INTRODUCTION TO PROTEOMICS

(HTTPS://WWW.BRUKER.COM/CONTENT/BRUKER/INT/JA/APPLICATIONS/ACADEMIA-LIFE-SCIENCE/PROTEOMICS.HTML)

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PROTEIN IDENTIFICATION WITH ANTIBODIES

IMMUNOFLUORESCENCE
(HTTPS://WWW.YOUTUBE.COM/WATCH?V=PTE06FRW03G)
&
IMMUNOHISTOCHEMISTRY
(HTTPS://WWW.YOUTUBE.COM/WATCH?V=ZO70NRWCAYK)

PROTEIN IDENTIFICATION WITH ANTIBODIES

- An antibody (Ab), also known as an immunoglobulin (Ig),
- Its a large**, Y-shaped protein** used by the immune system to identify and neutralize foreign objects such as pathogenic bacteria and viruses.
- The **antibody recognizes** a unique molecule of the pathogen, called an **antigen.**
- Each tip of the **"Y" of an antibody contains a paratope** (analogous to a lock) that is specific for one particular **epitope (analogous to a key) on an antigen.**
- This architecture allowing these **two structures to bind together with precision**.
- Using this **binding mechanism**, an **antibody** can tag a microbe or an infected cell for attack by other parts of the immune system, or can neutralize it directly (for example, by blocking a part of a virus that is essential for its invasion).
- Antibodies can be used to visualize the location of specific proteins within the cell

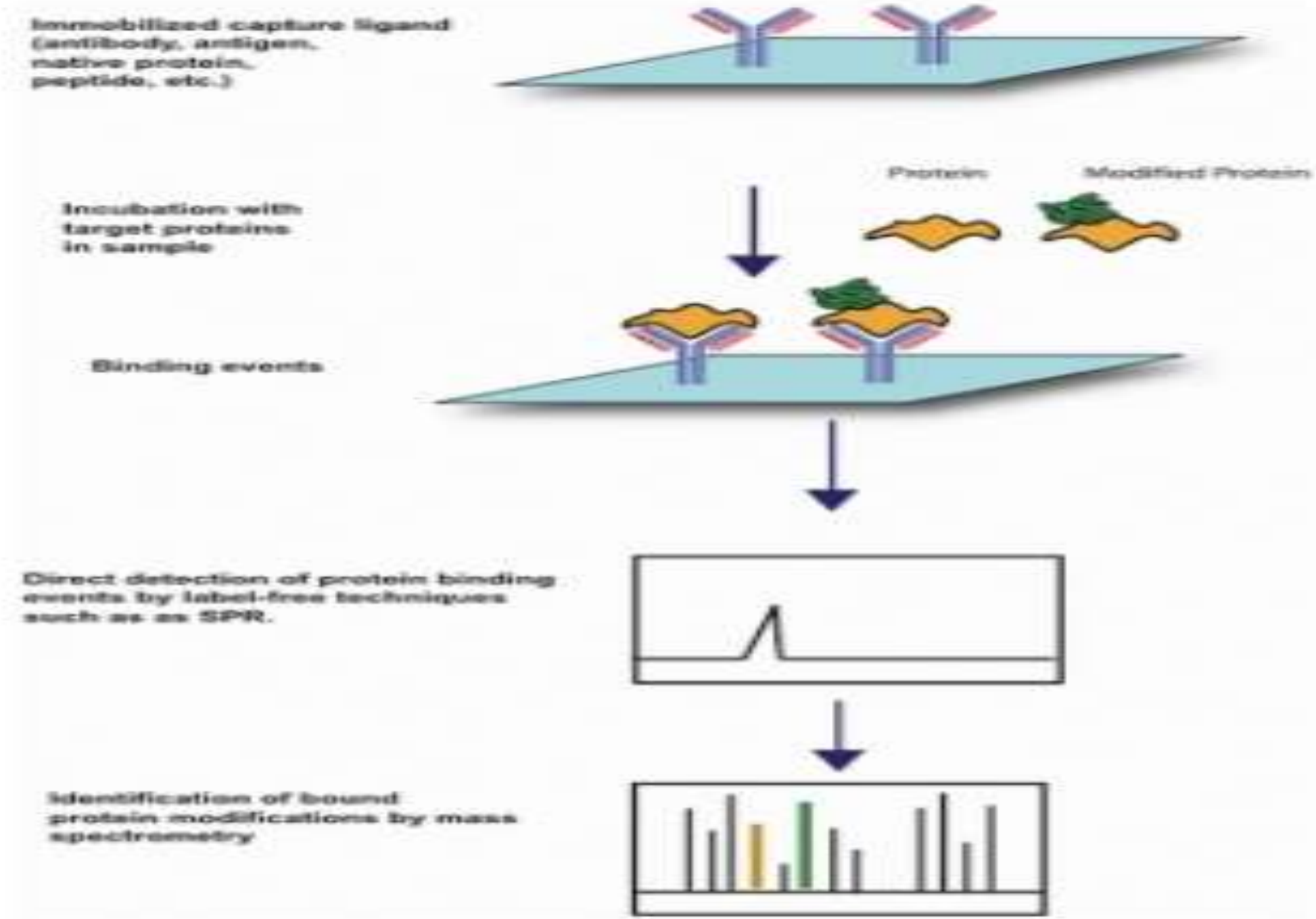
THE BENEFIT OF DETECTING ANTIBODIES & HOW

