

Quantitative Analysis of Immunohistochemistry in Melanoma Tumors

Jenna Lilyquist, PhD^{a,b}, Kirsten Anne Meyer White, PhD^a, Rebecca J. Lee, PhD^c, Genevieve K. Phillips, BS^c, Christopher R. Hughes, AAS^a, Salina M. Torres, PhD, MPH^{d,*}

Abstract

Identification of positive staining is often qualitative and subjective. This is particularly troublesome in pigmented melanoma lesions, because melanin is difficult to distinguish from the brown stain resulting from immunohistochemistry (IHC) using horse radish peroxidase developed with 3,3'-Diaminobenzidine (HRP-DAB). We sought to identify and quantify positive staining, particularly in melanoma lesions. We visualized G-protein coupled estrogen receptor (GPER) expression developed with HRP-DAB and counterstained with Azure B (stains melanin) in melanoma tissue sections (n=3). Matched sections (n=3), along with 22 unmatched sections, were stained only with Azure B as a control. Breast tissue (n=1) was used as a positive HRP-DAB control. Images of the stained tissues were generated using a Nuance Spectral Imaging Camera. Analysis of the images was performed using the Nuance Spectral Imaging software and SlideBook. Data was analyzed using a Kruskal–Wallis one way analysis of variance (ANOVA). We showed that a pigmented melanoma tissue doubly stained with anti-GPER HRP-DAB and Azure B can be unmixed using spectra derived from a matched, Azure B-only section, and an anti-GPER HRP-DAB control. We unmixed each of the melanoma lesions using each of the Azure B spectra, evaluated the mean intensity of positive staining, and examined the distribution of the mean intensities ($P = .73$; Kruskal–Wallis). These results suggest that this method does not require a matched Azure B-only stained control tissue for every melanoma lesion, allowing precious tissues to be conserved for other studies. Importantly, this quantification method reduces the subjectivity of protein expression analysis, and provides a valuable tool for accurate evaluation, particularly for pigmented tissues.

Abbreviations: ANOVA = Kruskal–Wallis one way analysis of variance, FFPE = formalin-fixed paraffin-embedded tissues, GPER = G protein-coupled estrogen receptor 1, H&E = hematoxylin and eosin stain, HRP-DAB = horse radish peroxidase developed with 3,3'-Diaminobenzidine, IHC = immunohistochemistry, Ki-67, S100, and HMB-45.

Keywords: Azure B, immunohistochemistry, melanoma, spectral imaging

Editor: Tobias Sinnberg.

JL and KAMW contributed equally to this work.

Financial support for this work was provided by NIH Grant Number 5K05CA131675-04. This work was also supported in part by the University of New Mexico CTSC NIH Grant Number UL1 TR000041; NIH IMSD #5R25GM060201, and the National Institutes of Health grant, R25 CA92049 (Mayo Cancer Genetic Epidemiology Training Program). The Los Angeles Residual Tissue Repository is supported by grants and contracts P30CA014089 and HHSN261201000035C from the National Cancer Institute awarded to the University of Southern California.

The authors have no conflicts of interest to disclose.

Supplemental Digital Content is available for this article.

^a Department of Internal Medicine, Division of Epidemiology, University of New Mexico, Albuquerque, NM, ^b Department of Health Sciences Research, Mayo Clinic, Rochester, MN, ^c Cancer Research and Treatment Center, ^d Center for HPV Prevention, Department of Pathology, University of New Mexico, Albuquerque, NM.

* Correspondence: Salina M. Torres, 1 University of New Mexico, MSC08 4640, Albuquerque, NM 87131-0001 (e-mail: storres@salud.unm.edu).

Copyright © 2017 the Author(s). Published by Wolters Kluwer Health, Inc. This is an open access article distributed under the terms of the Creative Commons Attribution-Non Commercial-No Derivatives License 4.0 (CCBY-NC-ND), where it is permissible to download and share the work provided it is properly cited. The work cannot be changed in any way or used commercially without permission from the journal.

