Structural Abstract

Background Creating permanent paraffin slides from Mohs layers takes considerably more time than staining fresh frozen sections using immunohistochemistry (IHC). The science of IHC has evolved to a point where, with the proper antibody and protocol, staining for atypical melanocytes can produce valuable results. Possible barriers to performing IHC include cost, efficiency, and institutional resistance. Most importantly there are hidden time constraints which could affect patient care.

Objective Those choosing to do IHC with frozen sections should be aware of several additional considerations which have not to our knowledge been previously mentioned. A protocol utilizing current technology to optimize immunostaining of fresh frozen tissue is suggested.

Methods The steps to performing IHC on frozen sections were reviewed and the time needed to perform it were evaluated.

Results Realistic antibody incubation times and more time for cutting additional frozen section slides and controls are only two of the additional time constraints to performing IHC. Using an alkaline phosphatase (AP) polymer detection protocol will avoid the deleterious effects of hydrogen peroxide on delicate frozen tissue. A permanent red chromogen can make visualization of the atypical melanocytes easier.

Conclusion While technology has improved the quality of IHC done on Mohs frozen sections, IHC is still costly and requires meticulous technique to perform. Though faster than preparing paraffin sections, there are still pitfalls to performing IHC which can invalidate its use. Institutional resistance to duplicity of service and other concerns may prohibit some Mohs practitioners from performing IHC in the Mohs lab.

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Text

Many papers have been published in recent years exploring the use of antibody staining otherwise known as immunohistochemistry (IHC) for detecting atypical melanocytes in Mohs frozen tissue sections. IHC can be performed more readily than what is often called “slow Mohs,” which has an approximately twenty-four hour turnaround time. [(1)] A number of studies have demonstrated impressive results with regards to the quality of the IHC stained sections and rapidity of the immunostaining techniques. [(2)] [(3)] [(4)] However, there is a disturbing lack of detail concerning the preparations of reagents and materials and the type of validation for the methods used. In order for IHC to become a feasible practice in a Mohs surgery office there needs to be high quality, reproducible staining of melanocytes done in a timely fashion for the convenience of patients. To increase awareness among Mohs surgeons and their staff about unmentioned time considerations and the complexity of the IHC process for Mohs frozen sections, we describe below several factors which should be considered when performing IHC.

Preparation of frozen sections for IHC

Extra time and effort are often needed to cut the frozen section slides for a melanoma case particularly if the Mohs layer is of considerable size and involves significant adipose tissue. Currently the acceptable margin from visible melanoma in situ ranges from 5-9 mm. Taking into account the visible tumor size, a resulting 20 mm diameter Mohs layer would require approximately four tissue sections. The same standards for a Mohs section (i.e. a complete section with all of the epidermal edge), applies to the sections used for IHC. Thus, time must be added for a deeper cut slide for each section to be used for IHC testing.

Testing fresh frozen tissue requires that some fresh frozen tissue, known to be positive for the specific testing antibody, be kept and maintained for use as controls. [(5)] [(6)] The best method for obtaining control tissue is to use debulked tumor that would normally have been discarded. This tissue is then embedded in freezing compound and then stored in a -30deg freezer until it needs to be cut for use in an antibody staining run. Additional time is needed for cutting all of the control slides. There needs to be a positive and negative control slide for each antibody being used and a negative methods control slide for each staining run. (A negative methods slide is a positive control slide that has no antibody applied to it during the staining procedure. The test tissue being stained will suffice as a positive methods control.) Using the example of a 20 mm diameter Mohs layer cut into four sections for fresh frozen processing, eleven slides would need to be cut with seven of those slides required for a single IHC run for one antibody. Cutting deeper into the frozen block to obtain these slides may yield false positive results.

The slides for IHC then need to be fixed, air dried, or a combination of both before staining. Cold (4° C) acetone is generally used as the fixative for fresh frozen tissue because it is non-additive and dehydrates and fixes the tissue quickly. Crucial enzymes such as phosphatases are thus preserved [(7)]. As most of the reagents used in the IHC process have a short shelf life, they ideally need to be prepared immediately before a staining procedure. This may take a variable amount of time to accomplish ranging between 10 to 20 minutes. In sum, a more pragmatic time estimate for performing an IHC procedure on a fresh tissue melanoma done with the Mohs procedure with all the necessary steps performed in advance and suitable incubation time would be approximately 2-4 hours. This time is dependent not only on the freshness and quality of the antibody, but on the skill and efficiency of whom or what device is performing the test as well.

