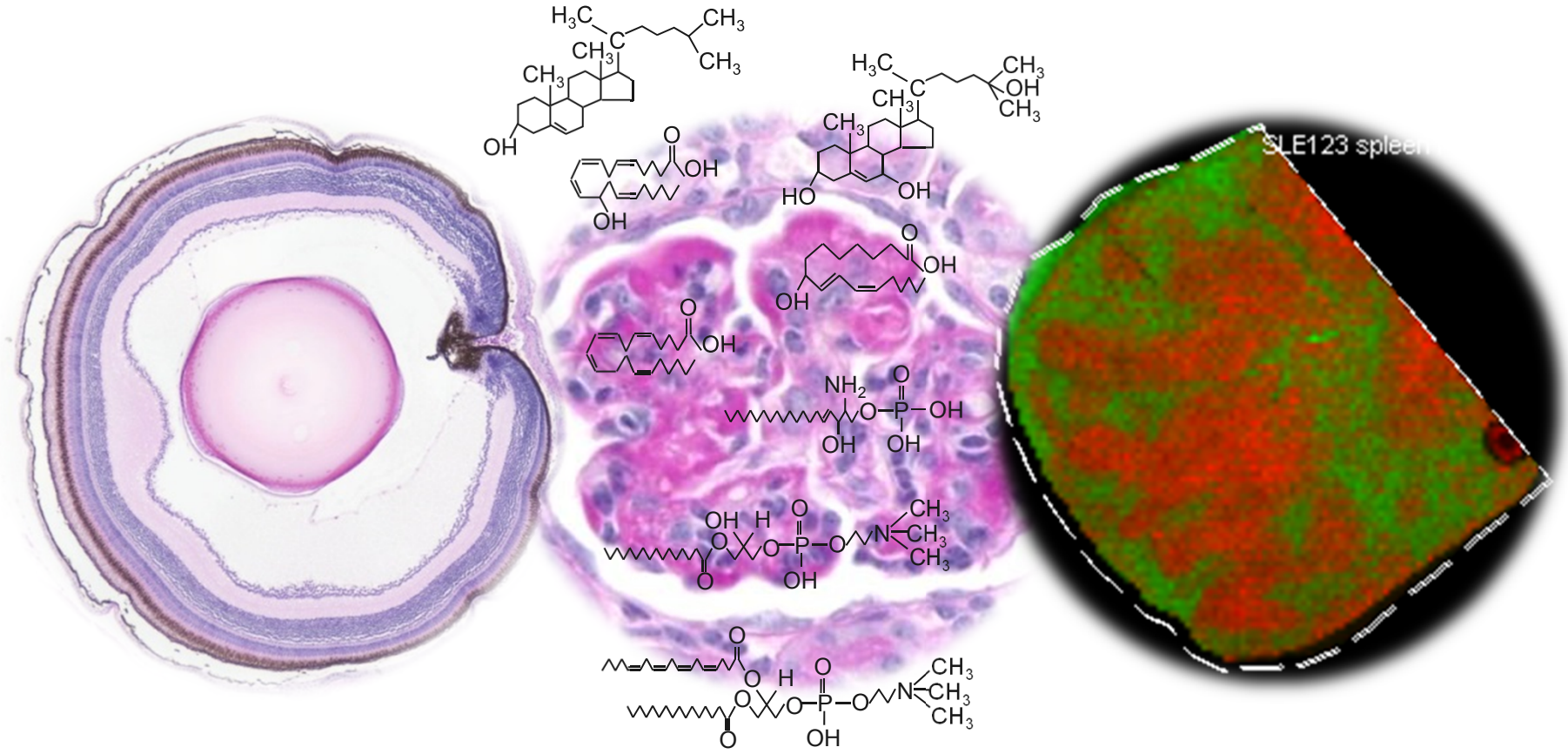
MALDI-IMS for spatial analysis of lipids and other small molecules.

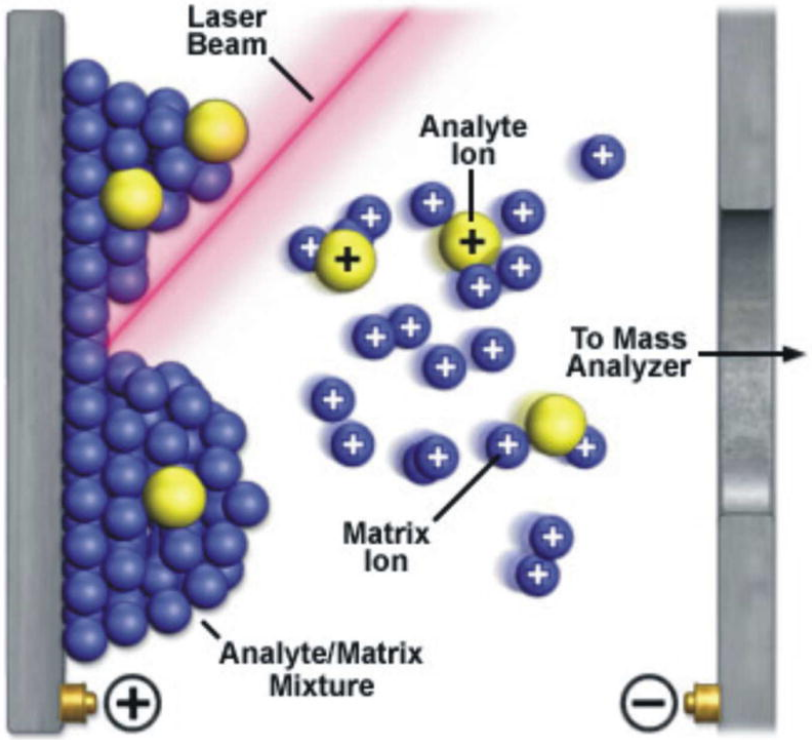


Metabolomics Workshop **6-2-2014**

Janusz Kabarowski, Dept. Microbiology, UAB.

Matrix-Assisted Laser Desorption/Ionization (MALDI):

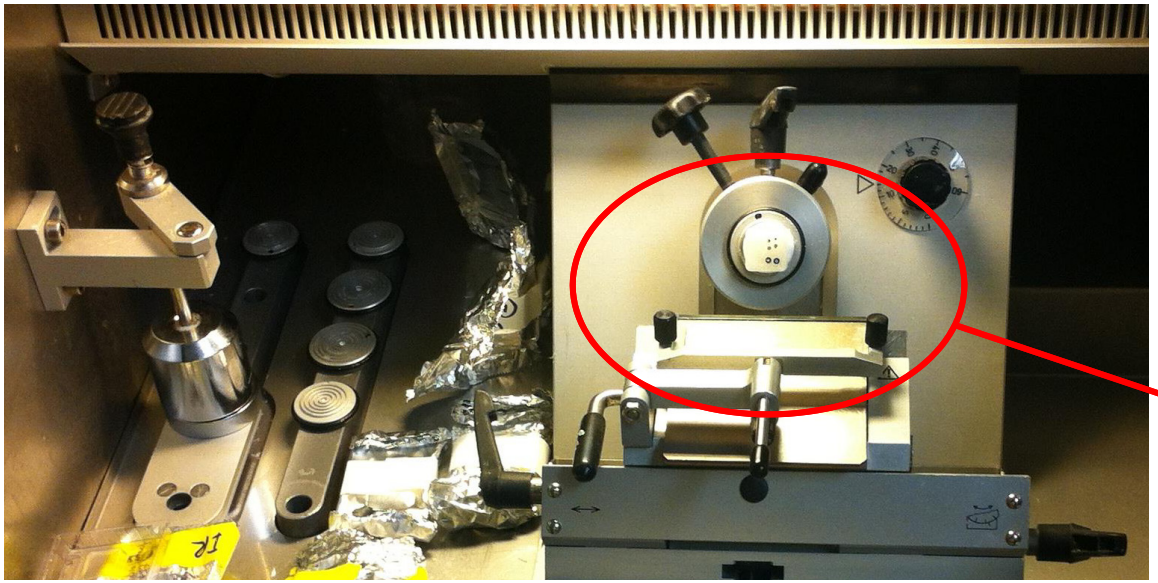
Matrix molecules absorb laser light, enter an excited state, and collide with sample molecules, facilitating charge transfer to create ions.

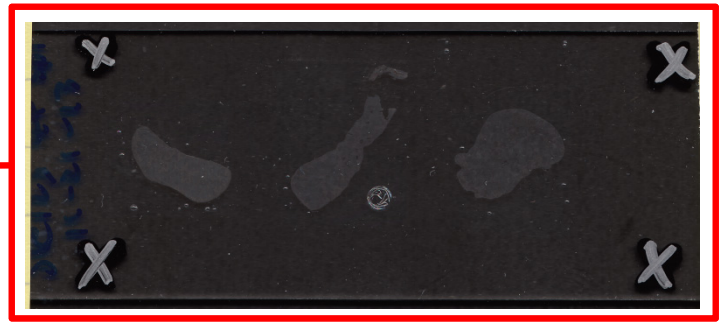


MALDI-TOF instrument

Mass Spectrometric Imaging for biomedical tissue analysis
Kamila Chughtai and Ron M.A. Heeren
Chem Rev. Vol.110(5): pp3237–3277, 2010.

Cryosection preparation onto ITO slides and scanning digital image for “teaching” FlexControl software on MALDI-TOF instrument.



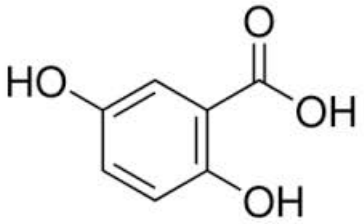


Matrix application

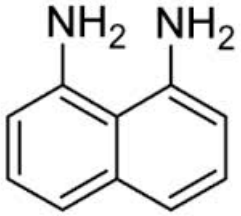
Vacuum sublimation is used to apply an even microscopically thin uniform layer of matrix compound onto tissue section without the need for solvents.

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 

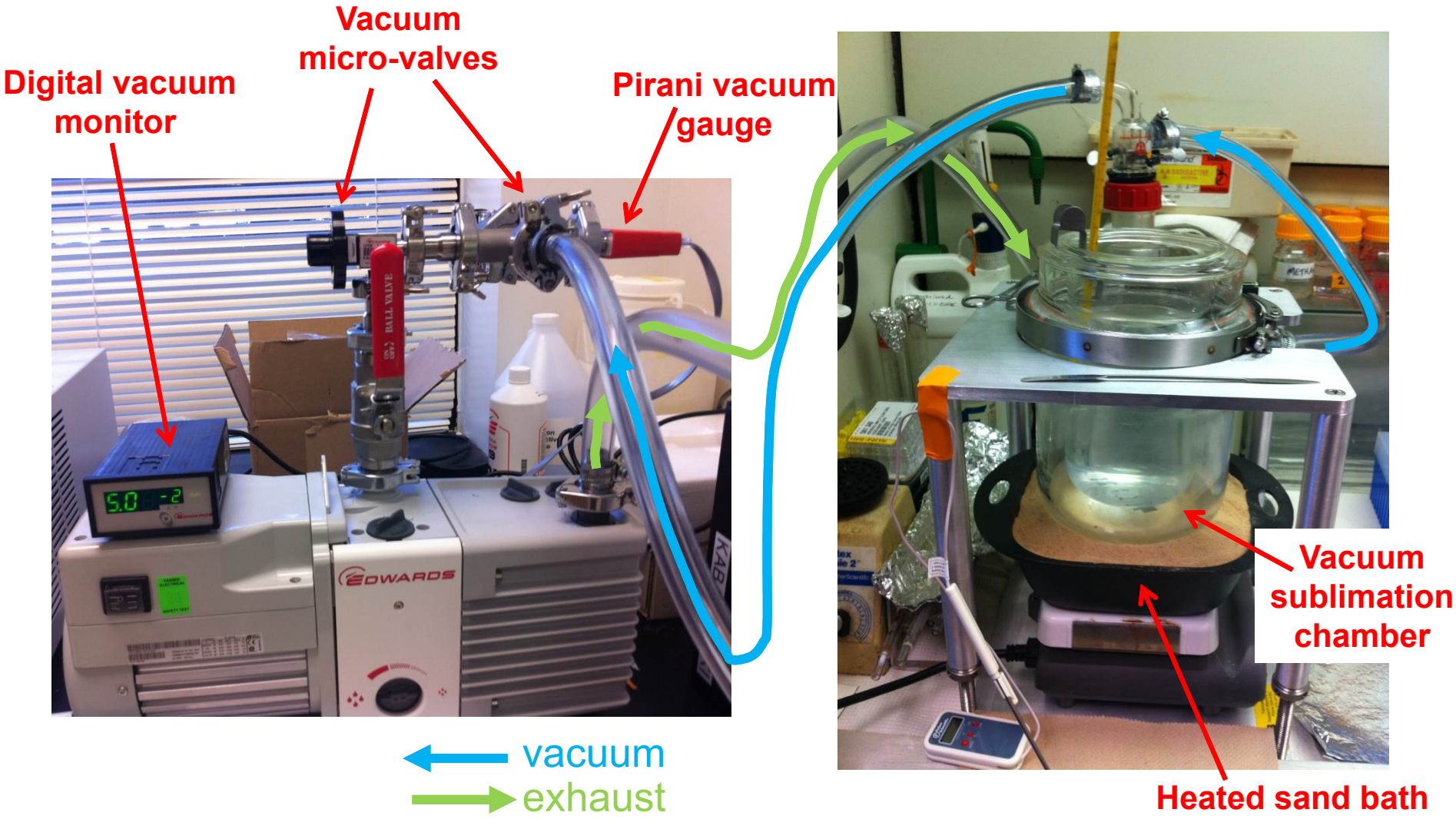
(+ve mode)

1,5-diaminonapthalene 

(-ve mode)

How do we apply matrix for MALDI Imaging ?

Vacuum sublimation apparatus.

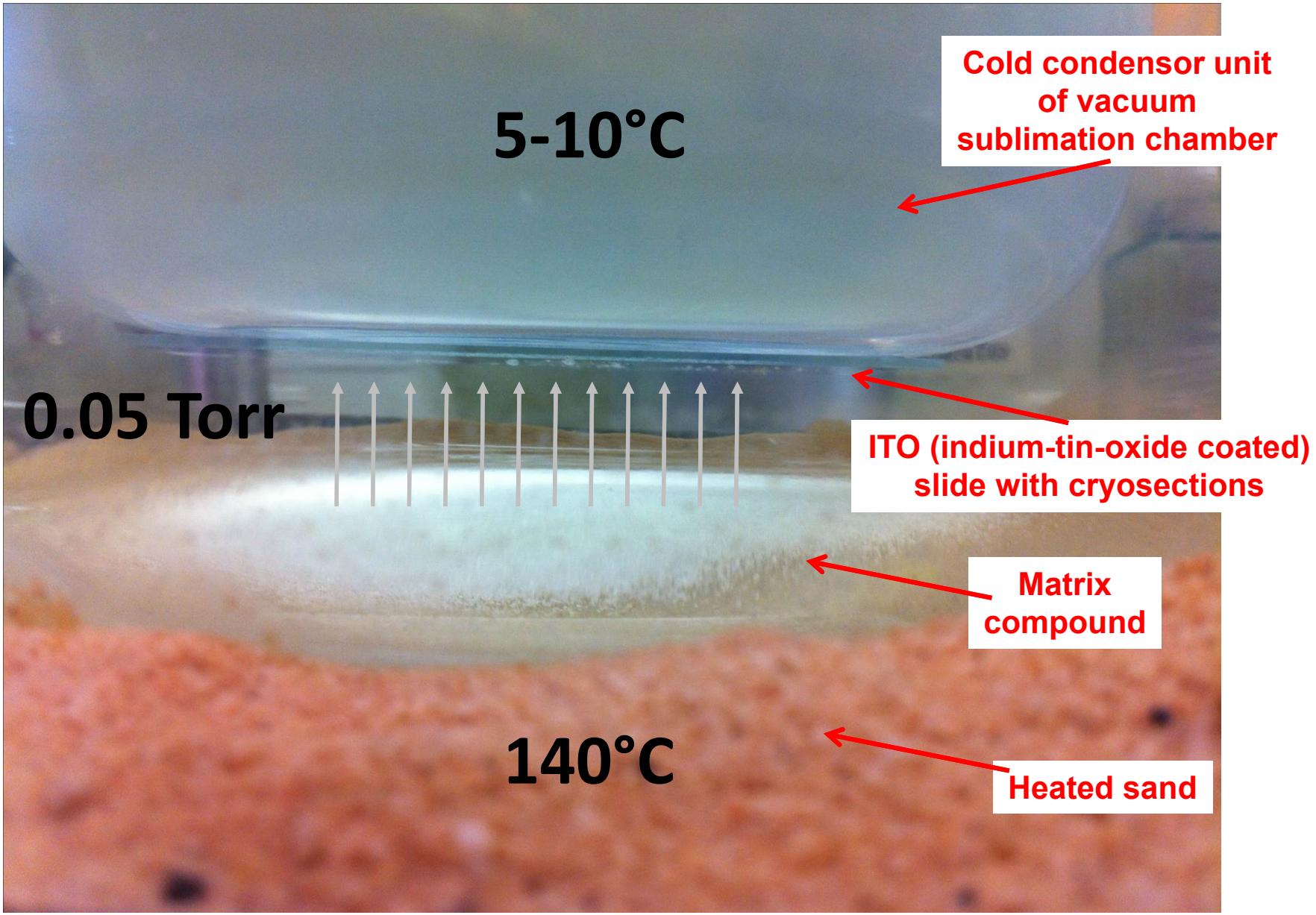


Vacuum at 0.05 Torr in sublimation chamber (atmospheric pressure is 750 Torr).

750 Torr
(atmospheric pressure)

0.05 Torr

Matrix deposition by vacuum sublimation.



