Microwave antigen retrieval in immunocytochemistry: a study of 80 antibodies

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Abstract

Aims—To evaluate the effect of microwave irradiation on the staining quality of a range of commonly used primary antibodies in archival, formalin fixed, paraffin wax embedded material, with emphasis on antibodies that have previously worked successfully only on frozen tissue.

Methods—Immunocytochemistry (streptavidin-biotin complex technique) was performed on histological sections of a range of normal and pathological tissues, after varying treatment with microwave irradiation. The staining quality of each antibody was compared with that achieved without prior treatment of the sections or after enzyme predigestion.

Results—Microwave irradiation permitted successful immunostaining with 20 antibodies that stained only frozen tissues before. The staining characteristics of 21 antibodies that were already known to stain formalin fixed, paraffin wax embedded material were improved. Another 39 antibodies did not show enhanced staining with microwave irradiation. The method preserves tissue morphology and produces more consistent staining than that achieved by enzyme predigestion with many antibodies. Microwave irradiation may also allow some primary antibodies to be used at higher working dilutions. The citrate

buffer used in this study avoids the necessity of exposure to heavy metal salts.

Conclusions—Microwave antigen retrieval represents an important technical advance within immunocytochemistry that will greatly increase the range of antibodies which can be used to study formalin fixed, paraffin wax embedded tissues.

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Table 1 Monoclonal antibodies previously not reactive with formalin fixed, paraffin wax embedded tissue, which work well after microwave irradiation

Antibody	CD/Specificity	Source
Lymphocytic and myelomonocytic markers		
E11	CD35	K Gatter, Oxford
AT13/5	CD38	A Tutt, Tenovus, Southampton
TU69	CD25	A Ziegler, Berlin
WR16	CD45R	Wessex Regional Immunology Unit
IB5	MHC class II	ICRF
IgD	IgD	Dako
QBEND 10	CD34	Novocastra
EBM 11	CD68	K Gatter, Oxford
CD5	CD5	K Gatter, Oxford
CD8	CD8	K Gatter, Oxford ⁹
8TCS	Human $\gamma\delta$ TCR	Becton Dickinson
BRIC 128	CD55	D Anstee, NBTS, Bristol ¹⁵
Proliferation markers		
MIB 1	Ki-67 antigen	J Gerdes, Forschungsinstitut Borstel, Germany ⁷
JC1	Proliferation marker	K Gatter, Oxford ¹⁶
Ki-67	Ki-67 antigen	J Gerdes, Forschungsinstitut Borstel, Germany ¹⁷
Adhesion molecules		
1-4C3	VCAM-1	D Haskard, Hammersmith
BRIC 235	CD44	D Anstee, NBTS, Bristol ¹⁵
2H1	CD44	S Jalkanen, Turku, Finland
IF1	CD44	S Jalkanen, Turku, Finland
IE8	CD44	S Jalkanen, Turku, Finland

Microwave irradiation has been used to improve tissue fixation,¹⁻² to enhance histological staining for light and electron microscopy,³⁻⁴ and, more recently, to enhance immunocytochemical staining.⁵⁻¹⁰ The latter technique is becoming increasingly popular and has several advantages over conventional methods such as enzymatic predigestion.¹¹ Shi *et al* have documented a range of antibodies in which performance in fixed tissue sections was improved by microwave irradiation.⁶ Many of the antibodies they investigated, however, were already known to be active in fixed tissue. The purpose of the present study was to extend the use of the technique specifically to antibodies previously found to react only with frozen sections or cytological preparations.

Methods

Eighty antibodies were studied, consisting of 47 reactive with lymphohistiocytic antigens, 20 reactive with cell adhesion molecules, four proliferation markers, and nine other antibodies directed against miscellaneous antigens. All except the CD3 reagent were monoclonal antibodies. A range of normal tissues (lymph node, tonsil, spleen, bone marrow, small bowel) and tissues showing a variety of pathologies (glomerulonephritis, Hashimoto's thyroiditis, Hodgkin's and non-Hodgkin's lymphomas, cerebral astrocytomas, sarcoidosis, Crohn's disease) were studied. All material, obtained from the files of the Department of Pathology at Southampton General Hospital, was fixed in 10% neutral buffered formalin and embedded in paraffin wax using routine methods.

