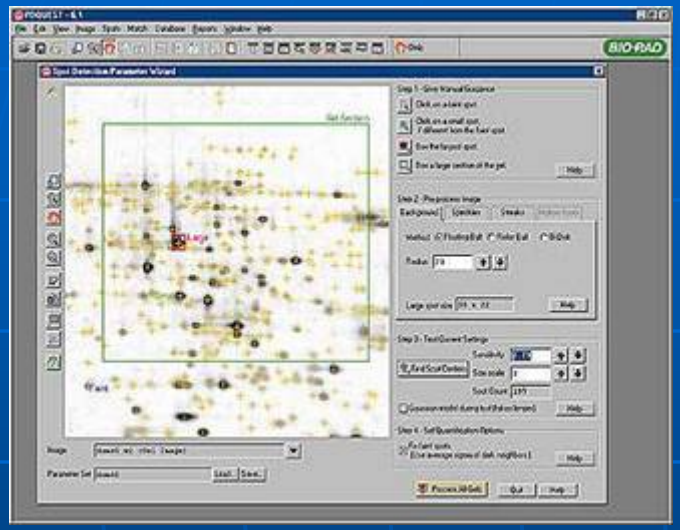
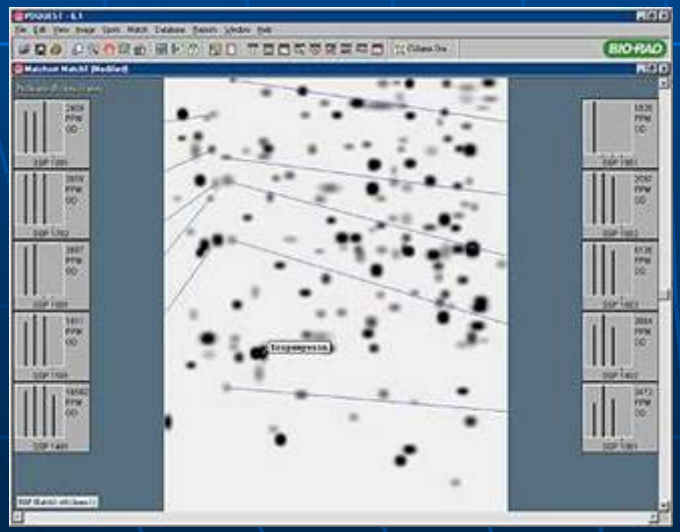
Image analysis

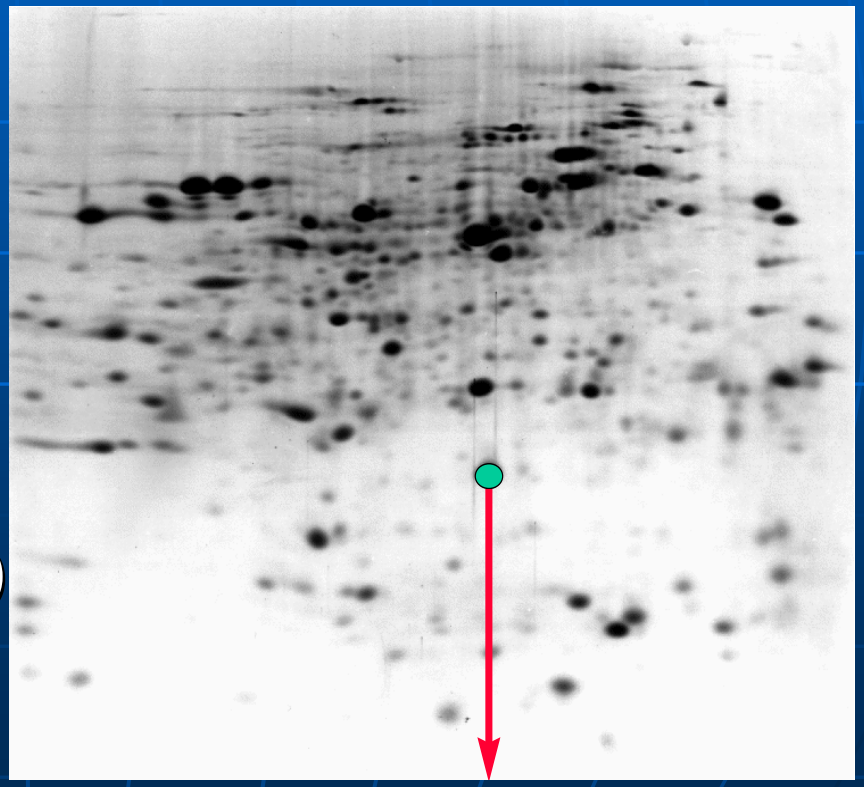




- e.g. PDQuest (Bio-Rad), ImageMaster (Pharmacia)
  - Database storage of many gel images
  - Multi-image manipulation and comparison
  - Creation of master gel image (“typical” profile)
  - Comparison of individual experimental gels to master
  - Identification of variant spots

Technology

- Gel spot excision and digestion
  - Individual variant spots
  - Washing (de-staining)
  - Digestion (trypsin)
  - Peptide extraction
  - Clean-up (desalting)

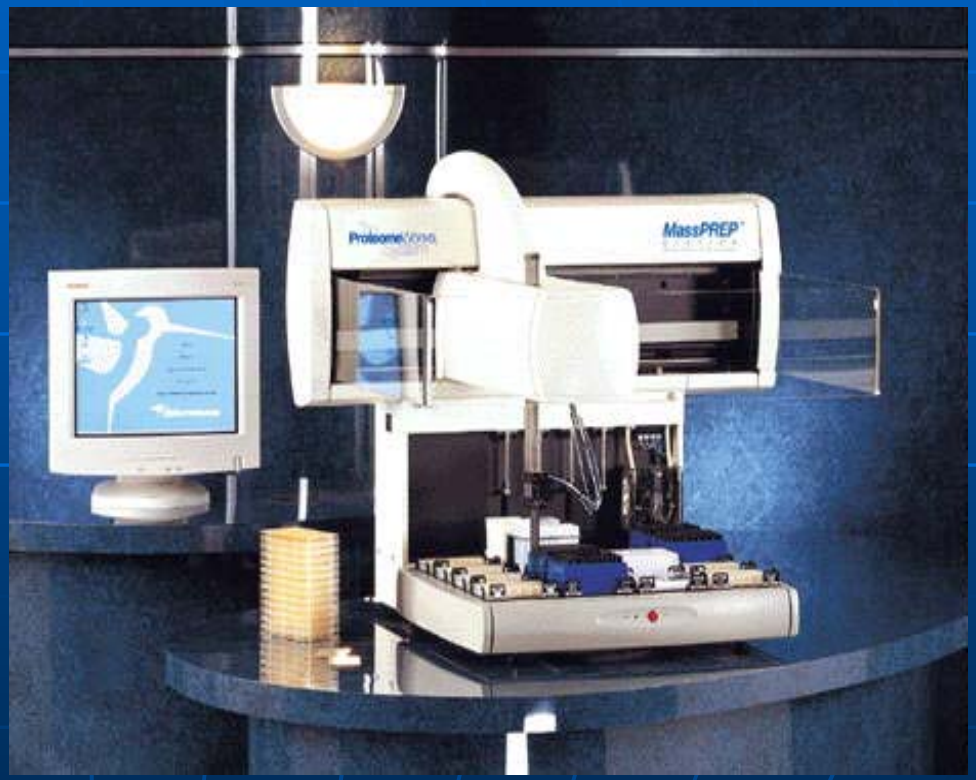


High-throughput analysis

- Robotics (1)
  - Gel-spot excision
    - Driven from gel image
    - Cuts out gel spots
    - Transfers to microtitre plates

High-throughput analysis

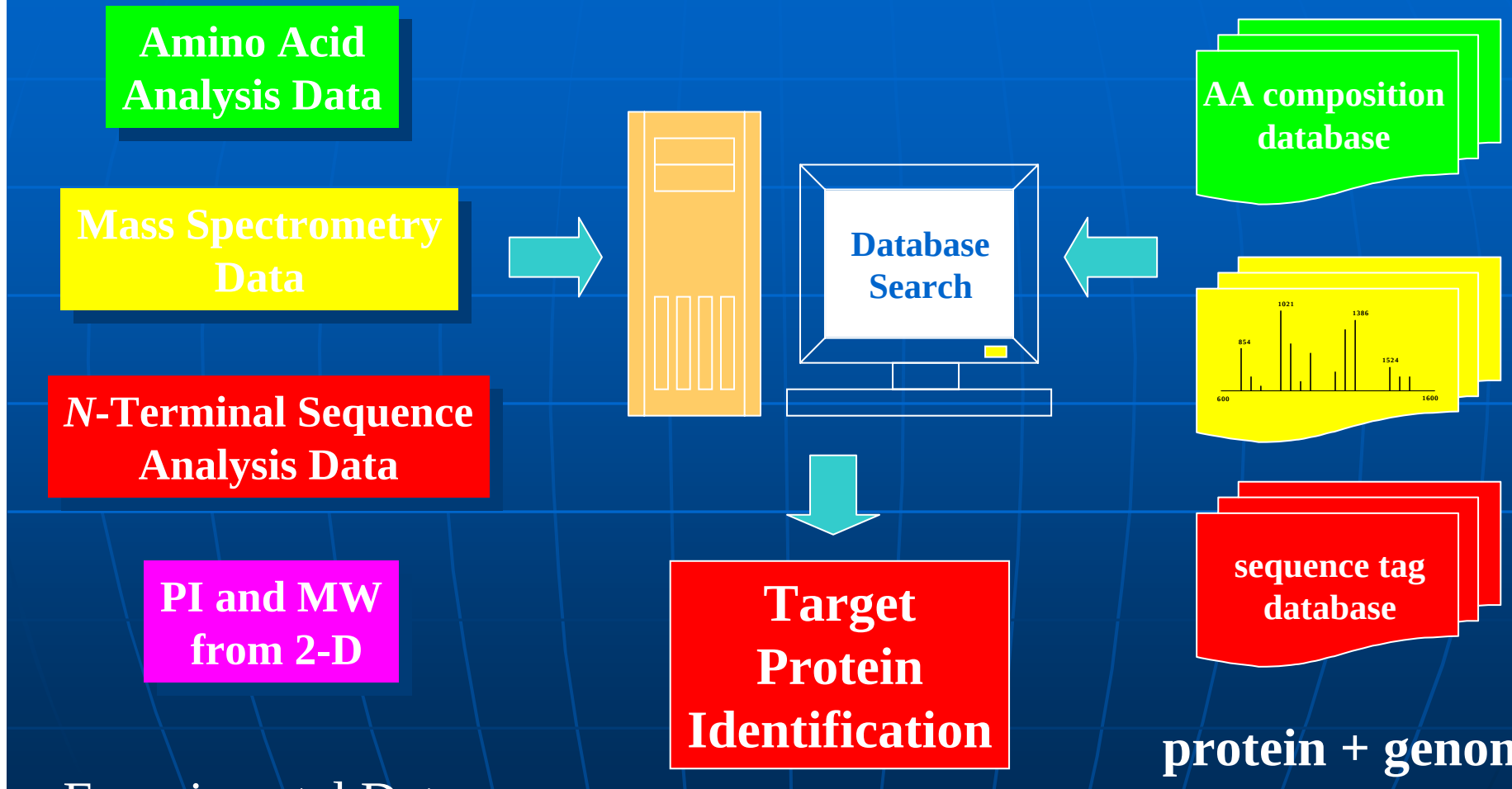
- Robotics (2)
  - Protein digestion
    - Washes gel pieces
    - Digests with trypsin
    - Extracts peptides
    - Desalts peptides
    - Applies peptides to MALDI plate



Technology

- Protein identification
  - Mass spectrometry
    - MALDI/TOF-MS
    - Q-Tof-MS/MS

Database Search (Bioinformatics)



利用其他分子生物技術配合分析:

Cell mapping, and identification of proteins in complexes:

- 共同沈澱法 or "pull-down" techniques using antibodies directed against one of the component proteins
  - Coprecipitation using affinity-tagged recombinant proteins and antibodies directed against the "tag" epitope
  - Protein-affinity-interaction chromatography (e.g., using recombinant glutathione S-transferase (GST)-fusion proteins and glutathione-affinity chromatography)
  - Isolation of intact multiprotein complexes (e.g., nuclear pore complexes, ribosome complexes, and spliceosomes).

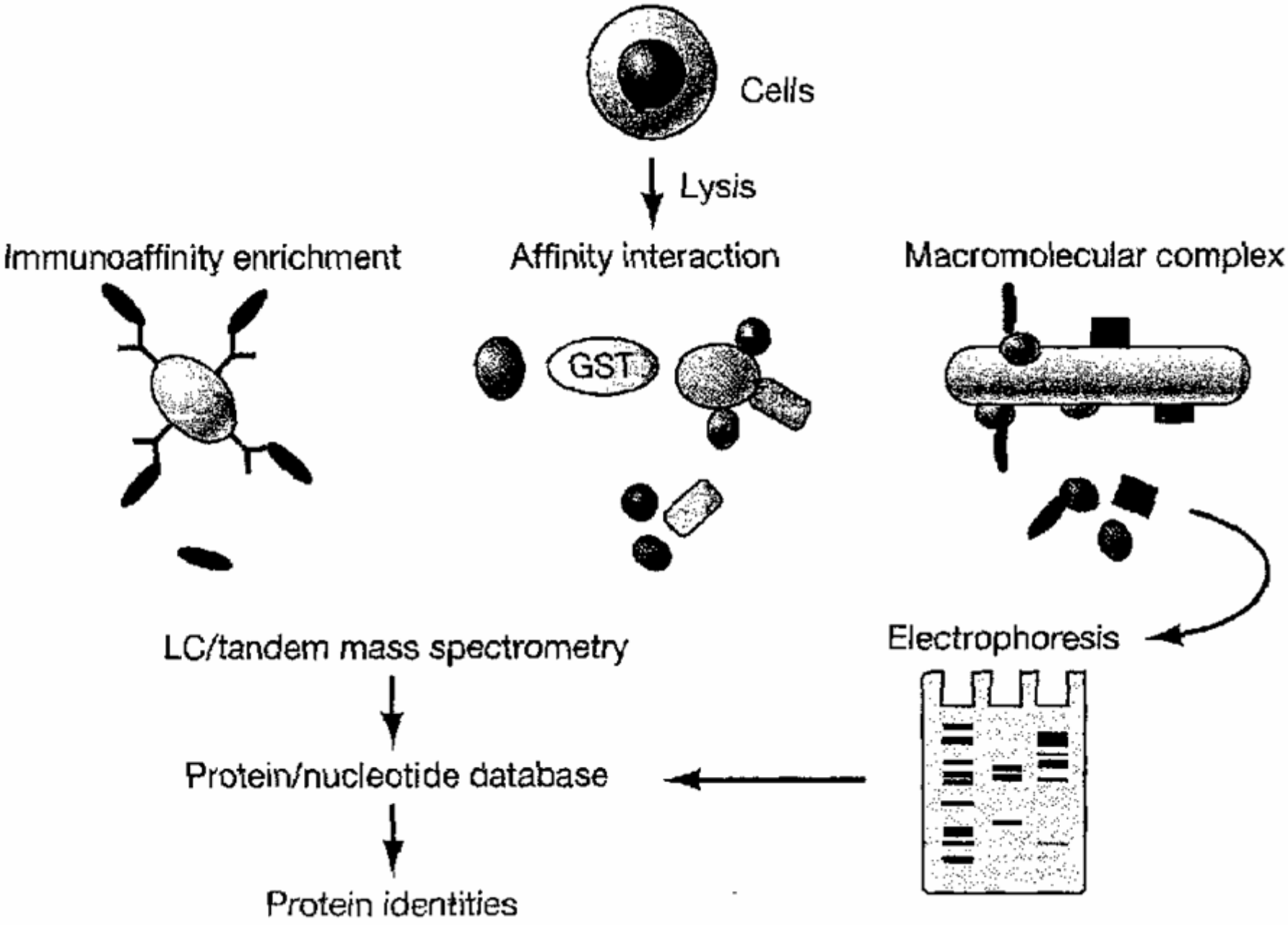


FIGURE 1.9. Cell mapping: Affinity capture methods.