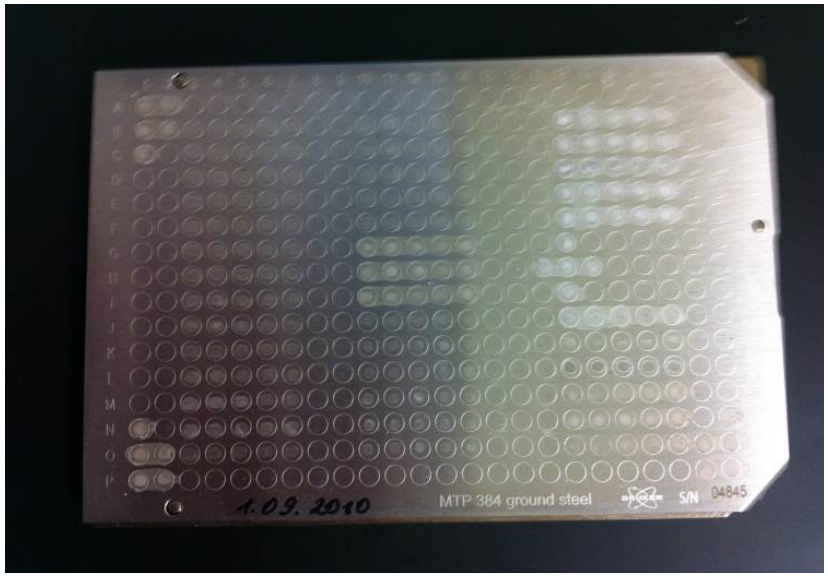
Slides with matrix applied by vacuum sublimation.

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.

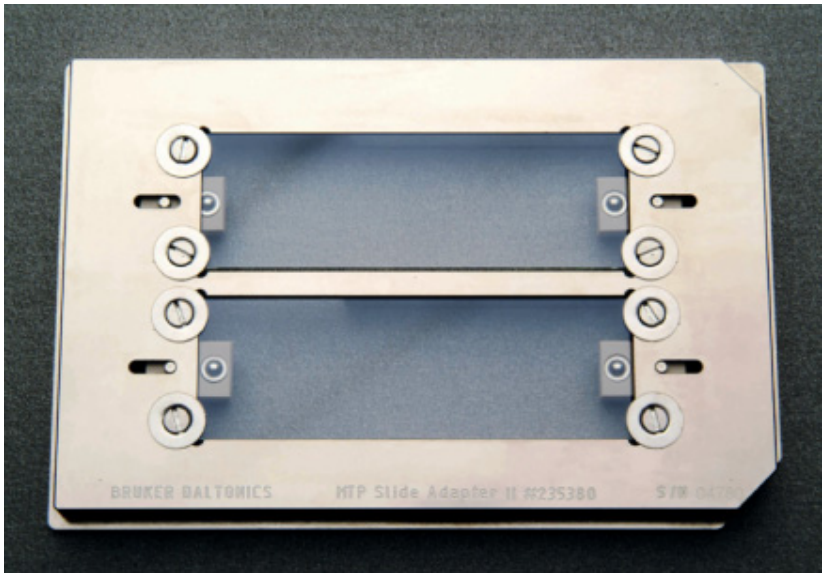
The uniformity of matrix deposition onto the slide attached to the cold condenser surface reflects the random Brownian motion of the released gaseous matrix molecules.

Adaptation of MALDI plate for imaging cryosections on slides.

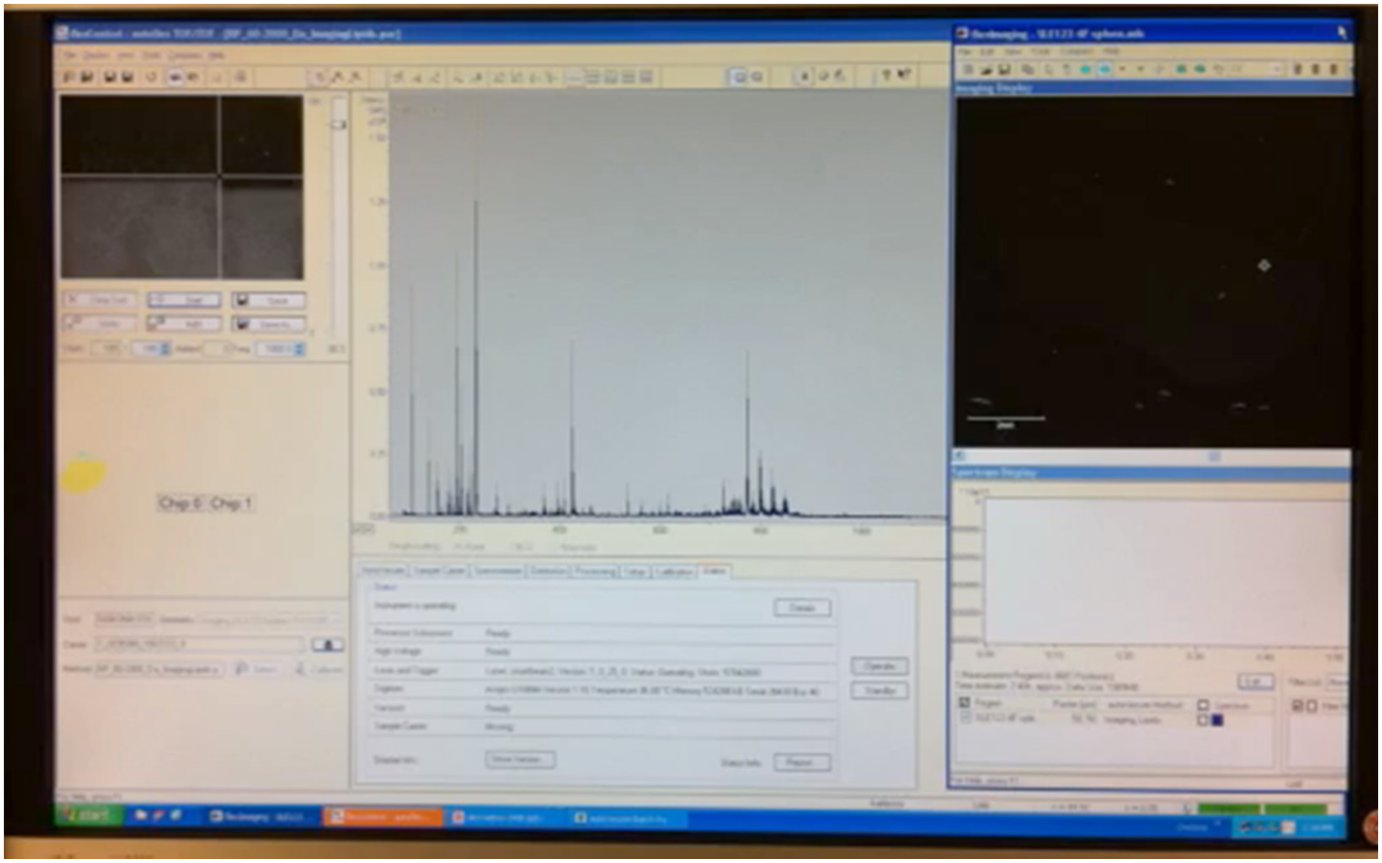
Conventional MALDI plate



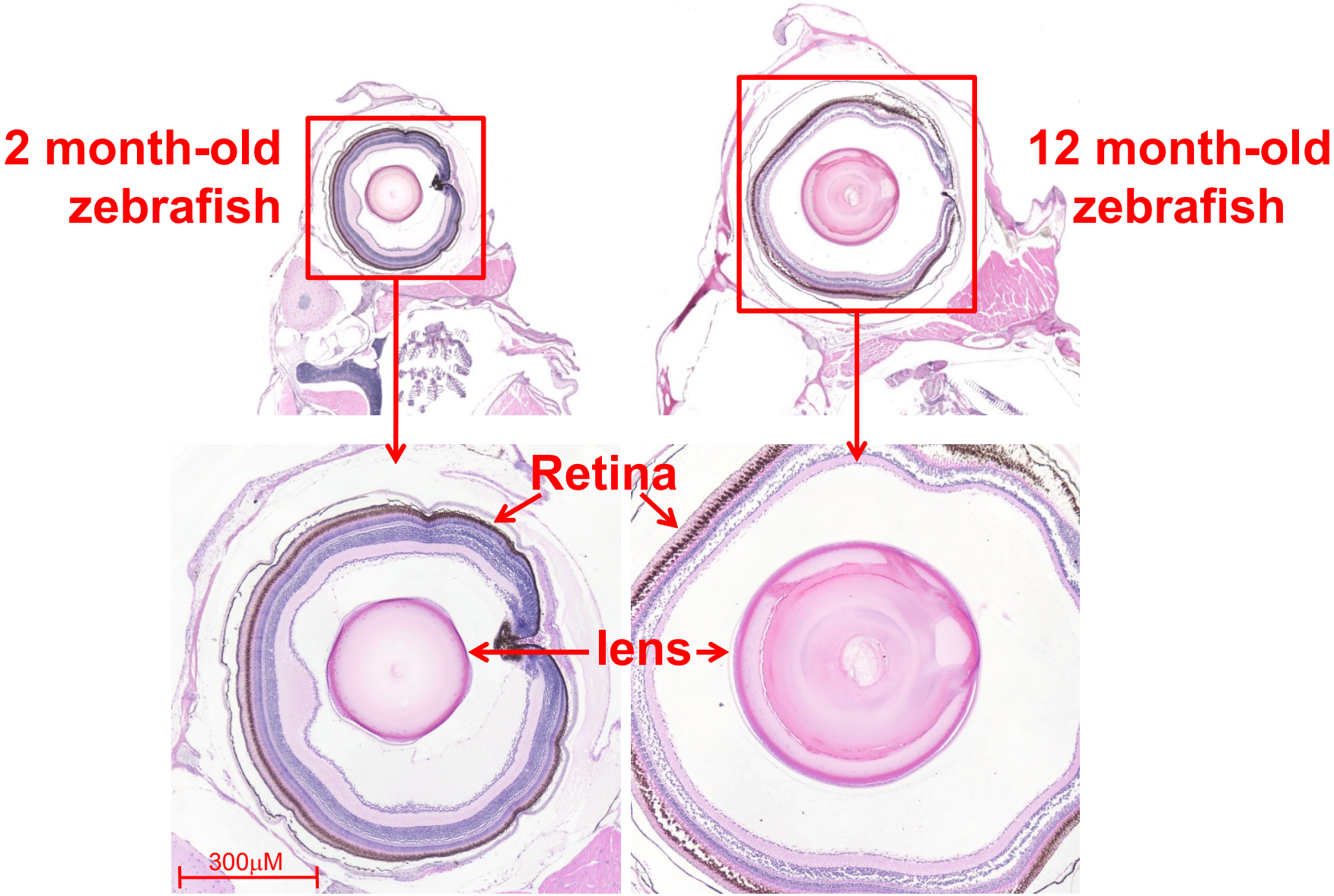
MALDI plate for cryosections



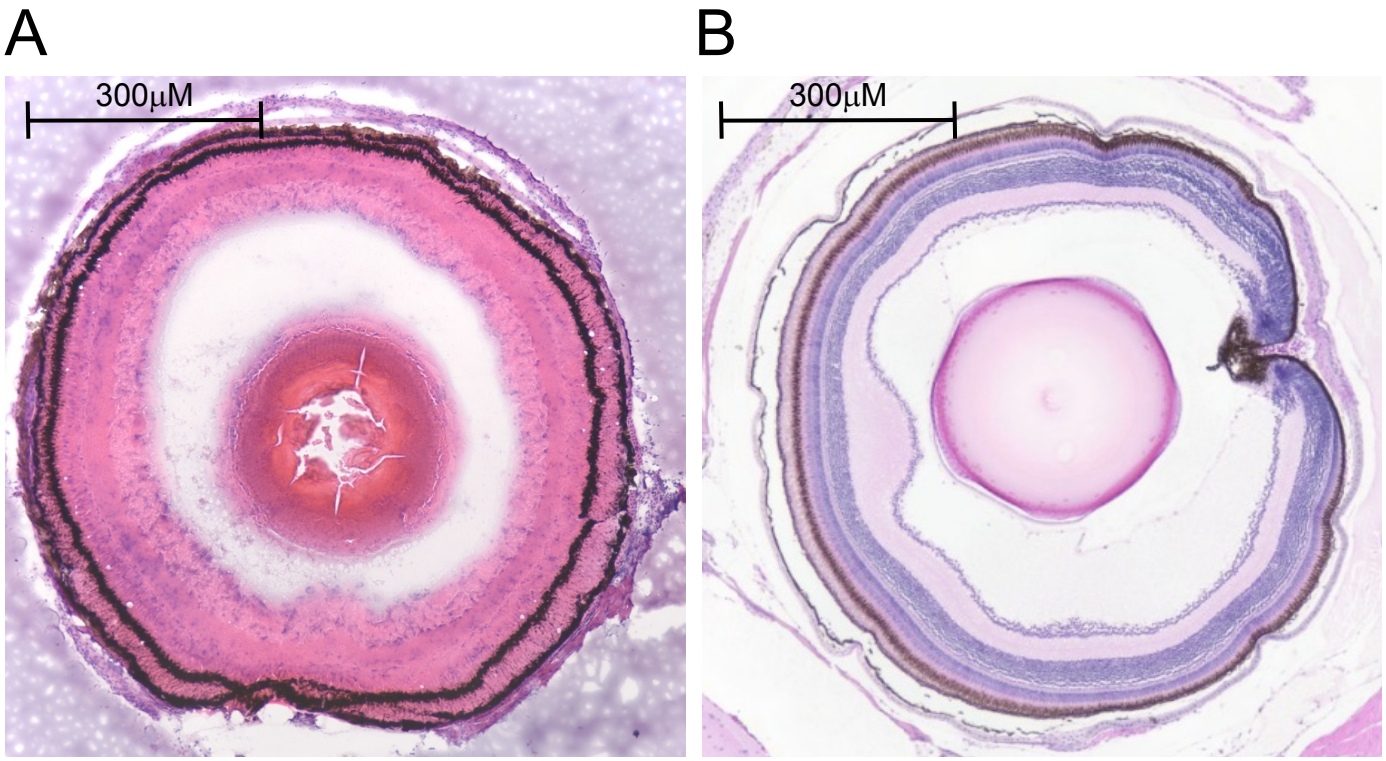
MALDI-IMS instrument running.



MALDI-IMS for studying lipids in eye.

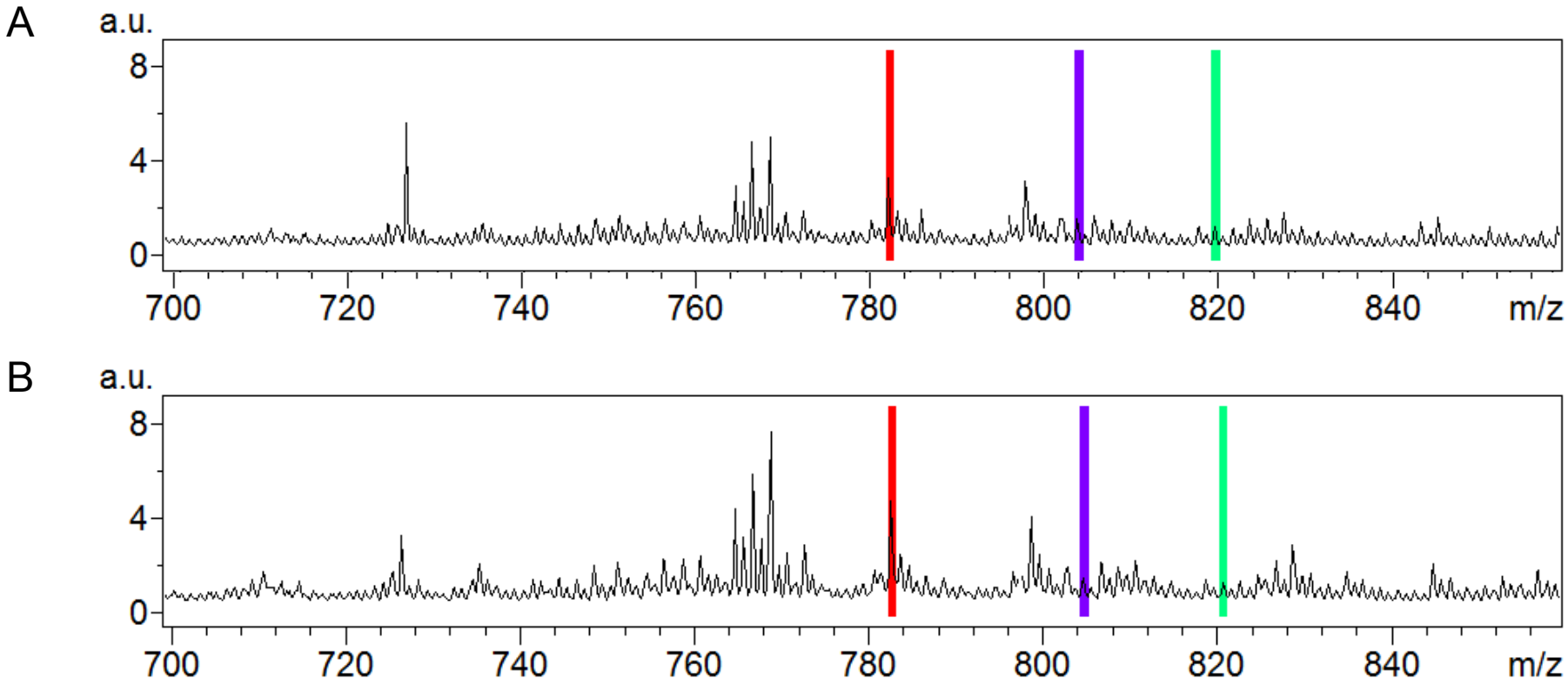


H&E stained paraffin sections from young and old zebrafish eyes to show optimal lens structural integrity.



