# Microwaves in the Retrieval of Proteins, RNA and DNA

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## Introduction

Microwaves (MWs) are a form of non-ionizing radiation with a typically standard frequency of 2.45 GHz, a wavelength of 12.2 cm and photon energy of $10^{-5}$ electron volts. When dipolar molecules such as water or the polar side chains of proteins are exposed to the rapidly alternating electromagnetic fields, they oscillate through $180^0$ at the rate of 2.45 billion cycles per second. The molecular movement or kinetics so induced results in the generation of instantaneous heat that is proportional to the energy flux and continues until radiation ceases. Molecules other than water and the polar side chains of proteins including molecules with an uneven distribution of electrical charge such as inorganic material and copper oxides may also be made to oscillate in the electromagnetic field generated by MWs.

## Antigen preservation and antigen retrieval

MWs have played a major role in the demonstration of tissue antigens in paraffin-embedded sections. Tissues fixed in formaldehyde display a distinct and progressive loss of staining of many antigens, frequently proportional to the duration of exposure to the fixative.[1] We found an appreciable loss of staining of some antigens after 3 days; many antigens being lost after 7 days and most not demonstrable after 14 days of fixation. Interestingly, enzymatic predigestion per se was useful for the unmasking of only some antigens, ineffective in many and even deleterious in others. By comparison, tissues immersed in normal saline and fixed by exposure to MWs showed uniformly superior antigen preservation compared to those tissues fixed by formaldehyde and some antigens which were not demonstrable in the latter preparations were readily labelled in MW-fixed sections.[2] Similarly, the enhancement of immunolabelling of cytological preparations[3] has been demonstrated with 0.1% formal saline as the optimal fixative[4] and air drying as the best method of preparation of such material.[5]

Shi et al[6] described MW heating of paraffin-embedded tissue sections in the presence of heavy metal solutions, such as lead thiocyanate, up to temperatures of $100^o$ C to “unmask” a wide variety of antigens for immunostaining. It was subsequently shown that MW-irradiation of deparaffinised-rehydrated sections in 10 mmol citrate buffer solution at pH 6.0, produced, with few exceptions, increased intensity and extent of immunostaining of a wide variety of tissue antigens.[7-9] This has proven to be one of the most important applications of MWs, and antigen retrieval has made it possible for the optimisation of immunostaining in fixed tissue sections.[10] The use of citrate buffer eliminated the need to employ heavy metal solutions which, when heated, generate toxic fumes. Several commercial antigen retrieval reagents are available but they mostly do not produce superior results than that obtained with citrate buffer.[11]

Other methods of generating heat, such as steaming, wet autoclave, pressure cooker, and even the hot sauna, have been advocated for antigen retrieval but MW irradiation remains among the most convenient.

A variety of factors influence the ability to produce antigen retrieval by these heat-induced methods. Among these are the osmolality, pH and chemical composition of the reagent, and the nature of the antigen of interest. It has been suggested that immunoreactivity is generally increased when the sections are irradiated in reagents of higher pH (pH 8-9). With variation of retrieval solution pH, most antigens fell into one of three patterns of reaction.[12] Antigens such as CD45 (leukocyte common antigen), PCNA (proliferating cell nuclear antigen), AE1, EMA (epithelial membrane antigen) and NSE (neuron specific enolase) showed excellent retrieval throughout the pH range. Others including ER (estrogen receptor) and MIB1 showed strong intensity of staining at very low pH and at neutral to high pH, but a dramatic decrease at moderately acidic pH (pH 3-6). The third group of antigens such as CD43 and HMB45 showed increasing intensity of immunostaining with increasing pH, but only weak staining at low pH. Thus, a high pH is most likely to produce optimal retrieval for the majority of antigens and when optimising a new antibody, it is best to start with a high pH.

We have demonstrated that exposure of cryostat sections briefly to MWs before of commencement of immunolabelling produces better quality cytomorphology and staining.[13] A similar procedure has been adopted for freshly frozen brain sections with notable enhancement of immunostaining, without affecting the integrity of cytomorphology. MWs have been applied between sequential rounds of a three-layer immunoenzyme staining (mouse Mab, goat anti-mouse IgG and mouse PAP or mouse APAAP) and color development technique for multiple antigen detection.[14] The MWs denatured bound antibody molecules resulting in the blocking of cross reactivity between the sequential staining steps, allowing the use of primary and other antibodies raised in the same species. Besides serving a role in antigen retrieval, MWs also inactivate peroxidase and alkaline phosphatase enzymes present in PAP and APAAP complexes, which would otherwise lead to inappropriate color development.