- Antibodies can be used to visualize the location of specific **proteins** within the cell.
- **Antibody** tests usually involve mixing the **patient's sample** with a **known antigen**, the substance that the **antibody** is directed against or produced in response to, and seeing if a reaction takes place.
- If an **antibody is present** and **binds to the known antigen**, the formation of the **antibody-antigen complex** can be measured
- Thus method used is:

1. **Immunocytochemistry/Immunofluorescence :** refers to the visualization of **specific antigens** in cultured cells.
2. **Immunohistochemistry** : refers to their visualization in prepared tissue sections

i.e.

- Immunofluorescence and immunohistochemistry : use antibodies for detection and localization of proteins and other antigens within biological samples.


Immobilized capture ligand
(antibody, antigen,
native protein,
peptide, etc.)
Protein
Modified Protein
Incubation with
target proteins
in sample
Binding events
Direct detection of protein binding
events by label-free techniques
such as as SPR.
Identification of bound
protein modifications by mass
spectrometry

- In addition, these methods **allow identification of regions** within cells that are important for **binding of proteins or specific antigens.**
- Important to highlight that these techniques have **the same basic steps as the ELISA** and **Western blot**.

- **Common Steps:**

a) fixation of the sample onto slides or plates, blocking in order to avoid unspecific signal, detection by the use of one or two antibodies, and analysis.

b) However, when the **sample is a tissue**, it is important to notice that when using **immunofluoresce** and **immunohistochemistry** methods, the sample must be blocked with **inactivated serum and not with BSA**.

c) This is an important step because **immune cells** may be present **within the tissue**.

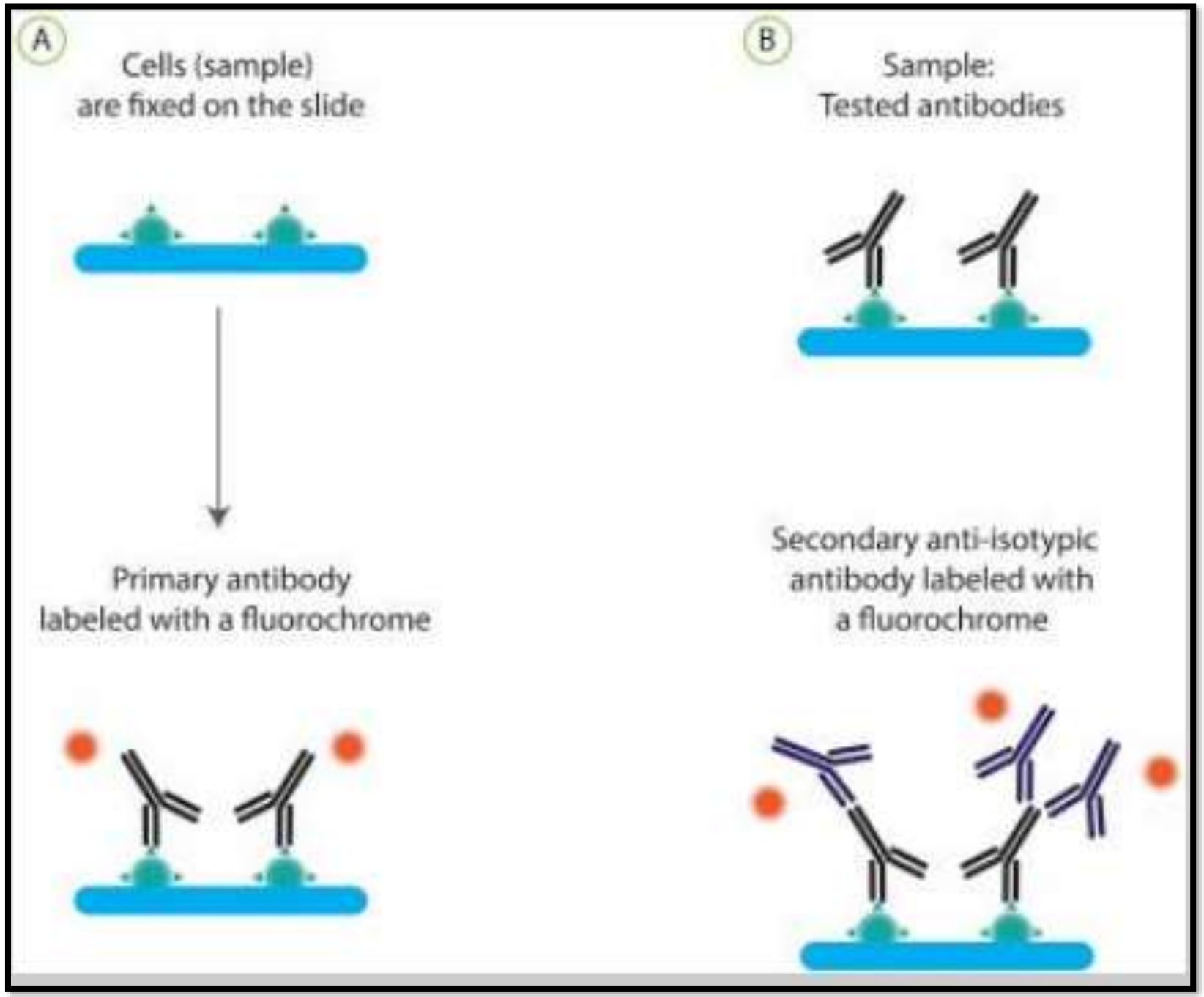
d) Such approach avoids an **unspecific response** because **primary antibodies can bind to antibody receptors present in immune cells' membrane. (e.g. Mast cell).**

