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The Basis of Dye Chemistry and Techniques Available For Identification of Adulterated or Mislabeled Dyes

PART II

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This is part two of the article "Basic Dye Chemistry and Techniques Available for Identification of Adulterated or Mislabeled Dyes". The first part of the article appeared in HistoLogic Winter Issue 1986.

Techniques Available for the Identification of Adulterated or Mislabeled Dyes

Dyes are used for many different staining purposes. They are important in industry as fabric, paper, leather and wood dyes, as well as for imparting color to oils, fats, waxes and numerous other substances. Such industrially important dyes contain many impurities and variations in the total dye content of different batches manufactured, hence variable coloration results when staining with different batches.

These dyes would not be useful for biological staining since impurities and different dye contents would result in variable staining and inconsistent results. Hence it is necessary for a dye to be consistent in content from batch to batch and to be virtually free of contaminants that will affect the dye's performance. Biological Stain Commissions ensure that all batches of a certain dye contain a definite weight of dye of a specified dye content.[1] Therefore, when variations in staining result, they can be attributed to a different amount of dye present and not due to impurities.

If we are presented with mislabeled or adulterated dyes, there are many methods, chemical and optical, that can be used in the identification of a dye, or mixture of dyes, in a staining solution. The following discussion will concentrate on the identification of mislabeled and adulterated dyes.

Let us first consider some chemical methods of testing for impurities, nature of dyes and their content.

Impurities in Dyes and Their Purification

Impure dyes may be purified by the following methods:

A. Reduction of the impure dye and then oxidation of the reduced solution to give the original dye; e.g. methylene blue.
B. Extraction by solvents: Dyes can be extracted from solvents and hence other impurities, if they are water soluble.
C. Recrystallization from solvents will also yield the pure dye.
D. Chromatography is the most common and useful method by which dyes can be separated from impurities, as well as from other dyes. By using known standards of Rf values, a dye can be analyzed and its Rf value, when compared to the standards, will indicate whether it is pure and what type of dye it is.

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Dye Content Analysis

A. Titration with titanous chloride. Lillie[1] suggests that this procedure depends on the fact that reducible radicals are present in almost all dyes, so that the dye content of a sample may be determined by titration with TiCl, provided that the endpoint is sharp and the molecular weight of the dye is known.

B. Nitrogen assays may also be used in the case of Azo dyes to find the total dye content, provided the nature of the dye is known; e.g. Kjeldahl Nitrogen Assay.

C. Acid dyes can be titrated with alkali or precipitated by alkali, therefore percentage yields can be calculated. The reverse is the case for basic dyes.

Identification of Dyes

A. Chromatography, as mentioned earlier, can be used for the separation and identification of dyes. A mixture of dyes can be separated on the chromatograph and individual dyes can be eluted to give a pure dye sample. Different chromatographic methods are available, from paper chromatography, paper partition chromatography, to thin-layer chromatography. Kirchner[5] provides a good explanation of the principles involved in thin-layer chromatography and gives examples of its uses in the identification and the separation of dyes.

TABLE V
SEPARATION OF DYES USING THIN-LAYER CHROMATOGRAPHY

(Dyes separated on Silica Gel using specific solvents)

DYES	RF VALUES
Acridine orange	0.41
Alkali blue	0.16, 0.34
Brilliant green	0.59
Erichromazurol	0.39
Gentian violet	0.43, 0.48
Crystal violet	0.43
Light green	0.11
Malachite green	0.35
Metanil yellow	0.39
Methylene blue	0.9
Methylene green	0.18
Victoria blue	0.51

B. Electrophoresis can also be used in the separation and identification of dyes. Kirchner[5] once again describes the methods of electrophoresis and Table VI gives examples of dyes that have been separated by electrophoresis in both an acid and a basic medium.

Optical identification of dyes using the Spectrophotometer is the most convenient and most rapid method of dye analysis. Where chemical analysis may be complicated with the fact that impurities are present and that some dyes may be similar in structure to others, therefore specific chemical assay methods must be adopted. However, the spectrophotomeric analysis of dyes provides both quantitative and qualitative results for nearly all dyes, even though their difference in chemical structure may be minor.