MWs have been applied for the acceleration of antibody-antigen reactions in the staining of labile lymphocyte membrane antigens in cryostat sections.[13] Similarly, we have employed irradiation to accelerate immunolabelling in paraffin-embedded sections[15] and the same technique has been applied for immunofluorescence labeling.[16]

## Applications in molecular techniques

The use of MWs in molecular analyses is a more recent development. Initial applications of MWs in molecular studies were for the achievement of the high temperatures necessary to denature probe and tissue DNA; MWs provided a method with ease of control and rapidity of heat generation. This application has also been adapted for the accelerated detection of mRNA.[17]

Formaldehyde-fixed tissues remain the most common source of material for molecular studies and protease digestion is an essential procedure for unmasking the cross-linking effects of this fixative before in situ hybridisation (ISH) can be performed. Recent studies demonstrate that the exposure of the formalin-fixed, paraffin-embedded sections to MWs in citrate

buffer in a manner similar to that applied for antigen retrieval produces enhanced signal detection for both DNA[18] and mRNA. Exposure to MWs for 15-20 min in 10 mmol citrate buffer at pH 6.0 was shown to be more effective than heating at 70$^{o}$ C for 30 min, in sodium chloride-sodium citrate. The procedure was performed in combination with proteinase K digestion (at reduced digestion time) which was applied before irradiation.[19] The same retrieval method was equally effective for mRNA as shown with Epstein-Barr virus EBER RNA with quantitative confirmation of the increased sensitivity render by MW pre-treatment.[20] Importantly, it renders RNA-ISH a more consistent and reliable procedure. The technique has also been successfully applied for terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) for apoptotic cells, proving more sensitive than proteolytic digestion and detergent treatment.[21]

We have recently demonstrated that microwave heating can also be employed to enhance the demonstration of DNA in the chromogenic in-situ hybridization (CISH) technique. By substituting MW irradiation for 10m at 98$^{0}$C for the pre-treatment step of boiling in a proprietary reagent as recommended by the manufacturer and repeating the irradiation after the enzyme digestion step we obtained consistent crisp staining for HER2 with the absence of background precipitation.[22]

**Ultrastructural antigen and enzyme preservation in MW-fixed tissues**

Numerous reports have described better ultrastructural preservation of various tissue enzymes and antigens in MW irradiated tissues compared to conventional fixation procedures. This is not unexpected considering the convincing documentation of the same results in paraffin-embedded tissues. MW irradiation in aldehyde fixatives result in drastically shortened durations of exposure to the fixative so that various lipolytic enzymes such as lipase and sphingomyelinase display much better ultrastructural definition and localization. Using rapid MW fixation, it has been possible to demonstrate, at ultrastructural level, various proteins, which are generally not demonstrable by conventional fixation protocols. Such molecules include tumour necrosis factor,[23] nuclear matrix proteins, glutathione peroxidase, various neuropeptides and myelinated fibres, and Ca2+ ions. In general, MW fixation improves antigenicity of glutaraldehyde-sensitive antigens while producing excellent preservation of ultrastructural details.[24]

**Antigen retrieval in 'thick' sections and immuno-electron microscopy**

Despite the now extensive use of MWs for antigen retrieval in paraffin sections, the technique has rarely been reported in resin embedded sections. Although there are numerous reports of immunolabelling in resin-embedded sections, the method has been generally regarded as capricious and difficult and is not routinely employed in diagnostic situations. Recent studies have shows that MW-stimulated antigen retrieval could be successfully applied to plastic-embedded tissues including acrylic glycol methacrylate (GMA),[25] epoxy Polarbed 812,[26] and methyl methacrylate (MMA).[27] All the three studies employed 10 mmol citrate buffer at pH 6.0 as the retrieval solution and demonstrated, with few exceptions, that immunogenicity was improved following MW antigen retrieval. More recently, we have extended the application of this procedure to sections embedded in acrylic resin (LR White

resin, London Resin Co, Basingstoke, Hampshire, UK). Antigen retrieval was performed by heating one mm sections mounted on 3-aminopropyltriethoxysilane slides to boiling in 10 mmol citrate buffer (300 ml) at high power setting in a 750-watt domestic microwave oven. The sections were kept in the simmering buffer for 10 min by maintaining at medium low power. Before immunostaining, the slides were allowed to cool in the hot buffer for 20 min. With this method we have been able to improve immuno-staining of vimentin, cytokeratin, smooth muscle actin, type IV collagen, laminin, β-catenin, IgA and IgG.[28]

