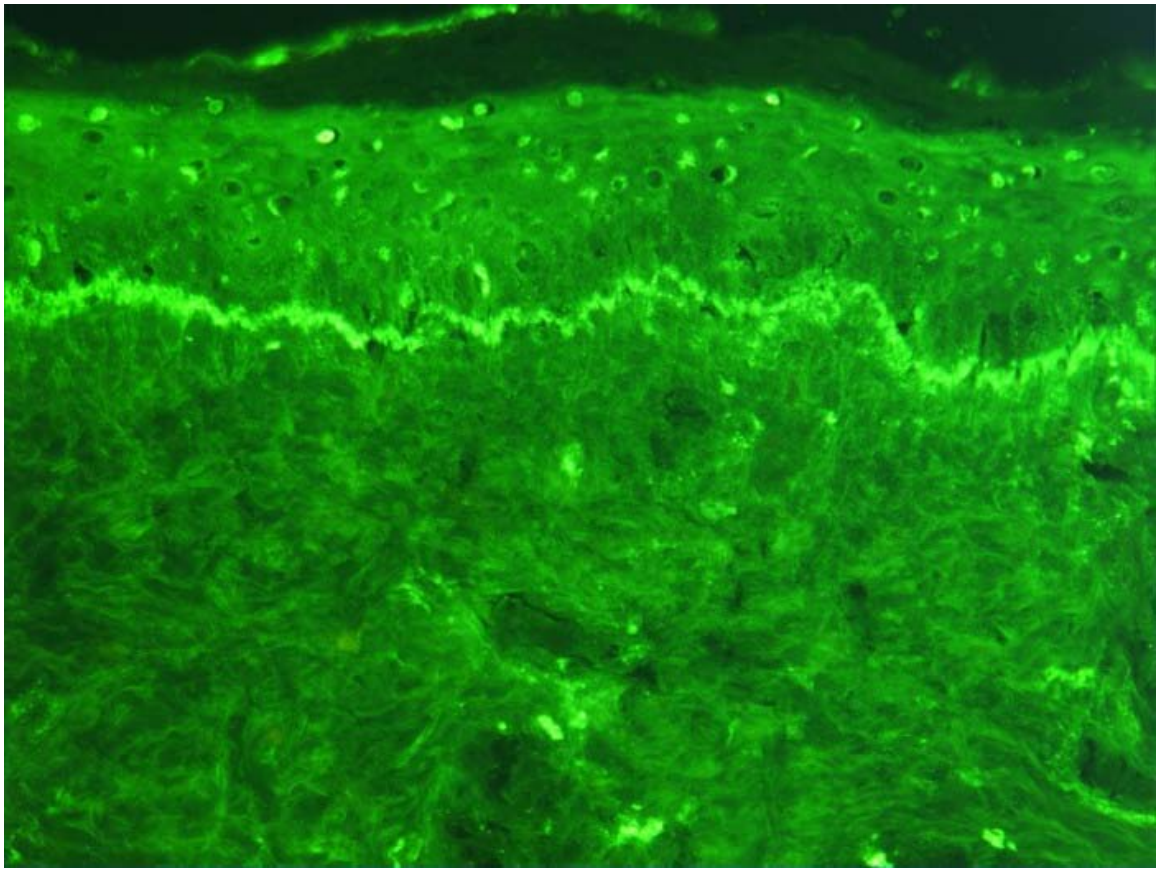
A big part of the examinations of clinical pathology, primarily in medical microbiology, use culture media. Those allow, for example, the description of one or several infectious agents responsible of the clinical signs.

Chapter 22

Immunofluorescence



Microphotograph of a histological section of human skin prepared for **direct immunofluorescence** using an anti-IgA antibody. The skin is from a patient with Henoch-Schonlein purpura: IgA deposits are found in the walls of small superficial capillaries (yellow arrows). The pale wavy green area on top is the epidermis, the bottom fibrous area is the dermis.



Microphotograph of a histological section of human skin prepared for **direct immunofluorescence** using an anti-IgG antibody. The skin is from a patient with systemic lupus erythematosus and shows IgG deposit at two different places: The first is a band-like deposit along the epidermal basement membrane ("lupus band test" is positive). The second is within the nuclei of the epidermal cells (anti-nuclear antibodies).

Immunofluorescence is a technique used for light microscopy with a fluorescence microscope and is used primarily on biological samples. This technique uses the specificity of antibodies to their antigen to target fluorescent dyes to specific biomolecule targets within a cell, and therefore allows visualisation of the distribution of the target molecule through the sample. Immunofluorescence is a widely used example of immunostaining and is a specific example of immunohistochemistry that makes use of fluorophores to visualise the location of the antibodies.

Immunofluorescence can be used on tissue sections, cultured cell lines, or individual cells, and may be used to analyse the distribution of proteins, glycans, and small biological and non-biological molecules. Immunofluoresence can be used in combination with other, non-antibody methods of fluorescent staining, for example, use of DAPI to label DNA. Several microscope designs can be used for analysis of immunofluorescence samples; the simplest is the epifluorescence microscope, and the confocal microscope is also widely used. Various super-resolution microscope designs that are capable of much higher resolution can also be used.

Types of immunofluorescence

There are two classes of immunofluorescence techniques, primary (or direct) and secondary (or indirect).

Primary (direct)

Primary, or direct, immunofluorescence uses a single antibody that is chemically linked to a fluorophore. The antibody recognises the target molecule and binds to it, and the fluorophore it carries can be detected via microscope. This technique has several advantages over the secondary (or indirect) protocol below because of the direct conjugation of the antibody to the fluorophore. This reduces the number of steps in the staining procedure, is therefore faster, and can avoid some issues with antibody cross-reactivity or non-specificity, which can lead to increased background signal.