比較蛋白體學
Comparative Proteomics

- 利用不同螢光標定法可正確定量樣品
- Fluroscence label (Cy3, Cy5)

DiGE: Quantitative 2D-PAGE

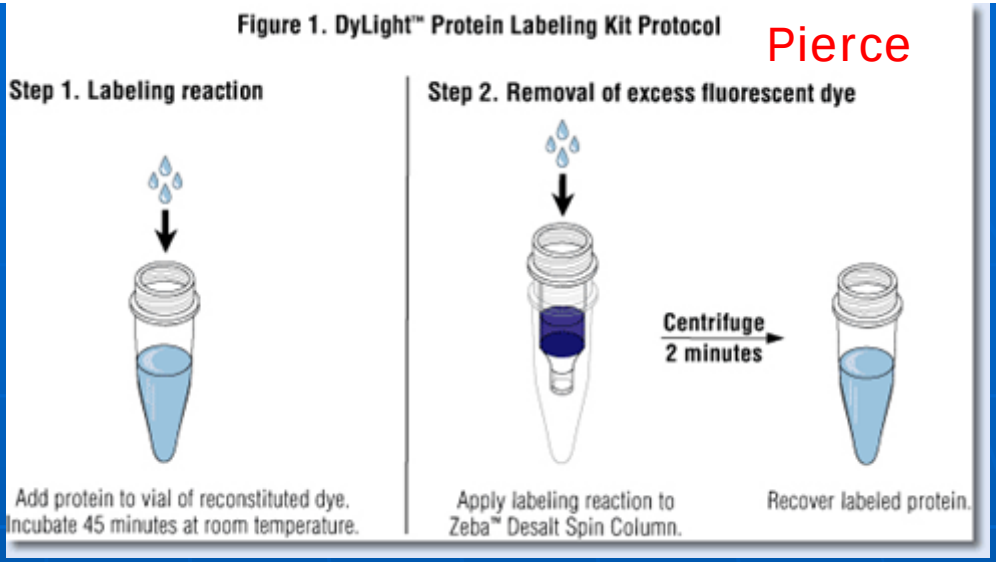
sensitivity = ~200pg -mgs

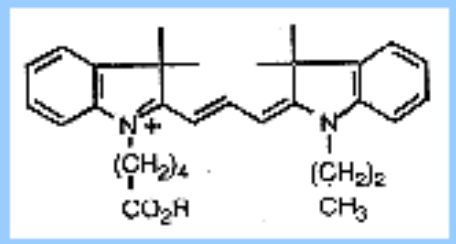
- Sample multiplexing: 由Minden group at Carnegie Mellon University in Pittsburg發表, 克服傳統蛋白體定量不準的問題.
- 待比較的不同樣本事先以 (Cy3, Cy5) 處理, 之後混合後在同一片膠體中分析-Difference Gel Electrophoresis (DiGE).

DIA: Difference in gel analysis

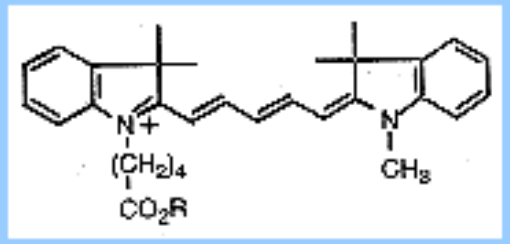
- For a DIA analysis, samples are minimally labelled with either Cy3 or Cy5 fluorescent dyes, and then pooled prior to 2D PAGE.
- The same isoform with the **different labels will co-migrate**
  - Fluorescent ratios can be compared after normalization.
  - The reported ratio indicates changes in expression levels.
- A reciprocal gel is run where the dye label is reversed.
  - Avoid differences in reactivity between dyes for the proteins.

- Ünlü,M., Morgan, M. E., and Minden, J. S. (1997). Difference gel electrophoresis: a single gel method for detecting changes in cell extracts. *Electrophoresis*,18, 2071-2077





Wildtype protein extract



Mutant protein extract

label protein extract with Cy3

label protein extract with Cy5

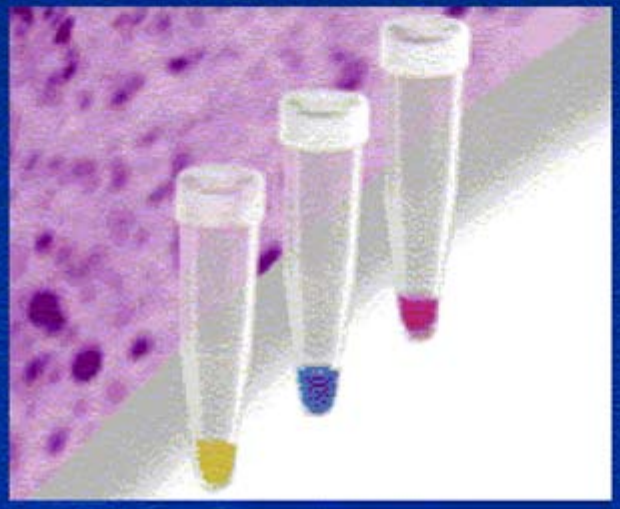
Fluorescent dyes

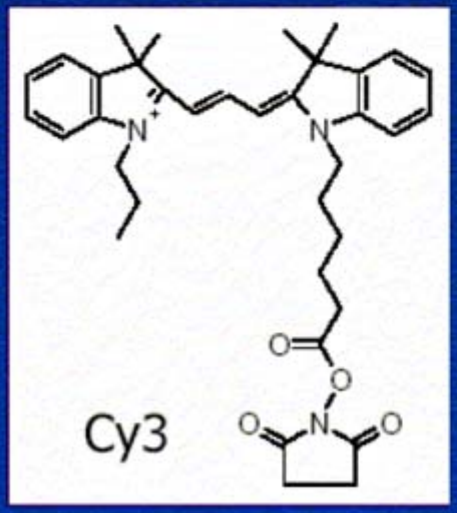
Cydye DIGE Fluors (Cy2, Cy3, and Cy5)

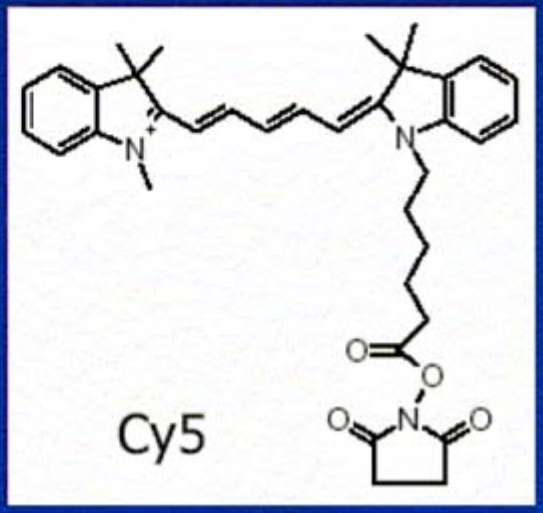
- cross-link to epsilon amine on lysine via amide linkage

- are size and charge-matched (positive charge on Cydye replaces charge on lysine - pI unchanged and proteins with different labels will overlay

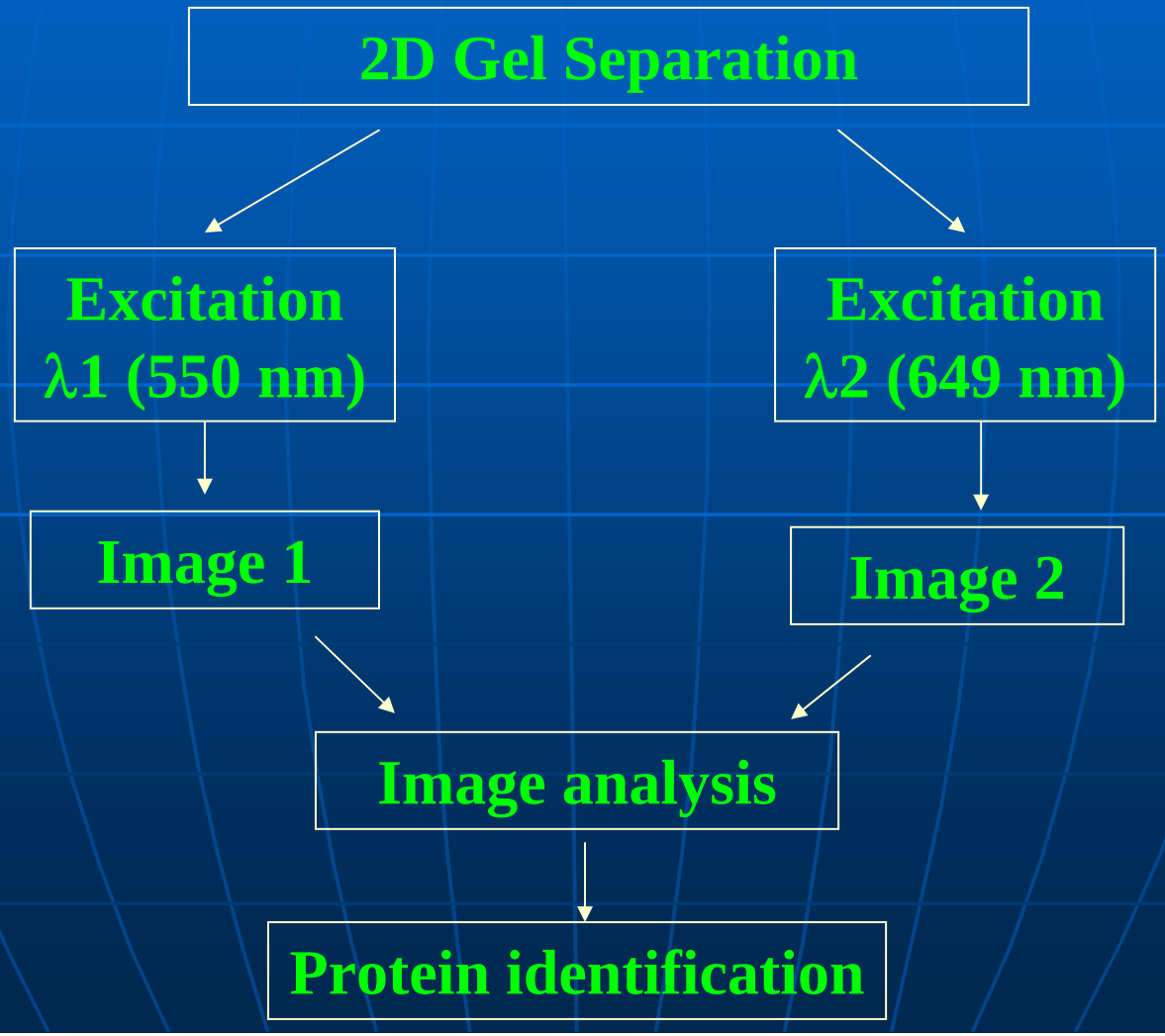
- have narrow excitation and emission bands



