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Semi–automated standardisation of melanin bleaching procedures of heavily pigmented melanocytic lesions for immunohistochemical analysis on an automated platform

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ABSTRACT

Background: The diagnosis of heavily pigmented melanocytic lesions is problematic. This is often compounded by lack of visibility of nuclear detail of tumour cells due to physical masking by melanin pigment. Similarly, there can be colour merging of chromogenic final reaction products with melanin, making an evidence of antigenic localisation problematic. There are a number of melanin bleaching techniques available for immunohistochemical assessments.

Material and methods: All methods to date have involved the bleaching of melanin as a manually performed primary step before loading subsequently bleached slides onto automated immunohistochemical platforms. Here we define a semi-automated bleaching procedure that allows full integration on one of the most widely employed automated IHC staining platforms (Roche Ventana BenchMark Ultra). The bleaching protocol was defined on the BenchMark Ultra and involved the assessment of 24 histological cases of heavily pigmented malignant melanoma lesions (13 cutaneous and 11 metastatic) routinely fixed processed and paraffin wax embedded.

Results: Completion of the bleaching was assessed on H&E preparations performed following the semi-automated bleaching step and employing the Roche Ventana BenchMark Ultra machine for 60 min at 42°C. Complete immunohistochemical staining was achieved on the automated platform within 5–6 h including the bleaching step. Results were consistent across all tissue evaluated.

Discussion: This data provides evidence that the hydrogen peroxide bleaching procedure can be adapted for integration on one of the most widely employed automated IHC staining platforms and as a result, improve the efficiency and reproducibility of the technique.

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Introduction

Diagnostically heavily pigmented melanocytic lesions can be highly problematic, often posing difficult interpretive challenges, especially in relation to defining the degree of atypia and establishing the benign or malignant nature of any given lesion. The presence of abundant deposits of melanin can pose two significant problems. Firstly it can have a direct physical masking effect on antigen–antibody interactions, mainly due to the intracellular nature of the pigment deposition. Secondly the most widely employed chromogen for most immunohistochemical evaluations of antigen–antibody interactions is still 3, 3 diaminobenzidene (DAB), which is also brown in colour and can be difficult to distinguish from brown melanin pigment [1,2]. The use of other colour chromogens which have a different final reaction colour to that of melanin has only partially addressed these problems and are not effective on the extensively pigmented melanocytic lesions, for example, as often seen in cases of atypical melanocytic naevi with extensive dermal melanosis [3]. Such different coloured chromogens include alkaline phosphatase (APAAP) (red) [4]. In addition, the use of other tinctorial counterstains such as azure B (green-blue) [5], has gained some popularity as they stain the melanin a different colour to that of the final DAB immunohistochemical reaction product (brown). However, in the authors experience this is not always satisfactory as they often produce a darker merging of the two colours making an accurate assessment of the exact localisation of the antibody interaction, i.e. cytoplasmic, nuclear or membranous difficult to determine. This problem can be compounded if the antibody employed is co-expressed on cell membranes as well as within the cytoplasm of the cells, for example, in cases of anti-S100 protein expression [6]. Similarly, an increasing number of new melanocytic markers used diagnostically are now expressed within the nucleus of the cell for example anti-MITFP, anti-SOX 10 and more recently the predictive marker anti-BRAF (V600E). In such cases, the presence of abundant melanin pigment can obscure direct visualisation of the nucleus making interpretation difficult. In such cases, histopathological use of melanin bleaching procedures is still widely popular.

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Previously the two key bleaching procedures employed in melanin bleaching included the use of either permanganate/oxalate or dilute hydrogen peroxide [6–8]. The former, although much faster (approximately 1 h) than the dilute hydrogen peroxide method adversely affects antigenic sites as a result of over oxidization of the tissue section and is therefore limited in terms of IHC analysis. Melanin bleaching using dilute hydrogen peroxide takes considerably longer (up to 24 h) to perform, yet it allows a full range of IHC investigations [6,8,9].

