MALDI-IMS for spatial analysis of lipids and other small molecules.

Metabolomics Workshop 6-2-2014
Janusz Kabarowski, Dept. Microbiology, UAB.
Matrix-Assisted Laser Desorption/Ionization (MALDI):
Matrix molecules absorb laser light, enter an excited state, and collide with sample molecules, facilitating charge transfer to create ions.

Mass Spectrometric Imaging for biomedical tissue analysis
Kamila Chughtai and Ron M.A. Heeren
Cryosection preparation onto ITO slides and scanning digital image for “teaching” FlexControl software on MALDI-TOF instrument.
Vacuum sublimation is used to apply an even microscopically thin uniform layer of matrix compound onto tissue section without the need for solvents.

Sublimation: *the transition of a substance from solid to gas phase without an intermediate liquid phase.*

**MALDI matrices for lipid imaging:**

- **DHB:** 2,5-dihydrobenzoic acid (+ve mode)

- **1,5-diaminonaphthalene** (-ve mode)
How do we apply matrix for MALDI Imaging?

Vacuum sublimation apparatus.
Vacuum at 0.05 Torr in sublimation chamber (atmospheric pressure is 750 Torr).
Matrix deposition by vacuum sublimation.

- **Cold condensor unit** of vacuum sublimation chamber
- **ITO (indium-tin-oxide coated)** slide with cryosections
- **Matrix compound**
- **Heated sand**

Temperature:
- 5-10°C
- 0.05 Torr
- 140°C
Deposition of the matrix compound is at the molecular level because gaseous molecules recrystallize at the relatively cold surface of the tissue section attached to the cold condenser.

The uniformity of matrix deposition onto the slide attached to the cold condenser surface reflects the random Brownian motion of the released gaseous matrix molecules.
Adaptation of MALDI plate for imaging cryosections on slides.
MALDI-IMS instrument running.
MALDI-IMS for studying lipids in eye.

H&E stained paraffin sections from young and old zebrafish eyes to show optimal lens structural integrity.
Formalin fixed paraffin sections such as that shown in (B) cannot be used for MALDI-IMS (although certain modifications of fixation protocols can allow for subsequent lipid MALDI-IMS). Cryosectioning (after tissue embedding in 10% gelatin) must therefore be optimized for the tissue being studied (A). In the case of eye lens, this is very challenging as the dense lens material has a propensity to crack.
Positive ion mode MALDI average mass spectra of (A) 8 week old and (B) 14 month old zebrafish eyes, showing the location of the peaks for which images were taken.

Peaks correspond to expected protonated, sodiated, and potassiated adducts of PC(36:4).
MALDI-IMS on protonated, sodiated, and potassiated adducts of PC(36:4) in zebrafish eye.
MS/MS on protonated adduct of PC(36:4) in zebrafish eye.

choline head group

loss of choline

[PC(36:4)+H]^+ =
MALDI-IMS on mouse spleen.

Cumulative MALDI-IMS spectrum from a normal (C57BL/6J) mouse spleen.

Peaks at 796.4 Da and 423.4 Da were found to give 184m/z phosphocholine fragments on MS/MS analysis. Together with the parent ion m/z, this information identifies 796.4m/z and 423.4m/z peaks as phosphatidylcholine and lysophosphatidylcholine respectively.
Acknowledgments.

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FLEX™ Series Training Manual for MALDI-TOF Mass Spectrometry

Bruker Daltonics, Inc.
40 Manning Road
Billerica, MA 01821
(978) 663-3660
www.bdai.com
Bruker Daltonics FLEX™ Series MALDI-TOF Systems

- **microflex™**
- **autoflex speed™**
- **ultraflExtreme™**
Layout of a FLEX™ Series MALDI-TOF/TOF MS

- Ion source
  - P1 Target plate
  - Ion source with P2 Plate (IS 2)
  - Lens
- Lift chamber
  - Pre-Cursor Ion-Selector
  - LIFT ion optics
  - LIFT 1 - Lift 2
  - LIFT XY Deflection
- Analyzer
  - Reflector Detector
  - Post Lift-Metastable Suppressor also matrix deflection
  - Reflector Ref 1 - Ref 2
  - Linear Detector
Diagrams of autoflex speed and ultrafleXtreme MALDI-TOF/TOF MS systems
MALDI-TOF applications

Proteomics
LC-based

Proteomics
Gel-based

TLC-MALDI

MALDI-biotype

MALDI-Molecular Imager

MALDI-TDS

Bruker Daltonics
MALDI-TOF mass spectrometry
general introduction
Materials to be analyzed are prepared with a matrix to aid ionization. Ions are created*, accelerated, and then allowed to drift through a free field toward a detector. The speed (time) of travel is proportional to the ion’s mass.