IMMUNOFLUORESCENCE (IF)

- The detection of **antigen-antibody complex in immunofluorescence** (IF) is based on the same principles of **flow cytometry**: as antibodies are dyes with **fluorochromes.**
- The cells are **fixed on a slide** instead of being suspended in a fluid, and the analysis is made by fluorescent microscopy.
- There are two kinds of **immunofluorescence** based upon the antibodies that are being used.

1st: just one antibody is used which binds directly to the target antigen; therefore, this technique is called direct immunofluorescence (DIF).

2nd: it is called indirect immunofluorescence (IIF) when two antibodies are used and because the antibody allowing the detection binds to a primary antibody that recognizes the antigen on the slide


A
Cells (sample)
are fixed on the slide
Primary antibody
labeled with a fluorochrome
B
Sample:
Tested antibodies
Secondary anti-isotypic
antibody labeled with
a fluorochrome

CONCLUSION

- Immunofluorescence : effective and sensitive tool for protein analysis.
- IF can be combined with structural and biochemical studies to increase its efficiency.
- Method is easy to perform and cost-effective,

But: important to pay attention to critical parameters such as **fixation, permeabilization, determination of antibody specificity and a careful selection of antibodies and type of tissues to ensure accurate results**.

- If the epitope is hidden, it will not be recognized and bound by the antibody.
- Therefore, necessary to

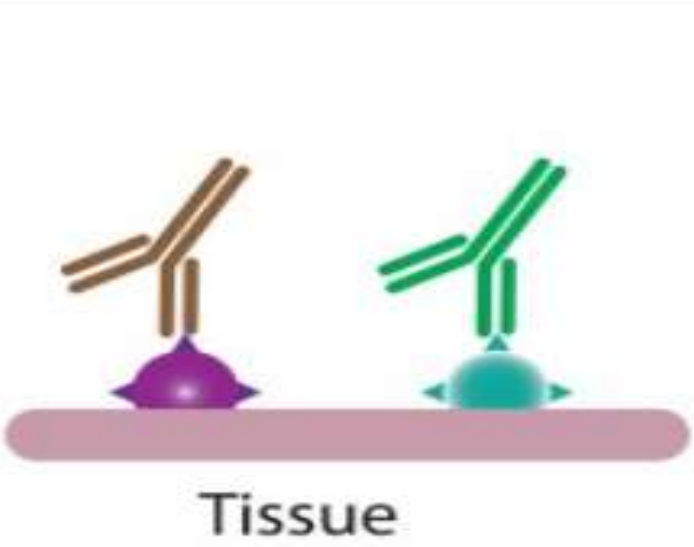
1) permeabilizing reagents which expose the epitope and highlight the localization of antigen because there are some antibodies which cannot go into the nucleus; therefore, the epitope will not be achieved and no signal will be produced.

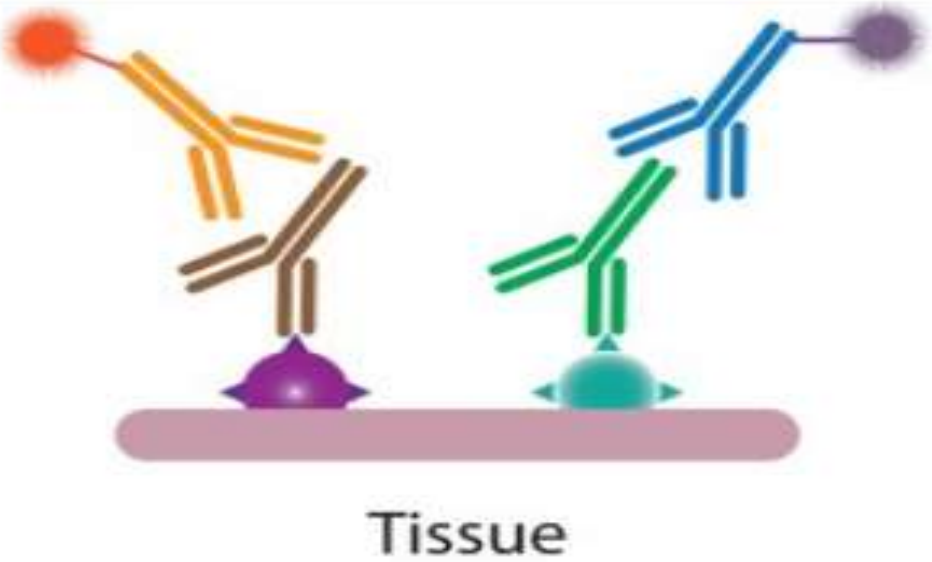
2) A sample incubated only with the secondary antibody to determine non-specific binding sites.