Medicine (2017) 96:15(e6432)

Received: 2 March 2016 / Received in final form: 28 February 2017 / Accepted: 1 March 2017

<http://dx.doi.org/10.1097/MD.0000000000006432>

1. Introduction

Immunohistochemistry (IHC) is an important technique to both researchers and clinicians,^[1] and is used to identify the presence and location of protein.^[2] For clinicians, IHC using formalin-fixed paraffin embedded (FFPE) tissues is important in the diagnosis of cancers as it allows for identification of overexpressed proteins known to be associated with carcinogenesis such as Ki-67, S100, and HMB-45 in melanoma. Researchers also frequently utilize IHC to identify new protein biomarkers for disease progression and to identify potential therapeutic targets.

Two common challenges exist in the field of IHC: visualization of multiple proteins in 1 tissue, often referred to as multiplexing and quantitative methods to evaluate protein levels in the tissues. Spectral imaging, where images can be unmixed based on their spectral properties, is 1 approach to addressing challenges with IHC. This approach allows for separation and quantification, even when using chromogens that do not have great visual contrast. As a result, chromogen combinations are not compromised and stains can be quadruple multiplexed.^[3–6]

IHC in melanoma tissues presents an additional challenge because the melanin pigment produced by melanocytes is a brown color that provides no visual contrast with the brown enzymatic product resulting from the most commonly utilized chromogen, 3,3'-Diaminobenzidine (DAB).^[7] In 1991, Kamino and Tam^[8] identified that Azure B, hereafter referred to as Azure, acts as an appropriate counterstain in melanoma sections. They reported that Azure stains melanin green-blue, allowing for its

identification in contrast to the surrounding tissue. However, this method was still quantified using the conventional scoring system, and therefore relies on subjective interpretation.

With advances in imaging and associated software, we sought to identify a quantitative method for measuring protein expression in melanoma tissues. In the current study, we used IHC HRP-DAB staining in melanoma tissues, with Azure as a counterstain, to develop a quantitative measurement of protein expression using spectral imaging technology.

2. Methods

2.1. Tissue samples

Melanoma tissue sections were obtained from 2 different sources: University of California Surgical Pathology Laboratory and the University of New Mexico Hospital (UNMH) Surgical Pathology Laboratory. California surgical tissues were selected from a residual biorepository of deidentified FFPE tumor blocks from patients diagnosed with malignant melanoma between 1990 and 1999 in Los Angeles County. UNMH surgical tissues were selected from the University of New Mexico Ultraviolet Light Exposure and Immunosuppression in Melanoma biorepository (INST 0815 HRRC 08-433). To serve as a nonpigmented control tissue, we obtained 1 tissue section from breast reduction mammoplasty surgery at UNMH between November 2007 and January 2011 as previously described.^[9] This sample was collected with IRB approval and was deidentified. An experienced Dermatopathologist, Dr Shelly A. Stepenaskie working at Tricore Reference Laboratory, defined regions of interest in the melanoma tissue sections. Images were taken incrementally over the slide to show that the unmixing process is effective across the tissue. Once defined for each tissue section, images were taken sequentially, representing a range of pigmentation and protein levels throughout the tissue.

2.2. Immunohistochemistry (IHC)

We used standard deparaffinization, rehydration, and antigen retrieval procedures. Briefly, we used xylene and ethanol to deparaffinize and rehydrate the tissues. For antigen retrieval, we incubated the tissues in 0.01 M sodium citrate buffer (pH 6.0) in a steamer for 20 minutes, followed by 10 minutes of cooling. Next, we blocked endogenous peroxidase using Peroxo-Block (Life Technologies, Carlsbad, CA).

Off-target antigens were blocked using 3% bovine serum albumin (Sigma, St. Louis, MO). Tissue sections were stained with antibody produced in rabbit and generated against a C-terminus peptide in GPER (clone number 8073) at a dilution of 1:200 for 1 hour and 15 minutes. The GPER antibody was a generous donation from the laboratory of Dr Eric Prossnitz. Tissue sections were then incubated with 1:100 dilution of secondary goat anti rabbit-HRP antibody for 1 hour (Sigma, St. Louis, MO).

HRP activity was visualized using the Liquid DAB Plus Substrate Kit (Life Technologies, Carlsbad, CA) according to manufacturer's instructions. Following incubation with DAB, sections were stained with Azure for 10 minutes^[8] (Sigma, St. Louis, MO). GPER IHC staining achieved by HRP developed with DAB will be referred to as GPER-DAB. For tissues that were stained with Azure-only, the only step of the staining procedure that was eliminated was the addition of primary antibody.