* Positive ionization mode shown
Desorption/Ionization and the Role of Matrix

- Laser light pulses
- Matrix molecules readily absorb laser light (photon energy), creating an excited energy state
- Localized heating causes micro-explosion of material
- Collisions with neutral sample facilitate charge transfer to/from excited matrix molecules

Positive ion mode: Protonation of sample

Negative ion mode: Deprotonation of sample

Formation of alternative adducts (in positive ion mode) depends on the presence of respective cations (either being ubiquitously present or actively added – depending on type of sample), e.g. –

Commonly Used MALDI Matrices

<table>
<thead>
<tr>
<th>Peptides:</th>
<th>α-Cyano-4-hydroxycinnamic acid (CHCA or HCCA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins:</td>
<td>Sinapinic acid (SA)</td>
</tr>
<tr>
<td></td>
<td>2,5-Dihydroxybenzoic acid (DHB)</td>
</tr>
<tr>
<td></td>
<td>2,5-Dihydroxyacetophenone (DHAP)</td>
</tr>
<tr>
<td></td>
<td>Super DHB (mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid) (SDHB) {recommended for ISD analysis}</td>
</tr>
<tr>
<td></td>
<td>1,5-Diaminonaphthalene (DAN) {recommended for ISD analysis}</td>
</tr>
<tr>
<td>Glycans:</td>
<td>2,5-Dihydroxybenzoic acid (DHB)</td>
</tr>
<tr>
<td></td>
<td>Super DHB (mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid) (SDHB)</td>
</tr>
<tr>
<td>Nucleic acids:</td>
<td>3-Hydroxypicolinic acid (HPA)¹</td>
</tr>
<tr>
<td></td>
<td>2,4,6-Trihydroxyacetophenone (THAP)¹</td>
</tr>
<tr>
<td>Polymers:</td>
<td>2′-(4-Hydroxyphenylazo)benzoic acid (HABA)</td>
</tr>
<tr>
<td></td>
<td>Dithranol (DIT)²</td>
</tr>
<tr>
<td></td>
<td>Trans-3-indoleacrylic acid (IAA)²</td>
</tr>
</tbody>
</table>

Additives:
1) Ammonium citrate
2) CF₃COOAg

Why different matrices for different types of samples?

- the amount of energy needed to ionize a particular sample compound varies (individual matrices show specific "energy threshold")

- samples have different stabilities (a matrix that is too "hot" may lead to undesirable fragmentation of sample compounds, but some instability may be of value, e.g. to promote in-source decay)
What about the Laser?
Lasers commonly used in MALDI

**Nitrogen laser:**
- **pro:** well structured energy profile
- **con:** slow (maximum 60Hz)

**Nd:YAG laser:**
- **pro:** fast (up to 1000Hz)
- **con:** Gaussian energy profile (non-structured)

**Smartbeam™/Smartbeam II (modified Nd:YAG laser):**
- **pro:** fast (up to 1000Hz)
- **pro:** well structured energy profile
- **pro:** compatible with all matrices developed for N$_2$ lasers
- **pro:** efficient sample consumption

Comparison between nitrogen, Nd:YAG and Smartbeam lasers

- Maximum laser frequency: 60 Hz
- Laser lifetime: $\sim 10^8$ shots
- Minimum laser focus: $\sim 50 \, \mu m$

Bruker systems:
- microflex
- autoflex I and II
- ultraflex I

- Maximum laser frequency: 1000 Hz
- Laser lifetime: $\sim 10^9$ shots
- Minimum laser focus: $\sim 10 \, \mu m$
- Smartbeam exclusive: variable beam profile

Bruker systems:
- ultrafleXtreme
- autoflex speed
- ultraflex II and III
- autoflex III
Time of Flight
principles and instrument specifics
MALDI-TOF Mass Analysis – Basic Principle

**Linear Mode**

High vacuum $10^{-7}$mbar

**IS1**

MALDI Ion Source

Acceleration

Field free TOF analyzer region (drift tube)

