



APPLICATION NOTE

An Alternative Paper Based Tissue Washing Method for Mass Spectrometry Imaging: Localized Washing and Fragile Tissue Analysis

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Abstract

Surface treatment of biological tissue sections improves detection of peptides and proteins for mass spectrometry imaging. However, liquid surface treatments can result in diffusion of surface analytes and fragile tissue sections can be easily damaged by typical washing solvents. Here, we present a new surface washing procedure for mass spectrometry imaging. This procedure uses solvent wetted fiber-free paper to enable local washing of tissue sections for mass spectrometry imaging and tissue profiling experiments. In addition, the method allows fragile tissues that cannot be treated by conventional washing techniques to be analyzed by mass spectrometry imaging.

Key words: Imaging Mass Spectrometry, Washing, Peptides and Proteins, MALDI, SIMS

Introduction

The quality of mass spectrometry (MS) imaging results depends heavily on the choice and execution of sample preparation methods. Specific molecular classes often require different sample preparation treatments, such as optimized washing steps and the correct choice of matrix for matrix assisted laser desorption ionization (MALDI). Several washing methods have been tested, optimized, and have become standard elements in many MS imaging workflows.[1–5] Popular washing methods include immersion of the sample in washing solution or pipette application of the solution onto the sample and subsequent removal by pipette. However, such washing steps often lead to undesired diffusion of molecules over the tissue surface. Delicate biological sections can also be severely damaged when washed with harsh solvents.

Blotting techniques have been applied to transfer intact proteins from biological sections onto polyvinylidene difluoride (PVDF) membranes, which are then measured in an indirect imaging experiment [6–8]. The “molecular

scanner” has been used to extract and digest proteins from 1D and 2D SDS-PAGE gels and from tissues onto capture membranes, while keeping their spatial organization [9–11].

Here, we present a surface cleaning method which also relies on the contact between biological material and a wetted fiber-free paper. Unlike the above-mentioned blotting techniques, here the biological tissue is directly analyzed by a MS imaging experiment. The solutions used in the surface cleaning are the same solutions already established for MS imaging sample preparation [1–5, 12]. The use of paper for surface cleaning allows its application on selected sample areas, allowing the simultaneous comparison of washed and unwashed areas on a single tissue sample. This gentle method allows surface cleaning of fragile biological samples, which cannot be washed by pipette or immersion methods.

Experimental

Rat heart and rat brain from male rats (type WU) and mouse brain from a female mouse (type 9CFW-1) were purchased from Harlan Laboratories (Boxmeer, The

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Netherlands). Rat heart and brain were sectioned at 10 μm thickness on a cryo-microtome (Microm International, Walldorf, Germany) and thaw mounted on indium-tin-oxide coated glass slides (ITO, 4–8 Ω resistance; Delta Technologies, Stillwater, MN, USA). Adjacent 12 μm thick mouse brain sections were collected for comparison of washing methods and thaw mounted on ITO slides. All samples were stored at $-80\text{ }^\circ\text{C}$ prior to use and dried in a vacuum desiccator for 30 min prior to washing, matrix coating, and MS analysis.

Fiber Free Paper Washing Procedure

The fiber-free paper washing procedure uses a common laboratory wipe (KIMTECH SCIENCE* Precision Wipes; Kimberly-Clark, Roswell, GA, USA), which is wetted with the wash solvent of choice and carefully placed on top of the tissue section. The paper is cut to a size slightly larger than the tissue section to allow for full coverage, or can be cut to any size for localized washing. Typically, a solvent volume of 100–200 μL is sufficient to completely wet the paper with a pipette. Alternatively, the papers can be soaked in the wash solvent before application, though care should be taken that the paper is

not too saturated, as this can cause flooding of the tissue section. Contact time is typically 30–60 s, and depends on the washing type and solvent of choice. Unlike submersion and pipette washing methods, volatile washing solvents (such as alcohols) will evaporate from the paper, which does not allow extensive contact periods (>60 s). Care should be taken when removing the wetted paper from the tissue section in order to reduce smearing effects from the application and lifting process.

Rat heart was washed twice with fiber-free paper wetted with 70% cold ethanol, each for 1 minute. The paper used to wash the rat heart was saved and imaged by metal enhanced secondary-ion mass spectrometry (MetA-SIMS). A rat brain section was locally washed with paper strips wetted with 70% cold ethanol for 1 minute per strip. A mouse brain section was washed with isopropanol (70% and 95%, in H_2O ; 30 s each) for comparison with conventional washing procedures for the enhancement of protein signals.

