MALDI-MSI on tissues

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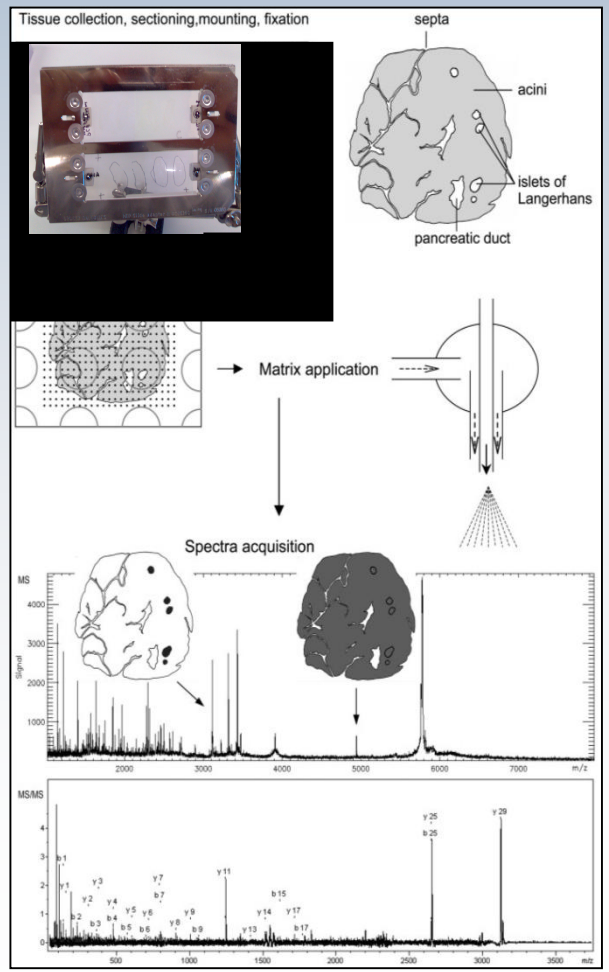
Introduction to Basic Protein Chemistry and Proteomics with Clinical Applications: 03.10.2014

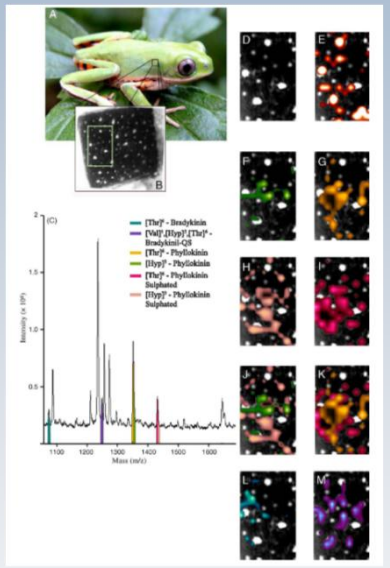


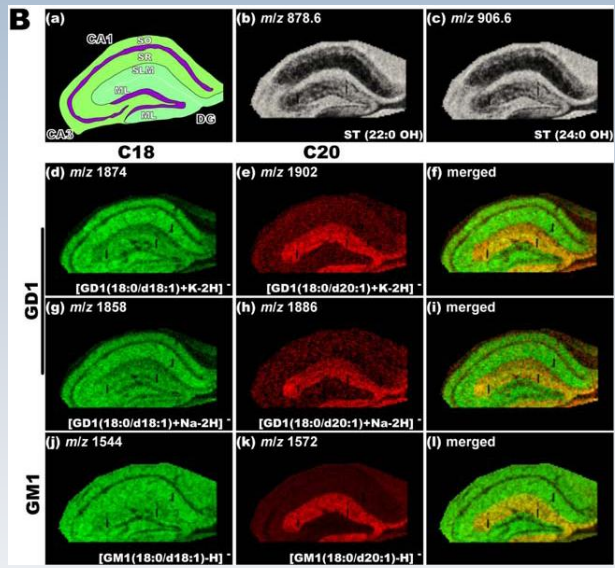
Mass Spectrometric Imaging: definitions

A technique for analyzing the spatial arrangement of proteins, peptides, lipids, and small molecules in biological tissues.

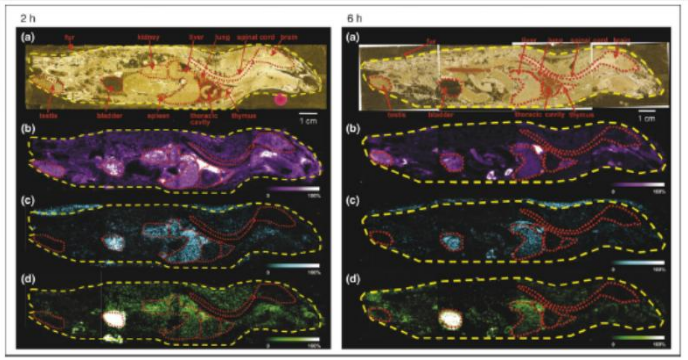
PROS:

1) No labeling required
2) Biomolecules are functionally unmodified
3) Imaging biomolecular modifications
   - PTM's
   - Metabolites
4) Detailed information on molecular identities
5) Vast array of different elements and molecules



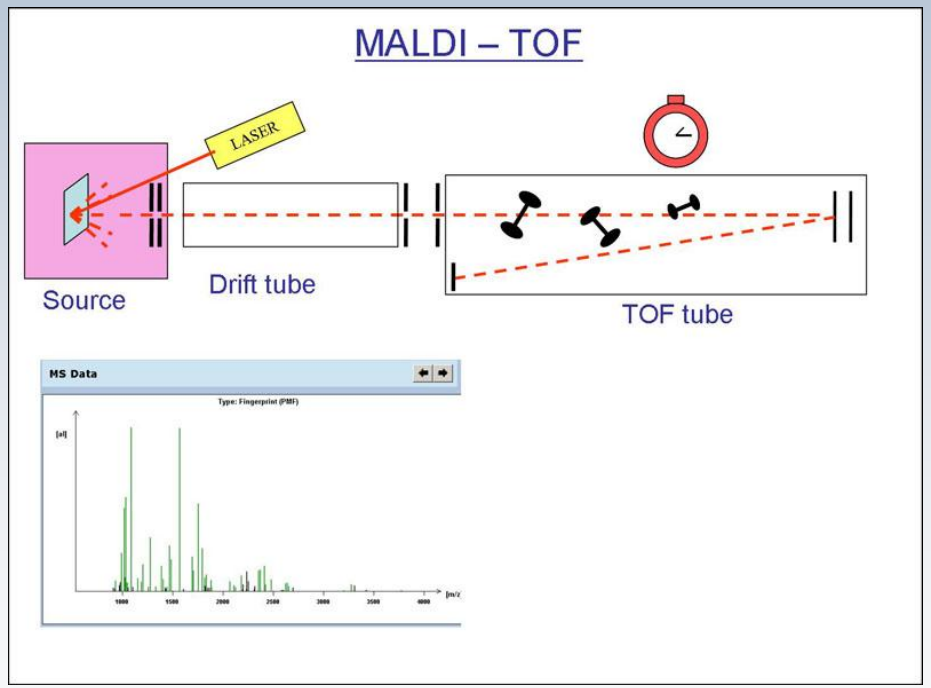


Endogenous peptides **Gangliosides**



Drugs

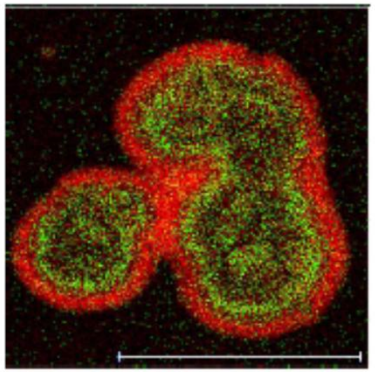
MALDI principle

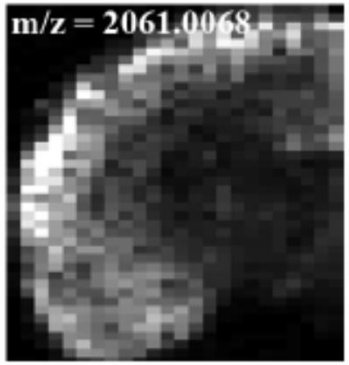


MALDI MS= Matrix assisted laser desorption ionization Mass spectrometry

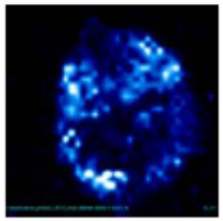
Resolving power in MSI

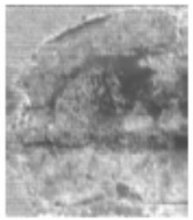
Combined high quality spatial and spectral detail