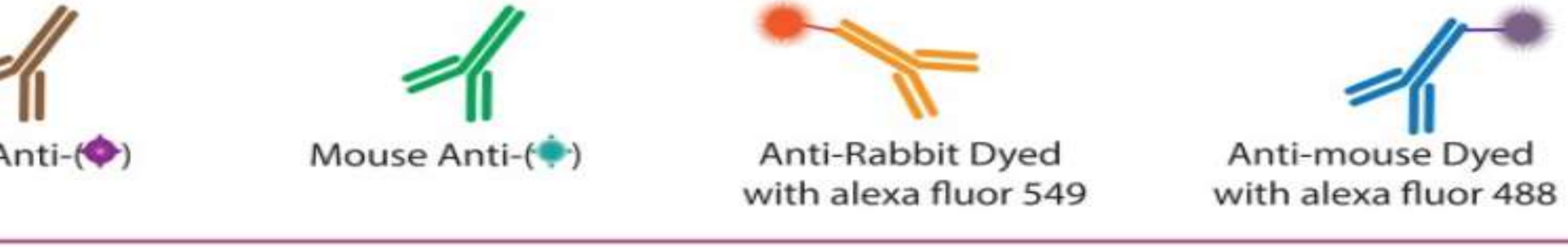
3) Control slide containing cells that either do not express and/or have high expression of the target protein.

IMMUNOHISTOCHEMISTRY

- Immunohistochemistry (IHC) same as immunofluorescence although with some differences.
- It allows the **analysis of tissue structure** and **localization of a specific marker** within it.
- The **antibody-antigen interaction** is visualized using either chromogenic detection, in which an enzyme conjugated to the antibody cleaves a substrate to produce a colored precipitate at the location of the protein or fluorescent detection, in which a fluorophore is conjugated to the antibody and can be visualized using fluorescence microscopy.
- This technique can be performed using samples with different previous treatments such as cryopreserved slides or paraffin-embedded tissue slides







Example of proper selection of antibodies for IHC.

hould be chosen considering the host species production and the dyes.

- IHC and immunofluorescence use similar methodology, there are some reasons that make IHC more accessible to researchers:

1) IHC does not require special equipment as IF; allows antigen localization and determination of cell or tissue morphology;
2) staining last for years, and enzymes or substrate used in IHC are light insensitive.
3) IHC is easier to perform than IF, but both have the same critical factors for performance as: **fixation, cautious antibody selection and dilutions, and use of appropriate controls**.
4) Negative controls could be slides previously exposed to antibody-specie specific serum (e.g., rabbit's serum if rabbit's antibody is used) or isotype-specific Ig as primary antibody.
5) Controls are also useful for determination of non-specific binding sites to secondary antibody and optimal primary antibody dilution.
6) Positive controls should also be included to validate proper activity of the different reagents.
7) IHC can be done also with fluorochrome labeled antibodies.

PROTEIN IDENTIFICATION

PROTEIN SEQUENCE DETERMINATION BY, EDMAN'S DEGRADATION (CHEMICAL DEGRADATION)

(HTTPS://WWW.YOUTUBE.COM/WATCH?V=A6SHYPXQTB8)