As reported by Orchard (2007) [10], the use of heat rapidly improves the time frame for bleaching using dilute hydrogen peroxide. This study revealed that complete bleaching of [5] cases of atypical dysplastic naevi with extensive dermal melanosis was achieved in all cases within 150 min when using 10% dilute hydrogen peroxide made in phosphate-buffered saline and heated to 60°C. Heating methods employed included a conventional fan assisted heating oven and also a water bath set at the required 60°C and subsequently employing a range of antibodies which included (antibodies against S100, HMB 45, NKIC3, Melan A, CD3, CD20, CD68, CD34, CD45, CD31 and SMA). Both heating options produced similarly satisfactory results indicating the method of heating was not significant but elevating the temperature to 60°C clearly improved the speed of completion of the bleaching effect. Subsequently, a more recent study published in 2013 [11] achieved bleaching within 30 min when the temperature was increased to 65°C.

Up to this stage, all these bleaching techniques were achieved when employing manual methods to complete the bleaching step before loading the bleached slides onto automated IHC platforms. A natural progression from this point would be to determine if the bleaching step could be completed on a range of heavily pigmented melanocytic lesion encompassing routinely fixed, processed and paraffin wax embedded histological sections and employing an automated IHC platform. The two popularly used automated IHC platforms include the Leica Bond and the Roche Ventana BenchMark Ultra. A previous report by Liu et al. [12] employed a Leica Bond platform and reported completion of bleaching within 40 min employing 10% dilute hydrogen peroxide on board the machine. The aim of this current study was to analyse 24 cases of heavily pigmented melanocytic lesions with a total of 29 blocks, which could be bleached successfully and subsequently stained immunohistochemical with a select panel of melanocytic and histiocytic markers and employing the Roche (Ventana) BenchMark Ultra platform.

Methods

In order to achieve the bleaching effects on the Roche Ventana BenchMark Ultra platform (Roche Diagnostics Ltd., UK), a review of the existing routine IHC programmes was assessed. The following protocol was adopted:

Roche (Ventana) benchmark ultra procedure

(1) Select paraffin protocol; (2) Select deparaffinization; (3) Warm up slides to (72°C) from medium temperatures (deparaffinization); (4) Select the primary antibody; (5) Select primary antibody temperature; (6) Warm the slides to 42°C for primary antibody (bleaching step); (7) Perform antibody titration[1] (if required).

Before the titration step can be performed the following steps need to be carried out:

(1) Open the slide draw; (2) Remove the liquid coverslip (LCS) which is covering the slide and place the slides into a rack; (3) Wash the slides in EZ prep to remove any residual LCS; (4) Wash the slides in running water to remove any residual EZ prep; (5) Place the slides back in the slide draws; (6) Prepare 10% Hydrogen peroxide solution.

Preparing 10% hydrogen peroxide solution

(Modified as previously described in Orchard (2007) [10].)

(1) Dissolve one PBS tablet (Sigma Aldrich Ltd.) in 200 ml of distilled water; (2) Ensure the tablet is fully dissolved; (3) Remove 34 ml of the solution that you have prepared and placed in a beaker; (4) Add 16 ml of hydrogen peroxide (30%) to the 34 ml solution of distilled water PBS tablet mix; (5) Mix thoroughly; (6) Apply solution to slides.

Hand application of bleaching solution on board benchmark ultra platform

Pipette the 10% hydrogen peroxide solution gently onto the slide. Make sure that enough is applied to fully cover the tissue sections completely. Incubate for 1 h.

All bleaching steps were introduced prior to antigen retrieval steps. Details of the antibodies used can be seen in (Table 1). The detection system employed was Roche (Ventana) Ultraview Red Alkaline Phosphatase (APAAP) kit (product code 760–501), Harris Haematoxlyin (Leica Microsystem UK Ltd. product code 3801560BBE and 1% aqueous eosin Leica Microsystems UK Ltd. product code 3801560BBE) H&E preparations using the standard protocol for routine HE staining were also performed on all cases prior to bleaching and directly following bleaching on the BenchMark Ultra platform as highlighted above and subsequently placed on the Leica Austostainer AL for completion of H&E staining. These were assessed by GEO and JG. Once the protocols for H&E staining

Table 1. Details of the antibodies and suppliers used in the assessment of 24 cases of heavily pigmented malignant melanoma.

