Frazionamento in Campo Flusso (FFF) e Spettrometria di massa

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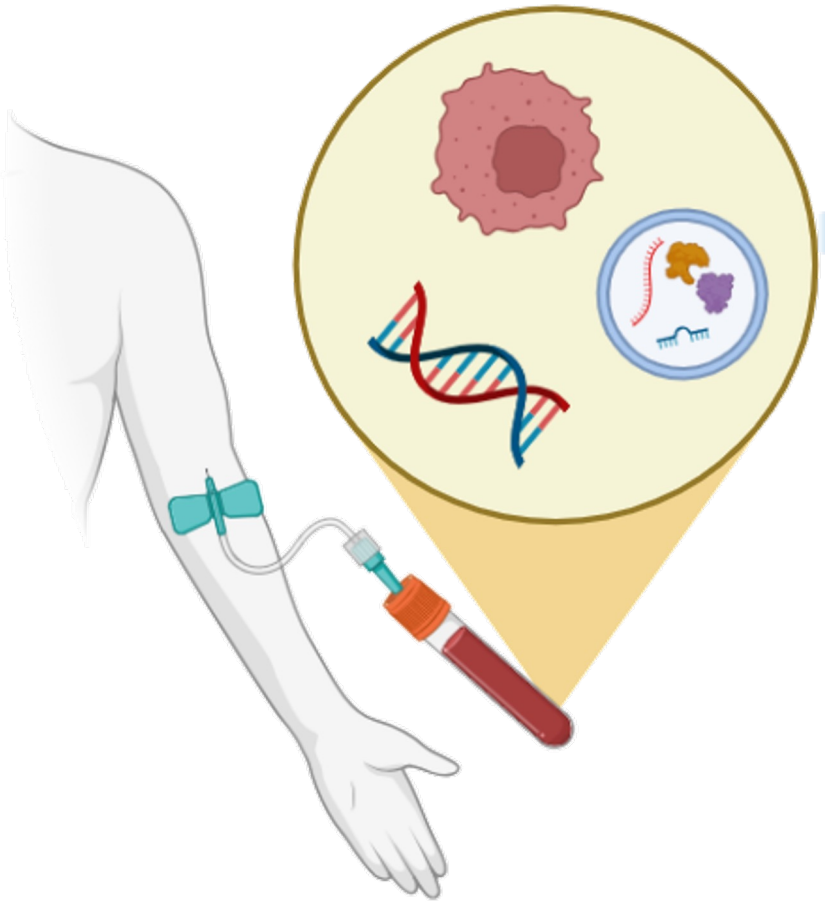
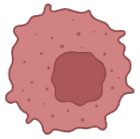
Disclosures of Valentina Marassi

Company name	Research support	Employee	Consultant	Stockholder	Speakers bureau	Advisory board	Other
Byflow srl				X			

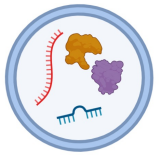
Liquid biopsy and precision medicine





Circulating tumor cells
Intact cancer cells, present as single or cluster, shed by primary and metastatic tumor

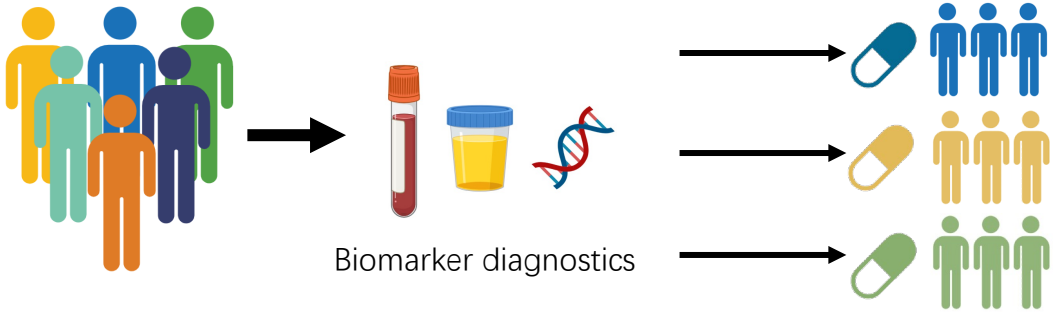
Circulating free and tumor DNA
Fragments of DNA released from cells routinary (cfDNA) and from tumor cells (ctDNA)



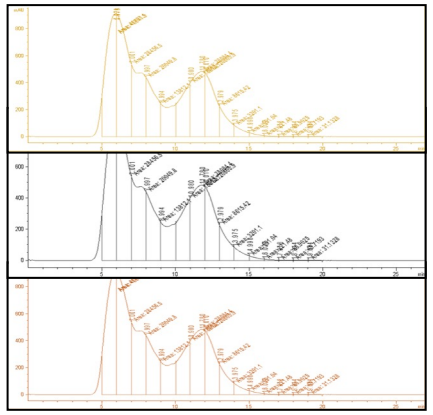
Extracellular vesicles
Membrane-bound, shed by cells carrying cargo (nucleid acids, proteins) and membrane markers

- Less invasive
- Early state information
- Based on cancer markers

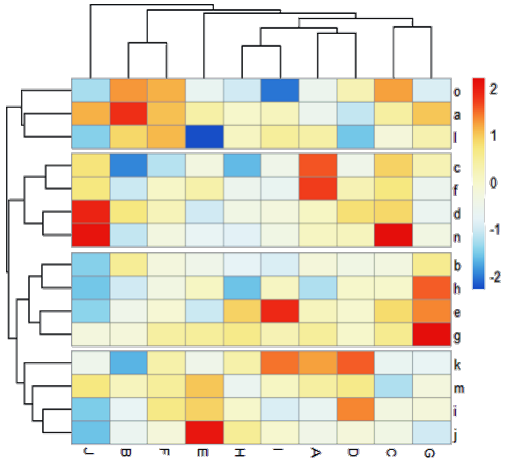
- Requires **isolation of analytes** or **matrix-independent approaches**
- Could enhance patient-centered medicine



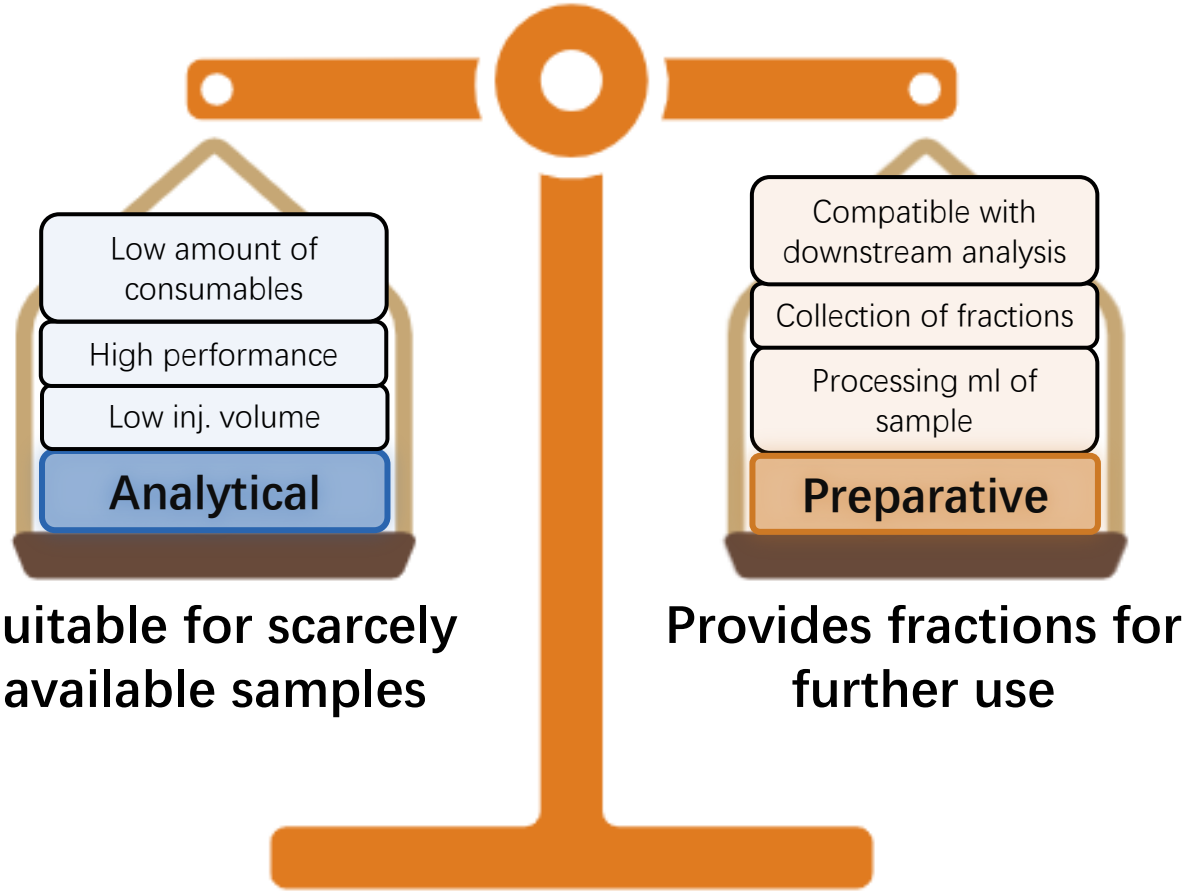
Liquid biopsy and precision medicine



- Simultaneous multispecies and multisize info on intact analytes



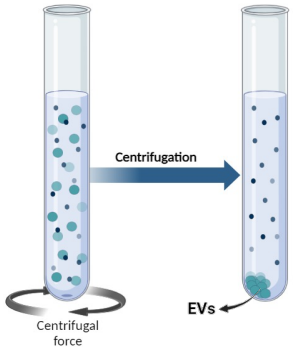
- Correlatable results
- Complete mapping
- Transversal signature



