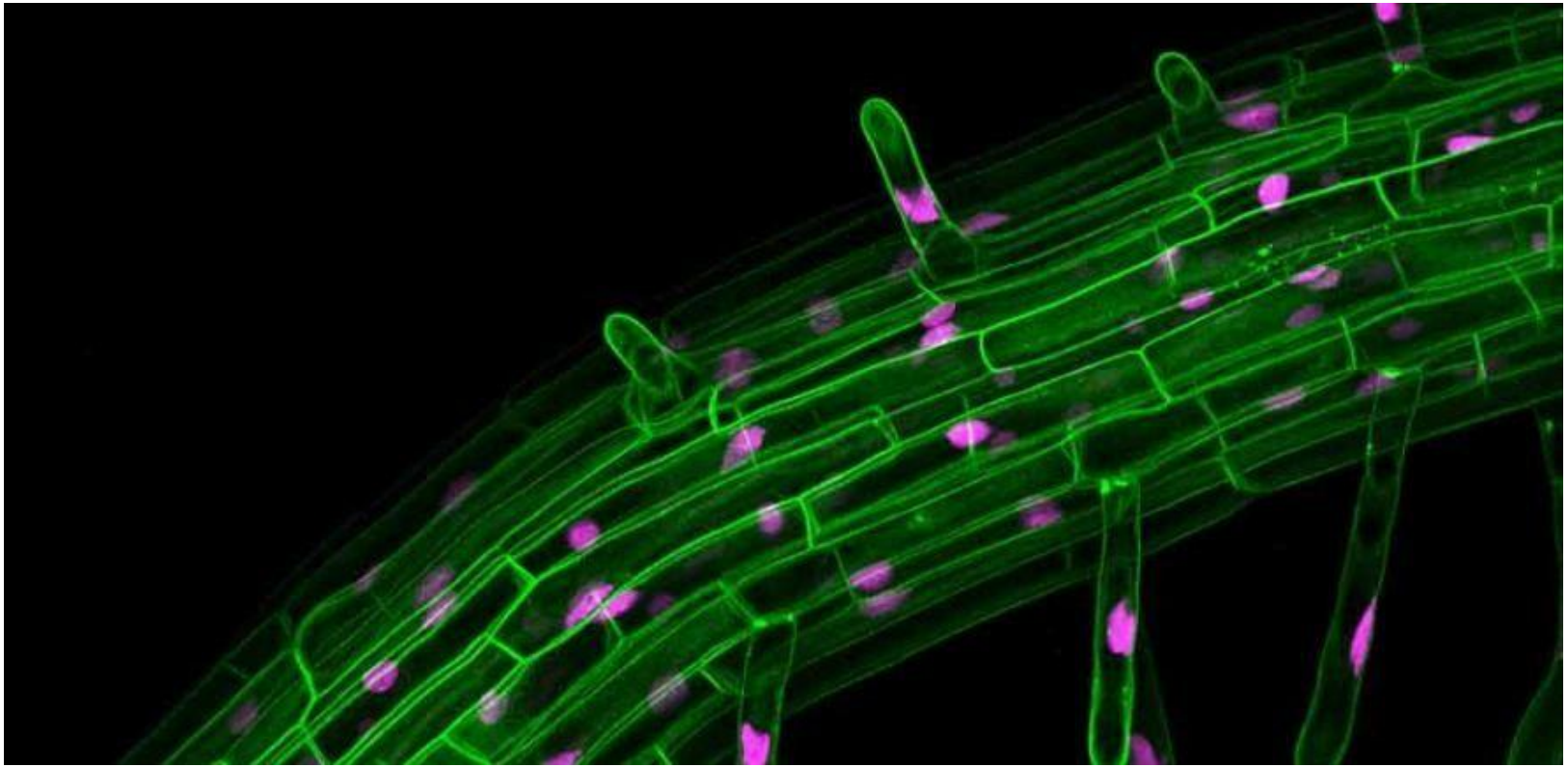
Review: Regulatory control of protein activity

You are studying the ability for a new drug to affect the activity of a certain enzyme:

- You note that your drug binds to a different site than the substrate does; how would you classify this drug?
- If you add an excess amount of substrate in the presence of your drug, would you expect the reaction rate to return to maximum speed of product formation? Why?
- Describe one change on a structural level that could be happening to the enzyme-substrate complex when your drug is bound.



Chapter 4 Part 2: Characterizing and Identifying Proteins in Scientific Research

Dr. Matthew Ellis

Learning Objectives for Chapter 4 Part 2:

Upon completing this module, **you should be able to**:

1) Understand ways in which we use antibodies to study proteins (immunofluorescence and flow cytometry)

2) Describe the process of isolating subcellular components and purifying specific proteins

3) Understand the different techniques used to study protein expression (western blotting) and determine protein structure (x-ray crystallography and cryo-EM)

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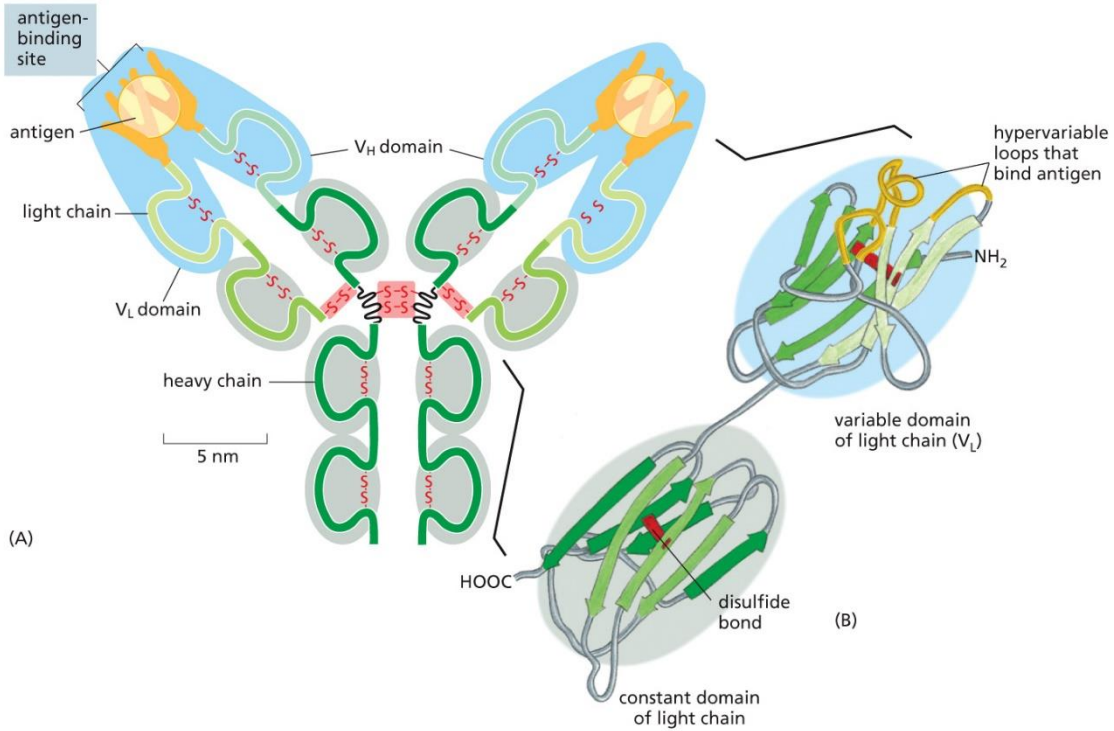
Antibodies are proteins that defend us against infection

