A great variety of sources has been used in the preparation of this lab manual, including textbooks, journals, other laboratory manuals, personal communications, etc. Since the material contained in this manual is still undergoing modification and revision, the references have not yet been included. When the evolution nears completion, proper sources will be cited.

I am especially indebted to Dr. A.A. Weaver of The College of Wooster, for initiating my interest in histology and microtechnique. It is his manual which provided a lot of the material contained in this book. I am also indebted to Sue Beaumier for her tireless typing and retyping of this text

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HISTOLOGY 333 - LABORATORY

The laboratory portion of this course includes two interrelated components. Firstly, we will learn how to make and stain slides for light-microscopy by a variety of techniques. This will begin by simply staining sections of tissue supplied to you. We will cover a number of different techniques in order for you to gain some insight into the techniques themselves and also what kinds of information can be derived from them. This portion of the course will culminate in a special project, where you carry out the entire process from the killing of an organism all the way through to the final stained slides. This will demonstrate to you the importance of fixation, the techniques of embedding and sectioning, and then allow you to put to use the knowledge you have gained about different staining procedures.

Secondly, we will be continually looking at a variety of slides from a collection of already prepared specimens. This will serve to illustrate to you all the structures, cell types, tissues, and their interrelationships as we discuss them in lecture. You will eventually be able to use the slides you prepare yourself in this manner as well.

TOXIC SUBSTANCES IN TEACHING AND RESEARCH LABORATORIES

taken from a report of the
American Association of Anatomists
by an ad hoc Committee
on Hazards in Research and
Teaching Laboratories, Arthur
LaVelle, Chairman

In this listing the commonly used term is usually followed by more frequently used synonyms in parentheses. Next is the sequence number assigned to it in the Registry of Toxic Effects of Chemical Substances (1977) published by the National Institute of Occupational Safety and Health (NIOSH). A technical usage is next identified. This is followed by the most likely route of entry into the body and by a Threshold Limit Value (TLV) given in the Registry as the recommended "time-weighted average concentration of a substance of which most workers can be exposed without adverse effect". Occasionally, this concentration may be indicated as a time-weighted average concentration (TWA). The structures most likely affected and some of the more apparent or known toxic effects are next. Finally it is indicated that the substance may be flammable or potentially explosive, or may react violently when mixed with certain other chemicals. Not all items are completely annotated in this manner, however, because available information was incomplete.

FIXATIVES

Acrolein (acrylic aldehyde, aquinite, etc.). AS 10,500. Air (TLV:.01 ppm), contact. Eyes, mucous membrane, respiratory system. Severe irritant, may cause unconsciousness. Highly flammable; may polymerize violently upon contact with strong acid or basic catalysts.

*Formaldehyde (as 37-41% solution; formalin = ca. 4% soln; formol, etc.). LP 89250. Fixative, preservative (usually contains 11-14% methanol.) Air (TLV: 2 ppm), contact. Mucous membrane, eyes, skin. Irritant; hardening or tanning effect on the skin; chronic effects lead to conjunctivitis, skin cracking and ulceration, laryngitis, bronchitis and bronchial pneumonia. CARCINOGENIC.

*Gluteraldehyde (glutaric dialdehyde, cidex). MA 24500. Fixative. Air (TLV: 2 ppm), contact. Eyes and mucous membrane. Irritant.

*Osmium tetroxide (osmic acid). RN 11400. Fixative, staining reagent. Air (TLV: 0.0002 ppm), contact. Respiratory system, eyes, skin. Vapor can cause severe irritation or burns of mucous membrane, eyes and skin.

Phenol (carbolic acid, hydroxybenzene, etc.). SJ 33250. Cadaver preservative, antiseptic, nucleic acid extractant. Air (TLV: 60 mg/m^3/15M), skin contact (TLV: 5 ppm or 19 mg/m^3). Skin, eyes, respiratory system, digestive tract, liver, kidneys. Vapor irritates eyes, respiratory mucosa; anesthetic effect on fingers of dissector; skin whitening and eventual possible burns may follow continued exposure by contact; skin absorption may result in headache, dizziness, possible collapse; prolonged exposure may cause digestive disturbances, liver and kidney damage, dermatitis.

*ONES YOU MAY ENCOUNTER IN THIS LAB

*Picric acid (2,4,6-trinitrophenol). TJ 78750. Fixative, commonly in alcoholic solutions. Skin contact (TLV: 0.1 mg/m^3). Skin, eyes, liver, kidney. Dermatitis; long exposure may result in skin eruptions, headache, nausea, vomiting, diarrhea. Explosive in greater than 50% solution with water; dry, it is a class A explosive which is readily detonated; forms heat, friction or impact sensitive salts with many metals (Pb, Hg, Cu, Zn).

*Potassium dichromate. HX 76800. Fixative. Air or dust (TWA: 25 mg/m^3). Eyes, skin, respiratory tract, liver, kidney. Irritating to eyes, respiratory tract; chronic exposure may cause skin ulceration, liver and kidney disease. CARCINOGENIC. Hydroxyamine reacts explosively with potassium dichromate.

SOLVENTS

*Acetone. AL 31500. Solvent. Air (TLV: 1000 ppm), contact. Eyes, mucous membrane, CNS - liquid contact highly damaging to eyes; dizziness, narcosis, coma. Highly flammable; violent reactions with chloroform plus base, also with $SC1_2$.

Benzene (benzol, coal naphtha, etc.). CY 14000. Solvent, clearing agent, etc. Air (TLV: 25 ppm), contact. Eyes, mucous membrane, nervous system, blood, liver, etc. Irritation; dizziness, headache, unconsciousness; tissue damage; chronic exposure may lead to blood disease. CARCINOGENIC. Highly flammable; may be explosive in combination with various oxidants.

Xylene or toluene can be substituted for benzene in most laboratory procedures. Benzene, therefore, should be eliminated from use insofar as possible.

*Carbon tetrachloride (benzinoform, tetrachloromethane). FG 49000. Solvent (including osmium tetroxide). (TLV: 10 ppm). Eyes, mucosa, liver, kidney, heart, CNS. Irritant; chronic effects include headache, nausea, stupor, vomiting, bronchitis, jaundice; dermatitis may follow repeated skin contact. CARCINOGENIC. May react explosively with metals.

*Chloroform (trichloromethane, methenyl trichloride, etc.). FS 91000. Solvent, anesthetic. Air (TLV: 25 ppm), contact. CNS, eyes. Drowsiness, headache, nausea, vomiting, unconsciousness; irritates eyes, causing conjunctivitis; suspected CARCINOGEN. May be explosive with Na/methanol, NaOH/methanol, or sodium methoxide.

Dioxane (diethylene dioxide, diethylene ether, etc.). JG 82250. Solvent, scintillation "cocktail". (TLV: 100 ppm), skin. Eyes, mucous membrane, kidney, liver. Irritant, tissue damage, nausea, vomiting. CARCINOGENIC. Highly flammable; forms explosive peroxides on exposure to air.

*Ether (diethyl ether, etc.). KI 57750. Solvent, anesthetic. Air (TLV: 400 ppm), contact. CNS. Drowsiness, dizziness and unconsciousness in high concentration; chronic effects lead to loss of appetite, fatigue and nausea. Highly flammable; can form explosive peroxides upon exposure to air and light.

2-ethoxyethanol (cellosolve, ethylene glycol ethyl ester, etc.). KK 80500. Clearing agent in histology. Air (TLV: 200 ppm), contact. Eyes, respiratory system. Vapor irritates eyes, respiratory system in high concentrations. Flammable, may form explosive peroxides.

Glycerol 0- see Explosives.

*Methanol (wood alcohol, etc.). PC 14000. Solvent, fixative. Air (TLV: 200 ppm), contact. Serious risk of poisoning by breathing vapor as well as by ingestion; effects may be delayed for many hours; ingestion causes blindness and damages nervous system, heart, liver, etc.; unconsciousness may develop hours after severe exposure followed by death. Flammable.

Propylene oxide (1,2-epoxypropane). TZ 29750. Dehydrant in EM. Air (TLV: 100 ppm). Eyes, respiratory system, skin. Vapor irritating to eyes and respiratory system epithelium; liquid rapidly absorbed by skin which can become blistered; suspected CARCINOGEN Extremely flammable.

Pyridine (azobenze, azine, etc.). UR 84000. Silver staining, fat solvent. Air (TLV: 5 ppm), contact. Eyes, skin, respiratory system, CNS. Irritant; conjunctivitis, dermatitis, headache, nausea, vomiting. Very flammable.

Tetrahydrofuran (THF; 1,4-epoxy-butane). LU 59500. Dehydrant in EM. Air (TLV: 200 ppm), contact. Eyes, respiratory system, liver. Irritant, narcosis at high concentration; liver damage. Very flammable; exposure to air and light may result in explosive peroxides.

*Toluene (methylbenzene, methacide, etc.). XS 52500. Solvent, clearing agent, scintillation "cocktail". Air (TLV: 200 ppm), contact. Eyes, skin, mucous membrane, CNS. Irritant to eyes, mucous membrane; inhalation of vapor may result in dizziness, nausea, mental confusion; prolonged exposure may cause dermatitis; because of benzene contamination, repeated exposure may cause blood disease. Very flammable.

Trichloroethylene (acetylene trichloride, algylen, benzinol). KX 45500. Vapors flatten sections in EM. Air (TLV: 100 ppm), skin contact. Eyes, CNS, liver, kidney. Irritant, headache, dizziness, nausea, unconsciousness, cellular damage. CARCINOGENIC.

*Xylene (xylol, dimethyl benzene). ZE 21000. Solvent, clearing agent. Air (TLV: 200 ppm), contact. Eyes, skin, mucous membrane, CNS. Irritant to eyes, mucous membrane; prolonged exposure may cause dermatitis; inhalation or absorption via skin may cause dizziness, headache, nausea, vomiting; benzene contamination: see Toluene.

EMBEDDING MEDIA

Acrylic resins [includes different proprietary names and/or formulations: methacrylic acid (MA) - OZ 29750; dimethylamino ethyl methacrylate (DMA) - OZ 42000; tetramethylene dimethacrylate (TMA); glycol methacrylate - OZ 47250; n-butyl methacrylate - OZ 36750]. Embedding material. These substances are irritating to eyes, skin and respiratory system. Avoid contact with skin.

Epoxy resins [includes different proprietary names and/or formulations: Epon, Araldite (VH 10500); DER 732, -736, -334; ERL 4206 or vinyl cyclohexene dioxide (RN 86400); Maraglas; Durcupan]. KD 42500. Embedding material in EM. Air (TLV: 5 ppm), skin contact. Eyes, skin, respiratory system. Irritation; blistering of skin; prolonged exposure can cause conjunctivitis, weariness, stomach upset; some identified as CARCINOGENIC (particularly vinyl cyclohexane dioxide). See Luft (1973) for further discussion.

Nitrocellulose - see Explosives.

Polyester resins. Contain styrene (phenylethylene). WL 36750. Air (TLV: 100 ppm), contact. Irritant to eyes and skin. Flammable.

EMBEDDING REAGENTS

Azodiisobutyronitrile (propionitrile, 2, 2'-azobis(2-methyl). UG 08000. Catalyst in EM embedding. Irritant; cyanide releasing.

Benzoyl peroxide (dibenzoyl peroxide, benzoic acid peroxide, etc.). DM 85750. Accelerator in EM embedding. Air (TWA: 5 mg/m^3). Skin, eyes, mucous membrane. Irritant. Explosive, possibly as a result of impact from friction when dry or upon heating above melting point (103_C).

Benzyldimethylamine (BDMA; N,N-dimethyl-benzylamine). DP 45000. Catalyst in EM embedding. Irritant.

Dibutyl phthalate (DBP). TI 08750. Plasticizer or softener in EM embedding. Air (TLV: 5 mg/m^3). Also used commercially as insect repellent. Biologic effects uncertain, although there is suggestive evidence that some phthalic esters are mutative in mice. Persistent exposure to fumes from any phthalic ester should be avoided.

2-4 dichlorobenzoyl peroxide (Luperco CDB). Explosive, flammable.

Dimethylaminoethanol (DMAE, S1, N,N-dimethylethanolamine, etc.). KK 61250. Catalyst in EM embedding. Contact. Eyes, skin. Irritation to eyes; causes lacrimation; splashing might cause serious injury to eyes. Flammable.

Dodecynyl succinic anhydride (DDSA). WN 12250. Hardener in EM embedding. Irritant.

Tridimethylaminomethylphenol (DMP-30; 2,4,6-tri-dimethylaminomethyl phenol). SN 35000. Catalyst in EM embedding. Skin irritant.

METALS (OR THEIR SALTS AND ACIDS)

*Chromic acid (chromium trioxide). GB 24500. Chromaffin reaction in histochemical technique. Air (TLV: 0.1 mg/m^3). Eyes, respiratory system, skin. Irritant; chronic exposure may cause skin ulcerations. Explosive with acetic acid, acetic anhydride; may ignite acetone, methanol, ethanol, butanol, glycerol and pyridine.

Lead acetate. OF 80500. Basic lead acetate in some formalin fixatives; Millonig's lead acetate has trihydrate in staining procedure. Air (TWA: 150 mg (Pb)/m^3), ingestion via inhalation, contact. Gastrointestinal mucosa, kidney tubules, CNS, PNS. Effects of lead accumulation, may lead to loss of appetite, vomiting, diarrhea, collapse, sever injury to CNS and PNS. CARCINOGENIC.

*Mercury. OV 45500. Thermometers, atmospheric pressure gauges, staining procedures. Air (TLV: 0.5 mg/m^3). Eyes, skin, CNS, kidneys. Vapor may lead to metallic taste, nausea, headache, vomiting, abdominal pain; chronic effects include loosening of teeth, excessive salivation, dermatitis, kidney damage, severe nervous disturbances. Escaping vapor from EM mercury high-vacuum diffusion pumps can be a hazard. Metallic mercury entrapped in porous surfaces continues to form harmful vapor.

*Osmium tetroxide - see Fixatives.

*Potassium permanganate (permanganic acid, potassium salt). SD 64750. Fixative, stain reagent. Contact. Repeated contact of skin with concentrations greater than 1:1,000 may result in a chemical burn. Explosive when combined with acetic acid, acetic anhydride, concentrated HC1 or H_2SO_4; fire can result on combining glycerol and solid potassium permanganate.

*Silver nitrate. VW 47250. Staining techniques, antiseptic. Contact. Skin, eyes. Repeated contact may irritate eyes or cause argyria or pigmentation of skin or skin burns. Possible explosive when combined with ethanol or charcoal.

Uranyl acetate (uranium, bis (aceta) dioxo). YP 36750. Stain in EM. Air (TWA: 50 mg (U)/m^3). Lungs, kidney. Inhalation of dust or vapor may irritate lungs, may lead to accumulative retention of uranium and damage to kidneys. Relatively large amounts of uranyl acetate (1 lb.) are significantly radioactive. These quantities should, perhaps, be handled sparingly.

Vanadium - vanadyl sulfate (oxysulfatovanadium). YW 19250. Stain in EM. Air (TLV: 0.5 mg/m^3). Skin, eyes, respiratory system. Dust or fumes irritating to skin, eyes (may cause conjunctivitis), respiratory system.

DYES

Acridine dyes - includes compounds of widely different molecular weights: quinacrine mustard (AR 75700); quinacrine (AR 77000); acridine orange (AR 76000); acriflavine hydrochloride (AR 96250). Quinacrine mustard is highly mutagenic and the others may be regarded as CANCER SUSPECT AGENTS.

Auramine O (Basic Yellow 2, etc.). BY 36750. Biological stain. CANCER SUSPECT AGENT.

Direct black 38 (chlorazol black E, etc.). JM 71700. Biological stain (benzidine derived). Air, ingestion (via inspiration into lungs). Liver, bladder. Possibility of benzidine release in body indicates this dye should be handled as a CARCINOGEN.

Direct blue 6 (Niagara blue, 2B, etc.). JM 71850. Biological stain (benzidine derived). Air, ingestion (via inspiration into lungs). Liver, bladder. Possibility of benzidine release in body indicates this dye should be handled as a CARCINOGEN.

EXPLOSIVE AGENTS

Ammonium persulfate (peroxydisulfuric acid, diammonium salt, ammonium peroxydisulfate). SE 03500. Gel electrophoresis, redox catalyst. May cause fire when combined with combustible material.

Benzene - see Solvents.

Dioxane - see Solvents.

*Ether - see Solvents.

*Glycerol (glycerin, etc.). MA 80500. Solvent, softening agent, mounting medium for light microscopy. Violent or explosive reactions with many oxidizing agents.

*Methanol - see Solvents.

Nitrocellulose (cellulose nitrate, cellulose tetranitrate, celloidin, collodion, etc.). FJ 60000. Embedding medium for light microscopy. Highly explosive when dry: flammable.

Perchloric acid. SC 75000. Histochemistry (e.g., nucleic acid extraction). Skin, eyes. As other acids, is highly corrosive and can cause severe burns. Potentially explosive at higher concentrations than 70%; thus spillage on porous surfaces, where it may build up in concentration, should be avoided; as perchlorate, may develop violent reaction when mixed with acetic acid, alcohols, analine/formaldehyde, dehydrating agents, carbon, ether, glycols and their ethers, iodides, etc.

*Picric acid - see Fixatives.

*Silver nitrate - see Metals.

Tetrahydrofuran - see Solvents.

MISCELLANEOUS

Acrylamide (acrylic acid). AS 33250. Gel electrophoresis. Air (TLV: 0.3 mg/m^3), contact. Irritating to skin, eyes, respiratory system and nervous system.

Diaminobenzidine (3, 3'4, 4'-tetraaminobiphenol). DV 87500; DC 96250. Used in the horseradish peroxidase neurological tracing technique. This substance is listed as CARCINOGENIC, as is 0-dianisidine (3, 3'-dimethoxybenzidine), DD 08750, which is also a developing reagent in the HRP technique. Of three other such reagents, benzidine dihydrochloride is listed (DD 06000) but is not indicated as a carcinogen in the Registry. The other two, tetramethoxy benzidine and paraphenylenediamine, are not listed in the Registry.

Hydroxylamine (oxammonium). NC 29750. Used in iron stain in EM. Air, skin absorption. Skin, mucous membrane, blood, liver, CNS. Acute effects: headache, dizziness, mental confusion; methemoglobin formation: severe exposure might cause asphyxia and neuronal damage; chronic exposure could cause weight loss, anemia, impairment of liver function. Reacts violently or explosively with many oxidants such as potassium dichromate.

*Polychlorinated biphenyls (2-chlorobiphenyl, DV 20650; 4-chlorobiphenyl, DV 20800). Some immersion oils for microscopy containing PCB's may still be on laboratory shelves, although current oils such as Cargilles' no longer contain them; can irritate skin, so contact should be avoided; suspected CARCINOGENS.

Infective agents in preserved human tissue. The virus (or viroid) responsible for Creutzfeldt-Jakob disease has been reported to survive in human brain tissue suspended in 10% formol-saline for 7 months (Gajdusek and Gibbs, 1976). The virus, which causes a progressive, eventually fatal degeneration of neurons in the CNS, is resistant to various chemical inactivators such as formaldehyde vapor and 70% alcohol. An allied viral form, scrapie, appears to be unstable in strong solutions of phenol, but some survival is present

in 2% solutions of phenol (Hunter and Gibbons, 1969). The agent infecting human tissue can possibly be transmitted via contact with breaks in the skin (Gajdusek et al., 1977). Although the disease is rare, the safest precaution is to insure that no human brain or cadaver accepted for dissection comes from a person who has been diagnosed to have the disease.

REFERENCES

Current Intelligence Bulletin 24 (Direct Blue 6, Direct Black 38, Direct Brown 95: Benzidine Derived Dyes) 1978 Center for Disease Control, National Institute for Occupational Safety and Health, Rockville, Maryland.

Dreisback, R. H. 1974 Handbook of Poisoning: Diagnostic Treatment.

Fairchild, E. J., F. J. Lewis, Sr., and R. L. Tatken (eds.) 1977 Registry of Toxic Effects of Chemical Substances, Vol. 1 and II. U. S. Dept. Health, Education and Welfare, National Institute for Occupational Safety and Health.

Gajdusek, D. C., and C. J. Gibbs 1976 Survival of Creutzfeldt-Jakob-disease virus in formol-fixed brain tissue. New Engl. J. Med., 294: 553.

Gajdusek, D. C. et al. 1977 Precautions in medical care of patients with Creutzfeldt-Jakob-disease. New Engl. J. Med. 297: 1253-1258.

General Industry Standards 1976 Occupational Safety and Health Administration. U.S. Dept. Labor.

Guidelines for the Laboratory Use of Chemical Substances Posing a Potential Occupational Carcinogenic Risk 1978 Laboratory Chemical Carcinogenic Safety Standards Subcommittee of DHEW Committee to Coordinate Toxicology and Related Programs.

Hunter, G. D., R. A. Gibbons, R. H. Kimberlin and G. C. Millson 1969 Further studies of the infectivity and stability of extracts of homogenates derived from scrapie affected mouse brains. J. Comp. Path., 79: 101-108.

Luft, J. H. 1973 Embedding media - old and new. In: Advanced Techniques in Biological Electron Microscopy. J. K. Koehler (ed.), Springer-Verlag, New York.

Muir, G. D. (ed.) 1977 Hazards in the Chemical Laboratory. The Chemical Society, London.

National Cancer Institute Safety Standards for Research Involving Chemical Carcinogens 1975 DHEW Publ. No. (NIH) 76-900.

OSHA News Briefs, October, 1976.

Smith, R. J. 1978 Laboratory chemicals may come under costly OSHA restrictions. Science, 202: 496-499.

Standards for Accreditation of Medical Laboratories 1974 Commission on Laboratory Inspection and Accreditation, College of American Pathologists, Skokie, Illinois.

TLVs® Threshold Limit Values for Chemical Substances in Workroom Air 1977 American Conference of Governmental Industrial Hygienists.

SECTION I

MICROTECHNIQUE:

TISSUE PREPARATION

KILLING AND FIXATION

Before tissues can be dissected out for examination the animals must be immobilized. Usually an anesthetic such as chloroform, ether, or Nembutal is satisfactory. If delicate cell organelles (e.g. mitochondria) are to be studied, the animal should be killed by a blow on the head. Anesthetics should be avoided, if possible. The organs to be studied should be removed immediately from the body and placed in an appropriate fixative solution. Degenerative cytological changes begin as soon as the animal dies. The faster the tissues can be placed in a fixative solution the more "life-like" will be the preservation of cellular structure.

Fixatives perform a number of important functions. They set or fix the parts of cells and tissues so that subsequent operations will not cause undue distortion and the micro-anatomical arrangements of the cellular components will not be disturbed. They arrest post-mortem autolytic, osmotic, bacterial, and other changes in the tissue. They render the substances of the cell insoluble. They bring out differences in the refractive indices of cellular components and thus make them more readily visible. They may enhance the stainability of certain tissue components.

In order that a fixative may act effectively all parts of a tissue must be exposed to the fixative solution within minutes after removal from the body. Many small invertebrates may be dropped directly into the fixative solution. Hypodermic injection of fixatives is very effective in insects. When working with larger animals we must rely on rapid removal of tissues from the body and immediate immersion of small pieces of tissue in the fixing agent. The rate of penetration of the fixative may be increased by heating the solution to 40˚ - 50˚C prior to fixation.

Hundreds of fixative mixtures have been devised. One of the nine solutions discussed below should provide satisfactory fixation for most tissues. The formulae for these and a number of other common fixatives will be found on the following pages. The choice of a particular solution will depend on the tissue and the specific techniques which are to follow fixation.

1. Bouin's and Brasil's Fluids

Procedure:

Tissue is fixed in either warm or cold solutions for 12-24 hours. Longer times are usually not harmful. They may be injected. Storage is in 70% alcohol. No special washing out required. Removal of the yellow color in several changes of 50% alcohol is advisable before staining whole mounts, but this color leaches out during normal procedures in preparation of sections.

Comments:

These are both widely used fixatives for routine histological studies. Brasil's has been much used for glycogen fixation. Both give moderately fine cytoplasmic precipitation and well differentiated nuclei. Tissue is of good consistency for dissection and sectioning. Mitochondria are destroyed. These fixatives are not very suitable for most histochemical procedures (except glycogen) and are not very satisfactory for whole mounts.