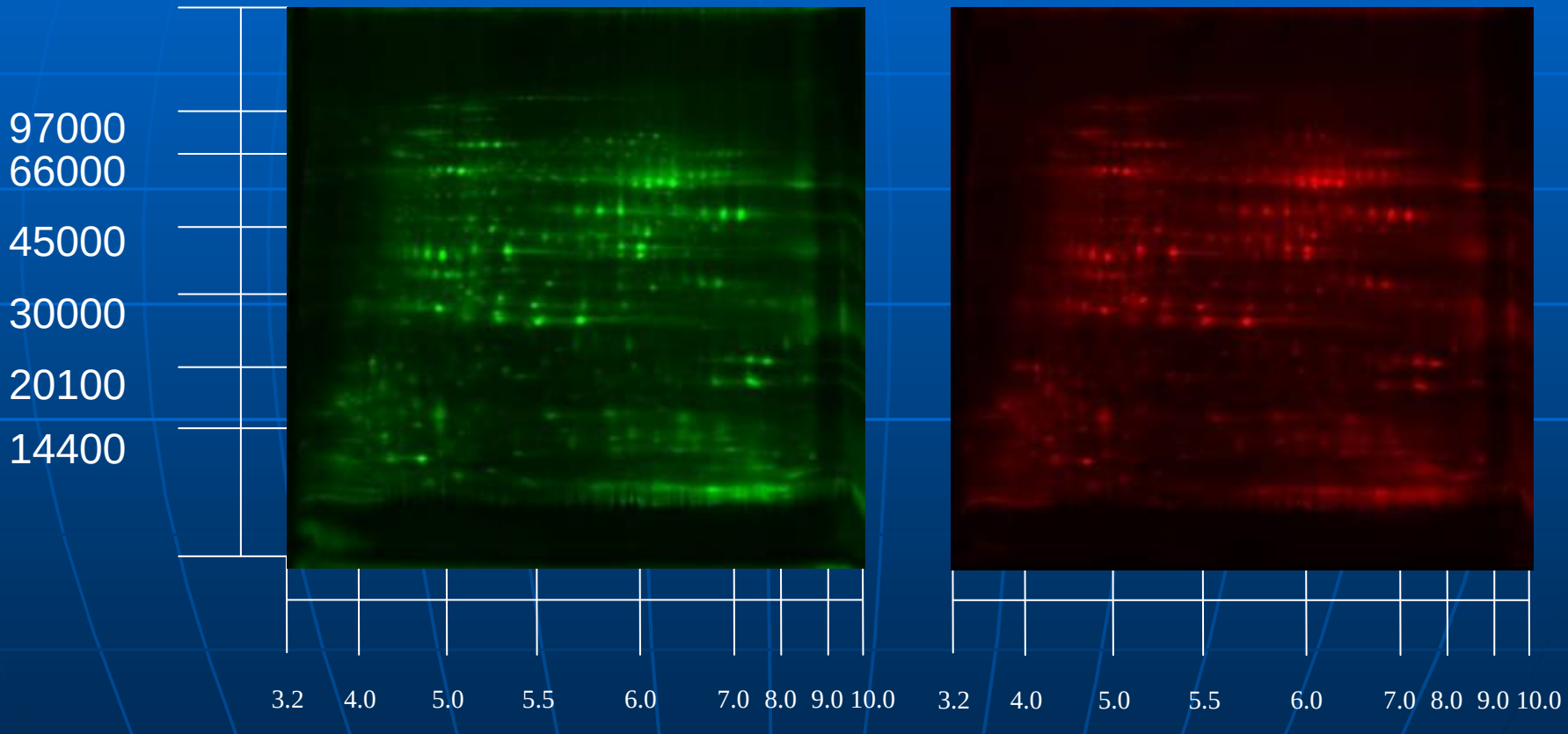




Gel Image


2D Gel Separation
Excitation λ1 (550 nm)
Excitation λ2 (649 nm)
Image 1
Image 2
Image analysis
Protein identification

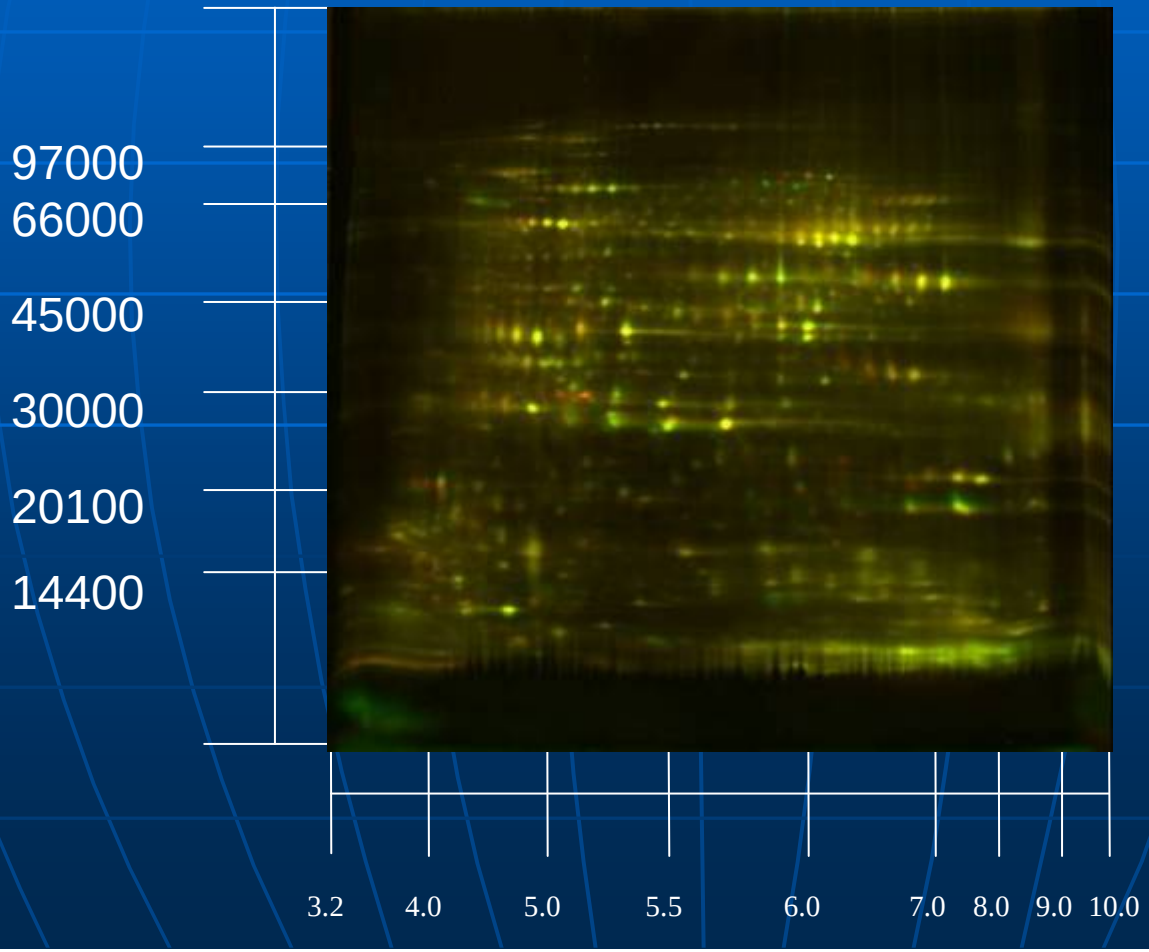
Gel Image



CNT1 labeled with Cy3

KDML105 labeled with Cy5

Combined 2 images



Imaging of Fluorescently labelled 2D gels

Fluorescently labelled gels are imaged using a Typhoon 9400 scanner

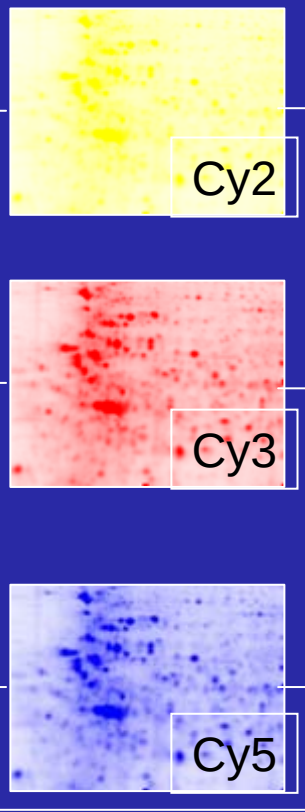
	excitation λ	emission λ
Cy2	490	510
Cy3	540	590
Cy5	620	680

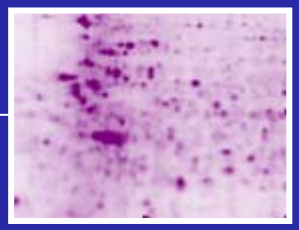
Sparking:Internal standard could be adopted

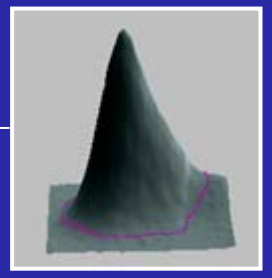
Pooled internal standard label with Cy2

Protein extract 1 label with Cy3

Protein extract 2 label with Cy5







Mix labelled extracts → 2D separation → Image gel with Typhoon Variable Mode Imager → Image analysis and data quantitation with DeCyder Differential Analysis Software

生物質譜
BIOLOGICAL MASS SPECTROMETRY

- **i)** 雷射輔助基質脫附游離法-飛行時間質譜 **MALDI-TOF**
- **ii)**電噴灑法-四極柱質譜 **ESI-Q-Tof**
- **iii)**液相電層分析-質譜 **LC-MS**
- iv) LC-MS/MS

- 自early 1990's, 質譜儀的兩大發現
  - 電噴灑游離法Electrospray ionisation (ESI) and雷射輔助基質脫附游離法 matrix-assisted laser desorption/ionisation (MALDI) were developed by Fenn *et al*. (1989) and Karas and Hillenkamp (1988), respectively.
  - 軟式游離法 Soft ionisation: very little internal energy is imparted into the ions during ionisation, resulting in the formation of intact ions, with minimal fragmentation.

MALDI-TOF mass spectrometry

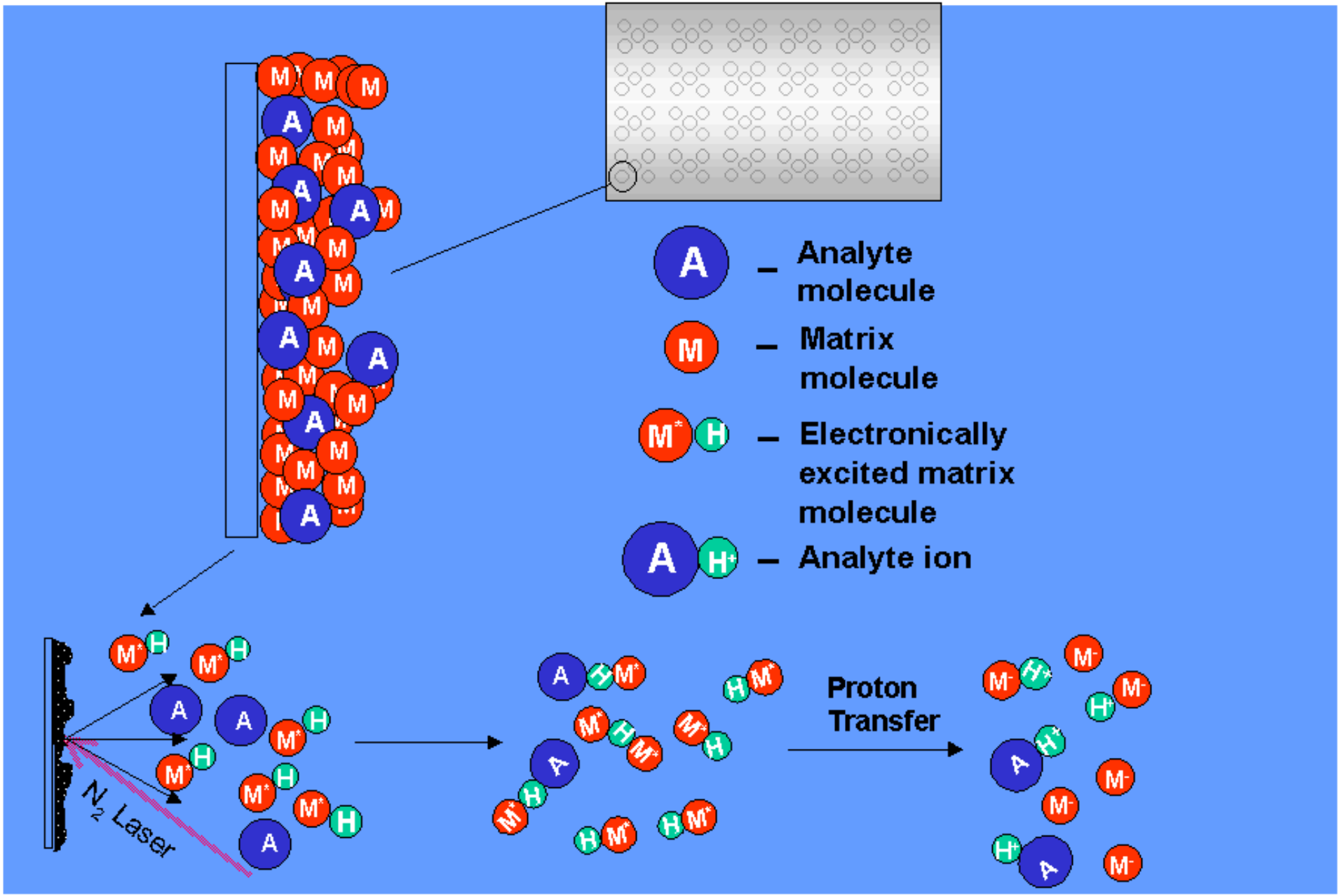
- 雷射輔助基質脫附游離法--Matrix-assisted laser desorption/ionizationtime of flight (MALDI-TOF) mass spectrometry
  - 適合分析蛋白等大分子, 應用範圍廣, 已發表如:
  - protein and nucleic acid sequence, structure, purity, heterogeneity, cleavage, post-translational modification, and a host of other molecular characteristics that are often difficult to study by other means.
- MALDI-TOF 也可用在 QC tool:
  - verify peptide, protein, and DNA syntheses, etc.

