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Evaluation of different haematoxylin stain subtypes for the optimal microscopic interpretation of cutaneous malignancy in Mohs frozen section histological procedure

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ABSTRACT

Background: The Mohs technique employs mainly H&E-stained frozen sections for surgical margin assessment of cutaneous excisions, utilising microscopic evaluation of the complete, circumferential, peripheral and deep margins. This study aimed to determine which mordant based haematoxylin (Ehrlich's, Cole's, Mayer's, Gill's I, Gill's II, Gill's III, Weigert's, Harris' or Carazzi's) produced the optimal morphological clarity of staining for the identification of cellular and tissue morphology of cutaneous basal cell carcinoma (BCC).

Material and methods: In total, 100 anonymised patient cases were selected, sectioned and stained with each haematoxylin subtype. The slides were independently evaluated microscopically by two assessors. A combined score was generated to determine the sensitivity (defined as the intensity of haematoxylin staining being too weak or too strong and the colour appearance of the haematoxylin not being blue/black) and specificity (defined as the appearance of background staining with haematoxylin, uneven staining and staining deposits) for each of the nine haematoxylin subtypes. The scoring criteria were based on the UKNEQAS CPT Mohs procedure assessment criteria.

Results: The scores generated for specificity identified Carazzi's haematoxylin as best performing (99.2%) followed by Gill's III (98.4%), Ehrlich's (98.2%) and Harris' (85.0%). The sensitivity score again identified Carazzi's as producing the best result (85.0%) followed by Weigert's (83.4%), Ehrlich's (81.6%) and Gill's III (80.4%).

Discussion: Carazzi's haematoxylin is the most optimal staining dye for the identification of BCC tumour for use as part of the Mohs micrographic surgery procedure.

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KEYWORDS
Mohs micrographic surgery; Mohs staining procedure; haematoxylin and eosin staining; H&E

Introduction

Non-melanoma skin carcinomas (NMSCs) are on the rise with around 105,000 cases diagnosed annually in the United Kingdom [1]. Basal cell carcinoma (BCC) is the most predominant tumour form with on average eighty per cent of keratinocyte carcinomas falling under this subtype [2]. The remaining twenty per cent of cases are attributed to squamous cell carcinomas (SCC) [2]. NMSCs are typically a slow-growing, locally invasive cutaneous tumour which occurs at a higher incidence in individuals with a fairer complexion [3]. Metastasis in these tumours types is rare, so that NMSC has a low mortality rate [3]. Morbidity is mainly due to the destruction of local tissue architecture due to tumour growth, which can severely impact the patient's quality of life.

Treatment of BCC traditionally involves the removal of the lesion with a margin of unaffected skin with the aim of completely excising the tumour. The sample, once removed, is sent for histological examination, which could take up to two weeks before a formalised report is generated. This results in the patients waiting an extended time before finding out whether they would require any additional surgical procedure to remove any remaining tumour due to positive margins in the cutaneous excision sample. In recent years Mohs micrographic surgery has gained popularity for the treatment of BCC particularly in cases of recurrence, a site where conservation of tissue is vital (e.g. face and neck regions) and where conventional treatment has not worked (e.g. excision or radiation) [4].

Mohs micrographic surgery (MMS) was established by Dr Frederic Mohs in the 1930s [4]. The technique used an intraoperative procedure involving the processing of fresh (unfixed) tissue for complete surgical margin examination, using microscopic evaluation of the complete, circumferential, peripheral and deep margin assessment of stained frozen sections. This process determines the successful excision of various cutaneous malignancies, including BCC and SCC. It has increased in popularity for the treatment for these conditions due to the high cure rate of 95% to 99% depending on tumour type and anatomical site [4] over conventional modalities, maximal preservation of healthy tissue and improved cosmetic result. However, a significant factor for the use of Mohs procedure is that it allows for the

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examination of the entire surgical margin, a facility not provided by conventional surgical techniques [5].

As a part of the Mohs procedure, H&E staining remains the staple method for microscopic evaluation for pathological diagnosis and interpretation of these tumour types. In most cases, the haematoxylin nuclear staining plays an essential role in determining neoplastic disease. The presence of basophilic, hyperchromatic nuclei, apoptotic bodies, mitotic figures and pleomorphism all rely on clear staining to allow the generation of unequivocal diagnoses. The haematoxylin dye is extracted from the bark of the logwood tree Haematoxylin *Campechianum*, originally located in the Mexican state Campeche [6]. The conversion of haematoxylin to haematin, vital for its ability to bind to nuclear components, is aided by the use of mordants. There are a broad range of mordants which can impact the tissue components stained and colour of staining, which is visualised. The mordants are usually a metal cation such as iron, aluminium, molybdenum, lead and tungsten [7].

