Methods of proteomic research.
Proteomics techniques (Part 1)

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Agenda

1. **The basics of mass spectrometry**
   - **Mass spectrometry (MS). Electrospray ionization mass spectrometry (ESI-MS)**
   - **Matrix-assisted laser desorption ionization (MALDI) mass spectrometry**

SAMPLE preparation methods:

2. **Filter aided sample preparation (FASP) - membrane-based method**
3. **Suspension trapping (S-trap) - membrane-based method**
4. **The MStern method**
5. **The solid-phase-enhanced sample preparation (SP3) method**
6. **The in-StageTip (iST) method**
7. **Protein Fragmentation and MS detection techniques:**
   - **Collision-induced dissociation (CID) - Диссоциация, вызванная столкновением**
   - **Electron-transfer dissociation (ETD) - Диссоциация с переносом электронов**

8. Protein quantitation:

Relative quantitation or absolute quantitation

Quantification strategies for peptides can be divided into two broad classes: label free quantification (LFQ) and label based quantification (or label-dependent quantitation) techniques

9. Tandem mass tag (TMT) and isobaric tag for relative and absolute quantitation (iTRAQ),

10. Western blot (protein immunoblot)

11. Immunoassay analysis

Introduction

- The proteome is the collection of proteins present in biofluids, cells and tissues and reflects the functional state of the biological system.
- Proteomics is the quantitative study of the proteome and is often used for contrasting different cellular conditions.

For Example,

- proteomic differences between **virus-infected** and **uninfected cells** would highlight cellular pathways and proteins needed for viral infection and replication.

- Drugs developed to target these proteins could slow down the infection.

The basics of mass spectrometry

- Mass spectrometry (MS)-based proteomics is the most comprehensive approach for the quantitative profiling of proteins, their interactions and modifications.

It is a challenging topic, because requires expertise in:

- biochemistry for sample preparation,
- analytical chemistry for instrumentation and
- computational biology for data analysis.

Since their inception in 1912, **mass spectrometers** have undergone continuous development, and these sophisticated bioanalytical instruments have now reached unrivalled detection limits, speed and diversity in applications.

They detect **the presence** and **abundance of peptides** (or other biomolecules such as metabolites, lipids and proteins**) using** fundamental **properties of molecules**, such as **mass,** and **net charge (суммарный заряд)**. **When peptides obtain a net charge** (usually through gain of protons), they are referred to as **peptide ions**.

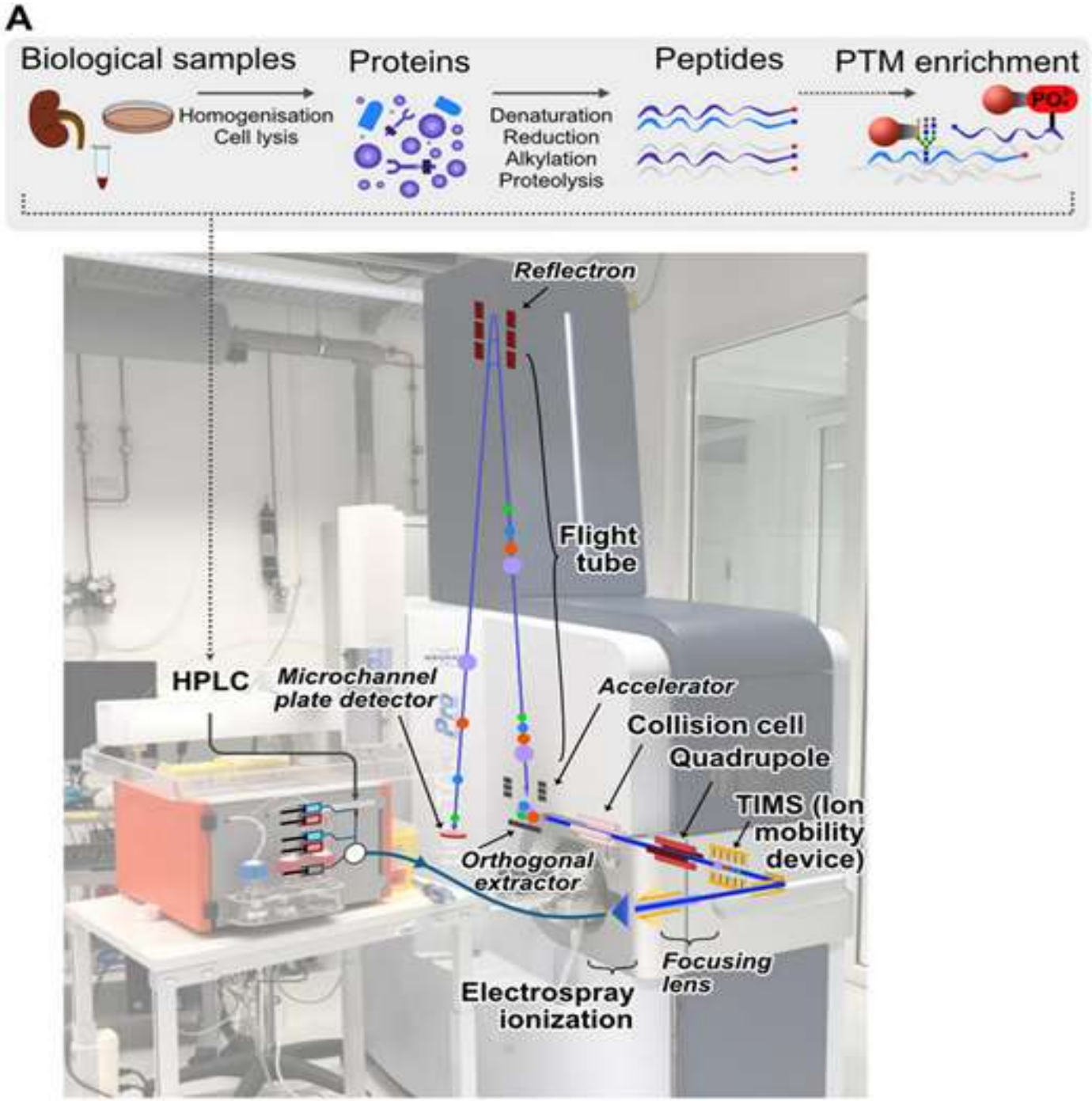
All mass spectrometers have three fundamental components:

- an ion source,
- mass analyser and
- detector (Figure A).

