HANDOUT

LECTURE-12

GEL-BASED PROTEOMICS

Slide 1:

In today's lecture we will discuss gel based proteomics. This methodology includes different types of techniques which can be used in different aspects of life science research. Regardless of the field studied, most labs use various gel-based approaches to separate proteins on a day-to-day basis.

Gel-based proteomics include different types of techniques such as Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis or SDS PAGE and 2 Dimensional Electrophoresis or 2DE.

Various advancements or variations in the 2DE technique have been developed. These include Difference in Gel Electrophoresis or DIGEand Blue Native PAGE or BN PAGE.

The gel-based proteomics module will cover different principles involved in operating each of these techniques and their applications. For example, the SDS PAGE can be used to separate the proteins based on their molecular weight,while 2DE separates proteins based on both isoelectric point and molecular weight in the 2 dimensions. The advanced form of 2DE (DIGE?) separates proteins in such a way that different types of artefacts and variations generated by 2DE can be eliminated/minimized. DIGE technology achieves this by the addition and mixing of different dyes.

This lecture on gel-based proteomics will introduce concepts of the different types of electrophoresis involved in 1DE & 2DE. The description of 2DE will also include a workflow of the steps involved.

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This module will cover the following areas, providing workflow, animations and finally laboratory demonstrations to clarify concepts.

- Gel-based proteomics,
- Applications of various electrophoresis techniques including 1DE or SDS PAGE for separation of simple protein mixtures and 2-DE to separate complex protein mixtures on the basis of 2 distinct physical properties.

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Different steps are involved in the analysis of the proteome. As discussed in the previous lecture, separation of proteins in the sample represents the first crucial step. This first requires very good protein preparation. The choices for protein separation then include 1DE, 2DE or various chromatographic methods. In other words, in proteomics, various options are available for protein analysismdepending on your applications, the biological questions to be posedand the information desired to be obtained from that particular technique. For example, SDS-PAGE would be sufficient if one just wishes to verify protein purity. Subunit compositions can be determined using a comparison of results obtained through SDS PAGE and native PAGE. If the goal is to resolve individual proteins from a complex mixture (such as a tissue or whole cell lysate) that contains 1000s of individual proteins, different pre-fractionation methods involving various chromatographic techniques as well as various advanced electrophorectic methods such as 2DE/DIGE can be used. Additionally, different mass spectrophotometric and microarray technologies can also be applied, these will be discussed at a later point.

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Gel-based proteomics involve different types of separation techniques including sodium dodecyl sulfate polyacrylamide gel electrophoresis or SDS-PAGE, 2 dimensional gel

electrophoresis or 2DE, the fluorescence 2-D difference in gel electrophoresis or DIGE, Blue native PAGE or BN-PAGE.

However, this is not a very descriptive list and these represent only a few important techniques available for analysis of proteome.

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Various methods have been developed to study the structure and functions of proteins. Electrophoresis, an important member of this cohort, is based on the principle of migration of charged proteins in a given electric field.

SDS-PAGE or different type of electrophoretic methods represent gold standards as they have the ability to provide information on protein structure and properties. Electrophoresis represents a powerful technique to separate and then visualize proteins by different types of staining methods.

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Historically, electrophoresis was invented by Professor Tiselius in 1930. He developed the moving boundary method to study electrophoresis of proteins. Since then a lot of development has occurred in this field and during the 1950s and 60s this process was widely adopted in different laboratories.

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See above Dr. Tiselius whois referred to as 'The father of electrophoresis'. He was awarded the Nobel prize in 1948 for his great contribution to protein chemistry.

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The following section will cover 1DE and the concepts involved.

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1DE involves separation based on the charge-to-mass ratio and the molecular weight of the protein. Therefore on a given gel smaller proteins in the electric field will move further down compared to the higher molecular weight proteins which remain on top.The application of some standard molecular weight markers then provides a reference range for the determination of the molecular weight of a sample protein separated by this process.

Commonly applied 1D techniques include SDS PAGE to study proteins under denaturing conditions) or Native PAGE, which permits study of the protein in its native conformation.

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Electrophoretic techniques can be used to study different properties, in the characterization of proteins. It can shed light on protein subunit composition, the molecular weight of subunits or the whole native proteins and different types of post-translational modifications. Again, only one of the techniques may not be sufficient to answer all of these questions, hence necessitating the involvement of more than one type of electrophoretic method. For example, while comparing native and subunit composition forms both Native- and SDS-PAGE are required. On the other hand, SDS-PAGE is useful for study of post-translational modifications. 2 DE is useful if the goal is protein separation based on isoelectric points, or to separate 2 isoforms or differentially post-translationally modified forms. The take home message is that knowledge of concepts and methodologies of these techniques is necessary to apply them correctly in different experimental settings.