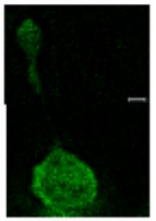




Highest spatial detail







300 nm <100 μm 100 μm 600 nm 200 nm

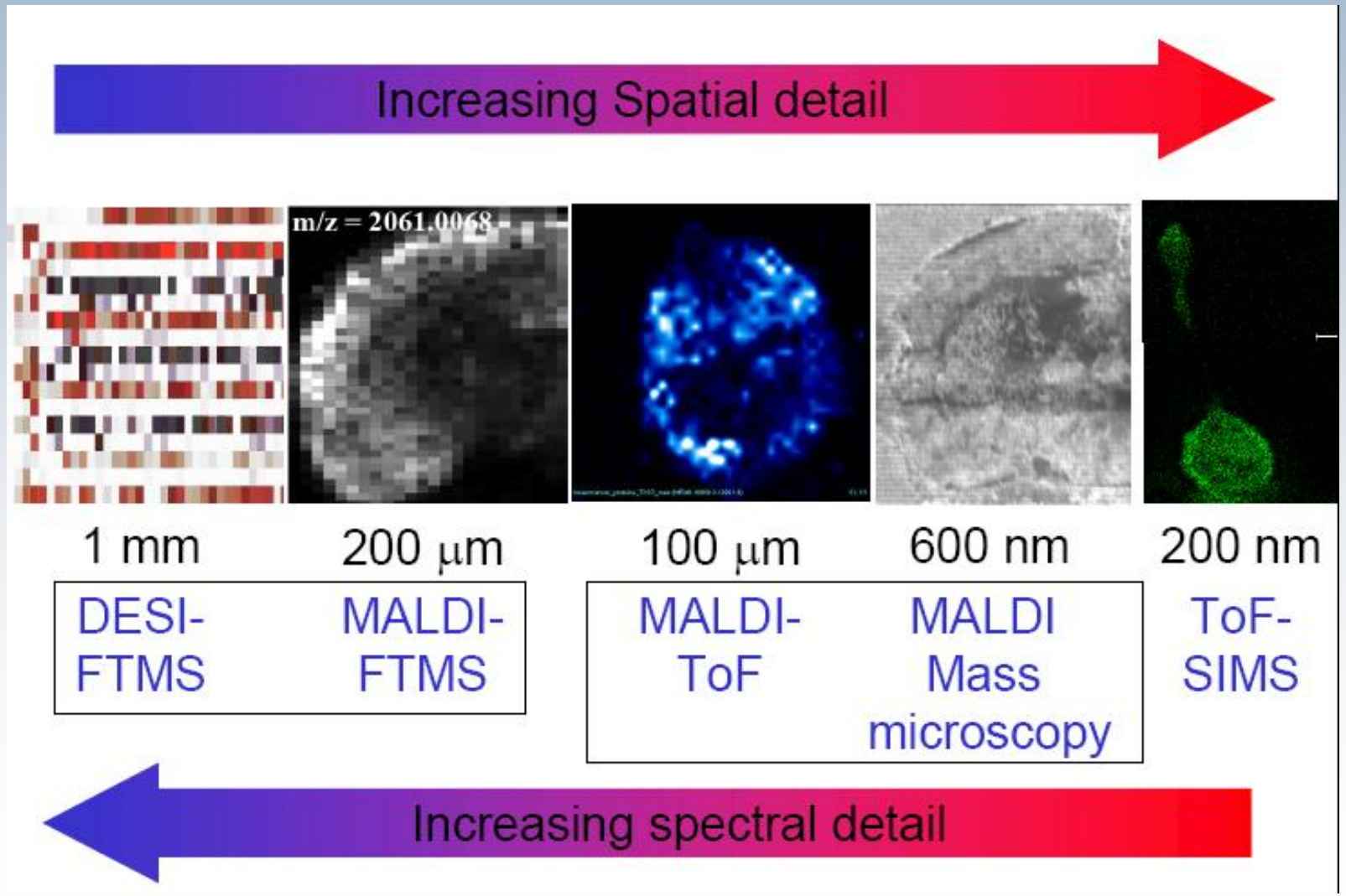
SIMS-FTMS | MALDI-FTMS

MALDI-ToF | MALDI Mass microscopy | ToF-SIMS

Highest spectral detail

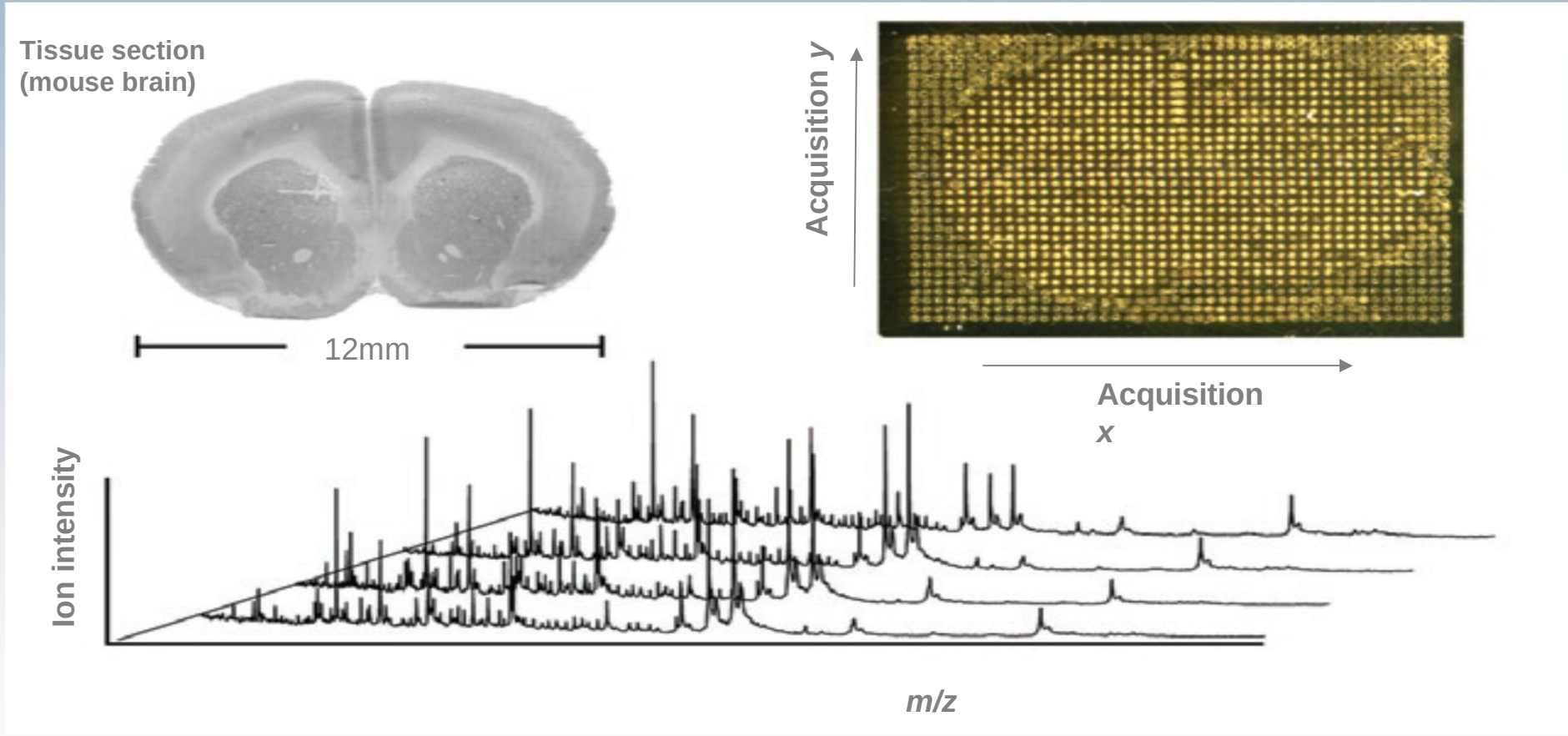
Courtesy by R.M. Heeren

Resolving power in MSI



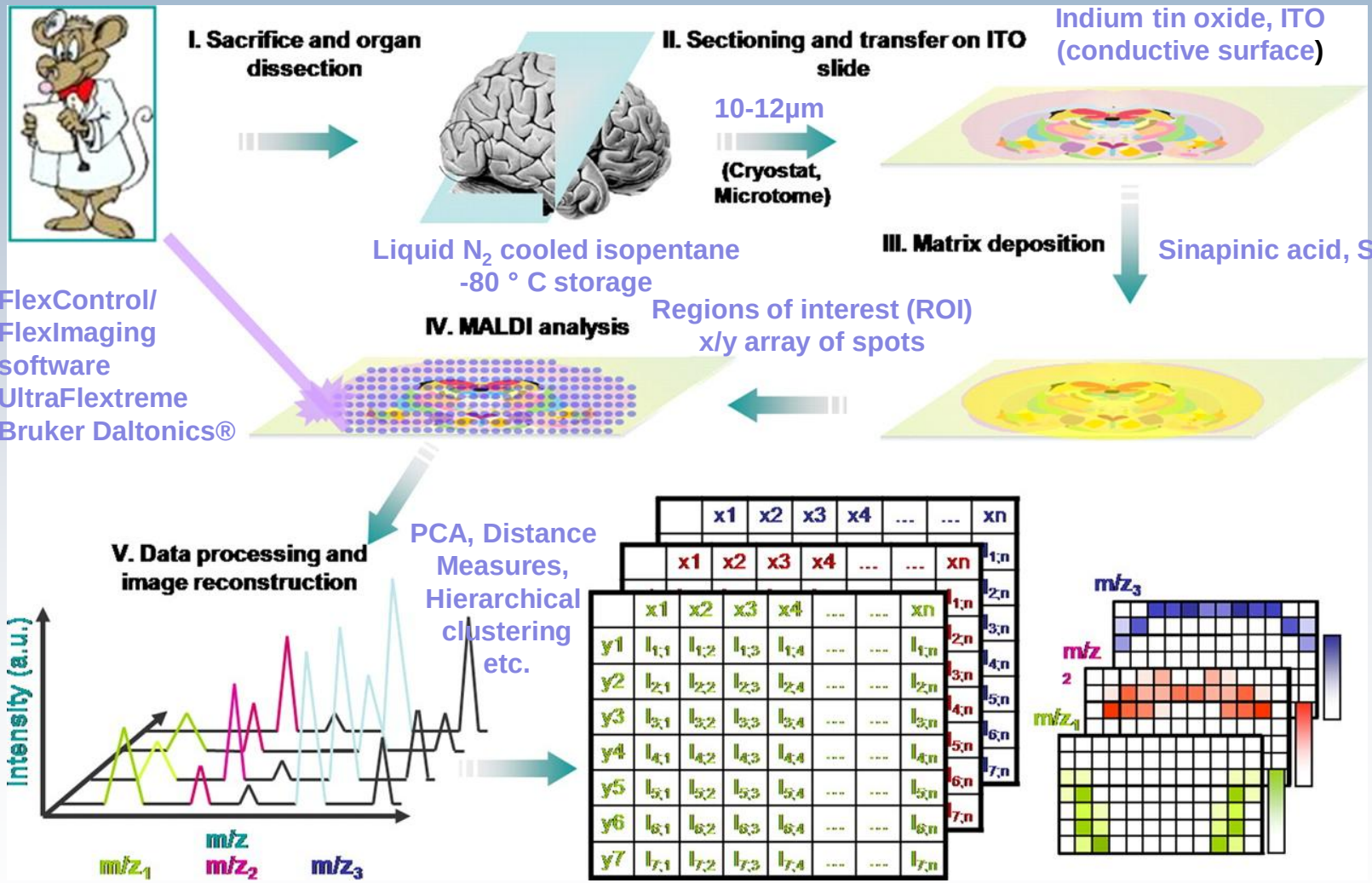
Courtesy by R.M. Heeren

Principles



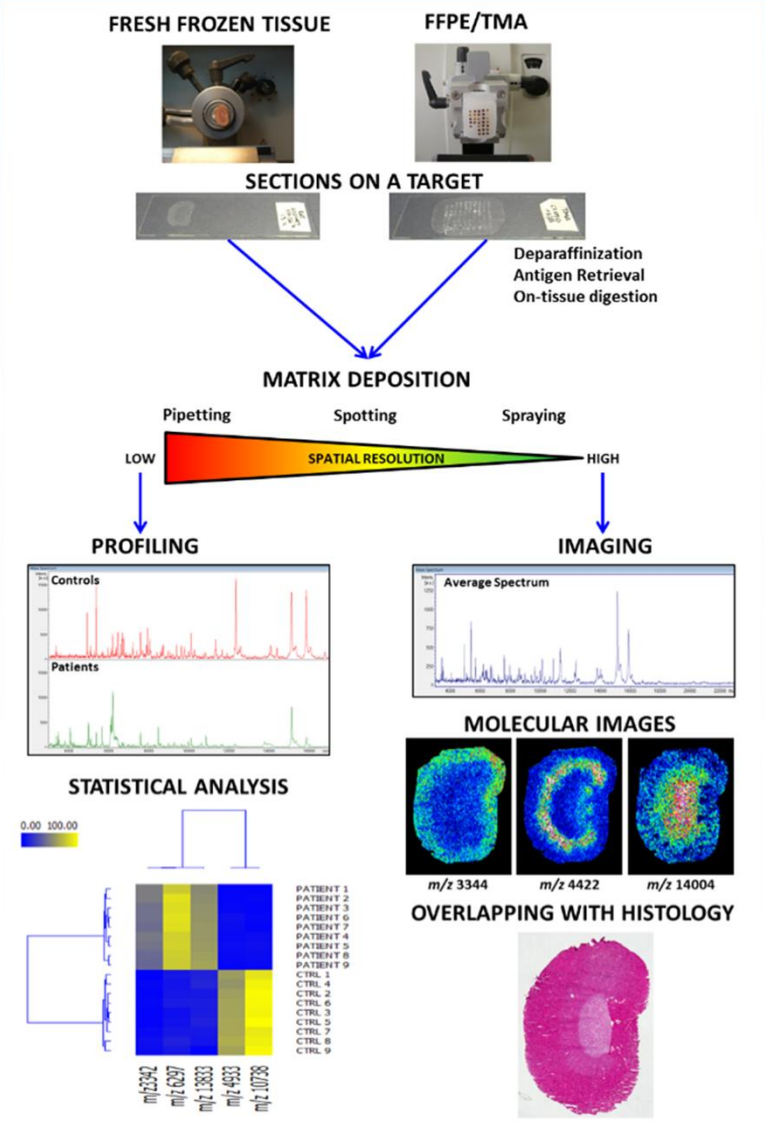
- A laser is rastered over a defined area while acquiring a complete mass spectrum from each position, resulting in molecular images for multiple analytes. Cornett, et al., *Nature Methods* 2007

Schematic representation of the MALDI-MSI work flow



Franck J et al. Mol Cell Proteomics 2009;8:2023-2033

MALDI-MSI: what can be analysed and 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-MSI analysis are cut on a cryomicrotome just before analysis.

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:**

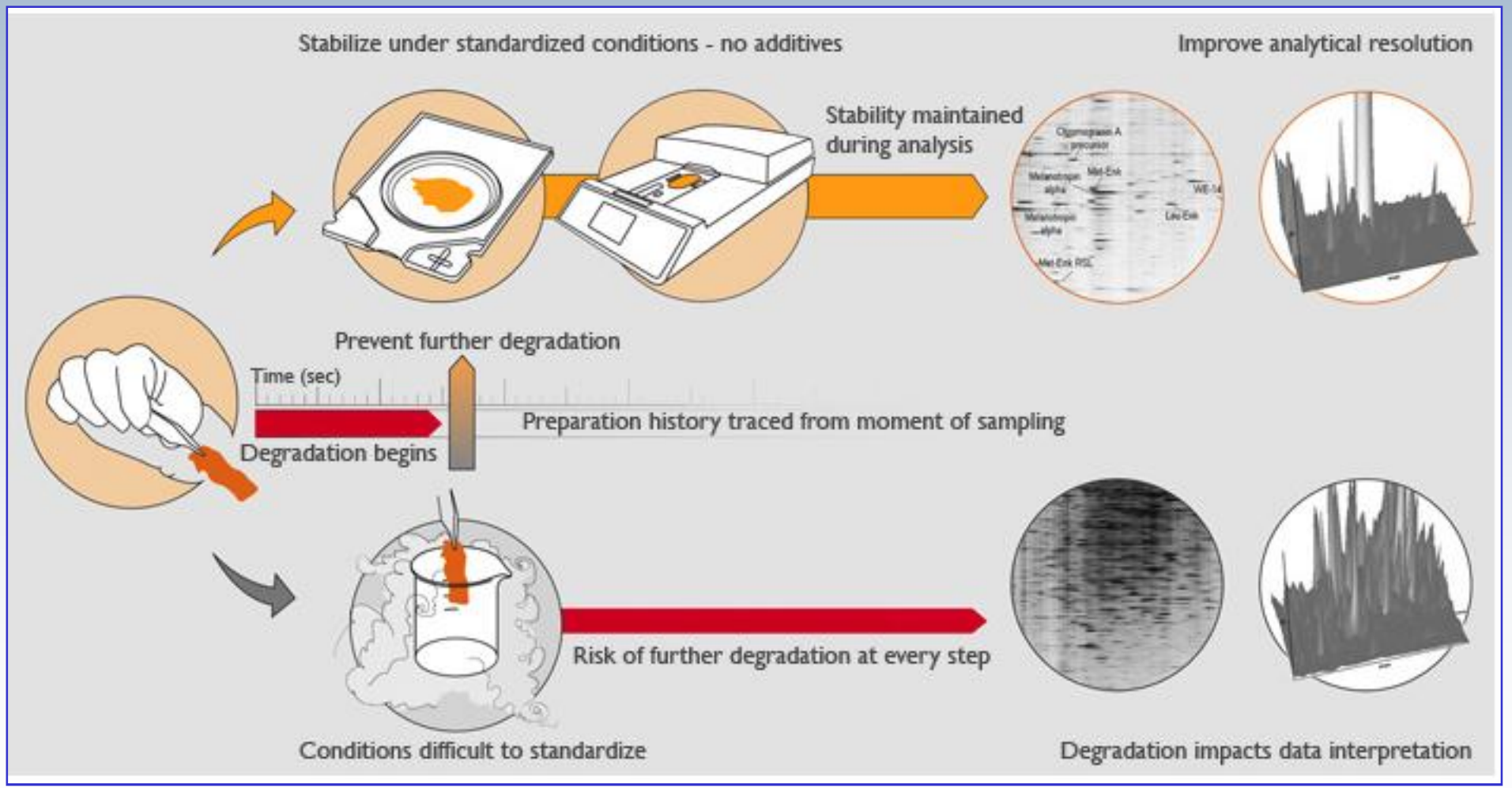
2. **The freezing process can lead to sample cracking and fragmentation, as different parts of the tissue cool down at different rates and ice crystals may form. To avoid sample damage, the tissue may be loosely wrapped in aluminum foil and frozen in liquid nitrogen, ethanol, or isopropanol at temperatures below -70 ° C by gently lowering the tissue into the liquid over a period of 30-60 s (Schwartz et al. *J Mass Spectr.*, 2003).**

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

4. **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**

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

Conductive heat transfer



www.denator.com

Sectioning the tissues I

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.

OCT effect on MSI spectra

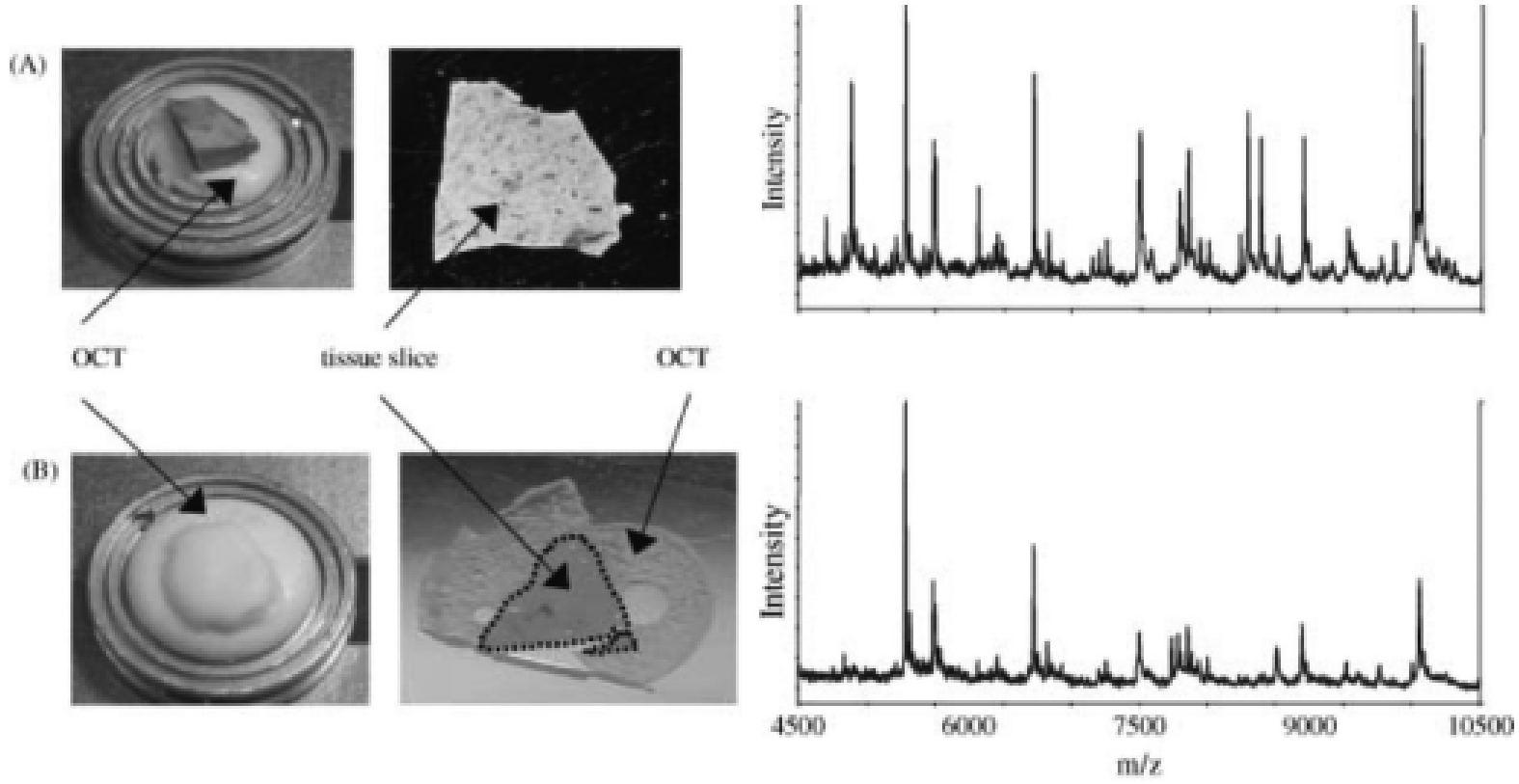


Figure 7. Analysis of the effect of OCT on MALDI signals from rat liver. (A) Procedure where OCT is used to adhere the tissue to the sample stage but does not come into contact with the sliced tissue. The resulting spectrum shows many intense signals between *m/z* 4500 and 10 500. (B) The tissue was embedded in OCT and attached to the sample stage. The resulting spectrum contains only about half of the signals as that in part A. Reprinted with permission from ref 95. Copyright 2003 Wiley Interscience.

(95) Schwartz, S. A.; Reyzer, M. L.; Caprioli, R. M. *J. Mass Spectrom.* **2003**, *38*, 699.

Sectioning the tissues II

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) **Typically, the sample stage temperature in the microtome is maintained between -5 ° C to -25 ° C. The tissue sections with higher amount of fat require (i.e. brain) lower temperature (-15 ° C to-20 ° C) for optimal cutting.**

3) **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 desiccators'). 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.**

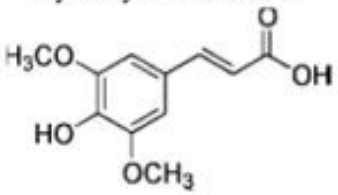
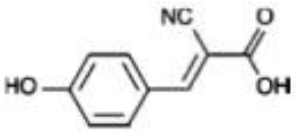
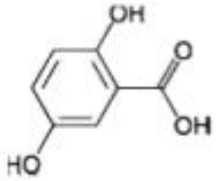
Tissue pre-treatment

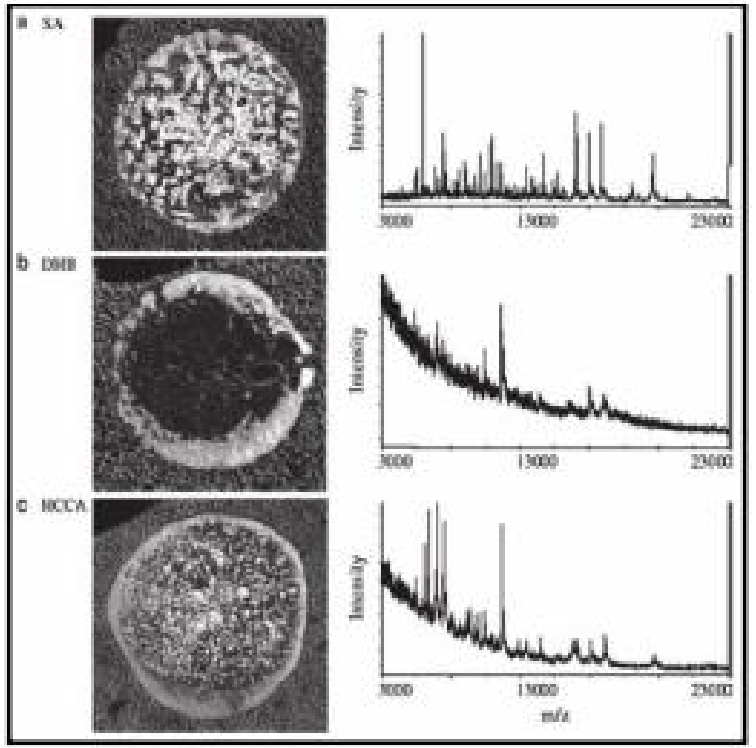
1) **Before protein/peptide imaging is executed, the tissue needs to be rinsed to fix proteins and remove contaminants such as endogenous molecular species (lipids or biological salts) and tissue-embedding media, which may affect protein desorption/ionization efficiency.**

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. 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.**