2. Carnoy's and Clarke's Fluids

Procedure:

Fix tissue 30 minutes to an hour in cold solution. Longer times are less satisfactory. No special washing out necessary. Store tissue in 95% alcohol.

Comments:

These penetrate very rapidly. Carnoy's fluid can be used to fix whole arthropods. Staining is excellent after both. Cytoplasm is rather coarsely precipitated, nuclei well differentiated, mitochondria destroyed. Most histochemical procedures can be used after these fixatives.

3. Zenker's and Helly's Fluids

Procedure:

Fix tissue for 12-24 hours. Wash out overnight in running water. Store in 70% alcohol. Before sections are stained they should be placed in a 0.5% iodine solution in 70% alcohol for 2 minutes. The iodine is rinsed out in 70% alcohol followed by a brief rinse in a 5% solution of sodium thiosulfate. These steps can be carried out on whole pieces of tissue, but can be done much more rapidly on sections.

Comments:

These fixatives make a useful pair since Zenker's does an excellent job as a general histological fixative and Helly's (which gives poor fixation of chromosomes) is a fine cytoplasmic fixative. Helly's preserves mitochondria, muscle striations, and other delicate cytoplasmic structures. Both give more brilliant staining than the other fixatives discussed here, but they both harden tissues badly so that sectioning is more difficult. Neither is suitable for injection.

4. San Felice's Fluid

Procedure:

Fix for 12-24 hours. Wash out overnight in running water. Store in 70% alcohol. Not suitable for injection.

Comments:

This is an excellent fixative for studies of chromosomes, but probably deserves wider use. Studies on cell shrinkage have shown that San Felice gives less shrinkage than any other fixative tested.

5. Kahle's Fluid

Procedure:

May fix overnight or longer. No washing out required. Store in 70% alcohol. May be injected.

Comments:

This is one of a long series of common fixative mixture containing various proportions of formalin, acetic acid, and alcohol. These all are nearly fool-proof for class use. Most stains will follow them. Kahle's has been widely used for arthropod tissues.

6. Gilson's Fluid

Procedure:

May fix overnight or longer. No washing out required. Store in 70% alcohol. Treat sections with iodine as directed for Zenker's.

Comments:

Gives bad cytoplasmic fixation, but is excellent as a fool-proof fixative for beginning work. Sections very easily.

7. Champy's Solution

Procedure:

Fix very small pieces 2-4 hours, wash 4-12 hours in running water. Store in 50% alcohol.

Comments:

Widely used to demonstrate mitochondria; also a nice, general fixative for staining with iron hematoxylin.

8. Susa's Fluid

Procedure:

Fix 5-8 mm slices in relatively large volume for 3-24 hours. Transfer to 50% alcohol, several changes, and then into 70% alcohol. Treat with iodine.

Comments:

An excellent general fixative for vertebrate and higher invertebrate tissues. Causes tissue to cut easily so it is useful for fixation of ovaries with yolky material as well as uterus and organs with lots of muscle and connective tissue. Causes mucin to swell, does not fix zymogen granules. Causes exceptionally nice staining.

9. Flemming's Solutions

Procedure:

Fix very small pieces of tissue for 24-48 hours; if using the "strong' solution use 5-6 times the tissue volume, if using the "weak" solution a large volume (50 times that of the tissue) is necessary. Wash 12-24 hours in running water. Store in 70% alcohol.

Comments:

The "strong" solution is suitable for all but very small and permeable specimens. The "weak" solution (25 G; 5K; 0.1D; 70Q) is good for very small & delicate tissue. Gives excellent preservation of nuclei, mitotic figures, and by omitting the acetic acid from the "strong" solution, mitochondria. Not suitable for general use, for it penetrates poorly, hardens & blackens the tissue, and may interfere with certain stains.

10. The Formalins

There is a tremendous diversity of formaldehyde-containing fixatives in use. Formalin is a saturated aqueous solution of the gas, formaldehyde; this turns out to be 37-40%. It is often used simply as a 10% solution (Note: a 1:9 dilution is 10%, not the 4% you might expect! Confusing enough?).

a. 10% formalin in water

37-40% formalin	10 ml
d-H_2O	90 ml

b. Formalin saline

37-40% formalin	10 ml
NaCl	0.9 gm
d-H_2O	90 ml

c. Neutral buffered formalin (NBF)

37-40% formalin	100 ml
d-H_2O	900 ml
Sodium phosphate monobasic, monohydrate	4 gm
Sodium phosphate dibasic, anhydrous	6.5 gm

d. Millonig's buffered formalin

37-40% formalin	10 ml
d-H_2O	90 ml
Sodium phosphate monobasic, monohydrate	1.86 gm
NaOH	0.42 gm

e. Alcoholic formalin

37-40% formalin	100 ml
95% Ethanol	906 ml

Generally tissues are fixed from 6 to 48 hours in these solutions. NBF is a great general stain; it is compatible with most stains.

FIXATIVE FORMULAE

Although many fixatives remain stable for years, experience seems to indicate that freshly mixed solutions of most give the best fixation. It is possible to prepare most common fixatives from a limited number of stock solutions. On the next page are given formulae for a group of widely used fixatives. All formulae are expressed in terms of number of milliliters of each of a number of stock solutions (letter coded). All formulae yield about 100 ml. of fixative solution.

Fixative	Stock Solutions
ALLEN B15 (1,3,7) -- 27.5C; 5.6D; 80E (add 1.7 g. chromium trioxide and 2.2 g. urea)	A. Absolute (100%) ethanol
ALTMANN (2,7) - 50K; 28M; 22Q	B. 95% ethanol
BOUIN (1) - 20C; 4D; 60E	C. Formalin (neutral)
BRASIL (1,4) -- 42B; 24C; 6D; 8F	D. Glacial acetic acid
CARNOY (1,4,5) - 60A; 10D; 30P	E. Picric acid (sat. aq. sol.) (1.5 g./100 ml.)
CHAMPY (2,7) - 35G; 20K; 12M; 24Q(9)	F. Picric acid (sat. abs. alc.). (5 g./100 ml.)
CIACCIO (6,7) - 10C; 0.4I; 56M	G. Chromic acid (1% aq. sol.)
CLARKE (1,4,5) - 60A; 20D	H. Nitric acid (conc.)
FLEMMING (1,2,3,7) - 5D; 75G; 20K	I. Formic acid (conc.)
GILSON (1,8) - 7B; 0.4D; 1.7H; 29C; 61Q	J. Trichloroacetic acid (20% aq.)
HELLY (1,2,7,8) - 2C; 56L; 21M; 3N (9)	K. Osmium tetroxide (2% aq. sol.)
KAHLE (1) - 30B; 12C; 2D; 60Q	L. Mercuric chloride (sat. aq. sol.) (7 g./100 ml.)
REGAUD (2,7) - 20C; 27M; 53Q	M. Potassium dichromate (sat. aq. sol.) (9 g./100 ml.)
ROSSMAN (4) - 10C; 90F	N. Sodium sulfate (25% aq. sol.)
SAN FELICE (3,7) - 32C; 4D; 64G (9)	O. Sodium chloride (10% aq. sol.)
SUSA (1,8) - 20C; 4D; 10J; 64L; 5Q	P. Chloroform
ZENKER (1,7,8) - 2D; 56L: 21M; 3N	Q. Distilled water

Key to numbers in parentheses after fixatives:

1. Histology
2. Cytoplasmic inclusions
3. Chromosomes
4. Glycogen
5. Nucleic acids
6. Lipids
7. Wash out with running water
8. Treat with iodine solution
9. Make up just before use

PRIMARY FIXATIVES: COMMENTS

ETHANOL: C_2H_5OH

- -coagulates cytoplasm, destroys mitochondria, fusion and destruction of lipid droplets, nucleolar shrinkage, chromosomes become indistinct.
- -causes shrinkage and contraction
- -compatible with picric acid, mercuric chloride, formaldehyde, and acetic acid
- -incompatible with chromium trioxide, potassium dichromate, and osmium tetroxide

PICRIC ACID: $C_6H_2(NO_2)_3OH$

- -may be EXPLOSIVE when dry
- -coagulates nucleoplasm, preserves chromosomes, cytoplasm homogeneous but badly shrunken and soft
- -very compatible with other primary fixatives

MERCURIC CHLORIDE: $HgCl_2$ TOXIC

- -preserves inclusions, e.g., mitochondria, distinct nucleolus with poorly fixed chromosomes, cytoplasm shrinkage, low cell distortion
- -black artifact to be removed with iodine in alcohol
- -prepares tissue nicely for most stains

CHROMIUM TRIOXIDE: $Cr0_3$ TOXIC

- -chromic acid in aqueous solution
- -strong oxidizer, wash out well with water
- -light unstable, reducing to insoluble green chromic oxide
- -very good for chromosomes, neurons, destroys mitochondria, cytoplasm is coarsely coagulated
- -incompatible with reducing fixatives, i.e., ethanol or formaldehyde

FORMALDEHYDE: CH_20

- -a gas, formalin is a 37-40% aq. solution
- -preserves mitochondria and protects them from acetic acid
- -used alone, a poor fixative for paraffin technique, offering no protection from shrinkage and distortion

OSMIUM TETROXIDE: $0s0_4$

- -TOXIC and VOLATILE, use in hood
- -slow penetration, reduced by light, does not protect from paraffin shrinkage
- -incompatible with formaldehyde and ethanol

POTASSIUM DICHROMATE: $K_2Cr_20_7$

- -use above pH 4, otherwise the same as chromium trioxide
- -wash well with running water to prevent reduction to insoluble chromic oxide
- -preserves mitochondria, dissolves nucleolus, moderately fixes chromosomes, cytoplasm and nucleoplasm are left homogeneous
- -compatible with picric acid, mercuric chloride, and osmium tetroxide
- -incompatible with (reduced by) formaldehyde and ethanol

ACETIC ACID: CH_3C00H

- -use below pH 4, otherwise tissue becomes macerated
- -dissolves out mitochondria and Golgi apparatus, good chromosome preservation
- -if mixed with potassium dichromate, fixes like chromium trioxide

DEHYDRATION

After the tissue has been properly fixed, washed, and stored in 70% or 90% alcohol, it must be embedded in some firm medium so that thin sections can be made. The most widely used embedding media are nitrocellulose (celloidin) or paraffin, but for special techniques (e.g. lipid staining) water-soluble media such as gelatin or Carbowax (polyethylene glycol) may be used. We will consider only the paraffin technique.

Paraffin is not miscible with water so all traces of water must first be removed from the tissue. This is the process of dehydration. Many dehydration agents are satisfactory. Absolute ethyl alcohol, acetone, tertiary butyl alcohol, isopropyl alcohol, methyl alcohol, and dioxane have been used. Dioxane and absolute alcohol are the two most widely used dehydrating agents. We will use alcohol. Dehydration must be gradual and thorough.

Procedure

1. If tissue is in water, it should be transferred to 30% alcohol for 1-2 hours.
2. 50% alcohol for 1-2 hours
3. 70% alcohol for 1-2 hours. If the fixative contained mercuric chloride, it formed minute blackish particles and crystals of unknown composition within the tissue. These are removed with an iodine solution while the tissue is in 70% alcohol. Add a saturated ˚ solution of iodine in 95% alcohol, drop by drop to the tissue in 70% alcohol until the solution is a deep amber color. The alcohol is decolored as the iodine works. If decoloration is complete, add more. When no more decoloration is noticed, discontinue iodine treatment and go on with the dehydration. The precipitate is converted to HgI_2, which is colorless and soluble.
4. 95% alcohol for 1-2 hours. If tissue has been stored in 70% or 90% alcohol, transfer it directly to 95% alcohol
5. 100% alcohol for 1-2 hours
6. 100% alcohol for 1-2 hours
7. The tissue should now be dehydrated and ready for "clearing" or removal of the alcohol by replacement with toluene

Comments:

Absolute alcohol picks up water quite rapidly, so be sure to keep the stock bottle and your sample bottles tightly closed.

CLEARING

This is the process of replacing the absolute alcohol with toluene (some labs use xylene, benzene, cedarwood oil, tertiary butyl alcohol, or isobutyl alcohol, as well as others). This is necessary because paraffin and ethyl alcohol do not mix, but toluene and paraffin do. Toluene in this instance is acting as an "antemedium" or as a "clearing" agent. Tissues permeated with this solvent appear more or less translucent due to changes in refractive index.

Procedure:

1. Transfer tissue from 100% alcohol to a mixture of 1 part toluene: 3 parts 100% alcohol for 1 hour.
2. Change to 1 part toluene: 1 part 100% alcohol for 1 hour
3. Change to 3 parts toluene: 1 part 100% alcohol for 1 hour
4. 100% toluene - 3 to 4 hours; tissue should clear
5. 100% toluene - 3 to 4 hours
6. After clearing, tissue is ready for infiltration

Comments:

The clearing agent, like the alcohol, must be introduced slowly to prevent disruption of the tissue. These mixtures of toluene and alcohol also help to remove any additional traces of moisture. Keep bottles tightly covered. Cloudiness in the fluid or opaque spots in the tissue are sure indicators of moisture. In either case, return to 100% alcohol for additional dehydration, then back to the toluene mixtures. Failure to remove all the alcohol will result in areas of the tissue which will not be infiltrated with paraffin. This will cause great difficulties when you attempt to section the material.

NOTE: There are numerous clearing agents available now that are considerably less toxic and better smelling than toluene or xylene. Histosolve, Americlear, etc. are commercially available products. We will be using one of these.

PARAFFIN INFILTRATION

After dehydration and clearing, we are ready to replace the antemedium (toluene) with paraffin. This will serve to support the tissue elements while thin sections are cut. Paraffin is soluble in toluene, especially when it is warm, so mixtures of the two are used to slowly replace all the toluene with paraffin (melted).

Procedure:

1. Transfer the tissue from the clearing agent to a new vial containing a warm mixture of 1 part paraffin: 2 parts toluene. Stopper tightly and keep in the 45˚ oven for 1 hour.
2. Transfer tissue to a second vial containing a warm mixture of 2 parts paraffin: 1 part toluene. Again cover and incubate at 45˚˚ for 1 hour.
3. Transfer to the first bath of pure paraffin. This is a vial of paraffin kept molten in an incubator set just above the melting point of the paraffin. Leave 2-4 hours.
4. Transfer to the second paraffin bath. Leave 2-4 hours.
5. Embed (next step)

Comments:

A. There is an alternate infiltration method:
    1. Place tissue in fresh clearing fluid and add warm paraffin to just saturate the fluid. Allow to stand an hour or so in a warm (30˚) place - like on top of the 60˚ oven. Then add a bit more paraffin and allow to stand overnight. The following morning add more paraffin, place it in the oven at 45˚ & in installments slowly add paraffin until the mixture is about 75% paraffin. Allow to sit 1 hour.
    2. Go through two pure paraffin baths.

B. Tissues should be transferred using warm forceps - handle tissue gently. The shorter the time the tissue is kept at the higher infiltration temperatures the better. These higher temperatures cause hardening of the tissues and subsequent difficulty in sectioning. Using the toluene-paraffin sludge cuts down the time necessary at these higher temperatures by beginning the process of replacing the toluene in the tissue with paraffin at a low temperature.

    The melting point of the paraffin used will depend on the tissue and thickness of the sections to be cut. High melting point (hard) paraffin is most useful for thin (less than 5 m) sections and/or hard tissue (e.g. insect cuticle). Higher melting point paraffin (60-62˚) is also useful in hot weather. A moderately hard paraffin (56-58˚C melting point) is ideal for cutting relatively thin sections (6-12m). It provides good support and the sections are easily managed.

    We will use Tissue Prep, a modified paraffin from Fisher Scientific Co. (m.p. 56.5˚C).

ALTERNATE SCHEDULE FOR DEHYDRATION, CLEARING AND EMBEDDING

There is one difficulty with the foregoing techniques, and that is that you have to find a stopping place to leave the tissue overnight. This usually means either leaving the tissue in toluene or melted paraffin, both of which can severely harden the tissue, making sectioning difficult. There is a material called CELLOSOLVE (ethylene glycol monoethyl ether) which can serve as a dehydrant and clearing agent, and it does not harden tissue, even after several days. It is a material which is miscible with water, ethanol, toluene and even melted paraffin. We will take advantage of its dehydrating capabilities and its nonhardening properties.

Procedure: (assuming reasonably small pieces of tissue)

1. If tissue is in water, 30% EtOH for 1 hour
2. 50% EtOH for 1 hour
3. 70% EtOH for 1 hour. Start here if tissue was stored in 50%. This is also the time to remove mercuric chloride crystals (see pg. 14).
4. 80% EtOH for 1 hour. If tissue has been stored in 70% EtOH, transfer it directly to 80%
5. Cellosolve: 80% EtOH (1:1) for 1 hour
6. Cellosolve I for 2 hours
7. Cellosolve II for 2 hours
8. Cellosolve III overnight
9. Cellosolve: toluene or Histosolve (1:1) for 1/2 hour
10. Toluene or Histosolve I for 1 hour
11. Toluene of Histosolve II for 1 hour
12. Paraffin I for 1 hour
13. Paraffin II for 1 hour
14. Embed

Comments:

Times may vary depending on tissue type, density, and size as well as the fixative. Use the minimal times possible.

EMBEDDING

Once the tissue is impregnated with paraffin, it is ready to be cased into a solid paraffin block. If large numbers of small pieces of tissue are being handled, use of a Syracuse watch glass, in which 4-6 pieces of tissue may be embedded at once, is advisable. A somewhat more troublesome method involves the use of paper or aluminum foil boxes. We also have some disposable paper molds.

Procedure:

1. Smear the inside of a Syracuse watch glass with a 9:1 mixture of 60% alcohol and glycerine. This will prevent the paraffin from sticking to the glass. This is not necessary if using paper molds.
2. Cool the watch glass by holding the bottom in ice water for a few minutes.
3. Remove a container of molten paraffin from the oven and pour some into the watch glass (fill not quite to the top of the watch glass.). Let a thin layer harden on the bottom.
4. Place the tissue in the watch glass with a pair of **heated forceps**. Be sure to think about proper orientation. Four to six pieces of tissue may be embedded at once if desired. Paper labels are placed in the paraffin at the edge of the glass opposite the pieces of tissue as necessary. **Heat the tips of the forceps (or a needle) and pass them over the tissue to melt any congealed paraffin and provide more even crystallization.**
5. Place the bottom of the watch glass in ice water and blow on the surface so that a film of congealed paraffin forms. When a tough film has formed, let the watch glass slip into the water and leave it until the paraffin completely solidifies. This procedure provides rapid cooling of the paraffin and small crystal formation. The disc of paraffin will float to the surface when it has solidified if the sides of the watch glass are parallel.

Comments:

The block of paraffin with the tissue in it should appear glassy clear after it has hardened. If white opaque areas appear around the tissue, the antemedium has not been removed through thorough infiltration with paraffin, or the tissue was not adequately dehydrated. Reinfiltration in a fresh paraffin bath should correct this situation if antemedium is the culprit; if water is the problem, the paraffin must be removed in xylene and the tissue dehydrated again. It is usually better to discard the tissue and start with freshly fixed tissue. If very much tissue is being handled, the paraffin baths should be frequently changed to avoid accumulation of antemedium.

SECTIONING

1. Cut a rectangular block containing the tissue out of the paraffin in which it is embedded. Be sure to allow several millimeters of paraffin on each side of the tissue for support.

2. Next shape the block by trimming with a single edge razor blade. First trim the face which will be cut down almost to the surface of the tissue. Next trim the sides of the block so they are parallel to one another, and finally trim the upper and lower edges of the block so that they are parallel to one another. This will provide a cutting face which is square or rectangular depending on the shape of the piece of tissue. If serial sections are to be made and it is necessary to recognize a particular end of the ribbon, nick off one corner of the block. Each section will then be marked at the upper edge. Leave enough paraffin at the base of the block so that it can be attached to the object disc. Trim the base so it is parallel to the front face of the block. When trimming, always remove a small amount of wax at a time or the block may crack. The upper and lower edges must be exactly parallel or the ribbon will not be straight.

3. Attach the paraffin block to the object disc. Place some paraffin on the disc and melt it with a hot section lifter. Allow this layer of paraffin to cool. Hold the object disc firmly in an upright position. Heat the section lifter again. Hold the paraffin block in one hand and the section lifter in the other in such a way that the hot lifter can be used to melt the paraffin on the surface of the disc and the paraffin at the base of the block at the same time. The lifter is quickly withdrawn and the paraffin block pressed against the disc. A hot needle may be used to melt the paraffin around the edges of the block to build a firmer base of attachment. Cool the object disc and attached block.

4. Fasten the object disc securely in the microtome chuck, adjusting the position so that the lower edge of the block is parallel to the microtome knife edge. (It is easy to check to see if the upper edge is also parallel at this time. If it is not, it can be trimmed while attached to the microtome, BE CAREFUL!!).

5. The proper adjustment of the microtome knife angle is in part a matter of trial and error. For most materials, the angle formed by the cutting facet (somewhat steeper than the remainder of the knife) and the face of the block should be around 5˚. If there is insufficient tilt, the face of the block is mashed into the cutting facet - compressing the tissue. If there is too much tilt, the knife acts more as a scraper.

6. Set the microtome for the thickness desired (8-10 microns for most tissue). Start sectioning at a moderate rate of speed. Hold the ribbon as it forms with a camel's hairbrush, or the section lifter. Cut about 25 sections, then lift the ribbon away from the knife and place it on a piece of paper. Folded paper towels work well for storage.

7. If very thin sections are to be cut (2-4 microns), or if very hard tissue is being sectioned, better results may be obtained by cooling the paraffin block and microtome knife with ice prior to sectioning. Hold one ice cube against the paraffin block so that the melting ice water will drip down over the front surface of the block. Another cube should be held against the front surface of the microtome knife. A cloth beneath the knife will serve to absorb any run-off water. Hold the cubes in this position for 1-2 minutes; remove them; wipe any water drops away from knife and block with a towel; start sectioning. Slow sectioning will give less compression of the thin sections.

8. **ALWAYS CLEAN THE MICROTOME AND MICROTOME KNIFE AFTER SECTIONING. NEVER LEAVE A KNIFE OR RAZOR BLADE IN THE MICROTOME WHEN NOT IN USE. ALWAYS LOCK THE BLOCK IN PLACE WHEN NOT ACTUALLY CUTTING.**

MOUNTING SECTIONS ON SLIDES

Paraffin sections can be mounted on slides in a number of ways. 1) For some stains an adhesive may not be advisable (e.g. Alizarin Red S calcium stain) so that sections may be simply expanded on warm distilled water on the slide, blotted, and allowed to dry flat against the slide. Such sections come off rather easily. 2) An adhesive may be used in several ways: a) it may be smeared in a thin layer on the slide and the sections pressed with a rolling motion of the thumb against the slide; b) it may be smeared on the slide, the sections placed on, water added at the edge of the sections to float them, warmed to expand sections, excel water blotted, dried; or c) the adhesive may be added to distilled water and this used to float and expand the sections. The first procedure is useful where water must be avoided (e.g. urate and glycogen techniques) but doesn't give a very well flattened section.

Solutions:

1. Baker's Adhesive Albumen

Egg White	50 cc.
Distilled water	50 cc.
NaCl	0.5 g.
Sodium p-hydroxybenzoate*	0.1 g.

Add egg white after mixing the other ingredients. Centrifuge until supernatant is clear, or filter through glass wool, or allow to stand until upper layers are clear. Use clear supernatant as adhesive. Store in refrigerator.

*Moldex, Tegosept, thymol, or sodium salicylate can be substituted as mold inhibitors.

2. Dilute Adhesive Albumen

Baker's Adhesive Albumen	4 drops
Distilled water	10 cc.

3. Commercial Bakers Adhesive (20%)

Adhesive	2.5 ml
Distilled water	100 ml

The solutions should be warming plate temperature or bubbles may form under sections. Solution should be mixed just before use and not stored for any length of time.