The spectrophotometer gives specific absorption spectra characteristics of a particular dye. This depends on the concept earlier discussed of absorption of certain wavelengths of light and the transmission of others. A certain dye may be tested for purity or a mixture of dyes identified by specific absorption curves that enable the identification of dyes by the general form of the curve and the absorption maximum. If the absorption maximum or peak is not in the accepted position, compared to a standard curve for that particular dye, then the dye may have been incorrectly labeled and the true identity can be found. Figure 5 gives a good example of the curves of a mislabeled dye believed to be methylene blue and thionin.

FIGURE 5.
ABSORPTION CURVES OF 1. THIONIN, 2. METHYLENE BLUE AND AN APPARENT MIXTURE OF THE TWO DYES[11] INCORRECTLY LABELLED

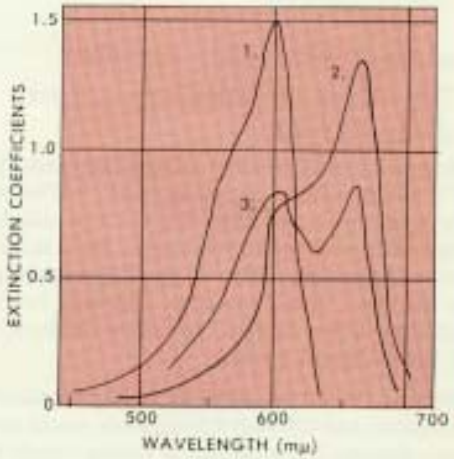
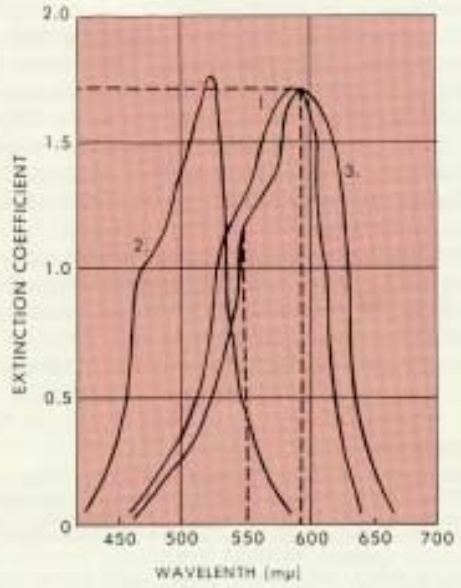


TABLE VI
SEPARATION OF DYES ON THIN LAYERS OF SILICA GEL

Acidic Electrophoresis				Basic Electrophoresis		
Color	Distance Traveled in mm	Direction of Movement	Dye	Direction of Movement	Distance Traveled in mm	Color
Yellow-Red	23	Anode	Methyl Orange		0	Orange
				Anode	7	
Red	0		Methyl Red	Anode	34	Yellow
Yellow	0		Dimethyl Yellow		0	Yellow
Blue	0		Congo Red	Anode	30	Red
Black	0		Eriochrome Black T		0	Red
Red	65	Anode	Crystal Ponceau	Anode	35	Red
Red	80	Anode				
Red with NaOH	14	Cathode	Phenolphthalein	Cathode	16	Red
Colorless	0		Thymolphthalein		0	Blue
Yellow	51	Anode	Bromphenol Blue	Anode	15	Blue
Yellow-Green	28	Anode	Fluorescein	Anode	22	Yellow
	0		Rhodamine B		0	Red
Red	25	Anode				
Visible in UV	37	Anode				
Visible in UV	56	Anode				
Visible in UV	75	Anode				
	0		Alizarine S		0	Visible in UV
Visible in UV	52	Anode				
Blue	0		Neocarmine W		0	Blue
Yellow	38	Anode		Anode	38	Yellow
Red	85	Anode		Anode	38	Red
				Anode	54	Red

Deliberate or accidental adulteration of dyes can also be detected using absorption curves. Adulteration may be identified by the production of multiple peaks, asymmetry or lack of sharpness of the peak, or the shift of the peak away from the known peak for that dye. Figure 6 provides an example of variations in absorption curve of crystal violet that enables distinction between an impure or mislabeled dye and the pure dye.

FIGURE 6.
ABSORPTION CURVE OF CRYSTAL VIOLET[1] EXAMPLES OF CURVES THAT ENABLE IDENTIFICATION OF ADULTERATED AND MISLABELLED DYES.



