THE CUTTING EDGE

REGION IX NEWSLETTER

Spring Newsletter – April 2005

Renew your 2005 membership

NSH Membership year is June 1st thru May 31st.

If you apply for membership or renew now at www.nsh.org , dues will be applied to 2005 – 2006 year!

The names of all the NSH members in each region will be placed into a draw to receive $500.00 U.S.

You could be a Winner for a $ 500.00 Regional Membership Scholarship

You can spend the scholarship funds on any NSH educational material or to attend any NSH sponsored event. You will have two years to spend the money.

Drawing to be held at:

Where: Region IX meeting
When: Sept 10, 2005
Time: 4:30 – 5:30 p.m.
Event: S/C Fort Lauderdale, Florida

Greetings from Region IX Director

The tulips and spring flowers are starting to bloom in Vancouver, B.C. and hopefully soon for all our dear members who have been frozen by the extremely cold weather and snow blizzards. By the time you receive our Spring Issue, the snow *"should"* have melted away in your area, and the signs of spring will have finally arrived.

Spring is also a sign of new beginnings and fresh starts. This statement reminds me of the movie "Groundhog Day" that takes place in Punxsutawney, Pennsylvania. Every year on February 2nd, the famous groundhog comes out of its burrow to predict when spring is going to arrive. The premise of the movie is based on a weatherman, Phil Connors, (portrayed by Bill Murray), who is bored with life and is anything but excited to cover the Groundhog Day festivities in Punxsutawney. Phil gets caught in a blizzard and is forced to return to the groundhog's hometown. Phil finds himself re-living his life exactly the same way everyday. For some reason, everyday he wakes up, it is still Groundhog Day. He finally realizes that the only real change must happen within himself, so he begins to change his behaviour and his life patterns. Instead of allowing situations or circumstances to take over his life, he decides to take control.

Lesson learned from this movie can be summed up in how we respond to a challenging or tough situation – do we look at this as an opportunity to see the glass half full….or as a problem….as in the glass is half empty?

This spring, let us try to be proactive by looking at our glass as being half full and taking the opportunities that come our way!!

Let us all take time to celebrate National Med Lab Week ~ April 24 – 30th – a special time to recognize and reflect on the importance of our profession and the vital role we all play in patient care.

If you are in Vancouver on June 25th, we would like to invite you to join us on our Education Day. We have such a keen education committee that has been having meetings at 7:00 a.m. to finalize details of this day – guaranteed to be an informative day!

We are proud to present our exciting spring issue of ***~ The Cutting Edge ~*** hope you enjoy reading it.

Rose Clarke
rose@nshregionix.org

Education Update

I would like to invite everyone to attend an exciting Education Day which we are organizing for Saturday, June 25, 2005 here in Vancouver. The event will take place in the Conference Center at St. Paul's Hospital, located in Vancouver's West End.

The day will begin with breakfast and will be followed by a number of lectures by prominent speakers on such topics as: Fixation and QA/QC; IHC; Basic Molecular Biology and ISH/FISH/CISH. Lunch will be provided. Speakers include Bryan Hewlett and Ethel Macrea. The lectures are eligible for NSH CEU credits and we are working on CSMLS credits as well. We have great vendor support and many may be on hand for you to meet with. Door prizes will be awarded.

Region IX NSH members will receive free Registration, with costs to others being very modest: non Region IX NSH members and non-NSH members $20.00; BCIT MedLab students $5.00; other students $10.00. Students will require proof of student status.

The Hospital is close to four major hotels; Sheraton Wall Centre and Century Plaza are directly across the street, while the Holiday Inn and Sandman are within a couple of blocks. Parking is available at St. Paul's as well as the Century Plaza and Wall Centre hotels.

Check the Region IX website for further updates. The complete schedule, registration forms and hotel information will be posted shortly. Registration deadline is FRIDAY, JUNE 3rd, 2005.
Any questions, suggestions, please contact me.

Mark Elliott mark@nshregionix.org
Region IX Education Committee, Chair

St. Paul's Hospital, Vancouver, B.C.

CSLMS 2005 Congress
Navigating the Tides of Change

28 May -1 June, 2005
Moncton, New Brunswick

"Changing Tides in Anatomic Pathology"

Date: Monday May 30 at 15:30
Presenter: Eleanor Hooley, Instructor from Vancouver Community College

Presentation on

- **Pathologists Assistant Program**
- **Autopsy Technician Program**

This session will introduce two new and innovative programs to Canada, the Pathologist's Assistant program from the University of Winnipeg and the Autopsy Technician program from VCC.

Most autopsy and anatomic pathology services in Canada face increasing pressures due to many factors including: a lack of trained and qualified personnel to fill vacant positions, the advent of SARS, vCJD and other emerging organisms and the increasing sophistication and complex needs of theses areas.

This session will outline both programs, showing how they will meet the increasing needs, on a national level, for formalized training, continuing education and the provision of highly trained individuals now and in the future.

Following the formal presentation a round table discussion moderated by Kurt Davis of the CSLMS will be held to discuss the changes occurring Anatomic Pathology.

Janet Tunnicliffe
janet@nshregionix.org

You've Got Mail...

Hello Fellow Region IX members,

I would like to commend our new director, Rose Clarke and Mark Elliott for their great accomplishments in our new Region IX website, and delivering the fall and winter Region IX Newsletters online. Fortunately my position of secretary has been minimal, **with the exception of stuffing envelopes.** My hand goes out to the person(s) that previously mailed all the Newsletters.

As the deadline draws near, I would like to remind everyone to renew your membership for 2005 as Rose and Mark have exciting plans for the coming year for Region IX. With your membership you will receive three Journals' of Histotechnology with excellent articles on numerous topics in Histology, educational material, and a reduced rate of registration when attending workshops at the NSH Convention in Florida this fall.

As the world is moving to electronic mail, I would like to encourage those of you that are not online, to come aboard and submit your email address to Rose Clarke at rose@nshregionix.org, or Mark Elliott at mark@regionix.org before the summer 2005 Newsletter. Visit our website: www.nshregionix.org and you will be very impressed.

Sincerely,
Cheryl Clarke
Secretary, Region IX

E-mail from: Regina Ross On Workload Benchmark

I am looking to establish a benchmark to determine what a reasonable workload would be in Histology-

- How many blocks to embed per person
- How many blocks/sections to cut per person/per 7.5 hour shift.

Currently, we run an average of 9500 surgicals/year; embed approximately 20,000 blocks/year and produce another 20,000 levels with only 2 cutters. We also do all manual staining (helped by another individual).

If anyone can share their workflow, or direct me, etc, it would be greatly appreciated. Thank you

Regina Ross rossrm@sah.on.ca
Sault Ste Marie, Ontario

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'New' on our Website: Employment Opportunities
http://www.nshregionix.org/employment.html

Region IX T-shirt Design Contest

2004 Region IX S/C T-shirt

Each year, NSH sponsors a

- **Best T-shirt Design Award**
- **Runner up for Best T-shirt Design**
- **Most number of attendees wearing a state or region T-shirt**

Design a T-shirt that Region IX members will be proud to wear to the S/C in Florida and you could win a

Prize: Bottles of beautiful B.C. Red and White wine

E-mail your design by: August 1, 2005 to:
Cheryl@nshregionix.org or janet@nshregionix.org

Please contact Cheryl or Janet if you wish to send your design by postal delivery.

Winning design will be printed in time for the S/C in Florida. We are hoping that the members attending the S/C will purchase these T-shirts to proudly represent our Region IX during the T-shirt competition.

Place: S/C Fort Lauderdale
Date and Time – TBA

T-shirt costs will be kept at a minimum and will be on sale at the Region IX meeting in September at Fort Lauderdale.

We are proud to be the Canadian NSH Histotechs!!

Leadership Corner

Carrie Diamond
New NSH Executive Director

Congratulations to Carrie Diamond, our new Executive Director! Carrie has unofficially filled this position after Peggy Micciche resigned at the close of the S/C in Toronto. Carrie has served the NSH previously as our Meeting Manager (did an excellent job!). Being a new member of the Board of Directors, I have worked very closely with Carrie and I am quite impressed with her professionalism.

More about Carrie in our summer issue of The Cutting Edge….

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2005 Awards & Scholarships
"Do not underestimate the value of your contribution"

An invitation to all Region IX members to

Nominate a colleague that you feel has contributed to the field of Histotechnology ~ and fits the criteria for any of the available awards and scholarships. These awards are also set up for self nomination – as a member, you can nominate yourself!

Criteria for eligibility and nomination forms can be downloaded from the website. Apply now and submit your nomination forms by the due date.

Region IX Awards & Deadline for submission of nominations:

- **Malcolm D. Silver Award – August 31, 2005**
- **Surgipath Award of Excellence – September 1, 2005**

http://www.nshregionix.org/awards.html

- **Numerous NSH awards & scholarships – May 1, 2005**

www.nsh.org

~

www.nsh.org

NSH

MISSION STATEMENT

The National Society for Histotechnology is a non-profit organization, committed to the advancement of Histotechnology, its practitioners and quality standards of practice through leadership, education and advocacy.

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Nominations and Membership

Region IX Website
www.nshregionix.org

NSH Website
www.nsh.org

Preservation and retrieval of antigens for immunohistochemistry – methods and mechanisms Part 2. Retrieving masked antigens

J. A. Kiernan
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The mechanisms of fixation by formaldehyde were described in a previous article (*The Cutting Edge*, January 2005, pp. 5-9), and it was noted that the cross-linking of protein molecules can render the epitopes (antibody-binding regions) of antigens inaccessible to large molecules, preventing detection by immunohistochemical methods. The lipoprotein membranes that enclose cells and organelles constitute another barrier to penetration of tissue by large molecules, especially when processing has not involved passage through organic solvents.