MALDI 主要三步聚:

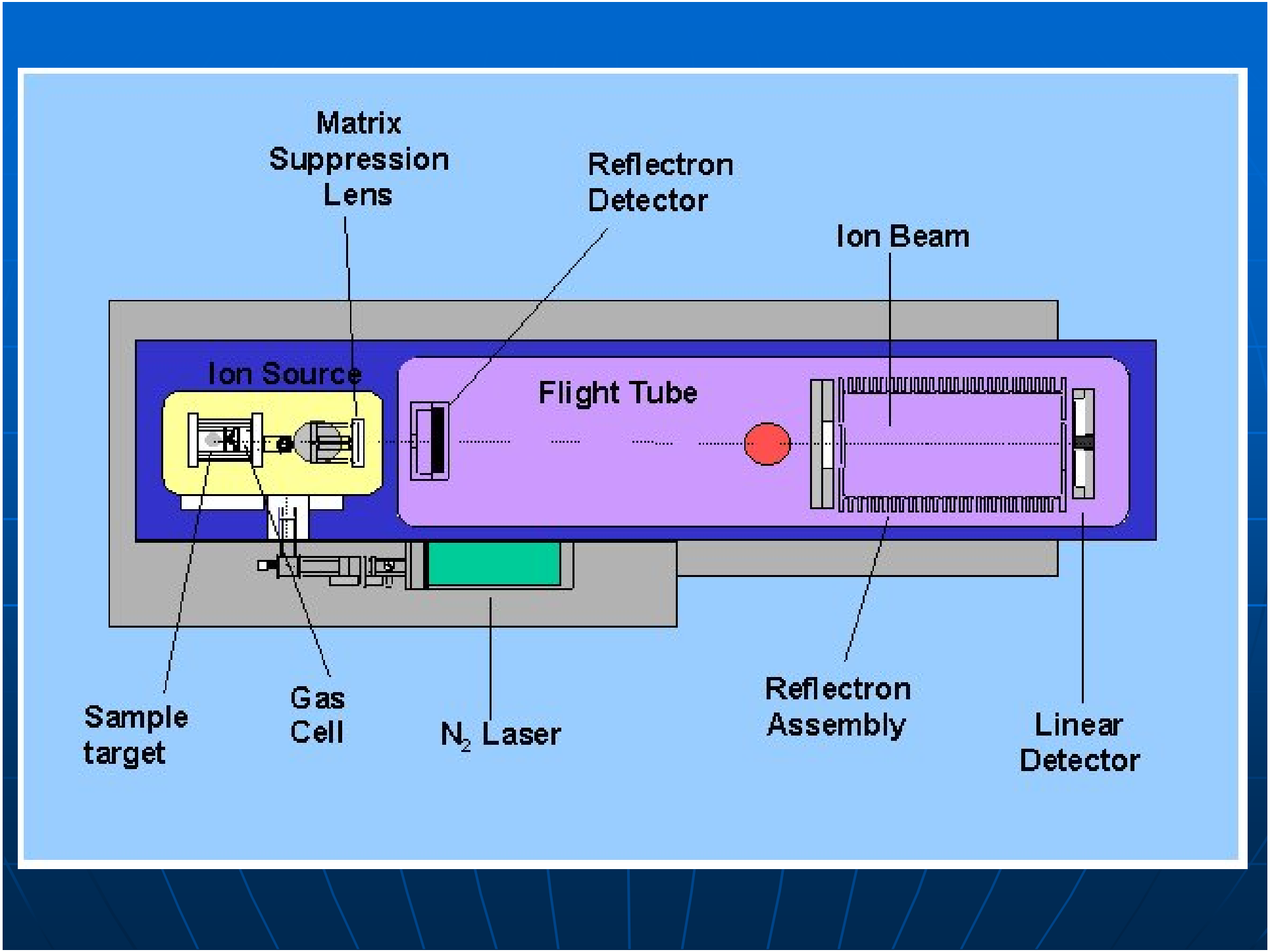
- 游離 Ionisation,
- 依質量分離 Mass separation, and
- 偵測 Detection.

- MALDI-TOF: 游離結果大部分帶一價電 i.e. $(M+H)^+$, where M = the biological molecule and H = H^+ (or a proton), 分子不會破碎.
- 離子由雷射激發
  - Sample mixed with matrix
  - α-cyano-4-hydroxycinnamic acid: commonly used for peptide analysis
  - 2,5-dihydroxybenzoic acid (2,5 DHB) – sugar analysis
  - Sample: pico gram

- 基質可吸收雷射能量後轉移給待測分子
  - laser (337 nm for N2 lasers)
  - A dense plume containing matrix and analyte molecules is produced and analyte molecules interact with hydrogen atoms from the matrix to form mainly singly charged $(M+H)^+$ ions.

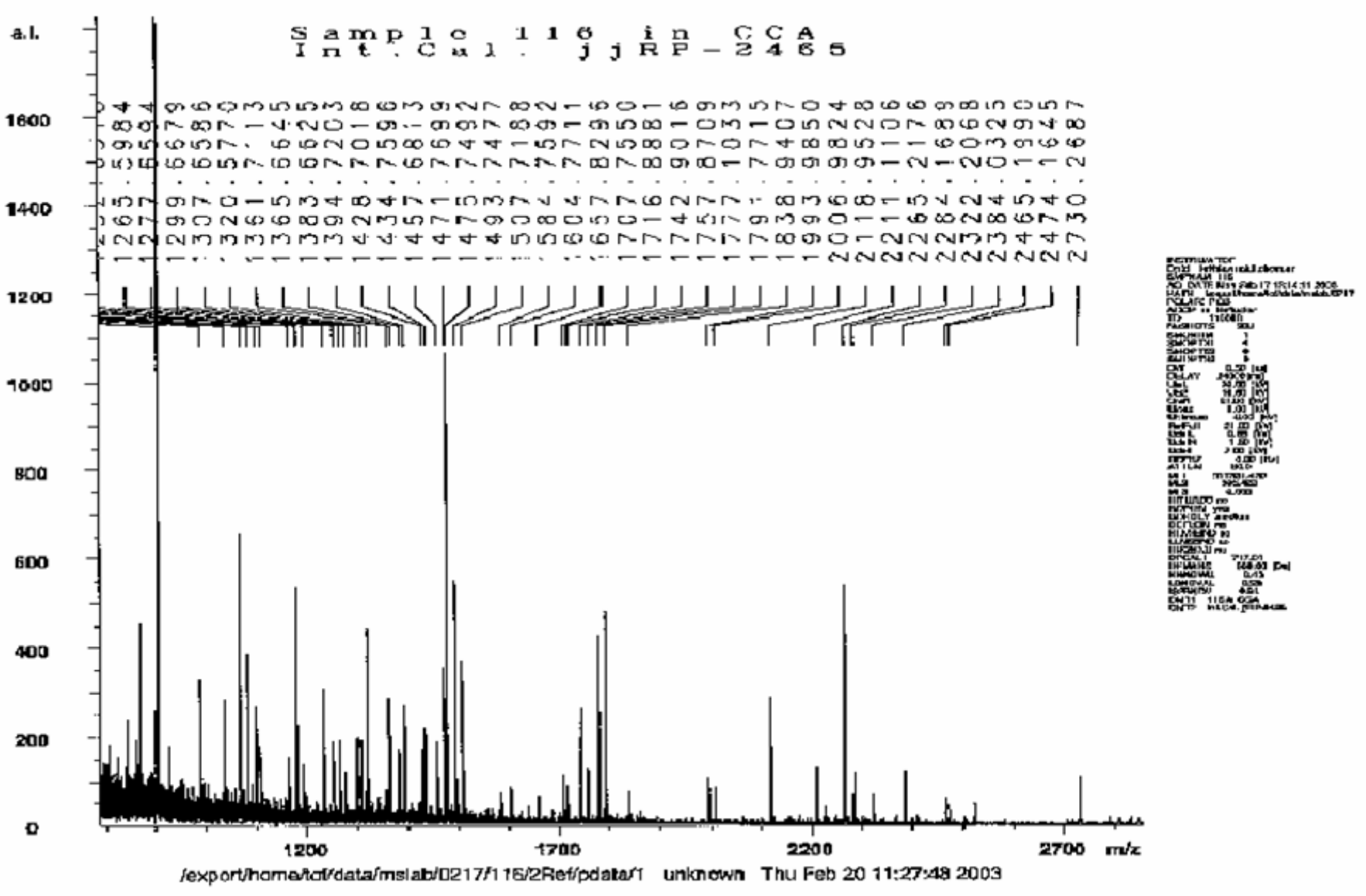

A – Analyte molecule
M – Matrix molecule
M* H – Electronically excited matrix molecule
A H+ – Analyte ion
N2 Laser
Proton Transfer

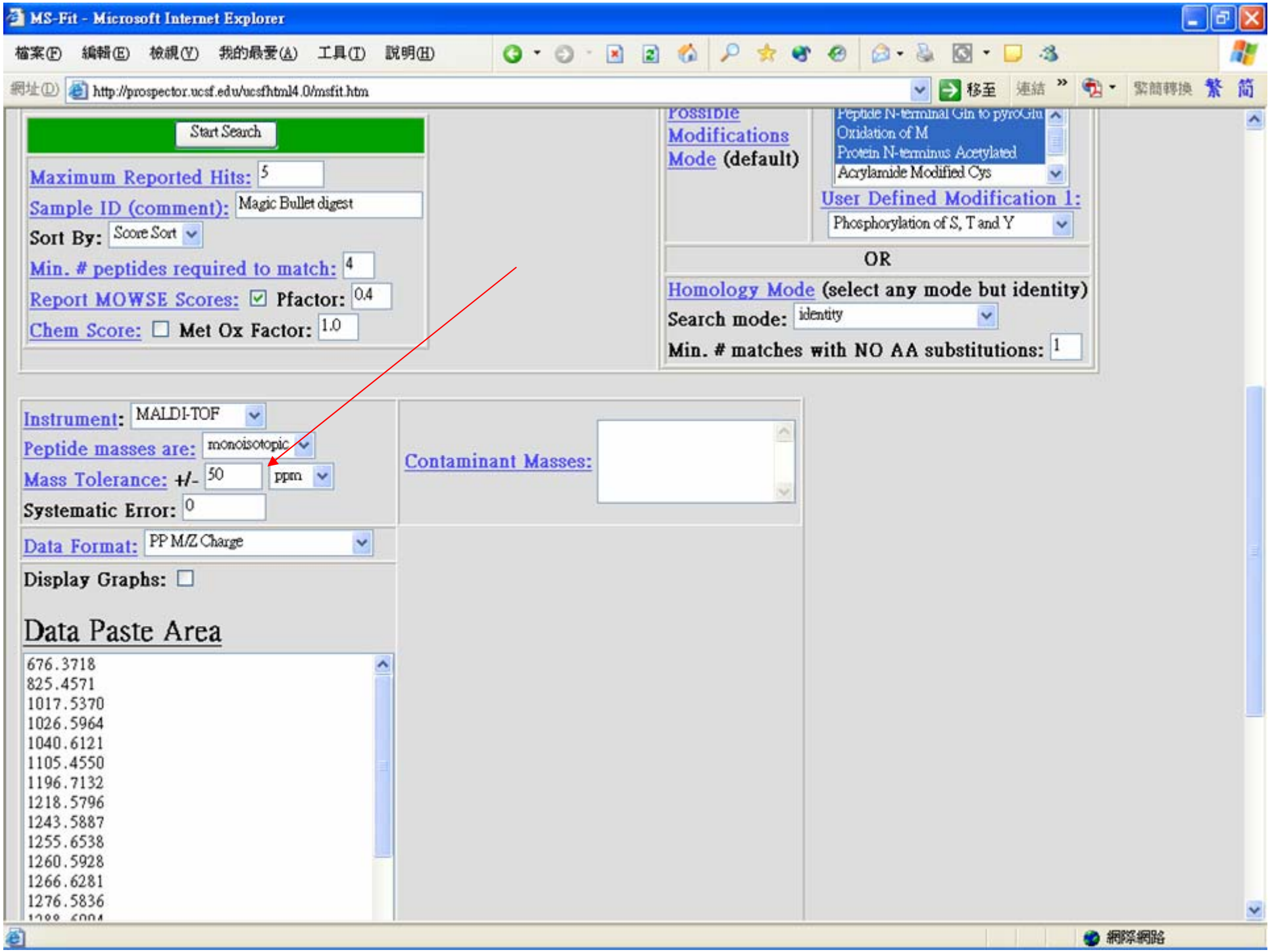
- 離子經TOF裝置後,由 microchannel plate detector (MCP)偵測.
- 反轉裝置 Reflection:a uniform electric field is generated at the end of the TOF tube which effectively pushes the ions back in the opposite direction.—可增加飛行時間.
- The mass range of a TOF analyser is, theoretically, infinite although, practically, it has an upper mass range of 750 kDa in linear mode and 100 kDa in reflectron mode.


Matrix
Suppression
Lens
Reflectron
Detector
Ion Beam
Ion Source
Flight Tube
Sample
target
Gas
Cell
N_2 Laser
Reflectron
Assembly
Linear
Detector

PROTEIN IDENTIFICATION BY PEPTIDE MASS MAPPING

- **COMPUTER EXERCISE #3: Investigate peptide mass mapping used for protein identification**




MS-Fit - Microsoft Internet Explorer
檔案(F) 編輯(E) 檢視(V) 我的最愛(A) 工具(T) 說明(H)
網址(D) http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm
移至 連結
Start Search
Maximum Reported Hits: 5
Sample ID (comment): Magic Bullet digest
Sort By: Score Sort
Min. # peptides required to match: 4
Report MOWSE Scores: Pfactor: 0.4
Chem Score: Met Ox Factor: 1.0
Possible Modifications Mode (default)
Peptide N-terminal Gln to pyroGlu
Oxidation of M
Protein N-terminus Acetylated
Acrylamide Modified Cys
User Defined Modification 1:
Phosphorylation of S, T and Y
OR
Homology Mode (select any mode but identity)
Search mode: identity
Min. # matches with NO AA substitutions: 1
Instrument: MALDI-TOF
Peptide masses are: monoisotopic
Mass Tolerance: +/- 50 ppm
Systematic Error: 0
Contaminant Masses:
Data Format: PP M/Z Charge
Display Graphs:
Data Paste Area
676.3718
825.4571
1017.5370
1026.5964
1040.6121
1105.4550
1196.7132
1218.5796
1243.5887
1255.6538
1260.5928
1266.6281
1276.5836
網際網路

Data Set 1 Results

MS-Fit search selects **240** entries (results displayed for top **5** matches).