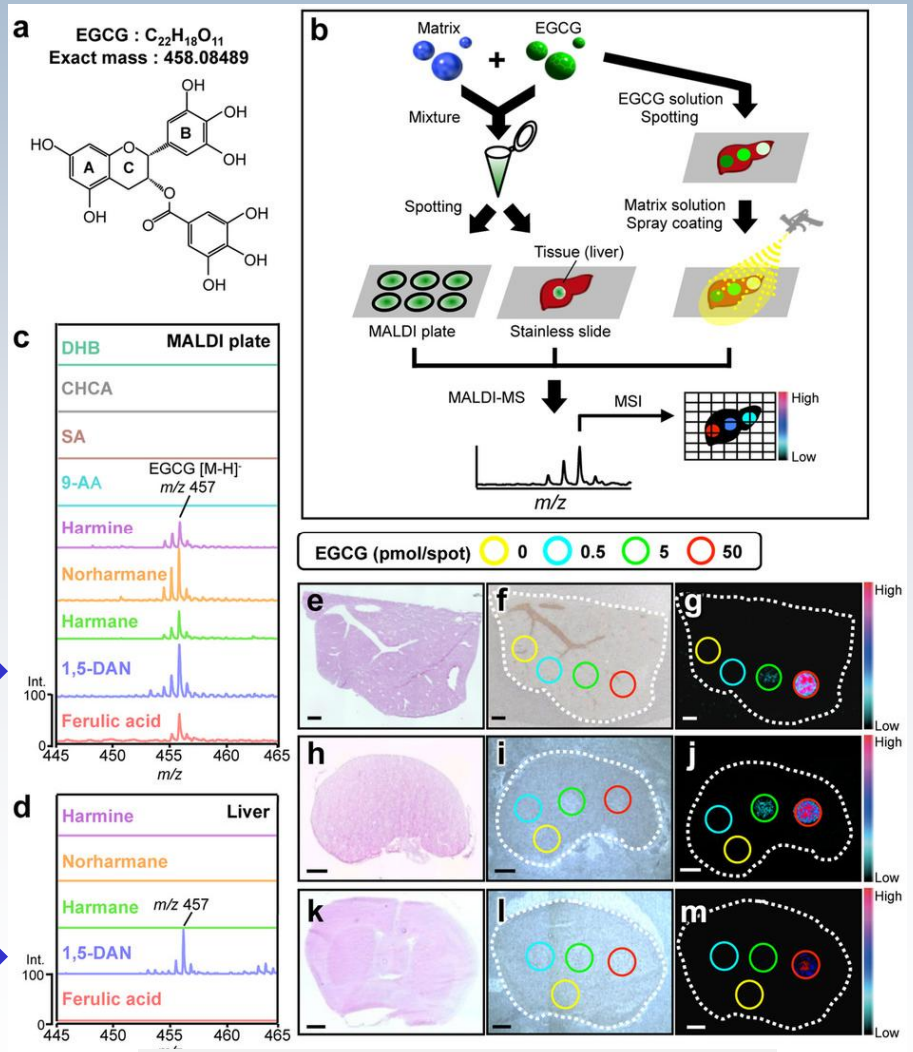
3) **Before (and after) the tissue washing procedure is implemented the sections are usually dried in a desiccator for 15-20 min., or briefly under a nitrogen stream.**

Common matrices

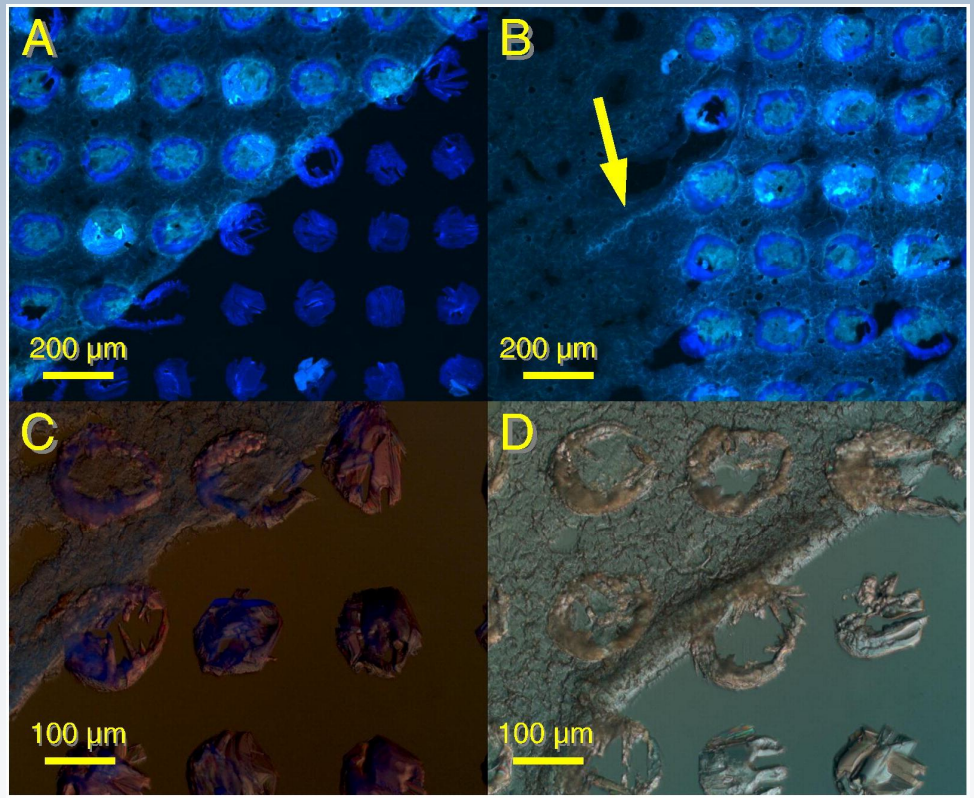
Matrix	SA	CHCA	DHB
Other name	• Sinapinic acid • 3,5-Dimethoxy-4-hydroxycinnamic acid	• α-Cyano-4-hydroxycinnamic acid	• 2,5-Dihydroxy benzoic acid
Structural formula			
MW	224.21	189.17	154.12
Chemical formula	$C_{11}H_{12}O_5$	$C_{10}H_7NO_3$	$C_7H_6O_4$
Solubility	• Low solubility in H_2O • Soluble in methanol/ H_2O and polar organic solvents	• Low solubility in H_2O • Soluble in methanol/ H_2O and polar organic solvents	• Soluble in H_2O • Soluble in methanol/H_2O and polar organic solvents
Feature	High signal-to-noise ratio		The quality of a mass spectrum largely depends on the quality of the matrix's crystal
Subject	Protein (4–30 kDa)	Lipid and peptides (~8 kDa)	Lipid and peptides (~5 kDa)



Other matrices and methods of application I



Kim *et al*. *Nature Sci Rep*, 2013, 3: 2805



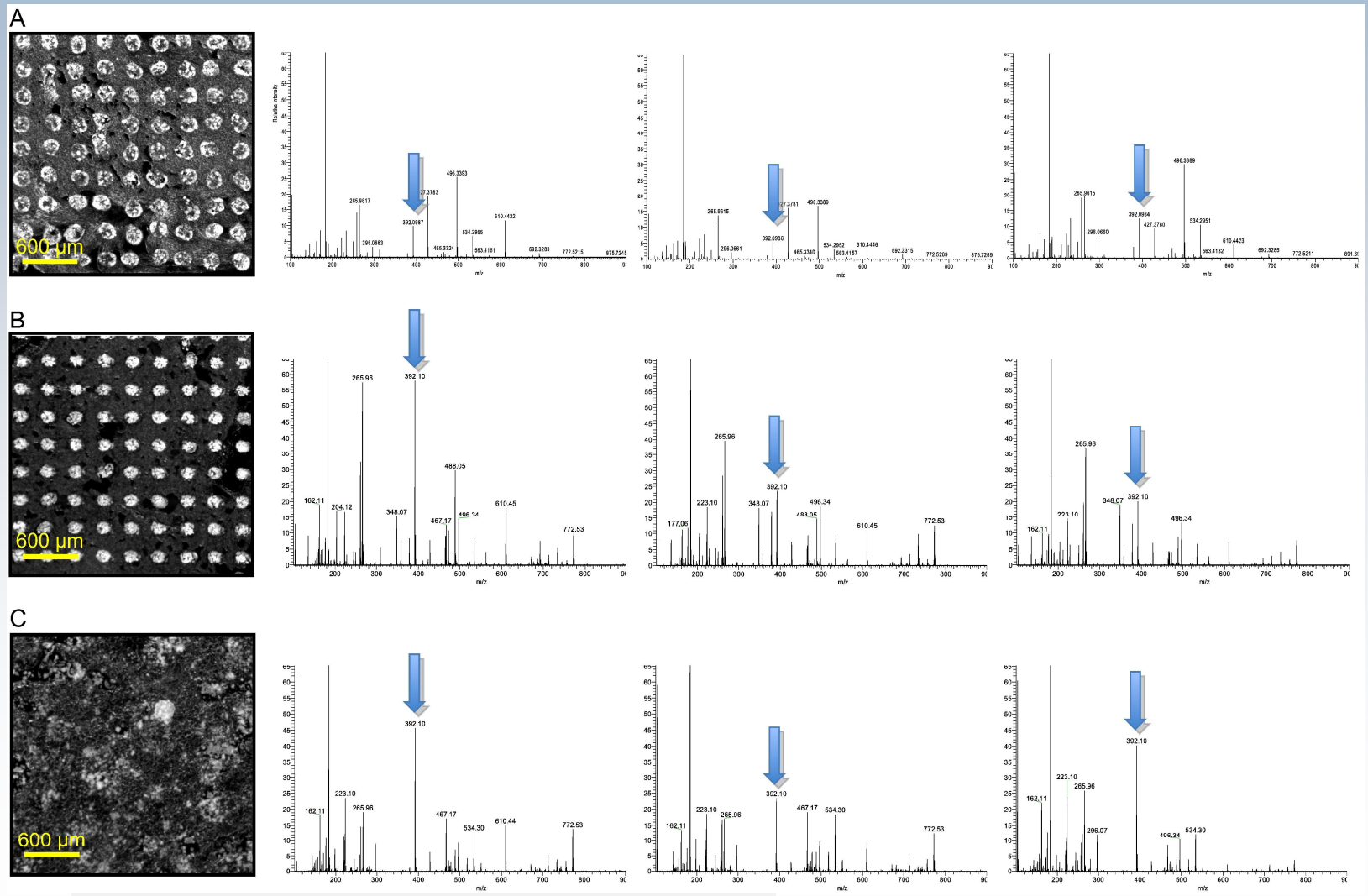
Micrographs of DBH matrix spots on a lung tissue section. DHB was dissolved at 20 mg/mL concentration in 50% MeOH and deposited as 5 droplets/position in 35 cycles (350 ng/spot).

Ákos Végvári *et al*. Proteomics, 2010, 73 (6), 1270-78

Matrix application: spraying

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).

Comparison: spotting vs spraying



Lung tissue sections covered with matrix by:

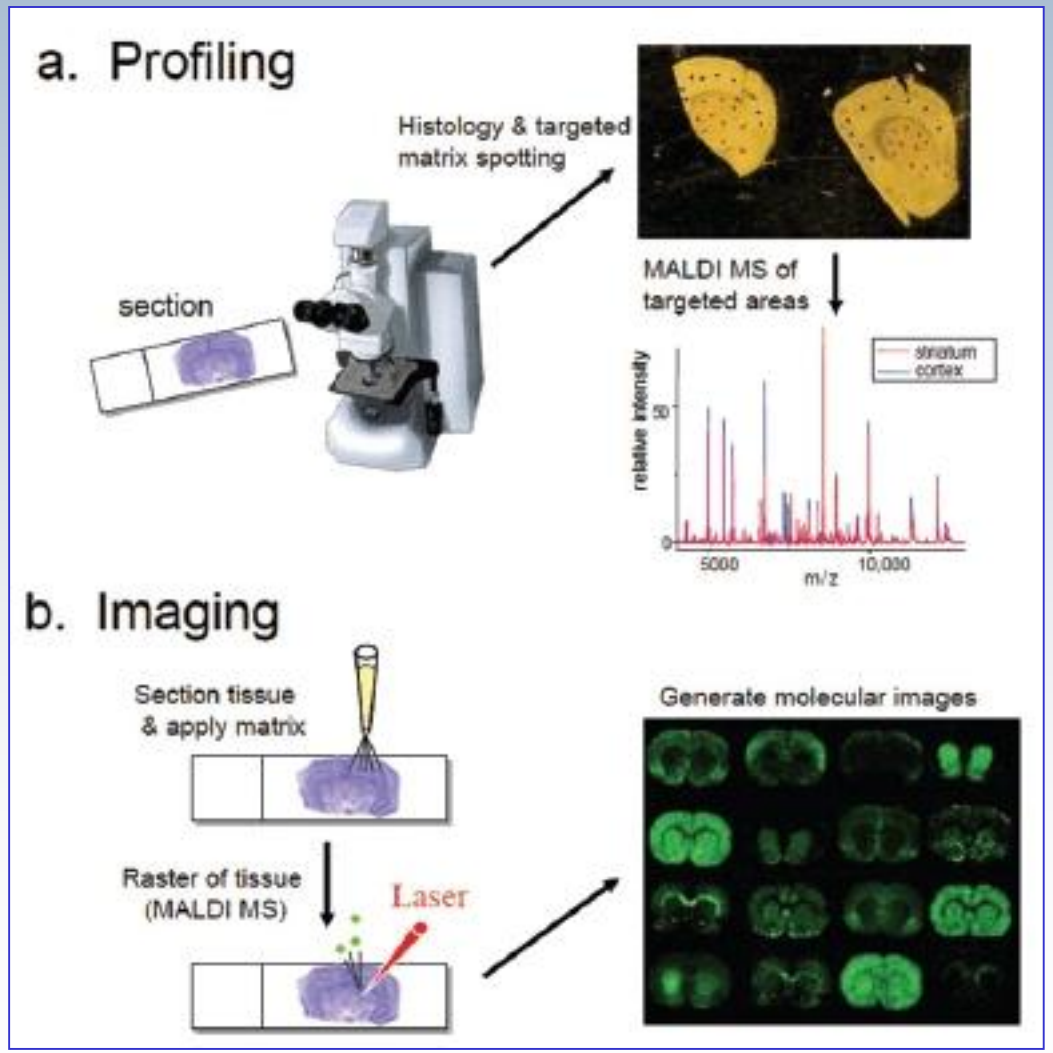
A.nano-spotter (4.5 mg/mL CHCA in 50% Acn/10% isopropanol/0.1% TFA)

B.Labcyte's acoustic spotter (10 mg/ml CHCA in 50% Acn/0.1% TFA)

C.ImagePrep sprayer (5 mg/mL CHCA in 50% Acn/0.1% TFA)
Arrows indicate the tiotropium signals, drug for treatment of *chronic obstructive pulmonary disease*

Ákos Végvári *et al*. Proteomics, 2010, 73 (6), 1270-78

Imaging versus profiling



1) In **MALDI-PMS experiments** matrix is either applied to a discrete spots on the tissue, by depositing matrix on defined regions of the tissue and selecting the zone of interest to where the laser pulses will be directed.

2) For **MALDI-MSI**, coating of the entire tissue with a homogenous layer of the matrix solution is utilized.

Main Andersson, Per Andren and Richard M. Caprioli

Defining *molecular signatures*

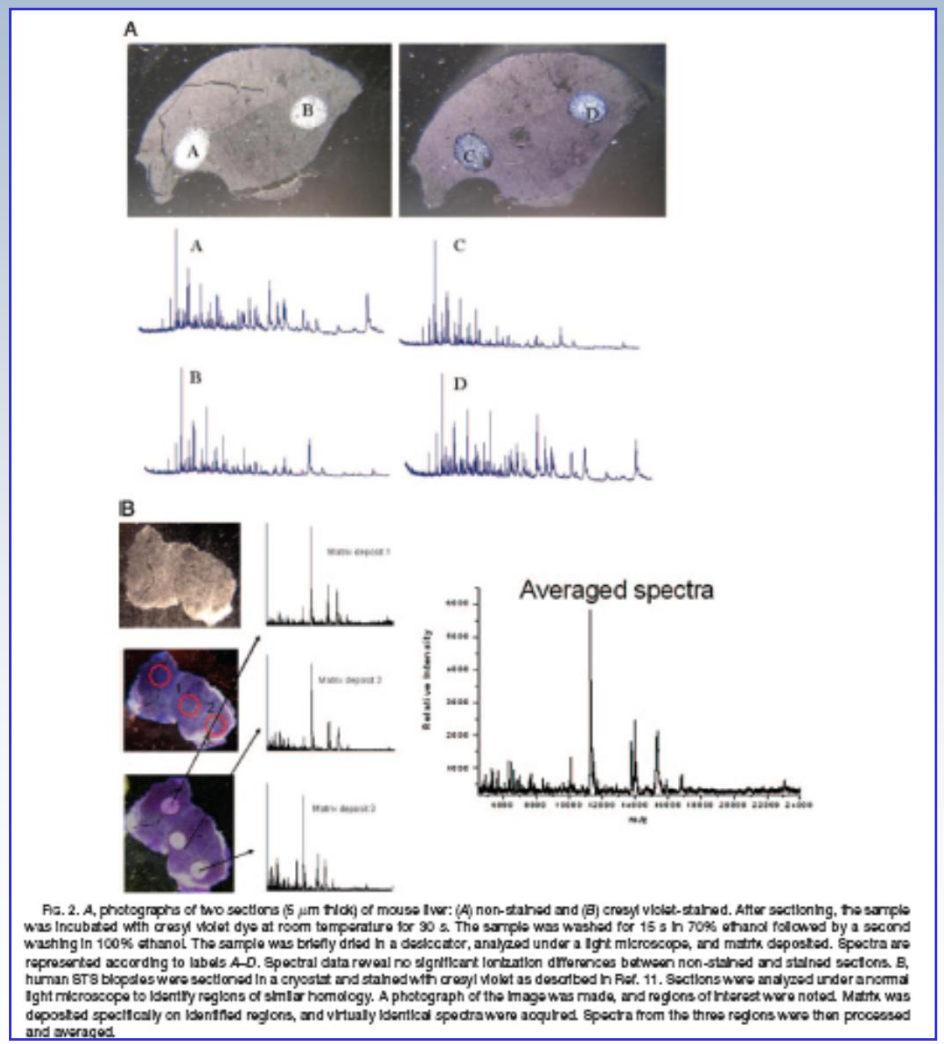
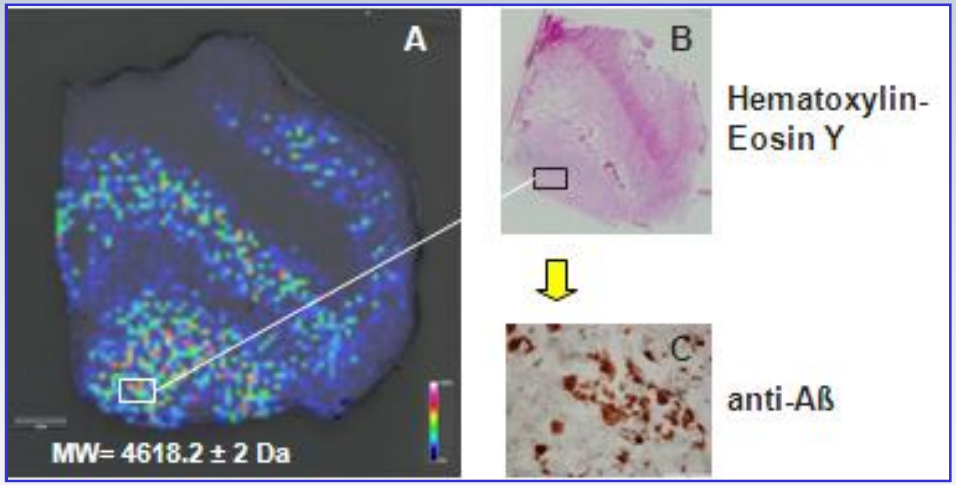


