Proteomics: Principles and Techniques

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Module No. # 12

Lecture No. # 12

Gel based Proteomics

Welcome to the Proteomics course, in today's lecture we will talk about Gel based Proteomics. The gel based proteomics includes different type of techniques, which can be used in different aspects of life science research. Regardless of whatever field you work in every lab uses different type of gel-based approach to separate proteins in their day to day research.

So, gel-based proteomics includes different type of techniques, such as sodium dodecyl sulfate, polyacrylamide, gel electrophoresis or SDS PAGE two dimensional electrophoresis or 2 DE. The advanced form of two dimensional electrophoresis also known as difference in gel electrophoresis or DIGE then, there are several advancement of it, variations of it such as Blue Native page or BN page.

So, in the gel-based proteomics, we will talk about different principles involved in operating each of this technique and their applications, for example, the SDS PAGE can be used to separate proteins based on their molecular weight. The two dimensional electrophoresis separates proteins based on both isoelectric point and molecular weight in the two dimension,

The advanced form of two dimensional electrophoresis separates proteins in such a way, so that different type of artifacts and variations from 2 DE can be overcome by adding different dyes, and mix them together in the dyes technology. So, all of these technologies will be discussed in the gel-based proteomics.

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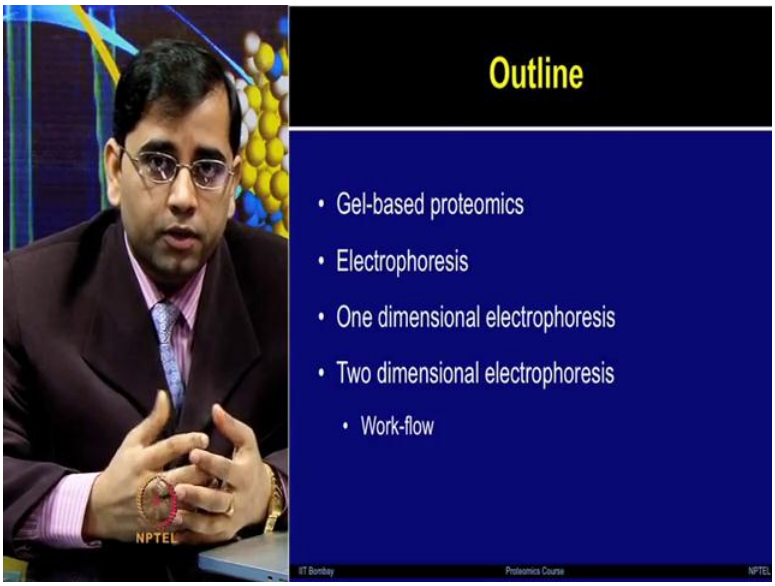

In today's lecture of gel-based proteomics, I will give you the concepts of different electrophoresis type for the one dimensional electrophoresis and two dimensions electrophoresis, during description of two dimensional electrophoresis, I will give you the work flow of several steps involved in two dimensional electrophoresis.

So, let me give you an outline for today's lecture, I will describe about a gel-based proteomics, how electrophoresis is used different type of electrophoresis such as one dimensional electrophoresis or SDS PAGE. Which is used to separate simple protein mixture but, when you have very complex mixture and you want to separate proteins based on two properties, then you can use two dimensional electrophoresis.

I will then describe you the work flow of two dimensional electrophoresis, there are different steps involved and we will walk you through on the steps, also during the process, I will show you some animations to clarify your concept. And similar semi laboratory demonstrations to give you the real feel of the laboratory work being carried for performing the two dimensional electrophoresis.

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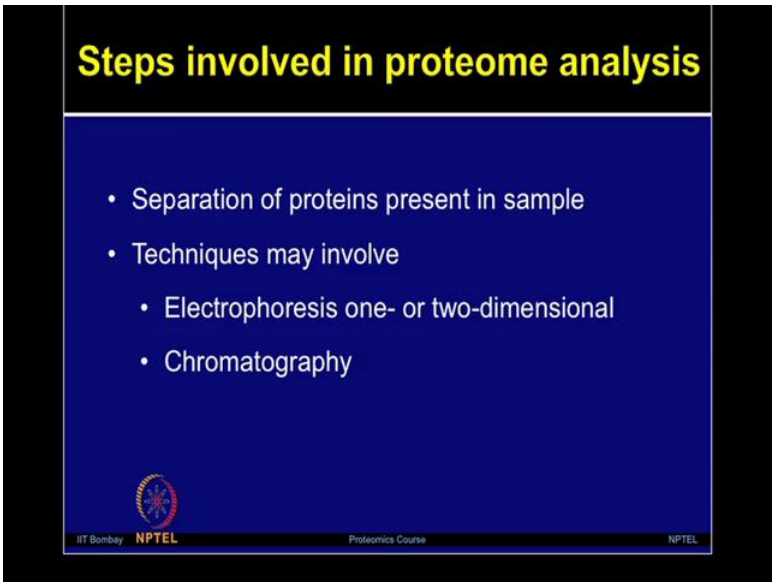

So, there are different steps which are involved in analysis of the proteome, as I talked in the previous lecture, that first of all it is very important to separate the proteins, which are present in the sample. Once you have a very good protein preparation made, the next thing is to separate the proteins by involving different techniques. You can use electrophoretic based method; such as one dimensional electrophoresis or two dimensional electrophoresis or one can also use different type of chromatography methods to separate the proteins.

So, in proteomics you always have various options available to analyze your protein samples, it depends on your applications and the biological question, which you want to ask, what information you want to obtain from that particular technique. So, if you want to have just want to verify, that your protein is very pure, maybe SDS PAGE electrophoresis is good enough, if you want to determine the subunit compositions you can compare that on SDS PAGE and native page.

If you want to resolve the proteins or very, very complex mature of the proteins obtained from wholesale (( )) or tissue or the cell and which contains thousands of protein mixture, at that time you can try different type of prefractionation methods, by involving different chromatography; as well as you can try different type of advanced electrophoretic methods such as two dimensional electrophoresis or DIGE. One can also use different mass spectrometry based microarray based technologies, to study those

proteins; given the course you will come across all of those techniques including mass spectrometry and microarray, but let us focus on the electrophoresis in this lecture.

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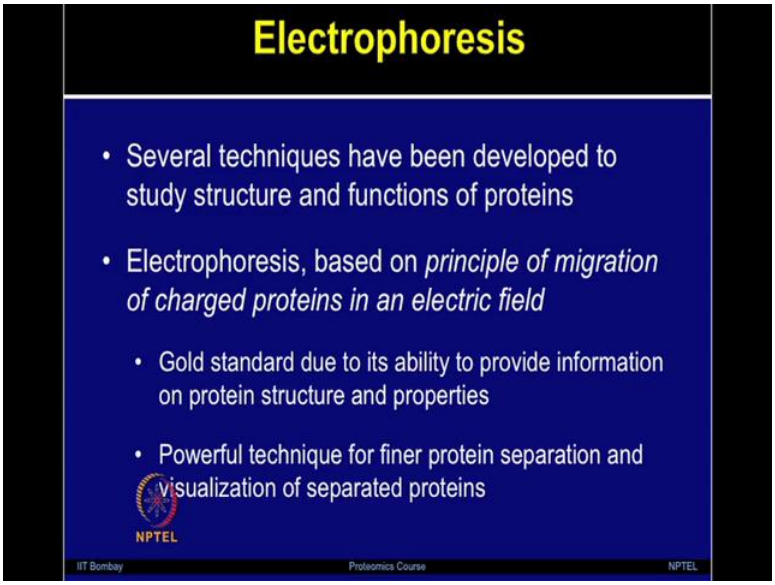

So, the gel-based proteomics involved different type separation techniques, which includes sodium dodecyl sulphate, polyacrylamide, gel electrophoresis or SDS PAGE, two dimensional gel electrophoresis or 2-DE; the fluorescence 2-DE difference in gel electrophoresis or DIGE. Blue native page or BN page, but let me remind you these is not a very descriptive list these are just few very important techniques used for analysis of proteome by applying gels.

There are different methods as well, which I have been developed to stable the structure and function of the proteins and electrophoresis is very important in that direction, which is based on the principle of migration of charge proteins in a given electric field. So, the very good standard of these SDS PAGE or different type of electrophoretic method is, because they have ability to provide the information on the protein structure and properties. This is one of the very powerful technique to separate the proteins and visualize in separate protein by applying different type of staining methods.

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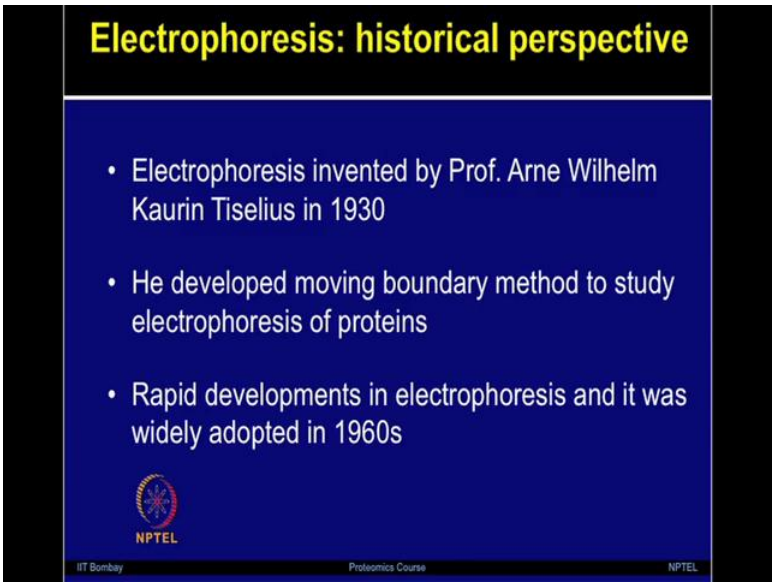

Let me give you the historical perspective of electrophoresis this process was invented by professor in Tiselius in 1930, he developed the moving boundary method to study the electrophoresis of proteins. Since then, lot of development happened in the field and during the 1950's and 60's, this process was highly adopted in the front laboratories.

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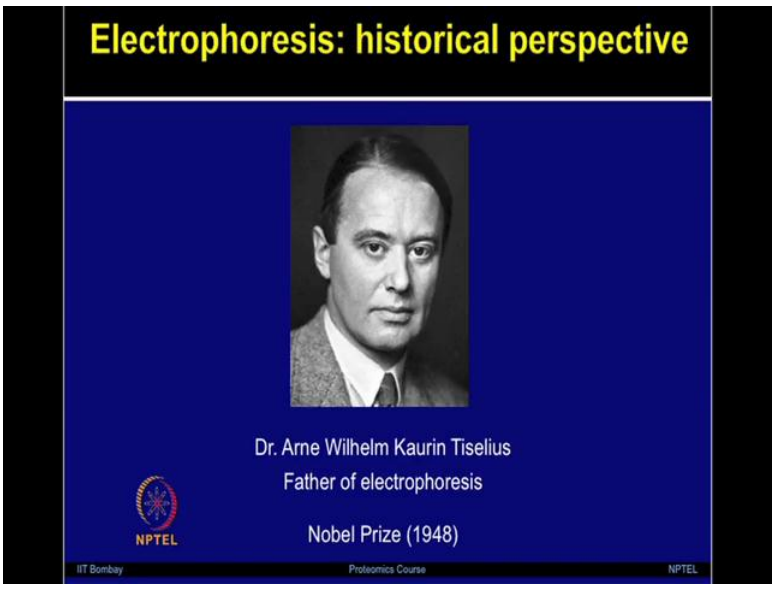

So, he is Doctor Tiselius who is also known as the father of electrophoresis for his great contribution in chemistry, he obtained the Nobel prize in 1948.