Antibody name	Antibody type	Supplier	Product code	Dilution (if applicable)
Melan A/Mart 1	Predilute	Roche Diagnostics LTD	5278350001	N/a
S100	Concentrate	Agilent Technologies LDA UK Ltd	Z031101	1:6000
Sox-10	Predilute	A Menarini Diagnostics LTD	MP-3099-PM6	N/a
HMB45	Concentrate	Launch Diagnostics	MU001A-UC	1:40
CD68	Concentrate	Agilent Technologies LDA UK Ltd	M081401-2	1:2000

following bleaching had been established, the IHC staining was performed.

All sections were cut at four microns and mounted on super frosted plus poly-L-lysine coated slides (VWR International) and dried down at 68°C for 1 h. A label for the melanin bleach protocol is affixed onto the slide (details of slide preparation prior to loading onto machine can be found under the Roche BenchMark Ultra procedure above). Once bleaching has been completed, print a new label for the antibody test being performed as a wet preparation procedure and affix the label to the slide.

Results

All cases examined showed successful bleaching of melanin had been achieved. There was minimal obscuring effect, and the melanin pigment colour was either completely eliminated or vastly reduced, so as not to cause any confusion on the visualisation of nuclear detail. The morphology and tissue integrity were preserved in all cases and tissue section detachment was only partially observed in two cases following 1 h bleaching at 42°C. All bleached HE stains were much improved in terms of identification and clarity of vision of morphological detail when compared to the unbleached section. Setting the optimal bleaching time ensured that in all cases a selected bleaching programme could successfully be developed on the Roche Ventana BenchMark Ultra platform. The procedure is virtually fully automated as all staining steps are as usually employed for the markers evaluated. The only manual aspect involves washing the LCS off and placing the bleaching solution on the slides aboard the machine for 1 hour incubation prior to subsequent normal IHC staining procedures.

The results for all IHC staining were easily interpreted giving excellent morphological clarity and the ability to determine antigenic localisation (cytoplasmic, membranous and also nuclear) (Figure 1(a–g), Figure 2(a–g)). The removal of pigmentation colour was particularly useful for those antigenic epitopes that labelled with a nuclear pattern, e.g. Sox-10, since surrounding cytoplasmic melanin pigmentation colour was completely removed improving visualisation. This is a consideration when assessing staining results for those antibodies that are co-expressed both in a nuclear and cytoplasmic manner, e.g. S100 protein.

Discussion

Classification of melanocytic lesions is often difficult and can be complicated by an inability to define the morphological characteristics of the melanocytes present as a result of excessive melanin which may be present and cause physical masking. In addition, this issue can also obscure visualisation of antigen–antibody interactions when performing IHC and make precise clarification of localisation of antibody binding to the antigenic sites difficult. The significance of being able to define if the antigen–antibody binding is membranous, cytoplasmic or nuclear or perhaps a combination of these can often be highly significant in determining if the IHC process has been performed correctly. In addition, it can help determine that the specificity and sensitivity of the antibody are as expected and as the manufacturers specified. The routine chromogen of choice for most IHC staining is 3, 3 diaminobenzidine (DAB) which is brown in colour like melanin. As previously described [1,7] this can cause confusion in determining the IHC staining patterns seen in heavily pigmented lesions. Alternative chromogens with different colour to that of DAB do not always resolve this issue of physical masking [9,10]. In addition, there are some dyes used such as Azure B [5] which can be employed as a counterstain to help define IHC staining reactions in heavily pigmented lesions; however, this is not popularly used. Melanin bleaching techniques involving the use of dilute H_2O_2 remain the most effective way of dealing with the physical masking effects of melanin. Up until recently the process of bleaching melanin was a manual procedure with many reports suggesting differing time frames for exposure to dilute H_2O_2 and also the improved efficiency and speed of the technique when heat is employed [10]. The first report of the use of automated IHC platforms being used for bleaching was that of Liu et al. [12]. That report described the use of a Leica Bond Polymer Refine Detection Automated Staining Module. The most widely employed Bond machines for diagnostic work are Bond Max machines. The authors do go on to state that the automated platform was based on a platform originally designed for automated IHC staining but with some additional heating and washing steps for depigmentation. However, specific details of these modifications are not given. Therefore, this is a restrictive option if such platform modifications are not widely available. The whole bleaching and staining procedure was fully automated, and slides were reported to be bleached and stained in 3 h.