As **mass spectrometers can only analyze gaseous ions**, methods such as electrospray ionization (ESI) are needed **to convert peptides from the liquid phase to gaseous ions**.

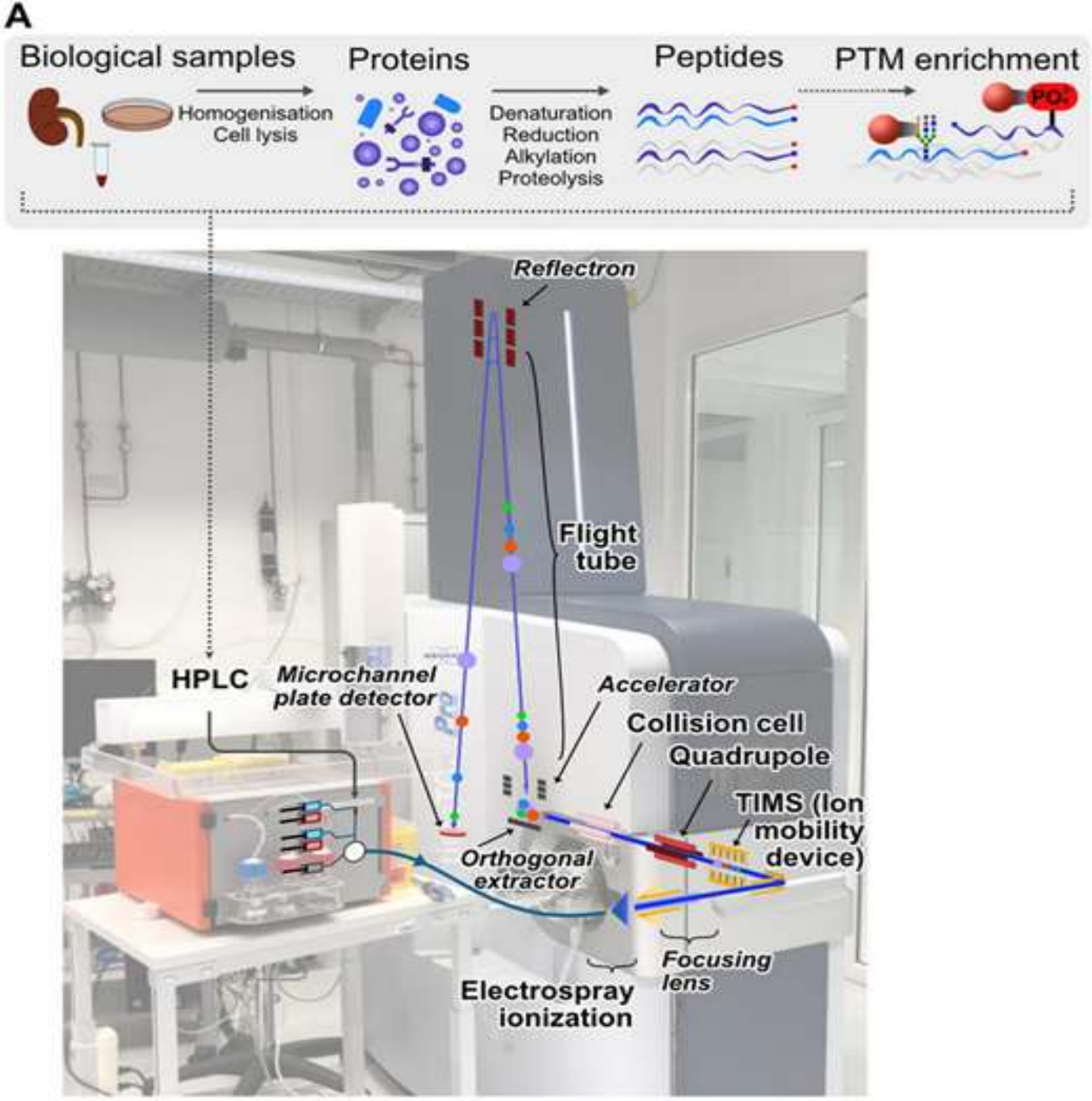
The liquid containing the peptides is pumped through a micrometre-sized orifice (щель) held at a high voltage (2-4 kV).

Upon reaching this emitter, the steady stream of liquid disintegrates into extremely small, highly charged and rapidly evaporating charged droplets, leaving peptide ions in the gas phase.



Proteins are digested into peptides using sequence-specific proteases. Optionally, post-translational modification (PTM)-containing peptides can be enriched using beads with specific surface chemistry or coupled antibodies.
High-performance liquid chromatography (HPLC) separates peptides based on hydrophobicity, and they are subsequently analysed by a TOF mass spectrometer.

The abundance of gaseous peptide ions is proportional to their original concentration, so it is beneficial **to use the lowest flow rates** possible, thereby maximizing sensitivity.
It is common in proteomics to separate peptide mixtures using high-performance liquid chromatography (HPLC) systems with flow rates of only a **few hundred nanolitres per minute** rather than millilitres in conventional HPLC.