Tissue sections were then dehydrated and mounted with a coverslip using permount (Life Technologies, Carlsbad, CA).

2.3. Image acquisition

Brightfield, spectral images of IHC labeled sections were generated at the UNM Cancer Center Fluorescence Microscopy and Cell Imaging Shared Resource using a Nikon TE2000 microscope (Melville, NY) in transmitted light mode, which had been adjusted for Koehler illumination. Spectral images were obtained using a Nuance Spectral Imaging Camera and software (Perkin Elmer). Images were taken using a $\times 60$ oil objective, at $\times 1.5$ intermediate magnification.

The Nuance camera uses a liquid crystal tunable filter (LCTF), set to collect transmitted light in 20 nm bandwidths, at 10 nm step intervals, from 420 to 720 nm. The resulting spectral image cube consists of 16 separate images each acquired at a different wavelength range. Each pixel in the resulting image cube has an absorbance spectrum that depends on the absorbing materials (labels) that are present at that pixel location. Nuance camera software controls both the LCTF and spectral image acquisition. Prior to imaging of the tissues, a 100% transmission reference image cube was acquired from a region of the slide with no tissue or other debris, and was used to convert all images to optical density images. Regions of interest for each tissue were manually selected in an area of the tissue including the epithelium. Three images were taken sequentially, representing a range of pigmentation and protein levels throughout the tissue.

Spectral image cubes collected from melanoma sections labeled only with Azure or anti-GPER-DAB were used to generate pure absorbance spectra for each of these labels. These pure spectra were then used by the Nuance software to unmix (using a linear unmixing algorithm) image cubes acquired from slides labeled with both Azure and DAB, generating single component images of each label. Absorbance spectrum imaging and unmixing of IHC labeled sections has been previously described.^[10]

2.4. Spectral library development

Three reference spectra (pure absorbance spectra) of Azure were generated from 3 separate patient tissues, each labeled only with Azure. A single reference spectrum of GPER-DAB was generated from a GPER-positive, nonpigmented tissue section labeled only with anti-GPER DAB. To quantify the staining, we generated 3 spectral libraries, each consisting of 2 spectra: GPER-DAB reference spectrum and Azure spectrum from patient tumor section A; GPER-DAB reference spectrum and Azure spectrum from patient tumor section B; GPER-DAB reference spectrum and Azure spectrum from patient tumor section C.

2.5. Spectral image cubes for analysis

Three additional tissue sections from the same patient tumors were doubly labeled with GPER-DAB and Azure and spectral image cubes were acquired with the Nuance camera. These spectral image cubes were unmixed with each of the 3 spectral libraries described above. The Azure-stained component, including melanocytes, melanin, and melanophages, was removed in the unmixing process. The resulting GPER-DAB component images allow for quantification of GPER protein levels in cells throughout the region of interest.

2.6. Analysis of GPER-DAB component images

GPER-DAB component images were exported from the Nuance software as TIF images, imported into Slidebook software 6.0 (3i, Intelligent Imaging Innovations, Denver, CO) and the

GPER-DAB staining was quantified. Component GPER-DAB images were inverted from absorbance to pseudofluorescence for analysis. In the inverted images, higher pseudofluorescence intensity corresponds to higher absorbance (DAB concentration).

A segment mask was created by setting a threshold to eliminate background. Intensity values above the threshold represent DAB labeling. A single threshold level was determined by the examination of multiple images, and was used for quantifying all of the DAB component images. Utilizing the mask statistics function in Slidebook, we exported the mean intensity value of the GPER-DAB component images for statistical analysis.

2.7. Statistics

Mean intensity of anti-GPER-DAB in the 3 sections stained with GPER-DAB and Azure was compared following unmixing with the 3 reference spectral libraries described above, generating 3 mean intensities of GPER-DAB staining for each of the participants. The distribution of mean intensities of GPER-DAB staining did not meet normality assumptions according to the Anderson–Darling test ($P = <.0005$). Therefore, we used the Kruskal–Wallis 1-way analysis of variance (ANOVA) to compare the distributions.

3. Results

3.1. Quantification of GPER-DAB staining in tissues sections with Azure counterstain

Figure 1 shows that the spectral image cube for the tissue doubly stained with GPER-DAB and Azure for participant C (Fig. 1A) can be successfully unmixed using a spectral library (Fig. 1C) generated using the Azure control from participant C and the GPER-DAB only unpigmented control tissue (Fig. 1B). GPER-DAB staining was then quantified from the resulting GPER-DAB component image (Fig. 1D; Table 1). Spectral image cubes for doubly stained tissues were also unmixed for participants A and B (data not shown).