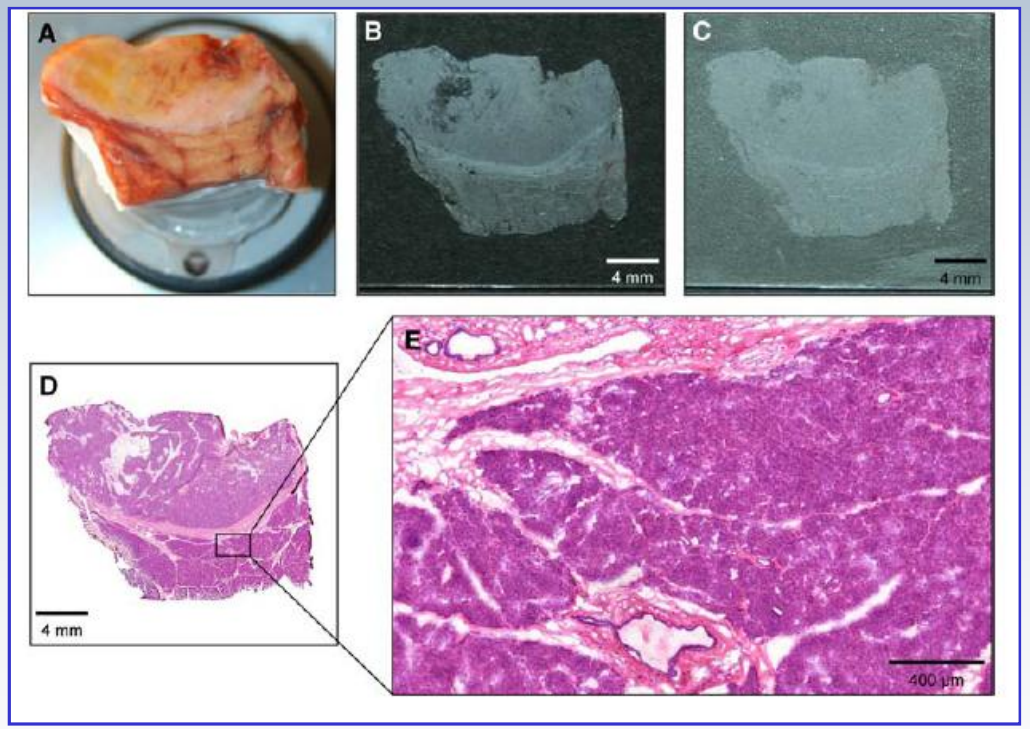
Fig. 2. A, photographs of two sections (5 μm thick) of mouse liver: (A) non-stained and (B) cresyl violet-stained. After sectioning, the sample was incubated with cresyl violet dye at room temperature for 30 s. The sample was washed for 15 s in 70% ethanol followed by a second washing in 100% ethanol. The sample was briefly dried in a desiccator, analyzed under a light microscope, and matrix deposited. Spectra are represented according to labels A–D. Spectral data reveal no significant ionization differences between non-stained and stained sections. B, human STS biopsies were sectioned in a cryostat and stained with cresyl violet as described in Ref. 11. Sections were analyzed under a normal light microscope to identify regions of similar homology. A photograph of the image was made, and regions of interest were noted. Matrix was deposited specifically on identified regions, and virtually identical spectra were acquired. Spectra from the three regions were then processed and averaged.

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.*

(Immuno)-histochemistry

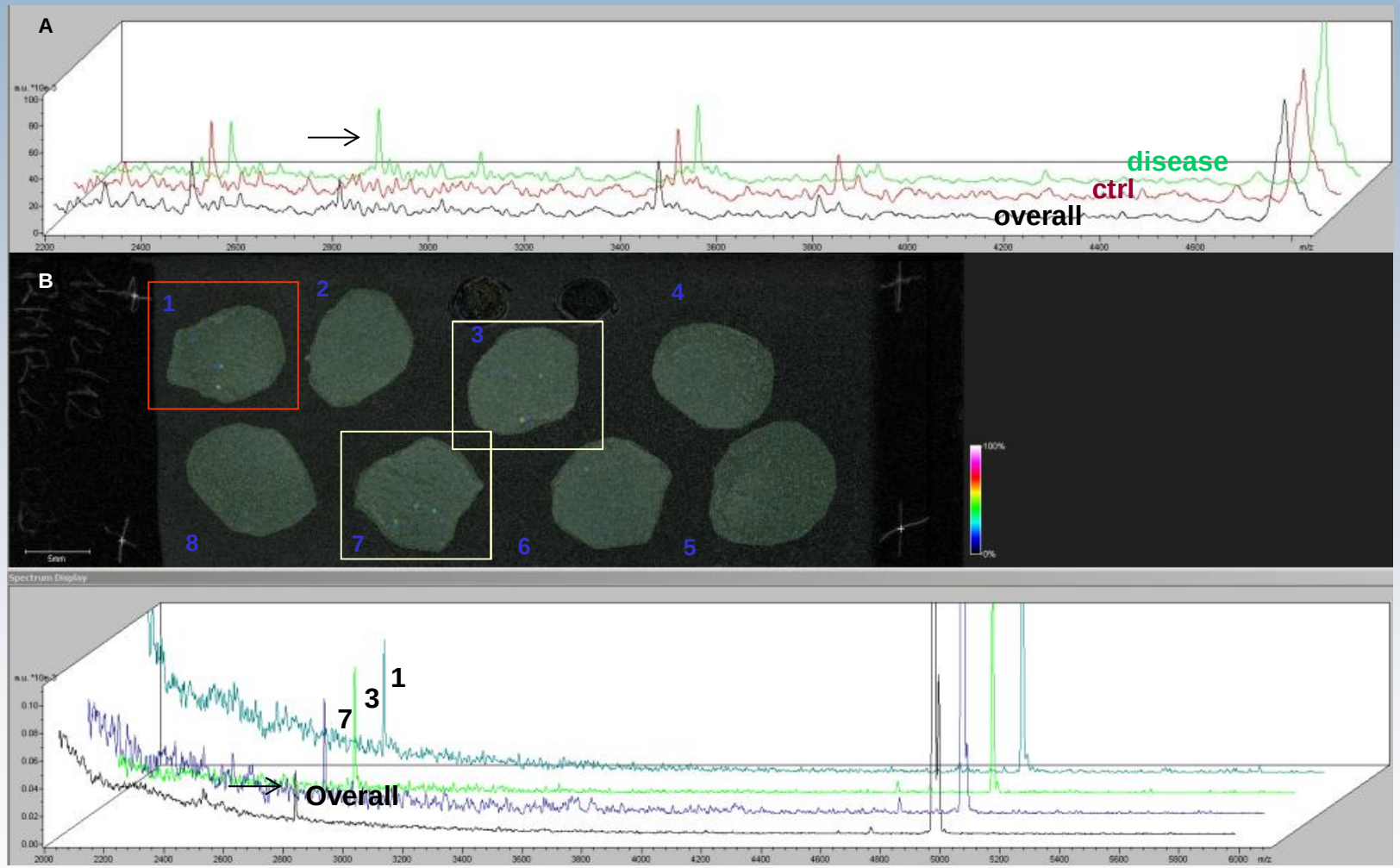


Consecutive sections staining



Post analysis HY staining

Lalowski et al. Nephrology Dialysis Transplantation, 2013
Magni, Lalowski*, et al. J Nephrology, 2013*

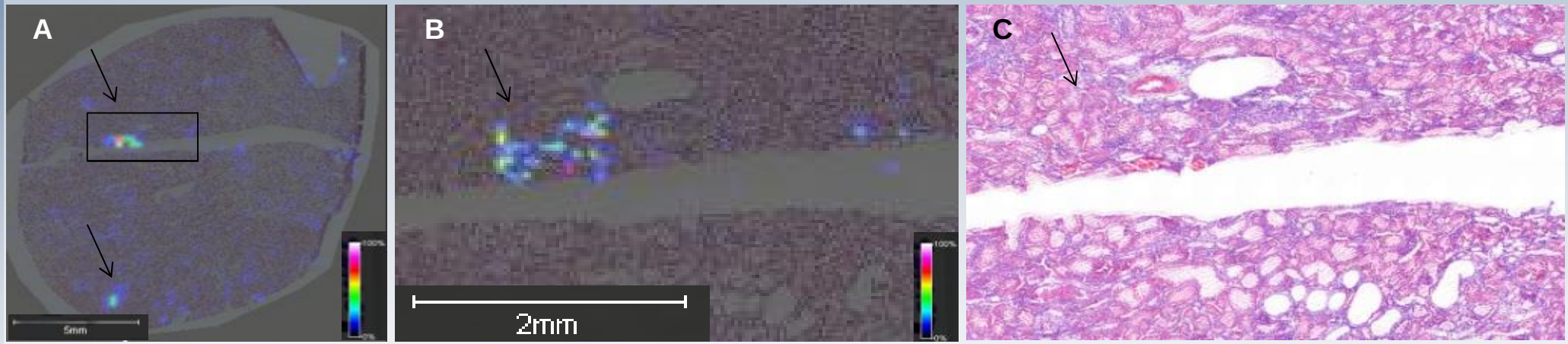


(A) Detection of a peptide with an average mass [Mav+H]+ of 2791.55 ± 1 Da, which corresponded well with the predicted [Mav+H]+ mass of a petide, in focal areas of the remnant kidneys of proteinuric rats (n=3), on several consecutively cut tissue sections (B), labeled as 1-8.

Lalowski, Macconi et al. in preparation

Peptide signal localizes to discrete regions in diseased kidneys

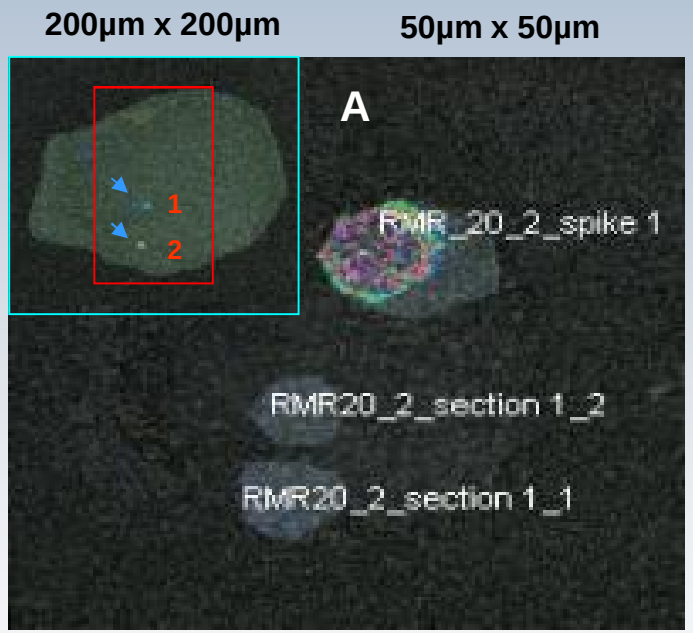
200µm x 200µm **50µm x 50µm** **HE post-analysis**



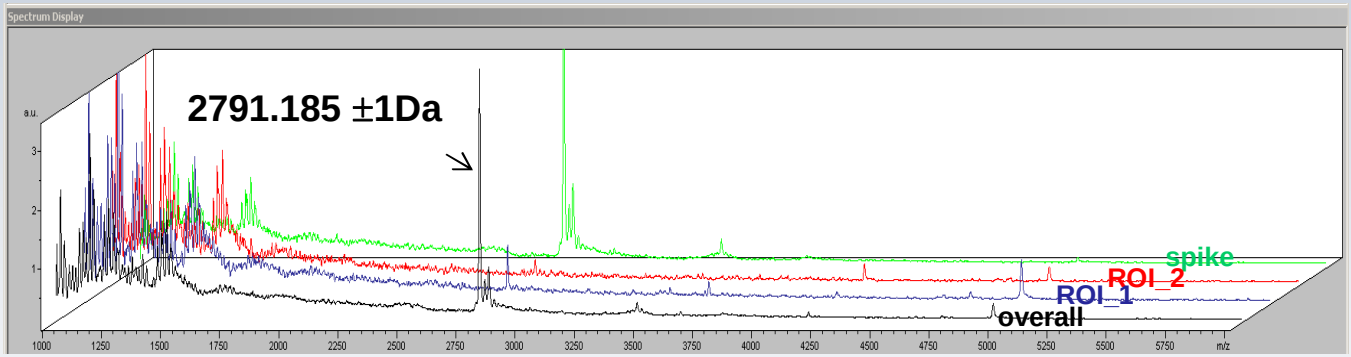
Higher density images (50µm x 50µm, B), obtained using Mirax micro-digital slide scanner (Carl Zeiss), from the signal-rich regions and matching regions from hematoxylin-eosin Y (C), post-analysis stained sections demonstrated a peptide localizing to the cortex, within and in proximity of tubular cells.

Lalowski, Macconi et al. in preparation

The observed *m/z* of endogenous peptide matches to *m/z* of synthetic rat peptide



B

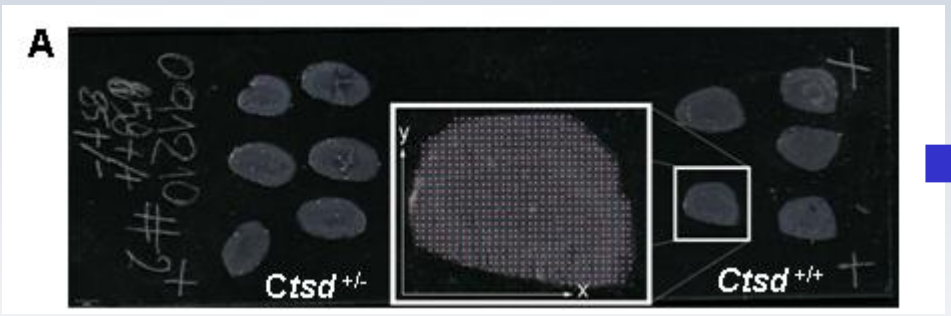


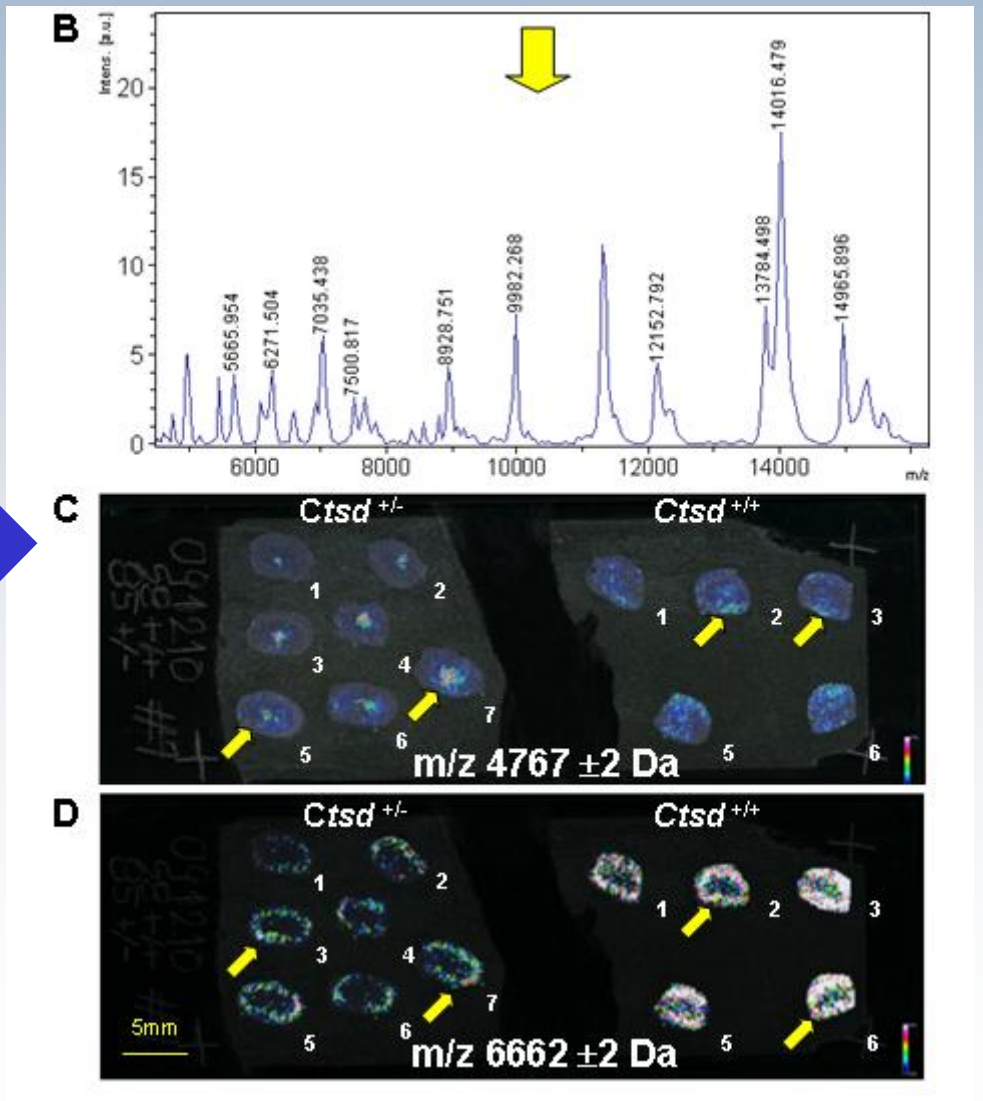
(A) Due to very low abundance of the endogenous peptide, that prevents its sequence identification we spiked the tissue sections with low amounts (10-^{4}µg) of synthetic peptide, in the vicinity of areas where the endogenous signal was detected and rescanned the regions of interest