We have also shown that MW-stimulated antigen retrieval can also be effectively employed on grids for immuno-electron microscopy[28] Briefly, thin sections cut on formvar-coated nickel grids were placed on plastic grid plates (Hiraoka Staining Kit, Fort Washington, PA, USA) and immersed in a glass beaker of 50 ml 10 mmol citrate buffer. Another beaker with 350 ml of water was placed in the oven cavity to absorb some of the MW radiation. The buffer was heated to 85-90° C and maintained at the same temperature for 5 min. The grids were allowed to remain in the buffer for a further 20 min then transferred to TBS-buffer before immunolabelling. The immunolabelling of most antigens studied was clearly enhanced. These included vimentin, cytokeratin, type IV collagen, IgA and IgG. There was clear staining for β-catenin, an antigen that is notoriously difficult to demonstrate by immunoelectron microscopy even in cryo-microtomy sections.

Besides enhancement of labeling, it is notable that background staining in irradiated specimens both for light and electron microscopy was reduced. Morphology of the tissue was assessed to be about the similar in quality to that of non-irradiated specimens and interestingly, staining of the endoplasmic reticulum appeared to be stronger and sharper following exposure to MWs.

## Antigen Retrieval and Superheating

Microwaves, pressure cookers, steamers, and autoclaves have been employed to generate the heat for antigen retrieval. For the majority of antigens heating above 100⁰C produces the greatest enhancement of immunostaining.[29] While the pressure cooker is claimed to develop temperatures above boiling point, as with all other methods of heating, the lead-up time and the temperature attained is too variable and it was not until the development of the Mega T/T (Milestone, s.r.l., Italy) that accurate control of time and temperature for retrieval processes became available. The glass pressure generator developed by Milestone allows temperatures of up to 120°C to be accurately controlled by a computer and maintained over the required duration. Our experience with this instrument reveals greater enhancement and consistency of staining for many antigens studied.[29] Importantly, it was possible to obtain consistent immunolabelling of antigens that were difficult to achieve with previous methods.

## Conclusions

Heat may increase the diffusibility of a fixative but high temperatures can also increase enzymatic activity and hasten the process of autolysis. For example, heating of 4% formaldehyde to 60-70° C can hasten the fixation process but the risk of tissue distortion is also greater. It is thought that formaldehyde and other aldehydes have the property of forming cross-links

between proteins, thereby forming a gel that retains cellular constituents in their in vivo relationships to one another. Soluble proteins are fixed to structural proteins and rendered insoluble, so that the entire structure is given some mechanical strength, allowing it to withstand the rigors of tissue processing. The cross-linking between protein molecules is mostly formed mostly by a reaction with the basic amino acid lysine, although other groups such as imino, amido, peptide, guanidyl, hydroxyl, carboxyl, sulphydryl groups and aromatic rings may also participate. Only those lysine residues on the exterior of the protein molecule react and they usually account for 40-60% of the total lysine residues.[30] Although the extent of protein denaturation which results from the fixation process is not of importance in light microscopy, denaturation is of particular relevance in immunolabelling, as well as in molecular analysis and high resolution electron microscopy. Similarly, the shape of molecules should not be changed significantly if they are to be recognized by biochemical analysis.[31] Glutaraldehyde causes a loss of up to 30% of the alpha helix structure of protein, depending on the type of protein and fixation with osmium tetroxide or post-osmification of glutaraldehyde-fixed material causes the complete denaturation of protein. As such, for immuno-electron microscopy, frozen sections should be employed or fixatives should be used in low concentrations and exposure is restricted to short durations.[32]

As with many laboratory stains and procedures, much of our knowledge concerning the actions of MWs is empirical. The accelerated staining of tissue sections, for example, is based on at least two important factors, namely the diffusion of the dye or antibody into the cells and it's binding to the substrate or antigen. Diffusion is a physical process that can be greatly accelerated by irradiation, but the influence of MWs on binding mechanisms is more complicated and less well understood.

