Technical tips.
Session 2

Chromatography: substances are placed in a system consisting of two physically distinguishable components -a *mobile phase* and a *stationary phase*- and molecular species separate because they differ in their distribution between these two phases. The stationary phase is usually held either in a layer or in a column. Methods described in the two papers use columns.

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Gel filtration chromatography: The stationary phase is prepared in a *column*. It is made of tiny particles of an inert substance that contains small pores. If a solution containing molecules of various dimensions is passed through the column, molecules larger than the pores move only in the space between the particles and hence are not retarded by the column material. Particles smaller than the pores diffuse in and out the particles with a probability that increases with lower molecular size, so they are slowed down in their movement down the column. Molecules are eluted from the column in order of decreasing size or, if the shape is relatively constant (globular, rod-like), decreasing molecular weight. *Sephadex G-75* (dextran polymer made of glucose) usually fractionates proteins within a range between 3000-70,000 Da.

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V_0 (*void volume*) is the volume of solvent needed to elute a substance completely excluded by the gel (the bigger proteins will elute in this fxn).

Diethylaminoethyl (DEAE)-cellulose chromatography: It is an anionic exchange chromatography. The stationary phase consists of an "ion exchanger": solid matrix (cellulose) that has chemically bound charged groups (DEAE) to which ions are *electrostatically* bound. It can exchange these ions for ions in aqueous solution. Molecules are separated according to their charge and size.

Charged molecules adsorb to ion exchangers reversibly so that molecules can be bound or eluted by changing the ionic environment (mobile phase). Separation is accomplished in two stages:

1) The substances to be separated are bound to the exchanger using conditions that give stable and tight binding.
2) Column is eluted with buffers that have different pH, ionic strength or composition, so that the components of the buffer compete with the bound material for the binding site.

An "anion exchanger" is positively charged and exchanges anions in the mobile phase. That means that proteins negatively charged can bind the exchanger and can usually be eluted in conditions such as a decreasing pH or an increase in ionic strength (i.e. increasing concentration of ions in solution that will compete for interaction with both the protein and the exchanger).

Sodium dodecyl-sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE): In every electrophoresis, molecules are separated in the presence of an electric field. In a gel electrophoresis, the molecules are placed onto a matrix of porous polymer (gel) so they can run on it by virtue of their charge and molecular size, shape and weight.

In a SDS-PAGE electrophoresis (electrophoresis in 'denaturing conditions', method developed by Laemli UK, 1970), protein samples are previously treated in ESB ('electrophoresis sample buffer') containing SDS (detergent that not only disrupts non-covalent interactions inside the protein and between proteins but also remains bound to them) and mercaptoethanol (β-ME) or dithiotreithol (DTT) (to break disulfide bonds). Because the SDS will remain bound to the proteins, it provides all of them with a negative charge. Since the proteins are this way denatured, they will run in the gel in a linear uniform shape and an identical charge-to-mass ratio so they will be separated just according to their molecular weight size (this is because the amount of SDS bound per unit weight of protein is constant). They will run down to the positive (anode) pole.

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Protein iodination by chloramine-T: It is a classical method to radio-label proteins by using their tyrosines amino acid residues. Chloramine-T is able to catalyze the reaction shown below.

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Protein labeling and "pulse-chase" analysis: Nowadays, visualization of most radioactively-labeled proteins usually gets accomplished by the use of S-containing amino acids (Cys, Met) labeled with S^{34} isotope in the -SH group present in their side chain. These labeled-amino acids are usually added in vivo to the cells so that they incorporate them into newly-translated proteins. This is called a "pulse" and you can have the cells x time in the presence of S^{34}- aas. At some point, the addition of the radioactive amino acid is stopped by adding an excess of 'cold' (meaning, non-labeled) amino acids. Then you start taking your samples at different time-points after that (this is what is called a "chase" period). There are two essential methods that are used to enrich your sample in the protein/s of interest:

-**Precipitation with TCA (trichloroacetic acid):** it precipitates the total bulk of proteins by salt-formation. A TCA-soluble fraction can be separated from a TCA-precipitable fraction by treatment with cold (0 °C) TCA, then hot (90°C) TCA, then centrifugation. The pellet (precipitable fxn) can be resuspended by dissolving into NaOH and then neutralizing with HCl. You can also separate the precipitable and soluble TCA fxns, simply by treating with cold TCA (the method of separation depends on your particular sample).