Currently, there is no standardised optimal protocol for H&E staining at a national level for the micrographic detection of cutaneous malignancies such as BCC as part of the Mohs procedure. This study aims to determine which mordant-based haematoxylin (Ehrlich, Coles, Mayer's, Gill's I, Gill's II, Gill's III, Weigert's, Harris or Carazzi's) produces the optimal morphological clarity of staining for the identification of cellular and tissue morphology of cutaneous BCC, as part of the Mohs frozen sectioning and staining procedure.

Materials and methods

In total, 100 patient cases were selected who had presented with and positively diagnosed as having basal cell carcinoma tumour. All patients were undergoing MMS at Guy's Cancer Centre in London. The tissues used were anonymised remaining tissue, that was no longer required for diagnostic purposes.

To help determine the optimal haematoxylin subtype for use as part of the Mohs procedure, all staining was performed on the linistat linistainer (Thermo Scientific) to allow for increased standardisation and reproducibility. Due to the need for rapid sample turnaround times, the linistainer allows for increased throughput for quicker staining times that ensure clinicians can microscopically evaluate samples to make a clinical judgement promptly. This is vital since the patient will be awaiting result to determine if further excisions are required before the surgical site is closed.

Initially, all nine haematoxylin subtypes (including Ehrlich, Coles, Mayer's, Gill's I, Gill's II, Gill's III, Weigert's, Harris and Carazzi's) were individually optimised on the linistat linear stainer within the parameters available on this platform, by increasing or decreasing immersion times in haematoxylin and acid alcohol. The concentration, time and volume of the remaining reagent constituents of the H&E staining process (Eosin (Leica Microsystems (UK) Ltd, Milton Keynes, UK: product code 3801590BBE), Scott's tap water (Leica Microsystems (UK) Ltd, Milton Keynes, UK: product code 3802901E), industrial-denatured spirit (IDS) (99%) (Genta Medical Ltd, York, UK: product code I99050) and xylene (Genta Medical Ltd, York, UK: product code XYL050)) remained identical for each haematoxylin staining protocol. As part of the staining process, each haematoxylin was filtered before use directly from the reagent bottle provided by the manufacturer. The only variation to this process was Weigert's haematoxylin which due to its strong oxidising effect was produced before each run by mixing equal quantities of Weigert's haematoxylin solution A and B. The optimisation procedure involved the use of positive BCC debulk specimens from anonymised patient samples where prior consent had been obtained. The cases were confirmed to be clear, as the tumour had been completely excised and the tissue sample was no longer required.

All samples were sectioned on the Leica CM1950 cryostat at 15um thickness and picked up on super frost plus poly-L-lysine coated slides (VWR International, Leics, UK: product code 631–0108). Before staining all slides were baked on a hot plate at 80 °C for 4 min, followed by 4 min in IDS 99% and water for an additional 4 minutes. After these steps, the slides were loaded onto the linistat linistainer and the H&E staining process was initiated. The optimal protocol was determined by two independent reviewers by microscopic evaluation of each slide to determine which factors (haematoxylin and/or acid alcohol immersion times) needed to be amended to produce the best result within the restricted parameters available on the linistainer. The finalised protocols for each haematoxylin subtype is shown in Table 1. The final step involved clearing the slides in xylene before the application of mountant (Leica CV mount) and a glass coverslip.

In the next stage, 100 anonymised patient cases were sectioned at 15um on a Leica CM1950 cryostat. Nine slides were generated for each case to ensure the same case could be stained with each haematoxylin subtype, which equated to 900 stained slides. The use of identical cases ensured consistency of tissue architecture and composition, which in turn allows for direct comparison of each haematoxylin subtypes, and any differences could be attributed to mordant used. All 100 slides for each haematoxylin subtype were stained according to the protocol set out in Table 1. All haematoxylin dyes were filtered before use to prevent precipitates being carried over on to the sections. Furthermore, to ensure consistency, all slides were stained at the same time to ensure the variables remained identical for all stained section. The reagents from the same batch were replaced in-

Table 1. H&E protocols times on the linistat linistainer for each haematoxylin subtype.

Name of Haematoxylin	Time in Haematoxylin (Seconds)	Wash in running water (Seconds)	Time in Acid alcohol (Seconds)	Wash in running water (Seconds)	Time in Scott's Tap water (Seconds)	Wash in running water (Seconds)	Time in Eosin (Seconds)	Wash in Water (Seconds)	IDS 99% (Seconds)
Carazzi's	20	10	10	10	10	10	10	10	20
Cole's Ehrlich's Gill's I Mayer's	50	10	10	10	10	10	10	10	20
Gill's II	40	10	10	10	10	10	10	10	20
Gill's III	10	10	20	10	10	10	10	10	20
Weigert's Harris	30	10	10	10	10	10	10	10	20

Table 2. Factors that assessed the sensitivity and specificity of all haematoxylin subtypes.