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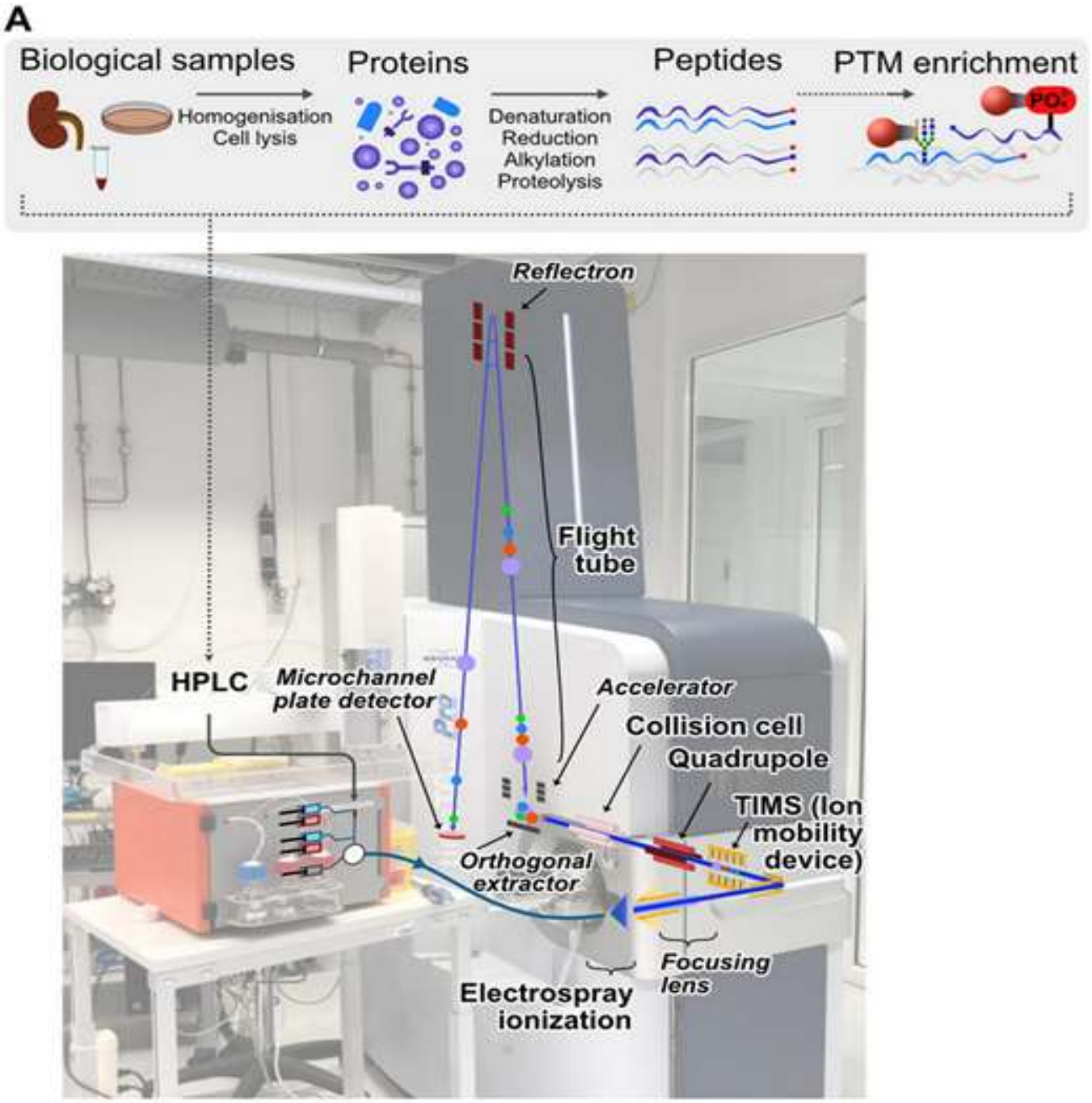
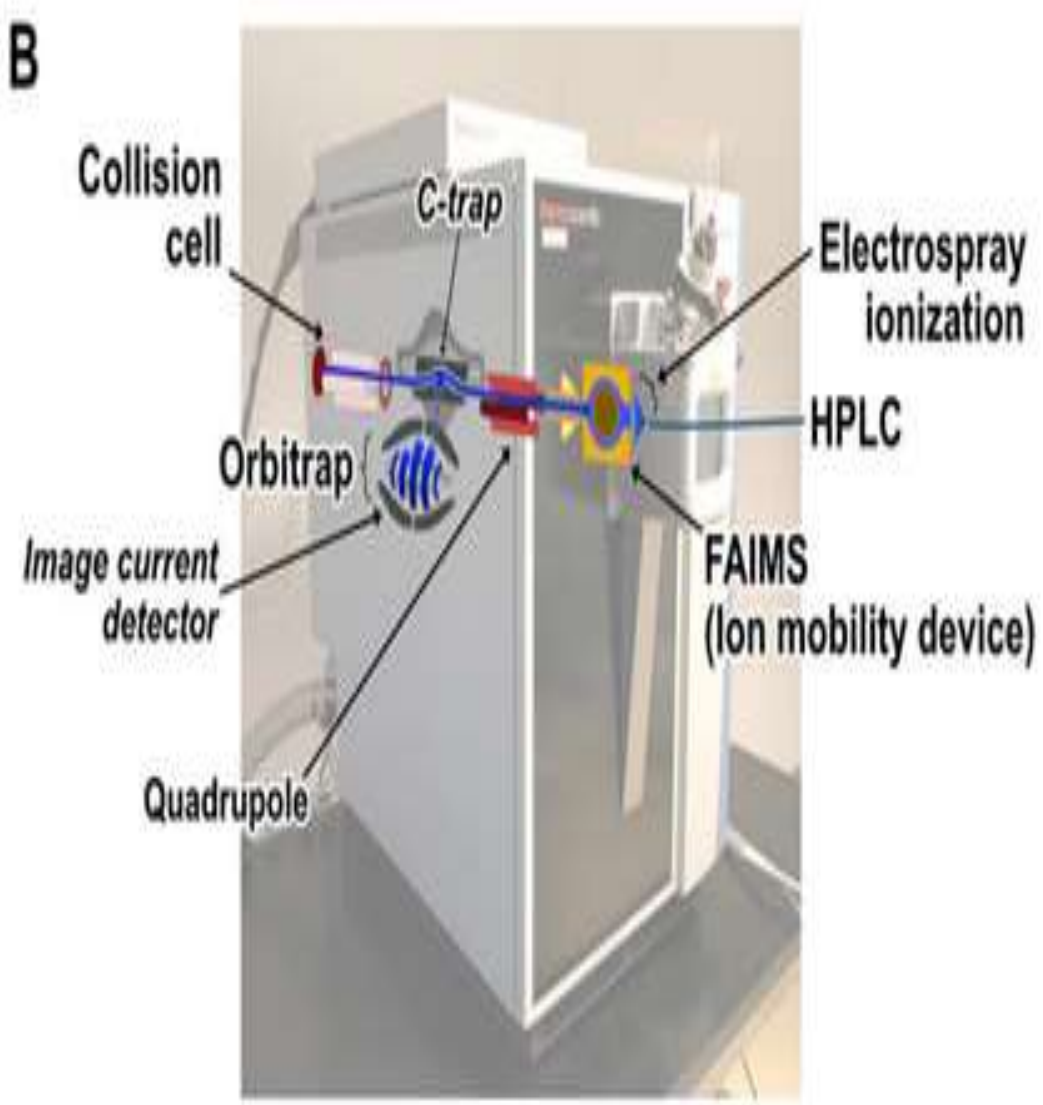