Results Summary

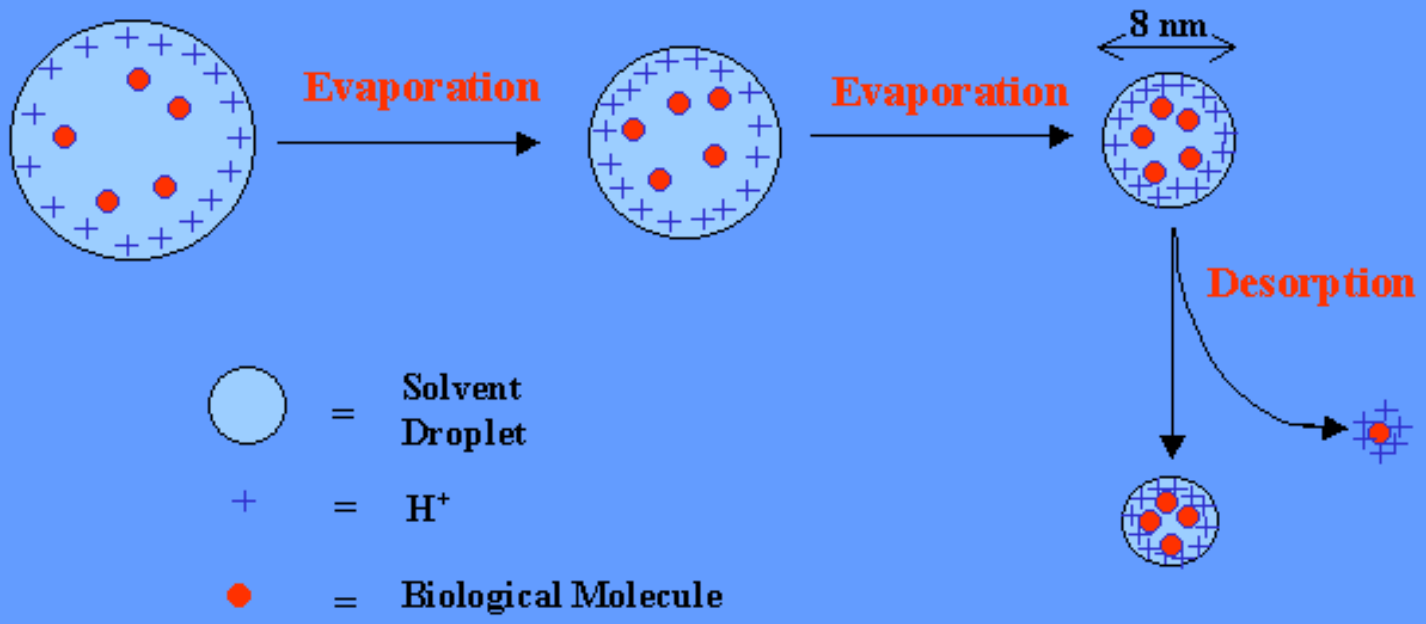
	MOWSE Score	#/50(%) Masses Matched	% Cov	% TIC	Mean Err ppm	Data Tol ppm	MS-Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name
1	3.211e+08	9 (18)	22.0	18.0	-3.54	49.7	32806	66018/8.2	P04264	HUMAN	Keratin, type II cytoskeletal 1 (Cytokeratin 1) (K1) (CK 1) (67 kDa cytokeratin) (Hair alpha protein)
2	9.544e+04	5 (10)	9.0	10.0	-2.33	47.8	148699	89866/6.6	O75582	HUMAN	Ribosomal protein S6 kinase alpha 5 (Nuclear mitogen- and stress-activated protein kinase-1) (90 kDa ribosomal protein S6 kinase 5) (RSK-like protein kinase) (RLSK)
3	9.465e+04	7 (14)	15.0	14.0	1.59	34.8	35320	65866/8.1	P35908	HUMAN	Keratin, type II cytoskeletal 2 epidermal (Cytokeratin 2e) (K2e) (CK 2e)
4	7.097e+04	5 (10)	8.0	10.0	-2.33	47.8	91738	96584/6.9	Q8C050	MOUSE	Ribosomal protein S6 kinase alpha 5 (Nuclear mitogen- and stress-activated protein kinase-1) (90 kDa ribosomal protein S6 kinase 5) (RSK-like protein kinase) (RLSK)
5	2.876e+04	5 (10)	18.0	10.0	-9.54	68.1	98869	30272/8.9	Q8ZNB9	SALTY	tRNA pseudouridine synthase A (Pseudouridylate synthase I) (Pseudouridine synthase I) (Uracil hydrolyase)

32806	. . . . . x . . . x x . . . . . x . . . . . x . . . . x . x . . . . . x . . . . . . x . . .
148699	. x . . . . x x . . . . . x . . . x . . . . .
35320	. . . x . . . x . . x . . . . . x . . . . x . . . . x . . . . x .
91738	. x . . . . x x . . . . . x . . . x . . . . .
98869	. . . . . x x . x . . . . x . . x . . . .

Electrospray Quadrupole-Time-of-Flight (ESI-Q-Tof)

- ESI-MS was first reported in 1968 by Dole *et al.* improved at 1984 (Yamashita and Fenn, 1994).
- 可串接在液相層析儀之後,目前最微量的 nanoES (Wilm and Mann (1994, 1996), nanoelectrospray) 使用 gold tipped glass capillaries. (<50 fmoles of total protein) 20-50 nL min^{-1}.

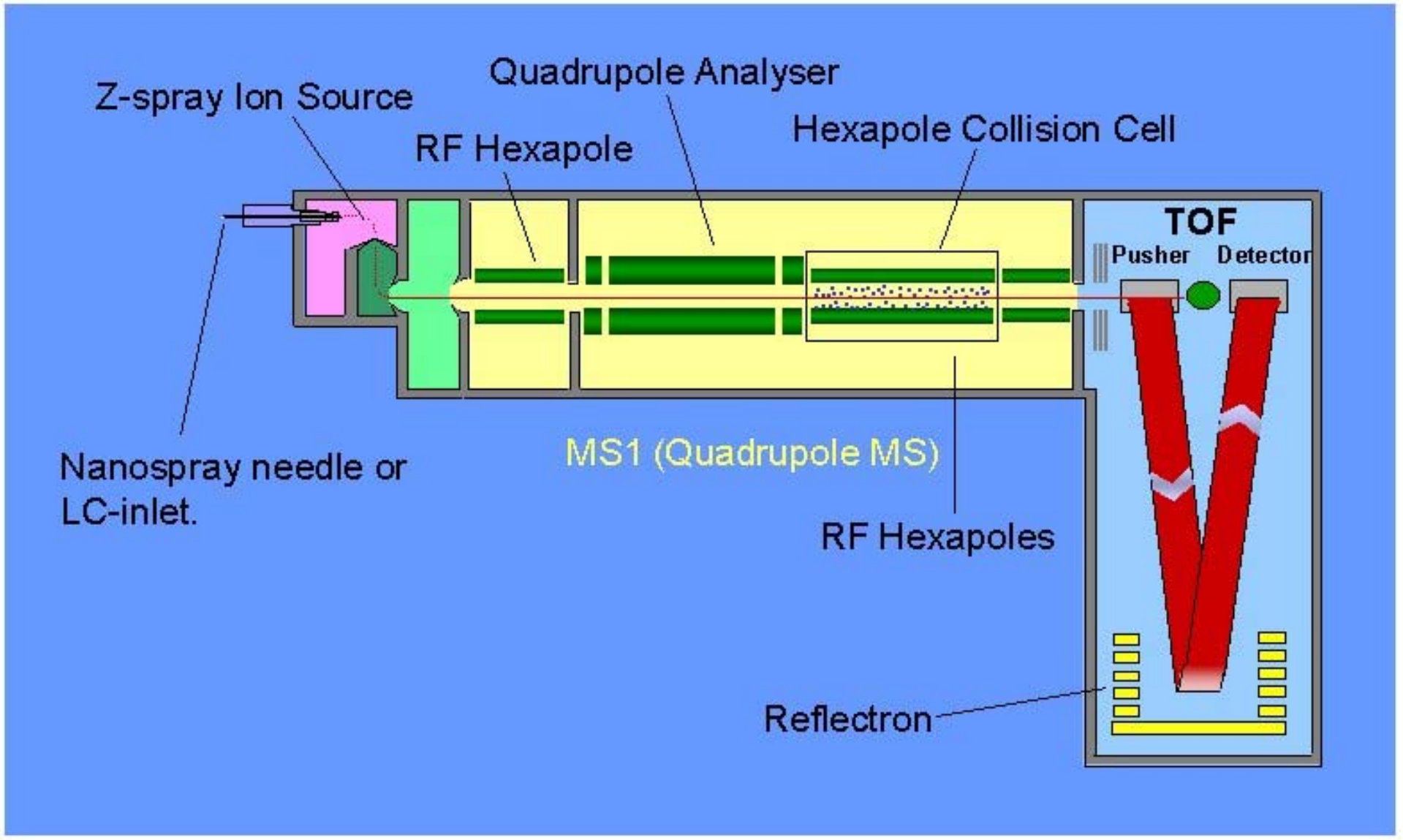
- 電噴灑法毛細管尖端使用高電壓（ ~ 3-4 kV), 導致形成極細的帶電液滴, 內含離子(ions of the type $(M+nH)^{n+}$, where M = the peptide molecule, *n*H is the number of protons attached to the molecule and *n*+ is the net charge of the biological ion.)
- 經由揮發後, 去除水份後分子帶電並進入分析儀 The multiply charged gas-phase ions are then formed as a result of desorption processes which occur due to evaporation of the solvent droplets (Iribarne and Thompson, 1976).



(1) Ions interact with dipole created by solvent at surface of droplet creating an electrostatic force

(2) Evaporation and coulombic explosions lead to an increase in the net charge of the droplet. This has the effect of inducing a strong electric field at the droplet surface

(3) Ions experience an increase in the potential energy barrier induced by the electrostatic force. This leads to field desorption of the $(M+nH)^{nH+}$ adduct ion

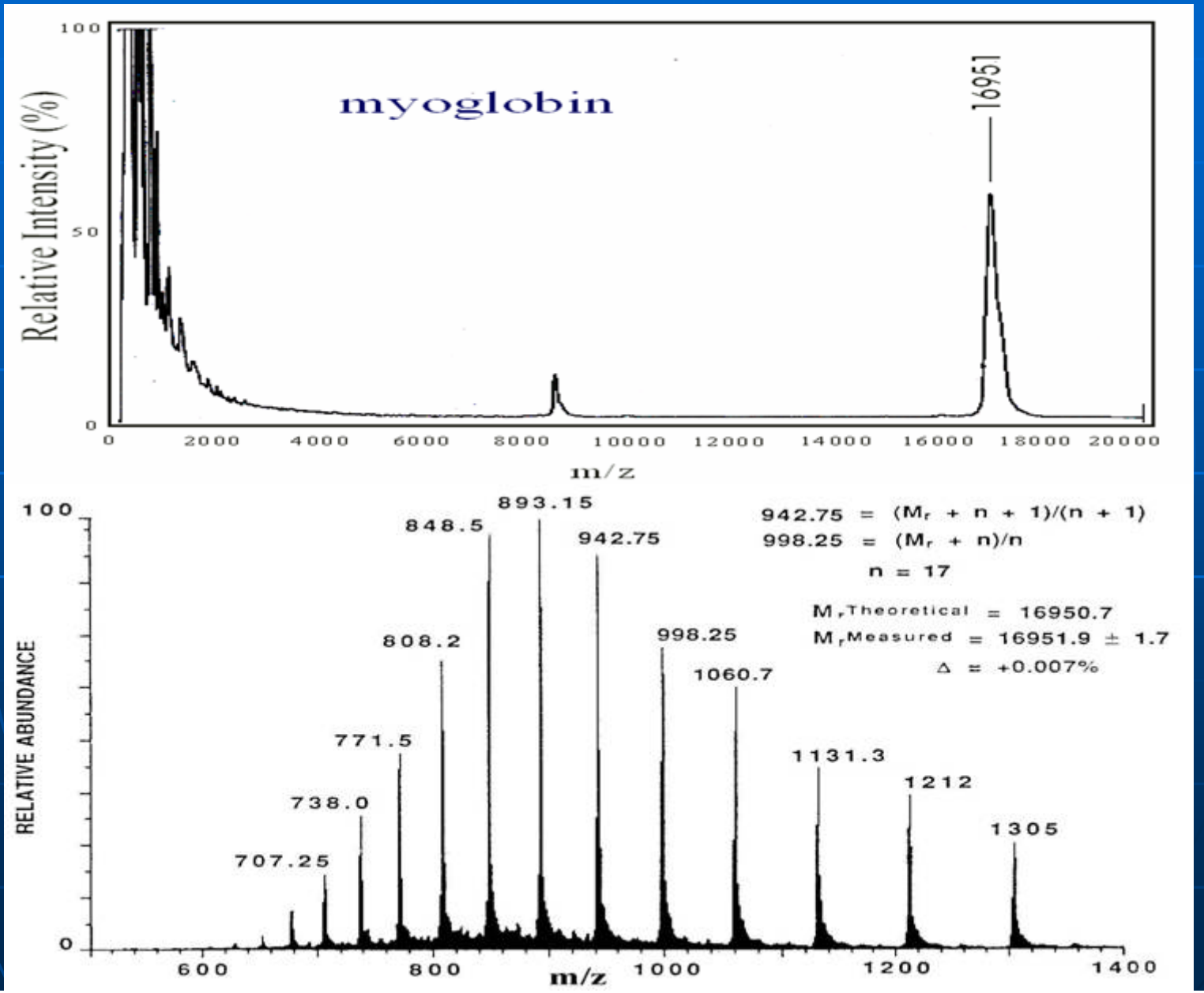

Z-spray Ion Source
Quadrupole Analyser
RF Hexapole
Hexapole Collision Cell
TOF
Pusher
Detector
Nanospray needle or
LC-inlet.
MS1 (Quadrupole MS)
RF Hexapoles
Reflectron

LC –MS/MS

- ESI 與 MALDI不同點:
  - 形成一系列帶不同電荷的離子.
  - 分子在液相中分離帶電.
- 可偵測高分子量的分子:
  - Relatively high molecular weight samples can be analysed by mass spectrometers with modest mass ranges (because mass spectrometry is concerned with the measurement of mass-to-charge (*m*/*z*) ratio, as opposed to mass).

- ESI可串接不同的質譜裝置,如:
  - quadrupoles, quadrupole ion traps, quadrupole time-of-flight (Q-tof) hybrid instruments and time-of-flight.
- 可使用串接成 tandem mass spectrometry or MS/MS, 可做定序並使用極少量的分子即可完成分析.