3.2. Azure reference spectra do not appear to differ among tissue sections

Figure 2 shows the 3 Azure reference spectra (blue, green, and red lines) generated from melanoma tissues sections labeled with Azure only from 3 different participants. A GPER-DAB reference spectrum (brown line) was generated in a nonpigmented tissue section stained only with GPER-DAB. Importantly, the 3 Azure spectra overlap and are visually comparable (Fig. 2).

Furthermore, Fig. 3 shows the original image for participant A unmixed using the Azure spectra generated from each participants' Azure-only control (Spectral library A–C, respectively). The resulting GPER-DAB component images appear nearly identical to the eye. Representative hematoxylin and eosin stain (H&E) images for each participant can be seen in Supplemental Figure 2, <http://links.lww.com/MD/B652>.

3.3. Distribution of mean intensities do not vary between participants

The spectral image cube for each participant (A–C) was unmixed with all 3 spectral libraries (depicted for participant A in Fig. 3). This resulted in 3 GPER-DAB component images for each participant (images not shown for participant B and C). An

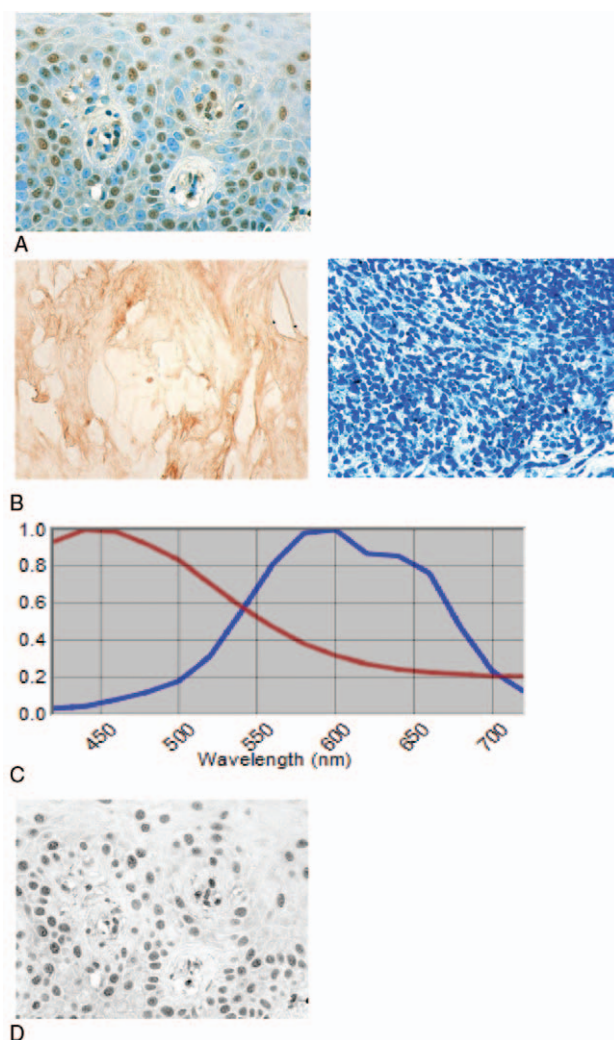


Figure 1. IHC using HRP-DAB with Azure as a counterstain can be unmixed and quantified using spectral imaging. The images presented in this figure, with the exception of the GPER-DAB only control, are from participant C. A, Original image: GPER-DAB and Azure in melanoma section (participant C). This is a single-image representation of a spectral image cube (containing 16 individual images). B, Images used to create spectral library from left to right: nonpigmented tissue from breast reduction mammoplasty surgery stained with GPER-DAB only. Pigmented melanoma section (participant C) stained with Azure only. C, Spectral library developed from images in (B) with GPER-DAB spectral library denoted by red-brown and the blue denoting the Azure spectral library. D, GPER-DAB component image resulting from unmixing of original image in (A) with the spectral library in (C) representing positive staining for GPER. GPER = G protein-coupled estrogen receptor 1, HRP-DAB = horse radish peroxidase developed with 3,3'-Diaminobenzidine, IHC = immunohistochemistry.

additional 22 sections from patients with a range of pigmentation levels were also stained with Azure only and unmixed using multiple Azure Spectral libraries to confirm the robust nature of our methodology but there were no significant differences in the distributions (Supplemental Table 1, <http://links.lww.com/MD/B652>, $P = .76$). Each GPER-DAB component image was analyzed to report the mean intensity of GPER-DAB expression (Table 1). The reported mean intensities within each participant vary slightly depending on the Azure reference spectra used for unmixing. However, there were no significant differences in the distributions (Table 1; $P = .73$).