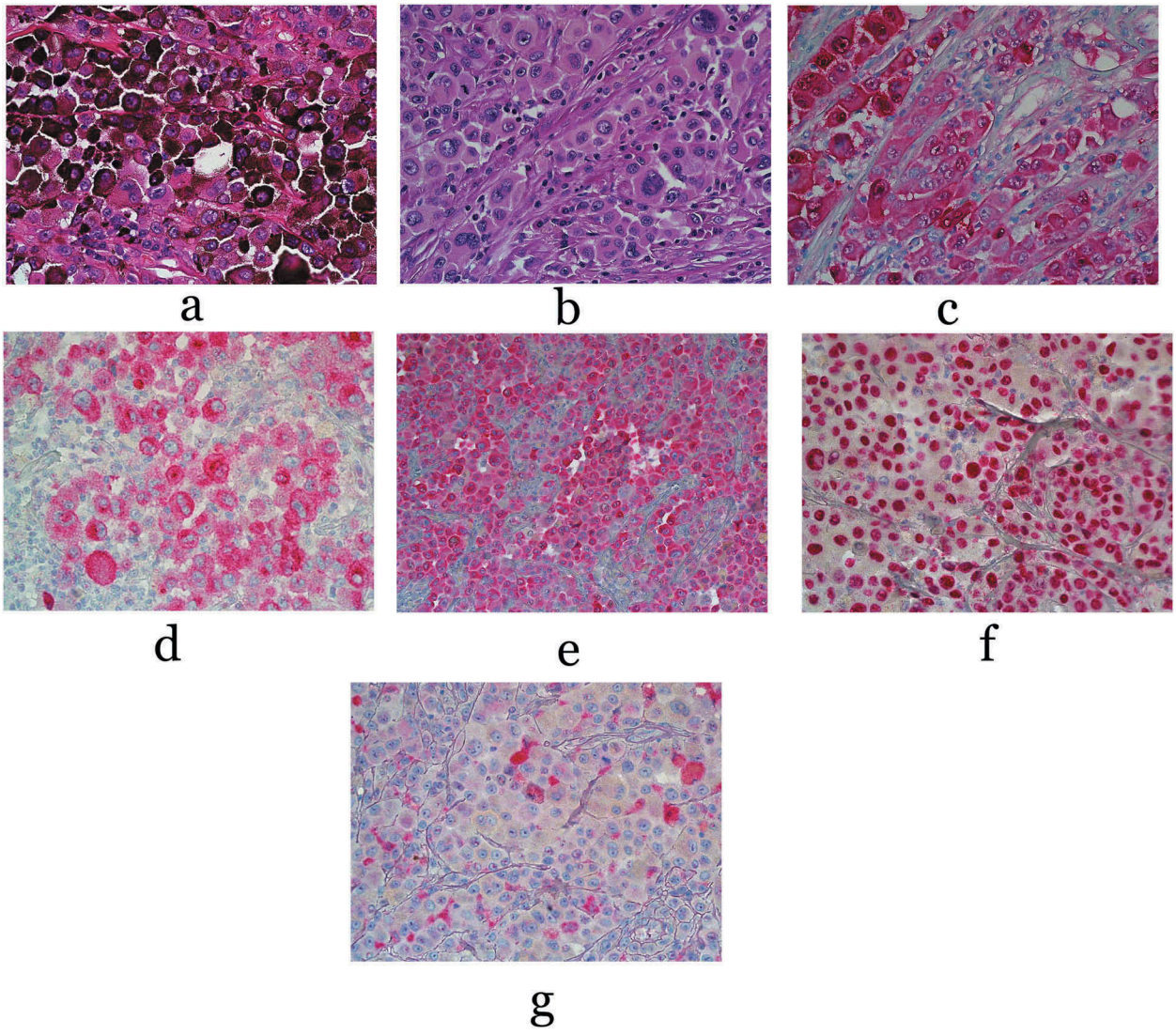

Figure 1. (a) H&E stained of metastatic malignant melanoma in a lymph node showing extensive melanin deposition Mag ×40. (b) Bleached H&E stained preparation of the same case as seen in (a) demonstrating the clarity of morphological detail seen following the bleaching of melanin pigment Mag ×40. (c) Same case as in (b) bleached and stained for anti-S100 protein demonstrating strong staining of the larger tumour cells. Mag ×40. (d) Same case as in (b) bleached and stained for anti-HMB 45 demonstrating strong cytoplasmic staining of the larger tumour cells. Mag ×40. (e) Same case as in (b) bleached and stained for anti-Melan A demonstrating strong cytoplasmic staining of the larger tumour cells. Mag ×40. (f) Same case as in (b) bleached and stained for anti-SOX 10 demonstrating strong nuclear staining of the larger tumour cells. Mag ×40. (g) Same case as in (b) bleached and stained for anti-CD68 demonstrating no staining of the larger tumour cells but positive staining of smaller inflammatory histiocytes. Mag ×40.

Here we describe the use of a popularly used widely available IHC staining machine (Roche (Ventana) BenchMark Ultra) without any onboard modifications in a semi – automated procedure which again can achieve the bleaching and staining of heavily pigmented melanocytic lesions in 5–6 h using the APAAP kit which requires longer time frames to complete staining compared to DAB kits. The two manual steps required were brief and did not take longer than 10 min each to perform simply involving the removal of the LCS and pipetting the dilute H_2O_2 directly onto the slides on the IHC staining machine platform. The procedure was then essentially an automated procedure; however, there remains a small manual requirement. The temperature setting was set for 42°C as this represents the maximum temperature setting on the Roche (Ventana BenchMark Ultra) machines, although this was less than 65°C as reported in Liu et al. [12]. Technically, it was still possible to achieve the bleaching effective within 1 h and completion of IHC staining within 4 h for those antibodies assessed in the study. The reason the bleaching effect could be achieved at a lower temperature setting and within the time frame of 1 h is potentially due to the more efficient heat distribution of the individual slide hot plates on the BenchMark Ultra machine. The individually bleached slides were resting directly on these hot plates, and this makes the heat distribution