Despite having been available for many years (Coons et al, 1942) immunostaining was still a "new" method in the 1960s and 1970s. The deleterious effects of fixation on enzymatic activity were well known, and it was widely assumed that fixation also destroyed the chemical basis of antigenicity. Cryostat sections of unfixed or minimally fixed tissue were commonly used for immunohistochemistry (e.g. Nairn, 1976). Some fixatives, including alcohol-based mixtures, Bouin's fluid and periodate-lysine-formaldehyde (McLean & Nakane, 1974), were said to "preserve" the antigenicity of certain peptides and proteins. Since about 1980 it has been recognized that distortion of macromolecular architecture by a fixative may either expose or conceal epitopes. Although antigens respond differently to fixatives it is generally true to say that epitopes are exposed by coagulation of proteins and masked by cross-linking. In terms of making antigens accessible to immunoreagents, neutral buffered formaldehyde was the worst of seven fixatives compared by Arnold et al (1996).

Nevertheless, neutral formaldehyde is the most frequently used fixative. Fortunately there are several ways to improve the access of antibodies to tissue antigens that have been masked by formaldehyde fixation.

Proteolytic enzymes

Probably the earliest way of unmasking formaldehyde-fixed antigens was to incubate the preparation, before exposing to the primary antibody, in a solution of a proteolytic enzyme (see Bullock & Petrusz, 1982). Usually an inexpensive grade of porcine trypsin (containing some chymotrypsin) is used at a concentration of 0.1% in 0.1 M aqueous $CaCl_2$, adjusted to pH 7.8 with TRIS or a few drops of 0.1M NaOH. An optimum incubation (in the range 10 to 60 minutes, at room temperature or 37°C) must be found for each tissue and antigen. The rationale of using a proteolytic enzyme is that breaking some peptide bonds will make holes in the matrix of cross-linked proteins, allowing the entry of antibody molecules (Fig. 1). Enzymes other than trypsin have been used in much the same way, including pronase and pepsin (Hume & Keat, 1990). Endogenous proteolytic enzymes, released from cells damaged by the microtome knife, have been shown by Mori et al (1992) to unmask certain epitopes of extracellular proteins.

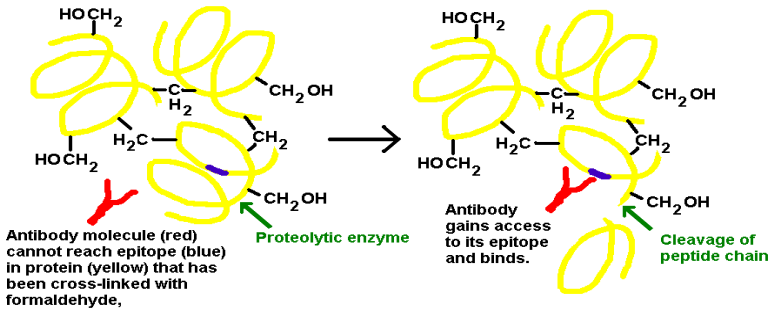

Fig.1. Unmasking of an epitope by the action of a proteolytic enzyme

The chief disadvantage of proteolytic enzymes is their propensity to digest the tissue, including the antigen one is attempting to demonstrate. A duration of exposure to the enzyme solution must be found that is just right for the job at hand (see Hayat, 2002). This requirement makes enzymatic treatment rather too labour-intensive for routine use.

Heat induced antigen retrieval

Most of the formaldehyde bound to a fixed tissue can be removed by prolonged washing (two to three weeks) in cold water (Helander, 1994; see also my previous article in *The Cutting Edge*). One may reasonably suspect that removal of formaldehyde would be accomplished more rapidly at high temperatures. Water alone, however, is seldom used for heat induced antigen retrieval. Other substances are nearly always dissolved in the water, and the reasons for trying the various solutes are not explained by the authors of most publications in this field. The first hot solutions to be used (Shi et al., 1991) contained either zinc sulfate or lead thiocyanate. The slides, bearing hydrated sections, were brought to 100°C in these solutions. The sensitivity of immunohistochemical staining was usefully increased for most of the 52 antibodies tested, and lead thiocyanate was generally more effective than zinc sulfate.

Subsequent studies of antigen retrieval in the 1990s focused especially on the pH of the hot water, and the type and duration of heating. The general consensus is that for most antigens pH 6 (nearly always obtained with a citrate buffer) is suitable. It is also generally agreed that a minority of antigens require either more acidic (pH 1) or more alkaline (pH 9) retrieval solutions. The source of heat may be a microwave oven, a boiling water bath or an autoclave. Some have argued that microwave heating may do more than simply raise the temperature, but the arguments are not convincing (see Hayat, 2002 for references and discussion). Effects of temperature have also been examined. It is evident that higher temperatures permit shorter times in an antigen retrieval solution. An overnight immersion in citrate buffer (pH 6) at 80°C is as effective as immersion for less than one hour in the same solution at 100°C (Koopal et al., 1998). With autoclaving (about 120°C), antigen retrieval is accomplished in about 10 minutes (Bankfalvi et al., 1994; Hunt et al., 1996), but much additional time is taken up waiting for the autoclave to cool without releasing the pressure. The usual procedure of decompression followed by closing the air intake valve causes boiling of the water in the jar containing the slides and detachment of all the sections (Kiernan – unpublished observation that should have been anticipated). A domestic pressure cooker is better suited to antigen retrieval than an institutional autoclave (Pileri et al., 1997). After 10 minutes at full steam remove it from the source of heat but do not release the pressure valve or cool the outside of the pressure cooker with cold water.

Ingredients of antigen retrieval solutions

No-one doubts the importance of pH (Shi et al., 1995; Boon, 1996), but ingredients other than buffer salts can also contribute to the efficacy of solutions for antigen retrieval. Before the advent of methods involving heating, techniques to improve immunostaining included treating sections at room temperature with 5M urea (Hausen & Dreyer, 1982) or with detergents (see Feldmann et al., 1983). Table 1 shows some of the substances that have been included in solutions for heat induced antigen retrieval.

Table 1. Possible functions of substances other than water in some solutions used for heat induced antigen retrieval. The formulations are listed in order of date of publication. The references should be consulted for exact details of composition of the solutions.

Solute	Possible function	Reference for formulation	Comments
Zinc sulfate [$ZnSO_4$], 1%	Protein coagulant cation	Shi et al., 1991, 1992	Shi et al. (1991) examined retrieval of 52 antigens. $ZnSO_4$ was generally less effective than $Pb(SCN)_2$

Lead thiocyanate [$Pb(SCN)_2$], 1%	Protein coagulant cation with chaotropic anion	Shi et al., 1991; Takahashi et al., 1993	Takahashi et al. found that heating with Pb $(SCN)_2$ improved immunostaining of Bouin-fixed tissue. Methacarn (a non aqueous fixative that does not contain formaldehyde) provided for even better immunostaining
Citrate buffer, 0.1M, pH 6	pH control; chelation of Ca^{2+}	Shi et al., 1993, 1994	Generally more effective than $Pb(SCN)_2$
Urea, 0.8M (5%)	Non-ionic chaotrope	Shi et al., 1994	Concentration lower than those used by other investigators
Glycine-HCl buffer, pH 3.5	Acidic medium	Shi et al., 1994	Less background immunostaining than after 0.8M urea
Aluminum chloride$_3$, 4% (pH2.5) *or* HCl, 0.1M (pH 1.0)	Acidic protein coagulants	Evers & Uylings, 1994	Vibratome sections of brain that had been in formaldehyde for more than a year.
Citrate buffers, pH 2.5, 4.5, 6.0	pH control; chelation of Ca^{2+} (at higher pH)	Evers & Uylings, 1994	Vibratome sections of brain that had been in formaldehyde for more than a year.
Citrate buffer, 0.1M, pH 6.0	pH control; chelation of Ca^{2+}	Beckstead, 1994	Applicable to most of the antigens tested
Urea, 3M (18%)	Chaotrope	Beckstead, 1994	Applicable to most of the antigens tested
Tris buffer, 0.05M, pH 10	Alkaline medium	Beckstead, 1994	Retrieved some antigens that could not be immunostained after pH 6 buffer or 3M urea
No solutes	Distilled water	Umemura et al., 1995	More effective than buffers for autoclave retrieval of Bcl-2 protein
EDTA, 0.001M, pH 8	Chelation of Ca^{2+}; pH control	Balaton et al., 1995	1.5 minutes in pressure cooker
Citrate buffer, 0.01M, pH 6	pH control; chelation of Ca^{2+}	Man & Tavassoli, 1996	70-80°C (oven)
Urea, 0.8M (5%) in Tris buffer, pH 9.5	Non-ionic chaotrope at high pH	Shi et al., 1996	Effective with 32 of 34 antigens examined
EDTA, 0.001M, pH 8	Chelation of Ca^{2+}; pH control	Pileri et al., 1997	2 minutes in pressure cooker. Tested with 61 antibodies, and found generally superior to citrate pH 6, Tris pH 9.5 or a proteolytic enzyme
Tris buffer, pH9.0	Alkaline medium	Koopal et al., 1998	Used for 16 antigens; overnight at 80°C
Formic acid, 19M (88%) followed by guanidine thiocyanate, 4M	Acid followed by chaotropic cation and anion	Everbroek et al., 1999	For unmasking prion protein. The sections were autoclaved before exposure to the reagents.
EDTA, 0.01M, pH 8	Chelation of Ca^{2+}; pH control	Gown & Willingham, 2002	Higher concentration of EDTA than in earlier studies; 10 minutes at 100°C

Citraconic anhydride, 4.5mM, pH 7.4	May reverse formaldehyde fixation	Namimatsu et al., 2005	Equal or superior to two other retrieval solutions, for 62 antigens tested

Mechanisms

At least four features of antigen retrieval solutions may be involved in their actions on formaldehyde-fixed tissue: hydrolysis of bonds to bound formaldehyde, actions of metal cations, chaotropic effects, and chelation of calcium ions.

