JOURNAL OF INTERDISCIPLINARY HISTOPATHOLOGY, 2018
VOL 6, NO. 2, PAGE 26–32
10.5455/jihp.20180528101354

eJManager

ORIGINAL RESEARCH

Open Access

The effect of antigen retrieval buffers on MALDI mass spectrometry imaging of peptide profiles in skin FFPE tissue

Pumza S. Magangane[1], Nonhlanhla P. Khumalo[2], Henry A. Adeola[2]

[1]Department of Anatomical Pathology, Faculty of Health Sciences, University of The Witwatersrand, Parktown, Johannesburg, South Africa

[2]Hair and Skin Research Laboratory, Division of Dermatology, Department of Medicine, Faculty of Health Sciences and Groote Schuur Hospital, University of Cape Town, Cape Town, South Africa

ABSTRACT

Objective: The success of novel *in situ* Immunohistoproteomic approaches that employ matrix-assisted laser desorption and ionization mass spectrometry imaging (MALDI-MSI) heavily depends on good antigen retrieval efficacy.

Methods: We have optimized the effect of various antigen retrieval buffers on peptide profiles acquired from formalin-fixed paraffin-embedded traction alopecia scalp tissue. Heat-induced epitope retrieval (HIER) was performed using citrate, Tris, and Tris-EDTA buffers under various conditions. MALDI-MSI analysis of the tissue was performed using a high-throughput, state-of-the-art RapiFlex MALDI TOF/TOF tissue-typer equipped with a smart beam laser.

Results: Background matrix peaks were found under most conditions except under Tris-EDTA buffer. Trypsin digestion periods longer than overnight and possibly higher concentrations are needed for citrate and Tris. The Tris-EDTA buffer performed best for MALDI-MSI antigen retrieval at an incubation period of overnight at 60°C.

Conclusions: We have identified in this study that antigen retrieval conditions need to be optimized prior to the MALDI-MSI analysis of different tissue types.

ARTICLE HISTORY

Received 28 May 2018
Accepted 19 June 2018
Published 27 June 2018

KEYWORDS

Skin; MALDI-MSI; antigen retrieval; buffer; mass spectrometry; FFPE

Introduction

Heat-induced epitope retrieval (HIER) as an antigen retrieval method allows for the detection of various proteins in formalin-fixed paraffin-embedded (FFPE) archival tissue [1–3]. FFPEs are stable for long periods because of crosslinking that is usually due to fixation *via* methylol adducts and methylene bridges [4]. Although the underlying mechanism for antigen retrieval is poorly understood, Vollert et al. [5], hypothesized that formaldehyde scavenging plays a pivotal role in epitope unmasking. The HIER technique has been known to produce better signals and has been most commonly used [6].

The success of HIER is time, pH, and temperature dependent [7]. Heat induces reversal of protein chemical modifications resulting from formalin fixation [8]. Time and heat are inversely correlated, in which higher temperatures need less time for reversal, and *vice versa* [8]. Not least, the type of buffer used is also an important factor in the success of antigen retrieval. The most common buffers available are: 0.01 M citrate buffer, pH 6.0; Tris buffer, pH 9.5–10.0; and 0.001 M Ethylenediaminetetraacetic acid (EDTA), pH 8 [1]. Prior to the use of HIER, proteolytic digestion (using enzymes such as trypsin, DNase, and proteinase K) breaks protein cross-linkages that have been commonly employed for antigen retrieval [8]. It has been noted that a combination of HIER with sodium citrate is the most commonly used protocol [5]. However, falling off of tissue from slide and tissue damage are common problems encountered during direct boiling of tissues in buffer [9]. Samples may still fall off, even after taking care of other factors such as improper sectioning and drying, insufficient fixation, unclean slides, and poor adherence, which are known to contribute to

Contact Henry A. Adeola ✉ henry.adeola@uct.ac.za Division of Dermatology, Department of Medicine, Faculty of Health Sciences and Groote Schuur Hospital, University of Cape Town, Cape Town, South Africa.

FFPE sample fall off and damage [9]. These factors require consideration prior to proteomic analysis of FFPE samples with matrix-assisted laser desorption and ionization mass spectrometry imaging (MALDI-MSI).