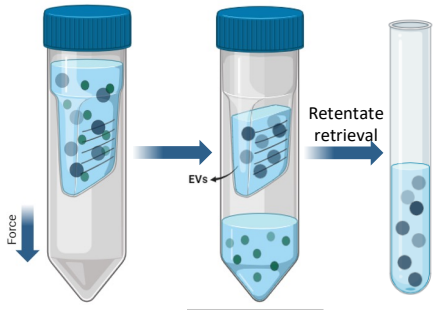
Extracellular vesicles: the challenge

Characterization needs **isolation**

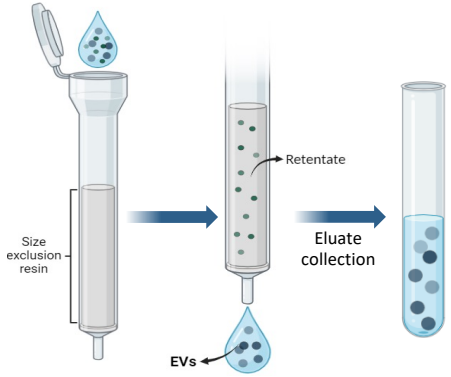
Ultracentrifugation (UC)



Ultrafiltration (UF)



Size-Exclusion Chromatography (SEC)

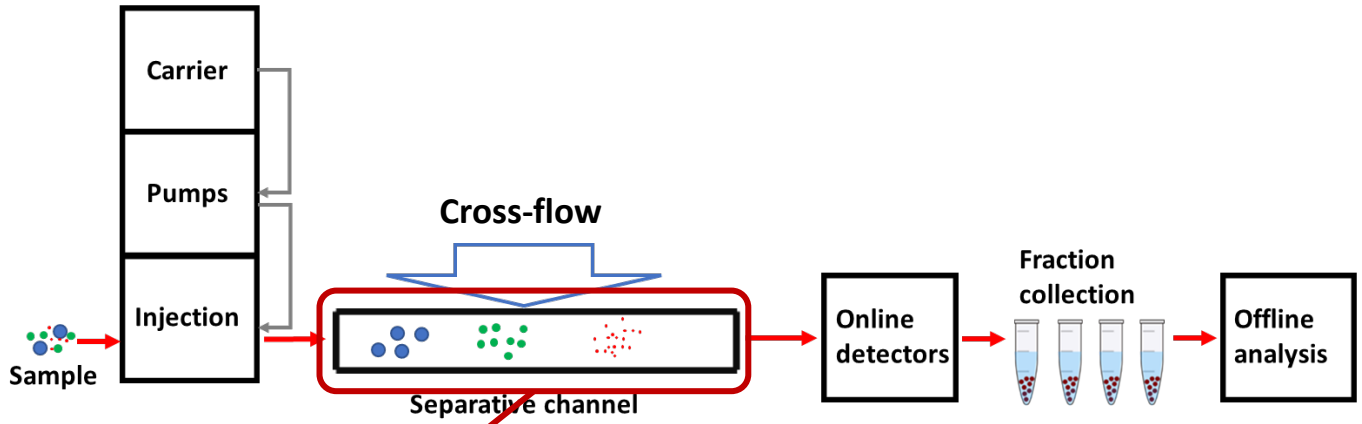


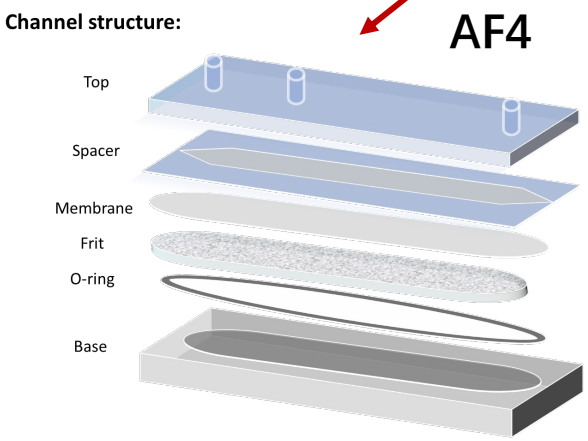
Limitations of conventional techniques (Ultracentrifugation, Size Exclusion, Ultrafiltration):

- Limited Sample flexibility (UC, UF)
- Lipoproteins co-isolation (UC, SEC)
- **EVs damage/Functionality loss** (UC, SEC, UF)
- Sample dilution (SEC)
- Sample Loss (UF, UC)
- Scale up

Adequate isolation technique required

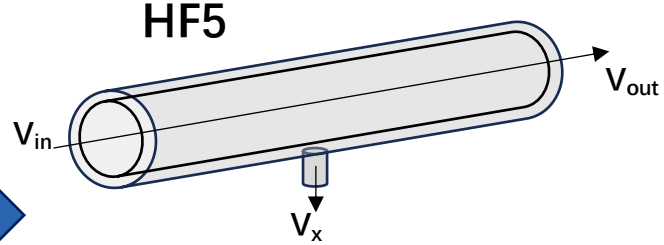
Flow Field-Flow Fractionation: the soft touch





AF4

HF5

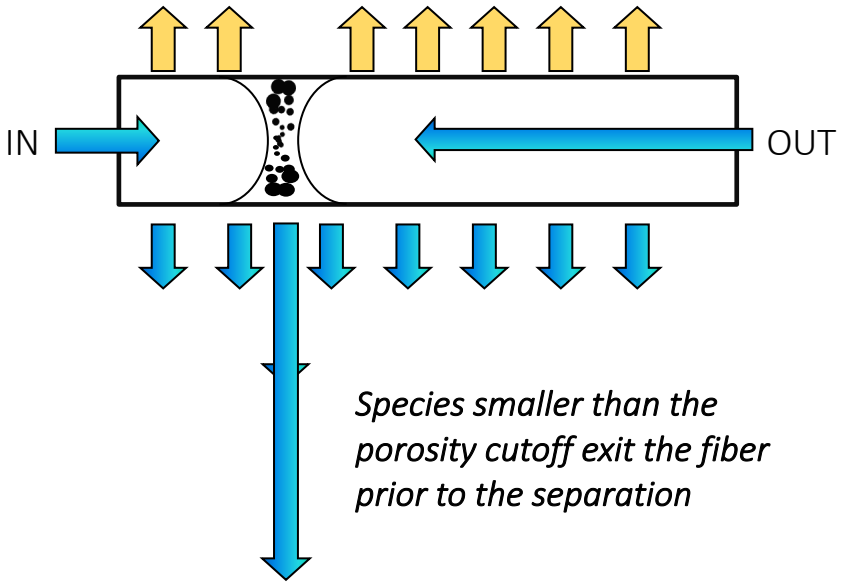


- Miniaturized version
  → minimal dilution
- Disposable cartridge

- Pseudo-chromatographic separation technique
- Hollow, porous channel
  → Sample flexibility
  → Minimal sample pretreatment
  → **Soft separation**
- Versatility on working conditions
  → Nativeness
- Analytes are size sorted
  → Hydrodynamic radius determination
- Compatible with several detectors

