

MY APPROACH

How we process trephine biopsy specimens: epoxy resin embedded bone marrow biopsies

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Improved cytomorphology of semithin resin sections over paraffin wax embedded sections may be important in diagnostic haematopathology. However, resin embedding can make immunohistochemical antigen detection or DNA isolation for clonal gene rearrangement assays difficult. This review describes the processing of bone marrow biopsies using buffered formaldehyde based fixation and epoxy resin embedding, with or without EDTA decalcification. Traditional semithin resin sections are completely rehydrated after etching in home made sodium methoxide solution. Resin elimination allows high resolution staining of tissue components with common histological stains. Efficient antigen retrieval and the Envision-HRP system permit the immunohistological detection of many antigens of diagnostic relevance, with retention of high quality cytomorphology. Furthermore, DNA can be extracted for clonality analysis. The technique can be completed within a similar time period to that of paraffin wax processing with only ~30% increase in cost. This technique has been used for diagnosis in over 4000 bone marrow biopsies over the past 14 years. By meeting traditional and contemporary demands on the haematopathologist, it offers a powerful alternative to paraffin wax processing for diagnosis and research.

There has been a variety of approaches used for bone marrow trephine biopsy processing including frozen sections, and diverse fixation, decalcification, and embedding protocols. Haematopathologists have to be familiar with the benefits and limitations of these techniques to make their best choice. Until now, formaldehyde fixation and ethylenediamine tetraacetic acid (EDTA) decalcification have been found to offer the best compromise for preserving both morphology and molecular targets reasonably well, allowing consistent *in situ* detection of proteins and polymerase chain reaction (PCR) amplification of DNA for diagnosis.³⁻⁸ The use of resin embedded semithin sections results in improved cytomorphology compared with that seen in paraffin wax embedded sections.⁹⁻¹¹ However, the polymerised resin usually hinders access of staining reagents to tissue molecules and the technology may require extra costs and skills.¹²

"The ideal diagnostic management of bone marrow biopsies should support good cytomorphology and histotopography, and at the same time protect proteins for immunohistochemistry and DNA for genetic studies"

We have set up a technique using formaldehyde fixation and epoxy resin embedding of bone marrow trephine biopsy specimens (Durcupan ACM; Fluka, Basel, Switzerland), with or without EDTA decalcification. The resin can be completely removed from the sections by etching with no adverse effects, so that rehydrated sections are suitable either for standard histological staining, immunohistochemistry, or DNA isolation. The combination of heat induced epitope retrieval, a selected antibody panel, and sensitive immunolabelling permits reliable immunodetection of a full range of antigens for diagnostic bone marrow pathology. The technique, which has been gradually improved in the light of the evolution of diagnostic classifications and antigen retrieval technology, has been used for diagnosing over 4000 bone marrow biopsies over the past 14 years. Here, we describe our protocol as an alternative approach for bone marrow pathology. Using a resin often used in electron microscopy, this technique fulfils traditional and recent diagnostic needs because it

Bone marrow trephine biopsies are essential when clinical data raise the suspicion of haematological disorders in which the diagnostic assessment of *in situ* tissue architecture, cellularity, and immunophenotyping can be decisive.¹ Bone marrow histology is also important for lymphoma staging, follow up, and residual disease detection and for detection of bone marrow involvement by metastatic tumours.¹⁻³ Because the recent histopathological classification of haematological disorders is based on the consideration of morphological, immunophenotypical, and genetic features, sample preparation for bone marrow archiving can be a crucial issue.⁴ Therefore, the ideal diagnostic management of bone marrow biopsies should support good cytomorphology and histotopography, and at the same time protect proteins for immunohistochemistry and DNA for genetic studies. In addition, the diagnostic procedure should be completed within a reasonable turnaround time and without exceeding a justifiable budget.

Abbreviations: DAB, 3,3'-diaminobenzidine tetrahydrochloride; EDTA, ethylenediamine tetraacetic acid; HRP, horseradish peroxidase; PCR, polymerase chain reaction