Procedure #1

1. Clean slide by dipping in acidified 95% alcohol (1 ml. conc. HC1 per 100 ml. alcohol) and wiping dry with cheesecloth. Sections will not adhere to slides that are dirty. Avoid contact of fingers with slide surface on which the sections are to be mounted.
2. Place sections on slide with section lifter. Float by adding several drops of dilute adhesive albumen at the edges of the ribbon. Be sure sections are mounted dull side up, shiny side down.
3. Place sections on the slide warming table set at about 5-10 degrees below the melting point of the paraffin being used.
4. The sections will expand and flatten quickly. The excess fluid should be blotted away with Kimwipes and the slides left only 5-10 minutes.
5. Transfer slides to a 30-40˚ oven and leave at least overnight to ensure complete drying.

Procedure #2

1. Use slides cleaned as above; place a small drop of undiluted adhesive on the slide and smear it around well with your finger. Wipe off excess with a second finger.
2. Place one or two sections on the surface of a water bath set about 5-10˚ below the melting point of the paraffin. Be sure they are dull side up. Allow a few minutes to flatten.
3. Lower the coated slide into the water at an angle; move the slide under the sections and use a needle to hold them against the slide while removing it from the bath.
4. Drain off excess water, dry overnight.

Comments:

Loss of sections from slides during staining is a common problem in beginning work in microtechnique. If the slides are clean, and the sections are allowed to dry thoroughly after flattening, there should be little if any problem with section losses.

Tissue will sometimes have folds in it because the paraffin does not expand enough. To correct this, break away the paraffin with a needle while the section is floating on water - but not while it is warm, for the paraffin may stick to the needle.

SECTION II

STAINING

PREPARATION OF PARAFFIN SECTIONS FOR STAINING

This will be covered in more detail later. Most stains utilize either water or alcohol as their solvent. This means that the paraffin surrounding and infiltrating the tissue section must be removed. This is done by placing the slide with sections on it into two changes of toluene (or other paraffin solvents which may be appropriate), then into absolute alcohol to remove the toluene, and then into lower concentrations of alcohol and perhaps into water, depending on the solvent for the first stain to be used. This is referred to as the Down series of alcohols, hydrating the slide by bringing it to water.

STAINING

Under the bright field microscope, most tissue components, i.e., extra cellular materials, cells and their organelles, etc. lack sufficient contrast to be distinguished by the human eye. Generally one uses two or three different stains which will selectively color different components of the tissue. No single stain can be used to reveal all there is to know about a tissue, just as no single primary fixative is adequate. For general work, two stains of contrasting color are used; typically these are known as the stain and the counterstain and differentiate between the nucleus and the cytoplasm respectively. Most commonly used in this manner is the hematoxylin nuclear stain and then any one of a number of counterstains to color the cytoplasm.

Some staining reactions are well understood; others are not. You should attempt to understand as much as possible about each one we will attempt. This will enable you to get a better stain and to know what to change when something goes wrong. Mechanisms will be given in this handout in an abbreviated manner.

DEHYDRATION, CLEARING AND MOUNTING

Materials:

1. Clean coverslips - #1 coverslips should be used if slides will be examined with oil immersion objectives; otherwise #2 coverslips are satisfactory. Coverslips are dipped in acidified 95% alcohol (1 ml. conc. HCl to 100 ml. alcohol) and wiped dry with cheesecloth to clean. A supply of clean coverslips should be kept in a Petri dish.

2. Permount bottle - Canada Balsam is a natural resin often used to seal the coverslip to the slide, thus protecting the sections, and providing high refractive index medium that makes the sections perfectly transparent. Canada Balsam tends to become acid (causing fading of stains) and darker in color with age. A number of neutral, colorless, synthetic resins (Permount, a Fisher Scientific Co. product, is the one we will use) are now available.

Procedure:

1. After the sections have been stained they must be dehydrated by passing through the Up Series of alcohols. (When passing slides up the alcohol series, the edge should be blotted on a paper towel to prevent contamination (with water) or the higher alcohols.) Slides should remain about 5 minutes in each of the alcohol jars. The absolute and 95% alcohols should be replaced at intervals depending on frequency of usage.

2. After dehydration transfer slides to toluene jar. Leave 10 minutes. If all the water has not been removed from the sections, a white cloudiness will be seen when they are transferred to toluene. Such poorly dehydrated sections will not make permanent slides, but will become opaque after a year or so.

3. Remove slides from toluene; blot edge to remove excess. DO NOT ALLOW SECTIONS TO DRY THROUGH EVAPORATION OF THE TOLUENE DURING APPLICATION OF THE COVERSLIP. A drop of mountant is placed on top of the sections and the coverslip is applied. If one edge of the coverslip is touched against the slide, moved to contact the drop of mountant, and then carefully lowered with the forceps, no air bubbles will be trapped beneath the coverslip. The correct amount of mountant can be judged only with practice.

4. Slides should be dried for several weeks at room temperature, preferably in a horizontal position.

CLEANING AND LABELING SLIDES

There is no point in going to all the trouble of making a good slide unless one is prepared to clean and label it properly.

Procedure:

1. Clean any excess mountant away from the edge of the coverslip with a single edge razor blade. Mountant must be dry and hard.

2. Wipe slide off with acidified 95% alcohol. This will remove any remaining mountant and smears of stain.

3. Dip slide in a warm detergent solution and polish dry with a cheesecloth. BE SURE MOUNTANT IS DRY BEFORE CLEANING!

4. The following information should be neatly printed on the label in India ink (use Rapidograph or similar fine pen):
    a. Name of organ
    b. Name of animal
    c. Fixative
    d. Stains
    e. Initials of slide-maker
    f. Thickness of section
    g. Date

5. Attach label to left end of slide. Be sure slide surface is perfectly clean before applying label.

CHARACTERISTICS OF A GOOD SLIDE:

1. The tissue is not cracked or distorted; there is neither excessive shrinkage nor swelling.

2. If an organ, it is cut in a definite direction - cross, longitudinal, frontal median, etc. Oblique sections are difficult to interpret.

3. Sections should be flat and complete, without knife marks.

4. The sections should be well arranged on the slide and should not be close to the edge of the coverslip.

5. Details are to be well shown, with sufficient contrast between the nuclear and cytoplasmic stains. Chromatin granules are visible in the nuclei.

6. The background is to be unstained. Muddiness may be due to excess albumin, a staining of the albumin, or formation of stain precipitate.

7. The edges of the coverslip are to be parallel with those of the slide, with no excess mountant leaking out. The layer of mountant should be thin, yet must completely fill the space between the coverslip and the slide.

8. The slide should be neatly labeled on the left. Consult your manual for details.

STAINING TECHNIQUES

STAINING: GENERAL PROCEDURE

Although it is possible to distinguish considerable detail in unstained (either living or fixed) cells, contrast between cell structures is usually poor. Application of suitable staining techniques will make cell organelles clearly visible and may aid in identification of the chemical components of these structures. Before the slide can be stained the paraffin must be removed from the sections and they must be hydrated since most stains are in aqueous solutions.

The student must provide herself with two series of Coplin jars, a DOWN SERIES for paraffin removal and hydration, and an UP SERIES for dehydration and clearing. Extensive graded series of alcohols may be used in each of these, but this is really not necessary if the sections are well affixed to the slides. If a graded series is to be used the best sequence is: absolute alcohol, 95% alcohol, 75% alcohol, 40% alcohol, 15% alcohol, water. This will give about the same diffusion effects from one solution to the next throughout the series. We will use the following sequences of jars:

DOWN SERIES - Toluene 1, toluene 2, absolute alcohol**, 95% alcohol, 70% alcohol, water.

UP SERIES - water, 70% alcohol, 95% alcohol 1, 95% alcohol 2, absolute alcohol 1, absolute alcohol 2, toluene.

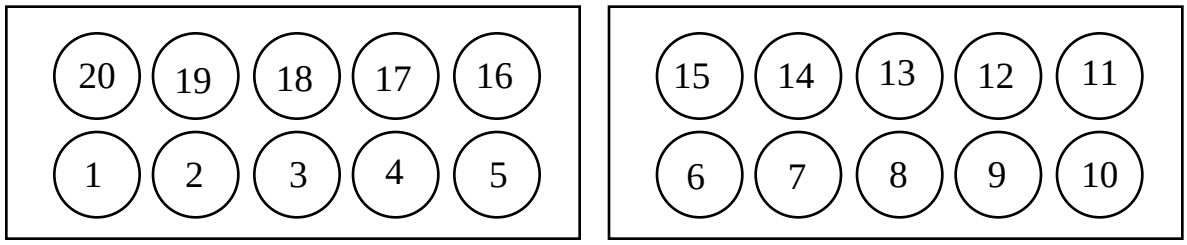
**ABSOLUTE ALCOHOL - this is a very hygroscopic reagent so it must be checked carefully to ensure absence of water.

Procedure:

1. Place the slide for 10 minutes in toluene 1; then transfer to toluene 2 for another 10 minutes. This will remove the paraffin. If the paraffin is not completely removed, staining will be irregular in the section or will not work at all. (Fresh toluene should be placed in these jars at frequent intervals if large numbers of slides are being handled.)
2. The toluene is next removed in absolute alcohol (5 min. is plenty).
3. Transfer to 95% alcohol for a few minutes, then 70%, then to water - 5 min. If the slides have been properly prepared, the diffusion currents encountered in this rather abrupt transfer will not cause any section loss.
4. Once the slides are in water, any of the staining procedures described on the following pages may be used. (Remember the special treatments for Helly's and Zenker's fixed tissues. See section on fixation.)

Literally hundreds of staining solutions are described in microtechnique texts. In general one uses a basic dye to stain the nucleus and an acid dye to stain the cytoplasm. Hematoxylin is the most widely used basic dye; eosin is the most widely used acid dye. Sometimes a more complex staining mixture such as Mallory's connective tissue stain is of use.

ARRANGEMENT OF COPLIN JARS ON STAINING TRAY



DOWN SERIES (removal of paraffin, hydration)	**STAINING SERIES**	**UP SERIES** (dehydration and "clearing")
1. Toluene #1	7. Direct Hematoxylin	15. 70% EtOH
2. Toluene #2	8. Differentiator (acid 70% EtOH)	16. 95% EtOH #1
3. Absolute EtOH	9. Bluing Solution (sodium bicarbonate)	17. 95% EtOH #2
4. 95% EtOH	10. Mordant (5% ferric alum)	18. Absolute EtOH #1
5. 70% EtOH	11. Indirect hematoxylin	19. Absolute EtOH #2
6. Acid 95% EtOH (for cleaning slides)	12. Differentiator (2.5% ferric alum or picric acid)	20. Toluene
	13. Counterstain #1	
	14. Counterstain #2	

COMMON NUCLEAR AND CYTOPLASMIC STAINS

NUCLEAR STAINS: HEMATOXYLINS

Although many basic dyes have been used as nuclear stains, hematoxylin still remains the standard nuclear stain for histological studies. Hematoxylin by itself is a very weak dye and is of no value in microtechnique. It is always used in conjunction with a mordant that causes it to act as a very strong basic dye. The mordant may be used separately from the hematoxylin solution (indirect staining hematoxylins) or mixed with the hematoxylin solution (direct staining hematoxylins).

INDIRECT STAINING HEMATOXYLINS

These require that the tissue be exposed to a mordant first. These are typically heavy metal ions (usually iron) that bind to the negatively charged sites on the tissue. Excess, unbound mordant is washed out, and then the section is exposed to the hematoxylin, which binds to the mordant and provides the coloration.

Fixation:

Most fixatives work well.

Solutions:

1. Hematoxylin Stock Solution

Hematoxylin	10 g
95% or absolute alcohol	100 ml.

Allow to ripen in light for 3-4 months with occasional admission of air. Keeps indefinitely.

2. Heidenhain's Hematoxylin

Stock solution	5 ml.
95% alcohol	5 ml.
Distilled water	90 ml.

Discard when it becomes muddy (this takes a long time).

3. Regaud's Hematoxylin

Stock solution	10 ml.
Glycerin	10 ml.
Distilled water	80 ml.

4. Ferric Alum Mordant* - 5% aq. ferric ammonium sulfate

5. Ferric Alum Differentiator*
   Dilute above solution with distilled water 1:1.

6. Picric Acid Differentiator*
   Saturated aqueous solution of picric acid.

*These solutions must be renewed at intervals that will depend on frequency of usage.

Procedure:

1. Deparaffinize sections in toluene; hydrate.
2. Transfer to ferric alum mordant. Leave for 15-20 minutes.
3. Rinse slides briefly (1 min.) by dipping up and down in distilled water.
4. Place slide in hematoxylin for 15-20 minutes. Sections should be black all over at the end of this time. If they are not, leave them in hematoxylin longer (1-3 hours), then rinse briefly in water.
5. Transfer to ferric alum differentiator or picric acid solution to differentiate. Control differentiation by removing slide, rinsing in running tap water for about 5 min., and examining under the microscope. The stain should be removed almost entirely from the cytoplasm, and the chromatin granules of the nucleus should be distinct with unstained spaces between them.
6. After differentiation wash slides in running tap water for an hour, or in several changes of water for several hours. The differentiator must be completely removed or the slides will fade later.
7. Counterstain if desired.
8. Dehydrate, clear, mount.

Results:

Nuclei, centrioles, muscle striations - black or dark blue
Cytoplasm, connective tissue - color of counterstain.

Comments:

Iron hematoxylin gives the best detail and sharpness of any of the hematoxylins. It can be used to stain almost any cytological structure (e.g. mitochondria or muscle striations) by manipulation of the times in the various solutions and the differentiation process. The iron from the mordant appears to be chemically bound by the free carboxyl groups of proteins and the phosphoric acid groups of nucleic acids. The hematoxylin then acts as an indicator for the presence of iron. Differential staining is a result of differential uptake of iron by cell structures. The stain is one of the most permanent stains known.

When staining tissues with high RNA concentrations in the cytoplasm*, it is wise to pretreat the sections with 1 N HC1 (10-12 min. at 60˚C) or perchloric acid (10% solution, 24 hours at 4-6˚C). This will give much better nuclear differentiation. Very short schedules (5 min. ferric alum, 10 min. rinse, 5 min. hematoxylin), while giving somewhat less intense stains, will also give less cytoplasmic staining.

Regaud's hematoxylin stains about half as fast as Heidenhain's. It also destains more rapidly. It is usually recommended for mitochondria, but for routine work Heidenhain's seems preferable.

The timing recommended above seems to give a stain which is as good as the much longer times often recommended. The length of time in ferric alum seems less critical than the time in hematoxylin. For careful cytological work the long schedule should be used (12-24 hours ferric alum; 12-24 hours in hematoxylin).

*High concentrations of RNA in the cytoplasm will result in strong basophilia, a marked affinity for basic dyes. This means the cytoplasm will stain with hematoxylin as strongly as the nuclei and one is unable to differentiate the stain properly.

Direct Staining Hematoxylins

The following hematoxylin stains incorporate the mordant into the stain formula and hence stain directly without any preliminary mordant treatment. They are much faster than iron hematoxylin and are the routine stains used in histological staining. They are not as good as iron hematoxylin for cytological studies.

Fixation:

Most fixatives work well

Solutions:

1. Carazzi's Hematoxylin

Distilled water	400 ml.
Potassium alum	25 gm.
Hematoxylin stock solution	5 ml.
Potassium iodate	.1 gm.
Glycerin	100 ml.

Add the reagents in the order given; stain is ready for immediate use and keeps indefinitely.

2. Harris' Hematoxylin

Distilled water	400 ml.
Potassium alum	40 gm.
Hematoxylin stock solution	20 ml.
Mercuric oxide	1 gm.
Glacial acetic acid	20 ml.

Dissolve the alum in the water and bring it to a boil. Add the hematoxylin stock and the mercuric oxide. Boil a few more minutes, with stirring. When solution turns dark purple, cool in cold water. Add acetic acid. Let stand overnight, filter and store in a tightly stoppered bottle. Ready immediately and keeps about a year.

3. Ehrlich's Hematoxylin

Distilled water	200 ml.
Aluminum or potassium alum	20 gm. (excess)
Hematoxylin stock solution	40 ml.
95% alcohol	160 ml.
Glycerin	200 ml.
Glacial acetic acid	20 ml.

Dissolve the alum in the water; there should be an excess - if not, add more. In a separate container, mix hematoxylin, alcohol and acetic acid. Add this and glycerin to the alum solution. Allow to ripen to a dark wine - red color with a fruity odor. May be ripened immediately by adding 0.2 gm. sodium iodate. Will keep for years.

4. Differentiating Solution

1 N HC1	12 drops
or	
Concentrated HC1	1 drop
70% alcohol	50 cc.

5. Bluing Solutions
   a) 70% alcohol saturated with lithium carbonate
   b) Sodium bicarbonate 0.1 g
      Distilled water 100 cc
   c) Ordinary tap water if alkaline.

Procedure: (Carazzi or Harris) (Regressive technique)

1. Remove paraffin in toluene, hydrate.
2. Place in stain. Leave for 5 minutes or longer if necessary (overstain). Sections should be dark purple or red. Can remove slides at any time and check progress of staining after rinsing the slide in water.
3. Rinse slide in tap water thoroughly and examine to see if differentiation is necessary. If it is, place slides in Coplin jar of the acid 70% alcohol. Alternate treatment with the acid alcohol and a bluing solution until the nuclei are well differentiated. The bicarbonate bluing solution will work most rapidly within 5 min.
4. When proper degree of differentiation is obtained, blue the slide carefully. If the acid is not all removed, the slide will fade later. Nuclei must never be reddish after hematoxylin staining, but a deep blue or purple. Can leave in bluing as long as you wish. Rinse 15-20 min. with running water.
5. Counterstain if desired.
6. Dehydrate, clear, mount.

Procedure: (Ehrlich) (Progressive technique) (This can be used regressively as well, i.e., overstain and differentiate in acid alcohol)

1. Remove paraffin in toluene, take to 95% alcohol.
2. Place in stain from the 95% alcohol. 1-2 minutes is usually sufficient.
3. Remove each slide, blot and rinse in up 95% alcohol until the excess stain has been removed.
4. Transfer slides to bluing solution (lithium carbonate probably best) and leave until blue. Rinse 15-20 min. with running water.
5. Counterstain if desired. Dehydrate, clear and mount.

Results:

Nuclei - black or dark blue
Cytoplasm, connective tissue - color of counterstain

Comments:

In the above procedures we are using the Carazzi and Harris stains regressively, i.e. we overstain, then remove the stain from the cytoplasm with the acid alcohol and leave it in the nuclei. Ehrlich's hematoxylin can be used in the same manner. The progressive procedure given produces a very sharp stain. It is also one of the most rapid hematoxylin procedures. In this procedure we are using the stain progressively, i.e. we leave the slide in the stain only long enough to stain the nuclei in order that we may avoid use of an acid differentiator. Ehrlich's works well with this kind of procedure since it is slow acting and doesn't readily overstain. This also makes it excellent for whole mounts.

CYTOPLASMIC STAINS; COUNTERSTAINS

These are acid dyes that are usually used to stain the cytoplasm a contrasting color to the nucleus. They are useful only when they increase the contrast, bring out details not otherwise visible, or selectively stain certain cell or tissue structures. Sometimes they add nothing to the preparation and are better omitted. Many of them tend to fade over the years.

Solutions:

1. Aqueous counterstains:
   - a. Aniline blue, WS — .5-2%; add 2 ml. glacial acetic acid per 100 ml.
   - b. Congo red — .5%; add .2 ml. .1N HC1 per 100 ml.
   - c. Naphthol green B — .25%; add .3 ml. .1% sulfuric acid per 100 ml.
   - d. Phloxine B (red-purple) — .5%; add .2 ml. glacial acetic acid per 100 ml.
   - e. Ponceau 2R — 1%
   - f. Eosin Y or B — .5%
   - g. Orange G — 1%

2. Ethanolic counterstains:
   - a. Eosin Y (orange) — 0.5% in 95% EtOH; add 4 ml. of .1N HC1 per 100 ml.
   - b. Fast green FCF — 0.03% in 95% EtOH.
   - c. Light green SF yellowish — 0.2% in 95% EtOH
   - d. Orange G — 1% in 95% EtOH; add 4 ml. of .1 N HC1 per 100 ml.
   - e. Ponceau 2R — saturated in 95% EtOH

   Note: When one consults the literature, one finds there are many different ways to make up these solutions. Eosin Y, for example, can be aqueous or ethanolic, and range in concentration from .1 to 1%. Many authors do not acidify the solution. In addition, there are many other stains, not listed here, available for use, and one also can use several in combination; an example of the latter is Gray's Double Contrast Stain with Orange II and Ponceau 2R.

Procedure:

A. Aqueous counterstaining solutions
   1. Finish staining with nuclear stain; take to water.
   2. Transfer to stain solution (generally around two minutes).
   3. Drain, blot, dip a few times in 95% EtOH to remove excess stain from surface of slide.
   4. Transfer to absolute EtOH and inspect. If overstained, you may remove some by differentiation in 70-95% EtOH.
   5. Dehydrate, clear and mount.

B. Ethanolic counterstaining solutions
   1. Finish staining with nuclear stain; take slide to 70%, then 95% EtOH, for a few minutes each
   2. Transfer to stain solution. The timing is variable: Eosin, Fast green, and Light green - less than 30 seconds, usually Orange G (or Naphthol green B or Ponceau 2R, Sat. in 95%) - 10-15 min.
   3. Drain, blot, dip once in 95% EtOH, then into absolute EtOH. Examine. If overstained, differentiate in 70-95% EtOH.
   4. Dehydrate, clear and mount.

Comments:

Eosin is the conventional contrast for hematoxylin. It tends to mask the blue of the nuclei, giving them a purplish cast. Orange G gives a pale orange cytoplasm and bright blue nuclei. Ponceau 2R gives a peach-colored cytoplasm and stains connective tissue from golden-orange-reddish; nuclei stand out brilliantly. Naphthol Green B gives a pale yellowish-green cytoplasm with connective tissue a darker shade. Fast Green and Light Green overstain very easily and tend to mask the nuclei. They are used with red nuclear stains (e.g. carmine or Schiff's reagent) but don't give a very satisfactory counterstain for hematoxylin. I find Orange G (alcohol) or Naphthol Green B (aqueous) give excellent results with hematoxylin. All the counterstains are most intense after fixatives containing mercuric chloride (Helly's, Zenker's, Gilson's, etc.)

MULTIPLE CONTRAST STAINS

Many combinations of stains have been employed to color different tissue components in contrasting shades so that they are readily differentiated from one another.

Picro-Mallory Stain

This is a modified Mallory's triple stain. It gives a much more brilliant result than the original Mallory's stain. Nuclei are bright red, white fibrous connective tissue blue, mucous pale blue, cartilage pale blue, muscles pink, erythrocytes orange, other cells various shades of violet-pink. This stain supposedly eventually fades, but I have slides about 30 years old that are still excellent. It is very helpful in recognizing the basic tissues in an organ.

Solutions:

1. Picro-Biebrich's Scarlet

Picric acid	0.2 g.
Biebrich's scarlet	0.8 g.
2% acetic acid	100 ml.

2. Aniline Blue

Aniline blue	2.5 g.
Distilled water	100 ml.
Glacial acetic acid	2.5 ml.

Boil water, add dye, boil again for a few minutes, add acid, cool and filter.

3. Phospho-Picric Differentiator Stock

Picric acid	2.5 g.
Phosphotungstic acid	25 g**
95% alcohol	100 ml.

DISSOLVE PTA FIRST, PICRIC ACID SECOND

**Enough to saturate; may be less than 25 g

4. Red Differentiator*

Differentiator stock	25 cc
Distilled water	25 cc

5. Blue Differentiator*

Differentiator stock	10 cc
Distilled water	60 cc

*Proportions may be manipulated to give faster or slower differentiation.

6. 2% acetic acid

Procedure:

1. Transfer the slide from water to Biebrich's Scarlet for 3-4 min. (until entire section is bright red). Hematoxylin stains may precede this step, but this produces a muddy-looking section.
2. Rinse in 2% acetic acid for about 30 seconds.
3. Place in red differentiator until connective tissue is colorless, muscles pink, red blood cells orange (3-5 minutes). Usually only the nuclei will be stained bright red.