Method basics

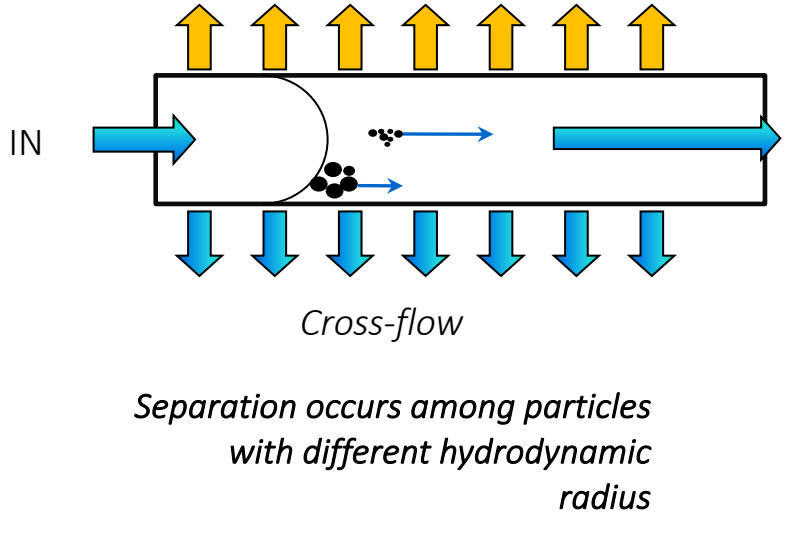
Focus injection



Species smaller than the porosity cutoff exit the fiber prior to the separation

Unreacted tag
Released molecules
Free ions

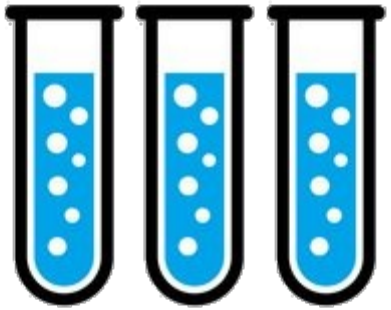
Elution



Separation occurs among particles with different hydrodynamic radius

Detectors

Separated species
Native aggregates
Purified fractions

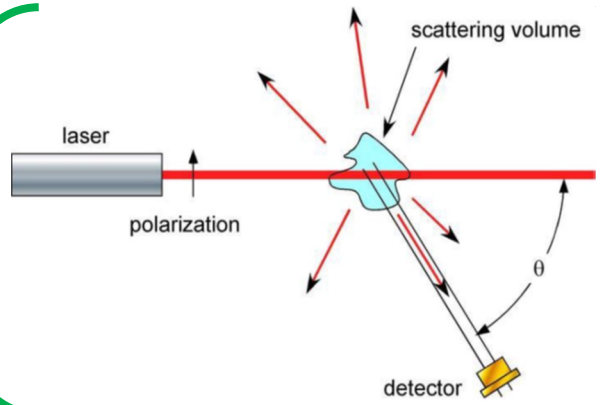


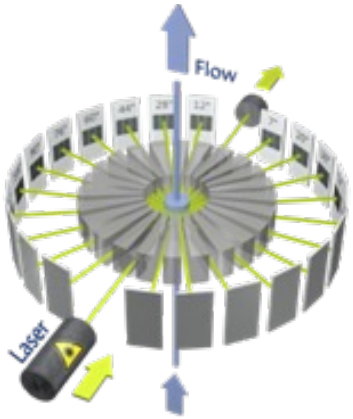
The focus flow can be exploited as a “sample outlet” for simultaneous recovery of filtered and unfiltered species

AF4 Separation

Online characterization

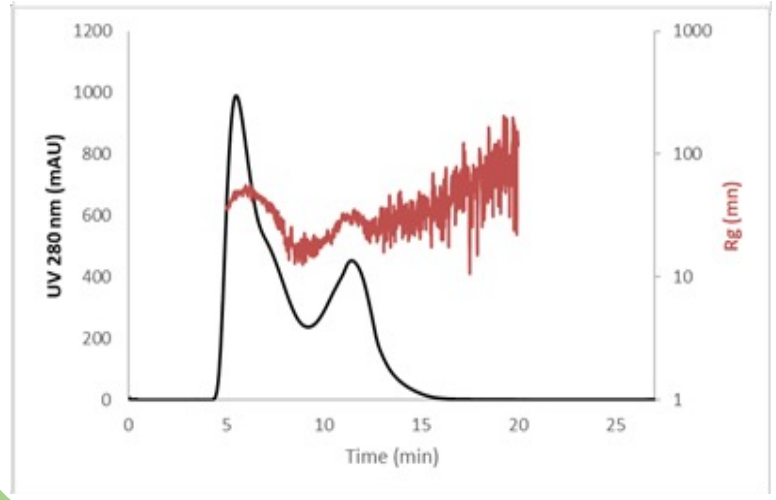
1) Multi Angle Light Scattering (MALS)



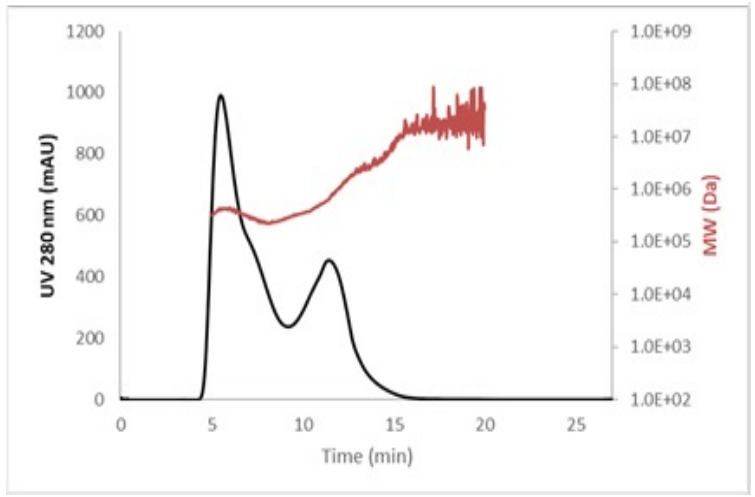


$$\frac{k^*C}{R(\theta)} = \frac{16\pi^2 n_0^2}{3\lambda_0^2}\langle r_g^2\rangle sin^2\left(\theta/2\right) + \frac{1}{M}$$