The principal role of a mass analyser is to separate ions by their mass-to-charge ratios (m/z)

- Fundamentally, all ions are separated by modulating their trajectories in electrical fields.
- Mass analysers differ in the principle they use for separating ions, and this defines their preferred application areas.
- Quadrupoles, usually combined with **time-of-flight (TOF)** or **Orbitrap analysers**, are the most common in proteomics.
- Quadrupole mass analysers separate ions using an oscillating electrical field between four cylindrical rods in a parallel arrangement, where each pair of rods produces a radio frequency electrical field with a phase offset.
- The resulting electrical fields define a pseudo-potential surface that is configured to allow the transmission of all ions, or to selectively transmit ions of a specific m/z window.

- **TOF mass analysers separate ions based on the differences in velocities** after acceleration to about 20 kV and subsequent **different arrival times at the detector**. A TOF can measure mass differences of one part per million (ppm) by detecting time differences of sub-microseconds.
- In contrast, the **Orbitrap mass analyser distinguishes ions based on their oscillation frequencies (частота колебаний).**
- Ions are tangentially injected and then trapped in the Orbitrap, and they move along the length axis of a central metal spindle (Figure B).
- Although an Orbitrap is only a few centimetres long, the ions can rapidly travel up to several kilometres, enabling very high resolution (typically tens of thousands) and low ppm mass accuracy.

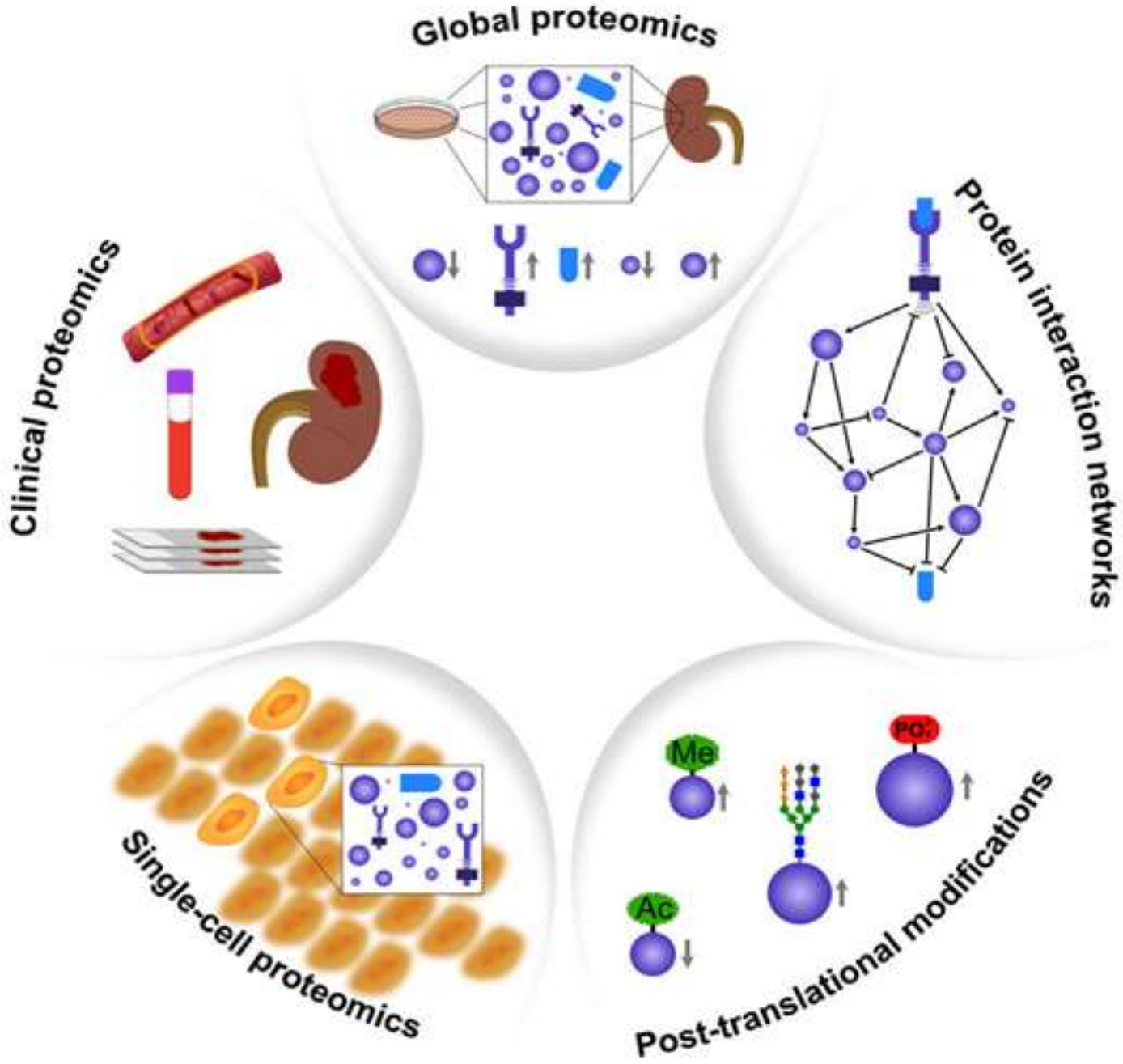


Alternatively, peptides can be analysed by an Orbitrap mass spectrometer, which is a mainstream instrument in proteomics

- In proteomics, the quadrupole element is normally followed by a ‘collision cell’, which is a quadrupole where the ions can be fragmented. Either intact peptide ions or fragment ions enter the final stage that also contains the detector – the resulting spectra are **called MS1** or **precursor ion spectra** in the former case and **MS2 or product or MS/MS spectra** in the latter.
- TOF instruments have microchannel plate (MCP) detectors, where each individual ion ejects electrons from a surface that are then amplified.
- In Orbitrap analysers, the ‘image current’ induced by the rapidly oscillating ions is measured, and it represents a quantitative readout of the strength of the individual ion packages. The current is recorded in the time domain and is converted into the frequency domain using Fourier transformation.