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While 1-DE is widely used and can address can address a limited number of applications (such as separation of a simple protein mixture), it has various limitations. It is:

- Not useful to study whole proteomes
- Not ideal to separate complex protein mixtures
- Not useful to analyse complex fluids such as serum or cell lysates.

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To overcome these limitations and to obtain better resolution of protein separation 2-DE was developed.

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The technique of 2-DE has been in wide use since the 1970s. In 1975,2 scientists, Klose and O'Farrell achieved success in applying 2-DE in protein separation based on both molecular weight and the isoelectric point. Since then this field has seen many ups and downs, upto the 1990s. Laboratories initiated use of this method, which was initially attractive due to its ability to enable visualization of all the protein spots on the gel. However, with widespread use in the separation of complex proteomes and protein mixtures, certain issues arose, especially when studying large (for example n=20) and unequal numbers of patient and control samples, with variations and low reproducibility being observed in the large numbers of gels produced during such analyses. Additionally, there were certain technical issues inherent to this concept in the beginning such as use of the tube gel. During the period of 1970s-1990s, advances were achieved by applying new methods such as the immobilized pH gradient strip. Additionally, in the rat race of the emerging field of proteomics, additional methods including mass spectrometry-based protein separation methods were developed and questions about the robustness of the 2DE technique in protein separation were raised. In the form of an answer to this then came the advanced versions of 2D gel electrophoresis such as DIGE.

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Overall, the point being made remains that from 1975 till date 2-D electrophoresis has received both criticism and appreciation. Nonetheless, till date it remains one of the core technologies to separate very complex protein mixtures as evidenced by the thousands of publications originating from utilization of this technique.

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- In 2-DE the first separation occurs based on the isoelectric point of the proteins and the second separation (at 90 degrees to the first) occurs based on the molecular weight. In the first dimension of separation you can separate the proteins using isoelectric focussing.
- Isoelectric focussing works on the principle that at all pH values other than their isoelectric point, proteins will be charged. If they are positively charged, they will be pulled towards the more negative end of the gel and if they are negatively charged they will be pulled to the more positive end of the gel. This technique this results in immobilization of the protein at its isoelectric point.
- In the second dimension, separation based on size occurs by SDS-PAGE.

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An overview of the 2DE process is provided, wherein in the first dimension the isoelectric focusing occurs by applying immobilized pH gradient strip in the iso-electric focussing unit (IEF) and in the second dimension, you can place the first dimension separated proteins in the SDS page gel and obtain separation based on molecular weight. So, on the given gel, you can separate the proteins based on both molecular weight and isoelectric point.

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An image of 2 DE gel is shown, where each spot represents a protein which was separated in this case from a bacterial sample and each spot already provides two pieces of information about molecular weight and its isoelectric point.

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2DE remains one of the core technologies to study the proteome and is applied in various biological applications. It is a relatively easy and affordable technique and is a highly sensitive method to visualize the proteins, making it a widely used method for protein separation in different labs.

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As previously mentioned, two scientists Klose and O'Farrell independently investigated different problems by applying 2DE and in 1975 they reported this method independently. Klose was investigating the heterogeneity of mice lactate dehydrogenase isozymes while O'Farrell was studying the complex proteins which are present in the crude extract of *E. coli.*

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See above the 2 scientists who developed the 2 dimensional electrophoresis method; Dr. Patrick O'Farrell and Dr. Joachim Klose. They made a great contribution to the field of proteomics, developing a technology which still is a core technique in the proteomics field.

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When initiated in the 1970s, 2DE was not a very easy process because protein separation in different dimensions involved casting the ampholyte containing

polyacrylamide gels in the glass tubes, making gel preparation a cumbersome process requiring a lot of care and attention. One of the major concerns was the reproducibility from gel to gel which posed issues in accurate comparison of protein composition between test and control samples.

