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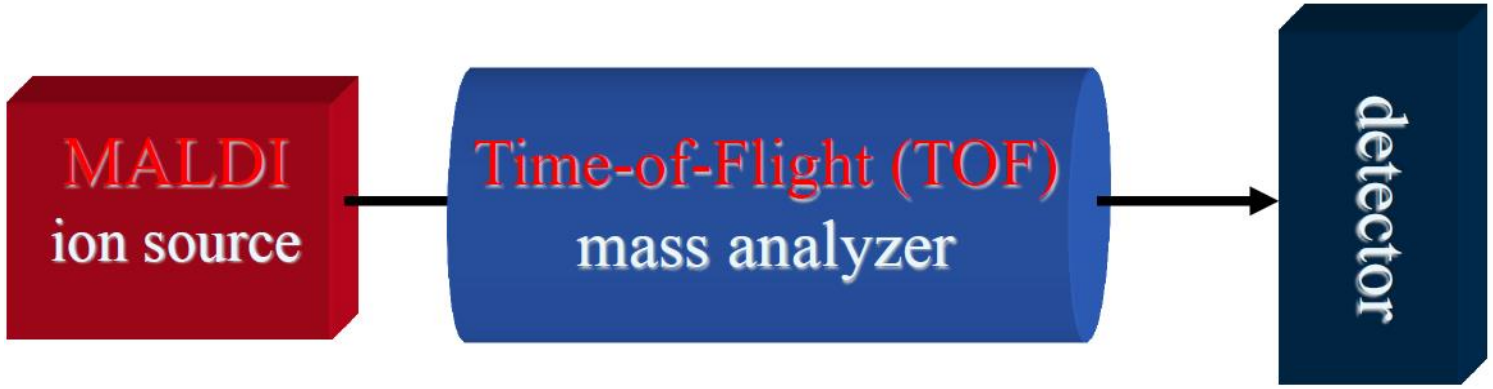
Upotreba MALDI* masene spektrometrije u oslikavanju metabolita na presecima tkiva-studija oslikavanja fenolnih jedinjenja na presecima biljnog tkiva

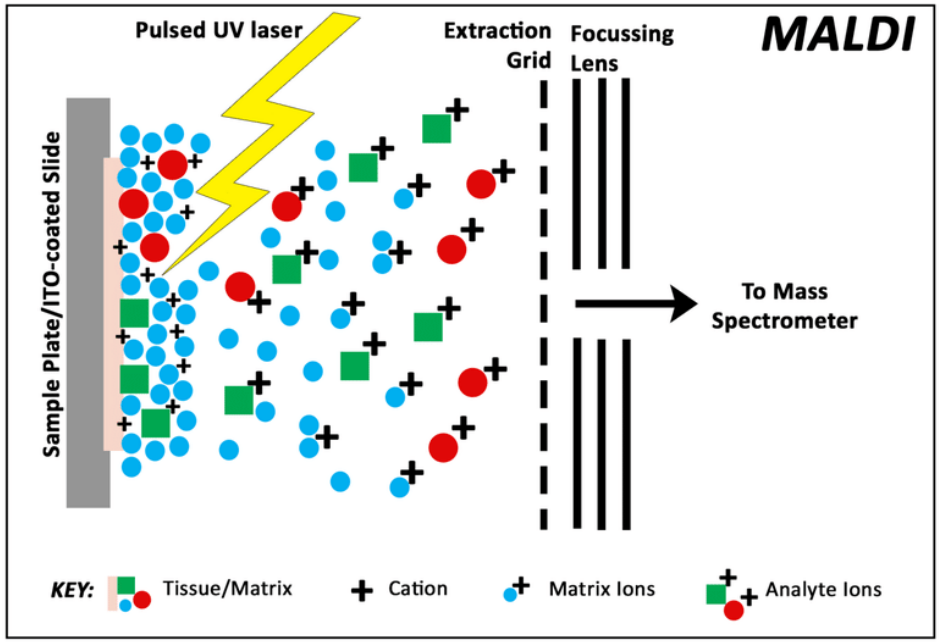
Marija Vidović

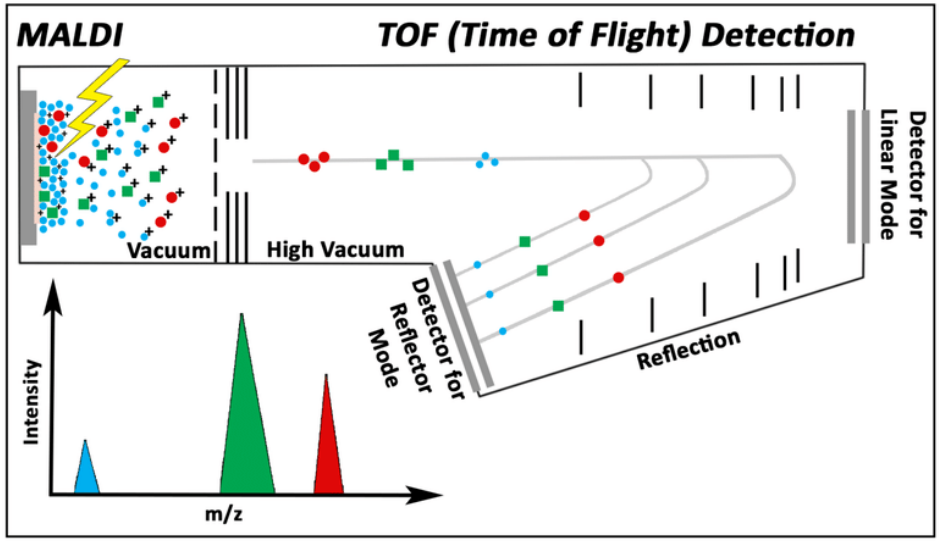
*Matricom potpomognuta laserska desorpcija/jonizacija. eng. matrix-assisted laser desorption/ionization

Šta je MALDI-TOF (/TOF) masena spektrometrija?

Tanaka Hillenkamp and Karas in the mid ´80s - Nobel prize awarded 2002



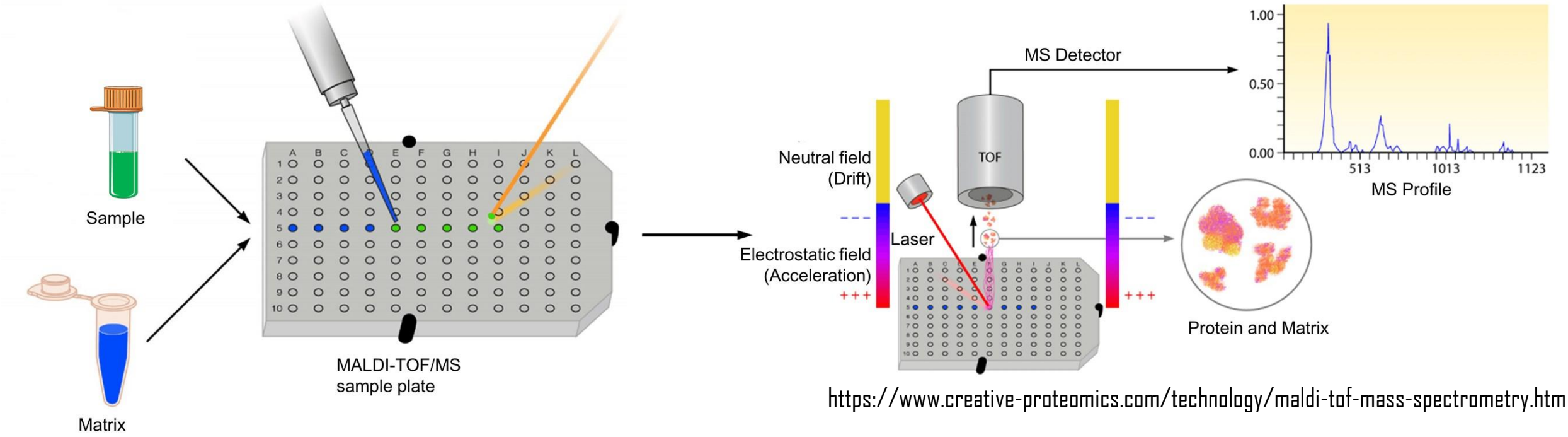




ToF:
relatively simple and inexpensive design and their excellent sensitivity and high-mass capability

Šta je MALDI-TOF (/TOF) masena spektrometrija?

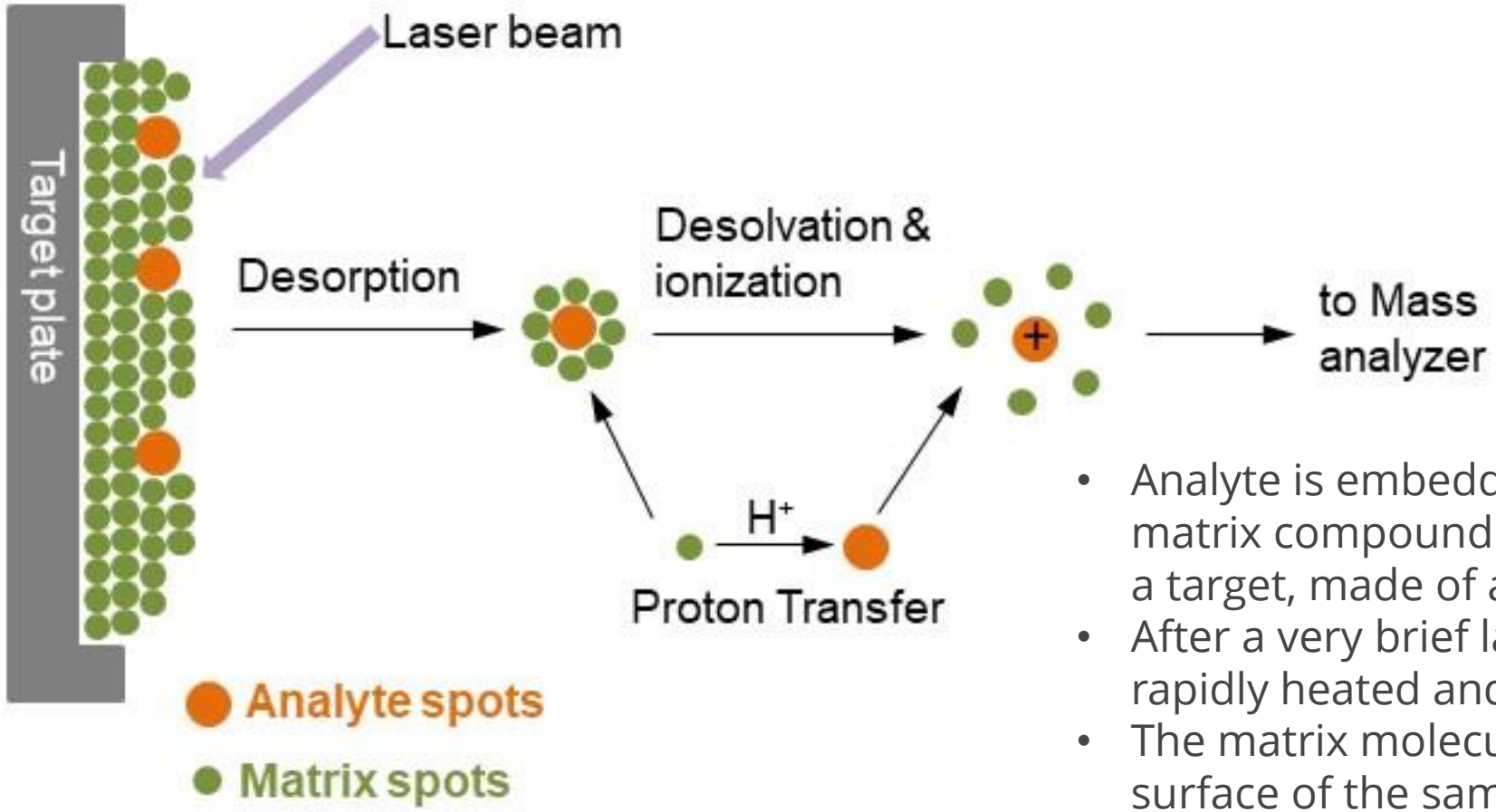
Osnovni princip



https://www.creative-proteomics.com/technology/maldi-tof-mass-spectrometry.htm

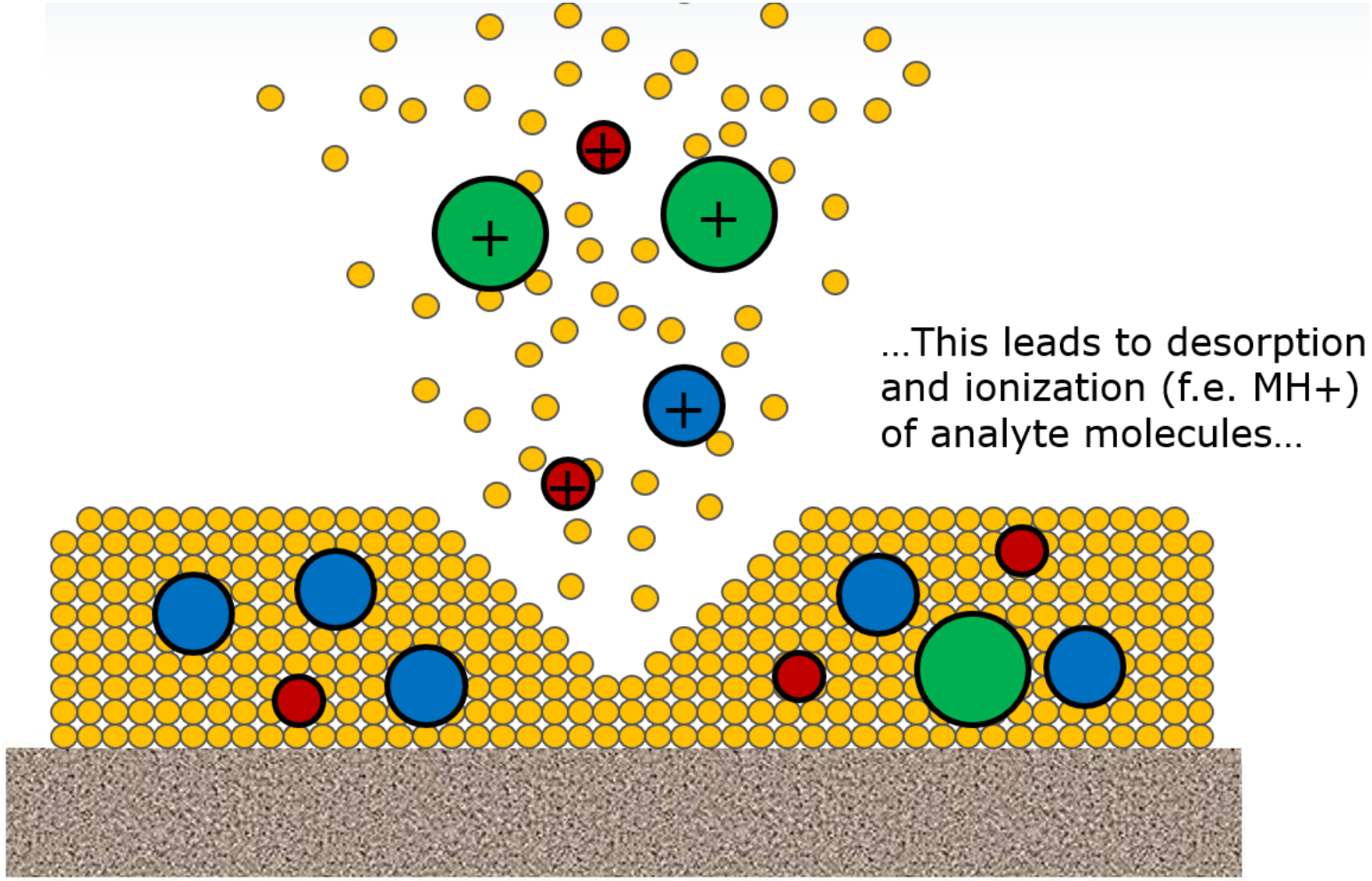
MALDI TOF-MS relies on the short laser pulse (typically 1-5 ns, depending on the laser) to produce discrete ion packets in the ion source, which are then continuously extracted from the ion source by the application of a large static electric potential (25-30 kv).

MALDI – uloga matrice



- Analyte is embedded in a very large excess of a matrix compound deposited on a solid surface called a target, made of a conducting metal.
- After a very brief laser pulse, the irradiated spot is rapidly heated and becomes vibrationally excited.
- The matrix molecules energetically ablated from the surface of the sample, absorb the laser energy and carry the analyte molecules into the gas phase.
- During the ablation process, the analyte molecules are usually ionized by being protonated or deprotonated with the nearby matrix molecules.

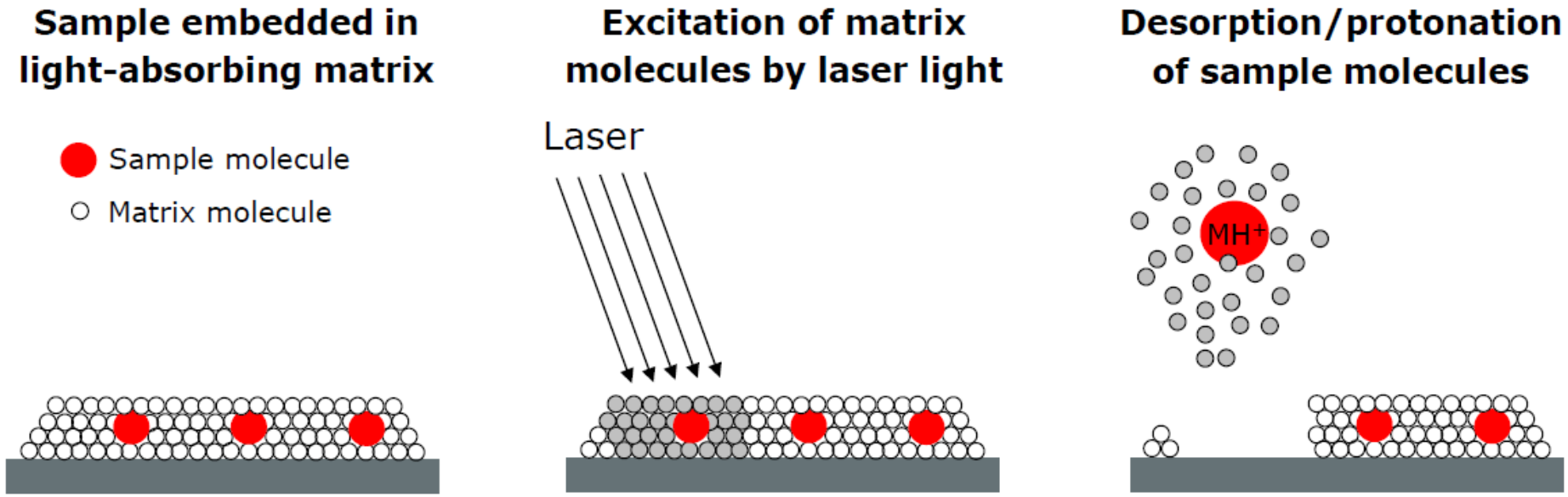
Kako MALDI radi?



MALDI matrice:

Peptides:	4-Hydroxy-α-cyanocinnamic acid (**HCCA**)
Proteins:	2,5-Dihydroxyacetophenone (**DHAP**) Sinapinic acid (**SA**) 2,5-Dihydroxybenzoic acid (**DHB**)
Glycans:	2,5-Dihydroxybenzoic acid (**DHB**)
Nucleic acids:	3-Hydroxypicolinic acid (**HPA**) 2,4,6-Trihydroxyacetophenone (**THAP**)

MALDI- Pozitivan jonski mod

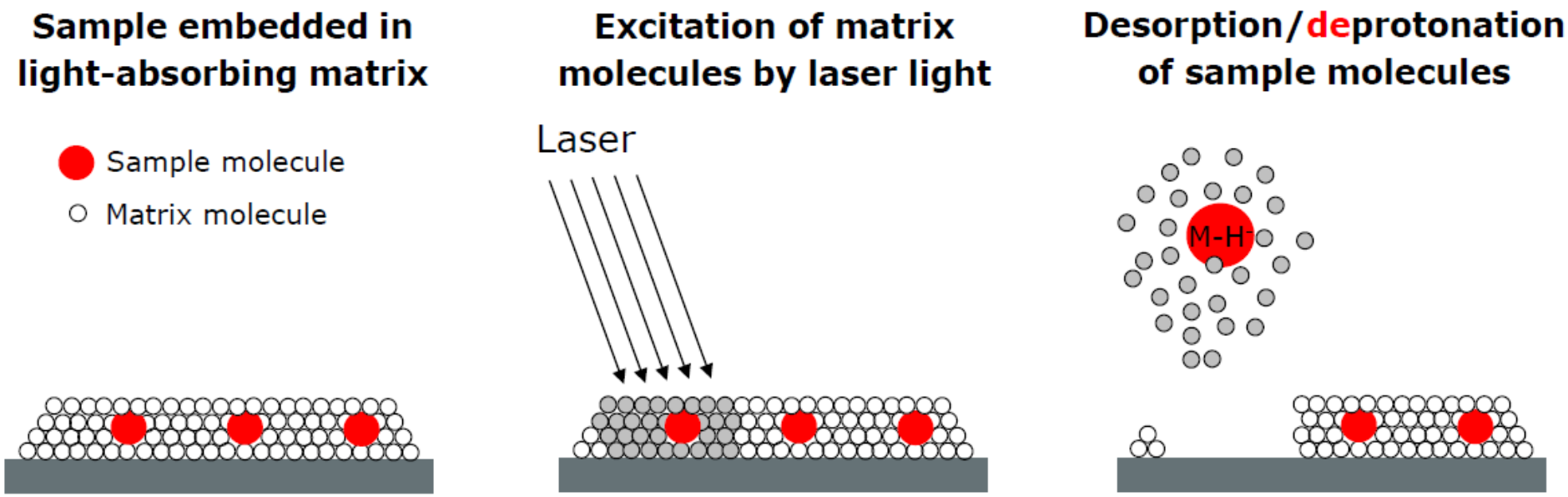


The matrix transfers the energy needed for ionization from the laser light to the sample molecules.

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

Formation of alternative adducts depends on the presence of respective cations (either being ubiquitary present or actively added – depending on type of sample):
$[M+Na]^+$; $[M+K]^+$; $[M+Cu]^+$; $[M+Li]^+$; $[M+Ag]^+$

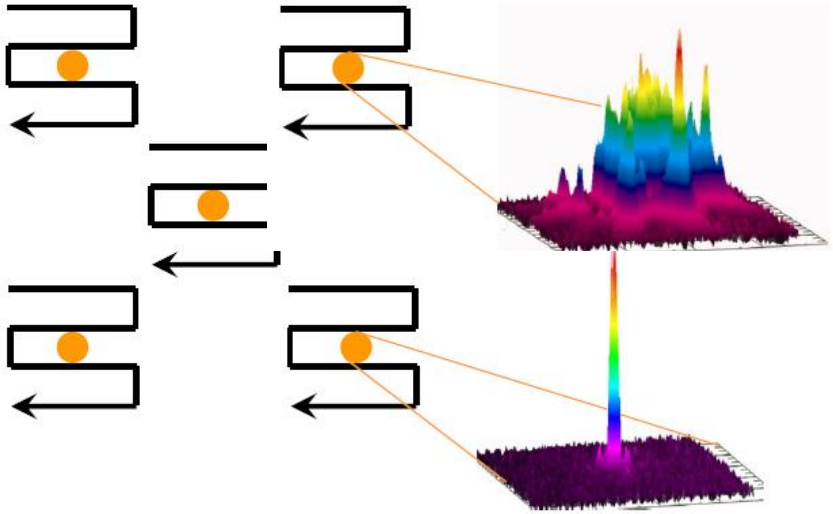
MALDI- Negativan jonski mod

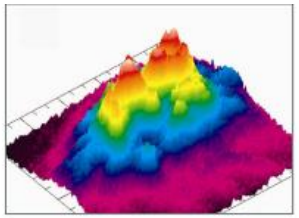


The matrix transfers the energy needed for ionization from the laser light to the sample molecules.