1. ABSORPTION CURVE - PURE CRYSTAL VIOLET - Absorption Max 590 mμ secondary absorption band 550 mμ
2. CURVE SHIFT - Although the overall shape of the curve is similar, the shift of the peak and differing position of max and secondary peaks may be due to impurities or dye being mislabelled.
3. LACK OF SHARPNESS - Impurities may be present in the dye, hence absorption peak lies between 580-600 mμ

The techniques described for the identification of dyes, unknown, mislabeled or adulterated, are only the basic methods and ideas used in dye analysis. Therefore, when one knows certain methods are available, detailed experimental procedures and data can be sought in specialized texts or manuals.

By the knowledge of the chemical and physical properties of dyes, the basis of dye chemistry can be explained. That is, by knowing the structure of the dye and the chemical nature of the many different dye groups, the way in which they bind to specific tissue sites has been understood. Also, the knowledge of the physical properties of the dye, such as its relation to white light and its absorptive capacities, has enabled us to understand why dyes are colored and hence, how they impart color or staining to tissues.

The methods available for the identification of dyes, such as chromatography and spectrophotometry, has enabled the separation of mixtures of dyes, the identification of mislabeled and adulterated dyes and the analysis of dye content.

References:

1. Lillie, R.D. (1977) *Biological Stains, H.J. Conn's.* Waverly Press: Baltimore, MD.
2. Bancroft, J.D. and Stevens, A. (1977) *Theory and Practice of Histological Techniques.* Churchill Livingstone:London, England.
3. Culling, C.F.A. (1974) *Handbook of Histopathological and Histochemical Techniques,* 3rd edition. Butterworth & Co:London, England.
4. Histotechnology Notes, New South Wales Institute of Technology. NSW, Australia.
5. Kirchner, J.G. (1967) *Technique of Organic Chemistry,* Vol. XII. Interscience Publishers:New York, N.Y.

A Combined Argyrophilic and Immunoperoxidase Technique

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Editor's Note: The articles or procedures cited in references 1, 2 and 3 should be obtained before an effort is made to attempt this procedure. The article contains come steps which are related to both the immunoperoxidase and Grimelius techniques. These steps are included here, since they have been somewhat modified from the original method.

The wide distribution of argyrophil cells of the neuroendocrine (APUD) system and their participation in pathologic processes, mainly of neoplastic origin, have drawn our attention to the nature of their neighboring cells. These cells, in normal as well as pathologic situations, may provide information regarding any differences and/or relationship that could exist between them.

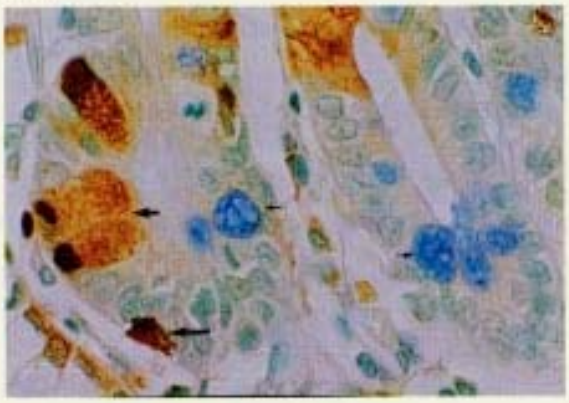

Figure 1: Note the good staining of goblet cells with alcian blue (small arrows), the paneth cell reaction to the immunochemical procedure (medium size arrow) and the silver reaction to the argentaffin cells (long arrow).

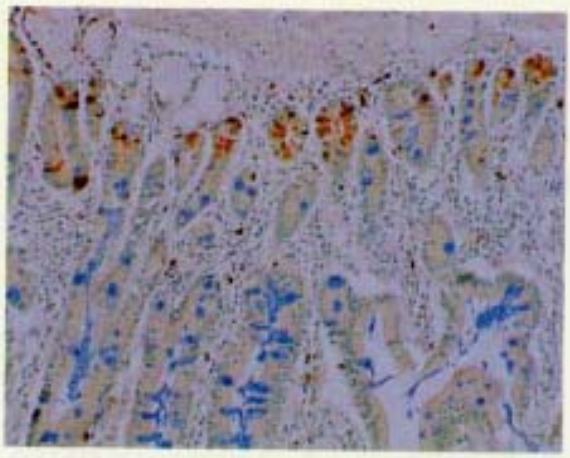

Figure 2: Low power view of same section to demonstrate the overall quality of this combined procedure.

