# Advances in Mass Spectrometry:

# Methods for structural biology and biophysics

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FELIX Free Electron Lasers for Infrared eXperiments

Master course Advances in MS - lecture 7

Radboud University

## Course Layout – biophysics, structural biology

**MS-based structural biology**

1) Intro / Bottom-up and Top-down techniques (for more info see Prof. Barran's lecture)
2) How to weigh a protein and determine its mass
3) Cross linking
4) FPOP

*also seen as biophysics*

**MS-based biophysics**

1) MALDI / MS-imaging
2) Medical applications

**Background literature structural biology & MS:**

- Albert Heck et al. the EMBO journal 35, 2634 (2016)
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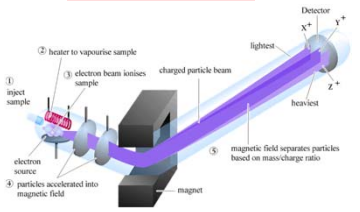
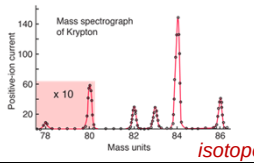
# MS for small and large molecules/complexes

## MS for physics



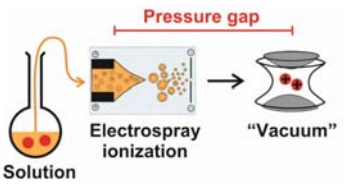

- EI to charge atoms (molecules)
- m/z ration by trajectories in electric/magnetic field




*isotope physics*

## MS for chemistry

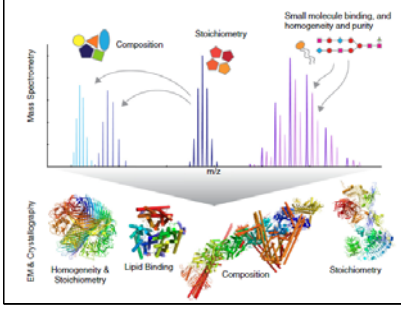
- Identify substances on accurate mass (first: crude oil)
- Chemical ionization: organic molecules into gas phase
- Uni- and bimolecular chemistry
- Tandem MS (CID), kinetics…
- IMS, spectroscopy: structure info




*complete chemical laboratory*

## MS for structural biology

- Introduction of MALDI and ESI: intact peptides and proteins
- Coupling with LC or electrophoresis
- Bottom-up vs Top-down

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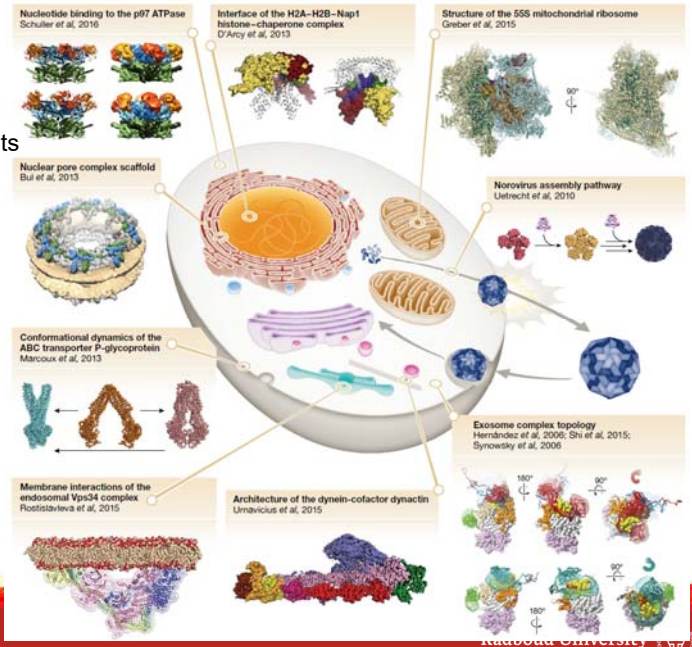
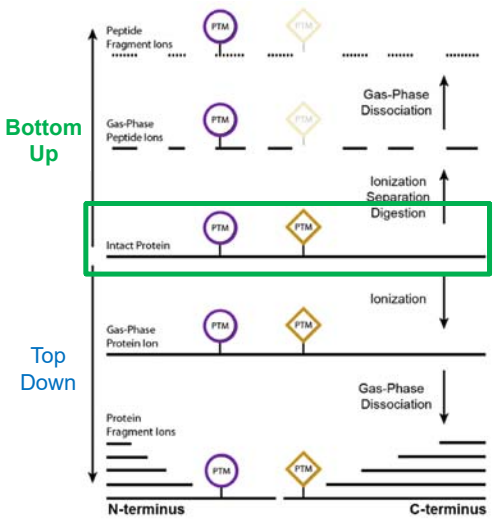
# MS-based molecular and structural biology

- Understanding large biomolecular assemblies
- Biomolecular complexes from all cellular compartments
  - Membrane interactions
  - Conformational (functional) dynamics
  - Nucleotide (ATP) binding
  - Structure of mitochondrial ribosome
  - Virus assembly (norovirus)




Heck et al. the EMBO journal **35**, 2634 (2016)

# Complementary MS-based methods




**Bottom-up:**

- Probe biomolecular structures in solution
- Proteins are manipulated in solution
  - chemical cross-linking
  - surface labeling (HDX)
  - limited proteolysis

*prior to their introduction to the MS*

- Proteins are digested into peptides
- LC-tandem MS to detect and identify resulting peptides
  - *Detection of digested peptides*
  - *Fragmentation of digested peptides*

**Goal:**

- to determine the structure of a protein
- to identify conformations
- interactions between proteins, proteins-nucleic acids
- ligand binding

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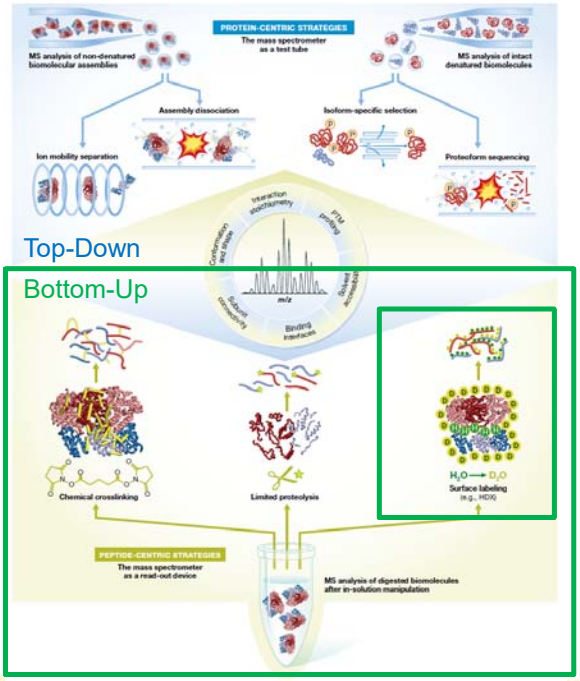
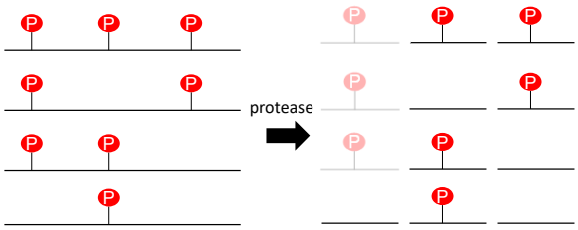