Secondary (indirect)

Secondary, or indirect, immunofluorescence uses two antibodies; the first (the primary antibody) recognises the target molecule and binds to it, and the second (the secondary antibody), which carries the fluorophore, recognises the primary antibody and binds to it. This protocol is more complex than the primary (or direct) protocol above and takes more time but allows more flexibility.

This protocol is possible because an antibody consists of two parts, a variable region (which recognizes the antigen) and an invariant region (which makes up the structure of the antibody molecule). A researcher can generate several primary antibodies that recognize various antigens (have different variable regions), but all share the same invariant region. All these antibodies may therefore be recognized by a single secondary antibody. This saves the cost of modifying the primary antibodies to directly carry a fluorophore.

Different primary antibodies with different invariant regions are typically generated by raising the antibody in different species. For example, a researcher might create primary antibodies in a goat that recognize several antigens, and then employ dye-coupled rabbit secondary antibodies that recognize the goat antibody invariant region ("rabbit anti-goat" antibodies). The researcher may then create a second set of primary antibodies in a mouse that could be recognised by a separate "donkey anti-mouse" secondary antibody. This allows re-use of the difficult-to-make dye-coupled antibodies in multiple experiments.

Limitations

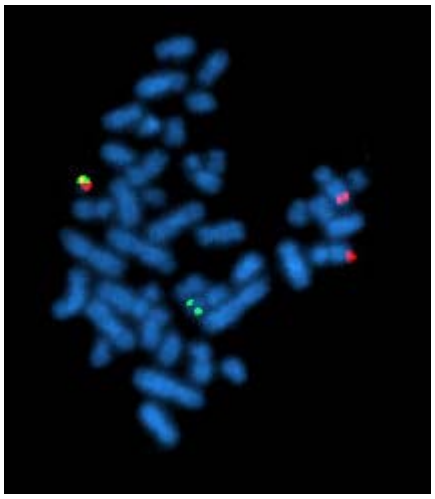
As with most fluorescence techniques, a significant problem with immunofluorescence is photobleaching. Loss of activity caused by photobleaching can be controlled by reducing the intensity or time-span of light exposure, by increasing the concentration of fluorophores, or by employing more robust fluorophores that are less prone to bleaching (e.g., Alexa Fluors, Seta Fluors, or DyLight Fluors).

Immunofluorescence is only limited to fixed (i.e., dead) cells when structures within the cell are to be visualized because antibodies cannot cross the cell membrane. Proteins in the supernatant or on the outside of the cell membrane can be bound by the antibodies, this allows for living cells can be stained. Depending on the fixative that is being used, proteins of interest might become cross-linked and this could result in either false positive or false negative signals due to unspecific binding.

An alternative approach is using recombinant proteins containing fluorescent protein domains, e.g., green fluorescent protein (GFP). Use of such "tagged" proteins allows determination of their localization in live cells. Even though this seems to be an elegant alternative to immunofluorescence, the cells have to be transfected or transduced with the GFP-tag, and as a consequence they become at least S1 or above organisms that require stricter security standards in a laboratory.