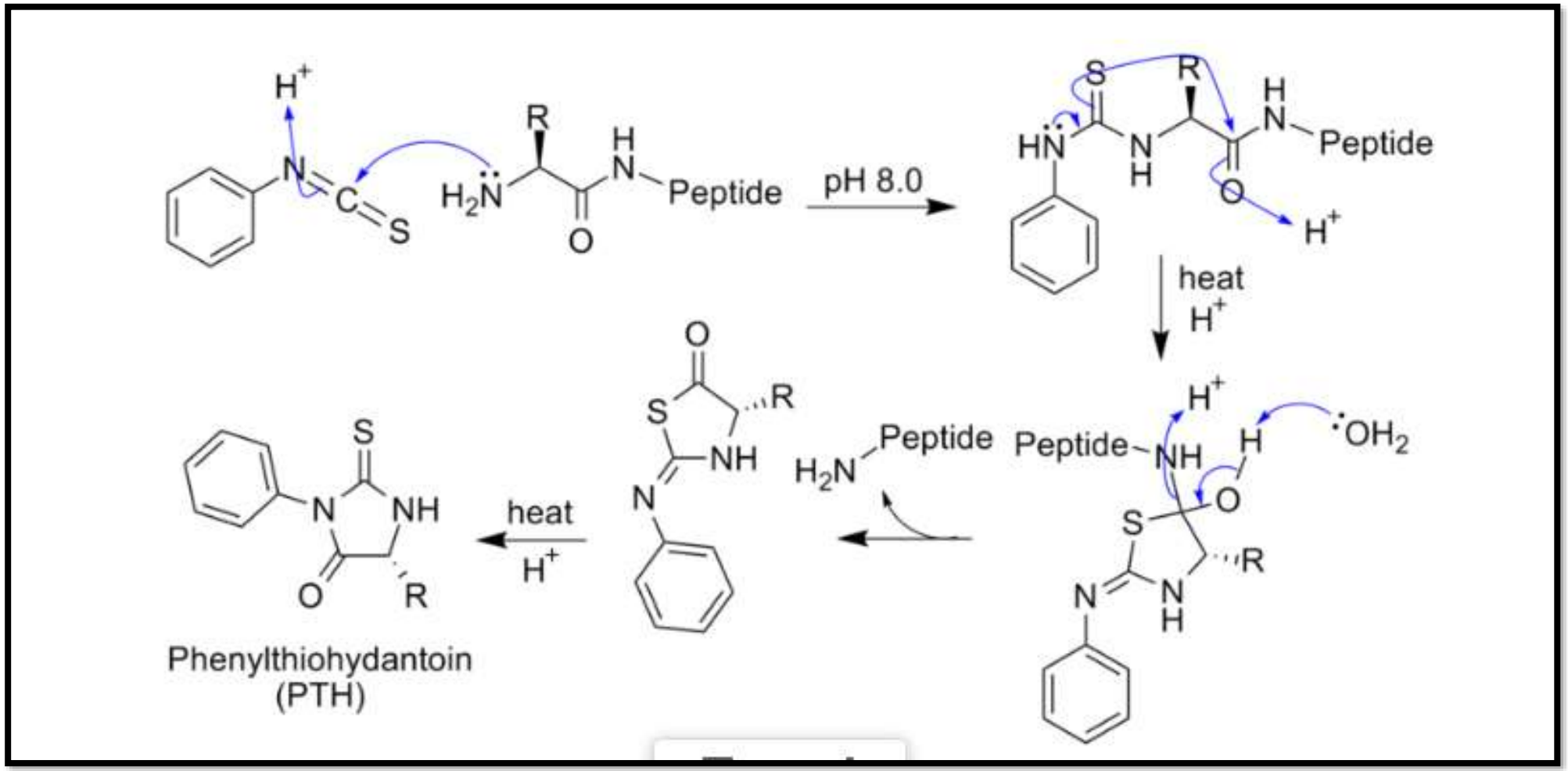
(HTTPS://WWW.YOUTUBE.COM/WATCH?V=GUHZONTCCP0)

EDMAN'S DEGRADATION (ED)

- **Edman degradation**, developed by **Pehr Edman**, is a **method** of **sequencing** amino acids in a **peptide**.
- In this **method**, the amino-terminal residue is labeled and cleaved from the **peptide** without disrupting the **peptide** bonds between other **amino acid** residues.
- The Edman degradation reaction was automated in 1967 by Edman and Beggs. Nowadays, the automated Edman degradation (the protein sequenator) is used widely, and it can sequence peptides up to 50 amino acids.
- Edman degradation is the process of purifying protein by sequentially removing one residue at a time from the amino end of a peptide.
- To solve the problem of damaging the protein by hydrolyzing conditions, Pehr Edman created a new way of labeling and cleaving the peptide. Edman thought of a way of removing only one residue at a time, which did not damage the overall sequencing.

Procedure:

1) Add Phenyl isothiocyanate, which creates a phenylthiocarbamoyl derivative with the N-terminal.
2) The N-terminal is then cleaved under less harsh acidic conditions, creating a cyclic compound of phenylthiohydantoin PTH-amino acid.
3) This does not damage the protein and leaves two constituents of the peptide.
4) This method can be repeated for the rest of the residues, separating one residue at a time.


pH 8.0
heat
H+
heat
H+
Peptide
Phenylthiohydantoin
(PTH)

- ED is very useful because it does not damage the protein.
- This allows sequencing of the protein to be done in less time.
- Edman sequencing is done best if the composition of the amino acid is known.
- To determine the composition of the amino acid, the peptide must be hydrolyzed.
- This can be done by denaturing the protein and heating it and adding HCl for a long time.
- This causes the individual amino acids to be separated, and they can be separated by ion exchange chromatography.
- They are then dyed with ninhydrin and the amount of amino acid can be determined by the amount of optical absorbance.
- This way, the composition but not the sequence can be determined

DRAWBACK'S

- Larger proteins cannot be sequenced by the Edman sequencing because of the less than perfect efficiency of the method.
- A strategy called divide and conquer successfully cleaves the larger protein into smaller, practical amino acids.
- This is done by using a certain chemical or enzyme which can cleave the protein at specific amino acid residues.
- The separated peptides can be isolated by chromatography.
- Then they can be sequenced using the Edman method, because of their smaller size.
- In order to put together all the sequences of the different peptides, a method of overlapping peptides is used.
- The strategy of divide and conquer followed by Edman sequencing is used again a second time, but using a different enzyme or chemical to cleave it into different residues.

- This allows two different sets of amino acid sequences of the same protein, but at different points.
- By comparing these two sequences and examining for any overlap between the two, the sequence can be known for the original protein.
- Overlapping of sequences can lad to find the original sequence of the initial protein.
- However, this method is limited in analyzing larger sized proteins (more than 100 amino acids) because of secondary hydrogen bond interference.
- Other weak intermolecular bonding such as hydrophobic interactions cannot be properly predicted.
- Only the linear sequence of a protein can be properly predicted assuming the sequence is small enough.