Tissue sections were cut at 5 μ m on to slides coated with poly L-lysine (PLL) or 3-aminopropyltriethoxysilane (APTS). Endogenous peroxidase was inhibited with 0.5% H₂O₂ in methanol for 10 minutes, followed by a single wash in TRIS-buffered saline (TBS).

Five slides were placed in a plastic Coplin jar, filled with citrate buffer⁷⁻⁸ (2.1 g citric acid monohydrate in 1 litre of distilled water, pH 6.0) and covered with perforated cling film to minimise evaporation. Three Coplin jars were placed, evenly spaced, in a domestic microwave oven (Panasonic NN-6450, 800W) and irradiated for five minutes at medium power. Evaporative fluid loss was replaced with fresh buffer and this cycle was repeated up to three times. After microwave irradiation the slides were allowed to cool to room temperature and incubation with pri-

mary antibody was then performed at 4°C for 18–24 hours. In some cases a short digestion step with trypsin (0.1% trypsin [Sigma T8128] in 0.1% CaCl₂ at pH 7.8, 37°C) was included after microwave irradiation and before the application of primary antibody. Bound primary antibodies were visualised using a peroxidase-labelled streptavidin-biotin complex method, with 3,3-diaminobenzidine as the chromogen.¹²

All antibodies were tested with and without microwave irradiation, and the staining quality was compared with that achieved using a

Figure 1 Examples of antibodies that previously showed no reactivity in formalin fixed, paraffin wax embedded material, but gave good results after microwave irradiation. (A) Staining quality with (right) and without (left) microwave pre-treatment: (i) MIB 1, tonsil; (ii) CD44, thyroid; (iii) CD8, tonsil.

(B) MIB 1 staining in enteropathy associated T cell lymphoma (left), Burkitt's lymphoma (centre), and Hodgkin's disease (right).

standard digestion with trypsin step (eight minutes). When lymphoid tissues were studied, the proliferation marker MIB-1 was used as a positive control for the microwave step as this antibody gives reliable and reproducible staining after microwave irradiation.¹³ Negative control sections from which primary antibody was omitted were included in all staining runs.

Results

Of the 80 antibodies tested, microwave pre-treatment resulted in high quality staining with 20 antibodies that previously produced no staining in formalin fixed, paraffin wax embedded material (table 1) (fig 1). Antibodies differed in the amount of microwave irradiation needed, but the best results were usually obtained with two or three five minute

cycles. Within this group the dilutions were generally similar or slightly more concentrated than those used in frozen sections.

A further 21 antibodies that already stain formalin fixed, paraffin wax embedded material showed significant improvement with microwave irradiation (table 2) (fig 2). Notably, the antibodies BerH2, CD3, and LeuM1 produced high quality staining after microwave irradiation without digestion with trypsin that would have otherwise been necessary. Many of these antibodies also produced improved staining at significantly higher working dilutions.

The remaining antibodies, of which WR17, 3-9, Ki M1, Y1/82A, UCHT1, OKT3, 4, 6, and 8, HB2, UCHL1, β F1, TCR δ 1, JOVI-1 and 3, PC10, TS2/77, and fibronectin are commercially available, did not show any improvement after microwave treatment. With some of these we combined microwave irradiation with a subsequent short digestion with trypsin stage, without any improvement in staining results.