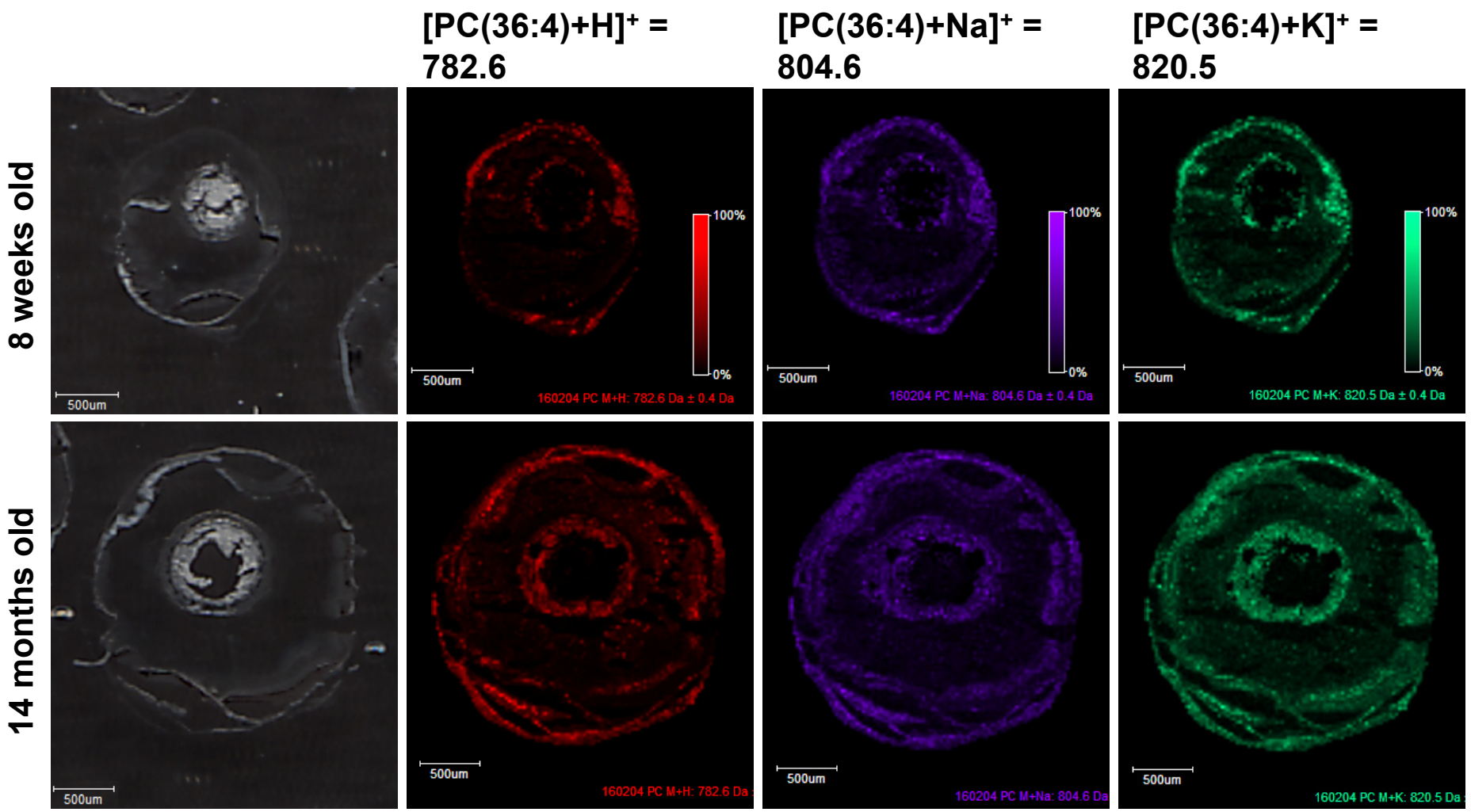
Formalin fixed paraffin sections such as that shown in (B) cannot be used for MALDI-IMS (although certain modifications of fixation protocols can allow for subsequent lipid MALDI-IMS). Cryosectioning (after tissue embedding in 10% gelatin) must therefore be optimized for the tissue being studied (A). 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.

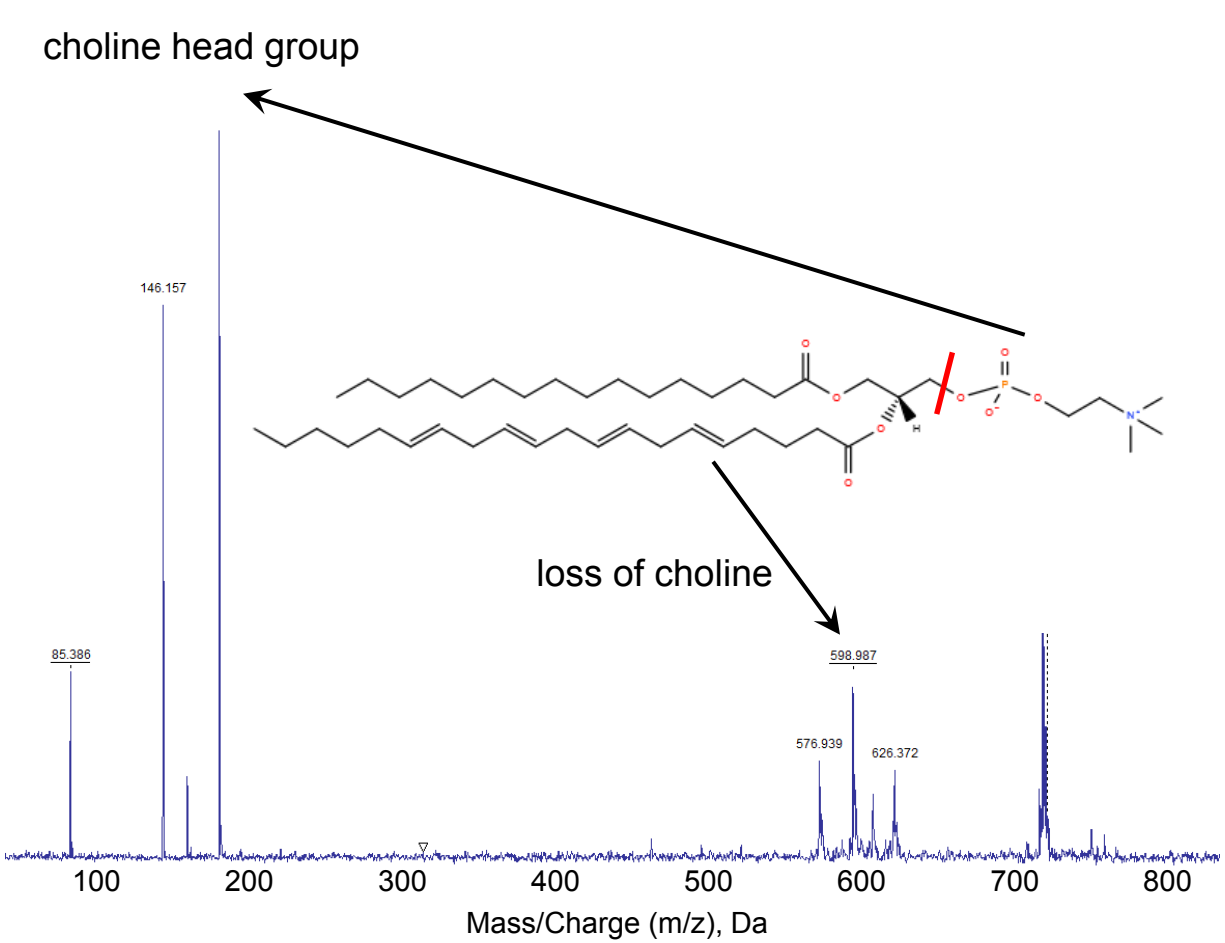


Peaks correspond to expected protonated, sodiated, and potassiated adducts of PC(36:4).

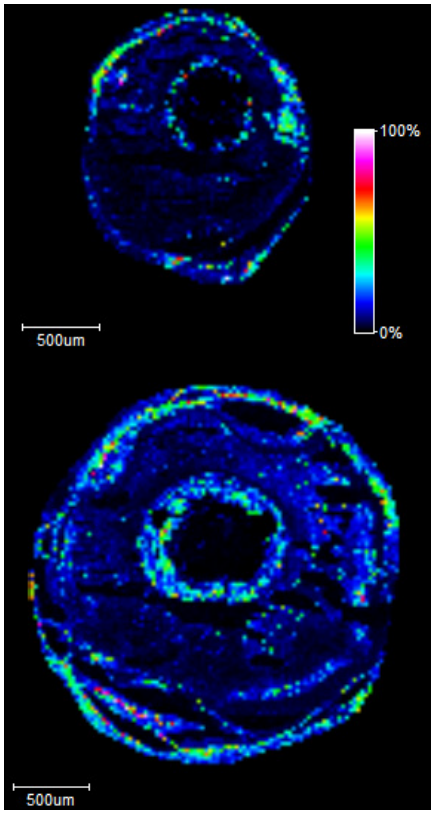
MALDI-IMS on protonated, sodiated, and potassiated adducts of PC(36:4) in zebrafish eye.



MS/MS on protonated adduct of PC(36:4) in zebrafish eye.



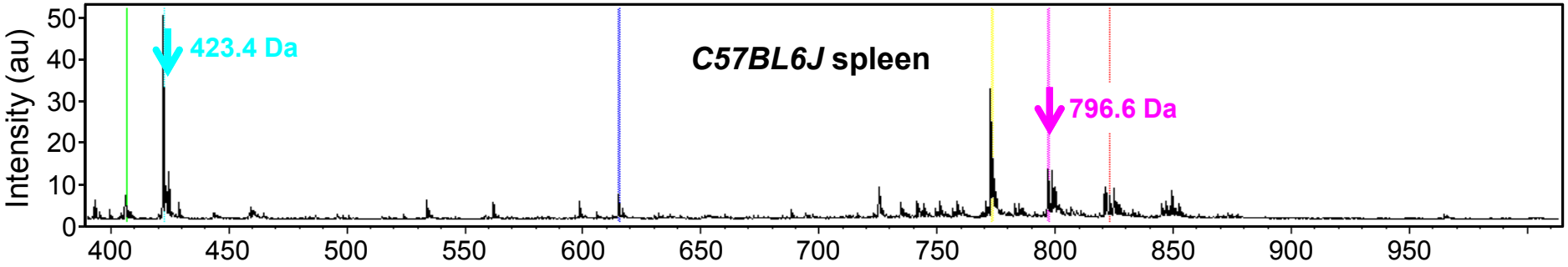
[PC(36:4)+H]$^+$ =



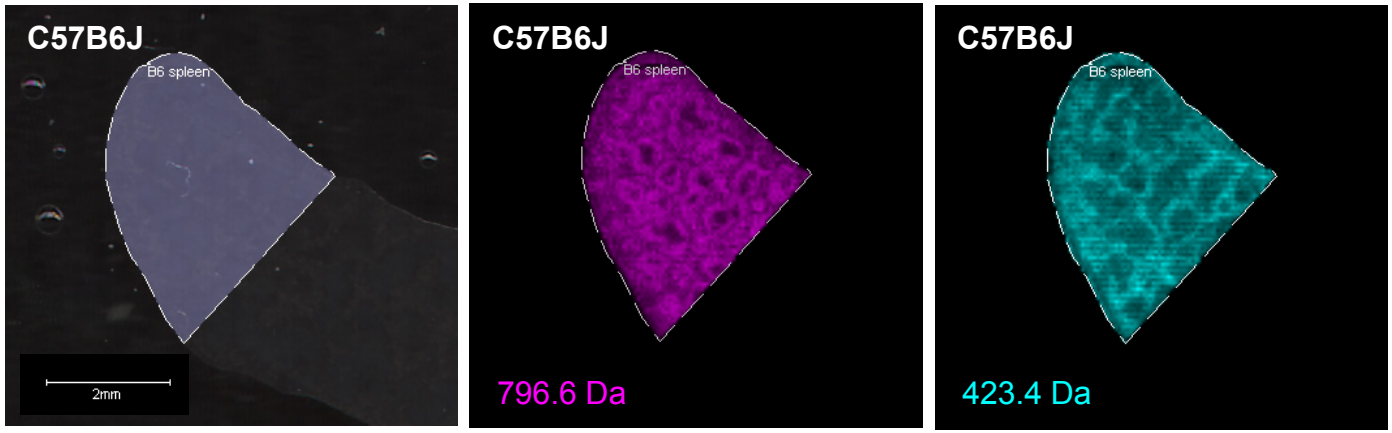
8 weeks old

14 months old

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

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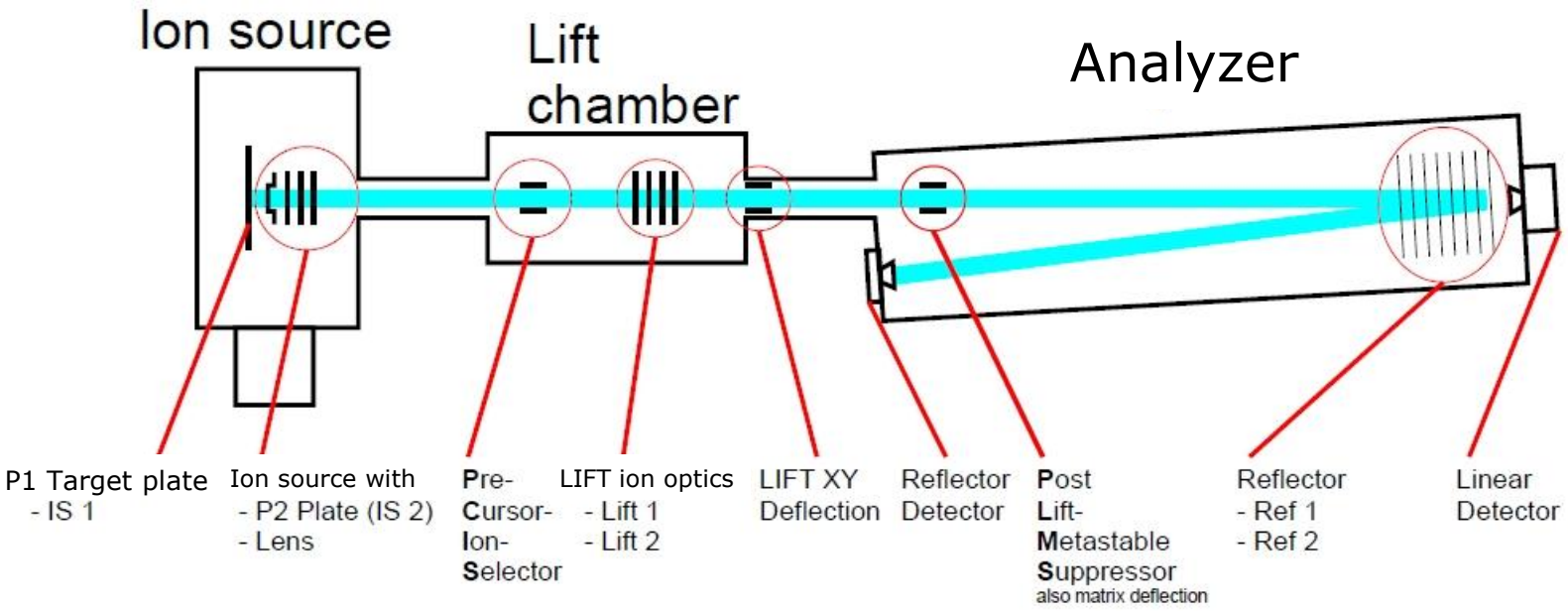
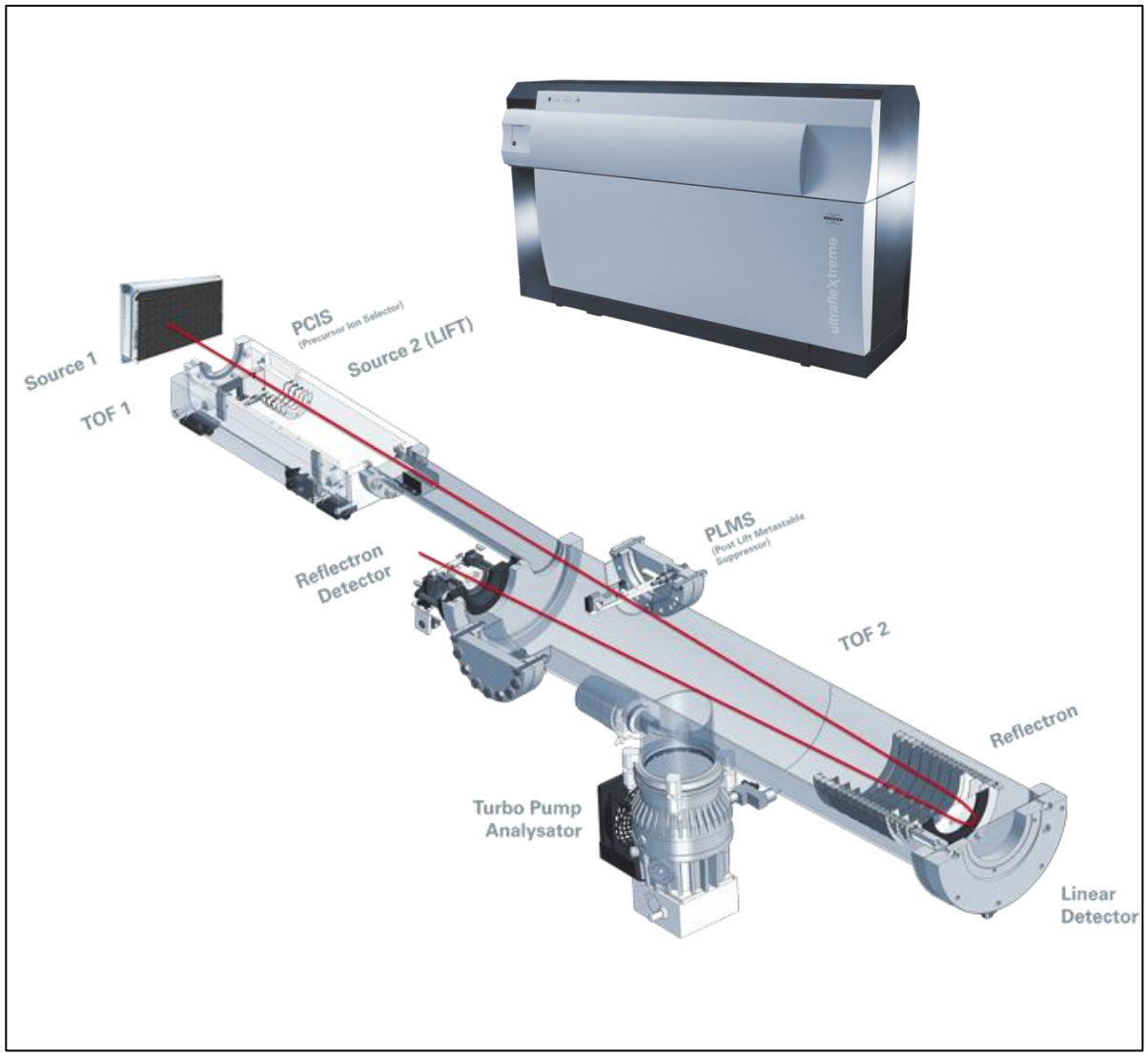
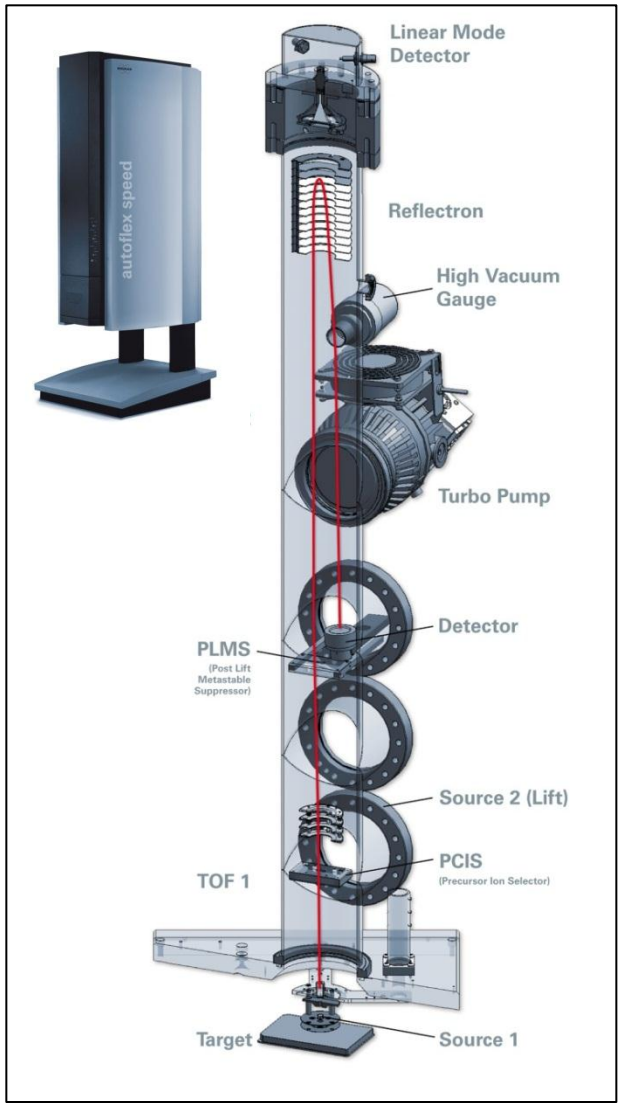
Layout of a FLEX™ Series MALDI-TOF/TOF MS