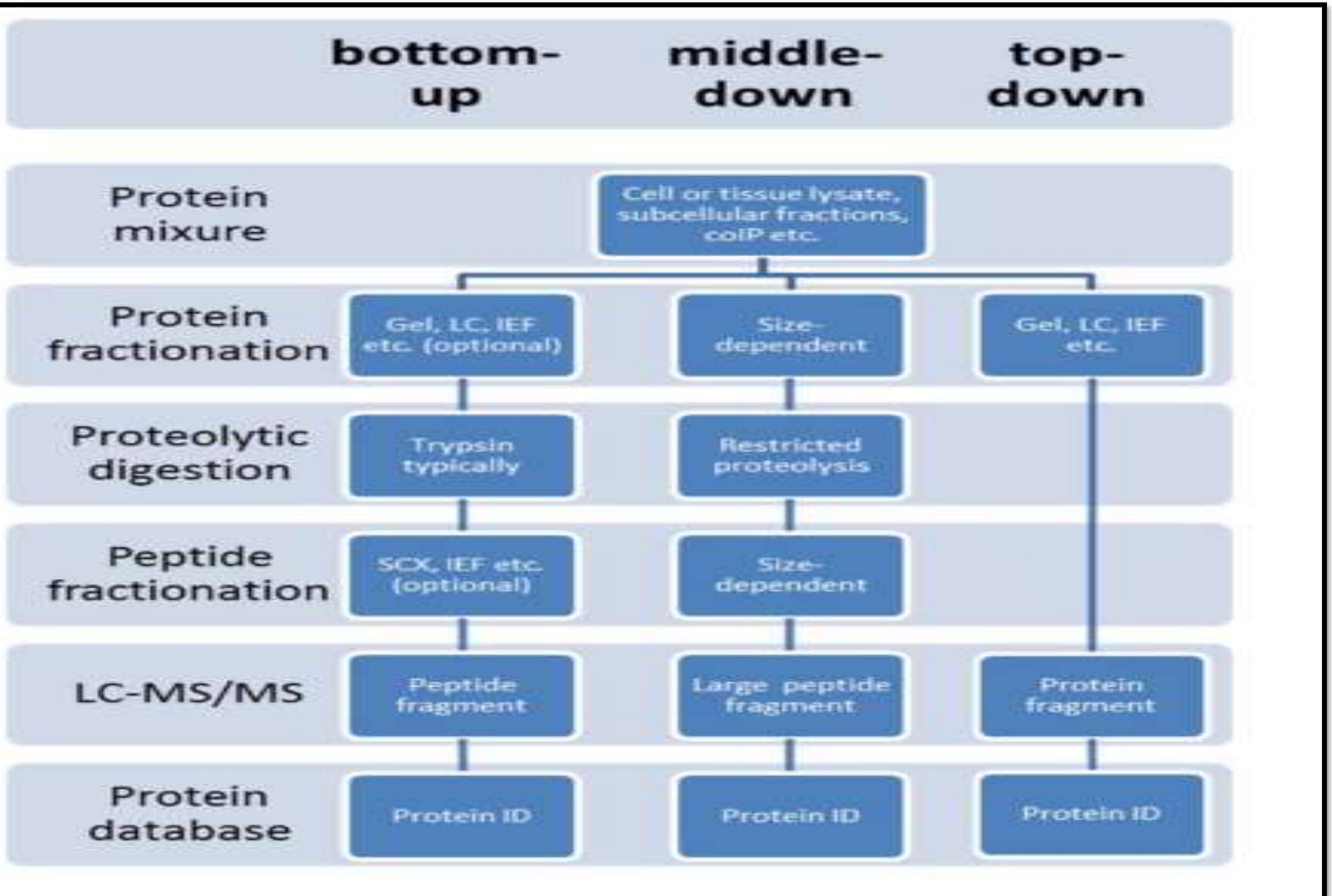
PROTEIN IDENTIFICATION

SHORT-GUN PROTEOMICS FOR PROTEOME PROFILE

MEMBRANE PROTEIN IDENTIFICATION BY SHOTGUN PROTEOMICS:
(HTTPS://WWW.YOUTUBE.COM/WATCH?V=BZCQKMG3DVM)

SHOTGUN PROTEOMICS/SHOTGUN PROTEIN IDENTIFICATION

- **Shotgun proteomics** allows global protein identification as well as the ability to systematically **profile** dynamic **proteomes**.
- It also avoids the modest separation efficiency and poor mass spectral sensitivity associated with intact protein analysis.
- Shotgun proteomics refers to a use of bottom-up proteomics techniques to study the whole proteins in a complex mixture, such as serum, urine, and cell lysates, etc.
- It utilizes the technology of high performance liquid chromatography (HPLC) in combination with mass spectrometry (MS) technology.
- The most distinctive feature of shotgun proteomics is that it enables identification and comparative quantification of a wide range of proteins at the same time with minimal protein separation needed.
- Protein mixtures are first digested by protease, and the resulting peptides are separated in HPLC, followed by tandem MS/MS analysis to identify the sequence of each peptide.
- The identified peptide sequences are compared with database, in searching for the corresponding protein identity.