A new method which combines argyrophilic and immunoperoxidase techniques in the same slide is described, to facilitate the staining of both argyrophilic and neighboring cells.

Material: The tissue used to evaluate this procedure was human duodenum because of its richness in both APUD cells and Paneth's cells. The latter contains lysozyme, which can be easily detected by means of an immunoperoxidase technique after the application of an antiserum raised in rabbit and diluted 1:200.

Fixation: 10% buffered formaldehyde

Microtomy: Cut sections at 6 microns.

Immunoperoxidase Techniques Used: Peroxidase-antiperoxidase (PAP) of Sternberger[1], or avidin-biotin complex (ABC) of Hsu[2].

Argyrophilic Technique: Grimelius stain[3].

Staining Procedure:

1. Deparaffinize sections to water.
2. Block endogenous peroxidase in 0.3% hydrogen peroxide in methyl alcohol.
3. Rinse slides in distilled water.
4. Apply the immunoperoxidase technique (either PAP or ABC) as described in references 2 and 3.
5. Develop the chromogenic reaction with 3,3'-diaminobenzidine (DAB) under microscopic observation.
6. Wash in distilled water twice, 1 minute each.
7. Place the slides in Grimelius acetate buffer-silver nitrate solution in a 60°C oven for 3 hours.
8. Place slides in Grimelius reducing solution in a 40°C oven for 1 minute. This solution should be preheated before use.
9. Wash slides in distilled water twice, 1 minute each.
10. **Optional:** Counterstaining of nuclei with 1% methyl green, 1 minute, and staining of mucosubstances with the alcian blue method, can be performed at this point if desired.
11. Dehydrate slides in alcohol, clear in xylene and mount coverglass with resinous media.

Results:

Neuroendocrine cells	black
Paneth's cells	brown
Nuclei	green
Mucosubstances	blue

Comments:

1. This combined procedure lengthens the usual immunoperoxidase techniques about three hours. Therefore, it is advisable to apply it in two days. The tissue sections are run to the primary antibody incubation step of the immunoperoxidase technique during the first day and left there overnight at 4°C. The remaining technique can be completed during the second day.
2. This combined technique yields high quality staining with minimum background staining. Counterstaining of the nuclei with hematoxylin increases nonspecific background staining.
3. Results obtained with both immunohistochemical techniques, PAP or ABC, are quite similar.

References:

1. Sternberger, L.A., Hardy, P.H., Cuculis, J.J. and Mayer, H.J.: The Unlabeled Antibody Enzyme Method of Immunohistochemistry: Preparation and Properties of Soluble Antigen-Antibody Complex (Horseradish Peroxidase-Antihorseradish Peroxidase) and its Use in Identification of Spirochetes. J. Histochem. Cytochem., 18:315-333, 1970.
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3. Grimelius, L.: A Silver Nitrate Stain for Alpha-2 Cells in Human Pancreatic Islets. Acta Soc. Med. Upsala, 73:243-270, 1968.

Figure 1: Microscopic view of a section of human duodenum. Neuroendocrine system cells (black), as well as Paneth's cells (brown) and mucosubstances (blue) are easily distinguishable. Nuclei appear green. (PAP-Grimelius-alcian blue-methyl green stain.) A-100X B-400X

Acid Extracted Eosin Y Stain*

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The following acid extracted eosin counterstain has been found very useful in my laboratory. It provides excellent counterstaining properties and therefore is being made available herein for the benefit of other histotechnologists.

Materials Needed:

1. Erlenmeyer flask (3,000 ml)

2. Side arm flask (3,000 ml)
3. Syphon
4. Funnel, large
5. Spatula
6. Filter paper
7. Drying oven (temperature not to exceed 60°C)
8. Glass mortar and pestle

Extraction Procedure:

1. Pour 1500 ml of distilled water into Erlenmeyer flask.
2. Keep the distilled water in semi-vigorous motion while slowly adding 7.5 gm of eosin Y powder. **Note:** A magnetic stirrer works well.
3. When dye is completely dissolved, add 12.0 ml of hydrochloric acid drop by drop. After each drop, agitate vigorously to enhance the formation of a flakey, water insoluble reddish-orange precipitate.
4. Allow the precipitate to settle for about 8 hours. Syphon off the supernatant water.
5. Add another 1500 ml of water. Briskly agitate the solution until the precipitate is well suspended. Allow the precipitate to settle again.
6. Repeat with two more washings as in 4 and 5 above, or until the supernatant water is light orange in color. **Note:** Too many washings will cause the precipitate to become water soluble and will necessitate reprecipitation with a small quantity (2-4 ml) of hydrochloric acid.
7. Set up side arm flask, funnel and stopper with appropriate sized hole and connect tubing to side arm and water aspirator. Agitate the mixture vigorously and pour into the filter paper lined funnel. Rapid filtration of the water is necessary to prevent packing of the wet dye.
8. Unfold the filter paper and with a spatula, spread the soft dye to a uniformly thin layer over the filter paper and allow to air dry overnight. Place filter paper with dye in oven for 24 hours. Break off a few pieces of the dye cake and inspect for dryness. If not completely dry, return to the drying oven for not more than 24 additional hours.
9. Do not expose the dye to above 60°C to hasten drying and do not exceed 48 hours total drying time, as either or both will result in loss of staining intensity and stability of the dye.
10. Remove the dye with the spatula from the filter paper. Pulverize the dye in a glass mortar.
11. Place the dye in a clean sealable container. A sterile urine specimen cup is ideal, if available.

Extracted Eosin (Stock) Solution

Extracted oesin (stock) solution ———— 0.5 gm
Ethanol, 95% ————————————100.0 ml
Filter the stain and store in refrigerator.
Solution is stable for 1 year.

1% Phloxine B (Stock) Solution

Phloxine B———————————— 1.0 gm
Distilled water ————————————100.0 ml

Extracted Eosin (Working) Solution

Extracted eosin (stock) solution ———— 50.0 ml
Ethanol, 95% ————————————400.0 ml
Acetic acid ———————————— 4.0 ml
1% phloxine B (stock) solution ———— 5.0 ml
Above solution is stable for several months. Some trial and error should be practiced to determine proper counterstaining properties.

Reference:

*Manual of Histologic and Special Staining Technics, 2nd ed., McGraw-Hill Book Co., New York, Pg. 28, 1960.

Preparation of Paraffin-Embedded Sections of the Eye

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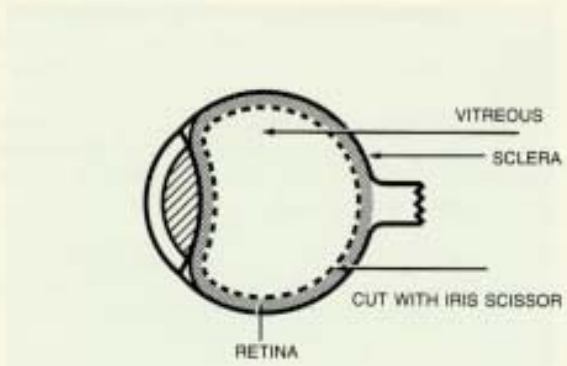
Good quality paraffin-embedded sections of the eye of dogs, monkeys and rabbits have been consistently obtained with the following procedure.

Fixation:

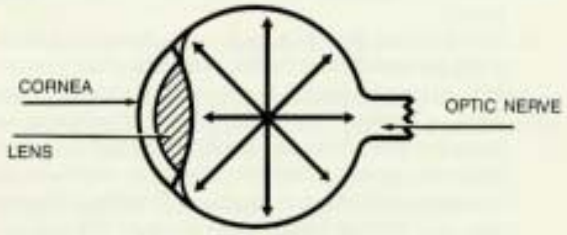
1. A small volume of 6% glutaraldehyde, sufficient to cause the eye to become rigid to the touch, is injected into the vitreous at the time of removal from the animal. The eye is then suspended in 6% glutaraldehyde for at least 24 hours.
2. After fixation, the eye is washed in running tap water overnight.
3. Place the eye in 70% ethyl alcohol for 16 to 24 hours.
4. Use a clean, double-edged razor blade and make a cut perpendicular to the posterior ciliary artery and approximately 1.5 mm away from the optic nerve. Continue this cut until the entire calotte has been cut free. After the calotte has been removed, place the portion containing the optic nerve on the cutting board, cut surface down. Starting at the cornea and cutting towards the back of the eye, the second calotte is removed. The center portion of the eye containing the optic nerve and pupil will remain for processing and sectioning.

   Using a fine-tipped iris scissor, a cut is made around the inside of the eye section to facilitiate removal of the vitreous body. (See illustration #1). The vitreous is then removed from the section using toothed forceps. During this procedure, care must be taken not to touch the retina with the scissors or the forceps.