-**Immunoprecipitation:** you can have antibodies specifically raised against your protein or you can also add *epitope tags* (small peptides such as -HA, -myc, -FLAG) from which antibodies specifically recognizing them, are commercially available. (The DNA encoding these tags is added in frame right before or right after the DNA-encoding sequence (ORF: open reading frame) of your protein of interest. This is usually performed with the gene encoding the protein previously inserted in a DNA vector (such as a plasmid)

and adding this additional sequence by insertion into a specific region of the DNA, by using restriction enzymes. PCR is normally used to amplify the DNA and to add the restriction enzyme sites to the specific regions to be modified).

The total protein sample is put in the presence of the antibody and the Ab will only bind your protein of interest. Then, tiny beads that specifically bind this Ab will be added. You will recover the Ab bound to the beads and also to your protein, by centrifugation. Then ESB buffer will be added to the beads to elute the protein bound to it and these samples will be then resolved by SDS-PAGE.

In a 'pulse-chase' analysis, proteins have been labeled with S^{34} so they can be visualized by drying the gel and carrying out fluorography (also called 'phosphorimaging' analysis (see below). But proteins that haven't been radiolabeled can also be visualized by *Western blot analysis* (which will be explained in further sessions).

Autorradiography: A sample containing a radioactive label (usually a membrane of nitrocellulose onto which you have transferred your proteins from a previously run electrophoresis gel) is put in direct contact with a thick layer of photographic emulsion (film). The film consists of a suspension of silver halide crystals in gelatine. Radioactive atoms decay in the sample and emit radiation. On exposure to light or radiation, the crystals on the film are "activated" so they can be chemically converted by reducing agents (present in the photographic developing liquids) into metallic silver. This shows as 'black spots' in the regions that were in contact with the radioactive material.

Fluorography and phosphorimaging analysis: The *fluorography* consists in the exposure of a film by secondary light that was generated by the excitation of a fluor or a screen by a β-particle or a γ-ray. The most generalized way to do this is by *phosphorimaging*. This consists in the use of plate/screens that are sensitive, as said, to light and radiochemical emissions. The screens are forced to an "excited" state upon exposure to the dried electrophoresis gel which contains the radioactively-labeled proteins. The *phosphorimager* exposes then the plate to a laser, causing the phosphor molecules to move back to a "relaxed' state by emitting light. This light is captured by a photo-multiplier tube inside the instrument and converted to an image similar to an X-ray photograph, by the computer.

Radiactivity measurement by scintillation counter: radioactivity can also be quantified by placing your sample (whole cells or TCA-precipitated proteins) into filters. These filters are dissolved inside tubes containing *scintillation liquid*. This is a solvent based on dioxane, toluene or p-xylene that contains a fluorescent substance (PPO or POPOP). The molecules in the solvent absorb the energy emitted by the radioactive sample and are raised to an excited state so when they return to ground state they emit a photon of light. Then, the fluorescent substrate absorbs these photons and re-emits photons at a longer wave-length. This will be detected by a photomultiplier attached to a computer which will quantify the amount of signal detected.
A similar approach is followed by **densitometry**.

ATP determination by luciferine-luciferase assay: The reagents for this reaction are commercially available and can detect the amount of ATP in a particular sample. The luciferase comes from Photinus pyralis (American firefly) and catalyzes this reaction:

$$ATP + \text{D-luciferin} + O_2 \rightarrow \text{oxyluciferin} + PPi + AMP + CO_2 + LIGHT$$

The light output is proportional to the concentration of ATP because you work in saturating amounts of the enzyme (Vmax). You first measure a standard with known concentrations of ATP; then you represent the luminescence units (detected in a fluorimeter) versus the ATP concentration and interpole your sample values into the standard slope. You have to work with concentrations that fall within a linear range (inside the linear part showed by the standard slope).

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Puromycin: causes the premature chain termination in protein translation because it resembles to the 3' end of aminoacyl-t-RNA.

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Chemical mutagenesis of DNA:
An example, treatment with <u>hydroxylamine</u> (NH_2OH)

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