Time Of Flight (depending on m/z)

Detector

High vacuum $10^{-7}$mbar

Intensity

$m/z$

MALDI-TOF Mass Analysis – Basic Principle

**Linear Mode**

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Time Of Flight (depending on m/z)

Detector

High vacuum $10^{-7}$mbar

Intensity

$m/z$
Time-of-Flight Equation

• After desorption, all charged ions (where \( q \) is the charge and \( z \) is the number of charges) have potential energy \( E_p \) proportional to the acceleration voltage \( U \).

\[ E_p = z e U \]

• The ions are accelerated out of the source and into the flight tube. Their potential energy is converted to kinetic energy \( E_k \).

\[ E_k = \frac{1}{2} m v^2 \]

\[ 1/2 m v^2 = z e U \]

• According to the first law of thermodynamics, the energy has to be conserved (\( E_k = E_p \)), therefore, this equation can be written as:

\[ 1/2 m (L/t)^2 = z e U \]

• Velocity (\( v \)) of ions equals to distance divided by time (where distance is the length of the flight tube \( L \) and the time is time-of-flight \( t \) (\( v = L/t \)). Substituting velocity with \( L/t \) leads to the following equation:

\[ m/z = (2eU/L^2) \ t^2 \]

\[ m/z = \text{const} \ t^2 \]

\[ t = \text{const} \sqrt{m/z} \]

In other words, the larger the \( m/z \), the longer the flight time...
How do we assess peak quality (and indirectly, instrument performance)?

- Peak resolution – how well are two neighboring peaks separated from each other (are they sufficiently narrow for baseline separation)?

- Mass accuracy – how close are the experimentally measured masses to the calculated (standard) values?

- Peak detection – are proteomic profiles or target masses reproducibly detectable at an acceptable signal/noise ratio?
Resolution, \( R = \frac{m}{\Delta m} \)

\( \Delta m \) is a mass peak's full width at half maximum (FWHM)

Example analysis of a mixture of two compounds \( X \) and \( Y \):

Mass spectrum of single compound \( X \) (yielding a signal @ \( m/z_X \))

Mass spectrum of single compound \( Y \) (yielding a signal @ \( m/z_Y \))

\( \Delta m \), FWHM

Mass spectrum obtained from mixture \([X+Y]\): Under these collection conditions, the resolution is insufficient for baseline separation of \( X \) and \( Y \). Narrower peaks (i.e. smaller FWHM = higher resolution) would be required for clear separation and accurate labeling.
Linear Mode Limitation:
Resolution limited due to spatial and energy spreads

Spatial spread:
• initial movement of ions in different directions
• ions are desorbed from different z-coordinates due to heterogeneity in size of matrix crystals

Initial energy (speed) spread:
• heterogeneous secondary reactions (ion-ion; ion-neutral)

Pulsed ion extraction (PIE): Increased resolution through efficient ion focusing in the MALDI ion source

- Pulsed ion extraction separates desorption and ionization from acceleration and mass separation
- Initially, there is a zero field voltage gradient in source (no gradient, IS1=IS2), during which time the ions have moved based on velocity spread
- After a suitable time delay, the ions are pulsed from source. Slower ions (those with less kinetic energy) ‘see’ higher field lines after the voltage pulse
- This higher field imparts greater kinetic energy to the slower ions thus slightly increasing the ion velocity relative to the faster ions
- By tuning the amplitude of the voltage pulse one can impart just enough ‘extra velocity’ to all slower ions so that they just catch up to the faster ones at the detector (less time spread - narrower peak)
Pulsed ion extraction (PIE): Increased resolution through efficient ion focusing in the MALDI ion source

- During extraction: IS1 voltage > IS2 voltage > ground
- Within flexControl, a number of preloaded data collection methods are appropriately tuned for particular mass ranges, applying the following considerations:
  - The time delay before application of the pulse
  - The amplitude of voltage pulse
  - The ion lens diameter (altered by changing its voltage)

Example ultrafleXtreme settings:

<table>
<thead>
<tr>
<th>flexControl method</th>
<th>IS1 (kV)</th>
<th>IS2 (kV)</th>
<th>Lens (kV)</th>
<th>PIE (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP_700-2000 Da</td>
<td>19.50</td>
<td>18.20</td>
<td>6.00</td>
<td>130</td>
</tr>
<tr>
<td>LP_5-20 kDa</td>
<td>19.50</td>
<td>18.20</td>
<td>6.50</td>
<td>340</td>
</tr>
</tbody>
</table>