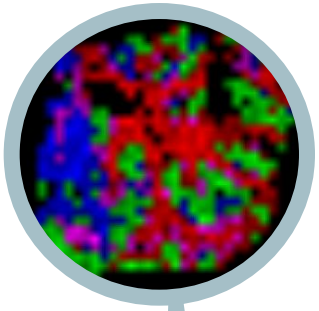


Diagrams of autoflex speed and ultrafleXtreme MALDI-TOF/TOF MS systems





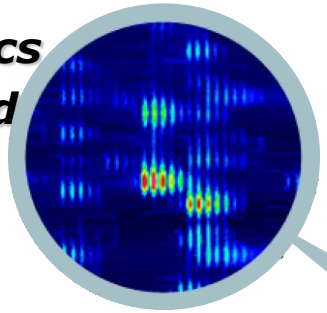
MALDI-TOF applications



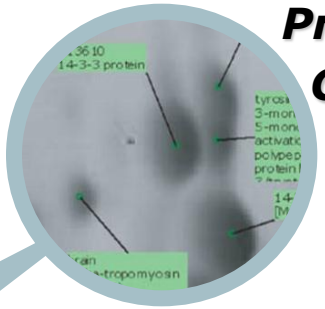
MALDI Molecular Imager

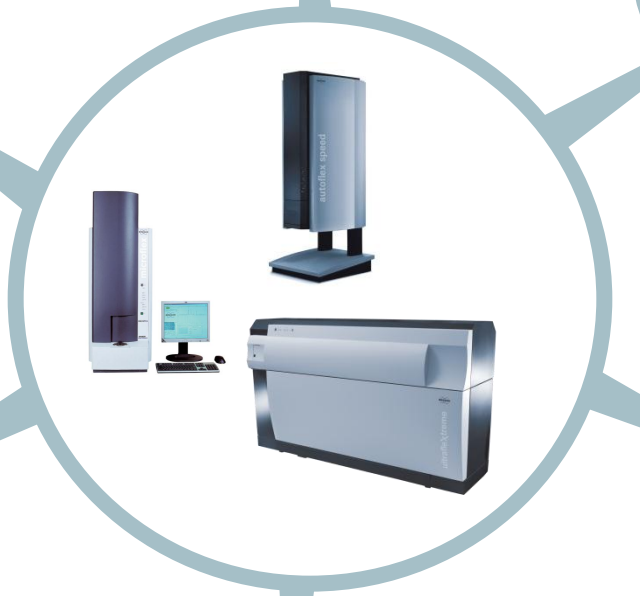
BRUKER

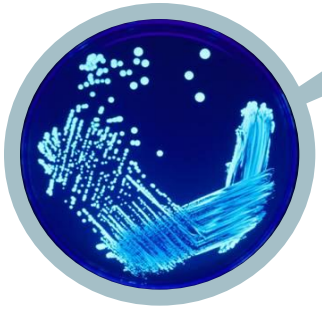
Proteomics LC-based



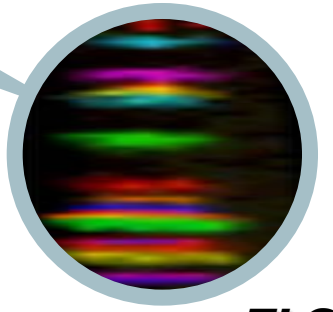
Proteomics Gel-based



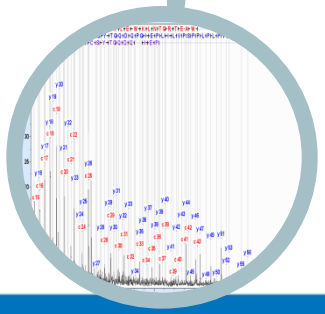




MALDI biotyper



TLC-MALDI

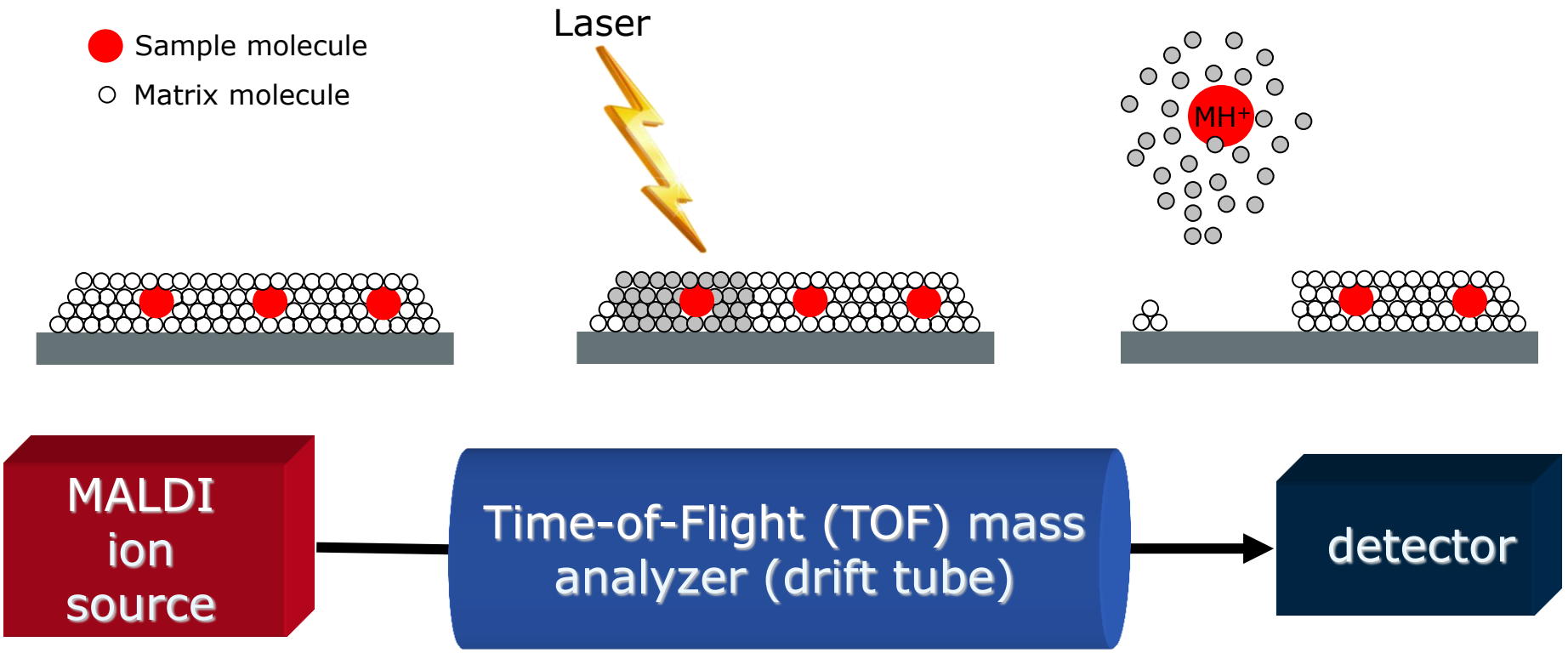


MALDI-TDS

MALDI-TOF mass spectrometry

general introduction

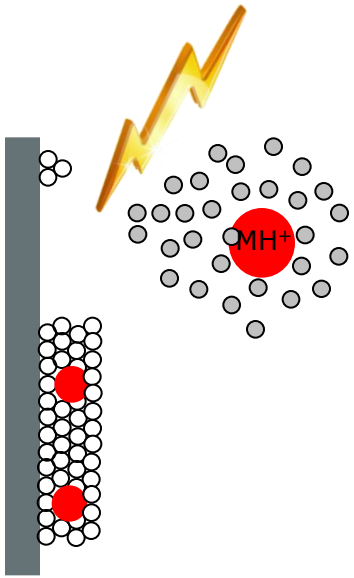
Fundamentals of Matrix-Assisted Laser Desorption/Ionization-Time of Flight MS



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

$$M \xrightarrow[\text{Matrix}]{\text{Laser energy}} [M+H]^+$$

Positive ion mode:
Protonation of sample

$$M \xrightarrow[\text{Matrix}]{\text{Laser energy}} [M-H]^-$$

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. –

[M+Na]+; [M+K]+; [M+Cu]+; [M+Li]+; [M+Ag]+

Positive ionization mode shown

Commonly Used MALDI Matrices

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

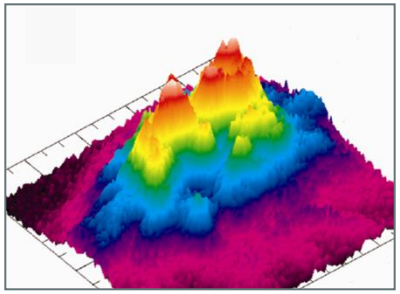
Additives:
1) Ammonium citrate
2) CF_3COOAg

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)

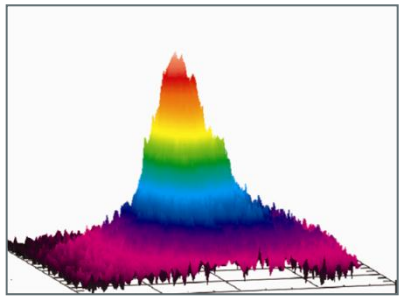
What about the Laser?
Lasers commonly used in MALDI



Nitrogen laser:

pro: well structured energy profile

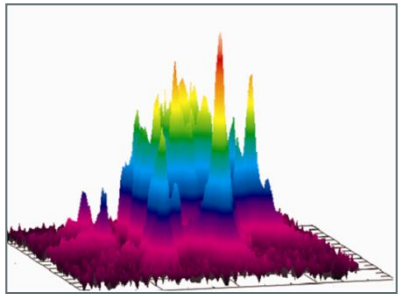
con: slow (maximum 60Hz)



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)

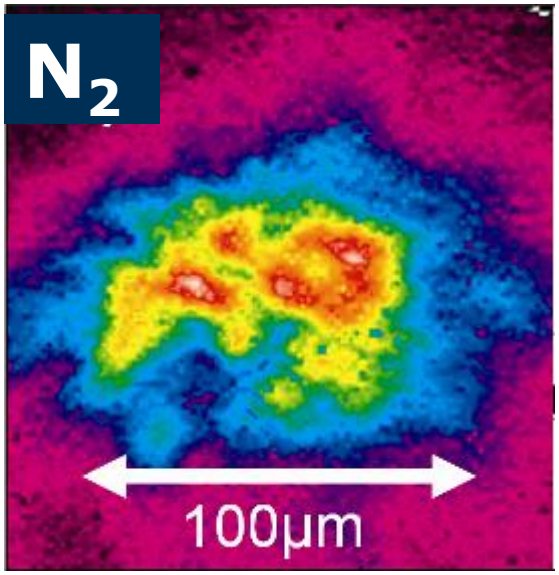
pro: well structured energy profile

pro: compatible with all matrices developed for N_2 lasers

pro: efficient sample consumption

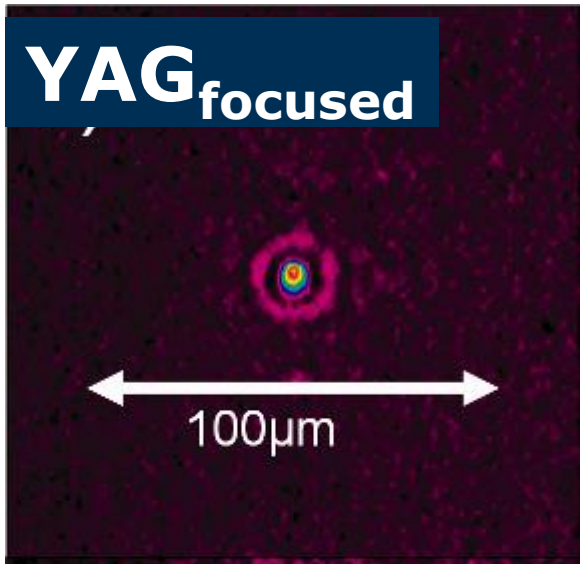
Reference: A. Holle, A. Haase, M. Kayser, J. Höhndorf, *Journal of Mass Spectrometry*, 41, 705-716 (2006)

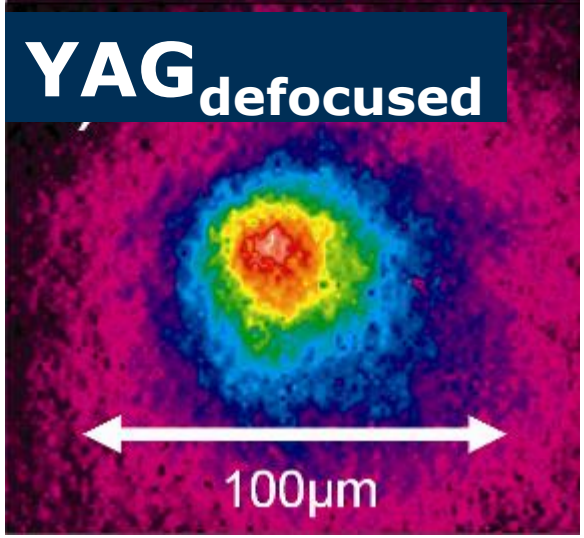
Comparison between nitrogen, Nd:YAG and Smartbeam lasers

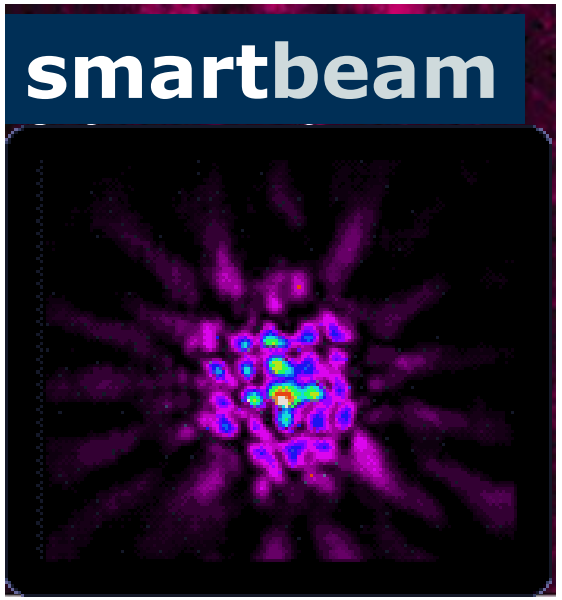


- Maximum laser frequency: 60 Hz
- Laser lifetime: ~10^8 shots
- Minimum laser focus: ~50 µm