Immersion Washing Procedure

The immersion washing procedure was adapted from Seeley et al. [4]. The ITO slide with the tissue section was immersed in a bath of the wash solvent of choice in a Petri

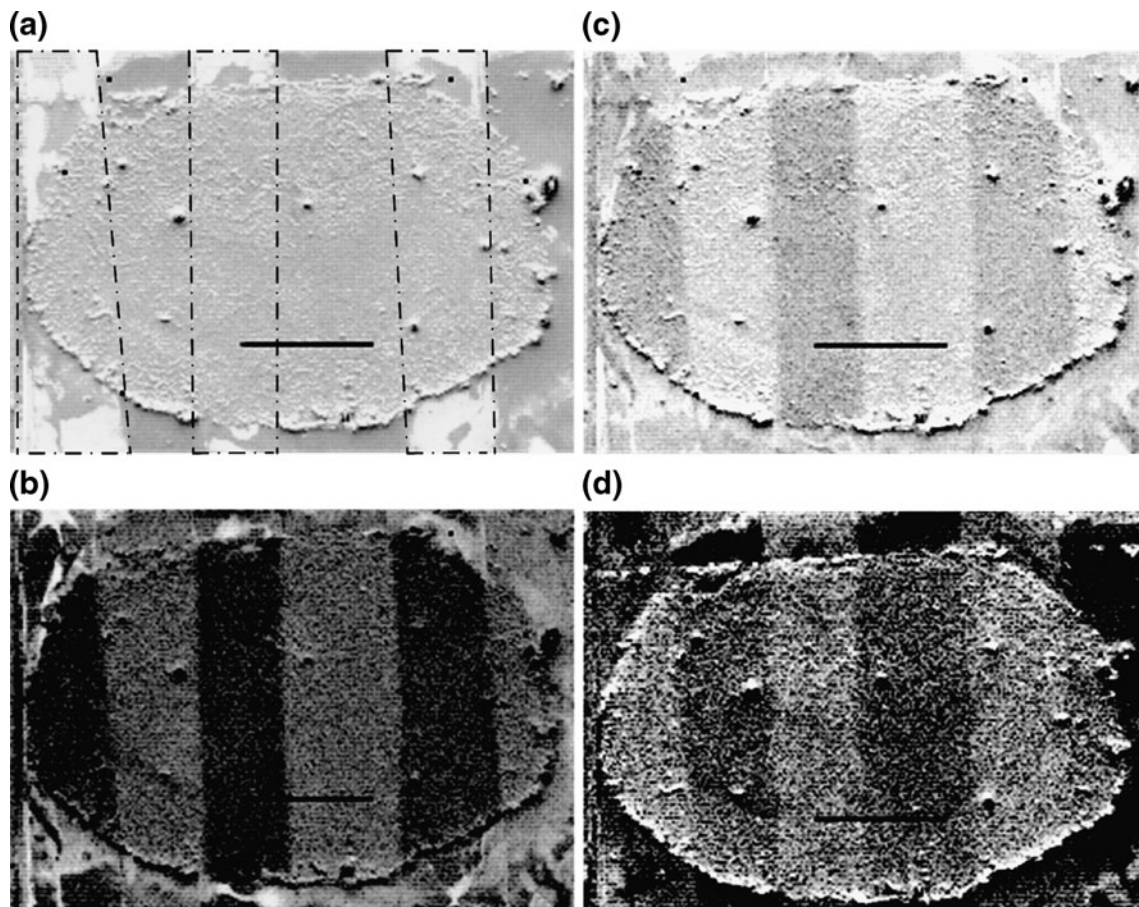


Figure 1. MetA-SIMS MS imaging experiment of a selectively washed rat brain section. The areas enclosed by the dotted lines in **(a)** indicate the areas selectively washed by wetted strips of paper. **(a)** Total ion image, **(b)** Na^+ (m/z 22.9), **(c)** K^+ (m/z 38.9), and **(d)** phosphocholine (m/z 184). Scale bar = 1 cm

dish (typically 50 mL solvent). No swirling or mixing was performed. A mouse brain section was immersed in isopropanol (70% and 95%, in H₂O; 30 s each) for comparison with wetted paper and pipette washing. A mouse brain section spotted with permanent markers was immersed in 70% ethanol (in H₂O) for 60 s to assess the degree of diffusion of small molecules.