MALDI vs ESI

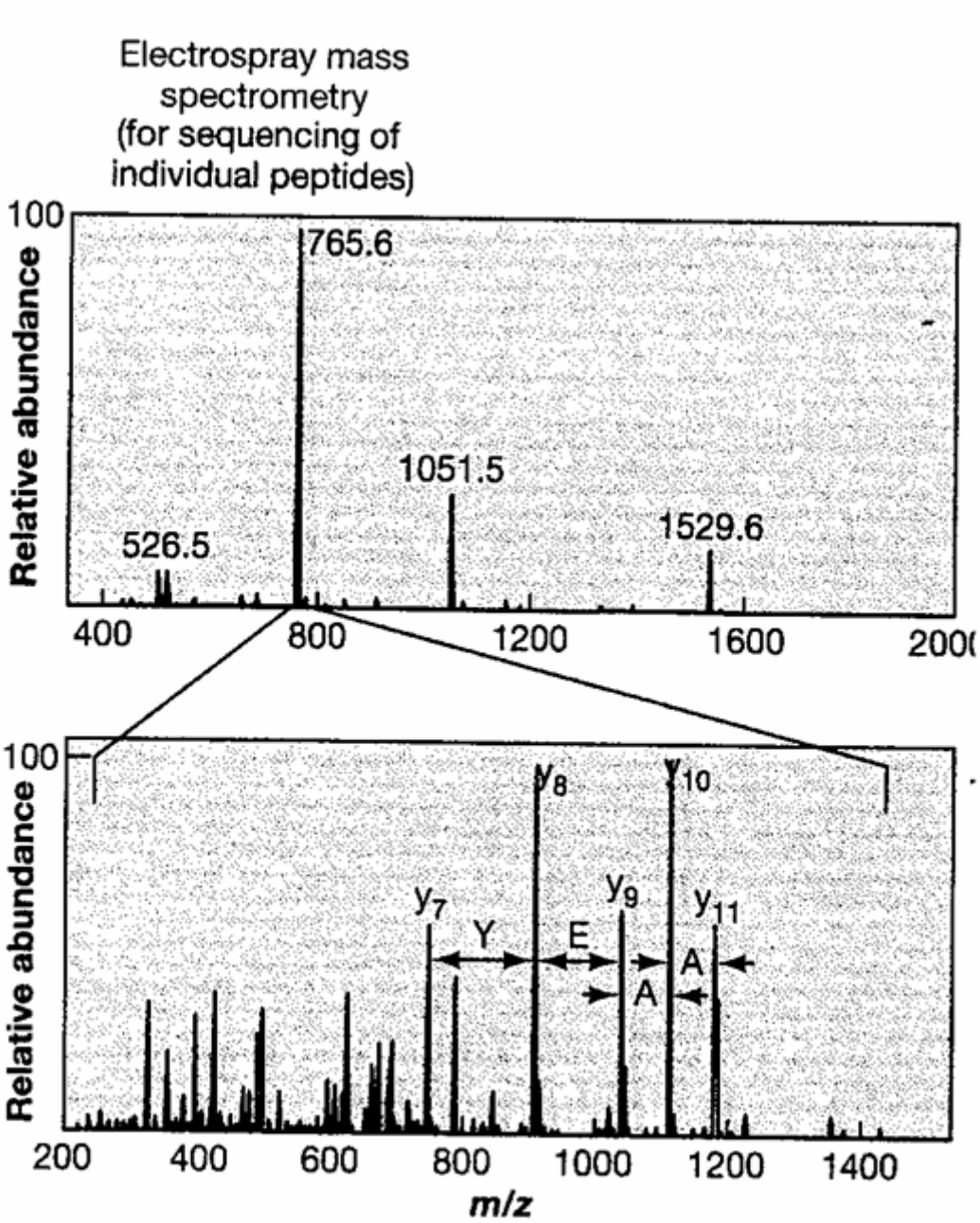

myoglobin
16951
Relative Intensity (%)
100
50
0
0
2000
4000
6000
8000
10000
12000
14000
16000
18000
20000
m/z
RELATIVE ABUNDANCE
100
0
893.15
848.5
942.75
808.2
998.25
1060.7
771.5
1131.3
1212
738.0
1305
707.25
600
800
1000
1200
1400
m/z
942.75 = (M$_r$ + n + 1)/(n + 1)
998.25 = (M$_r$ + n)/n
n = 17
M$_r$Theoretical = 16950.7
M$_r$Measured = 16951.9 ± 1.7
Δ = +0.007%

MS/MS

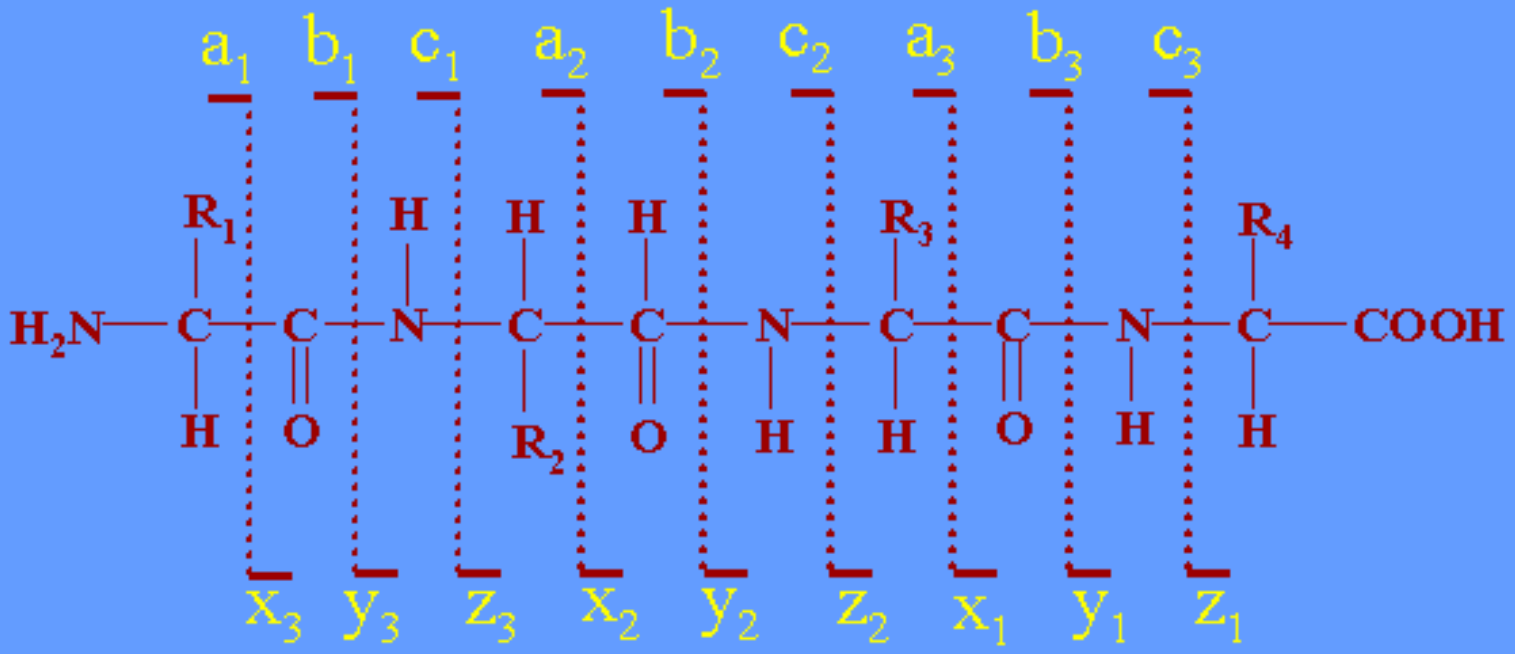
- 質譜指紋比對
  - 利用蛋白切除之小片段比對資料庫可得到吻合的蛋白
  - first developed by several groups in 1993 (Henzel *et al*., Mann *et al*., Pappin *et al*., Yates *et al*.).
- 指紋比對的缺點
  - (a) 某些蛋白在資料庫中沒有完整資料
  - (b) 指紋資料可能由多個蛋白混合 (the map represents a mixture of proteins).
- MS/MS: Mann and Wilm (1994) and Eng *et al.* (1994), peptide sequencing techniques using which compared database peptide sequences with MS/MS data.

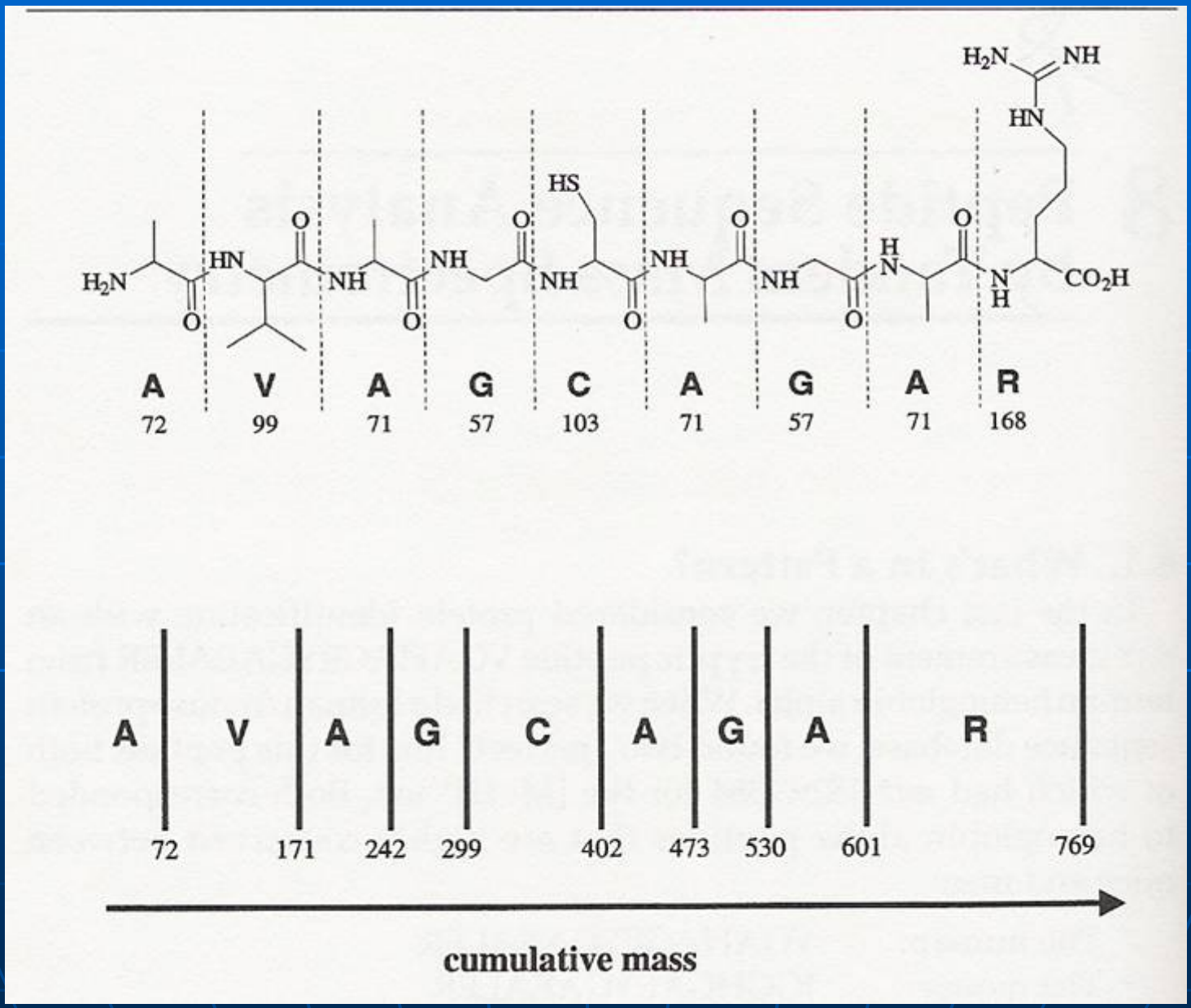
利用 MS/MS-TOF 做蛋白質定序

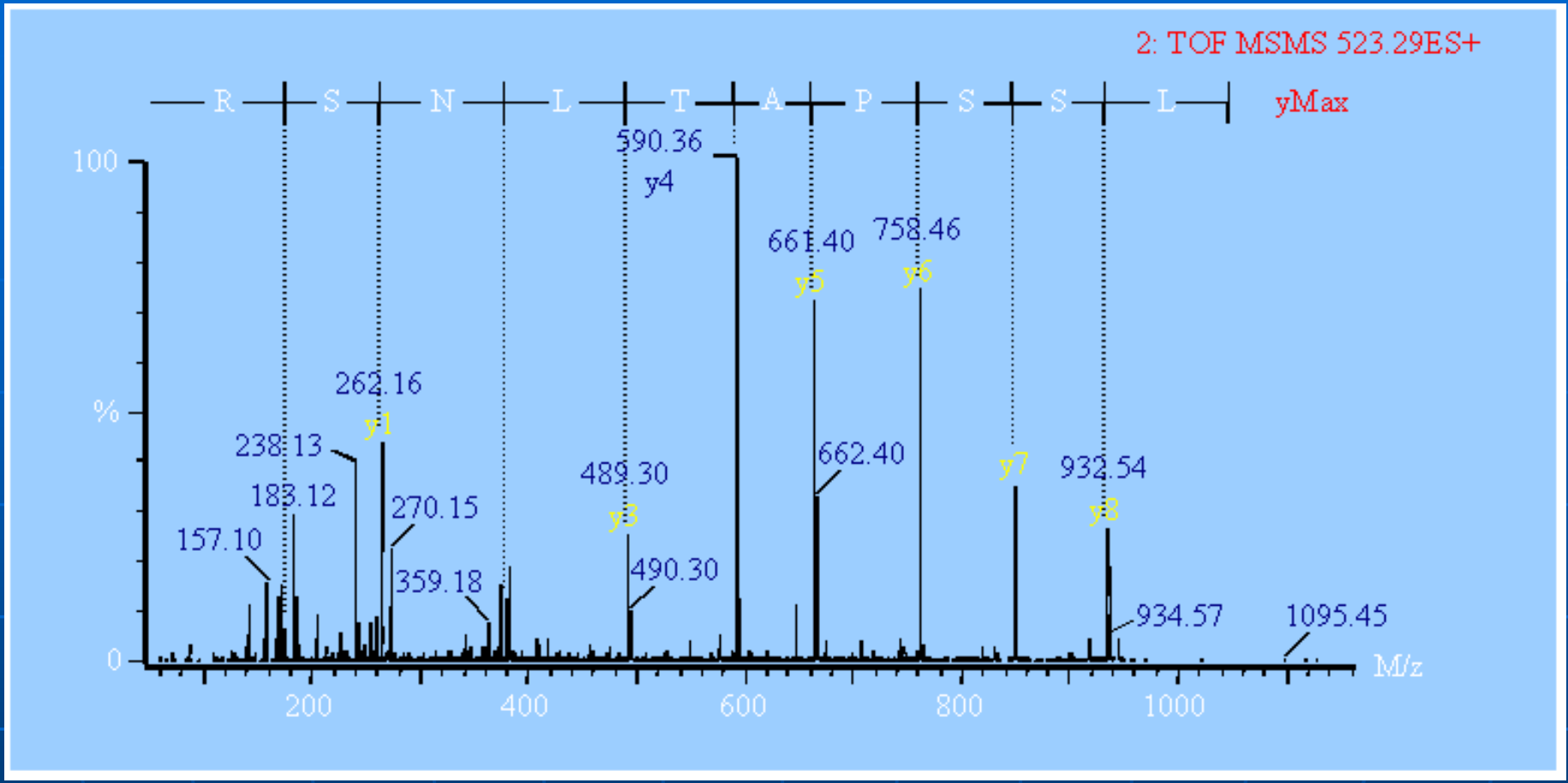
- 小片段 peptides 利用低能撞擊(low-energy collision-induced dissociation (CID) processes)使其分解.
- During low energy collisions in the 四極柱 (Q-Tof)或其他MS, *y* type ions最易出現 (C-N bond) (retention of the charge at the C-terminal side) and some low molecular weight *b* type ions (retention of the charge at the N-terminal side).