Sensitivity factors	Specificity Factors
-Haematoxylin intensity too strong (defined as excessive staining which obscures nuclear visualisation including chromatin and nucleoli detail). -Haematoxylin Intensity too weak (defined as reduced staining with pale nuclear visualisation including chromatin and nucleoli detail). -Haematoxylin colour not purple/blue -Clarity of chromatin detail -Crisp and clear demonstration of nucleoli.	-Haematoxylin background staining -Uneven staining -Stain deposit present -Non-specific staining of cells/tissue. -Poor haematoxylin to eosin balance.

between staining of each haematoxylin subtypes to ensure fresh reagents were utilised to assure poor reagent quality did not impact staining results. At the same time by utilising identical reagent batches, variability between different reagent compositions would not impede staining quality.

Upon completion of the staining process of all 100 cases with each haematoxylin subtype, the slides were independently evaluated by two independent observers. The scoring criteria were based on the UK National External Quality Assurance Cellular Pathology Techniques (UKNEQAS CPT) Mohs procedure assessment criteria [8]. Each observer allocated scores between 1 to 5 based on modified pre-set UKNEQAS scoring criteria [8]. The assessment focused mainly on the quality of the haematoxylin staining which are highlighted in Table 2.

The results assigned by each observer for the specificity and sensitivity of each slide were then combined to generate an overall score for each slide out of 10. These results were then added together and divided by 100 to calculate the mean to generate a sensitivity and specificity score as a percentage for each dye. These sensitivity and specificity scores were critically evaluated to determine if a particular haematoxylin preparation provides better morphological clarity and better pathological assessment of BCC tumours.

Results

All 900 slides stained as expected with each haematoxylin dye subtype demonstrating nuclear staining at different degrees of intensity. Staining was limited to the maximum capacity that was possible on the linistainer of 2 minutes 30 seconds.

The specificity and sensitivity results for each haematoxylin subtype based on the criteria that were set out in Table 2 are shown in Table 3. Figure 1 shows the sensitivity and specificity scores of all the haematoxylin subtype dyes graphically.

Carazzi's haematoxylin (Solmedia Ltd, Shrewsbury, UK: product code HST001) stained sections produced clear and crisp nuclear staining pattern. The nucleoli and mitotic figures were clearly visible. The intense nuclear staining was well balanced with the eosin counterstain. The staining allowed for clear differentiation and identification of BCC tumour mass (as shown in Figure 2(a) highlighted with an arrow). Carazzi's haematoxylin also showed no background or uneven staining. Carazzi's haematoxylin had the highest score by both observers, with a specificity and sensitivity score of 99.2% and 85.0%, respectively.

Cole's haematoxylin (Solmedia Ltd, Shrewsbury, UK: product code HST002) performed poorly with very weak nuclear staining. The weak haematoxylin staining resulted in some nuclear components displaying a pink hue due to the eosin counterstain overpowering the nuclear staining (Figure 3(a)), particularly when

Table 3. Breakdown of specificity and sensitivity result for each haematoxylin subtype.

Haematoxylin Subtype	Mordant	Specificity (%)	Sensitivity (%)
Carazzi's	Potassium Alum	99.2%	85.0%
Gill's III	Aluminium Sulphate	98.4%	80.4%
Ehrlich's	Potassium Alum	98.2%	81.6%
Harris	Potassium Alum	85.0%	80.2%
Weigert's	Ferric chloride	80.0%	83.4%
Gill's II	Aluminium Sulphate	62.2%	79.2%
Mayer's	Ammonium or Potassium Alum	60.6%	61.6%
Gill's I	Aluminium Sulphate	50.4%	59.8%
Cole's	Potassium Alum	40.0%	40.8%

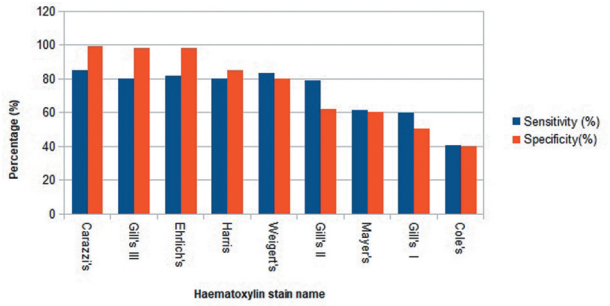


Figure 1. Sensitivity and specificity of haematoxylin stain subtype.

looking at the staining of the BCC tumour. There was also the appearance of background staining and uneven staining. Slides stained with Coles's haematoxylin suffered the presence of precipitates (highlighted by the arrows), visible in Figure 3(b). All these factors reflected in the low scoring of the slides, with Cole's haematoxylin scoring the lowest specificity and sensitivity with 40.0% and 40.8%, respectively.