Hydrolysis of bonds to bound formaldehyde

The possible action of hot water in undoing some of the cross-linking of protein molecules has already been mentioned, but this putative mechanism (Fig. 2) had not, until recently, been tested experimentally. Montero (2003) noted that prolonged fixation in formaldehyde results in poor stainability of tissue proteins by eosin, and that eosinophilia was restored by hot solutions used to retrieve antigens. Stronger evidence supporting the breaking of cross-links comes from the work of Yamashita & Okada (2005), who used SDS gel electrophoresis to study proteins that had reacted in solution with formaldehyde. Cross-linking resulted in the formation of dimers, trimers and other polymers; subsequent heating restored the original monomers. Eosin anions are electrostatically attracted to the basic side chains of proteins – the ones to which formaldehyde molecules bind covalently. Hot water may also alter the conformations of protein molecules. The latter process (cooking) can be expected to expose antigenic sites in much the same way as a coagulant fixative.

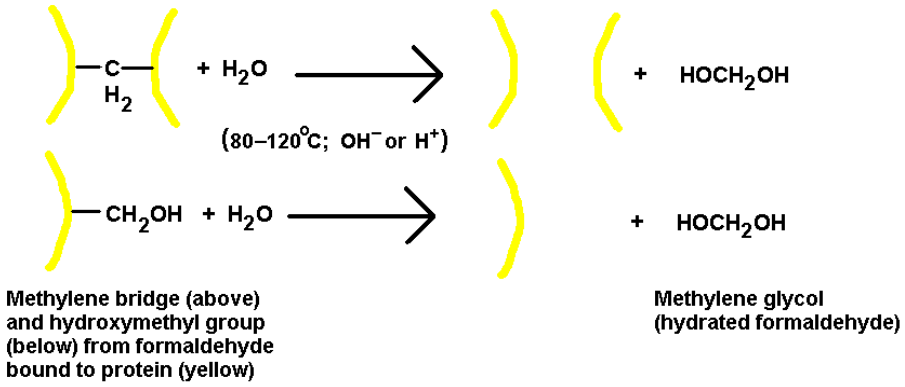

Fig. 2. Removal of bound formaldehyde and undoing of cross-links by base- or acid-catalyzed hydrolysis.

The effects of pH on different formaldehyde-fixed epitopes may be due to their different constituent amino acids, with some linkages to formaldehyde being more easily broken by hydrolysis in an acidic or alkaline medium. Shi et al. (1997) noted that some antigens could be heat-retrieved over a wide range of pH whereas others required an alkaline medium and yet others were retrievable at low or high but not at neutral pH.

Recently heating in an aqueous solution of citraconic anhydride (0.05%, pH 7.4, 45 minutes) has been proposed as a "universal antigen retrieval method" (Namimatsu et al., 2005). The procedure was equal or superior to citrate (pH 6.0) or Tris-HCl-5% urea (pH 9.0) for all 62 antibodies tested. The authors suggest that citraconic anhydride, which can combine reversibly with amino groups, attacked the bonds between formaldehyde-derived carbon atoms and protein nitrogens, thereby breaking the cross-links and removing the cross-links and giving antibodies access to epitopes.

Effects of metal cations

Solutions of the metal salts used in antigen retrieval solutions acidify the water. For aqueous solutions the pH is about 2 for 1% zinc sulfate, 5 for 1% lead thiocyanate, and 2.5 for 4% aluminum chloride. Zinc, lead and aluminum ions also coagulate proteins. The first are included in several modern fixatives, and the second in some older mixtures (see Gray, 1954). Solutions of zinc, lead and aluminum salts are traditional astringent lotions, which coagulate blood and proteinaceous exudates on inflamed skin or mucous membranes. Aluminum ions are not used in fixatives but they are used to harden the gelatin in photographic emulsions. Coagulant metal cations are not now considered major ingredients of antigen retrieval solutions, though they may be necessary for some particular antigens. The cations may cause changes in the conformations of protein molecules, especially in tissue that has not been adequately fixed by formaldehyde. Associated anions may have related effects; these will be discussed next.

Chaotropic effects

Water molecules occur in clusters of 280 molecules that can flip between an expanded and a collapsed structure (Fig. 3).

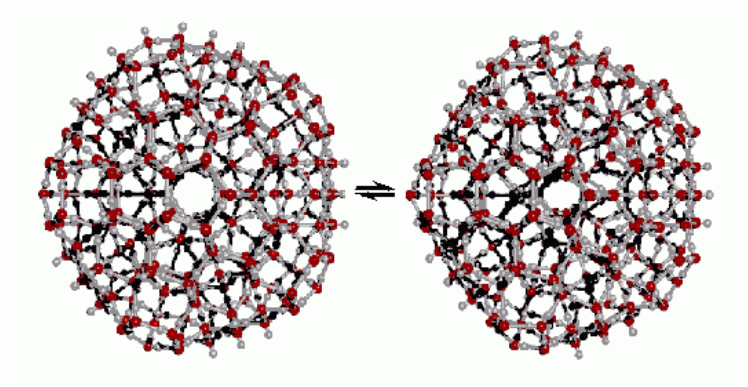

Fig. 3. Expanded (left) and collapsed (right) structures of water clusters. [Reproduced with permission from Chaplin (2004)]

Large molecules such as proteins dissolve by occupying the spaces between clusters. These spaces become wider when clusters change from the expanded to the collapsed form. The latter can therefore hold more macromolecules in solution. Smaller molecules or ions dissolved in water can alter the equilibrium between expanded and collapsed structures. Solutes that favor the collapsed structure are called chaotropes. Chaotropic ions, which include guanidinium, and thiocyanate, make the spaces bigger by inducing water clusters to flip to the collapsed state, making more room for dissolved macromolecules. Urea, a non-ionic compound, is a chaotrope when dissolved at high concentrations. Chaotropes are included in a number of solutions used for antigen retrieval (Table 1) but their modes of action have not been investigated. It is possible that these substances modify some proteins in fixed tissue to make them resemble proteins in solution, with more of their epitopes exposed.

Chelation of calcium

The most popular ingredient of antigen retrieval solutions is the citrate anion. This is a component of the buffer system that stabilizes the pH, but citrate ions can also form soluble complexes with calcium ions. Indeed, sodium or ammonium citrate is an ingredient of at least five solutions used for decalcifying bony specimens (see Lillie & Fullmer, 1976). A more powerful chelator of calcium is the EDTA anion, which is also widely used for decalcification. EDTA is included in several recently published antigen retrieval solutions (see Table 1 for a few examples), in which it serves to buffer the pH and to remove Ca^{2+} from the tissue. A chelating agent reacts with a metal ion, which becomes one of a ring of covalently bonded atoms in a stable, soluble, unreactive compound. Chelation reactions remove metal ions from liquid or solid materials.

A Ca^{2+} ion can form 4 coordinate bonds to other atoms such as oxygen or nitrogen. A coordinate bond is a covalent (strong) bond in which both electrons are donated by the O or N atom. Such a bond is often represented in a structural formula by an arrow; the head of the arrow pointing towards the metal atom indicates the electron donation. Some antigen-antibody reactions in solution are known to be inhibited in the presence of Ca^{2+} and facilitated by EDTA, presumably because the conformations of the proteins are changed by coordinate bonding between calcium and their amino, hydroxyl or carboxyl groups. Simple experiments have shown, for a few antigens, that addition of a calcium salt can impair immunostaining of sections of formaldehyde-fixed tissue. Heating in a Na_2EDTA buffer effectively retrieved these masked antigens, but a CaEDTA solution with the same pH was ineffective (Shi et al, 1999). It has been suggested that coordinate bonding of tissue-derived calcium occur with protein side-chains and also with bound hydroxymethylene groups derived from formaldehyde (Jasani et al., 1997; see also Hayat, 2002 for references and diagrams).

More recently, however, Yamashita & Okada (2005) have used SDS gel electrophoresis to examine the effects of some heat induced antigen retrieval procedures on five proteins in solution. This analytical procedure separates protein molecules according to size. Treatment with formaldehyde caused aggregation of protein molecules into dimers and trimers. Heating restored four of the proteins to their unfixed, predominantly monomeric, conditions. (The fifth protein was degraded by the heating procedure, yielding molecules smaller than the original monomers.) No effects of added calcium ions or of EDTA were detected, indicating that cross linking and deformation of antigens by calcium ions is not a major mechanism of epitope masking in formaldehyde-fixed proteins.

Conclusions

The large variety of ingredients in solutions for high temperature antigen retrieval indicates that more than one mechanism is probably involved. There is experimental evidence for temperature-dependent chemical reactions of water with formaldehyde-protein linkages, with breaking of cross-links. Most antigens can be retrieved at near-neutral pH, but a more alkaline medium is needed for some. In a few cases bonds to tissue-bound calcium ions may mask epitopes, necessitating removal of the metal ions by chelation. Other ingredients of retrieval solutions include heavy metal ions, which may expose epitopes by a coagulation-like action on proteins, and chaotropic substances which may modify the shapes of proteins by changing the structures of clusters of water molecules. Most recently, a hot citraconic anhydride solution has been introduced as a reagent to undo the fixation of proteins by formaldehyde (Namimatsu et al., 2005). Further work will be needed to determine if this is truly a universal antigen retrieval method as claimed, and to clarify the mechanism of action.

References

Arnold MM, Srivastava S, Fredenburgh J, Stockard CR, Myers RB, Grizzle, WE. 1996. Effects of fixation and tissue processing on immunohistochemical demonstration of specific antigens. *Biotechnic &.Histochemistry* **71**: 224-230.

Balaton AJ, Vaury P, Baviera EE, Vuong PN, Galet BA (1995). Protocole "EDTA-autocuiseur." Une technique immunohistochimique performante. *Annales de Pathologie* **15**: 295.