Advanced users may wish to create new flexControl collection methods by fine-tuning these values for maximum resolution of m/z(s) of interest
Reflector mode: Increased resolution through ion focusing with a two-stage ion reflector

- Designed for simultaneous time and spatial ion focusing by compensating for ions of different energies
- Provides for increased mass accuracy and resolution
- Consists of a series of plates with increasing applied voltages
- Re-directs the flight path of traveling ions to reflector detector
Linear vs. reflector mode: Cytochrome C
\[ MW_{avg} = 12360 \text{Da} \]

**Linear mode:** Low resolution
\[ R = 1,500 \]
Spectrum shows one broad peak representing the envelope of the non-resolved isotope peaks

**Reflector mode:** High resolution
\[ R = 30,000 \]
Spectrum shows all the isotope peaks well separated from each other
Increased resolution: PIE and reflector mode

**FAQ:**
If MALDI-TOF performed in reflector mode gives so much better resolution - why then use linear mode at all???

**Answer:**
Linear mode is used whenever analytes are not stable enough to survive the energetic stress which is inherent to passing the reflector (ions are deaccelerated/re-accelerated in the reflector by a high kV electric field within nanoseconds). Larger sized molecules, e.g. intact proteins, show limited stability when passing the reflector field, and may undergo serious fragmentation, which results in either badly resolved spectra (peak fronting due to non-resolved fragments) and/or drastic loss in sensitivity (low mass fragments will miss the reflector detector).
Mass Accuracy – Calibration Equations

Linear equation
\[ t = c_0 + c_1 \sqrt{m/z} \]

Quadratic equation
\[ t = c_0 + c_1 \sqrt{m/z} + c_2 m/z \]

Available calibration functions:
- Linear
- Quadratic
- Cubic enhanced
- Linear correction

Mass accuracy is calculated in ppm
\[
\frac{|M_{\text{calc}} - M_{\text{meas}}|}{M_{\text{calc}}} \times 10^6 = \text{ppm}
\]

Example:
\[
\begin{align*}
M_{\text{calc}} &= 3147.4710 \\
M_{\text{meas}} &= 3147.4670
\end{align*}
\]
\[
\frac{|3147.4710 - 3147.4670|}{3147.4710} \times 10^6 = 1.27 \text{ ppm}
\]
Mass Accuracy – Calibration Strategies

Option 1) External calibration

- Calibrants of known mass cover mass range of interest
- m/z vs. flight time is fitted using a polynomial of varying order (depending on size of mass range to be calibrated and number of available calibrant signals, respectively)

Option 2) Internal calibration

- Apply calibration fit to sample spectra obtained from near neighbor spots

* denotes compounds of known identity/mass
842.509 Da (trypsin artefact)
2211.104 Da (trypsin artefact)

Internal calibration (or re-calibration) allows for
- Optimum mass accuracy due to compensation of spot-to-spot heterogeneities that may cause mass errors after external calibration
Mass Accuracy – Digitization Rate
How many data points are required to see peak features?

Bovine Insulin collected with 3 different digitization rates

Comparison of Peptide Mass Accuracy from 0.5, 1 and 2 GHz data

<table>
<thead>
<tr>
<th>Peptide</th>
<th>0.5 GHz</th>
<th>1 GHz</th>
<th>2 GHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bradykinin</td>
<td>29 ppm</td>
<td>4.3 ppm</td>
<td>2.8 ppm</td>
</tr>
<tr>
<td>Angiotensin I</td>
<td>14.8 ppm</td>
<td>2.0 ppm</td>
<td>1.7 ppm</td>
</tr>
</tbody>
</table>
Factors affecting Peak Intensity

- Lower molecular weight ions generally more intense than larger ions – ionize more readily
- In mixtures, competition for charge affects ion abundances, such that equimolar mixtures may produce unequal peak intensities. More basic analytes are favored – greater ion abundance
- Matrix selection and preparation (concentration, ratio, solvent) often alters peak intensities
- Additives and contaminants (e.g., detergents, salts) can dramatically suppress peptide and protein ionization

Casein digest with 200 mM KCl and 0.1% Tween 20

Casein digest after clean up
TOF is theoretically limitless as a m/z analyzer, however, limitations arise from an inability to desorb/ionize very large analytes and from detectors which are inefficient for very large ion detection.