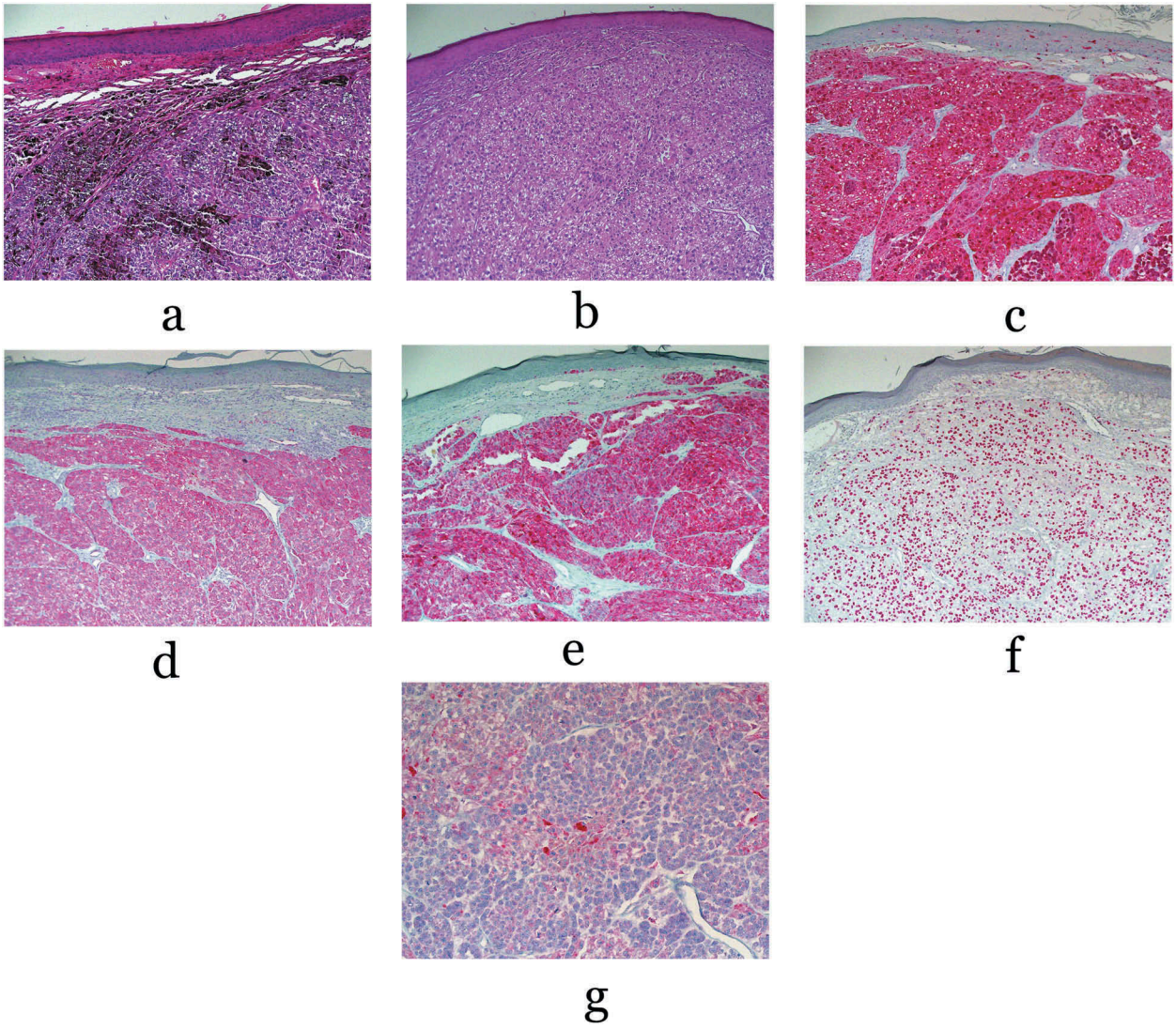

Figure 2. (a) H&E staining of an unbleached case of cutaneous nodular malignant melanoma, demonstrating an abundance of melanin pigment throughout the tumour cells. Mag ×10. (b) Bleached H&E staining of the same case as in (a), showing a complete absence of melanin pigment colour. Mag ×10. (c) Bleached anti-S100 protein staining the same case as in (a) showing uniform labelling of the entire nests of dermal tumour cells. Note the absence of any residual melanin pigment colour. Mag ×10. (d) Bleached anti-HMB45 staining of the same case as in (a) again showing uniform but not so intense cytoplasmic labelling of all tumour cells. Note the absence of any residual melanin pigment colour. Mag ×10. (e) Bleached anti-Melan A staining of the same case as in (a) showing uniform and intense cytoplasmic labelling of all tumour cells. Note the absence of any residual melanin pigment colour. Mag ×10. (f) Bleached anti-SOX 10 staining of the same case as in (a) showing intense nuclear labelling of all tumour cells. Note the absence of any residual melanin pigment colour. Mag ×10. (g) Bleached anti-CD68 staining of the same case as in (a) showing occasional histiocytes positive between the larger negative tumour cells. Mag ×20.

more efficient. The use of an automated or semi-automated approach will clearly give benefits of standardisation of the technique and will also reduce biomedical staff's time spent performing such a technique in a fully manual manner. It will also reduce health and safety risks as exposure to H_2O_2 is hazardous and does require appropriate personal protective equipment to be worn ex gloves and safety goggles. Bleaching slides and performing an H&E stain first are to be strongly recommended as it will enable evaluation of the bleaching process and determine if full bleaching is obtained and also enable evaluation of subsequent morphological features of the tissue sections. In all cases studied here, there were no undesirable morphological artefacts seen. In addition, tissue section adhesion was not compromised with only the occasional section lifting slightly. In a few cases studied here minor melanin pigment could still be seen following the procedure, although in a much reduced capacity, and this did not represent any physical masking of subsequent IHC staining. The heavily pigmented tissues used in this study were either skin or nodal deposits of malignant melanoma, it is possible that ocular tissue and other tissues with dense melanin

deposits may not bleach in the same manner due to variation in melanin pigment density between the tissue types.