Pipette Washing Procedure

The pipette washing procedure was adapted from Lemaire et al. [2]. A volume of $\sim 200 \mu\text{L}/\text{cm}^2$ was loaded into a pipette and rinsed over the tissue section while holding the ITO slide at an angle to allow the solvent to efficiently drain. A mouse brain section was rinsed with isopropanol (70% and

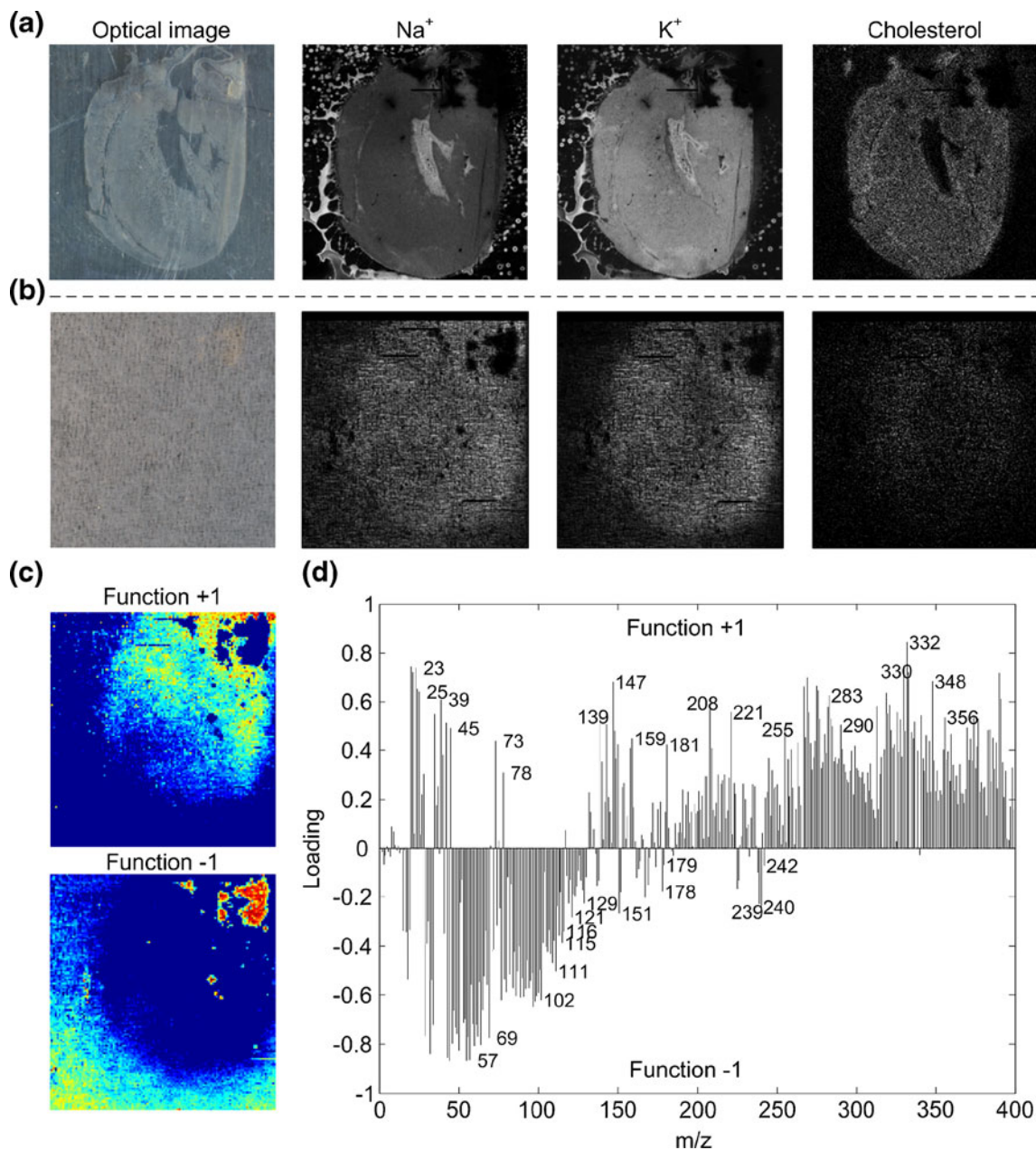


Figure 2. MetA-SIMS of a rat heart and the cleaning paper from a 70% ethanol treatment. **(a)** Ion selected images from the rat heart and **(b)** corresponding images of the same species from the washing paper. **(c)** Image of the first principal component of the MetA-SIMS spectra acquired from the paper used to wash the heart tissue and **(d)** the corresponding loading spectrum. Dominant species from the area in contact with the rat heart section show removal of salt cations, silicone contaminants such as polydimethylsiloxane, m/z 73 ($\text{C}_3\text{H}_9\text{Si}^+$) and m/z 147 ($\text{C}_5\text{H}_{15}\text{OSi}_3^+$), and a mounting compound (Optimal Cutting Temperature compound) related peak at m/z 332

95%, in H₂O; 200 μ L each) for comparison with wetted paper and immersion washing.