The rapidly oscillating electromagnetic field of MWs may itself have an effect on chemical reactions and proteins. While heat or thermal energy will increase molecular kinetics and hasten chemical reactions, the rapid rotation of molecules directly induced by the MWs will give rise to greatly increased collision of molecules, which will in turn accelerate chemical reactions. The heat generated may represent only an epiphenomenon secondary only to the kinetics. Hjerpe et al[33] examined MW stimulation of CEA/anti-CEA reaction in an enzyme-linked immunosorbent assay system. Despite continuous cooling by ice, MW stimulation increased reaction rates by a factor of 1000, allowing the investigators to conclude that such rate increases were far too large to be explained solely by the modest increase in temperature. Choi et al[34] went further to elucidate the existence of a "microwave effect". They showed that the rate of droplet temperature increase obtained in a thermal cycler was similar to that achieved by MW irradiation. However, the immunostaining obtained from a 3-minute incubation at 37$^{o}$ C in the thermal cycler followed by 2-minute incubation without heating was much weaker than that seen with MWs. Takes et al[35] similarly demonstrated that 7-s MW irradiation followed by 5-min room temperature incubation for each step of the avidin-biotin peroxidase complex procedure produced good immunolabelling. The droplet temperature will rise no more than 5$^{o}$ C following 7-s irradiation at 100% power in a 850-watt oven[34] so that temperature was not a significant component of the accelerated reaction. Others have argued that there is no significant microwave effect and the accelerated reactions are a function of

heat. Hopwood et al[36] concluded that MW irradiation did not produce cleavage or polymerization of proteins and irradiation resulted an electrophoretic pattern that was similar to that obtained when lysozyme and hemoglobin was heated in formaldehyde to 60$^{o}$ C for 30 min. Interestingly, Porcelli et al,[37] more recently, showed results to the contrary. In a study of S-adenosylhomocysteine hydrolase and 5'-methylthioadenosine phosphorylase, two thermophilic and thermostable enzymes, they found that exposure to MWs caused a non-thermal, irreversible and time-dependent inactivation of both enzymes. Conformational changes of S-adenosylhomocysteine hydrolase, detected by fluorescence and circular dichroism techniques, suggested that MWs induced protein structural rearrangements not related to temperature. In a study of cross linking of collagen induced by glutaraldehyde as reflected by the shrinkage of porcine collagen, Others have concluded that MWs did not induce a "substantial non-thermal effect on enhancement of glutaraldehyde cross linking of collagen" in the range of 4-20$^{o}$C.[38] Interestingly, one of the authors of this paper also co-authored the earlier work with CEA/anti-CEA described above.[33]

The recent demonstration that exposure to ultrasound can significantly increase antibody-antigen reaction in immunostaining lends further support to the relevance of molecular movement as an important factor in the acceleration of chemical reaction as the amount of heat generated by this physical modality is negligible.[39] A number of other hypothetical physical mechanisms may also play a role in the actions of MWs. Although the proton energy generated in MW fields is too small to alter covalent bonding, they may readily affect the integrity of non-covalent secondary bonding, including hydrophobic interactions, hydrogen bonds and van der Waal's interactions that make up the precise steric interactions at the cell membrane. Morgan et al[40] proposed that calcium forms a cage-like complex which masks antigenic sites during formalin fixation and the release of calcium requires a considerable amount of energy such as high temperature heating or the use of chelating agents like EDTA. When short synthetic peptides were employed to mimic antibody-binding sites of common protein targets, it was found that not all peptides exhibited the formalin fixation and antigen retrieval phenomenon and that this phenomenon was associated with a tyrosine in or near the antibody binding site and bound covalently to a nearby arginine, implicating a role for the complex Mannich reaction[41] which allows the hydrolysis of cross-linkages by heat or alkaline treatment. However, this only accounts for the reaction of formalin with some peptides but not the majority.[42] Many of the applications of MWs for the recovery of proteins, RNA and DNA remain empirical and their mechanisms remain to be explained.[43]

## References

1. Leong, A. S-Y, Gilham P N. The effect of progressive formaldehyde fixation on the preservation of tissue antigens. *Pathology,* 1989;**21,** 266-271.
2. Leong A.S-Y, Milios J, Duncis C G. Antigen preservation in microwave irradiated tissues. A comparison with formaldehyde fixation. *J Pathology,* 1988;**156,** 275-282.