Bankfalvi A, Riehemann K, Ofner D, Checci R, Morgan JM, Piffko J, Bocker W, Jasani B, Schmid KW (1994). Feuchtes Autoklavieren. Der einfachere Weg zur Antigendemaskierung. *Pathologie* **15**:345-349.

Beckstead JH (1994). Improved antigen retrieval in formalin-fixed, paraffin- embedded tissues. *Applied Immunohistochemistry* **2**:274-281.

Boon ME (1996). Microwave-antigen retrieval: The importance of pH of the retrieval solution for MiB-1 staining. *European Journal of Morphology* **34**:375-379.

Bullock GR, Petrusz P. 1982. *Techniques in Immunocytochemistry*. Vol. 1. London: Academic Press.

Chaplin M. 2004. Water Structure and Behavior. http://www.lsbu.ac.uk/water/index.html

Coons AH, Creech HJ, Jones RN, Berliner G. 1942. The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. *Journal of Immunology* **45**159-170.

Everbroek B, Pals P, Martin JJ, .Cras P (1999). Antigen retrieval in prion protein immunohistochemistry. *Journal of Histochemistry and Cytochemistry* **47**:1465-1470.

Evers, P. & Uylings, H.B.M. (1994). Microwave-stimulated antigen retrieval is pH and temperature dependent. *Journal of Histochemistry and Cytochemistry* **42**:1555-1563.

Feldmann G, Maurice M, Bernuau D, Rogier E, Durand AM (1983) Penetration of enzyme-labeled antibodies into tissues and cells: a review of the difficulties. In: S Avrameas, P Druet, R Masseyeff, G Feldmann, eds: *Immunoenzymatic Techniques.* Elsevier, Amsterdam. pp. 3-15.

Gown AM, Willingham MC (2002). Improved detection of apoptotic cells in archival paraffin sections: immunohistochemistry using antibodies to cleaved caspase 3. *Journal of Histochemistry and Cytochemistry* **50**:449-454.

Gray P (1954) *The Microtomist's Formulary and Guide.* New York: Blakiston.

Hausen P, Dreyer C (1982) Urea reactivates antigens in paraffin sections for immunofluorescent staining. *Stain Technology* **57**: 321-324.

Hayat MA. (2002) *Microscopy, Immunohistochemistry and Antigen Retrieval Methods for Light and Electron Microscopy.* New York: Kluwer Academic/Plenum Publishers.

Helander KG (1994) Kinetic studies of formaldehyde binding in tissue. *Biotechnic & Histochemistry* **69**:177-179.

Hume WJ, Keat S. 1990. Immunohistological optimization of detection of bromodeoxyuridine-labeled cells in decalcified tissue. *Journal of Histochemistry and Cytochemistry* **38**: 509-513.

Hunt NCA, Attanoos R, Jasan, B (1996). High temperature antigen retrieval and loss of nuclear morphology: A comparison of microwave and autoclave techniques. *Journal of Clinical Pathology* **49**:767-770.

Jasani B, Morgan JM, Navabi H (1997) Mechanism of high temperature antigen retrieval: role for calcium chelation. *Histochemical Journal* **29**: 433.

Koopal SA, Coma MI, Tiebosch ATMG, Suurmeijer AJH (1998) Low-temperature heating overnight in tris-HCl buffer pH 9 is a good alternative for antigen retrieval in formalin-fixed paraffin-embedded tissue. *Applied Immunohistochemistry* **6**:228-233.

Lillie RD, Fullmer HM (1976) Histopathologic Technic and Practical Histochemistry, 4th ed. New York: McGraw-Hill.

Man YG, Tavassoli FA (1996). A simple epitope retrieval method without the use of microwave oven or enzyme digestion. *Applied Immunohistochemistry* **4**:139-141.

McLean IW, Nakane PK. 1974. Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. *Journal of Histochemistry and Cytochemistry* **22**: 1077-1083.

Montero C. (2003) The antigen-antibody reaction in immunohistochemistry. *Journal of Histochemistry and Cytochemistry* **51**: 1-4.

Mori S, Sternberger NH, Hermann MM, Sternberger LA. (1992) Variability of laminin immunoreactivity in human autopsy brain. *Histochemistry* **97**: 237-241.

Müller A, Bögge H, Diemann E (2003) Structure of a cavity-encapsulated nanodrop of water. *Inorganic Chemical Communications* **6**: 52-53.

Nairn RC. 1976. *Fluorescent Protein Tracing*. 4th ed. London: Churchill-Livingstone.

Namimatsu S, Ghazizadeh M, Sugisaki Y (2005) Reversing the effects of formalin fixation with citraconic anhydride and heat: a universal antigen retrieval method. *Journal of Histochemistry and Cytochemistry* **53**: 3-11.

Pileri SA, Roncador G, Ceccarelli C, Piccioli M, Briskomatis A, Sabattini E, Ascani S, Santin, D, Piccaluga PP, Leone O, Damiani S, Ercolessi C, Sandri F, Pieri F, Leoncini L, Falini B (1997) Antigen retrieval techniques in immunohistochemistry: Comparison of different methods. *Journal of Pathology* **183**:116-123.

Shi SR., Key ME, Kalra KL (1991). Antigen retrieval in formalin-fixed, paraffin-embedded tissue: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *Journal of Histochemistry and Cytochemistry* **39**: 741-748.

Shi, SR, Imam SA, Young L, Cote RJ, Taylor CR (1995). Antigen retrieval histochemistry under the influence of pH using monoclonal antibodies. *Journal of Histochemistry and Cytochemistry* **43**:193-201.

Shi, SR, Cote C, Kalra KL, Taylor CR, Tandon AK (1992). A technique for retrieving antigens in formalin-fixed, routinely acid-decalcified, celloidin-embedded human temporal bone sections for immunohistochemistry. *Journal of Histochemistry and Cytochemistry* **40**:787-792.

Shi SR, Chaiwun B, Cote RJ. Taylor CR (1993). Antigen retrieval technique utilizing citrate buffer or urea solution for immunocytochemical demonstration of androgen receptor in formalin-fixed paraffin sections. *Journal of Histochemistry and Cytochemistry* **41**:1599-1604.

Shi SR, Chaiwun B, Young L, Imam A, Cote RJ, Taylor CR (1994). Antigen retrieval using pH 3.5 glycine-HCl buffer or urea solution for immunohistochemical localization of Ki-67. *Biotechnic & Histochemistry* **69**:213-218.

Shi SR, Cote RJ, Taylor CR (1997) Antigen retrieval immunohistochemistry: past, present and future. *Journal of Histochemistry and Cytochemistry* **45**: 327-343.

Shi SR, Cote RJ, Young L, Imam SA, Taylor CR (1996). Use of pH 9.5 tris-HCl buffer containing 5% urea for antigen retrieval histochemistry. *Biotechnic & Histochemistry* **71**:190-196.

Shi SR, Cote RJ, Hawes D, Thu S, Shi Y, Young LL, Taylor CR (1999) Calcium-induced modification of protein conformation demonstrated by immunohistochemistry. What is the signal? *Journal of Histochemistry and Cytochemistry* **47**: 463-470.

Takahashi H, Oishi Y, Oyaizu T, Tsubura A, Morii S (1993). Proliferating cell nuclear antigen (PCNA) immunohistochemistry: influence of tissue fixation, processing and effects of antigen retrieval. *Micron* **24**: \385-388.

Taylor CR, Shi SR., Chen C, Young L, Yang C, Cote RJ (1996) Comparative study of antigen retrieval heating methods: Microwave, microwave and pressure cooker, autoclave, and steamer. *Biotechnic & Histochemistry* **71**:263-270.

Umemura S, Kawai K, Osamura RY, Tsutsumi Y (1995). Antigen retrieval for bcl-2 protein in formalin-fixed, paraffin-embedded sections. *Pathology International* **45**:103-107.

Yamashita S, Okada Y (2005) Mechanisms of heat-induced antigen retrieval: analyses in vitro employing SDS-PAGE and immunohistochemistry. *Journal of Histochemistry and Cytochemistry* **53**: 12-21.

About the author ~ Dr. John A. Kiernan

(This is an excerpt from an article "Kiernan Presents Culling Lecture" written by Janet Dapson, NSH in Action Editor, Volume 30, Number 2 September 2003) Permission was obtained from Janet to print this excerpt.

"Since 1972, Dr. John Kiernan has been a professor in the Department of Anatomy and Cell Biology at the University of Western Ontario, Canada. He teaches undergraduate and graduate courses in neuroanatomy, a graduate histochemistry course, and conducts research in neuroscience. He is also involved in developing histological and histochemical techniques.

In conjunction with teaching and research, writing is another of Dr. Kiernan's activities. He has written, co-authored and contributed chapters to numerous books, published 100 papers, and submitted articles on a variety of topics to less formal publications. His scientific article, "Silver Staining for Spirochetes in Tissues" appeared in Laboratory Medicine, September 2002. At the top of the list of publications are "Histological and Histochemical Methods (three editions) and Conn's Biological Stains, 10th edition (co-authored with Richard Horobin, PhD). Histological and Histochemical Methods is thought by some to be the best reference available for explaining the mechanisms of fixation and staining. Conn's Biological Stains, 10th edition published 2002 is a completely revised version of the classic text.

Visit Dr. John Kiernan's website: http://publish.uwo.ca/~jkiernan/ . The histochemistry segment of the website is filled with FAQ (Frequently Asked Questions), inspired by the Histonet. You will find topics from fixation to photomicrography, techniques, recipes, chemistry and even history".

We have been very fortunate Dr. John Kiernan has shared his vast knowledge and expertise to our members in Region IX and we will look forward to more articles from him in the future!

Quality issues in histotechnology: The total quality approach to quality improvement. 1. Optimizing the Ziehl-Neelsen stain.