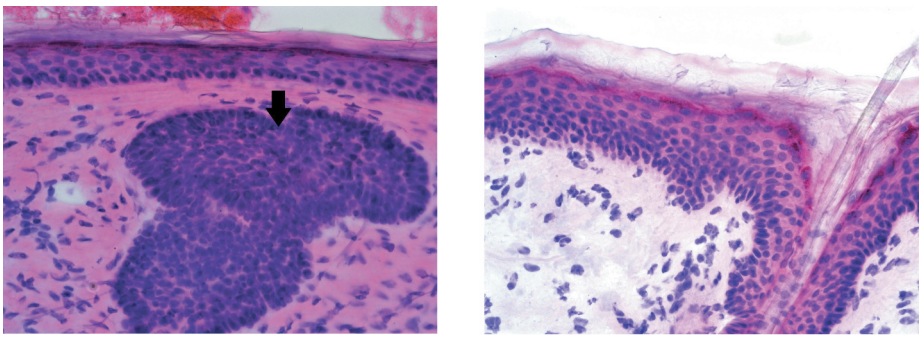
Ehrlich's haematoxylin (Solmedia Ltd: product code HST003) dye produced good quality staining with clear nuclear staining and good visualisation of chromatin and nucleoli detail. The haematoxylin to eosin balance was slightly skewed towards eosin shown by the mildly increased intensity of pink colour seen in the sections (Figure 4, highlighted in red). However, the BCC tumour was easily visualised and clearly differentiated from normal epithelial cells. Overall, Ehrlich's haematoxylin ranked third for both sensitivity and specificity with a score of 81.6% and 98.2%, respectively.

Gill's I haematoxylin (Solmedia Ltd: product code HST004) performed poorly with weak nuclear visualisation (Figure 5). The chromatin and nucleoli are difficult to observe due to the weak intensity of nuclear staining (Figure 5(a) highlighted with arrows). Gill's I haematoxylin also showed mild background staining with uneven staining patterns. The weak staining was reflected in the scores assigned by both observers and overall Gill's I ranked second to last with a sensitivity and specificity score of 59.8% and 50.4%, respectively.

Gill's II (Solmedia Ltd: product code HST005) performed moderately well. The intensity of nuclear staining was satisfactory. Chromatin and nucleoli visualisation was moderate with increased intensity of staining required to increase prominence. However, it is essential to highlight that the tumour (as shown in Figure 6a highlighted with black arrow) was clearly visualised and differentiated from non-malignant cells (as shown in Figure 6a highlighted with red arrow). While background staining is not prominent, uneven staining and skewed eosin to haematoxylin balance were noted. Gill's II ranked sixth overall with a specificity and sensitivity score of 62.2% and 79.2%, respectively.

Gill's III haematoxylin dye (Solmedia Ltd: product code HST006-A) produced good quality staining with a clear and crisp demonstration of chromatin and nucleoli detail. The BCC tumour was clearly identifiable and differentiated from epithelial cells. Gill's III did not exhibit background staining but did demonstrate moderate uneven staining patterns as shown by Figure 7. There was good eosin to haematoxylin staining balance which allowed for ease of morphological evaluation and interpretation. Gill's III ranked second for specificity and fourth for sensitivity with scores of 98.4% and 80.4%, respectively.

Harris haematoxylin (Leica Microsystems (UK) Ltd: product code 3801560BBE) produced satisfactory nuclear staining pattern that allowed for the interpretation and visualisation of chromatin and nucleoli detail. The intensity of staining could have been slightly more pronounced, as seen in Figure 8. Factors relating to specificity were mostly adhered to, such as no background staining and uneven staining demonstration. However, there was an increased intensity of eosin in places due to the weak intensity of haematoxylin staining. Overall, Harris haematoxylin ranked fourth for specificity and fifth for sensitivity with scores of 85.9% and 80.2%, respectively.



a b

Figure 2. Examples of cases stained with Carazzi's haematoxylin. 2A (X40 magnification) shows a photomicrograph of an H&E stained section of a case with tumour foci of infiltrative BCC in a debulk specimen. Figure 2B (X40 magnification) shows an H&E stained section of a normal uninvolved skin.

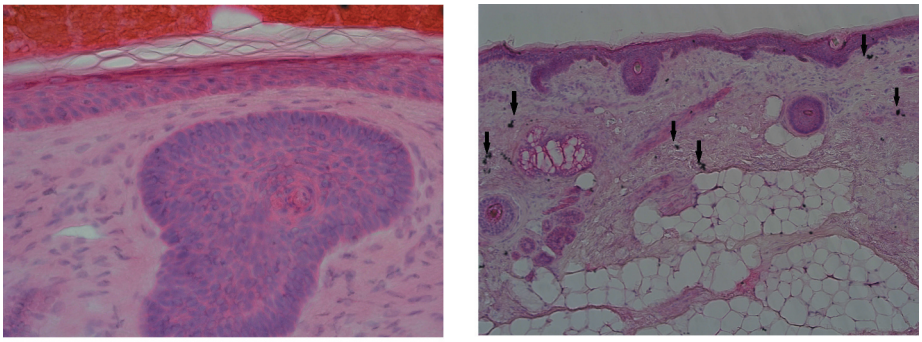


Figure 3. Examples of cases stained with Cole's haematoxylin. 3A (X40 magnification) shows a photomicrograph of an H&E stained section of a case showing tumour foci of infiltrative BCC in a debulk specimen. Figure 3B (X20 magnification) showing an H&E stained section of uninvolved skin. The arrows highlight the oxidative precipitates present.