bottom-up
middle-down
top-down
Protein mixure
Cell or tissue lysate, subcellular fractions, coIP etc.
Protein fractionation
Gel, LC, IEF etc. (optional)
Size-dependent
Gel, LC, IEF etc.
Proteolytic digestion
Trypsin typically
Restricted proteolysis
Peptide fractionation
SCX, IEF etc. (optional)
Size-dependent
LC-MS/MS
Peptide fragment
Large peptide fragment
Protein fragment
Protein database
Protein ID
Protein ID
Protein ID

FEATURES OF SHOTGUN PROTEOMICS

- Large scale and global protein identification and quantification
- Comparative analysis of different samples
- Ideal for discovery study of protein biomarker
- Dynamic proteomics profiling
- Identification and quantification of single proteins
- Identification and quantification of proteins in a complex
- Identification and quantification of post-translational modifications
- Protein-protein interaction analysis

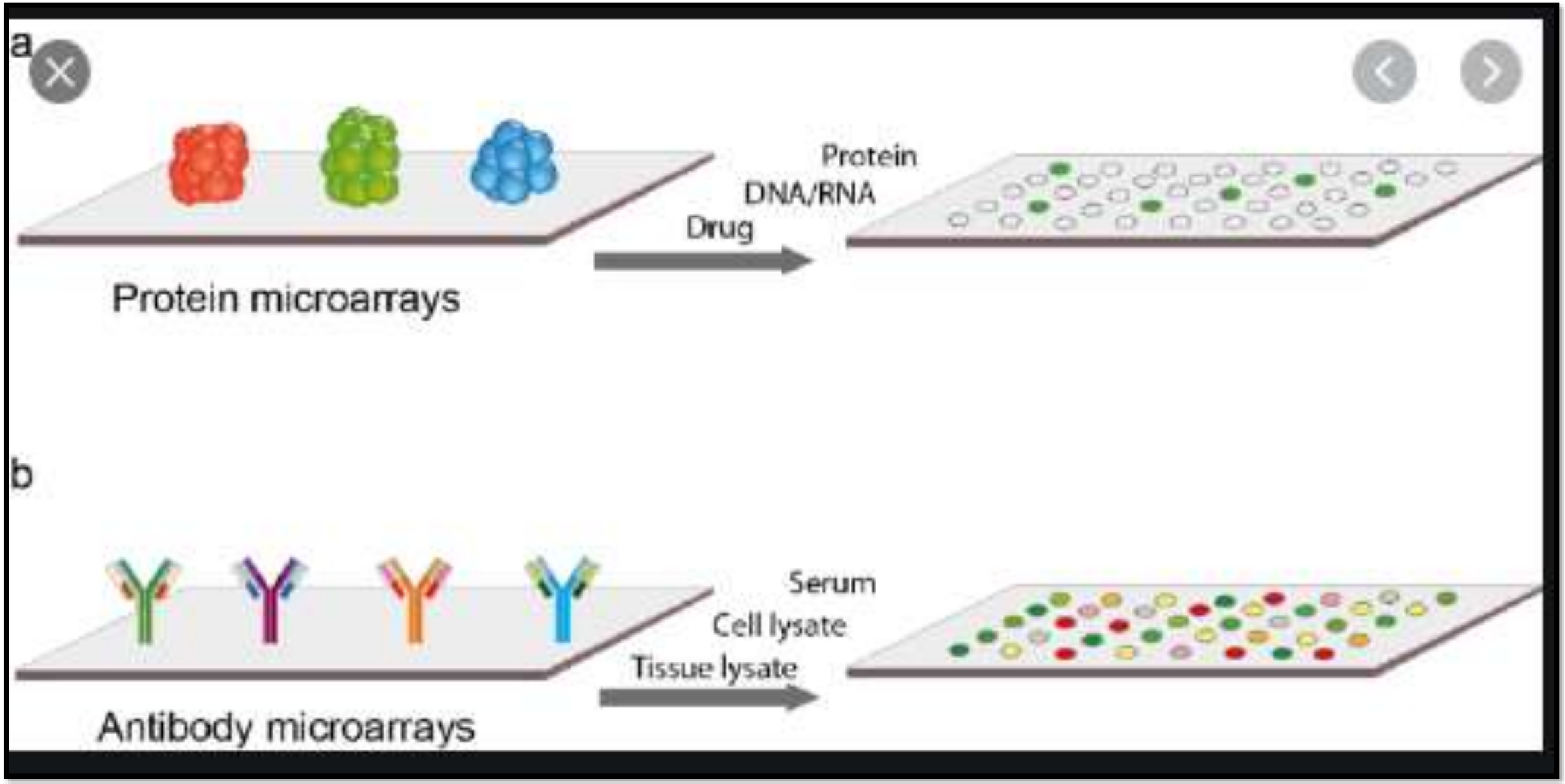
ADVANTAGE & DISADVANTAGE

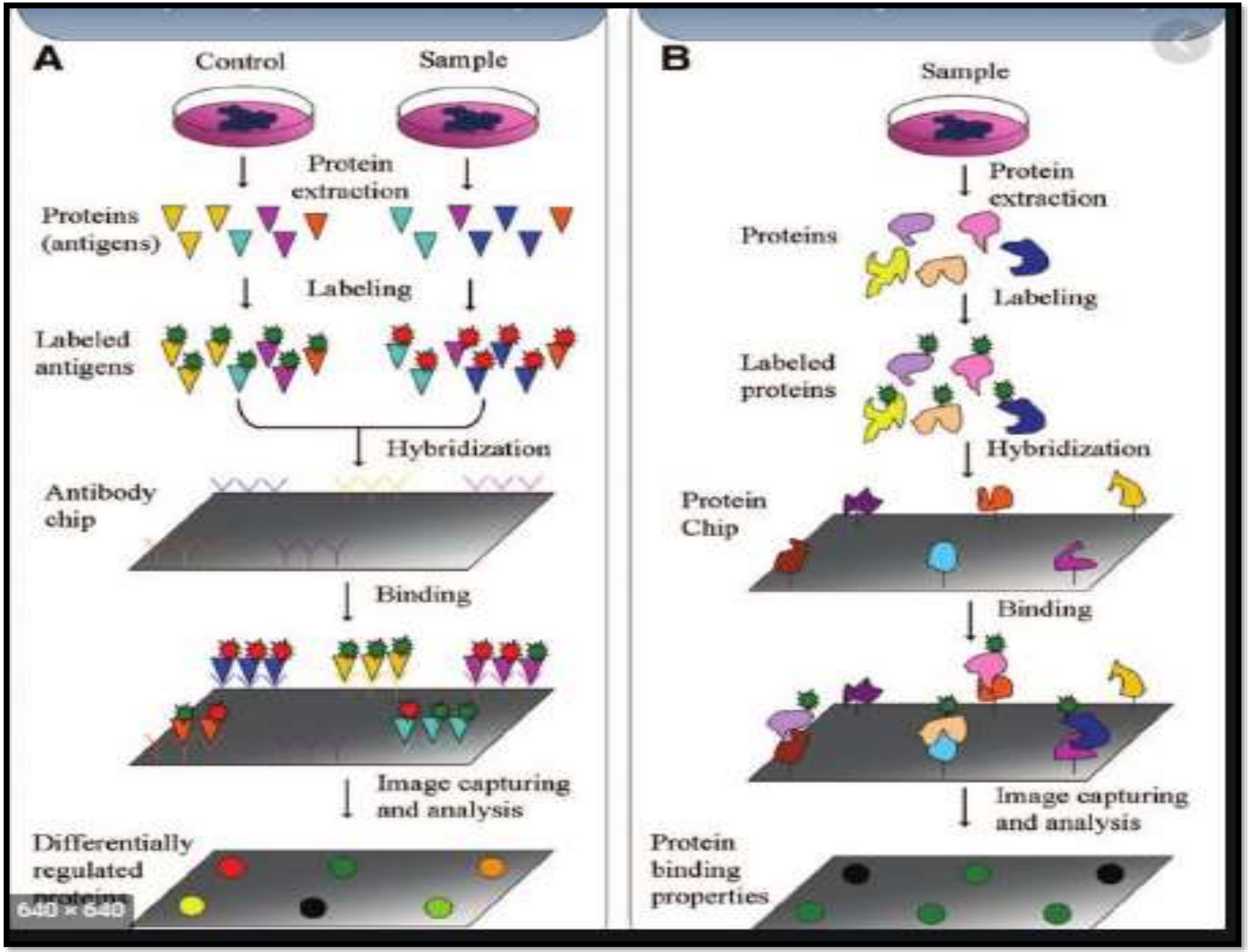
- Shotgun proteomics allows global protein identification as well as the ability to systematically profile dynamic proteomes.
- It also avoids the modest separation efficiency and poor mass spectral sensitivity associated with intact protein analysis