Protocols for imaging FFPE samples via MALDI-MSI have been reported for citrate buffer [10,11], Tris buffer [12,13], and enzymatic digestion [14] only. There are limited studies that compare MALDI imaging profiles of FFPE skin tissue sections under various antigen retrieval buffer conditions. The previous MALDI-MSI analyses on skin were using frozen samples that required pre-analytical washes in order to remove impurities [15–17]. The study by Taverna et al. [18] is the only available study performed on skin FFPE sections. The researchers performed antigen retrieval with Tris buffer pH 8.0 at high temperatures. They did, however, dry the sections for longer periods (overnight) at 60°C after sectioning to prevent detachment. Hence, the aim of this study is to determine and optimize the most suitable antigen retrieval buffer (using HIER method) for FFPE samples that are intended for MALDI-MSI.

Material and Methods

Study samples and ethical clearance

Traction alopecia scalp biopsies were obtained with ethical approval from the Faculty of Health Sciences, University of Cape Town (HREC REF# 398/2012). Tissues were processed into FFPE and were exposed to various antigen retrieval buffers under various experimental conditions.

FFPE slide section preparation

Chemical fixation of tissue samples in 10% phosphate-buffered formalin solution for a period of 24 hours was the routine histological sample collection protocol employed. The samples were then passed through an increasing concentration of ethanol (70%, 96%, and 100%), followed by 3-xylene washes at 40°C. Subsequently, the tissue was immersed in molten paraffin and cooled for 1 hour using an ice plate, before storage. For our study, serial 10 µM FFPE sections were cut from a single formalin-fixed paraffin wax embedded traction alopecia tissue block. These sections were place on indium tin oxide (ITO) coated glass slide (Sigma Aldrich, St. Louis, MO, USA), with square surface resistivity of 8–12 Ω/sq. The glass slides were then dewaxed using repeated (×3) immersion in xylene for 3 minutes followed by rehydration in decreasing alcohol concentrations. Considering the volatility and toxicity of these solvents, all processes were carried out in an extracted Class II biosafety cabinet.

Antigen retrieval

The FFPE tissue sections that were placed on poly-L-lysine and ITO-coated slides were then dewaxed and cleared prior to boiling in the appropriate buffer (10 mM citric acid (pH 6), 10 mM Tris (pH 8), and 10 mM Tris-1 mM EDTA (pH 9)) in a pressure cooker for 90 seconds as well as at 60°C overnight. These slides were then allowed to stand and cool for another 20 minutes before washing with distilled water. Controls were samples digested with trypsin as well as undigested samples.

Sample digestion for MALDI-MSI

Antigen-retrieved tissue sections were left to dry at room temperature. The digestion solution (tissue culture grade trypsin solution containing 50 mM ammonium bicarbonate, pH 8 and 5 ng/µL in 50% acetonitrile) was aerosolized into ca. 20 µM droplets and automatically sprayed on the tissue using optimized trypsin deposition settings on a TM-Sprayer system (HTX Technologies, LLC, Carrboro, NC, USA). The solvent flow rates, nozzle velocity, temperature, and number of passes were methodically altered and optimized to get a standardized spray method. Ten coatings were applied for a spray duration of 15 seconds and a drying time of 30 seconds between each coating. The slide containing the digested tissue was then incubated at 100% humidity for 15 hours at 37°C.

MALDI-MSI

The digested tissues were coated with matrix (α-cyano-4-hydroxycinnamic acid (HCCA) at 7 g/L in 60% acetonitrile and 0.2% trifluoroacetic acid) using optimized HCCA settings on a TM-Sprayer system (HTX Technologies, LLC, Carrboro, NC, USA). The matrix solution was sonicated for 5 minutes and centrifuged at 10,000 × g for 8 minutes before transferring it to the TM-Sprayer system. Bruker peptide calibration standard II was used for external calibration. For accurate positioning on the Bruker MALDI plate adaptor for glass slides (MTP Slide Adaptor II), high definition image of the slide was acquired using a slide scanner (MF500 Reflecta High-Resolution Tissue Scanner). The Imaging analysis of the tissue was performed by rastering at 200