Metal Deposition

For MetA-SIMS, a 1 nm gold layer was sputter-coated onto the locally washed rat brain section and the paper used to wash the heart section following the protocol described in reference [3].

Matrix Deposition

Sinapinic acid (25 mg/mL in 50% acetonitrile/0.1% trifluoroacetic acid) was used for protein MS imaging of the mouse brain sections washed with isopropanol. The matrix was applied using a vibrational spray coater (ImagePrep; Bruker Daltonics, Bremen, Germany). For the comparison of diffusion for small molecules on mouse brain tissues

subjected to different washing procedures, α -cyano-4-hydroxycinnamic acid (10 mg/mL in 50% acetonitrile/0.1% trifluoroacetic acid) was used and applied using a vibrational spray coater, as described above.

MS Imaging

SIMS experiments were performed on a Physical Electronics (Eden Prairie, MN, USA) TRIFT II time-of-flight secondary ion mass spectrometer with an Au¹⁺ primary ion beam. The data acquisition and visualization were performed by WinCadence ver. 4.4.0.17

MALDI experiments were performed on an Ultraflex III time-of-flight mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany), with an accumulation of 1000 shots per position for proteins and 700 shots for small molecules with a raster size of 200 μ m.

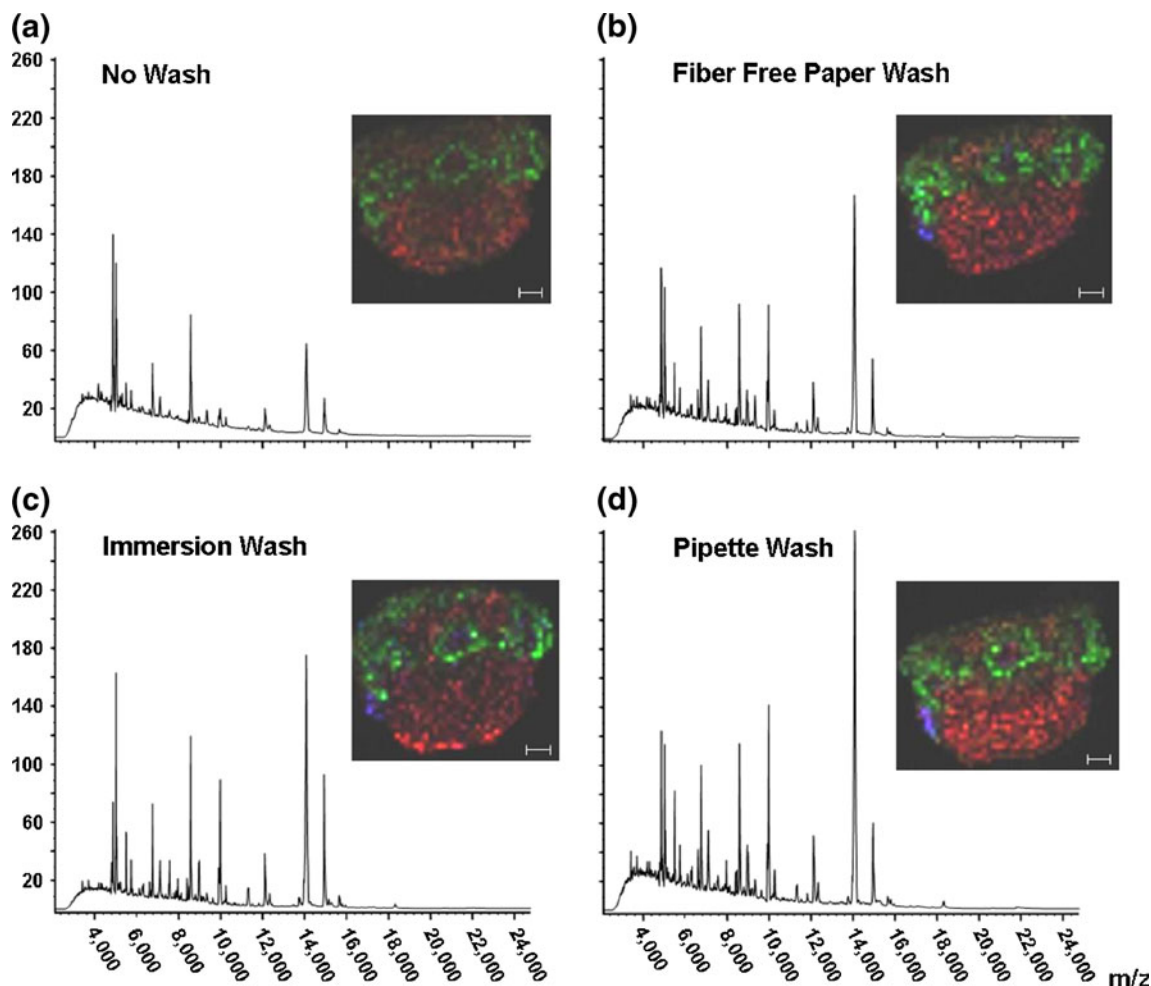


Figure 3. Spectral comparison of different washing methods of mouse brain sections using isopropanol (70% and 95% in H₂O, 30 s each, or 200 μ L each by the pipette method). The baseline subtracted, average mass spectra (over the entire image) are shown. **(a)** No washing step, **(b)** wetted fiber free paper wash, **(c)** immersion wash, **(d)** pipette wash. Inset images show three selected protein ions (blue: m/z 13,751 \pm 10, green: m/z 6,755 \pm 10, and red: m/z 7,093 \pm 10), which show that the spatial organization is retained by all washing methods. Scale bar = 1 mm

Results and Discussion

Tissue washing has been shown to improve the spectral wealth and quality for intact proteins, as well as improving peptide detection [1–5]. Here, a new method is presented that uses a fiber free paper, wetted with the wash solvent of choice, which allows localized washing and presents a number unique advantages over conventional methods. Samples deposited on substrates that cannot be washed by conventional methods due to sample mounting (e.g., whole-body animal section deposited on double sided adhesive tape) can be washed with this method, as well as fragile sections that may be damaged by conventional washing procedures.

The use of wetted paper allows surface cleaning of selected areas of a tissue section. Thus, analytes detected with and without surface washing can be compared on one tissue section. Paper strips wetted with 70% ethanol were applied on different parts of a rat brain and the section was measured by MetA-SIMS. Figure 1 shows the total ion image (Figure 1a) and mass selected ion images of sodium (m/z 22.9) (Figure 1b), potassium (m/z 38.9) (Figure 1c), and phosphocholine (m/z 184) (Figure 1d) from the localized washing experiment. The washed areas show a decrease in signal intensity for salt cations such as sodium and potassium, and an increase in signal intensity for small organic molecules such as phosphocholine (m/z 184).