Validation

The main goal of the antibody validation process is to optimize the specific staining while determining a reasonable incubation time and reducing background staining. The difference between the staining intensity of the antigen being targeted and unwanted background staining is called the "signal to noise" (SN) ratio. The sensitivity or attractiveness of an antibody to a specific antigen is called an "affinity" to that antigen. The minimal amount of time required for an antibody at optimal dilution, or the "functional affinity," to saturate a specific antigen is called the "equilibrium point". At this equilibrium point, not only are positive results easier to visualize, the antibody-antigen reaction is also less likely to disassociate during the subsequent washing cycles of the staining procedure. [(8)]

For smaller Mohs laboratories, validation requires a significant time-consuming effort but it is a necessary and critical component of the IHC procedure. [(9)] There are many parts of an IHC protocol that need regular validation. Each batch of antibody needs to be tested to determine whether the optimal incubation time is the same as the previous batch. If concentrated antibodies rather than prediluted staining kits are being used, their proper dilutions need to be determined and validated. Monoclonal antibodies have a lower antigen affinity but are more antigen-specific and more expensive to use than polyclonal antibodies. [(10)] The lack of specificity with polyclonal antibodies will shorten the incubation time but will contribute to additional background staining. Polyclonal antibodies are usually supplied in prediluted antibody kits. As such, the type of antibody used can make a difference in the staining protocol that is chosen. The temperature and humidity of the lab can be a factor in the consistency of the staining results so these aspects also need to be closely monitored and controlled. Written logs must be kept of all the validations to ensure reproducibility and satisfy the Clinical Laboratory Improvement Amendment (CLIA) standards. [(11)]

Selection of staining method

In addition to recognizing the importance of validation in the IHC process, becoming knowledgeable about the staining method to be used is essential. There are many methods

available to choose from but there are basically only two types of enzyme linkages. Peroxidase-labeled (horse-radish peroxidase or HRP, aka IPOX) antibodies were used at the outset and were designed to be used with paraffin slides. [6] [10] Although stacking these antibodies into a complex of antibodies provides better staining of the antigen of interest, this often increases endogenous background staining and can complicate accurate interpretation of the slides.

The avidin/biotin complex (ABC) staining method increases the SN ratio by providing four times as many binding sites for chromogenic attachment as compared to the standard peroxidase method. Combining the peroxidase-labeled secondary antibody with a biotin primary antibody to create what is called the labeled streptavidin/biotin (LSAB) method increases the SN ratio even more by providing greater antibody sensitivity and signal amplification. [12]

One challenge with the IPOX/HRP and ABC/LSAB methods is the presence of endogenous peroxidase and biotin in the tissue which are at even higher levels in fresh frozen tissue than in paraffin embedded tissue. Besides often being ruinous to the tissue, blocking or quenching of peroxidase and biotin adds more time and complexity to the IHC procedure. [12]

Using the polymer string method with an alkaline phosphatase enzyme (AP) labeled antibody provides a means of avoiding endogenous peroxidase and biotin, and eliminates the need for blocking. A dextran string contains secondary antibodies to any primary antibody of rabbit or mouse origin and provides a string of chromogenic attachments analogous to a string of lights. [13; 12] SN ratios are as good as or better than those achieved with the LSAB method. While there is no need to block or quench the tissue with an AP antibody kit, tris buffered saline (a combination of tris (hydroxymethyl aminomethane), sodium chloride, and sodium azide) should be used during the staining process instead of phosphate buffered saline to avoid potential for background staining.

Melanoma antibodies

This discussion is limited to immunostaining for melanoma, but still there is a wide choice of antibodies to choose from. (See table 1.) Microphthalmia transcription factor (MiTF) is perhaps the most popular of the melanoma detecting antibodies at the present time. [14] MiTF regulates the development and survival of melanocytes and is also involved in transcription of pigmentation enzyme genes such as tyrosinase. The MiTF antibody recognizes a nuclear protein which is expressed in the majority of primary and metastatic epithelioid malignant melanomas as well as in normal melanocytes, benign nevi and dysplastic nevi. There are antibodies which target surface and cytoplasmic proteins as well as nuclear proteins. Regardless of which antibody is used, the protocols for validation and staining should be the same. Specific information about any purchased antibody is listed on the datasheet that comes with it. These datasheets contain information such as the class of antibody and what the antibody target is, as well as the batch code and expiration date. The datasheet for each batch of antibody purchased must be kept for seven years. [11]

Chromogen color

It is unnecessary to use to diaminobenzidine (DAB) with an AP labeled antibody kit. Although DAB is a permanent chromogen, it is a carcinogen that requires special disposal protocols. It is also brown in color just like melanin. Alternate chromogen colors are available such as red, yellow, blue, or green, some of which are resistant to solvents like DAB so that the IHC slides can be cover slipped and stored like conventionally stained slides. Levamisole, which is mixed with the chromogen substance, suppresses any endogenous alkaline phosphatase in the tissue sections. There are AP antibody kits available that utilize permanent red or blue chromogen colors. Since counterstaining with hematoxylin is common practice with immunostains, a red chromogen would be preferable to a blue one.