× 200 μm2 pixel sizes. MALDI-MSI was performed using a RapiFlex MALDI Time of Flight (TOF)/TOF tissue typer (Bruker) equipped with a smart beam laser and controlled by the FlexControl 3.0 software package. The mass spectrometer was operated using positive polarity in reflectron mode and spectra were acquired in the range of m/z 600–3,500. MALDI parameters were set as follows: 8.00 kV lens voltage, 25–22.45 kV acceleration voltage, 13.35 kV final acceleration, and 26.71 kV reflector voltage, with up to 400 Da suppression. The folded regions of the tissues were not imaged.

Spectra acquisition

Spectra acquisition of the spotted arrays was carried out using the FlexImaging 2.0 (Bruker Daltonics) software package. The spectra were acquired by firing a 337 nm laser (20 μM laser spot size) at a rate of 50 shots per raster spot. A mass spectrum was acquired for each set of spatial coordinates based on 2,500 shots per pixel area accumulated in a random walk mode. A total of 1,600 spectra were acquired at each spot position in a customized spiral raster pattern in 200-shot increments at a laser frequency of 200 Hz. The customized raster pattern was used to sample the entire spot area. Acquired spectra were baseline-corrected and analyzed in flexAnalysis 3 (Bruker Daltonics). Ion images were assembled using the FlexImaging 2.0 software package.

Results

During our initial HIER optimization (dewaxing FFPE samples and boiling for 90 seconds), we observed that irrespective of the buffer system (citrate, pH 6; Tris, pH 8; and Tris-EDTA, pH 9) used, the tissue sections fell off from the glass slides during or after boiling. We observed that HEIR using citric acid buffer (pH 6) caused tissue damage and loss after 3 hours of digestion at 37°C. Therefore, we only proceeded with Tris and Tris-EDTA which could withstand 60°C overnight. We optimized by reducing the temperature (between 30°C and 80°C), but incubating for longer periods (1 hour to overnight). This approach was found to be better than boiling for 90 seconds. We observed that Tris and Tris-EDTA buffers were optimal at 60°C overnight, and this did not lead to loss of tissue or falling off the glass slide.

The presence of background peaks was found in samples that were measured directly on the RapiFlex instrument without proteolysis, antigen retrieval method, or matrix coating (Fig. 1). Similar trends were found for samples that were digested without carrying out the antigen retrieval step (Fig. 2a). The background matrix peaks interfered with the few peptide signals present. For samples that were digested and antigen retrieved using the Tris buffer (pH 8) at 60°C overnight, we observed that there were also background peaks, and poor peptide intensity during MALDI-MSI (Fig. 2b). We identified good peptide signals and increased intensity in matrix-coated samples that were digested and antigen retrieved using the Tris-EDTA (pH 9) buffer system. There was minimal background matrix signal and the signal-to-noise ratio was higher for samples that were antigen retrieved using Tris-EDTA (pH 9) (Fig. 2c).

Discussion

We investigated the efficacy of different antigen retrieval buffer using HIER for skin FFPE samples that are intended to be analyzed on an MALDI-MSI instrument. Furthermore, we identified the best buffer system and condition for good antigen retrieval for FFPE samples. Due to the tendency for tissue damage or falling off during HIER optimization (for most buffer systems), a study has described an improved HIER method that involves the use of overlapping another plain glass slide with an intervening gap between the slide and the tissue [9]. In our study, we eliminated this problem by reducing the temperature and incubating for longer periods.