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So, now we will describe one dimensional electrophoresis, some of the concepts involved in that.

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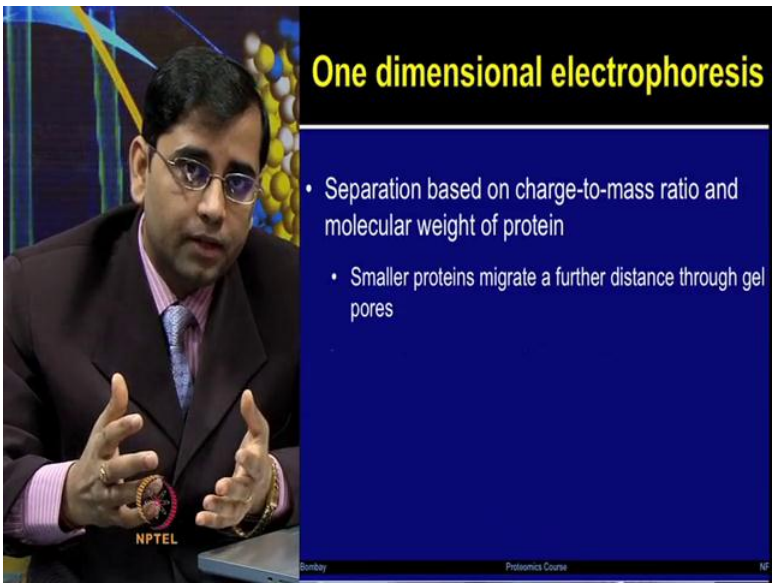

So, the one dimensional electrophoresis the separation is based on the charge-to-mass ratio and the molecular weight of the protein. Therefore, on a given gel if you have smaller the proteins in the electric field, they will move further down and the higher molecular weight proteins they will remain on the top. So, if you apply some standard molecular weight markers, which gives you the known molecular weight of the proteins

then, for a given unknown protein, you can determine the molecular weight. And it is expected that the higher molecular weight proteins will remain on the top and the smaller molecular weight proteins will reach towards the bottom of the gel.

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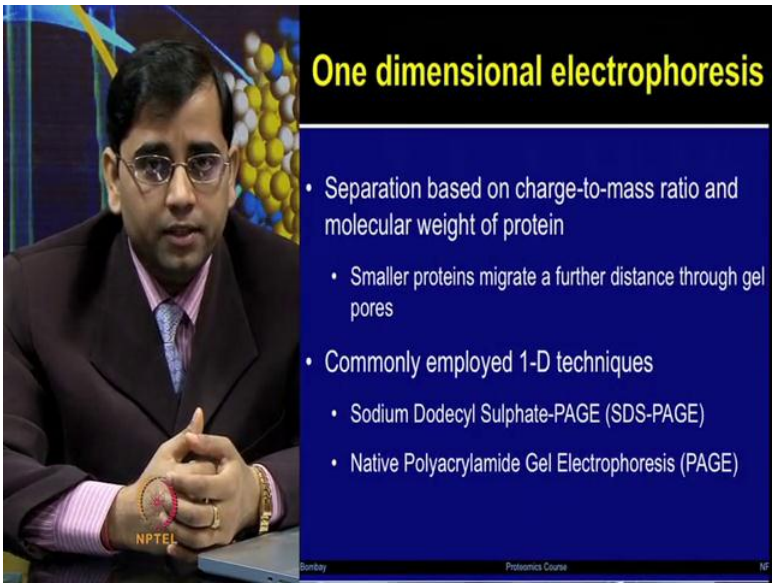

So, commonly employed one dimensional techniques include the sodium dodecyl sulphate PAGE, SDS PAGE or other technique, which is used for studying the protein in the native conformation native form, which is known a native polyacrylamide gel electrophoresis or PAGE.

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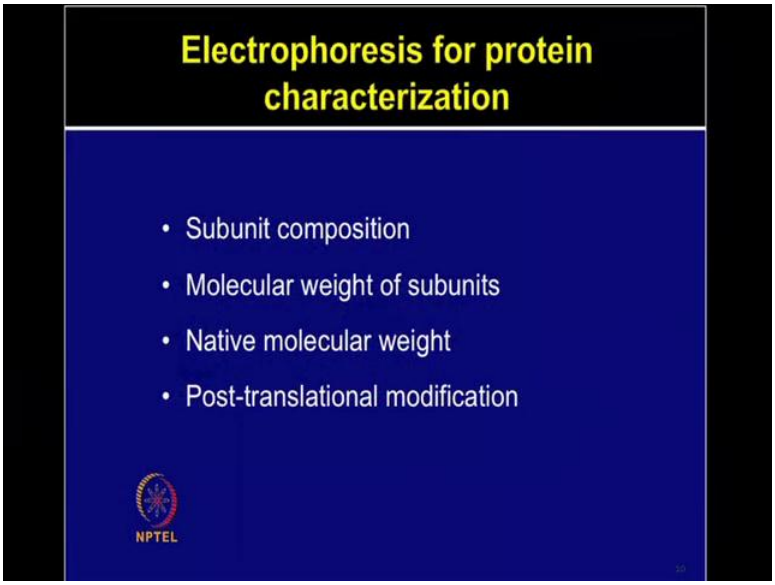

The electrophoretic techniques can be used for the protein characterization, to study different properties for example, the subunit composition of the proteins, what is the molecular weight of those subunits? What will the molecular weight of native proteins? Different type of post-translation modification again only one technique may not be able to answer all of this questions.

So, often you have to involve more than one type of electrophoretic method for example, while comparing native and subunit composition forms, you need both native electrophoresis and SDS PAGE if you want to look for post-translation modification, then SDS PAGE will be useful. If you want to compare the proteins based on the isoelectric point as well as the separation of isofroms or PTM form, based on the molecular weight again, two dimensional electrophoresis will be useful. So, one needs to know the concept and the laboratory way of using these technique, so that by applying a combination of the technique one can study different type of properties.

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However, the one dimensional electrophoresis has different limitations, you cannot separate your complex proteome, complex protein samples if you have thousands of protein and you want to study them then one dimensional electrophoresis has limitations.

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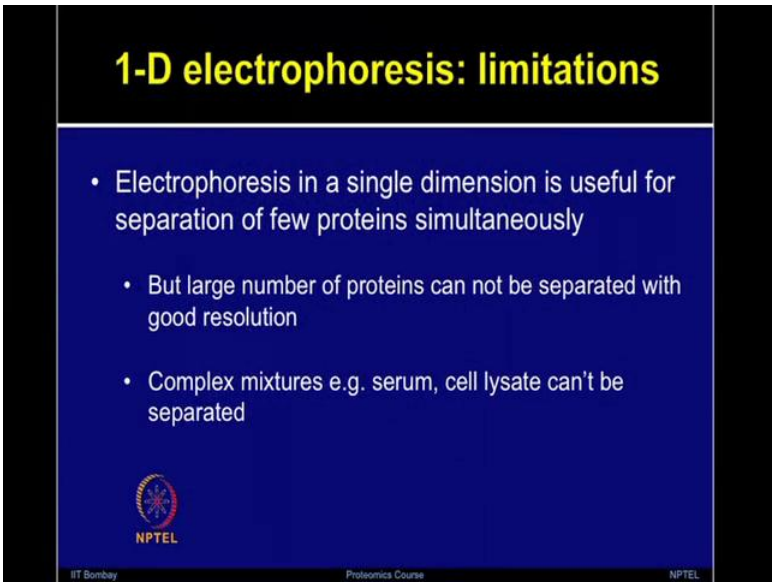

So, the one dimensional electrophoresis is useful to separate few proteins or a simple protein mixture but, when you are studying the complex mixture then two dimensional electrophoresis or DIGE or with different type of mass spectrometry based methods will b more useful. So, if you have very complex samples like cell lysates or you have serum then separating those and comparing different type of samples on SDS PAGE may not be a very good way in that case you should apply different methods.

So, one dimensional electrophoresis is very useful and its being used in almost all the laboratories working in the light sense area. But, it can give you few questions it can address only those questions but, if you want to get more characterization then you have to apply different techniques.

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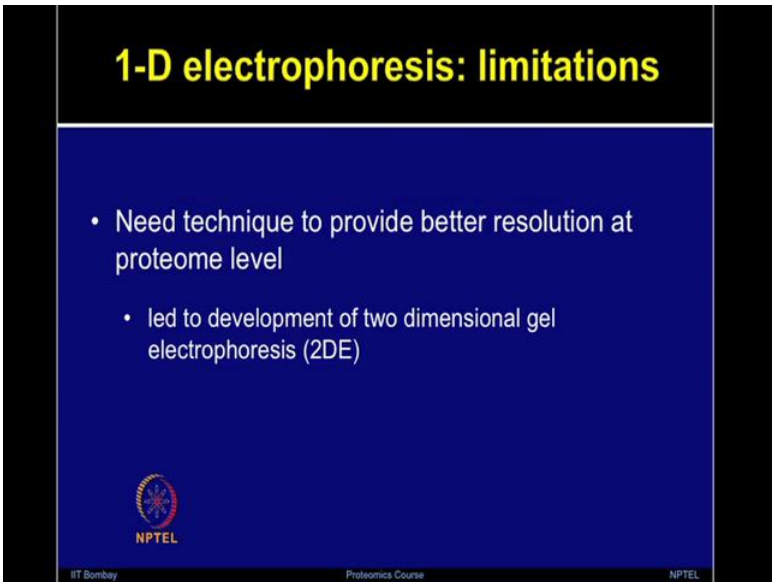

So, to overcome these type limitations and to obtain the better resolution of protein separation the two dimensional electrophoresis was applied.