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Various advancements have taken place in this field. During the optimization and advancement of this technology, different milestones were reached which accelerated the pace of its advancement. These included:

- The development of the immobilized pH gradient strip or IPG strip: This represents a major advance in this field.
- Solubilisation of hydrophobic proteins which were difficult to separate on the gel.
- Advancement in the gel electrophoretic apparatus and gel casting units, which helped improve reproducibility These improvements were pioneered by the many biotech supply companies supplying the scientific community.
- Staining methods: The development of staining methods with highly improved sensitivity helped in protein visualization.
- Image analysis: Improvement in image analysis came with the development of various softwares designed to perform this function

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The development of IPG strip as a replacement for the tube gel removed lot of earlier inconsistencies involved during the process of isoelectric focusing. The production of IPG strips involves computerized, computer-controlled gradient formation wherein pH gradient is covalently incorporated into acrylamide matrix and immobilized there. So reproducibility from strip to strip is high. They are now supplied commercially from different vendors and have made the 2DE process more efficient. Results obtained from them can be compared inter- and intra-laboratory wide in the world owing to the uniformity of computer-controlled gradient formation.

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IPG strips offer the following advantages over tube gels.

- They are more stable and durable as compared to the tube gels.
- They provide high resolution and higher reproducibility for inter or intra-laboratory comparisons.
- They demonstrate high loading capacity, useful during micropreparative 2 DE.
- They allow the separation of basic proteins under the equilibrium conditions.

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See above the scientist Prof. Angelika Görg who made a significant contribution to the development of the IPG strip. It was her pioneering research in the 1990s that eliminated a lot of criticism generated by low reproducibility in results obtained from gel-to-gel. However, several other developments other than the IPG strip also contributed to the elimination of variability in this field.

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IPG strips present the following advantages over tube gels

- They allow the application of concentrated protein samples without causing gradient disruption
- The IPG strip, which is dehydrated upon preparation, can be rehydrated upon protein application, which is followed by absorption on this chip, allowing for optimal separation in the electric field based on its isoelectric point.
- The IPG strip eliminates several problems which could be associated with top loading of a carrier ampholyte IEF tube gel which is more sensitive to overloading than the IPG strip.

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A variety of IPG strips of varying pH ranges are available, allowing for strip selection based upon the experiment type and nature of the sample to be separated. For

example, if no prior knowledge of the protein range to be separated is available, an IPG strip of broad pH range (ex: 3-10) should be selected. But if the protein mixture to be separated is maintained at a physiological pH, an IPG strip of a narrower pH range (such as 4-7) should be used, which may result in the loss of the few spots, but would provide increased resolution If the proteins to be separated are either acidic or basic, a strip with a narrower pH gradient range can be used, for example: 3-6, 4-7, 5-8 etc.

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The following slides are snapshots of an animation of the 2DE process.

Rehydration of the commercially supplied dehydrated strip: Can be done by passive or active rehydration. In passive rehydration the IPG strip is placed with the gel side facedown in a dish containing the protein sample reconstituted in a suitable buffer

Adding the protein sample and adding IPG strip to the protein solution

As seen in the animation the protein sample is first added followed by the placement of the IPG strip in the protein sample so that it can absorb the protein solution. This process takes between 10 to 20 hours depending upon the length of the IPG strip. This is then covered with mineral oil to prevent the gel from drying up and left over-night.

Active rehydration

In this alternate process, the protein sample is applied to the strip using a sample cup, followed by addition of the cover fluid to prevent the gel from drying up. Note: The protein sample application occurs under low voltage conditions. Following addition of the protein samples, the strip is then placed in the iso-electric focussing(IEF) unit, under low voltage conditions. Protein iso-electric focussing then occurs for a period between 10-20 hours, and is achieved by the passage of an electric current through the strip.

Isoelectric focusing instrument

The various proteins of the sample mixture migrate in the electric field and come to rest at the point when their pH is equal to their isoelectric point or pI. At this point they become neutral and are no longer are affected by the electric field. The progress of electrophoresis is monitored by adding a tracking dye, which as seen inthis snapshot, moves ahead of the proteins in the field

Progress of electrophoresis

2nd Dimension separation: The IPG strip is then equilibrated with a reducing agent such as DTT followed by an alkylating agent (iodoacetamide) which prevents reformation of reduced bonds. This strip containing the separated proteins is then placed on SDS-PAGE slab for further protein separation in the second dimension based upon molecular weight.

IPG strip on SDS-PAGE slab

The proteins on the IPG strip are subjected to SDS-PAGE by applying direct current between 100 to 350 volts depending upon the size of the gel. Any proteins that may have been present as a single band on the IPG strip (shown circled in red) due to similar isolelectric point can then be separated based on the molecular weight where the smaller proteins migrate further on the gel.

End results of 2DE

This is the view of a sample gel which has been run by 2 DEand stained with coomassie blue. Each spot provides information about the molecular weight and the isoelectric point of the protein.