Table 2 Antibodies previously reactive with formalin fixed, paraffin wax embedded tissue, but showing improvement after microwave irradiation

Antibody	CD/specificity	Source
<i>Lymphocytic and myelomonocytic markers</i>		
L26	CD20	Dako
LN1	CDw75	Biotest/Clonab
BU38	CD23	Binding Site, Birmingham
PGM1	CD68	B Falini, Perugia, Italy and Dako
Ki B3	B cells	Kiel
BCL-2	BCL-2 protein	K Gatter, Oxford and Dako
HB21	CD71	ATCC
BerH2	CD30	Dako
LeuM1	CD15	Becton Dickinson
WR14	CD43	Wessex Regional Immunology Unit
CD3	CD3	Dako
CD3	CD3	Dako
WR18	MHC class II	Wessex Regional Immunology Unit
<i>Adhesion molecules</i>		
BRIC 222	CD44	D Anstee, NBTs, Bristol ¹⁵
HECD 1	E-cadherin	S Hirohashi, Tokyo, Japan ¹⁸
NCC-CAD	P-cadherin	S Hirohashi, Tokyo, Japan ¹⁸
299		
Hermes 1	CD44	S Jalkanen, Turku, Finland
Hermes 3	CD44	S Jalkanen, Turku, Finland
<i>Miscellaneous</i>		
DO7	p53 protein	Dako
Collagen IV	Collagen type IV	Dako
Laminin	Laminin	Sigma
Vimentin	Vimentin	Euro-Path/Bio-nuclear Services

Discussion

Our study has shown that paraffin wax embedded material can be successfully immunostained using antibodies that were previously reported to stain only frozen tissues. Therefore, the number of antibodies available for the study of archival material can be greatly increased.

Microwave irradiation offers several advantages for routine immunocytochemistry. Recent studies have shown improved staining with several antibodies after microwave irradiation.^{6,14} Many of these antibodies already stain paraffin wax embedded tissue, and our study contains further antibodies in this category. In some cases the staining quality of such antibodies is improved; in others microwave treatment may allow cost savings to be made through the use of higher primary antibody dilutions. The need for enzymatic

Figure 2 Examples of antibodies that previously showed some reactivity in formalin fixed, paraffin wax embedded material, and gave much improved staining after microwave pre-treatment. (A) Hermes 3, with (right) and without (left) microwave pre-treatment (thyroid). (B) p53 staining in enteropathy associated T cell lymphoma (left), Burkitt's lymphoma (centre), and Hodgkin's disease (right).

predigestion of sections is obviated in some cases. Many antibodies (such as CD3) also benefit from a reduction in background staining.

The adverse effects of microwave treatment include detachment of sections from slides during irradiation, especially with higher numbers of cycles. This most often occurs with tissues containing prominent fibrous elements (such as nodular sclerosing Hodgkin's disease), and under these circumstances may occur despite coating slides with PLL or APTS. Background staining may also occur with some antibodies, particularly with longer exposure times to microwave irradiation.

We found that, using Coplin jars, the best results were obtained when sections were placed towards one end of the slide, to ensure continual immersion in buffer solution during microwave irradiation. With this method, a maximum of 15 slides can be irradiated during each five minute microwave cycle and the jars require repeated replenishment of evaporative loss. We are currently evaluating a method for the irradiation of larger numbers

of slides in an increased volume of buffer that may allow antigen retrieval with a single, longer, irradiation step.

The mechanism of antigen retrieval achieved by microwave irradiation remains obscure, but a possible explanation is that microwaves disrupt the cross-linking of proteins in a similar way to that achieved by enzymatic predigestion.⁶ At present, most laboratories use domestic microwave ovens for antigen retrieval, and the characteristics of microwave delivery may vary between different ovens. This could account for differences in the reproducibility of immunostaining results between centres.

It has been suggested that a combination of protease digestion and microwave treatment may improve the staining characteristics of a small number of antibodies, such as immunoglobulin light and heavy chains.¹⁹ We combined microwave irradiation with digestion with trypsin for several antibodies, but could not improve the staining quality achieved with microwave treatment alone. Additionally, tissue morphology was often

impaired when both forms of treatment were combined.

