Journal of Cancer Therapy, 2013, 4, 1082-1084
http://dx.doi.org/10.4236/jct.2013.46123 Published Online August 2013 (http://www.scirp.org/journal/jct)

The Emergence of Rapid Counter Immunostaining in the Controlled Narrow Excision of Malignant Melanoma—How We Do It

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Received May 22nd, 2013; revised June 25th, 2013; accepted July 3rd, 2013

ABSTRACT

Mohs Micrographic Surgery (MMS) is widely employed in the treatment of non-melanoma skin cancer and is a preferred treatment for many cutaneous malignancies, particularly in high risk locations and tumors [1,2]. It has also been used in the narrow excision of malignant melanoma with local control rates equivalent to standard margins [3]. It has gained acceptance in the treatment of noninvasive melanoma where standard 0.5 cm margins may be inadequate for local control [4]. The frozen section processing used in MMS has been assumed by some to be inadequate in assessing melanocyte populations or residual melanoma within excision margins. This difficulty has likely led to a majority of surgeons with fellowship training to process margins with slow, permanent hematoxylin and eosin sections ("slow-mohs") or to simply resort to standard 0.5, 1.0, or 2.0 cm margins with traditional excision and outside pathology confirmation of clear margins. A recent survey of practicing fellowship-trained Mohs surgeons revealed roughly one-third (35.9%) of Mohs surgeons felt comfortable interpreting MART-1 immunostains, and far fewer were actually performing immunostains in their labs [5]. Some Mohs surgeons currently refer melanoma to a colleague experienced in processing and reading melanoma with available rapid immunostaining. The development of rapid immunohistochemistry, which can be implemented into a traditional frozen section laboratory, has greatly improved the ease of interpreting margins in the excision of melanoma. Although the process is considerably more complicated than staining with H&E or Toluidine Blue (T-Blue), it easily falls within the skill-set and equipment of most busy frozen section laboratories. The additional cost of biologic reagents may be fully recovered by proper billing of immunohistochemical laboratory work and interpretation of slides.

Keywords: Moh's Surgery; Malignant Melanoma; Rapid MART-1

1. Introduction

Multiple immunohistochemical staining protocols for the melanoma antigen recognized by T-cells (MART-1) using frozen sections have been developed and presented over the last decade. Historically, melanoma was processed with en-face frozen H&E staining, or with permanent paraffin sections requiring overnight processing and multiple days between each Mohs layer. In 2004, Bricca [6] and associates presented a reproducible 1-hour MART-1 protocol for melanoma frozen sections. Later, Asadi [7] and colleagues reported a modified 20-minute protocol. Currently, the American College of Mohs Surgery has a suggested modified protocol available to its members online [8]. The third protocol resembles a combination of the Bricca and Asadi protocols.

While all three methods produce reliable staining, each has its respective strengths and weaknesses. The 1-hour protocol gives consistent dependable results, however the lab processing time is dramatically longer than that of a routine section stained with H&E. The 20-minute rapid protocol requires considerably less time, but demands the lab technician's full attention, excluding other casework. The clarity of the slides produced using this rapid protocol also may contain excessive chromogen "chatter" where much of the melanocytic detail may be lost. The weakness inherent in the Mohs College protocol is that it is only available to its members, and remains unpublished in the public domain.

We present an experimental 35-minute MART-1 staining protocol for melanoma frozen sections that

combines the best of the previously presented protocols, while eliminating many of the aforementioned obstacles (**Tables 1** and **2**). In addition, we propose the use of T-Blue as a reasonable alternative to the standard use of Hematoxylin, as a counterstain, although Hematoxylin may certainly be used. We have also substituted T-Blue in all of the published protocols with reproducible staining compatible with the suggested reagents. Lastly, we suggest the implementation of an automated stainer during the counterstaining process for consistent, reproducible, easily readable slides. As an added benefit, the use of the automated stainer unburdens the histo-technician freeing him/her for additional lab work.

Table 1. Happy medium protocol.