As found in the samples that were measured directly on the RapiFlex instrument without proteolysis, antigen retrieval method, or matrix coating, background matrix peaks have been found to interfere with good signals of small molecules/drugs (<1,000 Da) during MALDI TOF analysis [19–21]. However, alteration of the type of matrix applied can improve the signal [19,20]. Also as found here, background matrix peaks tend to affect samples processed without an antigen retrieval step, leading to fewer peptide signal present. It is established that antigen retrieval step is very essential for MALDI-MSI because it frees the proteins from formaldehyde, for detection [5]. In addition, antigen retrieval improves the number of peptide signals as well as the intensity of the signals [22]. We found poor peptide signals and background peaks signals in FFPE sample antigen retrieved in Tris buffer (pH 8) at 60°C overnight. Wiśniewski (2013) optimized Tris buffer (pH 8.0) for digestion of FFPE samples using the filter-aided sample preparation workflow [23]. Tris-EDTA buffer (pH

9) has previously been successfully used for antigen retrieval (at 95°C for 20 minutes) and high throughput analysis of a tissue microarray construct [24,25]. Similarly, we found good peptide signals and increased intensity in samples that were digested and antigen retrieved using the Tris-EDTA (pH 9) buffer system. Efficacious antigen retrieval has always been a very crucial step for getting good *in situ* spatial distribution of peptides on FFPE slide tissues. We investigated the efficacy of different antigen retrieval buffer using HIER for skin FFPE samples that are intended to be analyzed on an MALDI-MSI instrument. Furthermore, we identified the best buffer system and condition for good antigen retrieval for FFPE samples.

In conclusion, FFPEs represent a readily available treasure chest for genomic and proteomic materials that can shed better light on the pathogenesis of various disease. The advent of new high-end instruments further improves that depth of information that can be acquired from FFPEs to an extent that was never thought possible. Here,