Advances in MS

- Advances in MS have now reached a state where a multitude of conceptually novel applications have become feasible in proteome identification and quantification, protein–protein interactions (interactomics), organellar proteomics, PTM (post-translational modifications -PTMs) detection and many more (Figure 2).
- MS-based proteomics is a more complex technology than antibody-based methods, but its exquisite specificity of detection and global nature more than make up for this.



Clinical sample preparation methods for proteomic analysis

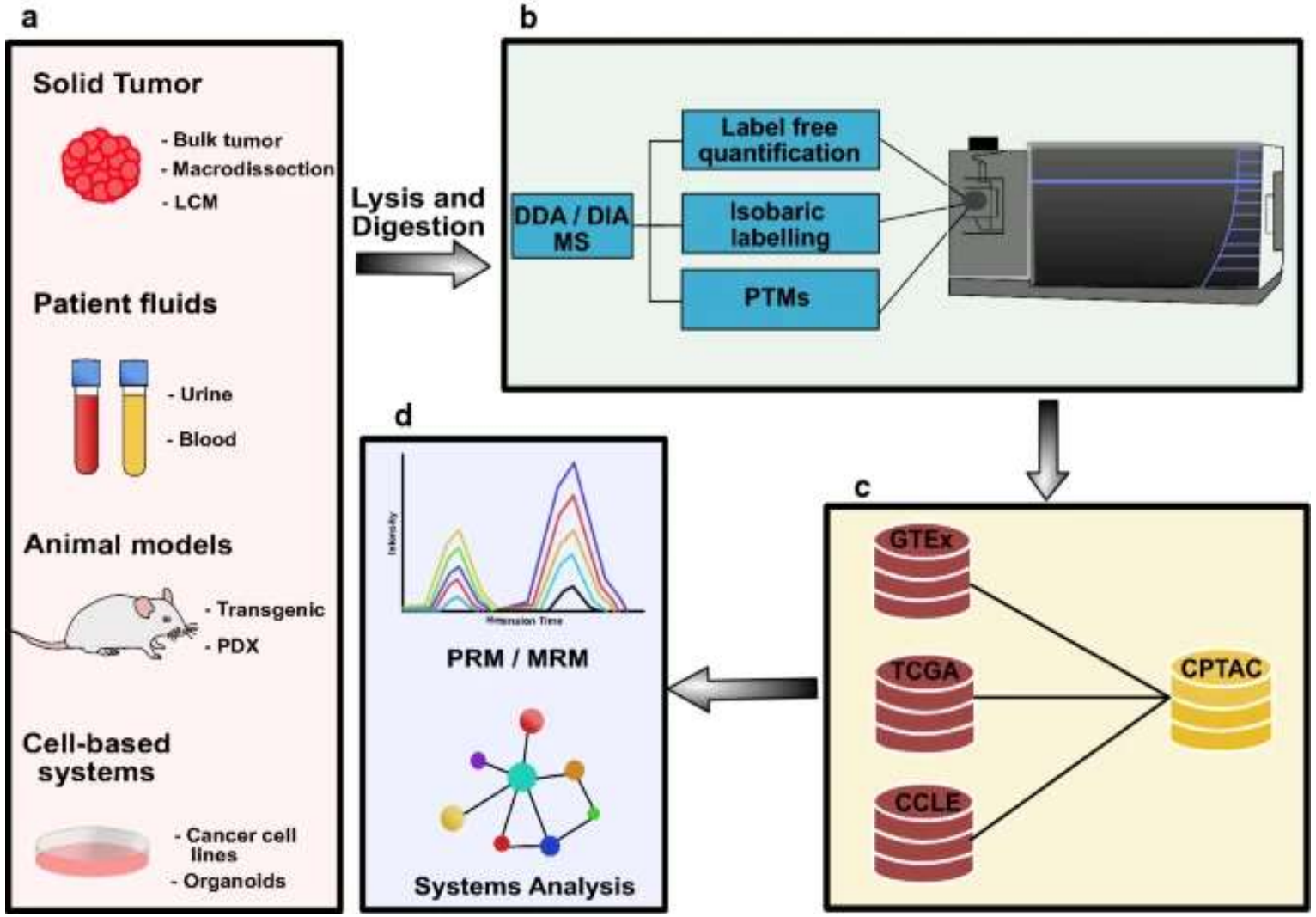
- Preservation of the tissue's proteome dynamics is critical from time of surgical resection to the protein digestion stage, and there are a few methods of doing so:
- - fresh frozen (FF),
- formalin-fixed paraffin embedded (FFPE), and
- optimal cutting temperature embedded (OCT).

More rapid procedures such as **needle biopsies** have the potential to overcome some of these complications but provide significantly lower amounts of tissue for proteome analysis.

