Chapter 27

Mass Spectrometry Imaging Using the Stretched Sample Approach

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Abstract

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) can determine tissue localization for a variety of analytes with high sensitivity, chemical specificity, and spatial resolution. MS image quality typically depends on the MALDI matrix application method used, particularly when the matrix solution or powder is applied directly to the tissue surface. Improper matrix application results in spatial redistribution of analytes and reduced MS signal quality. Here we present a stretched sample imaging protocol that removes the dependence of MS image quality on the matrix application process and improves analyte extraction and sample desalting. First, the tissue sample is placed on a monolayer of solid support beads that are embedded in a hydrophobic membrane. Stretching the membrane fragments the tissue into thousands of nearly single-cell sized islands, with the pieces physically isolated from each other by the membrane. This spatial isolation prevents analyte transfer between beads, allowing for longer exposure of the tissue fragments to the MALDI matrix, thereby improving detectability of small analyte quantities without sacrificing spatial resolution. When using this method to reconstruct chemical images, complications result from non-uniform stretching of the supporting membrane. Addressing this concern, several computational tools enable automated data acquisition at individual bead locations and allow reconstruction of ion images corresponding to the original spatial conformation of the tissue section. Using mouse pituitary, we demonstrate the utility of this stretched imaging technique for characterizing peptide distributions in heterogeneous tissues at nearly single-cell resolution.

Key words: Mass spectrometry imaging, matrix-assisted laser desorption/ionization, nervous tissue, pituitary, mouse, stretched sample, image reconstruction, automated data acquisition.

1. Introduction

Both invertebrate and mammalian nervous systems exhibit high levels of biochemical and morphological heterogeneity. Neighboring neurons often possess different sets of intercellular signaling peptides, with several prohormones encoding multiple peptides that are expressed differently among individual neurons. Investigation of the mammalian nervous system, where neurons number in the billions, presents a significant challenge when using classical labeling approaches to examine one or more cell-to-cell signaling molecules at a time. In contrast to other bioimaging techniques, mass spectrometry imaging (MSI) can uncover the distribution of a variety of analytes within tissues while simultaneously determining their chemical identities, without the need for specific labeling or immunostaining (1–5). MSI has broad applications in academic, clinical, and industrial research, having had significant impact on cancer studies (6–9), the search for new pharmaceuticals (10), and investigations of the nervous system (11). A variety of MSI approaches targeting different types of analytes have been developed over the years. MALDI MSI has become one of most successful technologies for investigation of peptide and protein distributions in fixed and freshly prepared tissues. The analyte desorption and ionization processes occurring during exposure of the MALDI matrix/analyte layer to UV or IR laser light allows detection of intact/unfragmented analytes. Not only is the entire sequence of ion desorption, formation, separation, and detection fast, the laser beam can be focused to sub-micrometer diameters. However, because the amount of proteins and peptides present decrease concomitantly with the size of the area being probed, the smallest laser diameters are not commonly used. Obviously, larger laser spot sizes allow desorption of increased amounts of analyte. Typical chemical images are generated with 25–50 μm spatial resolution.

In MALDI-MSI, a liquid or powder matrix is deposited on top of the sample, incorporating the analyte into the matrix crystals. When illuminated with the laser, the matrix and analyte are vaporized and ionized. Although longer exposure of the sample to matrix facilitates extraction of the analyte of interest from the tissue, it can also delocalize the analyte. Shorter extraction times ameliorate this problem, but result in poorer signal. This issue is particularly problematic when investigating small hydrophilic substances that diffuse during matrix application. Recent advances in MALDI matrix solution application approaches have helped to create uniform MALDI matrix layers; these include spray coating

(12), electrospray deposition (13), and automated acoustic deposition (14). Using these techniques, the imaging of fine analyte spatial distributions has been achieved. However, each type of sample and even class of analytes requires individualized optimization of the MALDI matrix exposure duration and drying time.