Mounting and Preparation	1. Cut thin (2 - 4 μm) sections-minimum two copies of each piece
	2. Mount on a positively charged slide facilitates better adhesion
	3. Air dry-2 minutes—room temperature
	4. Heat on a 60˚C hot plate (or equivalent)—3 minutes
	5. Fix in Acetone(in a Coplin jar) —2 minutes
	6. Air dry-2 minutes—room temperature
	7. Rehydrate in Tris-Buffered Saline (TBS) (in a Coplin Jar)—2.5 minutes
Staining*	1. Add protein blocking agent—3 minutes
	2. Shake off, do not rinse
	3. Apply "Ready to Use" MART-1 antibody—6 minutes
	4. Rinse in Tris Buffered Saline (TBS)—2 minutes
	5. Apply Polymer HRP (horse-radish peroxidase)—6 minutes
	6. Rinse in TBS–2 minutes (during this processing-mix up DAB Chromogen for step 7)
	7. Apply pre-mixed (1 drop substrate/1 drop solution/1 ml distilled water) DAB Chromogen—2 minutes
	8. Rinse in distilled water for 1.5 - 2 minutes (shorter time = darker chromogen)
Counterstain (with Toulidine Blue)+	1. Dip in T-Blue—10 seconds
	2. Rinse with running water-30 - 45 seconds
	3. Dip in 95% reagent alcohol-15 seconds
	4. Dip in 3 changes of 100% reagent alcohol—15 seconds each
	5. Dip in 3 changes containing a clearing agent—15 seconds each
OR	
Counterstain (with Hematoxylin)	1. Dip in Hematoxylin—2 seconds
	2. Rinse with running water-30 - 45 seconds‡
	3. Dip in 95% reagent alcohol—20 seconds
	4. Dip in 3 changes of 100% reagent alcohol—20 seconds each
	5. Dip in 2 - 3 changes of a clearing agent—20 seconds each

*Steps in this section take place in a humidity chamber-to achieve humidification by pouring 90˚C - 100˚C (boiling) water in the chamber beneath the slides and shutting the lid. +We perform the counterstain in a linear automated stainer—can be hand dipped using the same time increments. ‡If you require bluing in the H & E protocol, it will need to be added here.

We have also found inexpensive ways to implement this protocol. In addition to readily available supplies, we were able to utilize commonly found items in lieu of more costly lab equipment. Our 60˚C hot plate is a toaster oven in combination with a certified thermometer. A small percolator of boiling water replaced the need for an additional 100˚C hot plate that is used in the humidification process. Likewise, we eliminated the problematic issue of obtaining negative control tissue, that can be costly and difficult to store, by utilizing non-melanoma tissue from cases being treated at the same time as the melanoma(s). Positive/negative controls are subjected to the same protocol as the melanoma tissue, replacing the MART-1 antibody with distilled water for the negative control. By setting this as our standard operating procedure, we were able to satisfy CLIA requirements for positive/negative controls.

Lastly, we introduced a linear automated stainer to perform the counterstain. With our stainer set with T-Blue, it delivers a pleasing alternative to the traditional Hematoxylin counterstain. This simple step provides more opportunity for the histotechnician to continue processing other tissue while not sacrificing reproducible slide quality.

2. Conclusion

Most practices currently performing standard Mohs processing are well equipped to add rapid MART-1 immunostaining regardless of training or experience. Additional publications have elucidated simple interpretation of normal melanocytes, atypical melanocytic proliferations, or malignant melanoma and other cells which will stain positive with MART-1 [9,10]. Processing melanoma with MART-1 staining, due to the complexity and intensive hands-on nature of the staining process, can initially be a rate-limiting event, but may quickly become routine, reproducible, and valuable in the treatment of melanoma. As most practices have a single histotech, multi-tasking is essential to the efficiency and flow of the lab. Finding efficient, reproducible practices are central to the continued adeptness of the Mohs laboratory. Our experience with this protocol has produced well-defined, consistent readable slides in which the melanocytic detail is markedly distinct (**Figure 1**). Counterstaining with

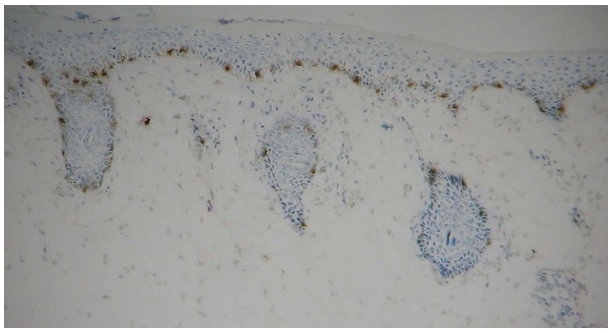

Figure 1. Example of frozen sections stained using our Happy Medium Protocol with a T-Blue counterstain.

Table 2. Product resource list[**].

1. Humidity trays/staining chamber—Evergreen Scientific www.evergreensci.com
2. Tris Buffered Saline-Dako—www.dakocytomation.com
3. Polymer based detection system—Leica Microsystems fax: 847-236-3009
4. MART-1 primary antibody, Coplin staining jars—ThermoFisher—800-828-1628
5. Positively charged slides, TBS mounting media, Toulidine Blue, Histoclear Clearing Agent, Reagent grade alcohol—Avantik—www.avantik-us.com

[**]We receive no commercial support from the above suppliers.

MART-1 has become a valuable tool in the controlled excision of malignant melanoma.

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