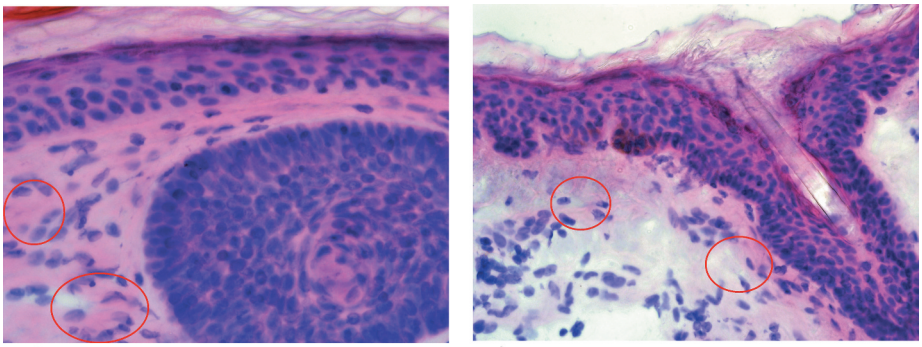


Figure 4. Examples of cases stained with Ehrlich's haematoxylin. 4A (X40 magnification) is a photomicrograph of a H&E stained section of a case showing tumour foci of infiltrative BCC in a debulk specimen. Figure 4B (X40 magnification) showing an H&E stained section of a case showing normal uninvolved skin.

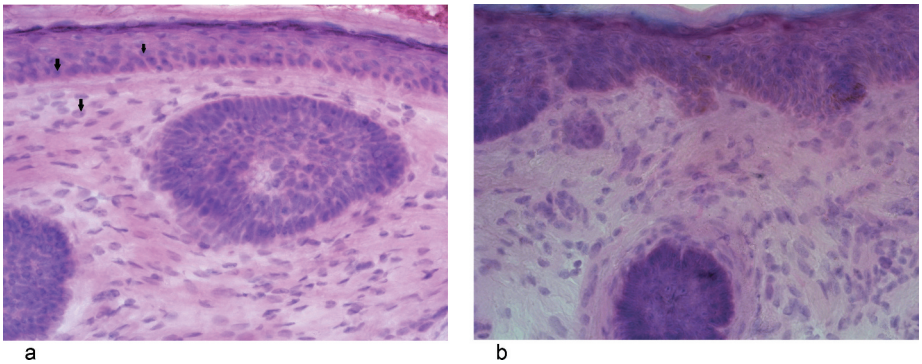


Figure 5. Examples of cases stained with Gill's I haematoxylin. 5A (X40 magnification) is a photomicrograph of an H&E stained section of a case showing tumour foci of infiltrative BCC in a debulk specimen. 5B (X40 magnification) showing an H&E stained section of normal uninvolved skin.

Mayer's haematoxylin (Solmedia Ltd: product code HST0011) staining resulted in weak nuclear staining with reduced intensity of colour. The nucleoli and chromatin detail was less clearly visualised due to weaker intensity of haematoxylin staining (Figure 9). The staining of BCC tumour can be distinguished from uninvolved epithelial cells. However, the intensity of staining was not as strong as should be expected. Mayer's haematoxylin did not show any background or non-specific staining. However, there was a presence of uneven staining noted. Mayer's haematoxylin ranked seventh overall with a specificity and sensitivity score of 60.6% and 61.6%, respectively.

Weigert's haematoxylin (Solmedia Ltd: product code HST204-A and HST205-A) produced a clear and crisp nuclear staining pattern with sections showing high-intensity staining. At times the staining appeared too strong, which resulted in the appearance of non-

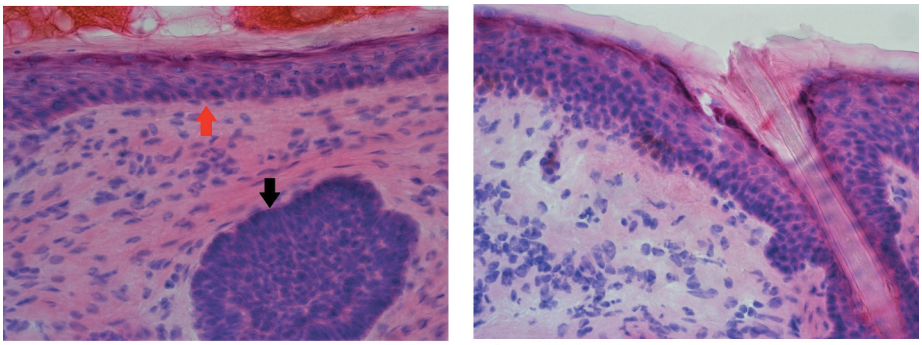


Figure 6. Examples of cases stained with Gill's II haematoxylin./ 6A (X40 magnification) is a photomicrograph of an H&E stained section of a case showing tumour foci of infiltrative BCC in a debulk specimen. Figure 6B (X40 magnification) showing an H&E stained section of normal uninvolved skin.