The impact of particles on the detector generates signal whose strength is related, in part, to the energy of impact.

\[ E_k = \frac{1}{2}mv^2 \]

If \( E_k \) is constant and determined by the ion charge and electric field strength then as mass gets larger then velocity becomes smaller. At some point the velocity will fall below that critical for generating signals. (For practical purposes this is 200-300 kDa but signals have been observed up to 1MDa)
Appendix A - Fundamental papers on the principle of MALDI

M. Karas, D. Bachmann, F. Hillenkamp
*Analytical Chemistry, 57, 2935-2939 (1985)*

K. Tanaka, H. Waiki, Y. Ido, S. Akita, Y. Yoshida, T. Yoshida
*Rapid Communications in Mass Spectrometry, 2, 151-153 (1988)*

R. C. Beavis, B. Chait, K.G. Standing
*Rapid Communications in Mass Spectrometry, 3, 233-237 (1989)*

M. Karas, M. Glückmann, J. Schäfer
*Journal of Mass Spectrometry, 35, 1-12, (2000)*

R. Zenobi and R. Knochenmuss
*Mass Spectrometry Reviews, 17, 337-366 (1998)*
Appendix B – MALDI matrix structures

- α-Cyano-4-hydroxycinnamic acid (CCA, CHCA)
- Sinapinic acid (SA)
- 2,5-Dihydroxybenzoic acid (DHB)
- 2,5-Dihydroxyacetophenone (DHAP)
- 2,4,6-Trihydroxyacetophenone (THAP)
- 3-Hydroxypicolinic acid (HPA)
- 2’-(4-Hydroxyphenylazo) benzoic acid (HABA)
- 1,5-Diaminonaphthalene (DAN)
- Trans-3-indoleacrylic acid (IAA)
- Dithranol (DIT)
## Appendix C – Common Detergents and Effect On MALDI Analyses

<table>
<thead>
<tr>
<th>Class</th>
<th>Effect on MALDI spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>no bad effect: may improve results in mixture</td>
</tr>
<tr>
<td>2</td>
<td>little effect</td>
</tr>
<tr>
<td>3</td>
<td>spectrum quality &amp; signal intensity reduced</td>
</tr>
<tr>
<td>4</td>
<td>spectrum suppressed: detergent must be removed</td>
</tr>
</tbody>
</table>

### Detergent

<table>
<thead>
<tr>
<th>Detergent</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-octyl-glucoside</td>
<td>1</td>
</tr>
<tr>
<td>n-dodecyl-glucoside</td>
<td>1</td>
</tr>
<tr>
<td>octanoyl-N-methylglucamide</td>
<td>1</td>
</tr>
<tr>
<td>decanoyl-n-methylglucamide</td>
<td>1</td>
</tr>
<tr>
<td>n-dodecyl-beta-D-maltoside</td>
<td>2</td>
</tr>
<tr>
<td>octylphenolpoly(ethyleneglycolether)$_{10}$ (Triton X-100)</td>
<td>3</td>
</tr>
<tr>
<td>octylphenolpoly(ethyleneglycolether)$_{7}$ (Triton X-114)</td>
<td>3</td>
</tr>
<tr>
<td>polyethylene glycol (PEG 2000)</td>
<td>3</td>
</tr>
<tr>
<td>dodecylpoly(ethyleneglycolether)$_{9}$ (Thesit)</td>
<td>4</td>
</tr>
<tr>
<td>isotridecylpoly(ethyleneglycolether)$_{8}$</td>
<td>4</td>
</tr>
<tr>
<td>CHAPS</td>
<td>4</td>
</tr>
<tr>
<td>CHAPSO</td>
<td>4</td>
</tr>
<tr>
<td>n-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate</td>
<td>4</td>
</tr>
<tr>
<td>sodium dodecylsulfate (SDS)</td>
<td>4</td>
</tr>
</tbody>
</table>
Preparation of tissues for MALDI imaging and profiling

