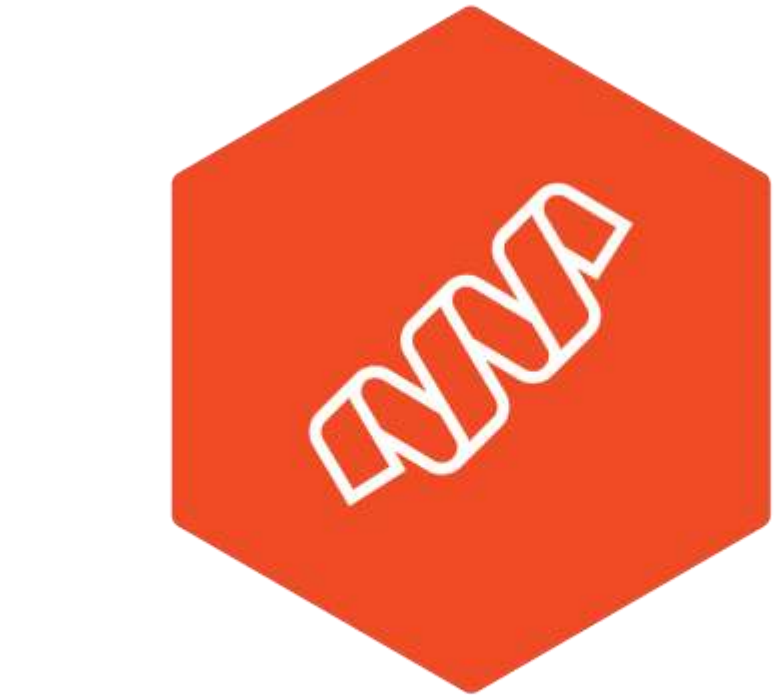
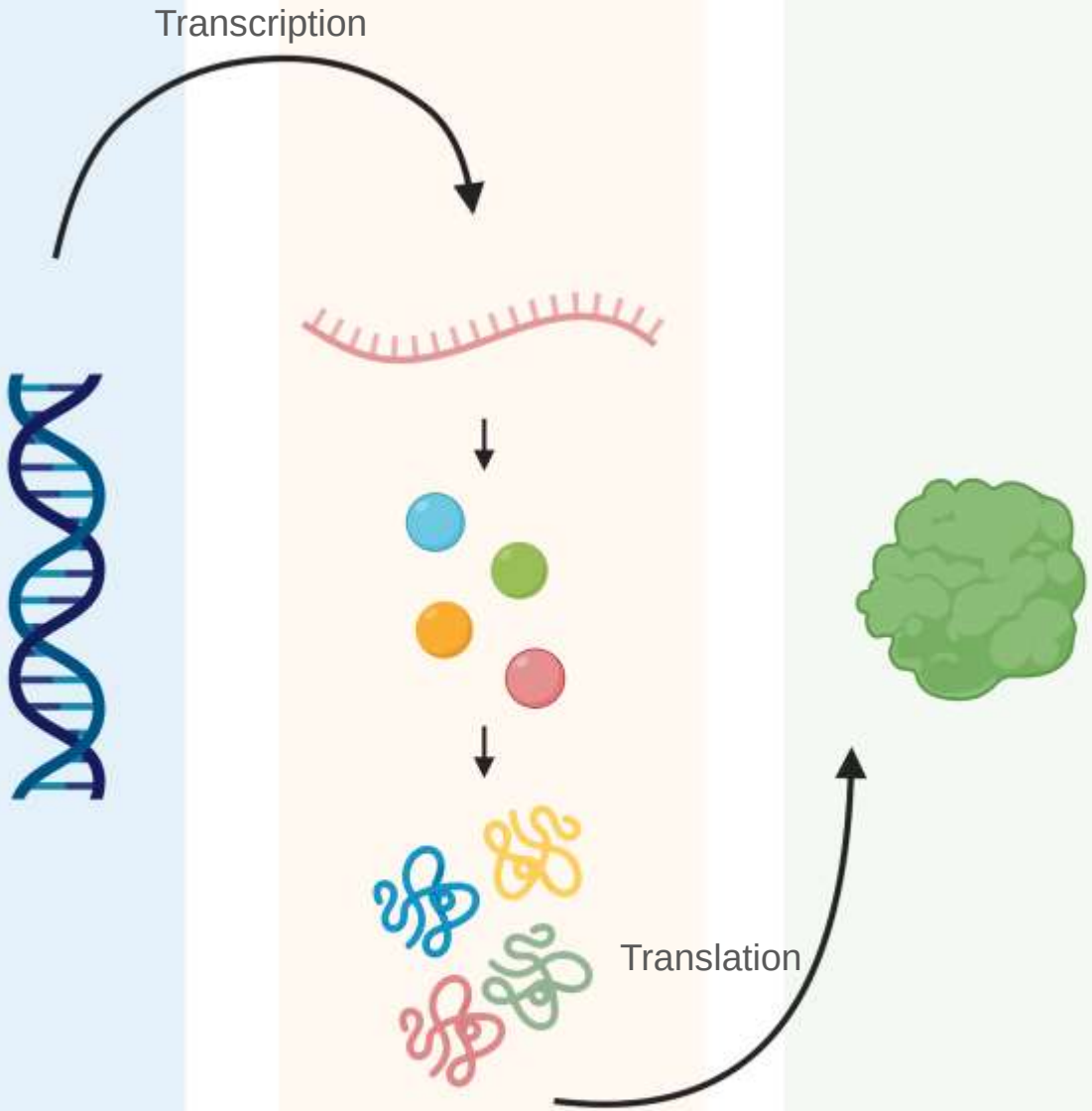
Proteomics III: iTRAQ/TMT

Liily Zhu

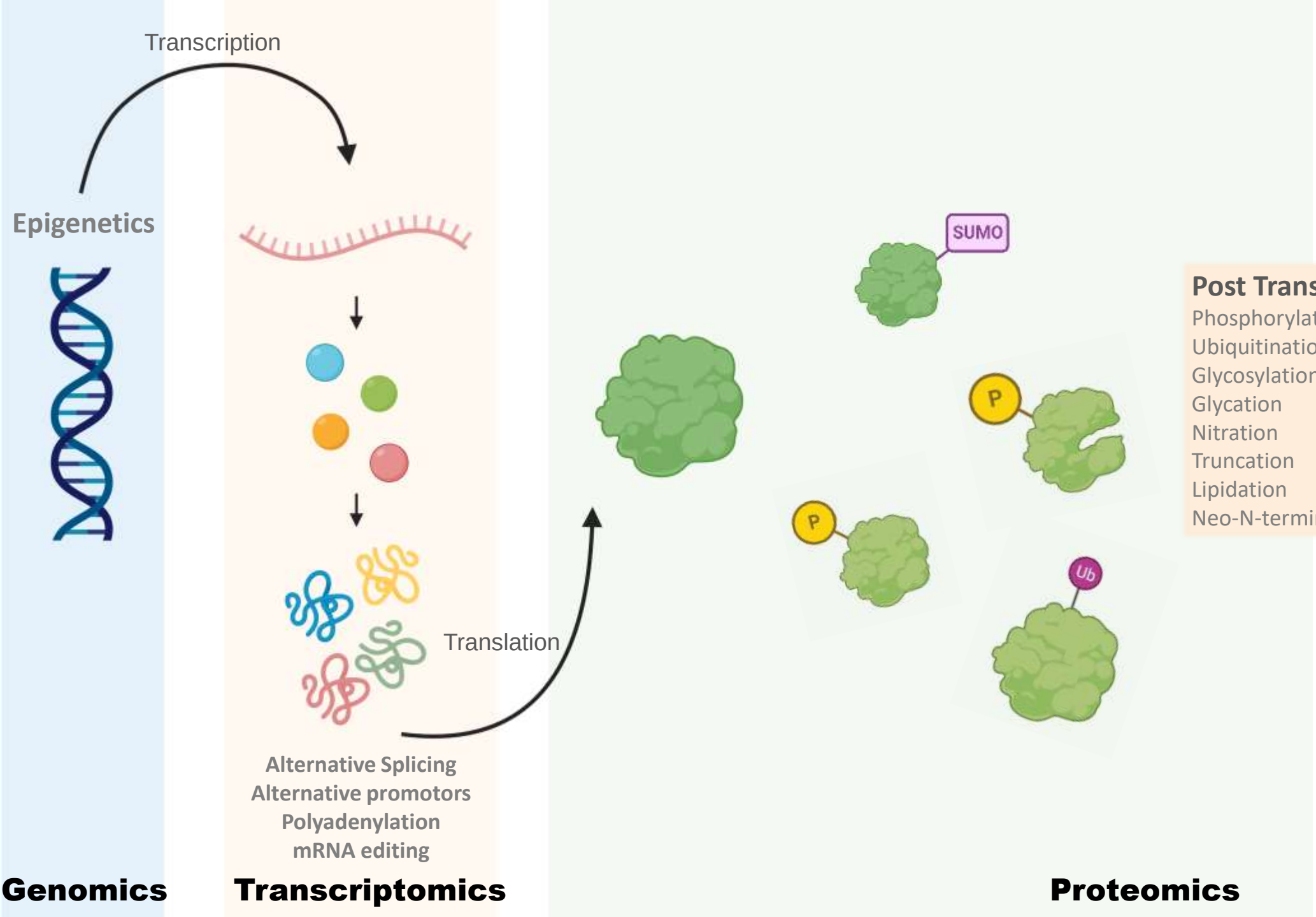


PROTEOMICS &
MASS SPECTROMETRY



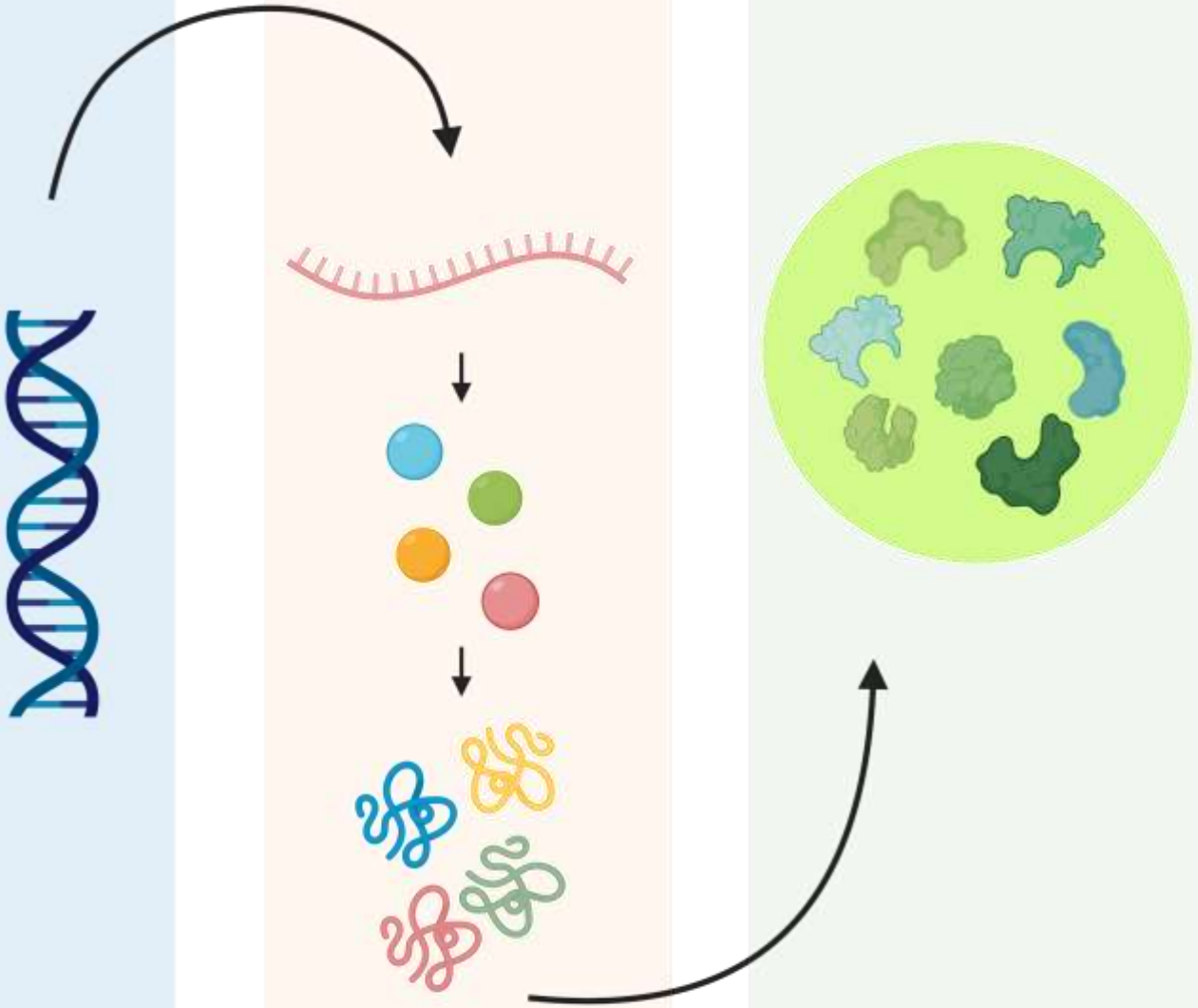
?

Genomics **Transcriptomics** **Proteomics**



Post Transcriptional modification

Phosphorylation
Ubiquitination
Glycosylation
Glycation
Nitration
Truncation
Lipidation
Neo-N-terminus etc.



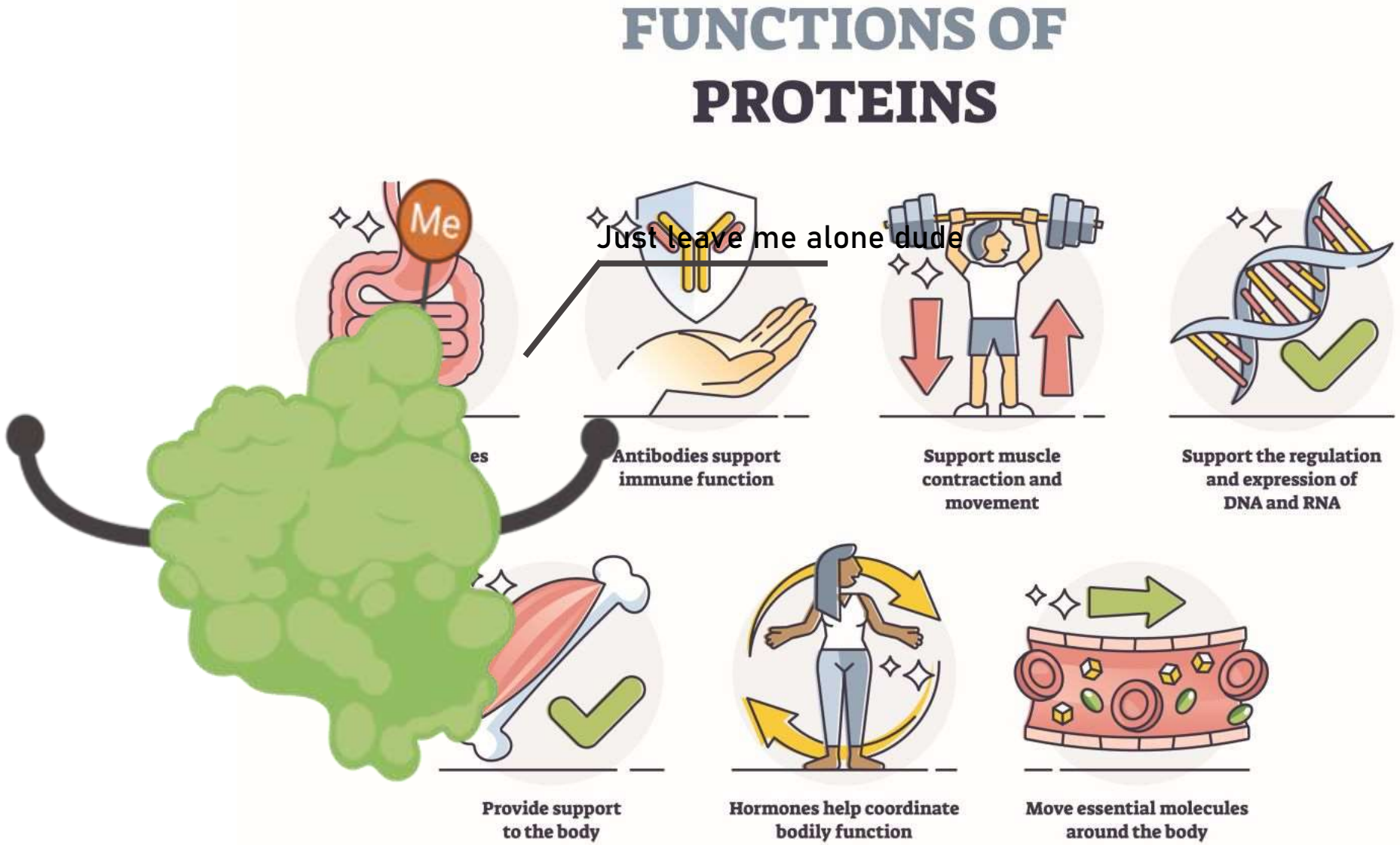
Protein Protein Interactions
Conformation
Activity State
Localization
Turnover

Directly correlate the involvement of specific proteins in a given state

Phenotype

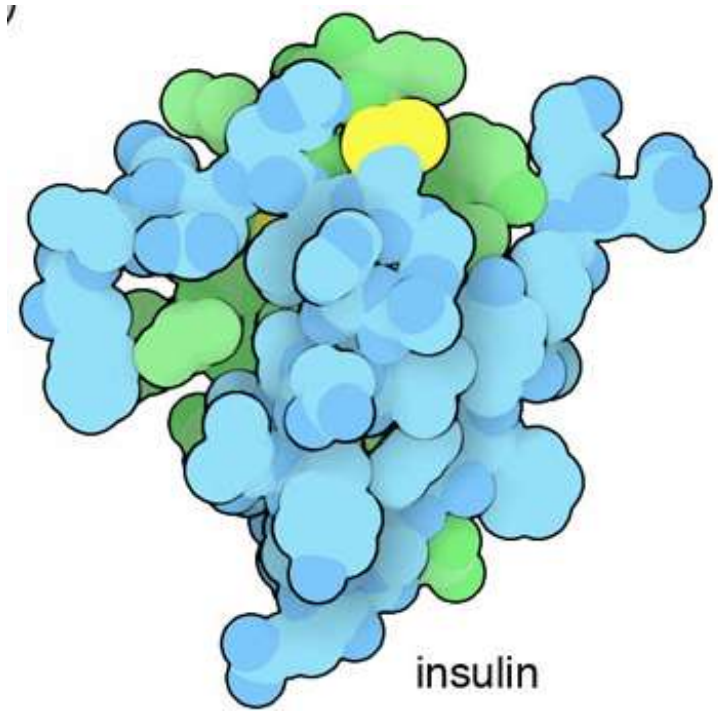
Genomics **Transcriptomics** **Proteomics**

So… Why should we quantify proteins?