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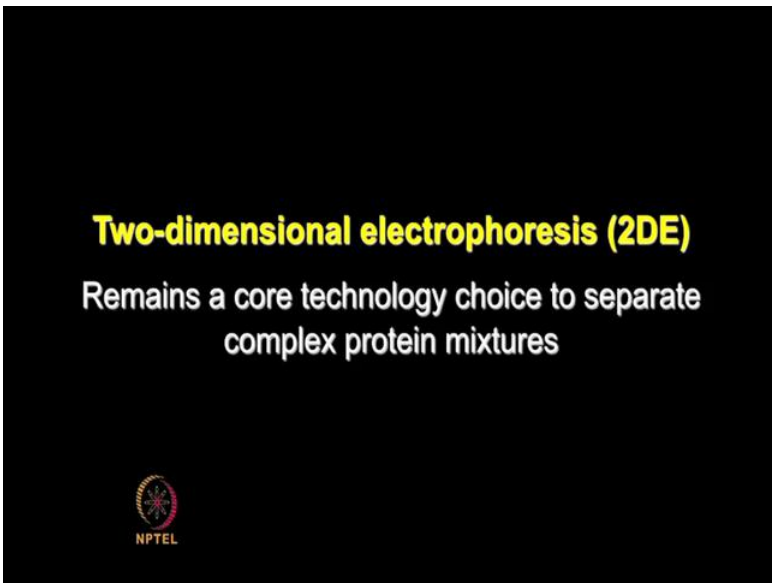

So, now let us talk about two dimensional electrophoresis or 2DE this technique was earlier used in 1970's, so it has a very long history of being used in laboratories from several years. And interestingly two scientists Klose and Farrell they applied two dimensional electrophoresis to study different biological problems and simultaneously

they reported independently in 1975, that two dimensional electrophoresis method can be used to separate the proteins, based on both molecular weight and isoelectric point.

Now, since then two dimensional electrophoresis field has seen many ups and down for example, several laboratories started using two dimensional electrophoresis, it was very easy method to be used. And since one can visualize all the protein spots on the gel it became very attractive way of separating the proteins. So, it was widely adopted people started using it very often but, when people started comparing the complex sample, complex proteome.

And for example, you have clinical sample, where you have to run maybe 20 patient samples and 20 healthy controls, in such a scenario, when you have token large number of gels then two dimensional electrophoresis, shows lot of variations and reproducibility issues. Therefore, this technique started getting lot of criticism, that there are some reproducibility issues, there are some manual artifacts, then there were some technical issues inherent to the this concept in the beginning like tube gel. So, all of this were part of the things from 1975's to 1990's and then during that time people started advancing this field by applying new methods, such as advancement of immobilized pH gradient (( )).

Further more than in the race of separating proteins in the complex proteomes, people started applying different methods, including mass spectrometry base methods. Then questions came that probably due to inherent issues of 2 D electrophoresis, this technique may not be a powerful technique to study the proteome. But, then advanced form of 2 D electrophoresis came such as difference in gel electrophoresis or DIGE.

So, in this discussion I am trying to convey you the fact that from 1975 till date 2 D electrophoresis has seen lot of criticism and appreciation of the technology but, still till date it remains one of the core technology to separate the very complex protein mixtures and thousands of publication are providing a very good evidence for this technology being widely used.

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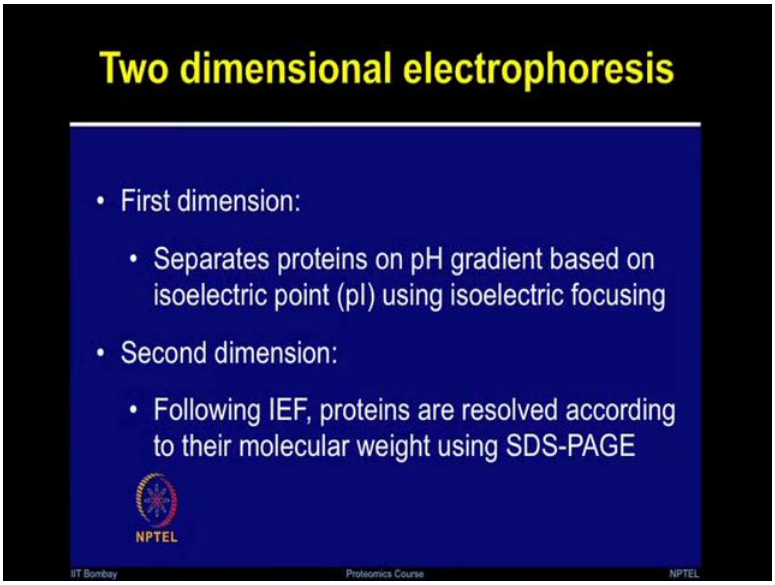

So, in the two dimensional electrophoresis the first separation occurs based on the isoelectric point of the proteins and the second dimension occurs based on the molecular weight. In the first dimension separation, you can separate the proteins in the isoelectric focusing unit, in the second dimension separation you can separate the proteins in SDS PAGE.

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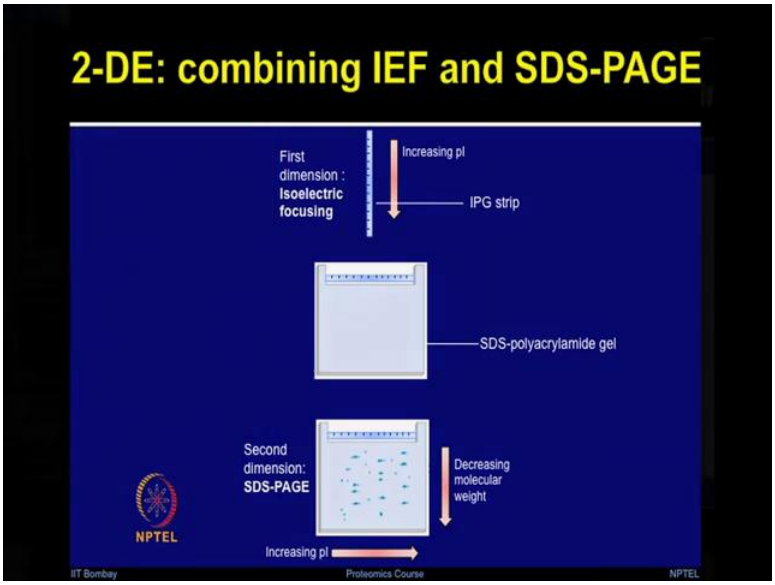

I am giving an overview of this process by showing this figure here, where in the first dimension the isoelectric focusing process is occurring by applying immobilized pH gradient strip. Now, I the second dimension the SDS polyacrylamide gel, you can place the first dimension separate protein and with the decreasing molecular weight the proteins are separated. So, on the given gel you can separate the proteins based on both molecular weight and isoelectric point.

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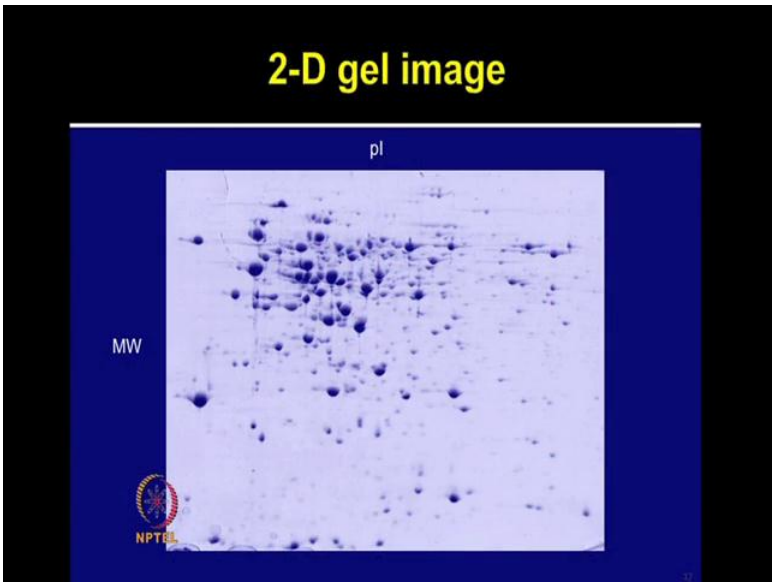

I am showing you an image of two dimensional electrophoresis gels or 2 D gel, where each spot is representing protein, which is separated in this case from a bacterial sample. And each spots already provides two information about it is molecular weight and it is isoelectric point.

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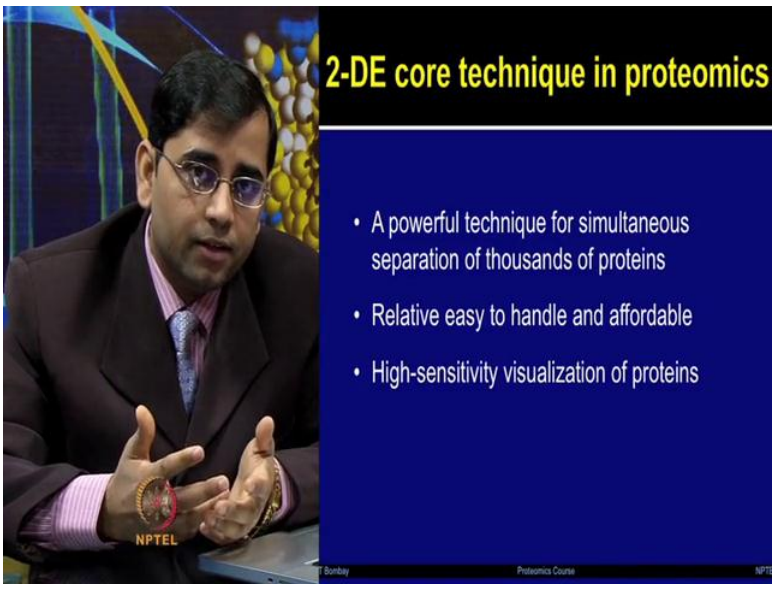

So, two dimensional electrophoresis, we means one of the core technology to study the proteome and it is applied in various biological applications, it is relatively very easy

technique to handle it is not, so costly it is still very much affordable. So, different laboratories use it widely, it is a very high sensitive methods to visualize the proteins.

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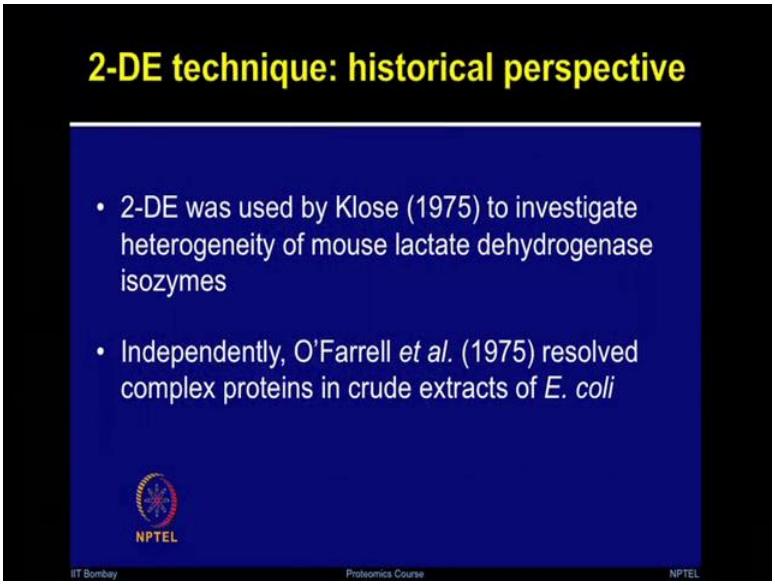

So, as I mentioned earlier two scientists Klose and Farrell independently investigated different problems by applying two dimensional electrophoresis and in 1975 they reported about this method independently. So, Klose he was investigating the heterogeneity of mouse lactate dehydrogenase isozymes, independently O'farrell was studying the complex proteins, which are present in the crude extract of E. coli.