Based on our experience with the antibodies studied, antibody specificity seems to be maintained after microwave treatment. However, the possibility of altered staining characteristics should be considered whenever antigen retrieval is used with new or previously untested antibodies, and results should be compared with those obtained using frozen section immunohistochemistry in initial evaluation.

In conclusion, microwave irradiation represents a major technical advance, enhancing tissue antigenicity while preserving morphology. It is straightforward to perform, and has wide application within research and diagnostic histopathology.

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- 1 Mayers CP. Histological fixation by microwave heating. *J Clin Pathol* 1970;23:273-5.
- 2 Login GR, Dvorak AM. Microwave energy fixation for electron microscopy. *Am J Pathol* 1985;120:230-43.
- 3 Brinn NT. Rapid metallic histological staining using the microwave oven. *J Histotechnol* 1983;6:125-9.
- 4 Estrada JC, Brinn NT, Bossen EH. A rapid method of staining ultrathin sections for surgical pathology TEM with the use of the microwave oven. *Am J Clin Pathol* 1985;83:639-41.
- 5 Chiu KY. Use of microwave for rapid immunoperoxidase staining of paraffin sections. *Med Lab Sci* 1987;44:3-5.
- 6 Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: An enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J Histochem Cytochem* 1991;39:741-8.
- 7 Gerdes J, Becker MHG, Key G, Cattoretti G. Immunohistological detection of tumour growth fraction (Ki-67 antigen) in formalin-fixed and routinely processed tissues. *J Pathol* 1992;168:85.
- 8 Cattoretti G, Becker MHG, Key G, Duchrow M, Schluter C, Galle J, et al. Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave processed formalin-fixed paraffin sections. *J Pathol* 1992;168:357-63.
- 9 Mason DY, Cordell JL, Gaulard P, Tse AGD, Brown MH. Immunohistological detection of human cytotoxic/suppressor T cells using antibodies to a CD8 peptide sequence. *J Clin Pathol* 1992;45:1084-8.
- 10 Sawhney N, Hall PA. Ki-67—structure, function and new antibodies. Editorial. *J Pathol* 1992;168:161-2.
- 11 Mephram BL, Frater W, Mitchel BS. The use of proteolytic enzymes to improve immunoglobulin staining by the PAP technique. *Histochem J* 1979;11:345-58.
- 12 Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. *J Histochem Cytochem* 1981;29:577-80.
- 13 Cuevas E, Jones DB, Wright DH. Immunohistochemical detection of tumour growth fraction (Ki-67 antigen) in formalin-fixed and routinely processed tissues. *J Pathol* 1993;169:477-8.
- 14 Cattoretti G, Pileri S, Parravicini C, Becker MHG, Poggi S, Key G, et al. Antigen unmasking on formalin-fixed, paraffin embedded tissue sections. *J Pathol* 1993;171:83-98.
- 15 Anstee DJ, Gardner B, Spring FA, Holmes CH, Simpson KL, Parsons SF, et al. New monoclonal antibodies in CD44 and CD58: their use to quantify CD44 and CD58 on normal human erythrocytes and to compare the distribution of CD44 and CD58 in human tissues. *Immunology* 1991;74:197-205.
- 16 Garrido MC, Cordell JL, Becker MHG, Key G, Gerdes J, Jones M, et al. Monoclonal antibody JCl: New reagent for studying cell proliferation. *J Clin Pathol* 1992;45:860-5.
- 17 Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* 1984;133:1710-5.
- 18 Shimoyama Y, Hirohashi S. Expression of E- and P-cadherin in gastric carcinomas. *Cancer Res* 1991;51:2185-92.
- 19 Merz H, Rickers O, Schrimel S, Orscheschek K, Feller AC. Constant detection of surface and cytoplasmic immunoglobulin heavy and light chain expression in formalin fixed and paraffin embedded material. *J Pathol* 1993;170:257-64.