Maciej Lalowski
Biomedicum Helsinki, Helsinki University

EuroKUP seminar, 12.10.2009
1. Collecting the tissue samples (usage of fresh frozen material or FFPE archives),
2. Slicing and tissue preparation,
3. Matrix deposition,
4. Rastering of an image,
5. Acquiring spectra,
6. Image processing
7. Statistical analyses.
8. In parallel, consecutive sections can be stained using classical (immuno)-histochemical methods allowing pinpointing regions of interest (i.e. tumour versus non tumour or focusing on defined anatomical structures).
9. MALDI-PMS can be performed on localized, “discrete” regions of the tissue, while MALDI-IMS requires larger, continuous areas of the tissues.
10. Subsequent validation analyses involve tissue microdissections, fractionations, enzyme digestions, MS and MS/MS runs and database searches. Alternatively, Tag-antibody approach (TAMSIM) might be utilized.
Collecting the samples

1. Sample handling and preparation of sections for image analysis are critical to the spatial integrity of measured molecular distributions.

2. Any molecular degradation that occurs in the time between sample collection and analysis can adversely affect the results.

3. A typical study may involve samples collected over a lengthy period of time, and standardized procedures are therefore required to minimize experimental variability over the time course of the study.

4. Good communication among all personnel involved with collecting, storing and analyzing samples is critical.

5. Ideally, samples are frozen immediately after collection and stored at -80°C until sections for MALDI-IMS analysis are cut on a cryomicrotome just before analysis.

Step 1: preserving the tissues

1. Animals are usually killed by cervical dislocation, after which the tissue of interest has to be rapidly removed and immediately processed:

A. Flash frozen in liquid nitrogen (30-60 sec) and stored at -80°C

B. Flash frozen in liquid nitrogen cooled isopentane and stored at -80°C until sectioning in order to minimize proteolysis and conserve PTMs of peptides and proteins.

C. Small sections can also be frozen using dry ice and ethanol

D. Alternatively, the tissue may be frozen in a mixture of dry ice and hexane at -75°C, embedded in a 2% gel of sodium carboxymethylcellulose (CMC) and stored at -80°C until further use.

E. Embedding in gelatine has been used to facilitate handling of small or fragile samples (e.g., biopsies).

Lalowski et al., *J Proteomics*, in revision
Step 1: preserving the tissues

1. Similarly, biopsy/autopsy human material can be stored at -80°C after being subjected to a conductive heat transfer. The methodology was developed to stabilize biological tissues and fluids at the moment of sampling (Denator AB, Gothenburg Sweden).

2. The tissue stabilization system utilizes a combination of heat and pressure under vacuum and its utility was demonstrated by monitoring the PTMs and stability of proteins and by checking the enzymatic activities in the mouse and human brain.
Step 1: preserving the tissues

- Stabilize under standardized conditions - no additives
- Improve analytical resolution
- Prevent further degradation
- Preparation history traced from moment of sampling
- Conditions difficult to standardize
- Degradation impacts data interpretation
- Risk of further degradation at every step

www.denator.com
Step 2: sectioning the tissues

1) Contamination with embedding media for cryosection, such as agar, a polysaccharide, Tissue-Tek® and OCT (optimal cutting temperature compound), a combination of polyvinyl alcohol and polyethylene glycol polymers, should be avoided as they suppress ion formation in MALDI MS.

2) To facilitate handling of small or fragile samples (i.e., biopsies), embedding in gelatine or agarose has also been used.

3) At present, the most widely used technique is to affix flash frozen tissue on a cold MALDI target plate or to a conductive surface, i.e. nickel or ITO-coated (indium-tin-oxide) glass slide with a minimal amount of OCT so that it is not in direct contact with the sectioned tissue or microtome blade during sectioning.

4) The microtome blades (preserved in mineral oil) should also be washed with acetone or methanol to prevent chemical contamination if no disposable blades are used.
Step 2: sectioning the tissues

1) The thickness of tissue for MALDI-PMS and MALDI-IMS lies within a range of 5-40 µm; however, for most of the applications 10-20 µm thin sections are used.

2) While thinner sections are difficult to handle, they provide higher quality mass spectra, especially in higher mass range, the thicker sections require longer drying times and have electrically insulating properties, which can adversely affect the image scanning performance.

3) Typically, the sample stage temperature in the microtome is maintained between -5°C to -20°C. The tissue sections with higher amount of fat require (i.e. brain) lower temperature (-15C to-20C) for optimal cutting.

4) The cut tissues are placed by forceps or an artist brush onto a cold surface and thaw-mounted with a warm finger (or placed in a desiccator). Alternatively, the tissue samples might be placed directly on a slide kept at room temperature; however, usage of the cold plate (slide) method is preferred as water-soluble compounds will remain within the tissue sample and the tissue alterations are minimal.