5. The sections are now processed through the tissue processor on a 16-hour cycle.
6. The sections are embedded in standard embedding molds.



7. Set the microtome at zero micrometers. To trim the blocks, the cutting wheel is turned very rapidly while the specimen feed arm is advanced very slowly. The trimming procedure is continued until the entire section, including the optic nerve, is exposed.
8. To obtain sections the microtome is set a 6 microns. Cut ribbons are floated onto a floatation bath, set at 48°C.
9. After the ribbons have been placed on the floatation bath, they may be stretched somewhat to obtain the same shape and size as the specimen in the paraffin block. To prevent retinal detachment during this process, the stretching must occur from the center outward as demonstrated in illustration #2. By stretching in this manner, artifactual retinal separation is reduced.



10. Sections are then mounted on glass slides, dried and stained.

Comments: Leaving the vitreous in the eye specimen causes artifactual retinal detachment and may change the shape of the eye. This occurs because the vitreous is 99% water and during dehydration and clearing, there can be a great deal of shrinkage. This shrinkage can, (a) pull the retina away from the sclera, or (b) the sclera will shrink on each side, thereby making it impossible to get a nice intact section that retains the normal shape of the eye.

Microtomy: Which Direction Are We Really Heading*

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Many people have felt that microtomy has progressed as far as possible and little improvement could be made to this area. This would be a fair assumption based on microtome developments during the past one hundred years. Prior to the 1970's, the basic design of microtomes had not changed significantly. During the 1970's and early 80's, some major changes have taken place with the development of "Universal" microtomes to handle many applications and these also included motorized systems.

The development of these modern microtomes seemed to indicate a further plateau had been reached and little future development could be envisaged.

This is not the case as major microtome manufacturers are presently committed to investing considerable sums of money into research and development to find ways of further improving microtomes and also to find new methods for specimen preparation. These efforts will result in improved section quality, section thickness, reproducibility and serial sections. The operator will also benefit from more comfortable and easier-to-operate equipment.

So we will continue to see improvements in all types of microtomes as new technology becomes available. This will be coupled with improvements in microtome knives, disposable blades, resins, paraffins, processors and embedding equipment.

To discuss some of the developments we will see in the future, microtomy can be divided into three distinct areas:

1. Preparation and sectioning of specimens for routine diagnosis.
2. Preparation and sectioning of difficult and special specimens.
3. Cryo-preparation and sectioning of specimens for:
   a. rapid diagnosis
   b. special applications

Preparation and Sectioning of Routine Specimens
Traditionally, routine specimens have been processed and embedded in paraffin prior to sectioning. Over recent years, a trend has developed towards plastic

*Basis of a presentation given at the Annual General Meeting of the Histotechnology Group of New South Wales, July 1984. The article was submitted by Mr. Bruce Munro, with the author's permission. Mr. Munro is associated with the Department of Pathology, University of Sydney, Sydney, N.S.W. Australia.

embedding which offered advantages such as the possibility of cutting thinner sections, better preservation of specimen detail and the possibility of using the same blocks for light and electron microscopy. Recent information indicates a slight trend away from plastics and back to paraffin. The reasons are:

1. Paraffin sections have improved in quality through better paraffin waxes, equipment and techniques, improved quality of disposable blades and the routine use of Ralph Knives.
2. Considerable cost in the purchasing of resins, equipment to process resins and motorized microtomes.
3. Problems associated with handling and processing resins.

In the future we can therefore expect to see a stagnation or partial regression in the light microscopy plastic technique. This will result in more research and development by the major microtome manufacturers in paraffin tissue processors, embedders and microtomes. The new generation microtomes will be precision instruments accepting a range of object clamping systems and knife holders including quick clamping systems. These microtomes will comprise manual models and simple motorized systems.

Preparation and Sectioning of Difficult and Special Specimens

There has been a gradual trend towards plastic embedding in this area with the exception of large organs which are still processed in paraffin. With the continued improvement in resins with low viscosities and improved sectioning capabilities, it will be possible for plastics to be also used for large embeddings.

Plastics will play an expanding role in the processing and sectioning of the tissue for special staining and immunological techniques.

They will also become increasingly important in the sectioning of hard materials such as bone and cartilage.