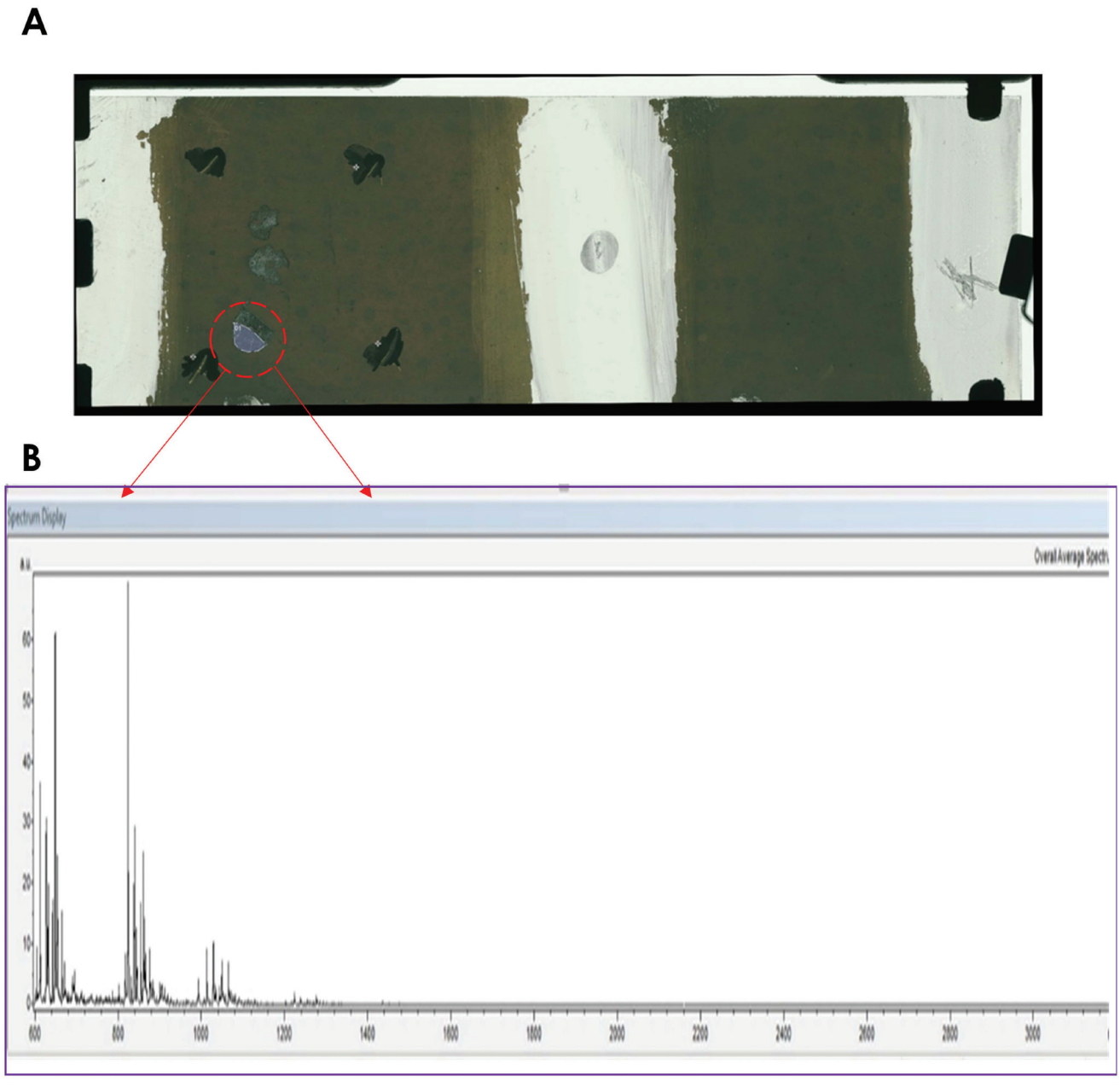

Figure 1. Background matrix peaks in FFPE skin samples. Matrix was applied without antigen retrieval or tissue digestion (a). Spectra were collected from the largest piece of tissue on the glass slide (Broken red circle) and spectra were acquired using the RapiFlex MALDI-TOF/TOF instrument. Background matrix peaks were found on the spectra generated (b).