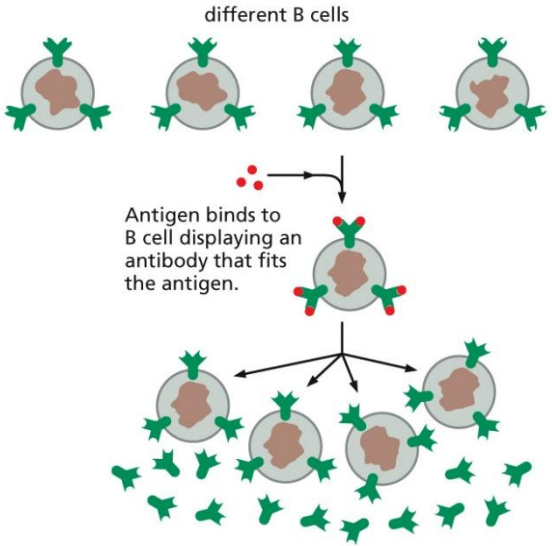
- Antibodies bind tightly to specific target molecules (*antigens*) to inactivate them or target them for destruction
- Comprised of 4 polypeptide chains (2 heavy and 2 light) all joined by disulfide bonds
  - What would we call this level of protein structure?
- Each has two identical antigen binding sites that can bind a small area on the surface of an antigen
  - Formed from several loops of polypeptide chains which can be *highly variable* and thus able to bind different antigens



Antibodies recognize very specific antigen targets

B CELLS PRODUCE ANTIBODIES

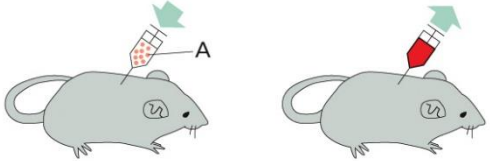
Antibodies are made by a class of white blood cells called B lymphocytes, or B cells. Each resting B cell carries a different membrane-bound antibody molecule on its surface that serves as a receptor for recognizing a specific antigen. When antigen binds to this receptor, the B cell is stimulated to divide and to secrete large amounts of the same antibody in a soluble form.



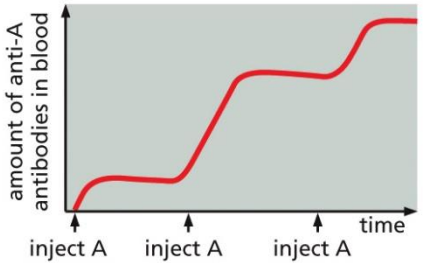
The B cell is stimulated both to proliferate and to make and secrete more of the same antibody.

RAISING ANTIBODIES IN ANIMALS

Antibodies can be made in the laboratory by injecting an animal (usually a mouse, rabbit, sheep, or goat) with antigen A.



Repeated injections of the same antigen at intervals of several weeks stimulate specific B cells to secrete large amounts of anti-A antibodies into the bloodstream.

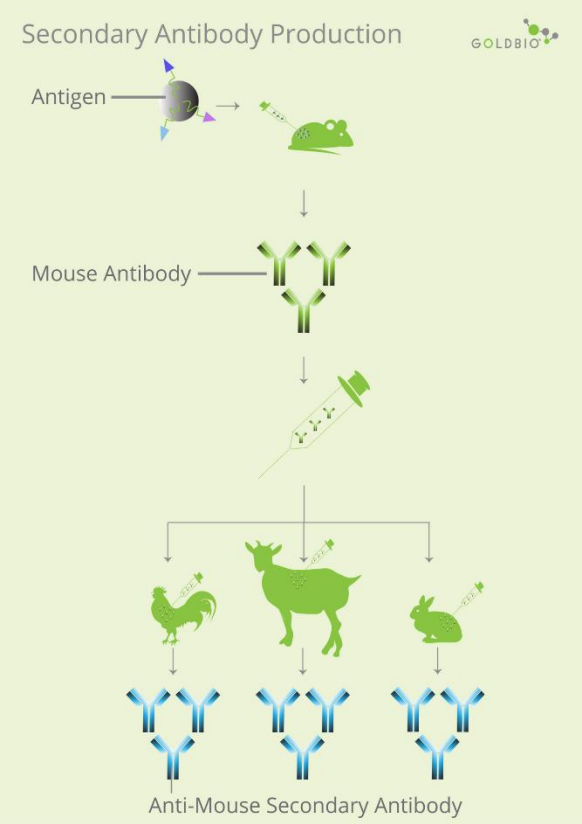


Because many different B cells are stimulated by antigen A, the blood will contain a variety of anti-A antibodies, each of which binds A in a slightly different way.

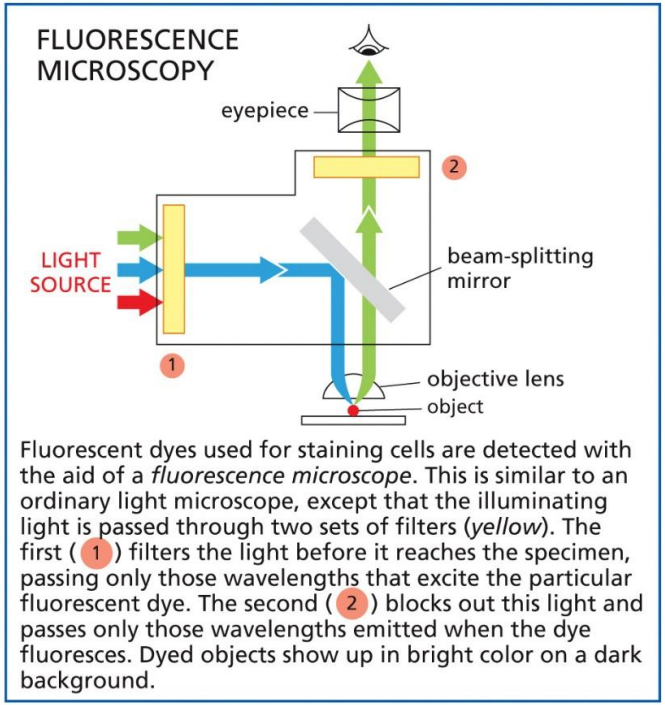
Primary and Secondary Antibodies

- *Primary antibodies* are produced in an animal host and recognize/bind to one specific antigen

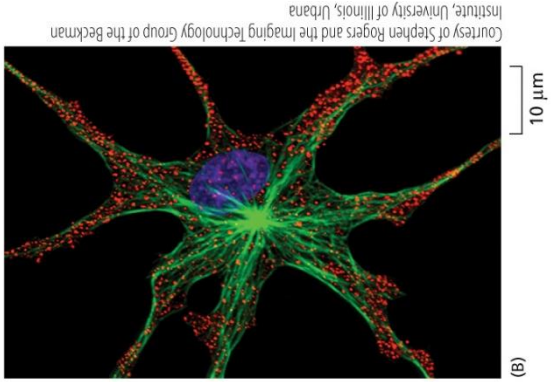
- *Secondary antibodies* are produced by injecting an antibody produced from the host into a completely different host species.
  - These antibodies are used in indirect immunofluorescence assays to recognize and bind to the injected antibodies