(B) The detected m/z of the endogenous peptide matched well with the observed and predicted average mass of synthetic peptide

Lalowski, Macconi et al. in preparation

Visualization of molecular species allows differential comparison

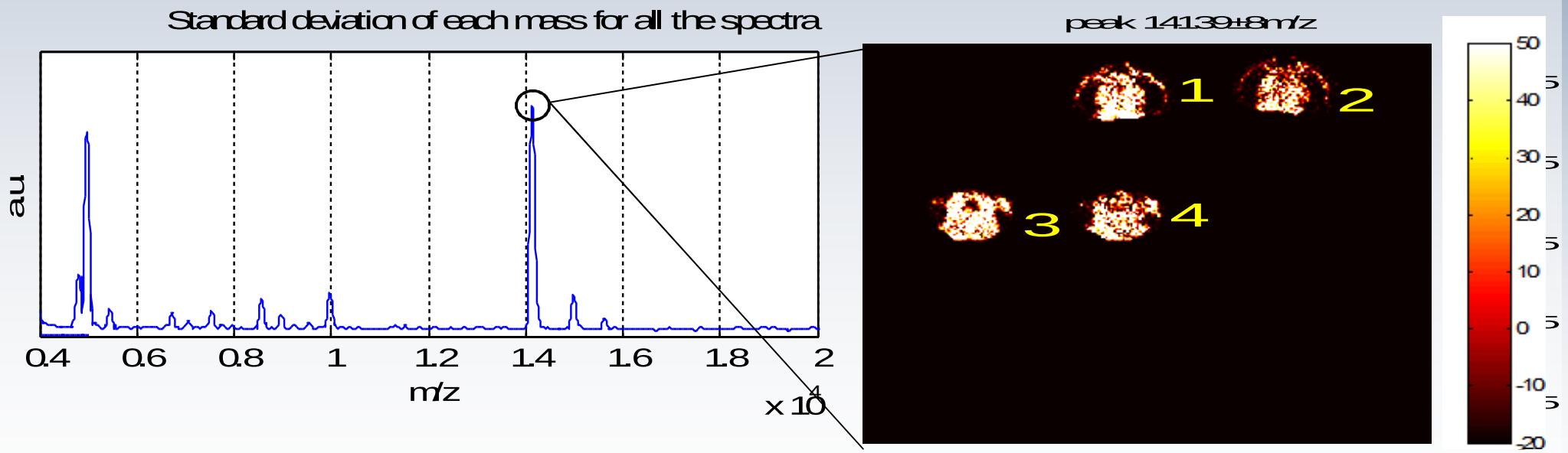




Lalowski et al. Nephrology Dialysis Transplantation, 2013

MALDI-MSI data analysis

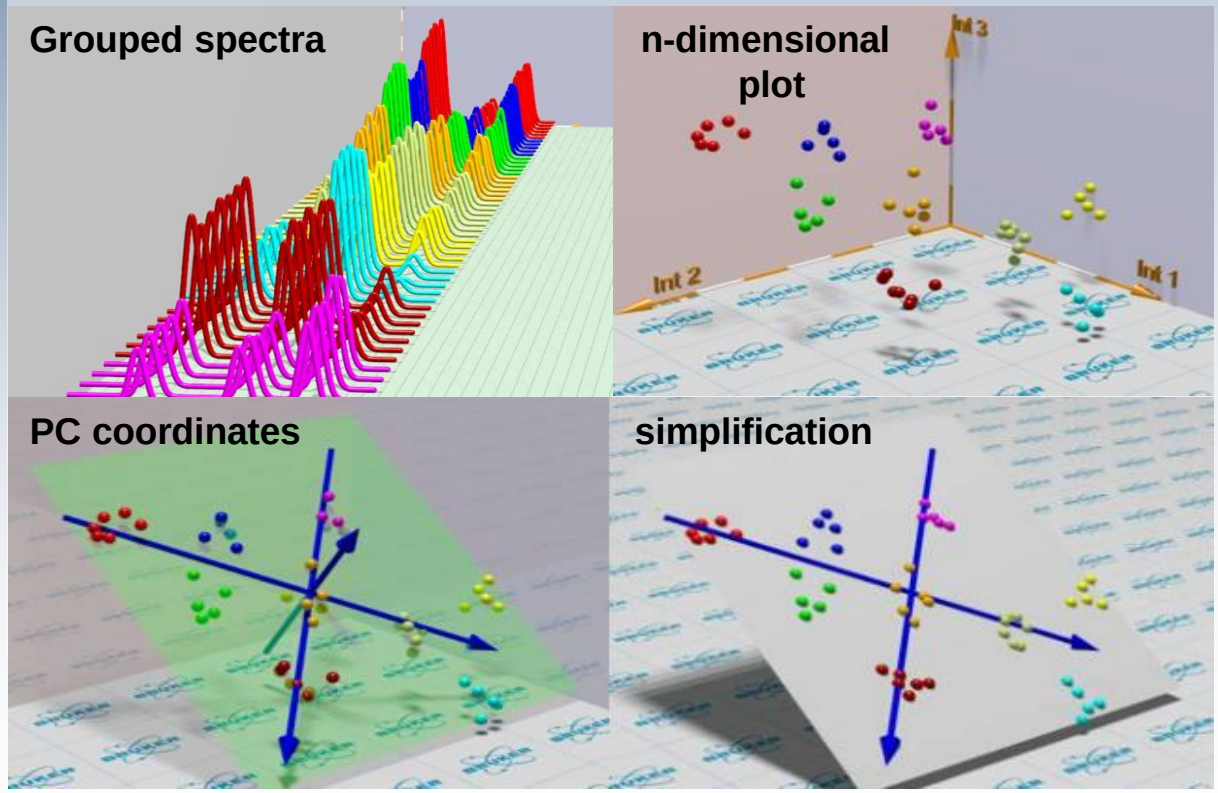
- **Data analysis allows pinpointing differences in protein localization in various conditions**
- **Statistical measures such as standard deviation, skewness and kurtosis unveil peaks of interest and describe the distribution (histograms) of the mass spectra**

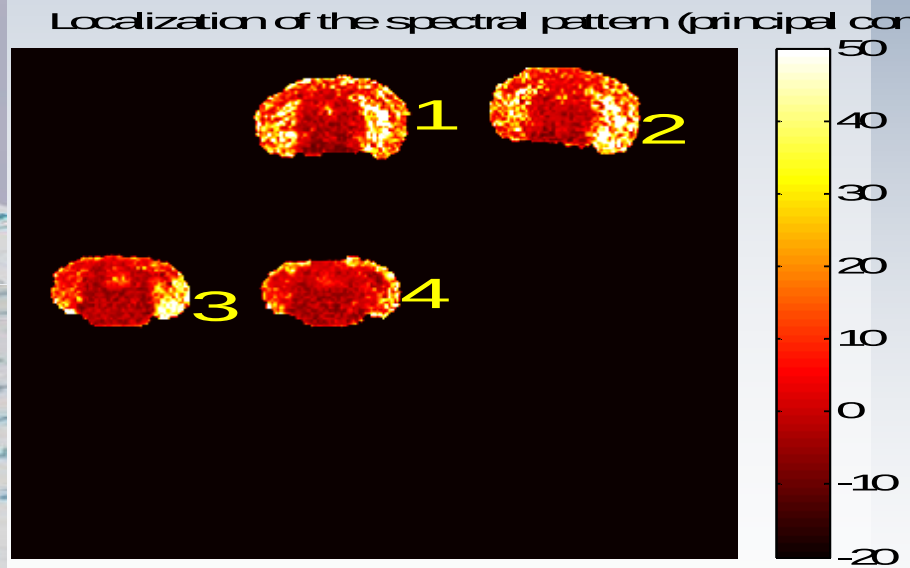


MALDI-MSI data analysis

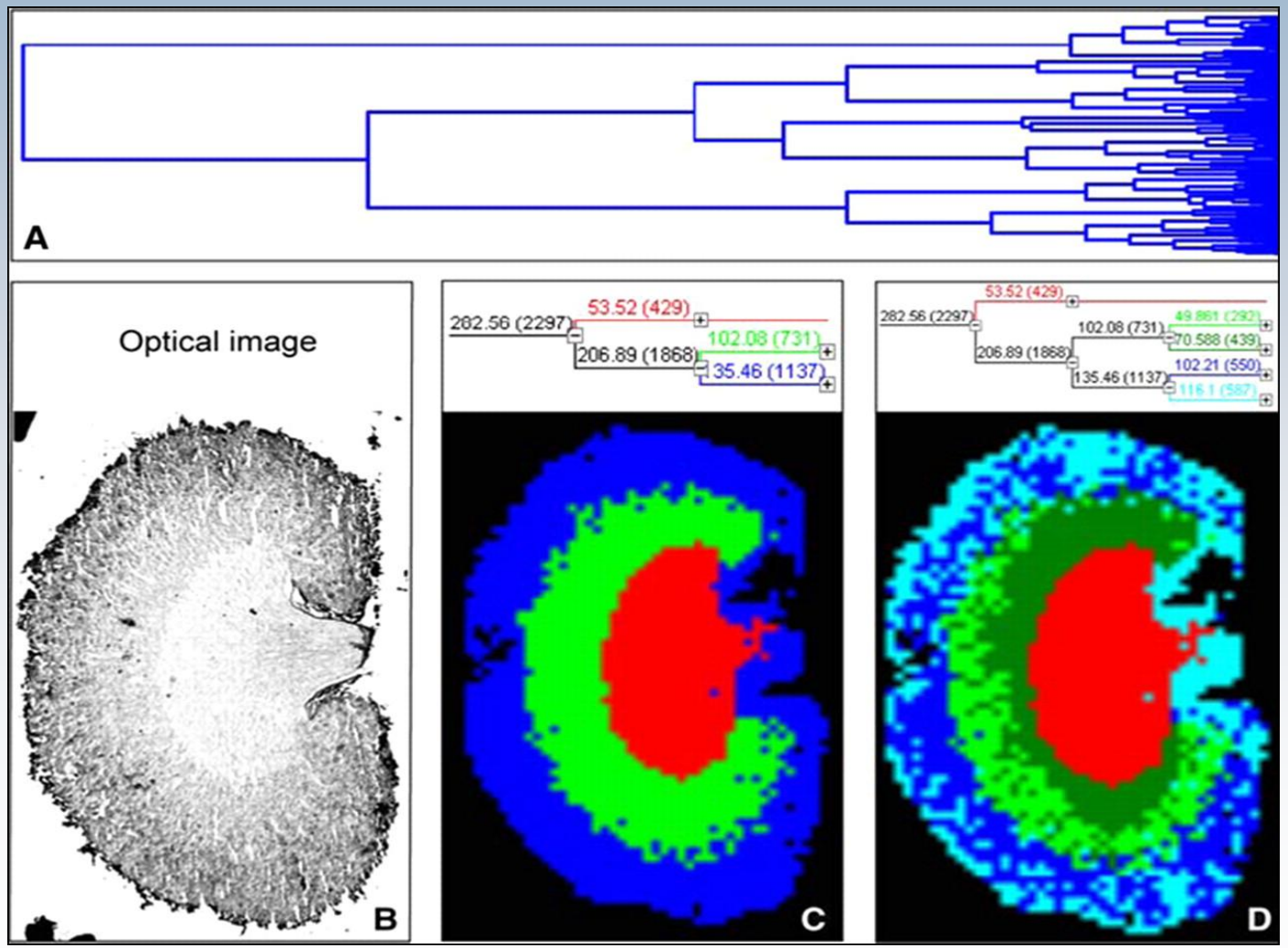
Principal Component Analysis (PCA) reveals spectral patterns instead of individual peaks

PCA: Localization corresponds to similarity of each spectrum to PC, called **PC score**





Hierarchical clustering of a mouse kidney data set achieved by MALDI-MSI

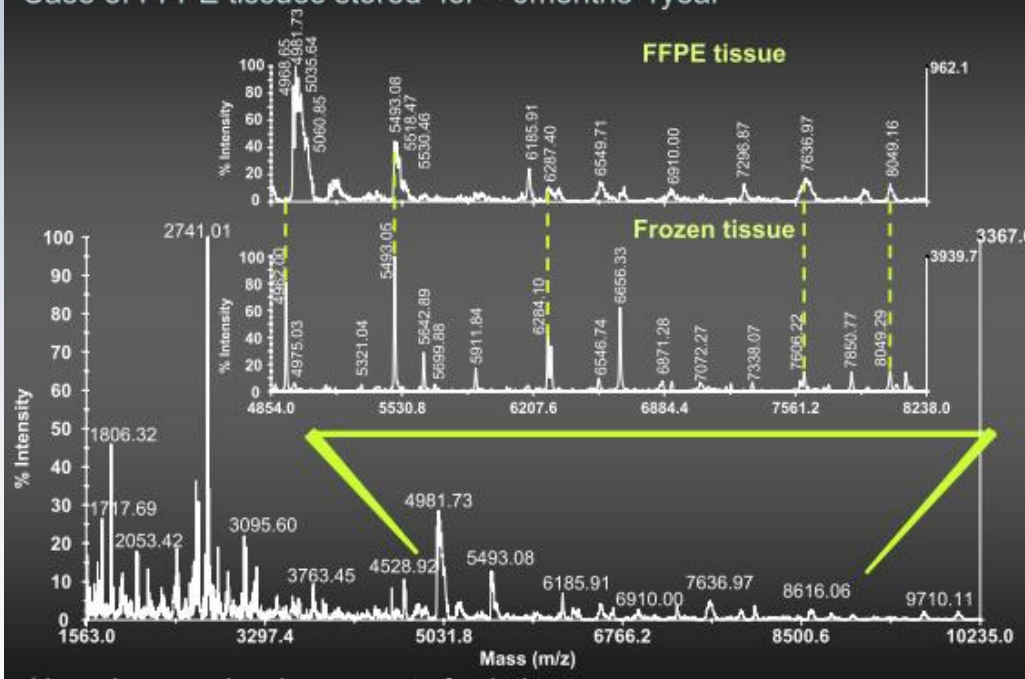


Franck J et al. Mol Cell Proteomics 2009;8:2023-2033

FFPE archaised tissues

ALONG TIME COURSE… FFPE < 1 year

Case of FFPE tissues stored for < 6months-1year



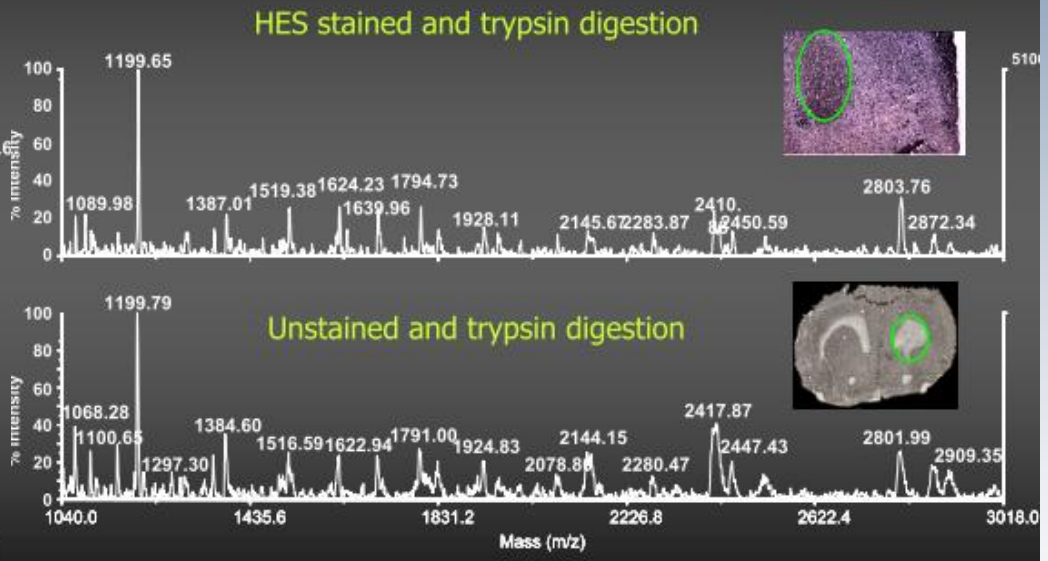
✓ Less intense signal compare to fresh tissue