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Few universal technical quality standards are applied to histopathological preparative techniques.
The standards used are usually of a local and purely subjective nature such as; 'our pathologist likes it this way', 'we've always done it this way' and/or 'it looks alright to me'. Variations in one histological technique are often introduced as a local response to a real or perceived problem, the root cause of which may actually lie in another histological technique. These variations are usually empirically derived, often spread anecdotally and can eventually make their way into the technical literature.

This is particularly true in the practice of histological staining, as is evident by the number of different modified staining methods which are in common use for any particular purpose. These modified staining methods are often only very minor variations of the original but, in turn, some of them undergo further multiple modifications until a truly bizarre and illogical variant occurs. To paraphrase John Kiernan, "these have the look of a method that has been passed down through generations of people who were not thinking about what they were doing!" Many of these modified staining methods proudly carry the name of their originator, further adding to the overall confusion when trying to choose a staining method. It is therefore not surprising that a wide range of reported result exists, making inter-laboratory comparison of such results difficult.

Concurrently, there is a long standing tradition of regarding staining as 'art' rather than science. However, the mechanisms of staining are well understood and, with the exception of some aesthetic considerations, staining is based on science not 'art'. It is possible to both design and standardize a staining method that is reproducible and easy to perform. The goal is to ensure run-to-run reproducibility of the final staining result on any patient sample, whether such stains are performed days, weeks or months apart. The reproducibility of result should be independent of individual technologists performing the stain within a single laboratory and robust enough to withstand the inevitable minor variations in the methodology, reagents and equipment utilized by other laboratories performing the same stain. Achieving this goal requires an understanding of the staining principles involved with careful attention to selection of method, preparation of reagents, strict adherence to the selected protocol and rigorous use of appropriate control material.
In addition, the method of tissue preparation can have dramatic effects upon the staining result.
In today's busy diagnostic laboratory, with the ever increasing demand for reduced turn-around- times, use of faster tissue processing and staining instrumentation and the introduction of more complex staining technology such as immunohistochemistry, these effects have been largely overlooked.

Quality in histotechnology

Many histopathology laboratories use both 'quality control' (QC) and 'quality assessment' (QA) activities for the various individual steps involved in completing the daily workload.
Briefly, QC activities are prospective i.e. they look forward at what will happen if all the steps in the process are followed. QC defines a product's quality and imparts to it the credibility needed for its intended purpose. Such QC activities are the result of advance planning and are prospectively applied to everything that contributes to the final product (on-line controls).

QA activities are retrospective i.e. they look backward at what has happened, with a view to measuring the degree to which the desired outcomes are successful. They provide the opportunity to modify the processes contributing to the final product (off-line controls). This is a laudable endeavor, however, little attention seems to be given to integration of the QA information obtained.

The 'total quality' or as Taylor (2000) calls it 'the total test' approach is a more holistic look at all the individual steps, in all of the various processes used, from initially obtaining the sample until the final reporting of results. It involves integrating the QC and QA findings, as well as understanding how changes to the various processes will affect the final outcome. The latter may involve experimentation in order to supply provenance for any proposed process change and consequent QC activity.

This article is intended to provide the reader with only a quick example of how the 'total quality approach' can provide improvement to the final result of a commonly used Ziehl-Neelsen stain for *mycobacteria*. A subsequent article will address a similar approach to the Gram stain. For brevity's sake these articles shortcut some of the necessary steps for a truly complete 'total quality approach'. It is hoped that readers will be stimulated to follow the path and finish the journey. A high level of knowledge, skill and experience of the histotechnologist is essential to a successful outcome with any histological stain. I encourage readers to acquire and critically study the references provided.

The Ziehl-Neelsen stain.

The acid and alcohol resistant nature of the tubercle bacillus was discovered by Paul Ehrlich in 1882 and he produced a staining method which used basic fuchsine. The procedure was slightly modified by others (Ziehl 1882 and Neelsen 1883) and subsequently misnamed the Ziehl-Neelsen method (Clarke. 1983). Many other modifications of Ehrlich's original method have been published and a vast number of papers record the use of these methods for both microbiological and histological purposes. Lillie (1965) devoted 7 pages to discussion of histological staining modifications. In modern histotechnology textbooks, despite the quite different methods of sample preparation used by microbiologists and histologists, little distinction is made between the original intended uses of these modified staining methods. The recent introduction of 'microwave-accelerated' staining procedures has introduced further modifications to this stain (Hafiz 1985 and Boon 1989).

A typical and commonly used modified Ziehl-Neelsen (ZN) stain may be found in Carleton (1962).
Briefly;
Paraffin sections are dewaxed and hydrated in the conventional manner.
Sections are placed in a Coplin jar containing pre-warmed carbol-fuchsine for 1 hour at 37°C.
Following a rinse in tap-water, sections are differentiated with acid alcohol (1% v/v Hydrochloric acid in 70% alcohol) until the section is pale pink and no more colour comes away (about 1-3 minutes).
After a further rinse in tap-water, sections are counterstained in 0.1% w/v aqueous methylene blue for one minute and rinsed in water. The counterstain is then differentiated until pale blue and the section simultaneously dehydrated with absolute alcohol, sections are cleared in xylene and mounted.

Although a differentiation time of 1-3 minutes is suggested in the method, in practice colour ceased to come away from the sections after 25 seconds and differentiation was stopped at this time. The result of following this procedure on both a surgical biopsy and the positive control section is illustrated in figures 1 and 2. (*All photomicrographs were taken using a 100X objective unless otherwise indicated*)

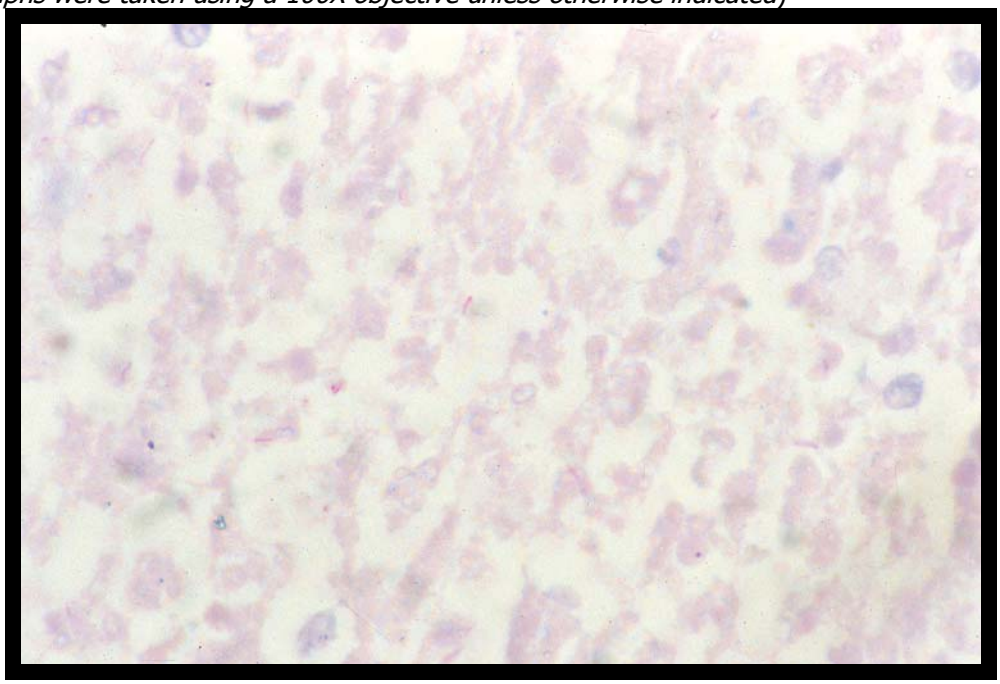

Figure 1. Section of 'routinely' NBF-fixed and processed lung biopsy tissue from a subsequently confirmed case of TB.

The result of a carefully performed commonly used ZN stain. Very few organisms are seen in this caseous granuloma. The organisms that are demonstrated exhibit weak staining and low contrast.

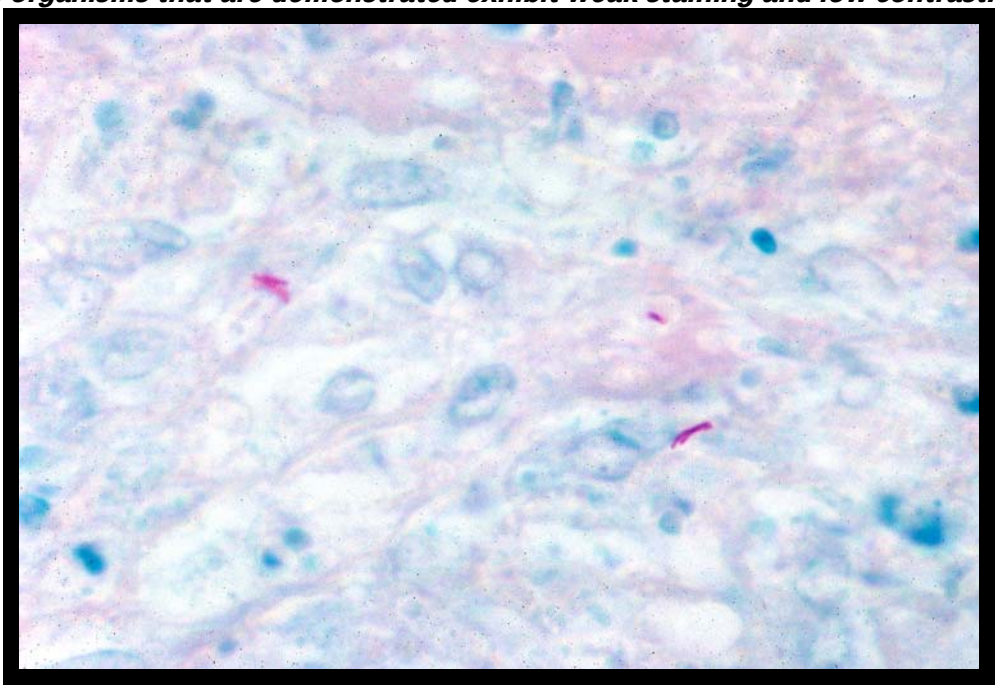

Figure 2. The positive tissue control section, stained at the same time, reveals organisms with more intensity and contrast.