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

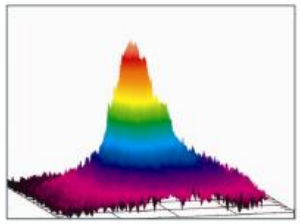
MALDI- laseri



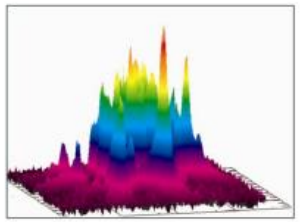


Nitrogen laser:
pro: well structured energy profile
contra: slow (maximum 50Hz)

N_2: 337 nm
YAG 335 nm



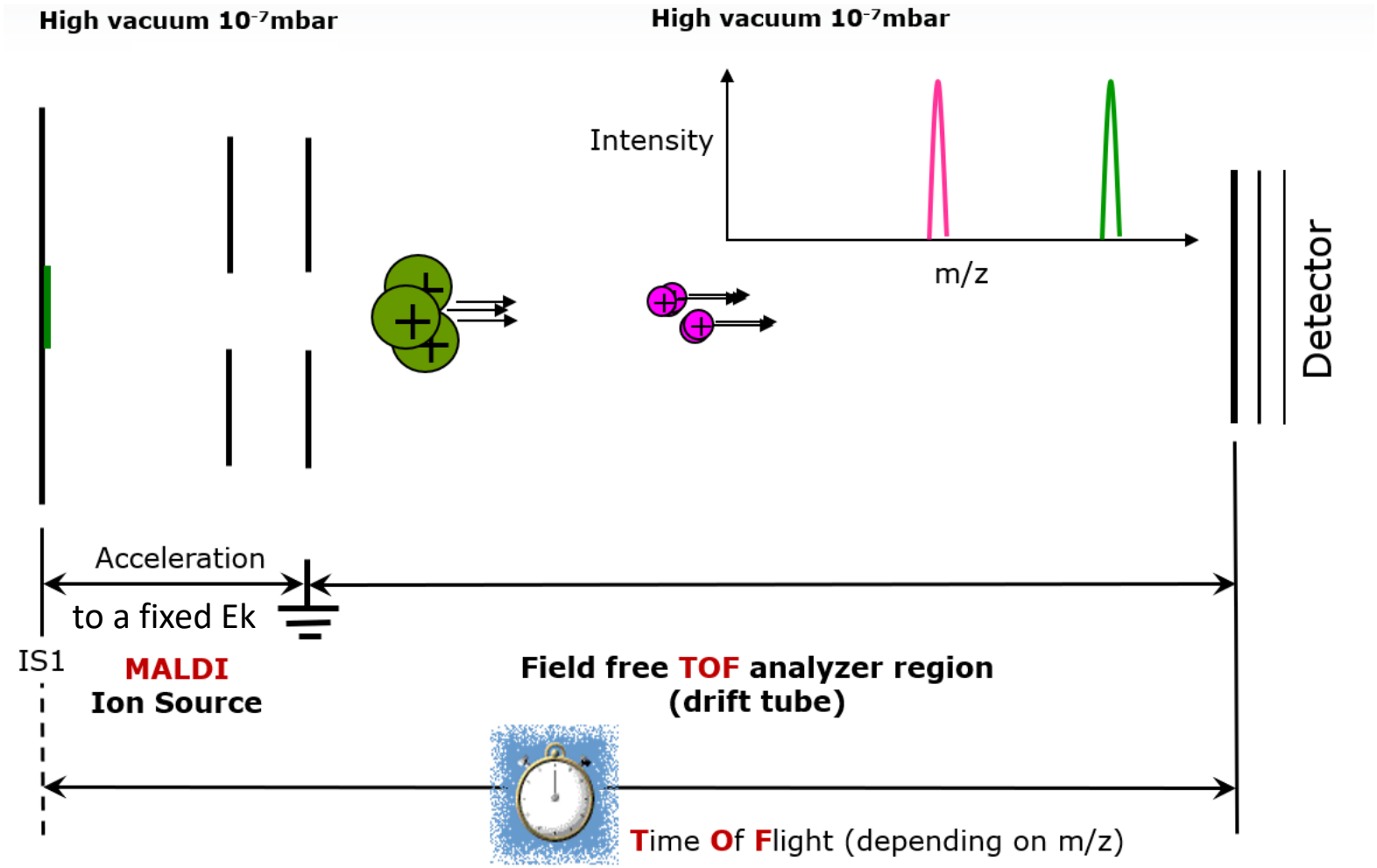
Nd:YAG laser:
pro: fast (up to 1000Hz)
contra: Gaussian energy profile (non-structured)



Smartbeam/Smartbeam II (modified Nd:YAG laser):
pro: fast (up to 1000Hz)
pro: well structured energy profile

Modified Nd:YAG laser, wavelength 355nm

MALDI ToF – Principi rada



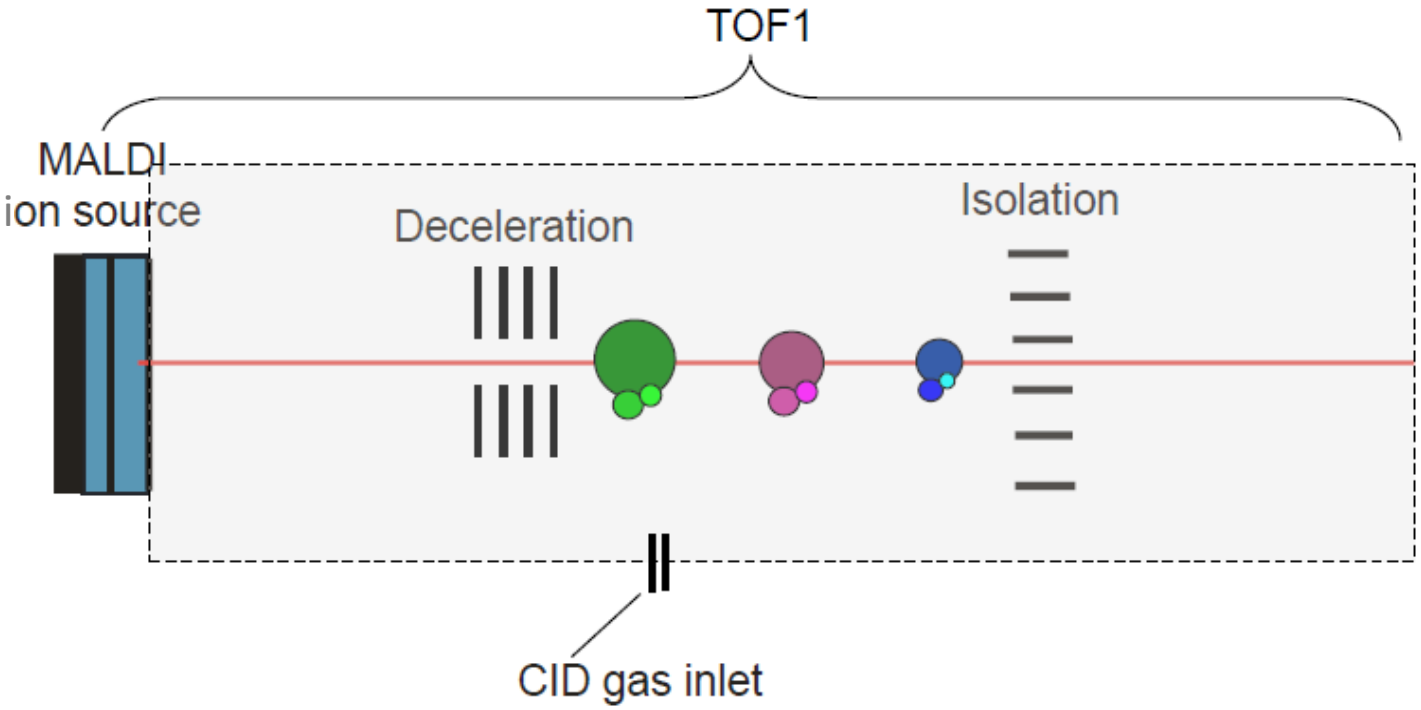
MALDI ToF – Principi rada

Analysis of a mixture containing 3 compounds (**green**,**red**, **blue**) being different in mass:

Molecular ions are being generated by means of MALDI ionization.

MALDI ToF – Principi rada

Analysis of a mixture containing 3 compounds (**green**,**red**, **blue**) being different in mass:



When performing CID experiments, the TOF1 stage is flushed with collision gas. This induces additional high-energy collision induced fragmentation (heCID).

Most important:

Fragment ions continue to travel at the same velocity as there precursors did.

LID vs. CID

LID: Laser-Induced Dissociation

Most straightforward way to peptide backbone fragmentation (b,y-type ions).
Used for protein identification by means of peptide sequencing.

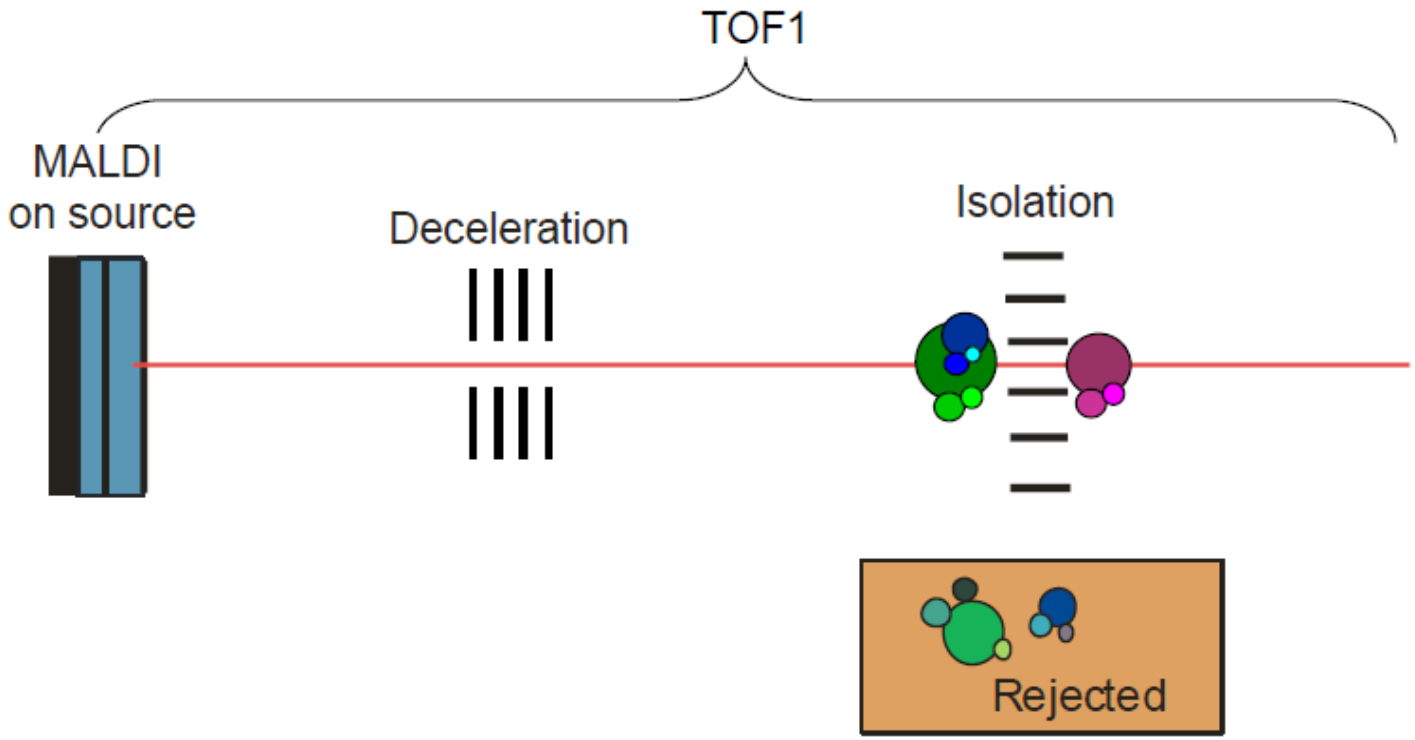
CID: Collision-Induced Dissociation (high energy)

Additional side chain cleavages in peptides. Higher relative intensity of internal fragments. Overall shift of average fragment size towards lower mass.
Used as an option in special applications, e.g.:

- de novo sequencing of peptides (enhanced immonium ions)
- differentiation of isobaric amino acids L and I in peptides by respective side chain cleavages
- detailed glycan analysis (cross ring cleavages occurring in heCID allow for linkage analysis)

MALDI ToF – Principi rada

Analysis of a mixture containing 3 compounds (**green**,**red**, **blue**) being different in mass:



Selecting the **red** colored precursor for fragment analysis

The isolation device allows for the selection of a specific group of fragment/molecular ions for further m/z separation and analysis.

MALDI-Time of Flight: Razdvajanje

$$E_{pot} = zeU$$

This defines the potential energy at which all ions start from the MALDI target.

$$E_{kin} = 1/2mv^2$$

This equation defines the kinetic energy of ions after acceleration into the flight tube.

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

In the process of ion acceleration, energy is preserved but turns from potential into kinetic energy.

$$v = \sqrt{2zeU/m}$$

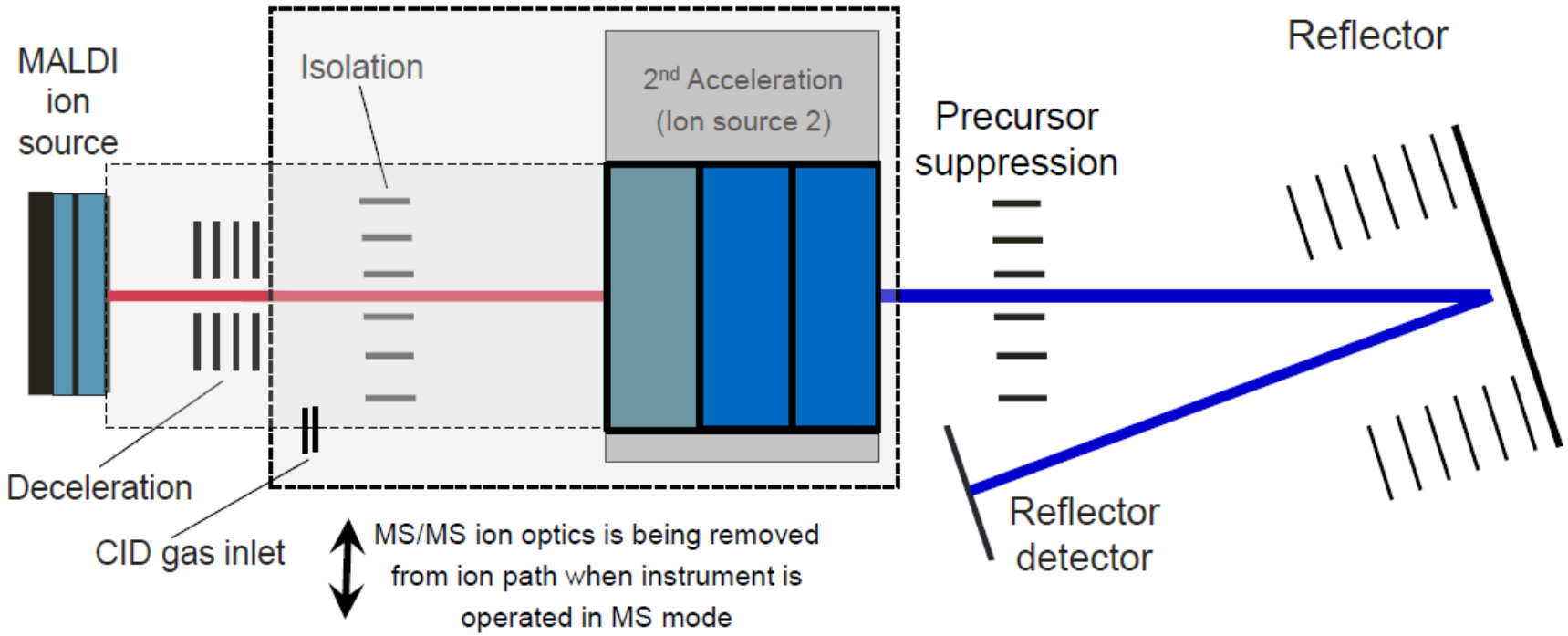
Transforming this equation shows the dependency of velocity of moving ions on their m/z value.

$$t = L\sqrt{m/2zeU}$$

With the linear velocity v being defined as $L_{drift\ tube}/t_{flight}$, the dependency of the ions´ flight time t_{flight} from m/z becomes obvious.

Principi ToF/ToF analize

Principal scheme: MS/MS operation mode

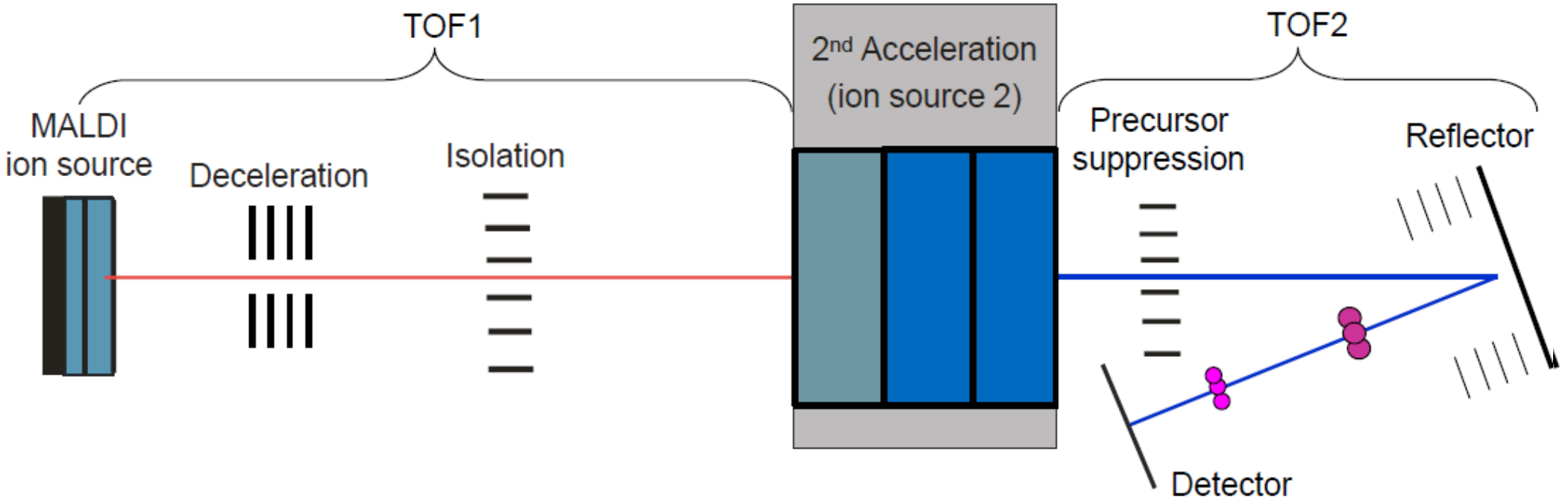


Ion path in TOF1 region (linear TOF)
Ion path in TOF2 region (reflector TOF)

Deceleration = removal of early metastables
Isolation = Timed ion gate
Ion source 2 = second acceleration cell
Precursor suppression = Timed ion gate for suppression of non-fragmented precursor ions

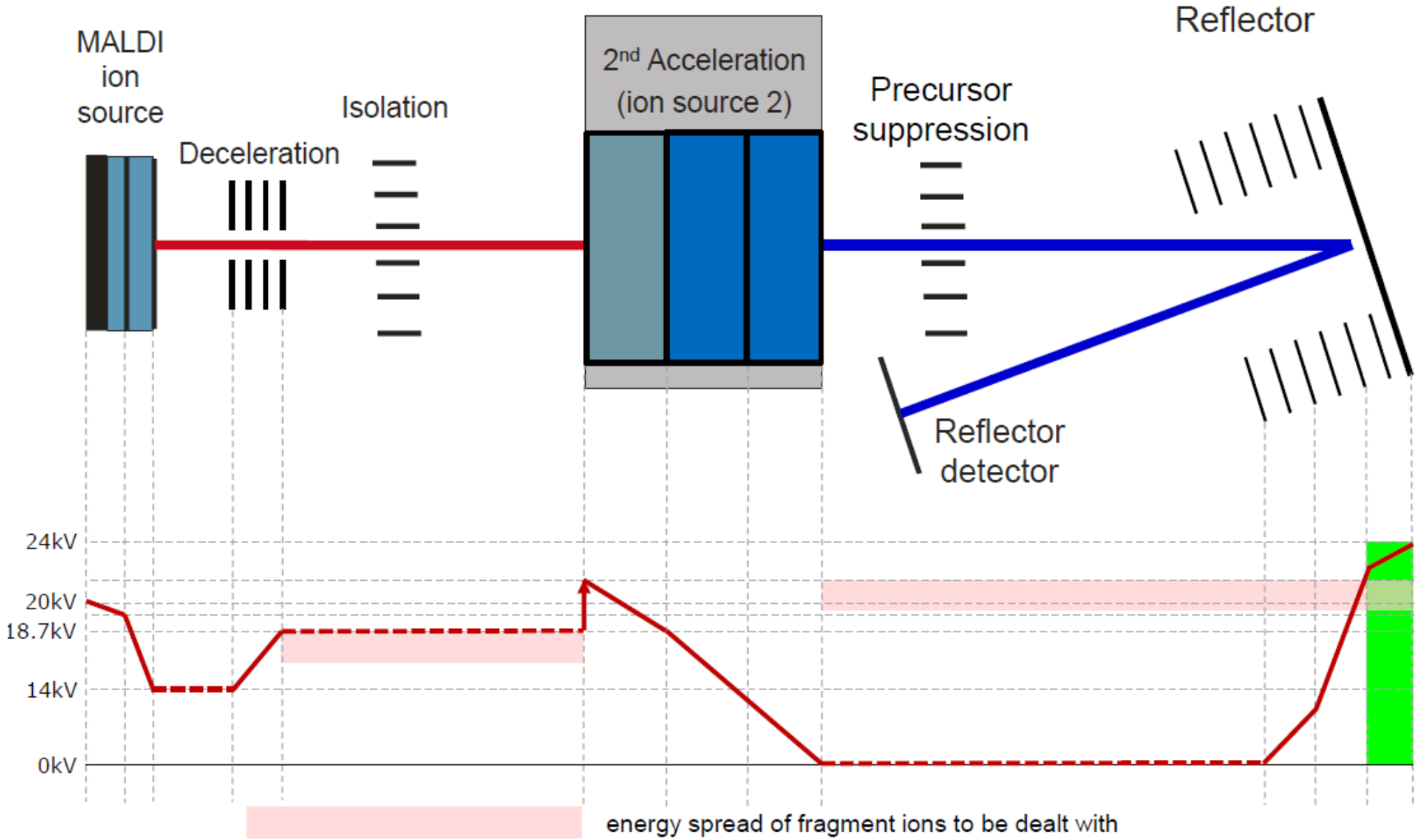
Principi ToF/ToF analize

Analysis of a mixture containing 3 compounds (**green**,**red**, **blue**) being different in mass:

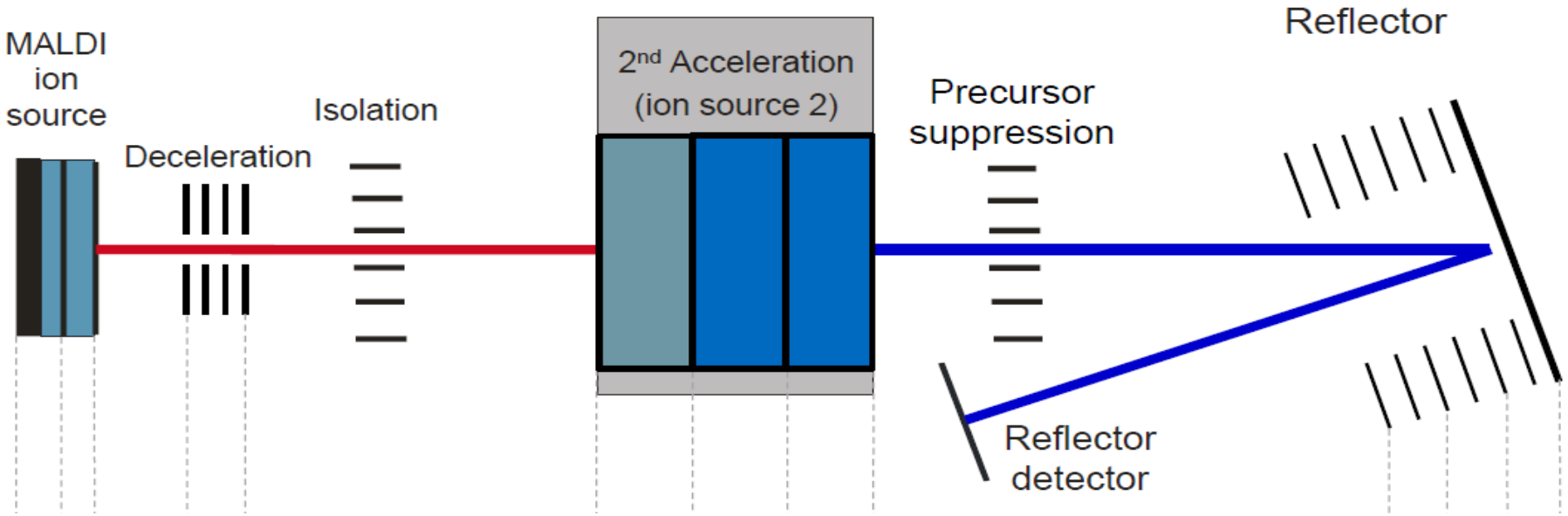


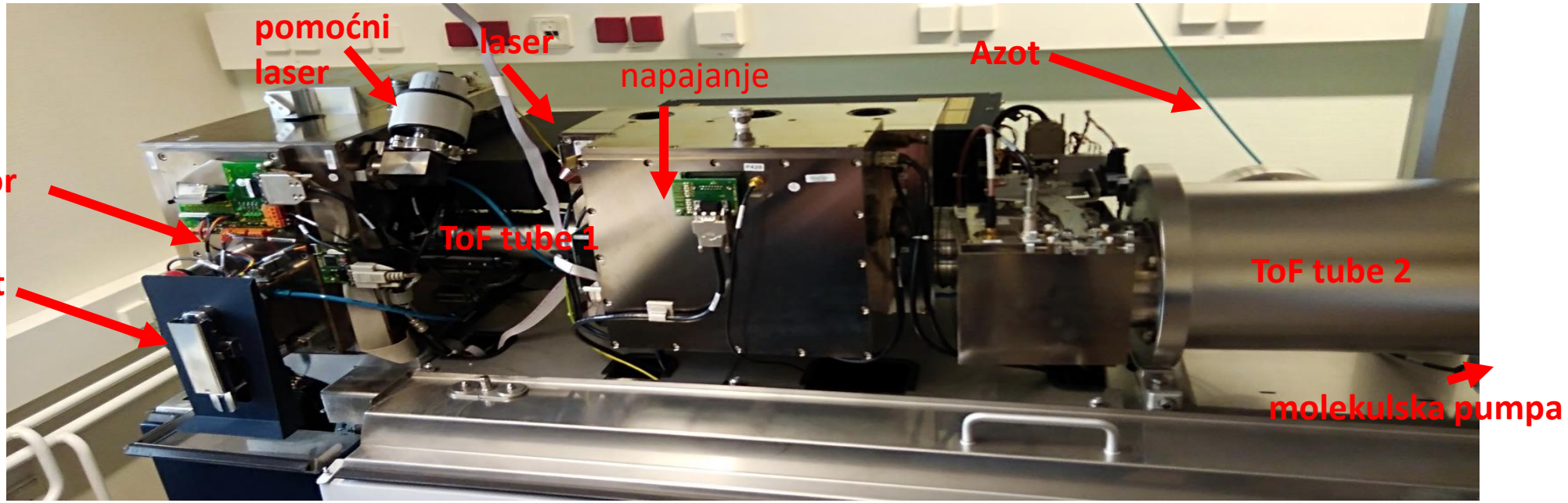
Precursor suppression prevents remaining intact molecular ions from passing on in TOF2, where they would otherwise undergo non-desired metastable decay again, which would yield wrongly calibrated, badly resolved fragment peaks.