# Advantages /Disadvantages of bottom-up MS

- lots of (detailed) information on the structure and function of biomolecular systems
- Key driver for technological development in biomolecular mass spectrometry
- regions of the protein may not be identified
  = leave behind important info regarding PTMs




Problem:

- missing peptides
- difficult to quantify proteoforms
- combinatorial effect between PTMs is lost

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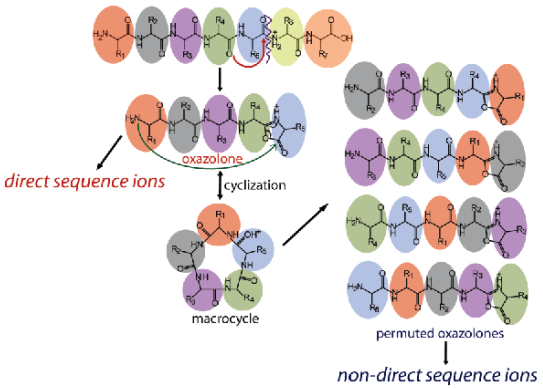
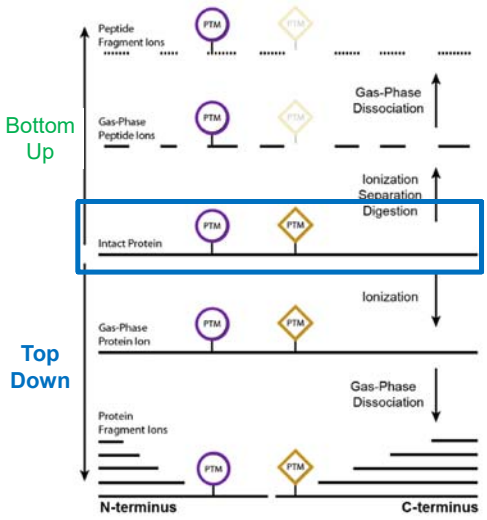
- lots of (detailed) information on the structure and function of biomolecular systems
- Key driver for technological development in biomolecular mass spectrometry
- regions of the protein may not be identified
  = leave behind important info regarding PTMs
- Peptide or even several peptides may not be specific to a protein






## Complementary MS-based methods




**Top-Down (protein-centric MS) :**

- Introducing the intact protein into the MS
- Identification of original protein
  - Detection of intact masses (m/z)
  - Detection of fragments (and secondary/tertiary etc)
- Info: composition and binding stoichiometry

# Top-down sequencing?

**Top-Down:**
- Introducing the intact protein into the MS
- Identification of original protein
  - Detection of intact masses (m/z)
  - Detection of fragments (and secondary/tertiary etc)

**Advantages**
- not limited by (1) digestion efficiency, (2) peptide ion detectability
- information about the whole protein
- combinatorial effect between PTMs remains intact
- comparable ionization efficiencies (quantification)
- assess conformational changes using ion mobility–mass spectrometry (IM-MS)

**disadvantages**
- Depends on efficiency fragmentation of peptide backbone
- More difficult for intact proteins than peptides
  Hence: fragmentation primarily happens in denatured regions
- sequence coverage << 100 %
- higher amounts of sample
- requires high mass resolution

How to improve this???



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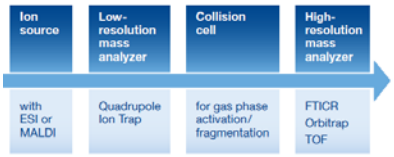
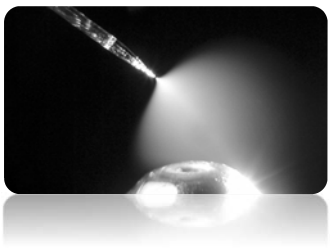
# MS of peptides and proteins: How to weigh a protein?

- MS: determine compound mass with unprecedented accuracy and precision
- MS experiment:
  - Analyte ionization ESI, MALDI



**ESI Process:**
Formation of small charged droplets
Solvent loss
Coulomb explosions
Desolvatation

**Nano Electrospray:**
Formation of smaller droplets
= faster desolvatation
= higher ion yields



# MS of peptides and proteins: How to weigh a protein?

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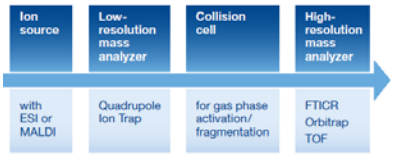
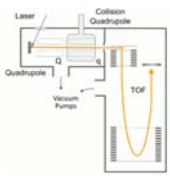
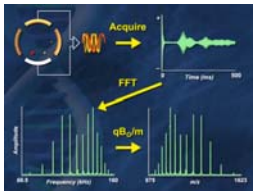
- MS experiment:
  - Analyte ionization ESI, MALDI
  - Mass determination TOF, FTICR, orbitraps



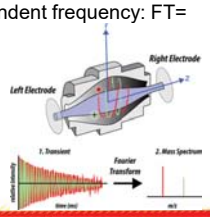

**Time of flight MS**
- Ions travel a fixed distance in a TOF tube
- Flight time – direct measure to determine their m/z




**FT-ICR**
- Ions are trapped in magnetic field and excited by an electric field oscillating at radio frequency
- ions rotate at an m/z-dependent frequency
- rotational motion is detected
- time – frequency: FT to m/z domain

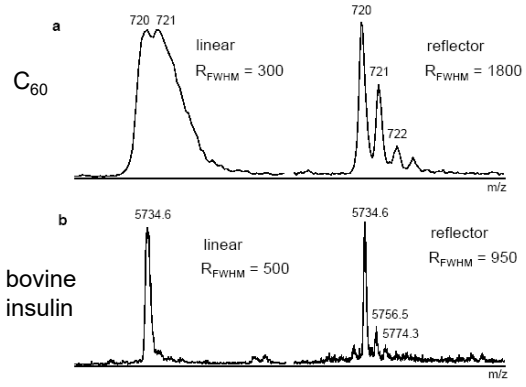
**Orbitrap**
- same principles as FTICR (no magnetic field)
- Ions are trapped by electric field between an outside barrel-like inner spindle-like electrode
- Ions oscillate along inner electrode
- oscillations: m/z dependent frequency: FT= m/z spectrum