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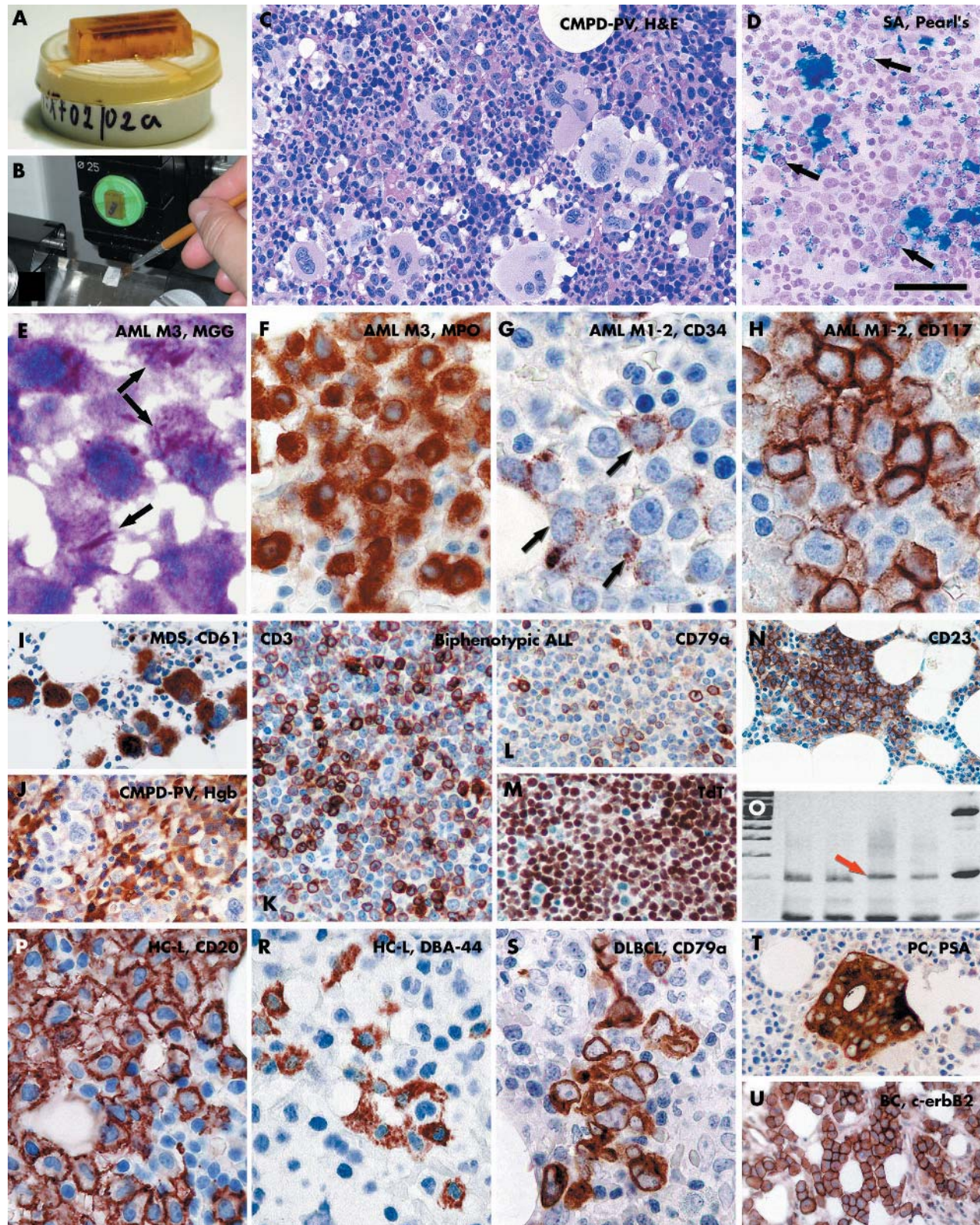


Figure 1 (A) Resin block of a bone marrow trephine stuck on to a labelled plastic stub. (B) Semithin sections are cut with a rotary microtome using a tungsten carbide edged knife. (C) Erythroid and megakaryocytic hyperplasia in chronic myeloproliferative disease (CMPD) polycythaemia vera (PV) using haematoxylin and eosin staining. Note the excellent cytomorphology and the absence of shrinkage artefacts. (D) Haemosiderin particles stained with Perl's Prussian blue in ring sideroblasts (arrows) of sideroblastic anaemia. (E) Auer rods (arrows) revealed with May-Grunwald-Giemsa in promyelocytic leukaemia, in undecalcified resin sections. (F) Promyelocytes of acute myeloid leukaemia (AML) FAB M3 stained for myeloperoxidase. (G) Granular cytoplasmic staining for CD34 (arrows) and (H) membrane staining for CD117 (c-kit) in myeloblastic tumour cells of AML FAB M1-2. (I) Increased numbers of immature megakaryocytes stained for CD61 in myelodysplastic syndrome. (J) Increased numbers of erythroid elements stained for haemoglobin A in CMPD PV. Biphenotypic acute lymphoblastic leukaemia stained for (K) CD3, (L) CD79 α , and (M) terminal deoxynucleotidyl transferase. (N) Interstitial colony of neoplastic lymphocytes in B cell chronic lymphocytic leukaemia/small lymphocytic lymphoma stained for CD23

supports high resolution immunostaining and reliable gene amplification assays in addition to providing superb cytomorphology.