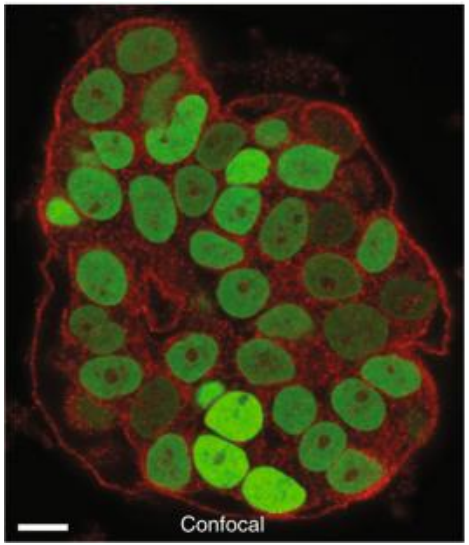
Recall: Fluorescence microscopy allows us to visualize protein subcellular localization using fluorescently tagged antibodies



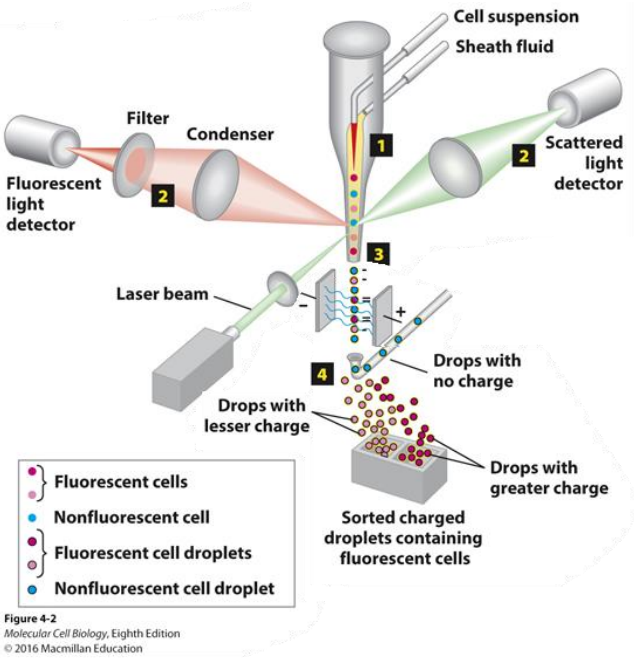
Fluorescent dyes used for staining cells are detected with the aid of a *fluorescence microscope*. This is similar to an ordinary light microscope, except that the illuminating light is passed through two sets of filters (*yellow*). The first (1) filters the light before it reaches the specimen, passing only those wavelengths that excite the particular fluorescent dye. The second (2) blocks out this light and passes only those wavelengths emitted when the dye fluoresces. Dyed objects show up in bright color on a dark background.

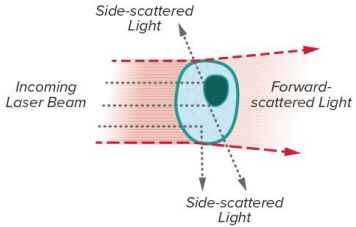


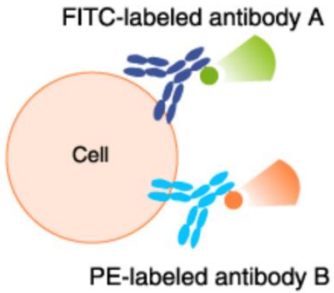
Courtesy of Stephen Rogers and the Imaging Technology Group of the Beckman Institute, University of Illinois, Urbana

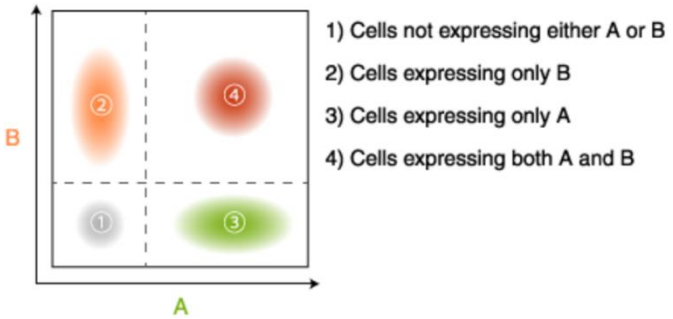


Flow cytometry









Flow cytometry uses fluorescent lasers and detectors to *rapidly* analyze/quantitate cells based on fluorescently-conjugated antibodies that bind to specific antigens.

Fluorescence-activated cell sorting (FACS) is a form of flow cytometry that physically sorts the cells based on specific antigens

Example flow cytometry data from a publication

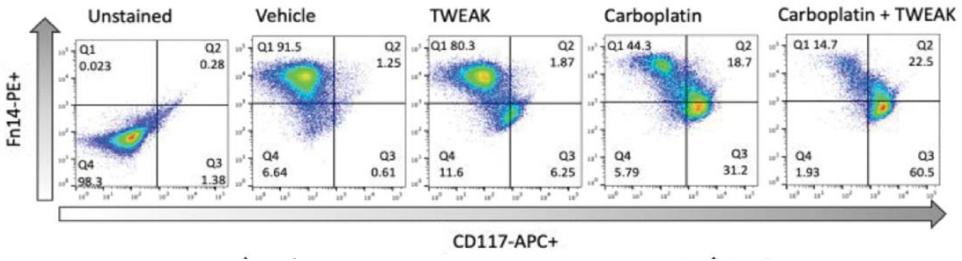
Each dot represents a cell:

1) What is the Y-axis Fn14-PE+? How about the X-axis CD117-APC+?

2) What do the different quadrants represent?

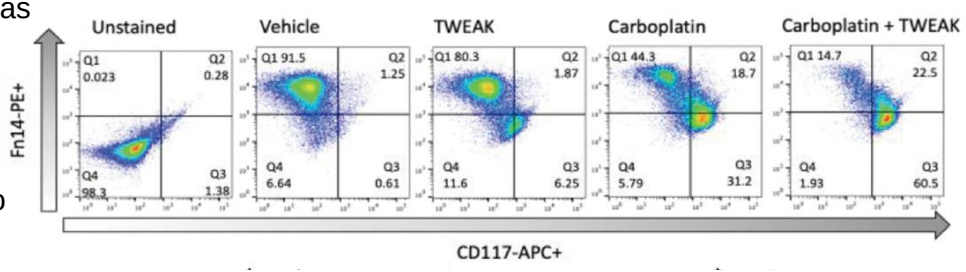
3) What are the labels across the top?

4) What might be some controls in this study?