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Here are these two scientist who developed the two dimensional electrophoresis method professor Patrick O'Farrell and professor Joachim Klose. So, they did a great contribution in the field of proteomics and a technology which is still remain one of the co technique in the proteomics field.

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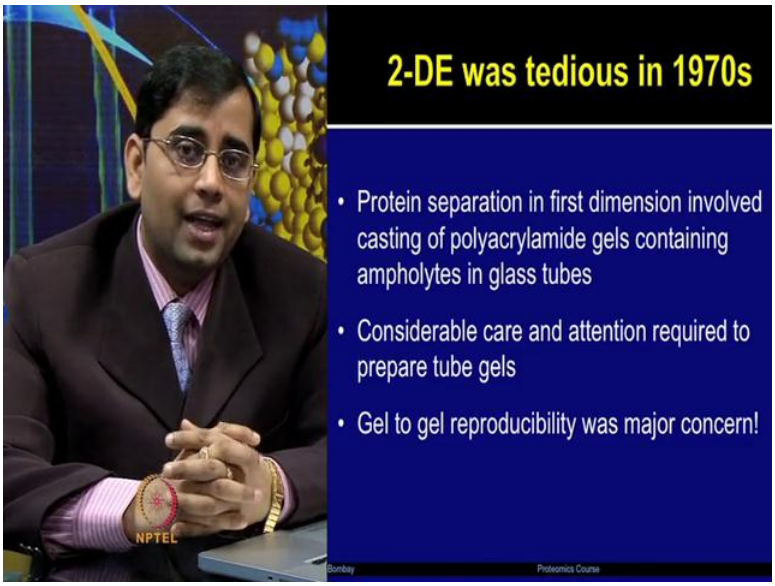

2-DE when it was started in the beginning in 1970's it was not very easy process, because protein separation the first dimension involved casting the polyacrylamide gels containing ampholytes in the glass tubes. Lot of care and attention have to be taken at that time to prepare the tube gels. But other major concern was the reproducibility of gels to gel for example, if want to compare a control and treatment and you want to analyze those two images, lot of variations will observed by using the tube gel method.

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So, question is how two dimensional electrophoresis became, one of the very powerful method.

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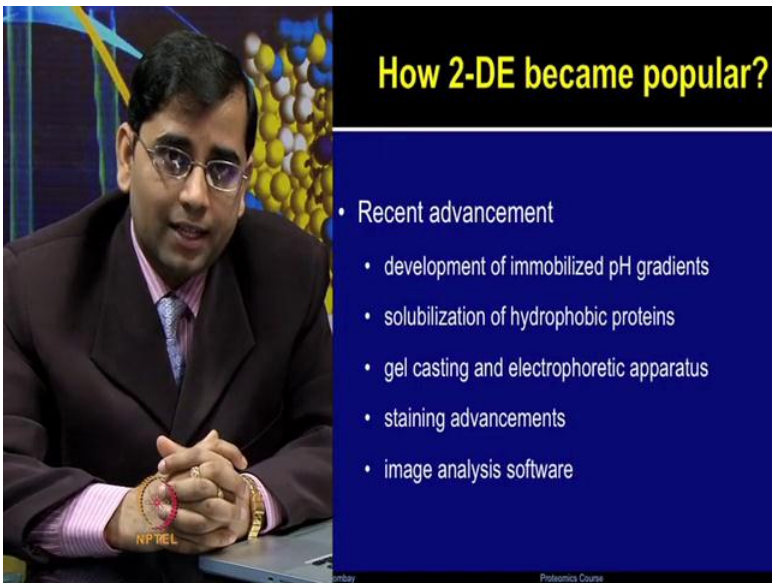

So, various advancement to play in the course of two dimensional electrophoresis, in that optimization and advancement of this technology different milestone research happened which accelerated the phase two dimensional electrophoresis research. For example, development of immobilized pH gradients, strip all IPG strip that was one of the major advancement in this field. Solubilization of hydrophobic proteins, which will difficult to

separate on the gel. Then reproducibility also advanced because of advancement in the gel electrophoretic apparatus and gel casting units again lot of companies manufacturers came into the play and they helped to improve the gel casting and electrophoretic units.

Different type of staining methods were developed, then we will talk about different type of stains and how they work during the course of two dimensional electrophoresis work flow; but very sensitive stains sphere head this process to visualize the proteins on the gel. Finally, how to analyze the images obtained from these gels that was one of the major advancement different type of softwares image in our systems, came into the play to analyze the gel.

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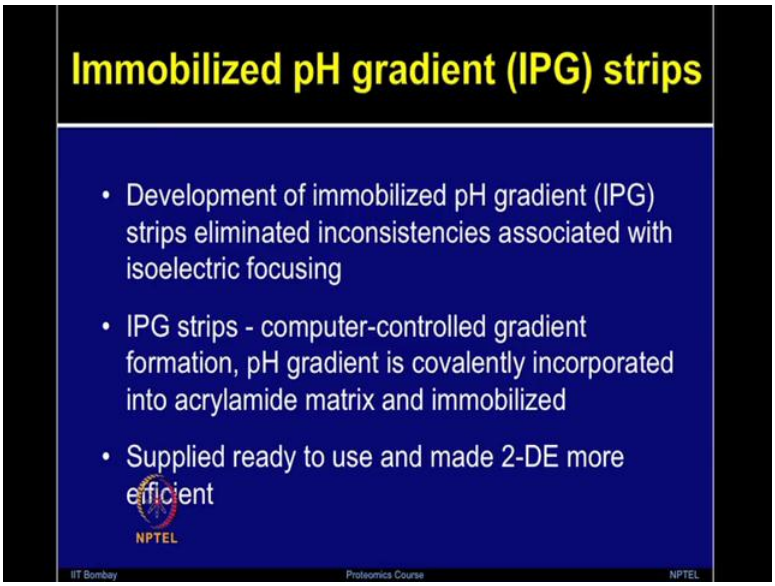

So, let us talk about immobilized pH gradients strip or the IPG strip, which was one of the major milestone developments in the 2-DE. So, the development of IPG strip as compare to the tube gel removed lot of inconsistency, which were involved earlier this is the process of isoelectric focusing.

So, the IPG strips, these are computerized computer controlled gradient formation and the pH gradient is covalently incorporated into the acrylamide matrix and it is immobilize there. So, reproducibility of one IPG strip, whether IPG strip is very high. Now, they are supply it commercially from different manufacturers and made the 2-D process more efficient and it can be compared inter and intralaboratory world wide because these are computer controlled gradient form.

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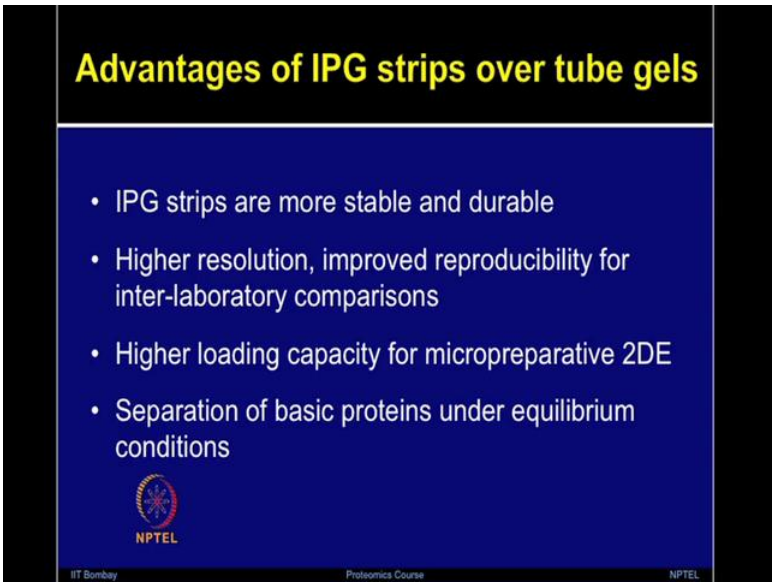

So, let us talk about of the advantages of IPG strips over tube gels, the IPG strips are more stable and durable as compare to the tube gel. They provide high resolution and much better reproducibility for inter or intralaboratory comparison, the higher loading capacity for micro preparative two dimensional electrophoresis is another advantage. Then separation of basic proteins under the equilibrium conditions, one of these are different advantages of using IPG strips over tube gels.

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Now, I am introducing you scientist professor Angelika Gorg, who made a significant contribution for the development of IPG strips. It was her pioneer work in 1990's which eliminated lot of flexism which people were showing due to the reproducibility of gel to gel; and along with IPG strip, so the other advancement occurred in the field which help to make this technology more reproducible.

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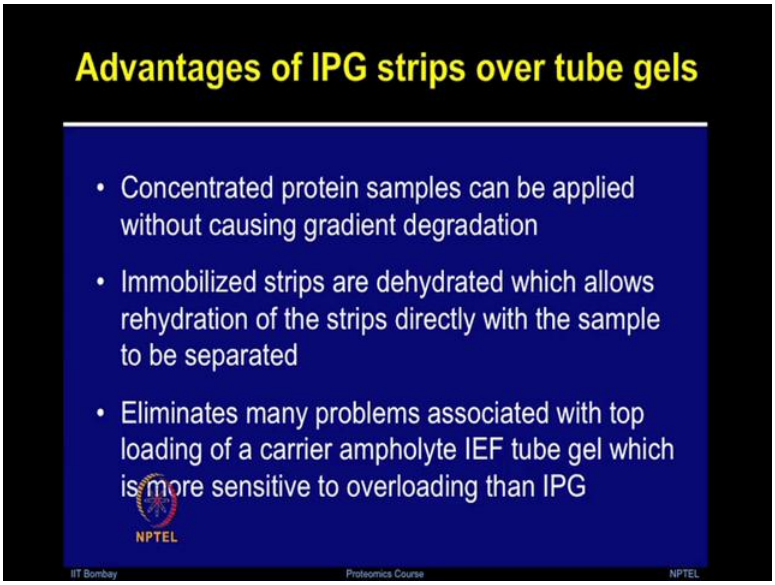

So, the concentrated protein samples can be applied in the IPG strip without causing gradient degradation which is another advantage of using this method over tube gel. You can take IPG strip and apply your protein sample, so that it is absorbed on the strip and then, protein can be separated in the electric field based on isoelectric point. So, the IPG strip eliminates several problems, which could be associated with the top loading of a carrier ampholyte IEF tube gel, which is more sensitive to overloading than the IPG strip.