Created with BioRender.com
https://s3.amazonaws.com/www.irunfar.com/wp-content/uploads/2022/09/19124230/Functions-of-protein-1.jpg

So… Why should we quantify proteins?



insulin

Diabetes

https://pdb101.rcsb.org/motm/14

How do we quantify proteins?

Bio Sample

- Break up tissue into cells
- Labeling

Protein

Lysis

Peptide

Digestion

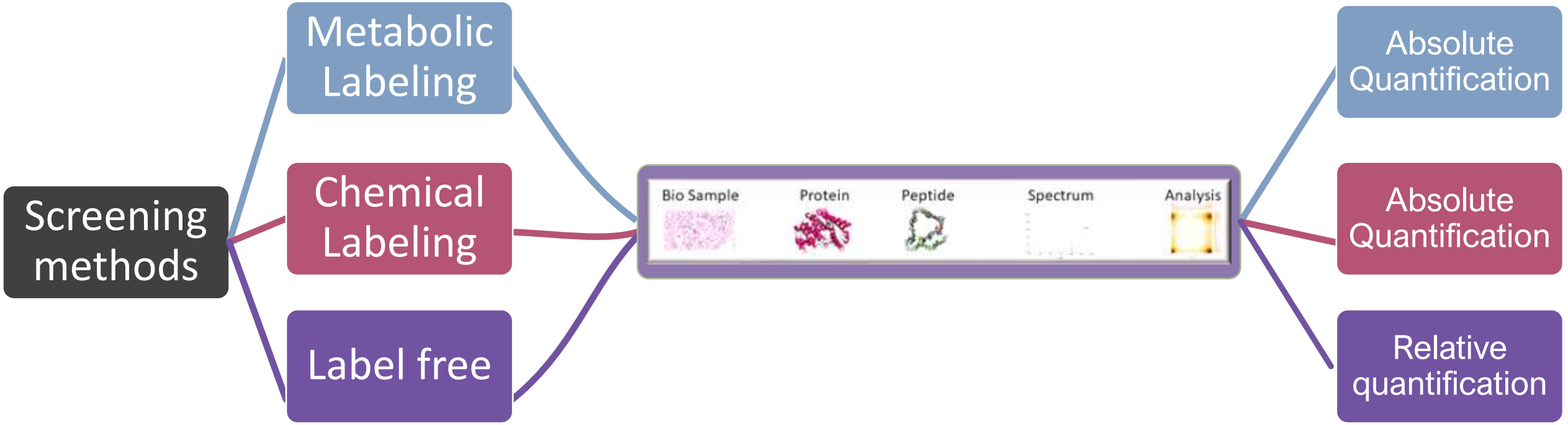
Spectrum

Liquid Chromatography
Tandem mass spectra

Analysis

Data analysis

How do we quantify proteins?



SILAC

Stable **I**sotope **L**abeling by **A**mino Acids in **C**ell Culture technique

Isobaric Labeling

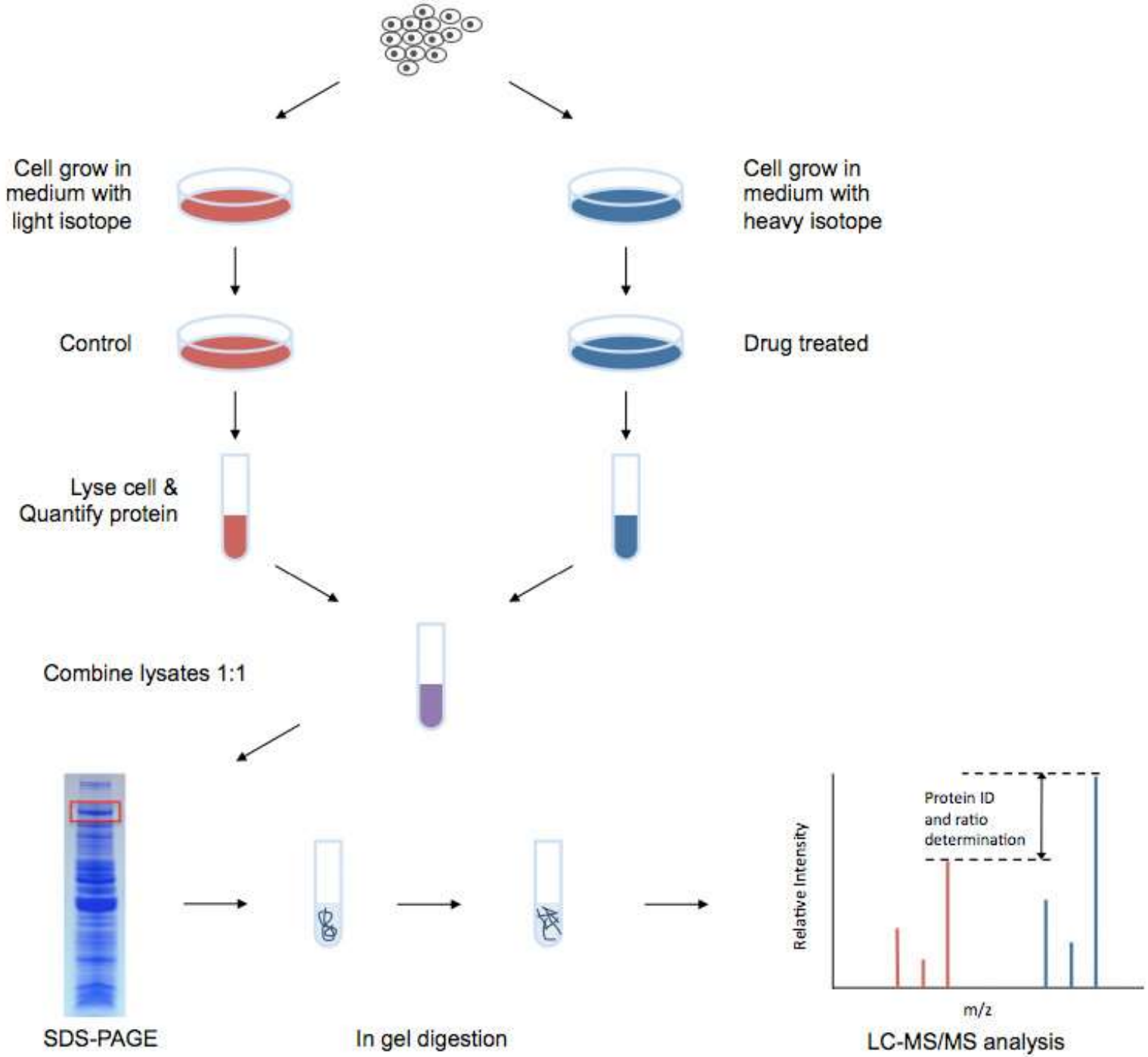
iTRAQ

Isobaric **T**ags for **R**elative and **A**bsolute **Q**uantitation

TMT

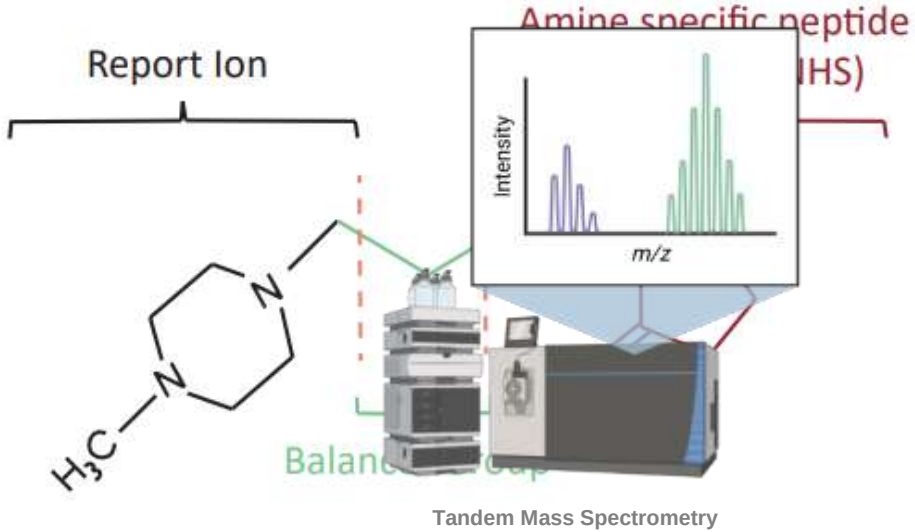
Tandem **M**ass **T**ags

What is SILAC?

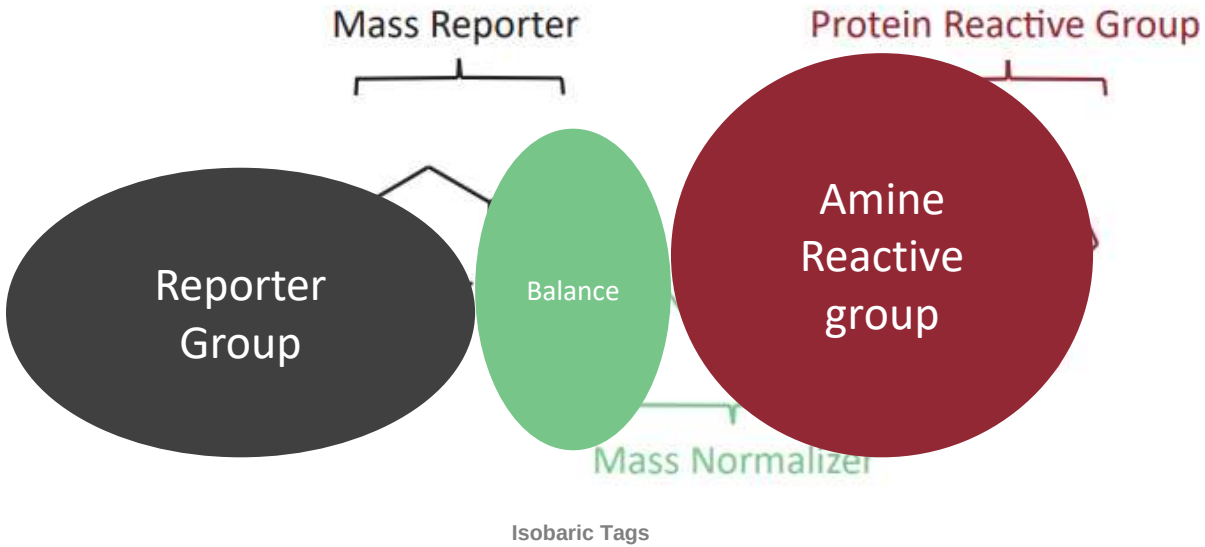


iTRAQ

Isobaric **T**ags for **R**elative and **A**bsolute **Q**uantitation



Tandem Mass Spectrometry



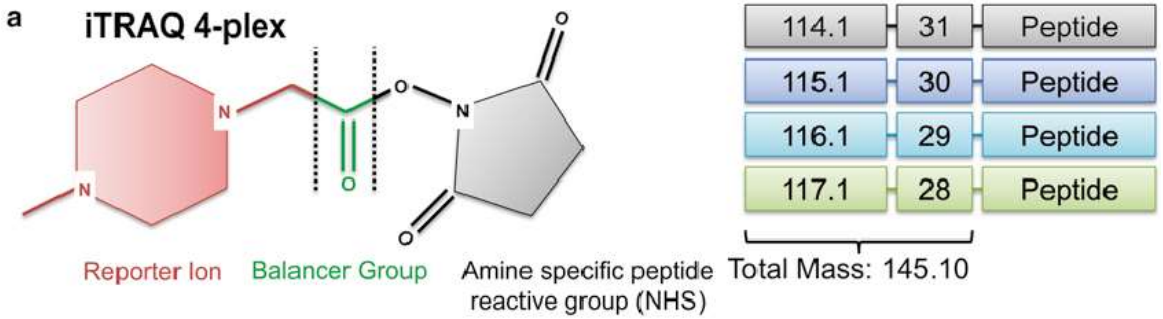
Isobaric Tags

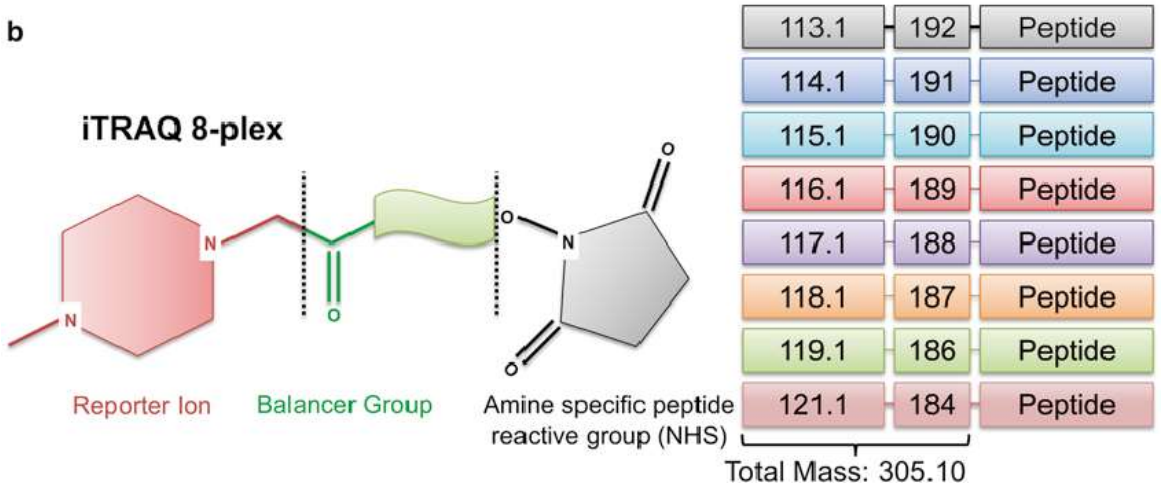
TMT

Tandem **M**ass **T**ags

iTRAQ

Isobaric **T**ags for **R**elative and **A**bsolute **Q**uantitation

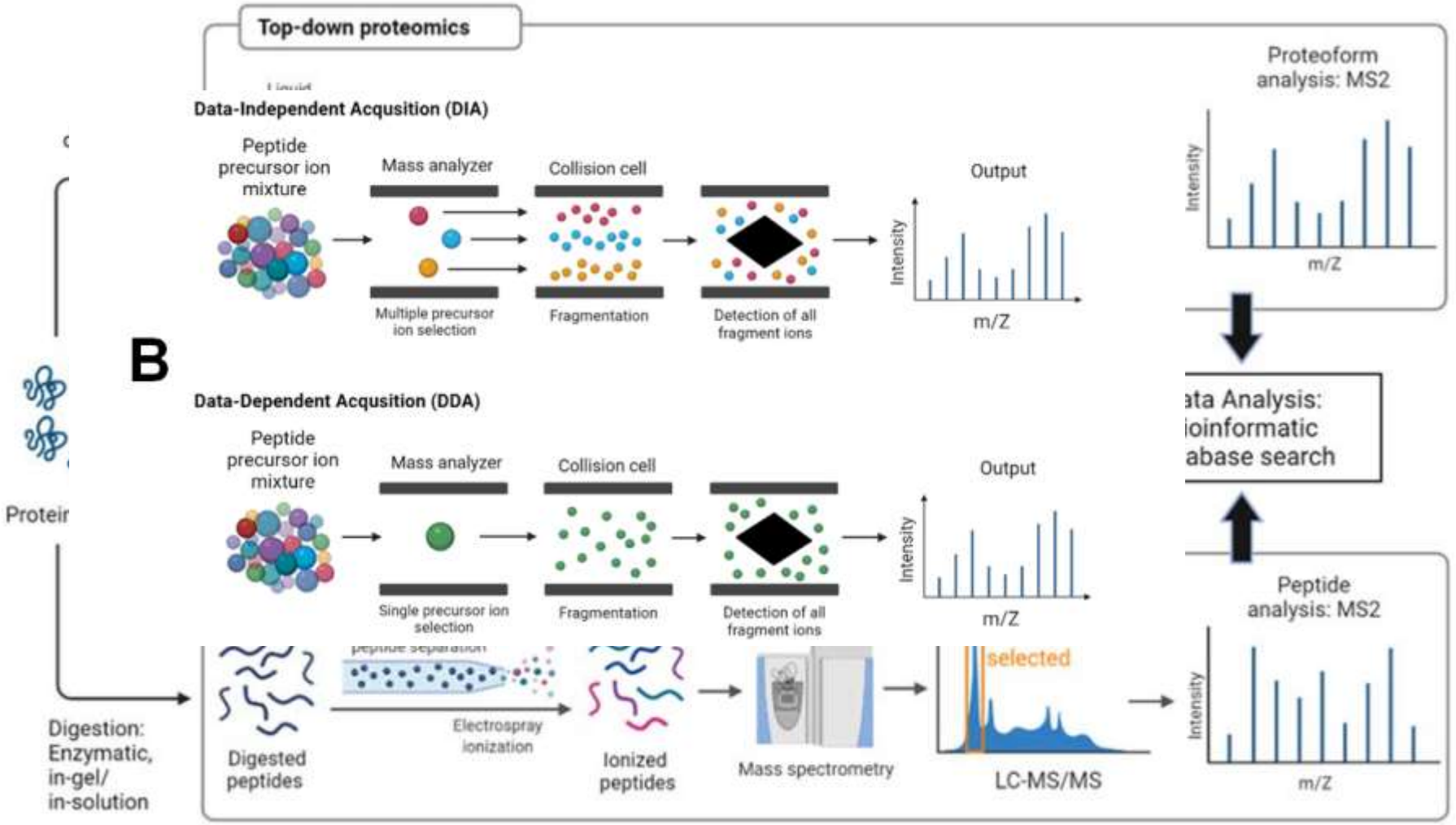




TMT

Tandem **M**ass **T**ags

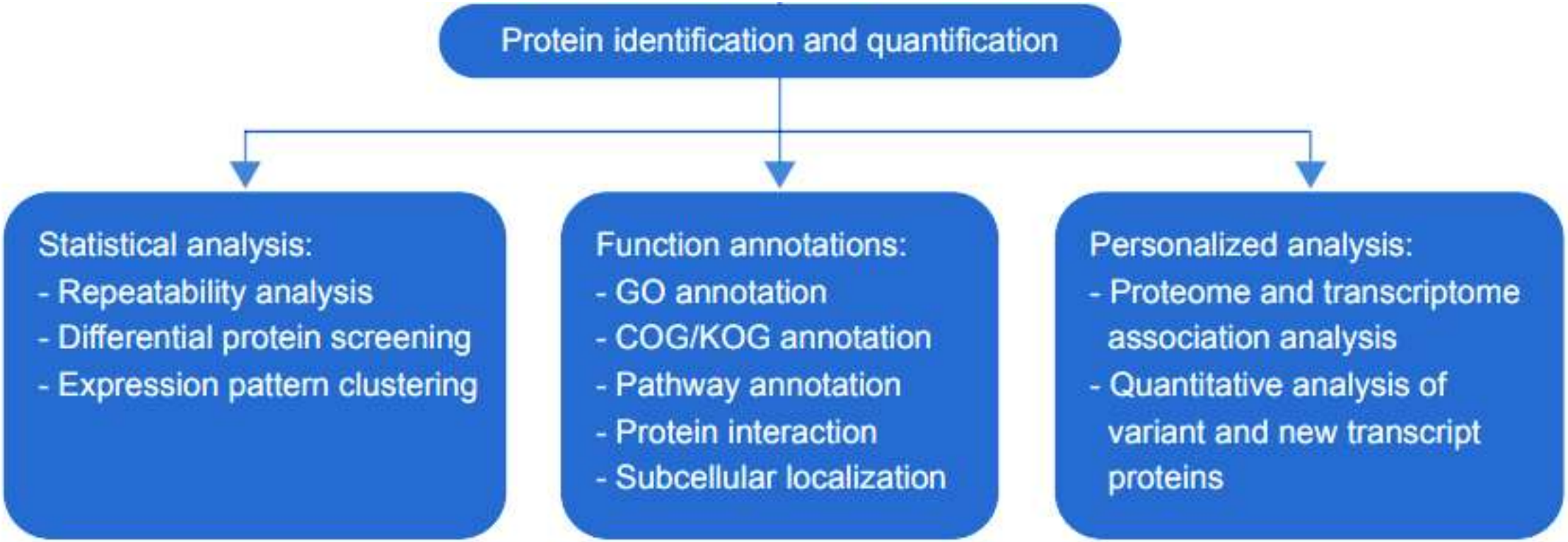
iTRAQ and TMT are very flexible



Why should we use iTRAQ/TMT to quantify proteins?