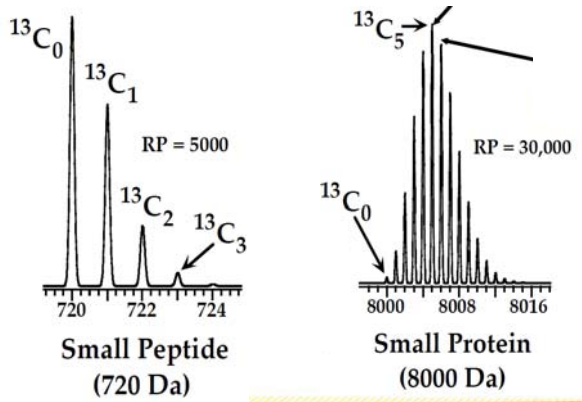
## How to weigh a protein: Resolution and Resolving power

**Resolution**: $\frac{m}{\Delta m}$ with m= mass of the ion (m/z) and Δm= peak width at FWHM (or max width the still have separation)
**Resolving power:** the instrument's ability to distinguish two adjacent ions with a small difference in m/z

linear versus reflector TOF




Intact protein analysis requires high resolving power






## How to determine the molecular mass from ESI spectra?

Time-of-flight: $t = \frac{L}{\sqrt{2U}}\sqrt{\frac{m}{z}}$

In general:

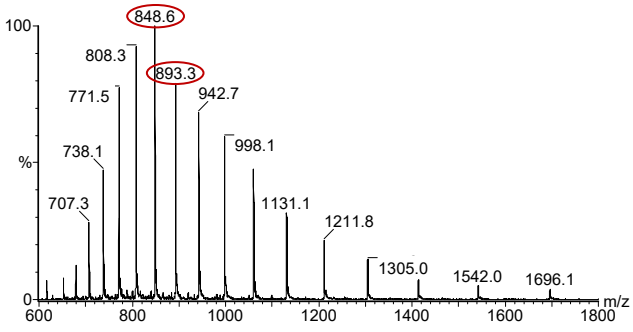
Consider adjacent peaks in a series corresponding to multiply protonated ions:

**Peak 1:**
mass/charge, $x_1$ = (M+n) / n
*where n = number of protons*

**Peak 2:**
mass/charge, $x_2$ = (M+n+1) / (n+1)

**Molecular mass:**
n = ($x_2$ – 1) / ($x_1$ – $x_2$) = (893.3 – 1) / (893.3 – 848.6) = 20

Mass = ~16972



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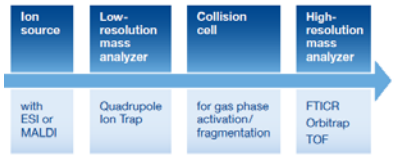
- MS: determine compound mass with unprecedented accuracy and precision
- MS experiment:
  - Analyte ionization — ESI, MALDI
  - Mass determination — TOF, FTICR, orbitraps
  - Selective manipulation — tandem-MS: CID (ETD, IRMPD, UVPD,…)





Gas-phase fragmentation of peptides - nomenclature

Peptide fragmentation channels:
- a–c describe N terminal fragment ions
- x–z describe C-terminal fragment ions

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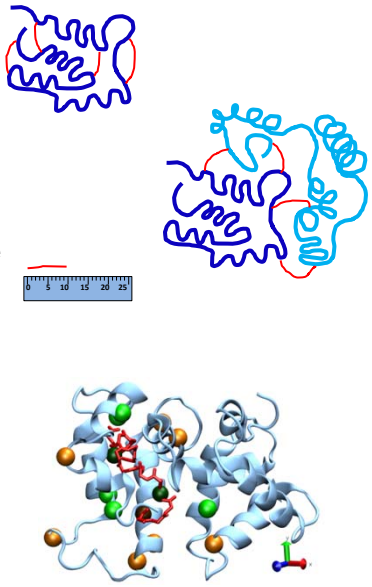
## Cross-linking MS

- Introduce covalent bonds within a protein complex by a chemical reagent
- The protein complex structure is fixed covalently
- Amino acids within the protein complex are bridged by the cross-linker. The cross-linker acts as a “molecular ruler“
- Use distance constraints to create a 3D- structure model of the protein complex

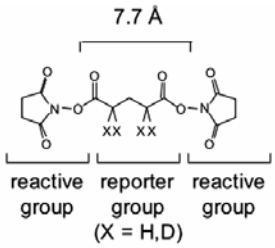


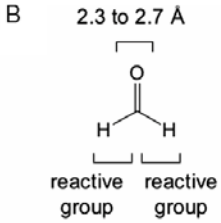




## The cross-linker

- Chemical reagents
- two reactive head groups
  = reactive to specific amino acid residues
- Spacer arm
  = defined length
  = connecting the two functional groups
  = molecular rules
- Introduce **covalent interactions** between functional groups of amino acid side chains
  = primary amines (N-terminus, Lys side chains)



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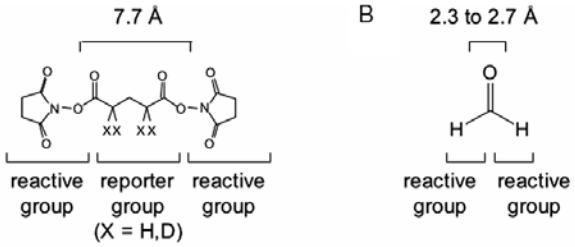
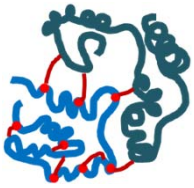
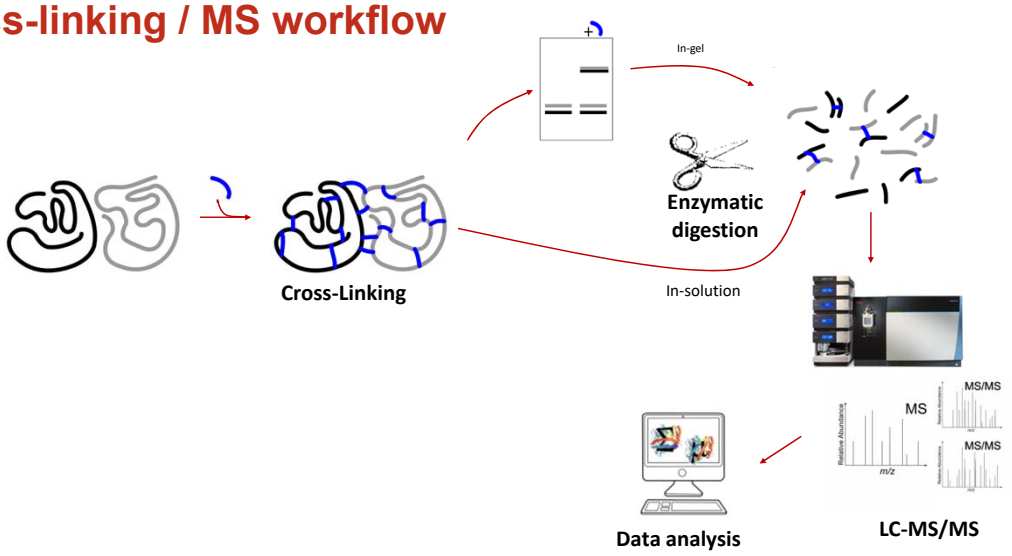
- Two residues only X-linked if mutual distance can be bridged
- X-links impose distance constraints on system