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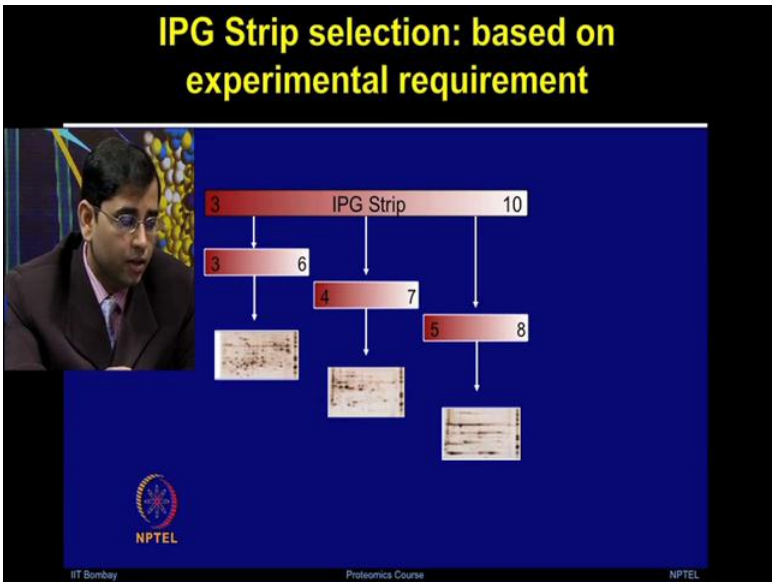

So, people perform different type of experiments and depending on the type of problem they want to study one can apply different type IPG strip. For example, if you have no clue about what a protein range you want to separate, based on the pH gradient. Then once we will start with a broad range of IPG strip, for example, 3 to 10 but, if you know that you are mainly interested in the a physiological pH may be 4 to 7 could be a better choice; where you may loose few proteins spot, but your resolution and separation will be much better.

Now, if you know your proteins are more which an acidic region or the basic region depending on those one can separate proteins on the narrower range of the IPG strips for example, one can use 3 to 6, 4 to 7, 5 to 8. And different type of gel patterns can be observed depending on your IPG strip and the preliminary weight obtain then one can apply that to separate the proteins, based on IEF the first dimensional electrophoresis and SDS page, second dimensional based on the molecular weight.

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So, I will now show you an animation for two dimensional electrophoresis, which will give you an over view of different steps involved 2-DE experiment.

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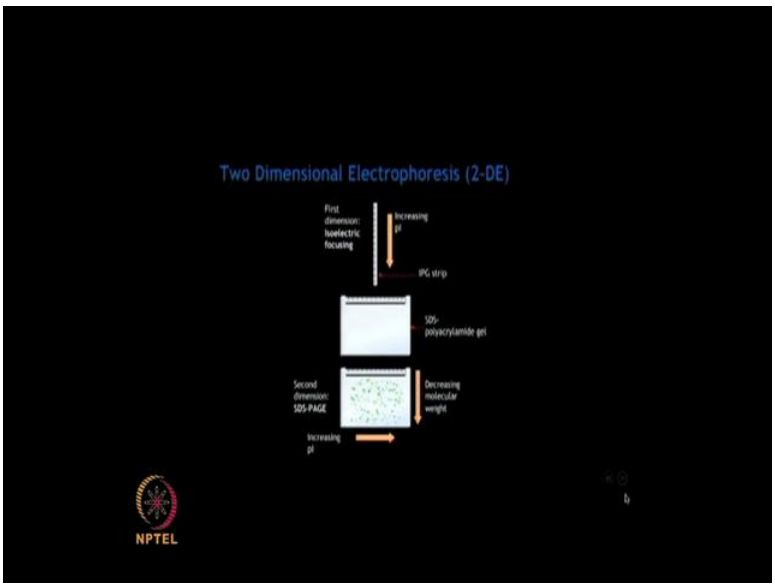

So, in this animation I will describe you the two dimensional electrophoresis process, pair to isoelectric focusing in two dimensional electrophoresis.

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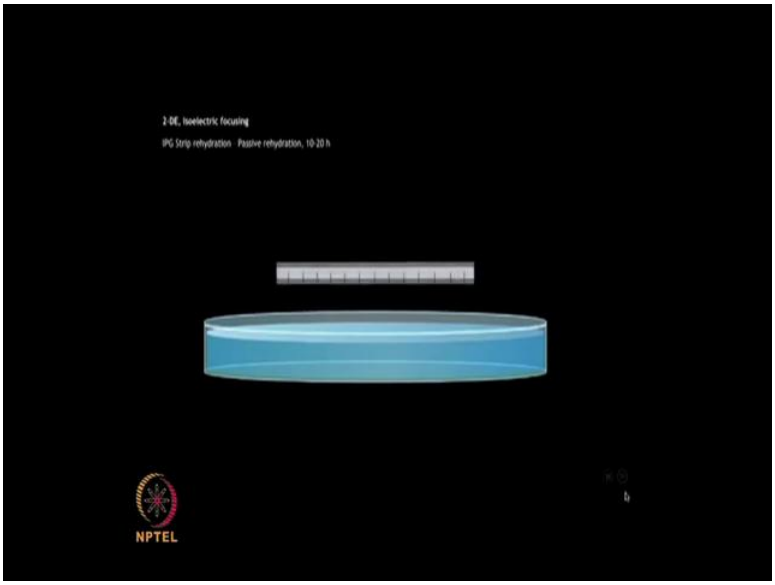

The commercially available IPG strips must be rehydrated, this process can be done either by the passive rehydration or active rehydration. In passive rehydration the IPG strip is placed with it gel site downwards in a well containing the protein sample reconstituted with a suitable buffer.

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As you can see in the animation, you can apply the protein sample and then add the IPG strip, so that it can absorb the protein solution, this process can be done for 10 to 20 hours depending upon your length of the IPG strip. This is then covered with mineral oil to prevent the gel for drying up and left over night, other process known as active rehydration, where the proteins sample is added to their strip via sample cup, followed by the cover fluid to prevent the gel from drying up. The protein sample being applied a very low voltage condition is provided and then cover fluid can be added, this is then placed in the isoelectric focusing instrument and low voltage is applied process can be perform from 10 to 20 hours.

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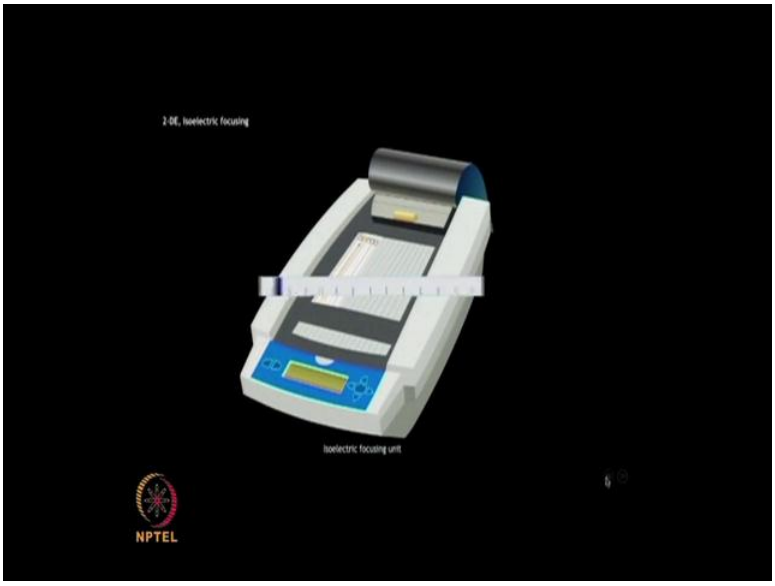

These loaded strips are then (Refer Slide Time: 29:07) focused on an isoelectric focusing unit by passing the current, the various proteins of the sample mixture migrate in the electric field and come to rest, when the pH is equal to their isoelectric point or PI. So, they become neutral and are no longer being affected by the electric field the progress of electrophoresis is monitored by adding a tracking dye, which you can see in the animation moving a head of the proteins.

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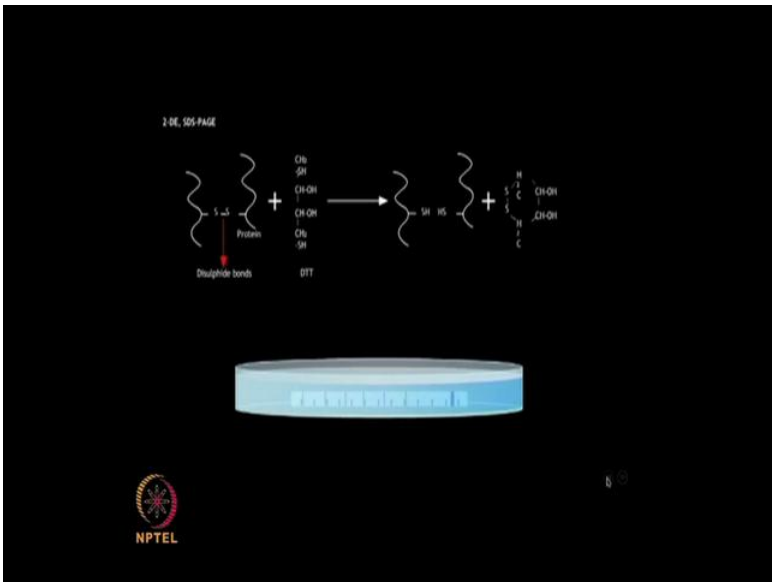

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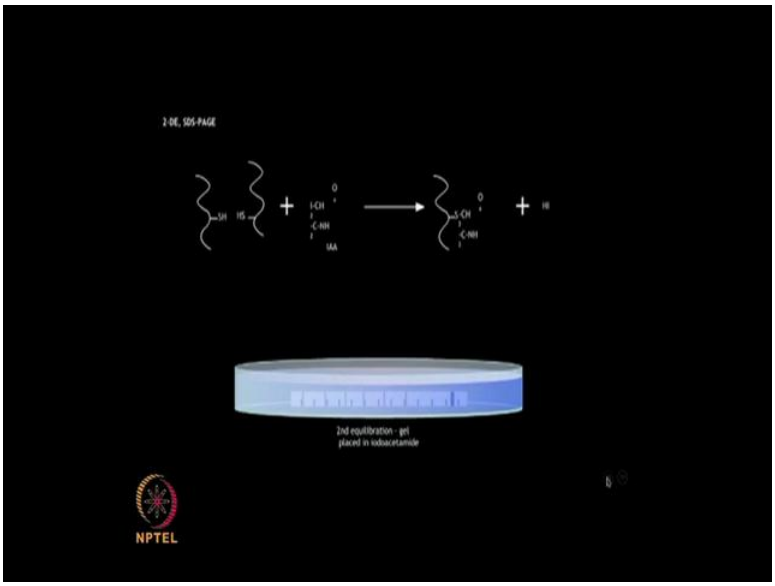

IPG strip is then equilibrated in a reducing agent, such as DTT followed by an alkylating agent iodoacetamide, which prevents reformation of reduce bonds.