- All protein can be analyzed, identified and quantified in one experiment.
- Can detect post-transcriptional modifications
- **Data-rich**

What does “Data Rich” mean?



SILAC

Advantages

- High labeling efficiency
- Good quantitative repeatability, low protein consumption
- Can identify all PTM

Disadvantages

- Can’t run multiple targets in the same sample
- Mainly suitable for passable cell-lines and bacteria
- Labor intensive

iTRAQ/TMT

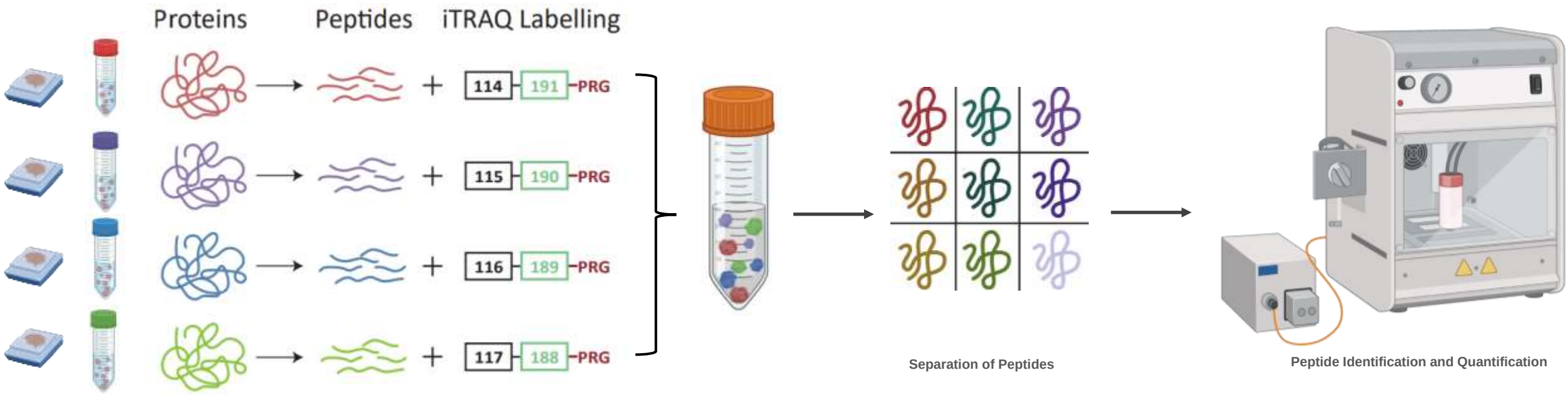
Advantages

- Can run multiple samples at the same time
- Suitable for most tissue types
- Semi- automated

Disadvantages

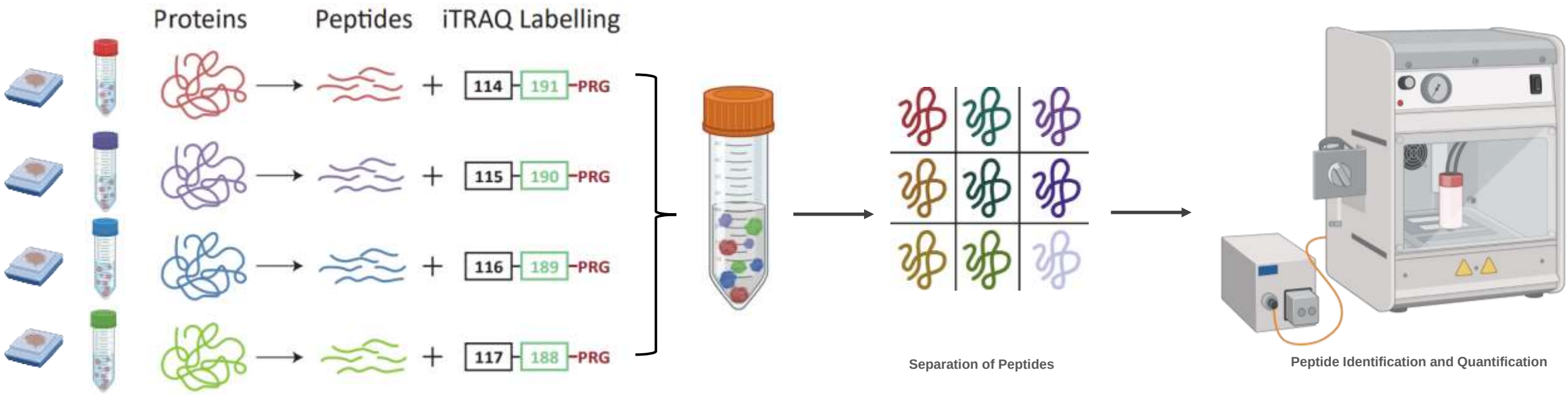
- Uses relative quantification, not absolute quantification.
- More demanding
- Can’t detect PTM at N-terminal

How does iTRAQ work?

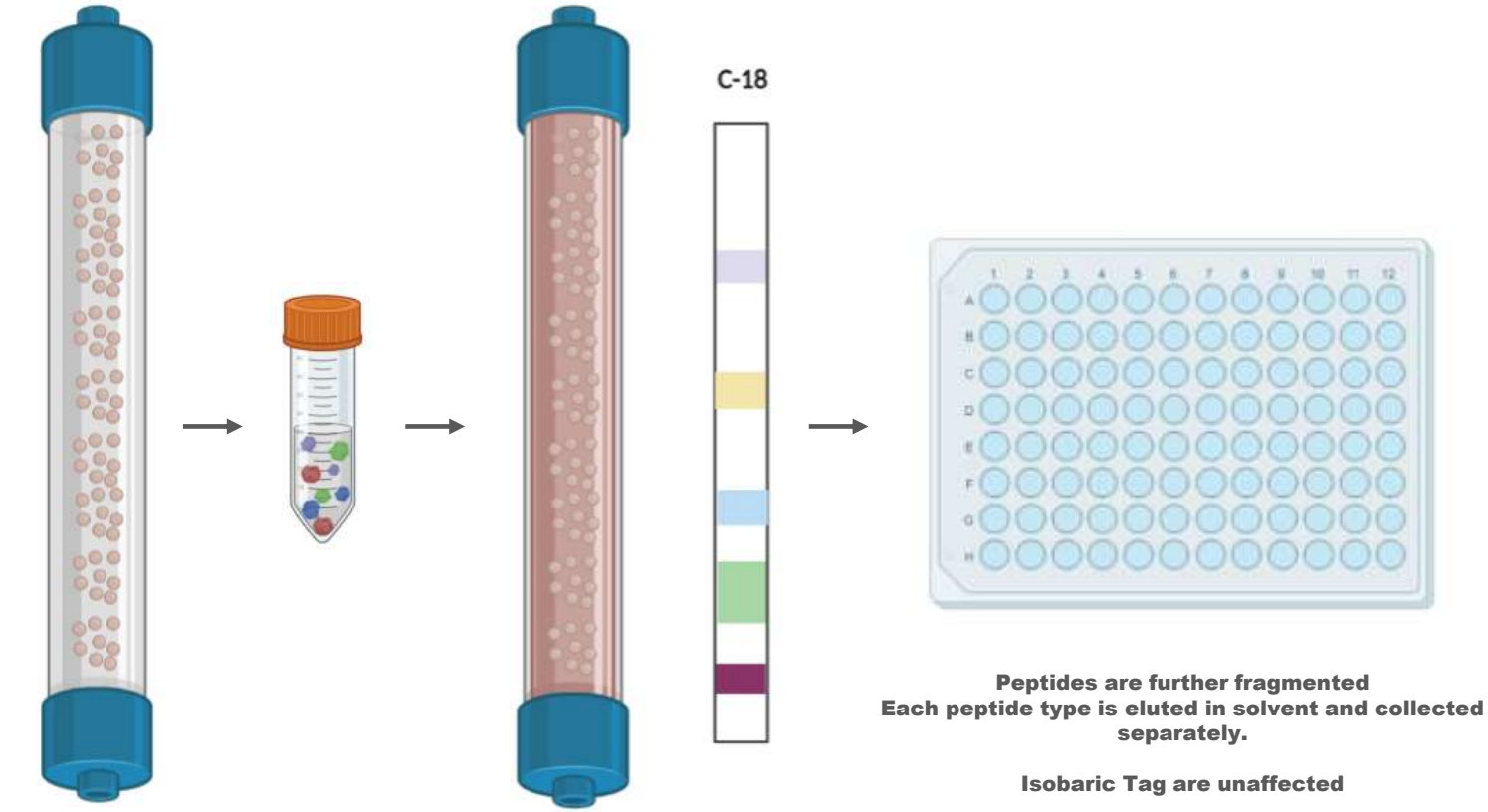


Bioinformatics | Data Validation | Protein identification & quantification

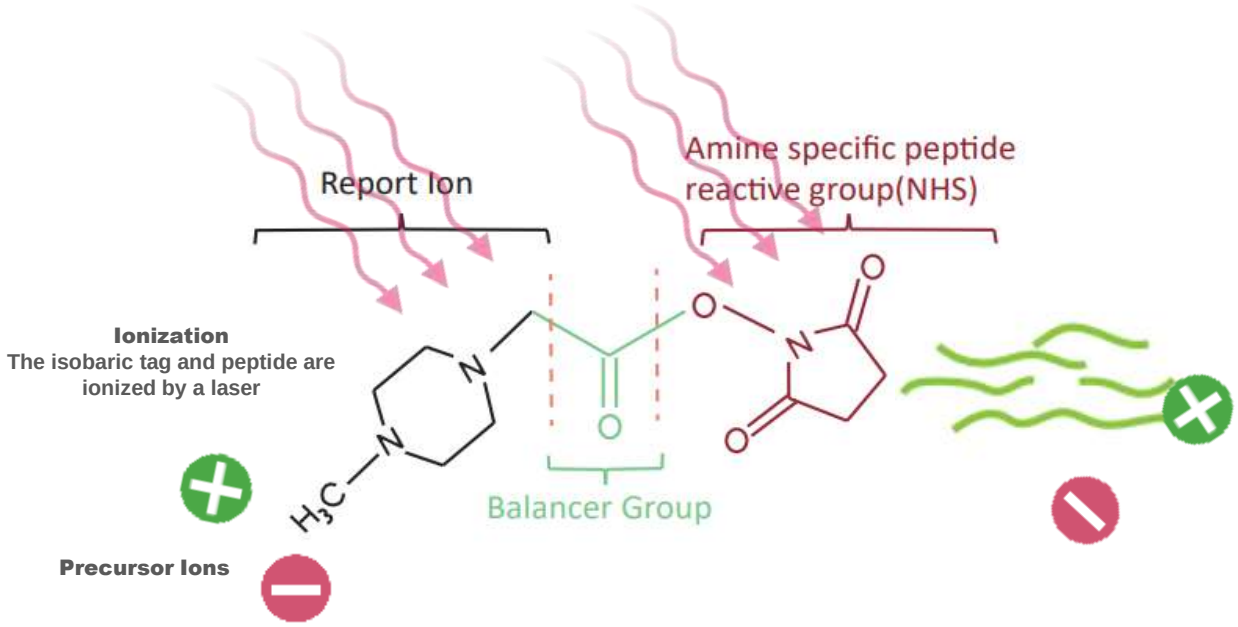
How does iTRAQ work?



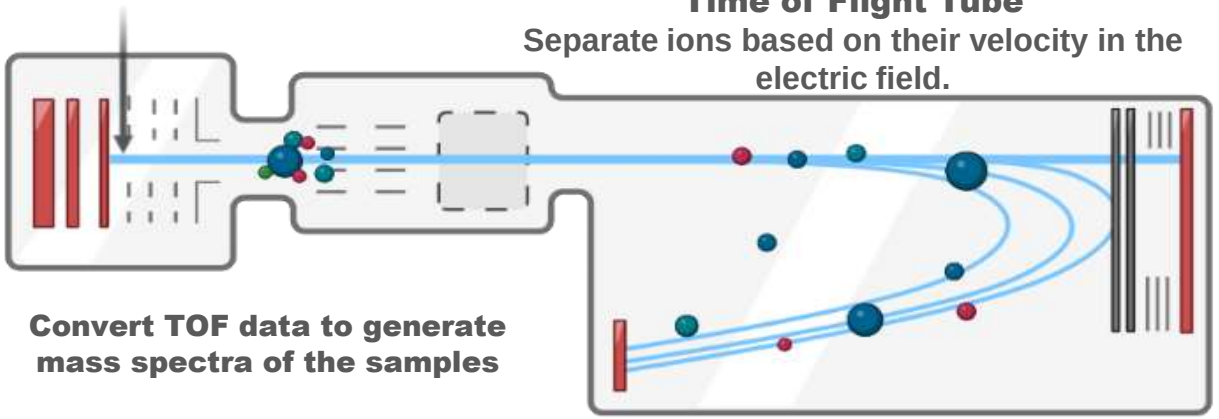
How did they sort the proteins after iTRAQ tagging?



How does MS/MS work?



Time of Flight Tube
Separate ions based on their velocity in the electric field.



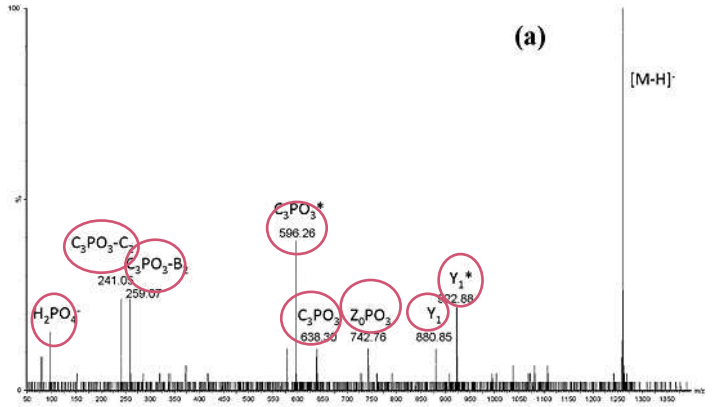
Convert TOF data to generate mass spectra of the samples

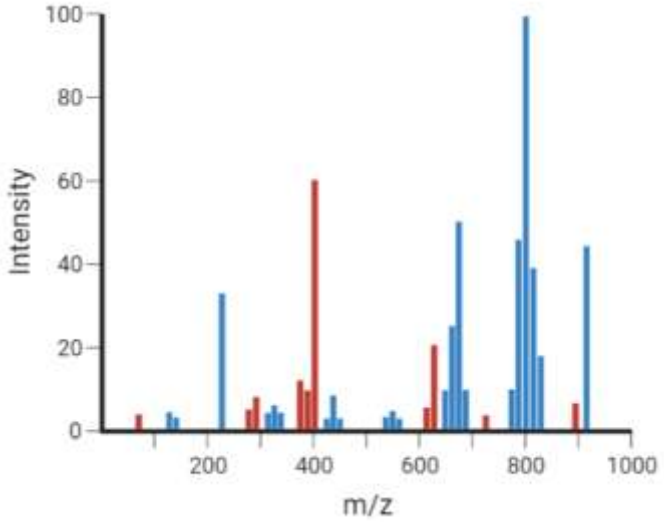
At this point, we know the relative abundance of peptides/proteins in each sample, and their molecular weight.