✓ Overlapping peaks : +12 u. adducts due to the PFA fixation

ALONG TIME COURSE… FFPE > 1 year

✓ No exploitable data whatever the matrix or treatment use

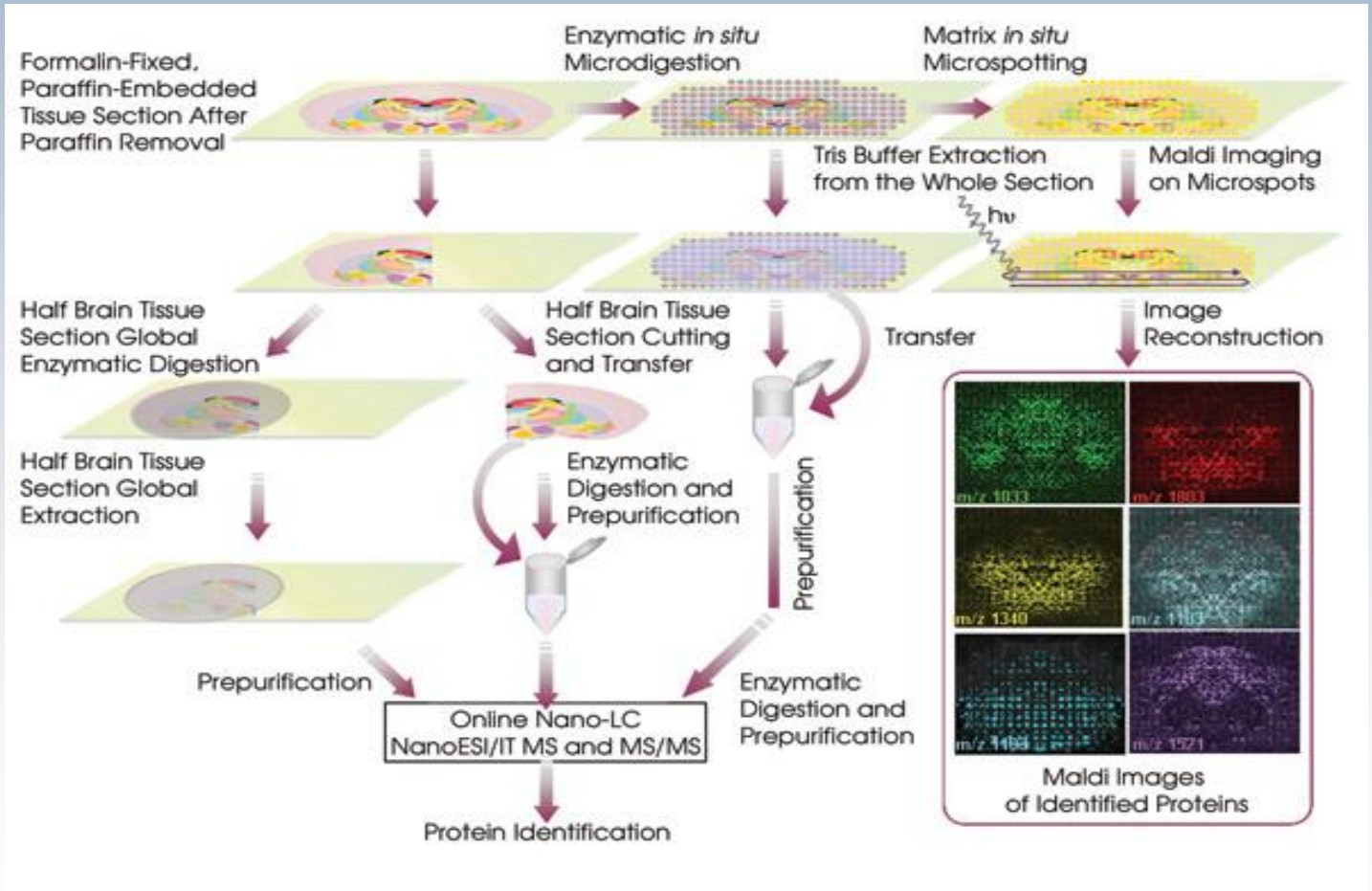
✓ Enzymatic digestion to obtain molecular information



✓ Important molecular information after digestion

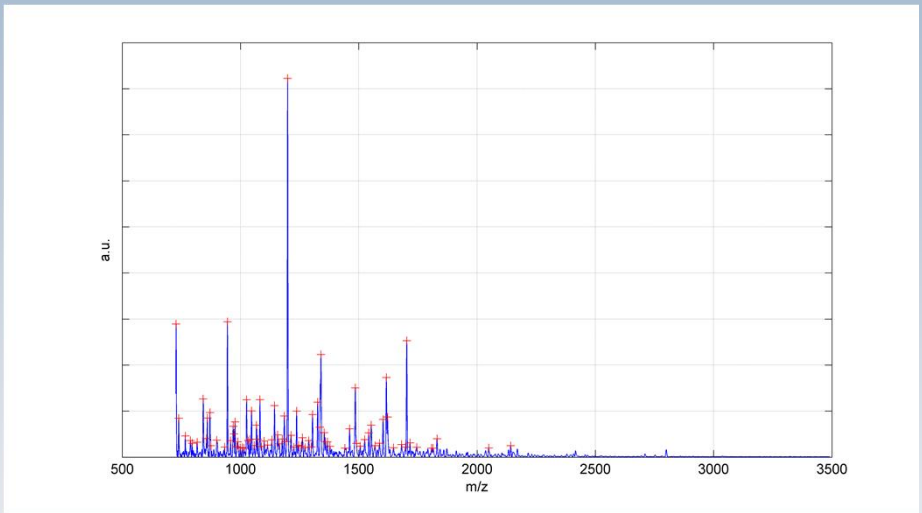
Fournier et al.; http//:www.maldi-imaging.com

Strategies for FFPE archaised tissues

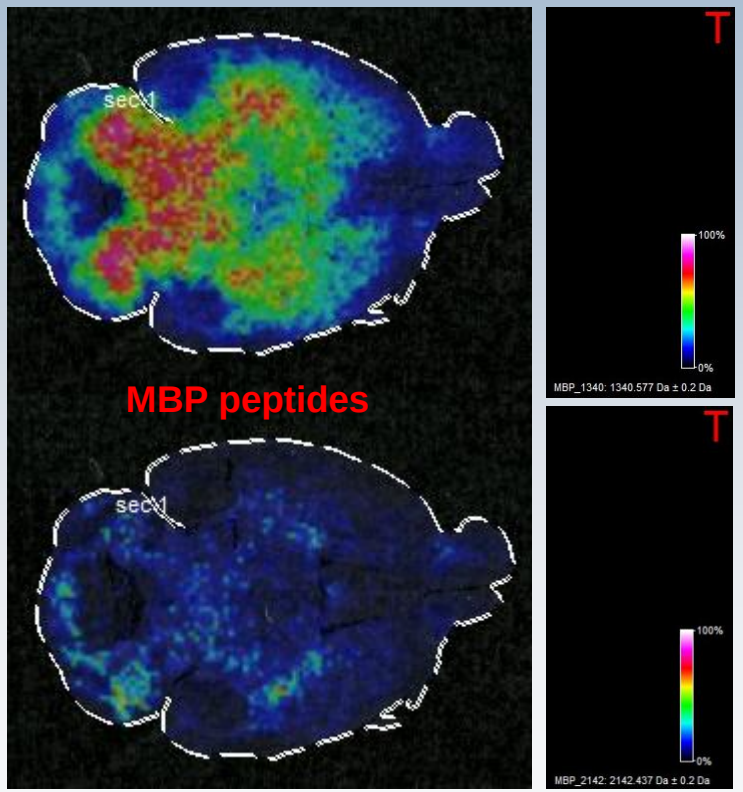


MALDI tissue imaging combined with *in situ* tissue enzymatic digestion by e.g. trypsin is mandatory for FFPE tissue analysis (Wisztorski et al., 2007).

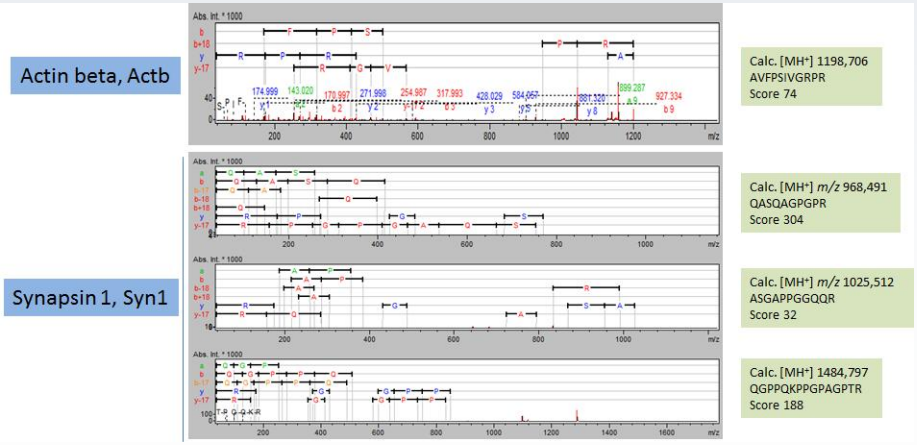
MS/MS on FFPE tissues



MS spectrum acquired from FFPE trypsin digested mouse brain tissue



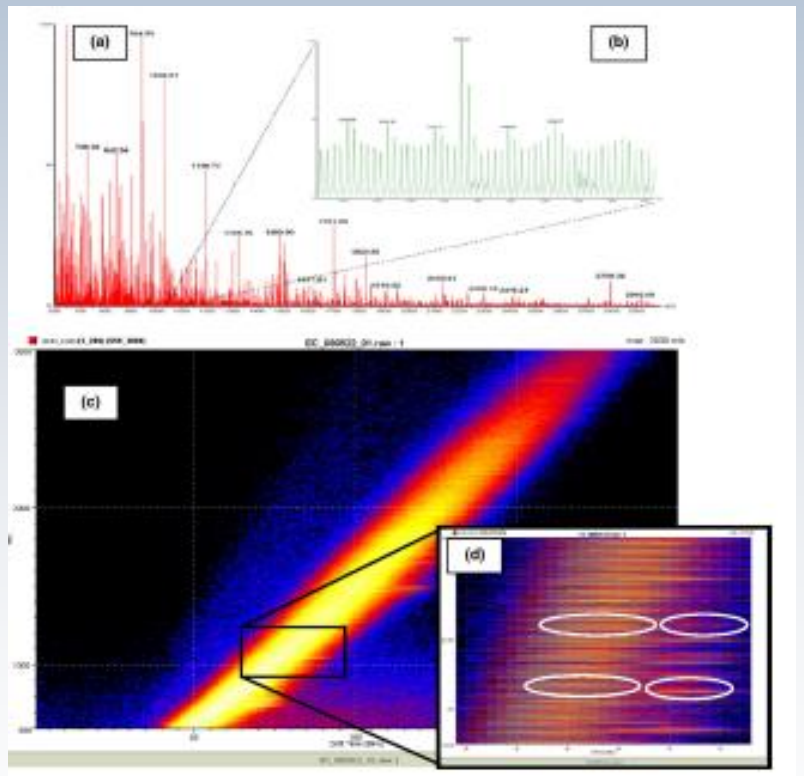
Number	m/z (mi)	m/z (av)	Modifications	Start	End	Missed Cleavages	Sequence
5	1339.708	1340.491		33	44	1	(R) HRDTGILDSIGR(F)
6	2141.114	2142.437		78	95	1	(R) TQDENPVVHFFKNIVTPR(T)

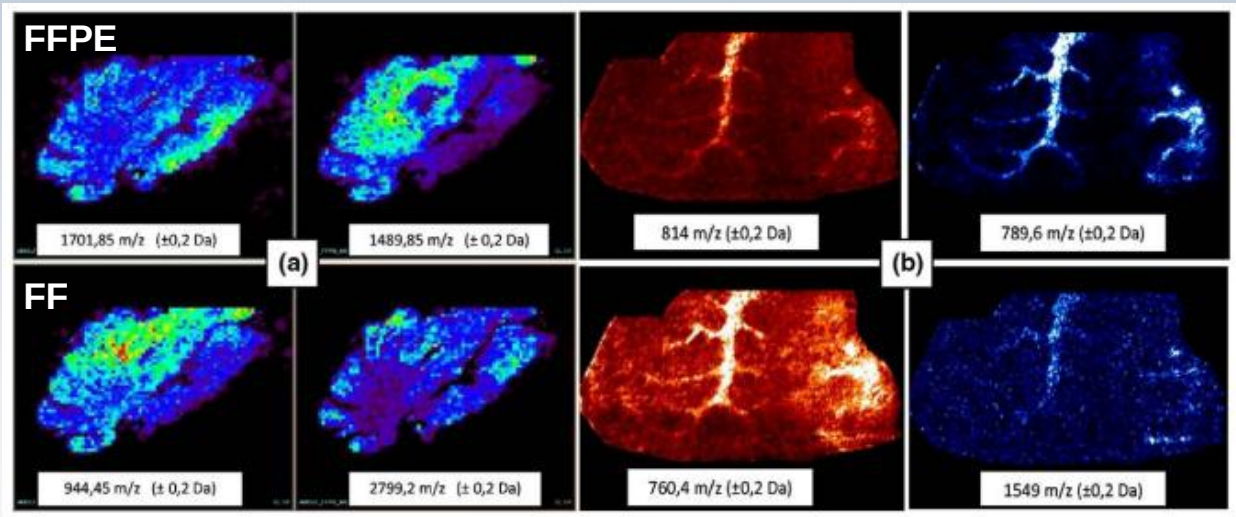


Examples of MS/MS spectra

Lalowski et al., unpublished

MALDI Imaging of different compounds in FFPE and frozen tissues with ion mobility separation

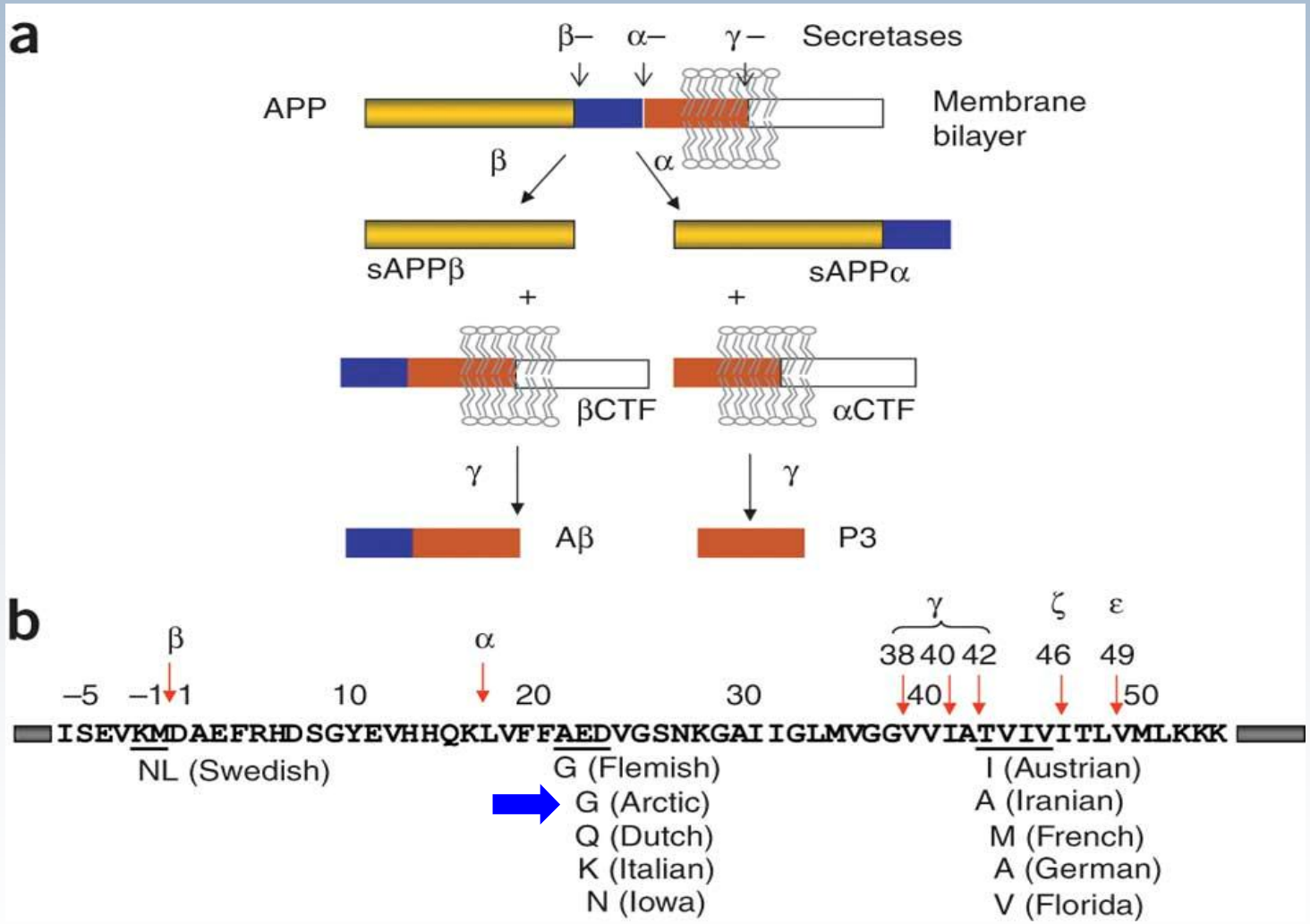




Stauber et al. J AmSoc Mass Spectrom, 2010

Mass spectra of digested FFPE tissue and the corresponding drift scope from MALDI-Ion Mobility Mass Spectrometry on Synapt G2 (Waters). The driftscope corresponds to the 3-dimensional visualization of the *m/z* value, signal intensities, and the retention time inside the ion mobility cell.