The stretched sample protocol resolves these issues by eliminating redistribution of analytes during the matrix application stage. A tissue slice is placed on top of a monolayer comprised of ~40 μm diameter glass beads, which has been partially embedded into a layer of Parafilm M (15). As the Parafilm M layer is manually stretched to a ~16-fold increase in area, the beads separate from each other and the tissue, which adheres to the beads, is fragmented into thousands of islands. Because each bead contains only one or a few cells, chemically and spatially separated by areas of hydrophobic membrane, the sample can be exposed to the MALDI matrix solution for a longer period of time without sacrificing spatial resolution. The spatial isolation of tissue fragments allows rare signals from small cellular clusters, single cells, and even subcellular regions to be better detected and spatially distinguished. Furthermore, multiple MALDI matrix wetting/recrystallization cycles can be accomplished via temperature-dependent condensation of solvents onto the stretched sample, which allows for increased incorporation of the analyte into the matrix, contributing to further signal enhancement.

Effective MS imaging of stretched samples demands new methods of data acquisition, along with image reconstruction protocols, to register the spectral data with the corresponding conformation of the tissue before stretching. Although classical MSI experiments collect data in a regular raster pattern over the sample (16), the small tissue/cell islands in the stretched sample occur in irregular spatial patterns on the Parafilm M membrane. Incorporating a step to identify bead positions from optical images of the sample via light thresholding allows automated MS data acquisition from the individual bead positions. Image reconstruction is done in silico with a free transform process that mimics the actual stretching process (17). During stretching, the beads tear sizable craters partially through the layer of Parafilm M, visible in an optical image of the stretched sample. Image reconstruction is performed by aligning an image of the initial positions of the embedded beads with an image of the tissue sample after stretching. The spectral data taken from the stretched sample are assigned to the nearest corresponding initial bead positions to reconstruct an ion image of the tissue in its original conformation. This novel stretched imaging method shows increased potential for identifying rare signals from heterogeneous tissue samples (18).

2. Materials

2.1. Preparation of Parafilm M Substrate

1. Parafilm M (Pechiney, Neenah, WI, USA).
2. Glass slides, $25 \times 75 \times 1.1$ mm (Delta Technologies, Stillwater, MN, USA).
3. At least 100 mg of ~ 40 μm diameter clear glass beads (Mo-Sci Corp., Rolla, MO, USA). Blue beads are optional and are used as markers to aid image reconstruction. Beads of other types and sizes including liquid chromatography solid phase materials can be also used.
4. A heated aluminum block.

2.2. Tissue Preparation and Sample Stretching

1. Four-month-old C57BL/6 mice obtained from an in-house colony bred by the Greenough group, University of Illinois at Urbana-Champaign, were used. Animals of similar strains can be purchased for research purposes from the Jackson (http://www.jax.org) or Harlan laboratories (http://www.harlan.com). A variety of tissues from different animals can be investigated using the protocol presented here.
2. SPECTRA-SONIC (or similar) solution, pH 7 (Spectrum Surgical Instruments Corp., Stow, OH, USA) for surgical instrument clean up.
3. Modified Gey's balanced salt solution (mGBSS): 1.5 mM $CaCl_2$, 4.9 mM KCl, 0.2 mM KH_2PO_4, 11 mM $MgCl_2$, 0.3 mM $MgSO_4$, 138 mM NaCl, 27.7 mM $NaHCO_3$, 0.8 mM Na_2HPO_4, 25 mM HEPES, and 10 mM glucose, pH 7.2 adjusted with NaOH.
4. Dissection tools including forceps, scissors (available online from Fine Science Tools http://www.finescience.com or World Precision Instruments – http://www.wpiinc.com), and a properly sharpened guillotine.
5. Cryostat capable of keeping the specimen temperature at -15 to -20°C and of cutting 10 μm sections, e.g., Microm HM550 (Thermo Scientific, Waltham, MA, USA).
6. Indium tin oxide (ITO)-coated glass slides, $25 \times 75 \times 1.1$ mm (Delta Technologies, Stillwater, MN, USA).
7. A piece of firm paper.
8. Liquid nitrogen and dry ice.
9. Vials for specimen storage.
10. Protective lab coat, gloves, and goggles.