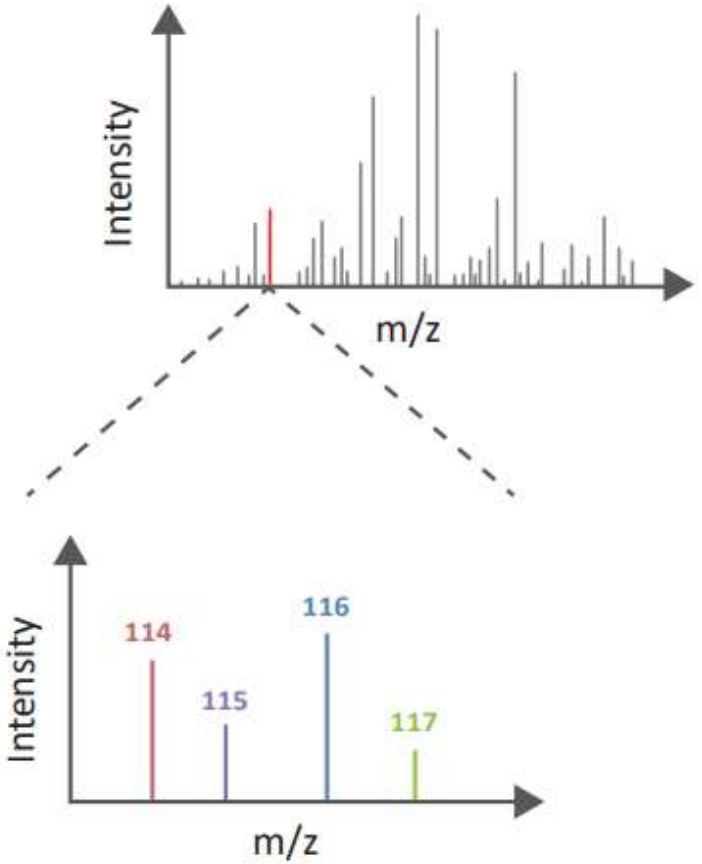
MALDI-MS MS/MS Analysis

Matrix-assisted laser desorption/ionization

Tandem Mass spectrometry







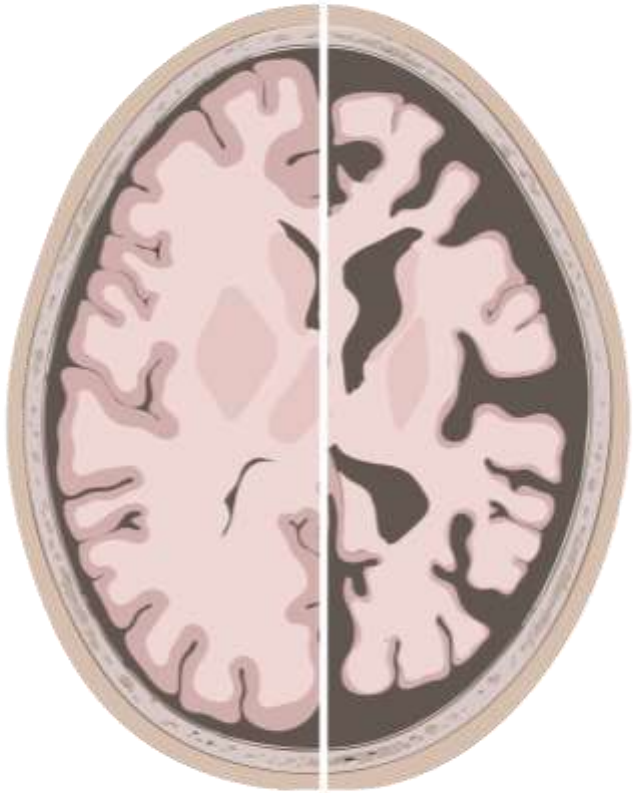
This Mass Spectra provides us with information on the fragmentation of the ions, informing us the Posttranslational Modifications and Amino Acid sequence.

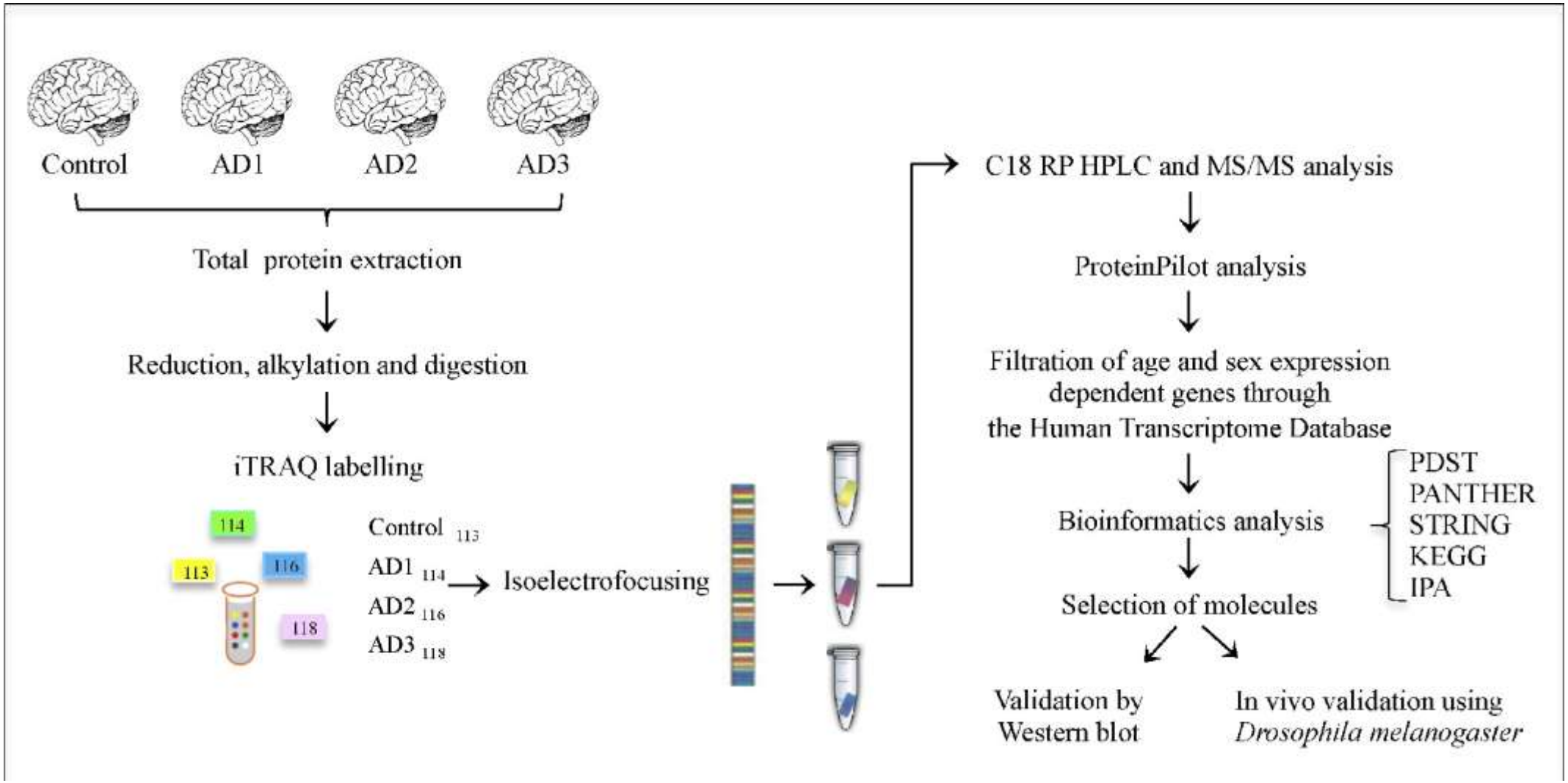
Juan Pedro Luna-Arias Lab

- Center for Research and Advanced Studies of the National Polytechnic Institute
- Cinvestav · Departamento de Biología Celular

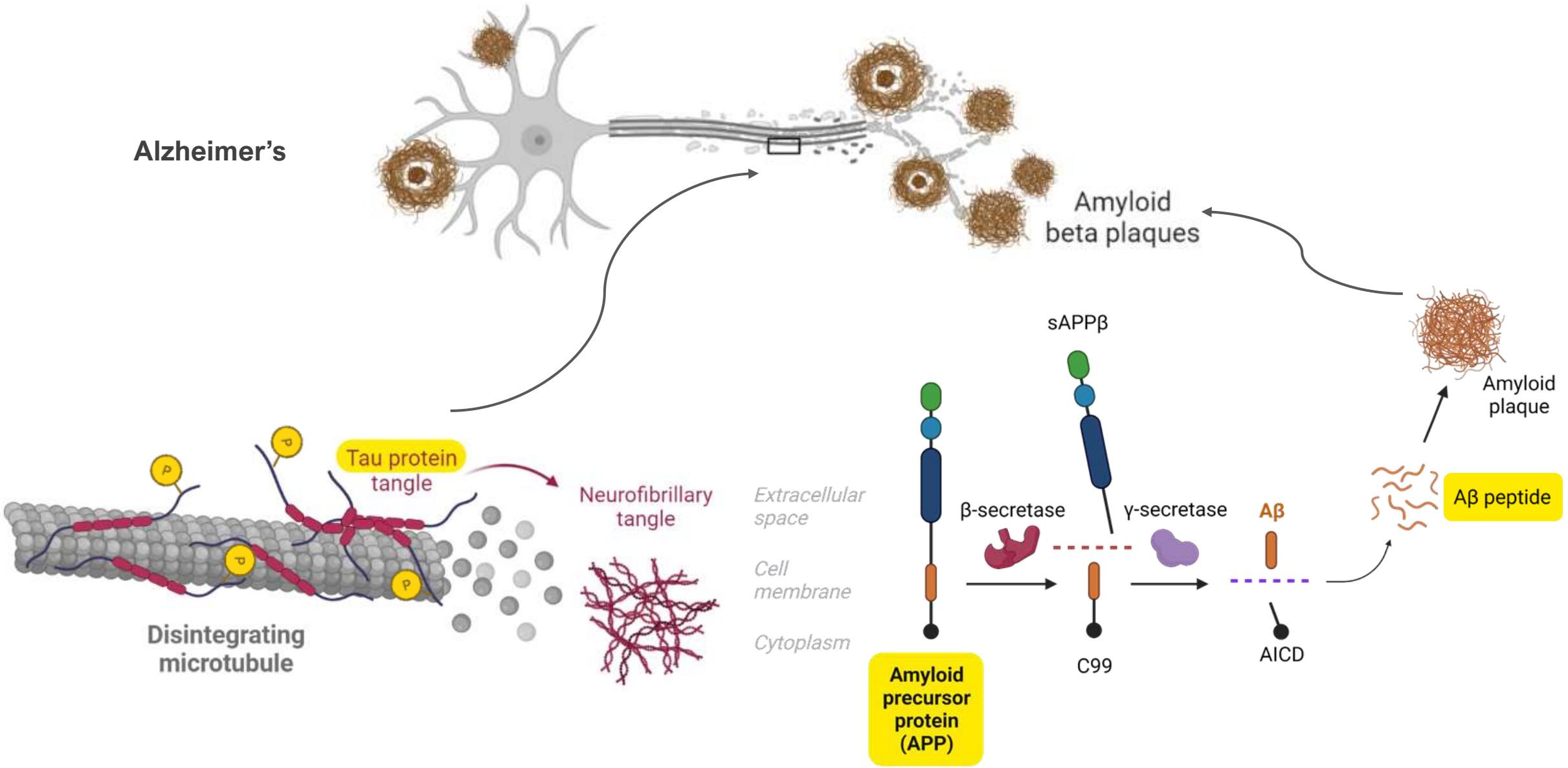
Identification of proteins that are differentially expressed in brains with Alzheimer's disease using iTRAQ labeling and tandem mass spectrometry

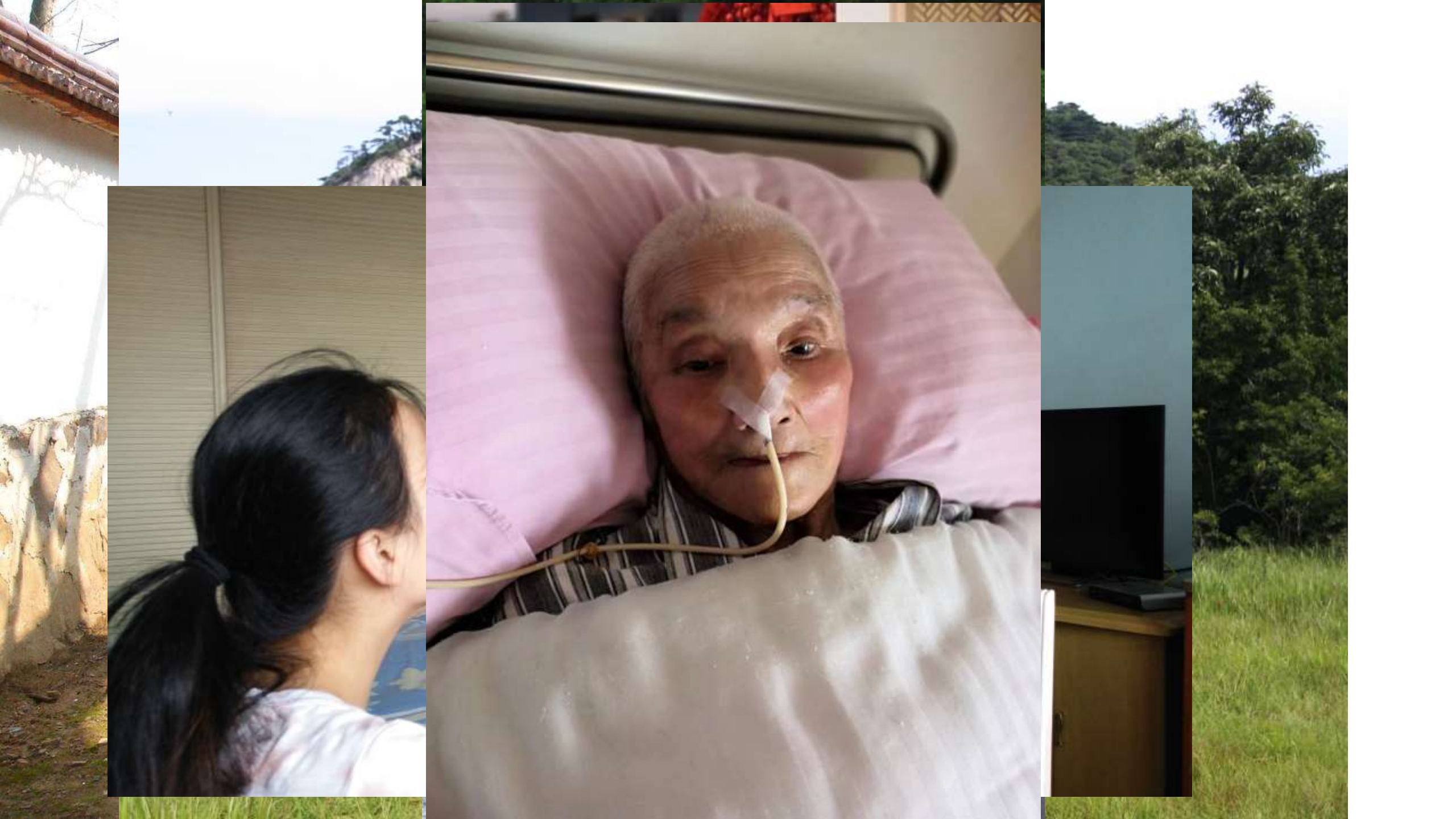
Benito Minjarez [a,1], Karla Grisel Calderón-González [a], Ma. Luz Valero Rustarazo [b,2], María Esther Herrera-Aguirre [a], María Luisa Labra-Barrios [a], Diego E. Rincon-Limas [c,d], Manuel M. Sánchez del Pino [b,3], Raul Mena [e,4], Juan Pedro Luna-Arias [a,*]



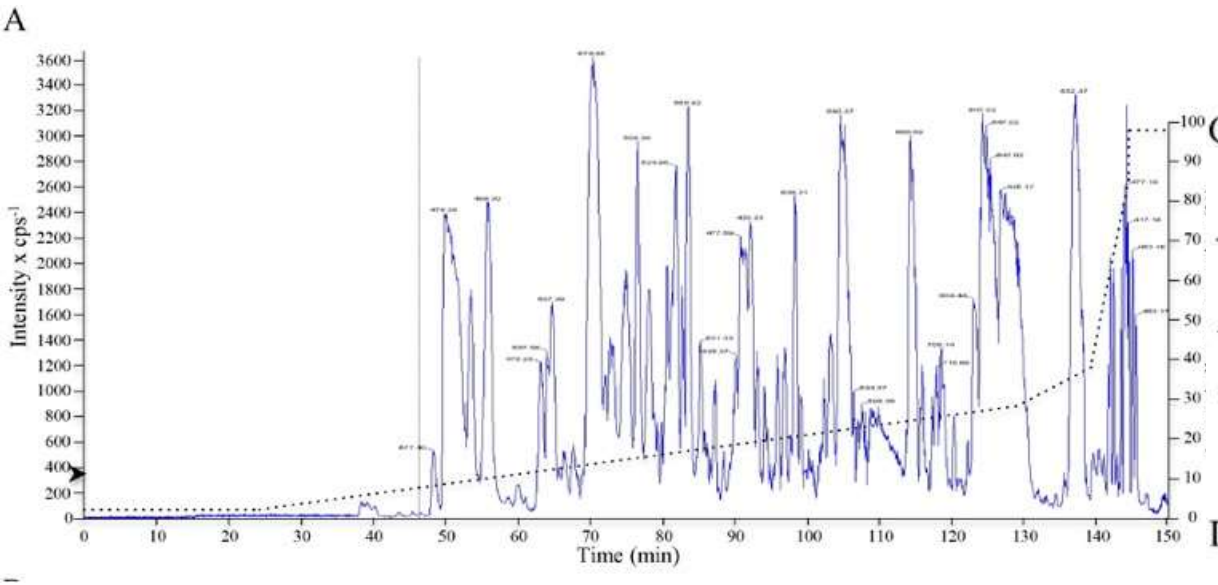

Control
AD1
AD2
AD3
Total protein extraction
Reduction, alkylation and digestion
iTRAQ labelling
114
113
116
118
Control 113
AD1 114
AD2 116
AD3 118
Isoelectrofocusing
C18 RP HPLC and MS/MS analysis
ProteinPilot analysis
Filtration of age and sex expression dependent genes through the Human Transcriptome Database
Bioinformatics analysis
PDST
PANTHER
STRING
KEGG
IPA
Selection of molecules
Validation by Western blot
In vivo validation using Drosophila melanogaster