Example flow cytometry data from a publication

1) What is the Y-axis Fn14-PE+? How about the X-axis CD117-APC+?
   - These are the cell surface markers, or proteins, that we are searching our mixed cell population for, the PE and APC are the fluorescent probes used to specifically identify these markers
2) What do the different quadrants represent?
   - The relative expression of Fn14 and CD117, e.g., Q4 has limited expression of either while Q2 has strong expression of both
3) What are the labels across the top?
   - These are the different treatment conditions prior to running the flow experiment (i.e., whatever was done to the cells prior to determining the relative expression of the markers of interest)
4) What might be some controls in this study?
   - *Unstained* is a technical control for the flow machine to make sure it is not picking up fluorescent background signals that do not exist. *Vehicle* is a solution control condition for the other three drug treatments, for example, if carboplatin was dissolved in an ethanol solution prior to adding it to the cells, the vehicle would be ethanol, and we need to test ethanol alone to ensure our observed effects are not due to the solvent alone



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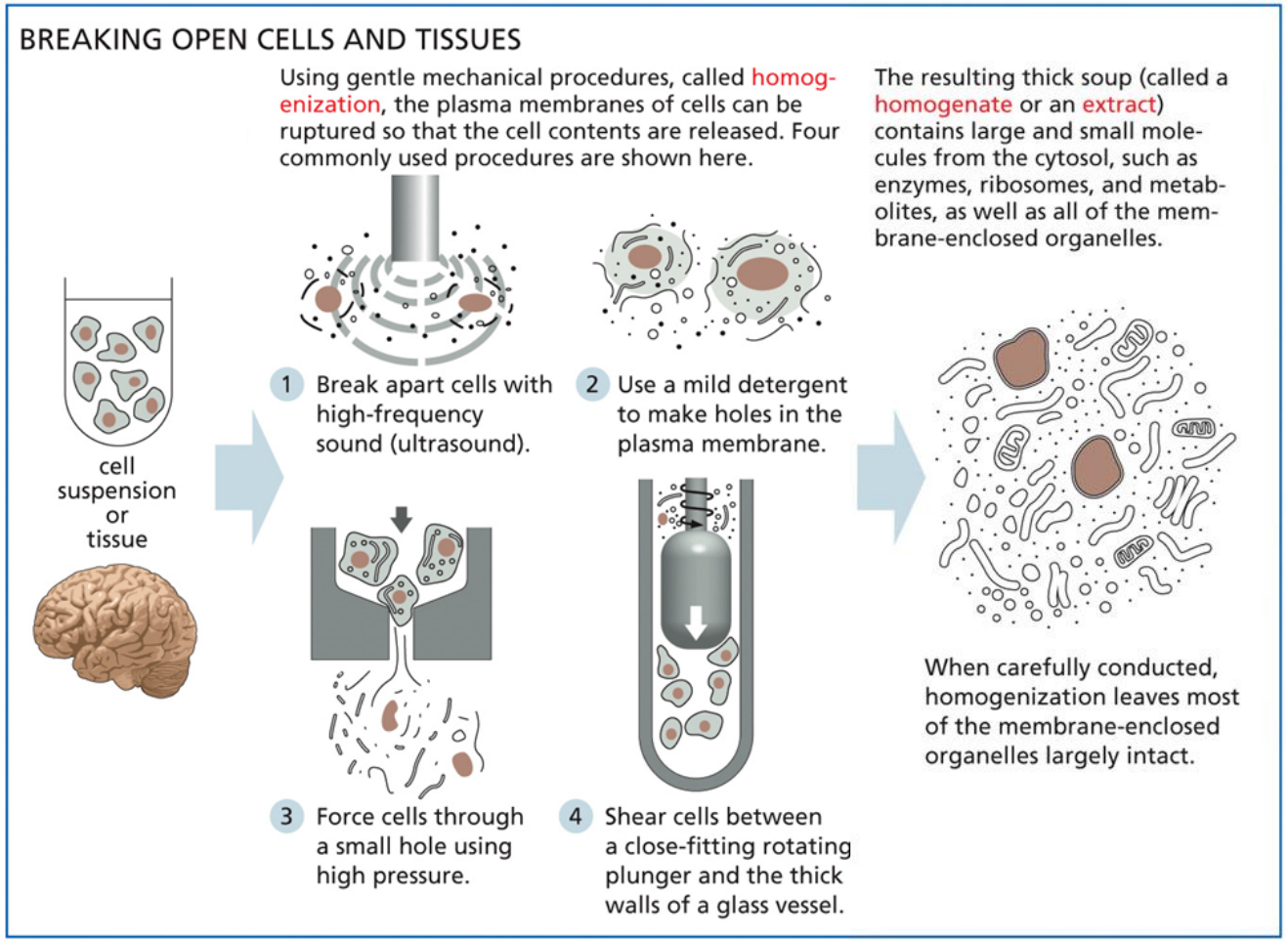
1) Understand ways in which we use antibodies to study proteins (immunofluorescence and flow cytometry)

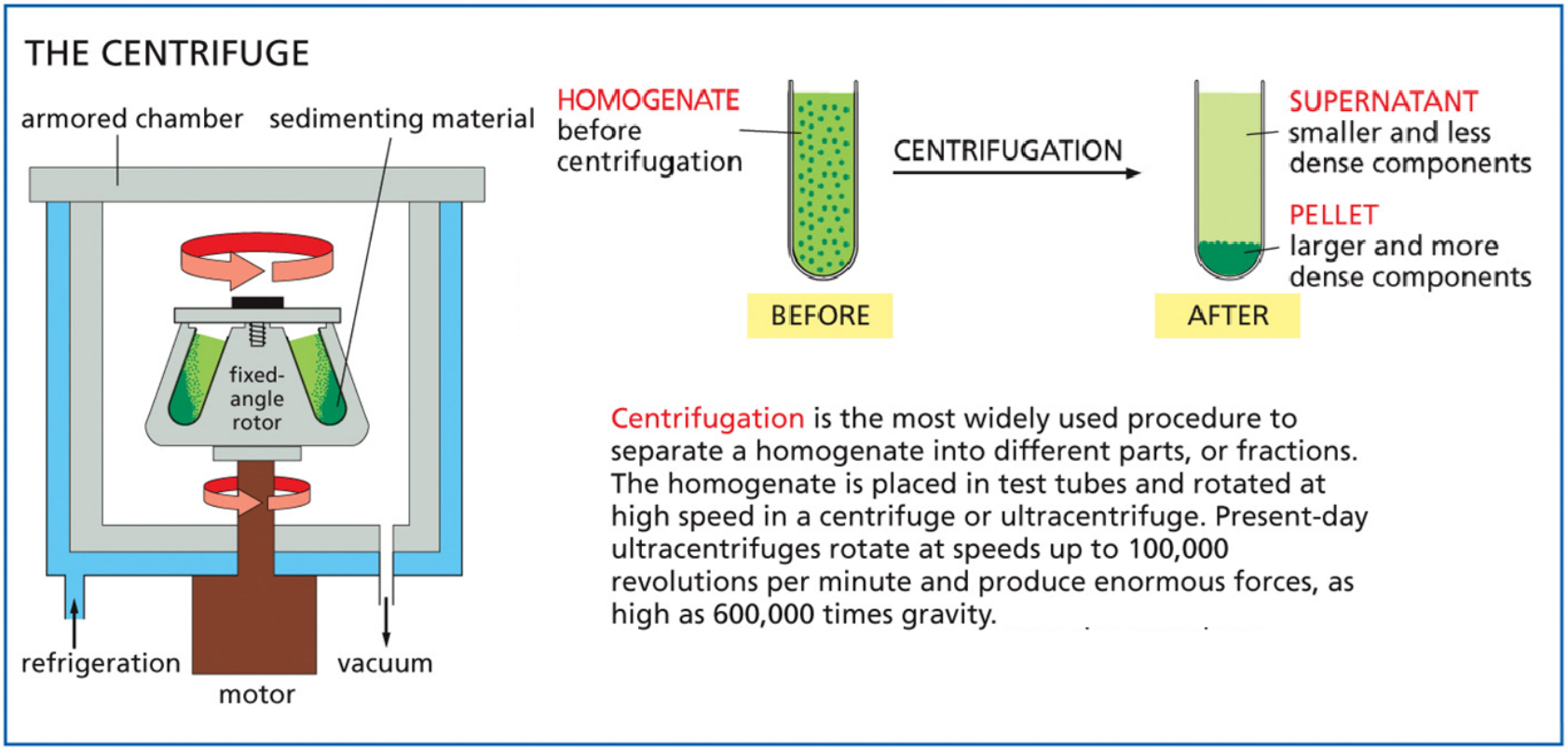
2) Describe the process of isolating subcellular components and purifying specific proteins