Chapter 23

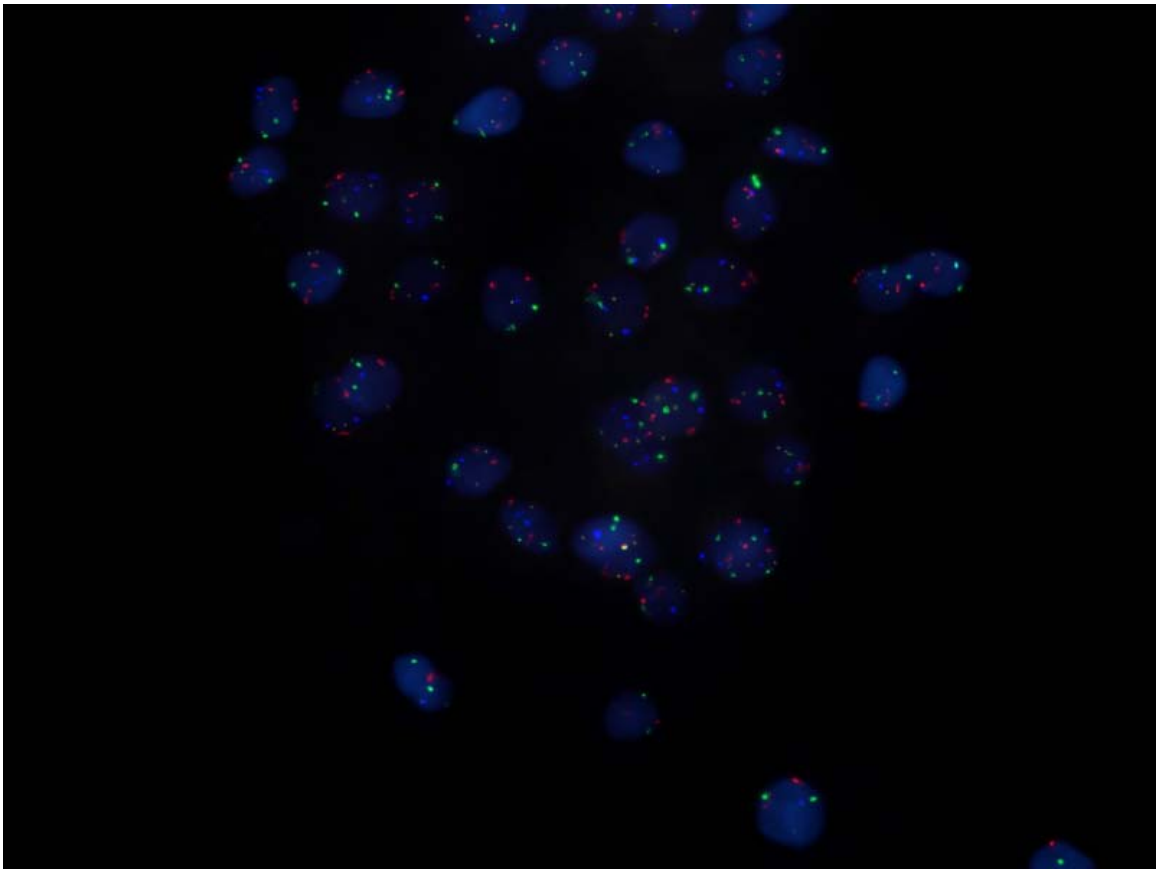
Fluorescence in Situ Hybridization



A metaphase cell positive for the *bcr/abl* rearrangement (associated with chronic myelogenous leukemia) using FISH. The chromosomes can be seen in blue. The chromosome that is labeled with green *and* red spots (upper left) is the one where the wrong rearrangement is present.

FISH (fluorescence *in situ* hybridization) is a cytogenetic technique developed by Christoph Lengauer that is used to detect and localize the presence or absence of specific DNA sequences on chromosomes. FISH uses fluorescent probes that bind to only those parts of the chromosome with which they show a high degree of sequence similarity. Fluorescence microscopy can be used to find out where the fluorescent probe bound to the chromosomes. FISH is often used for finding specific features in DNA for use in genetic counselling, medicine, and species identification. FISH can also be used to detect and localize specific mRNAs within tissue samples. In this context, it can help define the spatial-temporal patterns of gene expression within cells and tissues.

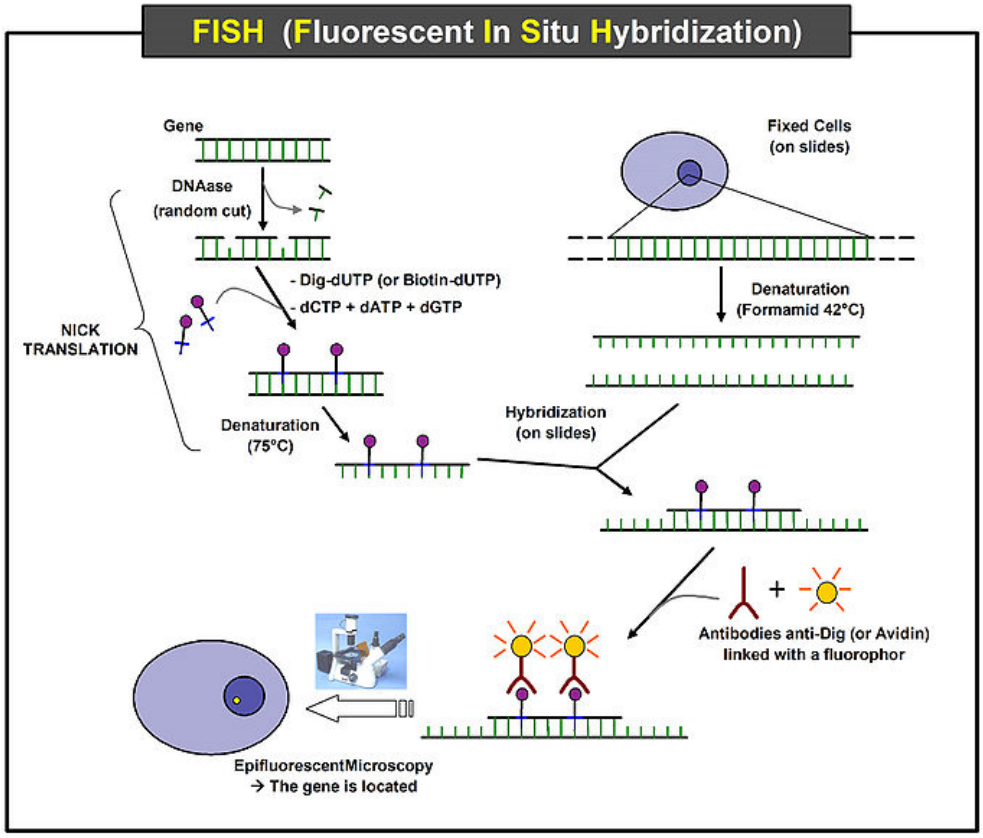
Probes



Urothelial cells marked with four different probes

Probes are often derived from fragments of DNA that were isolated, purified, and amplified for use in the Human Genome Project. The size of the human genome is so large, compared to the length that could be sequenced directly, that it was necessary to divide the genome into fragments. (In the eventual analysis, these fragments were put into order by digesting a copy of each fragment into still smaller fragments using sequence-specific endonucleases, measuring the size of each small fragment using size-exclusion chromatography, and using that information to determine where the large fragments overlapped one another.) To preserve the fragments with their individual DNA sequences, the fragments were added into a system of continually replicating bacteria populations. Clonal populations of bacteria, each population maintaining a single artificial chromosome, are stored in various laboratories around the world. The artificial chromosomes (BAC) can be grown, extracted, and labeled, in any lab. These fragments are on the order of 100 thousand base-pairs, and are the basis for most FISH probes.