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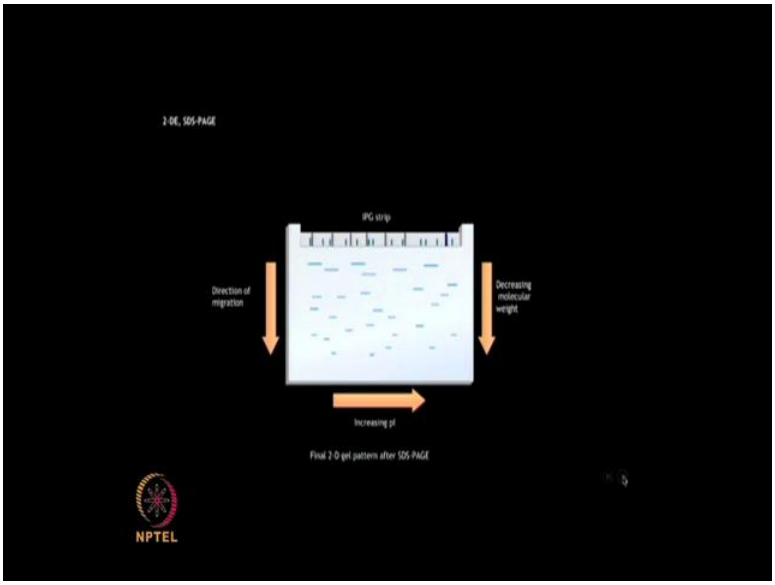

This strip containing the separated proteins is then placed on SDS page gel slab for further protein separation in the second dimension based on the molecular weight. The proteins on the IPG strip are subjected to SDS page by applying a direct current between 100 to 350 volt depending upon the size of the gel. Any protein that may have been present as a single band on the IPG strip as you can see in the red circle, due to similar isoelectric point can then be separated based on the molecular weight. Where the smaller proteins will migrate the (( ))

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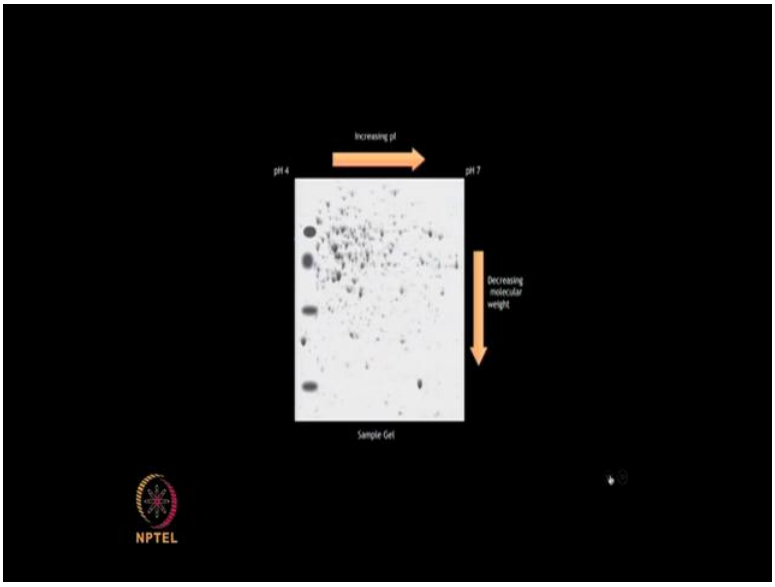

This is view of a sample gel, which has be run by two dimensional electrophoresis and stain with coomassie blue, each spots provide the information about the molecular weight and isoelectric point of the protein.

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I hope this animation was clear and you are able to understand the concepts involved in performing a 2-DE experiment. So, now let me give you few applications one can use by applying this technique.

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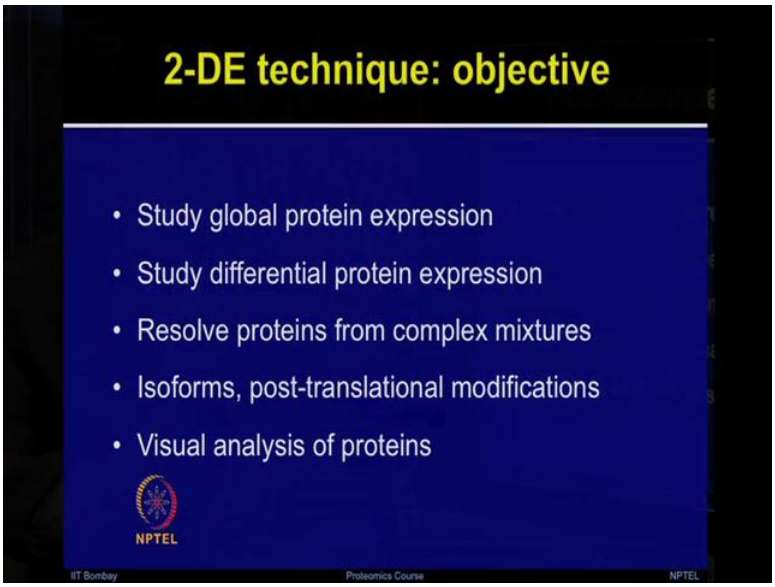

So, people apply it 2-DE for various objectives for example, studying the global protein expression, when I said global protein expression it means you want to identify or separate all the proteins present in a given protein sample. So, if you do not have information about all of the proteins, which could be present in your sample mixture then

first of all, when need create that finger print or blue print of all the proteins and use that by the process known as global protein expression.

Second thing which is most commonly being used, is you want to compare the protein abundance from a given sample to a treatment sample. So, that is known as differential protein expressions are also known as abundance base proteomics. You want to look at the proteins are either going up regulated or down regulated in their expression, because of a given treatment are because of a disease condition or because of visual experimental condition.

So, in both of these cases the two dimensional electrophoresis can be use to resolve the proteins from very complex mixtures, people also used to study different type of isoforms or the full translation modifications, which occur in that process. What are the major advantage of this technique is the visual analysis of protein, when you stain a gel after the two dimensional electrophoresis process, then you can see all the proteins spot on the gel, which is not possible from the gel flow approaches such as mass spectrometry. Over there most of your analysis depends on your spectra and you have to rely on your analysis; in this case here your all the protein spots are already present and you can visualize that and then you can use each of these spot to further analyze and compare the images.

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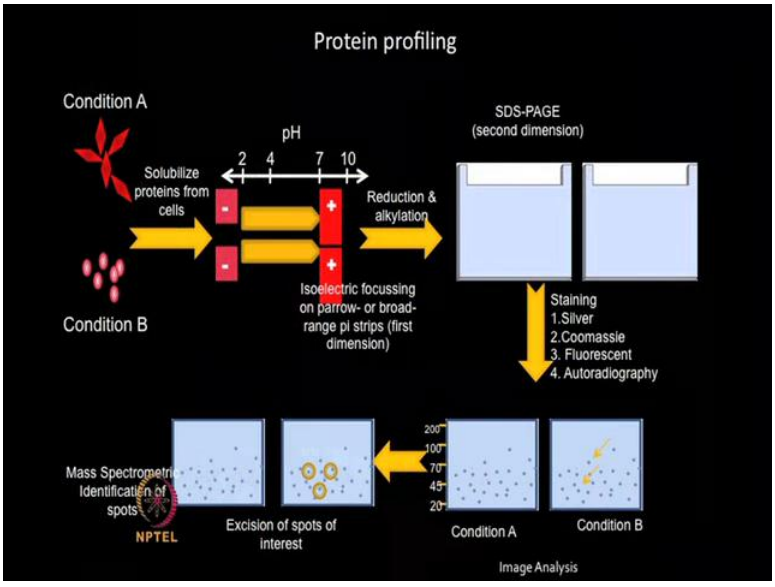

So, the protein profiling, if you want to compare your different sample types from condition A to condition B if we need to solubilize the proteins from the cell. And separate all the proteins by applying the pH which is shown here in the first dimension after that you need to reduce and alkylate your samples. And then separate that in the SDS page based on the second dimension molecular weight; and depending on your staining method being used, whether coomassie brilliant blue or silver stain, different type of fluorescent stain or autoradiography, you can visualize the protein spots.

Now, one can compare these images from condition A to condition B and if there are some proteins, which are different in their expression, those can be the proteins of your interest. One need to analyze that from different type of different replicates both technical and biological and then obtain the statistical information for the spot. And if these are significant then this is the protein of your interest, which you would like to identify by using mass spectrometry.

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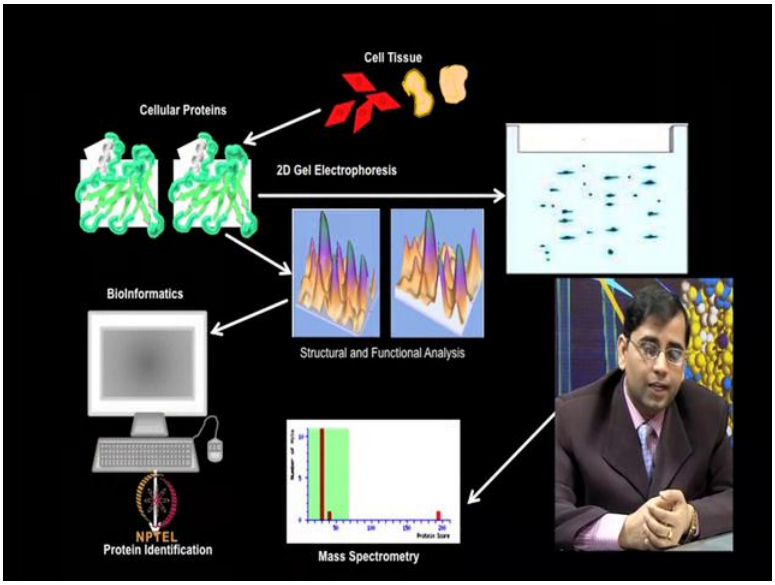

So, I am giving you a view of abundance base proteomics or differential expression profiling, where you can separate the proteins and you can compare the abundance of each spot as show in the 3D views. And then the spot of interest could be trypsinized and it can be identified the type of spectra that can be generated from mass break.

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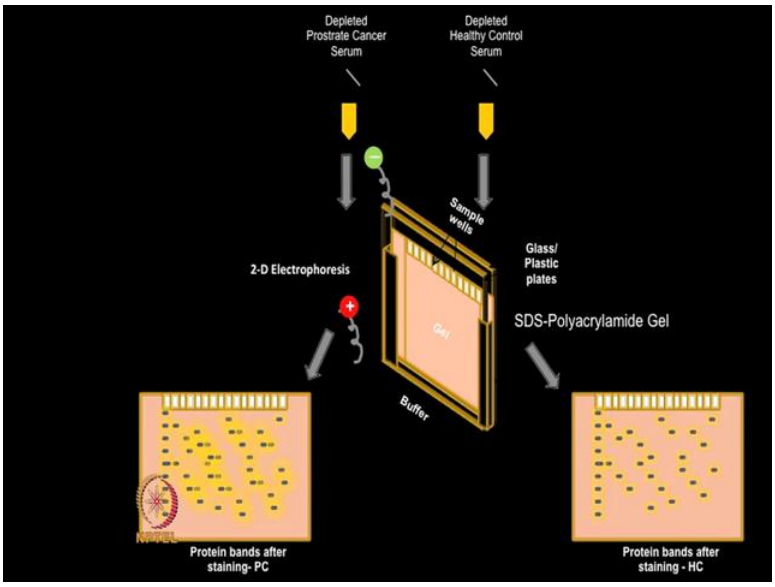

Now I am giving you one application example for example, you want to study the prostate cancer and we have obtained the serum sample from these patients suffering from prostate cancer and you want to compare that with individuals, who are healthy. So, both the samples I will talk about some of the details of these separation in the following 2D workflow and you have also studied different type of protein extraction and depression of serum. In the previous lecture, now you can apply those together here, that after obtaining the serum sample as you have learned in the last lecture, you can deplete those serum to remove the abundant proteins.