BONE MARROW PROCESSING AND STAINING

Epoxy resin embedding

Bone marrow trephine biopsies are fixed in 4% buffered formaldehyde supplemented with methanol and glucose¹³ (Schaefer's fixative; table 1). The normal duration of fixation is six to 10 hours, but it may be as long as 72 hours in the case of postage in formalin from a district hospital. Trephine biopsy specimens, ideally of 20–40 mm, are cut into no longer than 10 mm pieces and either processed directly into the resin or decalcified, at least partly, in a mixture of 4% buffered formaldehyde and 10% disodium-EDTA dihydrate (Sigma, St Louis, Missouri, USA) at 60°C, for 16–24 hours. Half of the biopsy is processed routinely into paraffin wax blocks for consultation purposes, and the other half is embedded in epoxy resin. For resin embedding, biopsy samples are dehydrated (for example, in an LKB Ultraprocessor; Bromma, Sweden), using a graded series of ethanol (50%, 70%, 96%, 2× 96%, and 2× absolute), then acetone, and finally equal parts of acetone and Durcupan ACM (Fluka), for 15 minutes each. The freshly prepared resin mix is composed of 12.2 g epoxy monomers, 10.0 g hardener (these components can be stored as a mixture for one month), 0.6 g accelerator, and 0.3 g plasticiser. Infiltration of the samples with the resin mix at 50°C for 40 minutes is followed by laying the trephine biopsy rods into polyethylene embedding moulds of 12 × 8 mm block size (TAAB Ltd, Aldermaston, UK), filled with fresh resin. Finally, labelled plastic cylindrical stubs (25 × 10 mm; TAAB Ltd) are put on top of each mould, and samples are kept at 56–60°C in a thermostat for 16–24 hours to allow the resin to polymerise (fig 1A).

Semithin sections (2 µm thick) are cut from the resin blocks on a rotary microtome (for example, Slee-Mainz 4055; Slee Technik GmbH, Mainz, Germany) equipped with a cylindrical specimen holder, and using either a tungsten carbide tipped knife or a "D" profile heavy duty knife (TAAB Ltd) (fig 1B). Sections are floated on an ~80°C waterbath and mounted on silane coated or charged glass slides, such as SuperFrost Plus (Menzel GmbH, Braunschweig, Germany) or alternative adhesive slides of similar quality, which are then kept at 80°C for 30 minutes or at 60°C for between two hours and overnight to activate adhesion. Semithin sections are deplasticised by immersing them in sodium methoxide for 15 minutes. This solution is prepared by saturating methanol or ISM (industrial methylated spirit) with sodium hydroxide pellets by regular mixing, so that some pellets still remain undissolved after two days. This solution can be stored at room temperature for two months and reused up to five times. The sections are washed twice in equal parts of alcohol and xylene for three minutes, then in alcohol alone for three minutes. Endogenous peroxidases are then blocked for 20 minutes in a methanolic/0.5% hydrogen peroxide solution. Finally, the sections are rehydrated and stained. Three 4 µm thick resin sections or three 5 µm thick paraffin sections are used for DNA isolation.

Immunohistochemistry

Before immunostaining, the deplasticised semithin sections (or dewaxed paraffin wax embedded sections) are exposed to wet heat at ~120°C for two minutes in a pressure cooker (for example, Prestige 6193 or 4.5 l Tefal Clipso) for antigenic epitope retrieval. The pH 6.1 target retrieval solution from DakoCytomation (Glostrup, Denmark), or a mixture of 0.01M Tris/0.1M EDTA, pH 9.0, is used as a retrieval solution (table 2). For a range of alternative antigen retrieval protocols see Krenacs *et al.*¹⁴ Immunostaining is performed in a vertical clip system (Tecan GmbH, Crailsheim, Germany), using the capillary force resulting from an approximately 150 µm gap between the section and a plastic coverplate. This force permits only 100 µl reagent to cover the whole glass slide until more buffer or a new reagent is added into the top funnel for replacement. The following incubation steps are carried out: (1) with primary antibodies for 60 minutes (for details see table 2); (2) with antirabbit (K4003; Dako, Glostrup, Denmark) or antimouse (K4007; Dako) immunoglobulin coupled horseradish peroxidase (HRP)-polymer conjugates (EnVision systems; Dako) for 30 minutes; (3) with a DAB (3,3'-diaminobenzidine tetrahydrochloride) hydrogen peroxide chromogen substrate kit (K3468; Dako), for 10–15 minutes to reveal antigen bound peroxidase activity; (4) with 5% copper sulfate for five minutes to improve the contrast of the DAB precipitate; and (5) with Gill's haematoxylin for two minutes as a counterstain. The sections are treated at room temperature and washed three times for three minutes in Tris buffered saline (pH 7.4) between the incubation steps. The primary antibody is replaced by normal mouse or rabbit serum as a negative control. When internal control cells (normal leucocytes) are not available parallel sections known to contain the tested antigen serve as a positive control.