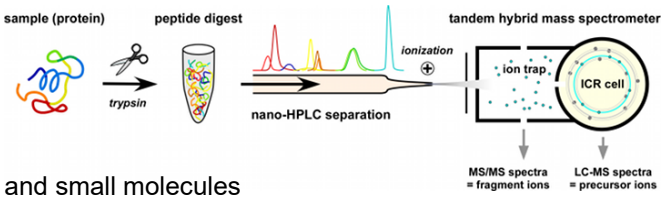





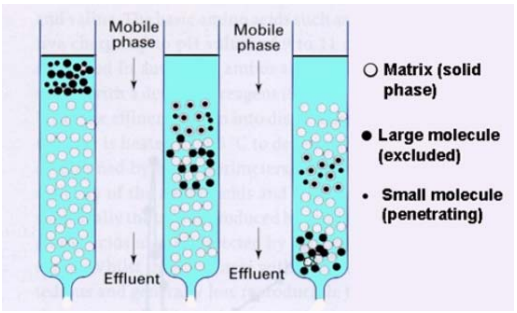
## Cross-linking / MS workflow

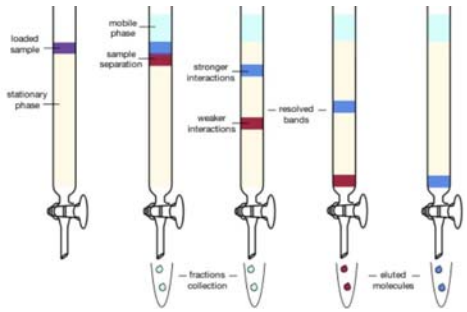


## Liquid Chromatography




- Used for separation of intact proteins, peptides and small molecules
- Separation based if interaction between protein wrt mobile / stationary phase

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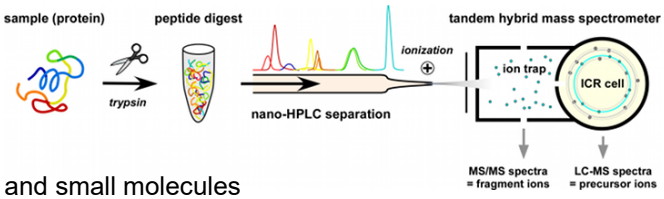
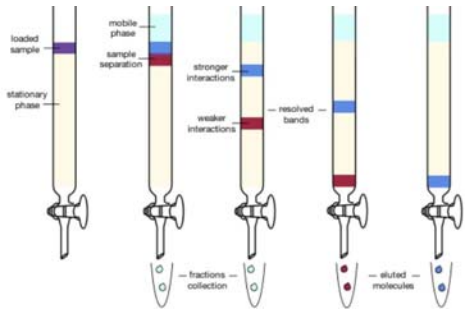



- Used for separation of intact proteins, peptides and small molecules
- Separation based if interaction between protein wrt mobile / stationary phase
- Reversed-phase LC
  - Non-polar stationary phase
  - Polar mobile phase
  - **Most hydrophilic analytes elute first**
- HILIC (hydrophobic interaction LC)
  - Polar stationary phase
  - Mobile: gradients of increasing water content
  - **Hydrophobic species elute first**

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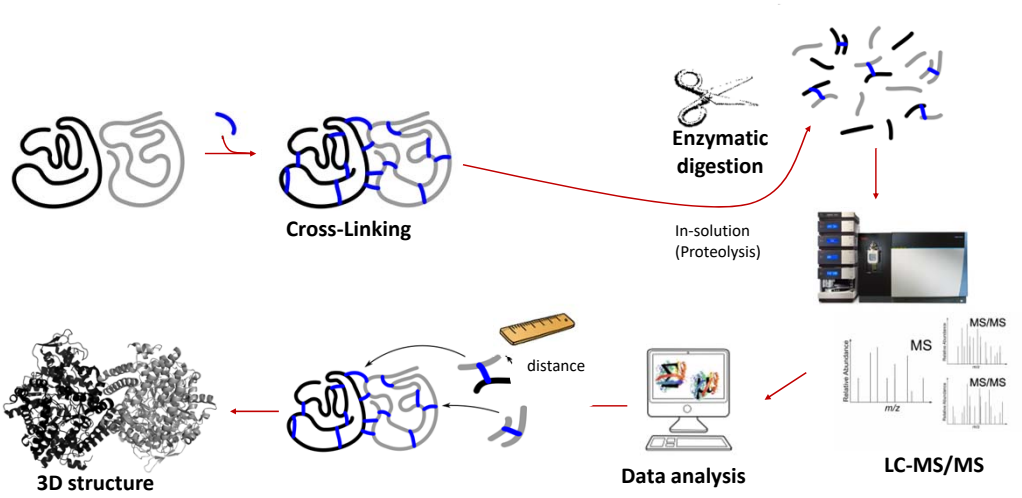
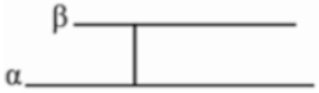
## Cross-linking / MS workflow






## Identification of cross-linked peptides

| Type 0 | Type 1 | Type 2 |
|---|---|---|
| "Dead-end", "mono-link" | Intrapeptide ("loop"-link) | Interpeptide |
|  |  |   |

Cross-link
- one reactive group reacted
- other has been hydrolyzed or reacted with quenching solvent

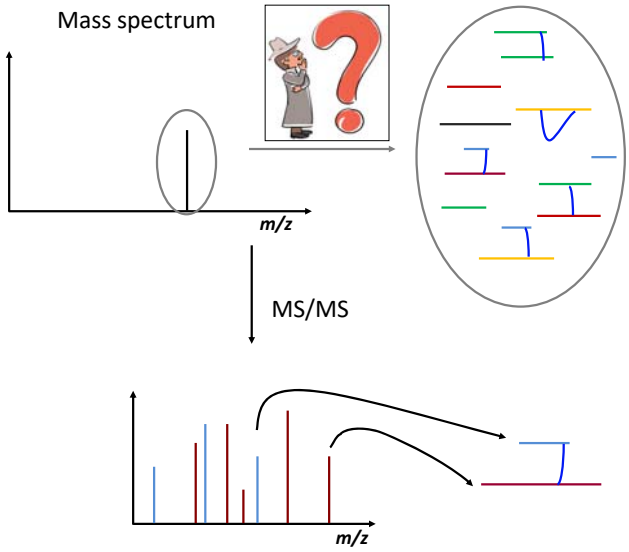
Mass Spec shows:
- Peptide where one amino acid is modified by cross-linker