Preparation and Hybridization Process



Scheme of the principle of the FISH Experiment to localize a gene in the nucleus

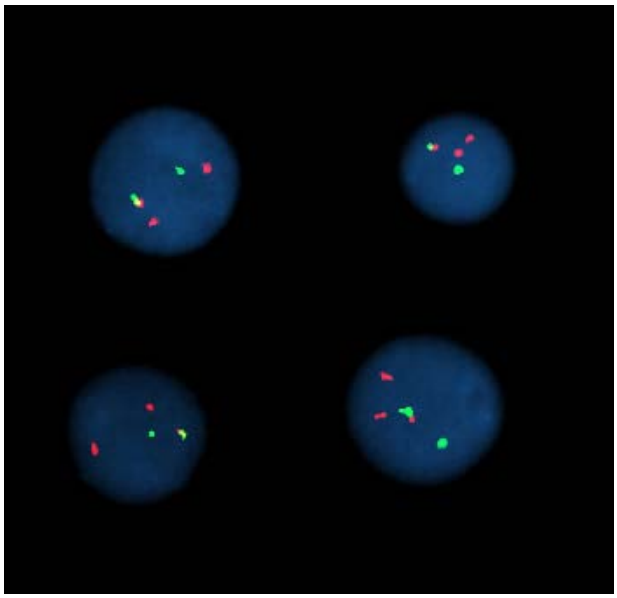
First, a probe is constructed. The probe must be large enough to hybridize specifically with its target but not so large as to impede the hybridization process. The probe is tagged directly with fluorophores, with targets for antibodies or with biotin. Tagging can be done in various ways, such as nick translation, or PCR using tagged nucleotides.

Then, an interphase or metaphase chromosome preparation is produced. The chromosomes are firmly attached to a substrate, usually glass. Repetitive DNA sequences must be blocked by adding short fragments of DNA to the sample. The probe is then applied to the chromosome DNA and incubated for approximately 12 hours while hybridizing. Several wash steps remove all unhybridized or partially-hybridized probes. The results are then visualized and quantified using a microscope that is capable of exciting the dye and recording images.

If the fluorescent signal is weak, amplification of the signal may be necessary in order to exceed the detection threshold of the microscope. Fluorescent signal strength depends on many factors such as probe labeling efficiency, the type of probe, and the type of dye. Fluorescently-tagged antibodies or streptavidin are bound to the dye molecule. These secondary components are selected so that they have a strong signal.

FISH experiments designed to detect or localize gene expression within cells and tissues rely on the use of a reporter gene, such as one expressing green fluorescent protein, to provide the fluorescence signal.

Variations on probes and analysis



Interphase cells positive for a chromosomal t(9;22) rearrangement

FISH is a very general technique. The differences between the various FISH techniques are usually due to variations in the sequence and labeling of the probes; and how they are used in combination. These few modifications make possible all FISH techniques.

Probe size is important because longer probes hybridize less specifically than shorter probes. The overlap defines the resolution of detectable features. For example, if the goal of an experiment is to detect the breakpoint of a translocation, then the overlap of the probes — the degree to which one DNA sequence is contained in the adjacent probes — defines the minimum window in which the breakpoint may be detected.

The mixture of probe sequences determines the type of feature the probe can detect. Probes that hybridize along an entire chromosome are used to count the number of a certain chromosome, show translocations, or identify extra-chromosomal fragments of chromatin. This is often called "whole-chromosome painting." If every possible probe is used, every chromosome, (the whole genome) would be marked fluorescently, which would not be particularly useful for determining features of individual sequences. However, a mixture of smaller probes can be created that is specific to a particular region (locus) of DNA; these mixtures are used to detect deletion mutations. When combined with a specific colour, a locus-specific probe mixture is used to detect very specific translocations. Special locus-specific probe mixtures are often used to count chromosomes, by binding to the centromeric regions of chromosomes, which are unique enough to identify each chromosome (with the exception of Chromosome 13, 14 21, 22.)

A variety of other techniques use mixtures of differently-colored probes. A range of colors in mixtures of fluorescent dyes can be detected, so each human chromosome can be identified by a characteristic color using whole-chromosome probe mixtures and a variety of ratios of colors. Although there are more chromosomes than easily-distinguishable fluorescent dye colors, ratios of probe mixtures can be used to create *secondary* colors. Similar to comparative genomic hybridization, the probe mixture for the secondary colors is created by mixing the correct ratio of two sets of differently-colored probes for the same chromosome. This technique is sometimes called M-FISH. The same physics that make a variety of colors possible for M-FISH can be used for the detection of translocations. That is, colors that are adjacent appear to overlap; a secondary color is observed. Some assays are designed so that the secondary color will be present or absent in cases of interest. An example is the detection of BCR/ABL translocations, where the secondary color indicates disease. This variation is often called double-fusion FISH or D-FISH. In the opposite situation---where the absence of the secondary color is pathological---is illustrated by an assay used to investigate translocations where only one of the breakpoints is known or constant. Locus-specific probes are made for one side of the breakpoint and the other intact chromosome. In normal cells, the secondary colour is observed, but only the primary colour is observed when the translocation occurs. This technique is sometimes called "break-apart FISH".

Single Molecule RNA FISH

Single Molecule RNA FISH is a method of detecting and quantifying mRNA and other long RNA molecules in a thin layer of tissue sample. Targets can be reliably imaged through the application of multiple short singly labeled oligonucleotide probes. The probes cooperatively bind to the target site. When each probe binds to the single stranded mRNA, it causes cooperative unwinding of the mRNA, promoting the binding of the next probe. The net result is the binding of 48 fluorescent labels to a single molecule of mRNA, providing sufficient fluorescence to reliably locate each target mRNA in a wide-field fluorescent microscopy image. Probes not binding to the intended sequence do not achieve sufficient localized fluorescence to be distinguished from the background. This technology is exclusively licensed to Biosearch Technologies as Stellaris™ FISH Probes.