2.3. MALDI Matrix Application	1. MALDI matrix solution: 300 mg of 2,5-dihydroxybenzoic acid (Sigma-Aldrich, St. Louis, MO, USA) in 10 ml of 75:25 acetone:water.
	2. Artist's spray brush (Badger, Franklin Park, IL, USA).
	3. In-house built condensation chamber consisting of a Peltier device (Melcor, Trenton, NJ, USA) connected to a cooling basin of water, and a thermocouple connected to a CN77000 temperature controller (Omega, Stamford, CT, USA).
	4. Acetone.
2.4. Mass Spectrometry and Automated Imaging	1. Optical stereomicroscope.
	2. Inverted transmission light microscope with 2.5–10× magnification and equipped with a digital camera (e.g., Axio-Cam MRc camera controlled by the AxioVision digital image processing software package, Carl Zeiss, Bernreid, Germany; AxioVision LE is free and a sample version of the full package is available at http://www.zeiss.com/).
	3. Ultraflex II MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA) with a solid-state UV laser.
	4. MTP slide adapter (Bruker Daltonics) for insertion of slides into the MS instrument.
	5. ImageJ, version 1.38 (National Institutes of Health, http://rsb.info.nih.gov/ij/).
	6. Java SDK, version 1.6.0 (Sun Microsystems, http://java.sun.com).
	7. FlexControl 3.0 (Bruker Daltonics).
	8. Bead geometry application (free at http://neuroproteomics.scs.illinois.edu/imaging.html).
2.5. Data Conversion	1. Software tool: CompassXport (Bruker Daltonics, free at http://www.brukerdaltonics.com; for more information see: http://www.ionsource.com/functional_reviews/CompassXport/CompassXport.htm).
	2. Software package: MATLAB R2006a, version 7.2, and the Bioinformatics Toolbox 3.0 (The MathWorks, Natick, MA, USA).
	3. Batch conversion MATLAB code (free at http://neuroproteomics.scs.illinois.edu/imaging.html).
2.6. Image Reconstruction	1. Photoshop CS, version 8.0 (Adobe Systems).
	2. Java-based code to create image of dots at the initial bead positions (free at http://neuroproteomics.scs.illinois.edu/imaging.html).

3. MSIReconstructor application (free at http://neuroproteo mics.scs.illinois.edu/imaging.html).

3. Methods

3.1. Preparation of Parafilm M Substrate

1. Parafilm M is cut into a square measuring approximately 5×5 cm and placed on top of a glass slide (*see* **Note 1**). In this step, the slide is used as a clean solid support and so does not require a conductive ITO-coated slide.

2. Approximately 100 mg of beads are transferred to the Parafilm M surface. Another glass slide is placed on top of the beads and vertical pressure is manually applied to partially embed the beads into the Parafilm M layer.

3. Application of a nitrogen stream to the substrate removes loose beads, ensuring an even monolayer is attached to the Parafilm M surface.

4. Placing the substrate between two glass slides and heating it on top of an aluminum block at ~60°C for 10–15 s with downward manual pressure allows the beads to become more strongly and uniformly attached to the Parafilm M. Care must be taken to ensure that peripheral parts of the Parafilm M section, which might touch the metal block, do not melt onto it. A small separate piece of Parafilm M can be used to test if the temperature is such that the Parafilm M might be melted by the metal block.

5. An optical image of the initial bead/Parafilm M substrate is taken in transmission mode (*see* **Note 2**).

3.2. Tissue Preparation and Sample Stretching

1. Experimental animals are selected and euthanized by decapitation. Importantly, work performed on animals should comply with local and federal rules and regulations for the humane care and treatment of animals.

2. Surgical/dissection instruments are cleaned and sterilized by ultrasonic treatment in SPECTRA-SONIC (or similar) solution for 5–10 min, followed by autoclaving according to the manufacturer's manual.

3. Vials and paper are prepared; protective lab coat, gloves, and goggles are worn.

4. The animal is decapitated using a sharp guillotine.

5. The cranium is exposed by pushing the skin in a rostral direction using a piece of firm paper.

6. The cranial bones in the frontal plane are cut using long, thin scissors.

7. The brain is carefully lifted and discarded after removal of the previously cut dorsal part of the cranium. The pituitary

typically remains in the scull, held in place by connective tissue.

8. The connective tissue surrounding the pituitary should be removed first; the pituitary is quickly removed using fine forceps.

9. The pituitary is briefly washed in ice-cold mGBSS. The tissue is quickly frozen in liquid nitrogen and stored in a vial over dry ice for transport to the cryostat environment.

10. The pituitary is placed on a cooled (to −20°C) sample stage inside of the cryostat, without addition of embedding solution (*see* **Note 3**).