Cross-link
- Connection of two neighboring amino acids within one peptide

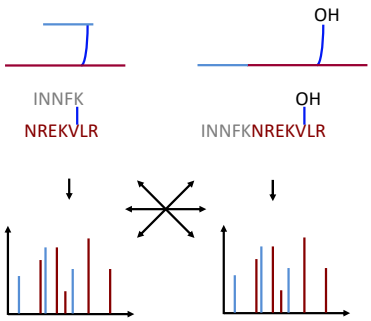
Cross-link
- Connects two peptides originating either from one protein or from interacting proteins

**Which one is the most informative for the tertiary structure?**

## Analysis of cross-linked products

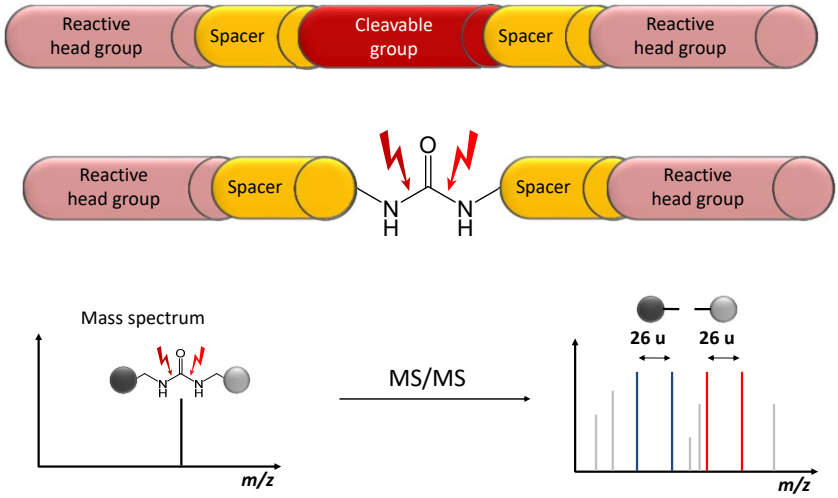



But how differentiate between isobaric species?

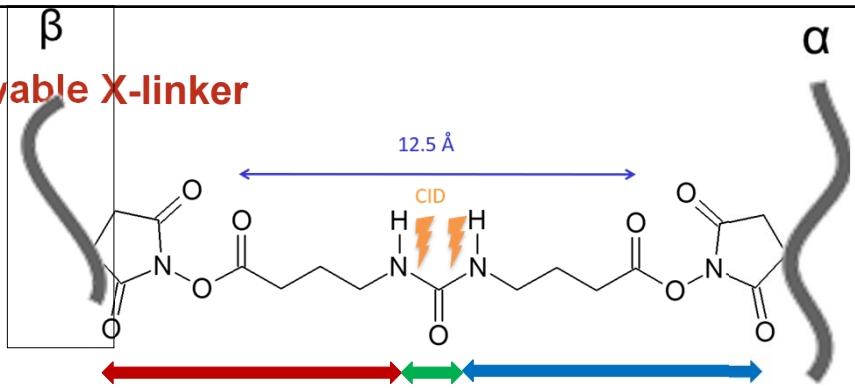


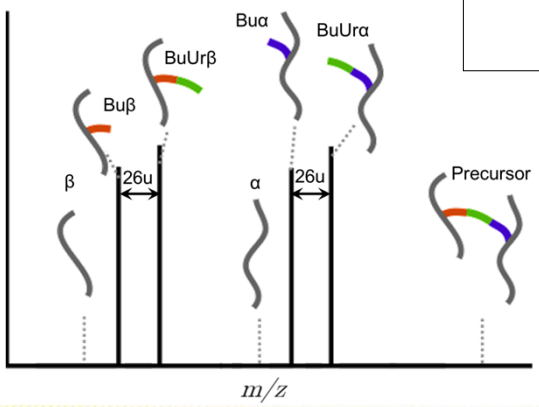



## MS-cleavable linker



## Example of CID-cleavable X-linker




MS-MS:
= doublet - cleavable group mass
= Note: you don't always observe all four peaks

Arlt, C., Götze, M., Ihling, C.H., Hage, C., Schäfer, M., Sinz, A., *Anal. Chem.* 88, 7930-7937 (2016)

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**MS-based biophysics**

1) MALDI / MS-imaging
2) Medical applications

**Background literature structural biology & MS:**

- Albert Heck et al. the EMBO journal 35, 2634 (2016)
- Neil Kelleher et al. Annu. Rev. Anal. Chem. 2016. 9:499–519
- Andrea Sinz et al. Adv Exp Med Biol. 1105:101-121 (2018)
- Lisa Jones et al. J. Biol.Chem. (2019) doi:10.1074/jbc.REV119.006218

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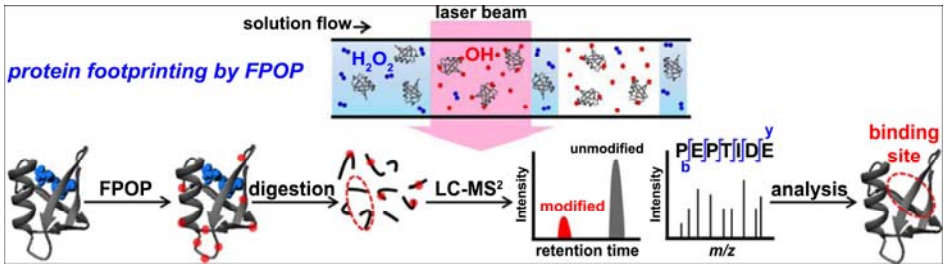
## Fast Photochemical Oxidation of Proteins (FPOP)

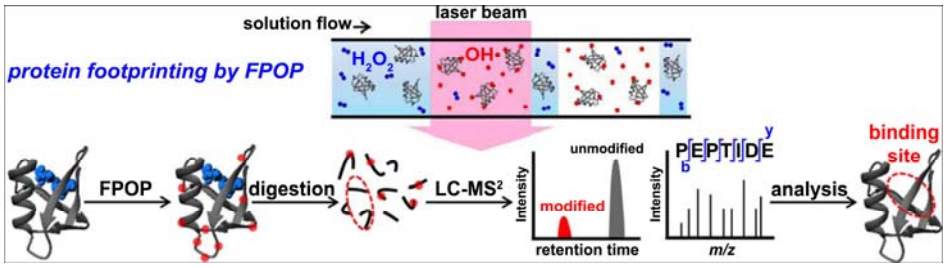
- MS-based foot printing method
  - Protein structures
  - Interactions
  - conformations
  - Protein folding
  - In vivo and in vitro
- Irreversible labeling of solvent exposed AA side chains by OH radicals
- Residue level resolution of protein structures
- Microsecond timescale