Single molecule RNA FISH assays can be performed in simplex or multiplex, and have potential applications in cancer diagnosis , neuroscience , gene expression analysis, and companion diagnostics.

Fiber FISH

In an alternative technique to interphase or metaphase preparations, fiber FISH, interphase chromosomes are attached to a slide in such a way that they are stretched out in a straight line, rather than being tightly coiled, as in conventional FISH, or adopting a random conformation, as in interphase FISH. This is accomplished by applying mechanical shear along the length of the slide, either to cells that have been fixed to the slide and then lysed, or to a solution of purified DNA. A technique known as chromosome combing is increasingly used for this purpose. The extended conformation

of the chromosomes allows dramatically higher resolution - even down to a few kilobases. The preparation of fiber FISH samples, although conceptually simple, is a rather skilled art, and only specialized laboratories use the technique routinely.

Q-FISH

Q-FISH combines FISH with PNAs and computer software to quantify fluorescence intensity. This technique is used routinely in telomere length research.

Flow-FISH

Flow-FISH uses flow cytometry to perform FISH automatically using per-cell fluorescence measurements.

Medical applications

Often parents of children with a developmental delay want to know more about their child's conditions before choosing to have another child. These concerns can be addressed by analysis of the parents' and child's DNA. In cases where the child's developmental delay is not understood, the cause of it can be determined using FISH and cytogenetic techniques. Examples of diseases that are diagnosed using FISH include Prader-Willi syndrome, Angelman syndrome, 22q13 deletion syndrome, chronic myelogenous leukemia, acute lymphoblastic leukemia, Cri-du-chat, Velocardiofacial syndrome, and Down syndrome. FISH on sperm cells is indicated for men with an abnormal somatic or meiotic karyotype as well as those with oligozoospermia, since approximately 50% of oligozoospermic men have an increased rate of sperm chromosome abnormalities. The analysis of chromosomes 21, X, and Y is enough to identify oligozoospermic individuals at risk.

In medicine, FISH can be used to form a diagnosis, to evaluate prognosis, or to evaluate remission of a disease, such as cancer. Treatment can then be specifically tailored. A traditional exam involving metaphase chromosome analysis is often unable to identify features that distinguish one disease from another, due to subtle chromosomal features; FISH can elucidate these differences. FISH can also be used to detect diseased cells more easily than standard Cytogenetic methods, which require dividing cells and requires labor and time-intensive manual preparation and analysis of the slides by a technologist. FISH, on the other hand, does not require living cells and can be quantified automatically, a computer counts the fluorescent dots present. However, a trained technologist is required to distinguish subtle differences in banding patterns on bent and twisted metaphase chromosomes.

Species identification

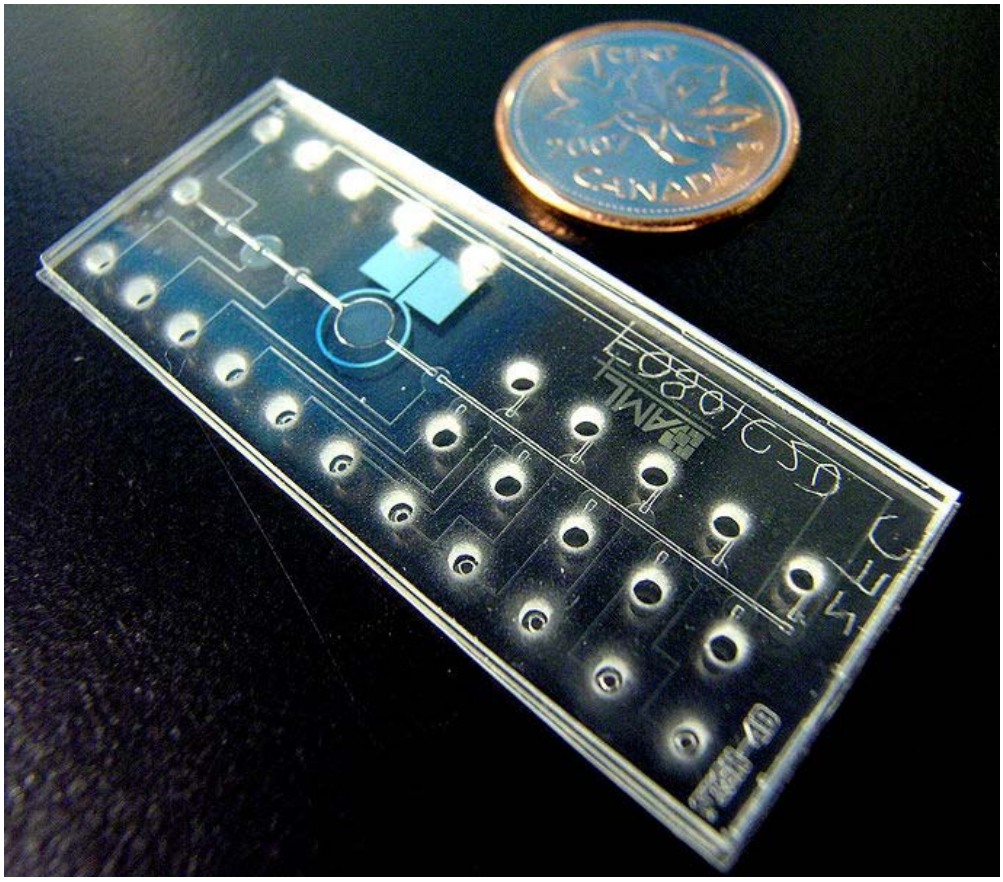
FISH is often used in clinical studies. If a patient is infected with a suspected pathogen, bacteria, from the patient's tissues or fluids, are typically grown on agar to determine the identity of the pathogen. Many bacteria, however, even well-known species, do not grow

well under laboratory conditions. FISH can be used to detect directly the presence of the suspect on small samples of patient's tissue.