Bruker systems:
microflex
autoflex I and II
ultraflex I







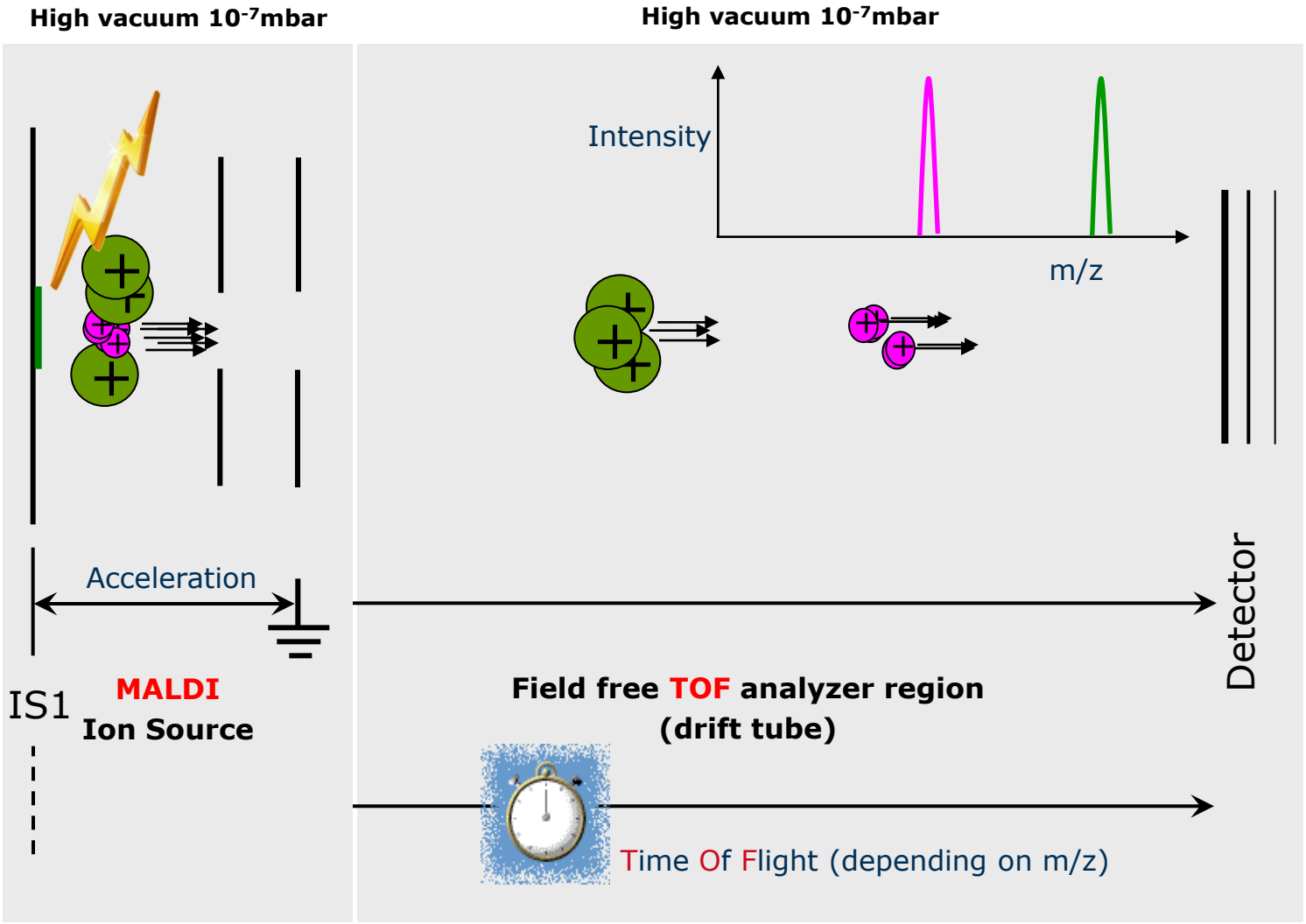
- Maximum laser frequency: 1000 Hz
- Laser lifetime: ~10^9 shots
- Minimum laser focus: ~10 µm
- Smartbeam exclusive: variable beam profile

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



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.

$$E_p = zeU$$

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

$$E_k = 1/2mv^2$$

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

$$1/2mv^2 = zeU$$

- 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:

$$1/2m(L/t)^2 = zeU$$

- From which the main TOF equation is derived…

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

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

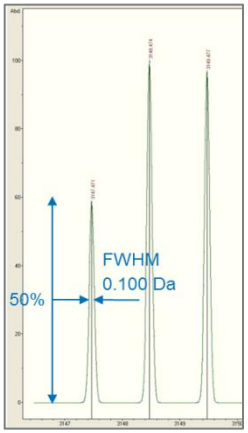
$$\mathbf{t = const\ \sqrt{m/z}}$$

In other words, the larger the m/z, the longer the flight time

How do we assess peak quality (and indirectly, instrument performance)?

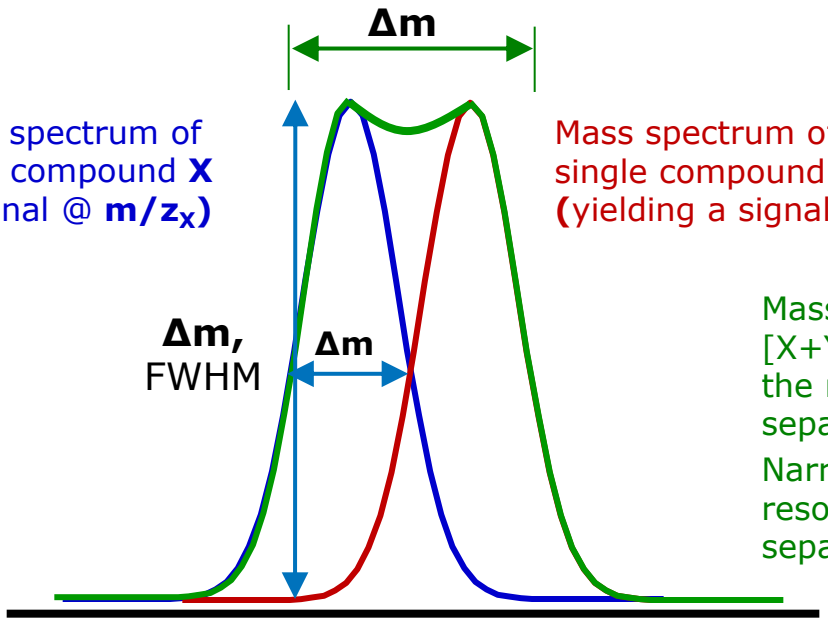
- Peak resolution – how well are two neighboring peaks separated from each other (are they sufficiently narrow for baseline separation)?
- Mass accuracy – how close are the experimentally measured masses to the calculated (standard) values?
- Peak detection – are proteomic profiles or target masses reproducibly detectable at an acceptable signal/noise ratio?

Resolution, R=m/Δm



Δm is a mass peak´s full width at half maximum **(FWHM)**

Example analysis of a mixture of two compounds X and Y:



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.

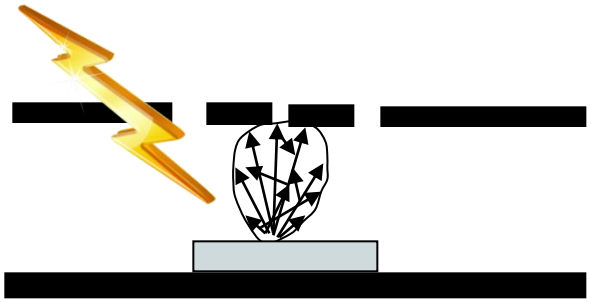
Linear Mode Limitation: Resolution limited due to spatial and energy spreads

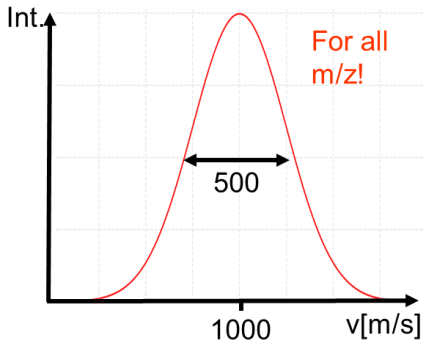
Spatial spread:

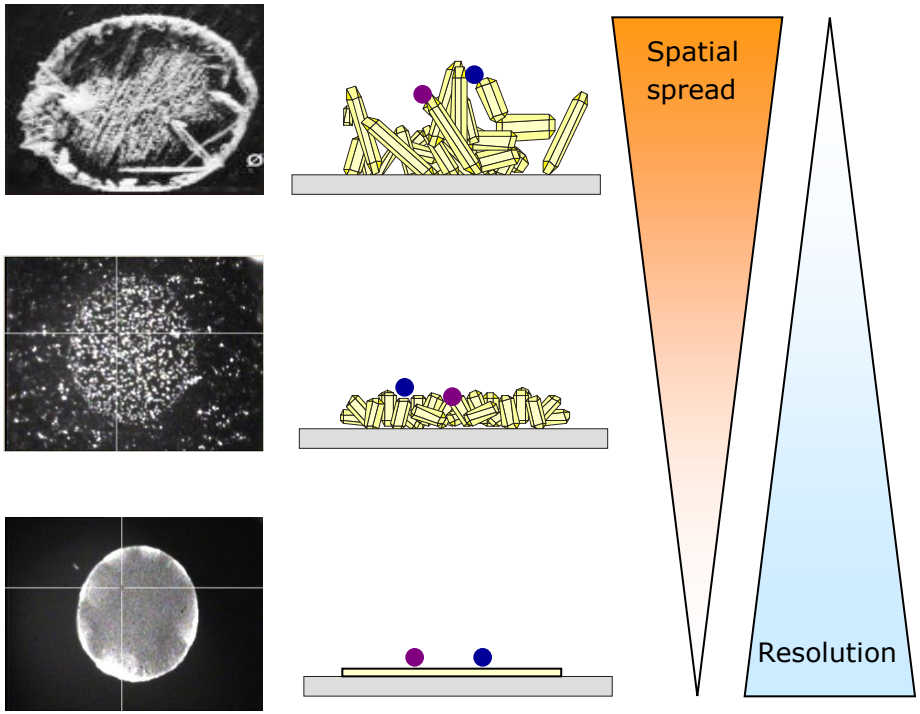
- initial movement of ions in different directions
- ions are desorbed from different z-coordinates due to heterogeneity in size of matrix crystals

Initial energy (speed) spread:

- heterogeneous secondary reactions (ion-ion; ion-neutral)

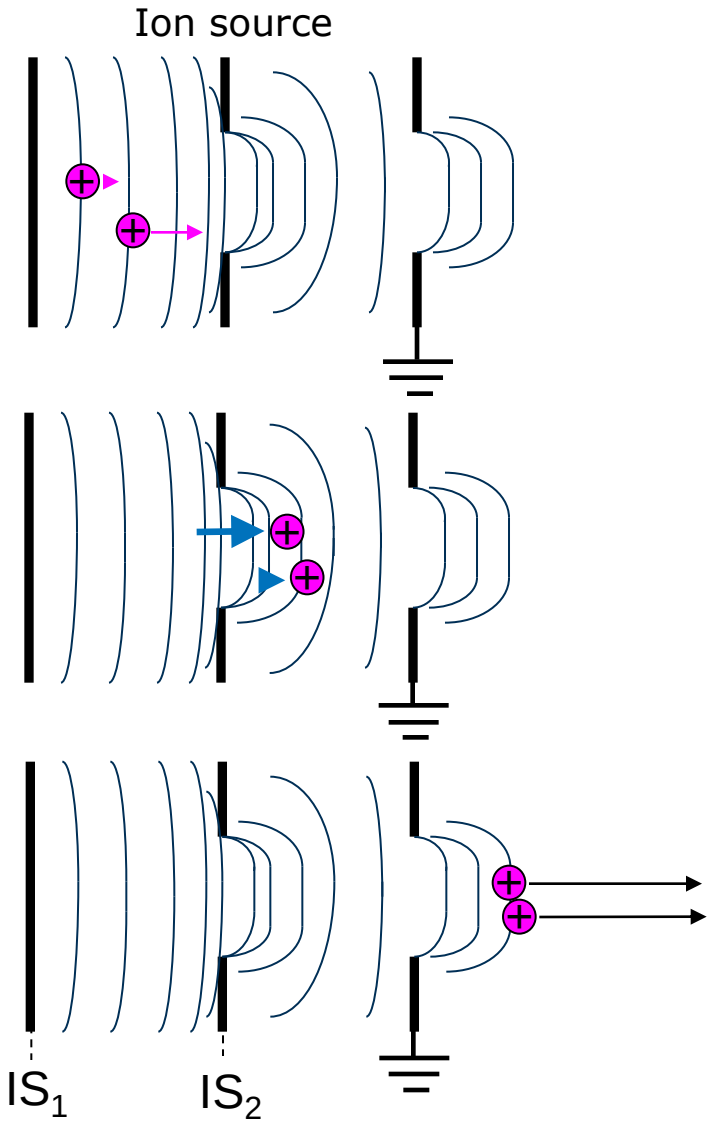






Reference: W. Ens, Y. Mao, F. Mayer, K.G. Standing,
Rapid Communications In Mass Spectrometry, 5, 117-123 (1991)

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)

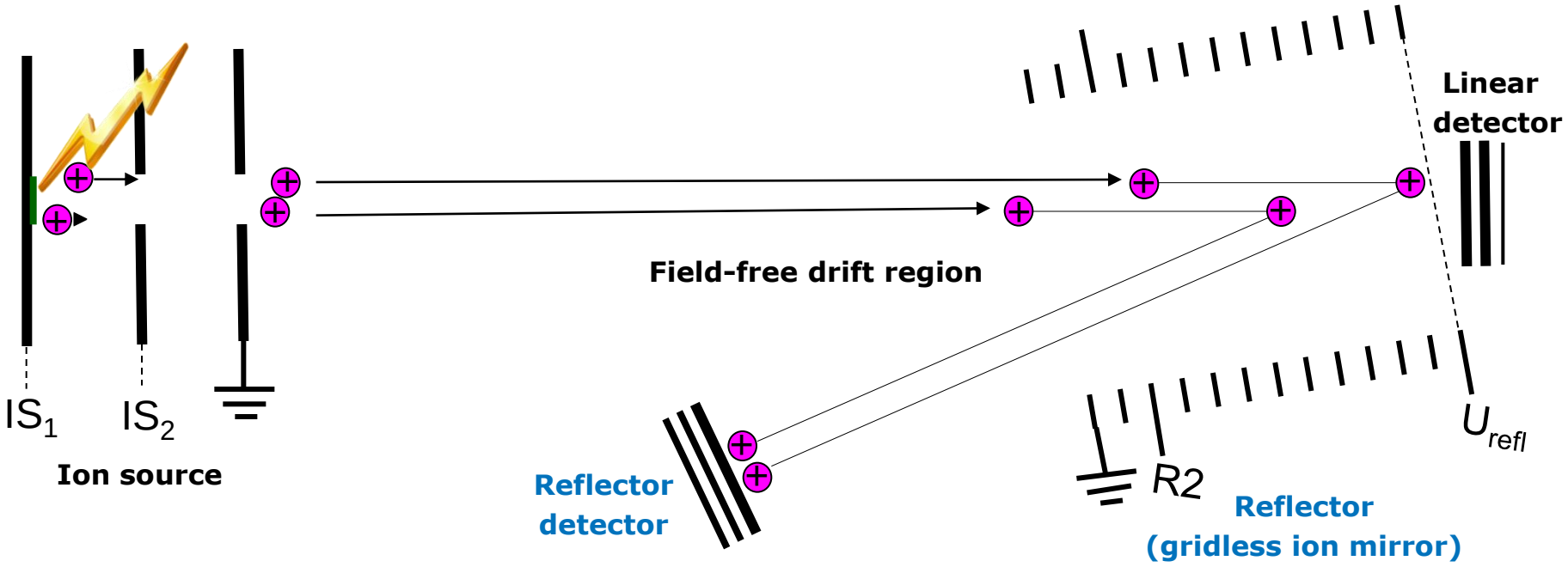
Example ultrafleXtreme settings:

flexControl method	IS1 (kV)	IS2 (kV)	Lens (kV)	PIE (ns)
LP_700-2000 Da	19.50	18.20	6.00	130
LP_5-20 kDa	19.50	18.20	6.50	340

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

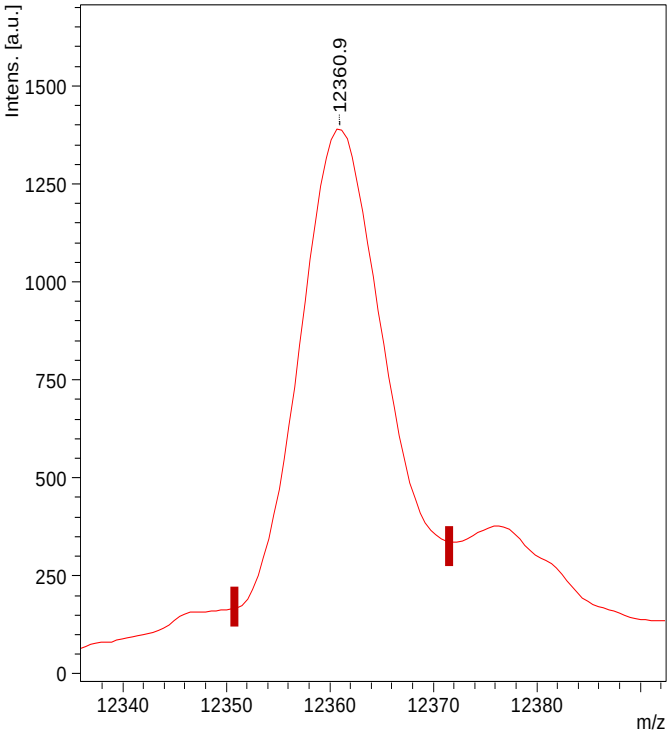
- Designed for simultaneous time and spatial ion focusing by compensating for ions of different energies
- Provides for increased mass accuracy and resolution
- Consists of a series of plates with increasing applied voltages
- Re-directs the flight path of traveling ions to reflector detector