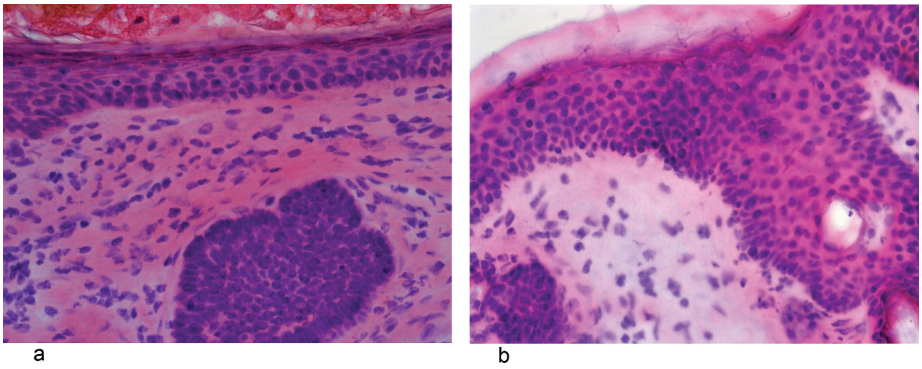


Figure 7. Examples of cases stained with Gill's III haematoxylin. 7A (X40 magnification) is a photomicrograph of an H&E stained section showing tumour foci of infiltrative BCC in a debulk specimen. Figure 7B (X40 magnification) showing an H&E stained section of normal uninvolved skin.

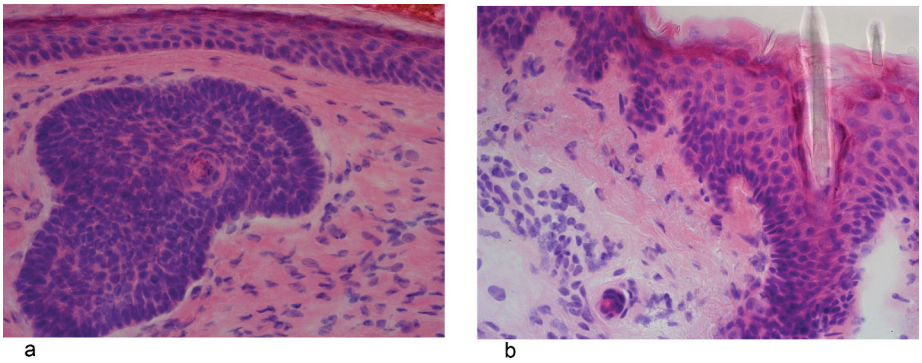


Figure 8. Examples of cases stained with Harris haematoxylin. 8A (X40 magnification) is a photomicrograph of an H&E stained section showing tumour foci of infiltrative BCC in a debulk specimen. Figure 8B (X40 magnification) showing an H&E stained section of normal uninvolved skin.

specific or background staining. It is noteworthy that the staining provided moderate visualisation of the nucleoli and chromatin detail, as seen in Figure 10. The staining of tumours cells was easily visualised due to intense nuclear staining, which can be seen in Figure 10a. However, the staining did appear to show uneven staining without any staining deposits. Overall, Weigert's haematoxylin ranked fifth for specificity and second for sensitivity with scores of 80.0% and 83.4%, respectively.

Discussion

The diagnosis and classification of most neoplastic disorders rely on the information gathered from the evaluation of H&E stained sections, with the interpretation of haematoxylin stained nuclear detail playing an essential role in determining morphological characteristics. The importance of clear demonstration of nuclear and chromatin detail is paramount for unequivocal diagnosis and provides confidence in the clinical

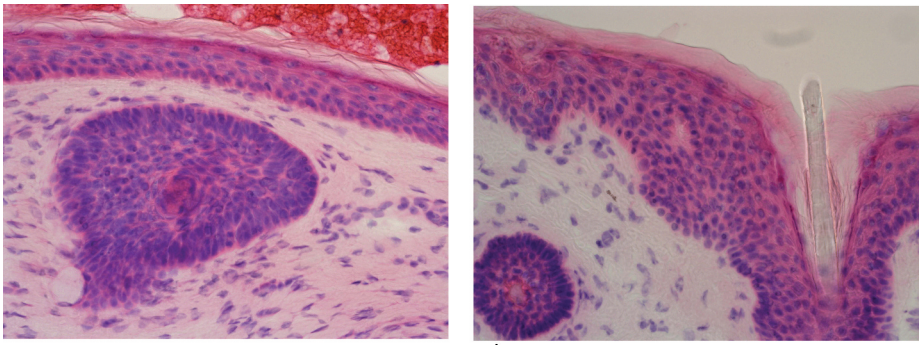


Figure 9. Examples of cases stained with Mayer's haematoxylin. 9A (X40 magnification) is a photomicrograph of an H&E stained section of a case showing tumour foci of infiltrative BCC in a debulk specimen. Figure 9B (X40 magnification) showing an H&E stained section of normal uninvolved skin.