FISH can also be used to compare the genomes of two biological species, to deduce evolutionary relationships. A similar hybridization technique is called a zoo blot. Bacterial FISH probes are often primers for the 16s rRNA region.

FISH is widely used in the field of microbial ecology, to identify microorganisms. Biofilms, for example, are composed of complex (often) multi-species bacterial organizations. Preparing DNA probes for one species and performing FISH with this probe allows one to visualize the distribution of this specific species within the biofilm. Preparing probes (in two different colors) for two species allows to visualize/study co-localization of these two species in the biofilm, and can be useful in determining the fine architecture of the biofilm.

Lab-on-a-chip and FISH



Microfluidic chip that automates the interphase FISH procedure. The microchip shown requires only minutes of setup time by technician, as opposed to the hours or days of labour needed to perform FISH with conventional equipment.

Although interphase fluorescence in situ hybridization (FISH) is a sensitive diagnostic tool used for the detection of chromosomal abnormalities on cell-by-cell basis, the cost-per-test and the technical complexity of current FISH protocols has inhibited its widespread utilization. Lab-on-a-chip or microfluidic devices, incorporate networks of microchannels that can miniaturize, integrate and automate conventional analytical techniques onto chip-style platforms. Since microchannels permit sophisticated levels of fluid control (down to picolitres), these devices can reduce analysis times, lower reagent consumption, and minimize human intervention.

Currently, FISH has been performed on glass microfluidic platforms that standardize much of the protocol offering repeatable results that are accurate, cost-effective and easier to obtain in a clinical setting.

Compared to conventional FISH methods, these first implementations of on-chip FISH provide a 10-fold higher throughput and a 10-fold reduction in the cost of testing, enabling the simultaneous assessment of several chromosomal abnormalities or patients. It is increasingly essential that diagnostic tests determine the type and extent of chromosomal abnormalities for more informed diagnosis and for appropriate choice of treatment strategies. Since the on-chip FISH technique is 10-20 times more cost-effective than conventional methods, and can be fully integrated and automated, this technology will make widespread genetic testing of patients more accessible in a clinical setting.

Recently, the first demonstration of Metaphase FISH on chip has led to renewed efforts towards automating the metaphase FISH protocol. Metaphase FISH had continued to be difficult to integrate owing to the complex sample preparation protocol often spanning over 3 weeks. New reports confirm that a research group in Denmark have tested successfully a novel lab on chip device to integrate the entire sample preparation protocol for Metaphase FISH called FISHprep.

Virtual Karyotype

Virtual karyotyping is another cost-effective, clinically available alternative to FISH panels uses thousands to millions of probes on a single array to detect copy number changes, genome-wide, at unprecedented resolution.

Chapter 24

High-Intensity Focused Ultrasound

HIFU (**High-Intensity Focused Ultrasound**) (sometimes **FUS** or **HIFUS**) is a highly precise medical procedure using high-intensity focused ultrasound to heat and destroy pathogenic tissue rapidly. It is one modality of therapeutic ultrasound, and, although it induces hyperthermia, it should not be confused with this technique, which heats much less rapidly and to much lower therapeutic temperatures (in general < 45°C).

Clinical HIFU procedures are typically image-guided to permit treatment planning and targeting before applying a therapeutic or ablative level of ultrasound energy. When MRI is used for guidance, the technique is sometimes called **Magnetic Resonance-guided Focused Ultrasound**, often shortened to **MRgFU**. When ultrasonography is used, the technique is sometimes called **Ultrasound-guided Focused Ultrasound**, often shortened to **USgFUS**. Magnetic resonance imaging (MRI) is used to identify tumors or fibroids in the body, before they are destroyed by the ultrasound. MRgFU is currently used in Australia, the United States, Canada, Israel, Europe, and Asia to treat uterine fibroids. Ultrasonography guided HIFU is currently used in the United Kingdom, Italy, Spain, Korea, Japan, Hong Kong, Malaysia, Russia, China, Romania and Bulgaria. Current clinical trials are underway, examining the possible use of HIFU in the treatment of cancers of the brain, breast, liver, bone, and prostate.

Therapeutic ultrasound is a minimally invasive or non-invasive method to deposit acoustic energy into tissue. Applications include tissue ablation (HIFU) (for tumor treatments, for example), hyperthermia treatments (low-level heating combined with radiation or chemotherapy), or the activation or enhanced delivery of drugs.

Aiming

The ultrasound beam can be focused in these ways:

- Geometrically, for example with a lens or with a spherically curved transducer.
- Electronically, by adjusting the relative phases of elements in an array of transducers (a "phased array"). By dynamically adjusting the electronic signals to the elements of a phased array, the beam can be steered to different locations, and aberrations due to tissue structures can be corrected.

How HIFU works

As an acoustic wave propagates through the tissue, part of it is absorbed and converted to heat. With focused beams, a very small focus can be achieved deep in tissues. When hot enough, the tissue is thermally coagulated. By focusing at more than one place or by scanning the focus, a volume can be thermally ablated. At high enough acoustic intensities, cavitation (microbubbles forming and interacting with the ultrasound field) can occur. Microbubbles produced in the field oscillate and grow (due to factors including rectified diffusion), and eventually implode (inertial or transient cavitation). During inertial cavitation, very high temperatures inside the bubbles occur, and the collapse is associated with a shock wave and jets that can mechanically damage tissue. Because the onset of cavitation and the resulting tissue damage can be unpredictable, it has generally been avoided in clinical applications. However, cavitation is currently being investigated as a means to enhance HIFU ablation and for other applications.