A sequence tag is derived via manual interpretation of the spectrum, which can now be used to search nucleotide and protein sequence databases.


a1 b1 c1 a2 b2 c2 a3 b3 c3
R1 H H H H R3 R4
H2N C C N C C N C C N C COOH
H O R2 O H H O H H
x3 y3 z3 x2 y2 z2 x1 y1 z1


A V A G C A G A R
72 99 71 57 103 71 57 71 168
A V A G C A G A R
72 171 242 299 402 473 530 601 769
cumulative mass



ESI-MS/MS spectrum of a doubly charged ion (*m/z* 523.29) of a trypsin autolylsis product from porcine trypsin. Subtraction of the masses of adjacent fragment ion peaks (*y*-type) corresponds to the masses of the amino acids in the peptide chain. Hence, the complete sequence of the peptide is LSSPATLNSR.

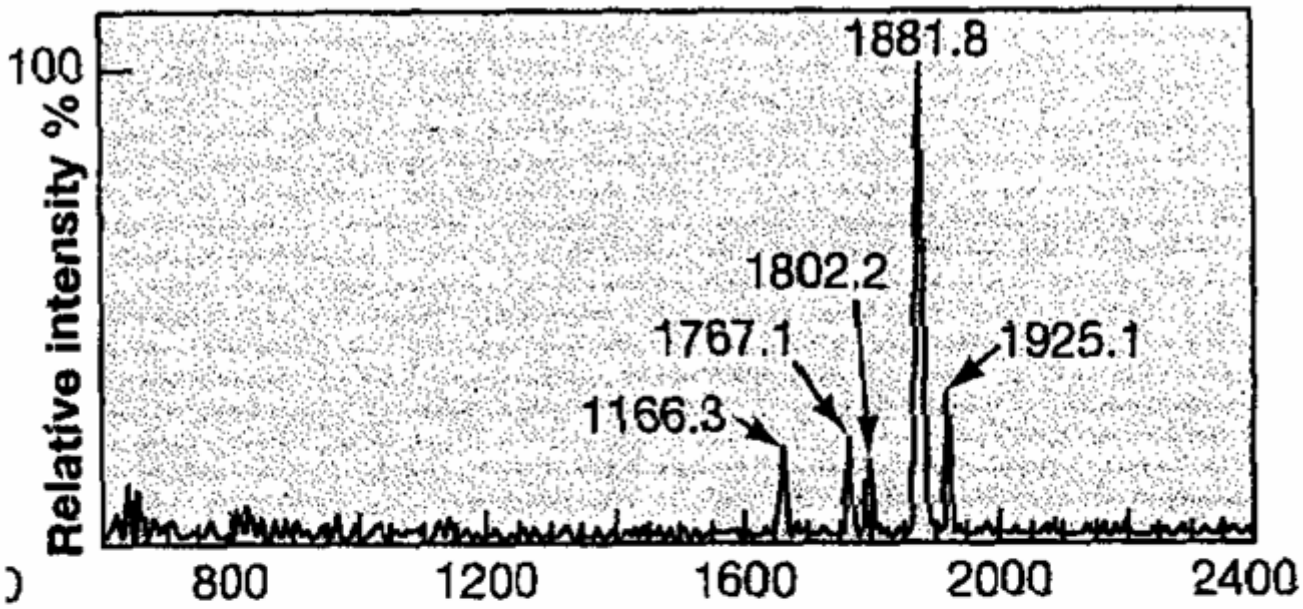
Posttranslational modification:

- More than 200 kinds of Posttranslational modifications (PTMs).
  - e.g., methylation: 14.0269 Mw, GalN: 161.1577,phosphorylation: 79.9799 Mw, etc.
- Two major PTMs of proteins: phosphorylation, and glycosylation.

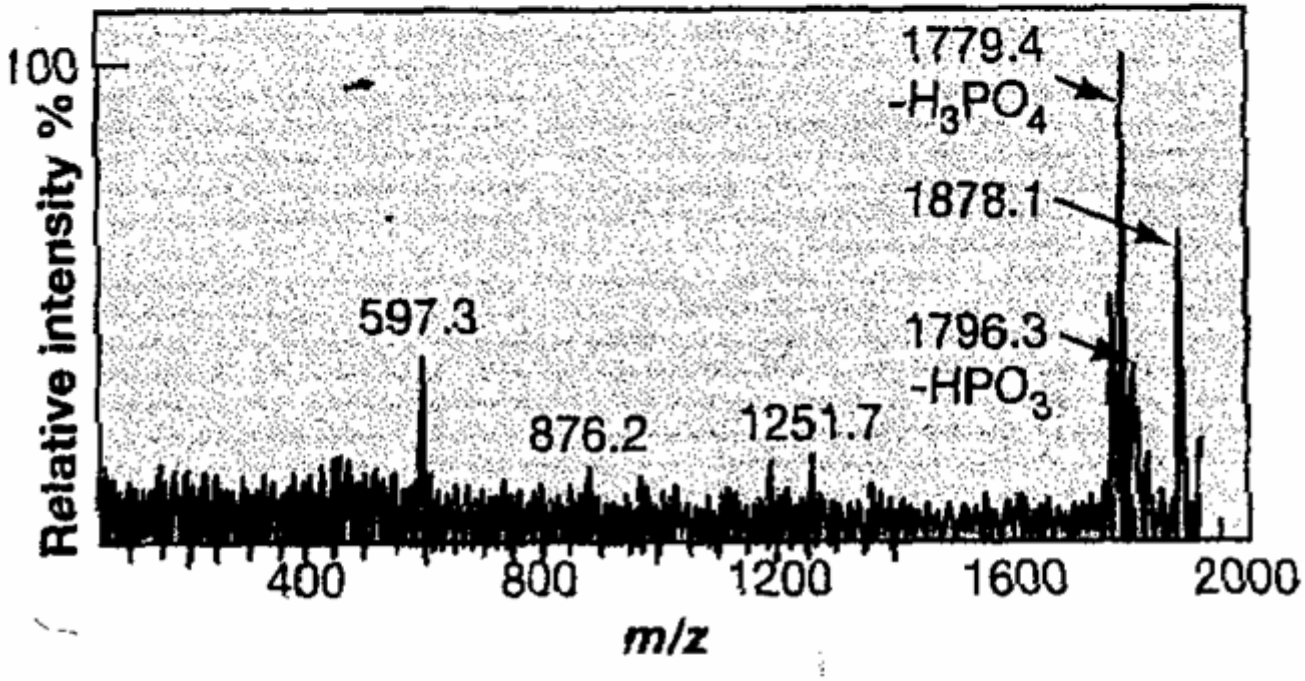
- 可利以MS/MS等質譜方法決定後修飾位置.

利用質譜儀偵測蛋白質修飾(e.g., Phosphorylation detecting by MS)

- MALDI:(example-利用PSD法)
  1. Post source decay (PSD): perform PSD on reflectron-equipped MALDI-TOF at first field free region.
     - P-Ser/Thr: loss of H_3PO_4 -- 98 Dalton.
     - P-Tyr: loss of HPO_3 -- 80 Dalton.
       - Annan and Carr, 1996
  2. Alkaline phosphate treatment:
     - MALDI before and after alkaline phosphate treatment.



inear
lode



98Da

SD, then
eflectron

80Da

蛋白質晶片 (Protein Microarray)

- Poor coorelation between mRNA and protein expression levels.
- DNA chips fail on PTMs signals.
- Applications:
  - Study enzyme-substrate, DNA-protein, protein-protein interactions on proteomic scale.
  - MacBeath and Schreiber (2000): protein arrays containing more than 10,000 proteins.

Classes of capture molecules for protein microarrays.

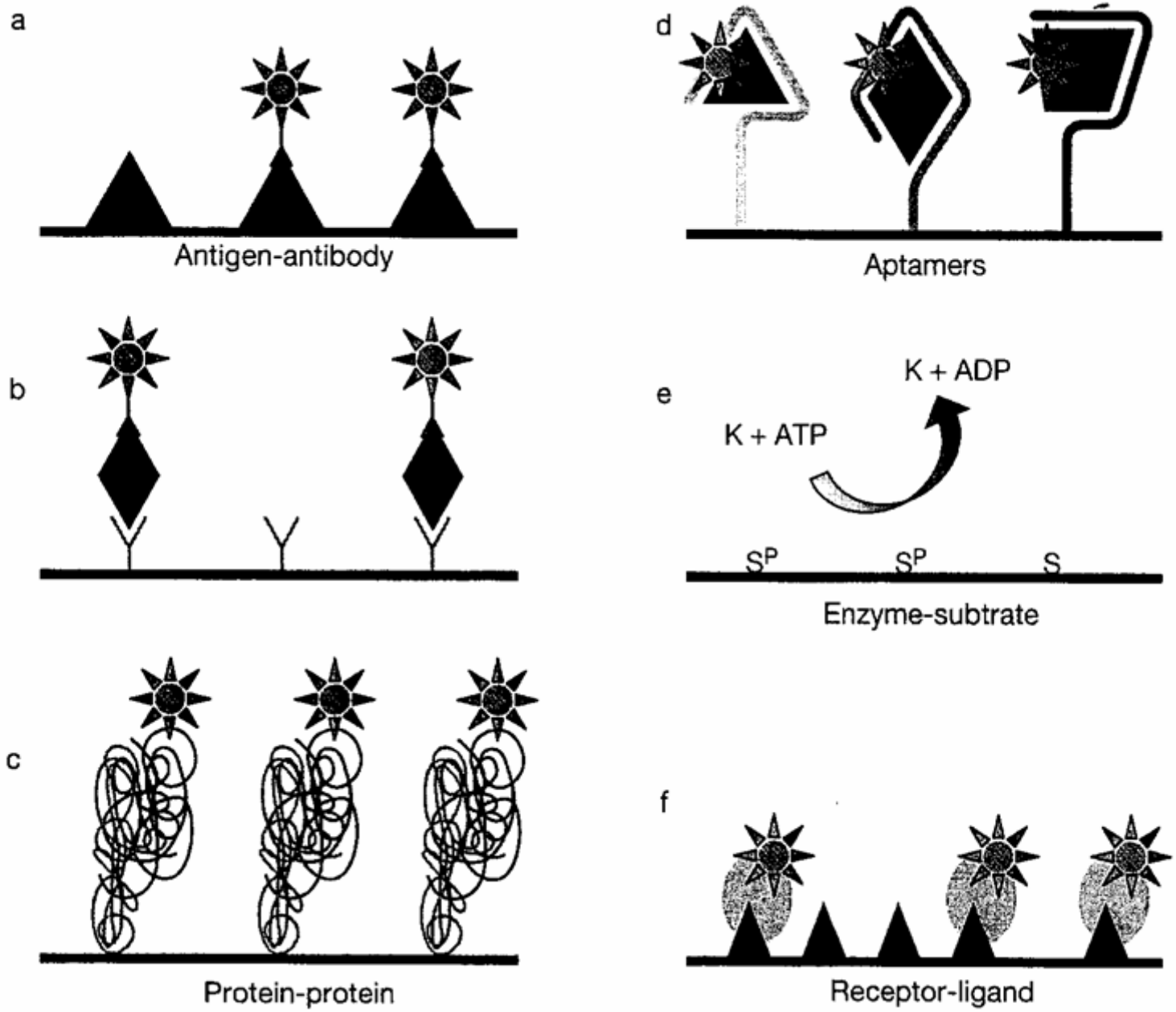
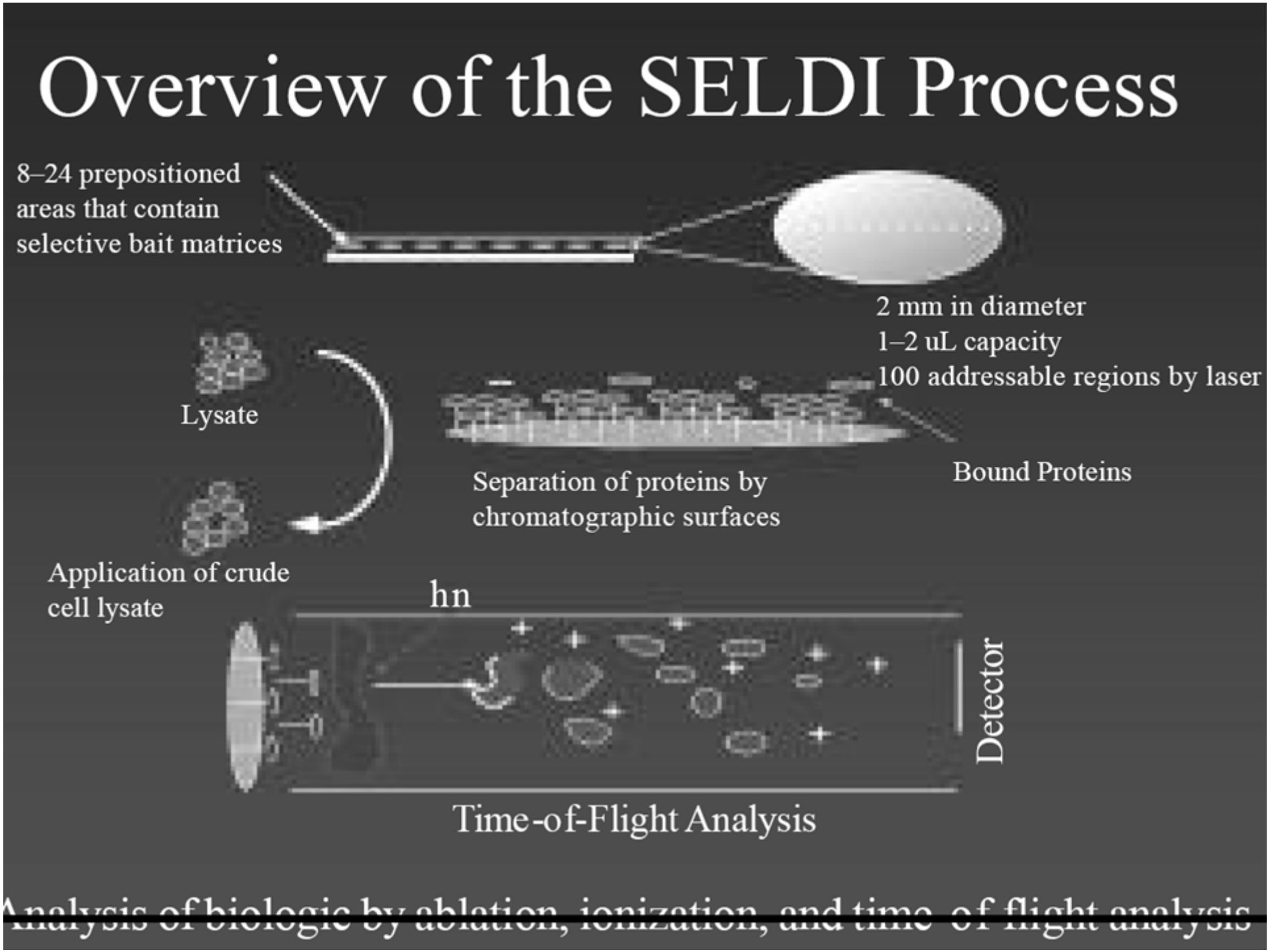