Principi ToF/ToF analize



Principi ToF/ToF analize



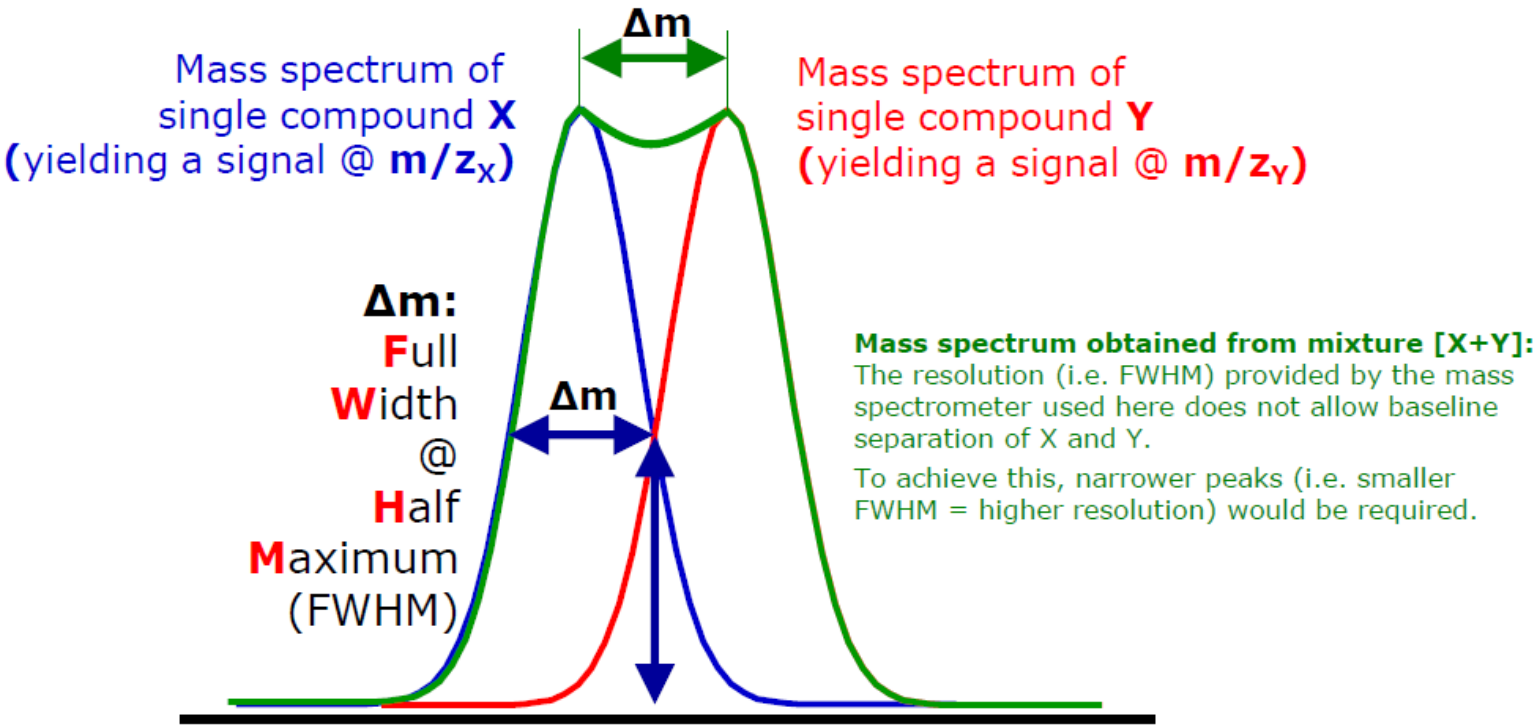


Rezolucija u MALDI-ToF analizi

Resolution R defines, how well two peaks are separated from each other:

$$R = m/\Delta m$$

Δm is usually derived from a mass peak´s full width at half maximum (FWHM), as shown below for the exemplaric analysis of a mixture of two compounds X and Y:



Rezolucija u MALDI-ToF analizi

$[M+H]^+$

m/z:

1046 Da

1047 Da

1048 Da

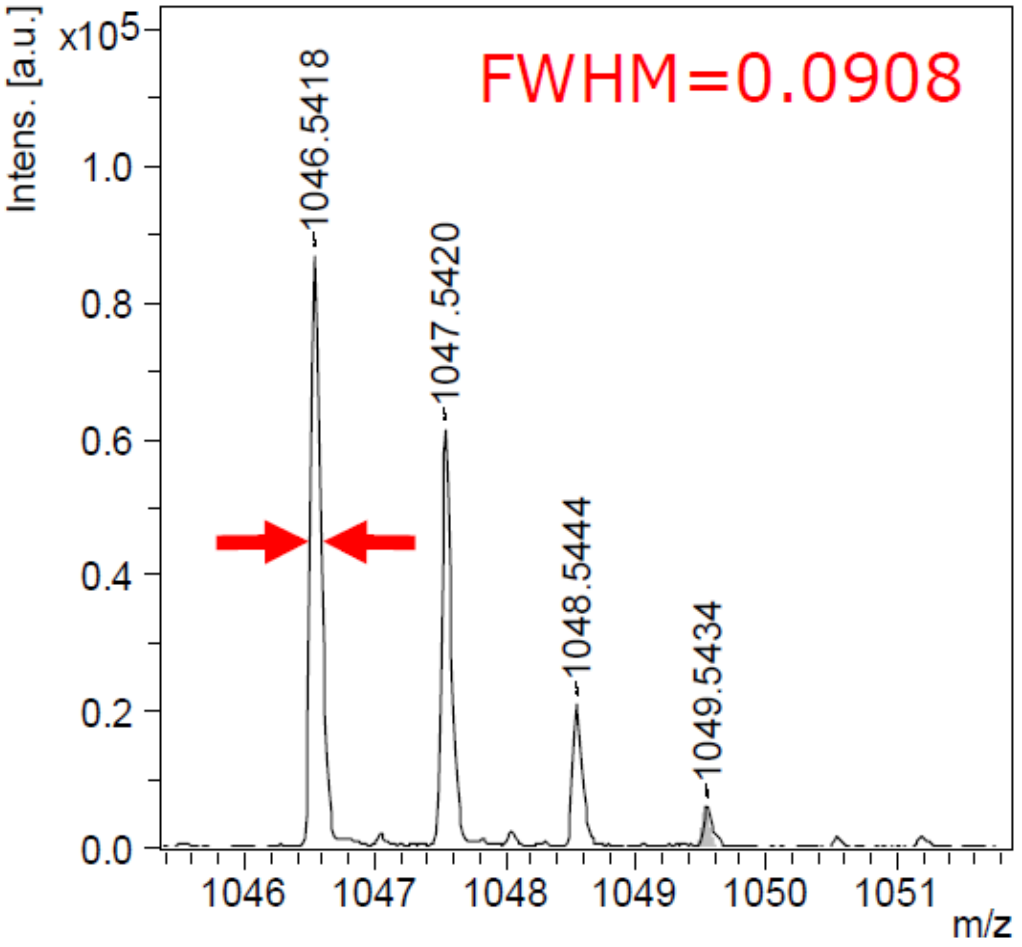
1049 Da

Resolution

= **m/Δm**

= 1046.5418/0.0908

= **11525**

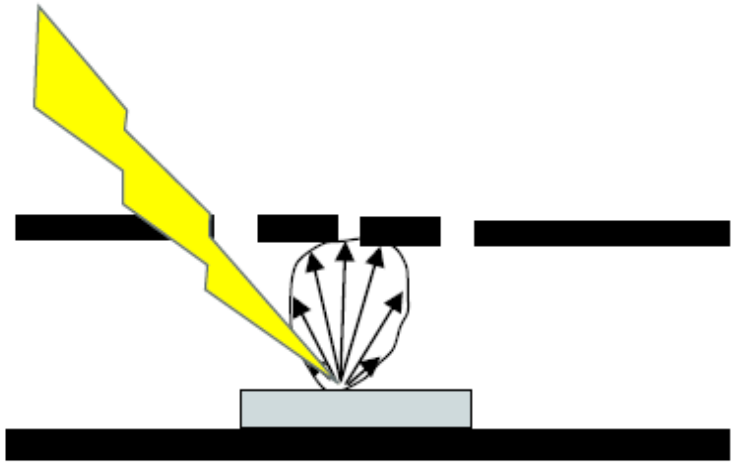


Rezolucija u MALDI-ToF analizi

Rezolucija je ograničena prostornim i energetskim širenjem

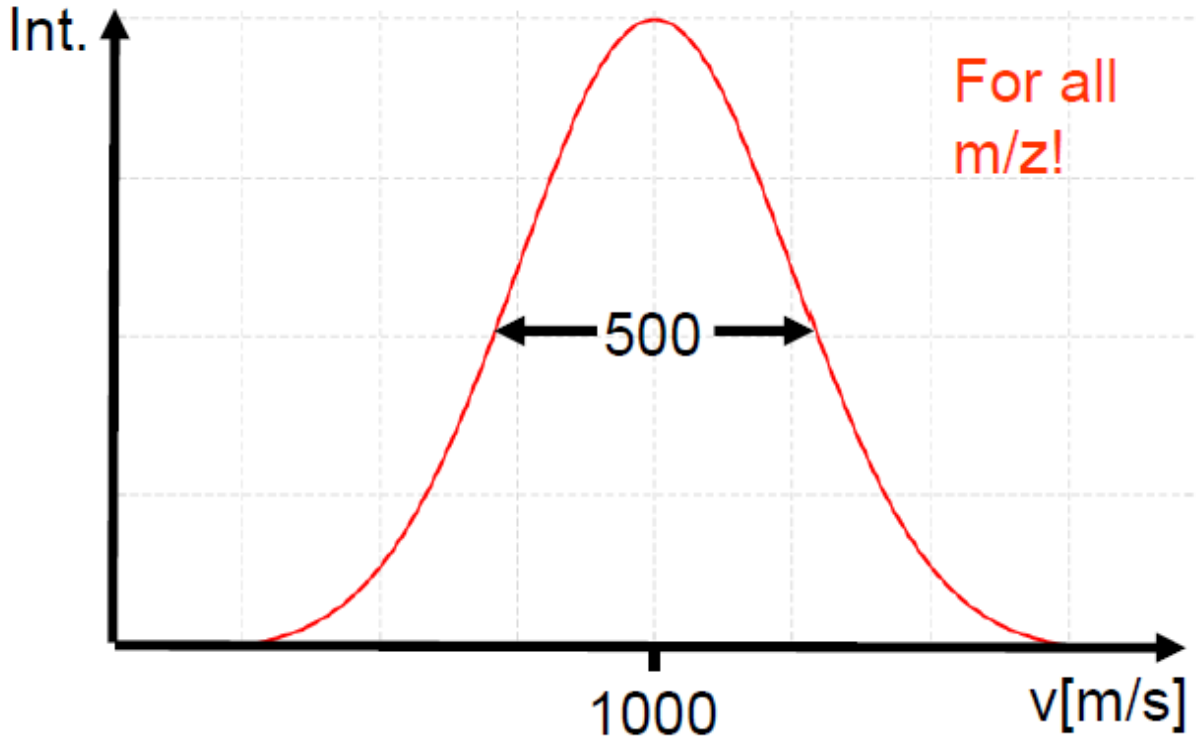
Spatial spread:

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



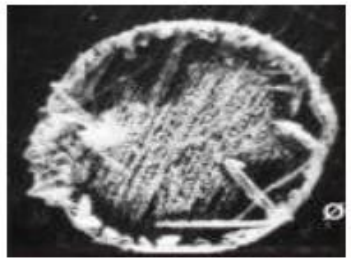
Initial energy (=speed) spread:

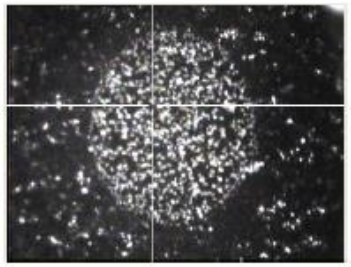
- heterogeneous secondary reactions (ion-ion; ion-neutral)

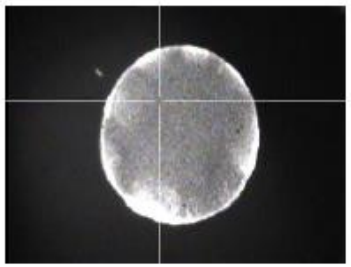


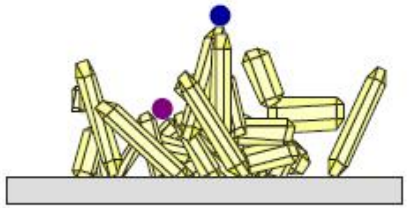
Rezolucija u MALDI-ToF analizi

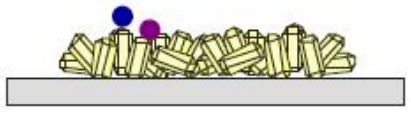
Homogenost kristala matrice

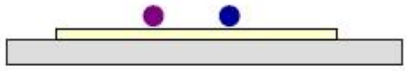


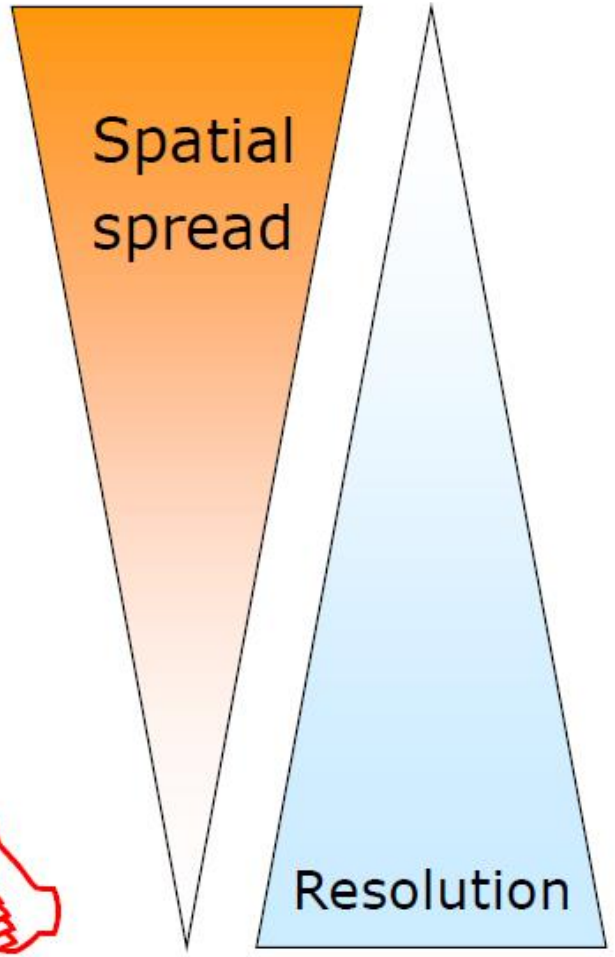






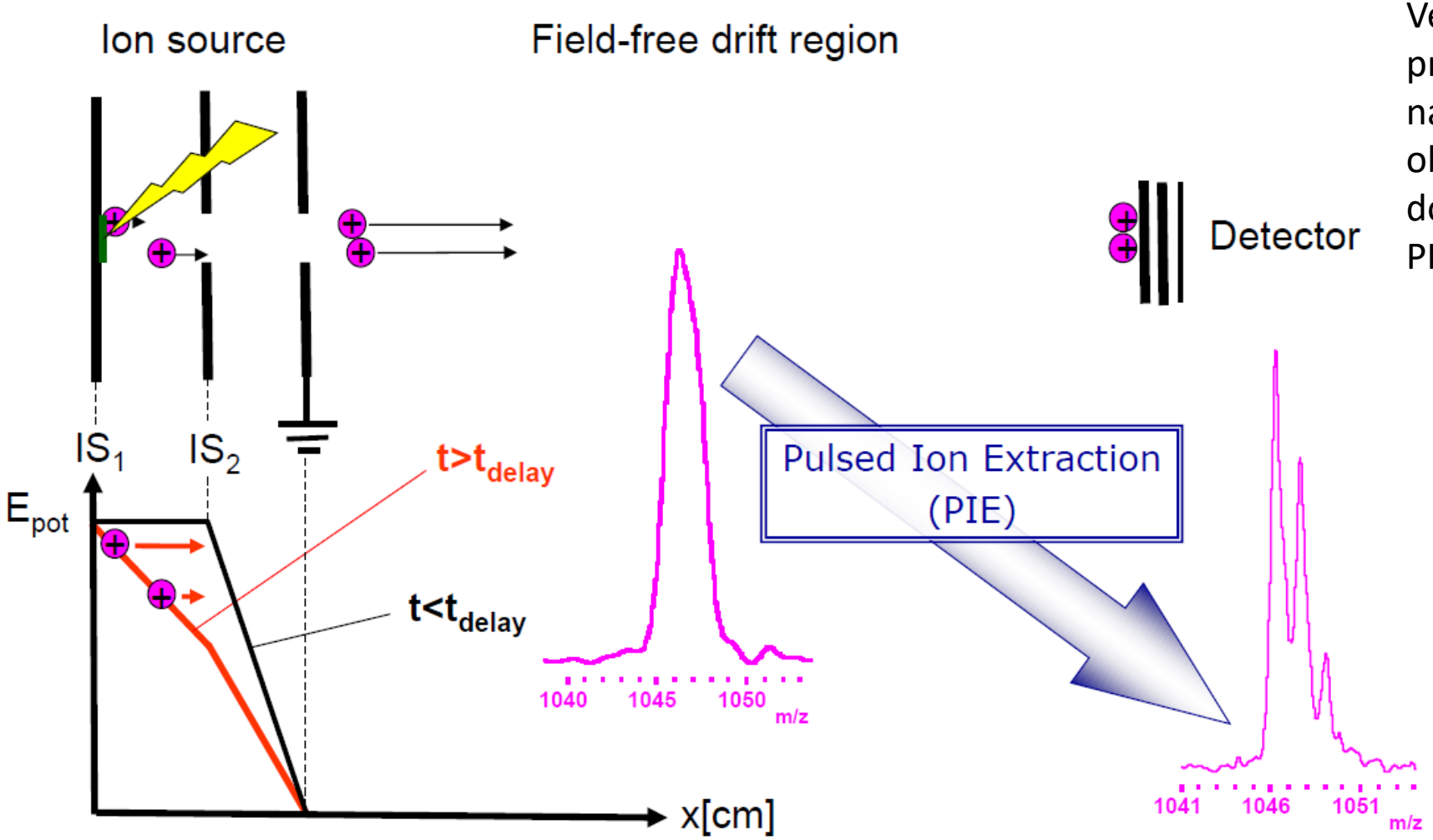






Rezolucija u MALDI-ToF analizi

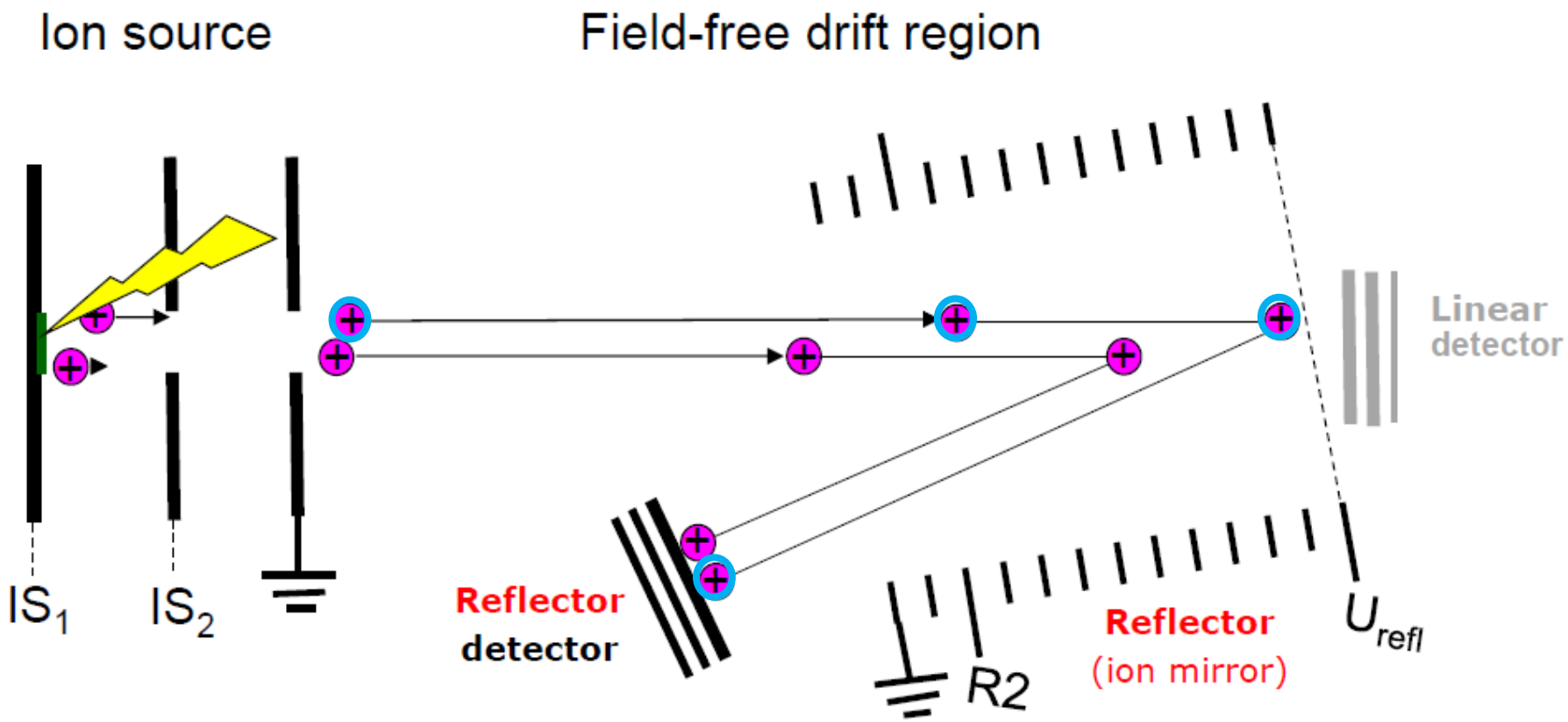
Pulsed ion Extraction for efficient ion focusing in the MALDI ion source



Veći napon / potencijalna E se primenjuje na sporiji jon kako bi se na IS2 –kapija 2 približili i na kraju oba jona sa istim m/z stižu zajedno do detektora.
PIE i IS2 se optimizuju za svaki jon

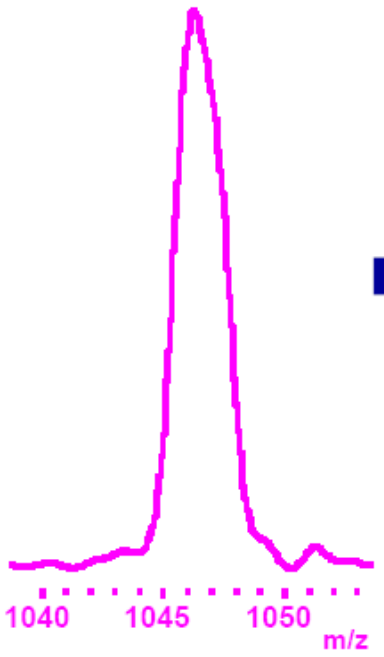
Rezolucija u MALDI-ToF analizi

- Further ion focusing by means of a **reflector** TOF setup

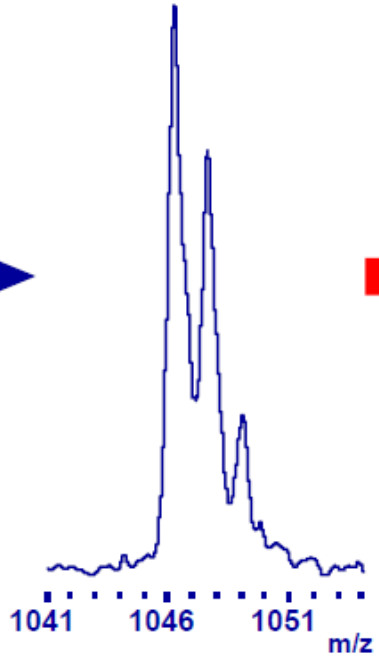


Rezolucija u MALDI-ToF analizi

MALDI-TOF,
linear

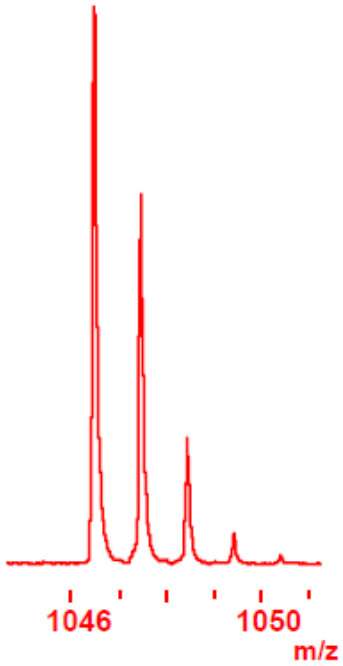


MALDI-TOF,
linear + PIE



PIE compensates
- initial energy spread
- initial spatial spread

MALDI-TOF,
reflector + PIE



Reflector compensates
- remaining energy spread

Linearni vs. reflektor mod

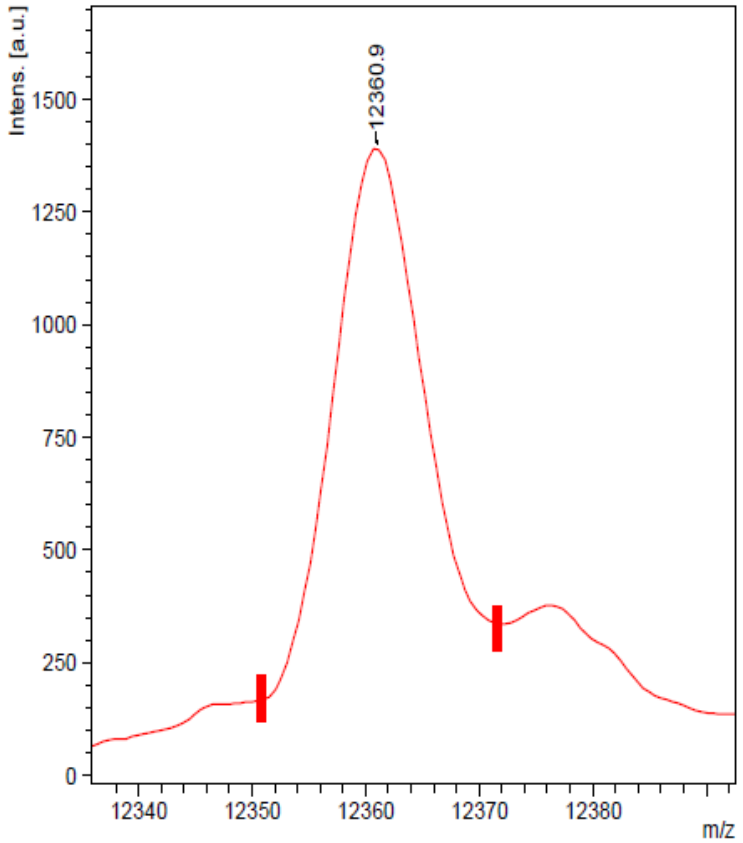
Ako reflektor mod daje mnogo bolju rezoluciju, zašto koristiti linearni uopšte ?