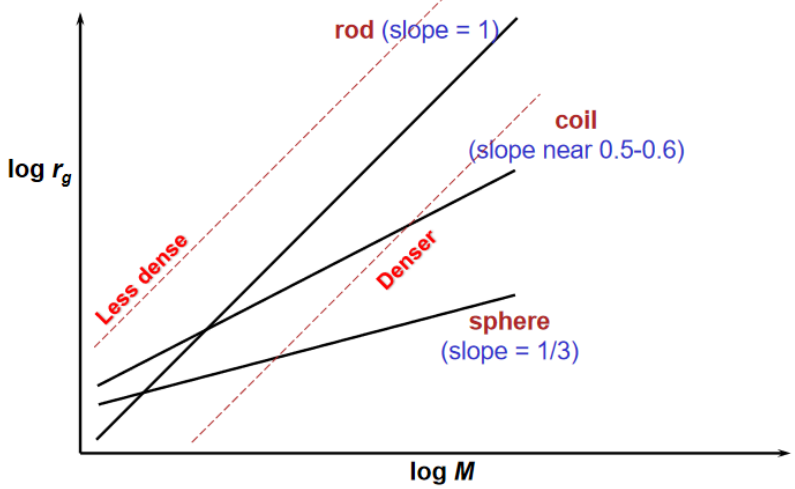
Gyration radius➔ size info



Molecular weight

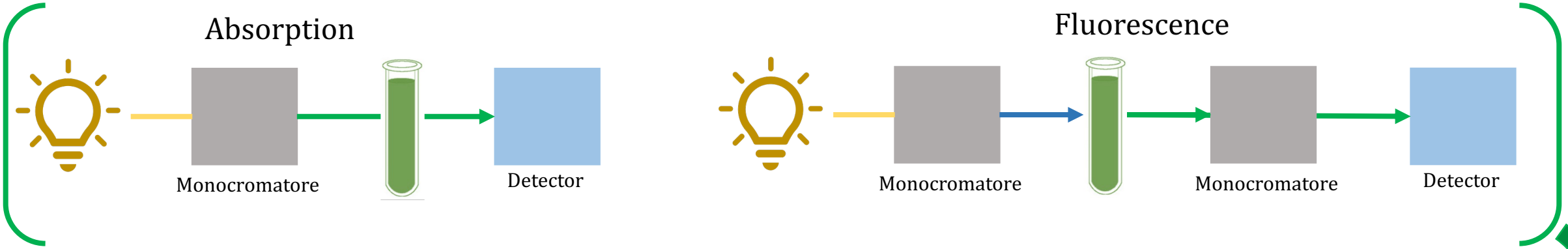


Conformation plot ➔ shape and morphlogy

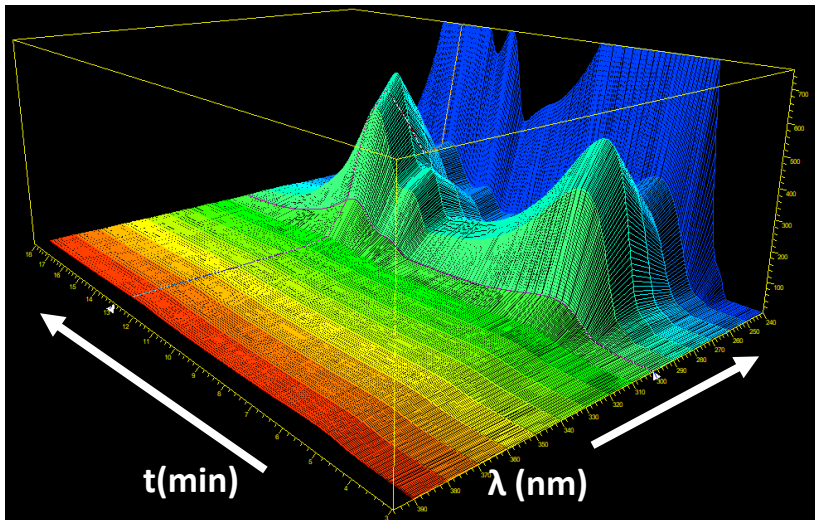


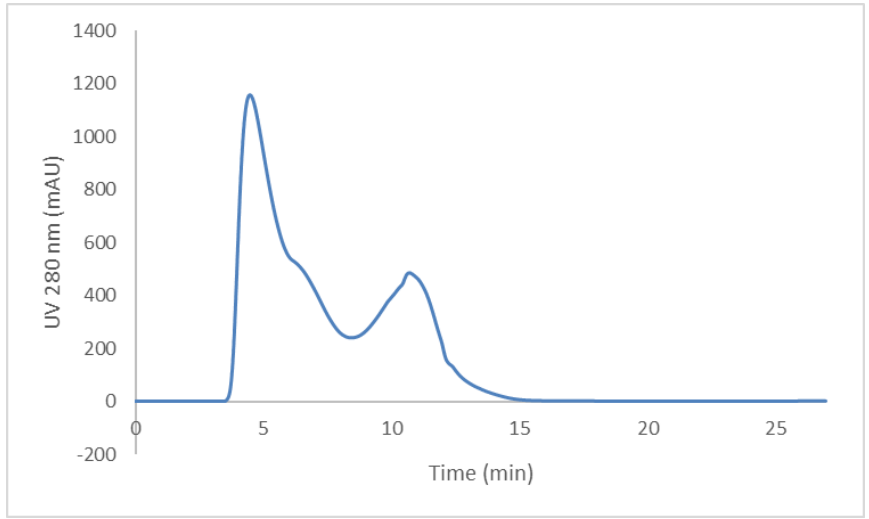
Online characterization

2) UV absorption and fluorescence



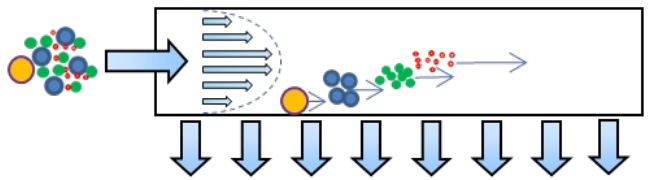
Spectral (composition) information





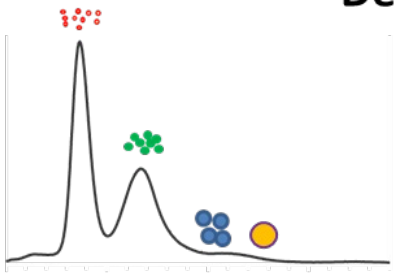
Platform overview

AF4 channel



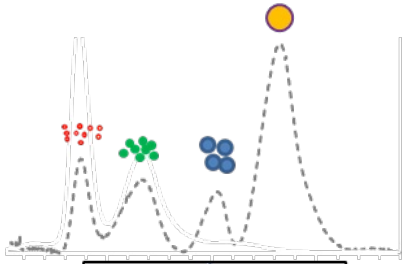
- Elution time **α** hydrodynamic radius
- Single phase, soft and native separation

Detectors



UV, dRI, FLD detector

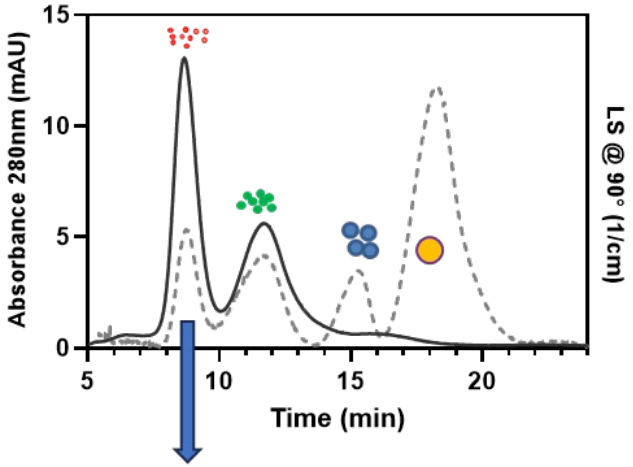
- Spectroscopical characterization
- Signal **α** to concentration (UV, dRI)



MALS detector

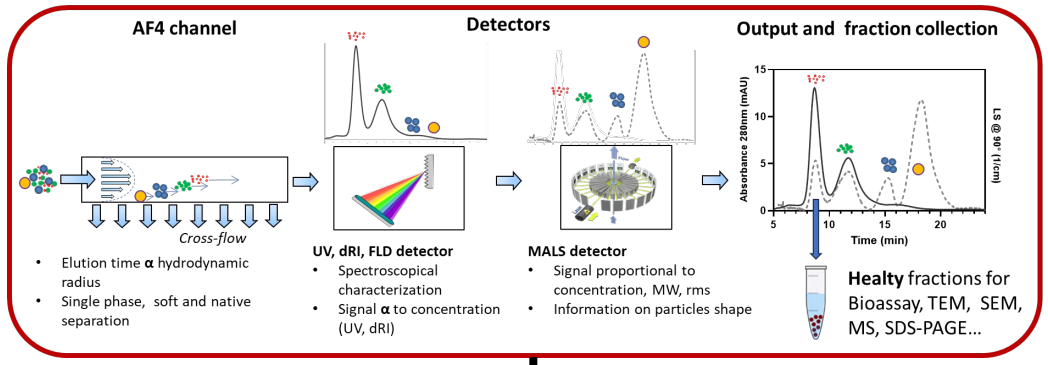
- Signal proportional to concentration, MW, rms
- Information on particles shape

Output and fraction collection



Healty fractions for Bioassay, TEM, SEM, MS, SDS-PAGE…

Platform overview



- Elution time **α** hydrodynamic radius
- Single phase, soft and native separation

UV, dRI, FLD detector
- Spectroscopical characterization
- Signal **α** to concentration (UV, dRI)