11. Tissue sections are made (10 μm thick).

12. Within the cryostat, the room temperature bead substrate is positioned onto the tissue section and briefly pressed using an index finger or an artist's brush handle. This ensures transfer of the tissue from the cryostat surface onto the substrate surface. Using a magic marker, the orientation and perimeter of the tissue section within the bead substrate are marked on the back of the Parafilm M substrate.

13. An ITO-coated glass slide is mounted onto a tall, thin vertical support (the slide box cover works well) with tape, conductive side facing upwards (**Fig. 27.1a**). A digital multi-
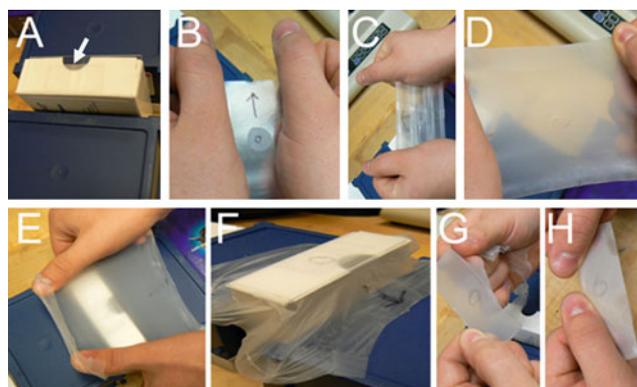

Fig. 27.1. Manual stretching of the sample. (**a**) An apparatus holds the sample, so that both hands can be used during stretching; here this device consists of the glass slide (*arrow*) taped to the thin side of the slide box cover, laterally stabilized by heavy objects. (**b**) The bead substrate, with the location and orientation of the tissue marked, is manually stretched along one axis, (**c**) rotated 90° and stretched, and (**d**) rotated and stretched again. (**e**) The stretched membrane is placed so that the marked sample area is on the glass slide. Use of thumbs provides a last bit of stretching before the sample is applied to the surface. (**f**) The arrow marking the orientation of the sample is clearly visible after stretching and can be marked again on the area of the glass slide if necessary. (**g**) The excess Parafilm M is removed from the edges of the glass slide. (**h**) A finished slide is ready for MALDI matrix coating.

meter can be used to determine which side is conductive. The glass slide box cover is the appropriate shape to support the slide as the stretched substrate is pushed onto the slide. This vertical support enables stretching without having to also hold the glass slide and can be stabilized by placing it between two holders such as large books, as illustrated in **Fig. 27.1a**.

14. The sample is stretched by hand and attached to the ITO-coated slide (**Fig. 27.1b–e**), and the excess Parafilm M is manually torn off of the sides of the slide (s*ee* **Note 4**). To ease the subsequent process of image reconstruction, the sample should be stretched with the maximum directional uniformity possible. The magic marker label along the perimeter (described above) helps when visually inspecting the sample to ensure it retains gross shape after the stretching process (*see* **Fig. 27.1f**).

3.3. MALDI Matrix Application

1. MALDI matrix is applied using the artist's spray brush at an ~25 cm distance from the sample (*see* **Note 5**). The spray brush is washed with pure acetone after use.

2. Water is condensed onto the sample with the condensation chamber at 14°C for 60 s and the sample evaporated at 28°C for 90 s. This cycle is repeated three times for increased analyte extraction, followed by returning the sample to room temperature (*see* **Note 6**).

3. The specimen is loaded into the mass spectrometer. Mass spectral profiling (15) is used to assess the quality of peptide signal received from the specimen before MS imaging of the sample.

3.4. Mass Spectrometry and Automated Imaging

1. The glass slide with the stretched sample is loaded into the mass spectrometer. Although a calibration bar is typically used (18), we determined that regularly spaced laser-melted holes in the Parafilm M serve as more accurate spatial calibration markers. Provided that the sample is stretched to sufficient thinness, the mass spectrometer's UV laser beam is used to melt several ~100 μm diameter holes through the Parafilm M surface at several of the ordered positions found in the "MTP Slide Adapter II" geometry file included in the Bruker FlexControl software. The location of these points should be chosen so that they span the area of the tissue sample; depending on the size of the sample, three to four points are sufficient.

2. The specimen is unloaded from the mass spectrometer and a transmission mode optical image is taken with a digital camera coupled to an optical microscope. If several optical

images are needed to cover the entire area of the sample, the Photomerge function in Photoshop can be used to stitch multiple images together.