Characteristics of Alzheimer's Disease



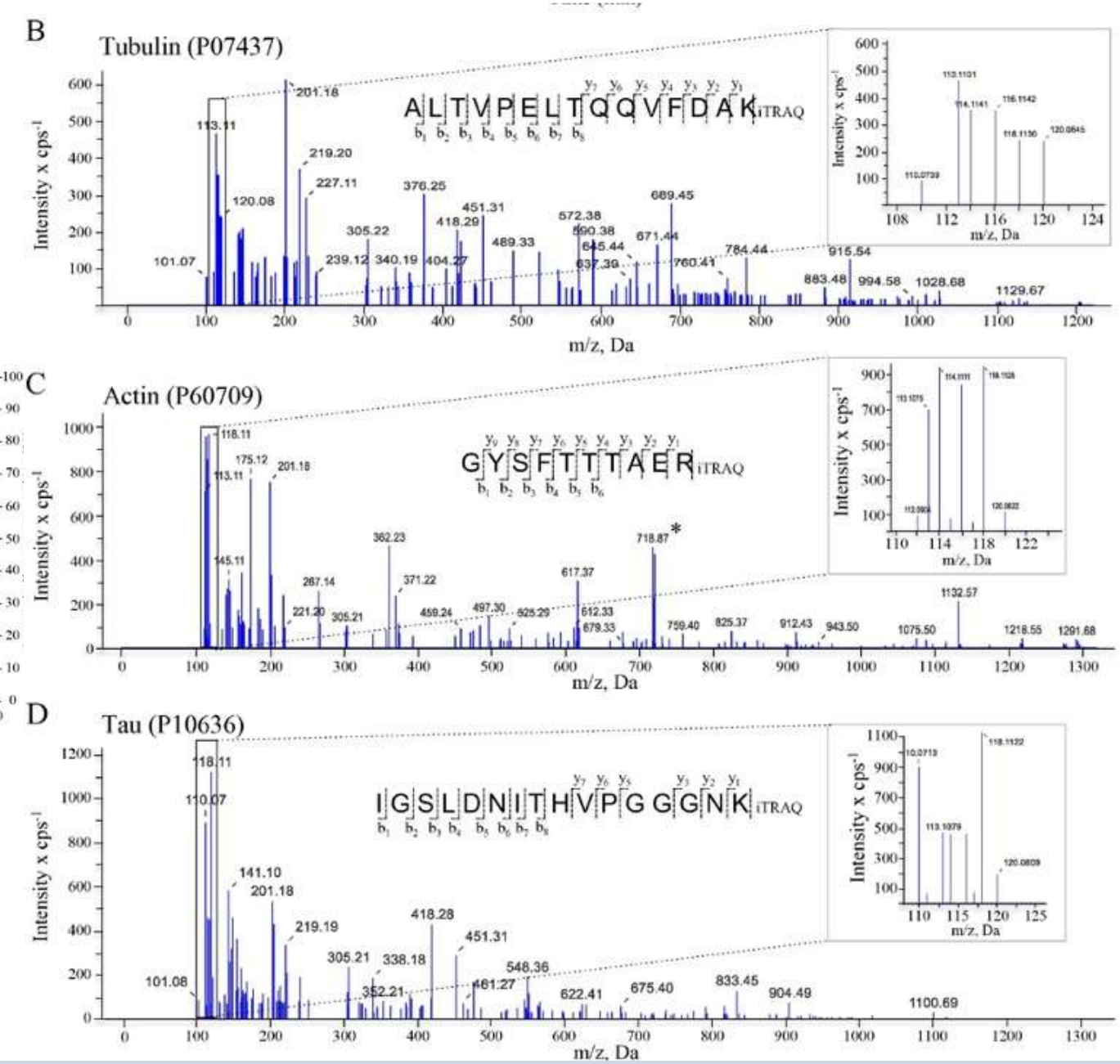


MS/MS esults

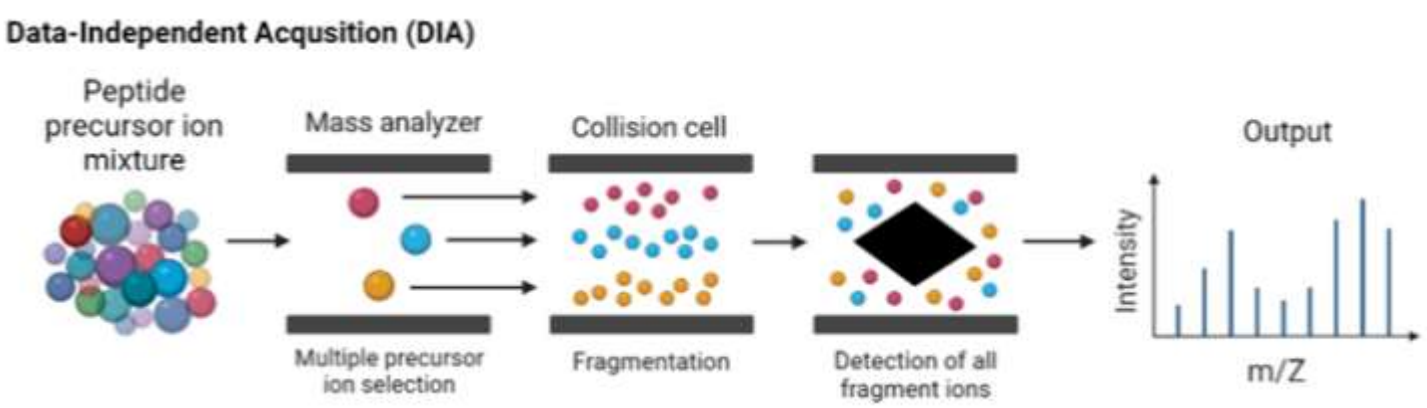


Time of Flight mass spectra

Total ion count of peptides contained in a segment as a function of the elution time as an example.



Bioinformatics Analysis



Identify the sequences detected in MS/MS, compared with sequence with reference genome

Proteins differentially expressed

Overexpressed

Table 1
Overexpressed polypeptides identified in whole protein extracts of brains with Alzheimer's disease by iTRAQ labeling and tandem mass spectrometry.

Protein name	Gene	UniProtKB[a] acc. no.	MW[b] (kDa)	pI[c]	Pep.[d] Identi. (≥95)	% Cov.[e] (≥95)	114:113[f]	116:113[g]	118:113[h]
1. Glial fibrillary acidic protein	GFAP	P14136	49.88	5.42	68	66.2	1.85	1.49	3.49
2. Collagen alpha-2(I) chain	COL1A2	P08123	129.31	9.08	50	36.2	1.41	1.97	6.10
3. **L-Lactate dehydrogenase B chain**	**LDHB**	**P07195**	36.64	5.71	18	42.5	1.53	1.24	1.21
4. **Hemoglobin subunit alpha**	**HBA1**	**P69905**	15.26	8.72	59	71.8	1.42	2.09	2.99
5. Alpha-1-antitrypsin	SERPINA1	P01009	46.74	5.37	11	29.4	2.84	1.26	1.62
6. Alpha-crystallin B chain	CRYAB	P02511	20.16	6.76	8	58.8	2.37	1.95	1.42
7. Ig gamma-1 chain C region	IGHG1	P01857	36.10	8.46	9	24.2	3.13	1.56	2.25
8. Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial	ALDH6A1	Q02252	57.84	8.72	5	9.9	1.29	1.31	1.33
9. Haptoglobin	HP	P00738	45.20	6.13	5	12.3	3.58	1.42	2.29
10. Ferritin light chain	FTL	P02792	20.02	5.50	7	25.7	1.50	1.37	2.65
11. Versican core protein	VCAN	P13611	372.82	4.43	7	2.59	2.35	1.60	2.06
12. Carbonic anhydrase 2	**CA2**	P00918	29.25	6.87	5	25.0	2.21	1.45	1.23
13. Peroxiredoxin-6	PRDX6	P30041	25.03	6.00	10	29.4	1.45	1.20	1.20
14. Ferritin heavy chain	FTH1	P02794	21.22	5.31	7	22.4	1.88	1.46	1.73
15. Gelsolin	GSN	P06396	85.70	5.90	6	7.6	1.54	1.39	1.89
16. Histone H2B type 1-O	HIST1H2BO	P23527	13.91	10.31	7	27.7	1.25	1.30	1.33
17. Glutathione *S*-transferase P	GSTP1	P09211	23.36	5.43	4	29.5	2.12	1.50	1.82
18. Histone *H3.3*	H3F3A	P84243	15.33	11.27	2	11.7	1.30	1.44	1.98
19. Ig alpha-1 chain C region	IGHA1	P01876	37.65	6.08	2	7.0	3.63	2.07	1.63

Overexpressed: 61

Proteins differentially expressed

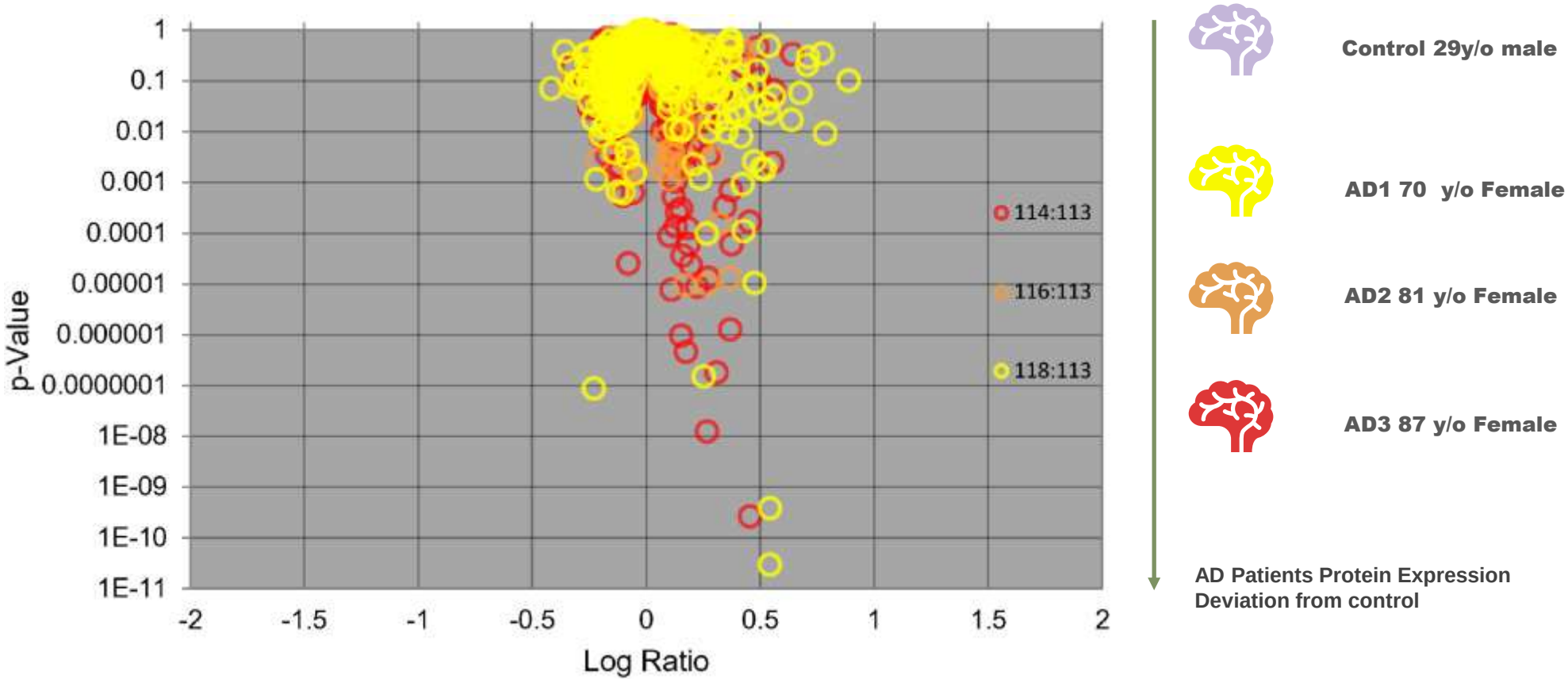
Subexpressed

Table 2
Subexpressed polypeptides identified in whole protein extracts of brains with Alzheimer's disease by iTRAQ labeling and tandem mass spectrometry.

Protein name	Gene	UniProtKB[a] acc. no.	MW[b] (kDa)	pI[c]	Pep.[d] ident. (≥95)	% Cov.[e] (≥95)	114:113[f]	116:113[g]	118:113[h]
1. Annexin A6	ANXA6	P08133	75.87	5.41	5	9.8	0.76	0.79	0.76
2. ***T*-complex protein 1 subunit beta**	**CCT2**	**P78371**	57.49	6.01	4	11.2	0.73	0.70	0.56
3. Synapsin-2	SYN2	Q92777	62.85	8.58	6	10.8	0.73	0.61	0.60
4. Neuronal cell adhesion molecule	NRCAM	Q92823	143.89	5.45	4	3.9	0.83	0.85	0.70
5. **NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial**	**NDUFS3**	**O75489**	30.24	6.98	5	16.6	0.71	0.82	0.76
6. Disks large homolog 4	DLG4	P78352	80.50	5.58	2	2.6	0.67	0.67	0.64
7. 2-oxoglutarate dehydrogenase-like, mitochondrial	OGDHL	Q9ULD0	114.48	6.18	2	2.8	0.72	0.82	0.70
8. ADP/ATP translocase 3	SLC25A6	P12236	32.87	9.76	5	19.1	0.57	0.69	0.59
9. DnaJ homolog subfamily C member 11	DNAJC11	Q9NVH1	63.28	8.54	2	5.9	0.79	0.67	0.67
10. NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, mitochondrial	NDUFS8	O00217	23.70	6.00	2	9.5	0.63	0.82	0.38
11. ATP synthase subunit f, mitochondrial	ATP5J2	P56134	10.92	9.70	2	25.5	0.62	0.74	0.81
12. V-type proton ATPase subunit D	ATP6V1D	Q9Y5K8	28.26	9.36	2	13.7	0.77	0.82	0.69
13. Glutaminase kidney isoform, mitochondrial	GLS	O94925	73.46	7.85	2	4.6	0.63	0.85	0.64
14. V-type proton ATPase 116 kDa subunit a isoform 1	ATP6V0A1	Q93050	96.41	6.01	2	3.3	0.70	0.81	0.85
15. Kinesin heavy chain isoform 5C	KIF5C	O60282	109.49	5.86	2	2.2	0.76	0.68	0.62
16. Copine-5	CPNE5	Q9HCH3	65.73	5.65	2	4.7	0.72	0.80	0.79
17. UMP-CMP kinase	CMPK1	P30085	22.22	5.44	4	20.9	0.61	0.61	0.46
18. **Tubulin alpha-1A**	**TUBA1A**	**Q71U36**	50.14	4.94	84	50.5	0.74	0.58	0.48
19. AP-2 complex subunit mu	AP2M1	Q96CW1	49.65	9.57	2	6.2	0.71	0.78	0.66

Subexpressed: 69

MS/MS Results



Volcano plot of proteins identified in Alzheimer's disease brains.

Gene Ontology

Classification of the overexpressed and subexpressed proteins found in common in all brains with Alzheimer's disease according to diseases and biofunctions with the IPA software (Core II analysis).

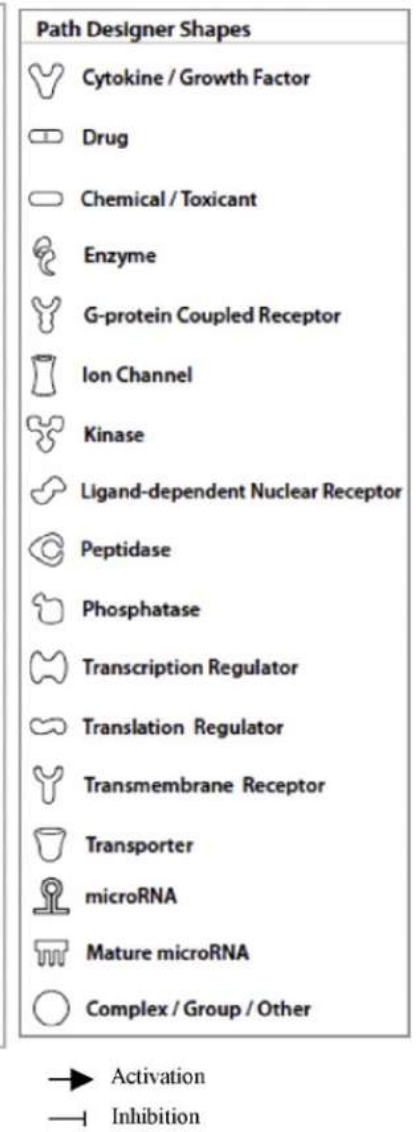
Category	*p*-Value	N	Molecules
Neurological disease	4.53×10^{-7} to 4.48×10^{-2}	32	**FTL**,*MAP2*,**PRDX1**,*GAK*,**SERPINA3**,**LDHB**,**VCAN**,*SLC12A5*,C4A/**C4B**,**CTSD**,*SLC25A6*,**SOD2**,**PREPL**,*NEDD8*,*OPA1*,*DLG4*,**GFAP**,*CAMK2B*,**S100A1**,*GLS*,**HNRNPDL**,**GSN**,**HP**,*TUBA1A*,*NDUFS8*,*NDUFV2*,**RTN4**,*SLC1A2*,**A2M**,*SYN2*,**FTH1**,**PRDX2**
Psychological disorders	1.81×10^{-6} to 4.48×10^{-2}	30	**FTL**,*MAP2*,**PRDX1**,*GAK*,**SERPINA3**,**VCAN**,*SLC12A5*,**LDHB**,C4A/**C4B**,**CTSD**,**SOD2**,*SLC25A6*,**PREPL**,*NEDD8*,*OPA1*,*DLG4*,**GFAP**,*CAMK2B*,**S100A1**,*GLS*,**GSN**,**HNRNPDL**,*TUBA1A*,*NDUFS8*,*NDUFV2*,**RTN4**,*SLC1A2*,*SYN2*,**PRDX2**,**FTH1**
Metabolic disease	2.54×10^{-6} to 9.12×10^{-3}	11	C4A/**C4B**,**CTSD**,**SOD2**,**PRDX1**,*GAK*,*SLC1A2*,*OPA1*,**GFAP**,**SERPINA3**,**GSN**,*CAMK2B*
Skeletal and muscular disorders	2.58×10^{-6} to 8.49×10^{-3}	13	**FTL**,*MAP2*,**SERPINA3**,**HNRNPDL**,**VCAN**,**LDHB**,C4A/**C4B**,*SLC25A6*,*TUBA1A*,**PREPL**,**GFAP**,**FTH1**,**PRDX2**
Cell morphology	1.5×10^{-4} to 4.48×10^{-2}	6	*DNM1*,**SOD2**,*RHOA*,**RTN4**,*OPA1*,**SYNM**
Cell death and survival	1.54×10^{-4} to 4.55×10^{-2}	10	**CRYAB**,*TUBA1A*,**SIRT2**,**SOD2**,**VTN**,**CAT**,**SERPINA3**,**SYNM**,**PRDX2**,**FTH1**
Hereditary disorder	4.05×10^{-4} to 1.82×10^{-2}	15	**S100A1**,*MAP2*,**SERPINA3**,**GSN**,*SLC12A5*,**VCAN**,C4A/**C4B**,**SOD2**,**PREPL**,*NEDD8*,**RTN4**,*SLC1A2*,*DLG4*,**GFAP**,*SYN2*
Nervous system development and function	1.68×10^{-3} to 3.6×10^{-2}	3	*TUBA1A*,*RHOA*,**RTN4**
Tissue morphology	1.68×10^{-3} to 9.12×10^{-3}	2	*RHOA*,**RTN4**
Cellular compromise	2.23×10^{-3} to 2.23×10^{-3}	2	*DNM1*,**RTN4**
Free radical scavenging	4.3×10^{-3} to 9.12×10^{-3}	2	**SOD2**,**PRDX2**
Amino acid metabolism	9.12×10^{-3} to 9.12×10^{-3}	1	*GLS*
Cellular assembly and organization	9.12×10^{-3} to 4.48×10^{-2}	2	*OPA1*,**GFAP**
Cellular development	9.12×10^{-3} to 9.12×10^{-3}	1	**SIRT2**
Cellular growth and proliferation	9.12×10^{-3} to 9.12×10^{-3}	1	**SIRT2**
Developmental disorder	9.12×10^{-3} to 9.12×10^{-3}	1	**GSN**
Ophthalmic disease	9.12×10^{-3} to 4.48×10^{-2}	2	**SERPINA3**,**GSN**
Small molecule biochemistry	9.12×10^{-3} to 1.82×10^{-2}	3	**APOA1**,*GLS*,**PRDX2**
Behavior	1.82×10^{-2} to 1.82×10^{-2}	1	**SOD2**
Lipid metabolism	1.82×10^{-2} to 1.82×10^{-2}	1	**APOA1**
Molecular transport	1.82×10^{-2} to 1.82×10^{-2}	1	**APOA1**
Cell-to-cell signaling and interaction	3.6×10^{-2} to 3.6×10^{-2}	1	**VTN**
Organ morphology	3.6×10^{-2} to 3.6×10^{-2}	1	*TUBA1A*
Tissue development	3.6×10^{-2} to 3.6×10^{-2}	1	**VTN**
Cancer	4.48×10^{-2} to 4.48×10^{-2}	1	**SOD2**
Cellular function and maintenance	4.48×10^{-2} to 4.48×10^{-2}	1	**SOD2**