3) Understand the different techniques used to study protein expression (western blotting) and determine protein structure (x-ray crystallography and cryo-EM)

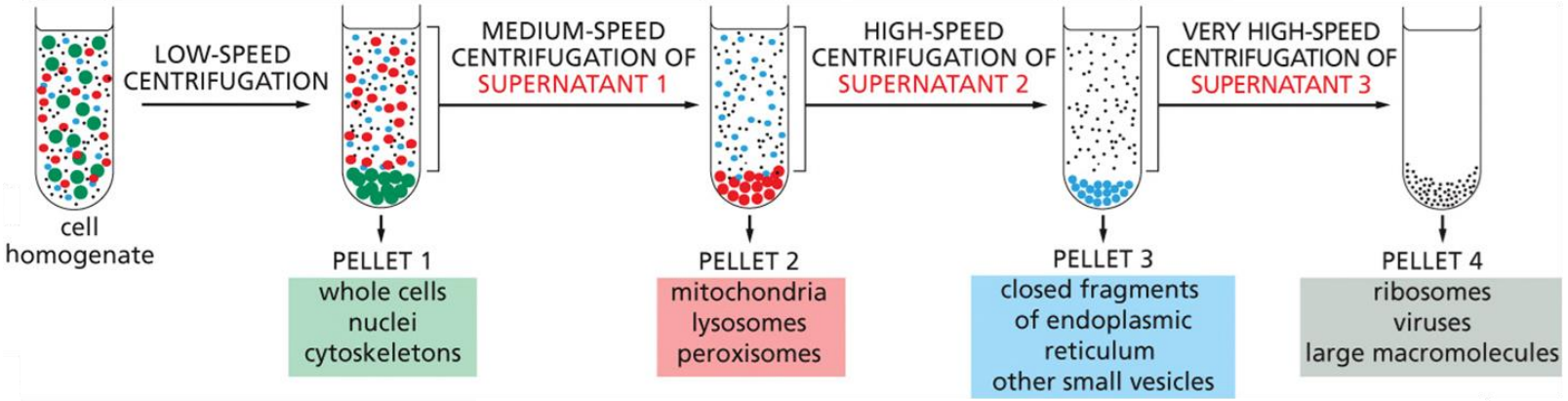
To access the internal components of our cells, we must break them apart



We can separate cellular components through centrifugation



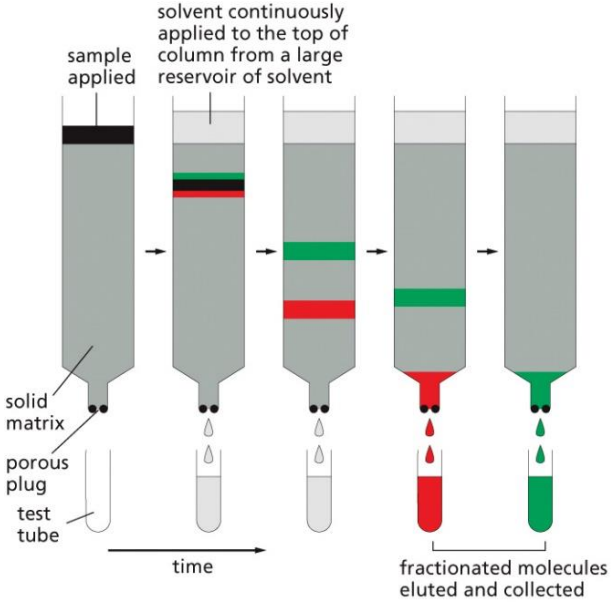
Differential centrifugation (repeated centrifugations at increasing speed) can separate cellular components by size

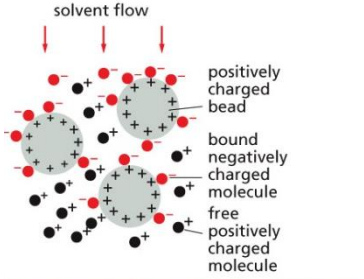


Column Chromatography: Purifying Proteins

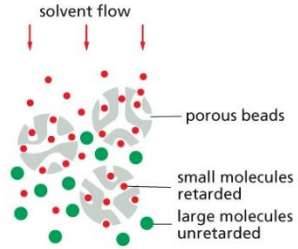
COLUMN CHROMATOGRAPHY

Proteins are often fractionated by column chromatography. A mixture of proteins in solution is applied to the top of a cylindrical column filled with a permeable solid matrix immersed in solvent. A large amount of solvent is then pumped through the column. Because different proteins are retarded to different extents by their interaction with the matrix, they can be collected separately as they flow out from the bottom. According to the choice of matrix, proteins can be separated according to their charge, hydrophobicity, size, or ability to bind to particular chemical groups (see below).