And then you can separate the proteins on the gel after obtaining different gel images, you can compare and if there are some proteins, which are showing difference in the abundance based on up or down regulation and statistical significance then these are the spots for further characterization.

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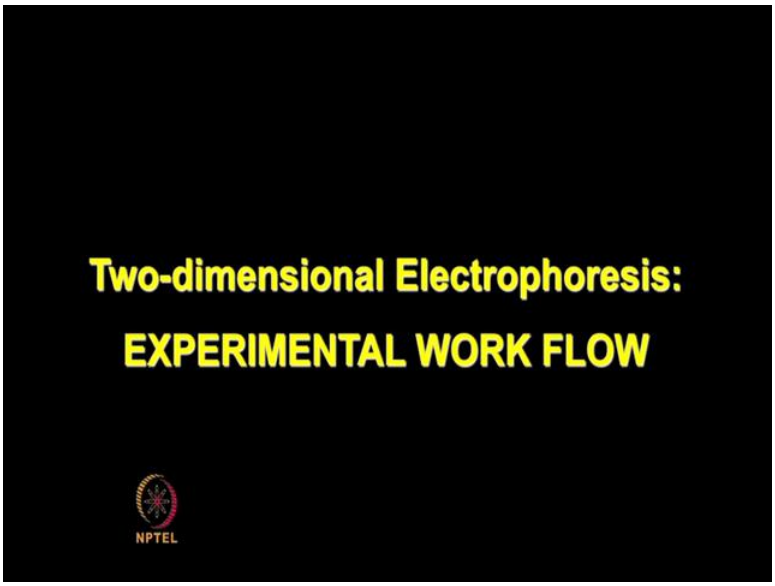

So, now let us go to the detail part of each of the processes involved in studying two dimensional electrophoresis, the experimental workflow, the different steps which are involved in making a good gel.

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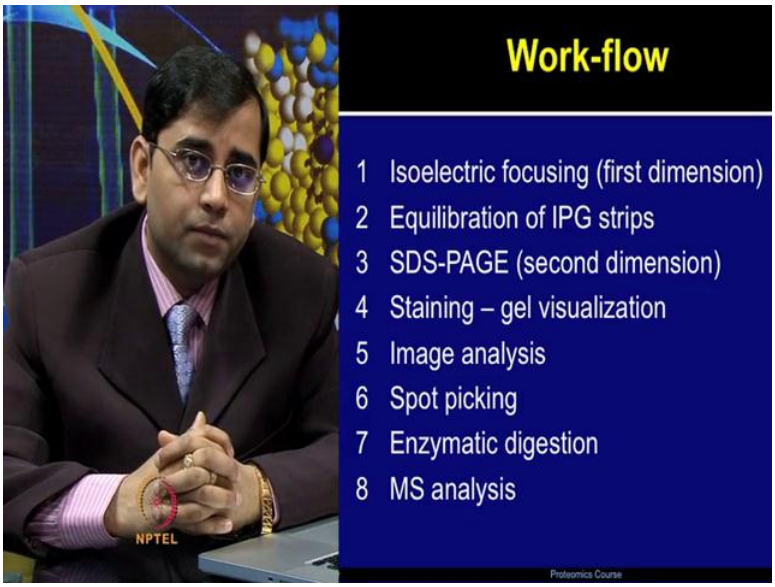

So, the first point in that workflow is isoelectric focusing, which is the first dimension separation of the protein based on isoelectric point. Second step is equilibration of immobilized pH gradient strip, when you are running an SDS PAGE gel you make your

protein denatured you want to separate that based on the molecular weight and you heat the samples you apply different type of denaturant.

Now, in this case when you are doing 2-DE your first dimension separation has occurred on the IEF in the immobilized p h gradient strip. Now you want to take strip and want to apply that on an s d s page gel you cannot do the heat treatment. So, you want to make the strips prepared to be separated based on the molecular weight, in the second dimension and that is where the equilibration process is peaceful. Once you have done the equilibration of these strips then protein can be further separated based on the SDS page the third point.

Now, fourth in the workflow is staining the gels and visualizing the gel images after staining with different type of stains image analysis, the fifth point is very crucial, because you need to analyze the all the protein spots, so that the bindings of each of these proteins can be calculated and more detailed statistical analysis can be performed.

Once you are confident that, these are the proteins which are highly significant then, you would like to pick those spots by a process mentioned as sixth point, which is spot picking. After that if you want to identify and know that, what this protein is then you need to do the in gel digestion or enzymatic digestion of these proteins obtained from the gels. Then the last point will be the mass spectrometry analysis which will be different series of lectures in the other module based on the mass spectrometry.

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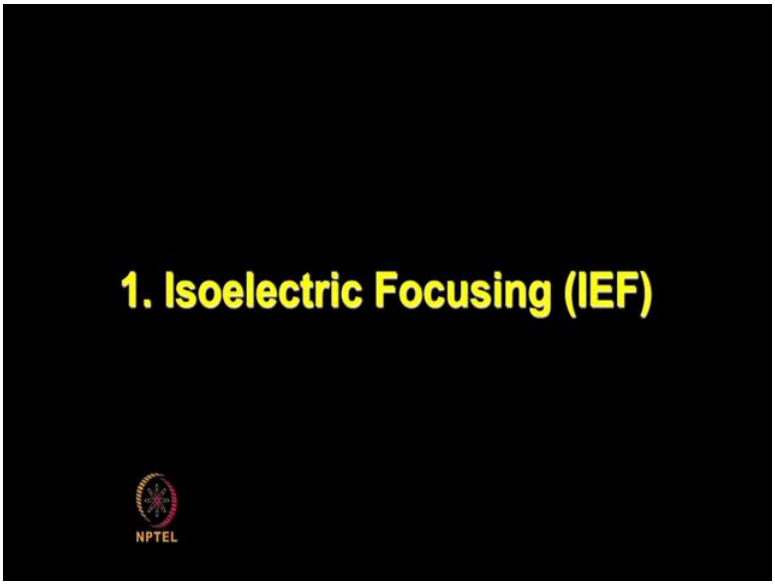

Say let us talk about the first point isoelectric focusing or IEF.

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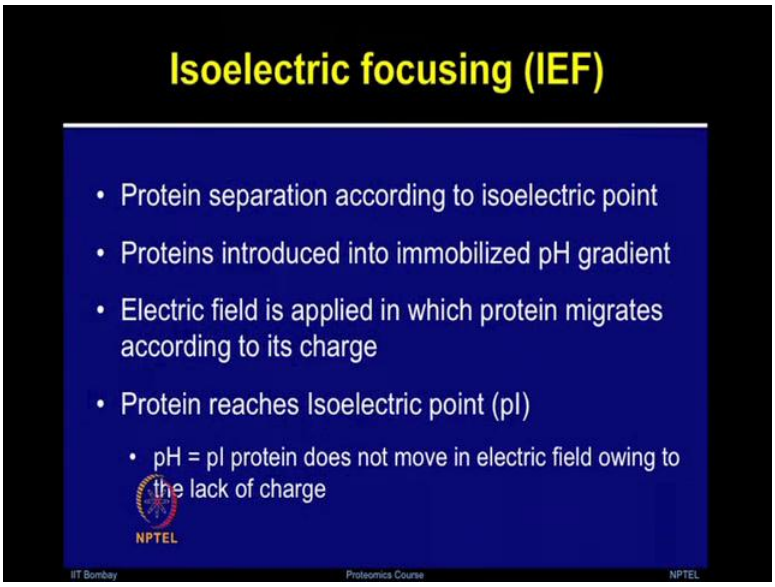

In the IEF process proteins are separated according to the isoelectric point you can take the immobilized pH gradient strip. And apply the protein solution on the IPG strip, so that protein is absorbed on the strip and then after applying the electric field the proteins will migrate according to its charge. When protein is reaching to it is isoelectric point where pH becomes equal to its isoelectric point or PI, then proteins do not move in the electric field because of the lack of charge. So, when p h request to PI then protein will not move any further.

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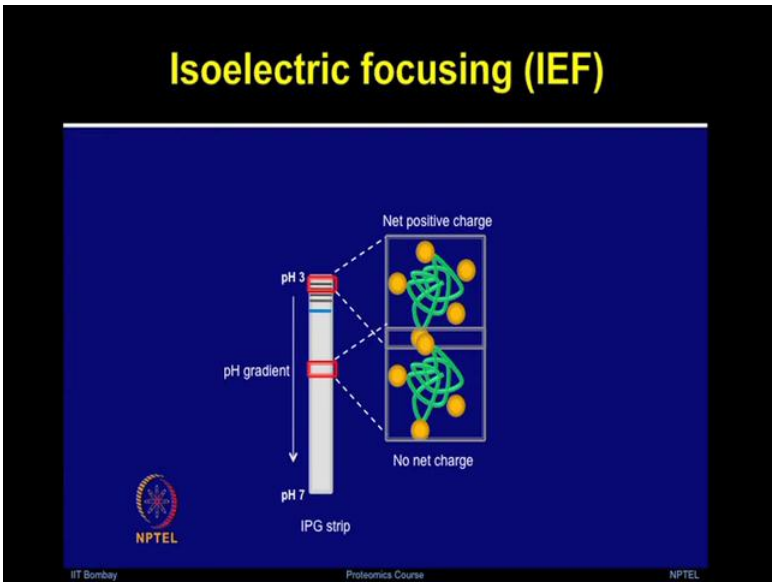

Let me show with this process in this animation here, you have any IPG strip with pH 3 to 7 gradient, when you are moving in the electric field from the positive charge towards the negative charge. When pH becomes request to it is P I then there is no net charge and protein will remain over there, so now, this will be the isoelectric point.