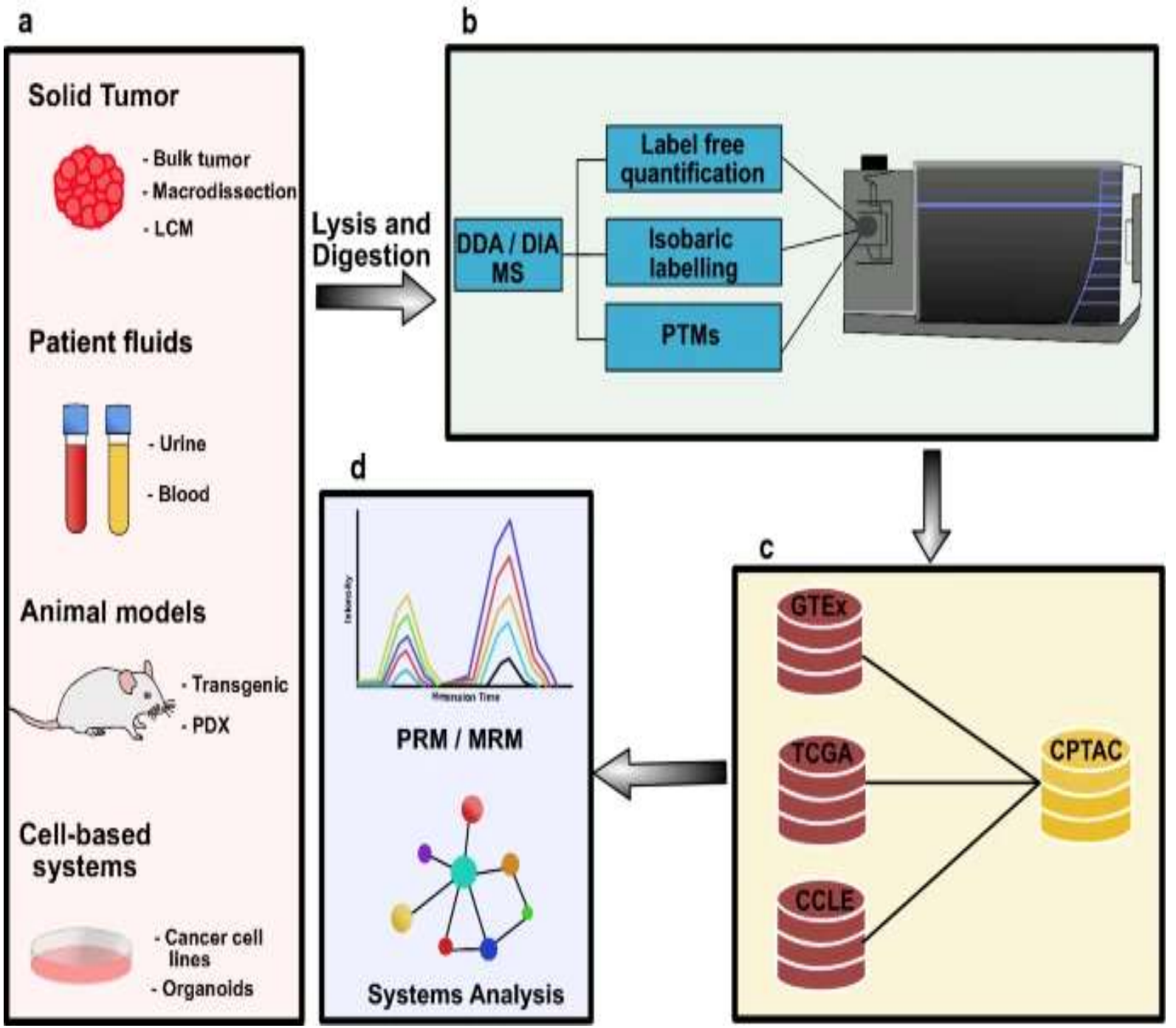
While **FF** is the preservation method of choice from a proteome coverage perspective, **FFPE** tissues have been banked for decades, providing extensive clinical follow-up and an invaluable resource for clinical proteomics.

- Clinical sample cohorts of the past were often underpowered due to biobanking limitations (i.e. availability of high-quality, richly annotated samples).
- As a result, various model systems have been developed to facilitate the discovery of new biomarkers or aid in characterizing proteins of interest, as in Figure 3.

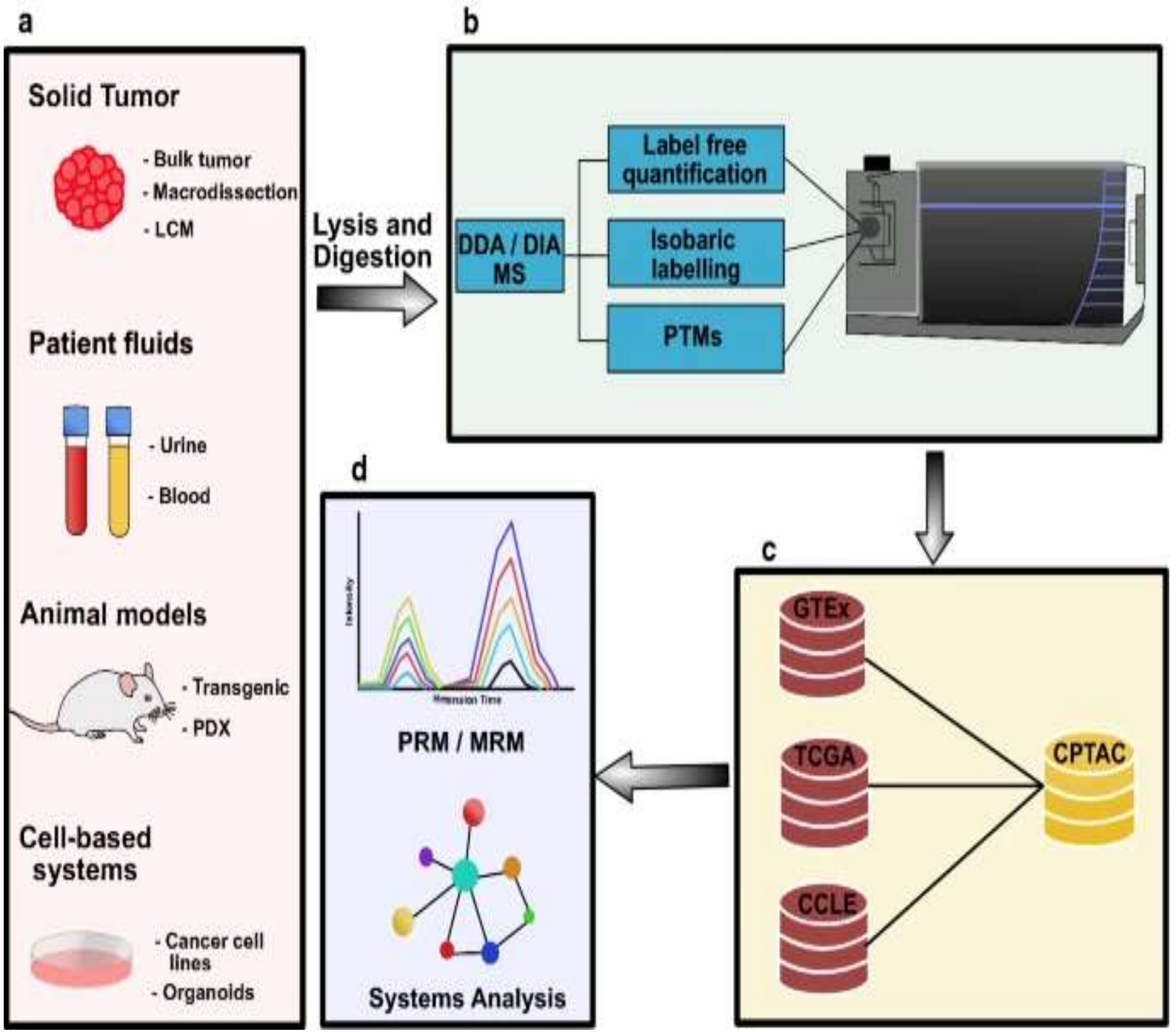
These models include transgenic animal models, immortalized cancer cell lines, primary cell lines etc. Cell lines of various cancer subtypes can be grown in 2D on cell culture dishes, either directly on plastic or on various matrices (i.e. collagen), or under more sophisticated 3D conditions (i.e. embedded in Matrigel).