Recently the application of predictive markers within the field of melanoma assessments has become popular and includes markers such as BRAF (V600E) [13] and antibodies raised against programme cell death ligand (PDL1). One of the significant interpretive drawbacks has been the presence of melanin in such tissue and the visual obstruction this pigment can cause when assessing final IHC reaction products. The option now exists to use a bleaching procedure on an automated IHC platform that could improve the efficiency of interpretation and also ensure the rapid turn around times required are met.

In conclusion, we believe a semi-automated IHC staining platform that is routinely available and requires no modifications provides the most useful approach to dealing with modern day IHC investigations of heavily pigmented melanocytic lesions. It is easily incorporated into routine IHC staining protocols and results in no loss of morphological details enabling improved visualisation of melanocytic tissue cell types making the IHC distinction of melanocytes from melanophages easier to achieve.

Summary table

What is known about this subject:

- Manual bleaching techniques for the assessment heavily pigmented melanocytic lesions is best addressed using dilute hydrogen peroxide and employing heat.
- Improving on the speed, efficiency of this procedure and also developing automated strategies to tackle this requirement remains a focus for attention.

What this study adds:

- Demonstrates the use of conventional automated IHC machines to assist in delivering an on board bleaching platform to improve the efficiency of bleaching of melanin pigment.
- Demonstrates an improved strategy for melanin bleaching of histological tissue sections in an integrated fashion for automated IHC staining.

Note

1. The antibodies used are purchased as either concentrated antibodies or pre-diluted RTU (ready to use). The concentrated antibodies will require a dilution factor to be determined for optimal use.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

[1] Alexander RA, Hiscott PS, Hart RL, et al. Effect of melanin bleaching on immunoperoxidase, with reference to ocular tissues and lesions. Med Lab Sci. 1986;43:121–127.
[2] McGovern J, Crocker J. The effect of melanin pigment removal on the peroxidase- antiperoxidase immunoperoxidase technique. Am J Clin Pathol. 1987;88:480–483.
[3] Orchard GE, Calonje E. The effect of melanin bleaching on immunohistochemical staining in heavily pigmented melanocytic neoplasms. Am J Dermatopathol. 1998;20:357–361.
[4] Alexander RA, Cree IA, Foss AJ. The immunoalkaline phosphatase technique in immunohistochemistry: the effect of permanaganate- oxalate melanin bleaching upon four final reaction products. Br J Biomed Sci. 1996;53:170–171.
[5] Kamino H, Tam ST. Immunoperoxidase technique modified by counterstain with azure B as a diagnostic aid in evaluating heavily pigmented melanocytic neoplasms. J Cutan Pathol. 1991;18:436–439.
[6] Orchard GE. Heavily pigmented melanocytic neoplasms: comparison of two melanin bleaching techniques and subsequent immunohistochemical staining. Br J Biomed Sci. 1999;56:188–193.
[7] Foss AJ, Alexander RA, Jefferies LW, et al. Immunohistochemical techniques: the effect of melanin bleaching. Br J Biomed Sci. 1995;52:22–25.
[8] Kivela T. Immunohistochemical staining followed by bleaching of melanin; a practical method for ophthalmic pathology (letter). Br J Biomed Sci. 1995;52:325.
[9] Orchard GE, Calonje E. Immunohistochemical staining following the melanin bleaching technique in an atypical junctional melanocytic lesion with prominent dermal melanosis (letter). Br J Biomed Sci. 1997;54:326.
[10] Orchard GE. Use of heat provides a fast and efficient way to undertake melanin bleaching with dilute hydrogen peroxide. Br J Biomed Sci. 2007;64:89–90.
[11] Liu CH, Lin CH, Tsai MJ, et al. Melanin bleaching with dilute hydrogen peroxide: a simple and rapid method. Appl Immunohistochem Mol Morphol. 2013;21:275–279.
[12] Liu CH, Lin CH, Tsai MJ, et al. Melanin bleaching with warm hydrogen peroxide and integrated immunohistochemical analysis: an automated platform. Int J Surg Pathol. 2018;26:410–416.
[13] Orchard GE, Wojcik KE, Rickaby W, et al. Immunohistochemical detection of V600E BRAF mutation is a useful primary screening tool for malignant melanoma. Br J Biomed Sci 2019;76:77–82.