4. Rinse thoroughly (5 minutes) in tap water, then 1 minute in distilled water.
5. Place in aniline blue 5 minutes or less.
6. Rinse off excess stain in 2% acetic acid (30 seconds) after blotting slide.
7. Place in blue differentiator. Dip up and down a few times.
8. Rinse briefly in 2% acetic acid, then distilled water.
9. Blot slide and place in 95% alcohol. Dip up and down a few times and quickly transfer to absolute alcohol. The blue comes out quickly in 95% alcohol, but only very slowly in absolute alcohol.
10. Dehydrate, clear, mount.

Results and Comments:

The differing rates of diffusion of dyes, in combination with differing permeabilities of tissue components, can provide a basis for differential staining.

Permeabilities		Dye Diffusion Rates	
High	collagen	Fast	Picric acid
	cytoplasm		Orange G
	nuclear material		Acid fuchsin
	muscle		Biebrich scarlet
Low	RBC		Aniline blue
		Slow	Phosphomolybdic acid (PMA)

The slowest diffusing dyes end up in the most permeable tissue; the faster diffusing dyes end up in the least permeable tissue. The latter get into the more permeable tissue as well, of course, but are subsequently removed. Certain charge effects are superimposed on these.

a. Picro-Biebrich Scarlet. Given sufficient time, the scarlet stains everything except the RBC's. Picric acid is mixed with the Biebrich scarlet and it gets into the RBC's rapidly, excludes the scarlet, and stains them yellow-red, or orange.

b. Differentiation. The PTA displaces the scarlet from the collagen. The picric acid compete with the scarlet everywhere, both with respect to the diffusion/density considerations, but also with respect to their charges. The scarlet is removed from the cytoplasm before the nucleus because of these considerations. This leaves:
   PTA - in collagen
   Picric acid - in cytoplasm and RBC's
   Biebrich scarlet - in nucleus

c. Water rinse serves to remove the PTA and a little bit of the picric acid.

d. Aniline blue is a very slow diffuser; if used only a short time, it will stain only the collagen. Rapid differentiation serves to remove only the excess blue.

Masson Trichrome Stain

This is another multiple contrast stain. Although there are four dyes used, in the original description it was called a trichrome stain. There are many modifications of this. The one presented here, by Gurr, is very good since there is good differentiational control of the two red dyes.

Fixation:

Any general fixative

Solutions:

1. Ferric ammonium sulfate, 4% aq.
2. Hematoxylin (direct)
3. Acid Fuchsin, 1% in 1% aq. acetic acid
4. Ponceau de xylidine, 1% in 1% acetic acid***
5. Fast green, 2% in 2% acetic acid
6. Phosphomolybdic acid, 1% aq.
7. Acidified water, 1% acetic acid
8. Sat. aq. picric acid

***can substitute Ponceau 2R, Biebrich scarlet, Bordeaux red, Chromotrope 2R, Chrysoidin, Orange, G, Eosin Y.

Procedure:

1. Deparaffinize slide and bring to water.
a. Remove $HgCl_2$ if necessary
b. Mordant formalin fixed tissue in sat. aq. $HgCl_2$ for 5 min., rinse in running water for 5 min., treat with Lugol's and sodium thiosulfate.
2. Alum mordant for 30 min., then wash in running water for 5 min.
3. Stain in hematoxylin (Delafield, Harris, Ehrlich) for 30 min., wash in running water for 5 min.
4. Differentiate in sat. aq. picric acid (2-20 min), wash in running water 10 min.
5. Stain in acid fuchsin for 5 min., then rinse and differentiate in d H_2O.
6. Stain in Ponceau de xylidine for 1-5 min., then differentiate in tap water. Check intensity of both red stains.
7. Treat in phosphomolybdic acid for 5 min., do not rinse.
8. Stain in fast green for 2 minutes, then differentiate in acidified water and dehydrating alcohols.
9. Dehydrate in absolute, 2x @ 3 min. each, clear and mount.

Results:

nuclei - mauve to black
cytoplasmic material - shades of red to mauve
muscle - red
collagen, mucus - green

Gabe's Trichrome Stain
(from Kiernan, 1990)

The original polycontrast stains are spectacular; they benefit from the application and differentiation of each color singly. This makes them lengthy and somewhat tedious and has pushed their replacement by a number of "one-step methods" which are simpler and less time consuming. However, these shorter procedures don't always work quite as well, for control of individual staining reactions is lost.

Fixation:

The fixative should not be formaldehyde alone, nor should it contain glutaraldehyde. Bouin's is satisfactory; fixatives containing mercury are excellent (if you use a Hg-containing fixative, don't forget to treat with iodine and thiosulfate).

Solutions:

1. An iron hematoxylin

2. One step trichrome solution:

Amaranth	2.5 g
Phosphomolybdic acid	2.5 g
Fast Green FCF	1.0 g
Water	500 ml
Glacial acetic acid	5 ml
Stir until all is dissolved, then add:	
Martius yellow	0.5 g

Stir for 1 hour, then filter. Ready for immediate use; may be used repeatedly; stable for years.
May substitute Naphthol yellow S (0.05 g) for the Martius yellow.

3. Acidified water:

Water (distilled)	1000 ml
Glacial acetic acid	5 ml

Procedure:

1. Deparaffinize and hydrate sections.
2. (Optional) Stain nuclei* with hematoxylin; wash in running tap water, 2 minutes.
3. Stain in Gabe's Trichrome mixture for 10 - 15 minutes.
4. Wash in two changes of acidified water.
5. Shake off water, dehydrate directly in absolute alcohol (up series), clear and mount.

Results:

*Nuclei: Black if stained with hematoxylin, otherwise red
Cytoplasm: pink, red or grayish purple
Erythrocytes: yellow, sometimes pink
Collagen: bluish green
Mucus, collagen matrix, some secretory granules: green

Acidic and basic tissue components

This is a simple method for distinguishing between the acidic (basophilic) and the basic (acidophilic) reacting components of the cell and tissue. It is also effective in demonstrating the effects that different fixatives have on the various tissues.

Fixation:

Any general fixative

Solutions:

1. Phloxine Solution

Phloxine B	0.125 gm.
d. H_2O	100 ml.
glacial acetic acid	0.2 ml.

will form slight ppt., filter before use.

2. Methylene Blue Solution

Methylene blue	0.25 gm.
Azure B	0.25 gm.
borax	0.25 gm.
d. H_2O	100 ml.

3. Acetic acid, 0.2% aq.

Procedure:

1. Deparaffinize and bring to water. Remove the mercury deposits if necessary.
2. Stain in Phloxine solution, (5-10 sec., up to 1 minute)
3. Rinse well in distilled water.
4. Stain in Methylene blue solution, 1 min.
5. Dip once or twice in 0.2% acetic acid to remove excess blue stain.
6. Dehydrate rapidly through 95% EtOH and into absolute, clear and mount.

Results:

Nuclei, cytoplasmic basophilia (RNA), mucin (Goblet cells) - blue
Cytoplasm, collagen, muscle fibers - pink

Comments:

The structures of the dyes are given below; you can figure out their action. The basophilic reactions could be elucidated further by selective removal of DNA, RNA, or both.

THE EFFECT OF pH ON STAINING

Because of differences in amino acid composition proteins may have quite different properties. By staining with an acid stain at different pH's we can stain different proteins. At low pH (2.0-2.2) all proteins of the cell stain. In an acid medium the positively charged (basic) groups on the proteins are all available for combination with the acid (negatively charged) dye. The proteins associated with the DNA of the chromosomes are very basic, a result of a very high concentration of lysine and arginine. These proteins are called histones. At high pH (8.0-8.1) only the very basic proteins of the nucleus have any available basic groups ionized so as to be capable of forming a salt linkage with the dye. We are thus able to use one dye as a general protein stain, or a histone stain, by adjusting the pH of the dye solution.

Fixation:

Any general fixative is OK

Solutions:

1. 0.1% fast green stock solution (aqueous)

2. 0.1% acidified (pH 2.2) fast green (Prepare and check pH just before use)

Fast green stock	50 cc.
1 N HC1	0.5 cc. (10 drops)

3. 0.1% basic fast green (pH 8.1) (Prepare and check pH just before use)

Fast green stock	50 cc.
0.05 N NaOH	0.35 ml.. (7 drops)

4. 5% trichloroacetic acid solution (aqueous)

Procedure:

1. Deparaffinize and rehydrate sections (any tissue and fixative may be used.).
2. Immerse two slides in hot 5% aqueous TCA (trichloroacetic acid) for 15 min., the TCA should be heated in a boiling water bath. This will remove all the nucleic acids (DNA and RNA). Save two slides in which the nucleic acids have not been removed to see how they will stain.
3. Rinse out TCA in 70% alcohol followed by distilled water.
4. Stain a TCA-treated slide and a non-treated slide in each of the staining solutions (both acid and basic fast green). Staining time should be 30 min.
5. Wash sections 5 min. in distilled water; transfer directly to 95% alcohol; complete dehydration, clear, mount.

Results: Fill in the table below:

Stain	Nucleic Acids		Cytoplasm Color	Nuclear Color	What components are staining?
Acid fast green	Present	(No TCA)	_____	_____	_____
Basic fast green	Present	(No TCA)	_____	_____	_____
Acid fast green	Removed	(TCA)	_____	_____	_____
Basic fast green	Removed	(TCA)	_____	_____	_____

Be sure you can explain these results?

Comments:

1. TCA has a dual usage:
    a. 5% aq. at 60˚C for 10 minutes removes RNA.
    b. 5% aq. at 90˚C for 15 minutes removes RNA and DNA.

   TCA hydrolyzes the phosphate-ester bond between the phosphate group and the sugar. This solubilizes the DNA and RNA, so it is washed out. The histones stay.

2. Fast green is an acid (-) dye. When used at a low or acid pH, all proteins stain. At a high pH, only the very basic proteins of the nucleus (histones with high concentrations of lysine and arginine) have any available basic groups to bind the stain. TCA removes the DNA and RNA, thereby unmasking additional basic sites on the histones.

3. Additional information: the approximate isoelectric point of DNA is pH 4, that of the histones is about pH 9-10.

TOLUIDINE BLUE

Toluidine blue is a basic (cationic) dye that exhibits metachromasia. When certain tissue components are stained, the orthochromatic color of the dye (blue) changes to a reddish purple (the metachromatic color). Typically the tissue components exhibiting this metachromatic color are heavily negatively charged (mast cell granules, cartilage matrix, mucin, etc.). Often the metachromatic color is lost during dehydration, so it is a good idea to examine the slide after staining but before passing it up to the final clearing agent. To help prevent this loss, we will try dehydrating in acetone instead of ethanol.

Fixative:

Any general fixative, 10% formalin, or one with alcohol in it. Avoid dichromate containing fixatives.

Solutions:

2% aqueous Toluidine Blue O

Procedure:

1. Deparaffinize and hydrate sections.
2. Stain in Toluidine Blue O for 1 minute.
3. Wash in water for 2 - 3 minutes; examine to see metachromasia.
4. Dehydrate in 2 changes of 100% acetone, 3 - 5 minutes each.
5. Clear and mount as routine.

Results:

Mucin, mast cell granules - reddish violet
Cartilage matrix, some cell granules - bluish violet
Nuclei - deep blue
Cytoplasm, bone - bluish green

Taken from Humason, 1972

TECHNIQUES FOR NUCLEIC ACIDS

THE FEULGEN REACTION FOR DNA (NUCLEAL TEST)

This is a specific histochemical test for DNA. It gives excellent whole mounts, sections, and squashes for nuclear studies. Acid hydrolysis removes purine bases from DNA to free sugar groups so that aldehyde groups are formed. These react with Schiff's reagent to give a colored product; the mechanism at this interaction is not well characterized. RNA is destroyed by the acid hydrolysis. DNA can also be successfully localized with basic dyes (e.g. methyl green) usually in conjunction with selective removal procedures.

Fixation:

Many fixatives may be used as long as they are washed out well; Helly's or Zenker's or Brasil's are excellent. The two most used fixatives are 10% neutral formalin and Clarke's fluid.

1. 10% Neutral Formalin - (1 part neutral commercial formalin to 9 parts distilled water.) Fix three hours; wash in running water three hours.
2. Clarke's fluid - fix 1-2 hours; rinse in 95% alcohol. Whole mounts should be rinsed finally in water before hydrolysis.

Solutions:

1. Schiff's Reagent
   a. Pour 200 ml. boiling distilled water over 1 g. basic fuchsin; stir.
   b. Cool and filter.
   c. Add 2 g. potassium metabisulfite and 20 ml. 1N HC1 (86 ml. conc. HC1 to 914 ml. distilled water). This forms an ugly precipitate that goes away in 5-6 hours.
   d. Bleach 24 hours in dark. Color at end of 24 hours will vary from light yellow to orange-brown depending on dye purity.
   e. Add 0.5 g. activated charcoal (Norite) and shake for one minute. Filter through coarse filter paper to remove charcoal.
   f. Store at about 5˚C in the dark in a glass stoppered, filled bottle. Will keep about 6 months.

2. Sulfite Rinse

1N HC1	5 ml.
10% potassium metabisulfite aq. sol.	5 ml.
Distilled water	100 ml.

Prepare fresh just before use. Keep a stock supply of 1N HC1 and 10% potassium metabisulfite for preparation of the sulfite rinse.

Procedure:

1. Take sections to water.
2. Hydrolyze in 1N HC1 at 60˚C. Time of hydrolysis will depend on fixation; try 15 min. for formalin, 16-17 min. for Clarke's. Most other fixatives are about this time or half this time. Rinse in distilled water for 5 min.
3. Schiff's reagent (room temperature) for 45 min.
4. Sulfite rinse - three changes, 2-5 min. each.
5. Tap water 5-10 minutes.
6. Distilled water 5-10 minutes.
7. May counterstain with orange G, light green, fast green, or Naphthol green B.

8. Dehydrate, clear, mount.

Results:

DNA - Magenta

Comments:

Specific and intense coloration of the nucleus and chromosomes - purple to red. This is a very quantitative test. The acid hydrolysis in 1.0 N HC1 removes the purine bases from the DNA and thus frees the aldehyde group at the C_1-site of the deoxyribose sugar. This aldehyde group then can react with the colorless Schiff's reagent to generate the magenta colored compound. Apparently RNA is destroyed by the hydrolysis.

BASIC DYE STAINING OF RNA

Ribonucleic acids are found many places in the cell, but are in high concentration in the rough endoplasmic reticulum and the nucleolus where they are of central importance in protein synthesis. Localization of RNA depends on use of basic dyes at a pH that inhibits protein staining, in conjunction with selective removal of RNA with acids or enzymes as a control. Three basic dyes have been widely used; pyronin Y, Toluidine blue, and Azure B. The methyl green/pyronin technique is described later. In my experience it is the most tricky of the stains for RNA. The use of Toluidine blue and azure B is described below.

Fixation:

The same fixatives as for DNA may be used. Clarke's or Carnoy's are most easily used. Three precautions should be taken:

1. Use a short time of fixation (not more than 1 hour).
2. Don't store long in alcohol, embed as soon as possible.
3. Rinse out acetic acid thoroughly in several changes of absolute alcohol.

Agents for Selective Removal of RNA:

The specific enzymes for both DNA and RNA have been crystallized and are available, though rather expensive. These are much to be preferred over acid extractants. 10% perchloric acid has been used as a substitute for the enzyme ribonuclease. It must be used at low temperatures or DNA is also removed. 1N HC1 hydrolysis, as used in the nucleal reaction for DNA, will also extract RNA.

Solutions:

1. Toluidine Blue O Stain

Toluidine blue	.032 g.
Acetate buffer, pH 4.2	100 ml.

Prepare 24 hours before use. Will keep several days.

2. Azure B Bromide Stain

Azure B bromide	.025 g.
Acetate buffer, pH 4.0	100 ml.

Prepare 24 hours before use. Will keep several days.

3. 10% Perchloric Acid

Perchloric acid, conc. (70%)	14.3 ml.
Distilled water	85.7 ml.

4. Ribonuclease

Ribonuclease	O.2 mg/ml. H_2O

Procedure:

1. Deparaffinize sections and hydrate.
2. Stain 3 hours at room temperature with Toluidine blue or 2 hours at 37-40˚C with Azure B.
3. Rinse briefly in distilled water. (Observe slide under microscope here to see maximum metachromasia).
4. Blot slide, transfer to tertiary butyl alcohol. Differentiate (keep butyl alcohol at 30-35˚C) overnight, changing the alcohol 2-3 times.
5. Blot slide, transfer to toluene, mount.
6. One control slide should be treated with enzyme or acid extractant and then stained.
   a. Treat with 10% perchloric acid 18-24 hours at 4-6˚C; rinse in several changes of distilled water: stain as above.
   b. Incubate slides in ribonuclease 1 hour at 37˚C; rinse in distilled water about 1 minute; stain as above.
   c. 2 hrs at room temperature or 1 hour at 40˚C;
7. Another control slide should be treated in distilled water at the same temperature and for the same time as the slide treated with extractant. This is primarily a control for proteolytic activity and selectivity of the extractant.

Results:

The control slide from which the RNA has been extracted should show only the nuclei staining. This demonstrates that there is no protein staining, that the RNA has been removed, and DNA (in the nuclei) is the only basophilic substance left to take the stain. The distilled water slides should show no loss of stain intensity. If there is an appreciable loss of staining in this slide, there is a non-specific removal of RNA taking place as a result of something other than the enzyme or acid. Both Toluidine blue and azure B are metachromatic dyes, i.e., they stain different cellular components in different colors. Toluidine blue stains RNA reddish purple and DNA blue-green but these color differences are obscured when the slide is mounted in resinous media (shows best in water mounts). A yellow filter helps bring out the difference. Azure B stains RNA purple and DNA blue-green and there is good metachromasia after mounting. These two stains will also stain acid mucopolysaccharides metachromatically. Mucopolysaccharides stain a bright pink. The color persists somewhat after mounting but is most striking in aqueous medium.

Comments:

Localization of RNA depends on the use of basic dyes at a pH that inhibits protein staining. This is done in conjunction with the selective removal of RNA from the tissue with acid or RNAse. Control slides should show only the nuclei staining; no protein because of the pH, no RNA because of its removal, and so nuclear DNA is the only remaining basophilic substance (phosphoric acid groups) left for binding the basic dye. The two dyes used in the experiment are also metachromatic; they stain DNA and RNA different colors. This is an additional method of distinguishing them.

METHYL GREEN - PYRONIN Y FOR NUCLEIC ACIDS

This is a frequently used technique, one that apparently distinguishes between the states of polymerization of the two classes of nucleic acids, not between the acids as such, although there is some question of this as well. Other pairs of basic dyes appear to work as well, as long as their molecular weights are sufficiently different and their relative amounts in the staining solutions are properly controlled. It can be tricky, depending to some degree on the dye lot used.

Fixation:

It is somewhat critical, as apparently any fixative that alters the state of polymerization of the nucleic acids, such as heat, picric acid, Zenkers, etc. must be avoided. Use absolute alcohol, cold acetone, brief exposure to Carnoy's, or neutral formalin (pH 7.0).

Solutions:

Pyronin Y, 2% aq.	12.5 ml.
Methyl green, 2% aq.*	7.5 ml.
Distilled water	30.0 ml.

*Purify this solution by numerous extractions with equal volumes of chloroform using a separatory funnel, to remove methyl violet.

Procedure:

1. Deparaffinize and hydrate slides.
2. Stain in methyl green-pyronin for 5-30 minutes.
3. Carefully blot with filter paper.
4. Dehydrate in n-butyl alcohol, two 5 minute changes.
5. Clear in toluene, 5 minutes; mount.

Results:

RNA (cytoplasm, nucleoli)-red;
DNA - green

Comments:

It seems that there is a great deal of uncertainty with regard to how this stain works but the shape and size of the dye molecules are important. Methyl green is very specific for DNA alone; it seems to stain highly polymerized nucleic acids. Two amino groups of the dye bind electrostatically to two phosphoric acid groups of the DNA. Pyronin stains polymers of both, but methyl green competes with it for the highly polymerized DNA. Pyronin alone stains nuclei red, but in combination with methyl green, nuclei are green. Methyl green's position on the outside of the helix prevents the pyronin from reaching the inner regions, where it would otherwise intercalate between the base pairs. RNA has a more open structure and the pyronin cation binds between the base pairs, neutralizing the phosphate anion and preventing the binding of methyl green. Control slides (such as?) are obviously important.

Another theory is that it is the dye of the higher molecular weight which stains the DNA while the smaller molecule does the RNA. Perhaps the higher density of the ribosomes and nucleoli as opposed to the chromatin are critical. The thinking is that the larger cation cannot penetrate the denser materials, but it can replace (compete) the smaller cation from less dense materials.

TECHNIQUES FOR POLYSACCHARIDES

MAYER'S MUCICARMINE STAIN FOR MUCIN

Mucicarmine is a selective stain for mucins staining them bright red. Alcian blue at an acid pH and mucihematin are two other mucin stains which may be used. You have seen the metachromatic stain reaction of toluidine blue and azure B.

Fixation:

Not critical.

Solutions:

1. Mayer's Mucicarmine Stock Solution

Carmine	1 g.
Aluminum hydroxide, powdered	1 g.
Aluminum chloride, anhydrous	0.5 g.
50% alcohol	100 ml.

Heat in boiling water bath with frequent stirring: 2.5 min. Cool under tap water, let stand 24 hours, and filter through #1 paper. Store in refrigerator, the stock keeps for several months.

2. Mucicarmine Stain

Mucicarmine stock solution	15 ml.
Distilled water	30 ml.

The stain is stable for only a few hours.

3. 0.25% Naphthol Green B with 0.3 ml of 0.1%H_2SO_4 in 100 ml.

Procedure:

1. Hydrate sections (or to 95% if going into Ehrlich's); may stain nuclei *progressively* with a direct staining hematoxylin (like Ehrlich's). Rinse well in distilled water.
2. Place sections in mucicarmine stain for 15-45 minutes - until the mucin stains bright red. You can **precede** this with an aq. light green counterstain if desired (discovered by a student error in 1996).
3. Rinse quickly in 95% alcohol and finish dehydration in absolute alcohol.
4. Clear in toluene and mount.

Results:

mucins - bright red
nuclei - black or dark blue

Comments:

Carminic acid is a naturally occurring dye found in the fat bodies of the females of the coccid insect Dactylopius cacti. This is a scale insect found in subtropical regions where the plant Nopalea coccinellifera is cultivated to provide these insects with food. The ground up, dried insects constitute cochineal. Carmine is a crude form of the dye, resulting from a precipitation of an aqueous extract of cochineal with potassium aluminum sulfate (alum). In this stain, the Carmine forms a "lake" with the aluminum mordant, and the aluminum binds to the acidic groups of the mucopolysaccharides; like other positively charged dyes, it can stain nuclei and other extranuclear structures (ex., mast cell granules) that are negatively charged if given sufficient time.

BEST'S CARMINE STAIN FOR GLYCOGEN

This stain is quite selective for glycogen; some other carbohydrates may stain, but very faintly compared to the bright red glycogen particles.

Fixation:

See the PA/S test.

Solutions:

1. Best's Carmine Stock

Carmine	2 g
Potassium carbonate	1 g
Potassium chloride	5 g
Distilled water	60 ml.
Ammonium hydroxide, conc.	20 ml.

Dissolve the carmine, KC1, and K_2CO_3 in the water. Boil 3-5 min. in a large flask. Cool and filter. Add ammonia. Store in refrigerator. Will keep several months in a dark place.

2. Best's Carmine Staining Solution

Best's carmine stock	15 ml.
Ammonium hydroxide, conc.	12.5 ml.
100% methanol	12.5 ml.

Mix just before use. **Filter**. Lasts 2-3 weeks

3. Differentiator solution

Absol. EtOH	20 ml.
Absol. Methanol	10 ml.
d·H_2O	25 ml.

Mix just before use.

Procedure:

1. Hydrate sections (or to 95% if going into Ehrlich's); and stain progressively in a direct hematoxylin (like Ehrlich's).
2. Rinse in tap water; stain 10-25 min. in carmine stain
3. Differentiate in differentiator solution, if necessary, for about 10 seconds - NO WATER!
4. Dehydrate briefly in 95% EtOH, 100% EtOH's clear and mount.
5. Controls: see the PA/S test [or do an amylase (saliva) digestion control].