Gene rearrangement analysis using PCR

DNA is extracted from resin embedded bone marrow sections using sodium methoxide treatment for 15–20 minutes and proteinase K digestion without subsequent organic extraction. For immunoglobulin heavy chain gene rearrangement tests a simple pair of primers is used with the forward primer

Table 1 Schaefer's fixative for bone marrow trephine biopsies

| | |
|---|--------------|
| Methanol | 480 ml |
| Glucose phosphate buffer (pH 7.4) | 20 ml |
| Neutralised formalin | 250 ml |
| Glucose phosphate buffer (pH 7.4) | |
| Sodium dihydrogen phosphate | 0.96 g |
| Potassium dihydrogen phosphate | 0.17 g |
| Glucose | 3.08 g |
| Distilled water | Up to 100 ml |
| Neutralised formalin | |
| Formalin (~35% formaldehyde) | 87.5 ml |
| Distilled water | 12.5 ml |
| Calcium carbonate (~50 g/l) | 5 g |
| EDTA-formaldehyde for overnight decalcification | |
| Disodium EDTA dihydrate | 25 g |
| 4% formaldehyde (~10% formalin) | Up to 250 ml |

showing (O) monoclonal IgH gene rearrangement with polymerase chain reaction amplification (arrow). Large lymphocytes presenting (P) villous membrane staining with anti-CD20 antibody and (R) a granular intracytoplasmic reaction with DBA-44 antibody in hairy cell leukaemia. The histogenetic origin of tumours metastatic to the bone marrow is identified by immunostaining for (S) CD79a in a diffuse large B cell lymphoma, (T) prostate specific antigen in prostate cancer, and (U) Her2/c-erbB2 in a breast cancer. Note the excellent morphology and chromatin details, in addition to the high resolution antigen localisation in all immunostained cases. (C, F–U) Show EDTA decalcified tissues. The length of the bar shown in D is equal to 50 µm in D, K, L, and M; 100 µm in C and N; 20 µm in E; 35 µm in F; 25 µm in G and H; 70 µm in I, J, and T; 30 µm in P, R, and S; and 120 µm in U.

directed at the VH region (FR3) and the reverse primer at the JH segment.¹⁵ For T cell receptor γ chain gene rearrangement tests, triplicate aliquots of each sample are used with three forward primers (V1-8, V9, and V10-11 regions) and one reverse primer (J1-2 segments).¹⁶ In all cases, 40 cycles of PCR of 30 seconds at 94°C, 30 seconds at 56°C, and 30 seconds at 72°C are performed after preheating to 94°C. The final extension time is 10 minutes at 72°C. DNA extracted from a B and T cell lymphoma is used as a positive control and a reaction without template DNA is run as a negative control. Amplified products are run on a 10% polyacrylamide gel, which is fixed in a 0.5% acetic acid, 10% ethanol solution for six minutes, impregnated in a 0.1% silver nitrate solution for 15 minutes, and developed in a 0.15% formaldehyde, 1.5% sodium hydroxide solution to be photographed under visible light.¹⁷

RESULTS AND COMMENTS

The diagnostic interpretation of bone marrow biopsies is based on histopathological tests, which are largely dependent on tissue processing from the time of obtaining the sample to the detection of target molecules.¹⁸ Crucial factors thought to influence the end result include fixation, decalcification, embedding, cutting, antigen retrieval, the specificity and sensitivity of the antibodies used, the immunodetection system used, DNA amplification, and gel detection.

Whatever fixative is used, it is crucial to start fixation if not immediately then at least within 10 minutes of the biopsy procedure. Acidic fixatives such as Bouin's, or those using mercuric chloride, such as B5 or Zenker's, offer fair cytomorphology but their value for immunohisto/cytochemistry (these terms may be used as alternatives) and particularly for DNA preservation is limited.¹⁹⁻²² Fixation of trephine biopsies in buffered formaldehyde has been found a useful compromise supporting, when followed by appropriate decalcification and paraffin wax embedding, both adequate morphology and the detection of proteins and nucleic acids.³⁻⁵⁻⁸ The addition of calcium carbonate and glucose to the fixative in the Schaefer's formula (table 1) helps to improve chromatin details and prevents shrinkage artefacts (fig 1), making this fixative preferable for bone marrow histopathology.¹³

To ensure structural and molecular preservation it is also essential to complete fixation before starting decalcification.²³ If there is an increased amount of cortical bone it is necessary to extend fixation and decalcification beyond the usual six to 10 hour and 16-24 hour ranges, respectively. The pressure on pathologists to speed up diagnostic reporting has led some laboratories to use acid based decalcifiers. However, both inorganic acids (such as hydrochloric acid) and organic acids (such as formic acid at 5-10% concentration) can damage tissue architecture and stainability.²³ Acid treatment requires endpoint checking of calcium removal to avoid tissue damage, and it may also damage antigenic epitopes and DNA. In contrast, decalcification of properly fixed trephine biopsy specimens in 10% neutral EDTA solution for 16-24 hours at 60°C usually allows sufficient elimination of calcium from tiny bone trabecules without noticeable morphological and molecular damage. Moreover, EDTA as one of the most efficient antigen retrieval agents may add to the beneficial effect of heat induced epitope retrieval treatment used before immunohistochemistry.²⁴ Formaldehyde postfixation during decalcification may be crucial in preserving tissue morphology after heat induced epitope retrieval.