3. Suthipintawong C, Leong AS-Y, Chan KW, Vinyuvat S. Immunostaining of estrogen receptor, progesterone receptor, MIB1 antigen and c-erbB-2 oncoprotein in cytologic specimens. *Diagn Cytopathol* 1997;17:127-133.
4. Suthipintawong C, Leong,A. S-Y, Vinyuvat S. Immunostaining of cell preparations: a comparative evaluation of common fixatives and protocols. *Diagn Cytopathol* 1996;**15,** 167-174.
5. Leong AS-Y, Suthipintawong C, Vinyuvat S. Immunostaining of cytological preparations: A review of technical problems. *Appl Immunohistochem* 1999; 7:214-220.
6. Shi S, Key E, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: An enhancement method for immunohistological staining based on microwave oven heating of tissue sections. *J Histochem Cytochem* 1991;**39,** 741-748.
7. Leong A.S-Y, Milios J. An assessment of the efficacy of the microwave antigen-retrieval procedure on a range of tissue antigens. *Appl Immunohistochem* 1993;**1,** 267-274.
8. Gown A.M., de Wever N, Battifora H. Microwave-based antigenic unmasking. A revolutionary new technique for routine immunohistochemistry. *Appl Immunohistochem* 1993;**1,** 256-266.
9. Cuevas EC, Bateman AC, Wilkins BS, et al. Microwave antigen retrieval immunocytochemistry: a study of 80 antibodies. *J Clin Pathol* 1994*;***47,** 448-452.
10. Leong A.S-Y. Microwave technology in immunohistology – the past 12 years. *Journal of Cellular Pathology,* 1996;**1,** 99-108.
11. Leong A.S-Y, Milios J, Leong FJW-M. Epitope retrieval with microwaves. A comparison of citrate buffer and EDTA with three commercial retrieval solutions. *Applied Immunohistochemistry,* 1996;**4,** 201-207.
12. Shi S-R, Imam SA, Young L, et al. Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. *J Histochem Cytochem* 1995;**43,** 193-201.
13. Leong, A. S-Y Milios J. Rapid immunoperoxidase staining of lymphocyte antigens using microwave irradiation. *J Pathol* 1986;**148,** 183-187.
14. Lan HY, Mu W, Nikolic-Paterson DJ, Atkins RC. A novel, simple, reliable and sensitive method for multiple immunoenzyme staining: use of microwave oven heating to block antibody cross reactivity and retrieve antigens. *J Histochem Cytochem* 1995;**43**, 97-102.
15. Leong AS-Y, Milios J. Accelerated immunohistochemical staining by microwaves. J Pathol 1990; 161:327-34.
16. Chiu, KY, Chin K W. Rapid immunofluorescence staining of human renal biopsy specimens using microwave irradiation. *J Clin Pathol* 1987;**40,** 689-692
17. Besancon R, Bencsik A, Voutsinos B, et al. Rapid in situ hybridisation using digoxigenin probe and microwave oven. *Cell Mol Biol (Noisy-le-grand),* 1995;**41**, 975-977.
18. Allan GM, Smyth JA, Todd D, McNulty MS. In situ hybridization for the detection of chicken anaemia virus in formalin-fixed, paraffin-embedded sections. *Avian Diseases* 1993; **37,** 177-182.
19. Sperry A. Jin L, Lloyd RV. Microwave treatment enhances detection of RNA and DNA by in situ hybridization. *Diagn Mol Pathol* 1996**;5,** 291-296.