Results:

glycogen - red
mucin, fibrin mast cell granules - light pink
nuclei - blue or black

Comments:

Stain only briefly in hematoxylin, not allowing the cytoplasm to stain, for we do not want to differentiate. There is no need to "blue" the hematoxylin, for the carmine is made with ammonia. After differentiation of the carmine in methanol, go directly into absolute ethanol. This staining technique is empirical but highly specific for glycogen. It can be rather unpredictable, working at some times and not at others. It is dependent on factors not fully explained, is not necessarily as sensitive as the PA/S technique, and really must be accompanied by a salivary amylase control. It is suggested that hydrogen bonding is involved in the reaction carmine at pH 4.5 is converted to carminic acid - a large molecule with carboxyl, phenol and hydroxyl groups present. Stain may depend on the hydrogen bonding of the stain to the many hydroxyl groups of the glycogen polymer.

THE PERIODIC ACID-SCHIFF (PA/S) TEST FOR POLYSACCHARIDES

This is a generic test for polysaccharides, though some polysaccharides don't give a positive reaction (e.g. cartilage ground substance), and some non-carbohydrates may give the reaction. It can be used to localize certain polysaccharides, e.g. glycogen, by using it in conjunction with selective extraction. Glycogen, mucin, and glycoproteins give strong positive reactions - rose to purplish red.

Fixation:

If glycogen is to be studied, all fixatives should be ice cold. Fix for 24 hours in the refrigerator. Rossman's Fixative has been widely used. It consists of 9 parts absolute alcohol saturated with picric acid to 1 part neutral formalin. Other fixatives which have been recommended are: Bouin's, Brasil's, Clarke's, 10% formalin. Rossman's, Brasil's and Clarke's have given the best results for me. Tissues should be kept out of water. When mounting the sections on slides, pressure alone may be used to avoid floating the sections on water. Sections so prepared come off easily, so that tests should be run to see if this is a necessary procedure to avoid loss of glycogen.

Solutions:

1. Periodic acid

Periodic acid	0.25 g
Distilled water	50 ml.

Keeps 2-3 days if stored in refrigerator.

2. Schiff's Reagent - see Feulgen Reaction.

3. Sulfite Rinse

1N HC1	5 ml.
10% K metabisulfite	5 ml.
d·H_2O	100 ml.

Procedure:

1. Bring sections to water and oxidize for 10 minutes at room temperature in the periodic acid.
2. Wash 5 minutes in running water and rinse in distilled water.
3. Immerse for 10 minutes in Schiff's reagent.
4. Rinse in three changes of sulfite rinse, 2-3 min. in each rinse.
5. Wash in tap water for 5 minutes.
6. Stain nuclei with direct hematoxylin, going to 95% EtOH if using Ehrlich's. This should be done progressively, watching carefully, for the periodic acid oxidation seems to increase the affinity of the nucleic acids for the hematoxylin. Staining time will probably be less than for untreated sections. Blue and rinse well, counterstain with a suitable counterstain (fast green) - if appropriate.
7. Dehydrate and mount.
8. For control slides (if glycogen localization is important), digest slide for 30-60 min. at 30˚C in saliva. Change saliva several times. Rinse saliva off with warm (30-37˚C) water. A more specific digestion can be obtained using a 0.1% solution of malt or animal diastase buffered at pH 6.8 (see appendix). After digestion, the slide is treated

as above. By comparing a digested slide and a non-digested slide, the location of glycogen can be ascertained.

Results:

Hexose sugar containing substances - reddish purple

Comments:

Saliva contains a diastatic enzyme which dissolves glycogen and starch. After saliva digestion, be sure to rinse the slide well; otherwise, the mucus of the saliva will stain.

The PA/S test detects polysaccharides, including glycogen, starch and cellulose. It generally stains mucins, cartilage matrix material, collagen and other tissue constituents because they contain appreciable amounts of polysaccharide. Strongly PA/S positive substances, such as glycogen and other hexose sugar containing substances stain a deep reddish purple. Other less PA/S positive material (collagen, cartilage matrix material) stain rose. The test depends upon the detection of aldehyde groups with the Schiff's reagent, but the specificity is determined by the way the sugar residues are oxidized to yield the aldehydes. Here we use periodic acid, whereas in the Feulgen reaction (Nucleal test), HCl was used.

ALCIAN BLUE

Alcian blue is another basic (cationic) dye with a blue-green color. It binds to carboxylated and sulfated mucosubstances at pH 2.5, but only to the sulfated ones at pH 1.0. In the more acid condition, the carboxyl groups are not ionized and thus don't bind the cationic dye. This of course makes the stain useful for partially dissecting the composition of certain glycosaminoglycans (GAG's, or acid mucopolysaccharides).

Fixative:

Any general fixative (formalin:99% EtOH - 1:9 recommended by Presnell and Schreibman, 1997.

Solutions:

Alcian blue, pH 1.0: 1% Alcian blue in 0.1 M HCl; filter before use. Keeps several weeks.
Alcian blue, pH 2.5: 1% Alcian blue in 3% aqueous acetic acid. Keeps for years.

Procedure:

1. Deparaffinize and hydrate sections.
2. Stain in either solution for 30 minutes.
3. Rinse in the solvent for the dye (either 0.1 M HCl or 3% aqueous acetic acid) to get rid of excess, then wash in running tap water for three minutes.
4. Optional:
    a. Stain with PA/S
    b. Stain with Feulgen technique
    c. Stain nuclei with hematoxylin or Nuclear fast red
    d. Stain with a cytoplasmic counterstain
5. Dehydrate, clear and mount as routine.

Results:

a. All acid mucosubstances at pH 2.5 stained blue/green
b. Sulfated mucosubstances at pH 1.0 stained blue/green

Taken from Presnell and Schreibman, 1997 and Kiernan, 1990.

TECHNIQUES FOR CONNECTIVE TISSUE FIBERS

ORCEIN STAIN FOR ELASTIN

Fixation:

Almost any type will do.

Solution:

1. Argaud's Orcein

95% alcohol	100 ml.
HCl, conc.	1 ml.
Orcein	to saturation (approx.0.1%)

Make up just before use

Procedure:

1. Take sections to 95% alcohol.
2. Stain sections a few hours (until elastic fibers are deep red), rinse off excess stain in 95% alcohol; counterstain lightly with fast green or another alcoholic counter stain if desired.
3. Rinse in absolute alcohol.
4. Clear, mount.

Results:

Elastin - deep red

Comments:

Orcein is a natural dye, extracted from certain lichens. The lichens contain a phenolic compound, orcinol. When the lichens are treated with ammonia and then exposed to air, a blue or violet color develops; this is due to the conversion of the orcinol into a new compound, orcein. This compound is now synthesized synthetically. The dye is alcohol soluble, is amphoteric with a pI of 5.7, and has no special affinity for elastin. The staining mechanism is unclear. It is a weakly basic dye at acid pH, so it is capable of staining negatively charged tissue components, including elastin. It has been suggested that there is the formation of hydrogen bonds between elastin and orcein. The compound is quite diffusable in alcohol and readily penetrates elastic fibers, cartilage, acid mucous and chromatin, coloring them strongly. It is not sufficiently basic to bind real strongly, especially at the acid pH, so it can be extracted from all components with alcohol. Therefore tissue density seems to control the staining. Elastin, being quite dense, holds the dye, while other, less dense components do not. Others suggest hydrophobic interactions.

FRENCH'S ELASTIC FIBER STAIN

Fixation:

Not critical

Solutions:

1. Dry Stock

Crystal violet	5 g.
Basic fuchsin	5 g.
Resorcinol	20 g.
Dextrin	2.5 g.
Ferric chloride (30% aq. wt/vol.)	125 ml.
Distilled water	1000 ml.

Boil everything together except for the ferric chloride which is added to the boiling solution. Continue boiling 2-5 min. after addition of ferric chloride. Cool. Filter; Wash (with water) and dry precipitate. This can be stored indefinitely. About 15 g. of the dry stock will be obtained with the above amounts of the ingredients.

2. Staining Solution

Dry stock	3 g.
Hydrochloric acid (conc.)	2 ml.
95% alcohol	100 ml.

Boil the dry stock in the alcohol for 5 min. on a hot plate. Cool. Filter. Add acid and make up to 100 with alcohol. This staining solution keeps well for at least a month.

Procedure:

1. Deparaffinize sections, take down to 95% alcohol.
2. Stain for several hours in the staining solution.
3. Differentiate in 95% alcohol. (Usually involves simply washing off excess stain).
4. May counterstain with eosin or orange G (alcoholic counterstain).
5. Dehydrate, clear, mount.

Results:

Elastin - deep purple

VERHOEFF'S METHOD FOR ELASTIC TISSUE

This is a third technique for elastin, considered by some to be the best.

Fixative:

Zenker's, Helly's, 10% formalin, or Bouin's; if mercuric chloride was in the fixative, it will be removed during the staining procedure.

Solutions:

1. Verhoeff's Elastic Fiber Stain

Hematoxylin, powder		1.0 g.
Absolute alcohol		20 ml.
Dissolve hematoxylin in EtOH on electric hot plate then add:		
Ferric chloride, 10% aq.		8 ml.
Lugol's solution		8 ml.
iodine	1 g.	
potassium iodide	2 g.	
distilled water	100 ml.	

2. 1% aq. ferric chloride
3. cytoplasmic counterstain, alcoholic

Procedure:

1. Deparaffinize and bring down to 70% alcohol
2. Stain for 15 minutes in Verhoeff's elastic fiber stain
3. Wash 2-3 minutes in running water. Then place in the 1% ferric chloride until stain is removed from all structures except the nuclei and the elastic fibers. The fibers will be black and the nuclei gray. Keep agitating the slide and watch carefully for the differentiation occurs rapidly (if too fast, use a weaker solution of ferric chloride).
4. Wash 30 minutes in running water, then dehydrate up to 70% alcohol. Leave slides in 70% alcohol for ten minutes to remove all traces of iodine (a component of the stain).
5. Counterstain lightly with an alcoholic counterstain, preferably eosin.
6. Complete dehydration, clear and mount.

Results:

Elastic fibers - jet black
Nuclei - gray
Cytoplasm, etc. - the color of the counterstain.

Comments:

The entire section is overstained with the Verhoeff' stain (this is a hematoxylin-ferric chloride-iodide lake) which precipitates within the elastic fibers. Differentiation is accomplished by the use of excess mordant (ferric chloride). The amount of mordant in the tissue is small compared to that in the differentiator solution, so the stain will first and most easily be removed from those tissue elements which have the least affinity for it by generation of a soluble lake with the mordant. The elastic tissue elements retain the stain the longest because they have great affinity for it. If you destain too much, you can go back into the Verhoeff's stain.

PICRO-PONCEAU STAIN FOR COLLAGEN

This stain will give collagen a red color. It can be combined with Verhoeff's method for elastin to demonstrate both in the same section.

Fixative:

Any general fixative

Solutions:

1. Hematoxylin stain
2. Picro-Ponceau S

Ponceau S, 1% aq.	10 ml.
Picric acid, sat. aq.	86 ml.
acetic acid, 1% aq.	4 ml.

Procedure:

1. Deparaffinize and hydrate sections.
2. Overstain in hematoxylin. Wash well in running tap water until slides are completely blued: 10 minutes or longer.
3. Stain in the Picro-Ponceau S for 3-5 minutes. Rinse briefly in distilled water and examine. Stain longer if the nuclei are not sharp. This stain, in addition to staining, also serves as a destaining agent or differentiator of the hematoxylin, by virtue of its picric acid content.
4. Dip several times in 70% alcohol. Dehydrate in 95% alcohol, two changes, to remove all the excess picric acid.
5. Dehydrate in absolute alcohol, clear and mount.

Results:

Nuclei: black to blue;
collagen - red;
other tissue components - yellow to orange.

Comments:

Collagen fibers have an affinity for acid stains which is strongly dependent on acidic solutions (why?). In this counterstain, the picric acid is responsible for the reduced pH and hence the action of the acid dye. It has been stated that the picric acid must be in a saturated solution, or else the collagen fibers stain pink to orange instead of red. The picric acid also serves as a counterstain, imparting a yellow color to the cytoplasm (mechanism?).

COMBINED VERHOEFF ELASTIN STAIN WITH A PICRO-PONCEAU COUNTERSTAIN FOR COLLAGEN

Fixation:

Not critical

Solutions:

1. Verhoeff's Stain
2. 1% ferric chloride
3. 5% sodium thiosulfate
4. Picro-Ponceau counterstain

Procedure:

1. Deparaffinize slide and run down to 70% alcohol.
2. Stain in Verhoeff stain for 15 minutes.
3. Rinse in distilled water, then differentiation in 1% ferric chloride for a few minutes; elastic fibers should be sharp black, nuclei a muddy black-brown. If you destain too far, return slides to Verhoeff's for another 5-10 minutes, then redifferentiate.
4. Wash 10 minutes or so in running water.
5. Transfer to 5% aq. sodium thiosulfate for 1 minute, wash in running water, 5 min.
6. Counterstain in Picro-Ponceau counterstain for 1 minute or less. Do not go too long here or the acid of the counterstain will draw out the hematoxylin.
7. Differentiation in 95% alcohol, two changes for a few seconds each.
8. Dehydrate in absolute alcohol, clear and mount.

Results:

Elastic fibers - brilliant blue-black
Collagen - red
Nuclei - blue to brownish-black
Other tissue elements - yellow

Comments:

See preceding section

STAINING OF RETICULAR FIBERS

Reticular fibers have a strong affinity for silver (argentaffin or argyrophilic), and a large number of silver stains have been devised to demonstrate these fibers.

Fixation:

Formalin is the recommended fixative, but most any standard fixative will work.

Solutions:

1. 0.25% Potassium Permanganate Solution
2. 2% Oxalic Acid Solution
3. 5% Sodium Thiosulfate Solution
4. 0.1% Gold Chloride Solution
5. 10% Neutral Formalin
6. 10% Silver Nitrate Solution (keeps only a few days)
7. Ammoniacal Silver Nitrate

   Take 15 ml. of the 10% silver nitrate solution and add dropwise concentrated ammonium hydroxide diluted with distilled water (2 parts distilled water to 1 part ammonia). Keep adding the ammonia until the precipitate which forms is nearly dissolved. A few black grains should remain. Wait 30 sec. until you are sure the reaction is final. If reaction goes too far, add more silver nitrate. Filter into Coplin jar. Add enough distilled water to cover sections. Discard this solution at end of each lab period.

Procedure:

1. Deparaffinize and hydrate sections.
2. Place for 5 min. in 0.25% potassium permanganate.
3. Rinse in distilled water and place in 2% oxalic acid until decolorized (1 min. or less).
4. Wash in running water for 5 min.
5. Two min. each in two changes of distilled water.
6. 45 min. in 10% silver nitrate.
7. Then rinse in two changes of distilled water (wave back and forth about 10 times in the first jar of water; 30 times in the second jar of water.)
8. 30 min. in ammoniacal silver nitrate - turn pale brownish color.
9. Rinse as in step 7.
10. 5 min. in 10% neutral formalin. (color first appears here-yellow under microscope, brown to eye).
11. Wash 5 min. in running water; rinse in distilled.
12. 3 min. in 0.1% gold chloride (until sections turn gray)
13. Rinse in distilled water.
14. Place in 2% oxalic acid for 5 min. or until sections turn purple.
15. Rinse in distilled water.
16. 3 min. in 5% sodium thiosulfate.
17. Wash 5 min. in running water.
18. (OPTIONAL) May stain nuclei with Picro-Biebrich's Scarlet, or a direct hematoxylin (stain progressively); counterstain if desired.
19. Dehydrate, mount.

Results:

Reticular fibers, black

Comments:

Reticular fibers can be stained by taking advantage of their carbohydrate content. This means one could use the PA/S technique. There are also older, interesting techniques which take advantage of the deposition of metallic silver at the sites of reticular fibers. They often stain the reticulin more darkly than does the PA/S approach. The histochemical basis of the two are somewhat similar. In the silver technique, the adjacent hydroxyls of the hexoses of the reticulin glycoprotein are oxidized by potassium permanganate and converted to aldehydes. These aldehyde groups then reduce the silver diamine ions of the ammoniacal silver solution, $[Ag(NH_3)_2]^+$, to metallic silver. There are four atoms of silver bound to each hexose residue; this is not enough to allow one to visualize the material. One precipitates more silver by transferring partially washed sections to the formaldehyde solution. This causes reduction of remaining diamine silver ions. This reaction is catalyzed by metallic silver, so additional precipitation takes place at the site of the original sugar residues of the reticulin. Further contrast can be generated by gold toning. Here gold chloride is reduced by the silver metal with the formation of gold and silver chloride. Treatment with oxalic acid further reduces the gold chloride which accumulated around the original silver particles. Sodium thiosulfate solubilizes any remaining silver chloride, further enhancing the contrast of the reticular fibers against the background.

TECHNIQUES FOR NERVOUS TISSUE

PROTARGOL METHOD FOR NERVES IN SECTIONS
(ACTIVATED SILVER ALBUMOSE)

When silver albumose (Protargol) is activated by metallic copper, silver is selectively accumulated by certain structures in the section (both neural and connective tissue, but the copper apparently displaces it from the connective tissue!). This forms the basis of this staining technique. The accumulated silver is then reduced by hydroquinone, an ingredient in most photographic developing solutions, and then replaced or "toned" by gold to improve the differentiation of the various cellular or histological elements. Oxalic acid reduces the gold, turning the section purple, and the thiosulfate removes the unreacted silver from the section.

Fixative:

Bouin's, 10% formalin, alcohol-formalin (9 pts. 95% EtOH:1 pt 40% formaldehyde

Solutions:

1. 1% aq. Protargol, activated with 5 gm. copper wire/100 ml. (make up fresh)
2. Reducing solution*

Sodium sulfite, anhydrous	5 g.
Distilled water	100 ml.
Dissolve completely, then add:	
Hydroquinone	1 g.

3. 1% aq. gold chloride**
4. 2% aq. oxalic acid
5. 5% aq. sodium thiosulfate

Procedure:

1. Deparaffinize and hydrate to water.
2. Put in the activated Protargol for 12 to 24 hours at 20˚C to 37˚C.
   We will try 24 hours at around 30˚C.
3. Wash briefly in distilled water (a few quick dips), then transfer to the reducing solution for 10 minutes.
4. Wash in running water for 5 minutes, then rinse well in distilled water.
5. Tone the sections in gold chloride for 5 minutes (they should turn from brown to gray). Rinse for about 15 seconds in distilled water.
6. Place the sections in the oxalic acid solution for about 10 minutes. They should take on a purplish color.
7. Wash in running water for about 5 minutes, then remove the excess silver and gold by rinsing in the sodium thiosulfate solution for about 5 minutes.
8. Wash thoroughly in running tap water.
9. Dehydrate, clear and mount.

*Or undiluted photographic developer - DEKTOL; or Sprint-Quicksilver (1+9 with water)

**add 3 drops glacial acetic acid/100 ml

Results:

Neural elements, such as neurofibrils, myelinated and unmyelinated fibers, and end plates - purplish black.

HOLMES' SILVER NITRATE METHOD FOR NERVES IN SECTION

Silver impregnation methods have been used to demonstrate a variety of tissue constituents: reticular fibers, Golgi apparatus, and nerve cells and processes. The silver does not act as a stain. It impregnates the tissue [the buffer provides the optimal pH (8.4) for this to occur, and pyridine "modifies the electrostatic condition of the tissues] and then, when reduced by a reducing solution, it forms a black metal deposit that is quite selective. Gold toning serves to substitute part of the dead black silver color and creates a variable colored image with an improved contrast. See Protargol method for oxalic acid and thiosulfate effects.

Fixative:

Bouin's, Carnoy's. Must not contain potassium dichromate, chromic acid, or osmium tetroxide. 24 hours in neutral buffered formalin is also recommended.

Solutions:

1. Silver nitrate, 20% aq.
2. Developer

Boric acid, 1.24% aq.	55 ml.
Borax (sodium borate), 1.9% aq.	45 ml.
Distilled water	394 ml.
Silver nitrate, 1% aq.	2 ml.
Pyridine solution, 10% aq. (v/v)	5 ml.

Keep in dark, make up just before use.

3. Reducer

Sodium sulfite	10 g.
Distilled water	100 ml.

Dissolve completely, then add:

Hydroquinone	1 g.

4. Gold chloride, 0.2% aq.
5. Oxalic acid, 2% aq.
6. Sodium thiosulfate, 5% aq.

Procedure:

1. Deparaffinize and hydrate to water. Soak in tap water for ten minutes.
2. Place in glass distilled water, two changes, five minutes each.
3. Transfer slides to the 20% silver nitrate solution for two hours, IN THE DARK.
4. Rinse again in distilled water, three times, ten minutes each. Following this rinse, place the sections in the staining solution overnight at 37˚C, again in the dark.
5. Transfer slides directly to the developer for two minutes. This should be at 25˚ - 30˚C.
6. Rinse in running tap water for three minutes, then distilled water, one minute.
7. Place the slide in the gold chloride solution for 3 minutes or until colorless. Rinse in distilled water, one minute.
8. Place the slide in the oxalic acid for 3-10 minutes until the nervous tissue is dark black, then rinse in distilled water for one minute.
9. Transfer to the sodium thiosulfate for five minutes, then running tap water for ten minutes. This is followed by one minute in distilled water.
10. Dehydrate, clear and mount.

Results:

Neurons, especially axons - black
Muscle and other cellular elements - rose-brown

COLE'S METHOD FOR MOTOR END PLATES (GOLD IMPREGNATION)

Metallic impregnations are of great importance in our study of the nervous system, for they outline the cell shape more definitively than the more routine histological preparations. Salts of silver, gold, mercury, and osmium are frequently used. Staining is accomplished by precipitation of metallic compounds on or within cell boundaries, causing them to stand out. The metal salts are first impregnated into the tissue, and then they are chemically reduced, which means that the metal is precipitated within the cells.

Solutions:

1. 0.001 M citric acid
2. 1% aq. gold chloride (make up 24 hours in advance)
3. 15-20% formic acid

Procedure:

1. Tease intercostal muscle from mammal or reptile into small pieces (1x1x2-3 mm)
2. Place in distilled water for a brief rinse
3. Place in 0.001 M citric acid for 10-30 minutes
4. Rinse in several changes of distilled water: 5 minutes
5. Transfer to clean glass container containing 1% gold chloride using wooden applicator stick (not metal). Keep in the dark (in your drawer) for around 60 minutes. The tissue should turn dark yellow.
6. Wash in several changes of distilled water
7. Transfer, again using wooden applicator sticks, to 15% formic acid. Keep in the dark for 8-24 hours. The acid serves to slowly reduce the gold salt. Check at intervals of several hours by examination under dissecting microscope. Proper reduction results in:
   nerve fibers and endings - black
   muscle fibers - reddish or light purple
   connective tissue - colorless
   nuclei - unstained
8. Rinse in tap water
9. Transfer to 95% methyl alcohol - glycerol (1:1) for several hours in a covered container. Then remove the cover and allow the alcohol to evaporate
10. Transfer to pure glycerin for one hour
11. To mount: Transfer muscle in a tiny bit of glycerine to the center of a 7/8 inch diameter cover slip. Place a smaller (18 mm) round coverslip over it. Spread the muscle to single fiber thickness by applying gentle pressure and slight movements (at right angles to the fibers) to the smaller coverslip. Be neat, and then carefully wipe away excess glycerol. Invert this coverslip sandwich so that the smaller one is on the bottom and carefully lower it onto a slide with a drop of Permount on it. This will hold the slips to the slide, as well as seal the edges so that, with time, the glycerol will not dry out.

Results:

nerve endings-black
muscle cells-red to purple

Illustration of Double Mounting Technique:

Comments:

Metal impregnation techniques are tricky. You should run several pieces of muscle and look at them all until you find one (hopefully) that has worked. Clean glassware is important.

NOTE:

There is a variation on this same theme which seems to be slightly less complex and perhaps more reliable. I am told that failures generally result from dirty glassware rather than differences in schedules or reagents.