The decrease in relative abundance of (salt) cations is attributed to the removal of these species from the tissue section into the wetted paper. Evidence of this migration is presented in Figure 2, where an entire rat heart section was cleaned once with wetted paper (cold 70% ethanol, 60 s). The analysis of the paper surface by MetA-SIMS shows that Na^+ and K^+ were blotted from the tissue section onto the paper (Figure 2b). The signal enhancement of certain ions after this localized surface cleaning procedure is attributed to reduced ion suppression resulting from the removal of the salts. The method does result in transfer of some organic molecules, such as cholesterol, to the washing paper. Principal component analysis (PCA) was performed on the MetA-SIMS data acquired from the paper surface. The first principal component spectra in Figure 2c demonstrates the removal of silicone contaminates at m/z 73 and 147 [13], which can be detrimental to SIMS experiments.

In order to compare washing methods, serial mouse brain sections were freshly prepared and submitted to three washing protocols with isopropanol (70% and 95% in H_2O), coated with sinapinic acid MALDI matrix and imaged. Figure 3 shows the average, baseline subtracted, mass spectra from the MS imaging runs and an overlay of three selected protein ion images. Without a washing procedure (Figure 3a), proteins are still detected from the tissue, but fewer peaks are observed. All three washing procedures improve the protein ion detection and retain their spatial distribution. We conclude that the fiber-free wetted paper method provides similar spectral results when com-

pared with accepted standard washing procedures for proteins. The spatial distribution of intact proteins has been shown to be affected little by washing procedures prior to MS imaging [4] (Figure 3).

Conclusions

The method described in this application note is a simple, effective method for the washing of biological tissue sections for MALDI and SIMS mass spectrometry imaging. The method uses a common laboratory paper wetted with washing solvent that is blotted onto the tissue sample surface. Fragile tissue sections, not amenable to conventional wet washing methods (thus resulting in poor MALDI MS imaging data quality), can be cleaned with this method and show good MALDI MS imaging spectral and image quality. Localized areas of tissue sections can be selectively washed, and SIMS imaging of a selectively washed tissue section shows increase in signal intensity of small organic molecules and a decrease in signal intensity of salt cations in the washed areas. Contaminant agents such as PDMS can be removed from the analyte surface, improving the data quality. The surface cleaning procedure described here is a reproducible, reliable, and efficient method that can be applied in any type of sample preparation for SIMS and MALDI-MS imaging.

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