Cost considerations with IHC

There are three classes of antibodies specified by Food and Drug Administration (FDA) regulations: analyte specific reagent (ASR), in vitro diagnostic (IVD), and research use only (RUO). [(15)] Care must be taken to ensure that one of the three types of IVD class antibodies and not the more readily available and inexpensive RUO antibodies are used by the Mohs lab. This is important to realize if expecting compensation for performing the IHC test as Medicare and most health insurers will not reimburse for the use of non IVD class antibodies. [(16)] The class of antibody being used as well as other important descriptive information is listed on the antibody datasheet supplied by the antibody manufacturer with each antibody. A copy of the datasheet for each batch of antibody purchased must be kept for at least two years as per CLIA standards. [(11)]

Antibody staining is a more expensive endeavor compared to conventional staining. The standard stains used in a Mohs lab such as hematoxylin, eosin, and toluidine blue are certified for standard consistency by the International Biological Stain Commission. There is no such commission regulating the available antibodies. Antibodies can therefore vary from batch to batch and from wholesaler to wholesaler. The price of monoclonal antibodies is greater than for polyclonal antibodies. To test and standardize these antibodies would put their price beyond any practical utilization.

With Medicare reimbursements becoming more stringent, cost efficiency may be a significant issue, particularly in a hospital or academic setting. Large hospitals will usually have their own fully automated IHC specialized lab which can produce excellent work, although results may not be available for several days. A dermatopathologist would need to request the IHC testing and would have to read the slides as well. In this case the institution may question the additional cost to the patient of IHC being done in the Mohs clinic lab, and not have concerns over the time required to obtain results.

Conclusion

Mohs surgery needs a quantitation of IHC procedures in order to adapt a standardized method for those who choose to use antibodies to detect residual melanoma. We suggest the use of an IVD class monoclonal antibody with an anti-mouse polymer string AP-labeled secondary antibody and a permanent red chromogen appears to be an optimal staining method.

Expectations for the time needed to do a conscientious and meticulous IHC process need to be more realistic. While the total amount of time needed to get valid results may only be around two hours, much more time is needed to prepare to do the testing. The Mohs surgeon must also be treating a fair number of melanoma cases to assure that the reagents are fresh and to justify the cost of performing IHC. If the melanoma demonstrates discontiguous growth then IHC staining results may be inaccurate.

Certainly performing IHC staining by hand is not difficult, but it requires hours of practice to develop sufficient proficiency. There may be a need to purchase some special equipment like pipettes, wash bottles, and a staining tray. The meticulous nature of IHC staining lends itself to robotic/automated staining in order to achieve consistent results, improve efficiency, and reduce costs in the process. This is why expensive robotic or automated strainers are now performing the immunohistochemical staining in most of today’s modern labs. The extra work required in order to obtain reproducible results might well put this procedure, for practical purposes involving time, effort, and expense, beyond the scope of most Mohs practices.

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Table 1

Antibody	What it labels	Possible limitations
S100	S100 stains for Schwannomas, ependymomas, astrogliomas, almost all benign and malignant melanomas and their metastases. S100 protein is also expressed in the antigen presenting cells such as the Langerhans cells in skin and interdigitating reticulum cells in the paracortex of lymph nodes.	Stains paraffin sections better than frozen sections. [17]
HMB-45	This antibody reacts with a neuraminidase sensitive oligosaccharide side chain of a glycoconjugate present in immature melanosomes. It reacts with junctional and blue nevus cells. Non-melanocytic cells are negative.	Inconsistent staining of melanocytes creates variability in staining patterns. [17]
Tyrosinase	Tyrosinase is a key enzyme involved in the initial stages of melanin biosynthesis. Studies have shown Tyrosinase to be a more sensitive marker when compared to HMB45 and MART-1. It has also been shown to label a higher percentage of desmoplastic melanomas than HMB45.	Cytoplasmic and/or perinuclear localization. Also marks benign nevi.
MART-1/Melan-A	Involved in melanosome biogenesis by ensuring the stability of GPR143. Plays a vital role in the expression, stability, trafficking, and processing of melanocyte protein SILV/PMEL17, which is critical to the formation of stage II melanosomes. The MART-1/Melan A recognizes a protein of 18kDa, identified at MART-1 (Melanoma Antigen Recognized by T cells 1) or Melan-A. Studies have also shown that MART-1 is more sensitive than HMB45 when labeling metastatic melanomas	Also stains sun damaged skin which creates a potential for false positives. [18]
Pan Melanoma	The combination of S100, HMB45, MART-1 and Tyrosinase make this quadruple antibody cocktail a first-order pan melanoma screener.	Has a potential for increased background staining and false positives.
MiTF	MiTF regulates the development and survival of melanocytes and is also involved in transcription of pigmentation enzyme genes such as tyrosinase. The MiTF antibody recognizes a nuclear protein which is expressed in the majority of primary and metastatic epithelioid malignant melanomas as well as in normal melanocytes, benign nevi and dysplastic nevi.	Interpretations have some false positive nonmelanocytic cells and false negative spindle cells. [19]
Mel-5	Mel-5 targets a tyrosinase related protein which is associated with stage III and IV melanosomes.	May not stain the dermal components of melanoma. [20]