Solutions:

1. 1% gold chloride

2. 25% formic acid

3. Glycerine Jelly
   a. Gelatin 3 g.
   b. Water 80 ml
      Dissolve in hot water bath; before cooling add:
   c. Glycerol 20 ml.
   d. Chrome alum., 0.7% aq. 30 ml.
      Filter while hot, add 0.1 g. camphor

This mountant must be used warm, as it solidifies when it cools.

Procedure:

1. Tease strips of intercostal muscle while immersed in the formic acid. They should be in the acid for 15 minutes.
2. Blot dry with filter paper and transfer to the gold chloride solution. DO NOT ALLOW ANY METAL TO CONTACT THE GOLD SOLUTION. Store in the dark for 10 minutes.
3. Blot dry again and immerse in excess formic acid, in the dark, for 48 hours. Blot dry, store in pure glycerol.
4. Semipermanent mounts can be made using glycerine jelly as the mountant.

Results:

Nerve endings - black

THE THIOCHOLINE - COPPER - FERROCYANIDE METHOD FOR DEMONSTRATION OF CHOLINESTERASES

This is a classic technique for cholinesterases, one which is suitable for light and electron microscopy (see Karnovsky and Roots, 1964. J. Histochem. Cytochem., 12(3):219). A thiocholine ester is used as a substrate (in this case we will use acetylthiocholine iodide), and action of the enzyme releases the thiocholine. This compound reduces ferricyanide to ferrocyanide which is capable of pulling copper ions out of chelation to form the insoluble precipitate copper ferrocyanide (Hatchett's Brown). The copper is complexed with citrate or tartrate to prevent the precipitation of copper ferricyanide. This is but one of a variety of techniques available for acetylcholinesterase.

Fixation:

Tissue is fixed in 4% formaldehyde buffered to pH 7.4 with 0.1 M phosphate or cacodylate buffer for 2-3 hours at 4˚C. Wash the tissue for several hours or overnight in 0.1 M phosphate or cacodylate buffer, pH 7.4, at 4˚C. If thick pieces of tissue are to be used, one must cut frozen sections in order to get thin enough material to allow the reactants to penetrate. The enzyme will not withstand our usual preparative techniques. Preincubate the tissue for two hours in the pH 6 maleate buffer.

Solutions:

1. 0.1 M sodium hydrogen maleate buffer, pH 6.0

2. 0.1 M sodium citrate (2.94 g/100 ml)

3. 30 mM copper sulfate (.48 g/100 ml)

4. 5 mM potassium ferricyanide (.165 g/100 ml)

Incubation medium:

Dissolve 5 mg of acetylthiocholine iodide (Sigma Chemical Co.) in 6.5 ml of the buffer. Add the following in the order given, with thorough mixing between each addition:

1. sodium citrate	0.5 ml
2. copper sulfate	1.0 ml
3. water	1.0 ml
4. potassium ferricyanide	1.0 ml

Inhibitors:

If eserene sulfate (Sigma Chemical Co.) is used as an inhibitor (it blocks the active site of the enzyme), add it as 1.0 ml of a freshly prepared 1 mM (3.6 mg/10 ml)solution in place of the water, but in the same position as the water in the sequence. Tissue must be preincubated in cytochemical medium without substrate but with inhibitor for 15 minutes in order to allow time for the inhibitor to penetrate. An additional control, which we will not run, would be to incubate the tissue in complete medium without substrate (what would this tell you?).

Procedure:

1. You will be presented with mouse diaphragm or intercostal muscle which has been fixed and is in the preincubation buffer rinse. Transfer to the incubation medium of choice.
2. a) Experimental: Use medium containing substrate but no inhibitor. Incubation time will have to be determined. It should be 15-60 minutes at 37˚; inspect the tissue under the dissecting microscope for signs of reaction product accumulation.
   b) Control: Preincubate 15 minutes at 37˚ in medium without substrate but with inhibitor. Transfer directly to medium with substrate and with inhibitor. Incubate at 37˚ for the same length of time as the Experimental.
3. Rinse tissue briefly in water. Transfer to 70% alcohol for 20 minutes. During this time cut the tissue into squares about 3-4 mm on a side. Do not allow the tissue to dry out. Replace the 70% with absolute, two changes, 20 minutes each. KEEP COVERED. Go through two similar changes of toluene to clear the tissue.
4. Mount two pieces of tissue side by side - one control and one experimental. Place a large drop of mountant on the tissue and, using several chips of broken coverglass as supports, carefully lower a coverslip into place over the tissue and chips, being careful not to disturb them. This preparation will take time to dry due to the thickness; you may need to add extra mountant at the edges as the solvent evaporates.

Results:

Sites of acetylcholinesterase activity are indicated by an accumulation of brownish precipitate.

Comments:

Acetylcholinesterase (AChE) catalyzes the hydrolysis of acetylcholinesters. The enzyme is found bound to the membranes of excitable tissues. Choline is important in nerve impulse conduction and bioelectric current generation. It is found in nerve tissue, synaptic junctions, mammalian RBC's, snake venom, and the electric "organs" of fish and eels.

MISCELLANEOUS TECHNIQUES

THE CHAMPY-KULL MODIFICATION OF ALTMANN'S TECHNIQUE

FOR STAINING MITOCHONDRIA

Mitochondria are organelles present in all eukaryotic cells. Cytologists worked very hard to develop techniques for their demonstration, for they are preserved in fixed tissue with difficulty and are not easily selectively stained. Their significance as centers of activity of enzymes involved in cellular respiration is well known, as is their physical organization as viewed through the electron microscope. In view of this, simple recognition of them under the higher magnifications of the light microscope may seem a bit old-fashioned. In certain situations, however, this approach can be very useful. Identification of mitochondria and correlation of their abundance and distribution with other cellular components can be accomplished very effectively with the light microscope. The demonstration of mitochondria requires fixation in a fixative that will preserve them and is usually a lengthy process; after fixation it is necessary to treat the tissue with a saturated potassium dichromate solution at 35˚-37˚C for several days to stabilize the phospholipids; staining is done by one of a variety of techniques.

This is probably the most widely used procedure for staining mitochondria. It is essentially a three stain process. First the tissue is overstained with acid fuchsin. Next this is displaced from the cytoplasm and from the nucleus with toluidine blue. Finally the toluidine blue is displaced from the cytoplasm with aurantia. A good preparation will have bright blue nuclei, clear yellow cytoplasm, and bright scarlet mitochondria.

I. Tissue Preparation:

Solutions:

1. Fixatives
   - Helly's
   - Regaud's
   - Flemming's (without acetic acid)
   - Champy's
   - Altmann's
2. Potassium dichromate solution (sat. aq.)

Procedure:

1. Kill animal by cervical dislocation (AVOID ANESTHETICS)
2. Dissect tissue out rapidly
3. Place in fixative to harden slightly, then cut off thin pieces and leave in the fixative.
4. Fix for four or more hours (depending on the size and density of the tissue) except for Regaud's fluid. Fix for four days with Regaud's, changing the fixative daily.
5. Transfer the tissue to a saturated dichromate solution at 35˚-37˚C. Leave for three days. Regaud's solution is usually followed by treatment with 3% potassium dichromate for seven days, changing the solution every other day.
6. Wash overnight in running water.
7. Dehydrate, infiltrate and embed in a higher melting point paraffin and section as thin as you can, 2-6 microns being best.

II. Staining:

Solutions:

1. Altmann's Acid Fuchsin (BE CAREFUL - CONTAINS SUSPECTED CARCINOGEN)
   Distilled water 100 ml.
   Aniline5 ml.
   Mix well by shaking, then add:
   Acid fuchsin 8 g.
   Keep warm, shake at intervals for 24 hours.
2. 0.5% aq. Toluidine blue - fresh
3. 0.5% aurantia in 70% EtOH - keeps indefinitely

Procedure:

1. Deparaffinize and hydrate a slide, dry with a Kimwipe up to edge of section
2. Cover section with several drops of acid fuchsin. Pass back and forth through the flame of your alcohol lamp until the stain steams. DO NOT LET BUBBLES FORM - DO NOT ALLOW TO DRY.
3. Cool thoroughly (5"). Rinse off excess stain with a stream of distilled water. Sections should appear deep purple-red.
4. Transfer slide to a Coplin jar of toluidine blue and leave for 4 minutes. This has two effects. It stains the chromatin blue and partly differentiates the acid fuchsin.
5. Remove slide, rinse in distilled water, and then place in a Coplin jar of aurantia for 4-10 minutes. This dye serves to clear up the cytoplasm and transforms it from a densely stained mass to a transparent pale yellow. CAUTION - AURANTIA IS TOXIC AND CAN CAUSE SEVERE DERMATITIS. Avoid contact.
6. Remove slide. Dip in 95% EtOH for 30-90 seconds. Go into absolute alcohol #1 for 30 seconds, then #2 for the same amount of time. Alcohol will remove the stains. Get the slide into toluene.
7. Mount

Results:

Mitochondria-red;
nuclei-blue;
cytoplasm-yellow

Comments:

1. Examine slide under your oil immersion lens
   a. If the cytoplasm is reddish - increase time in toluidine blue
   b. If mitochondria are not red enough, decrease the time in the toluidine blue
   c. If the cytoplasm is bluish - increase time in aurantia
2. You will not be able to correct an improperly stained slide. Throw it away and start over.

MITOCHONDRIAL STAINING ACCORDING TO BENDA'S TECHNIQUE:

Tissue Preparation:

Solutions:

1. Champy's fixative
2. Pyroligneous acid
3. Chromium trioxide (1% aq.)
4. Potassium dichromate (3% aq.)

Procedure:

1. Kill by cervical dislocation; dissect
2. Fix 18-24 hours in Champy's; wash in distilled water
3. 24 hours in a 1:1 solution of pyroligneous acid and 1% $Cr0_3$, wash
4. 3 days in 3% $K_2Cr_20_7$, wash 24 hours in running water
5. Embed, cut 1-3 micron sections (use ice on knife and block)

Staining:

Solutions:

1. 3-4% iron alum
2. Sodium sulfalizarinate (sat. in ethanol)
3. Crystal violet (sat. in 70% ethanol)
4. Aniline water (5% aq., with shaking)
5. 30% acetic acid
6. Tertiary butyl alcohol

Procedure:

1. Mordant 24 hours in iron alum, rinse and treat 24 hours in sodium sulfalizarinate (2-3 ml of saturated ethanolic solution in 100 ml water), wash with distilled water
2. Stain with crystal violet (equal parts of sat. solution in 70% ethanol and aniline water). Flood section and heat over small flame until it "steams". Rinse briefly with water.
3. Differentiate 15-30 seconds in the acetic acid, wash 5 min in running water. Blot slide dry (except sections).
4. Place in tert. butyl alcohol, clear and mount.

Results:

Mitochondria - intense blue.

Comments:

Presumably, the poorly diffusing dye is heated until the ion aggregates are dispersed. The hot dye solution enters the tissue in this form and readily penetrates membranes of tissue and cell components that normally would be fairly resistant. Cooling reforms the ion aggregates of the dye, and the dye which as penetrated into such components is unable to escape easily. Differentiation first removes the dye from the tissue components which are more easily penetrated, leaving the other components stained. If continued for sufficient time, even the dye trapped in the less readily penetrated components will be removed.

STAINING BLOOD SMEARS WITH NEUTRAL STAINS (COMPOUND STAINS)

Acid and basic dyes, when mixed together, interchange ions in exactly the same way as do other salts, and new compounds are thus formed by the chemical union of color acids with color bases. These are called neutral stains. Compounds in the cell that have an affinity for the neutral stain are said to be "neutrophilic", while those with an affinity for either the color base or color acid are said to be "basophilic" or "acidophilic", respectively. Giemsa made a compound called methylene azure or Azure I, known as Azure A or B by today's terminology. Originally he combined this with Eosin Y, generating as eosinate, a neutral dye. This left the basophilic cytoplasm unstained, so in order to get better differentiation, he first mixed Azure I with methylene blue (forming Azure II). This was precipitated from solution by the addition of Eosin Y. This precipitate, "Azure II-eosin", an eosinate, is then mixed with a specific amount of Azure II, which helps solubilize it, to form Giemsa's stain. Wright's stain is similar.

Preparation of Smears:

1. Place drop of blood on **clean** slide. Blood of lower vertebrates is best obtained directly from the heart to avoid contamination with mucus.
2. Place end of another clean slide on the first slide just in front of the drop.
3. Back up into the drop until the blood spreads evenly along the edge. Step 1.
4. Move the slide forward along the surface of the slide with a smooth, steady motion, keeping the spreader slide at a constant angle to the surface, (about 45˚ angle). Step 2.
5. The red corpuscles should be close together, but not touching. The thickness of the smear is controlled by the size of the drop, the angle of the spreading slide, and the speed of motion used in making the smear. If smear is too thick, decrease angle and increase speed. If smear is too thin, increase angle and decrease speed.

Solutions:

1. Wright Blood Stain
   Dissolve 0.2-0.3 gm Wright stain powder in 100 ml methanol (absolute, neutral, acetone free). Stand 1-2 days before use.

2. Giemsa Blood Stain - stock
   Dissolve 0.8 gm Giemsa stain in 50 ml of methanol mixed with 50 ml of glycerol. Shake mechanically for 2 or 3 days to dissolve.

3. Buffered water, pH 6.4

Monobasic potassium phosphate	6.63 gm
Anhydrous dibasic potassium phosphate	2.56 gm
Distilled water	1 liter

4. Giemsa Stain - working solution
   Dilute stock 1:50 with buffered water

5. Absolute Methyl Alcohol

Procedure: (Wright Stain)

1. Air dry smear. Should stain within 6 hours after preparation.
2. Lay slides on pair of glass rods over sink.
3. Flood slides with Wright Stain. Keep track of number of drops added!

4. Wait 2 min. then add twice the number of drops of buffered water, wait 4 min.
5. Pour water over slide to wash off stain quickly.
6. Blot slide between two sheets of filter paper and allow to air dry thoroughly.
7. Mount in Permount using a #1 coverslip. Dip slide in xylene just before mounting to get fewer bubbles.

Procedure: (Giemsa Stain)

1. Air dry smear. Fix for 3-4 minutes in absolute methyl alcohol in a Coplin jar.
2. Remove and blot; allow to air dry.
3. Stain slide 30 minutes (or longer) in the dilute Giemsa stain. This can either be done as for Wright stain or in a Coplin jar.
4. Wash in distilled water.
5. Blot; air dry thoroughly.
6. Mount in Permount using a #1 coverslip. Dip slide in xylene just before mounting to get fewer bubbles.

Results: Giemsa Blood Stain

Type	Nuclei	Granules	Cytoplasm
Erythrocyte	yellow-red		
PMN (Neutrophil)	reddish-purple	reddish lilac	pale pink
Eosinophil	reddish-purple	red/orange-red	blue
Basophil	reddish-purple	dark purple	
Lymphocyte	dark purple		sky blue
Platelet		violet/purple	
Monocytes	med. red/purple		pale blue

Wright's Blood Stain

Type	Nuclei	Granules	Cytoplasm
Erythrocyte	yellow-red		
PMN (Neutrophil)	dark blue	reddish lilac	pale pink
Eosinophil	blue	red/orange-red	blue
Basophil	purple/dark blue	dark purple	
Lymphocyte	dark purple		sky blue
Platelets		violet/purple	
Monocytes	blue		pale blue

Comments:

The chemistry of blood stains is not well understood. In the case of Giemsa's stain, you are using a polychromic stain containing a mixture of Azure A, Methylene blue, and Eosin Y. The azure dyes are metachromatic, and the one used here has a strong tendency to impart a reddish color to the chromatin. Methylene blue has a special affinity for the basophilic cytoplasm of lymphocytes and monocytes (presumably staining the RNA). The coloration of the granules is largely dependent on the diversity of their contents.

I personally prefer Giemsa's stain to Wright's. It seems to give a more intense, more permanent stain. Both Giemsa's and Wright's can also be used on sections. They are particularly useful on organs such as spleen, bone marrow, and others associated with blood formation.

PHOSPHOTUNGSTIC ACID HEMATOXYLIN

This method gives a differential stain in two colors. It stains the entire mitotic complex (chromosomes, spindles and asters) rather uniformly, making it useful for testes or ovaries. It stains myofibrils and differentiates them from collagen. It is affected by the fixative used.

Fixation: One with mercuric chloride is preferred. If not, see comments.

Solutions:

1. Mallory's Phosphotungstic Acid Hematoxylin

Hematoxylin (dry)	0.1 g
Phosphotungstic acid	2.0 g
Distilled water	100 ml

   Dissolve the ingredients in separate portions of water, using gentle heat on the hematoxylin. Combine when cool. Ripens after several weeks. Can ripen rapidly by adding a few drops of 0.01% potassium permanganate. Should be brownish-red in color. Mixture keeps for years.
2. 5% oxalic acid (aqueous)
3. 0.25% potassium permanganate (aqueous)

Procedure:

1. Hydrate sections.
2. Place in 0.25% potassium permanganate for 10 min. Wash in running water 1 min.
3. Place in 5% oxalic acid for 15 minutes. Wash in several changes of distilled water.
   NOTE: If formalin fixed, mordant before staining in sat. aq. mercuric chloride, rinse, and treat with iodine. If other fixative used, mordant in 5% aq. ferric alum for 1 hour, wash 10 min. and stain.
4. Stain in PTA Hematoxylin until the desired results have been attained. This will vary from 1 to 48 hours.
5. Drain slide, dip several times in 95% alcohol, then into absolute alcohols for 15-20 seconds each. Transfer quickly to Toluene.
6. Finish clearing, mount.

Results:

Chromatin, centrioles, asters, smooth muscle fibers, Z lines of straited muscle, mitochondria - blue.
Collagen, cartilage, elastin, bone - yellowish or brownish-red.
Cytoplasm - gray or pink.

Comments:

Fixatives containing mercuric chloride are preferred.

Note: Humason gives a faster stain preparation protocol.

a) Heat 10g PTA in 300 ml. water
b) At the same time, heat 0.5g hematoxylin in 200 ml. water
c) Combine the two when dissolved, bring to a boil, add 0.05g HgO and cautiously cool.
d) Add 2 ml. of 3% H_2O_2. Ready to use in 5-7 days.

THIAZINE RED R FOR STRIATED MUSCLE

This acid dye has occasionally been used as a counterstain in animal histology. It was recommended by Heidenhain as useful for enhancing the visibility of the striations of skeletal and cardiac muscle.

Solutions:

1% aq. thiazine red

Procedure:

1. Stain with either alum hematoxylin of iron hematoxylin, differentiate and blue. Rinse well. Heidenhain's is good.
2. Place tissue in a 1% aq. solution of thiazine red for 30 minutes.
3. (Rinse very briefly) dehydrate in 100% EtOH, clear and mount.

Results:

This stain brings out the cross striations of skeletal and cardiac muscle.

HISTOCHEMICAL DETECTION OF INORGANIC IONS - IRON
PERLS' METHODS FOR FERRIC IONS - PRUSSIAN BLUE

from Kiernan, 1981

Because of their solubility, many of the inorganic ions are not detectable by histochemical methods. When they are present in insoluble compounds, such as salts or protein complexes, they are not washed out during tissue preparation and therefore may be localized accurately. There are two classes of tests for these materials. First, the reaction product may be a colored or black inorganic material, or the compound formed may be a visible organic one. In the latter case, think of what you already know of mordants. In addition, these tests detect metals present as ions in the tissue; if they are bound by strong coordination bonds to organic substances, they cannot be detected. Iron within the heme group of hemoglobin cannot be detected in this manner.

Most of the iron in the mammalian body is found either in the red blood cells as hemoglobin or in the cells of the mononuclear phagocyte system, chiefly in the form of ferritin or hemosiderin. The metal is present in the ferric ($^{+++}$) form, but it is complexed to protein. Treatment with dilute mineral acid will release the ferric ions from the ferritin and hemosiderin. In the presence of ferrocyanide, Prussian blue is precipitated. This is known as Perls' Reaction. One could also reduce all the ferric ions to the ferrous state with ammonium sulfide, then dissolve the ferrous sulfide in a dilute mineral acid and capture the ferrous ions with ferricyanide ions, generating Turnbull's blue.

Fixation:

The fixative must not be acidic or contain chromium.

Solutions: (**use very clean glassware**)

1. Acid ferrocyanide reagent - prepare just before use

potassium ferrocyanide	2.0 g
water	100.0 ml
Dissolve and add:	
Conc. HCl	2.0 ml

2. Nuclear stain:
   .5% aq. neutral red in 1% acetic acid

Procedure:

1. Deparaffinize and hydrate slides
2. Place in the acid ferrocyanide solution for 10 minutes, 56˚C.
3. Wash in 4 changes of water (distilled)
4. Stain nuclei in neutral red for 15-45 seconds
5. Rinse briefly in 70% EtOH
6. Dehydrate in 100% EtOH, clear and mount

Results:

Ferric ions, liberated from ferritin or hemosiderin - blue-green
Nuclei - pink or red
Hemoglobin - unstained
Other tissue elements - shades of red or rose

Remarks:

As a control, iron can be removed before staining by treatment with 5% aq. oxalic acid for six hours or fresh 1% sodium dithionite in acetate buffer, pH 4.5, for 5 minutes.

LUXOL FAST BLUE MBSN FOR MYELIN (EXPERIMENTAL)

This technique is for myelin sheaths of fibers in both the central and peripheral nervous system.

Fixative: 10% formalin is best; calcium-formalin is recommended by some.

Solutions:

1. Luxol Fast Blue MBSN

Stain	0.l gram
95% ethanol	100 ml
10% acetic acid	0.5 ml

2. Lithium carbonate differentiator (0.05%)

lithium carbonate	0.05 gram
distilled water	100 ml

Procedure:

1. Remove paraffin, take slides through several changes of 95% ethanol.
2. Place in stain for 24 hours at 57˚C.
3. Rinse off excess in 95% ethanol, rinse well in distilled water.
4. Differentiate by dipping slide for 5 - 10 seconds in the lithium carbonate solution, washing thoroughly in 70% ethanol, and placing in water. Examine. Most of the differentiation occurs in the alcohol, so be careful not to over-differentiate. Repeat the process if necessary, until the white matter shows greenish-blue against a colorless gray matter.
5. Wash well with distilled water, dehydrate, clear and mount.

Results: Myelinated fibers - blue green

Comments:

This stain seems to be somewhat specific for phospholipids; the staining mechanism is unclear, but it may depend to some degree on hydrophobic interactions and the fact that it is an acid dye. The stain is insoluble in water, but soluble in common alcohols (ethanol and methanol), and very soluble in glycols, ethanolamine, choline, and phospholipids. According to Lillie (1977), this solubility spectrum likely accounts for its staining of myelin. The mechanism of differentiation is also unclear. This stain will also color stores of phospholipid and enter the hydrophobic domains of some proteins.

SECTION III

PROJECTS

PROCEDURE FOR FINAL MICROTECHNIQUE PROJECT

The purpose of the project is threefold: 1) practice in the techniques you have learned using a variety of tissues and organs from a variety of animals; 2) provide experience in full utilization of techniques in the study of the histology of one organ; 3) provide a modest collection of slides of tissues and organs of various vertebrates for each student in the lab.

1. Animals - selected representative vertebrates
2. Organs and tissues - you should think about preparing slides of almost any type of tissue or organ in the body. These can include connective tissue, glandular tissue, nervous tissue, muscle, sense organs, digestive organs, respiratory organs, excretory and/or reproductive organs.
3. Fixation - a variety of fixatives will be available. Each organ or tissue should be fixed in a different one; give some thought to selecting one that is appropriate for the tissue or what you want to show. Keep careful records as to the identification of the organs, and in the case of the alimentary track, of the precise region of the tract that was sampled.