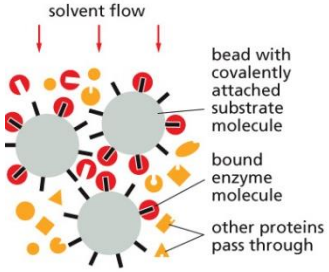




(A) ION-EXCHANGE CHROMATOGRAPHY



(B) GEL-FILTRATION CHROMATOGRAPHY



(C) AFFINITY CHROMATOGRAPHY

Column is filled with charged beads to collect proteins of a specific charge

Column is filled with porous beads to collect proteins of a specific size

Column is filled with antibody bound beads to collect specific proteins

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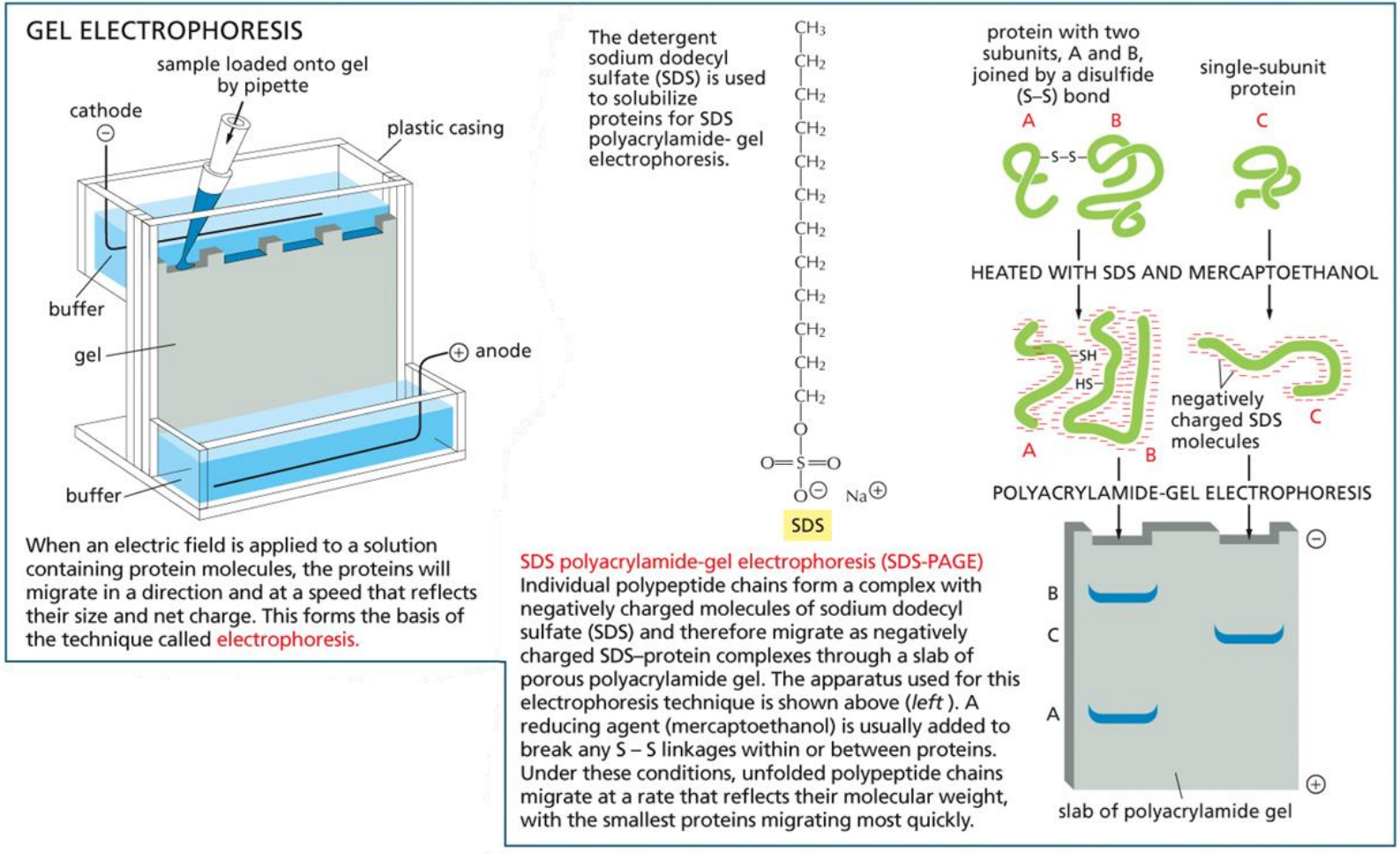
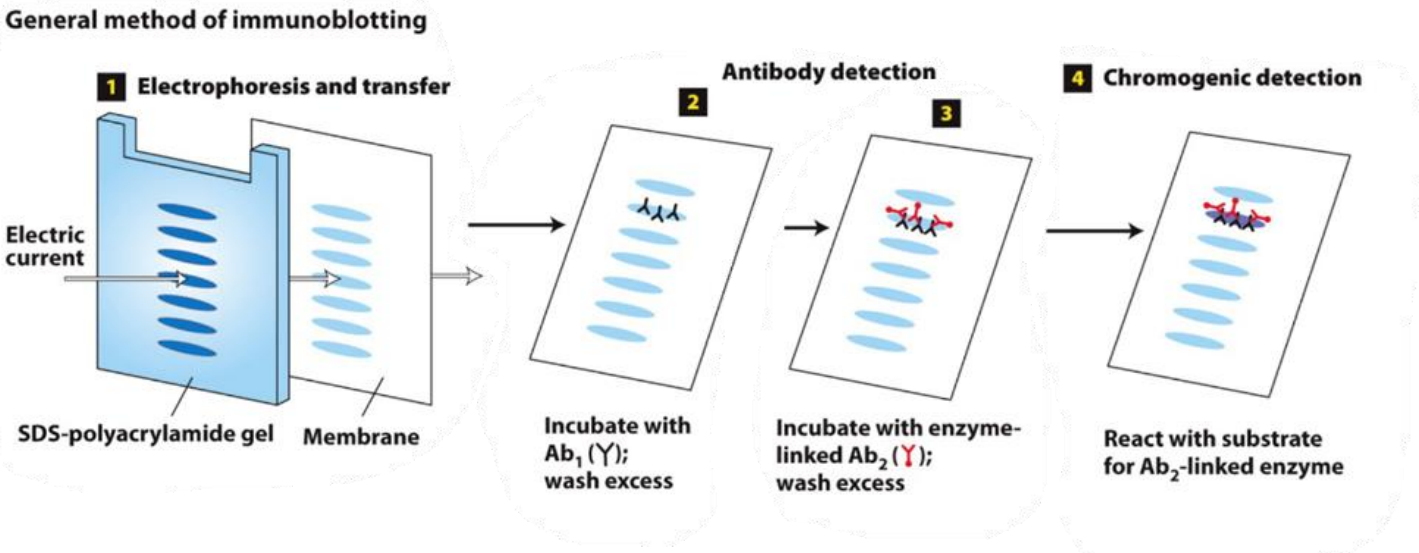
Gel electrophoresis: Separating proteins by size



Western Blotting: Using antibodies to quantify levels of protein being *expressed* (how much is present in the cell/tissue)

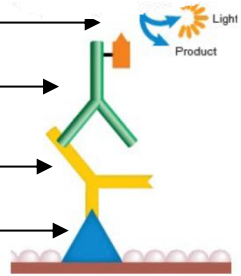


Chemical detection of intensity of antibody signal

Secondary antibody recognizing primary antibody

Primary antibody recognizing protein

Specific protein on membrane

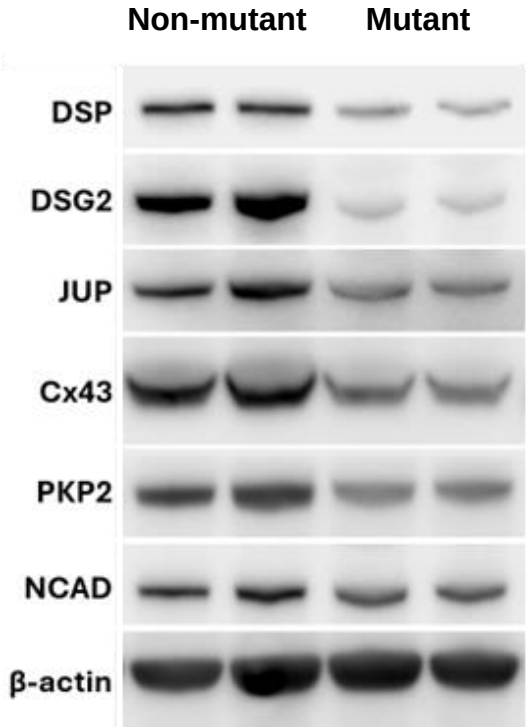


Example western blot from my research

1) What do DSP, DSG2, JUP, etc., represent? How did we specifically find these within our collected cells?