For bone marrow diagnosis, some laboratories prefer plastic embedded semithin sections, because they provide superior cell and tissue morphology to paraffin wax embedded sections.⁹⁻¹¹⁻¹³ In addition, the hard resin allows cutting of sections without eliminating tissue calcium, which may shorten tissue processing and is better suited to the

detection of fine haemosiderin particles with a Perls' stain or the detection of Auer rods with a May-Grünwald Giemsa stain (fig 1D, E). Nevertheless, even moderate decalcification helps to preserve bone trabeculae and the surrounding endosteal haemopoietic margin during staining procedures; thus, recently we have been using EDTA treatment. However, resin embedding may have inherent limitations, such as the need for special instruments, tools, and conditions for resin processing and cutting. Furthermore, reactive resin monomers may add to the antigen masking effect of the fixative, and most acrylic resins cannot be removed from the sections, which may prevent immunoreagents from accessing their targets. Therefore, laboratories requiring superb semithin resin histology may divide trephine biopsies and use resin sections for histology and paraffin embedded or frozen sections for immunohistochemistry.¹¹⁻¹³ Alternatively, resin protocols can be adjusted to the requirements of antigen and even DNA detection.²⁵⁻²⁸ We have run parallel resin and paraffin wax embedding for bone marrow trephine biopsy specimens. Paraffin wax embedded specimens may be used for consulting with centres not familiar with resin technology and for routine DNA extraction. Paraffin wax embedded tissues may also provide a safe stock for research in case an important new marker is not detectable in resin sections. Durcupan ACM is an aromatic polyepoxide resin of uniform hardening character and low risk of tissue shrinkage²⁹ (fig 1). Serial sections can be cut from the resin blocks using an ordinary rotary microtome and heavy duty cutting knives and the sections are fully rehydrated after etching.

Wet heat induced epitope retrieval is thought to loosen crosslinks formed by formaldehyde in and around antigenic epitopes, allowing them to be exposed later by immunoreagents.³⁰⁻³¹ Usually, more efficient antigen retrieval buffers, such as the high pH Tris/EDTA buffer, have a higher risk of damaging section integrity. Therefore, firm and standard section adhesion provided by adhesive glass slides, such as SuperFrost Plus, is essential for efficient antigen detection. We have continuously refined our antigen retrieval protocol to achieve sensitive and reproducible antigen detection in bone marrow semithin sections (fig 1). If manually stained, the two step EnVision-HRP conjugate system saves up to several technician weeks each year compared with a three step ABC-HRP detection, without compromising reaction sensitivity.³² The use of a standard DAB chromogen substrate kit and copper sulfate contrasting are also important parts of a reliable protocol.³³ Sensitivity is a specific issue here, in view of the fact that semithin sections contain significantly fewer antigens than regular paraffin wax embedded sections.

“Wet heat induced epitope retrieval is thought to loosen crosslinks formed by formaldehyde in and around antigenic epitopes, allowing them to be exposed later by immunoreagents”

During the diagnostic process, deplastified semithin sections are regularly stained with haematoxylin and eosin for routine histology. Giemsa, periodic acid Schiff, Gomori's silver stain, or Perls' stain are used for specific components, on request. Based on clinical data and assessment of cytomorphology and histotopography, targeted immunohistochemical marker analysis is performed using a well referenced panel of antibodies (table 2). Improved cytomorphology provided by semithin sections greatly facilitates making an initial provisional diagnosis. In some cases, paraffin wax embedded sections, peripheral blood smears, or touch prints may also be immunostained. Antigen receptor gene rearrangement assays may also be initiated after immunostaining.