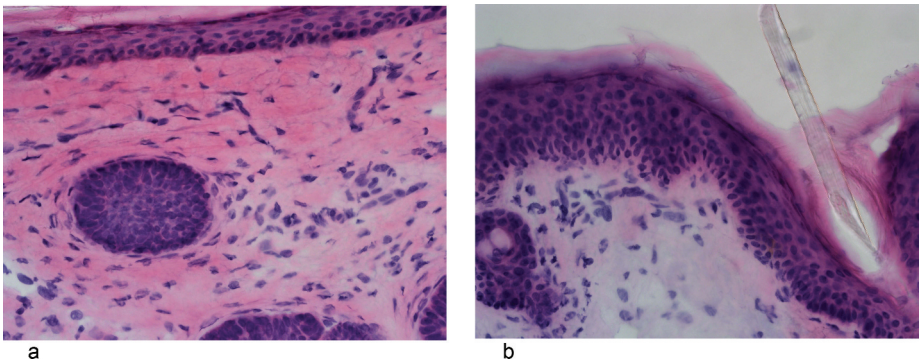


Figure 10. Examples of cases stained with Weigert's haematoxylin. 10A (X40 magnification) is a photomicrograph of an H&E stained section showing tumour foci of infiltrative BCC in a debulk specimen. Figure 10B (X40 magnification) showing an H&E stained section of a normal uninvolved skin.

decisions made. This study highlights how the performances of different haematoxylin dye subtypes can produce varying results even while maintaining identical variables such as slide preparation, reagent batch and staining platform.

Analysis of the results identified Carazzi's haematoxylin as performing the best when used as part of the H&E staining process during MMS procedure. In a rapid intraoperative clinical setting where clear and precise results need to be generated in a quick turn around, Carazzi's which uses a potassium aluminium mordant proves to be the most optimal choice for haematoxylin dye[9]. Evaluation of slides highlighted the high intensity of nuclear staining, which ensured ease of confirmation of BCC tumours due to the clear hyperchromatic staining, which allows for tumour cell separation from uninvolved epithelial cells. It has been widely accepted that Carazzi's haematoxylin performs better when used as part of a frozen section procedure. However, this study affirms that it is undoubtedly the case when used as part of the Mohs staining procedure [9].

Ehrlich's haematoxylin, which also uses a potassium alum-based mordant, performed consistently well ranking third overall. This is in contrary to current literature available which states that Ehrlich's haematoxylin performs less well when used as part of a frozen section staining process [9]. It is found to work best on tissue such as bone and cartilage, as well as tissues that have undergone decalcification utilising acids [9]. However, this study has highlighted that while Ehrlich's haematoxylin did not perform as well as Carazzi's haematoxylin, it did perform consistently well which reinforces the possibility of this haematoxylin being used as part of the MMS technique.

Harris haematoxylin is another subtype which utilises Potassium alum-based mordant [9]. Overall, Harris' haematoxylin stained slides performed moderately well with clear nuclear detail which allowed to distinguish malignant BCC neoplastic cells from non-malignant epithelial cells. One would expect due to the already established use of Harris haematoxylin it would perform better, with the remaining haematoxylin facing a slight disadvantage. On the contrary, this study has identified three other subtypes which have performed better than Harris, which reinforces robustness of the optimisation and impartiality of the judging process.

Potassium alum is utilised as the primary mordant for Carrzai's, Ehrlich's and Harris' haematoxylin which all performed well, however, Cole's haematoxylin which utilises the same mordant ranked the lowest in this study. This

raises the question of whether other ingredients present in the haematoxylin could have an impact on the staining quality rather than solely the mordant used. Another limitation that was observed with Cole's haematoxylin, in particular, was the presence of staining deposits due to oxidative precipitation. Before initiation of the staining procedure, all haematoxylins were filtered to prevent the transfer of precipitates. However, in Cole's, the issue of staining deposits was still encountered mostly due to further oxidisation when exposed to air. This further reinforces the conclusion that Cole's haematoxylin is not suitable for use as part of the MMS procedure.

Mayer's haematoxylin which utilises ammonium alum-based mordant is another variant of the aluminium mordant subtype [9]. Visually the solution appears as a paler purple colour compared to some of the other haematoxylins, and this is also reflected in the staining pattern observed microscopically which is of a weaker intensity. Larson and colleagues recommended a staining time of 15 minutes to achieve optimal nuclear staining utilising Mayer's haematoxylin as part of the Mohs procedure [10]. Therefore, it could be argued that it is possible to achieve a pronounced nuclear staining pattern if left in the haematoxylin solution for longer. Normally, section thickness variation can also have an impact staining, with thinner section's showing weaker staining and thicker sections showing darker staining. However, in this study all sections were cut at 15µm to ensure consistency and any variation in staining can be attributed to the haematoxylin dye staining times. Overall, due to the need for rapid result generation that is required as part of the Mohs procedure, haematoxylin subtypes which require a ~15 minute incubation period such as Mayer's haematoxylin are not suitable for use.