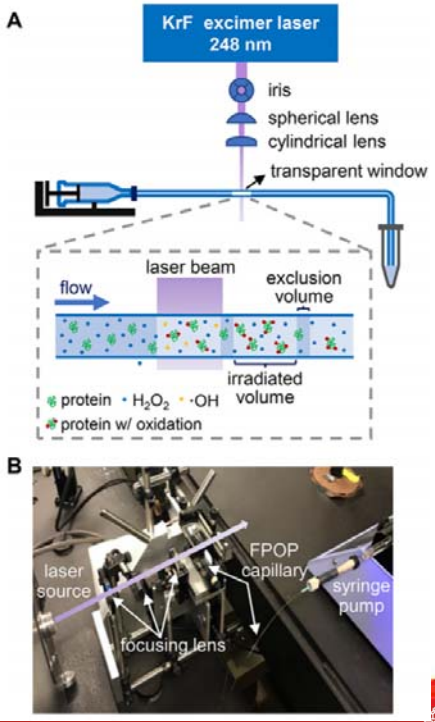

## Fast Photochemical Oxidation of Proteins (FPOP)

- FPOP
  - Hydroxide radical formation from H2O2
  - Laser activation
  - Formed •OH irreversible modification on protein
  - Labeling/modify side chains of amino acids



## FPOP experiment (1)

- Solution of protein and $H_2O_2$ + scavenger (mixed before or via T-junction)
- 248 nm KrF laser to generate hydroxyl radicals
- Laser focused: expose 2-3 mm of ~200 micron capillary (flow tube)
- Sample irradiated, laser (high flux) ensures max. yield of •OH
- Photolysis of $H_2O_2$ into •OH in nanosecond (laser pulse duration)
- Flowrate / laser pulse sync: each protein irradiated once
- Afterwards reaction quenched
- On/off: correct for back ground oxidation + avoid double laser shooting

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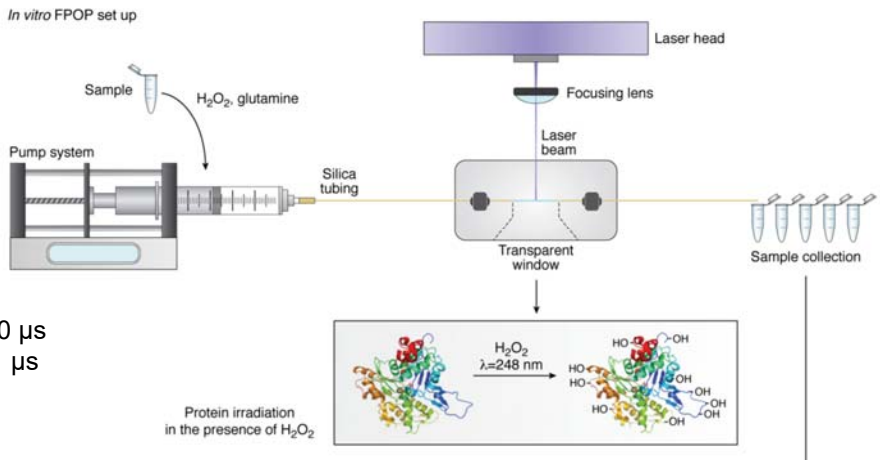
## FPOP experiment (2)

- Outlet FPOP capillary connected to sample collection tube
- Catalase and free methionine in buffer: remove leftover $H_2O_2$
- Exterior of protein foot printed
- Time-scale
  - Life time •OH about 100 µs
  - Scavenger: reduce to 1 µs

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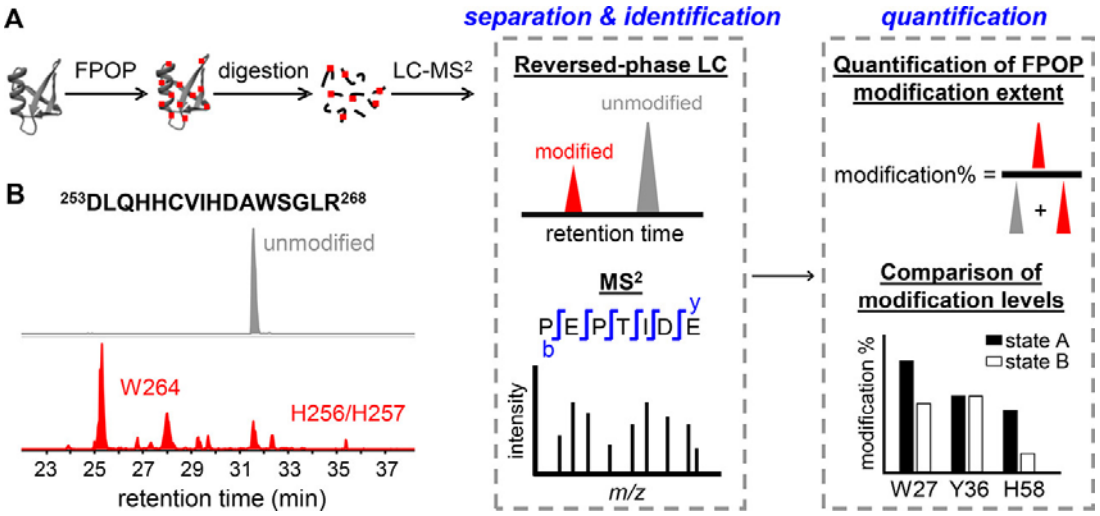
## Post-FPOP workflow




*Acc. Chem. Res.* 2018, 51, 3, 736-744

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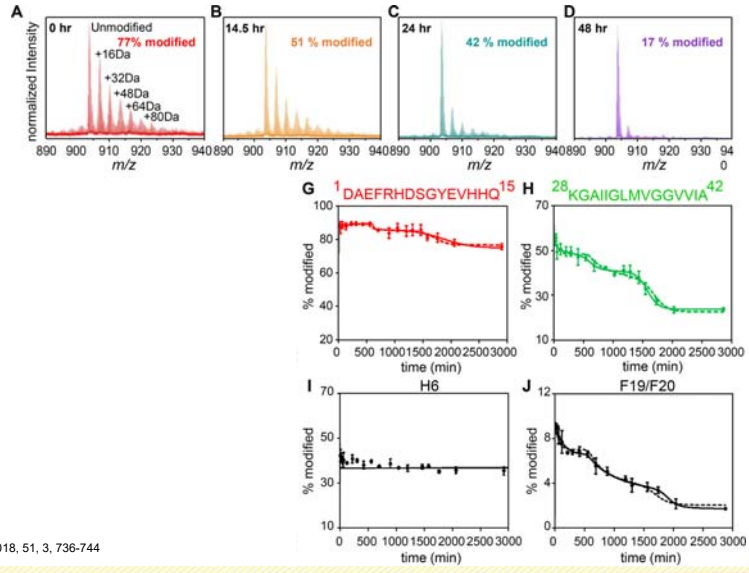
## An example: Protein aggregation