   When the animal is sacrificed and dissected, you should get your tissue quickly and cut it with your razor blade into sections not much more than about 2 mm. thick in one dimension. The thin slices allow for the rapid and even penetration of the fixative, dehydrating agents, clearing agents, paraffin, etc. Also be sure to pay attention to the orientation you want when you cut your sections. If you are unsure about this, ask for assistance.

   a. Label all jars with the fixative, tissue and time and date.
   b. Fix for the appropriate time period. Don't skimp here or in the washes.
   c. The running water wash requires that you cover the jar with a piece of cheesecloth held in place with a rubber band. This is then placed in the sink under a gentle stream of water.
4. You are to follow standard procedures for dehydration, infiltration, and embedding. Cut sections at 8 microns, unless there is good reason not to, and mount enough slides to provide one for each student in the class and still have enough left over for your own use and to replace a few if others destroy their own. If you find a tissue is making especially good slides, it is wise to make a few extra. A DEADLINE for completion of this task will be set by the instructor. At this time each student will give a slide of each of her organs to every member of the class. These slides will be identified by a code number marked on the slide with the diamond marker, and a sheet identifying the animal, tissue or organ, fixation, and student preparing the slide will be posted.
5. Staining - Select one of the organs you have fixed and make slides using as many different techniques as necessary to provide information about the histology of that organ. Special stains will be made available on a weekly basis. Write a histological description of that organ (to hand in) based on the slides you have prepared. A minimum of five differently stained slides will be required for this. For the other organs <u>you</u> <u>have</u> <u>fixed</u>, try a number of stains and hand in the best two slides of each organ. Stain as many of the other slides you have obtained from class members as you can and hand in the best ones you get, to bring the total number of slides to be handed in up to _____number depends on size of class. After examination these will be returned to you to keep (you have to buy the slide box if you want it) unless the instructor obtains your permission to keep one or more for the permanent slide collection.

6. Summary of requirements:
   a) _____ slides handed in (5 or more from one organ you fixed, two or more from each of the organs you fixed, one each of other organs prepared by others to bring the total to __.
   b) Written descriptive report of the histology of one organ you prepared. **This report is based on <u>your</u> slides and what <u>they</u> show, not a text**!.
   c) Slides and report due date will be set. It will be close to last day of class.
   d) Slides will be graded on the basis of quality of fixation, sectioning, staining, neatness of mounting and labeling.
   e) Start now. Do not wait until the last few weeks of school. Some class time will be set aside for this staining; some of the work will be done outside of class.

FIXATIVE STUDIES

This exercise is designed to serve a number of purposes: 1) It will introduce you to the basic operations of microtomy. Each student will be fixing, dehydrating, infiltrating, embedding, sectioning, and mounting tissue. 2) You will gain experience in judging quality of fixation since you will be asked to compare the fixation produced by a series of standard fixatives acting on one tissue. 3) You will also be making a study of the effects of fixation on stainability.

Fixatives:

A variety of different fixatives of similar and dissimilar properties will be evaluated.

Test Tissues:

We will use kidney or testis of some animal.

Procedure:

1. Each student will be assigned a fixative (identified only by a code designation) and a tissue. The animals will be sacrificed and the tissues distributed to each student for fixation. By the next lab period all tissues should be in the storage solutions ready for further treatment.
2. Dehydrate, infiltrate, and embed tissue.
3. Prepare sections (8 microns) and mount. Mount about 20 slides. This will provide enough slides for the rest of the exercise and all extra ones for practice of staining techniques. Mount two sections of the tissue per slide. KEEP CAREFUL NOTES ON THE QUALITY OF SECTIONS AND EASE OF SECTIONING WITH YOUR FIXATIVE.
4. Turn in a set of slides to the instructor. These will be labeled with a code designation to indicate the fixative used. Each student will then be given a set of slides, each slide fixed with a different fixative.
5. Each student will next be assigned a test stain and will stain all slides with the test stain. (Practice the stain you are assigned on some of your extra slides before trying it on the test slides.) DETAILED NOTES SHOULD BE KEPT ON THE STAINING OF EACH TEST SLIDE. The exact approach taken will vary with the test stain and should be worked out with the guidance of the instructor, but the following types of observations should be made:
   a. How much time (in minutes) is required for the stain(s) to `take', or conversely what are the differences in intensity of staining after each slide has been exposed to the stain for the same period of time?
   b. Are there differences in selectivity?
   c. Do the stains (especially counterstains) come out more readily after certain fixatives?
   d. Are the staining results more brilliant after certain fixatives?

   Rate the slides as to overall stainability. The best slide would be #1, second best #2, etc. This rating is based only on stainability, not on quality of fixation.
6. Next you will be shown some demonstration slides illustrating the effect of several fixatives on quality of fixation of a tissue. Examine you own slide series with an eye to detecting similar differences in quality of fixation. Take notes on your observations concerning fixation quality for each slide. Examples of the sort of thing you will be looking for are given below:
   a. Shrinkage - spaces between cells in epithelium; pulling away of cytoplasm from the nuclei; differences in diameter of tubular structures.
   b. Fineness of cytoplasmic precipitate - are there large vacuoles and/or coarse fibrous structures, or is the cytoplasm very homogeneous with small, evenly distributed granules?

c. Distortion - especially at the surface of organs and cells, e.g., brush border of kidney tubules.
d. Preservation of cellular components - especially applicable to histochemical stains.
e. Fineness of nucleoprotein precipitate - size of chromatin granules in the nucleus.

7. Rate your slides as to quality of fixation. The best slide would be #1, second best #2, etc. Reasons for your ratings are important.
8. Write up a short report giving all pertinent data, observations, and conclusions including your ratings as regards stainability and quality of fixation. Hand in your slides with the report.
9 The instructor will prepare a composite report and ditto copies for each student.
10. PLEASE DO YOUR OWN WORK ON THIS! DO NOT ATTEMPT TO MAKE COMPARISONS WITH THE SLIDES AND RATINGS OF OTHER MEMBERS OF THE CLASS! Such comparisons would destroy the objectivity of the ratings and make them of little interest.

STAINS FOR FIXATION STUDY

The following is a list of the test stains we will be using for the fixative test series.

1. Carazzi's Hematoxylin (pg. 29) and Ponceau 2R. Use a standard staining time of two minutes for all slides (slides must be handled individually) and note differences in staining. If staining is inadequate after two minutes, increase the time (record the change). Counterstain with Ponceau 2R (aqueous) for a standard time of two minutes.
2. Delafield's Hematoxylin (pg. 29) and Eosin Y. Use a standard time of 5 minutes, note differences in intensity, then differentiate (record times). If staining is not intense enough, increase staining time. Use standard time (15 seconds) for Eosin also, followed by standard times in the alcohols. Observe results. If necessary restain or destain longer, noting times in various solutions.
3. Heidenhain's Hematoxylin (pg. 27) and Orange G (in ethanol). Use standard times and observe times needed for differentiation. Use 15 min. in ferric alum, 15 min. in the Heidenhain's hematoxylin, and 15 min. in Orange G.
4. Harris' Hematoxylin (pg. 29) and Fast Green (ethanol). Follow the instructions for Delafield's Hematoxylin and Eosin Y.
5. Phosphotungstic Acid Hematoxylin. This is a slow-acting, polychromatic stain (see pg. 65). Leave slides in stain until nuclei are dark blue, connective tissue - yellowish red. Stain progressively - time for a particular slide may be from 1 to 8 hours, possibly up to 24 hours. No counterstain.
6. Picro-Mallory Stain (pg. 33). Use the standard times of 5 min. in Picro-Biebrich's Scarlet and 1 min. in aniline blue. Record times for proper differentiation.
7. PA/S and Carazzi's Hematoxylin and Orange G (ethanol). Follow instructions for PA/S on page 45. Do not run saliva digested controls; use standard times. Stain with Carazzi's Hematoxylin progressively, see #1. Stain 15 min. with Orange G. Observe differences in staining (especially the Schiff staining) after mounting.
8. Nucleal Test (pg. 38) and Naphthol Green B (aqueous). Use a 15 min. hydrolysis time in 1.ON HCl for all slides. Keep all other times standard. Stain 2 min. in counterstain; note how much, if any, destaining is necessary.
9. Best's Carmine Stain (pg. 44). Stain nuclei progressively using Carazzi's Hematoxylin, noting the time necessary. Stain 15 minutes in carmine stain and note the amount of differentiation (time) necessary in the methanol. Do not run controls; do not counterstain.
10. Azure B, pH4.2 (pg. 40). Stain 2 hours at 37˚, differentiate overnight in tertiary butyl alcohol. Observe staining differences immediately after mounting. Do not run controls.

SECTION IV

MICROSCOPY AND EXAMINATION OF PREPARED SLIDES

THE LIGHT MICROSCOPE

For well over a century, the light microscope has been one of the most important instruments available to biologists of all disciplines. In fact, a modern research worker in many fields of science besides biology and medicine must rely on microscopes to unlock the secrets of things too small to be perceived with the naked eye.

In the past two or three decades the light microscope has been modified and improved in many ways. Lenses have not improved very much, but various contrast generating devices have been added to improve the image quality, and many ways have been found for making microscopes into measuring instruments capable of yielding quantitative information of various kinds. It is now possible to measure quite accurately a number of physical properties of living cells and other microscopic objects. From these data it is often possible to study or infer processes or events at the molecular level. A modern research microscope equipped with phase contrast, interference and polarized light optics with attachments for microspectrophotometry and microfluorimetry is a far cry from the simple and compound microscopes of even a half century ago.

Research biologists often find that they spend their entire lives studying the background information in physics, chemistry and mathematics required to explore biological phenomena. For a person who must use microscopes in research, a course in optics would be very worthwhile. A course in optics normally is divided into three sections, in which light is considered first as rays, then as waves, then finally as particles. Geometrical optics deals with light as rays emanating from the source in straight lines unless they enter a medium of different refractive index (e.g. a lens) or are reflected by a mirror. Geometrical optics thus deals with how lenses work. Physical optics treats light as waves. Historically, the wave theory of light was introduced in order to account for the phenomena of diffraction (scattering), interference, and polarization. Physical optics forms the basis for our understanding of phase contrast, interference, and polarized light microscopy. Quantum optics is the 20th Century's contribution to the science of light, and deals with light as particles called photons (or quanta). Quantum optics forms the basis for understanding absorption and fluorescence and many other interactions between light and matter. All of these optical properties yield important chemical information about kinds of substances present and their concentrations.

In the most general sense, lenses are devices for altering the direction of trains of waves or particles (usually photons or electrons) in such a way as to form images. The magnification of a lens or of a system of lenses, such as a microscope, is simply the factor by which the image is made larger than the original object. There is virtually no limit to the magnification that can be achieved with any microscope. However, there is a real limit to the useful magnification, because beyond a certain point the image appears "fuzzy", and it is said to have "empty magnification".

The magnification at which you are observing details through your microscope is computed simply as the product of the figures printed on the objective and ocular. (For example: using a 40x objective with a 10x ocular gives an image magnified 400 times and appearing 10 inches in front of the eyes.)

The resolving power of a microscope lens is defined as the ability of the lens to separate two tiny spots of light in a dark field (or two dark specks against a bright background) and render them as two spots rather than one. The resolution is the smallest such distance that can be detected. These two properties, that is, magnification and resolution, are independent of one another in the sense that magnification may be attained without resolution. It is the resolving power that is so highly dependent upon proper lens adjustment and control of illumination. Early microscopists learned that the resolving power depended upon what they called the `light gathering power' of the objective lens. We now call this the angular aperture. The angular aperture is half the widest angle subtended by any two rays entering the lens from an object in focus. An objective capable of utilizing a large angular cone of light coming from the specimen (i.e., a larger angular

aperture) will have better resolving power than an objective limited to a smaller cone of light. It was found that the resolving power could be increased by immersing the object and the lower objective surface in a medium of high refractive index. This was the basis for the vastly improved performance obtained when Ernst Abbe introduced his `homogeneous immersion system' in about 1875. Abbe introduced the concept of numerical aperture, defined as the refractive index of the medium multiplied by the sine of the half angular aperture.

$$\text{Refractive index} = \frac{\text{Velocity of light in vacuum}}{\text{Velocity of light in medium}}$$

Since the refractive index of oil is 1.515, and air 1.000, there was obviously a 50% increase in numerical aperture with an oil immersion lens (objective).

Numerical aperture (NA) = refractive index of medium x sine q (q is half the angular aperture)

Abbe also demonstrated that the resolving power (R) for an objective lens depends on the numerical aperture (NA) of the lens system and also upon a second variable, the wave length (l) of the light used for illuminating the specimen, according to the relationship:

$$R = \frac{0.612l}{NA}$$

Resolution is highest when the value for R is smallest and indicates the size of the smallest particle which can be clearly resolved. Resolution can be improved in two ways: 1) By decreasing the wave length of light employed; 2) by increasing the numerical aperture of the objective. Under ordinary laboratory conditions the wave length available is relatively constant, therefore it follows that resolving power may be increased only by utilizing a system with a higher NA. Remember that there are two factors involved in NA. Accordingly one can select an objective lens with a higher sine q and then properly adjust the illumination so that the cone of light will fill the available aperture of the objective. This will give the maximum value of q. One should also consider the other variable involved in NA and this involves changing the refractive index of the system.

For example, when the medium between the specimen and the objective lens is air, the refractive index = 1; therefore, the NA is less than one because it is impractical to make the one-half angular aperture as large as 90_. Therefore, the NA of such a dry system is less than 1, and usually less than 0.95. By using immersion oil with a refractive index as high as 1.5, one gets a 50% increase in the NA.

The limits of resolution can be readily calculated from the above equations. Since the eye is most sensitive to the color apple-green (wave length = 0.55m), we will use this in our calculations.

1. Standard 10x objective - NA = 0.25 $R_{10x} = \frac{.6x0.55}{0.25} = 1.3mm$

2. Standard 40x objective - NA = 0.65 $R_{40x} = \frac{.6x0.55}{0.65} = 0.51mm$

3. Standard 90x objective - NA = 1.25 $R_{90x} = \frac{.6x0.55}{1.25} = 0.27mm$

If we use blue light (0.49m) and an apochromatic oil immersion objective (NA = 1.4) we can get a theoretical resolution of 0.18mm. In practice this would be about 0.2m. By using ultraviolet light and quartz optics in conjunction with photographic emulsions we could theoretically get a resolution of 0.1m. This represents the absolute limit of the light microscope. Many of the smaller cell organelles are at or below these limits. The electron microscope, by employing extremely short wave lengths (0.000005mm), gives a resolution of about 10-15 _ (0.001mm).

If a very thin "pencil" of parallel light rays strikes a microscopic object, some of the light continues in the same path as the "undiffracted light". Some of the light is scattered or diffracted by object details and diverges at various angles from the undiffracted light. The finer the detail in the object, the greater is the diffraction angle (Figure I).

FIGURE I
Light diffracted by a microscopic object

Object detail appears in a microscope image only when the diffracted light from that detail passes into the objective. This is why the numerical aperture is so important in resolving power: it limits the angle at which diffracted light can be accepted.

Microscopic objects are perceived if they alter the distribution of brightness in the image. For this reason, we adjust the microscope in such a way that the brightness of the empty field is as uniform as possible (by Köehler illumination: see below).

An object that is visible only because it absorbs light is called an amplitude object. One that does not absorb, but rather bends, retards, or advances light waves in relation to others nearby is said to be a phase object (because it phase-shifts light waves). Many objects are mixed phase-amplitude objects.

FIGURE II

Light waves passing through a cell: A. a fixed and stained cell (amplitude object) and B. a living, unstained cell (phase object). In A absorption reduces wave amplitude (a). In B refractive index differences in the cytoplasm and nucleus produce different amounts of retardation (from Ross).

The human visual apparatus (eye plus brain) and its extensions, photographic film and television cameras, are capable of recording images in brightness contrast when objects are illuminated with monochromatic light (light of one wave length). Both photographic and television images can be quantified - measured. These same types of devices can also register in color contrast - the differential behavior of different wave lengths (polychromatic illumination). Color contrast is what you see when you observe a green Euglena (the chloroplast absorbs red and blue light, leaving the green to be perceived) or a red blood cell (containing haemoglobin, which absorbs blue and green wave lengths, leaving the red wave lengths to be perceived), or when you look at a specimen that has been stained with special dyes.

The important thing to remember is that just because some detail in a microscopic image may not be visible does not mean it is not there! Later we shall consider an entire family of contrast generating devices that make visible many details, the presence of which was never suspected from observation with an ordinary microscope.

THE BRIGHT FIELD OR SIMPLE COMPOUND MICROSCOPE*

(from A.A. Weaver, Histology Lab Guide, 1964)

As you should know by now, the bright field microscope is most useful for viewing amplitude objects. It is most frequently used for looking at brightly stained sections although naturally pigmented cells do show some detail. Remember that the amount of available contrast can be just as important a limitation as resolving power.

To obtain the maximum resolution from a compound microscope one must employ Köehler Illumination (see next section). This produces a beam of parallel light rays which will just fill the back lens of an objective and thus will give the full sine q for that particular objective. Köehler illumination requires a rather elaborate microscope illuminator. There are, however, a number of important rules whose application will result in much improved resolution even with an ordinary desk lamp as the source of illumination:

1. KEEP THE IRIS OPEN TO FILL THE BACK LENS OF THE OBJECTIVE WITH LIGHT. This can be checked by removing the ocular and looking down the tube of the microscope. The iris will be seen in the plane of the back lens of the objective. It should be open so as to almost coincide with the edge of the back lens.

2. NEVER USE THE IRIS TO REDUCE LIGHT INTENSITY. If the light is too bright at full aperture, reduce the wattage of the bulb.

3. USE THE PLANE SURFACE OF THE MIRROR (if there is a separate light source). Use of the concave surface reduces the resolution and increases glare.

4. KEEP THE CONDENSER RACKED UP LEVEL WITH THE STAGE. Using the condenser in a lower position has the same effect as stopping down the iris: contrast and depth of field are increased, but resolution is decreased.

5. KEEP ALL OPTICAL SURFACES CLEAN. This will reduce glare and markedly improve quality of image.

Most student microscopes cannot be adjusted properly for Köehler illumination, for one reason because they are equipped with built-in illumination systems. These are permanently aligned so that centering is unnecessary. Optically they are the equivalent of the Köehler system. The only adjustments to be made are in the substage condenser, whose function is to direct a light beam of the desired numerical aperture and field size onto the specimen. The iris diaphragm in the condenser controls the effective numerical aperture of condenser and therefore the angle of the cone of illuminating rays. The theoretical resolution is highest when the iris is wide open, because the working aperture is then highest. However, the contrast is highest when the iris is fully closed. The adjustment of the iris is essentially a compromise between high contrast and high resolution, and the proper setting is different for every type of microscope and for every object. In dealing with thick objects (for example a Paramecium or rotifer under high power) it is important to realize that the depth of field is far greater when the iris diaphragm is closed than when it is open. In practical terms, this means that closing the iris may bring into the image a number of out of focus details that were previously not observed.

To prepare the microscope for viewing with any of the objectives place a slide on the stage with the cover slip up and follow these steps, in sequence:

1. Swing the 16mm objective (10x) into position.
2. Raise the condenser to its highest position; on microscopes with condensers having movable components, follow the manufacturer's instructions.

3. Place a slide on the stage, center the object over the condenser lens with the stage control knobs and turn the lamp on. If your microscope is equipped with a low voltage lamp and transformer, the first intensity stop on the transformer will usually suffice.
4. With the coarse adjustment, bring the object to within a few millimeters of the front of the 16mm objective.
5. Looking into the oculars, use the coarse adjustment to move the specimen away from the front of the objective until the specimen comes into focus.
6. If you are using a binocular microscope, adjust the interpupillary distance and compensate for any difference which might exist in the focal length of your two eyes.
   Interpupillary distance: Adjust by grasping knurled plates which hold oculars and set oculars to most comfortable position for your eyes. Note reading on Interpupillary Scale for future settings.
   Compensation for difference in focus of eyes: There is a knurled collar on the left eyepiece. So that the image will be sharply in focus for both eyes, close the left eye and focus the image with the fine adjustment for the right eye. Now close the right eye and focus the image sharply for the left eye by rotating the knurled collar. This setting need not be adjusted further if it is not disturbed.
7. If your microscope is equipped with a centerable lamp and/or field diaphragm, check the instruction manual, or with an instructor, for proper positioning. If your substage has a swing out, or slide out lens element it will usually be set one way for the scanner and the other for all other objectives. The setting is dependent on the design of the particular instrument. The purpose of this removable lens is to provide even illumination over the field of the scanning lens.
8. With the specimen in focus, vary the condenser height until you can see the image of the frosted glass (or pieces of lint in the glass) in sharp focus on the specimen. This usually involves only a slight deviation from its uppermost position. After focusing, one usually moves the condenser down slightly from its focused position to even out the illumination.
9. Remove one ocular and look into the tube, keeping your eye at least several inches from the top of the tube. Open and close the iris diaphragm and note that its image can be seen at the back lens of the objective. Open the diaphragm so that approximately 9/10ths of the area of the back lens of the objective is illuminated (see note 2) and both definition and resolution should be maximum for your microscope with the lighting available.
10. Your objectives should be parfocal, that is, when any one is in focus, the others, when placed in position, should be within one turn of the fine adjustment of being in focus. Swing the 4mm objective (medium length, 43x) into position and bring the specimen into focus with the fine adjustment only.
11. Repeat step 9.
12. If greater resolution and magnification are desired, swing aside the 4mm objective, place a single drop of immersion oil on the coverslip at the illuminated area, and swing the 1.8mm objective (longest, 97x) into position in the oil drop. Bring the specimen into focus with the fine adjustment only.
13. You may wish to begin your observations with the scanner instead of the 10x objective. If the scanner is parfocal, the procedure outlined above can be initiated with it except for the adjustment of the movable condenser element when appropriate. If your scanner is not parfocal, you will quickly learn in which direction to move the body or stage in order to switch from 10x to scanner.
14. When finished, clean oil from the objective with clean lens tissue, from the slide with lens tissue or a cloth. A little toluene will help to clean the slide but should not be used on the objectives except when the "dry" objectives become accidentally oiled. In this case, moisten a piece of lens tissue with toluene, and allow most of it to evaporate (until the paper is barely moist) before cleaning the lens.

Supplementary Notes.

1. Never attempt to focus the high dry or oil immersion objectives without having first observed the field with the 10x objective or scanner. The free working distance of the higher powered objectives is very short and damage to their front lenses, or to the slide, may easily result from trying to bring them into focus without having taken advantage of their parfocality with the 10x objective. Use only the fine adjustment for the 4 and 1.8mm objectives.
2. The iris diaphragm controls the angle of the beam of light leaving the condenser, traversing the specimen, and entering the objective. This angle is intimately involved in the resolution obtainable by the microscope and should not be used merely to control the intensity of the light or the contrast of the image. By closing the diaphragm more than the 9/10 necessary for maximum resolution, more contrast may be gained in poorly stained sections, but only at the expense of resolving power and definition. To prevent this image deterioration use only the variable light control and/or neutral density filters to reduce the light.
3. Keep all exposed lens surfaces clean by using lens tissue.
4. There are two convenient "rules of thumb" for setting the iris diaphragm. The first is to look down the microscope tube with the ocular removed and close the diaphragm to approximately 90% of the objective diameter. The second is to observe the object with the partly stopped-down field-diaphragm in view. If the iris is too wide, stray light "leaks over the edges of the field diaphragm border" as a bluish haze. The iris is stopped down until this haze is visible but dim.
5. It is possible to obtain much higher magnifications with the light microscope than we ordinarily employ, but we can see no more detail at these higher magnifications. In fact usually the quality of the image deteriorates since we also magnify any imperfections in the objectives. We call magnification which is beyond the useful magnification for a particular objective empty magnification. As a practical rule we can usually employ a magnification of about 750x the numerical aperture of the objective. For a 10x objective this would give a useful magnification of 187x; for a 40x objective, 488x; for a 90x objective, 935x. This means that use of eyepieces with more than 10-12.5 magnification will be detrimental to the image quality. With high resolution objectives, more powerful eyepieces are useful.