Lalowski et al., J Proteomics, in revision
Step 3: tissue pre-treatment

1) Before protein/peptide imaging is executed, the tissue needs to be rinsed to fix proteins and remove contaminants such as endogenous molecular species (lipids or biological salts) and tissue-embedding media, which may affect protein desorption/ionization efficiency.

2) Usually washing increases the intensity of observed signals 3-10 fold, depending on the sample. For example HPLC-grade ethanol-based tissue rinsing, performed for approximately 30 seconds, improves the quality of mass spectra and preserves the tissue over time. Usually the first washing step in 70% of ethanol is followed by 95% ethanol or a mixture of 90% ethanol, 9% glacial acetic acid, and 1% deionized water.

3) Before (and after) the tissue washing procedure is implemented the sections are usually dried in a desiccator for 15-20 min., or briefly under a nitrogen stream.

Lalowski et al., *J Proteomics*, in revision
To improve signal sensitivity in MALDI profiling experiments, Lemaire et al., 2006 have developed a tissue-washing procedure using organic solvents traditionally used for lipid extraction, i.e., chloroform, hexane, toluene, acetone, and xylene. The increased detection for peptides/proteins (m/z 5-30 kDa) was close to 40% with chloroform or xylene, and 25% with hexane, while also improving sample reproducibility for each solvent used in the study.
Step 3: tissue pre-treatment

Systematic study exploring the effects of 11 different solvent combinations in tissue-washing approaches, for their effect on protein and lipid signals was performed by Seeley et al., 2008. In that study, alcohol-based washes of sections, in particular consecutive washes with isopropanol (70% and 95%), were found to be most effective for protein analysis when considering MS signal quality, matrix deposition regularity, and preservation and histological integrity of the tissue.

Figure 2. (a) TIC variations recorded from the MALDI-TOF MS protein profiles acquired from serial mouse liver tissue sections not washed or washed with different solvent systems in the m/z range from 500 to 1100 (lipid component) and m/z range from 2000 to 25,000 (protein component). (b) Number of peak variations as a function of the same washes for the protein component.
Step 4: Matrices

One of the major requirements of successful MALDI-PMS and MALDI-IMS is the proper incorporation of tissue analytes into a thin matrix layer deposited directly on the tissue and the choice of suitable matrices for different molecular classes.

1. Sinapinic acid (3, 5-dimethoxy-4-hydroxycinnamic acid, SA) at ~10-30 mg/ml, has been reported as a matrix of choice for protein analysis both in the linear MALDI-TOF MS and higher resolution MALDI-IMS. It has a high gas-phase basicity (206 kcal/mol) that is particularly suitable for protein MALDI ionization, given its low tendency in analyte fragmentation.

2. CHCA, α-cyano-4-hydroxycinnamic acid, on the other hand is more suitable for the analysis of smaller molecules, especially peptides (below 4 kDa).

3. DHB, 2,5-dihydroxybenzoic acid, ordinarily known to be suitable for negatively charged less than 4 kDa molecules, such as carbohydrates, is less commonly used as the crystals it forms are larger and mainly suited for certain profiling experiments requiring lower resolution images.
The typical solvent used to dissolve the matrix: 50% acetonitrile/0.1% trifluoroacetic acid, also solubilises proteins, such that the application of matrix solution to tissue is thought to delocalize analytes and disturb tissue integrity if no prior fixation step is performed. In the course we will utilize 60% acetonitrile/0.2% TFA, which in our hands performs best.
### Table 2. Matrix application techniques for imaging MS