MALS detector
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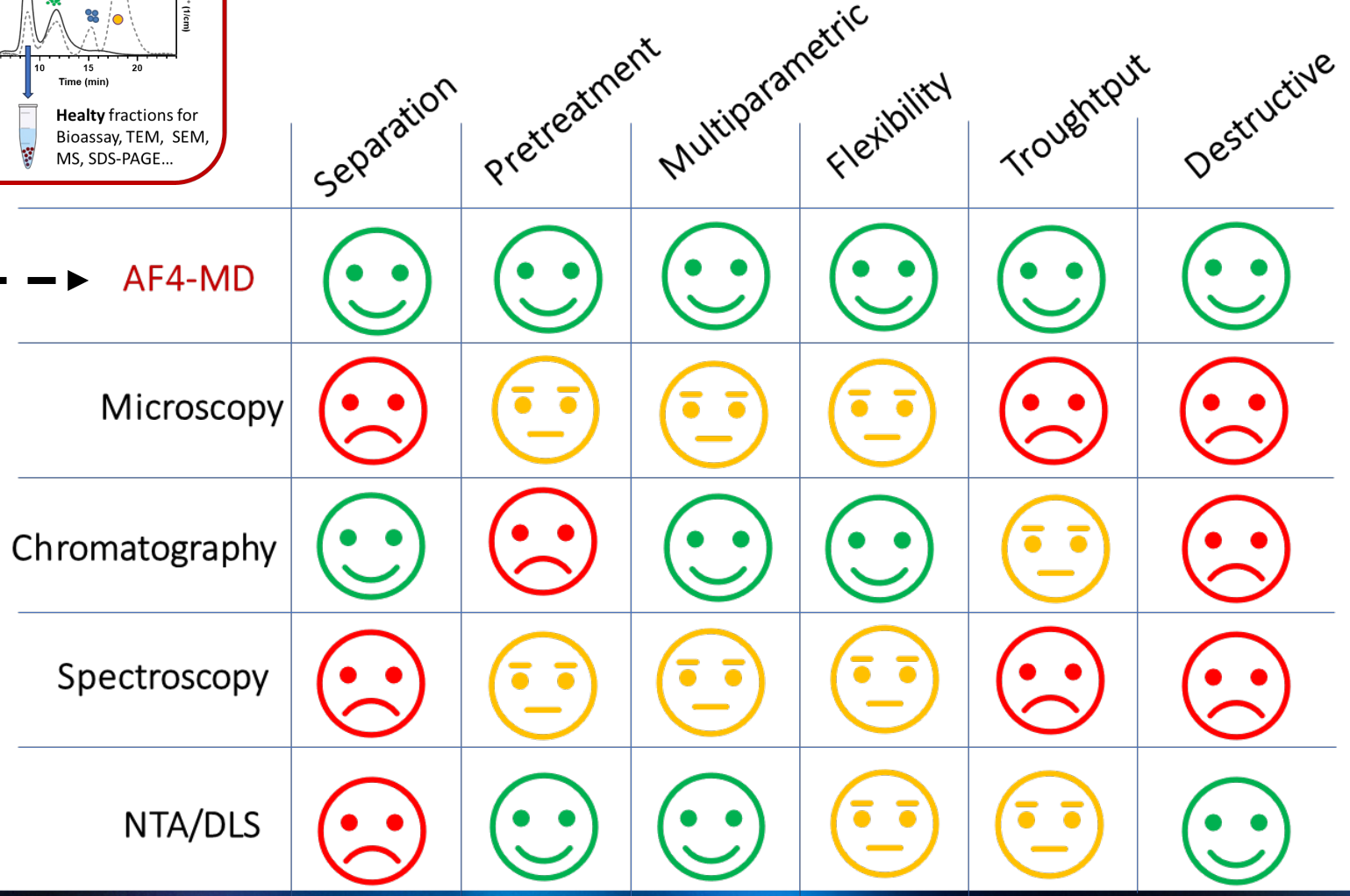
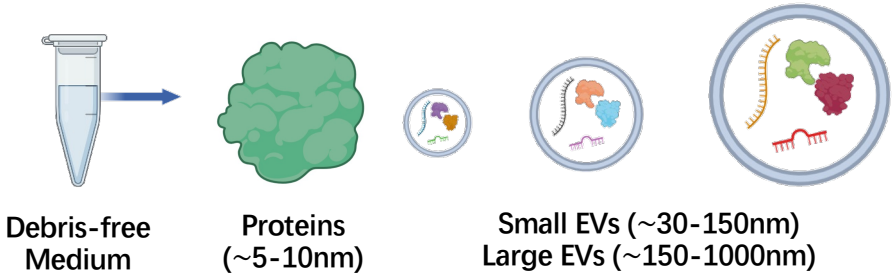
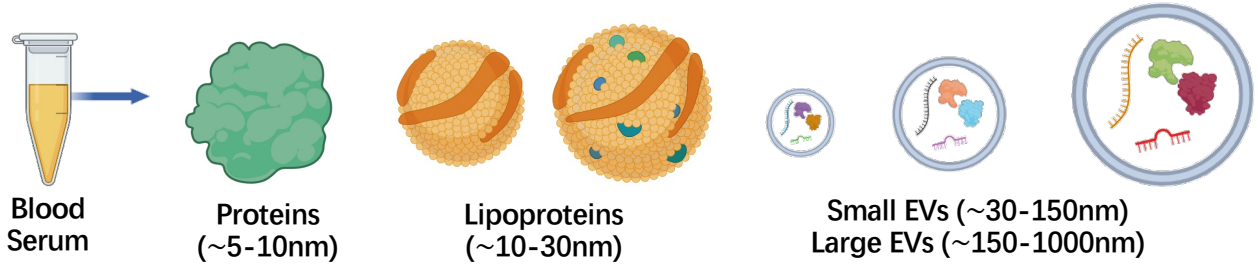
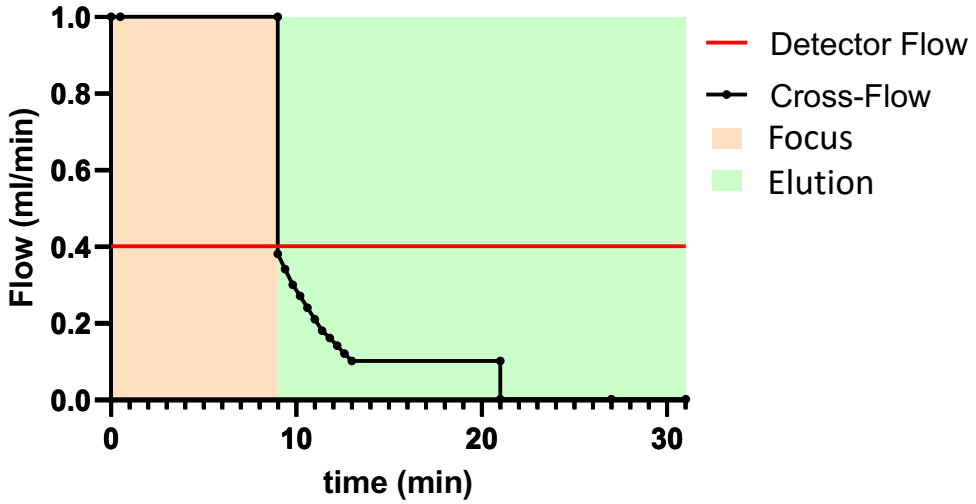
Healty fractions for Bioassay, TEM, SEM, MS, SDS-PAGE…