- Pojedini molekulski joni formirani u MALDI izvoru nisu stabilni i tokom prolaska kroz *field-free* region fragmentišu. To se posebno odnosi na velike molekule, npr proteine koji prolaze kroz prirodan gubitak H_2O, NH_3, CO_2 čime postaju slabije pokretni što utiče na senzitivnost i rezoluciju ako se analizira u refraktor modu.
- Linearne metode imaju manju masenu rezoluciju(nisu razdvojeni izotopski pikovi, ali mogu da omoguće m/Δm FZ 500 ili manje, zavismo od m/z vrste), ali veću osetljivost i pogodni su za jone većih masa (>200 000 Da).
- Metode u reflektor modu omogućavaju najbolje masene rezolucije (razdvojeni su i izotopski pikovi) ali se preporučuju za m/z < 6000.
- Reflektor mod nije preporučljiv za analite koji ne mogu dugo da prežive u električnom polju.

Linearni vs. reflektor mod

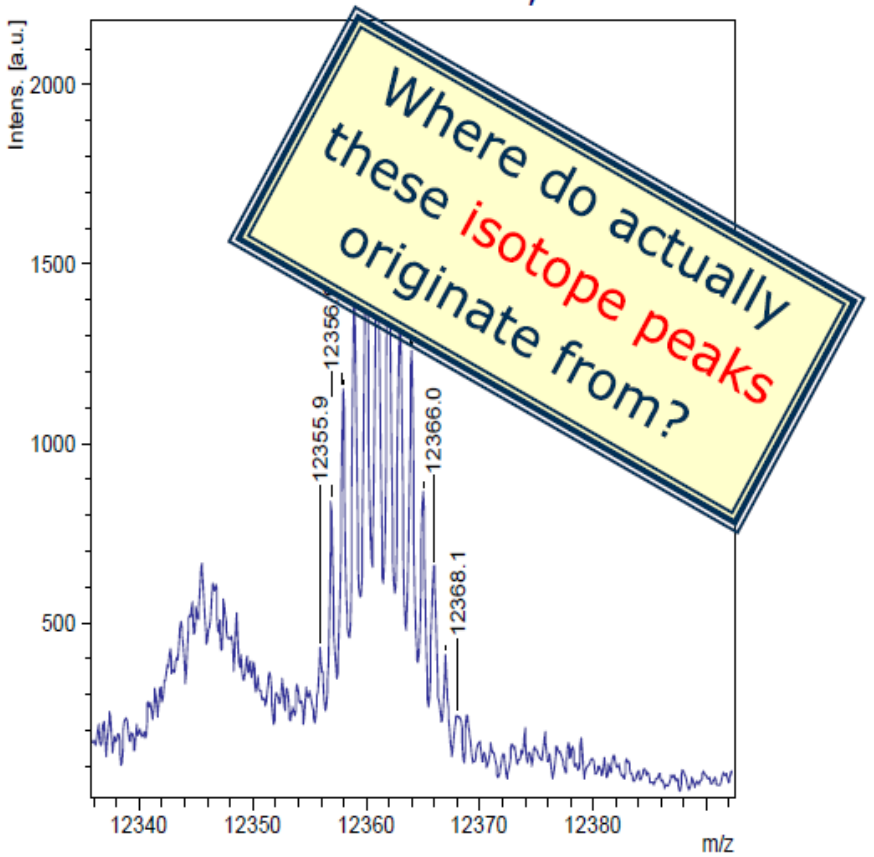
cyt c

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

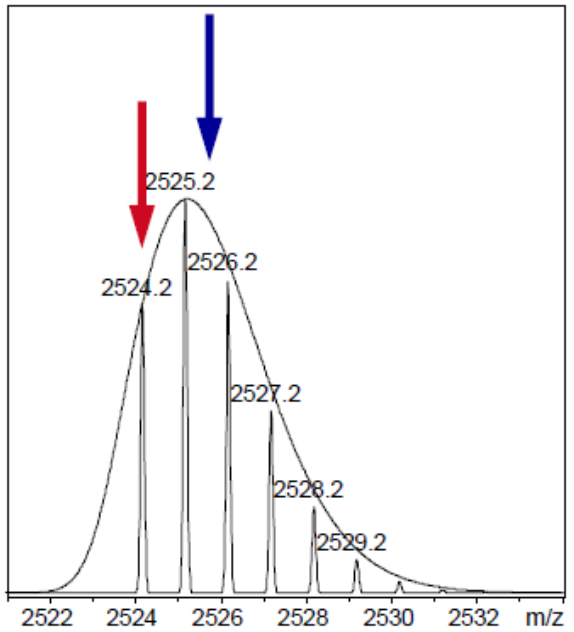


Spectrum shows all the isotope peaks well separated from each other.

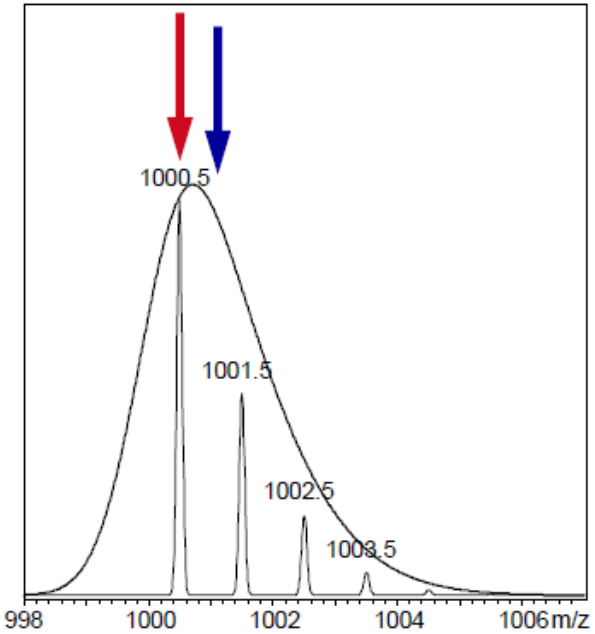
MALDI-ToF: Izotopni pikovi

Isotope	Mass	[%] Abundance
1-H	1.007825	99.985
2-H (Deuterium)	2.014000	0.015
12-C	12.00000	98.90
13-C	13.00336	1.10
14-N	14.00307	99.63
15-N	15.00011	0.37
16-O	15.99491	99.76
18-O	17.99916	0.20
19-F	18.99840	100
23-Na	22.98977	100
31-P	30.97376	100
32-S	31.97207	95.03
34-S	33.96787	4.22
35-Cl	34.96885	76.77
37-Cl	36.96590	31.98
39-K	38.96371	93.26
79-Br	78.91834	50.69
81-Br	80.91629	49.31

Element composition: $C_{112}H_{164}N_{29}O_{34}S_2$
Monoisotopic mass $[M+H]^+$: 2524.1510
Average mass $[M+H]^+$: 2525.8196



Element composition: $C_{41}H_{69}N_{13}O_{14}S$
Monoisotopic mass $[M+H]^+$: 1000.4880
Average mass $[M+H]^+$: 1001.1409



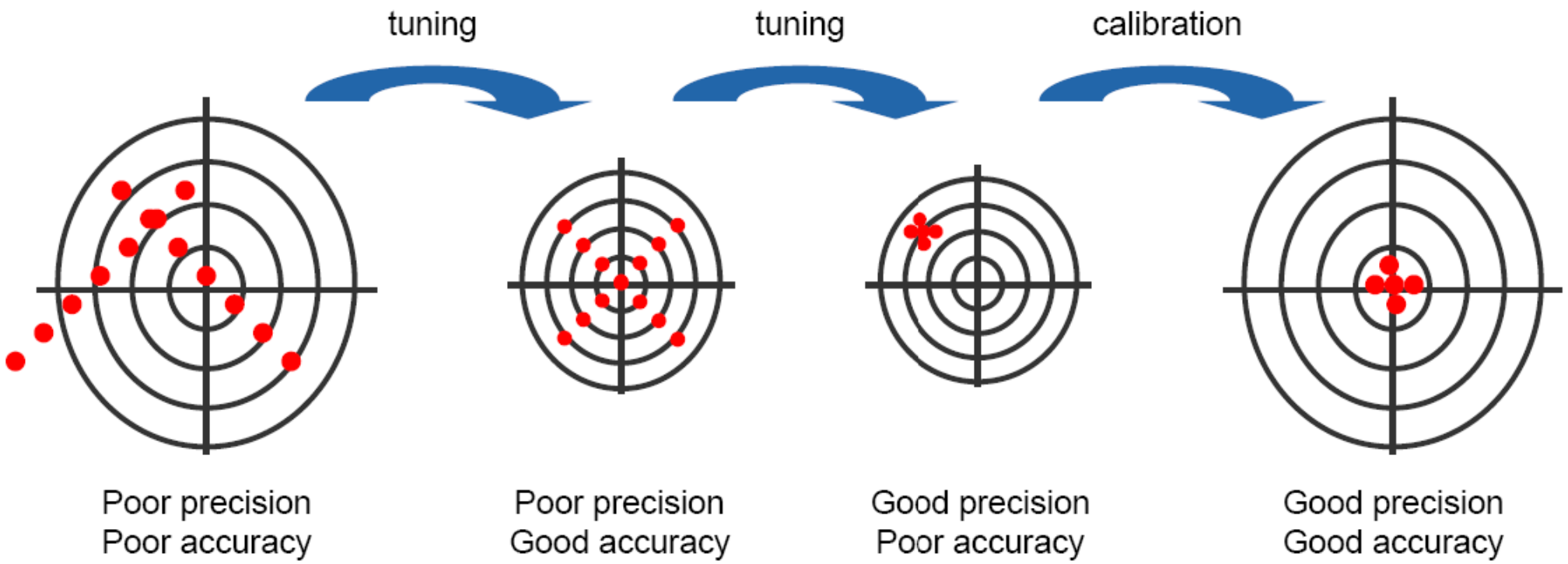
The **monoisotopic mass** is the sum of the masses of all the atoms present in a molecule using the mass of the most abundant isotope for each element.
The **average mass** of a molecule is the sum of elemental masses using the average weighted over all stable isotopes of each element contained in the molecule.

Elements that are found in nature in form of only one single isotope, are called monoisotopic elements.

MALDI-ToF: Kalibracija

Precision: Variation of values obtained from repetitive measurements performed under identical conditions (*random error*)

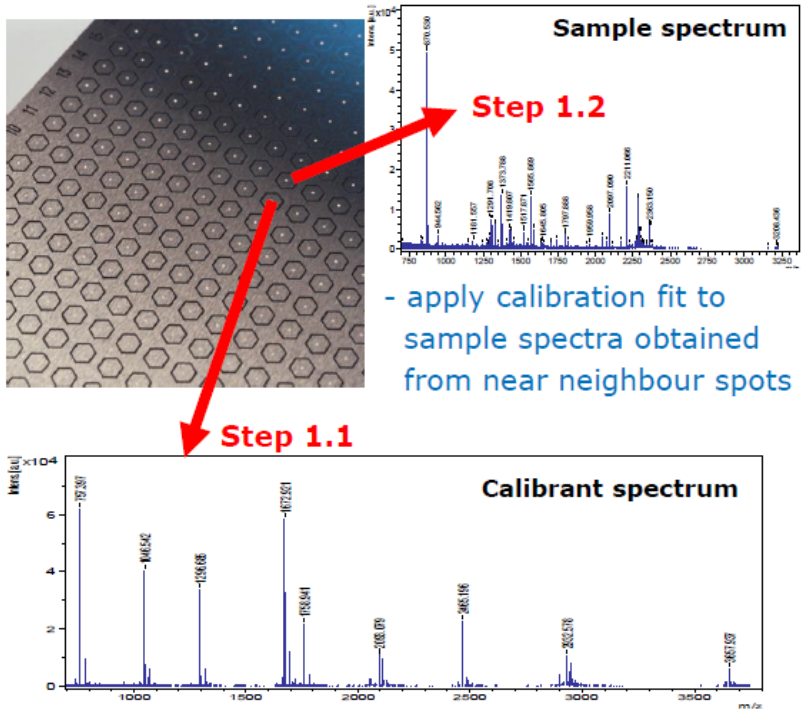
Accuracy: Deviation of a measured value from the reference value (*systematic error*)



MALDI-ToF: Kalibracija

MALDI-TOF: Calibration strategies

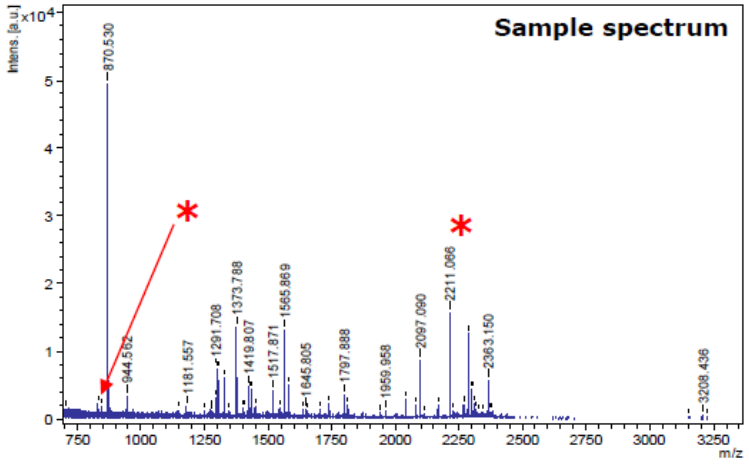
Step 1) External calibration



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

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

Step 2) Internal re-calibration (optionally)



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

Internal re-calibration allows for

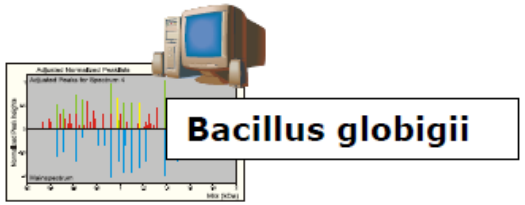
- optimum mass accuracy due to compensation of spot-to-spot heterogeneities that typically cause mass errors after external calibration

MALDI-ToF: Primena

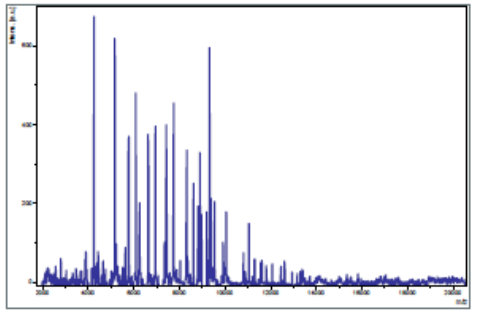
MALDI-TOFMS profiling of microorganisms:

- linear MALDI-TOF
- mass range: 2000 ... 20000Da

Identified species

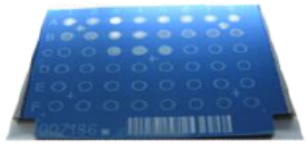


Fingerprint search against proprietary spectra library



Generate MALDI-TOF profile spectrum

Mix with MALDI matrix (HCCA), prepare onto a MALDI target plate



Select a colony

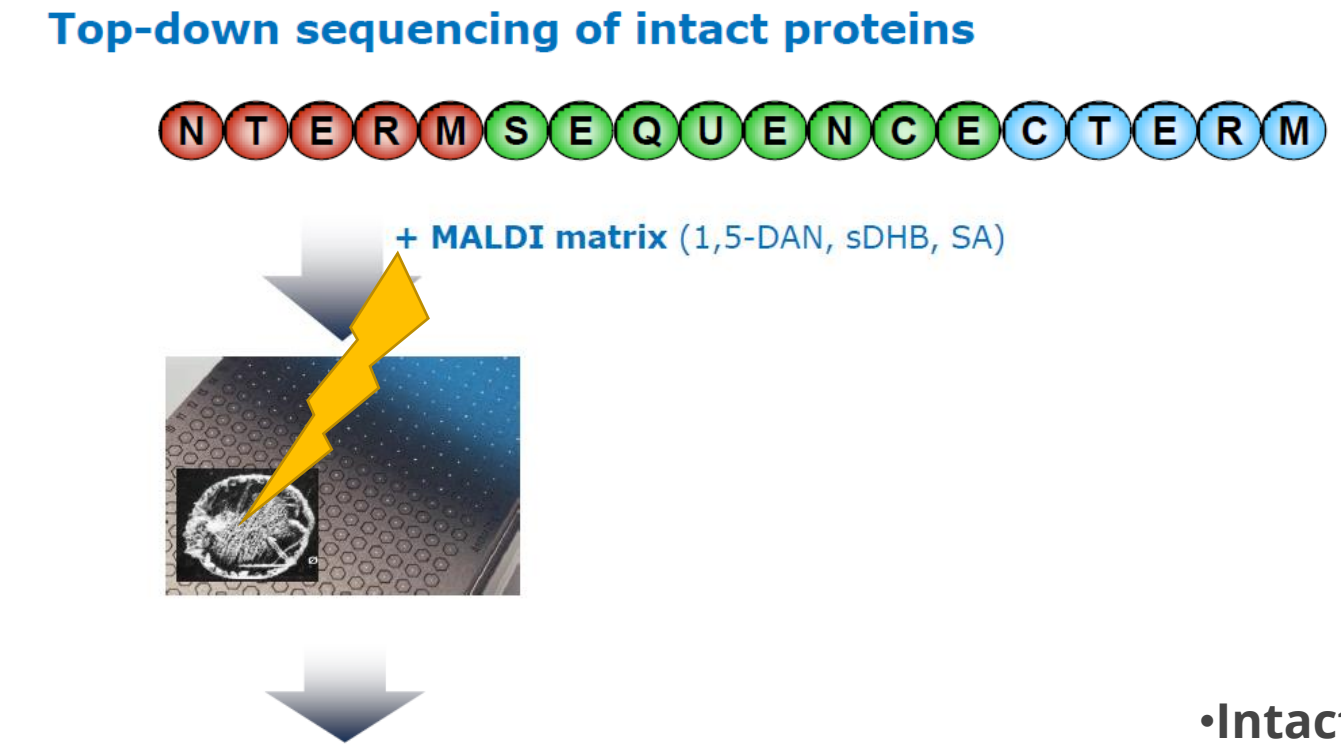
Unknown microrganism

?

- **Oligonucleotides analysis**

Primena MALDI ToF/ToF

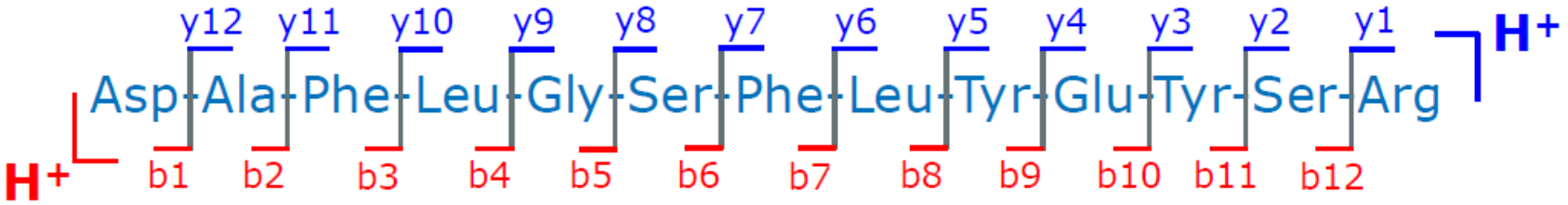
MALDI Fragmentation of peptides:



- Intact Mass determination
- Peptide mass fingerprinting (PMF)
- Post source decay (PSD) MALDI-TOF analysis

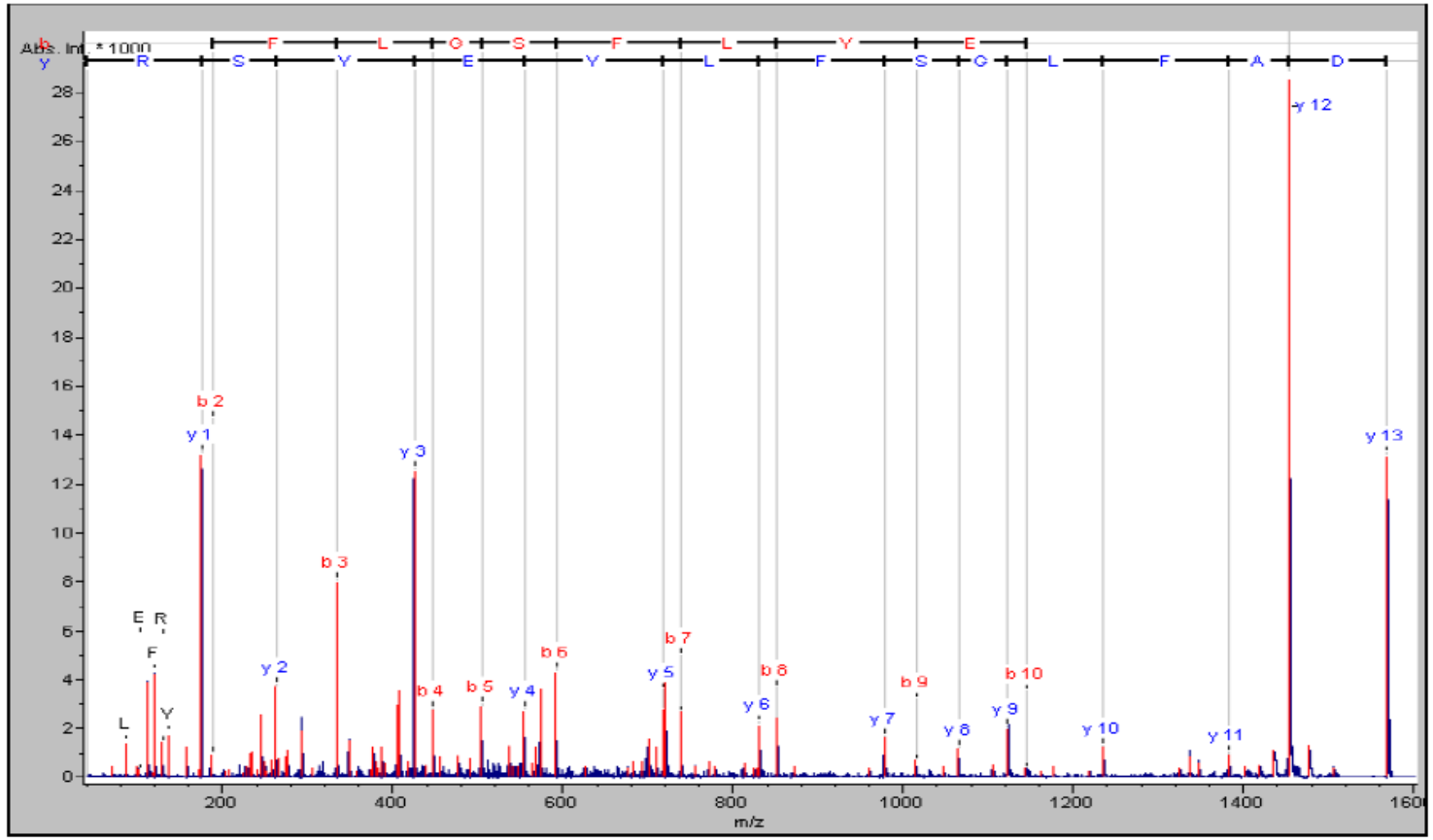
Primena MALDI ToF/ToF

Example MS/MS spectrum obtained from a peptide:



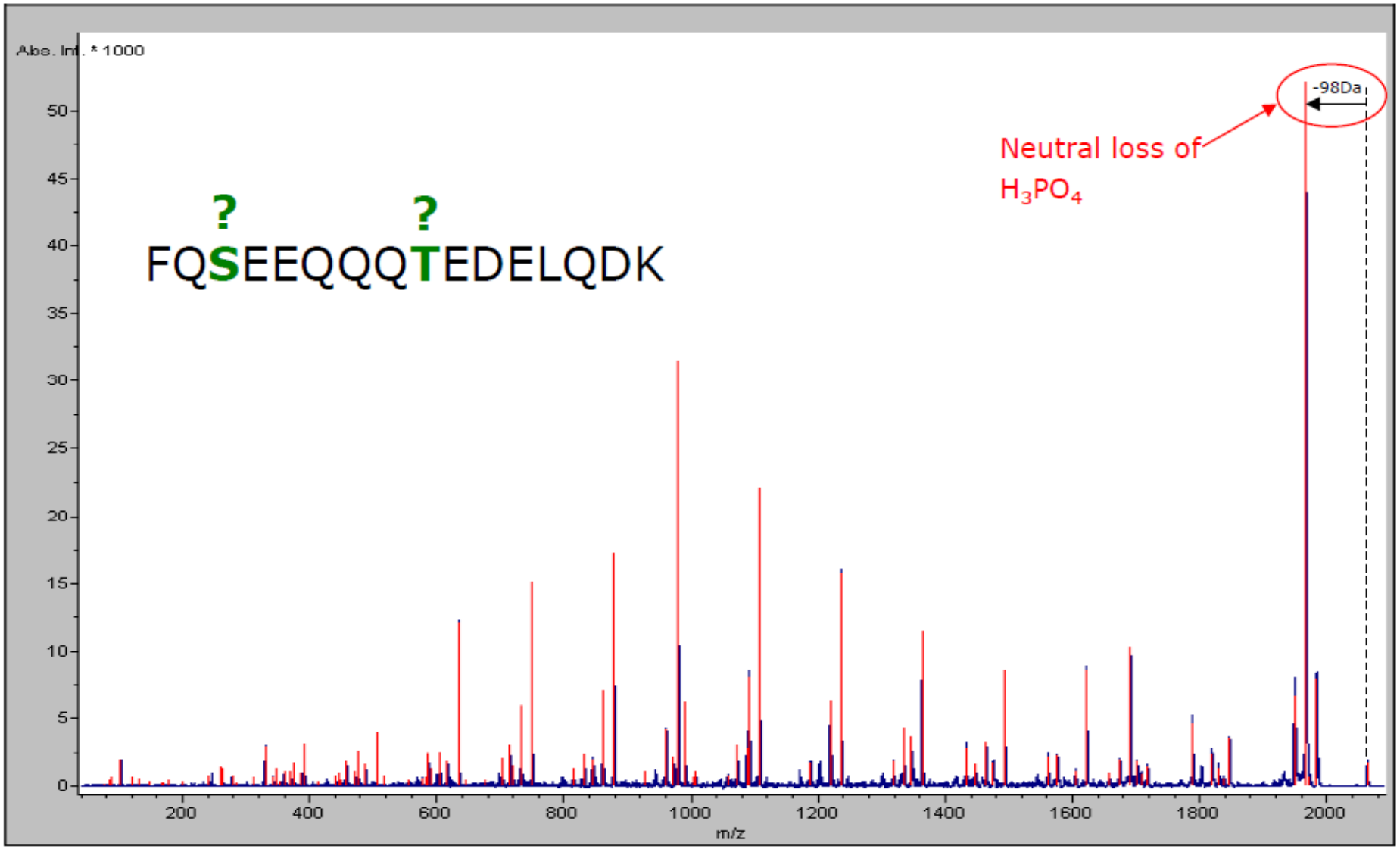
y- C terminus

b- N-terminus



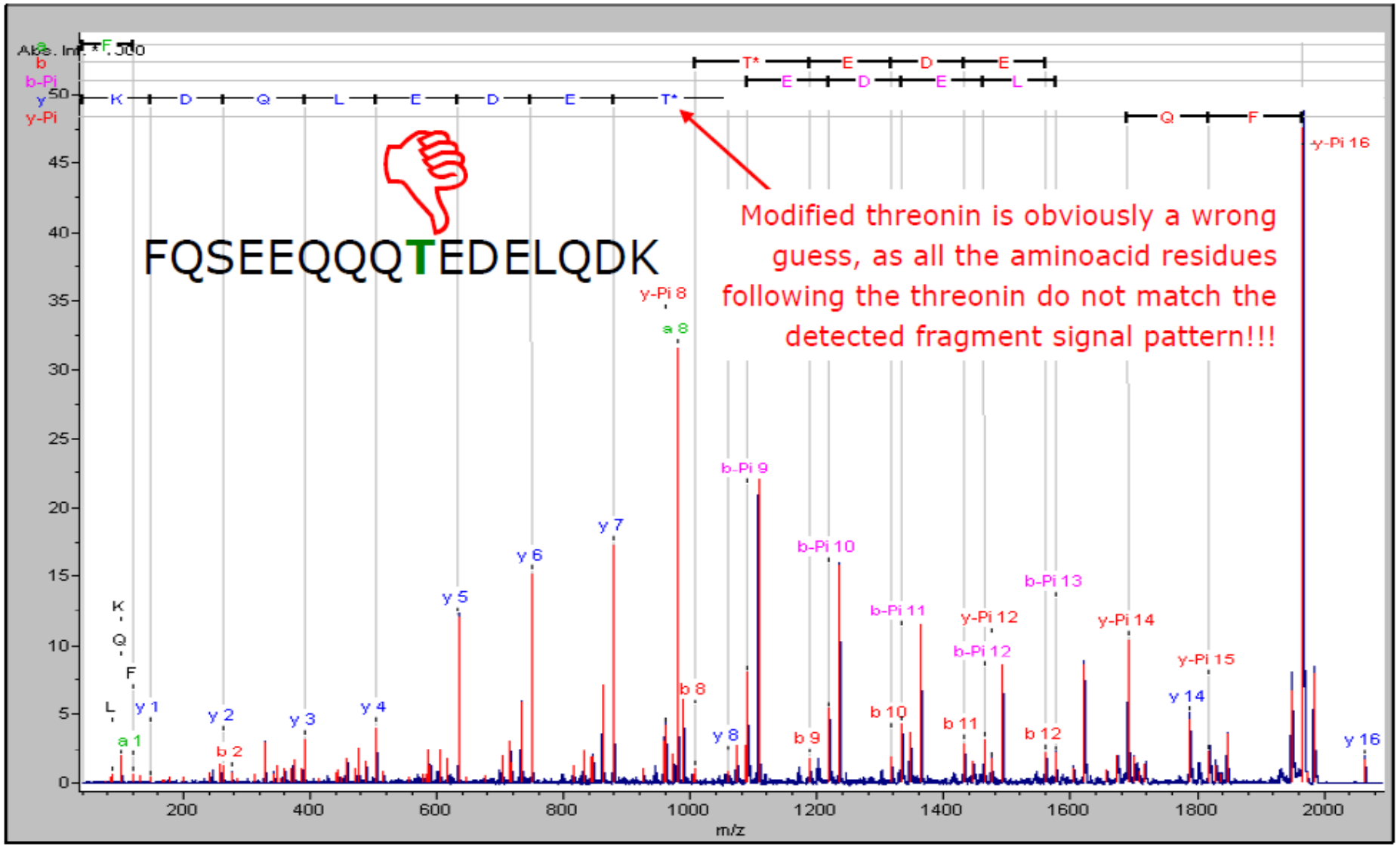
Primena MALDI ToF/ToF

MALDI Analysis of posttranslational modifications: Phosphorylation



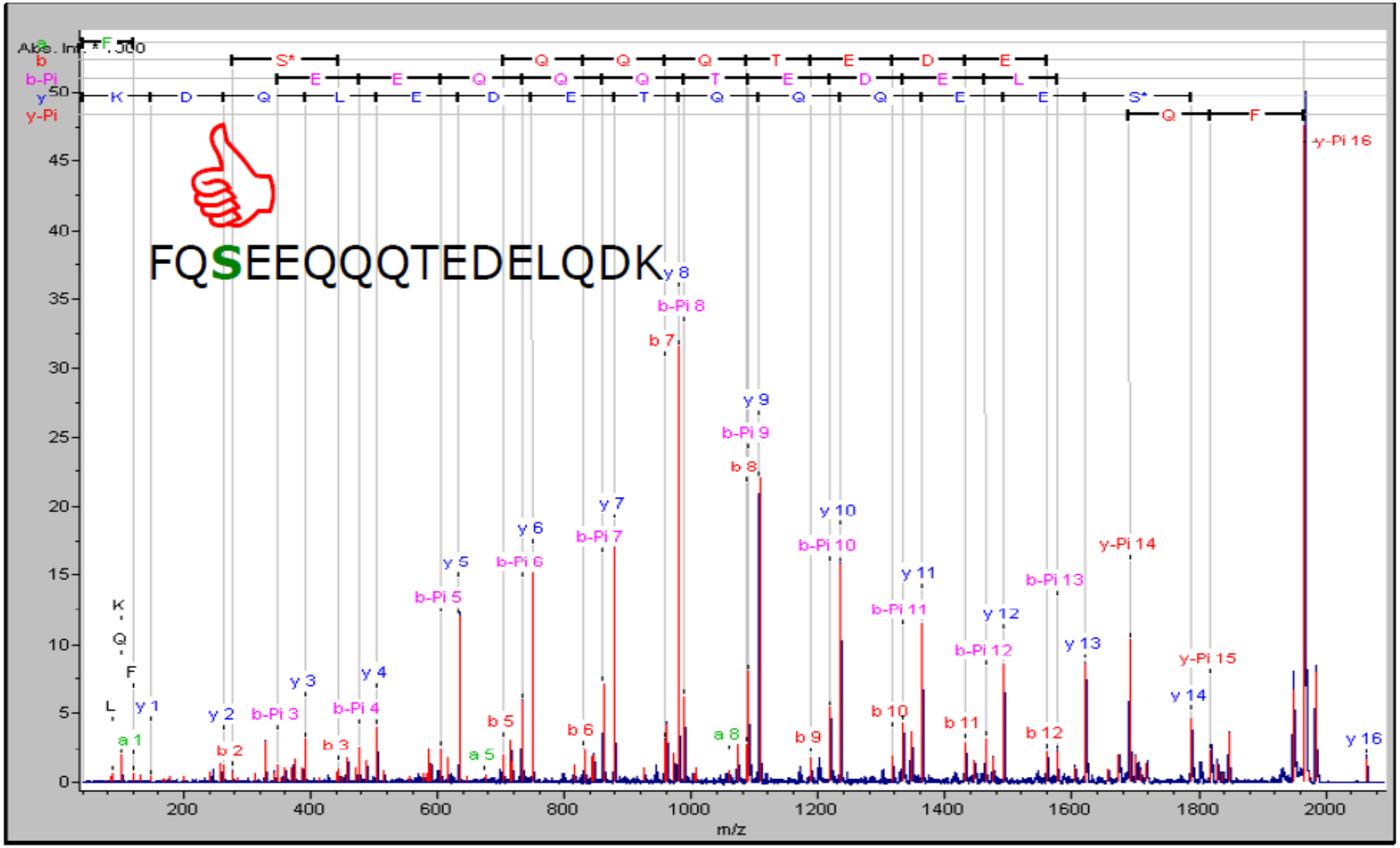
Primena MALDI ToF/ToF

MALDI Analysis of posttranslational modifications: Phosphorylation



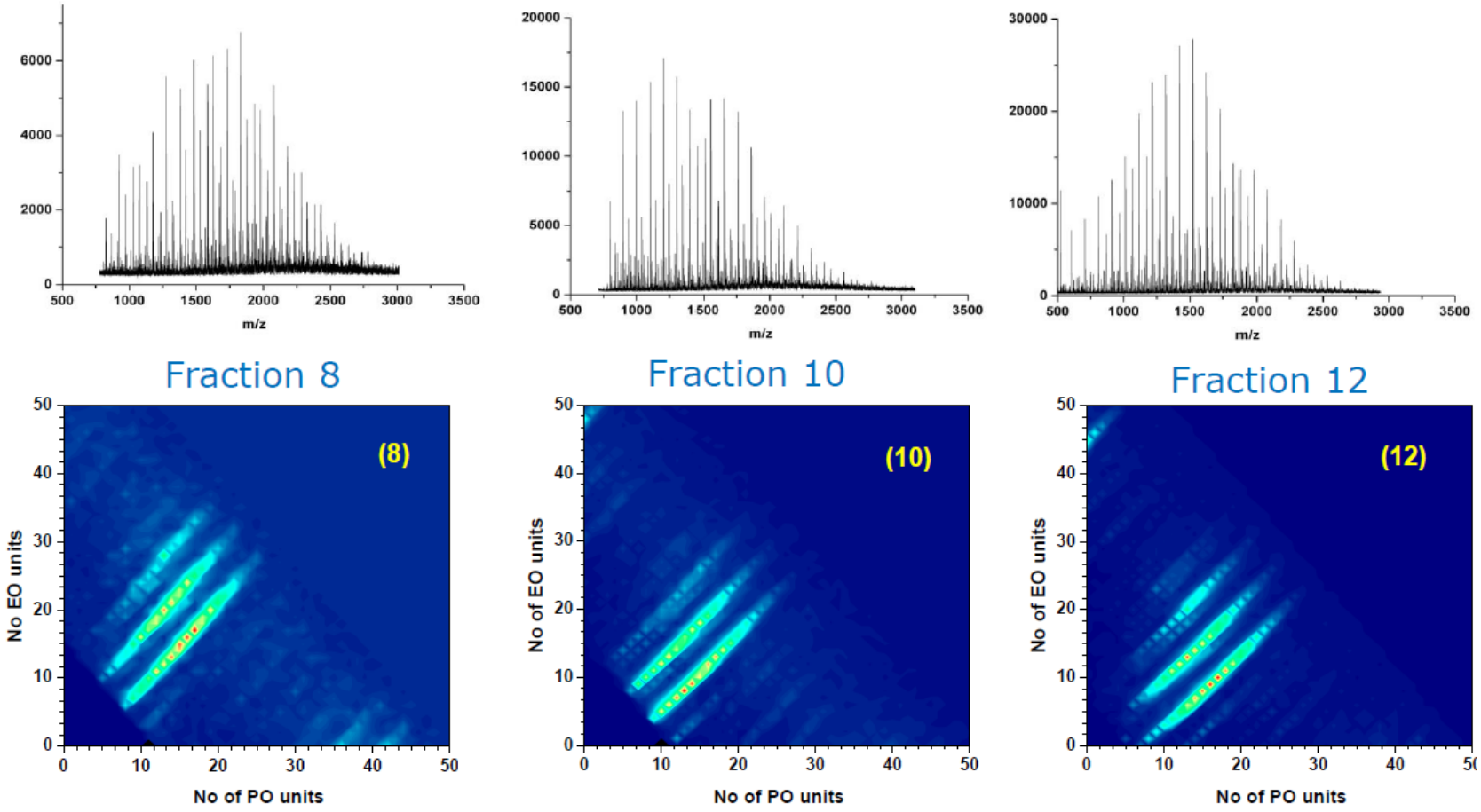
Primena MALDI ToF/ToF

MALDI Analysis of posttranslational modifications: Phosphorylation



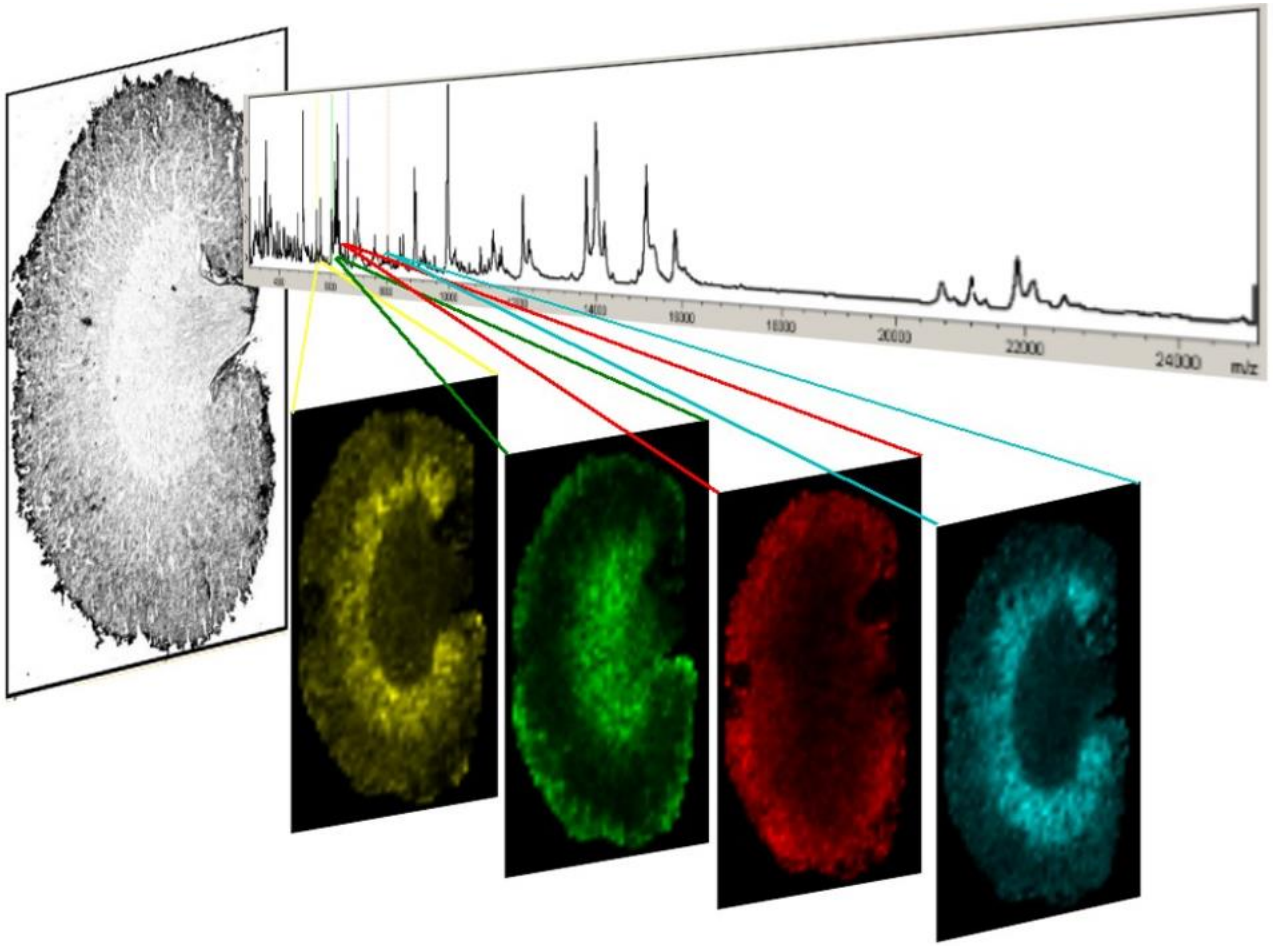
Primena MALDI ToF/ToF

Polymer characterization: Pre-separated PEO/PPO Copolymer



Weidner S.M., Falkenhagen J., Maltsev S., Sauerland V., Rinken M. *Rapid Commun. Mass Spectrom.* 2007; **21**: 2750-2758

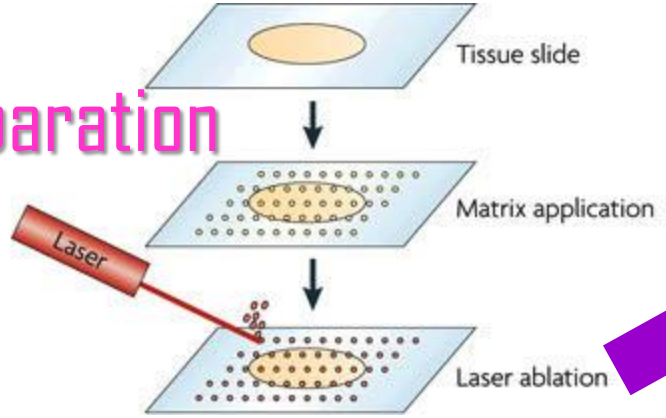
Šta je MALDI MS oslikavanje?



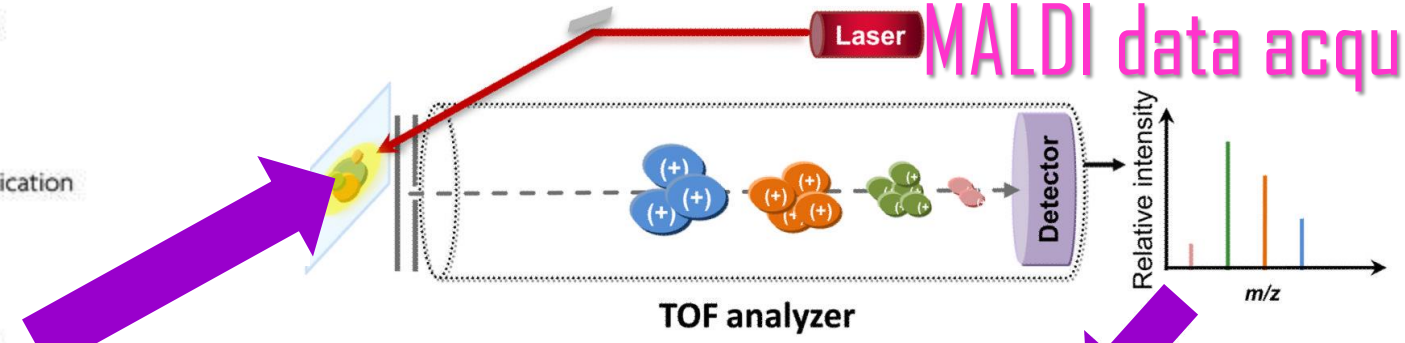
- Spatially resolved mass spectra are being recorded

MALDI MSI -workflow

Sample preparation



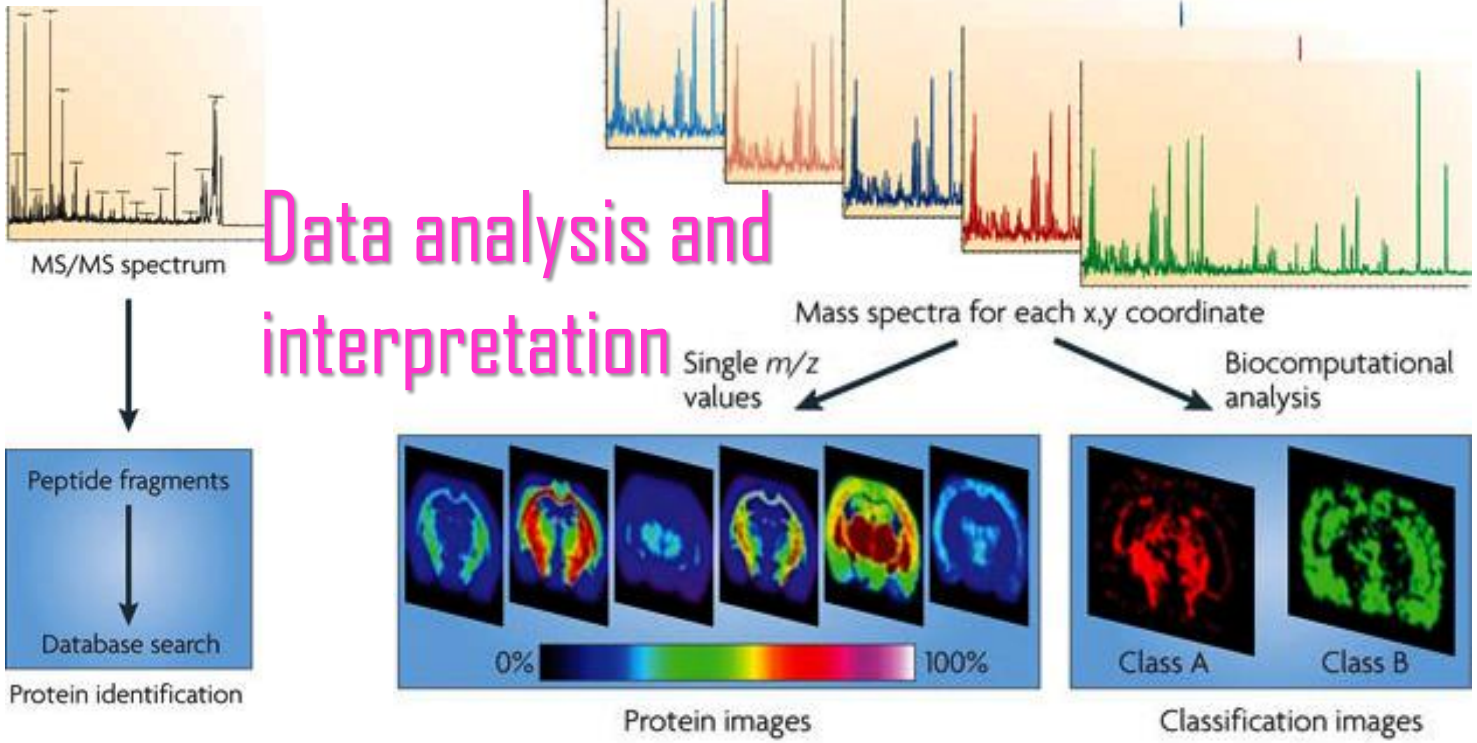
MALDI data acquisition



Tandem MS

Single MS

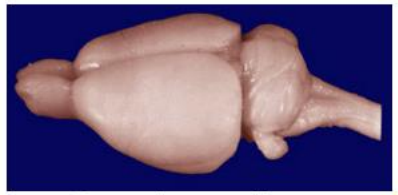
Data analysis and interpretation



Nature Reviews Cancer 10, 639-646 (September 2010) doi:10.1038/nrc2917
Seeley and Caprioli. Trends Biotechnol. 2011 March ; 29(3): 136–143. doi:10.1016/j.tibtech.2010.12.002

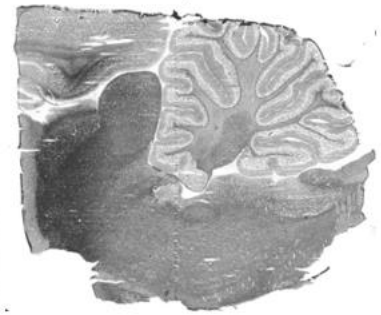
MALDI –priprema uzoraka – sveže zamrznuto tkivo

Tissue preparation



Snap-frozen tissue
FFPE tissue

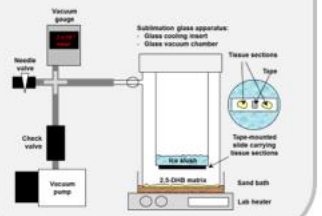
Cryo-microtome



Tissue section mounted
on conductive ITO slide
(preferred thickness ≤ 10µm)

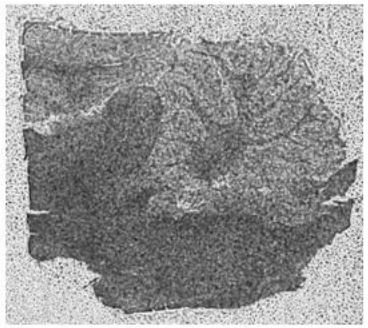
Pneumatic spray

Sublimation



Nebulization
(Bruker Imageprep)