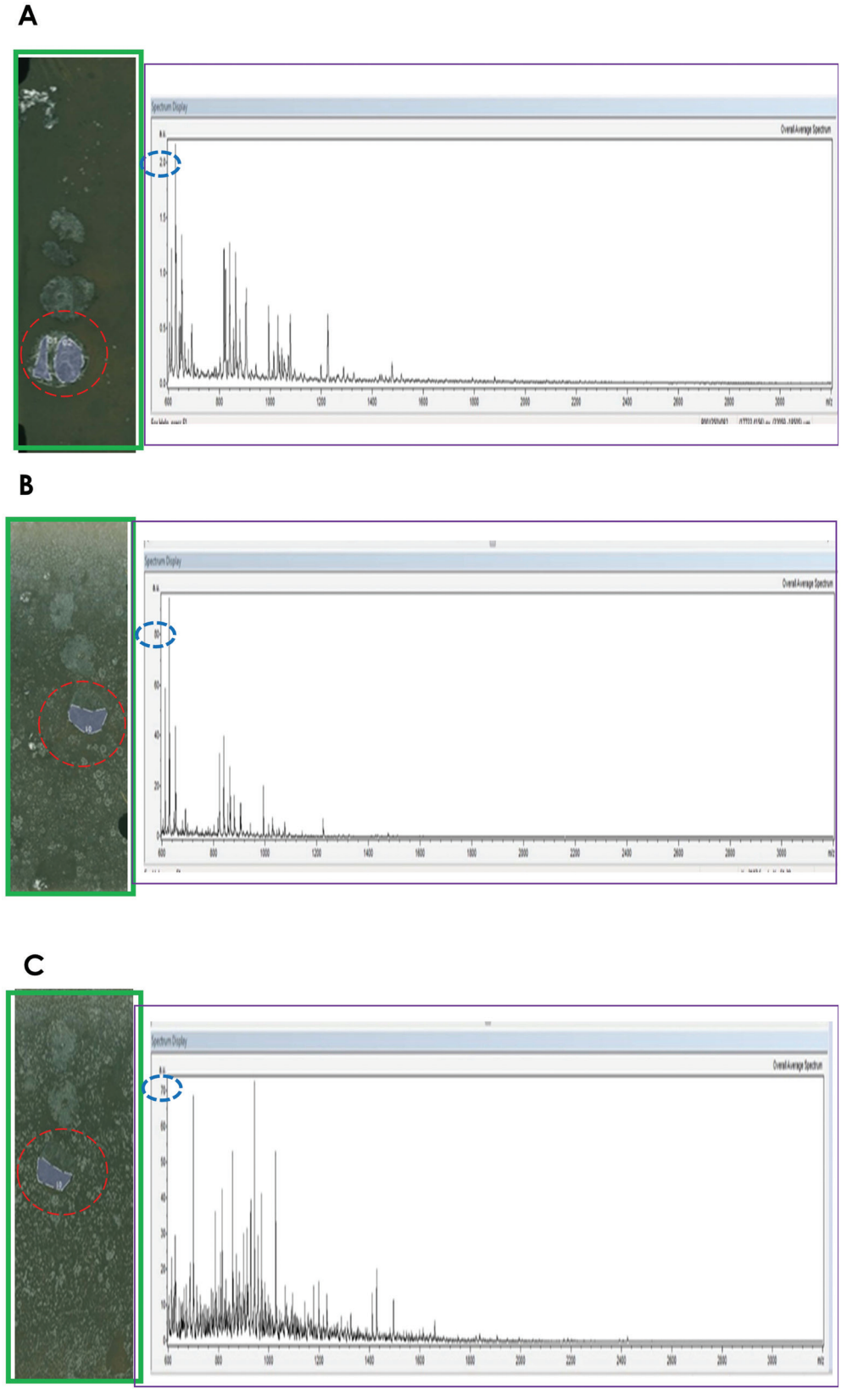

Figure 2. Prepared slides for MALDI-MSI and spectra for different antigen retrieval buffers. Background matrix peaks were found for samples that were digested without carrying out an antigen retrieval step (a). Peptide signals were very low due to matrix signal interference (Broken blue circle on *Y*-axis). For samples that were digested and antigen retrieved using Tris buffer (pH 8) at 60°C overnight, we observed that there was limited extraction of peptides and we could observe poor peptide signal due to background matrix peak interference during MALDI-MSI (b). Tris-EDTA buffer (pH 9) demonstrated the best-extracted peptide signals with minimal background matrix peaks (c).

we have highlighted the need to determine the best antigen retrieval method prior to MALDI-MSI analysis of FFPE tissues. A limitation of our study is that the experiment was only performed on a single type of tissue. The MALDI-MSI analysis is poised to reveal a robust detail of *in situ* differential proteomic profiles of FFPE tissue samples in the future, and hence, selecting the most appropriate antigen retrieval methods is essential.

Acknowledgments

HAA thanks the South African Medical Research Council for a mid-career scientist research fellowship. NPK thanks the SAMRC and the National Research Foundation South African Research Chair Initiative.

Authors' Contributions

PSM designed the study, performed experiments, and prepared the manuscript. HAA and NPK were involved in the conceptualization and design of the study, prepared the manuscript and figures, and critically reviewed the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