<table>
<thead>
<tr>
<th>Technique</th>
<th>Droplet diameter</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Droplet deposition by hand [94, 91]</td>
<td>Variable, mostly large (&gt; 900 μm)</td>
<td>Fast, simple, cheap</td>
<td>No spatial information, poor reproducibility</td>
</tr>
<tr>
<td>Pneumatic nebulization (airbrush) [16, 94]</td>
<td>Variable, mostly small (aerosols)</td>
<td>Fast, simple, homogenous layer, cheap</td>
<td>Limited environmental control, low concentrations of matrix solution can be used, quality varies from person to person, droplet size not constant</td>
</tr>
<tr>
<td>ChIP [97, 99]</td>
<td>~150 μm (100 pL)</td>
<td>Uniform droplets, precision of placement, conditions can be controlled, automated, high signal quality, reproducible</td>
<td>Slow, nozzle tip clogging, expensive</td>
</tr>
<tr>
<td>Acoustic reagent multi-spotter [94, 98]</td>
<td>180–230 μm (170 pL)</td>
<td>Uniform droplets, precision of placement, automated, no clogging, fast, good reproducibility</td>
<td>Matrix applied as droplets, few experiences in use of it</td>
</tr>
<tr>
<td>Electrosprey deposition [94, 92]</td>
<td>Small</td>
<td>Homogenous layer, equally sized crystals</td>
<td>Limited time for analyte–matrix interaction, quality varies from person to person</td>
</tr>
<tr>
<td>Pneumatic Sprayer [96]</td>
<td>Variable, but small</td>
<td>Homogenous layer, automated, controlled environment, suitable for large area</td>
<td>Very large amounts of matrix solution used (50–150 mL), droplet size not constant</td>
</tr>
<tr>
<td>ImagePrep [100]</td>
<td>Variable, but small (~20–50 μm)</td>
<td>Conditions can be varied and controlled, automated, homogenous layer</td>
<td>Slow, small area, membrane clogging, droplet size not constant, expensive</td>
</tr>
<tr>
<td>Dry-coating [102]</td>
<td>20 μm (crystal size)</td>
<td>Cheap, very homogenous, high purity of organic matrix, reproducible, fast</td>
<td>Limited time for analyte–matrix interaction, only lipids detected so far</td>
</tr>
<tr>
<td>Sublimation [101]</td>
<td>Very small</td>
<td>Cheap, very homogenous, high purity of organic matrix, reproducible, fast</td>
<td>Limited time for analyte–matrix interaction, only lipids detected so far</td>
</tr>
<tr>
<td>Desktop inkjet printer [93]</td>
<td>Very small (droplet volume 3 pL)</td>
<td>Uniform droplets, precision of placement, automated, simultaneous deposition of different solutions (multi-channel), cheap</td>
<td>Slow, not compatible with all solvents, clogging</td>
</tr>
</tbody>
</table>

Kaletas et al., *Proteomics* 2009
Vibrational vaporization of the matrix with a piezo-electric spray head is utilized in the Imageprep from Bruker Daltonics. An optical light-scattering sensor assesses matrix thickness, tissue wetness and drying rate during the whole procedure (approximately 120 minutes for one slide).
Step 6: matrix application

1) In **MALDI-PMS experiments** matrix is either applied to a discrete spots on the tissue, by depositing small droplets of matrix on defined regions of the tissue (using pipette, syringe pump or an automated robotic spotter) or fully covering the tissue section with fine matrix layers selecting the zone of interest to where the laser pulses will be directed.

2) For **MALDI-IMS**, coating of the entire tissue with a homogenous layer of the matrix solution is utilized. The techniques for matrix deposition in MALDI-IMS include manual protocols, which suffer from low reproducibility: i.e. spraying using an airbrush or TLC sprayer, dipping the tissue sections into matrix containing solutions or automated ones.

Caldwell and Capriolli., *MCP* 2005
Step 7: (Immuno)-Histochemistry

Consecutive sections staining

Post analysis HY staining
Step 7: (Immuno)-Histochemistry

The regions of interest can be well defined by histopathology directed profiling using classical histopathology stains, with preferential usage of hematoxylin-eosin Y (H and Y stain), methylene blue, cresyl violet, DAPI and/or immunohistochemistry allowing the recognition of tissue, region specific molecular signatures.
MALDI tissue profiling was combined with *in situ* tissue enzymatic digestion, which appears to be mandatory for FFPE tissue analysis Wisztorski et al, 2007.
Alternative protocol: TAMSIM

Thiery et al. developed TArgeted Multiplex MS IMaging (TAMSIM), utilizing photocleavable mass tags that are covalently coupled to antibodies. With the usage of MALDI laser pulses, those tags are cleaved off generating ions of known masses, which enable further tracing of the immunodetected structures in the tissues.

Comparative MALDI mass spectra in the linear positive mode recorded on two adjacent rat brain sections in the same region of the brain after ICC experiment with a primary antibody directed against carboxypeptidase D protein and an anti-rabbit FITC polyclonal secondary antibody (a) or bearing the photocleavable linker/tag system (b).