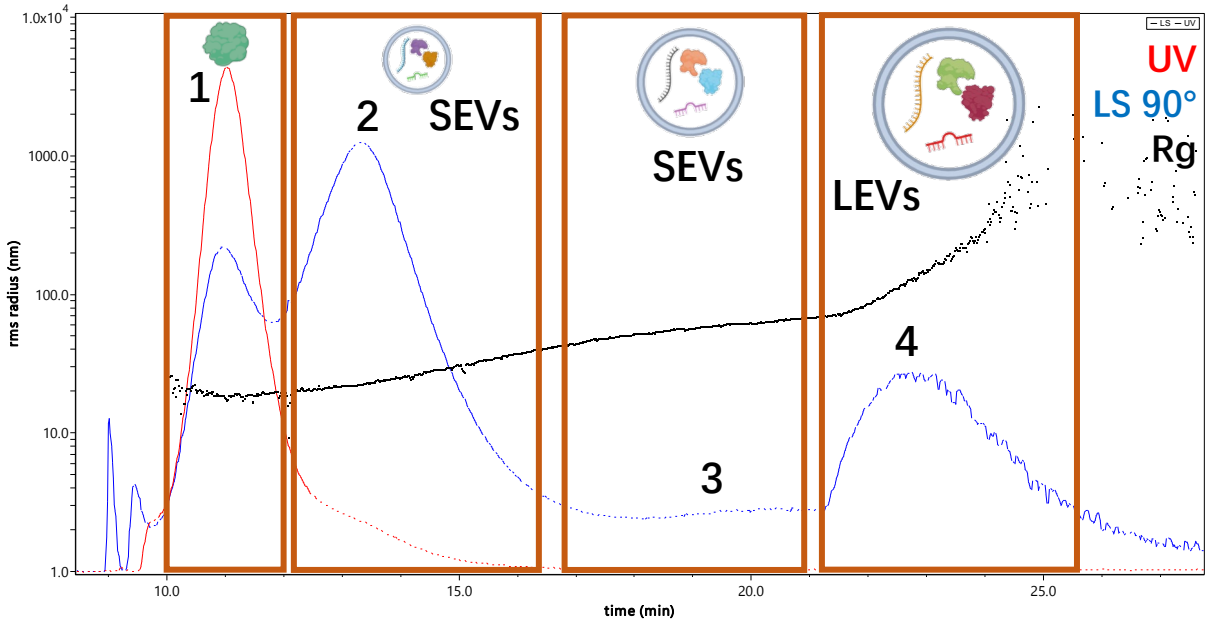


Subpopulations sorting

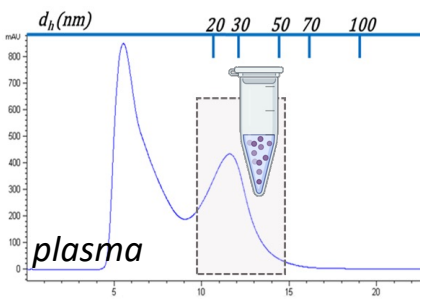


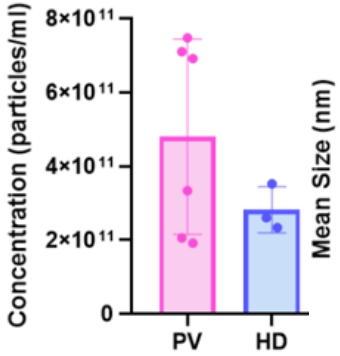


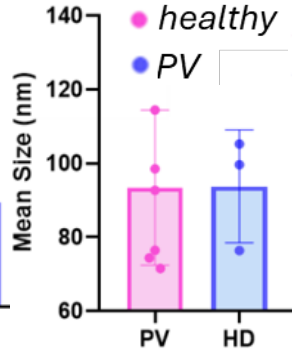


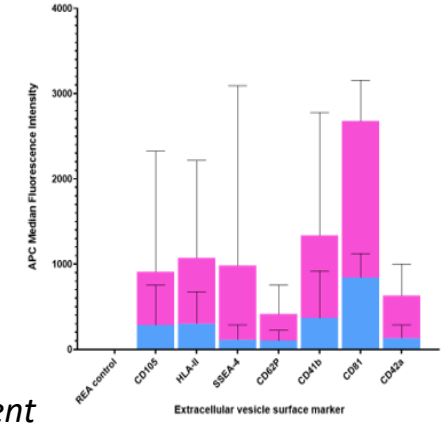


FFF sorting of Evs from serum, plasma and cell cultures

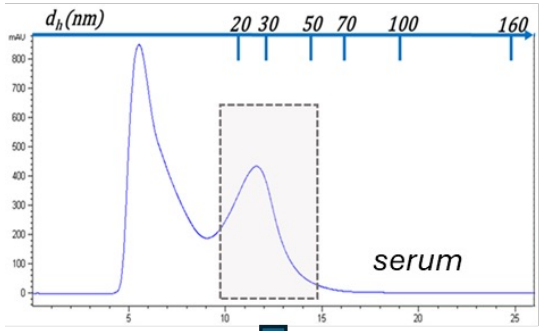




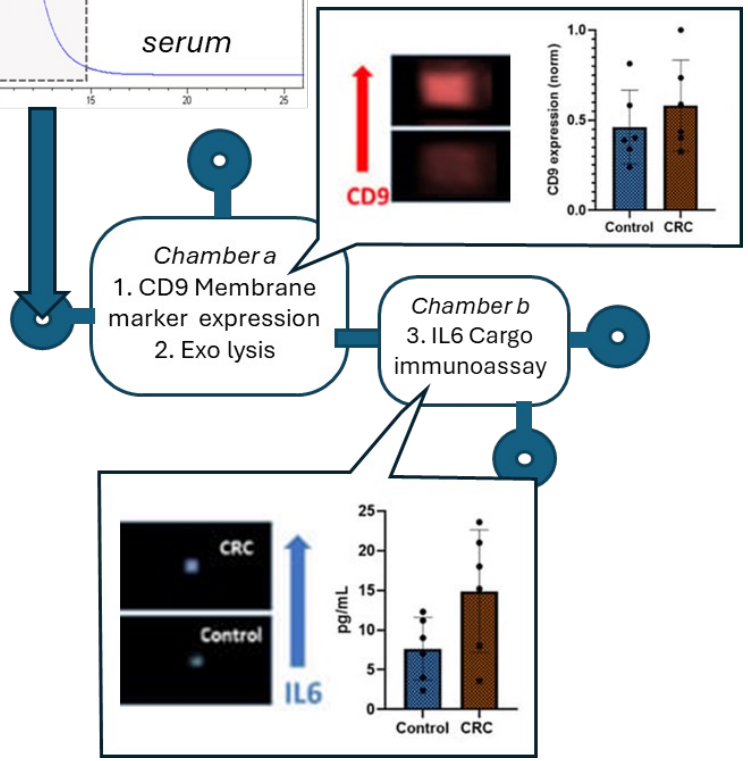


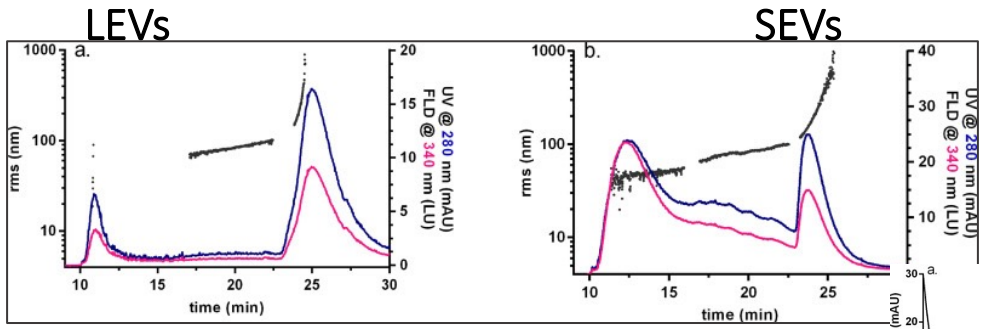


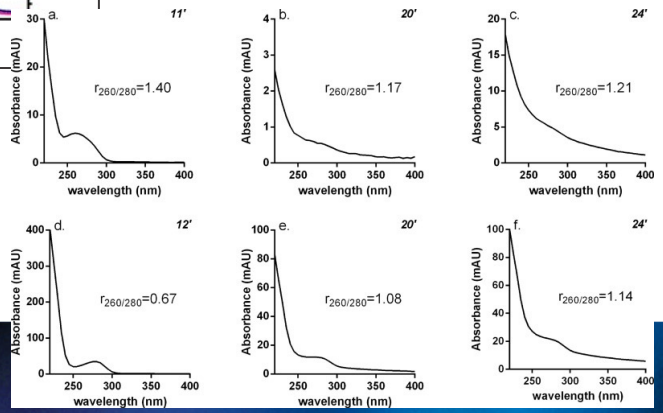
cancer-specific EV-based signature of inflammatory microenvironment