Table 1

Kruskal–Wallis analysis of mean intensities in melanoma GPER stained tissues.

Melanoma tissues	Mean intensities of DAB-labeled GPER			P value
	Spectral library A*	Spectral library B†	Spectral library C‡	
Section A	1466.82	1420	1392.53	.73
Section B	1508.88	1458.26	1427.57	
Section C	1317.89	1371.52	1409.93	

* Azure spectra generated from Azure-only control matched to melanoma section A.

† Azure spectra generated from Azure-only control matched to melanoma section B.

‡ Azure spectra generated from Azure-only control matched to melanoma section C.

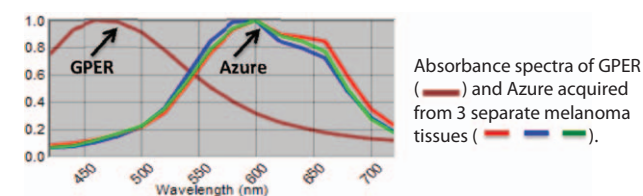


Figure 2. Azure spectra do not vary between melanoma tissue sections. Spectra derived from unpigmented control stained for GPER-DAB (n=1) and Azure-only matched controls for each melanoma lesion stained with GPER-DAB + Azure (n=3). Each image is unmixed with the GPER-DAB spectra and an Azure-only spectra. GPER = G protein-coupled estrogen receptor 1-Diaminobenzidine.

4. Discussion

4.1. Azure-melanin binding mechanism

Selective binding of Azure to melanin is not well characterized in the literature. We aimed to address this gap with a theoretical

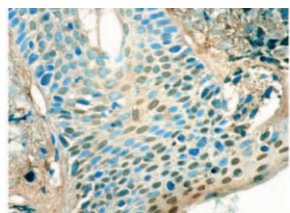
model, to support using Azure as a counterstain in melanoma tissues, particularly in regard to spectral imaging analysis.

Azure, a major metabolite of methylene blue, is a cationic dye.^[11] As a provisional mechanism, we propose that the heterocyclic nitrogen in Azure functions as a base^[12] to deprotonate carboxylic acids found in melanin. This would allow for ionic interactions between melanin's anion and the cations in Azure. Acidic interplay, in conjunction with hydrogen bonding, likely results in the preferential staining of the melanin (Supplemental Figure 1, <http://links.lww.com/MD/B652>).

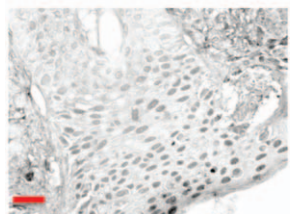
We have shown that using Azure as a counterstain in pigmented tissue sections, such as melanoma tumors, allows for quantification of HRP-DAB staining using spectral imaging techniques. Importantly, this method eliminates inter-reader variability in the evaluation of pigmented sections because unmixing is based on the absorbance spectra of the labels and then quantified, rather than depending on subjective scoring. The clinical utility of this should be explored. Current clinical scoring systems use a simple positive or negative score for stains such as KI-67 or MART-1.^[13] However, intensity of staining may also prove to be an important clinical prognostic indicator.

Another way to obtain good visual contrast in pigmented tissue sections is to develop the HRP substrate with a red chromogen such as 3-amino-9-ethylcarbazole (AEC) or FastRed.^[10] However, eliminating the option of using a brown chromogen to obtain good visual contrast will limit multiplexing potential in a pigmented tissue. If a red chromogen is preferred, our quantification technique is robust, and should allow for AEC staining with Azure counterstain if AEC is the preferred chromogen. Alternatively, without Azure counterstain, reference spectra could be developed using melanin pigment to allow for unmixing and quantification of AEC staining, but this may require a reference spectrum for each tissue since melanin pigment varies from person to person.