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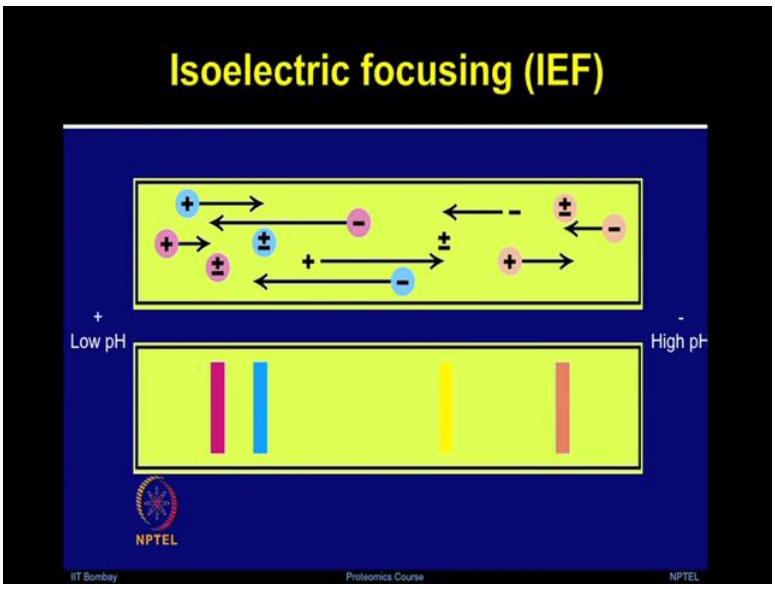

And this view you can see from the low pH to the high pH, proteins are moving in electric field and once they reach to a stage, where there is no net charge or pH becomes request to their isoelectric point P I then these discrete band can be seen.

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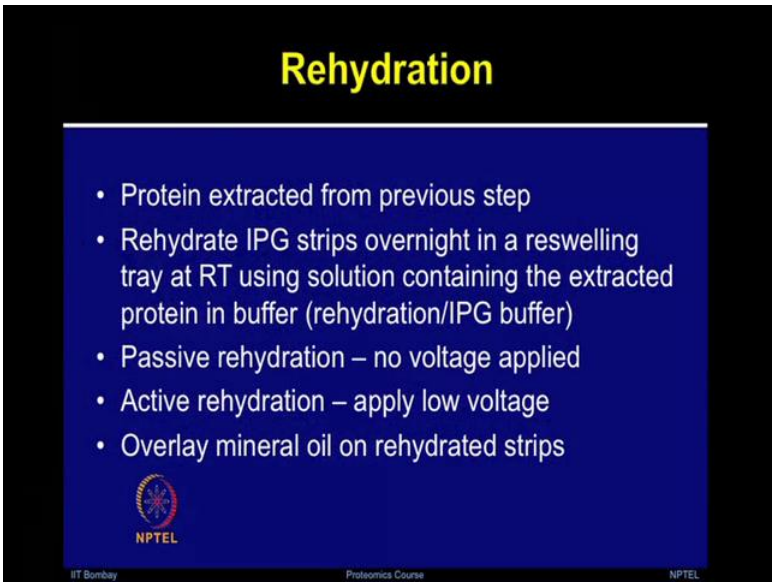

So, the rehydration is one of the important step before applying the electric field in the IEF process as you have seen in the previous animation, that to rehydrate the IPG strip you can apply the protein solution on the IPG strips. And different methods can be used for this whether passive rehydration, where no voltage is applied or active rehydration where you apply the protein sample and a very low voltage is applied to resolve the proteins.

Now, we have already discussed that none of these methods can be compared and said as the superior method, one has to really use that in their own biological sample and see where the protein separation can be better, based on whether passive rehydration or active rehydration. Once you have done the rehydration process then one can apply the mineral oil to avoid any protein evaporation from these IPG strips.

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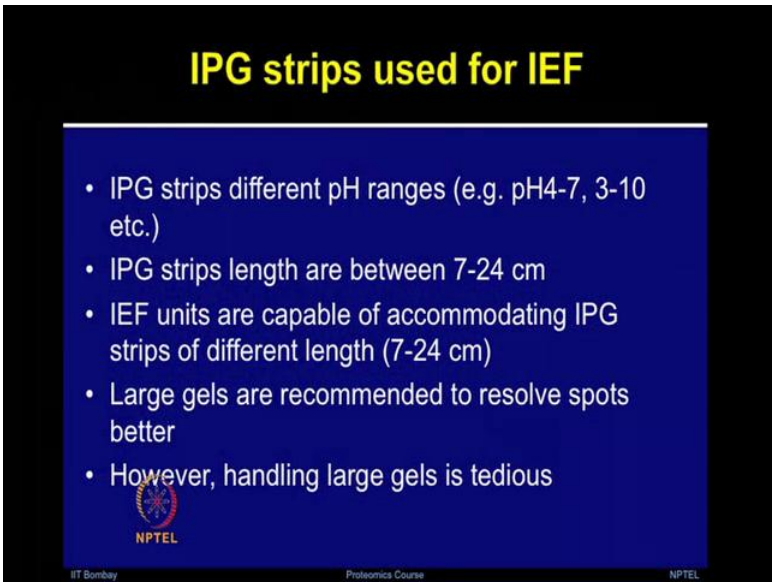

Now, different type of strips can be used during the IEF process whether its p h variant of 4 to 7 or 3 to 10 or some other pH range as I described in my previous slide depending on your biological question, if you are mainly interested in separating the proteins in their biological pH range. Then 4 to 7 could be a good choice where you can have better separation of the protein but, you may lose few proteins which could be in the stream region of acidic and basic.

Now, if you want to know all the proteins which could be present in your sample, you can apply a very broad range strip like 3 to 10. Now what type of length of IPG strip should be used one can use starting from 7 centimeter till 24 centimeter, there are different type of strips being manufactured from different commercial companies.

Now, when you are optimizing a protein extraction protocol, it is better if you use a small strip, so that you know that your IEF process is going fine and your protocol is looking good you can separate the proteins. Now once we know that your extract is good then, you can apply that to separate the proteins in the long strip length for example, 17 or 24 centimeter.

Now, these IEF units or isoelectric focusing units obtained from different vendors commercial manufacturer, they are capable of taking the IPG strips of different varying pH length. Few instruments take a flexible length from 7 to 24 centimeter other

commercial instruments, they are able to take different type of trays designed for each type of strip length.

Now, depending on your proteins how much protein sample you have in your protein in the sample mixture, it is always better or good idea to resolve that on the very large gels. For example, 18 or 24 should be a better choice but, if you do not have enough protein to separate, then you have to restrict yourself to smaller strips or one can still go with the large strip but, apply different more sensitive stream.

So, when we will talk about different type of staining methods at that time we will talk, even if you have low protein you can separate on the larger but, then apply more sensitive stain such as silver or SYPRO ruby. But overall the large gels are recommended for doing any type of differential proteomic analysis, when you want to compare your controls and treatment spot but, handling the large gel is very tedious. So, I show you some lab demonstration the video for that. So, that you can be well prepared to perform these experiments in your lab.

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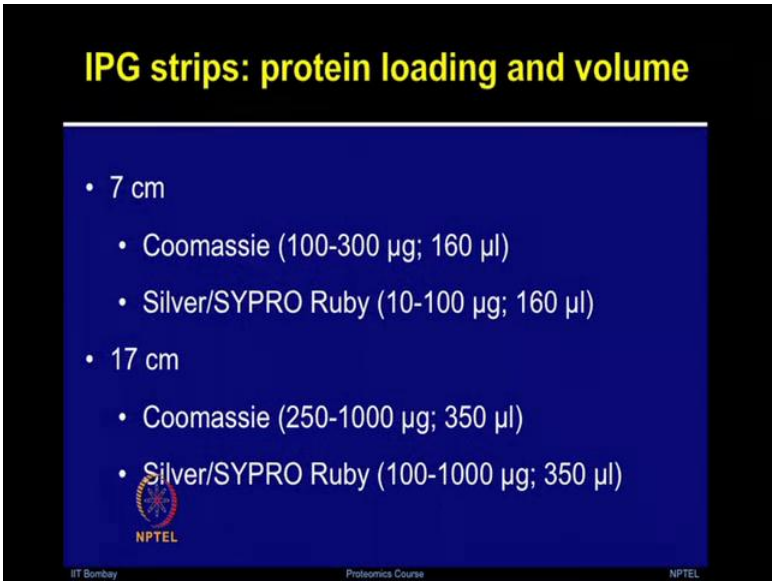

These are some recommendations for how much protein one should load on the IPG strips for example, if you have small strip like 7 centimeter length, you can apply between 100 to 300 micro gram of protein and maximum volume could be 150 to 160 microliters. Usually Coomassie stain is a good choice and if you have Coomassie stain

you can stain that, with this much protein it is your protein sample is having very less amount of protein.

For example, 10 to 100 micro gram at that time we have to apply the sensitive stain, such as silver or sypro ruby one can also apply the large strip like 17 centimeter or 24 centimeter strip but, then you have to increase the protein amount to be loaded and separated on the IPG strip. For example, between 250 to 1000 micro gram of protein can be loaded on the large strip and one can visualize that by using coomassie stain. Total reaction volume should not be more than 350 microleters.

But if you do not have too much protein present in the sample then, still you can apply these method you can apply the larger strip but, then you have to stain with silver or SYPRO ruby in that case you can load between hundred to 100 to 1000 micro gram of protein. So, I will give you the laboratory demonstration of isoelectric focusing, so that you can be very clear about different intercreative steps involved in performing IEF.

Isoelectric focusing this process involves two major steps, rehydration of the IPG strips and focusing of the rehydrated strips. IPG strip rehydration clean all the apparatus thoroughly before use to avoid any contamination, add the reconstituted protein sample in a well of the rehydration tray. Then remove the IPG strip from its cover and place it carefully in the well, IPG strips are used for separation of proteins based on their isoelectric point, these are acoelomate coated plastic strips containing immobilance of various pH spread across it, they have successfully replaced conventional tube gels due to their reproducibility they can achieve.

After around 30 minutes, pour some mineral oil over the strip to prevent it from drying up cover the tray and leave it overnight to allow rehydration to occur. Isoelectric focusing initialize the instrument and clean the surface well with a dust free cloth, place the focusing tray on the instrument and ensure that it is properly balanced. Cut the paper wicks required for focusing to a suitable length and wet them with a small amount of water before use, carefully remove the IPG strip from the rehydration tray and drain out any excess oil by blotting it on a tissue paper, place the strip in focusing tray and immerse it with mineral oil.

Then place the two wicks at either end of the IPG strip followed by an electrode at each end fill all the adjacent wells with mineral oil to ensure uniform current flow. 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 on the basis of net charge of the protein, proteins will migrate along the strip and come to rest at a point when the net charge becomes 0, known as their isoelectric point.

So, in summary in today's lecture, we have discussed about a gel based proteomics different type of techniques being used in gel based proteomic process. We have talked about different type of electrophoresis methods being used including SDS page and two dimensional electrophoresis. Then we started discussing about the details of 2D electrophoresis process and I have given you the work-flow.

In that work-flow we talked about isoelectric focusing or IEF process in more detail you have seen animation for 2D electrophoresis process as well as the laboratory demonstration of IEF process. I hope by now you are cleared that what are different type of technique being used in gel based proteomics. And what are different work-flow steps involved in performing this process. We will continue our next lecture on the 2D work-flow and we will study about the next steps, including the equilibration of the strips SDS page, staining gels obtaining image analysis of those images is spot picking, etcetera thank you.