[1] Tacha D, Teixeira M. History and overview of antigen retrieval: methodologies and critical aspects. J Histotechnol 2002; 25:237–42.
[2] Shi SR, Shi Y, Taylor CR. Antigen retrieval immunohistochemistry: review and future prospects in research and diagnosis over two decades. J Histochem Cytochem 2011; 59:13–32.
[3] Krenacs L, Krenacs T, Stelkovics E, Raffeld M. Heat-induced antigen retrieval for immunohistochemical reactions in routinely processed paraffin sections. Methods Mol Biol 2010; 588:103–19.
[4] Adeola H, Goosen RW, Godlberg P, Blackburn J. Prospects of omics based molecular approaches in colorectal cancer diagnosis and treatment in the developing world: a case study in Cape Town, South Africa. In: Khan DJS (ed). Colorectal cancer—surgery, diagnostics and treatment, Vol. 1, InTech, London, pp. 1–41, 2014.
[5] Vollert CT, Moree WJ, Gregory S, Bark SJ, Eriksen JL. Formaldehyde scavengers function as novel antigen retrieval agents. Sci Rep 2015; 5:17322.
[6] O'Hurley G, Sjostedt E, Rahman A, Li B, Kampf C, Ponten F, et al. Garbage in, garbage out: a critical evaluation of strategies used for validation of immunohistochemical biomarkers. Mol Oncol 2014; 8:783–98.
[7] Paulsen IM, Dimke H, Frische S. A single simple procedure for dewaxing, hydration and heat-induced epitope retrieval (HIER) for immunohistochemistry in formalin fixed paraffin-embedded tissue. Eur J Histochem 2015; 59:2532.
[8] Leong TYM, Leong ASY. How does antigen retrieval work? Adv Anat Pathol 2007; 14:129–31.
[9] Vinod KR, Jones D, Udupa V. A simple and effective heat induced antigen retrieval method. MethodsX 2016; 3:315–9.
[10] Schwamborn K, Caprioli RM. MALDI imaging mass spectrometry—painting molecular pictures. Mol Oncol 2010; 4:529–38.
[11] Jeon YE, Lee SC, Paik SS, Lee KG, Jin SY, Kim HR, et al. Histology-directed matrix-assisted laser desorption/ionization analysis reveals tissue origin and p53 status of primary liver cancers. Pathol Int 2011; 61:449–55.
[12] Casadonte R, Caprioli RM. Proteomic analysis of formalin-fixed paraffin-embedded tissue by MALDI imaging mass spectrometry. Nat Protoc 2011; 6:1695–709.
[13] Mainini V, Lalowski M, Gotsopoulos A, Bitsika V, Baumann M, Magni F. MALDI-imaging mass spectrometry on tissues. Methods Mol Biol 2015; 1243:139–64.
[14] Magangane P, Sookhayi R, Govender D, Naidoo R. Determining protein biomarkers for DLBCL using FFPE tissues from HIV negative and HIV positive patients. J Mol Histol 2016; 47:565–77.
[15] Enthaler B, Bussmann T, Pruns JK, Rapp C, Fischer M, Vietzke JP. Influence of various on-tissue washing procedures on the entire protein quantity and the quality of matrix-assisted laser desorption/ionization spectra. Rapid Commun Mass Sp 2013; 27:878–84.
[16] Hart P, Francese S, Woodroofe MN, Clench MR. Matrix assisted laser desorption ionisation ion mobility separation mass spectrometry imaging of ex-vivo human skin. Int J Ion Mobil Spec 2013; 16:71–83.
[17] Harvey A, Cole LM, Day R, Bartlett M, Warwick J, Bojar R, et al. MALDI-MSI for the analysis of a 3D tissue-engineered psoriatic skin model. Proteomics 2016; 16:1718–25.
[18] Taverna D, Pollins AC, Nanney LB, Sindona G, Caprioli RM. Histology-guided protein digestion/extraction from formalin-fixed and paraffin-embedded pressure ulcer biopsies. Exp Dermatol 2016; 25:143–6.
[19] Hrabak J, Chudackova E, Walkova R. Matrix-assisted laser desorption ionization-time of flight (maldi-tof) mass spectrometry for detection of antibiotic resistance mechanisms: from research to routine diagnosis. Clin Microbiol Rev 2013; 26:103–14.

[20] Penno MA, Ernst M, Hoffmann P. Optimal preparation methods for automated matrix-assisted laser desorption/ionization time-of-flight mass spectrometry profiling of low molecular weight proteins and peptides. Rapid Commun Mass Spectrom 2009; 23:2656–62.
[21] Hrabak J, Walkova R, Studentova V, Chudackova E, Bergerova T. Carbapenemase activity detection by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 2011; 49:3222–7.
[22] Djidja MC, Claude E, Scriven P, Allen DW, Carolan VA, Clench MR. Antigen retrieval prior to on-tissue digestion of formalin-fixed paraffin-embedded tumour tissue sections yields oxidation of proline residues. Biochim Biophys Acta 2017; 1865:901–6.
[23] Wisniewski, JR., Proteomic sample preparation from formalin fixed and paraffin embedded tissue. *Journal of visualized experiments : JoVE* 2013.
[24] Fowler CB, O'Leary TJ, Mason JT. Toward improving the proteomic analysis of formalin-fixed, paraffin-embedded tissue. Expert Rev Proteomics 2013; 10:389–400.
[25] Groseclose MR, Massion PP, Chaurand P, Caprioli RM. High-throughput proteomic analysis of formalin-fixed paraffin-embedded tissue microarrays using MALDI imaging mass spectrometry. Proteomics 2008; 8:3715–24.