Table 2 Immunohistochemical markers useful in the diagnostic interpretation of Durcupan embedded bone marrow trephine biopsies

| Antibody/clone | Source | Dilution | HIER | Tissue/cell specificity | Diagnostic note |
|---|------------|---|------|--|---------------------------------------|
| m-CD1a; O10 | Dako | 1/100 | TRS | Cortical thymocytes, activated interdigitating cells | Langerhans cell histiocytosis |
| m-CD2; AB75 | NovoCastra | 1/50 | TRS | Thymocytes, T cells, NK cells | TCL, NK-CL |
| r-CD3e; or rmo-CD3; SP7 | Dako | 1/200 | T/E | T cells | TCL, ALL |
| m-CD4; 4B12 | NeoMarkers | 1/100 | T/E | Some activated NK cells | |
| r-CD5 | NeoMarkers | 1/300 | T/E | Thymocytes, helper T cells | |
| m-CD8; C8/114B | Dako | 1/100 | TRS | T cells; B cell subset | B-CLL, MCL |
| m-CD10; 56C6 | NovoCastra | 1/20 | T/E | Cytotoxic cells, T cells | TCL |
| m-CD15; C3D-1 | Dako | 1/30 | TRS | CALLA, pre-B cells, GC B cells | ALL, FL |
| | | | | Myelomonocytic cells (IgM type) | Hodgkin's R-S cells |
| m-CD20; L-26 | Dako | 1/200 | TRS | B cells, except lymphoblasts and plasma cells | |
| m-CD23; 1B12 | NovoCastra | 1/40 | TRS | B cell subsets, FDCs | B-CLL |
| m-CD30; MP-1 | NovoCastra | 1/50 | TRS | Some activated lymphocytes | ALCL, Hodgkin's R-S cell |
| m-CD31; JC70A | Dako | 1/50 | TRS | PECAM-1, endothelial cells, platelets, megakaryocytes | |
| m-CD34; QBEnd10 | Dako | 1/50 | TRS | Haemopoietic stem cells, endothelial cells | Precursor cells |
| m-CD38; SPC32 | NovoCastra | 1/2000 | T/E | Early B cells, plasma cells | MM, ALL |
| m-CD43; DF-T1 | NovoCastra | 1/50 | TRS | T cells, B cell subsets | B-CLL |
| m-CD45; 2B11+PD7/26 | Dako | 1/100 | TRS | Most leucocytes of lymphoid or myeloid origin | |
| m-CD56; 1B6 | NovoCastra | 1/50 | TRS | N-CAM, NK cells, T cell subsets | MM, NK-CL |
| m-CD61; 2f2 | NovoCastra | 1/100 | TRS | Megakaryocytes, platelets | CMPD |
| m-CD68; PG-M1 | Dako | 1/100 | TRS | Monocytes, macrophages | AML FAB M4-5; CML |
| m-CD79a; JCB117 | Dako | 1/50 | TRS | All B cells including pre-B cells and plasma cells | B-CLL, ALL, MM |
| r-CD117 | Dako | 1/300 | TRS | Mast cells, myeloblasts (c-kit) | AML, mastocytosis |
| m-CD138; MI15 | Dako | 1/100 | TRS | Syndecan-1, plasma cells | MM |
| m-CD163; 10D6 | NovoCastra | 1/100 | TRS | Activated monocytes, macrophages | |
| m-CD235a; JC159 | Dako | 1/100 | TRS | Glycophorin A, erythroid cell | AML, CMPD |
| m-CD246; ALK-1 | Dako | 1/100 | TRS | ALK protein, t(2;5) | ALCL |
| m-Androgen receptor; AR441 | Dako | 1/100 | TRS | Glandular and ductal prostate epithelium | Prostate cancer metastasis |
| r-Chromogranin A | Dako | 1/800 | TRS | Neuroendocrine cells | Metastasis |
| r-C-erbB2/Her2 | Dako | 1/100 | TRS | EGF-R family, breast cancer | Metastasis |
| rmo-Cyclin D1; SP1 | NeoMarkers | 1/100 | T/E | Cell cycle regulation | MCL/t(11;14), MM |
| m-Cytokeratin 7; OV-TL 12/30 | Dako | 1/100 | T/E | Ductal and glandular epithelium (e.g. breast) | Breast cancer metastasis |
| m-Cytokeratin 20; Ks.20.8 | Dako | 1/100 | T/E | Transitional and gastrointestinal epithelium | Colon cancer metastasis |
| m-Cytokeratin pan; AE1/ AE3 | Dako | 1/200 | T/E | Pan-epithelial marker | Carcinoma metastasis |
| m-EBV-LMP-1; CS1-4 | Dako | 1/100 | TRS | EBV latent infection | Hodgkin's R-S cell |
| m-DBA-44 | Dako | 1/100 | TRS | B cell subsets (IgM type) | Hairy cell leukaemia |
| m-Desmin; D33 | Dako | 1/100 | TRS | Muscle cells (skeletal, smooth, and cardiac) | Rhabdomyosarcoma metastasis |
| m-EMA; E29 | Dako | 1/100 | TRS | Epithelial membrane antigen, plasma cells | Carcinoma metastasis |
| m-Oestrogen receptor; 6F11 | NovoCastra | 1/100 | TRS | Breast epithelium | Breast cancer metastasis |
| m-GCDFP15; 23A3 | NovoCastra | 1/50 | TRS | Ductal epithelium, breast and salivary glands | Carcinoma metastasis |
| m-Granzyme B; GrB-7 | Dako | 1/100 | TRS | Cytotoxic T cells, NK cells, large granular lymphocytes | LGL leukaemia |
| r-Haemoglobin | Dako | 1/500 | TRS | Erythroblasts, erythrocytes | CMPD, MDS, AML |
| m-Melanosome; HMB-45 | Dako | 1/100 | TRS | Melanocytic cells | Melanoma metastasis |
| IgH (r- α , - γ , - μ) | Dako | IgA, 1/800 IgG, 1/10000 IgM, 1/1000 | TRS | Plasmacytoid cells, plasma cells, immunocytes | MM, immunocytoma |
| IgL (r- κ , - λ) | Dako | 1/100000 | TRS | B cells: perinuclear/membrane or cytoplasmic | B cell clonality |
| m-Ki67; Mib-1 | Dako | 1/100 | TRS | Cell cycle (G1, S, G2, M) | Proliferation |
| r-Lysozyme | Dako | 1/2000 | TRS | Myeloid cells, histiocytes | AML, CML |
| r-Myeloperoxidase | Dako | 1/5000 | TRS | Myelopoietic cells | AML, CML |
| r-PSA or m-PSA; ER-PR8 | Dako | 1/100 | TRS | Prostate secretory and ductal epithelium | Prostate cancer metastasis |
| r-S100 (AB) | Dako | 1/500 | TRS | Neoplastic melanocytes, Langerhans cells, fat cells | Melanoma metastasis |
| m-Spectrin; RBC2/3D5 | NovoCastra | 1/100 | T/E | Erythroid cells | CMPD, AML |
| r-TdT | Dako | 1/50 | T/E | Lymphoid precursor cells | B/T-ALL |
| m-TTF-1; 8G7G3/1 | Dako | 1/100 | TRS | Lung and thyroid epithelia | Lung, or thyroid cancer metastasis |
| r-VWF (FVIII related) | Dako | 1/1000 | TRS | Endothelial cells, megakaryocytes | MPD, CMPD |