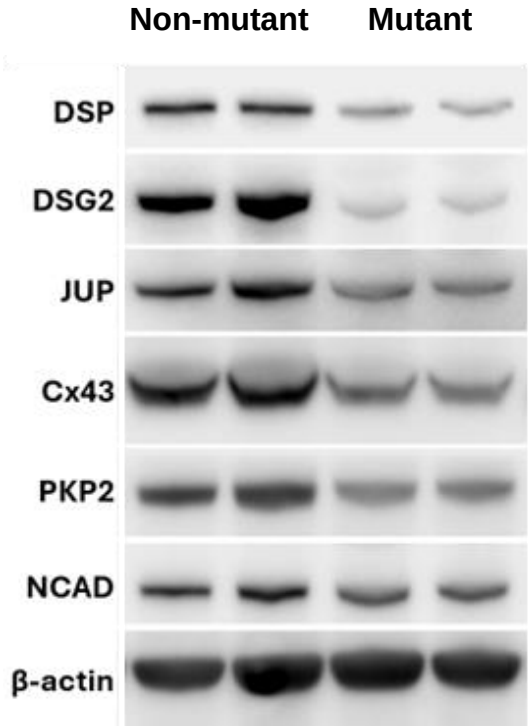
2) What are the differences between the non-mutant and mutant lanes? What might this mean?

3) What do you notice about the row labelled β-actin? This is a “housekeeping gene” or “loading control,” what might be the purpose of this row in western blotting?



Example western blot from my research

1) What do DSP, DSG2, JUP, etc., represent? How did we specifically find these within our collected cells?
   - Each of these represents a different protein of interest within our cell homogenate/extract. We locate these ones specifically out of all proteins attached to our membrane by using specific targeting antibodies.
2) What are the differences between the non-mutant and mutant lanes? What might this mean?
   - The mutant lane appears to show reduced expression of these proteins of interest in almost every row. This likely means that something to do with the mutant cells is affecting their ability to produce these specific proteins in abundance and can be a focus of further research.
3) What do you notice about the row labelled β-actin? This is a “housekeeping gene” or “loading control,” what might be the purpose of this row in western blotting?
   - This row shows highly comparable protein expression between the mutant and non-mutant cells. A housekeeping gene is one that is known to have relatively constant expression across a wide number of conditions and so it can be used as a loading control to keep researchers honest and know that the same amount of protein in total was loaded into each column, so we can accurately compare the expression levels of other proteins.



Mass Spectrometry separates peptide samples by size to determine protein identity

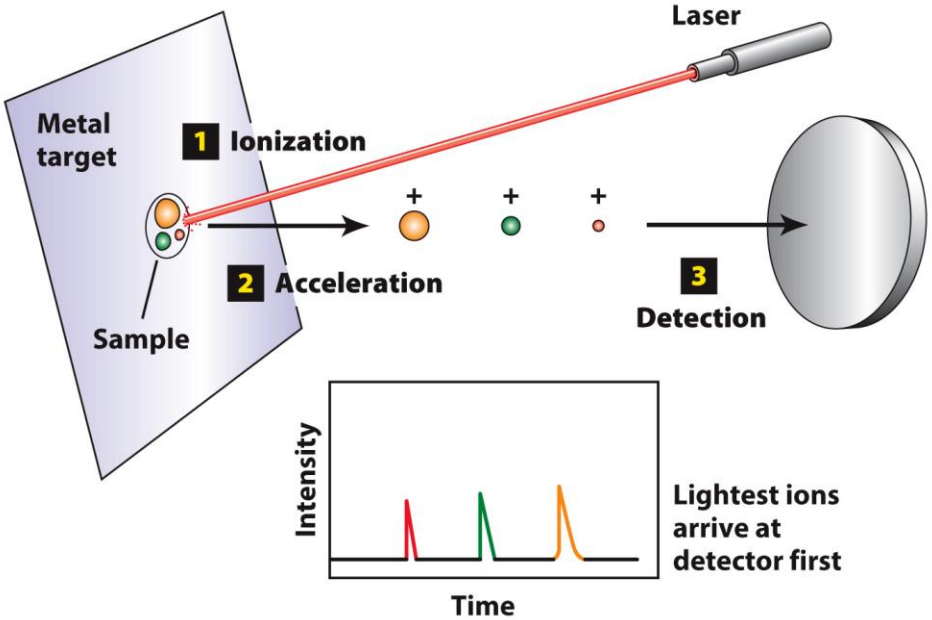


Figure 3-43
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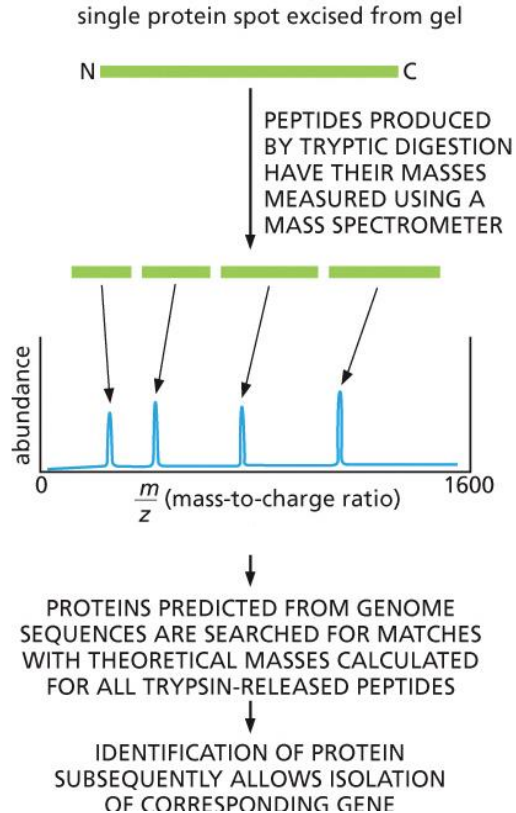

Proteomics: Can identify proteins in a complex biological sample – example organelle proteome