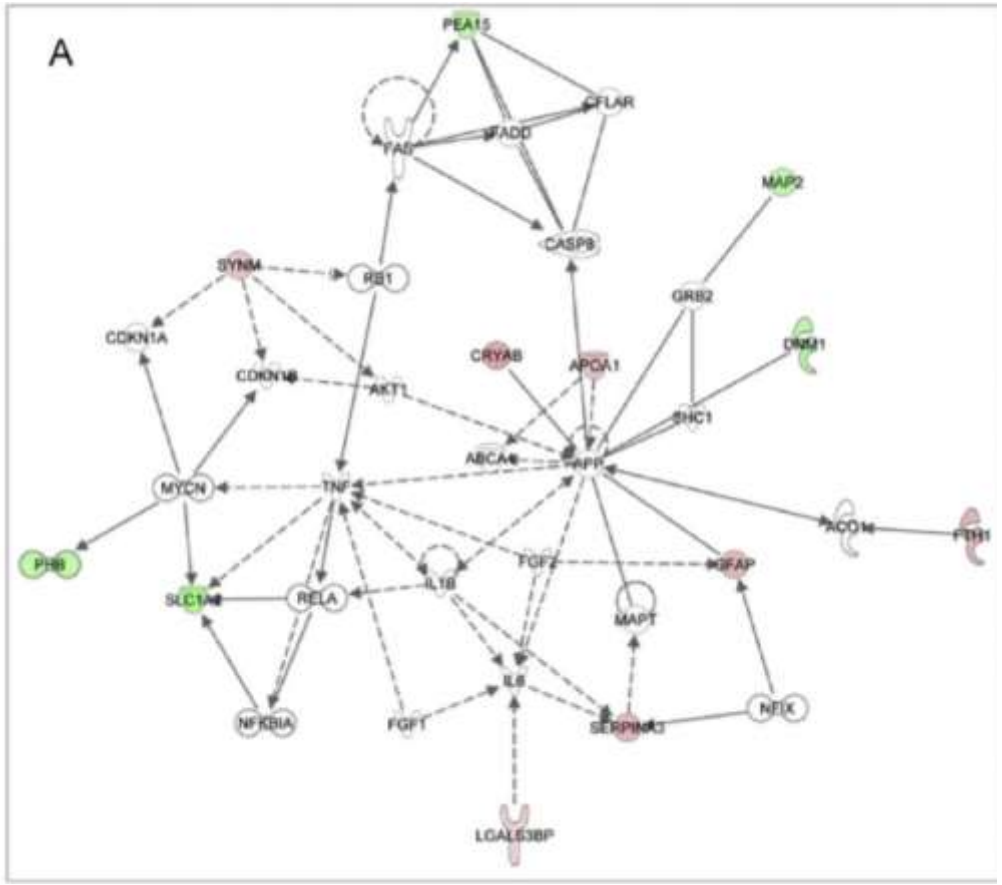
Out of **130** differentially expressed proteins
24% are related to Neurological Disease
23% are related to psychological disorders
8% are related to Metabolic disease

tissues and primary cells: astrocytes and neurons, nervous system CNS and neuroblastoma cell lines; neurological diseases and psychological disorders

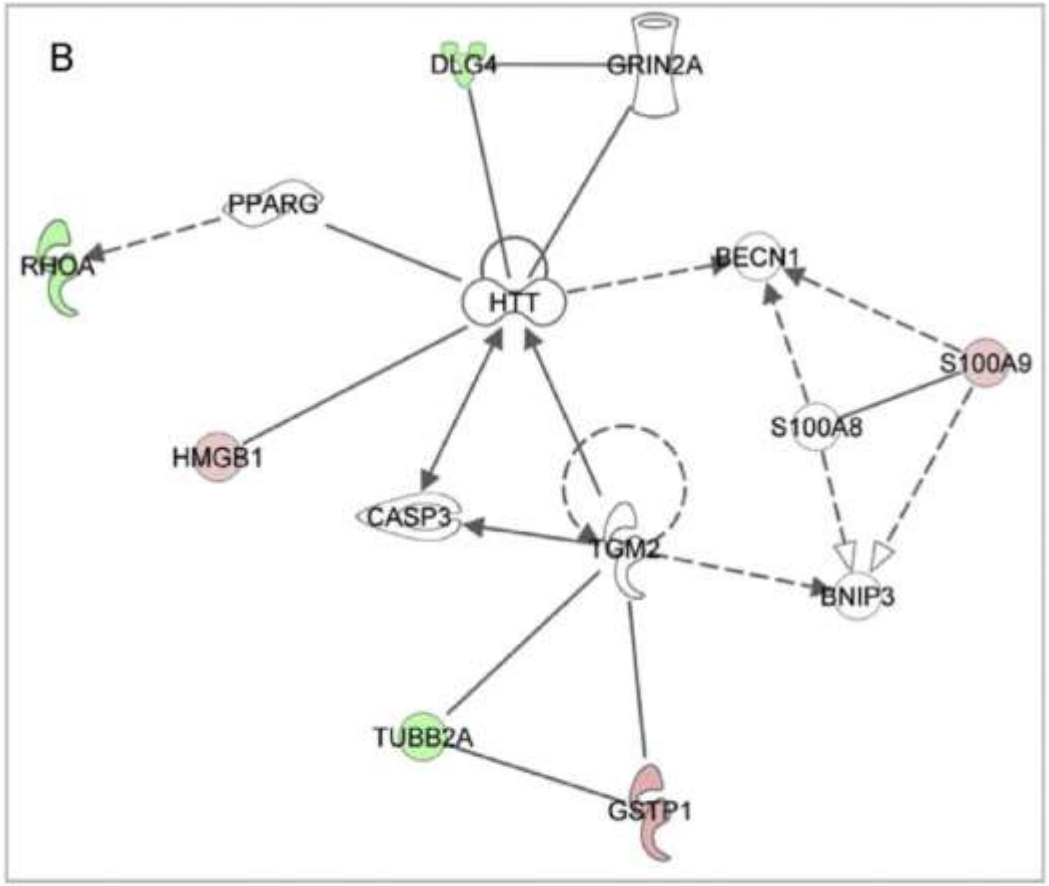
IPA Core Analysis shows how the differentially expressed interact

Ingenuity Pathway Analysis





Cell death and survival, free radical scavenging, cellular growth and proliferation



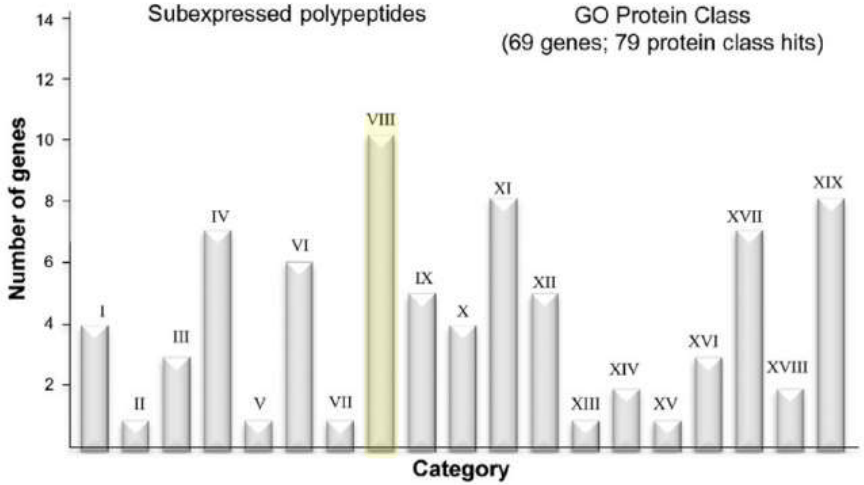
Free radical scavenging, cardiovascular disease, cell death and survival, cancer, and cell morphology

tissues and primary cells: astrocytes and neurons, nervous system CNS and neuroblastoma cell lines; neurological diseases and psychological disorders

Gene Ontology classification results are consistent with AD symptoms

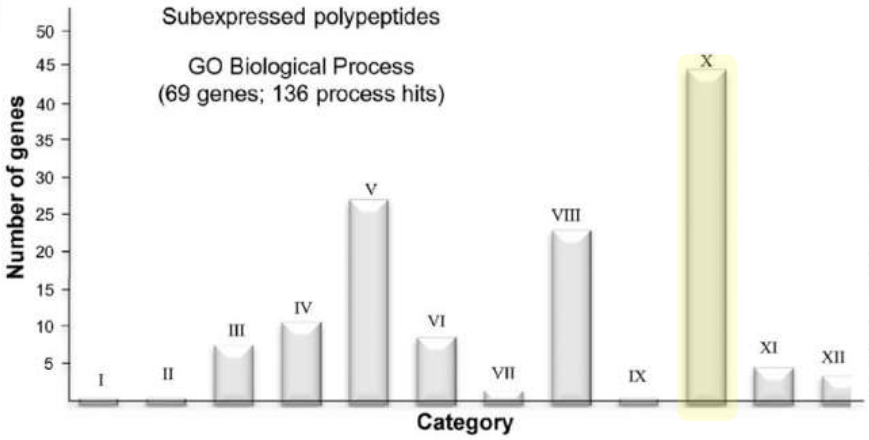
Subexpressed

C

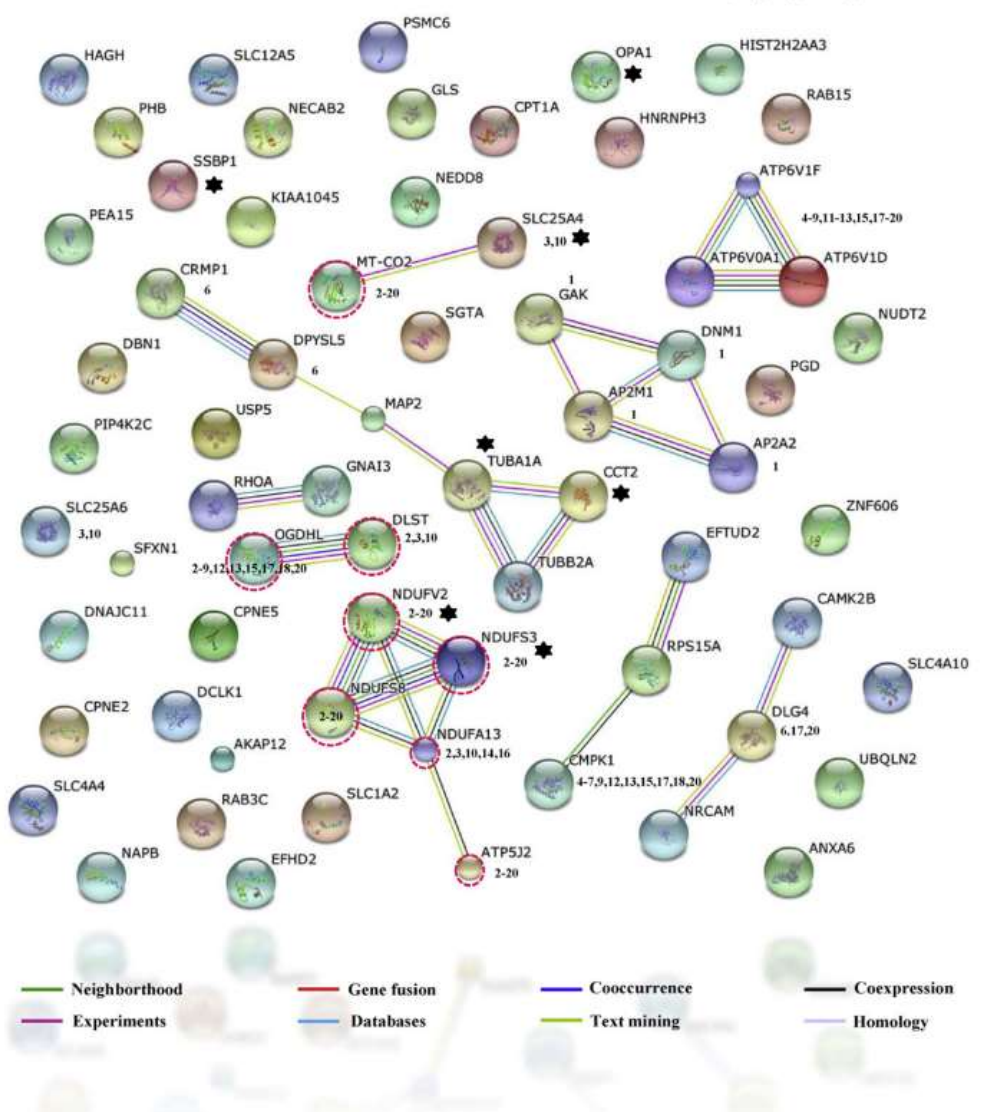


I. Calcium-binding protein (PC00060)
II. Cell adhesion molecule (PC00069)
III. Chaperone (PC00072)
IV. Cytoskeletal protein (PC00085)
V. Defense/immunity protein (PC00090)
VI. Enzyme modulator (PC00095)
VII. Extracellular matrix protein (PC00102)
VIII. Hydrolase (PC00121)
IX. Kinase (PC00137)
X. Membrane traffic protein (PC00150)
XI. Nucleic acid binding (PC00171)
XII. Oxidoreductase (PC00176)
XIII. Phosphatase (PC00181)
XIV. Receptor (PC00197)
XV. Signaling molecule (PC00207)
XVI. Transfer/carrier protein (PC00219)
XVII. Transferase (PC00220)
XVIII. Transmembrane receptor regulatory/adaptor protein (PC00226)
XIX. Transporter (PC00227)

D



I. Apoptotic process (GO: 0006915)
II. Biological adhesion (GO:0022610)
III. Biological regulation (GO:0065007)
IV. Cellular component organization or biogenesis (GO:0071840)
V. Cellular process (GO:0009987)
VI. Developmental process (GO:0032502)
VII. Immune system process (GO:0002376)
VIII. Localization (GO: 0051179)
IX. Locomotion (GO:00400011)
X. Metabolic process (GO:0008152)
XI. Multicellular organismal process (GO:0032501)
XII. Response to stimulus (GO:0050896)