20. Oliver KR, Heavens RP, Sirinathsinghji DJ. Quantitative comparison of pre-treatment regimens used to sensitise in situ hybridisation using oligonucleotide probes on paraffin-embedded brain tissue. *J Histochem Cytochem* 1997;**45,** 1707-171
21. Negoescu A, Lorimier P, Labat-Moleur F, et al. In situ apoptotic cell labelling by the TUNEL method: improvement and evaluation of cell preparations. *J Histochem Cytochem* 1996;**44,** 959-968.
22. Leong AS-Y, Haffajee Z, Clarke M. Microwave enhancement of CISH for HER2 oncogene in breast cancer. *Appl Immunohistochem Mol Morph* (In press).
23. Beil W.J, Login GR, Galli SJ, Dvorak A.M. Ultrastructural immunogold localisation of tumor necrosis factor- alpha to the cytoplasmic granules of rat peritoneal mast cells with rapid microwave fixation. *J Allergy Clin Immunol* 1994; **94 (3 Pt 1),** 531-536.
24. Jamur MC, Feraco CD, Lunardi LO, et al. Microwave fixation improves antigenicity of glutaldehyde-sensitive antigens while preserving ultrastructural detail. *J Histochem Cytochem* 1995*;***43,** 307-311
25. Suurmeijer A.JH, Boon ME. Notes on the application of microwaves for antigen retrieval in paraffin and plastic tissue sections. *Eur J Morph* 1993;**31**, 144-150.
26. McCluggage WG, Roddy S, Whiteside C, et al. Immunohistochemical staining of plastic embedded bone marrow trephine biopsy specimens after microwave heating. *J Clin Pathol,* 1995;**48,** 840-844
27. Hand NM, Blythe D, Jackson P. Antigen unmasking using microwave heating on formalin fixed tissue embedded in methyl methacrylate. *J Cell Pathol* 1996;**1**, 31-37.
28. Sormunen RT, Leong AS-Y. Microwave-induced antigen retrieval for immunohistology and immunoelectron microscopy of resin-embedded sections. *Appl Immunohistochem* 1998; 6:234-237.
29. Leong AS-Y, Lee ES, Yin H, et al. Superheating antigen retrieval. *Appl Immunohistochem Mol Morph* 2002;10:263-8.
30. Leong A.S-Y, Leong FJW-M. Applications and protocols for microwave technology for morphological analyses. In *Analytical morphology: Theory, Applications and Protocols,* ed. Gu J. 1997;pp 69-90. Eaton Publishing Co., Natick, MA, USA.
31. Leong A.S-Y. *Principles and Practice of Medical Laboratory Science. Basic Histotechnology.* 1996*;*p 25, pp 33-100, Churchill Livingstone, London.
32. Leong A.S-Y, Sormunen R. Invited Review: Microwave procedures for electron microscopy and resin-embedded sections. *Micron* 1998;**29**:397-409.
33. Hjerpe A, Boon ME, Kok LP. Microwave stimulation of an immunological reaction (CEA/anti-CEA) and its use in immunohistochemistry. *Histochem J* 1988;**20**, 388-396.
34. Choi T-S, Whittlesey MM., Slap SE, et al. Microwave immunohistochemistry. Advances in temperature control. In *Analytical morphology: Theory, Applications and Protocols,* ed. Gu, J. 1997, pp 91-114, Eaton Publishing Co., Natick, MA, USA.

35. Takes PA, Kohrs J, Krug R, Kewley S. Microwaver technology in immunohistochemistry: application to avidin-biotin staining of diverse antigens. *J Histotechnol* 1989;**12**, 95-98.
36. Hopwood D, Yeaman G, Milne G. Differentiating the effects of microwave and heat on tissue proteins and their cross linking by formaldehyde. *Histochem J,* 1988;**20**, 341-346.
37. Porcelli M, Cacciapuoti G, Fusco S, et al. Non-thermal effects of microwaves on proteins: thermophilic enzymes as a model system. *FEBS Letters*, 1997;**402**, 102-106.
38. Ruijgrok JM, Boon ME, Feirabend HK, Ploeger S.Does microwave irradiation have other than thermal effects on glutaraldehyde crosslinking of collagen? *Eur J Morphol* 1993;**31**, 290-297.
39. Portiansky E, Gimeno EJ. A new epitope retrieval method for the detection of structural sytokeratins in the bovine prostate tissue. *Appl Immunohistochem* 1996;**4,** 208-214.
40. Morgan JM, Navabi H, Jasani B. Role of calcium chelation in high temperature antigen retrieval at different pH values. *J Pathol* 1997;**182**:233-7.
41. Sompuram SR, Vani K, Messana E, Bogen SA. A molecular mechanism of formalin fixation and antigen retrieval*. Am J Clin Pathol* 2004;**121**:190-9.
42. Gown AM. Unmasking the mysteries of antigen or epitope retrieval and formalin fixation. *Am J Clin Pathol* 2004;**121**:172-4.
43. Leong AS-Y*. Microwave technology for light microscopy and ultrastructural studies.* Bangkok: Amarin Press, 2005.