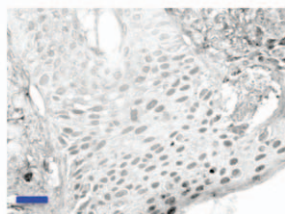
Other approaches, such as color deconvolution, have been developed to address the challenges of multiplexing and



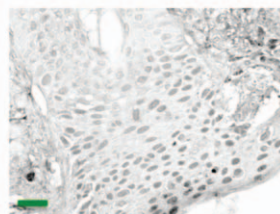
A



Original image A unmixed with Spectral Library A



Original image A unmixed with Spectral Library B



Original image A unmixed with Spectral Library C

B

Figure 3. Component images unmixed with different spectral libraries. A, Original image: GPER-DAB and Azure in melanoma (participant A). This is a single-image representation of a spectral image cube (containing 16 individual images). B, Component images representing GPER-DAB in melanoma: original image (participant A) unmixed with 3 spectral libraries. GPER = G protein-coupled estrogen receptor 1-Diaminobenzidine.

quantification of positive staining.^[14] However, these algorithms require good visual contrast between the enzymatic products, limiting chromogen selection. Additionally, these algorithm approaches address density of staining, but do not address intensity of staining.^[15] Previous studies have successfully paired IHC and spectral imaging, noting that spectral imaging is ideal for multiplexing because it does not require good visual contrast.^[3,10,16]

In this study, we have shown that we can effectively unmix the Azure-stained pigment from DAB-stained protein. Pigment changes from person to person are difficult to unmix without the Azure stain. Therefore, studies without Azure counterstain would be prohibited from using the DAB chromogen, limiting their multiplexing potential. Utilizing Azure as a counterstain eliminates this concern.

There are many difficulties in accurately assessing pigmented skin lesions. The ABCD rule based on the criteria asymmetry (A), border (B), color (C), and differentiation (D) has been shown to improve diagnosis.^[17] However, the use of multiplex IHC staining to clinically stage the lesions is often subjective and cannot be reliably reproduced. This leads to discrepancy in results and interindividual variations. Ki-67, p16, and HMB45 are some examples of stains that are typically utilized in clinical staging. Unfortunately, none of these single markers have been considered accurate enough in isolation to determine clinical staging.^[18] With the additional confounder of pigmentation, we hypothesize that a combination of these markers with Azure staining and spectral imaging for quantification could benefit the pathologists in clinical staging of malignant melanoma. In the future, it would be worthwhile evaluating the potential of tumor markers counterstained with Azure to distinguish between tumor and nontumor in an automated fashion as shown by Fiore et al^[6] 2012 in nonpigmented tissues.

Multiplexed IHC and quantitation of imaging by spectral imaging have been discussed previously and there is evidence that combining these improves analysis from qualitative analysis which is based on visual perception.^[3,4,16] Additionally, the discovery that one Azure reference spectrum can be used for every participant has important implications. First, this will reduce the number of tissue sections needed from each participant, thereby allowing precious samples to be used for additional biomarker testing. Second, it will reduce the amount of time needed to image tissue sections by eliminating the need to image an Azure reference for every participant. This will also improve the unmixing process, as each participant's dual-stained image will be unmixed using the same spectral library, allowing for batch unmixing. One limitation of our study is that we did not investigate melanoma lesions from patients with naturally heavy pigmentation, such as African Americans. Therefore, it remains unknown if one Azure reference image can be used to unmix across racial/ethnic groups.

Although not demonstrated here, this method is applicable in tissues using multiple substrate-chromogen combinations.^[3] Similarly, it can also be useful to quantify colocalization of proteins in tissues. In addition, when multiplexing stains with both a red and a brown chromogen, pigmentation can be very problematic. One additional benefit of the incorporation of our methodology is that Azure B could act as a counter stain with instead of the usual hematoxylin. Therefore, our findings have the potential to impact the field of IHC and biomarker development beyond our focus of pigmented melanoma lesions.

To our knowledge, this is the first study to use spectral imaging to quantify protein expression in pigmented tissue sections. This quantification method reduces the subjectivity and hastens analysis of protein expression detected by IHC in pigmented melanoma lesions, and in broader IHC tissue studies. These improvements in IHC quantification have potential to impact both clinicians and researchers.