Method of use

In HIFU therapy, ultrasound beams are focused on diseased tissue, and due to the significant energy deposition at the focus, temperature within the tissue rises to 65° to 85°C, destroying the diseased tissue by coagulation necrosis. Each sonication of the beams treats a precisely defined portion of the targeted tissue. The entire therapeutic target is treated by moving the applicator on its robotic arm in order to juxtapose multiple shots, according to a protocol designed by the physician. This technology can achieve precise ablation of diseased tissue, therefore it is called HIFU surgery. Because it destroys the diseased tissue non-invasively, it is also known as "Non-invasive HIFU surgery". Anesthesia is not required, but should be recommended. The treatment can be combined with radiotherapy or chemotherapy.

Uses

Uterine fibroids

Development of this therapy significantly broadened the range of treatment options for patients suffering from uterine fibroids. HIFU treatment for uterine fibroids was approved by the Food and Drug Administration (FDA) in October 2004. This is a non-invasive treatment option for patients suffering from symptomatic fibroids. Most patients benefit from HIFU and symptomatic relief is sustained for two plus years. Up to 16-20% of patient will require an additional treatment .

Currently available approved uterine fibroids HIFU treatment devices are Philips Sonalleve MR-HIFU and GE Insightec ExAblate 2000 and ExAblate 2100. And CE approved Haifu model JC and JC200.

Prostate cancer

The earliest widespread use of HIFU ablation was as a treatment for prostate cancer. This treatment is administered through a trans-rectal probe and relies on heat developed by focusing ultrasound waves into the prostate to kill the tumor. Promising results approaching those of surgery have been reported in large series of prostate cancer patients. These treatments are performed under ultrasound imaging guidance, which allows for treatment planning and some minimal indication of the energy deposition. HIFU may also be used to ablate the entire prostate gland using a transrectal probe. This is an outpatient procedure that usually last 1–3 hours. Results show that it greatly reduces some of the side effects common with other treatments for prostate cancer.

During HIFU, the entire prostate is ablated, including the prostatic urethra. The urethra has regenerative ability because it is derived from a different type of tissue (bladder squamous-type epithelium) rather than prostatic tissue (glandular, fibrotic and muscular). While the urethra is an important anatomical structure, the sphincter and bladder neck are more important to maintaining the urinary function. During HIFU the sphincter and bladder neck are identified and avoided.

Available devices for prostate cancer treatment

Ablatherm Robotic HIFU

Developed in 1989 in France with Inserm (French National Institute of Medical Research), Edouard Herriot Hospital in Lyon and EDAP TMS (Nasdaq : EDAP), Ablatherm HIFU was the first prostate cancer HIFU device to receive CE marking in 2000. The first "Ablathermy" treatments on men were performed in 1993 and as of January, 2010, more than 21,000 treatments have been performed worldwide.

Sonablate 500

Developed at the early 90s for the treatment of benign prostate hyperplasia (BPH) in the US by Misonix (Nasdaq : MSON), Sonablate was then modified to treat prostate cancer at the end of the 90s. Sonablate 500 received CE marking in 2001. As of January 2010, a total of more than 9,000 treatments have been performed for benign prostate hyperplasia and over 7,000 prostate cancer treatments.

During Sonablate HIFU, the physician obtains real-time ultrasound images of the prostate and surrounding areas. From these images, a customized plan for delivering the ultrasound energy is created. The Sonablate software allows the physician to precisely define the treatment zones in order to destroy the entire gland.

Sonablate HIFU is minimally invasive, performed on an outpatient basis and typically lasts 2–4 hours, depending on the size of the prostate. There is no surgery or radiation involved. Patients wear a catheter post-procedure but are able to resume normal activities almost immediately. The Sonablate is the only HIFU device for prostate cancer that does

not require an advance surgical procedure (known as a TURP) in order to achieve effective results when treating enlarged prostate glands. Sonablate HIFU can treat large prostates up to 40 grams.

The Sonablate incorporates three-dimensional imaging to provide better visuals of the prostate, especially any irregularities, and allow the physician to create the most effective treatment plan possible. The newest technological enhancement to the Sonablate is tissue change monitoring (TCM) software, which gives real-time feedback to the physician, thus confirming if sufficient energy has been delivered to completely ablate the tissue.

Other cancers

HIFU has been successfully applied in treatment of cancer to destroy solid tumors of the bone, brain, breast, liver, pancreas, rectum, kidney, testes, prostate. At this stage cancer treatments are still in the investigatory phases as there is a need to find more about their effectiveness.

HIFU may be used to create high temperatures not necessarily to treat the cancer alone, but in conjunction with targeted delivery of cancer drugs. For example, HIFU and other devices may be used to activate temperature-sensitive liposomes, filled with cancer drug "cargo" to release the drug in high concentrations only at the tumor site(s) only where triggered to do so by the hyperthermia device. This novel approach is resulting in drug concentrations 10 times or more than traditional chemo with a fraction of the side effects since the drug is not released system-wide.

In addition, several thousand patients with different types of tumors have been treated in China with HIFU using ultrasound imaging-guided devices built by several different companies.

Delivering drugs to brain

In current research, HIFU is being used to temporarily break up the blood-brain barrier, allowing an influx of drugs into the brain. It is most effective when used in combination with an inhibitor like verapamil.

Treatment of atrial fibrillation

HIFU has been used to treat the most common heart arrhythmia, atrial fibrillation (AF). A minimally invasive catheter based system designed to ablate heart tissue responsible for propagating AF has been approved for use in Europe and is undergoing an FDA approved phase III pivotal efficacy trial in the United States.