ALCL, anaplastic large cell lymphoma; ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; B5, mercuric chloride containing fixative; B-CLL, B cell chronic lymphocytic leukaemia/small lymphocytic lymphoma; CALLA, common acute leukaemia lymphoma antigen (CD10); CML, chronic myeloid leukaemia; CMPD, chronic myeloproliferative disease; EBV-LMP-1, Epstein-Barr virus latent membrane protein 1; EMA, epithelial membrane antigen; FDC, follicular dendritic cell; FL, follicular lymphoma; GC, germinal centre; HIER, heat induced epitope retrieval; IgH/L, immunoglobulin heavy/light chain; LGL, large granular lymphocyte; m, mouse monoclonal immunoglobulin; MCL, mantle cell lymphoma; MDS, myelodysplastic syndrome; MM, multiple myeloma; MPD, myeloproliferative disease; N-CAM, neural cell adhesion molecule; NK-CL, natural killer cell lymphoma; PECAM-1, platelet endothelial cell adhesion molecule-1; PSA, prostate specific antigen; r, rabbit polyclonal immunoglobulin; rmo, rabbit monoclonal immunoglobulin; R-S cell, Reed-Sternberg cell; TCL, T cell lymphoma; TdT, terminal deoxynucleotidyl transferase; T/E, Tris EDTA pH 9; TRC, T cell receptor; TRS, target retrieval solution pH 6.1; TTF-1, thyroid transcription factor 1; vWF, von Willebrand factor.

Dako, Glostrup, Denmark; NeoMarkers, Fremont, California, USA; NovoCastra, Newcastle, UK.

Table 3 Comparison between Durcupan and paraffin wax processing of bone marrow trephine biopsies