Linear vs. reflector mode: Cytochrome C

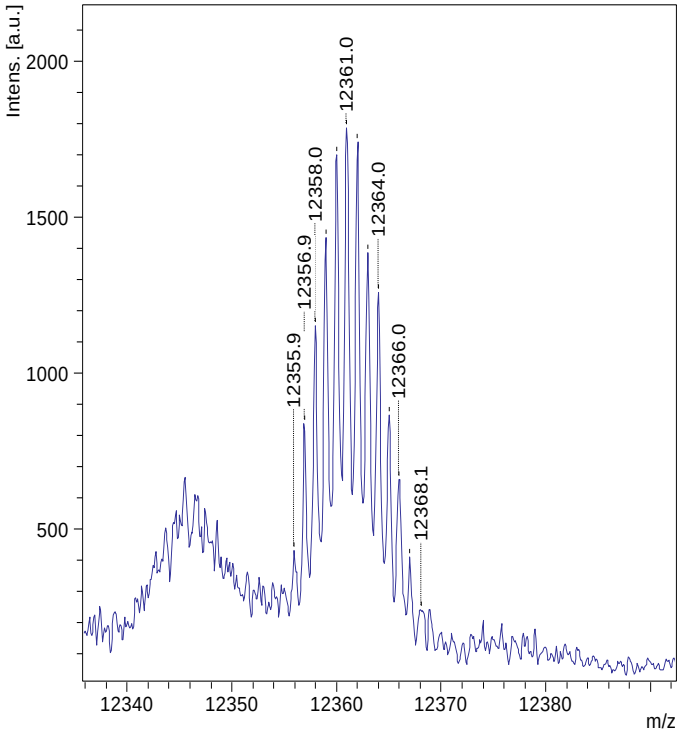
MW_{avg}=12360Da

Linear mode: Low resolution
R=1,500



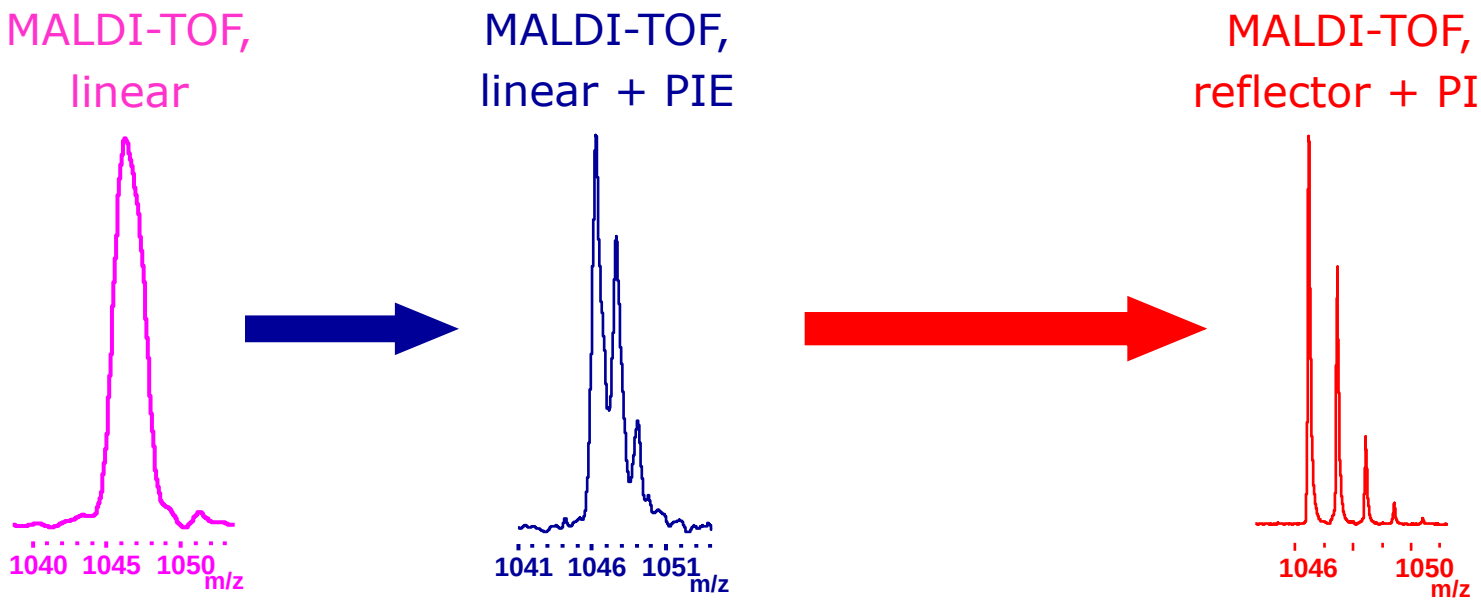
Spectrum shows one broad peak representing the envelope of the non-resolved isotope peaks

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 deccelerated/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

t : time-of-flight

c_0 and c_1 : calibration constants

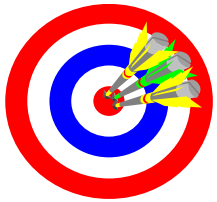
C_0 and C_1 are determined by measuring the TOF of two signals of known standard masses m_1 and m_2

Mass accuracy is calculated in ppm

$$\frac{|M_{calc} - M_{meas}|}{M_{calc}} \times 10^6 = \text{ppm}$$

M_{calc} – calculated mass

M_{meas} – measured mass



Example:

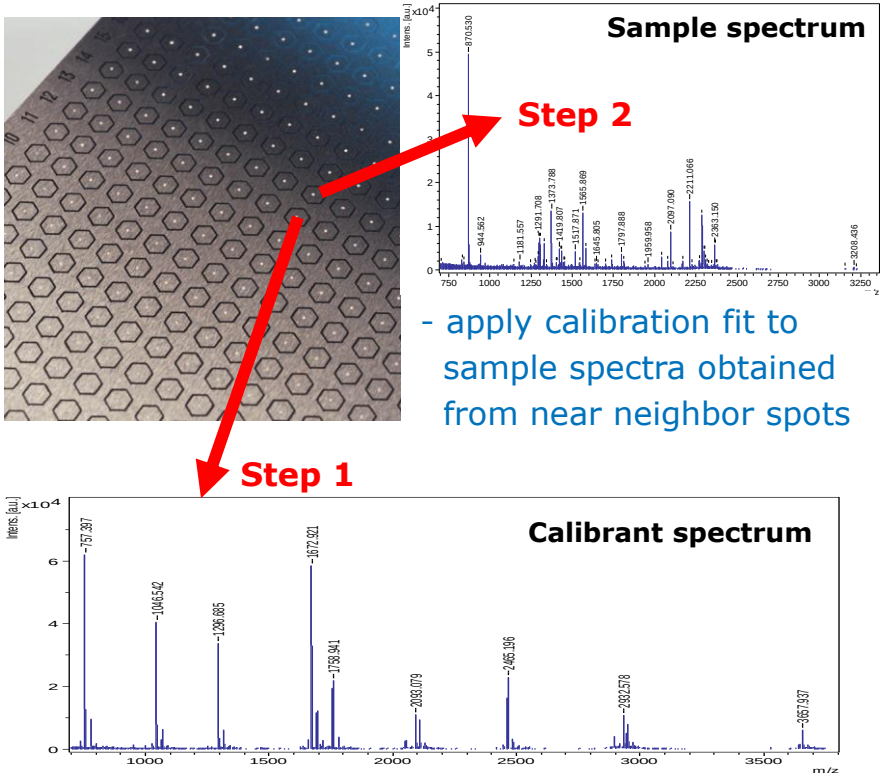
$M_{calc} = 3147.4710$

$M_{meas} = 3147.4670$

$(3147.4710-3147.4670)/3147.4710 \times 10^6 = 1.27$ ppm

Mass Accuracy – Calibration Strategies

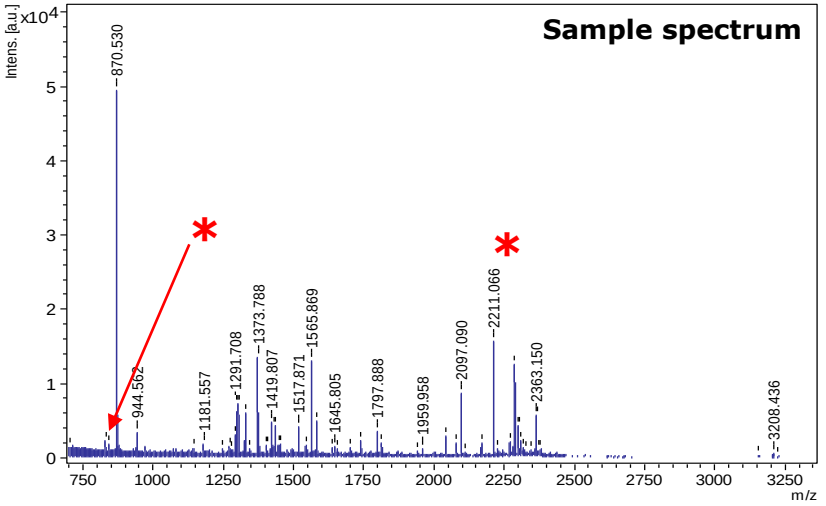
Option 1) External calibration



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

- 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



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

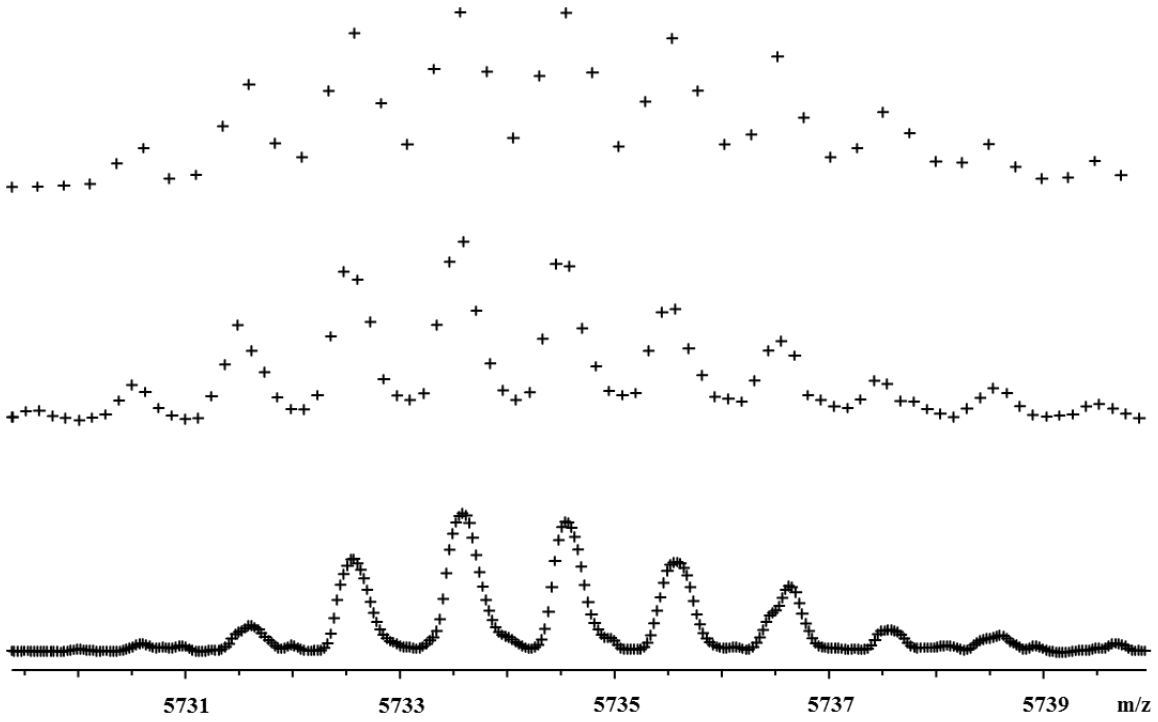
Internal calibration (or re-calibration) allows for

- 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



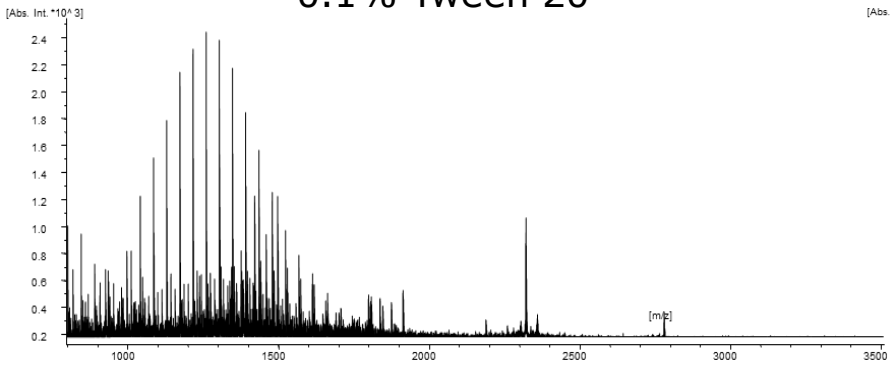
Comparison of Peptide Mass Accuracy from 0.5, 1 and 2 GHz data

	0.5 GHz	1 GHz	2 GHz
Bradykinin	29 ppm	4.3 ppm	2.8 ppm
Angiotensin I	14.8 ppm	2.0 ppm	1.7 ppm

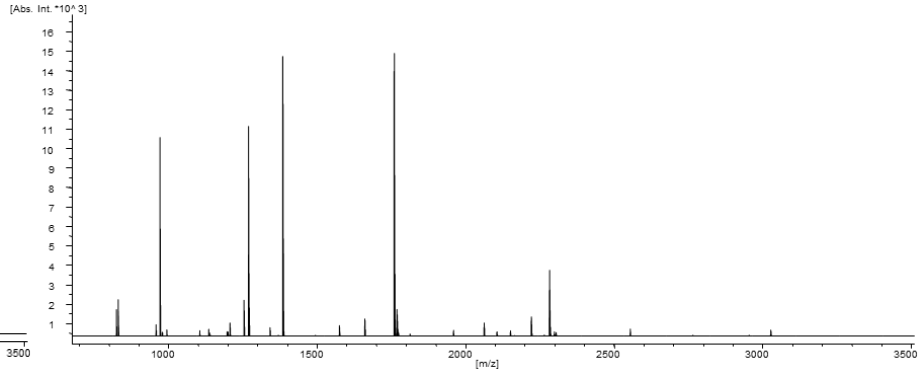
Factors affecting Peak Intensity

- 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

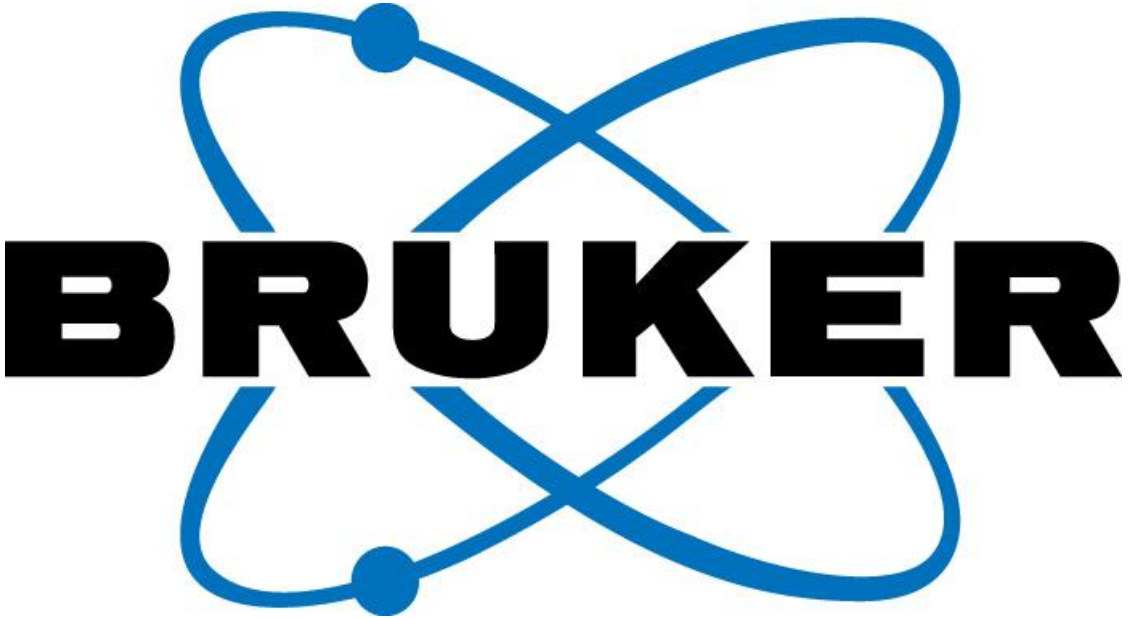


Mass Limits – Limits of Peak Detection

- 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.

$$E_k = 1/2mv^2$$

- 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)



www.bdal.com

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)

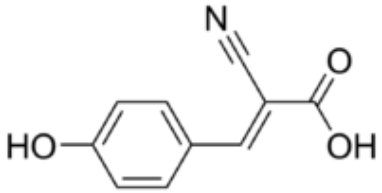
M. Karas, M. Glückmann, J. Schäfer

Journal of Mass Spectrometry, 35, 1-12, (2000)

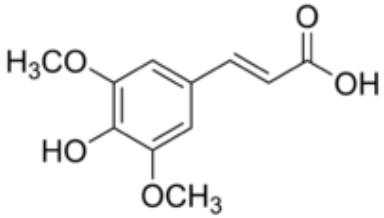
R. Zenobi and R. Knochenmuss

Mass Spectrometry Reviews, 17, 337-366 (1998)

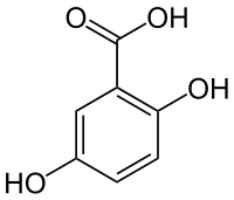
Appendix B – MALDI matrix structures



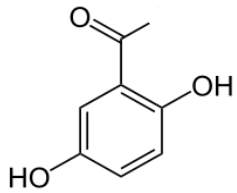
α-Cyano-4-hydroxycinnamic acid
(CCA,CHCA)



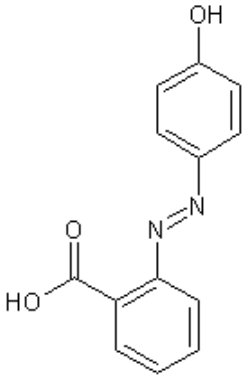
Sinapinic acid
(SA)



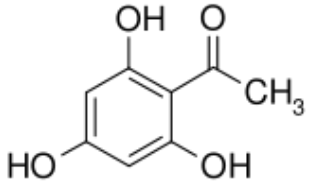
2,5-Dihydroxybenzoic acid
(DHB)



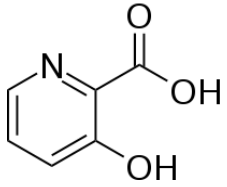
2,5-Dihydroxyacetophenone
(DHAP)



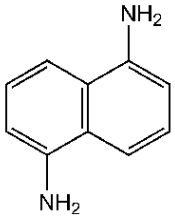
2'-(4-Hydroxyphenylazo)
benzoic acid
(HABA)



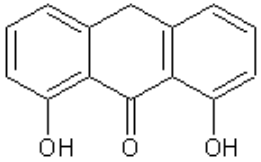
2,4,6-Trihydroxyacetophenone
(THAP)



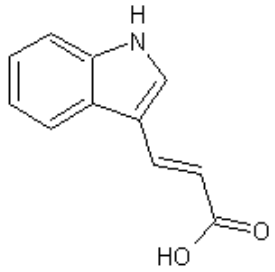
3-Hydroxypicolinic acid
(HPA)



1,5-Diaminonaphthalene
(DAN)



Dithranol
(DIT)