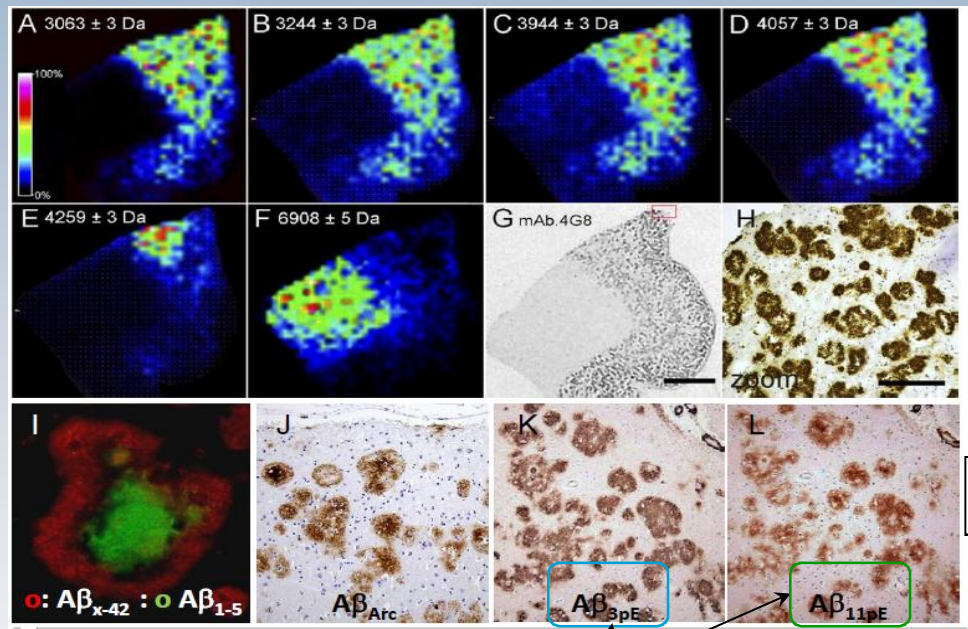
Alzheimer's disease and Aβ processing



Tian et al. NSMB, 2010

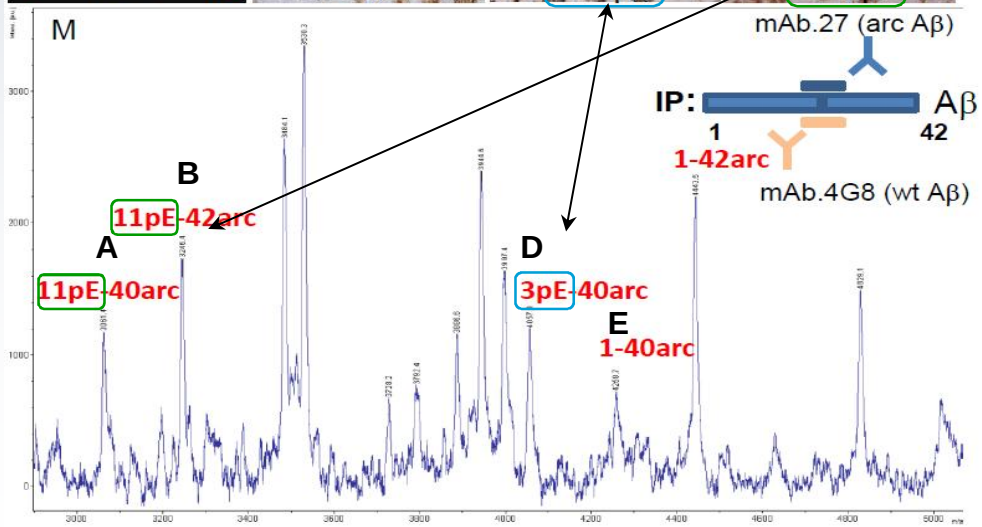
MALDI-MSI on AD brain tissues: technology and validation

1



3

2



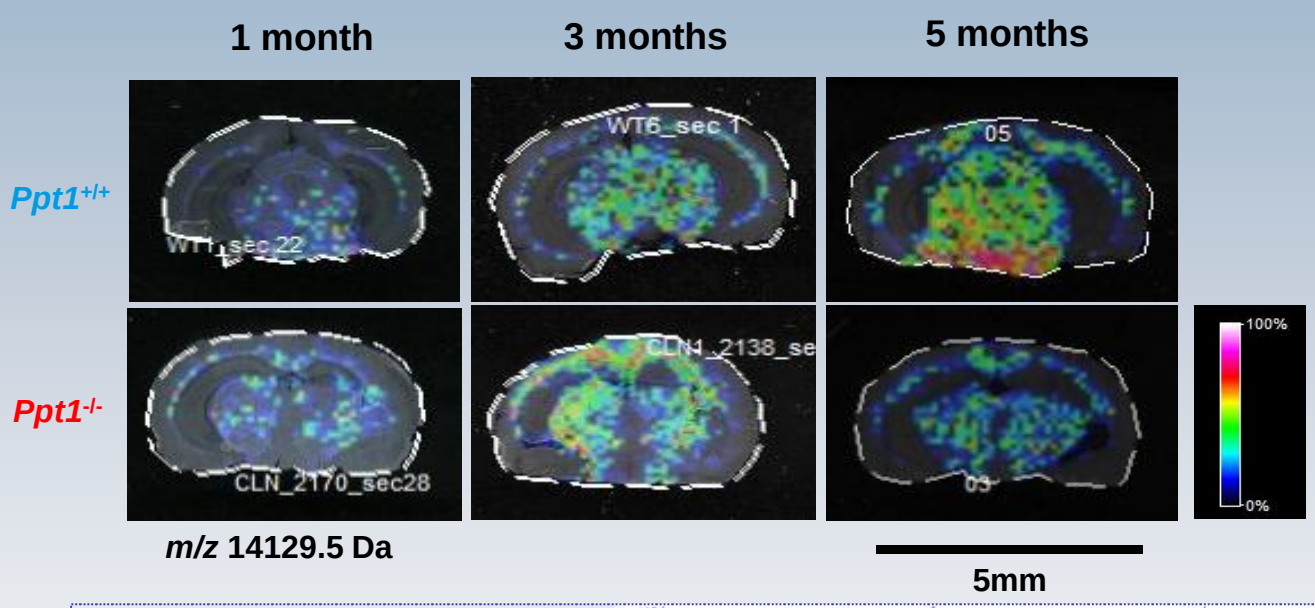
Strategy:

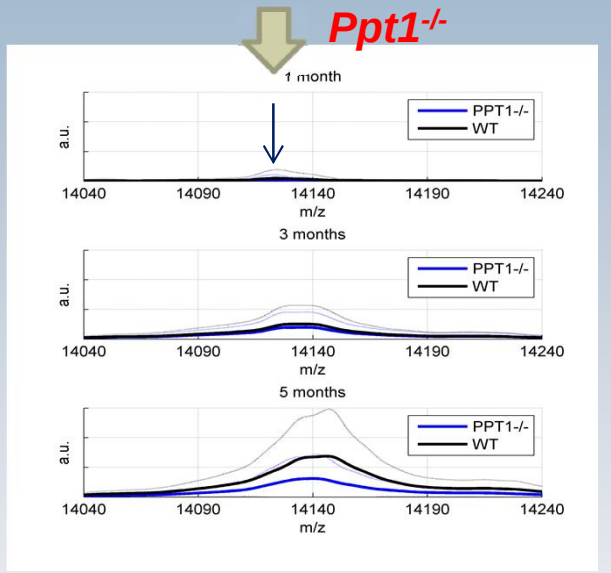
1. **MALDI-MSI**
2. **Extraction/IP with specific antibodies**
3. **MALDI-MS / IHC**

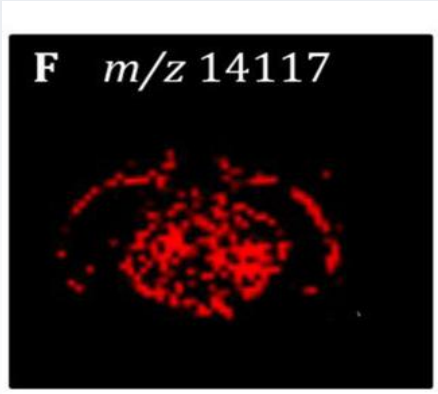
"Arctic" Familial Alzheimer's Disease
Mutation within Aβ sequence:
Position 22E ⇨ G
pE- pyroglutamate (PTM)

Philipson et al. Neurobiology of Aging, 2012
Kalimo, Lalowski* et al. Acta Neuropathologica Communications, 2013*

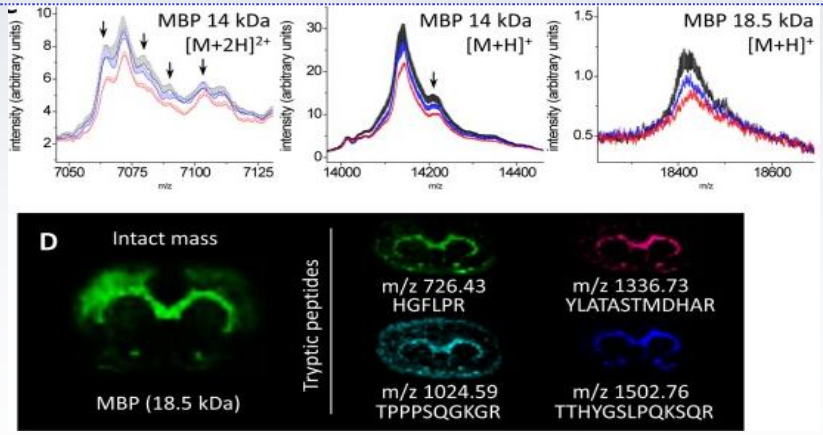
Myelin basic protein isoform 8 is downregulated in the *Ppt1*$^{-/-}$ brain



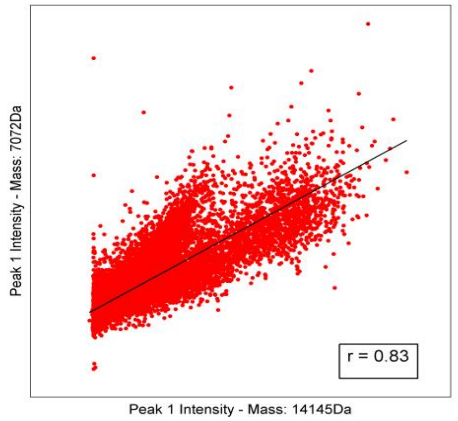




Eberlin et al., Anal Chem 2011



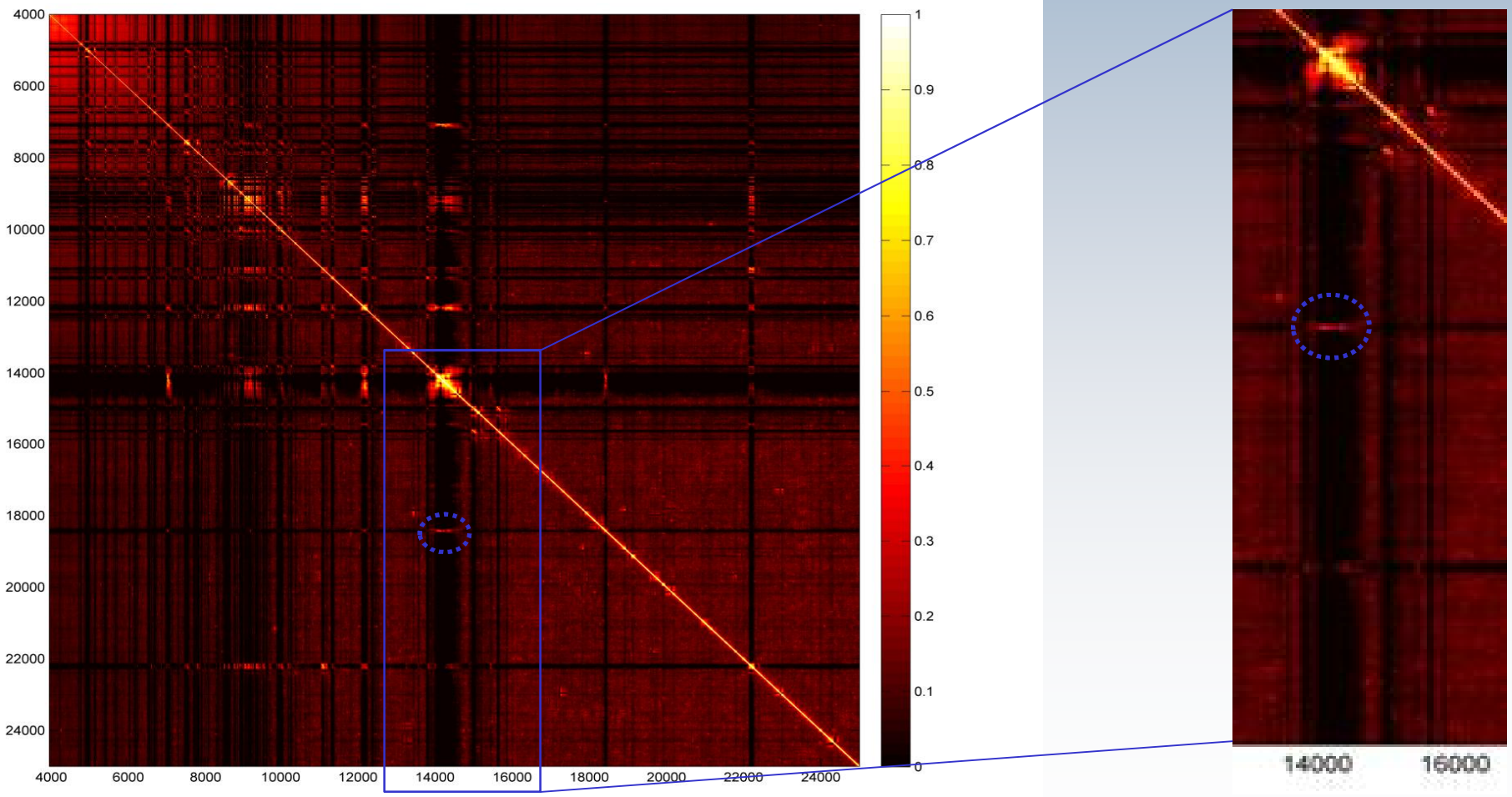
Karlsson et al., Mol Cell Proteomics, 2014



Pearson correlation

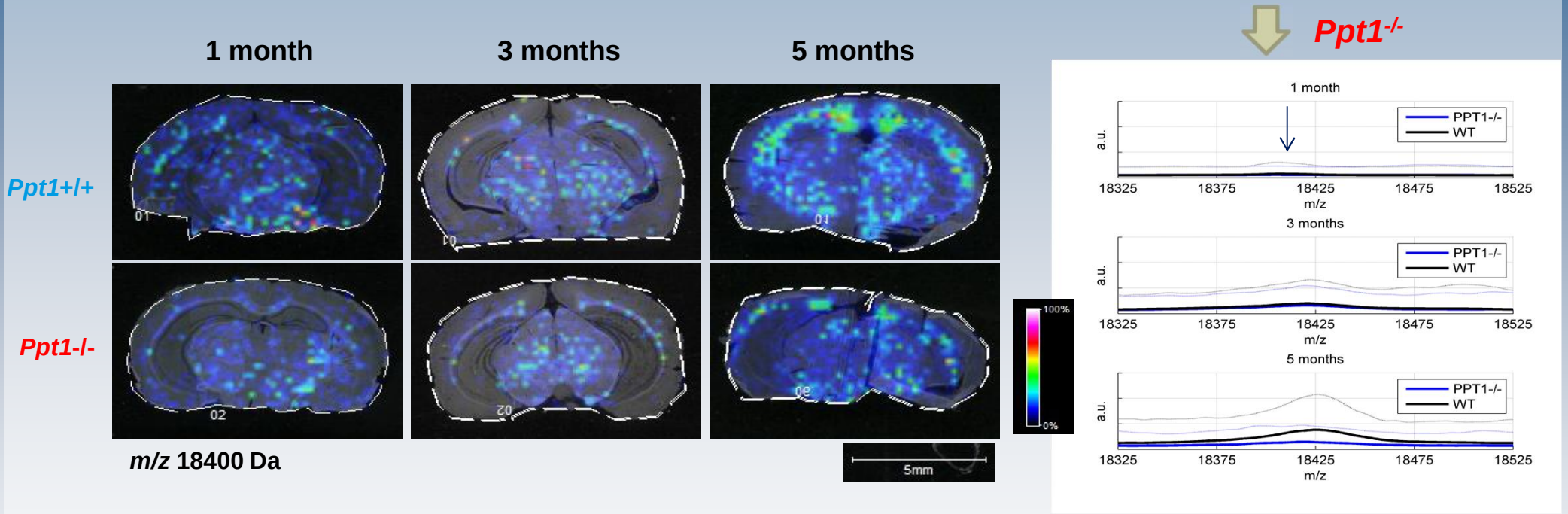
Tikka, Monogioudi et al. in preparation

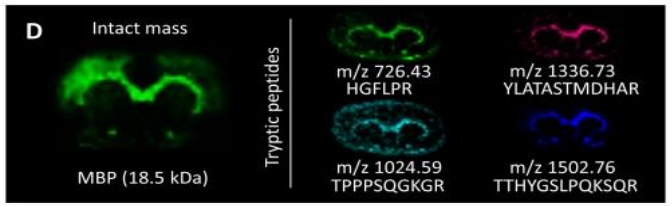
14.1 kDa *m/z* is linked to 18.4 kDa m/z in the Pearson correlation matrix



Tikka, Monogioudi et al. in preparation

Myelin basic protein isoform 5 is downregulated in the *Ppt1*$^{-/-}$ brain





Karlsson et al., Mol Cell Proteomics, 2014

Tikka, Monogioudi et al. in preparation

Downregulation of MBP proteins is independently confirmed by LC-MS^E

Isoform 8 (identifier: P04370-8) and Isoform 5 (identifier: P04370-5)

The sequence of this isoform differs from the canonical sequence as follows:
1-133: Missing.
236-236: K → KGRGLSLSRFSW (isoform 8)
236-236: K → KGRGLSLSRFSWGAEGQKPGFGYGGRASDYKSAHKGFKGAYDAQGTLSKIFKL (isoform 5)
Note: Initiator Met-1 is removed. Both contain a N-acetylalanine at position 2 and a unique P-Threonine at 96.

P04370-8, Isoform 8 of Myelin basic protein, *Mus musculus* (MW_{av} [MH+]= 14122.0 Da)

```
          10         20         30         40         50         60
MASQKRPSQR SKYLATASTM DHARHGFLPR HRDTGILDSI GRFFSGDRGA PKRGSGKDSH
          70         80         90        100        110        120
TRTTHYGSLP QKSQHGRTQD ENPVVHFFKN IVTPRTPPPS QGKGRGLSLS RFSWGGRDSR
SGSPMARR
```

P04370-5, Isoform 5 of Myelin basic protein, *Mus musculus* (MW_{av} [MH+]= 18399.5 Da)

```
          10         20         30         40         50         60
MASQKRPSQR SKYLATASTM DHARHGFLPR HRDTGILDSI GRFFSGDRGA PKRGSGKDSH
          70         80         90        100        110        120
TRTTHYGSLP QKSQHGRTQD ENPVVHFFKN IVTPRTPPPS QGKGRGLSLS RFSWGAEGQK
         130        140        150        160
PGFGYGGRAS DYKSAHKGFK GAYDAQGTLS KIFKLGGRDS RSGSPMARR
```

Sequence covered by peptides identified in LC-MS^e is underlined, identified Phospho-T at 96 aa is browsed in blue, while NIVTPRTPPSQGK peptide in green.