- The dynamic exclusion filtering that is often used in shotgun proteomics maximizes the number of identified proteins at the expense of random sampling.
- This problem may be exacerbated by the under sampling inherent in shotgun proteomics

PROTEIN IDENTIFICATION

PROTEIN MICROARRAY/PROTEIN CHIP/PROTEIN ARRAY


a
Protein
DNA/RNA
Drug
Protein microarrays
b
Serum
Cell lysate
Tissue lysate
Antibody microarrays


A
Control
Sample
Protein extraction
Proteins (antigens)
Labeling
Labeled antigens
Hybridization
Antibody chip
Binding
Image capturing and analysis
Differentially regulated proteins
B
Sample
Protein extraction
Proteins
Labeling
Labeled proteins
Hybridization
Protein Chip
Binding
Image capturing and analysis
Protein binding properties

PROTEIN MICROARRAY

- Protein Microarray Part 1: Introduction, Probes on Chip, sources of probes and detection system: https://www.youtube.com/watch?v=gfTcp3UuNlc

- Protein Microarray Part 2; Types of Protein Microarray, Applications and challenges: https://www.youtube.com/watch?v=AasUsFG8pmI

TOOLS AVAILABLE AT EXPASY PROTEOMICS SERVER

- http://www.pdg.cnb.uam.es/cursos/Leon_2003/pages/visualizacion/programas_manuales/spdbv_userguide/us.expasy.org/tools/index.html

- Compute pI/Mw **Tool**
- ProtParam **Tool**
- PeptideMass
- Peptide Cutter
- ProtScale

- ETC……

THANK YOU