When comparing the three Gill's (I, II and III) haematoxylin subtypes, we can see that performance of Gill's increases with the concentration of haematoxylin, with Gill's III performing the best followed by Gill's II and Gill's I performing least well. However, it is evident from this study that the concentration of haematoxylin does not impact the quality of nuclear staining beyond a certain point, as Gill's II and Gill's III scores for sensitivity varied by only 1.2%. The most significant impact the variations of the concentration of haematoxylin was on the factors relating to specificity. Gill's III did not present with some of the issues observed with Gill's II, which included; uneven staining pattern and more pronounced eosin staining patterns due to weaker haematoxylin staining. These factors contributed to the difference of 30.2% observed in the specificity result between Gills III and Gill's II.

In this study, Weigert's haematoxylin was the sole iron mordant based (ferric chloride) haematoxylin [9]. Traditionally, iron-based haematoxylins are not commonly utilised as part routine diagnostic staining due to its strong oxidative nature which can result in increased intensity/unpredictable staining patterns. This was reflected in the sensitivity and specificity scores that were observed. The high-intensity nuclear staining was quite pronounced, which made the distinction of BCC tumours from non-malignant cells quite straight forward, this contributed to the high sensitivity score. However, due to its strong oxidative nature, a drawback was the increased background staining and uneven staining pattern observed, which resulted in a lower specificity score. Due to these limiting factors, Weigert's haematoxylin is not suitable for use as part of the MMS procedure.

As described, the linistat linistainer is restricted in any alteration that can be made in the immersion timings of reagents that are used. The maximum possible immersion time for haematoxylin is limited to 50 seconds. This proved challenging when optimising the three lowest-ranking haematoxylin's (Mayer's, Gill's I and Cole's). All three lowest ranking haematoxylins were run on a protocol that included a maximum immersion time. However, it was still not sufficient to achieve an optimal level of staining. It could be argued that if the sections were left in the haematoxylin dyes for longer by employing a manual method, the level of staining achieved could be vastly improved. However, MMS is an intraoperative procedure where time is a critical factor and results should be generated promptly. As a result, Mayer's, Gill's I and Cole's are not a viable option to use as part of the MMS procedure, as alternatives are available.

The use of Carazzi's haematoxylin as part of any frozen section procedure, including Mohs, has not been widely assessed. However, this study has highlighted the vastly improved and clear visualisation of nuclear and chromatin detail of Carazzi's haematoxylin when used as part of the H&E staining process. This was reflected in the higher sensitivity and specificity scores that Carazzi's obtained overall in this study. Nationally in the UK, there is no standardised staining protocol for use in the MMS procedure. This study helps towards quantifiably determining an optimal H&E staining protocol that can be used as part of this procedure.

Summary table

What is known about this subject?

- Carazzi's haematoxylin performs well when used as part of a frozen section procedure.
- The use of Carazzi's haematoxylin as part of the Mohs procedure has not been widely assessed.

What this study adds

- In the United Kingdom, there is no standardised staining protocol for use in Mohs Micrographic surgery procedure.
- This study helps towards quantifiably determining an optimal H&E staining protocol that can be used as part of this procedure.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

[1] Griffin L. Non-melanoma skin cancer. Clin Med. 2016;16:62–65.
[2] Cameron MC, Lee E, Hibler BP, et al. Basal cell carcinoma Epidemiology; pathophysiology; clinical and histological subtypes; and disease associations. J Am Acad Dermatol. 2019;80:303–317.
[3] Telfer NR, Colver GB, Morton CA. Guidelines for the management of basal cell carcinoma. Br J Dermatol. 2008;159:35–48.
[4] Orchard GE, Wojcik W, Shams F, et al. Pan-cytokeratin markers for rapid frozen section immunocytochemistry from head and facial Mohs cases of basal cell carcinoma: a comparison and evaluation to determine the marker of choice. Br J Biomed Sci. 2016;72:61–66.
[5] Mansouri B, Bicknell LM, Hill D, et al. Mohs micrographic surgery for the management of cutaneous malignancies. Facial Plast Surg Clin North Am. 2017;25:291–301.
[6] Titford M. The long history of hematoxylin. Biotech Histochem. 2005;80:73–78.
[7] GE O. Hematoxylin – the story of the blues. Br J Biomed Sci. 2018;75:149–152.
[8] UKNEQAS. Staining criteria handbook Mohs' procedure. 2 ed. Gateshead: UKNEQAS CPT; 2017.
[9] Bancroft J, Suvarna K, Layton C, et al. Bancroft's theory and practice of histological techniques. Oxford: Elsevier; 2015. 126–138.
[10] Larson K, Ho HH, Anumolu PL, et al. Hematoxylin and eosin tissue stain in mohs micrographic surgery: a review. Dermatol Surg. 2011;37:1089–1099.