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After the runthough of 2DE procedure, its applications are as follows.

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2DE can be used for various purposes. These include

- Study of global protein expression, which is the separation and identification of all the proteins present in a given sample. This involves 'finger-printing' or blueprinting' all the proteins present in that sample .
- Comparison of protein abundance between test and control samples: This is a very common application, also called differential protein expression or abundance based proteomics. And is useful to examine up- or down-regulation of proteins in the context of treatment/experimental conditions or any particular disease.
- Resolution of proteins in complex mixtures
- Study of different types of isoforms or the post-translational modifications. One of the major advantages of this technique is the visual analysis of the proteins. When you stain a gel after 2DE all the protein spots on the gel can be observed which is not possible from the gel-free approaches such as mass spectrometry wherein most of the analyses depend on protein spectra and you have to rely on your analysis.

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Protein profiling refers to the process of comparison of separated proteins from 2 sample conditions. It involves the following steps.

- Solubilzation of proteins from sample being studied.
- Sepeartion of proteins in the first dimension on the pH gradiet through IEF.
- Reduce and alkylate samples and then separate that bySDS-PAGE in the second dimension, that is based on molecular
- Visualization of individual protein spots by different straining methods. The choices include coomassie xxxx blue or silver strain, variousfluorescent strains or autoradiography

- Comparison of the images generated in Condition A vs. Condition B. Any proteins which are present in one condition and not the other represent proteins of interest. One needs to consider the results of analysis of the same 2 samples from different gels, using various replicates (both technical and biologica), then analyse the data using statistical methods. Any spot difference reaching significance reperesents a protein of interest, which can be further characterized using mass spectrometry.

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The above is a representation of abundance based proteomics or differential expression profiling wherein, following protein separation, the abundance of each spot as shown in the 3D views can be compared, followed by trypsinization and identification by mass spectrometry.

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This slide shows an application example, where the workflow is explored.

- For example, if one wishes to study proteins in the serum of individuals with prostate cancer, samples from a patient and a age matched healthy control would be obtained.
- The processes of protein extraction and serum depletion have been covered in the previous lectures.
- These methodologies are first applied here, to remove the abundant proteins by depletion, followed by separation of the remaining serum proteins.
- Following the completion of 2-DE, compare the resultant gels to determine if any proteins are up or downregulated while comparing patient to the control.
- If any results reach statistical significance these are further examined by mass spectrometry.

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So now let us go to the detailed part of the each of the processes involved in studying 2 DE: the experimental workflow.

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The steps in the workflow are as follows:

1. Isoelectric focusing: This is the first dimension separation of the proteins based on isoelectric point.
2. Equilibration of IPG strips.: This is required to deal with the heat generated during gel denaturing during preparation for running the second dimension of SDS PAGE.
3. SDS-PAGE: This is the second dimension where proteins immobilized at their isoelectric point are separated on the basis of their molecular weight.
4. Gel Staining and Visualization
5. Imaging: This is a crucial point wherein all the protein spots are analysed so that the abundance of each of these proteins can be calculated and detailed statistical analysis can be performed.
6. Spot Picking: Once one is confident that there is a significant difference between the 2 samples, the protein spot that has significantly changed in expression is picked up
7. Enzymatic digestion: This is required to extract the proteins of analysis for examination by mass spectrometry.
8. Mass Spectrometry: Discussed in detail in another module.

Each point is now discussed in detail.

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IEF: in this process, proteins are separated according to their isoelectric points. The protein is first applied to the IPG strip. Following absorption, then the electric field is applied, making the proteins migrate on the basis of their charge till the point where they

become neutrally charged upon reaching their isoelectric point. They remain immobilized at this point and can be further separated in the second dimension.

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This animation details the process of IEF. An IPG strip with a pH 3 - 7 gradient is shown. The protein migrates in the field (positively charged proteins will migrate towards the negative end and so forth), till they reach the point where pH becomes equivalent to the pI, at which they are immobilized.

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In this view, the gradient ranges from low pH to high pH At the point where pH becomes equal to pI, discrete bands form.

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The process of rehydration is an important one and is discussed below.

1. Protein extraction
2. Rehydration of IPG strip by applying the extracted protein solution. This can be done the following ways, as previously discussed.
   - Passive rehydration: IPG strip added facedown in the protein solution, no voltage applied.
   - Active rehydration: IPG strip is faceup. Proteins are applied during the application of a low voltage, which helps to resolve proteins.
3. Application of mineral oil to prevent protein evaporation and gel drying.