3. ImageJ, along with the color threshold plugin, is used to automatically report the pixel coordinates of the beads (*see* **Note 7**). The computational steps for bead identification by thresholding are summarized in **Fig. 27.2a**. The Analyze–>Set Scale function is used to specify the units of the coordinates as pixels. The results of the threshold are viewed by
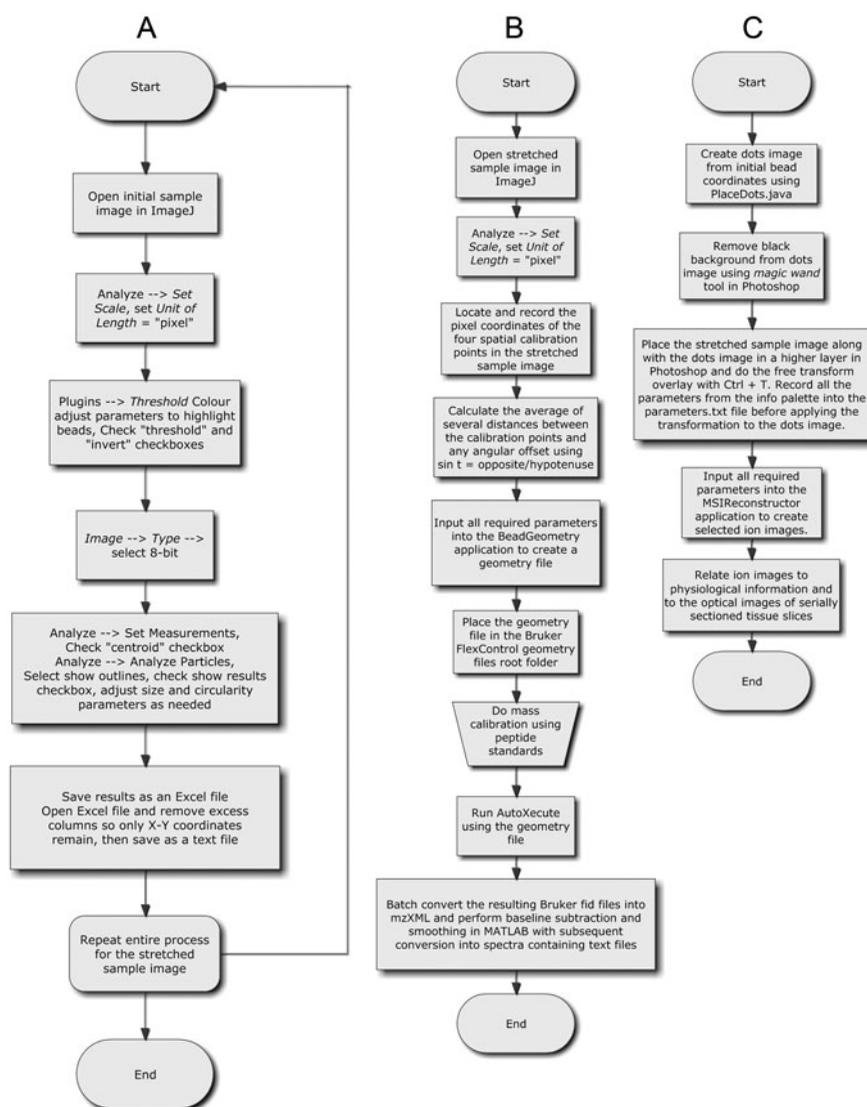
Fig. 27.2. Flow chart for the computational steps in the stretched imaging method, including the process of (**a**) bead identification by light thresholding, (**b**) geometry file creation, and (**c**) image reconstruction. The custom software routines can be found online at http://neuroproteomics.scs.illinois.edu/imaging.html.

selecting Analyze–>Analyze Particles–>Show Outlines. The circularity and size parameters can be adjusted and the process repeated until the outputted outlines image appears not to be highlighting non-bead regions and irregular shapes.

4. In ImageJ, the pixel coordinates of the center of the melted calibration regions are recorded and the distances between them are calculated using the Cartesian distance formula (*see* **Note 8**). If several equivalent distances can be calculated between the various calibration points, the variations in these distances are averaged (*see* **Note 9**).

5. The coordinates of the calibration points and stretched sample bead positions are entered into an in-house written Java-based application available on the Web (*see* **Note 10**). The steps for geometry file creation are summarized in **Fig. 27.2b**.