KÖEHLER ILLUMINATION

Equipment:

1. Microscope with a focusable substage condenser.
2. Microscope lamp with an iris diaphragm and a focusable condenser.

Procedure:

1. Place lamp in front of microscope about 10 inches away. Tilt lamp to direct light on the microscope mirror.
2. Focus the condenser of the lamp so the filament image is in sharp focus (this must be done with no filter or a clear filter) on the leaves of the iris diaphragm of the microscope condenser. The condenser iris can be seen by looking down into the microscope mirror.
3. Focus the microscope on a specimen (using the 10x objective) and then focus the microscope condenser until the lamp iris is in focus in the plane of the specimen as you look through the ocular. The lamp iris should be closed down to facilitate this step. It also is helpful to have the condenser iris partly closed.
4. Manipulate the microscope mirror to center the image of the lamp iris in the field of view as you look through the microscope.
5. Examine the mirror to make sure the light from the lamp falls on the center of the mirror and is centered around the opening of the condenser iris. If it is not so centered, move the lamp until it is. Recheck centration of the lamp iris image.
6. Open (or close) the lamp iris until the edges of the iris image seen through the microscope just coincide with the field of view.
7. Remove the eyepiece and examine the back lens of the objective. Close the condenser diaphragm to just coincide with the aperture of the back lens of the objective. (May be closed slightly beyond this to reduce glare.) An image of the lamp filament will be seen and this image should be large enough to fill the back lens of the objective. If it is not, moving the lamp further away and refocusing may correct this. Use of an auxiliary condenser lens may be necessary on some microscopes. Use of a ground glass filter with the light source or on the microscope may be advisable in some cases.
8. If the light density is too great, it may be reduced with neutral density filters or by turning down the transformer if it is a variable one. NEVER USE THE CONDENSER IRIS TO REDUCE LIGHT INTENSITY!
9. When the objective is switched, the following adjustments will be necessary:
   a) Get specimen in sharp focus, close lamp iris.
   b) Refocus condenser, set condenser iris at correct setting.
   c) Open lamp iris to coincide with field to view.

PHASE CONTRAST MICROSCOPY

Until the phase contrast microscope was generally available (not until the late 1940's, because of World War II), biologists had to rely on fixed and stained material for nearly all their information about tissue and cell structure. Fritz Zernike, who invented the phase contrast microscope, was awarded the Nobel Prize for his invention, which gave biologists their first look at the activities of some kinds of cells and cell organelles.

The phase contrast microscope was the first contrast generating system to become available for the study of microscopic objects. It is useful for the examination of phase objects; that is, objects which shift light waves in relation to each other.

To understand how contrast generating systems work we must consider light as waves rather than as rays or photons. Light is emitted from a source either at a characteristic frequency (monochromatic light) or at a series of frequencies (polychromatic light). The frequency (f) is invariant, but as the light travels through various media (e.g. air, water, oil, cytoplasm) of different refractive indices, its velocity (v) and wave length (l) vary reciprocally.

$$f = \frac{v}{l}$$

The refractive index (n) of a medium is the ratio of the velocity of light i*n vacuo* to that in the medium.

$$n = v_{vacuum}/v_{medium}$$

As light enters water from the air, it is slowed down, or retarded. On re-emerging it assumes its original velocity.

A phase object is simply one composed of material with a refractive index unlike its surround (environment). The term "phase" object refers to the fact that the light waves passing through such an object are shifted in phase (or "phase-shifted") relative to those passing near the object through the surrounding area (Figure II). The phase contrast microscope works by separating the diffracted and undiffracted light from the object, and then phase-shifting and partly absorbing the undiffracted light.

There are only two special parts added to a bright-field microscope to convert it into a phase contrast microscope. The first is a phase annulus, a fully silvered (or black painted) plate in place of the iris diaphragm in the condenser with an annular (ring-shaped) window. Light passes through the condenser from this annulus as a hollow cone of light coming to a focus at the object (specimen) plane. This light then diverges after the object plane into an inverted hollow cone of the same angle which passes through the objective.

FIGURE III
Path of Diffracted and Undiffracted Waves
in a Phase Contrast Microscope

The second component peculiar to a phase contrast microscope is the phase plate (or phase ring) in the objective. This "plate" usually is a curved lens surface (often the inner surface of a cemented doublet) at which a ring-shaped area has been built up (by vacuum deposition of a transparent material) or eroded away (by acids and polishing) to retard or advance the light waves passing through that area. The same ring-shaped area is also coated with a light absorbing material.

The phase microscope, when adjusted for proper illumination (see handout on Compound Microscope) needs only one further adjustment: centration of the phase annulus (by screws provided in the condenser mount) so that the bright annulus is superimposed over the dark ring of the phase plate (Figure IV).

FIGURE IV
View Through "Phase Telescope"
of Back Focal Plane of Objective

This adjustment is usually made while looking down the empty microscope tube (ocular removed) or while looking through a "phase telescope" or Bertrand lens focused on the annulus and phase ring. It is worthwhile to look at a living cell or other specimen while centering and decentering the phase annulus to see how the image behaves when adjustment is correct. As the annulus moves over the phase ring, the field darkens considerably (due to the absorbency of the ring) and the object suddenly takes on a much higher contrast.

For the present, it will suffice to say that contrast in a phase contrast microscope is achieved by the interference of diffracted and undiffracted waves. The undiffracted waves pass through the phase plate, where they are partly absorbed and phase-shifted. The diffracted waves do not and are phase-shifted only through the object. The blacks, grays, and whites of phase contrast images are caused by interference of diffracted and undiffracted waves when recombined to form the image (see Figure V).

FIGURE V

Phase contrast microscopy produces optical artifacts. An optical artifact is part of an image that does not correspond to any real part of the original object. For example, in a phase contrast microscope every object detail is surrounded by a "halo". Halos sometimes intrude on real structures making them distorted or even invisible. Another feature of phase contrast microscopes that is disturbing is positive and negative out-of-focus images. If one examines a diffraction grating in a phase contrast system using a monochromatic light source, one finds a "stack" of images of the grating, each 0.25 micron and of opposite contrast from its nearest neighbor. The result of this is that images of living cells may be quite "busy" with out-of-focus (and thus spurious or incorrect) information about the optical plane of focus in the specimen.

HOW TO STUDY TISSUE SECTIONS

(from Harvard Medical School Pathology Manual)

I. Examination of a slide:

1. Hold the slide up to the light and examine with the unaided eye and/or with an inverted ocular to bring out more detail. This will help you to orient yourself and to locate the important fields for further examination under the microscope. You will be able to tell a lot in this manner.
2. Your 4x scanning objective will be very useful.
3. The 10x objective is the most important lens for general histological examinations. Study first the apparently important fields you located by steps A or B. Then scan the entire slide SYSTEMATICALLY and note the overall architectural pattern, aberrant regions, if present, defects in staining, artifacts, etc. Be sure to look at the periphery of the tissue and any natural surfaces which may be present. You should strive to learn to identify tissue and cell types and their interrelationships at this power.
4. The high dry objective is more than sufficient to study most detail such as cell structure and type, interstitial material, etc. The oil immersion lens is important for studying bacterial, certain cellular organelles, and perhaps some parasites, etc., but it is rarely if ever needed for tissue morphology or cell type identification.
5. THE MOST COMMON DIFFICULTY IS USING TOO HIGH AN OBJECTIVE LENS. TOO MUCH MAGNIFICATION AND TOO SMALL A FIELD OF VIEW CAUSE LOSS OF ORIENTATION AND PERSPECTIVE.

II. Description of a slide: It is important that you learn to write a description of what you see so that the reader can visualize the specimen as you saw it, without being influenced by your interpretation of its nature. Your description should be as accurate, systematic, and brief as possible with emphasis on the pertinent observations. The description might proceed as follows:

1. Name of organ or tissue.
2. General architecture, e.g., normal, altered, etc.
3. Surface of the organ - capsule, etc. If the normal surface is missing, state so. In hollow organs, examine both surfaces.
4. Parenchymatous cells of the tissue. Note their arrangement, size, shape, staining reaction, foreign contents, etc.
5. Condition of the framework, i.e. stroma. Abundance, cellularity, types of cells present, foreign materials.
6. Conditions of blood vessels - types and abundance.

III. Drawing a microscope object: This is not emphasized in Histology as a means of providing busy work for students, but rather to train you to see better what it is you are viewing. Drawing requires intense attention to size, shape, proportion and detail, and your drawings should attempt to be photographic, with every little detail included.

IV. Learning from a slide: In order to learn rapidly from a slide it is important to know what you are looking for. It is almost essential to have at hand a textbook or atlas containing microscopic detail which can be consulted during the study of the slide. It is of equal importance to make some notation of what one has observed on each slide. This may be a brief written description or a labeled sketch (see above). To gain an appreciation of the microscopic observations in terms of their functional significance, which really should be your objective, you should always ask yourself what is the meaning of what you are seeing. Attempt to answer these questions yourself before asking the instructor.

KEY TO HISTOLOGY SLIDES SET

1. Stomach (3 regions)
2. Small Intestine
3. Uterus (human)
4. Uterus (human-proliferative)
5. Uterus (secretory stage)
6. Mammary Gland
7. Ovary (immature)
8. Ovary (maturing follicle)
9. Ovary (corpus luteum)
10. Salivary Glands (3 types)
11. Placenta (human)
12. Developing intra-membranous bone
13. Meissner's touch corpuscle-skin
14. Corpuscle of Vater-Pacini-pancreas
15. Retina
16. Artery, vein & nerve
17. Peyer's patches-ileum
18. Palatine tonsil (human)
19. Adenoid (human)
20. Spleen
21. Lung
22. Pancreas
23. Appendix (human)
24. Large Intestine
25. Hard Palate
26. Soft Palate
27. Liver-Kupffer cells with carbon
28. Liver
29. Ureter
30. Epididymis
31. Vas deferens
32. Trachea & esophagus
33. Larynx
34. Nasal mucosa
35. Lung with carbon particles (human)
36. Tongue
37. Seminal vesicle
38. Prostrate
39. Mammary gland-nipple (human)
40. Oviduct-ampulla (human)
41. Mammary gland-cancer (human)
42. Parathyroid & thyroid
43. Liver (pig)
44. Intestine
45. Stomach (rabbit)
46. Tongue (injected)
47. Esophagus (human)
48. Esophagus (human)
49. Stomach (cat)
50. Small Intestine
51. Gall bladder
52. Sciatic nerve-mammal
53. Nerve
54. Sensory nerve ending (cat)
55. Spinal cord (cat)
56. Spinal cord & ganglion
57. Nerve cells (ox)
58. Cerebral cortex
59. Cerebellum (rabbit)
60. Cerebellum (Hs. Golgi)
61. Purkinje cells (cerebellum)
62. Spinal cord (silver impregnation)
63. Cochlea (ear)
64. Skin (finger tip)
65. Liver (Rat, bile canaliculi)
66. Pituitary gland (human)
67. Adrenal gland (cat)
68. Kidney (rat)
69. Kidney (kitten-injected)
70. Elastic Cartilage-Epiglottis (dog)
71. Lung (dog-injected)
72. Trachea
73. Olfactory membrane
74. Spermatogenesis (rat testis)
75. Vagina section (human)
76. Carotid Artery (human)
77. Vena Cava (human)
78. Cardiac muscle (monkey)
79. Striated muscle (kitten)
80. Striated muscle (teased)
81. Smooth muscle
82. Squamous epithelium (amphibian)
83. Areolar connective tissue
84. Reticular tissue
85. Hyaline cartilage
86. Tendon
87. Red Bone Marrow
88. Bone, decalcified (cat)
89. Ground bone
90. Dry bone
91. Bone (foot of pig)
92. Urethea (female)
93. Urinary bladder (contracted/expanded)
94. Lymph node (human)
95. Yellow elastic tissue
96. Finger or Toe Nail
97. Thymus
98. Thymus (infant)
99. Cardiac muscle (Purkinje fibers)
100. Sympathetic ganglion
101. Fibrous cartilage
102. Scalp (human)

103. Bone marrow, smear (human)
104. Artery, vein, & nerve-elastic tissue
105. Mucous tissue (human umbilical cord)
106. Voluntary muscle, injected
107. Tooth
108. Axillary skin
109. Tendon/bone
110. Cervix uteri
111. Uterus, early pregnancy
112. Senile ovary
113. Senile uterus
114. Mammary gland, pregnancy
115. Ovary, corp. leut. of pregnancy
116. Ovary, corpus luteum
117. External ear, Elastin Stain
118. Parotid (human)
119. Submax. (human)
120. Sublingual (human)
121. Vagina (human)
122. Lingual tonsil (human)
123. Tattooed skin
124. Lymph vessel, with valve?
125. Sq. cell carcinoma of lymph node
126. Emphysema, lung
127. Cirrhosis of liver
128. Esophagus, lower
129. Vena cava
130. Aorta
131. Cerebral cortex, silver
132. Blood smear
133. Spongy bone, section
134. Lymph node
135. Testis
136. Frog skin
137. Liver, Kupffer cells with carbon
138. Yellow elastic tissue xs. & l.s
139. Eyelid
140. Spleen, phagocytic cells
141. Stomach, cardiac region
142. Stomach, fundic region
143. Stomach, pyloric region
144. Brown adipose tissue (multilocular)
145. Yellow elastic tissue
146. Atherosclerosis
147. Malignant melanoma

COVERING EPITHELIUM

1. True squamous epithelium - parietal layer of Bowman's capsule, thin region of loop of Henle-kidney (68), lung (21, but it's tough to see), amphibian mesentery (82).
    a. mesothelium - outer surface; stomach (49), intestine (2), heart (78).
    b. endothelium - lining of blood vessels (2, 16, 129, 130), heart (78), lymphatics (124).
2. Cuboidal epithelium - many glands and ducts - thyroid (42), salivary gland (10, 118, 119, 120), kidney tubules(68), pancreas (22), covering of ovary (8).
3. Columnar epithelium
    a. non-ciliated - intestine (striated border) (2), stomach (1), gall bladder (51), kidney tubules (brush border) (68), pancreatic duct (2).
    b. ciliated - oviduct (40).
4. Pseudostratified columnar epithelium
    a. ciliated - trachea (32, 72).
    b. stereociliated - epididymis (30).
5. Stratified squamous epithelium
    a. keratinized - skin (64, 102, 108, 139).
    b. non-keratinized - esophagus (47, 48), vagina (75, 121), frog skin (136, may be very lightly keratinized), eyelid (139).
6. Stratified cuboidal and stratified columnar epithelium - ducts of salivary glands (10, 118, 119, 120), sweat gland ducts (102, 108).
7. Transitional epithelium - kidney pelvis (68), ureter (29), bladder (93).
8. Neuroepithelium (another loose category) - taste buds of tongue (36), olfactory epithelium of nose (73).

GLANDULAR EPITHELIUM

1. Unicellular - Goblet cells of intestine (2,24,50) and respiratory tract (21?,32,72): frog skin (136)
2. Secretory sheet - gastric mucosa (49)
3. Simple:
    a. Tubular:
        1) Straight - Small intestine (Crypts of Lieberkühn) (2,44)
        2) Coiled - Eccrine and apocrine sweat gland (102,108), cardiac stomach (1,141)
        3) Branched - Duodenum (Brunner's gland-tubuloalveolar to some) (2), cardiac and pyloric stomach (1,141,143)
    b. Alveolar:
        1) Unbranched - frog skin (136) - not in mammals
        2) Branched - sebaceous gland (102,108)
4. Compound:
    a. Tubular - Brunner's gland? (2,24), kidney (68), Cardiac glands of stomach? (1)
    b. Tubuloalveolar - prostate an aggregate of 30-50 compound tubuloalveolar glands (38), mammary gland, as with the prostate, this too is an aggregate (6,114), salivary glands (118,119,120)), pancreas (22), Brunner's gland? (2,24)
5. Serous glands - parotid salivary gland (human) (118), pancreas (22)
6. Mixed - submaxillary (submandibular) salivary gland (119), sublingual salivary gland (120) (look for demilunes).
7. Endocrine glands - thyroid follicles (and parafollicular cells) (42); Islets of Langerhans (clumps) (22), adrenal cortex (cords) and medulla (clumps) (67)

CONNECTIVE TISSUE

1. Mesenchyme (embryonic) - (12)
2. Mucous - umbilical cord (105).
3. Loose, areolar - special elastin stain (83, 30).
4. Adipose - (32, 68, 108, 144).
5. Reticular - spleen, specially stained (84).
6. Dense, regular - tendon (86, 109).
7. Dense, irregular - dermis of skin (102, 108, 64), perichondrium (85), periosteum (88).
8. Elastic - (95, 104, 138, 145).

CARTILAGE

1. Hyaline cartilage - (85), lung (21), trachea (32), developing bone (91).
2. Elastic cartilage - outer ear (117), epiglottis (70).
3. Fibrous cartilage - (109), pubic symphysis (101).

BONE

1. Long bone - decalcified: cross-section (88, 133)
   longitudinal section (91)
   ground: cross-section (89)
   longitudinal section (90)

2. Bone development: Long Bone - pig embryo (91)
   membrane bone - (12)

BLOOD

1. Human blood - use the smears you made of your own blood to look for all the cell types, or l32.

   Make a differential white blood cell after you have learned to differentiate the various cell types. To do this, start in one corner of the coverslip, at the top edge. Move across the slide in a straight line, parallel to the top edge. When you reach the end of a countable area, drop down the width of one field of view, and begin scanning and counting in the opposite direction. Continue this until you have counted at least 100 cells. Record the numbers of each kind and figure out their respective frequencies.
2. Non-human blood - a variety of types are available for examination including bird, reptile, amphibian, fish and camel!
3. Blood formation - examine red bone marrow smear (103) and section (87) and try to find as many of the stages as possible. Oil immersion may be necessary.

MUSCLE

1. Smooth muscle:
   a. digestive tract - inner layer of esophagus (47,48), stomach (1), intestine (2), gall bladder (51)
   b. excretory system - ureter (29), urethea (92), bladder (93)
   c. reproductive system - uterus (3), oviduct (40), vas deferens (31)
   d. respiratory system - trachea (32), larynx (33)
   e. circulatory system - artery (76), vein (77)
   f. special slide of isolated cells (81)
   g. miscellaneous - skin (associated with hair follicles) (102), spleen (20)
2. Skeletal muscle - special slides (79, 80), tongue (36), esophagus, outer layers (47,48), injected (106)
3. Cardiac muscle - heart (78, 99); look for striations, branching, and intercalated disks

NERVOUS TISSUE

1. Neurons
   a. motor neurons - spinal cord, (smears and sections) (55, 56, 57)
   b. pyramidal cells - cerebrum (58, 131)
   c. Purkinje cells - cerebellum (59, 60)
   d. Autonomic neurons - various regions of the digestive tract (2,17,24,44,50) show small groups of neurons in the connective tissue between the longitudinal and circular smooth muscle layers and between the circular smooth muscle layer and the submucosa.
2. Nerve sections - there are special sections (cross and longitudinal) of isolated nerves. You should also look for these in various tissues, such as tongue, salivary gland, artery, vein and nerve slides, etc. You should see the nerve fibers, both myelinated and unmyelinated, and their associated cells, as well as the epi-, peri- and endoneurim. (16, 52, 53, 104)
3. Spinal ganglion (sensory) - single preparation or in conjunction with the (56) spinal cord. Look for pseudounipolar nerve cells, satellite cells, capsule and fibers. Note their arrangement within the ganglion.
4. Autonomic ganglion - sympathetic chain ganglion showing multipolar neurons, fibers, reduced number of satellite cells, capsule etc. (100) Parasympathetic ganglia can be found in the wall of the gut (2,17,24,44,50).
5. Spinal cord - gray and white matter, motor neurons in the ventral horn, fibers, ependymal cells. With special stains, look for astrocytes, oligondendrocytes, microglia. (55, 56, 62)
6. Cerebrum - pyramidal cells, fibrous and protoplasmic astrocytes (58, 131)
7. Cerebellum - white matter consisting primarily of fibers and gray matter with the three layers - molecular (mostly fibers). Purkinje layer, and granular layer. (59, 60, 61)
8. Sensory endings: Meissner's (13)
   Corpuscles of Vater-Pacini (14, 54)
   Retina (15)
   Cochlea (63)
   Olfactory (73)
   Taste buds (36)

The nervous system is a bit more difficult to study histologically, for a variety of stains must be used to demonstrate the various components. H and E shows mainly nuclei; basic dyes will show the Nissl substance. To see myelin, use OsO_4 or the Pal-Weigert method. Very commonly, investigators use metal impregnation techniques, as we did in lab, to demonstrate the glial cells, neurilemmal cells, and the neurofibrils within the neurons. You should examine a variety of preparations of each tissue in order to see all that is present.

CIRCULATORY SYSTEM

1. Artery and vein - carotid artery, vena cava (16, 76, 77, 104, 129, 130)
2. Cardiac muscle (78, 99)
3. Lymphatic (with valve) (124)
4. Small arteries and veins - liver, salivary gland, and various other tissues.
5. Atherosclerosis (146)

LYMPHATIC TISSUES

1. Diffuse lymphatic tissue - appendix (23)
2. Solitary lymph nodules - appendix (corona, germinal center) (23)
3. Peyer's patches - ileum (large nodules in submucosa) (17)
4. Tonsils - palatine (18), adenoid (19), lingual (122)
5. Lymph nodes - capsule, cortex, medulla, germinal centers, medullary cords, lymph sinuses (subcapsular, intermediate, medullary), trabeculae, reticular cells (especially in sinuses), lymph vessels (94, 134), squamous cell carcinoma (metastatic) (125).
6. Thymus - lobules, cortex, medulla, no germinal centers, Hassal's bodies (adult and infant, 97, 98)
7. Spleen - capsule and trabeculae (note large amount of smooth muscle), germinal centers, red pulp, white pulp, central arteries, sheathed arterioles (20), phagocytic cells (140).

INTEGUMENT

1. Thick skin - no hair, shows all layers: with Meissner's corpuscles (13), with Meissner's and Pacinian corpuscles (64).
2. Thin skin - scalp (102), mammary gland (6, 39), frog skin (136), eyelid (139).
3. Scalp - sweat glands with secretory portions and ducts, hair follicles, sebaceous glands, as well as the other features of the epidermis, dermis and hypodermis (102).
4. Fingernail - 96, this is embryonic and thus not as informative as it might be.
5. Axillary skin - 108.
6. Tattoo - 123.
7. Melanoma - 147.

DIGESTIVE TRACT AND ASSOCIATED STRUCTURES

1. Hard palate - 25
2. Soft palate - 26
3. Tongue - 36, injected (46)
4. Esophagus - 32, 47, 48, 128
5. Stomach - cardiac, fundic and pyloric (1, 45, 49, 141, 142 143)
6. Small intestine - duodenum, jejunum, ileum (2, 17, 44, 50)
7. Large intestine - 24
8. Appendix - 23
9. Salivary glands - parotid (118), submaxillary (119), sublingual (120), mix (10)
10. Pancreas - 14, 22
11. Liver: pig, with classic lobules (43)
    human (28)
    Kupffer cells with carbon (27, 137)
    bile canaliculi (65)
    cirrhosis (127)
12. gall bladder - 51
13. Tooth - 107

RESPIRATORY SYSTEM

1. Nasal mucosa - 34
2. Olfactory epithelium - 73
3. Hard and soft palate - 25, 26
4. Epiglottis - 70 (especially from boxes 2, 3, and 4)
5. Larynx - 33
6. Trachea - 32, 72
7. Lung - 21, 35 (smoker), 71 (circulation injected), emphysema (126)

URINARY TRACT

1. Kidney - 68, 69 (circulation injected)
2. Ureter - 29
3. Urethra - 92
4. Bladder - 93

ENDOCRINE SYSTEM

1. Pituitary gland - 6
2. Thyroid gland - 42
3. Parathyroid gland - 42
4. Adrenal gland - 67 (trade around)
5. Pineal gland - demonstration
6. Pancreas, Islets of Langerhans - 2
7. Liver - 43

MALE REPRODUCTIVE SYSTEM

1. Testis - 74, 135
2. Epididymis - 30
3. Vas deferens - 31
4. Seminal vesicle - 37
5. Prostate - 38

FEMALE REPRODUCTIVE SYSTEM

1. Ovary: immature (7)
   maturing (8)
   corpus luteum (9, 116)
   corpus luteum of pregnancy (115)
   senile (112)
2. Oviduct - 40
3. Uterus: proliferative (4)
   secretory (5)
   menstrual (3)
   early pregnancy (111)
   senile (113)
4. Vagina - 75, 121
5. Mammary gland: lactating and nonlactating (6)
   pregnancy (114)
   nipple (39)
   ductal carcinoma (41)
6. Placenta - 11
7. Cervix - 110