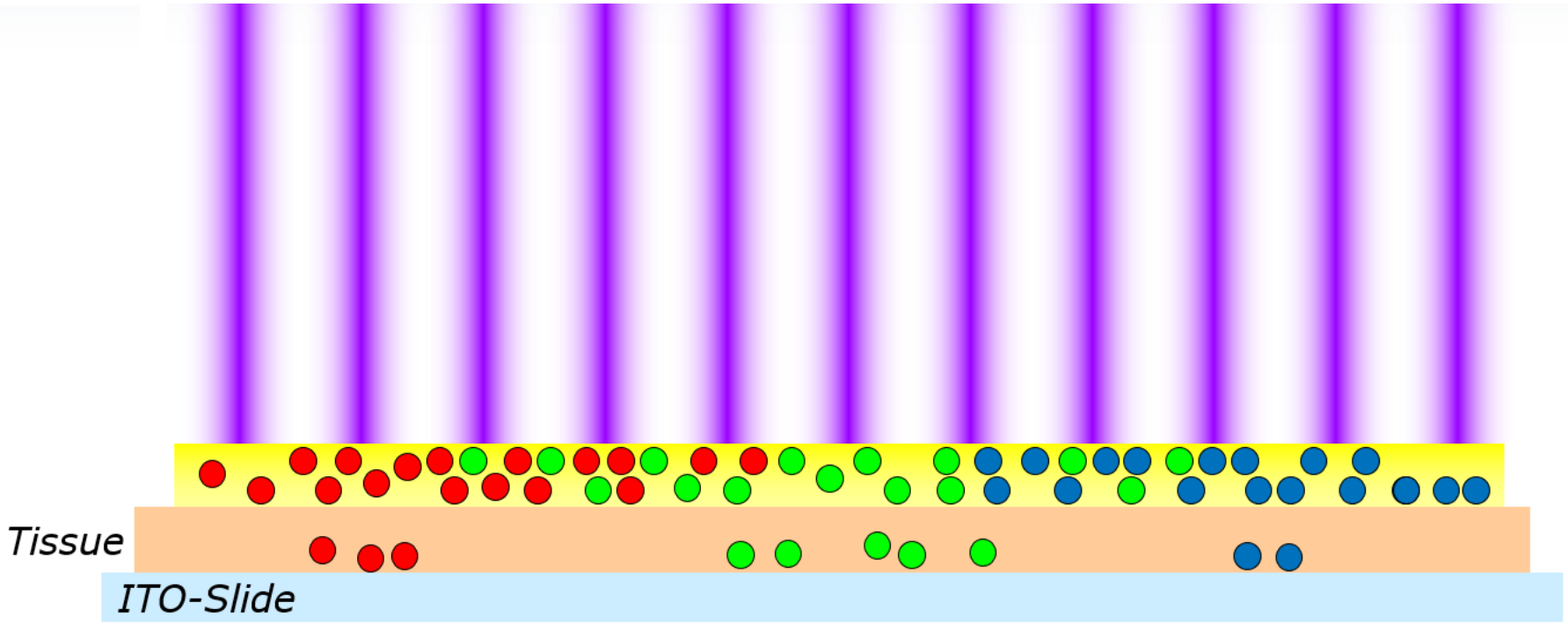
MALDI matrix preparation



Matrix coated section

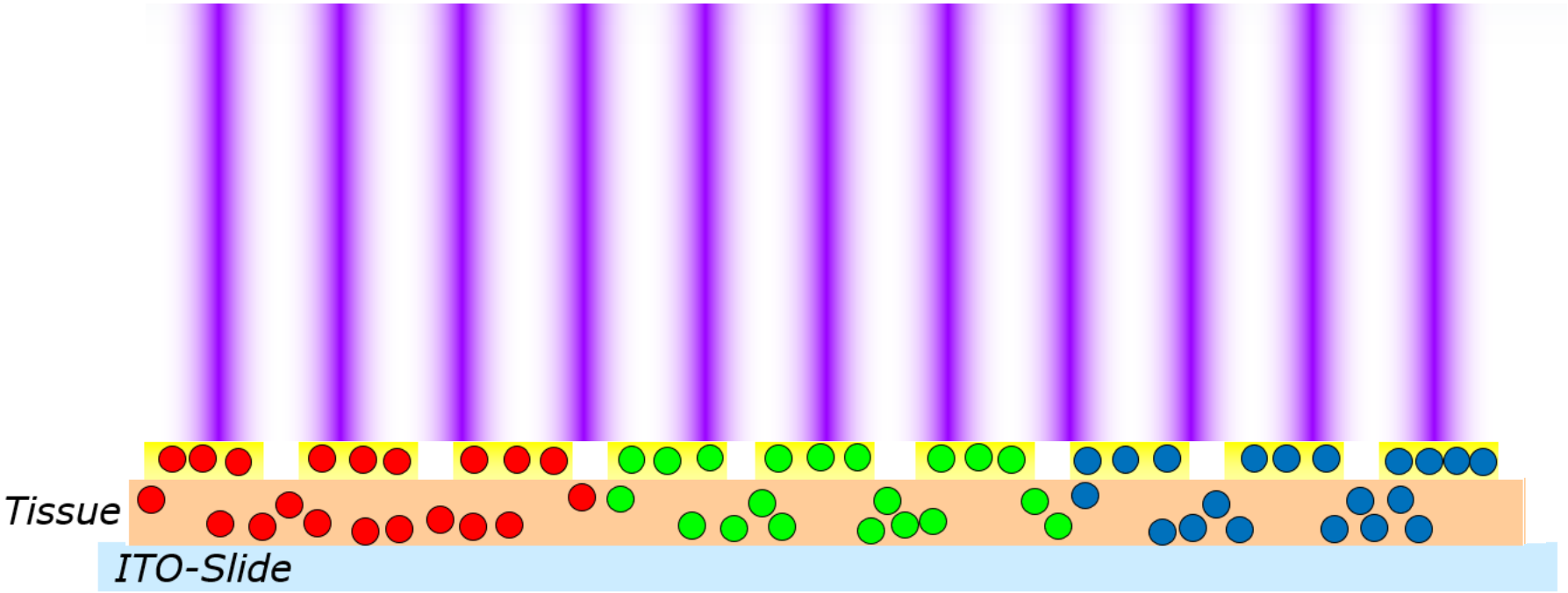
Nanošenje matrice

Wet matrix deposition method



Large droplets: good extraction, low spatial resolution

Nanošenje matrice



Small droplets: reduced extraction, high resolution

Metode vlažno za nanošenje matrice

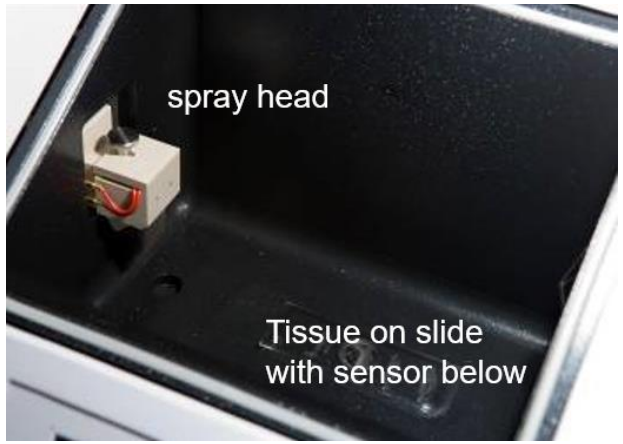
➢ ***Nebulization*** (f.e. **Bruker imageprep**):

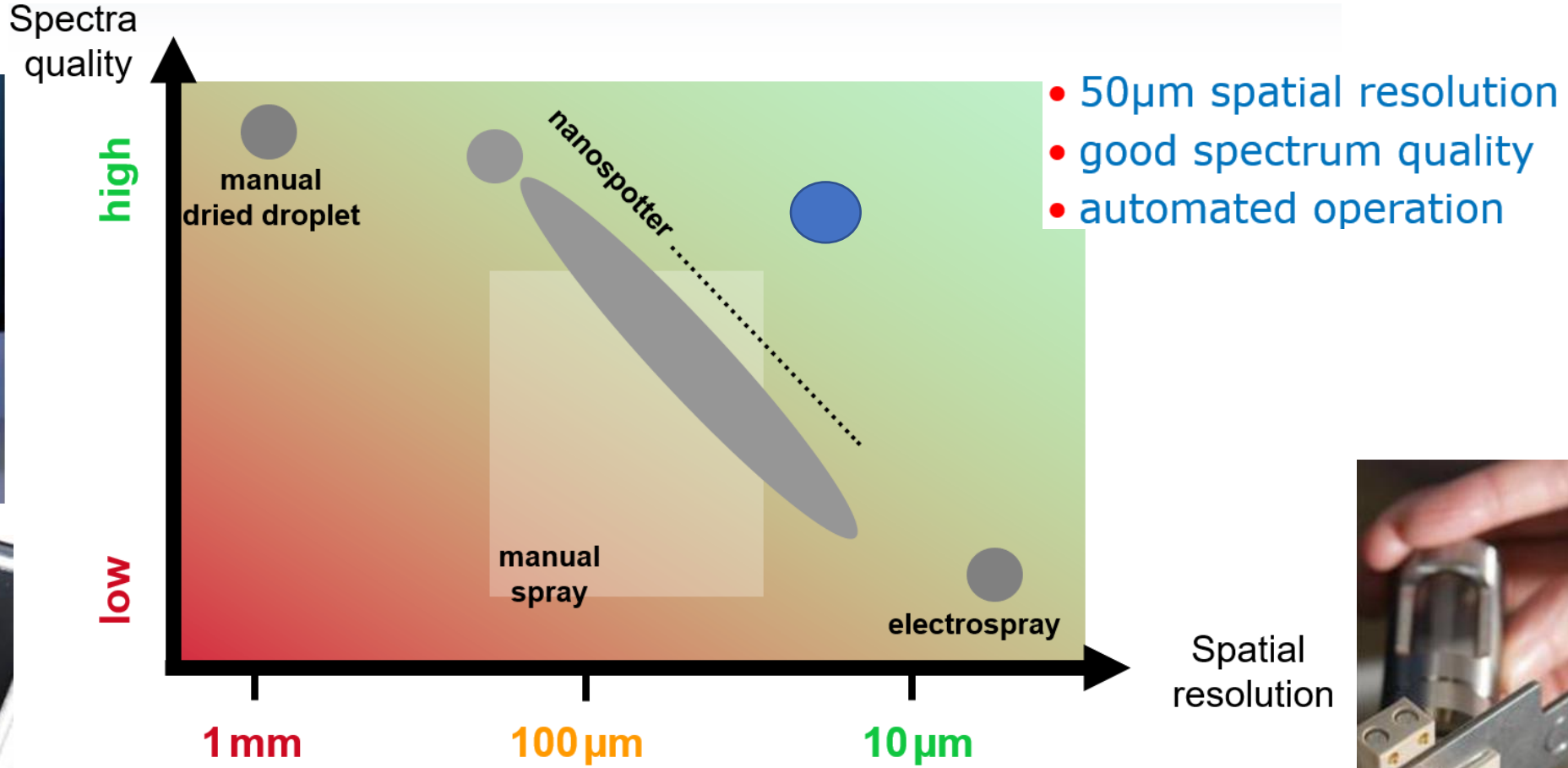
- Very good spectrum quality especially for intact proteins, due to efficient extraction of analyte molecules from tissue
- 50-70µm resolution routinely achievable
- Rather slow (takes approx. 1hour)

➢ ***Pneumatic spray*** (f.e. **HTX TM-sprayer, suncollect**)

- Applicable to rather broad range of analytes, MALDI matrices and applications
- Capable of generating very thin matrix layers consisting of very small crystals
- Careful method optimization required, to find best compromise between spatial resolution and spectral quality
- Depending on analyte of interest, spatial resolution of 10-20µm achievable
- Rather fast (5 – 15min depending on method setup)
- best suited instrument type for *deposition of enzymes* (trypsin f.e.) when dealing with FFPE tissue.

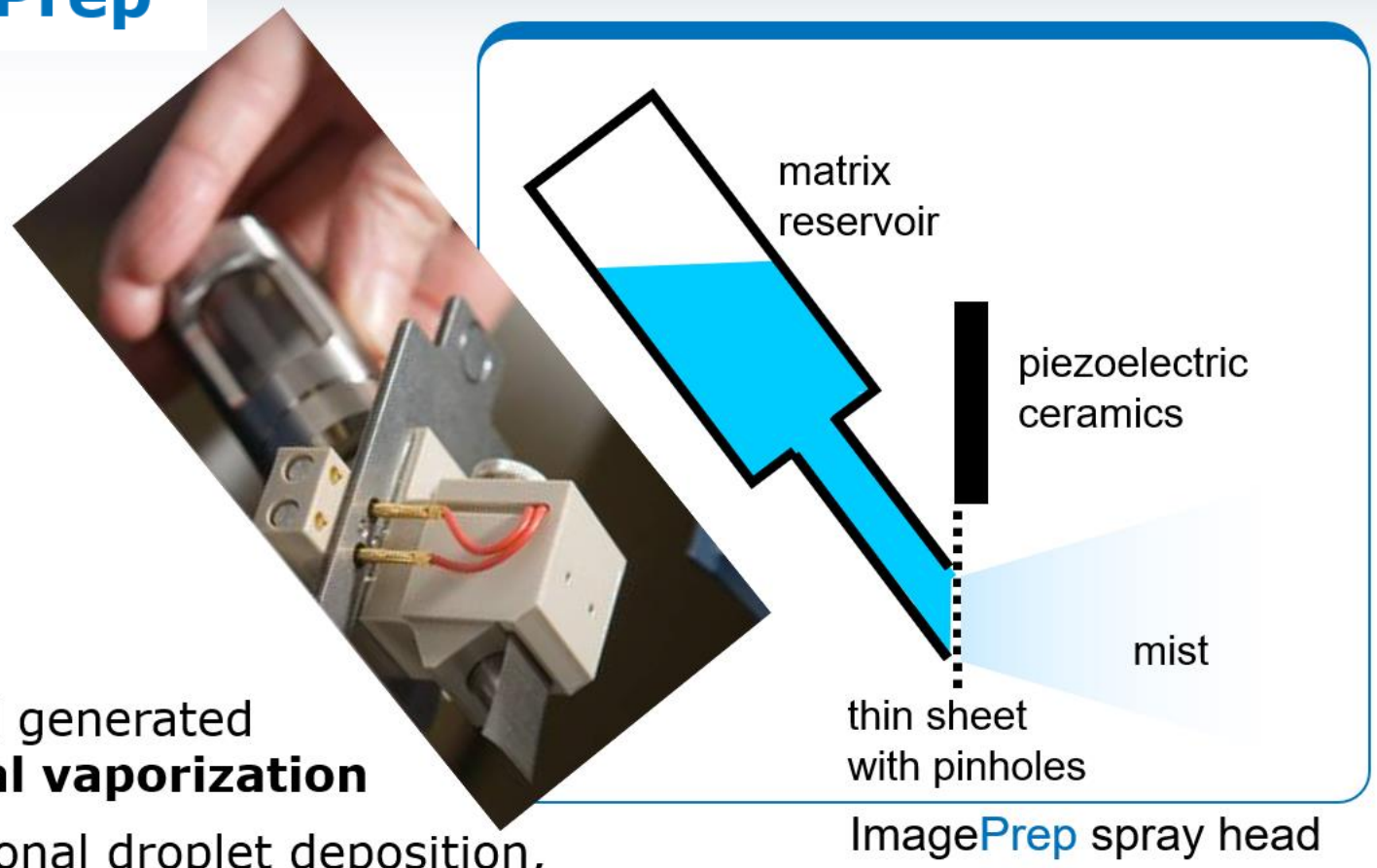
Bruker ImagePrep





- 50µm spatial resolution
- good spectrum quality
- automated operation

Bruker ImagePrep

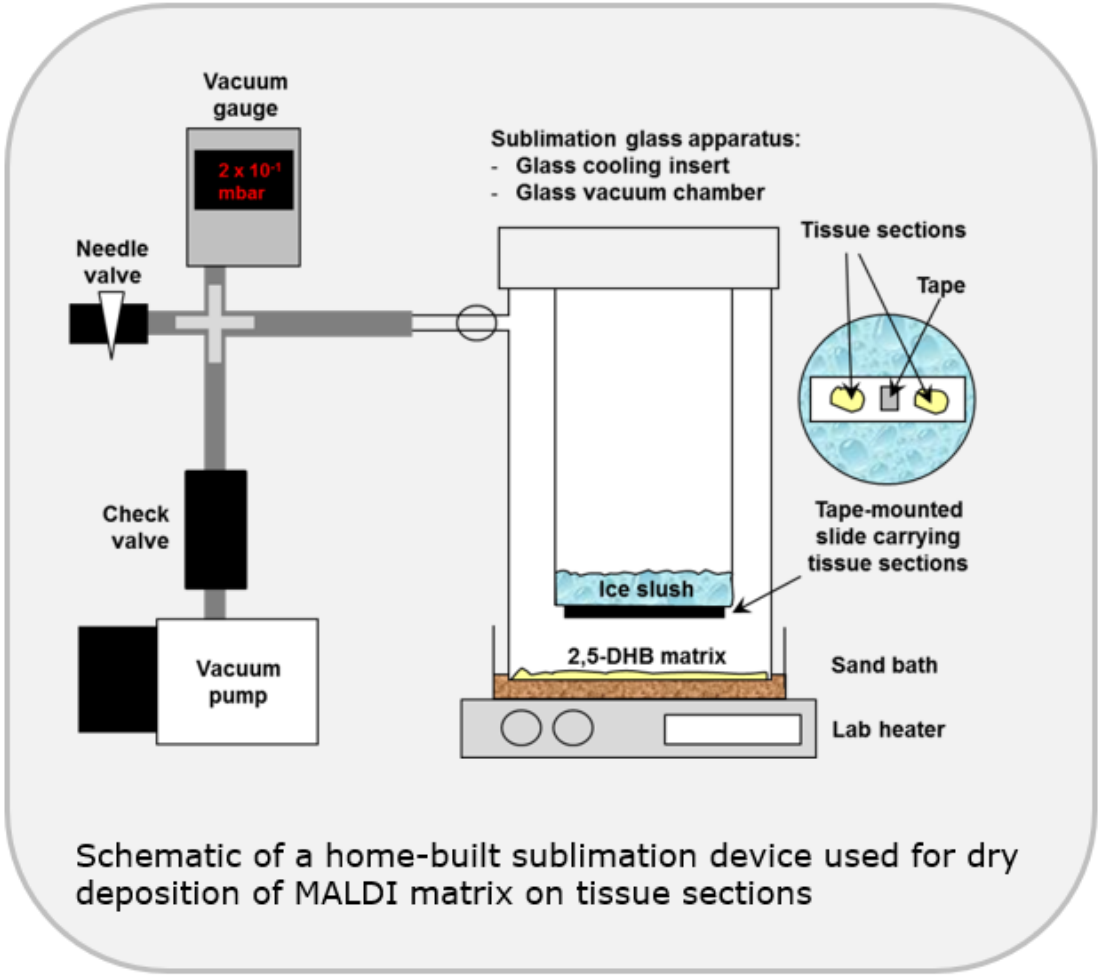


- Matrix aerosol generated by **vibrational vaporization**
- Soft, gravitational droplet deposition, **controlled atmosphere**
- **Reproducible crystal size**

Metode za suvo nanošenje matrice

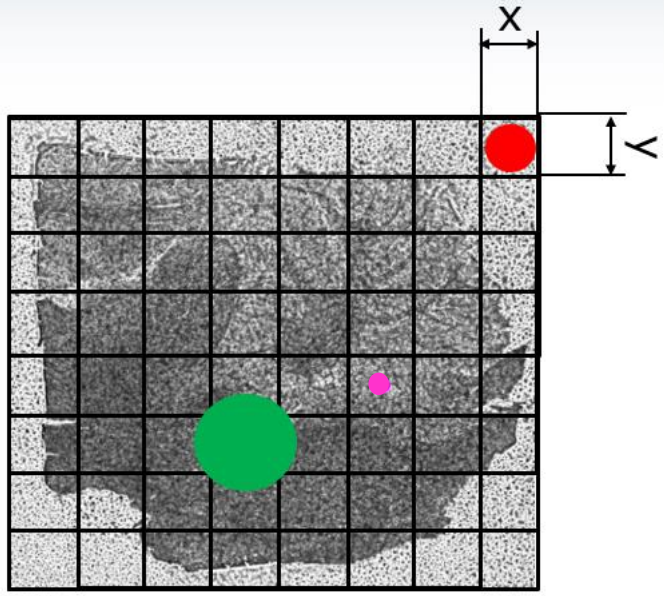
➢ *Sublimation*

- Very thin matrix layers consisting of extremely small crystal size
- No artificial delocalization of analyte molecules due to absence of solvents
- Ultimate level of spatial resolution achievable (<10μm)
- Works best for sufficiently small analyte molecules that ionize very well in MALDI (f.e. lipids, various other small molecules)
- Simple and fast technique
- For larger analyte molecules (f.e. peptides, proteins), dry matrix deposition may require an additional rehydration step, or may even not work at all



Schematic of a home-built sublimation device used for dry deposition of MALDI matrix on tissue sections

Prostorna rezolucija u MALDI MSI



Spatial resolution is determined by the following MALDI instrument parameters:

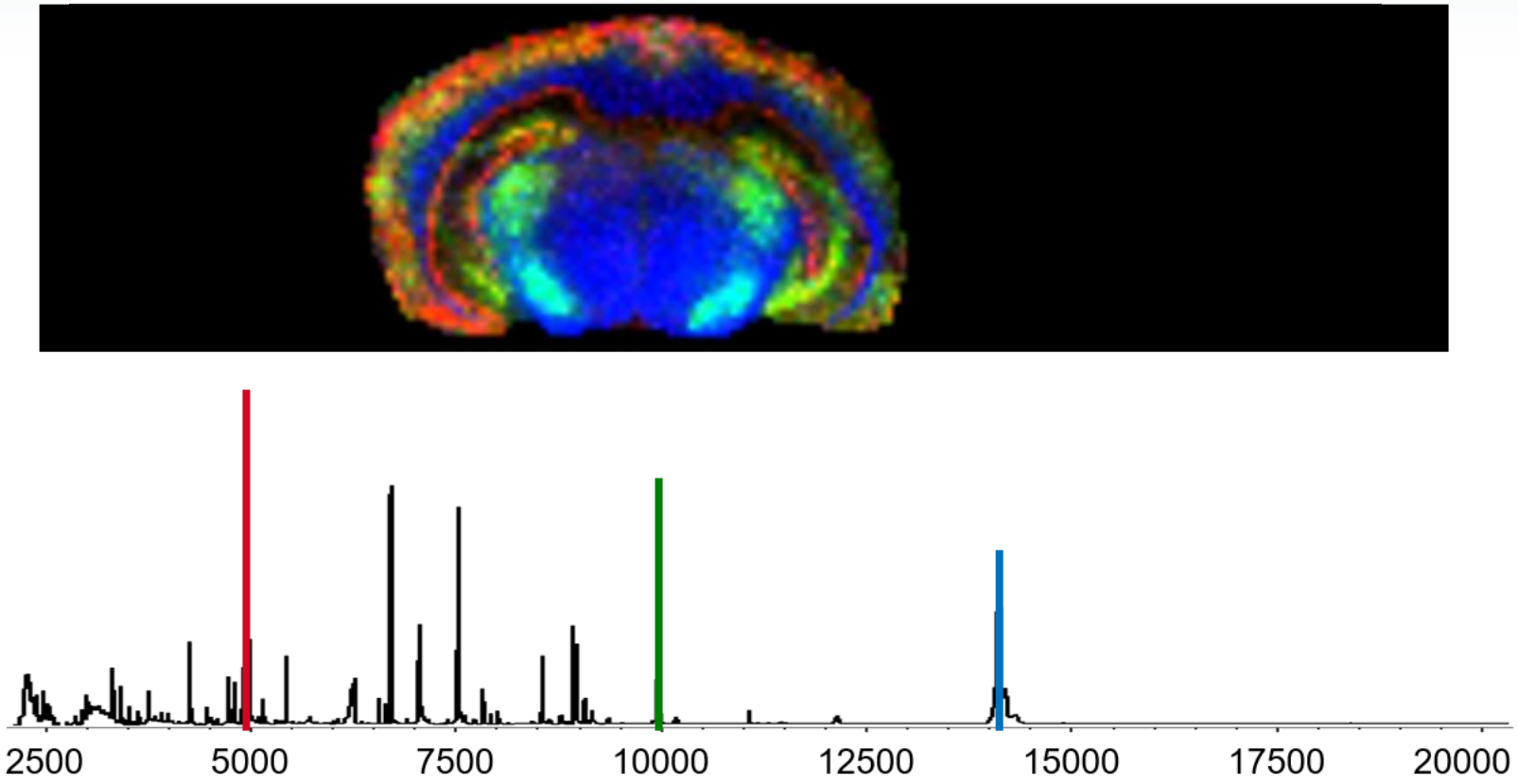
- raster width (i.e. pixel size) defined for MALDI sample stage movement in x and y direction
- size of the area within a pixel ablated by the laser

Ideally, the laser ablation area should cover the defined pixel area as complete as possible to enable high yield of MALDI ions.

Laser ablation area smaller than desired pixel dimensions will cause **undersampling** (lowered MALDI ion yield because of low pixel coverage).

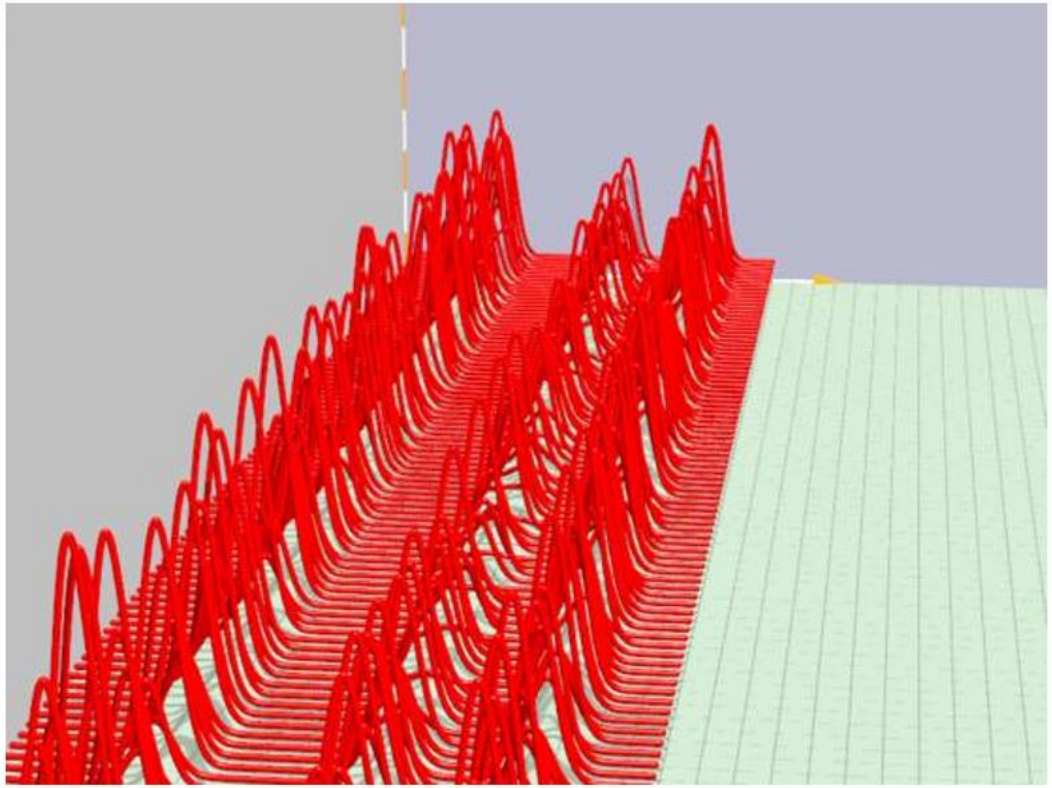
Laser ablation area larger than defined pixel dimensions will cause **oversampling** (leading to diminished spatial resolution of resulting MALDI images).