*Michael Gross et al, Acc. Chem. Res.* 2018, 51, 3, 736-744

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2) Medical applications

**Background literature MSI:**
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Special thanks to prof. Claire Eyers (Liverpool) and Andrea Sinz (Halle) for sharing slides of their work

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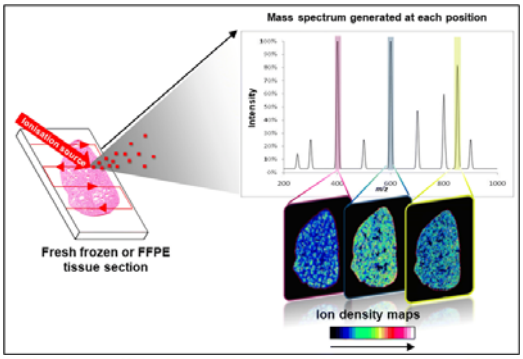
## Mass Spectrometry Imaging

- MS imaging (MSI) research areas
  - Biomedical
    - Oncology
    - Neurological disorders
    - …
  - Pharmaceutical research
    - Drug distribution
    - Metabolite distribution
  - Biomarker

- Microscopy technique
- Label free, multiplex technique
- Spatial distribution: visualize 2D (and 3D) molecular distribution
- Analyze tissue sections

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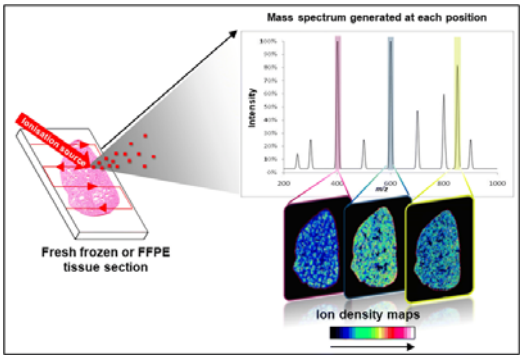
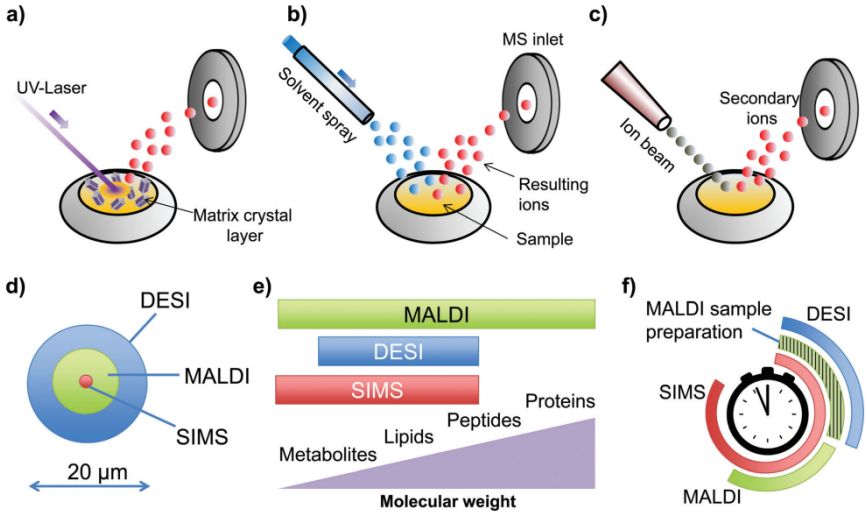
## Mass Spectrometry Imaging

- Visualize spatial distribution of molecules by molecular mass
- Collecting mass spectrum of selected spot, move to next spot, etc.
- By selecting a m/z of interest: MS data converted into distribution map across the sample



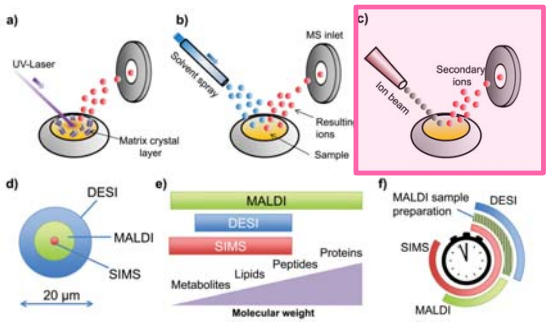


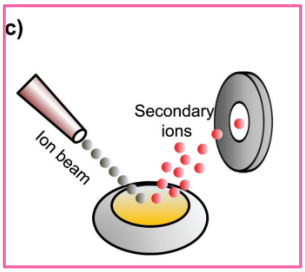

## MSI techniques



## MSI techniques (1)

- **SIMS (secondary ion mass spectrometry)**
  - Focused primary ion beam (pulsed) moves across surface
  - Secondary ions are generated locally
  - Secondary mass spectra recorded
  - Used for elemental and molecular composition of surfaces and spatial resolutions in subcellular scale
  - SIMS: highest spatial resolution

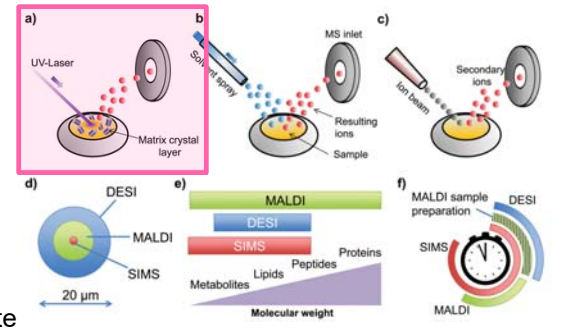


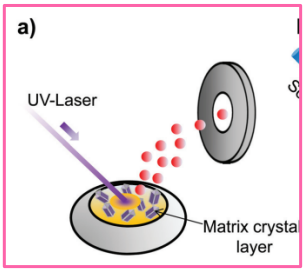






## MSI techniques (2)

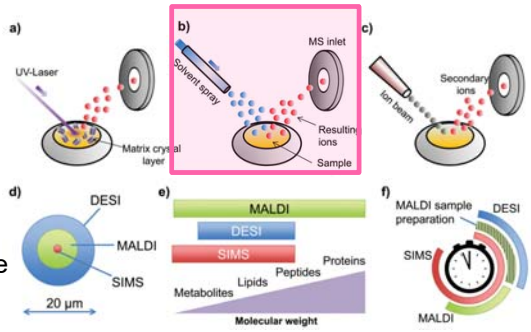
- **MALDI (Matrix Assisted Laser Desorption Ionization)**
  - Used for biological tissue sections
  - Soft ionization technique
  - Laser to probe a specific section of a surface
  - Use of matrix
    - to act as a mediator between laser energy and analyte
    - To promote ionization
  - Sample preparation: homogeneous matrix deposition
  - MALDI-MSI: highest versatility in mass range (thus large variety of samples possible)