Quality assessment of this result

Disparity in staining between a known positive control and the test sample is a common problem in histopathology. In many cases of both known and suspected tuberculosis, the result of ZN staining on tissue sections is completely negative with only 1 in 5 or fewer cases showing positivity, which may vary from many to just a few organisms. To account for these observations a number of suggestions have been made including; the presence of only a few organisms can result in a large tissue response, uneven distribution of organisms in tissue (both are sampling errors); decrepit organisms lose their acid-fastness (either as a result of treatment or immune response). Undoubtedly, sometimes these suggestions may apply, however, in this case, a portion of the same biopsy had been submitted to microbiology and the direct smear of this sample had stained strongly positive with both ZN and fluorescent methodology. Subsequently, culture had confirmed the presence of *M. tuberculosis.*

QC of the staining methodology indicated that the stain had been performed correctly. A repeat stain performed by another experienced senior technologist with similar results seemed to confirm that. Therefore, it was likely that another cause existed for the poor staining of the biopsy. Both the biopsy and the positive tissue control had been fixed in 4% w/v formaldehyde in phosphate buffer pH7.2 (NBF) and processed using similar reagents and timing schedules. However, there was one significant difference in preparation; the fixation time. The positive tissue control had been fixed in NBF for 22 days prior to processing whereas the biopsy material had been fixed in NBF for only approximately 8-9 hours, a very common situation for biopsy materials. Good laboratory practice dictates that the QC tissue samples should be prepared in a manner similar to that of the test tissues. This dictum is extremely difficult if not impossible to follow in reality. Histology QC tissues are commonly acquired from other patient samples destined for disposal at some time after the diagnosis has been made. Could this difference in fixation time be the cause of the poor staining of the organisms seen in this biopsy? If so, how can we test this?

The organism

The genus *Mycobacterium* is the only genus in the family *Mycobacteriaceae* and is related to other mycolic acid-containing genera. With the advent of better biochemical and molecular techniques for culture and identification, approximately 100 mycobacterial species have been identified. The *M. tuberculosis* complex, which includes *M.tuberculosis, M. bovis (including M. bovis* BCG*), M. africanum, M. microti, and M. canettii*, is identified as responsible for tuberculosis in humans.
M.leprae is responsible for leprosy in humans and naturally occurring infections have been documented in the nine-banded armadillo (a possible source of positive control material?).

Many other mycobacterial species, some associated with human disease, are found in water, soil, plants or other environmental sources (Pfyffer 2003). These are a potential source of section contamination and false positivity (Dizon 1976).

The peptidoglycolipids in the mycobacterial cell wall contain *meso*-diamin-opimelic acid, alanine, glutamic acid, glucosamine, muramic acid, arabinose, and galactose. Mycolic acids (number of carbon atoms ranging from 60 to 90), together with free lipids such as trehalose-6,6'-dimycolate, and the presence of other important fatty acids such as waxes, phospholipids, mycoserosic, and phthienoic acids, provide a hydrophobic permeability barrier. The large variety of lipids and glycolipids of the cell wall differ among mycobacterial species or groups of species. Various patterns of cellular fatty acids (number of carbon atoms ranging from 10 to 20) including tuberculostearic acid is also found. (Besra 1994, Brennan 1994)

Mechanism of staining

The high content of complex lipids of the mycobacterial cell wall provides a hydrophobic permeability barrier. This prevents easy access by aqueous solutions of the common cationic dyes used to stain other microorganisms. Access may be gained by increasing the amount of dye available in solution, by addition of a cosolute to the primary dye solution or by staining at elevated temperatures. Traditionally, all three strategies are employed to speed diffusion of dye into the *Mycobacterium*. Access may also be gained by greatly prolonging the staining time.

Once access is gained, the affinity of the primary dye for mycobacteria is due to basic dyeing of the various anionic components found within both the cell and the cell wall. This first step is non-selective; anionic components of both the mycobacteria and the tissues are stained.

The selective nature of the stain depends on the subsequent differentiation step. This relies on the low permeability of the mycobacterial cell wall and the resulting slow dye diffusion which prevents easy and rapid loss of dye from the organism. Differentiation or destaining is the controlled removal of dye from tissue until dye only remains in the structure/s desired. This controlled removal may be accomplished by a variety of mechanisms.

Solvent differentiation works by extracting the dye from tissues with a liquid in which the dye is readily soluble. A differential removal relies on the fact that dyes generally have a higher affinity for, or are present at higher concentration in, some components of cells or tissues than others.

Acid differentiation works by disruption of the electrovalent bonds between the oppositely charged ionized groups in the dye and the tissue component. A differential release of dye relies on the relative acidic strength of the ionized groups in various tissue components, stronger anions retaining the dye.

Mycobacteria are resistant to both these methods of differentiation although some species, most notably *M. leprae,* are less resistant than others. *M. tuberculosis* is highly resistant to both methods of differentiation and is referred to as being both acid- and alcohol-fast (AAFB). This high resistance to both methods of differentiation led to the common practice of combining them into a single step, as acid-alcohol, and simply calling the organism acid-fast (AFB). The acid-alcohol combination is more aggressive in removal of dye than when the differentiators are applied separately (Pfyffer 2003).
In histopathology this practice is regrettable, since the ZN stain may be used to demonstrate structures other than mycobacteria. The distinction between acid-fast and alcohol-fast may be important in their identification. Members of other mycolic acid containing genera such as *Nocardia* and *Actinomycetales* , are acid-fast but may not be alcohol-fast. Other low permeability tissue structures such as hair shafts, red blood cells, spermatozoa heads and some of the lipofuscins also demonstrate variable acid and/or alcohol-fastness.

Fixation of tissues

The mechanisms of fixation by formaldehyde were described by John Kiernan in the last issue of this newsletter (*The Cutting Edge,* January 2005, pp. 5-9), more detailed information may be found in the references at the end of that article and in his textbook (Kiernan 1999). It was pointed out that the main chemical reactions of formaldehyde are with proteins, resulting in the formation of methylene bridge cross-links. The reactions are very slow and require a minimum of 24 hours at ambient temperatures for reasonable structural preservation. More complete structural stabilization requires at least 3 to 7 days and continues very slowly after that.

Formaldehyde may also combine with some lipids, but does not react significantly with soluble carbohydrates. These other reactions are not considered to participate to any great extent in the fixation of tissues. Even so, some lipids and carbohydrates are satisfactorily preserved by fixation in formaldehyde. This has been attributed to their entanglement in a meshwork of closely associated cross-linked proteins (Baker 1958).
Some of these lipids even largely resist extraction by the solvents used in processing tissue to paraffin wax. Inadequate formaldehyde fixation followed by paraffin processing distorts proteinaceous structures, damages cell membranes and significantly increases their permeability. Since low permeability of the mycobacterial cell wall is the controlling mechanism for differentiation of the ZN stain, increased permeability could have a significant effect on the final result.

A model for testing fixation and staining of mycobacteria

Plasma clot control blocks provide a novel method of obtaining known controls. The cells to be stained are suspended in pooled human plasma, thrombin is added to clot the plasma and the resulting model tissue fixed and processed as a routine tissue block. If microbiology samples are to be used as the target cells, the addition of blood cells from a Buffy layer will provide additional non-organism control targets for the counterstain. **Microbiology control samples should only be prepared by trained staff using the appropriate biosafety level practices.** After fixation such samples may be handled in the same way as other tissues. Detailed preparation instructions may be obtained by contacting the author.

Several plasma clot control blocks were prepared, each containing four different species of *mycobacterium*, *M. bovis, M. avium, M. fortuitum* and *M. gordonae.* The organisms were obtained from the stock cultures available in the department of microbiology and they vary in both size and degree of acid- and alcohol-fastness. Each organism was present at a concentration of approximately 10^8 cells/mL. Clots were fixed in NBF for 24 hours, 72 hours, 7 days and 21 days, then processed to paraffin wax using the same 'routine' reagents and schedule as the tissue biopsies. **Note that a short fixation (<24 hours) control was not included!** It would have been scientifically more valid to also test a fixation time matching that of the biopsy but for safety reasons, the author decided not to obtain a control fixed for only 8-9 hours. In view of a recent publication (Gerston 2004), that appears to have been a wise decision.
ZN stained sections of the 24 hour and 72 hour fixed controls are illustrated in figures 3 and 4, the controls fixed for 7 and 21 days gave results similar to that shown in figure 4.