MALDI- Data analysis and interpretation

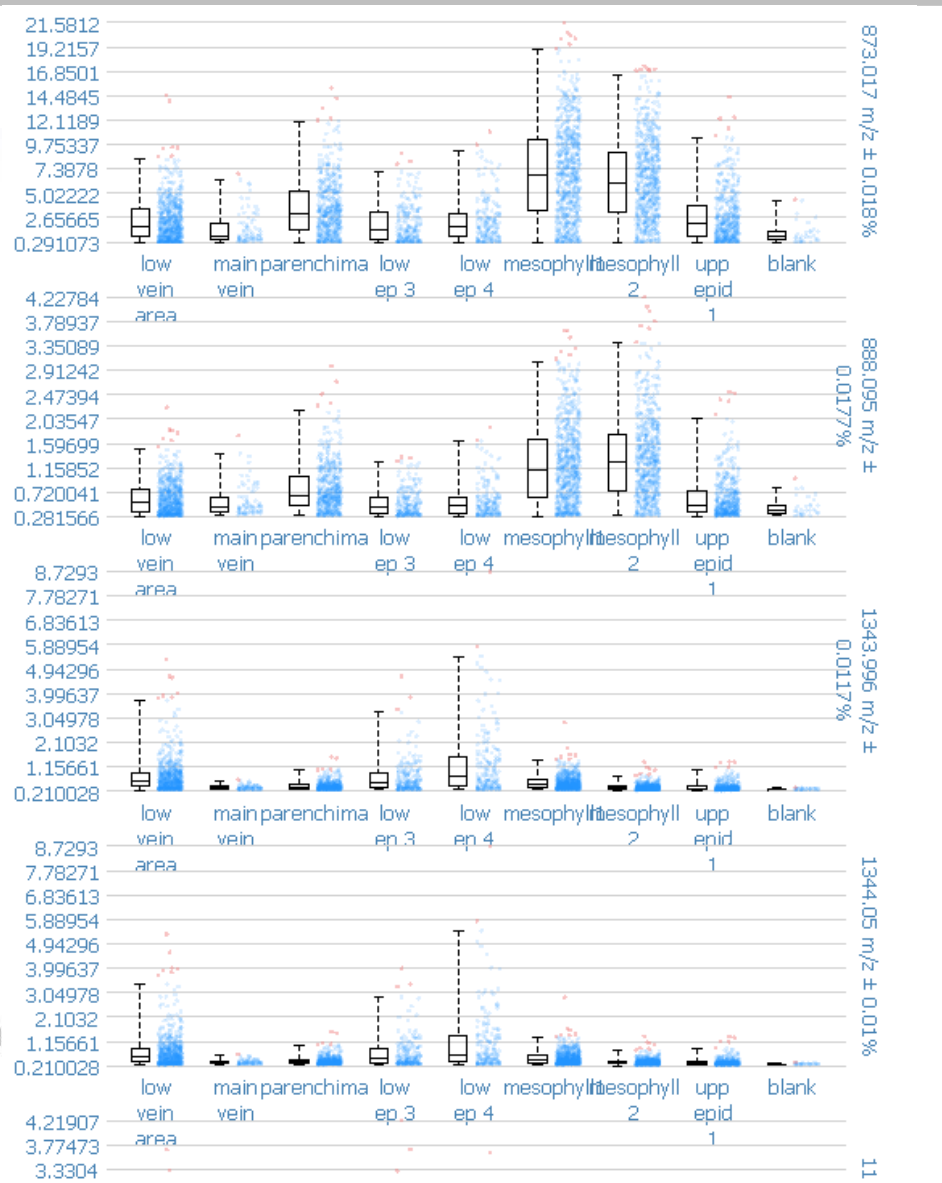


MALDI-MS imaging is a truly multiplexing technique. Ion images can be extracted from a data set for any mass feature of interest within the detection range covered.

MALDI-MSI Obrada podataka



We start with this set of spectra, e.g. individual pixels from a MALDI ima



Tissue-specific accumulation of phenolics in crops and variegated plants as a part of adaptation processes to changing climate conditions

Leibniz Association

DAAD Deutscher Akademischer Austauschdienst German Academic Exchange Service

BRUKER

IPK LEIBNIZ INSTITUTE

Fenolna jedinjenja

Physiological roles (solar irradiance):

1. UV/light screening - epidermal phenolics (flavonoids-Flav and hydroxycinnamic acids-HCAs).

2. Antioxidants- flavonoids with *ortho*-dihydroxylated B-ring in the mesophyll cells → against oxidative damage induced by high light, UV-A and UV-B radiation, and cold stress.

3. Sink of reduced carbon (an energy escape valve).

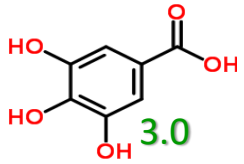
The TEAC reflects the ability of hydrogen-donating antioxidants to scavenge the $ABTS^{\bullet+}$ radical cation

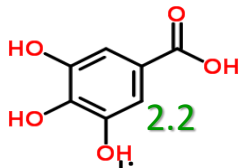
Trolox=Asc=1

Rice-Evans *et al. 1996*

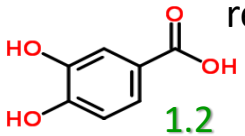
HBAs

gallic 3.0





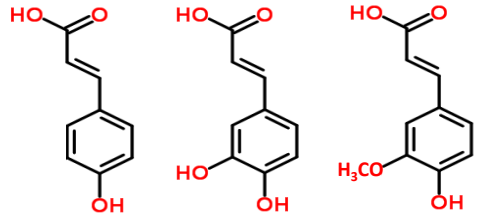
resorcylic 2.2



protocatechuic 1.2

λ_{max}= 260-280 nm

HCAs



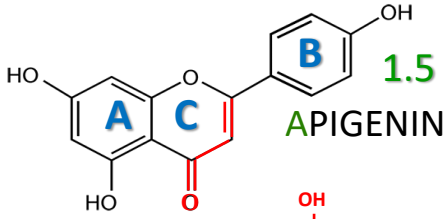
p-coumaric 2.2

caffeic 1.3

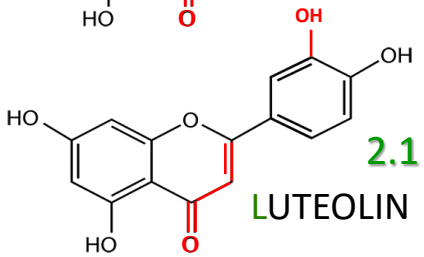
ferulic 1.9

λ_{max}= 310-330 nm

FLAVONES



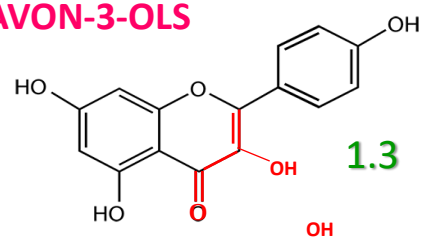
APIGENIN 1.5



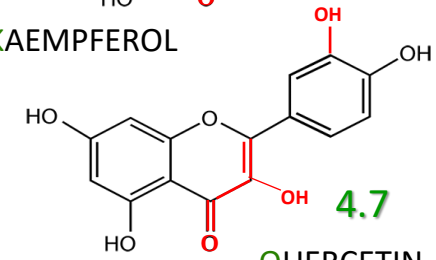
LUTEOLIN 2.1

λ_{max}= 340-350 nm

FLAVON-3-OLS



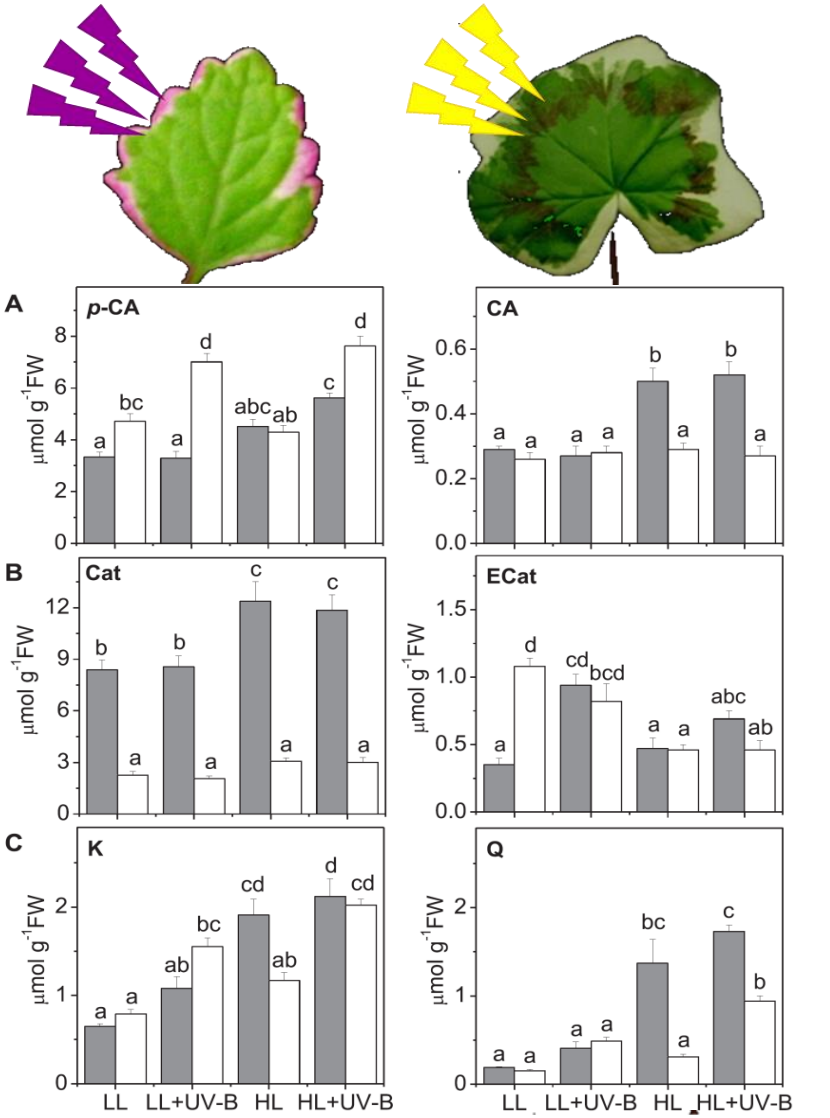
KAEMPFEROL 1.3

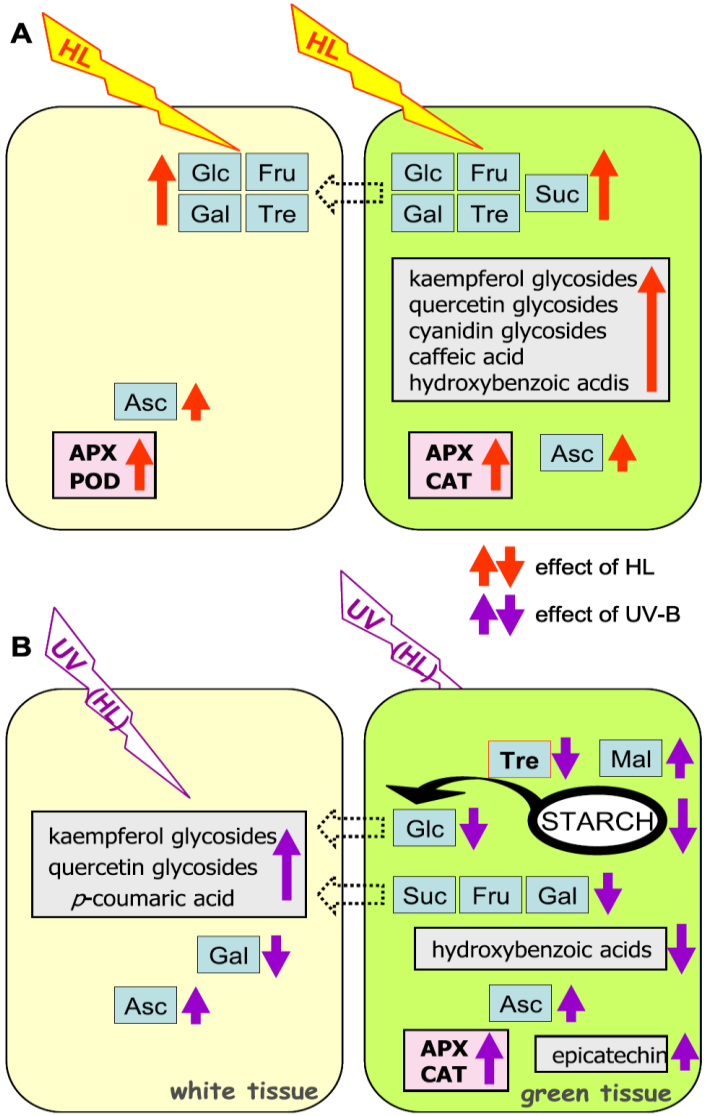


QUERCETIN 4.7

λ_{max}= 360-370 nm

"Sector-specific" leaf phenolics in *P. zonale*

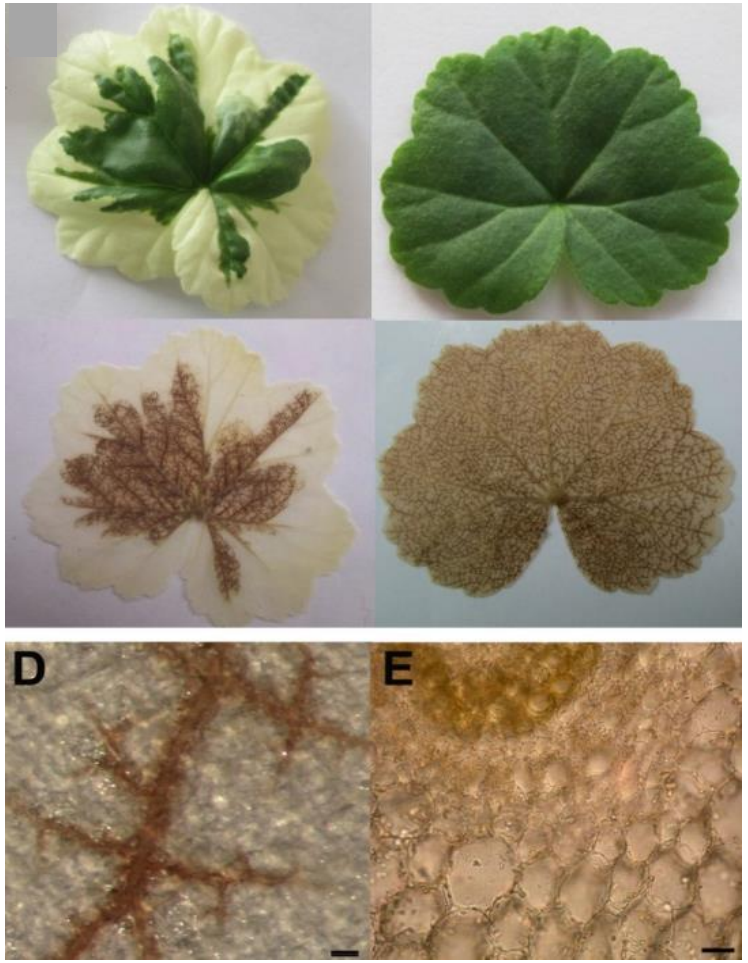




Vidović et al. 2015 *Plant Physiol. Biochem.* 93:44-55
Vidović *et al.* 2015 *Plant Cell Environ.* 38:968–979

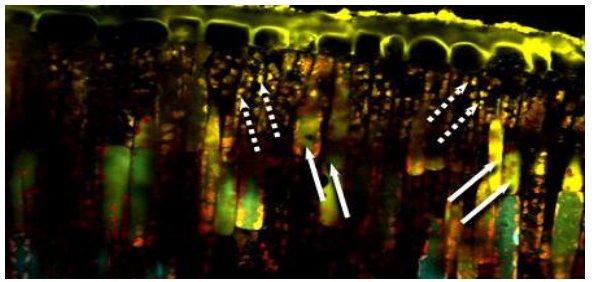
Spatial distribution

Hydrogen peroxide/HL stress



Vidović et al. 2016 *J.Plant Physiol*. 206:25–39

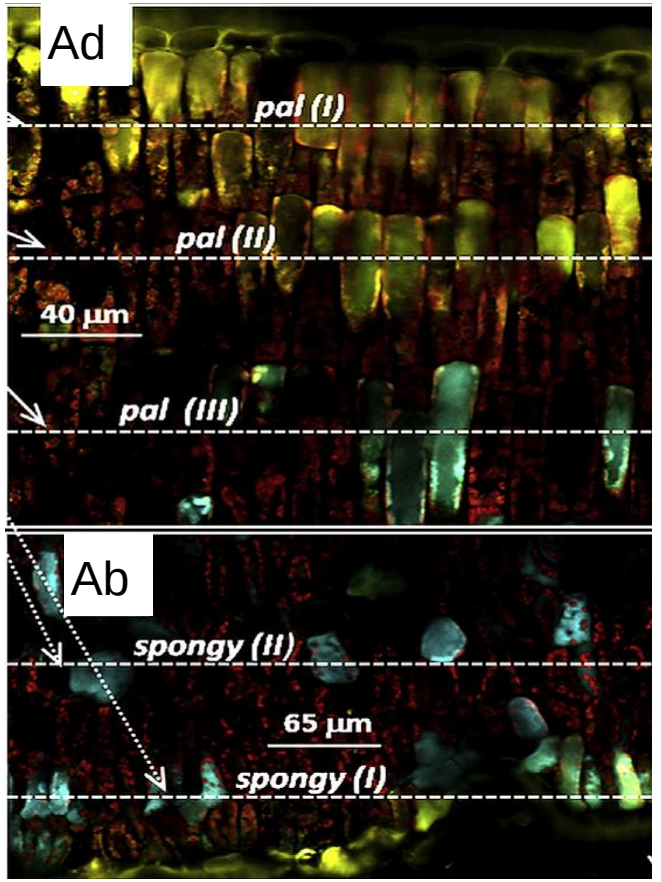
Flavonoids*/full sun exposure:



in the vacuole

in the chloroplast

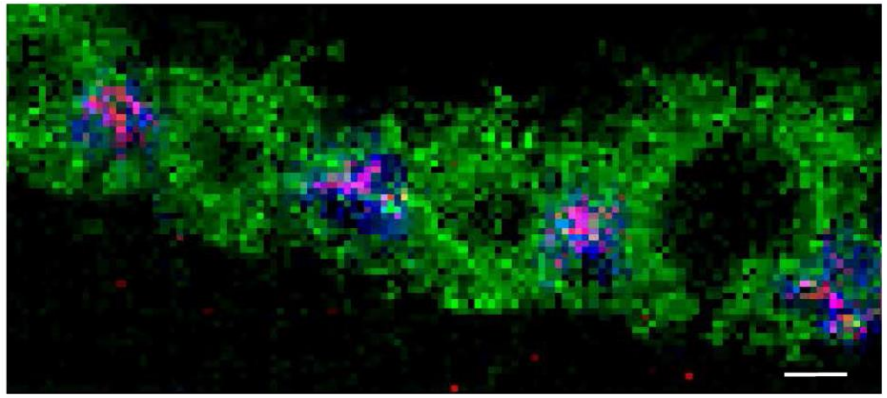
Distribution of Flav, and HCA in *Phyllirea latifolia* leaves

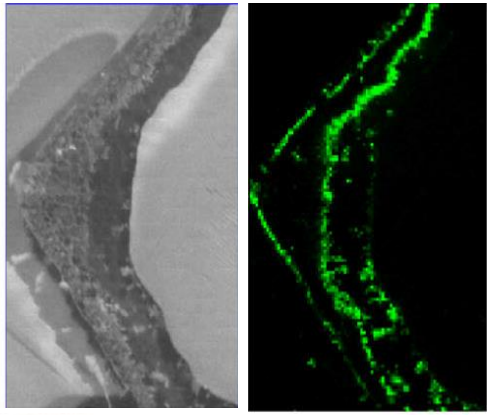


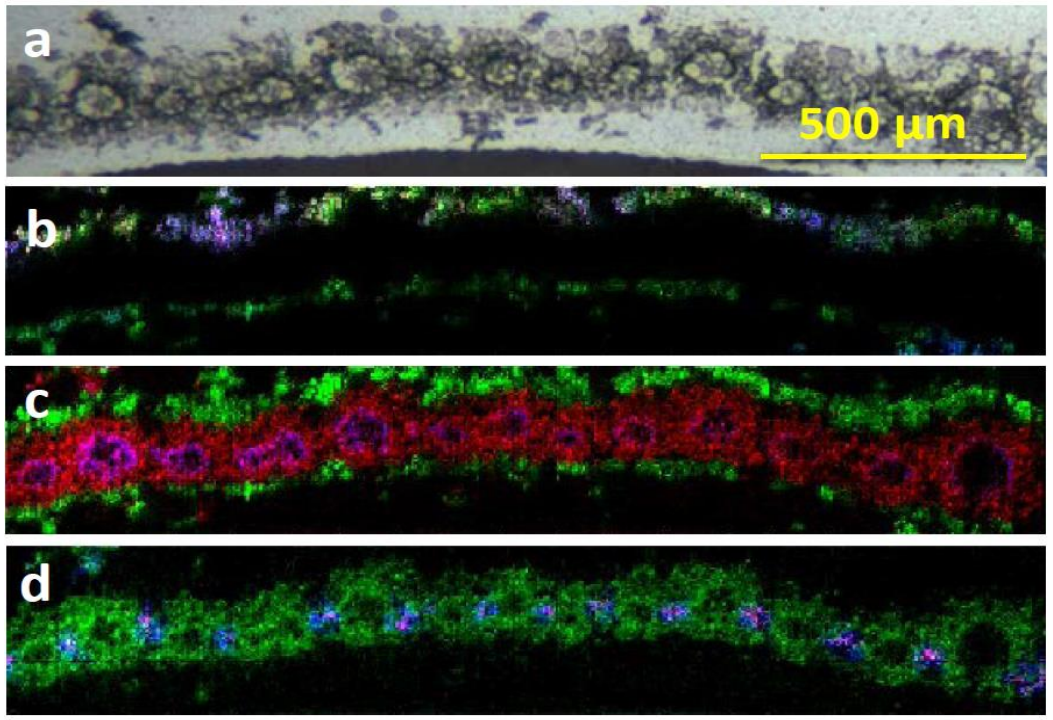
*Naturstoff reagent

Agati et al. 2013 *Plant Physiol Biochem* 72:35-45
Agati et al. 2017 New Phytologist 174:77–89

Tissue-specific distribution







Maize leaf cross section

al., 2015

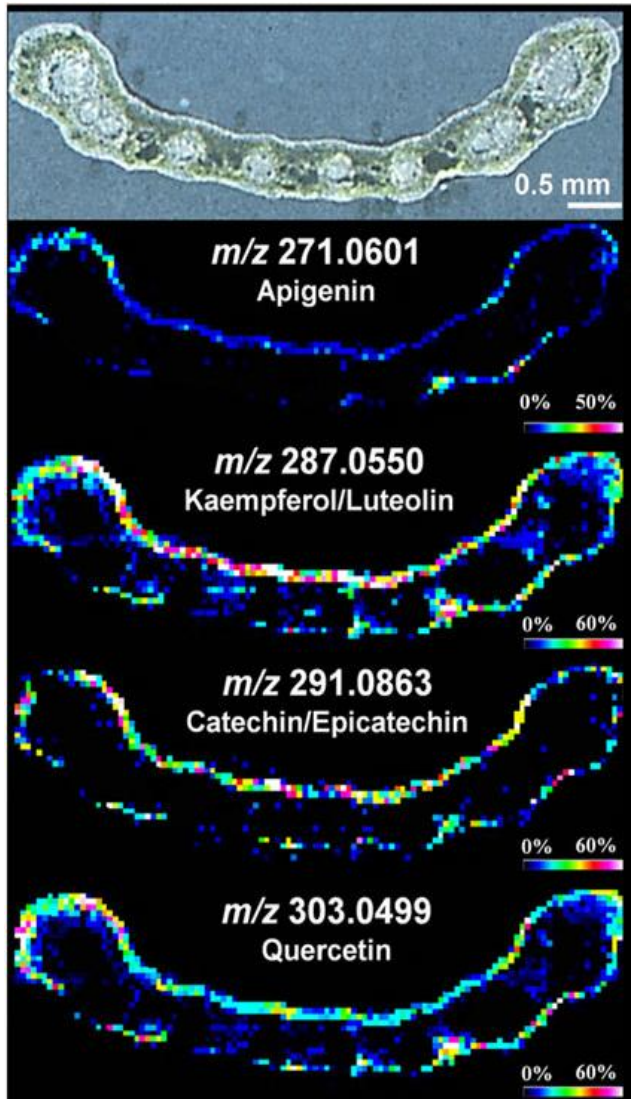
Ru#n
Maysin
Luteolin/
Kaempferol

PG(34:2)
SQDG(34:3)
Luteolin/
Kaempferol

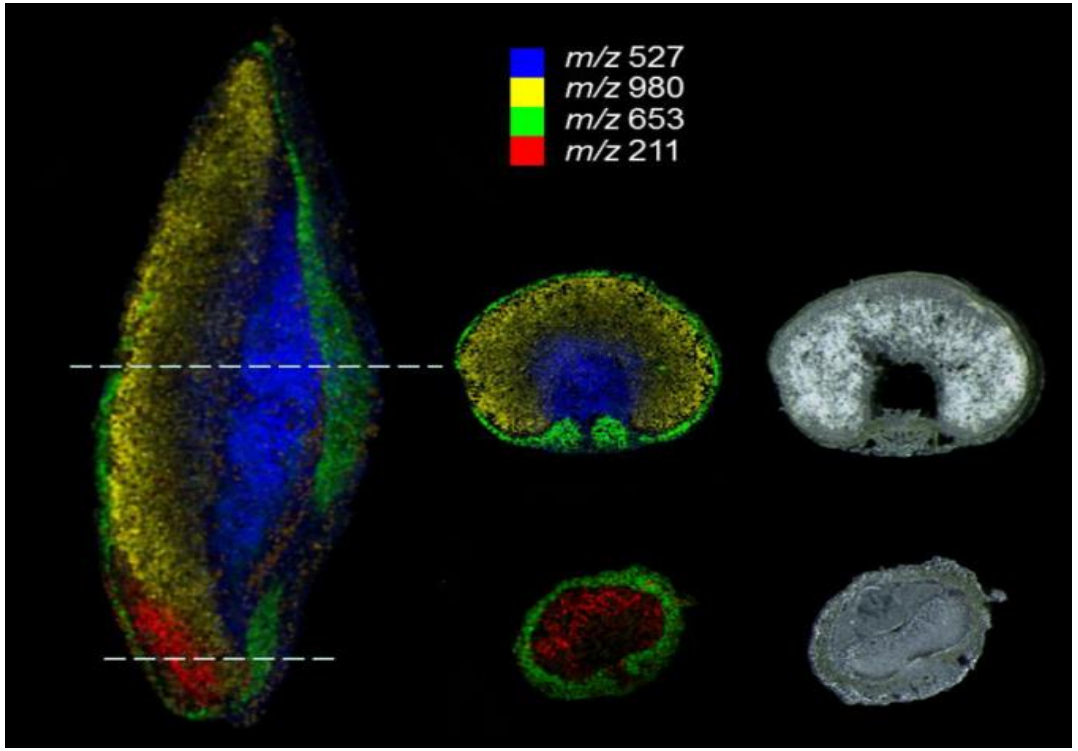
HMBOAEGlc
DIMBOAEGlc
SQDG(34:3)