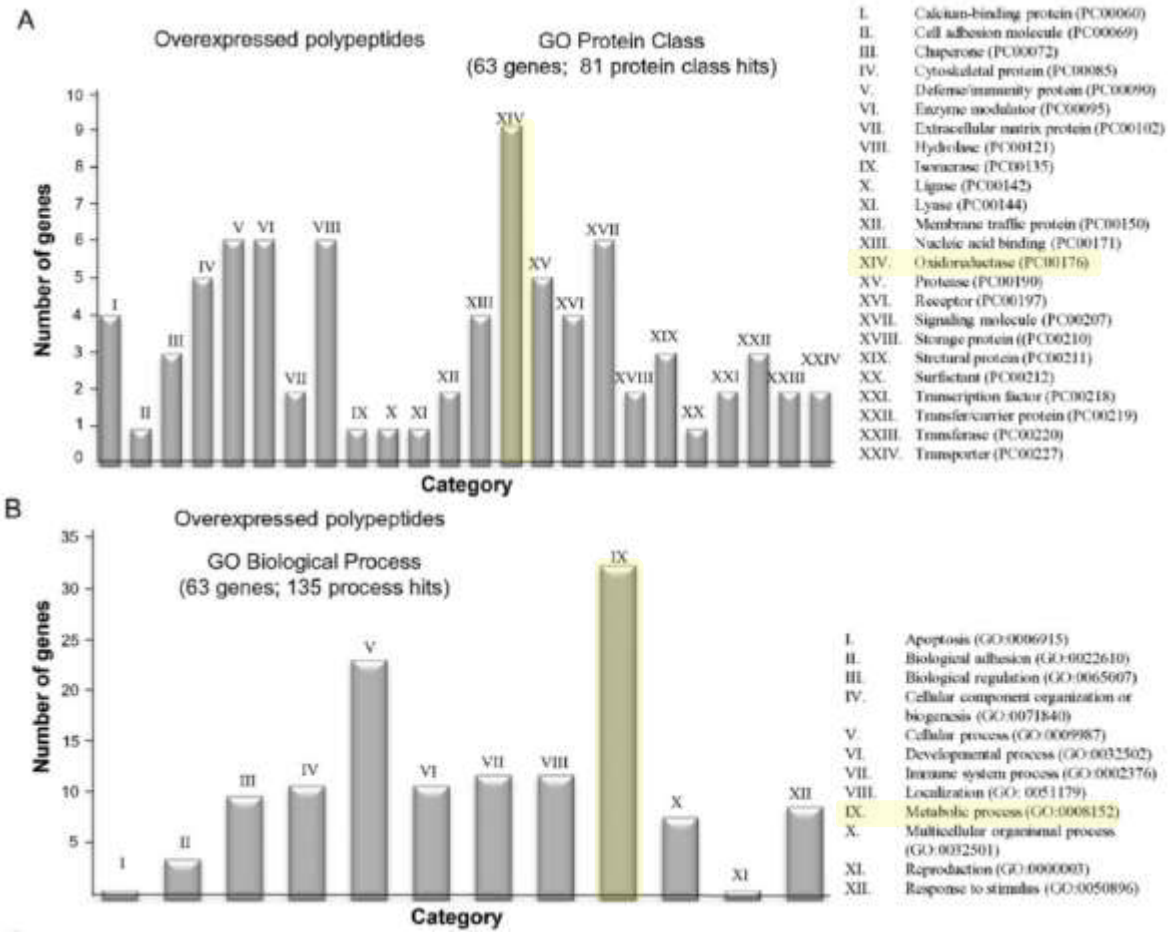


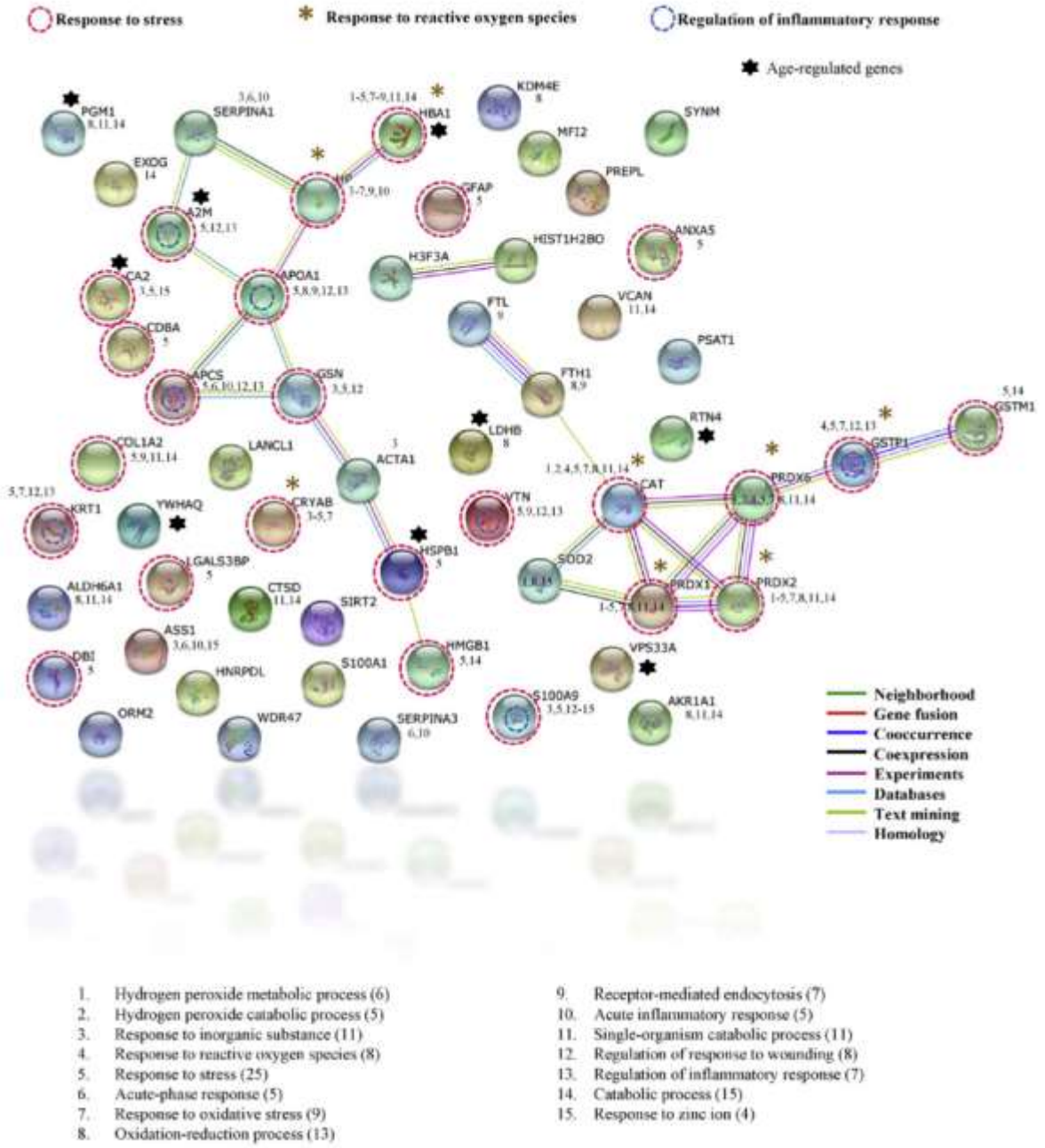
1. Clathrin-mediated endocytosis (4)
2. Cellular respiration (8)
3. Energy derivation by oxidation of organic compounds (10)
4. Ribonucleoside monophosphate metabolic process (8)
5. Nucleoside monophosphate metabolic process (8)
6. Nucleobase-containing small molecule metabolic process (11)
7. Nucleoside triphosphate metabolic process (7)
8. Purine ribonucleoside monophosphate metabolic process (7)
9. Ribonucleoside metabolic process (8)
10. Generation of precursor metabolites and energy (9)
11. ATP metabolic process (6)
12. Nucleoside metabolic process (8)
13. Ribonucleotide metabolic process (8)
14. Electron transport chain (6)
15. Ribose phosphate metabolic process (8)
16. Respiratory electron transport chain (6)
17. Nucleoside phosphate metabolic process (9)
18. Glycosyl compound metabolic process (8)
19. Purine ribonucleoside triphosphate metabolic process (6)
20. Nucleotide metabolic process (9)

tissues and primary cells: astrocytes and neurons, nervous system CNS and neuroblastoma cell lines; neurological diseases and psychological disorders

Gene Ontology classification results are consistent with AD symptoms

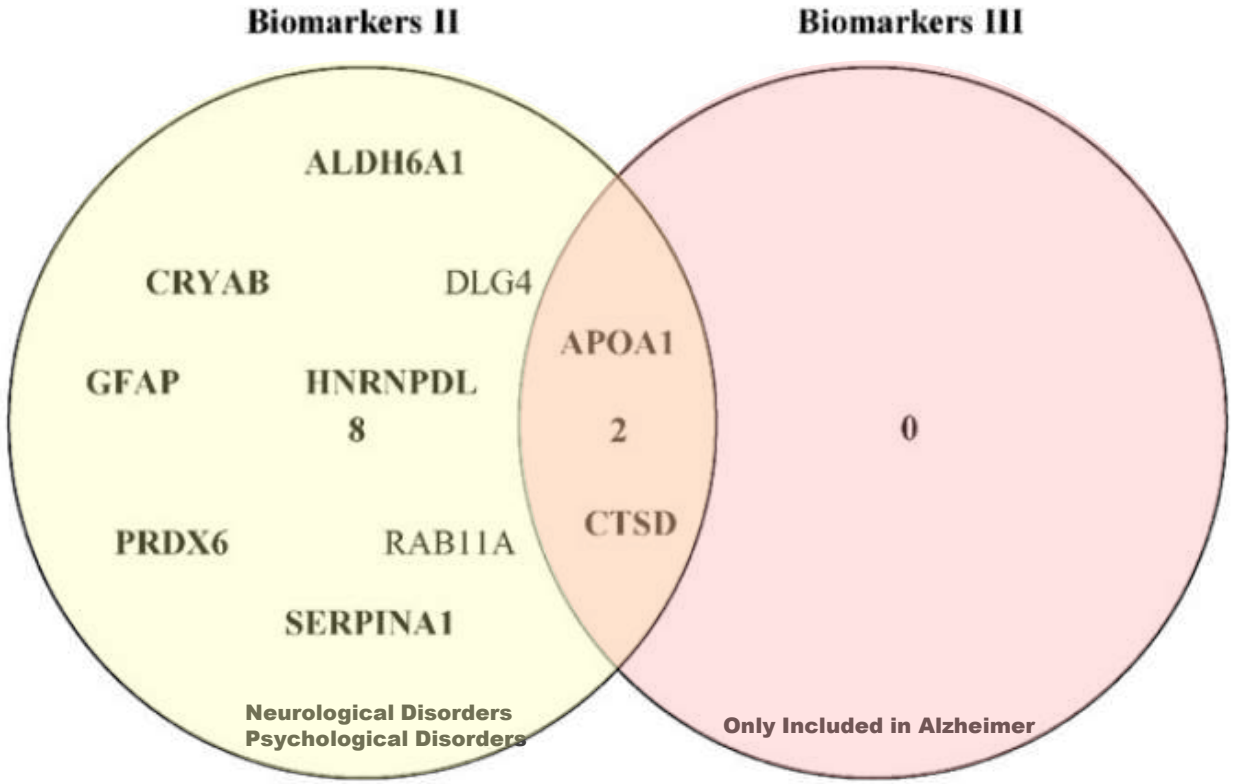
Overexpressed





tissues and primary cells: astrocytes and neurons, nervous system CNS and neuroblastoma cell lines; neurological diseases and psychological disorders

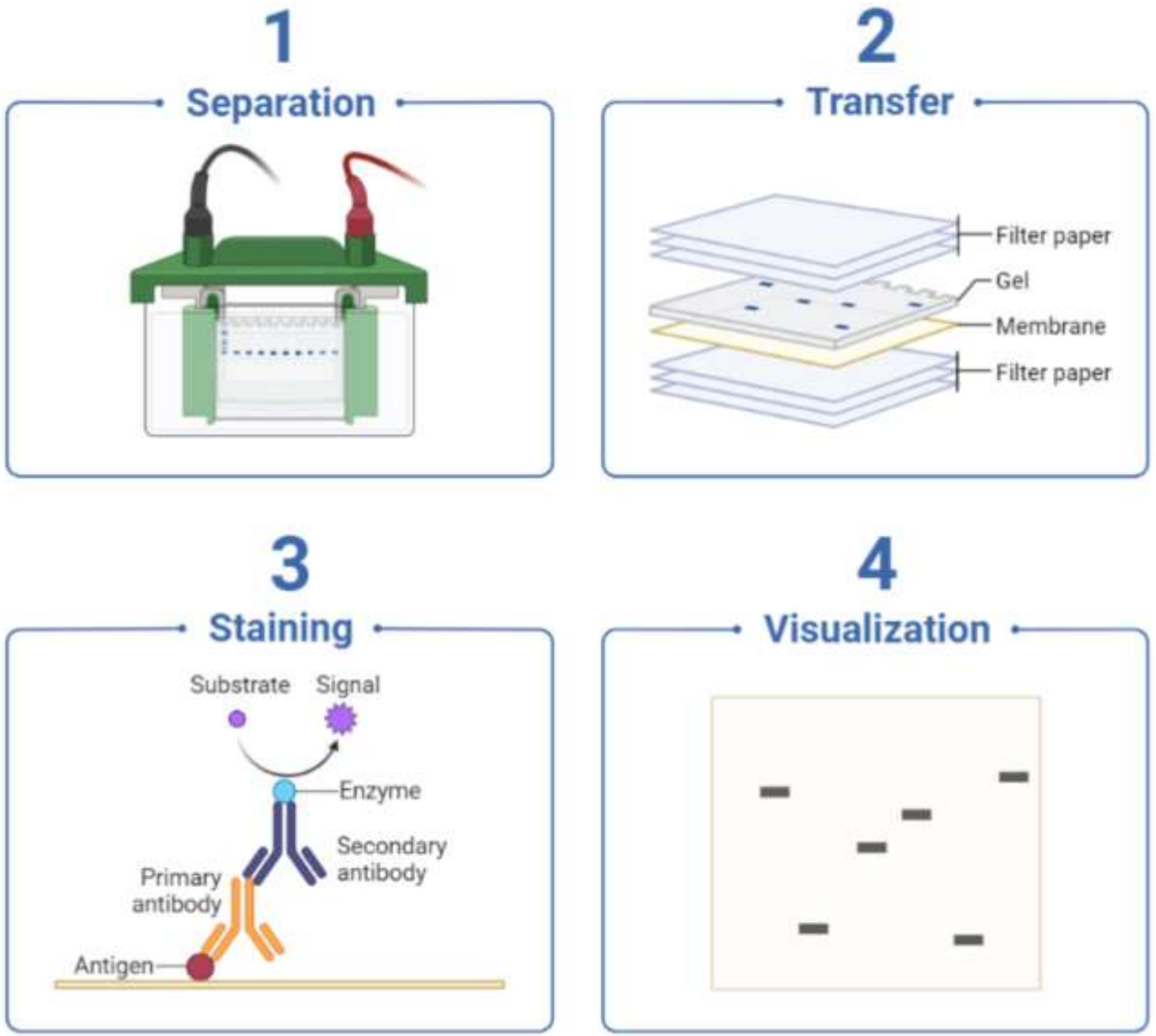
Proof of concept, identifying biomarkers with iTRAQ



Biomarkers found in brains with Alzheimer's disease with IPA

tissues and primary cells: astrocytes and neurons, nervous system CNS and neuroblastoma cell lines; neurological diseases and psychological disorders

Protein Validation with western blot



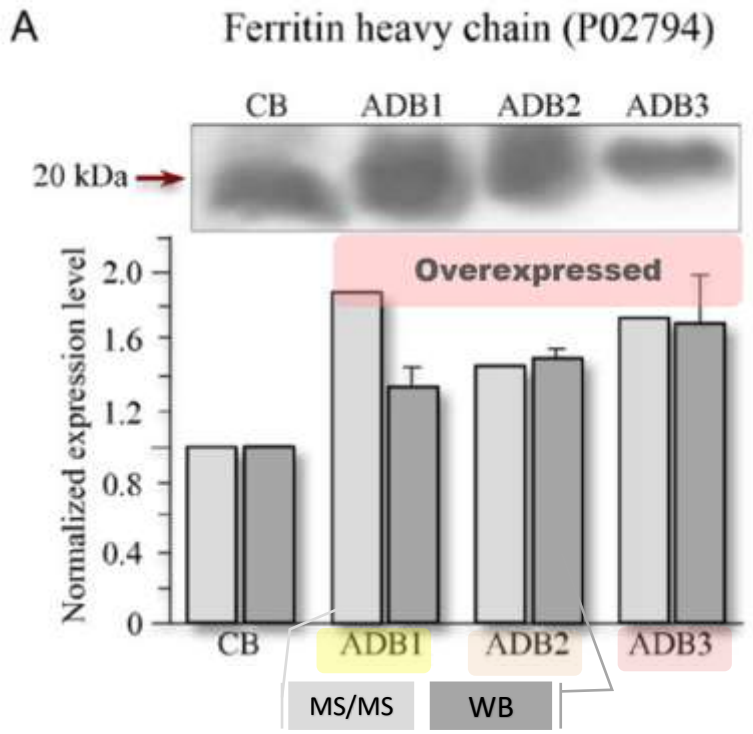
Ferritin heavy chain level is elevated in Protein Validation of elderly patients

Control 29y/o male

AD1 70 y/o Female

AD2 81 y/o Female

AD3 87 y/o Female



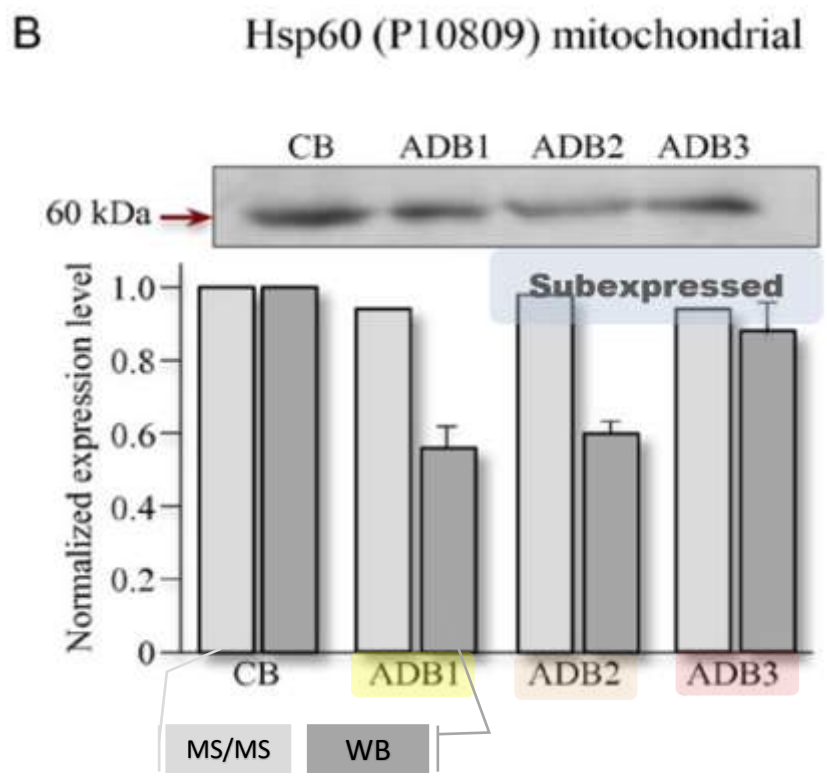
Hsp60 is initially decreased in in AD patients,, but the level elevates in elderly patients