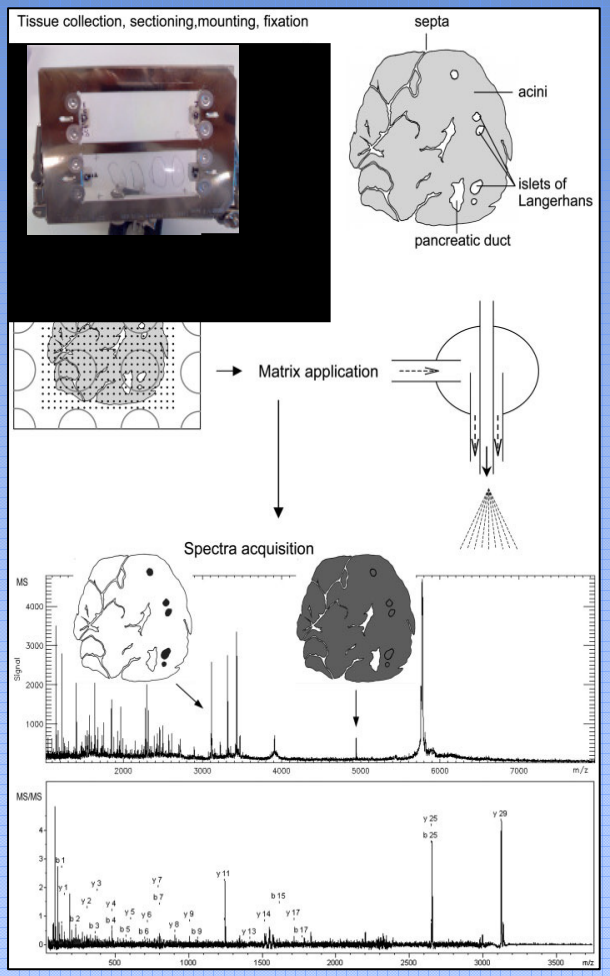
Trans-3-indoleacrylic acid
(IAA)

Appendix C – Common Detergents and Effect On MALDI Analyses

Class	Effect on MALDI spectrum
1	no bad effect: may improve results in mixture
2	little effect
3	spectrum quality & signal intensity reduced
4	spectrum suppressed: detergent must be removed

Detergent	Class
n-octyl-glucoside	1
n-dodecyl-glucoside	1
octanoyl-N-methylglucamide	1
decanoyl-n-methylglucamide	1
n-dodecyl-beta-D-maltoside	2
octylphenolpoly(ethyleneglycolether)$_{10}$ (Triton X-100)	3
octylphenolpoly(ethyleneglycolether)$_{7}$ (Triton X-114)	3
polyethylene glycol (PEG 2000)	3
dodecylpoly(ethyleneglycolether)$_{9}$ (Thesit)	4
isotridecylpoly(ethyleneglycolether)$_{8}$	4
CHAPS	4
CHAPSO	4
n-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate	4
sodium dodecylsulfate (SDS)	4

Preparation of tissues for MALDI imaging and profiling

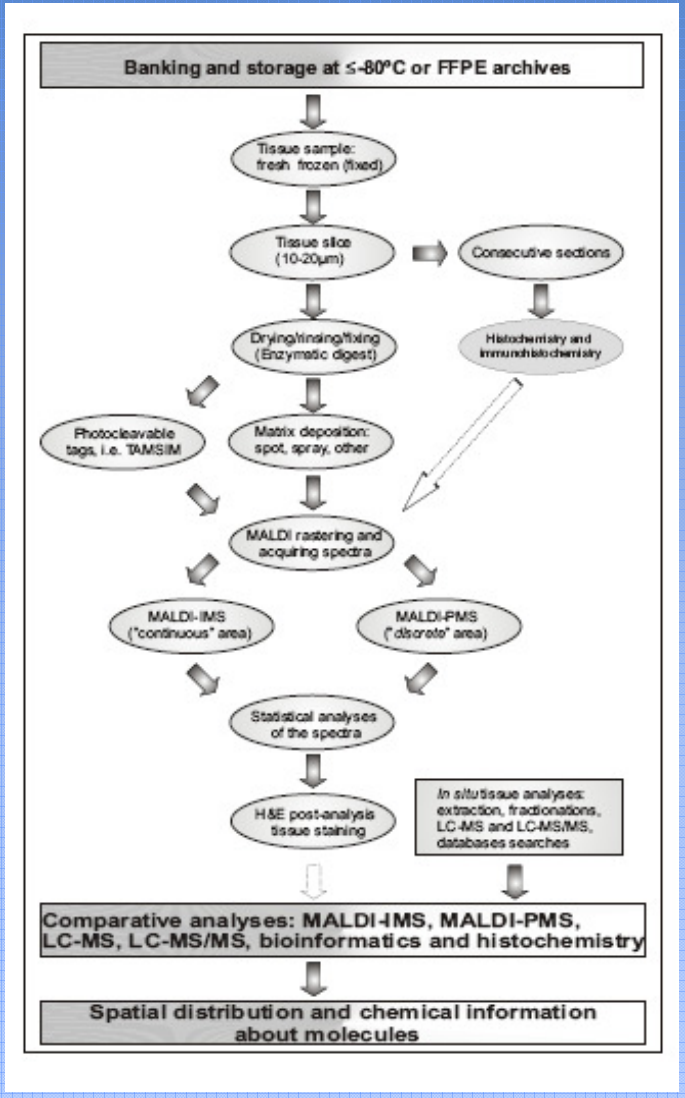


Maciej Lalowski

Biomedicum Helsinki, Helsinki University

EuroKUP seminar, 12.10.2009

Scheme of utilization



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.

Cornett et al., *Nature Methods,* 2007

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

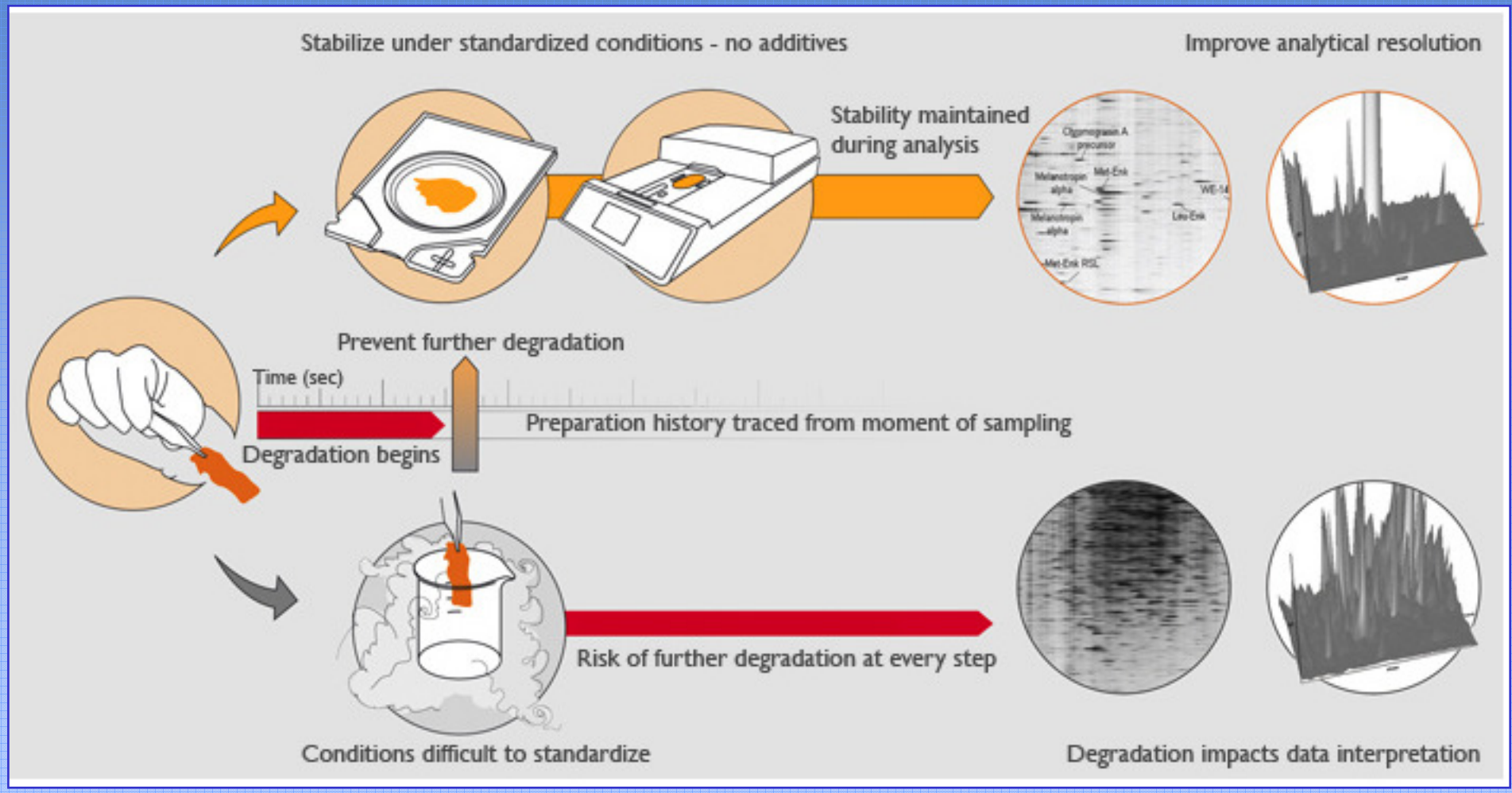
Step 1: preserving the tissues

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.**

www.denator.com

Step 1: preserving the tissues



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

Step 3: tissue pre-treatment

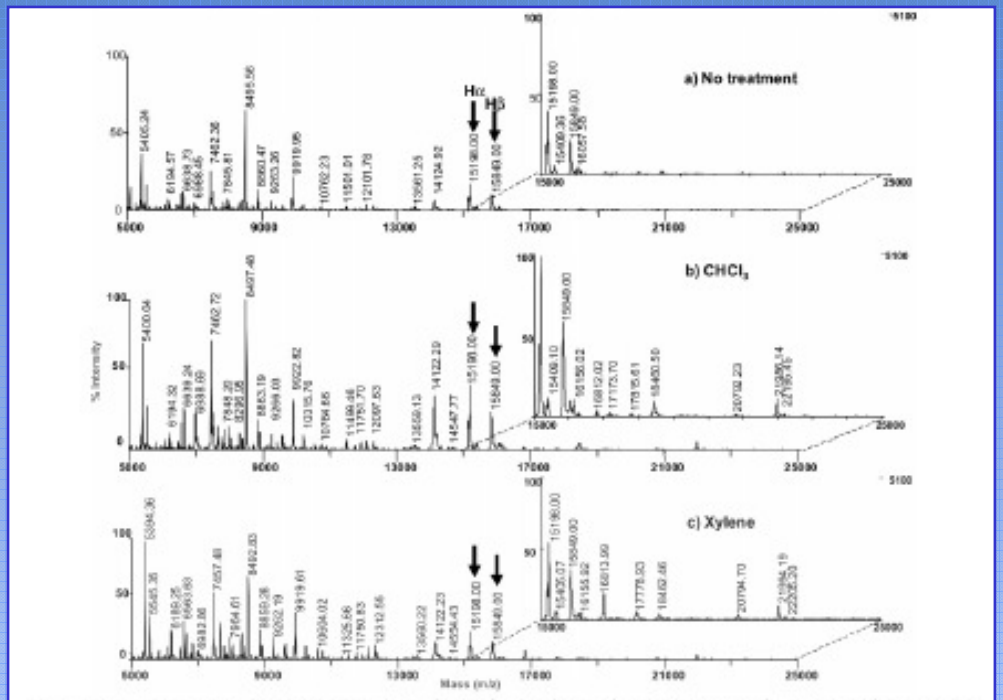


Figure 2. m/z >5000 region of the MALDI-TOF mass spectra resulting from the direct analysis in linear positive ion mode with SA as matrix of rat brain tissue sections from a tissue block conserved 6 months at −80 °C. (a) Untreated sample, after tissue treatment with (b) chloroform and (c) xylene (the arrows indicate the peaks corresponding to hemoglobin α chain (major) or Hα and hemoglobin β chain (major) or Hβ used as internal calibrants).

Table 1. Average Number of Detected Compounds, Standard Deviation, and Calculated Increase Detection for Peptides/Proteins of m/z > 5000 Determined from the Mass Spectra Recorded on Untreated Rat Brain Sections versus Organic Solvent Treated Ones

treatment	n[a]	no. of detected compds	standard deviation (%)	increase in detection (%)
chloroform	10	81	22	34
hexane	5	75	38	25
toluene	5	68	32	13
xylene	5	86	13	44
acetone	5	64	29	7
untreated	10	60	34	0

[a] Number of experiments.

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

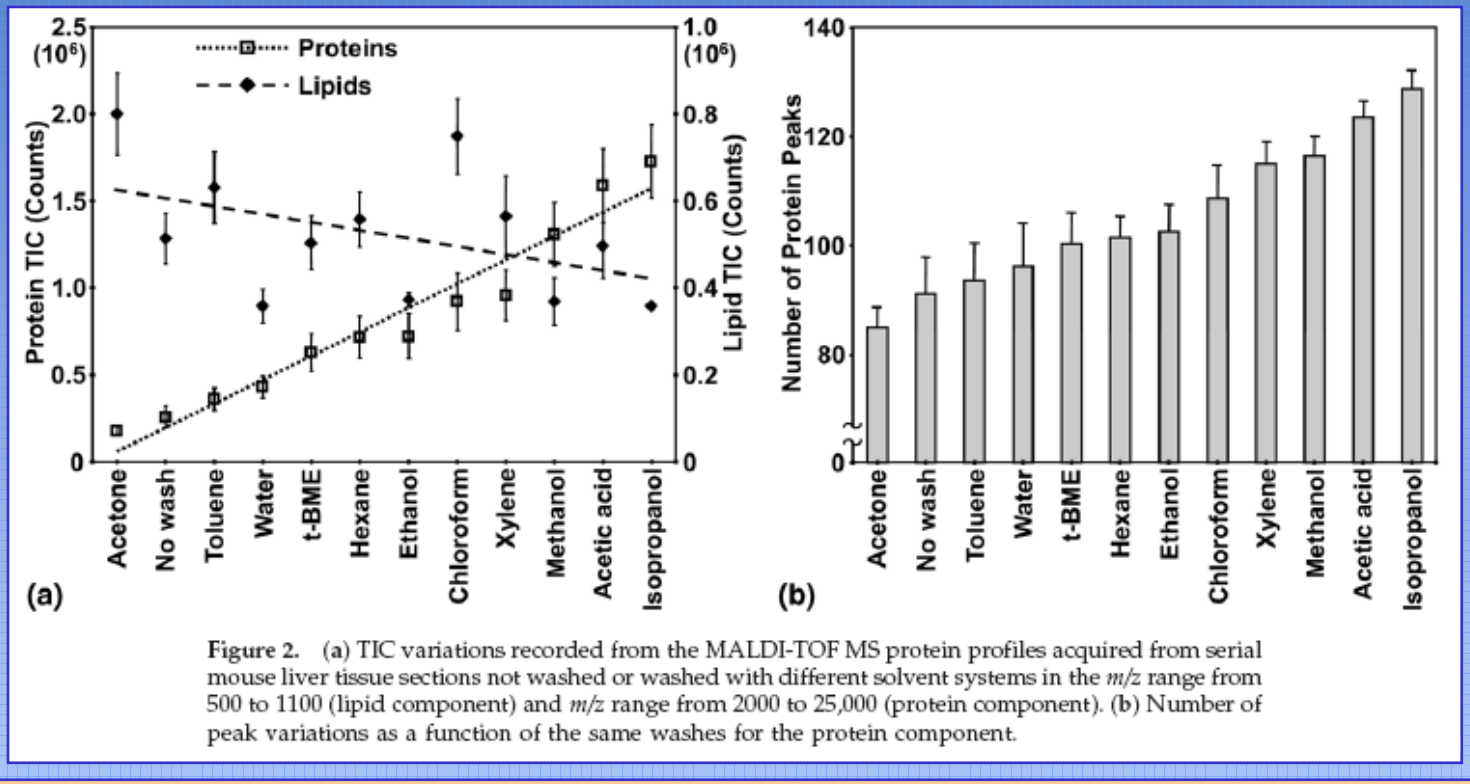


Figure 2. (a) TIC variations recorded from the MALDI-TOF MS protein profiles acquired from serial mouse liver tissue sections not washed or washed with different solvent systems in the *m/z* range from 500 to 1100 (lipid component) and *m/z* range from 2000 to 25,000 (protein component). (b) Number of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

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.

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.

Step 4: Matrices (other)

Table 1. Common MALDI matrices used in imaging MS

Matrix	Abbreviations	Applications	Type
2,5-Dihydroxybenzoic acid [68–70]	DHB	Sugars, peptides, nucleotides, glycopeptides, glycoproteins and small proteins	Crystalline
α-Cyano-4-hydroxycinnamic acid [70, 71]	CHCA	Peptides, small proteins and glycopeptides	Crystalline
3,5-Dimethoxy-4-hydroxycinnamic acid [70, 72, 73]	SA	Peptides and large proteins, lipids	Crystalline
2,4,6-Trihydroxyacetophenone [70, 74]	THAP	Oligonucleotides	Crystalline
3-Hydroxypicolinic acid [70, 75]	3-HPA	Oligonucleotides, peptides and glycoproteins	Crystalline
2,6-Dihydroxyacetophenone [9, 50, 70]	DHA	Phospholipids	Crystalline
2,4-Dinitrophenylhydrazine [48]	2,4-DNPH	Peptides, proteins (also for FFPE tissues)	Reactive
CHCA/aniline [49]	—	Peptides	Ionic solid
CHCA/*N*,*N*-dimethylaniline [49]	—	Peptides	Ionic solid
CHCA/2-amino-4-methyl-5-nitropyridine [49]	—	Peptides	Ionic liquid

Kaletas et al., *Proteomics* 2009

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.