6. The resulting geometry file is placed in the FlexControl software's geometry files root folder and can be easily found and automatically loaded by the software.

7. The sample is loaded into the mass spectrometer and mass calibration is performed using peptide standards.

8. An AutoXecute sequence is created using the geometry file, specifying an appropriate maximum laser intensity, a value of 100 laser shots per spot, and a 50 Hz repetition rate before starting the MS imaging run.

9. The region of interest is imaged using MALDI-TOF MS.

3.5. Data Conversion

1. The data must be converted from the Bruker ftd file format to the more general mzXML format. The CompassX-port software is run along with the – multiName tag at an MS-DOS prompt to create a file called new.mzXML within each spectrum directory.

2. MATLAB is used along with the bioinformatics toolbox and the batch conversion wrapper code available online (*see* **Note 10)** to convert mzXML files into spectra-containing text files. During this step, the data may be processed by baseline subtraction and smoothing with the Bioinformatics Toolbox functions in MATLAB to eliminate noise and create more uniform ion images.

3.6. Image Reconstruction

1. The coordinates of the initial bead positions are found in the same manner as for the stretched sample image, as outlined above in **Section 3.4**, Step 3 (*see* **Note 11**).

2. A simple code (*see* **Note 10**) is compiled in Java and used to create a separate image that places small dots at the initial positions. These dots are easier to see and aid the free

transform process. The computational steps for image reconstruction are summarized in **Fig. 27.2c**.

3. The small dots image and the stretched sample optical images are opened into Photoshop. The free transform command (Ctrl + T) is used to report the centroid coordinates of each of the two images from the options bar. A duplicate background layer is created, and the black background is removed from the small dots image to create a transparent image using the Magic Wand tool in Photoshop. A new blank Photoshop image file is created, large enough (in the range of 5,000×5,000 pixels) to hold both images when placed side-by-side in separate layers, allowing adequate work space to manipulate the images when aligning on top of each other. The small dots image must be placed in a layer above the stretched image layer. The new centroid positions of each image in the blank image file are recorded from the options bar. The free transform command is used to translate, rotate, and resize the small dots image until it is
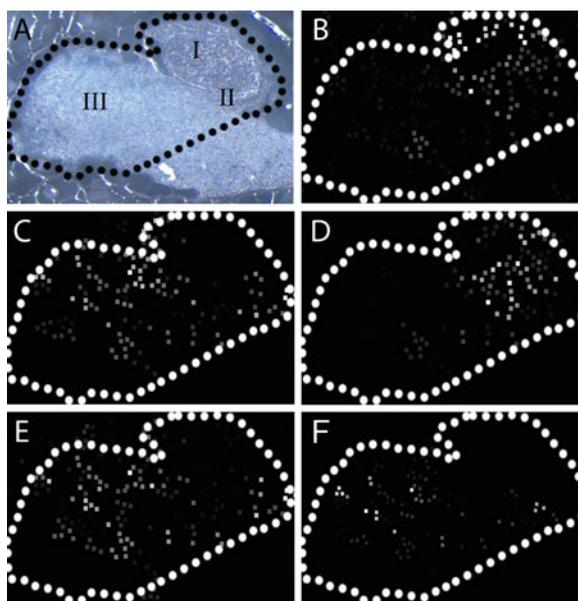

Fig. 27.3. Reconstructed MALDI-MSI ion images from a 10 μm section of mouse pituitary prepared with the stretched sample method. (**a**) Optical photomicrograph of the pituitary section showing (I) the posterior lobe, (II) a darker band corresponding to the intermediate lobe, and (III) the anterior lobe. Only the outlined region tissue was imaged. Reconstructed ion images correspond to the outlined tissue area showing: (**b**) oxytocin, 1,007 *m/z*; (**c**) di-Ac-α-MSH, 1,707 *m/z*; (**d**) vasopressin, 1,083 *m/z*; (**e**) POMC J-peptide, 1,883 *m/z*; and (**f**) Arg-CLIP [1–22], 2,505 *m/z*. The intermediate lobe is a small band that is highlighted by signals from the di-acetylated-α-MSH and J-peptide ion images.

appropriately aligned on top of the stretched sample image. The small dots should each align within one of the bead-torn regions of the stretched sample image.