Future resins will have low viscosities, low toxicity, simple mixing of components, simple polymerization and be capable of infiltration and polymerization at low temperature and have good sectioning capabilities.

To handle these specimens, future generation microtomes will have powerful constant torque motors, generating very high cutting forces (double the present cutting forces in some models) to enable sectioning of the hardest materials and also large block faces. The rotary microtomes will also have a large range of specimen holders and object holders to make them as versatile as possible. The use of electronics will also increase to provide more flexibility and precise control.

Cryo Preparation and Sectioning of Specimens

Cryo preparation offers a lot of exciting possibilities for the future. Recent research at the European Molecular Biology Laboratory in Heidelberg has produced some interesting results in this area. Three forms of ice have been identified: Hexagonal (large crystal patterns), Cubic (fine powder structure) and Vitreous (no structure). Electron diffractograms are used to identify the ice crystal state.

The cooling rate initiates these three ice forms. Slow freezing rates such as in the conventional cryostat would produce ice crystals of the hexagonal type which have a damaging effect on the tissue components and structure. Faster cooling rates such as the use of liquid nitrogen can produce cubic ice crystals which will also affect the specimen.

Very high cooling rates are required to produce vitreous ice (liquid propane or ethane). To maintain the vitreous state, the temperature would have to remain below approximately −140 °C. Sectioning at these temperatures is possible using the latest Cryo-ultramicrotomes. Should the temperature go above −140 °C, the ice will revert to the cubic structure.

These are important developments in the understanding of low temperature sectioning. As further research is undertaken in this area, routine methods will develop to enable good cryopreservation of specimens providing significant improvement in the quality of the resulting sections and specimen detail.

1. **Rapid Diagnosis:** Improvements that can be envisaged in this field are:
   —Better cryo-preservation of the specimen using more efficient freezing compounds and cryo-protectants.
   —More efficient cryostats with improved refrigeration systems, better microtomes and more comfortable conditions for the operator.
   —The development of disposable blades for cryo applications.

2. **Special Applications:** This field offers considerable potential for future developments. In conjunction with electron microscopy and the use of cryo-ultramicrotomes, major advances will be made in the areas of identification of viruses and cancer tissue. Future research will give us a better understanding of the structure and functioning of body tissues as it will be possible to carry out investigations without tissue damage caused by chemicals and processing techniques. Specialized cryo-microtomy offers an exciting future.

With all this information in mind, one need not ask—Microtomy, which direction are we really heading?

References:

1. Reichert-Jung (R. Jung - Heidelbert, Federal Republic of Germany)
2. Dr. A.W. McDowall, European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany.

A Mounting Medium Which Retards Fading of Fluorescent Dyes

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Since Coons[1] first used fluorescent-labeled antibodies to stain sections of *pneumococcus*-infected tissue, the technique of immunofluorescence has become an invaluable tool, both in research and in clinical diagnosis. Many technicians now routinely perform fluorescent techniques in histology laboratories on a day-to-day basis.

Despite its usefulness, the fading of fluorescent-stained preparations has always been an accepted drawback to the technique. Exposure to ultraviolet light sources results in very rapid fading of tetramethylrhodamine isothiocyanate and fluorescein isothiocyanate, the most commonly used fluorescent dyes. This can be an extreme nuisance when attempting to photograph these preparations.

Utilizing a report by Giloh and Sedat,[2] we have confirmed their finding that the use of antioxidant n-propyl gallate in a buffered glycerol mounting medium prevents fading of fluorescent-labeled material. We have routinely used this mounting medium for over two years with great success. By enabling longer exposure times, more sensitive fluorescent photomicroscopy is now possible. The method is being presented here to make more histologists aware of its existence. However, it is strongly recommended that the original paper cited in reference 2 be obtained and studied for complete detailed information.

Fluorescent Mounting Medium

90% glycerol
10% phosphate-buffered saline
4% n-propyl gallate*
pH to 8.0
Store in light-proof bottle.

*Sigma Chemical Co.

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1. Coons AH, Kaplan MH: Localization of Antigens in Tissue Cells. II. Improvement in a Method for the Detection of Antigen by Means of Fluorescent Antibodies. *Journal of Experimental Medicine* 1950; 91:1.
2. Giloh H, Sedat JW: Fluorescent Microscopy: Reduced Photobleaching of Rhodamine and Fluorescein Protein Conjugates by n-propyl gallate. *Science* 1982; 217: 1252.

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