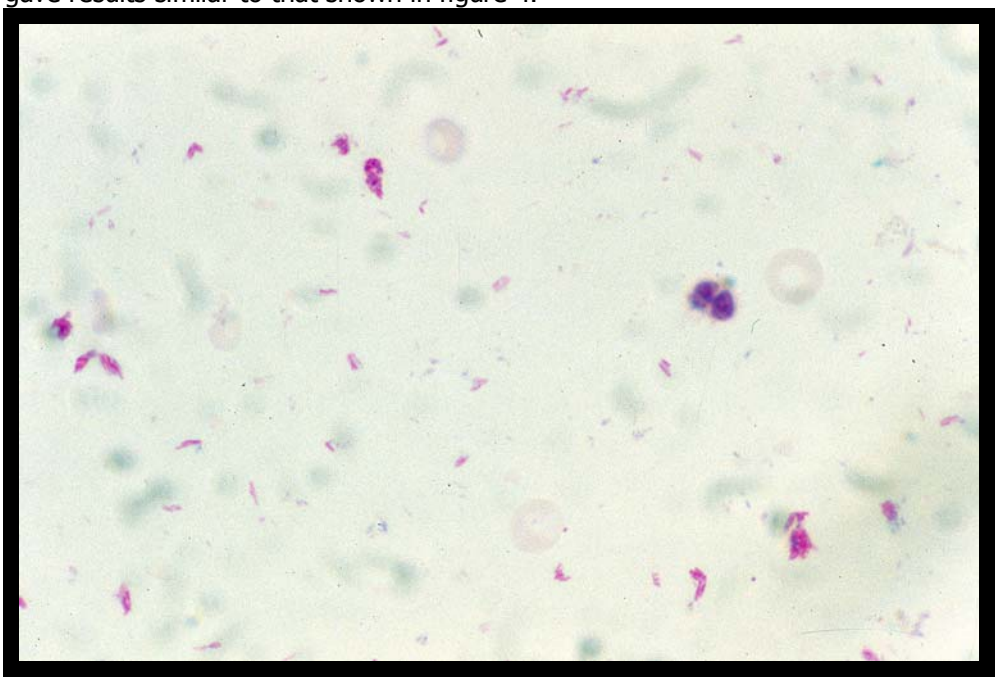

Figure 3. A standardized procedural control section of a 'plasma clot' suspension, containing four different species of mycobacterium, fixed in 4% w/v formaldehyde in phosphate buffer pH7.4 for 24 hours, processed to paraffin wax via a 'routine' overnight process. This carefully performed commonly used ZN stain utilized acid-alcohol as the differentiator for 25 seconds. Note the considerable variation in intensity and contrast of the various mycobacteria, some being visualized only as 'ghosts'. The red cells also exhibit loss of staining.

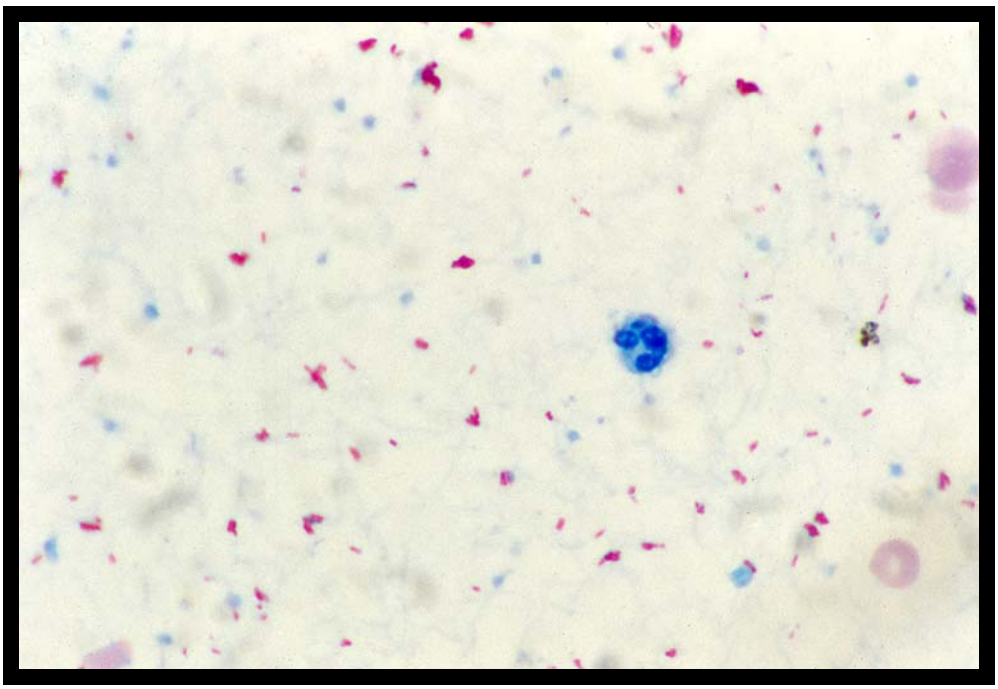

Figure 4. A standardized procedural control section of a 'plasma clot' suspension, containing four different species of mycobacterium, fixed in 4% w/v formaldehyde in phosphate buffer pH7.4 for 72 hours, processed in the same manner as the sample in figure 3. This ZN stain was performed simultaneously with the slide in figure 3. Note the more uniform intensity and contrast of the various mycobacteria. The red cells are showing their normal degree of staining.

Modification of the staining method

Comparison of the staining results shown in figures 3 and 4 would suggest that fixation in NBF for 24 hours only provides partial stabilization of the cell walls of *mycobacterium* and that, despite careful control of the differentiation time, there is loss of acid-and alcohol fastness of some of the organisms. It would seem reasonable to conclude that shorter fixation times would only exacerbate the problem. Fixation times of 72 hours or longer appears to provide a more complete stabilization of the cell wall. In fact, differentiation in acid alcohol could be safely extended to 2-3 minutes with no loss of staining (not shown). It was decided to change the method of differentiation to one less aggressive and see if this would improve staining in both the original biopsy and clot sections fixed for less than 72 hours. In addition, the counterstain was acidified to improve selectivity and reduce subsequent differentiation time in alcohol (Lillie 1944).

Paraffin sections are dewaxed and hydrated in the conventional manner.
Sections are placed in a Coplin jar containing pre-warmed carbol-fuchsine for 1 hour at 37˚C.
Following a rinse in tap-water, sections are differentiated with two changes of 10% v/v aqueous sulphuric acid for 5 minutes each. Sections may appear yellow-colourless during the second change of sulphuric acid due to temporary conversion of the dye to a colourless form. Wash well in tap-water for 5-10 minutes, sections will become pink as the dye colour is restored. Sections are counterstained in 0.1% w/v methylene blue in 1% v/v aqueous acetic acid, for two minutes and rinsed in water. Dehydrate with 3 changes of absolute alcohol for 1 minute each, sections are cleared in xylene and mounted. (*N.B. Sulphuric acid was introduced by Neelsen (1883) in place of the nitric acid used by Ehrlich. The author has used this separate acid and alcohol procedure for more than 40 years and found it superior for both M. tuberculosis and M. leprae in hundreds of cases.*)

Results of this staining procedure are illustrated in figures 5 through 8

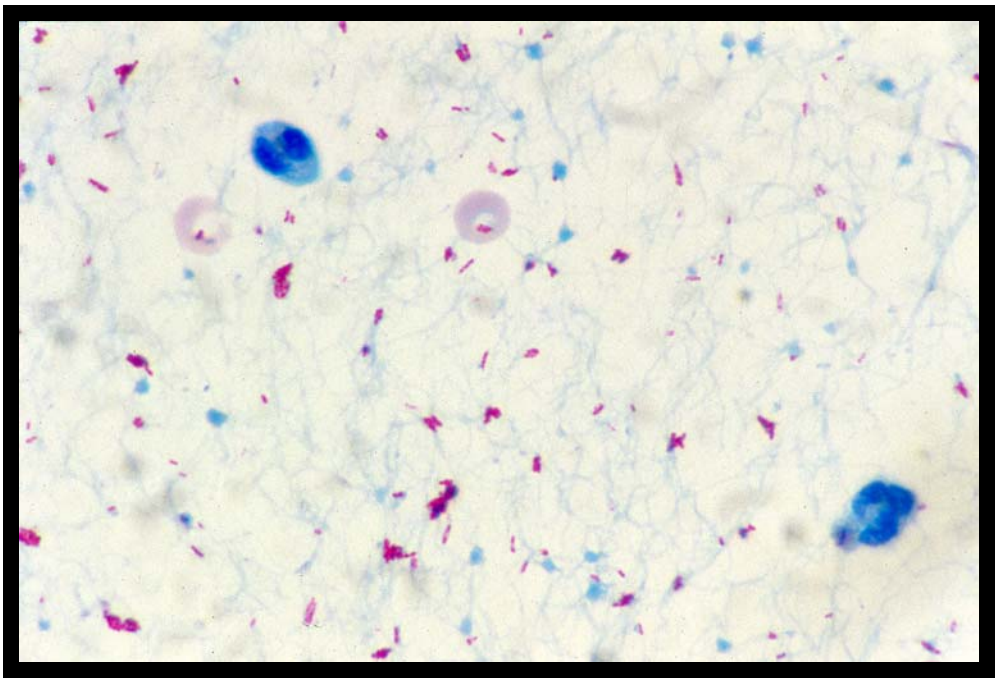

Figure 5. A standardized procedural control section of a 'plasma clot' suspension, containing four different species of mycobacterium, fixed in 4% w/v formaldehyde in phosphate buffer pH7.4 for 24 hours, processed to paraffin wax via a 'routine' overnight process. This ZN stain utilized 10% aqueous sulphuric acid as the acid differentiator for a total of 10 minutes, the alcohol differentiation was performed separately (a total of 3 minutes) during the dehydration stage following counterstaining. Note the high intensity and contrast of the various mycobacteria, a considerable improvement over figure 3. The red cells are showing their normal degree of staining.