History

The first investigations of HIFU for non-invasive ablation were reported by Lynn et al. in the early 1940s. Extensive important early work was performed in the 1950s and 1960s

by William Fry and Francis Fry at the University of Illinois and Carl Townsend, Howard White and George Gardner at the Interscience Research Institute of Champaign, Ill., culminating in clinical treatments of neurological disorders. In particular High Intensity ultrasound and ultrasound visualization was accomplished stereotaxically with a Cincinnati precision milling machine to perform accurate ablation of brain tumors. Until recently, clinical trials of HIFU for ablation were few (although significant work in hyperthermia was performed with ultrasonic heating), perhaps due to the complexity of the treatments and the difficulty of targeting the beam noninvasively. With recent advances in medical imaging and ultrasound technology, interest in HIFU ablation of tumors has increased.

The first commercial HIFU machine, called the Sonablate 200, was developed by the American company Focus Surgery, Inc. (Milipitas, CA) and launched in Europe in 1994 after receiving CE approval, bringing a first medical validation of the technology for benign prostatic hyperplasia (BPH). Comprehensive studies by practitioners at more than one site using the device demonstrated clinical efficacy for the destruction of prostatic tissue without bloodloss or long term side effects. Later studies on localized prostate cancer by Murat and colleagues at the Edouard Herriot Hospital in Lyon in 2006 showed that after treatment with the Ablatherm (EDAP TMS, Lyon, France), progression-free survival rates are very high for low- and intermediate- risk patients with recurrent prostate cancer (70% and 50% respectively) HIFU treatment of prostate cancer is currently an approved therapy in Europe, Canada, South Korea, Australia, and elsewhere. Clinical trials in the United States are expected to begin in 2006. Prostate cancer trials for the new Sonablate 500 are ongoing in the U.S.A. currently. Magnetic Resonance Guided Focused Ultrasound MRgFU was first developed by Harvey Cline and Ronald Watkins at GE Corporate R&D lab in Niskayuna NY and Kullervo Hynynen at the University of Arizona, Tucson AZ. starting in 1991. This is described in U.S. Patent #5247935.(1992) The technology was later transferred to InsighTec in Haifa Israel in 2000. The InsighTec ExAblate 2000 was the first MR Guided focused ultrasound system to obtain FDA market approval and sold commercially in the United States. Haifu Model JC and JC200 by ChongQing Haifu Ltd. are complete ultrasound guided tumor treatment systems, and they are only CE approved for beningn and malignant tumors. HIFU-2001(By Sumo Corporation Ltd) is an enhanced technology treatment system that does not require anesthesia since 2001, which are famous in Asia countries. The treatment area included Liver/Pancreas/Bladder/Uterus/Kidney.

Advantages over other techniques

High Intensity Focused Ultrasound is often considered a promising technology within the non-invasive or minimally invasive therapy segments of medical technology. HIFU's capacity to generate in-depth precise tissue necrosis using an external applicator, with no effect on the surrounding structures, is unique. The history of using therapeutic ultrasound dates back to early in the 20th century. Technology has continually improved and additional clinical applications, both diagnostic and therapeutic, have become an integral part of medicine today.

An important difference between HIFU and many other forms of focused energy, such as radiation therapy or radio surgery, is that the passage of ultrasound energy through intervening tissue has no apparent cumulative effect on that tissue.

The absence of cumulative effect of HIFU on the treated tissue means that the treatment can be repeated in case of first HIFU treatment failure or partial treatment of the prostate. As a clean treatment (= non-ionizing) HIFU is also an option to treat prostate cancer recurrence after radiation therapy failure.

Discoveries during use

Currently, the only proven imaging method to accurately quantify the heating produced during HIFU *in vivo* is Magnetic Resonance Imaging (MRI). MRI also has superior soft tissue contrast and can image in any orientation, making it the state of the art for guiding HIFU treatments. But MRI can't operate in *real-time* with HIFU, with the current state of the art being one image acquisition approximately every six seconds using a full scan of k-space. Researchers are working to reduce this image acquisition time through some of the speed enhancements common in other areas of MRI, including pulse sequences to scan a reduced k-space, constrained reconstruction, and model-based filtering using data from the bioheat equation.

Clinically, MRI-guided HIFU treatments have been tested for uterine fibroids, breast fibroadenomas, breast cancer, bone metastases, and liver tumors. The largest number of patients treated with MRI-guided HIFU have been with uterine fibroids.

USgFUS treatments have been approved with CE for wider range of benign and malignant tumors due to its higher power, precision and realtime monitoring system. The largest number of patients are uterine fibroids.

Ultrasound-guided HIFU treatments have been approved in Europe and Asia. MRI-guided treatments of uterine fibroids have been approved in Europe and Asia, and were granted FDA approval in the US in 2004.

Focal HIFU treatment

With the latest improvements in biopsy techniques enabling to better locate cancer, focal HIFU treatment (i.e. partial HIFU ablation) is now starting to be investigated to further reduce the side effects of cancer treatment.

Organizations

The International Society for Therapeutic Ultrasound, founded in 2001, aims to promote clinical, academic and industrial advancement in Therapeutic Ultrasound. Its primary activity is the annual International Symposium on Therapeutic Ultrasound, which has attracted experts in HIFU from throughout the world.

The Foundation for Focused Ultrasound Research is an unincorporated association promoting research into medical uses of high intensity focused ultrasound, including HIFU.

The Focused Ultrasound Surgery Foundation (**FUSF**) is working to shorten the time from technology development to patient treatment, develop new applications and accelerate the worldwide adoption of MR-guided focused ultrasound surgery.