Li et al., 2018

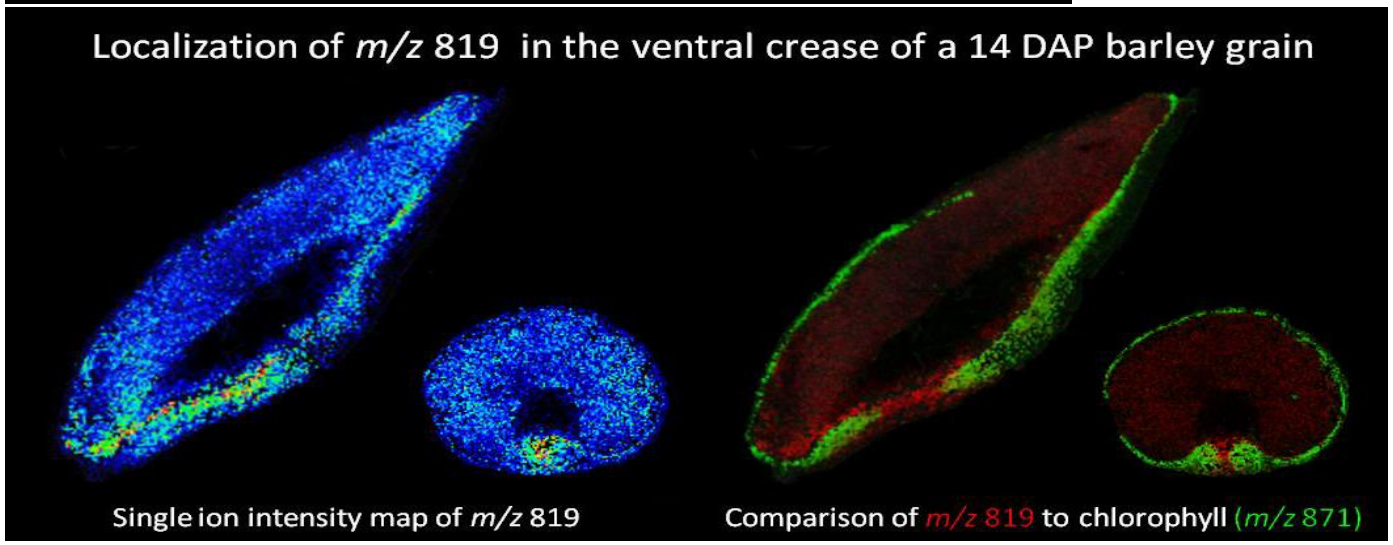
Ginkgo leaf cross section



Tissue-specific distribution



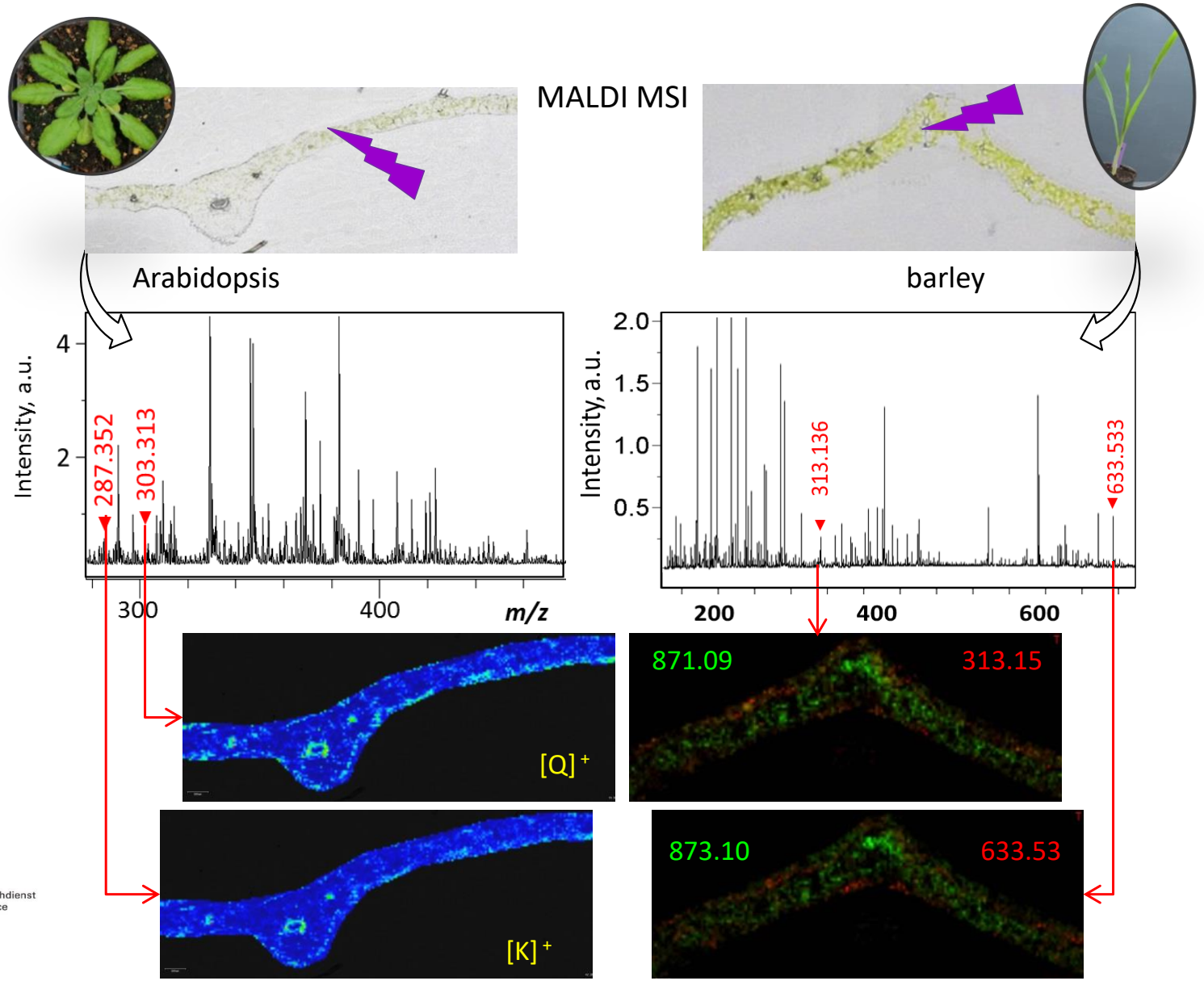
Longitudinal and cross sections of barley grains. 211, 527, 653 and 980.
m/z = 211: JA.
m/z = 527: raffinose
m/z = 653; 980: not identified



Peukert 2013, *Dissertation*

A unified method for mass spectrometry (MS) imaging of polyphenols in the leaf cross- sections

Marija Vidovic,
Hans-Peter Mock

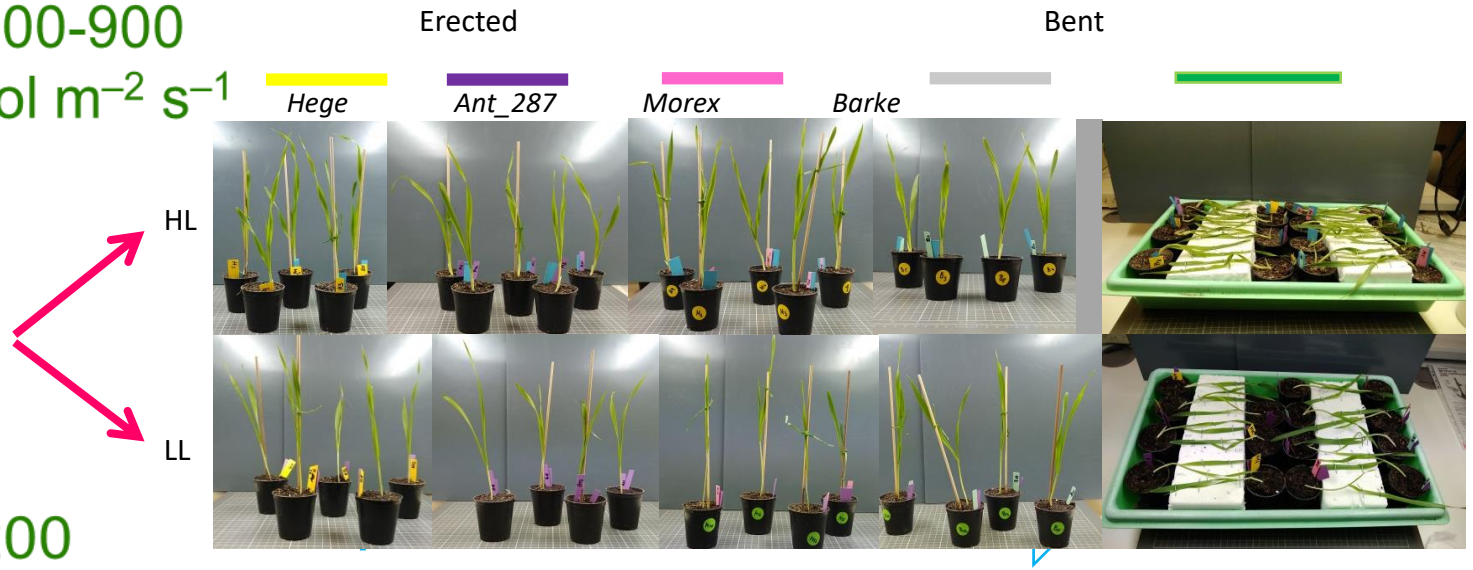


IPK LEIBNIZ INSTITUTE
Leibniz Association
DAAD Deutscher Akademischer Austauschdienst German Academic Exchange Service

Experimental set-up-barley twist

HL: 800-900
µmol m^{-2} s^{-1}

21°C/18°C
12 h photoperiod
200 µmol m^{-2} s^{-1}
PAR
10 days



LL: 200
µmol m^{-2} s^{-1}

5 days

Experimental set-up

Growth conditions:

20°C/18°C

12 h photoperiod

300 µmol m^{-2} s^{-1} PAR

10 days

LL

HL

LL: 300-340 µmol m^{-2} s^{-1}

HL: 500-550 µmol m^{-2} s^{-1}

5 days

Plants were watered daily

*A. t.*wist

21°C/18°C
12 h photoperiod
200 µmol m^{-2} s^{-1}
PAR
10 days

LL: 200
µmol m^{-2} s^{-1}

2, 3, 4, 6 days

HL: 800-900
µmol m^{-2} s^{-1}

A. thaliana: cold

Phenylalanine
↓ PAL
Cinnamate
↓ C4H
p-coumaric acid
↓ 4CL
p-coumaroyl CoA → (C3H) Caffeoyl-CoA → (CCoAOMT) Feruloyl-CoA

LIGNINS

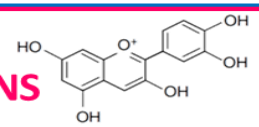
Feruloyl-CoA
↓ F6'H1
Scopolin
COUMARINS

p-coumaroyl CoA
↓ CHS

FLAVONOIDS

Naringenin chalcone
↓ CHI
Naringenin
↓ F3H
Dyhydroxykaempferol → (FLS1) Kaempferol
↓ F3'H
Dyhydroxyquercetin → (FLS1) Quercetin

Dyhydroxyquercetin
↓ DFR
Leucocyanidin
↓ LDOX
Cyanidin → **ANTHOCYANINS**



ldox

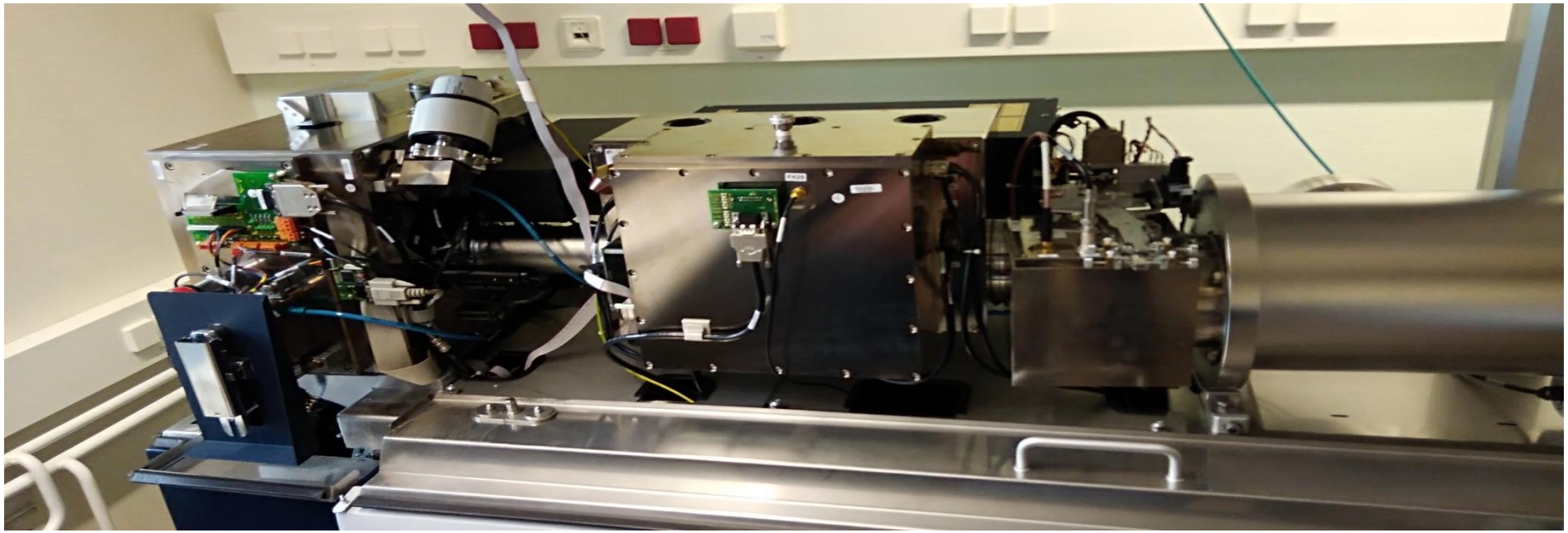
fls

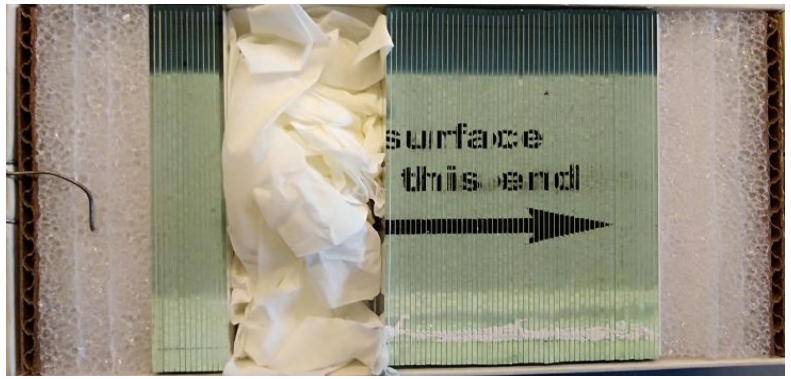
fls x ldox

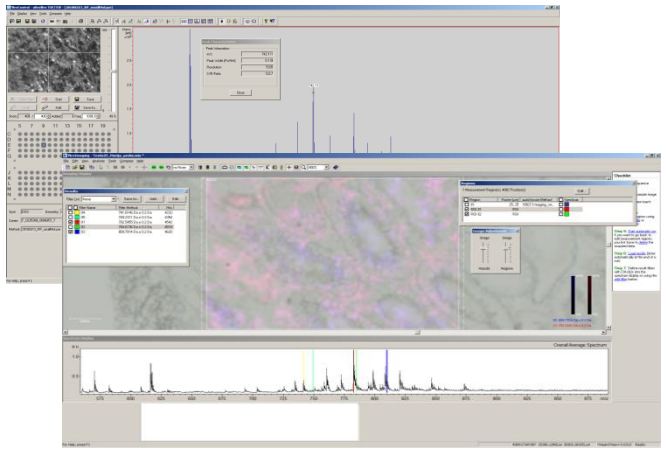
wt

dfr

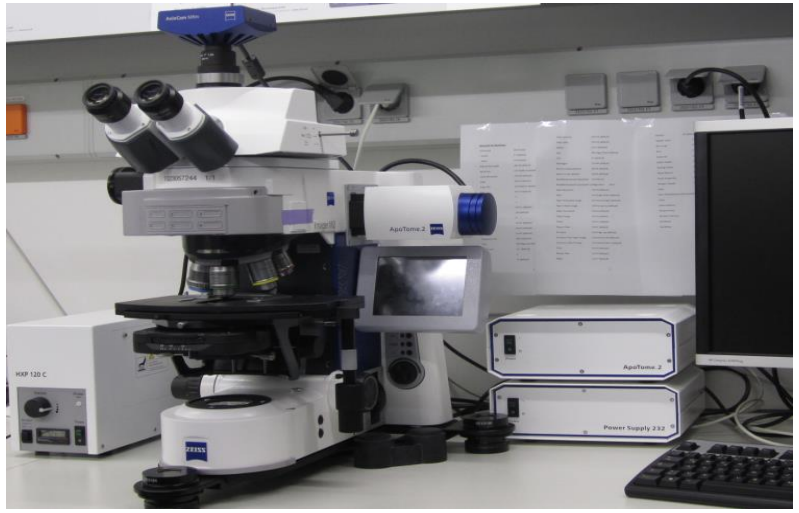
What do we need:

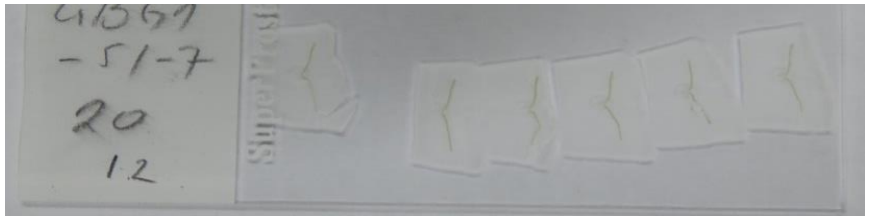


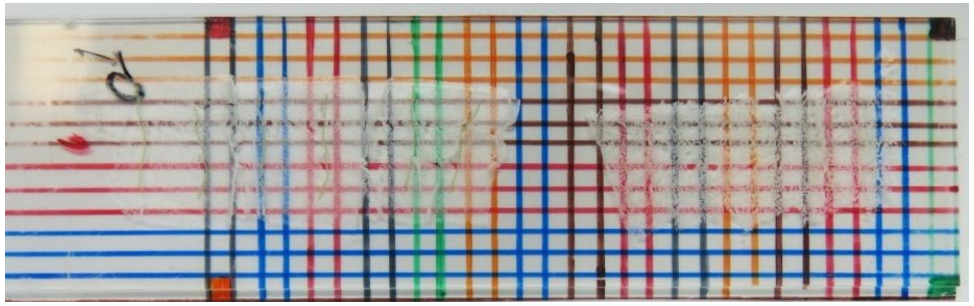




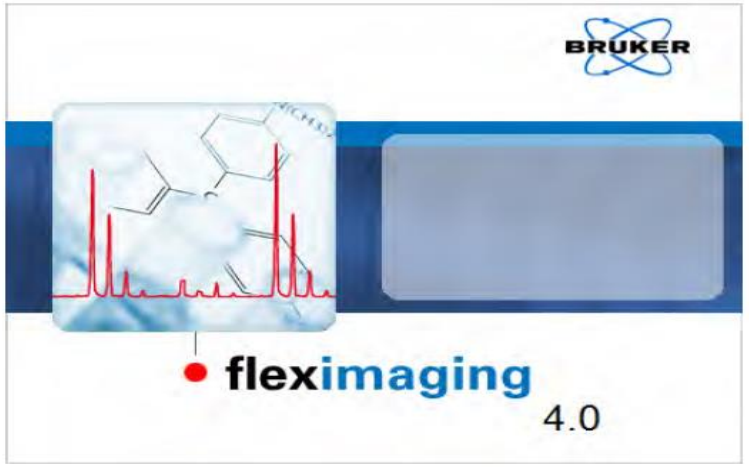
What do we need:

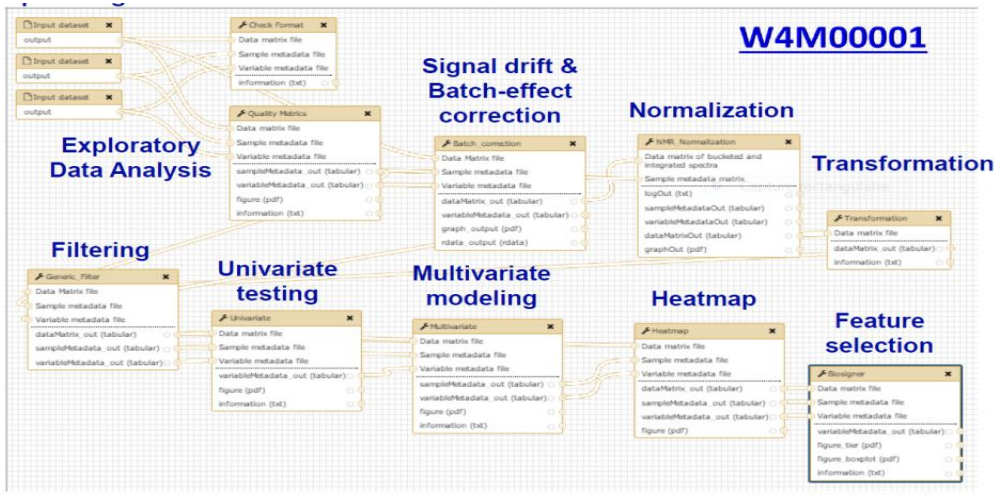




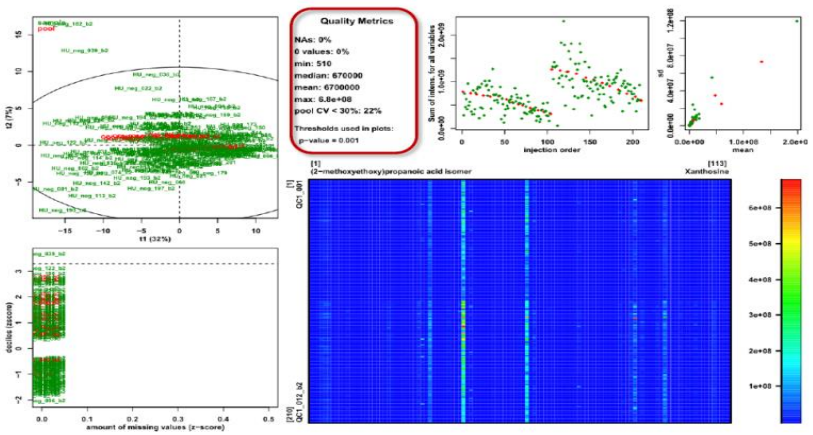


What do we need:



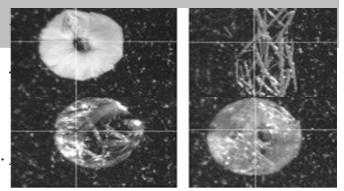


Summary of the intensities in the dataMatrix



Workflow

Complications:
- physico-chem. prop.
- spectral complex.
- ionization prop.



Luteolin
Q-3-O-**R**ut
K-3-O-Rut
Isovitexin
Saponarin

MALDI MSI

Embedding
↓
Sectioning
↓
Inspection
↓
Matrix deposition
↓
MALDI MSI
↓
Compound annotation

MALDI MS

Selection of a matrix solution
↓
Selection of an embedding medium
↓
Identifying the referent m/z fragments in standard polyphenol solutions
↓
Annotation of polyphenols in plant extracts

LC/MS-MS

Defining a fragmentation pattern of polyphenol standard solution
↓
Annotation of polyphenols in plant extracts

Complications:
- decoration pattern
- isobaric isomers
- matrix-influence
- interferes/chl
- ion suppression