4. Before applying the transformation to the transformed images in Photoshop, the final width, height, and rotation angle are recorded into a text file from the Info palette, along with the final centroid position of each image.

5. The text files of the initial positions and the image reconstruction parameters, recorded both before and after the free transformation, are inputted into another in-house Java program (*see* **Note 10**) to create reconstructed ion images at select *m/z* ratios of interest as seen in **Fig. 27.3** (*see* **Note 12**). The example shown in **Fig. 27.3** is with a thin tissue section from mouse pituitary.

4. Notes

1. Optionally, Parafilm M may be soaked for 1 h in either acetic acid (100%) or ammonium hydroxide solution (28.8%) to soften the film (19). After drying, this treatment allows the film to be stretched by a greater degree into an approximately sevenfold increase in each dimension. Use of the more elastic film results in formation of small, concentrated droplets of solution upon matrix application with less spatial spreading. In addition, soaking can reduce polymer signals resulting from the Parafilm M.

2. If only performing mass spectral profiling without imaging on stretched samples, as described in (15), this step and the steps related to image reconstruction and geometry file creation can be omitted.

3. Most embedding media interferes with obtaining good signals in mass spectrometry investigations. One exception to this is embedding of tissues in low melting point agarose gel blocks or gelatin. It was found that a block of solidified saturated agarose solution that is freeze mounted onto the dissection stage, followed by sectioning through the top layers of the gel, creates a flat surface for better freeze mounting and orienting of the tissue.

4. For mass spectrometry imaging, it is important to prevent the tissue from completely drying as this will cause bead clumping and may reduce incorporation of analyte into the MALDI matrix crystals. Excess sample drying is prevented by immediately applying MALDI matrix after stretching while the tissue is still partially wet. Thus, if more than one

section is to be taken from the tissue, these sections are sectioned with the microtome after immediately applying MALDI matrix to the preceding stretched section.

5. A larger sprayer-to-sample distance helps in not over-wetting the sample, as larger ($\sim$0.5 mm) droplets can cause spreading, even in a stretched sample. A light microscope can be used to monitor the drying process, so that the sample is completely dry before the next spray application. The light microscope also helps to visually monitor the amount of matrix applied. Generally, several spraying-drying cycles over 10–20 min is sufficient. Alternatively, matrix application can be done by capillary deposition to control the size of the matrix spots and prevent the spatial redistribution of analytes ([19]).

6. The solvent condensation/MALDI matrix recrystallization procedure has shown the ability to improve mass spectra quality by reducing the number and intensity of inorganic salt ion adducts typical for traditional MS imaging sample preparations. This reduction in potassium and sodium salt adducts creates less complex mass spectra ([20]).

7. The success rate of the bead position identification depends on the quality of the optical images. Transmission-mode images are easier to threshold for bead positions as they appear brighter than the background Parafilm M.

8. As the FlexControl "MTP Slide Adapter II" geometry file uses a fractional distance coordinate system where the distance between each point in the regular array is separated by exactly 0.086957 units, a new geometry file can be created in this coordinate system to acquire data at the bead positions.

9. Variations in the distances between calibration points that are larger than bead diameter signify inaccuracies in the optical image of the stretched sample such that the resulting geometry file may not accurately represent the bead positions. Inaccuracies sometimes occur because of errors in stitching of images by Photoshop and can be prevented by taking well-focused images with sufficient spatial overlap.

10. All in-house written Java software is available online, along with an example dataset with step-by-step instructions, at http://neuroproteomics.scs.illinois.edu/imaging.html.

11. Alternatively, image reconstruction can be done before geometry file creation so that some time is saved in the rare event that image reconstruction is not successful, upon which the sample is discarded. Any difficulties with image reconstruction using the free transform approach arise from

highly non-uniform stretching of the Parafilm M that can be prevented by visually adjusting for the shape of the marked perimeter of the sample while stretching. Overall, image reconstruction is fairly reproducible, as a set of six samples resulted in a classification rate of 84.1% for bead position matching between the stretched and the initial samples, with the remaining portion being only near-neighbor mismatches (18).

12. The success of image reconstruction can be verified using an in-house written code (http://neuroproteomics.scs.illinois.edu/imaging.html) that plots an image of the calculated transformed initial positions. This image can be checked against the transformed image in Photoshop to verify for any calculation or positional errors.

Acknowledgments

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