TABLE 1.7. Protein arrays: Classes of capture molecules

Capture molecules	Source	Technique	References
mAb	mouse	hybridoma	Goldman (2000)
scFv/Fab diabodies	antibody libraries	phage display, in vitro evolution	Gao et al. (1999); Ryu and Nam (2000); Krebs et al. (2001); Lecerf et al. (2001); Raum el al. (2001)
Affinity binding agents	recombinant fibronectin structures	in vitro evolution	Kreider (2000)
Affibodies	microorganism	heterologous expression	Gunneriussion et al. (1999a,b)
Aptamers (DNA/RNA/ peptide)	library	SELEX/mRNA display, in vitro evolution	Jayasena (1999); Brody and Gold (2000); Hoppe-Seyler and Butz (2000); Lee and Walt (2000); Lohse and Wright (2001); Wilson et al. (2001)
Receptor ligands	synthetic	combinatorial chemistry	MacBeath et al. (1999); Lee and Walt (2000)
Substrates of enzymes	synthetic; pro- and eukaryotic organisms	protein purification, recombinant protein technology (bacterial fusion proteins, baculo-virus, peptide synthesis)	Arenkov et al. (2000); MacBeath and Schreiber (2000); Zhu et al.(2000)

This table summarizes classes of molecules that have the potential to be used or are actually used as capture molecules in protein microarray systems. Abbreviations: (Fab) Antigen-binding fragment; (scFv) single-chain variable region fragment; (mAb) monoclonal antibody.
Reproduced, with permission, from Templin et al. (2002).

Overview of the SELDI Process

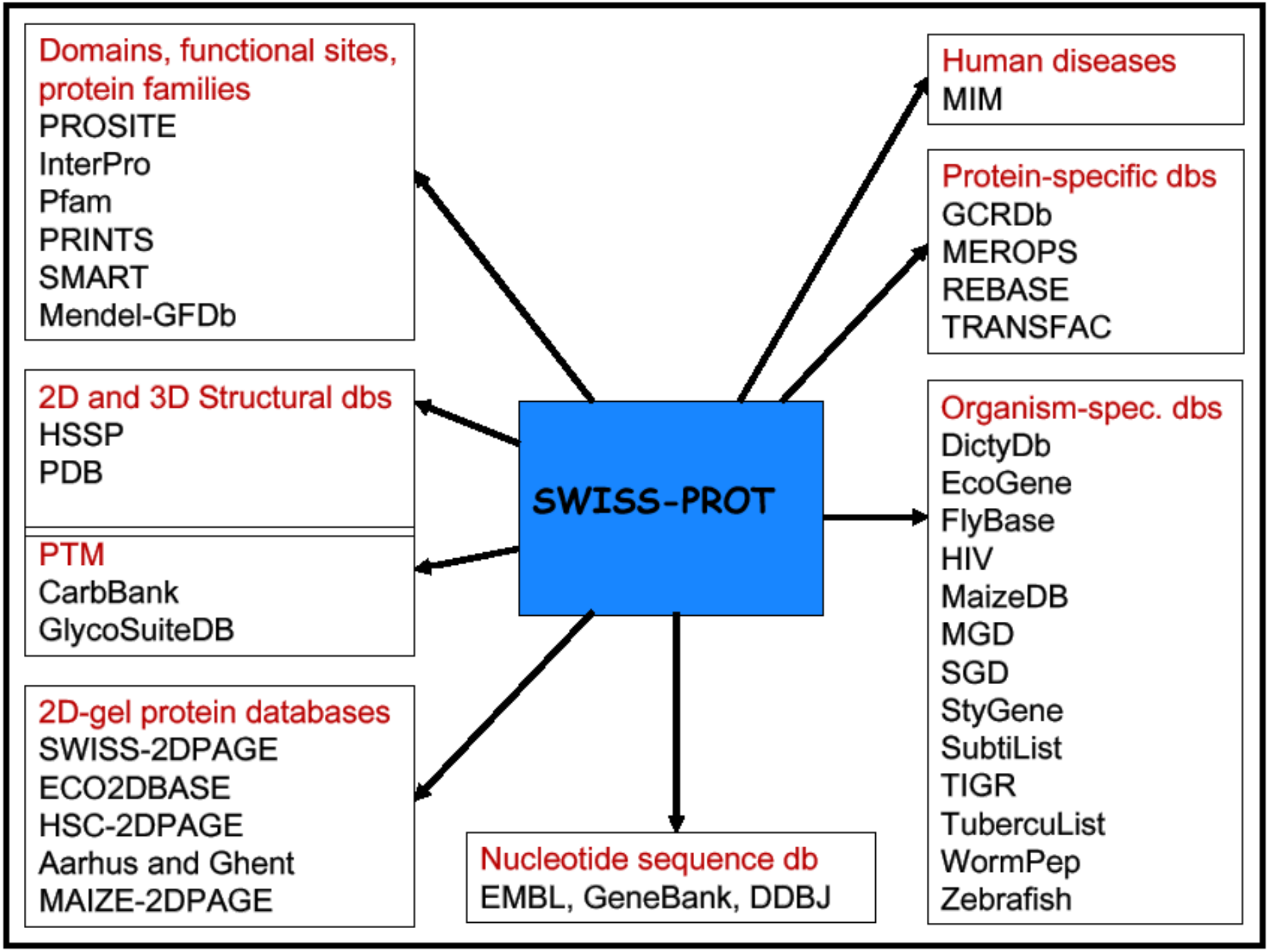


Analysis of biologic by ablation, ionization, and time-of-flight analysis

- http://www.ciphergen.com

蛋白質體網站

- Expasy (http://tw.expasy.org)
- etc...


Domains, functional sites, protein families
PROSITE
InterPro
Pfam
PRINTS
SMART
Mendel-GFDb
2D and 3D Structural dbs
HSSP
PDB
PTM
CarbBank
GlycoSuiteDB
2D-gel protein databases
SWISS-2DPAGE
ECO2DBASE
HSC-2DPAGE
Aarhus and Ghent
MAIZE-2DPAGE
SWISS-PROT
Nucleotide sequence db
EMBL, GeneBank, DDBJ
Human diseases
MIM
Protein-specific dbs
GCRDb
MEROPS
REBASE
TRANSFAC
Organism-spec. dbs
DictyDb
EcoGene
FlyBase
HIV
MaizeDB
MGD
SGD
StyGene
SubtiList
TIGR
TubercuList
WormPep
Zebrafish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ExPASy web site
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database
SWISS-2DPAGE contains data on proteins identified on various 2-D PAGE and SDS-PAGE reference maps. You can locate these proteins on the 2-D PAGE maps or display the region of a 2-D PAGE map where one might expect to find a protein from Swiss-Prot [More details / References / Linking to SWISS-2DPAGE / Commercial users / Disclaimer].
Release 17.3, March 2004 and updates up to 08-Apr-2005 (contains 1265 entries in 36 reference maps from human, mouse, Arabidopsis thaliana, Dictyostelium discoideum, Escherichia coli, Saccharomyces cerevisiae, and Staphylococcus aureus (N315)).
Recent additions: 2-D PAGE reference maps for Staphylococcus aureus (N315) new and for Human lymphocytes new
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by accession number (AC lines)
by clicking on a spot: select one of our 2-D PAGE or SDS-PAGE reference maps, click on a spot and then get the corresponding information from the SWISS-2DPAGE database.
by author (RA lines)
by spot serial number (2D and 1D lines)
by full text search
retrieve all the protein entries identified on a given reference map
SWISS-2DPAGE documents
User manual
Release notes (March 31, 2004)
FAQ (Frequently Asked Questions about SWISS-2DPAGE)
Protocols:
Technical information about 2-D PAGE (IPG's, silver staining, protocols, etc)
High performance 2-D gel comparison

http://proteome.sinica.edu.tw/pro_technology.asp

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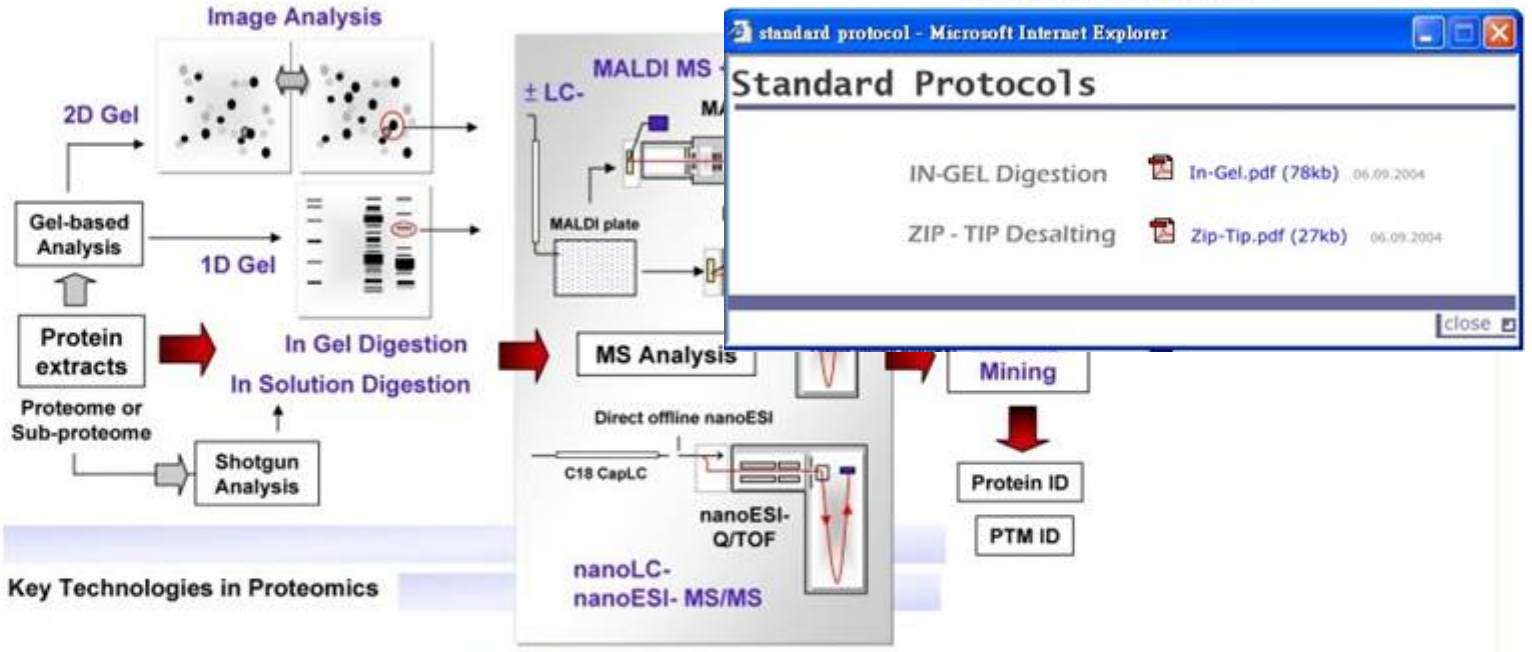

Text Description of the MS Experimental Details and Acknowledgement Statement !!

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服務範圍說明與聲明

使用者對象分類與順序

使用單位分佈圖表

	服務項目	規格與單位	基因體醫學國家型計劃	其他學術計劃	產業界計劃
1	二維凝膠蛋白質電分析	18 x 18 cm /片(gel) Sypro Ruby Stain	5,000	6,500	7,000
2	蛋白質膠內酵素水解(自動化)	Trypsin Digestion / 個	200	300	400
3	樣品去鹽純化濃縮(自動化)	Zip-Tip純化/個	200	300	400
4	純蛋白質分子量測定	直接MS進樣/個	400	500	600
5	混合蛋白質之個別分子量測定	LC-MS/個	1,000	1,500	2,000
6	蛋白質身分鑑定(based peptide mass fingerprint)+部分internal sequence	MALDI-MS and MS/MS	400	500	600
7	蛋白質身分鑑定(based on internal sequence)	LC-MS/MS / 個	3,000	4,000	6,000

附註：其他進階分析(例如：蛋白質修飾鑑定、蛋白質定量分析或其他特殊分析)及生物資訊服務 (SRS 資料庫整合查詢系統、VectorNTI 及 EMBOSS 序列分析系統) 需進一步討論並以學術合作方式執行。

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