a) Various sample types are used for clinical proteomics. These include solid tumor tissues, patient body fluids, animal models and cell-based systems. Tumor tissues are obtained either as surgically resected samples or are biopsy based. There are a number of tissue processing approaches available, which include the analysis of “bulk” tissue or preferentially after pathological inspection, tissue macro-dissection or laser capture microdissection (LCM).

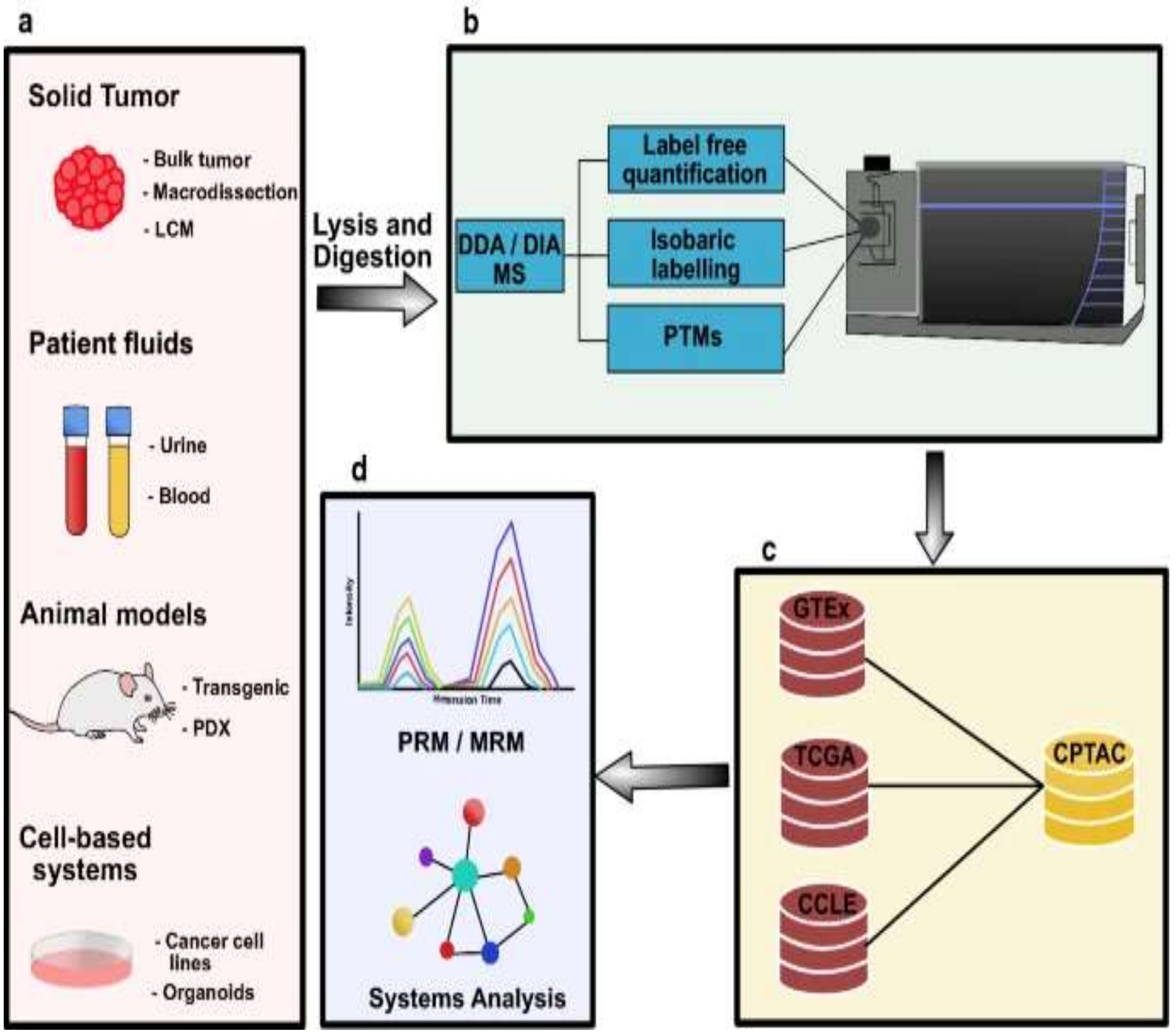


b) Proteomic analyses can use several well-established workflows. These include label-free proteomics (LFQ), isobaric labelling strategies or the specific enrichment of post-translational modification such as phosphorylation, ubiquitination, glycosylation, etc.



c) Integration of proteomics data with publicly available resources such as the CPTAC proteomics data or transcriptional profiles from GTEx, CCLE and TCGA can be used for biomarker prioritization.

d) Bioinformatics analyses (clustering, enrichment, pathways, etc.) are used to extract biological content or further prioritize candidates for targeted proteomics validation, using **multiple reaction monitoring (MRM)** and **Parallel reaction monitoring (PRM**).