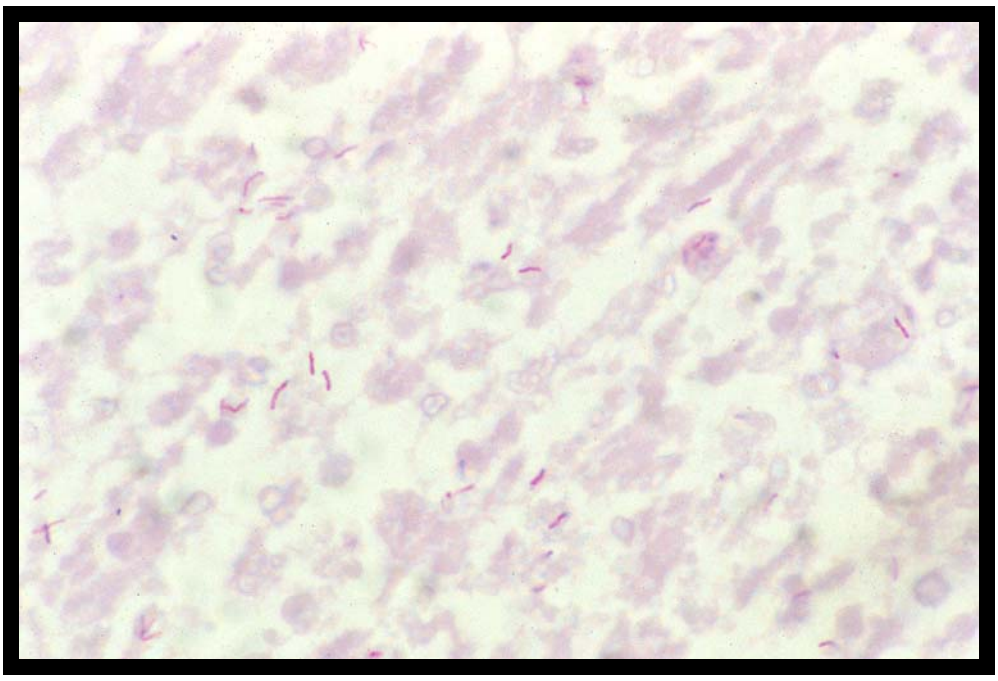

Figure 6. Adjacent section from the same block illustrated in figure 1. The result of a ZN stain performed simultaneously with the standardized procedural control shown in figure 5 and using the same separate acid and alcohol differentiation procedure. Impressive numbers of organisms are clearly demonstrated in the same area of the granuloma shown in figure 1.

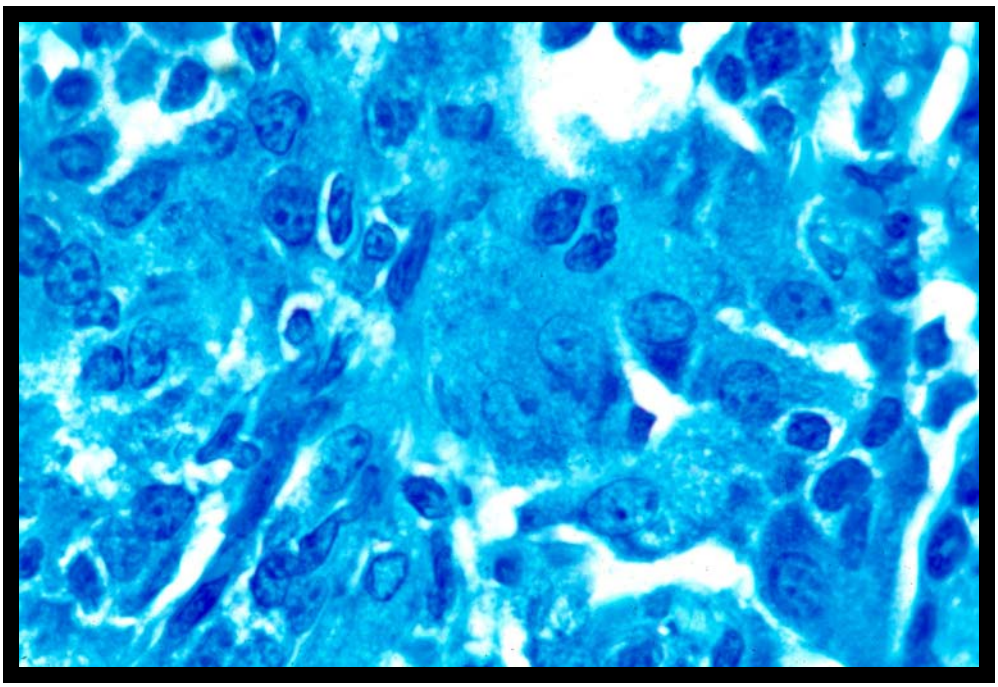

Figure 7. The use of an overly intense and non-selective counterstain almost completely masks the presence of the 2 or 3 mycobacteria in the central portion of this field.

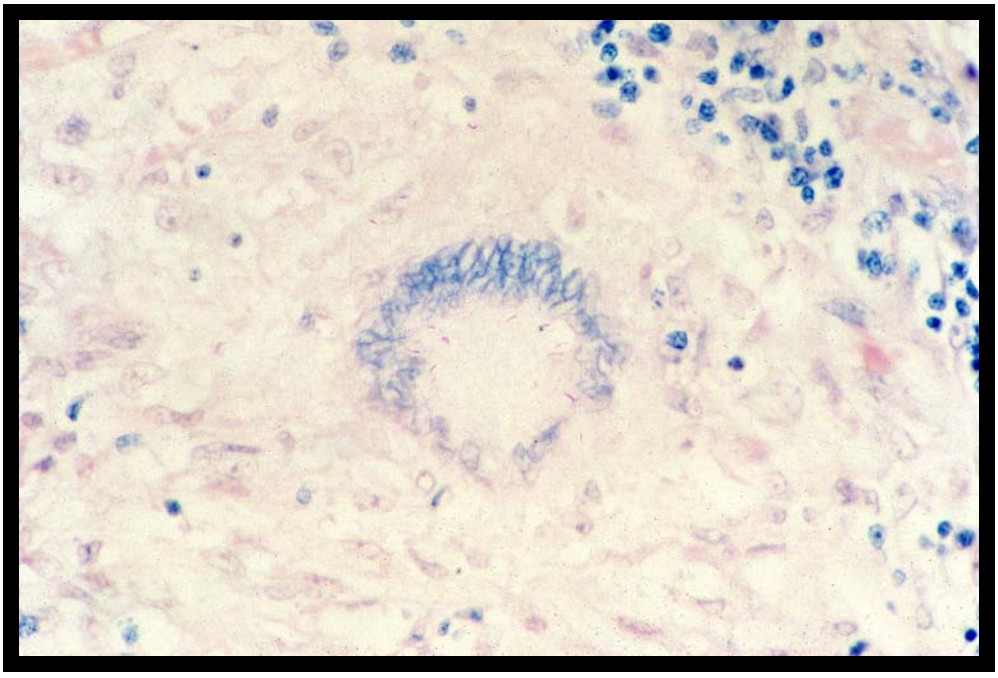

Figure 8. When using a counterstain of appropriate intensity and selectivity, even at the lower magnification used in this photomicrograph (40X objective), mycobacteria in the cytoplasm of the Langerhans' giant cell and elsewhere in the field are readily visible.

Further Quality improvement

Many other opportunities exist for further quality improvement of this ZN staining procedure, both generally and for specific purposes. Readers are encouraged to pursue them.

References

Quality issues in histotechnology: The total quality approach to quality improvement. 1. Optimizing the Ziehl-Neelsen stain.

Bryan R. Hewlett

Baker JR. (1958) *Principles of biological microtechnique* (Reprinted 1970, with corrections). Methuen, London

Besra GS and Chatterjee D. (1994) Lipids and carbohydrates of *Mycobacterium tuberculosis*, Ch.20 In: Bloom BR. *Tuberculosis, Pathogenesis, Protection and Control*. ASM Press, Washington, D.C.

Boon ME and Kok LP (1989). *Microwave cookbook of pathology: The art of microscopic visualization*. 2nd revised ed. Coulomb Press, Leiden. ISBN 90-71421-10-4

Brennan PJ and Draper P (1994) Ultrastructure of *Mycobacterium tuberculosis,* Ch 19 In: Bloom BR *Tuberculosis, Pathogenesis, Protection and Control*. ASM Press, Washington, D.C.

Carleton HM and Drury RAB. (1962) *Histological technique for normal and pathological tissues and the identification of parasites*. 3rd ed. Oxford University Press, London

Clarke G and Kasten FH (1983) *History of staining*. 3rd ed. Williams & Wilkins, Baltimore. ISBN 0 683 01705-5

Dizon D, Mihailescu C and Bae HC. (1976) Simple procedure for detection of *Mycobacterium gordonae* in water causing false-positive acid-fast smears. *J Clin Microbiol*. **3**(2): 211

Ehrlich P (1882) *Dtsch. Med. Wochenschr*. **8**: 269-270

Gerston KF, et al. (2004) Viability of mycobacteria in formalin-fixed lungs. *Hum. Pathol*. **35**(5): 571-575

Hafiz S, et al. (1985) Use of microwaves for acid and alcohol fast staining. *J Clin Pathol*. **38**: 1073-1076

Horobin RW and Bancroft JD (1998). *Troubleshooting histology stains*. Churchill Livingstone, London. ISBN 0 443 05312 X.

Kiernan JA. (1999) *Histological and histochemical methods: Theory and practice*. 3rd ed. Butterworth Heinemann, Oxford. ISBN 0 75064936 4

Lillie RD (1944) Acetic methylene blue counterstain in staining tissues for acid-fast bacilli. *Stain Technology*, **19**: 45

Lillie RD (1965) *Histopathological technic and practical histochemistry*. 3rd ed. McGraw-Hill, New York.

Neelsen F. (1883) Ein casuistischer Beitrag zur Lehre von der Tuberkulose. *Zentralbl. Med. Wiss*. **21**: 497-501

Pfyffer GE, et al. (2003) Mycobacterium: General characteristics, isolation, and staining procedures. Ch.36 In: Murray PR, Baron EJ, et al. *Manual of clinical microbiology*. 8th ed. ASM Press, Washington, ISBN 1 55581 255-4

Somoskővi Á, et al. (2001) Lessons from a proficiency testing event for acid-fast microscopy. *Chest* **120**: 250-257

Taylor CR (2000). A total test approach to standardization of immunohistochemistry. *Arch. Pathol. Lab. Med*. **124**: 945-951.

Ziehl F. (1882) Zur färbung des Tuberkelbacillus. *Dsch. Med. Wochenschr*. **8**: 451

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- Reflect both Canadian and North American articles pertinent to the practice of Histotechnology
- Publish articles that are beneficial to our discipline .
- Promote communication between Region IX members and members of the other NSH Regions
- Provide a medium for exchange of information among members.

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