| Durcupan sections | | | Cost/case (€) | Paraffin wax embedded sections | | | Cost/case (€) |
|--|---|----------------------------------|---------------|---|----------------------------------|--|---------------|
| Mode | Timing (hours) | | | Mode | Timing | | |
| Fixation | 4% buffered formaldehyde | 6–10 h 1st day, or until arrival | ~1.2 | 4% buffered formaldehyde | 6–10 h 1st day, or until arrival | | ~1.2 |
| Decalcification | 10% EDTA at 60°C (with 4% formaldehyde) | 16–24 h, 1st night | ~1.2 | 10% EDTA at 60°C (with 4% formaldehyde) | 16–24 h, 1st night | | ~1.2 |
| Processing (dehydration) | Ethanol (IMS) series and acetone | 2 h 2nd day | ~1 | Ethanol (IMS) series and xylene | 3 h 2nd day | | ~1 |
| Embedding | Durcupan ACM resin (hardening at 60°C) | 16–24 h 2nd night | ~5 | Paraplast (for infiltration and block making) | 3 h 2nd day | | ~1.8 |
| Cutting and section mounting (1 section for H&E) | Tungsten-carbide knife (on rotary microtome) + activation of adhesion | 0.3 h/block/6 sections 3rd night | ~6* | Disposable blade (on rotary microtome) + activation of adhesion | 0.2 h/block/6 sections 3rd night | | ~2 |
| Rehydration | Sodium methoxide | 0.5 h 4th day | ~1 | Xylene/ethanol series | 0.4 h 4th day | | ~2 |
| Immunohistochemistry (5 sections/case) | Standard antibody panel (table 2), HIER-EnVision | 3 h 4th day | ~10 | Standard antibody panel (table 2), HIER-EnVision | 3 h 4th day | | ~10 |
| PCR technique (excluding DNA isolation) | Standard amplification and detection | 6 h occasionally | (~10) | Standard amplification and detection | 6 h occasionally | | ~10 |
| Sum total | | | 25.4 | Sum total | | | 19.2 |

The calculation is based on economical use and bulk processing and the costs relate only to the reagents and consumables. Because PCR is occasional its costs have not been included in the totals.

*Heavy duty knives are used for cutting ~80 cases before sharpening.

H&E, haematoxylin and eosin; HIER, heat induced epitope retrieval; PCR, polymerase chain reaction

Durcupan embedding following formaldehyde fixation, with or without decalcification, permits the detection of a wide range of antigens of diagnostic interest in the bone marrow (table 2). These include: (1) lineage restricted antigens identifying haemopoietic and lymphoid cells (for example, CD2, CD3, CD20, CD61, CD79a, haemoglobin, myeloperoxidase); (2) markers indicating cell differentiation stages, such as immature (for example, CD1a, CD10, CD34, CD38, CD117, and TdT), middle stage (for example, CD20), or late differentiation (for example, CD138); (3) markers of clonal lymphoid populations (immunoglobulin light and heavy chains; or monoclonal gene rearrangement by PCR); and (4) markers not detected normally in archived bone marrow indicating lymphoreticular disease (for example, CD23, CD30, CD246, cyclin-D1, Epstein-Barr virus latent membrane protein, and DBA-44), or metastatic tumours, such as carcinoma (for example, pancytokeratin and epithelial membrane antigen), melanoma (for example, HMB-45), paediatric small cell tumours (for example, desmin and chromogranin A), or even specific sites of metastatic carcinoma (for example, oestrogen receptor, thyroid transcription factor 1, and prostate specific antigen). Figure 1 shows some examples.

The same protocol can also be used to detect a range of other antigens in Durcupan embedded tissues—for example, peptide hormones and intermediate filaments.³⁴ In addition, aliphatic epoxy resins such as those based on the Epon 812 formula can be used for immunohistochemistry in a similar way to that described here.²⁷ Before the era of heat induce antigen retrieval, we exploited the etching effect of sodium methoxide on epoxy resins (thought to be the result of transesterification and/or oxidation²⁹) for retrieving antigens and enhancing the sensitivity of immunodetection in paraffin wax embedded sections.³⁵ Recently, this approach has been advocated as a non-heat mediated antigen retrieval technique.³⁶

The advantage of using Durcupan over an acrylate resin is that its processing does not require special conditions (such as ultraviolet light) and the rehydration of sections needs no modification of the original resin formula, which may be necessary when using acrylates.^{25 28} The benefit of Durcupan

over paraffin wax embedding includes less shrinkage artefact, better cytomorphology and cytotopography, easier indication of targeted immunocytochemistry, improved resolution of photographic archives, and more efficient utilisation of the tissue (at least three times as many sections can be cut from the same biopsy specimen). Conversely, there are drawbacks to Durcupan embedding related to the extra costs (resin, special cutting knife, sodium methoxide) and the need for embedding and cutting skills. The diagnostic turnaround time does not differ greatly between the two systems. Table 3 summarises the comparison between Durcupan and paraffin wax processing of bone marrow trephines. This resin technique may be worth introducing into laboratories where several hundred bone marrows are examined each year and where the budget for bone marrow histopathology can be extended by ~30% compared with that for routine paraffin wax processing.

The Durcupan resin processing of bone marrow trephine biopsies described here offers very satisfactory histomorphology and cytomorphology, sensitive and reliable immunodetection of an ever growing number of antigens, and reliable DNA analysis. Considering the advantages and disadvantages in comparison with paraffin wax embedded sections, this resin technology can provide a powerful support in the diagnostic interpretation and research into primary and secondary disorders of the bone marrow.

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