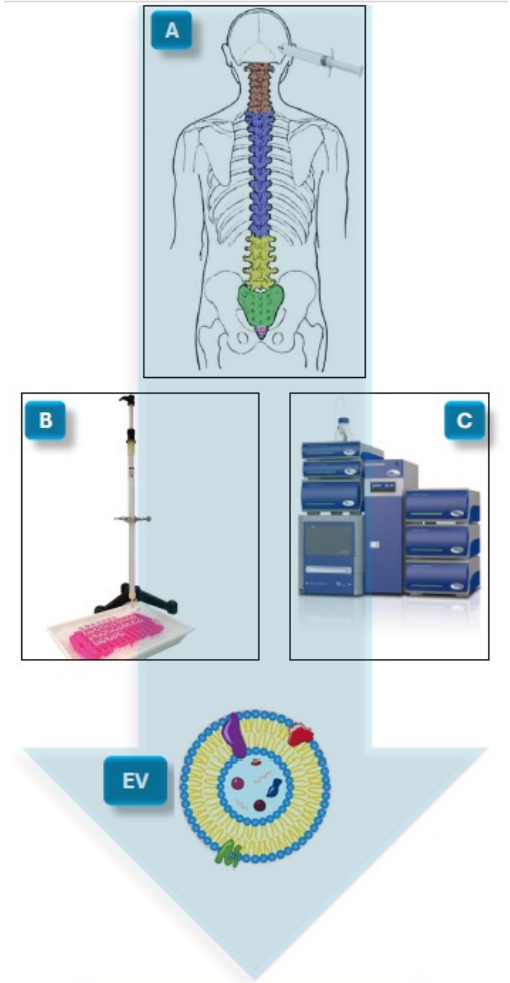
Marassi et al, Sensors, 2023

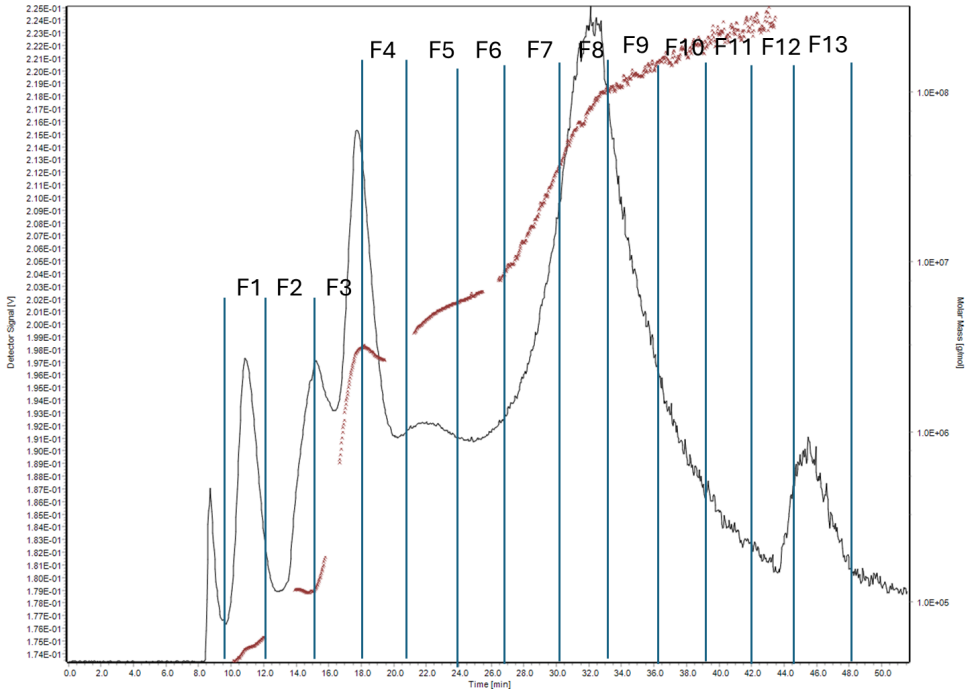




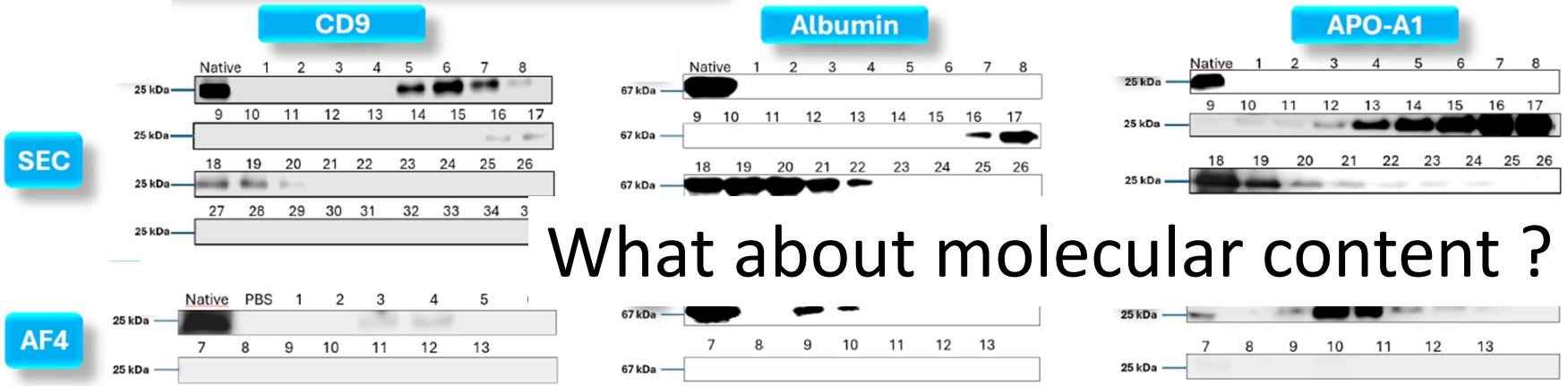


FFF sorting of Evs from traumatic brain injury





Unlike SEC, AF4 uses a smaller sample input volume and ensures less sample dilution in the fractionation process, and thus higher molecular signal and yield of EVs. AF4 fractionation shows a more efficient separation of protein from EV fractions with simultaneous monitoring of protein concentration, size distribution and molecular weight of particles in the sample



What about molecular content ?

Mass spectrometry

To discover novel biomarkers EVs derived microRNA ,metabolites, lipids, and proteins have been investigated .

→ Among the biological molecules, exosomal proteins are most intensively investigated as biomarkers for various diseases

→ LC-MS/MS-based proteome analysis of exosomes has become the most popular fundamental tool for the identification and characterization of exosomal proteins

Bottom-Up Approach (Proteolytic Digestion-Based)

- Proteins are enzymatically digested (e.g., with trypsin) into smaller peptides before MS analysis.
- Peptides are then analyzed using liquid chromatography-MS (LC-MS/MS) and identified by database searches.

✅**Advantages:**

High sensitivity and deeper proteome coverage
Well-established workflows and databases for peptide identification
Suitable for complex exosome samples

❌**Disadvantages:**

Loss of information on post-translational modifications (PTMs) and protein isoforms
Requires protein digestion, increasing sample preparation complexity
Peptide reconstruction to full-length proteins can be challenging

Top-Down Approach (Intact Protein Analysis)

- Intact proteins (without digestion) are directly analyzed using high-resolution MS.
- Enables identification of proteoforms, including PTMs and sequence variations.

✅**Advantages:**

Preserves complete protein structure, providing full proteoform characterization
Direct detection of PTMs and isoforms
Reduces sample processing steps compared to bottom-up

❌**Disadvantages:**

Lower sensitivity, especially for complex exosome samples
Requires advanced MS instrumentation (e.g., Orbitrap, FT-ICR MS)
Challenging data analysis due to complex spectra

Steps for EV Analysis via Mass spectrometry

EV Isolation & Purification
•Techniques: **Ultracentrifugation, size-exclusion chromatography (SEC), precipitation, immunoaffinity, or field-flow fractionation (FFF)**
→Remove contaminants (e.g., proteins, lipoproteins) while preserving EV integrity

EV Lysis & Protein Extraction
•Methods: **Detergent-based lysis, ultrasonication, freeze-thaw cycles**
→ Release **EV proteins** for MS analysis

Proteomic Sample Preparation
•**Bottom-Up MS:** Protein digestion (e.g., trypsin) → Peptide cleanup
•**Top-Down MS:** Intact protein preparation without digestion
•Enrichment for **low-abundance proteins** may be necessary

Mass Spectrometry Analysis
•**Liquid Chromatography-MS (LC-MS/MS)** for peptide separation
•**High-resolution MS (e.g., Orbitrap, FT-ICR, TOF-MS)** for protein/peptide identification
•**DIA (Data-Independent Acquisition) or DDA (Data-Dependent Acquisition)** strategies

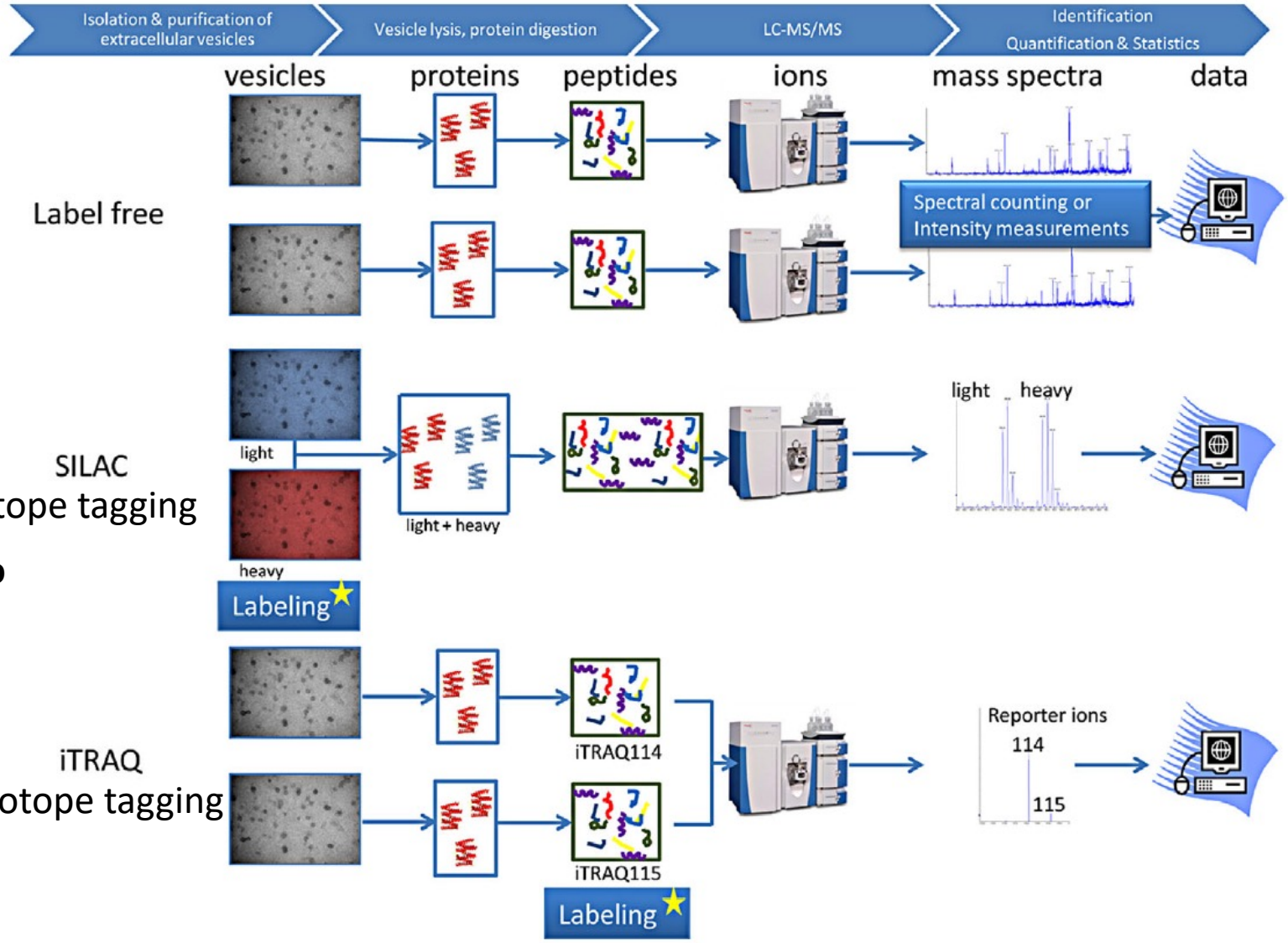
Data Processing & Bioinformatics
•**Peptide/Protein Identification** (database search, spectral matching)
•**Proteoform & PTM Analysis** (for top-down MS)
•**Quantitative Proteomics** (Label-free, SILAC, TMT/iTRAQ)
•Pathway & biomarker discovery analysis