Fold change : 1.63 (down-regulation in 3 month *Ppt1*$^{-/-}$ brain)
22 peptides identified in LC-MS^e; 18 used for quantitation analysis

Tikka, Monogioudi et al. in preparation

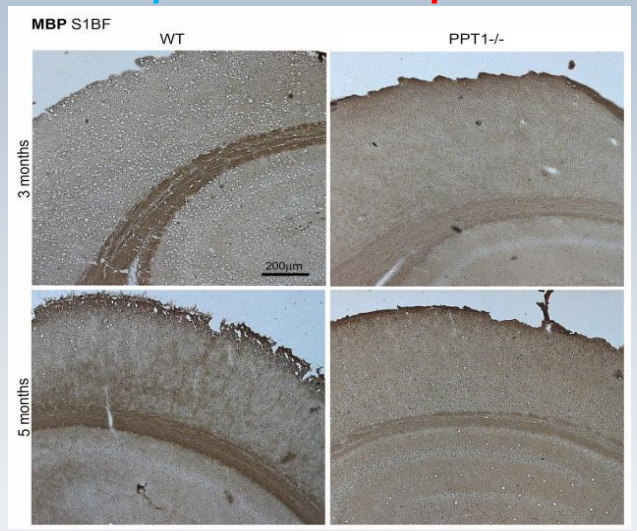
Myelin basic proteins are downregulated in the *Ppt1*$^{-/-}$ brain

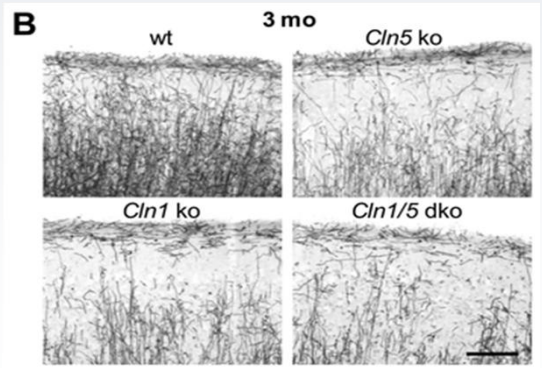
SB1F

Ppt1 $^{+/+}$ *Ppt1* $^{-/-}$

3 months

5 months





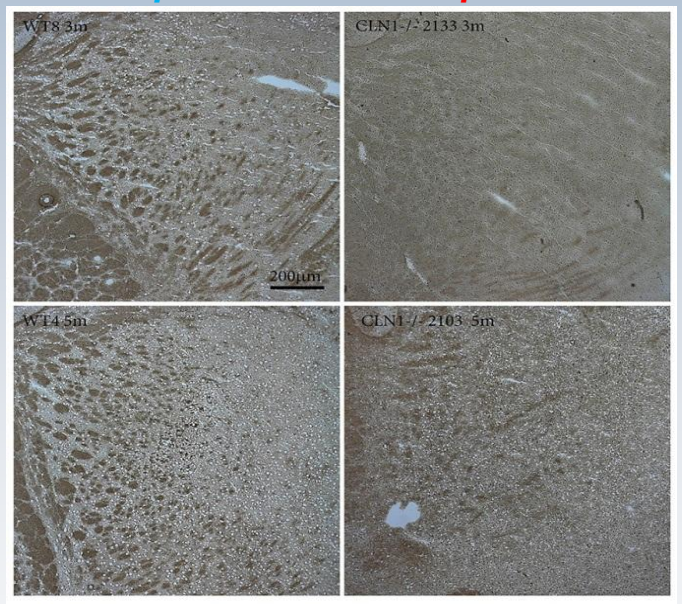
Blom et al., DMM 2013

Thalamus

Ppt1 $^{+/+}$ *Ppt1* $^{-/-}$

3 months

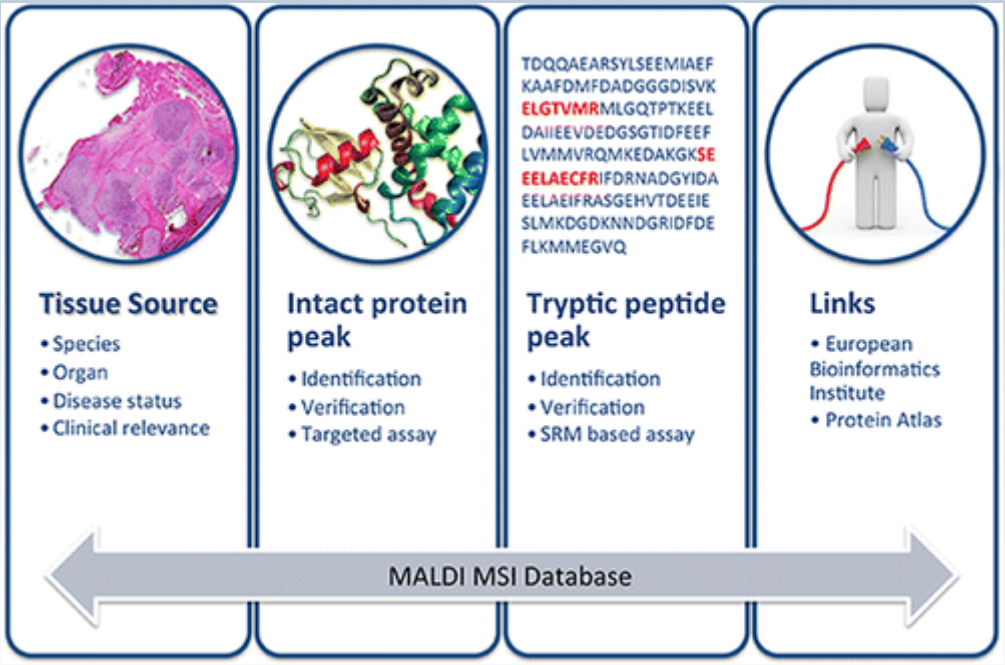
5 months



Tikka, Monogioudi et al. in preparation

Databases, websites, consortia

COST Action On MS Imaging



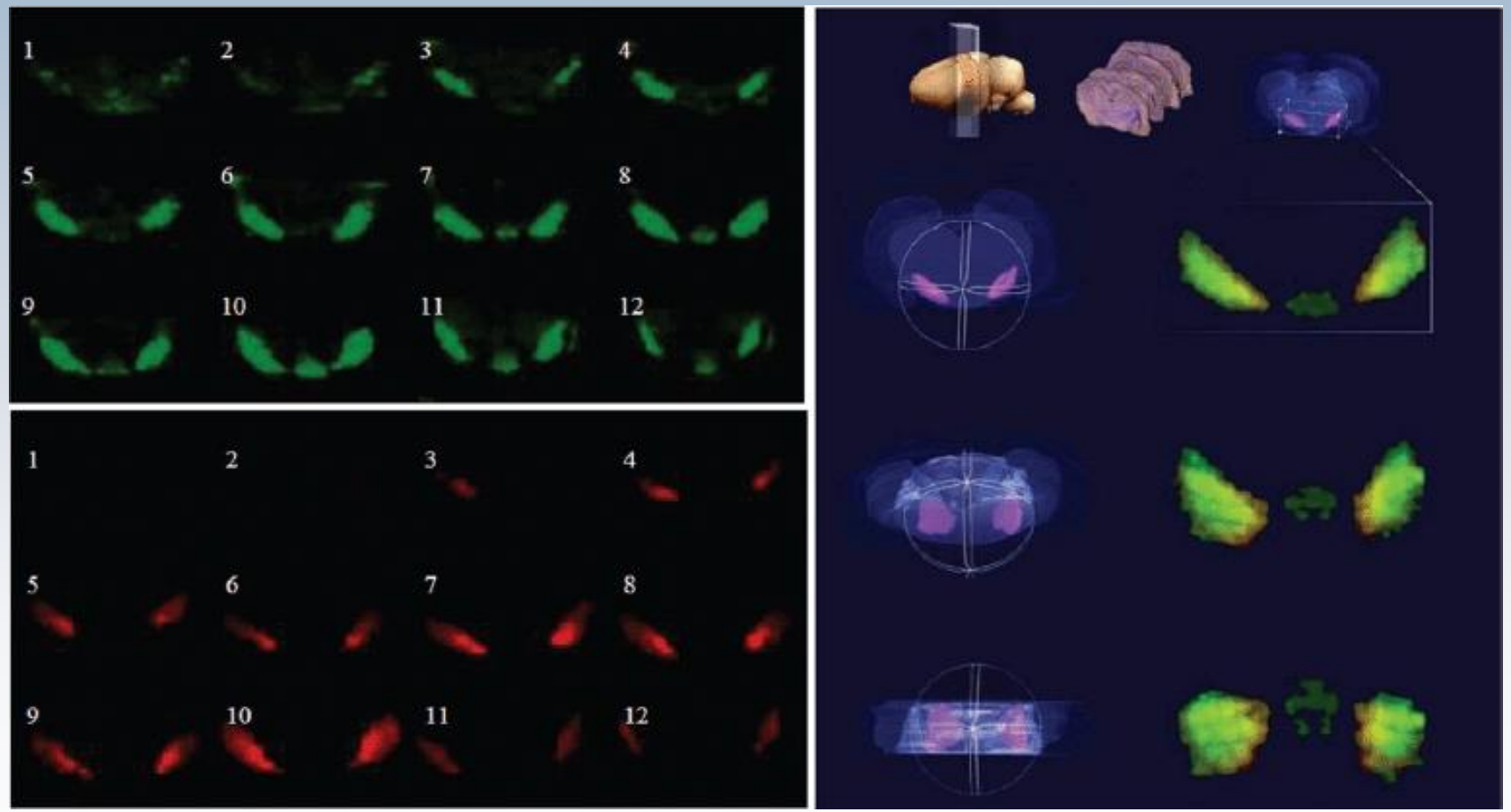
MSiMass List: A Public Database of Identifications for Protein MALDI MS Imaging, Mc Donnel, LA. et al. J Prot Research, 2014

A public database of identifications has been initiated to aid the clinical development and implementation of mass spectrometry imaging. The MSiMass list database (www.maldi-msi.org/mass) enables users to assign identities to the peaks observed in their experiments and provides the methods by which the identifications were obtained. In contrast with existing protein databases, this list is designed as a community effort without a formal review panel.

Towards 3D reconstruction

PEP-19

Unknown 7.4 kDa



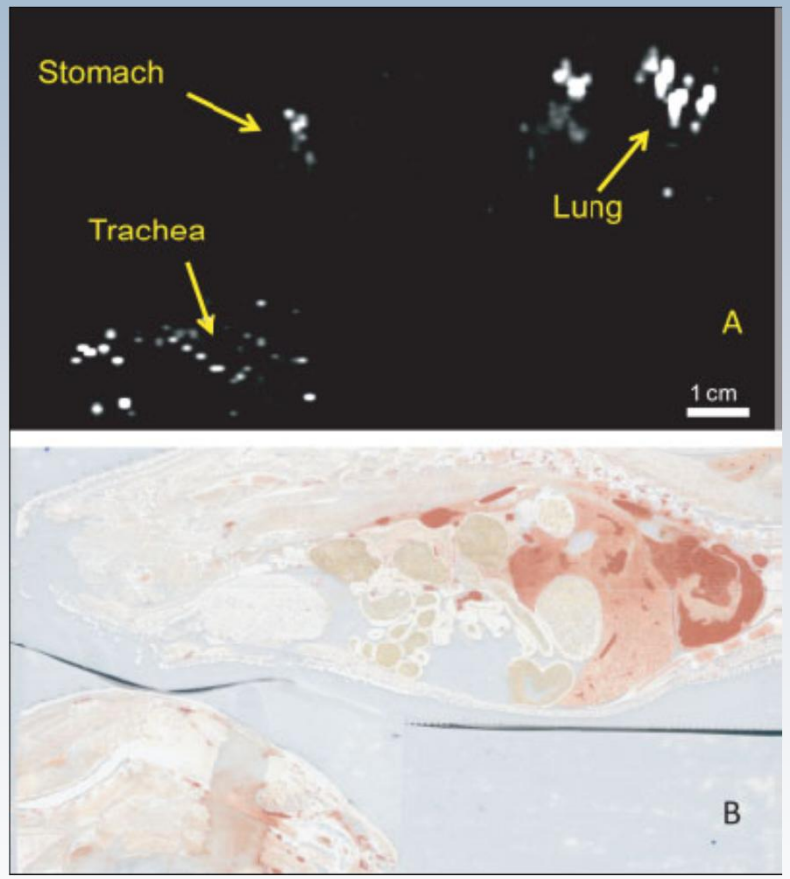
Rat ventral midbrain. 3D reconstructions of histologically stained sections provide the volume coordinates *(right panel, left column*) to insert the two MALDI-MSI images into the same volume reconstruction *(right panel, right column).* Co-localization = yellow color.

Main Andersson, Per Andren, and Richard M. Caprioli

Limitations, so far …

- **MSI Scans are best performed <30 kDa *m/z* range**
- **Sample preparations challenges:**
  - **Tissue cryosections (mostly)**
  - **Autolysis possible**
  - **Tissue fixation**
  - **Suppression effect**
  - **Reproducibility is a concern**
- **Interpretation of complex spectra is not straightforward**
  - **Protein identifications represent a challenge**
  - **Possible displacement or loss of small molecules after trypsinization for MS/MS**
- **Minor proteins enrichment is a handicap**
- **Requires other staining (visualization) methods for direct comparison**

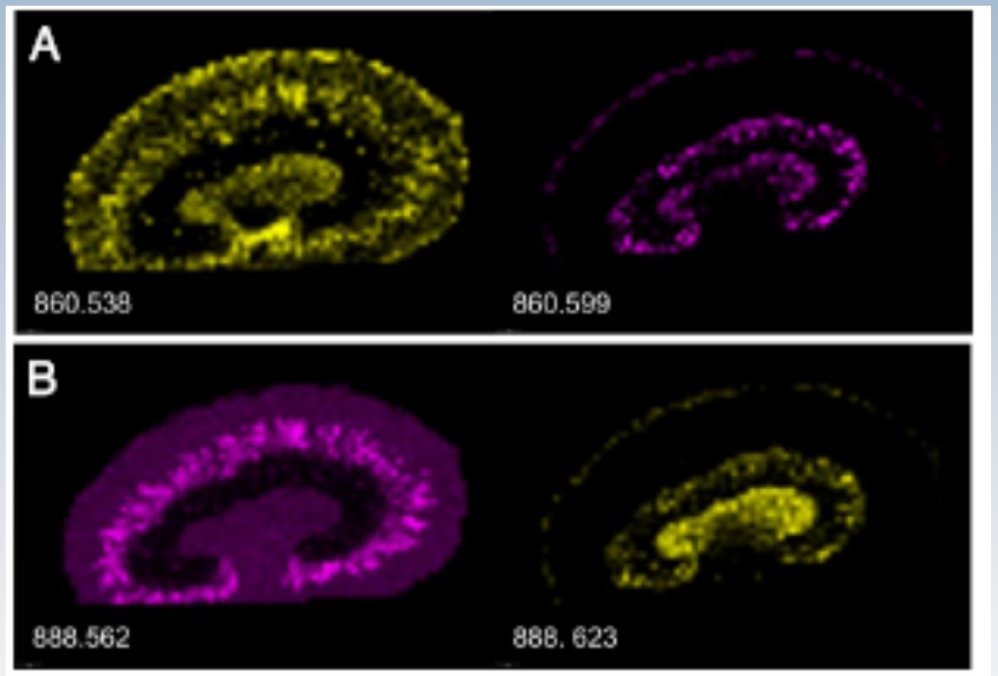
Drug imaging in a whole mouse



(A) SRM image (m/z 468.5 Da) showing the compound distribution (B) Optical image of the rat section.

Hopfgartner et al. RCMS 2009;23:733-736

FT-ICR

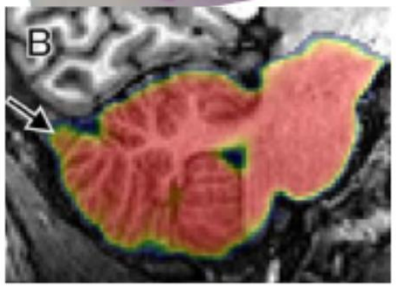


Fourier transform ion cyclotron resonance (FT-ICR) imaging of a mouse kidney. Two examples (*A* and *B*) are shown of lipid species of the same nominal mass, but that display very different spatial distributions. The 0.06-Da mass difference between these species is easily resolved in MALDI-FT-ICR.

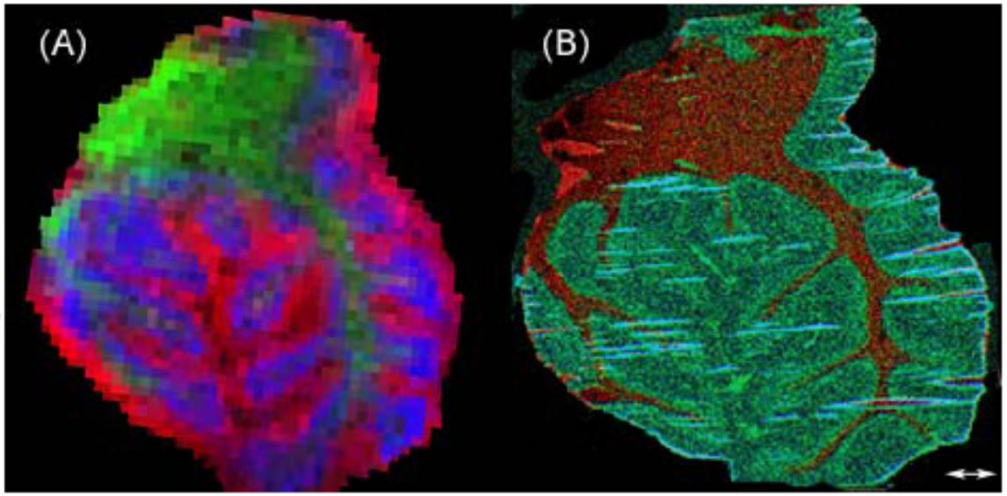
Seeley and Caprioli PNAS 2008;105(47):18126-18131

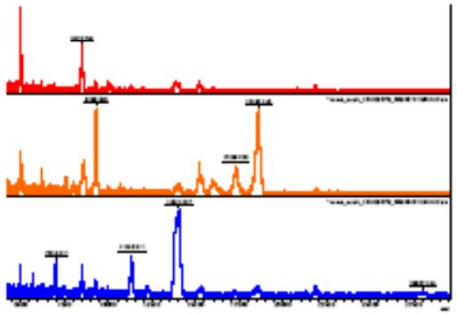
Comparison of different imaging technologies

HE



MRI





MALDI-ToFMS	SIMS -ToFMS
Matrix, heavy washing!	No matrix, no washing!

- Washing steps remove lipids and salts and reduces ion surpression

Courtesy by R.M. Heeren