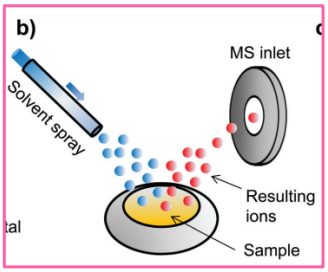


## MSI techniques (3)

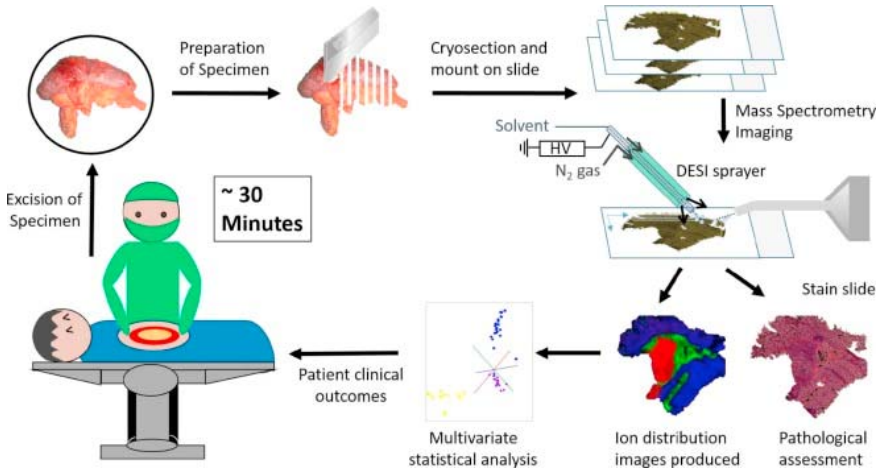
- DESI (Desorption ElectroSpray Ionization)
  - Imaging under ambient conditions
  - Approach:
    - Solvent of charged droplets is directed towards surface
    - Desorption of analyte
    - Ionization similar fashion ESI
  - Spatial resolution: diameter of aerosol hitting surface of sample
  - scanning:
    - Spray continuously, sample stage continuously
    - Horizontal resolution: defined analyzer scan time
  - DESI fastest method
  - Mainly focuses on small molecule reporters
    - Metabolites, neurotransmitters, drugs, lipids






## MSI: medical applications



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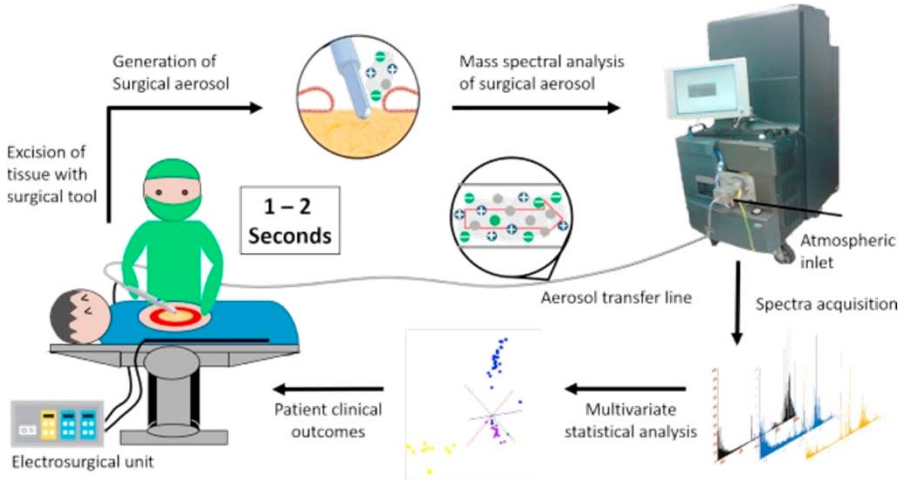


MSI & iknife: https://youtu.be/tHdvxB23U4Y

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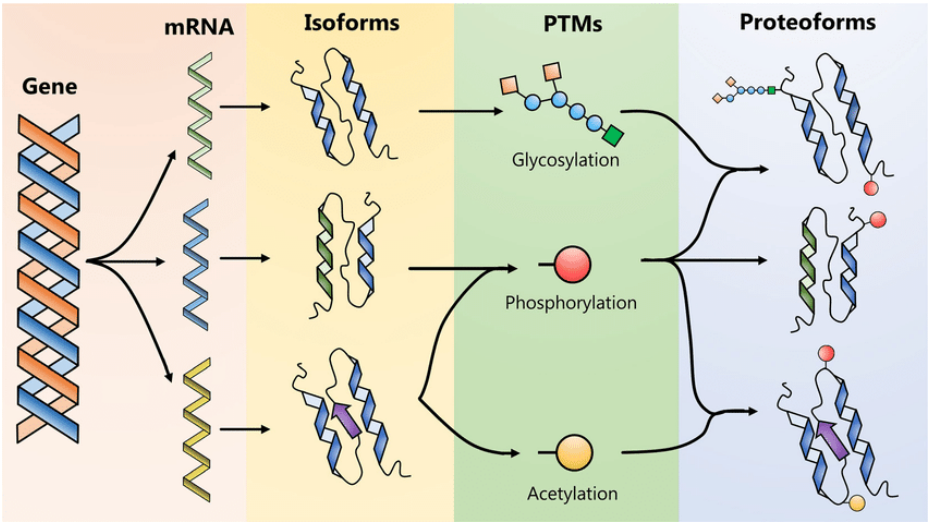
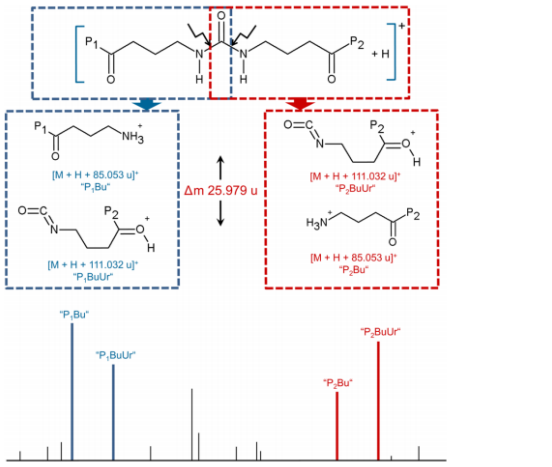
## proteoforms



**Scheme 1. Chemical Structure of BuUrBu and Theoretical Fragmentation Pathway after Cleavage under Collision-Induced Dissociation Conditions (CID- and HCD-MS/MS)[a]**




[a]Bu: γ-aminobutyric acid; BuUr: isocyanate; P1: peptide 1; P2: peptide 2. Doublet patterns ($\Delta m \sim 26$ u) of reporter ions in the fragment ion mass spectra indicate the presence of a cross-link.