Mass spectrometry

Mass spectrometry (MS), can identify and characterize molecular composition of vesicles

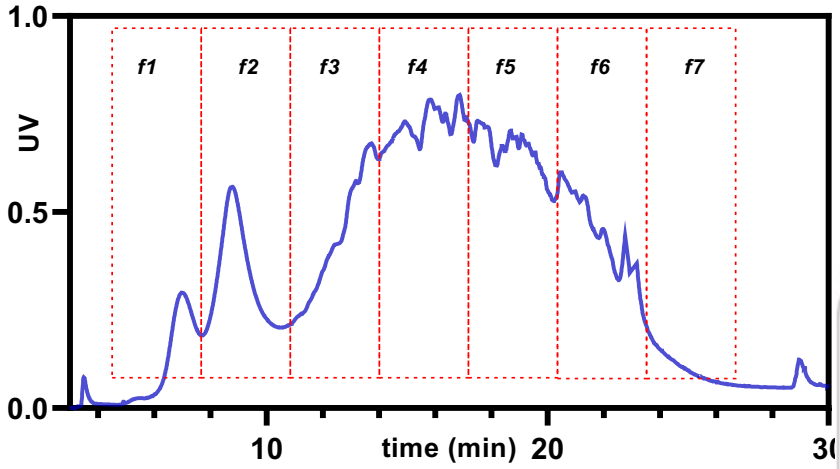
→ MS is the major tool to assess protein composition of Evs in qualitative and quantitative proteomics approaches

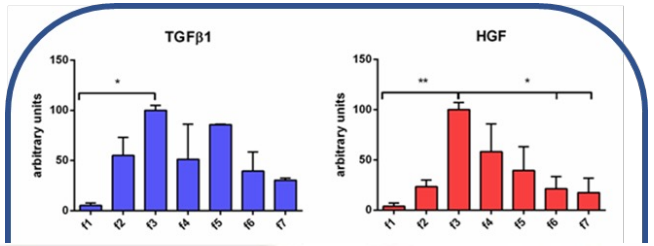
→ lipid and metabolite composition of vesicles might also be best assessed by MS techniques

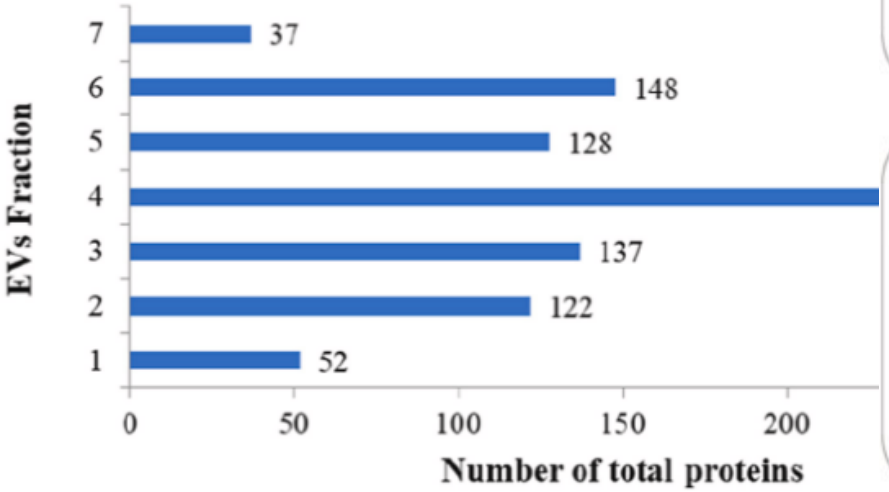


to **track EV protein cargo dynamics** by labeling donor cells

FFF + Mass spectrometry

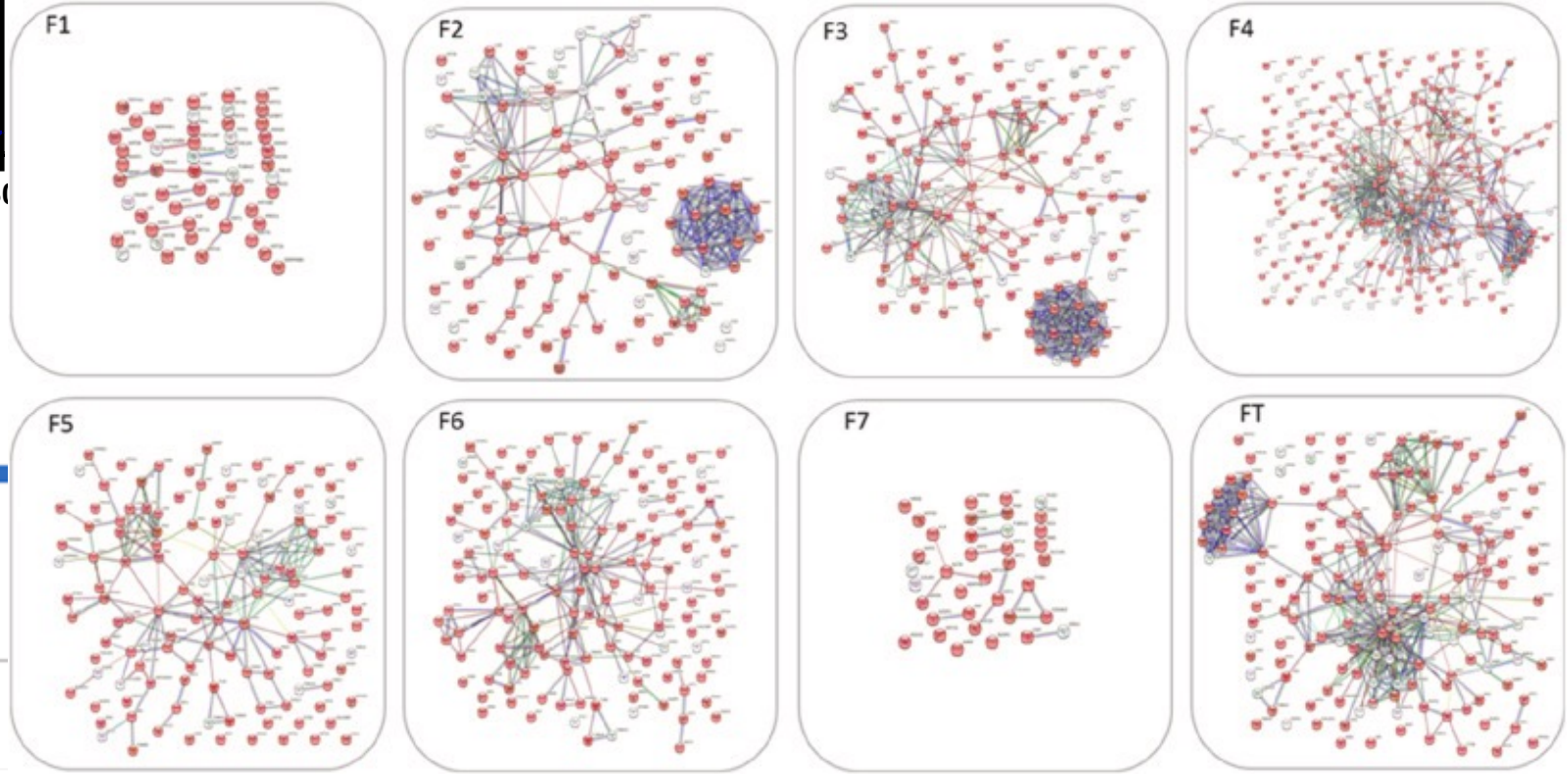






ELSEVIER
Talanta
Volume 276, 15 August 2024, 126216

Native characterization and QC profiling of human amniotic mesenchymal stromal cell vesicular fractions for secretome-based therapy



ASSEMbLe, *"Immunomodulatory properties of the Amniotic Stromal cell SEcretome: from Multi-omics profiling to nanotechnoLogy-aided delivery for controlled release in osteoarthritis" (PRIN 2017)*

Summary

•**Field-Flow Fractionation (FFF)** is a powerful technique for **size-based isolation** of EVs, enabling better separation from contaminants like lipoproteins and protein aggregates.

•**Mass Spectrometry (MS)** plays a crucial role in **EV characterization**, offering insights into **protein composition, biomarkers, and post-translational modifications (PTMs)**.

•**Bottom-Up vs. Top-Down MS Approaches**:

- •Bottom-up provides **higher sensitivity** but loses information on intact proteoforms.
- •Top-down preserves **full protein structure and PTMs** but faces challenges in sensitivity and data complexity.

•**FFF-MS Integration**: combining **FFF with MS** enhances the resolution and depth of EV proteomics.

•**Future Perspectives**: Further advancements in **FFF and MS methodologies, instrumentation, and data analysis** will improve **biomarker discovery** and **clinical applications of EVs**.