Control 29y/o male

AD1 70 y/o Female

AD2 81 y/o Female

AD3 87 y/o Female



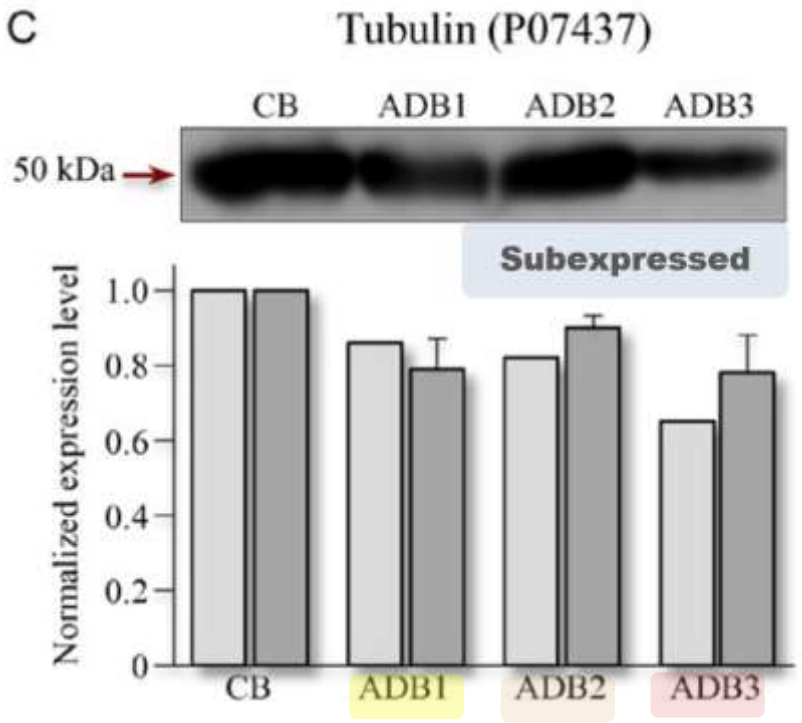
Tubulin is decreased in in AD patients

Control 29y/o male

AD1 70 y/o Female

AD2 81 y/o Female

AD3 87 y/o Female



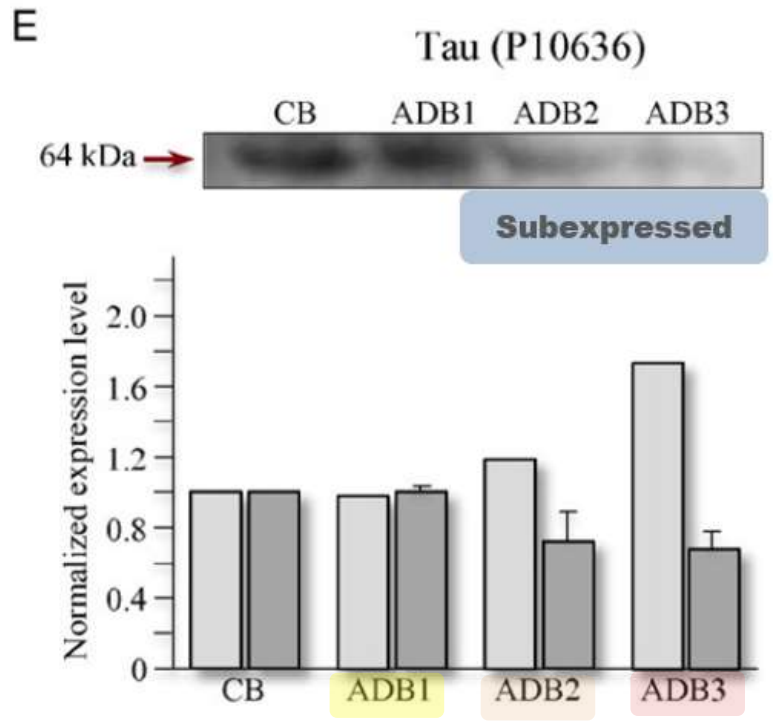
MS/MS predicted increased expression of Tau, but WB showed a decrease in Tau

Control 29y/o male

AD1 70 y/o Female

AD2 81 y/o Female

AD3 87 y/o Female



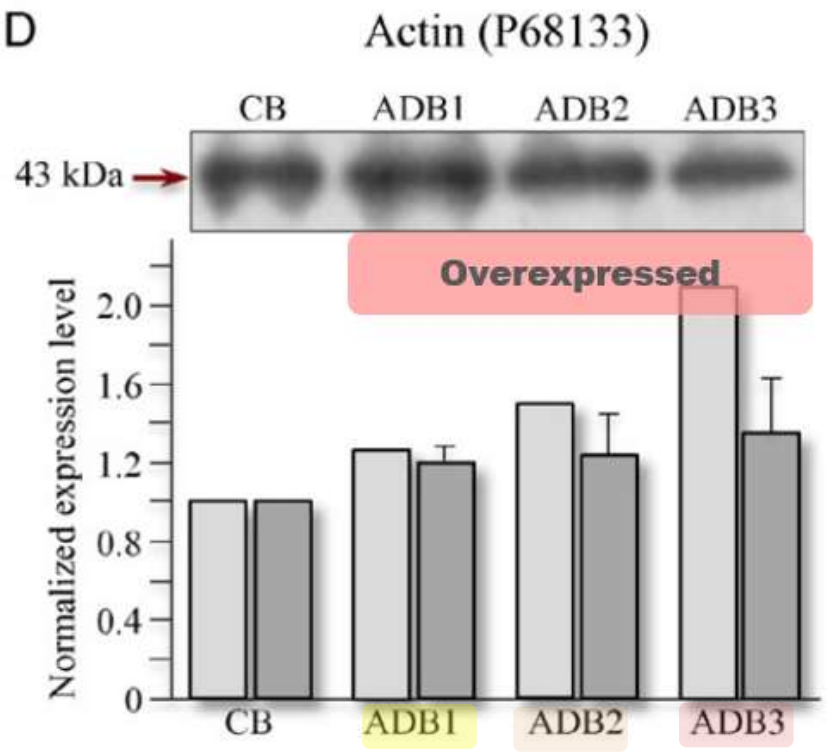
Actin is overexpressed in in AD patients

Control 29y/o male

AD1 70 y/o Female

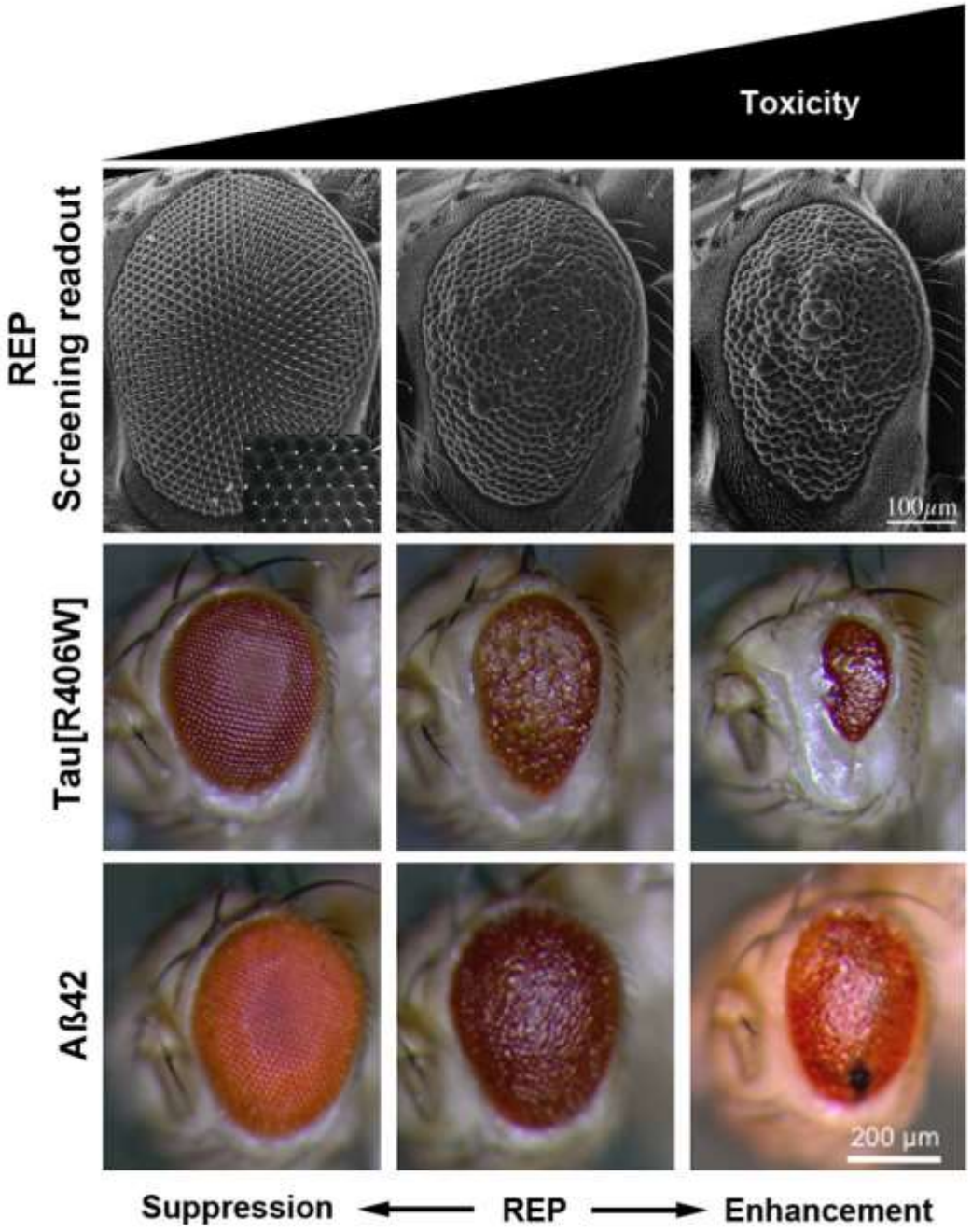
AD2 81 y/o Female

AD3 87 y/o Female



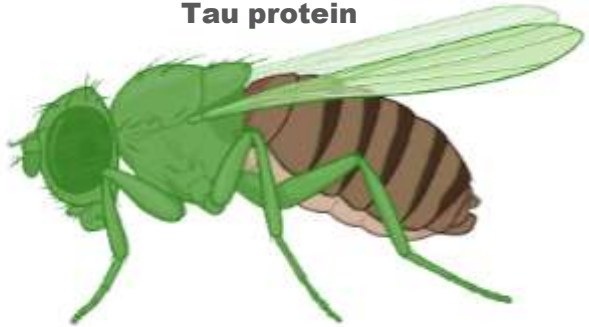
29. Actin, alpha skeletal muscle	ACTA1	P68133	42.05	5.23	37	50.4	1.27	1.38	2.09

In-vivo Validation



UAS flies

UAS-Tau flies: Longest human Tau isoform (4N2R) Tau protein



UAS- Aβ42 flies: produced signal peptide for secretion of Aβ42 Amyloid Beta peptide

Silenced
PGM1 (BDSC#34345)
Rho1 (BDSC#9909)

Drosophila melanogaster as a model organism for Alzheimer's disease - Scientific Figure on ResearchGate. Available from: https://www.researchgate.net/figure/Exemplified-rough-eye-phenotypes-REP-used-as-readout-for-modifier-screens-Scanning_fig2_258851716 [accessed 5 Apr, 2023]

In-vivo Validation

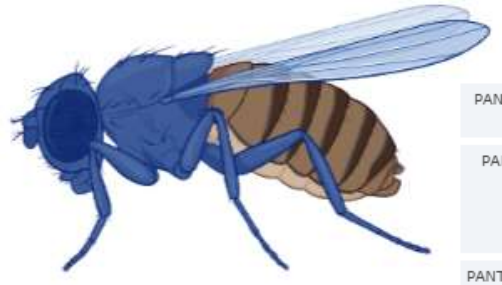
UAS flies

RNAi Silencing Rho

PANTHER GO-slim Molecular Function:	neutral amino acid transmembrane transporter activity organic anion transmembrane transporter activity cation transmembrane transporter activity
PANTHER GO-slim Biological Process:	anion transmembrane transport carboxylic acid transport cation transmembrane transport neutral amino acid transport mitochondrial transmembrane transport amino acid transmembrane transport
PANTHER GO-slim Cellular Component:	integral component of mitochondrial inner membrane

32. Transforming protein RhoA — RHOA

Subexpressed



Sideroflexin

PANTHER GO-slim Molecular Function:	neutral amino acid transmembrane transporter activity organic anion transmembrane transporter activity cation transmembrane transporter activity
PANTHER GO-slim Biological Process:	anion transmembrane transport carboxylic acid transport cation transmembrane transport neutral amino acid transport mitochondrial transmembrane transport amino acid transmembrane transport
PANTHER GO-slim Cellular Component:	integral component of mitochondrial inner membrane

33. Sideroflexin-1 — SFXN1

Subexpressed

RNAi Silencing Phosphoglucomutase 1 PGM1

PANTHER GO-slim Molecular Function:	intramolecular transferase activity
PANTHER GO-slim Biological Process:	carbohydrate metabolic process
PANTHER GO-slim Cellular Component:	cytosol

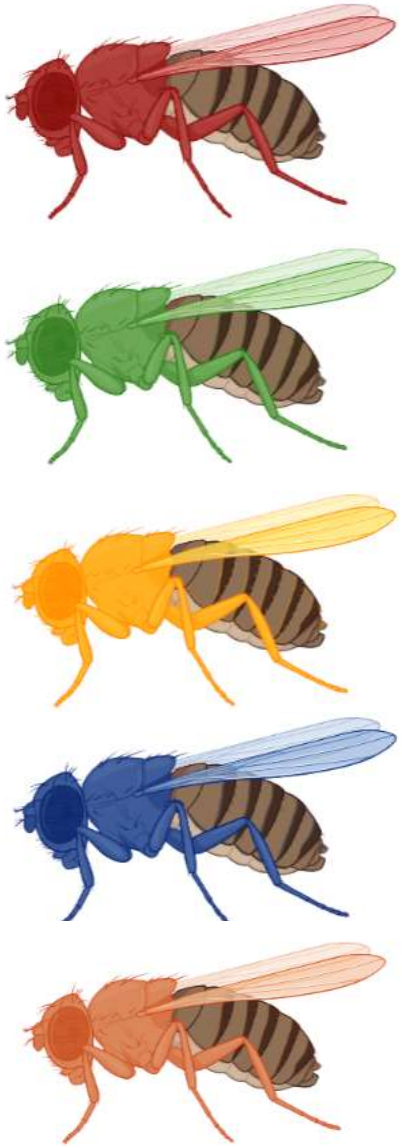
35. Phosphoglucomutase-1 — PGM1

Differentially expressed as a function of age

Overexpressed

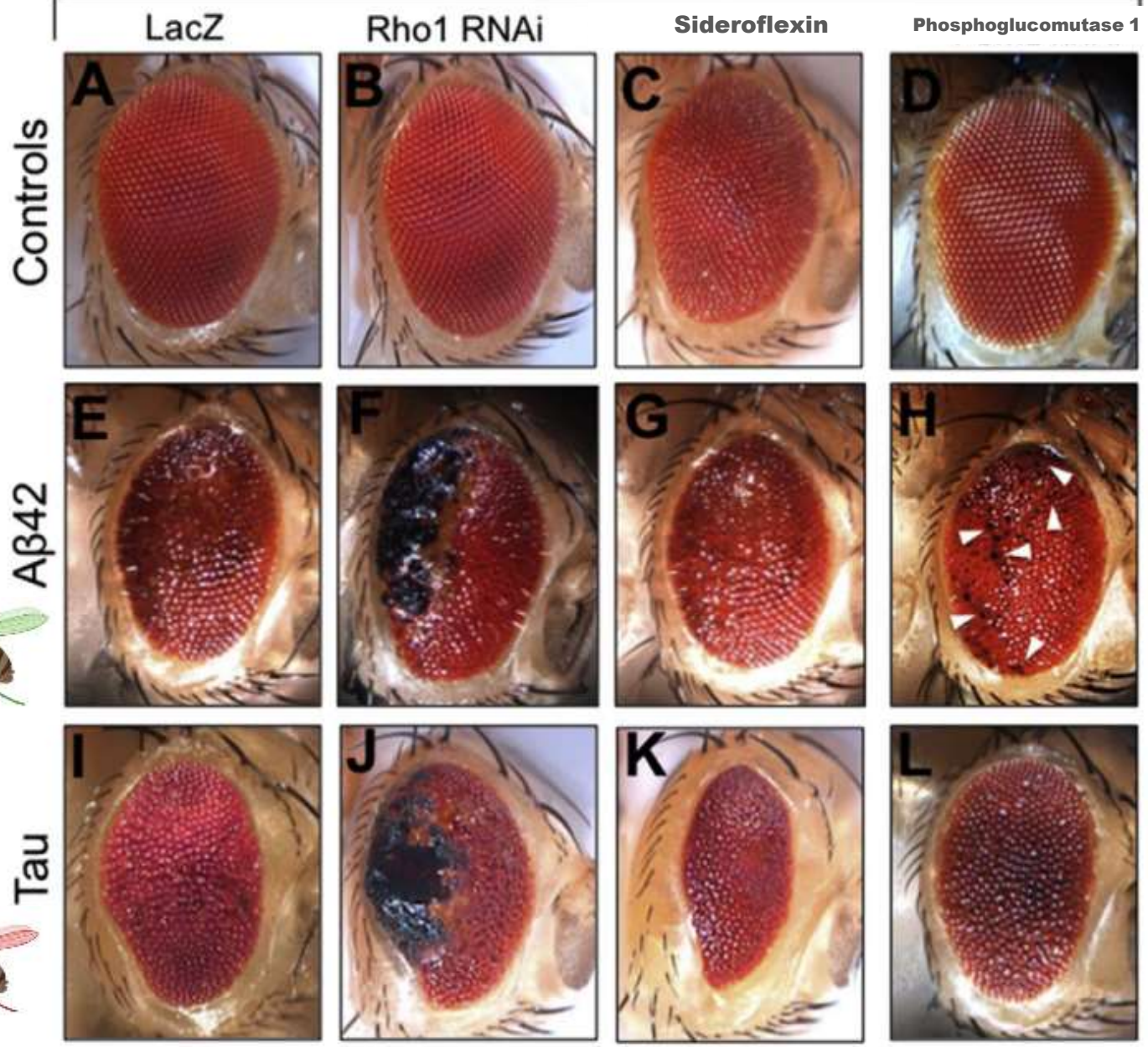
In-vivo Validation

UAS flies



Control

gmr-Gal4



Summary

- Through iTRAQ it is possible to determine differentially expressed proteins,and biomarkers.

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- The **overexpressed** polypeptides affected ROS and stress responses, while the **subexpressed** polypeptides affected oxidative phosphorylation, organellar acidification, and cytoskeleton.

- Drosophila is an excellent model to study Tau and Amyloid beta toxicity.
  - **Sideroflexin** and **Phosphoglucomutase-1**

Sources:

Images:

https://biotech.ufl.edu/proteomics/

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