Note: Neither Active nor Passive rehydration offers any global advantage over the other, but each are better suited for certain applications, which has to be gauged by the scientist.

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Overview of IPG strips:

Different types of strips of varying pH ranges can be used during the IEF process. Optimal strip selection, as previously discussed, depends on knowledge (or lack thereof) of the pH ranges in the protein mixture to be separated. The length of IPG strip commercially available ranges from 7 cm to 24 cm.

- A smaller sized strip is more suitable during optimization of protein extraction and separation protocols.
- Following optimization, longer strips(17-24 cm range) can be used.

- Importantly, IEF units obtained from various manufacturers are capable of accommodating IPG strips of varying lengths. Few instruments accommodate a flexible length range of 7-24 cm while the other instruments come with different types of trays designed individual strip lengths.
- Samples of high protein concentration are better resolved using longer strips. (for eg. 18 or 24 cm.). Samples of low or inadequate protein amounts can be resolved on small strips but can also be applied to longer strips with the modification of a more sensitive stain selection(such as the silver stain or SYPRO Ruby stain). But overall the longer gels are suitable doing a range of different proteomic analysis. However, handling longer gels is tedious and cumbersome.

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These are certain recommendations for how much protein one should load on the IPG strip. For example, a 100-300 µg of protein in a volume of 150-160 µL is suitable for a. Usually coomassie is a good choice to stain this much protein. If you have a very low amount of protein in your sample, (for eg. 10-100 µg) more sensitive stains such as silver or SYPRO Ruby are suitable. Use of the longer strip (in the 17 -24 cm range) will necessitate an increase in the amount of protein to be added For example, 250-100 µg of protein can be applied on the long strip and visualize by using coomassie stain. The total reaction volume should not be more than 350 µL.

Even when you do not have much protein in your sample you still apply the sample to the long strip but you have stain it with silver or SYPRO Ruby. In that case you can load between 100 to 1000 µg of protein.

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The following is a laboratory demonstration of isoelectric focusing so the intricate steps in performing IEF become clear.

Isoelectric focusing involves two major steps: rehydration of the IPG strips and IEF of rehydrated strips.

Rehydration of IPG strips

Step 1: Clean all the apparatus thoroughly to avoid any contamination.
Step 2: Add the reconstituted protein sample in a well of the rehydration tray.
Step 3: Then remove the IPG strip from its cover and place it carefully in the well.
Note: IPG strips are used to separate proteins based on their isoelectric points. These are acrylamide-coated plastic strips containing immobilins of various pH spread across it. They have successfully replaced conventionally used tube gels due to the higher reproducibility they can achieve.
Step 4: After around 30 min pour some mineral oil over the strip to prevent it from drying up.
Step 5: Cover the tray and leave it over night to allow rehydration to occur.

Isoelectric focusing

Step 1: Initialize the instrument and clean the instrument with a dust-free cloth.
Step 2: Pace the focusing tray on the instrument and ensure that it is properly balanced.
Step 3: Cut the paper wicks required for focusing to a suitable length and wet them with a small amount of water before use.
Step 4: Carefully remove the IPG strip from the rehydration tray and drain any excessive oil by blotting it with a tissue paper.

Step 5: Place the strip in the focusing tray and immerse it in mineral oil.

Step 6: Then place the 2 wicks at either ends of the IPG strip followed by an electrode at each end.

Step 7: Fill the adjacent wells with mineral oil to ensure uniform current flow.

Step 8: Then input the desired protocol on the instrument software along with details of strip length, pH range and number of strips and start the focusing process. The voltage time curve will appear based on the protocol that has been set.

Protein separation occurs based on the net charge of the protein. Protein will migrate along the IPG strip and come to rest at a point when the net charge becomes zero known as the isoelectric point.

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In summary, today's lecture has covered the following points:

- Gel-based proteomics,
- The different types of techniques being used in gel-based proteomics:. The different types of electrophoresis including SDS-PAGE and 2 dimensional electrophoresis.
- 2-DE process in detail, including the workflow which discusses 2-DE at length, with a focus on. isoelectric focusing., with an animation and laboratory demonstration.
- A clarity on the e different types of techniques being used in gel-based proteomics and the different workflow steps involved in performing this process should have been achieved. the next lecture will continue the discussion on 2-DE workflow with focus on equilibration of the strips, SDS-PAGE, staining gels, obtaining images, image analysis, spot picking, etc.