Acknowledgments

The authors gratefully acknowledge the support of the UNM Clinical Translational Science Center staff, particularly Susan Tigert and Amy Overby. Images in this paper were generated in the University of New Mexico and Cancer Center Fluorescence Microscopy Shared Resource, funded as detailed at: <http://hsc.unm.edu/crtc/microscopy/acknowledgement.shtml>. The authors appreciate the generous donation of the GPER antibody from Dr. Eric Prossnitz laboratory, particularly assistance received from Dr. Natalie Fredette. They also appreciate the generous donation of melanoma tissue sections from USC, as well as the donation of the mammoplasty tissue from the laboratory of Dr. Helen Hathaway with assistance from Tamara Howard. They appreciate the expert guidance by Dr. Shelly A. Stepenaskie from Tricore Reference laboratory.

References

- [1] Matos LL, Truffelli DC, de Matos MG, et al. Immunohistochemistry as an important tool in biomarkers detection and clinical practice. *Biomark Insights* 2010;5:9–20.
- [2] Brandtzaeg P. The increasing power of immunohistochemistry and immunocytochemistry. *J Immunol Methods* 1998;216:49–67.
- [3] Stack EC, Wang C, Roman KA, et al. Multiplexed immunohistochemistry, imaging, and quantitation: a review, with an assessment of Tyramide signal amplification, multispectral imaging and multiplex analysis. *Methods* 2014;70:46–58.
- [4] van der Loos CM. Multiple immunoenzyme staining: methods and visualizations for the observation with spectral imaging. *J Histochem Cytochem* 2007;56:313–28.
- [5] Bauman TM, Ricke EA, Drew SA, et al. Quantitation of protein expression and co-localization using multiplexed immuno-histochemical staining and multispectral imaging. *J Vis Exp* 2016;110:e53837–153837.
- [6] Fiore C, Bailey D, Conlon N, et al. Utility of multispectral imaging in automated quantitative scoring of immunohistochemistry. *J Clin Pathol* 2012;65:496–502.
- [7] Chromogens in Multiple Immunohistochemical Staining Used for Visual Assessment and Spectral Imaging: The Colorful Future. Available at: <http://www.nsh.org/sites/default/files/March2010Article.pdf>. Accessed July 28, 2015.
- [8] 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;18:436–9.
- [9] Scaling AL, Prossnitz ER, Hathaway HJ. GPER mediates estrogen-induced signaling and proliferation in human breast epithelial cells and normal and malignant breast. *Horm Cancer* 2014;5:146–60.
- [10] Levenson RM, Mansfield JR. Multispectral imaging in biology and medicine: slices of life. *Cytometry A* 2006;69:748–58.
- [11] Horobin RW. How Romanowsky stains work and why they remain valuable—including a proposed universal Romanowsky staining mechanism and a rational troubleshooting scheme. *Biotech Histochem* 2011;86:36–51.
- [12] Sipponen MH, Pihlajaniemi V, Littunen K, et al. Determination of surface-accessible acidic hydroxyls and surface area of lignin by cationic dye adsorption. *Bioresour Technol* 2014;169:80–7.
- [13] Rothberg BEG, Bracken MB, Rimm DL. Tissue biomarkers for prognosis in cutaneous melanoma: a systematic review and meta-analysis. *J Natl Cancer Inst* 2009;101:452–74.
- [14] Ruifrok AC, Johnston DA. Quantification of histochemical staining by color deconvolution. *Anal Quant Cytol Histol* 2001;23:291–9.

- [15] Onder D, Zengin S, Sarioglu S. A review on color normalization and color deconvolution methods in histopathology. *Appl Immunohistochem Mol Morphol* 2014;22:713–9.
- [16] Taylor CR, Levenson RM. Quantification of immunohistochemistry—issues concerning methods, utility and semiquantitative assessment II. *Histopathology* 2006;49:411–24.
- [17] Nachbar F, Stolz W, Merkle T, et al. The ABCD rule of dermatoscopy. High prospective value in the diagnosis of doubtful melanocytic skin lesions. *J Am Acad Dermatol* 1994;30:551–9.
- [18] Uguen A, Talagas M, Costa S, et al. A p16-Ki-67-HMB45 immunohistochemistry scoring system as an ancillary diagnostic tool in the diagnosis of melanoma. *Diagn Pathol* 2015;10:195.