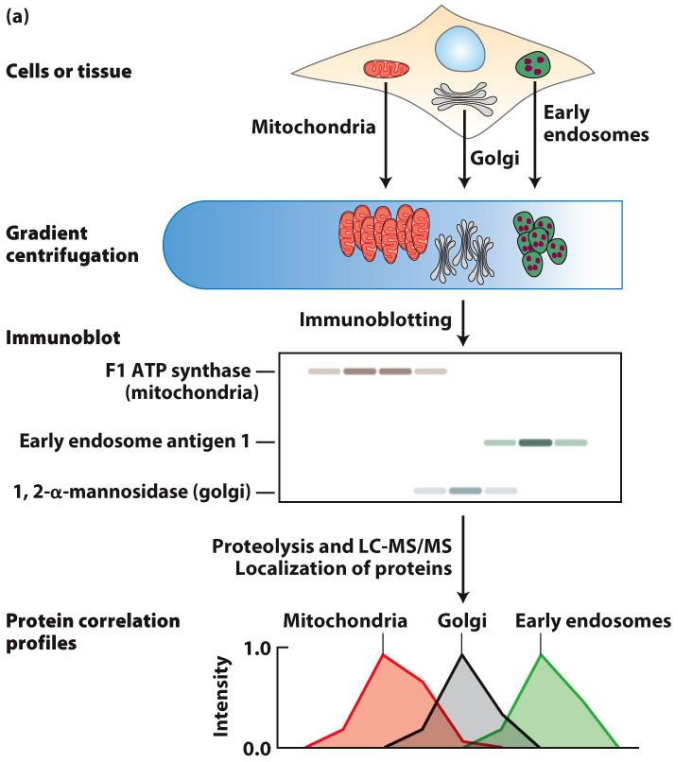

Figure 3-48
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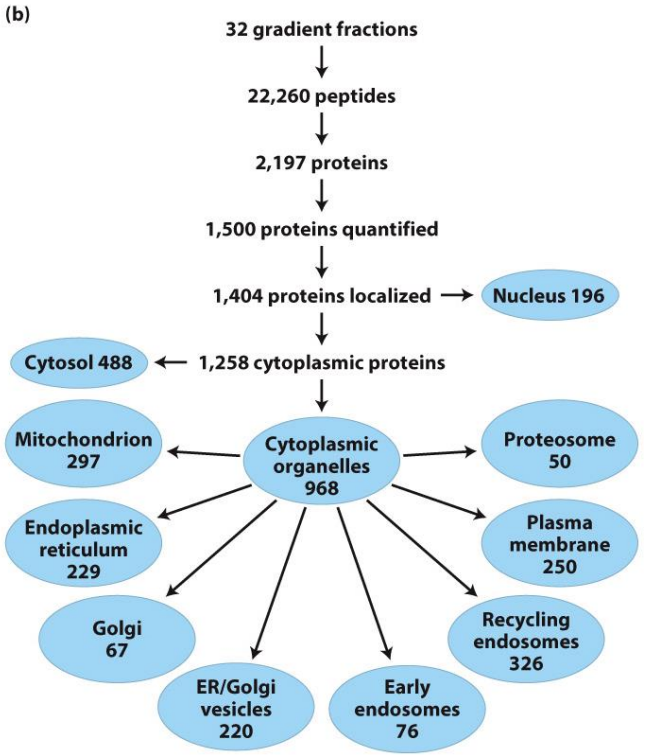
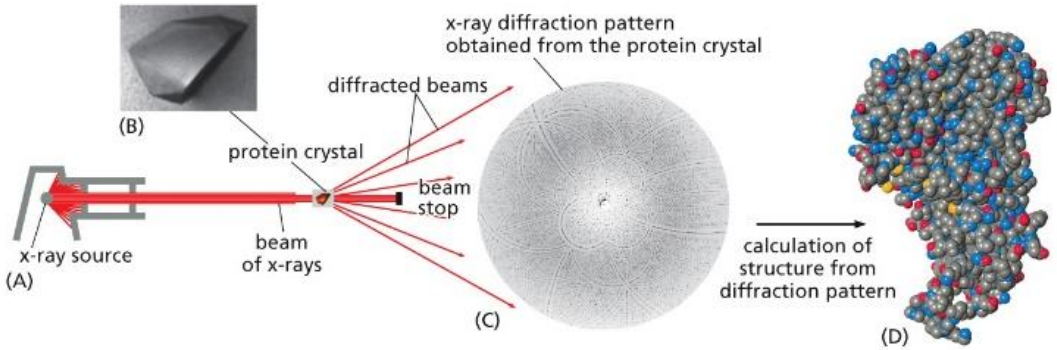


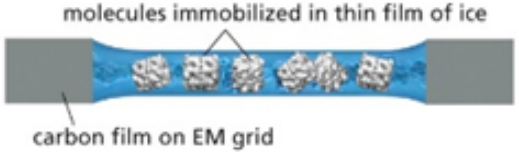
X-Ray Crystallography: Determination of protein structure

- Purified proteins are dehydrated to form crystals in which every protein has the same conformation in perfect alignment with neighbors
  - Time intensive trial-and-error process to determine correct conditions for crystal formation
- A beam of x-rays directed at the crystal produces a specific diffraction pattern due to wave interference
  - The position and intensity of each spot on this pattern contains information about the position of atoms in the protein crystal
- Computers are then used to render an image of the proteins structure at atomic level resolution

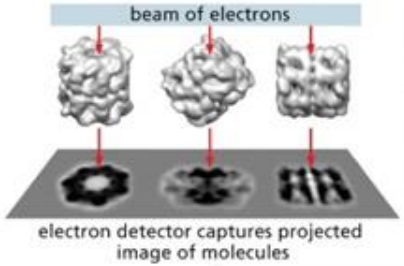


Cryo-Electron Microscopy offers high resolution structural information for proteins, especially those proving difficult to crystallize

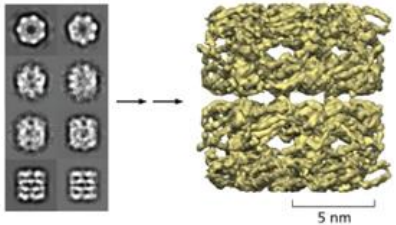
Snap freeze samples to immobilize purified protein of interest

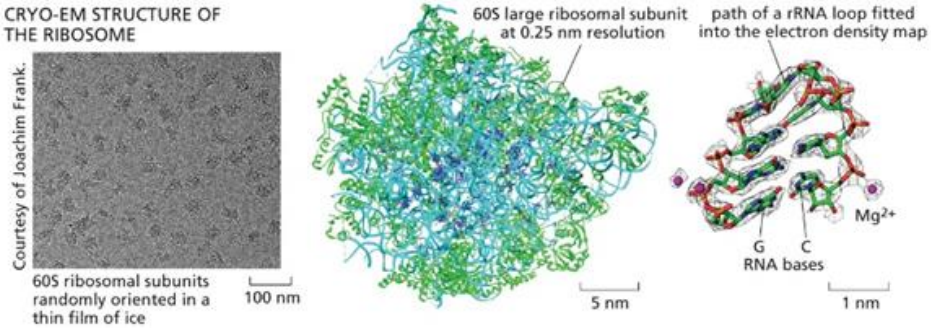


Shoot beam of electrons through sample to detect various conformational states of protein



Computational 3D rendering of overall protein shape and structure





Squarecap #1-3

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Feedback/Reflection

https://forms.gle/vbdZXee89Dh9diY78