Sample preparation plays an important role in the proteomic characterization

- There is no universal protocol for proteomic sample preparation, but rather the selected strategy should be optimized/selected based on the proteomic complexity, the available quantity of sample and the goal of the study.
- The first step in sample preparation for MS includes **lysis and extraction of proteins** from the clinical samples.
- This includes extraction reagents such as different organic solvents and detergents followed by tissue disruption techniques such as freeze–thaw cycles, sonication or mechanical disruption to maximise the protein extraction and solubilisation.

Denaturants (**urea and guanidine HCl),** ionic detergents (SDS, SDC), and non-ionic detergents (Triton X-100, NP-40) act to efficiently lyse cells and solubilise protein complexes, especially membrane proteins.
The disadvantage of detergent use is their difficult removal from samples for downstream MS applications that could lead to peptide ion suppression.
Detergents also tend to deposit in the electrospray emitters and liquid chromatography lines, C18 chromatography columns, and in the MS instrument front-end causing added maintenance. Many MS-compatible, commercially available detergents have been reported and widely used including Rapigest (Waters), ProteaseMax (Promega), Invitrosol (Thermo). These detergents degrade with the addition of heat or acidic pH conditions; hence reducing problems described above.

FASP, MStern and S-trap

- The anionic surfactant sodium dodecyl sulfate (SDS) is an excellent agent to solubilise proteins but possess limited compatibility with MS applications. The removal of SDS from the peptide sample has proven to be a major barrier with conventional methods.
- Recently developed sample preparation methods focus on using SDS and other denaturants as the solubilization agent and their removal through various membrane-based protein capture techniques.
- One of these developed methods is filter aided sample preparation **(FASP)**

FASP

- FASP uses molecular weight (MW) filtration to bind proteins to a nitrocellulose filter, while lower MW analytes pass through the filter. Consecutive urea washes facilitate SDS removal, followed by on-filter digestion and peptide elution.
- This technique reduces sample preparation time and sample loss while maintaining the advantages of using SDS for improved proteome coverage. One of FASP's limitations is a reduced binding efficiency with small quantities of starting material with greater sample losses.
- Additionally, the small membrane pore size in FASP requires higher spinning speeds which makes it time consuming in the 96-well format.
- Alkylation and reduction were performed on-filter and further washes with MS-compatible volatile salts such as ammonium bicarbonate leading to highly purified samples.
- FASP has been utilised in different clinical tissue proteomics studies including colorectal cancer (CRC) FFPE samples.

The MStern method

- The MStern method was developed to overcome the problem of slow liquid transfer through nitrocellulose membrane.
- MStern uses hydrophobic PVDF membranes with significantly larger pore sizes allowing for improved liquid transfer and more efficient protein adsorption relative to nitrocellulose.
- A vacuum system is used for passing the samples through the membrane more effectively than centrifugation. Similarly to FASP, MStern involves reduction, alkylation and digestion on the same membrane. Apart from the speed and efficiency of MStern, peptides are not eluted with a high salt concentration. Rather, they are eluted by acetonitrile and formic acid which limits the need for extra desalting steps.
- The one **limitation of MStern** is that the binding capacity of each well is 25 μg compared to < 400 μg for FASP.

Suspension trapping (S-trap)

- Another recently developed membrane-based method that uses a similar principle as FASP is called suspension trapping (S-trap).
- S-Trap packed filters consist of quartz fibers packed with a larger pore size compared to FASP. The other protocol modifications include the use of higher SDS concentrations (5%) in the lysis method.
- The addition of methanol and phosphoric acid causes the formation of protein particulates which are trapped by the filter. Similarly, to FASP and MStern, reduction, alkylation and digestion are done directly on the filters.
- S-Trap and FASP provided the greatest number of protein detections compared to the polyvinylidene difluoride (PVDF) method.

SP3 and iST

- Clinical tissue samples are sometimes challenging to process due to their **small size,** particularly LCM samples.
- These samples require efficient sample processing techniques to ensure limited sample losses and maximal extraction of the proteins to maximize proteome coverage.
- The **solid-phase-enhanced sample preparation (SP3)** method was developed with these limitations in mind.
- the method uses paramagnetic beads which are coated with hydrophilic carboxylate groups. The beads are compatible with various detergents and organic solvents including SDS, urea, TFE and acetonitrile (ACN).
- The **proteins are immobilized to the charged carboxylate groups** in the presence of an organic solvent with acidic or basic pH. After the immobilisation of the proteins, detergents are removed with high organic content washes, followed by on-bead digestion. Eluted peptides can be directly introduced into the MS without the need for desalting. SP3 protocol has been further modified to improve the efficiency and its reproducibility.
- Studies showed that binding efficiency of proteins are lower in acidic conditions as compared to neutral pH.

The **in-StageTip (iST)** method

- The **in-StageTip (iST) method** developed by Kulak et al. **-** is another sample preparation workflow, which is compatible with low input material.
- The method focuses on using **a single stage tip enclosed by a barrier** to perform multiple sample processing steps **to minimize sample losses** and to provide better proteome coverage.
- In the iST workflow, **a pipette tip is inserted** with a **reversed-phase membrane** barrier at the bottom. The sample in the tip is introduced from the top, where they can be lysed through heating or sonication.
- The sample is then denatured, alkylated and digested. The membrane at the bottom of the tip is then used for peptide clean-up. Alternatively, samples can also be fractionated on the same tip.
- The iST workflow shows high performance handling ultra-low amount of material, but due to the reverse-phase membrane barrier the iST is incompatible to use detergents (SDS) and organic solvents (TFE) for lysis.