Step 5: Methods of matrix application

Table 2. Matrix application techniques for imaging MS

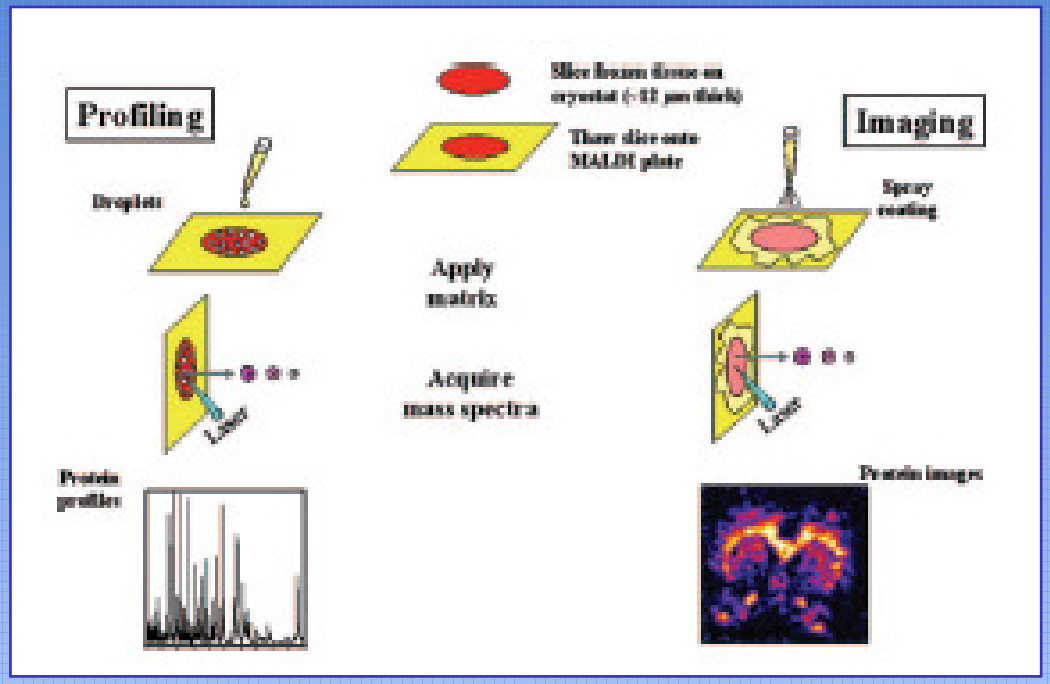
Technique	Droplet diameter	Advantages	Disadvantages
Droplet deposition by hand [94, 91]	Variable, mostly large (>900 μm)	Fast, simple, cheap	No spatial information, poor reproducibility
Pneumatic nebulization (airbrush) [16, 94]	Variable, mostly small (aerosols)	Fast, simple, homogenous layer, cheap	Limited environmental control, low concentrations of matrix solution can be used, quality varies from person to person, droplet size not constant
ChIP [97, 99]	~150 μm (100 pL)	Uniform droplets, precision of placement, conditions can be controlled, automated, high signal quality, reproducible	Slow, nozzle tip clogging, expensive
Acoustic reagent multi-spotter [94, 98]	180–230 μm (170 pL)	Uniform droplets, precision of placement, automated, no clogging, fast, good reproducibility	Matrix applied as droplets, few experiences in use of it
Electrospray deposition [94, 92]	Small	Homogenous layer, equally sized crystals	Limited time for analyte–matrix interaction, quality varies from person to person
Pneumatic Sprayer [95]	Variable, but small	Homogenous layer, automated, controlled environment, suitable for large area	Very large amounts of matrix solution used (50–150 mL), droplet size not constant
ImagePrep [100]	Variable, but small (~20–50 μm)	Conditions can be varied and controlled, automated, homogenous layer	Slow, small area, membrane clogging, droplet size not constant, expensive
Dry-coating [102]	20 μm (crystal size)	Cheap, very homogenous, high purity of organic matrix, reproducible, fast	Limited time for analyte–matrix interaction, only lipids detected so far
Sublimation [101]	Very small	Cheap, very homogenous, high purity of organic matrix, reproducible, fast	Limited time for analyte–matrix interaction, only lipids detected so far
Desktop inktjet printer [93]	Very small (droplet volume 3 pL)	Uniform droplets, precision of placement, automated, simultaneous deposition of different solutions (multi-channel), cheap	Slow, not compatible with all solvents, clogging

Kaletas et al., *Proteomics* 2009

Step 5: Methods of matrix application

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).

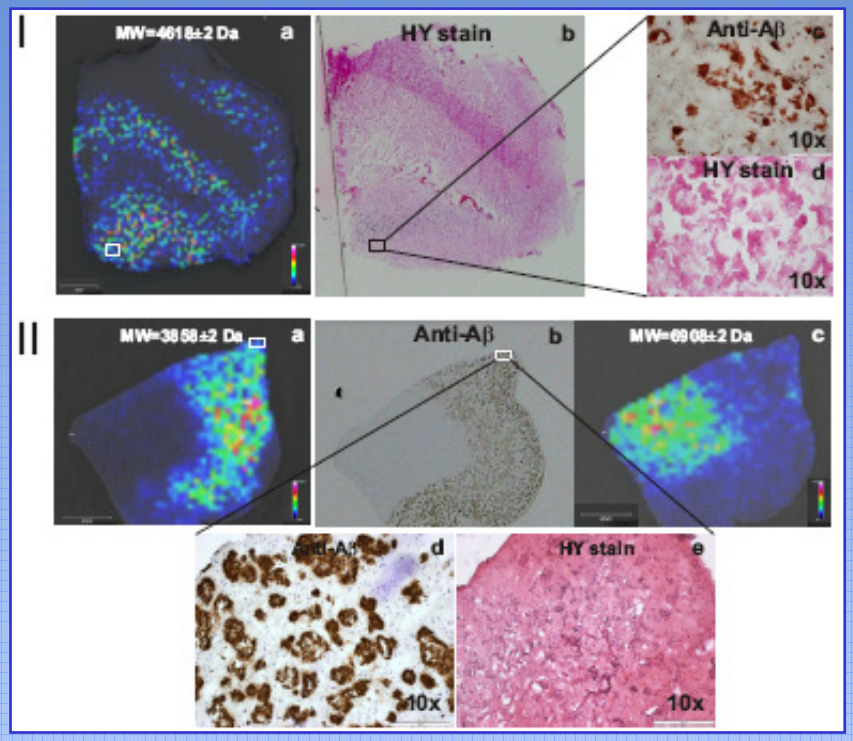
Step 6: matrix application



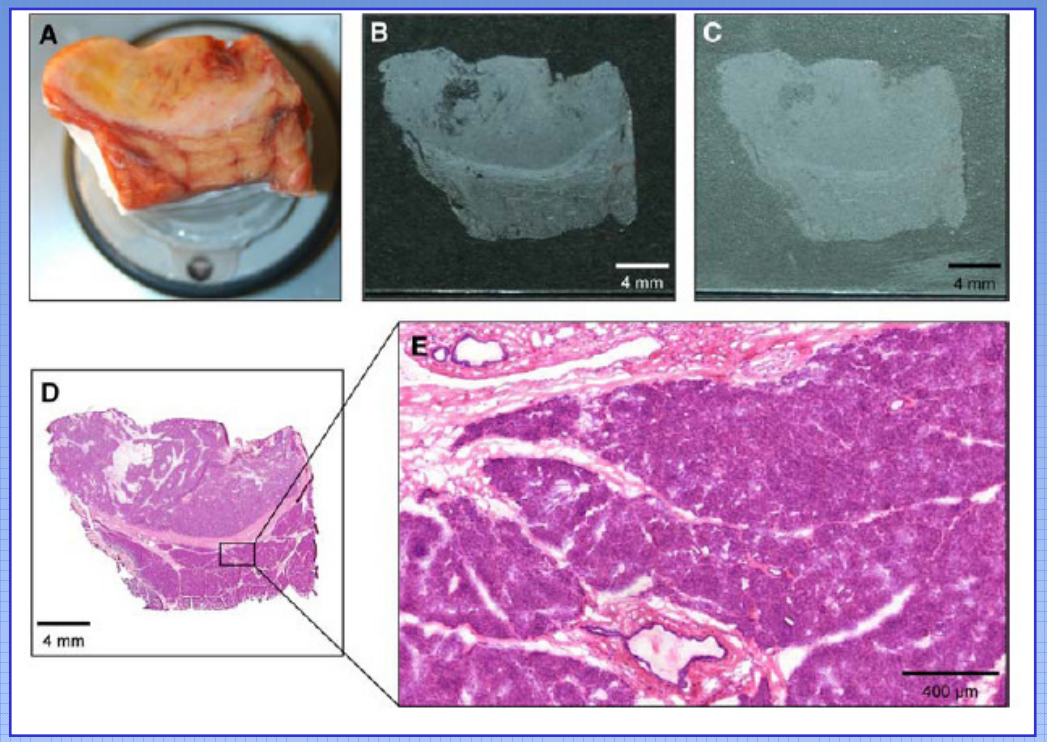
Caldwell and Capriolli., *MCP* 2005

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.

Step 7: (Immuno)-Histochemistry



Consecutive sections staining



Post analysis HY staining

Step 7: (Immuno)-Histochemistry

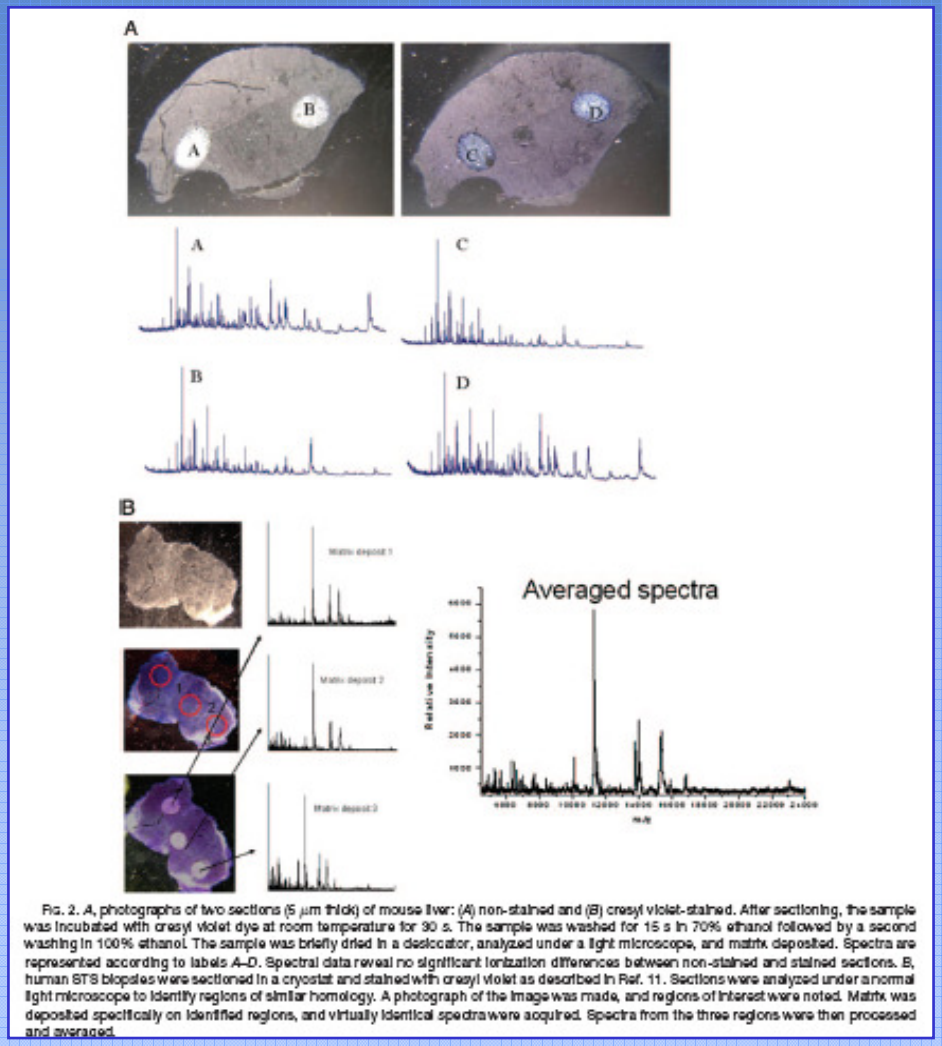


FIG. 2. A, photographs of two sections (5 μm thick) of mouse liver: (A) non-stained and (B) cresyl violet-stained. After sectioning, the sample was incubated with cresyl violet dye at room temperature for 30 s. The sample was washed for 15 s in 70% ethanol followed by a second washing in 100% ethanol. 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.*

FFPE archaised tissues

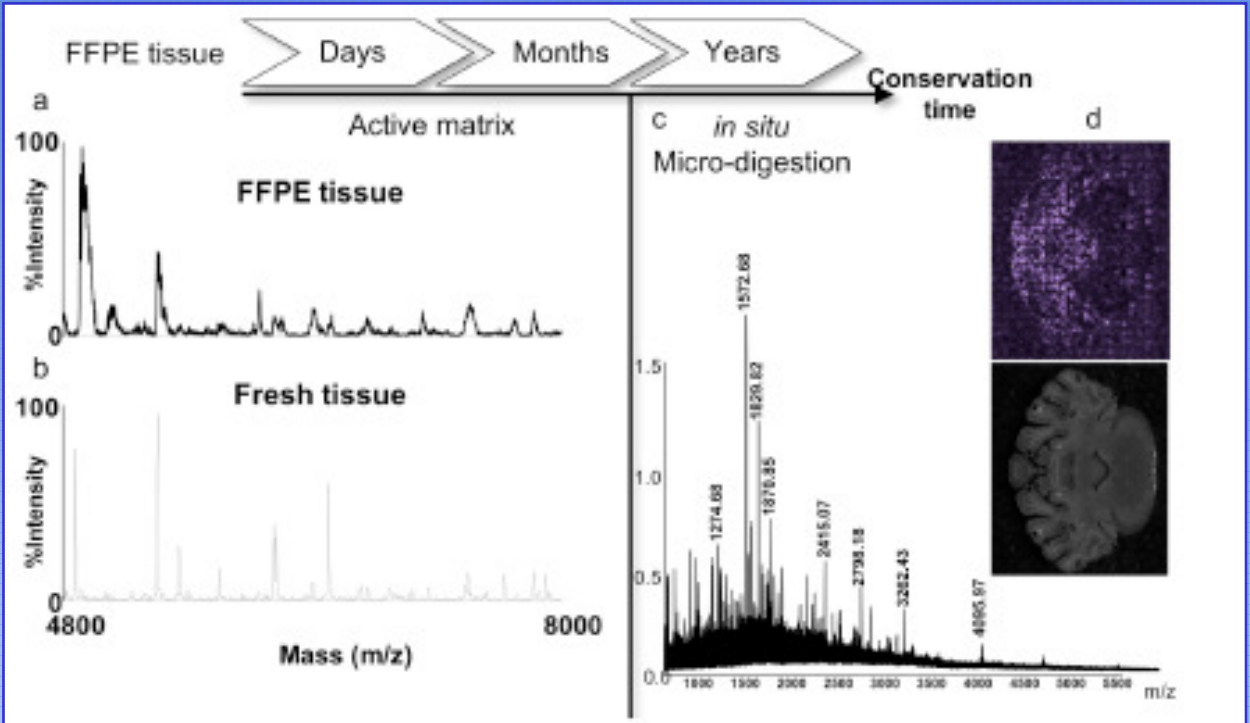


Fig. (4). Strategies used for FFPE tissue (a) stored less than 6 months, (b) stored more than 6 months.

a: Compared MALDI mass spectra in the linear positive mode of the direct analysis of a <1 year old FFPE and fresh frozen rat brain tissues recorded in the same region With sinapinic acid as matrix.

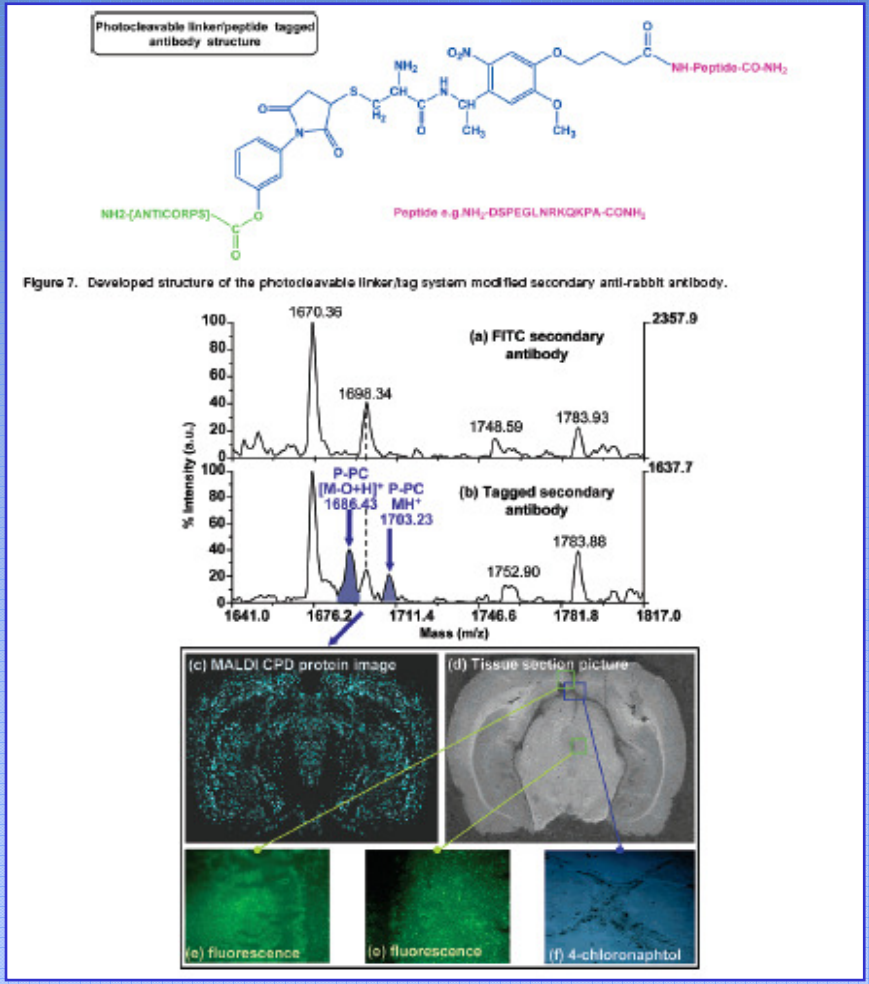
b: MALDI mass spectrum in the linear positive mode of the direct analysis of a <1 year old FFPE tissue using 2,4-DNPH as matrix. Zooming compared this spectrum to the one recorded in the same conditions and in the same region of the rat brain of a fresh frozen tissue.

c: MALDI mass spectrum in the linear positive mode of the direct analysis of a 2 years old FFPE tissue section after *in situ* trypsin digestion of the whole tissue section (15 min).

d: MALDI molecular images reconstructed from the data recorded on the 2 years old FFPE rat brain tissue section after micro-spotted *in situ* trypsin digestion followed by extraction and performed on MALDI-TOF-TOF/TOF using HCCA as matrix and compared to rat brain picture and morphology.

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 *TA*rgeted *M*ultiplex *MS IMa*ging (*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).
Lemaire, et al. J Proteomce Research 2007

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