Bioengineering: An Interface with Biology and Medicine

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Lecture – 34
Techniques to Study Protein & Proteome-II

Welcome to MOOC-NPTEL course on Bioengineering, an Interface with Biology and Medicine. In the last couple of lectures, we have started studying about amino acids and proteins and how studying the complex protein mixtures in totality comes under the field of proteomics. There are different tools and techniques which are being employed to study the proteins and we started discussing about the electrophoretic techniques in the last lecture.

We talked about various type of protein separation technologies, especially SDS page and 2-dimensional electrophoresis. We have seen that, you know, 2-dimensional electrophoresis is very elegant technology which can separate proteins based on its isoelectric point and molecular weight and very simple for any laboratory to perform these kind of experiments. However, when it comes to the large number of samples to be realized and especially the clinical sample kind of analysis, it starts posing some challenges.

And one of the major challenge is gel to gel variation. Many times there are issues of data analysis and there can be some user bias in data analysis because the gels have to cropped. It has to be matched. Two gels cannot be exactly same. So there could be some bias of analysis, how you are defining the spot boundaries in the analysis process, can be subjective. There are other systemic variations as well in this technology and of course, there you have to handle the inherent biological variations of individuals or biological samples.

So considering these facts that, you know, on one hand we have biological variations. Then if human technology has some variability, then it becomes very difficult to draw conclusions from, you know, in quantitative manner. Say if intention is to do quantitative proteomics, when you want to compare every protein from the healthy individual to disease individual or control under test conditions, then you have to look for some better alternative approaches.

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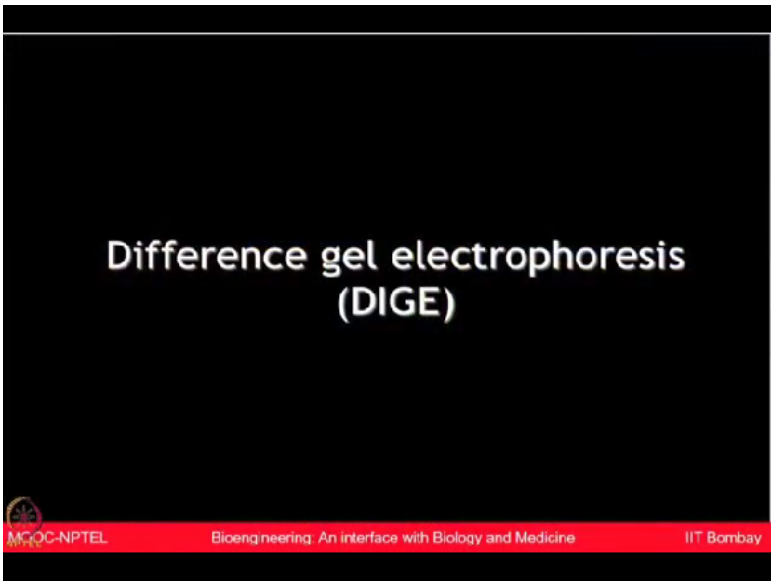

And that set of technique DIGE or difference gel electrophoresis has come forward and started playing an important role in area of gel based quantitative proteomics.

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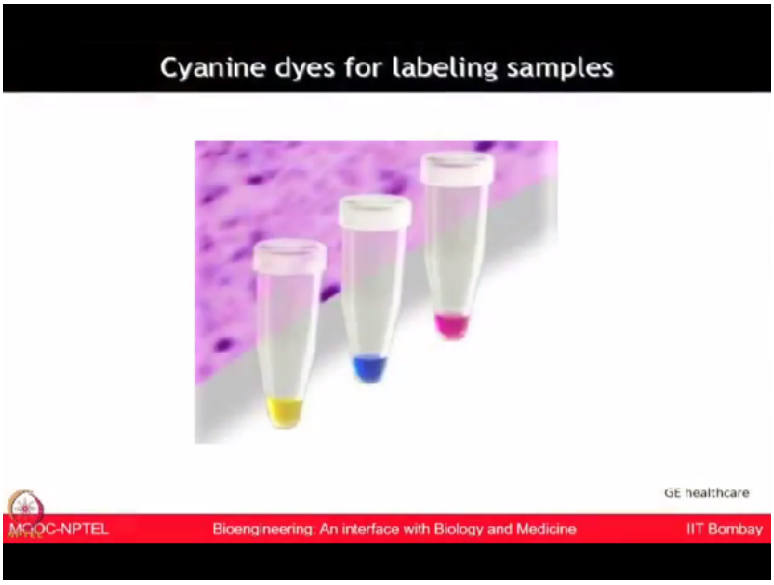

So DIGE technology, different protein samples are labelled with different cyanine dyes. They are then mixed together and then run again like a 2D gel. So you are in some way utilizing the 2D gel properties but to avoid the variability of one to other sample, you are labelling them with some dyes which are cyanine dyes. So what are cyanine dyes? These are derivatives of N-hydroxysuccinimide which covalently binds the epsilon amino group of proteins on the lysine residues.

They are respectively resolved. They fluoresce at distinct wavelength and therefore, even when you run the samples on 1 gel which are labelled with different Cy dyes, you can scan them on different wavelengths. So the labelled samples can be a mixed organ on one single gel and you can now eliminate the major problem of gel to gel variability by employing the cyanine dyes based DIGE technology.

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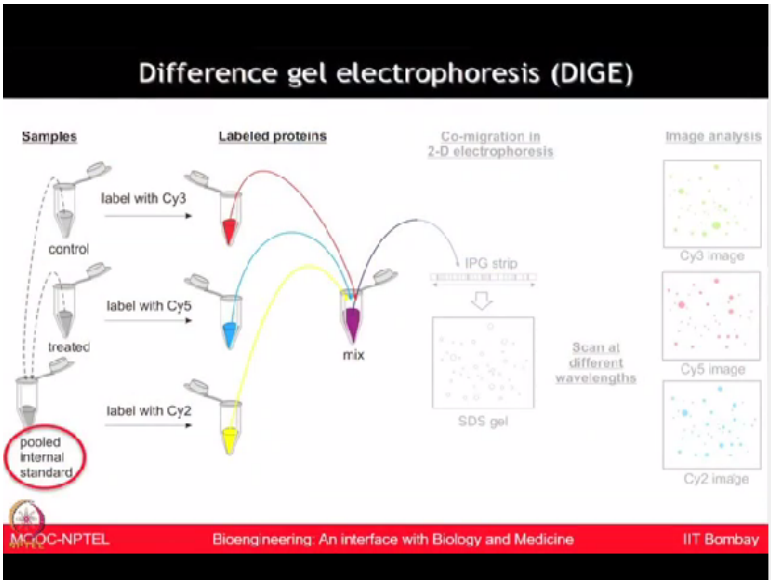

So let us look at the overview of difference gel electrophoresis. Imagine your control sample is labelled with one of the Cy dyes which is Cy3 dye and your treated sample is labelled with Cy5 dye and like that you have, you know, 10 healthy individuals and 10 of the disease individuals, so you can label them separately with Cy3 and Cy5 dyes but additionally because you are going to run on 1 gel, 1 healthy and 1 of the disease individual samples, you are going to separate them on 1 gel.

So like that you have to run 10 gels. So if you want to cross compare these 10 gels or for that matter, you know, protein profiles of different patients, then you need to have some sort of, you know, the common parameter to be analysed. And that is internal standard. So you can take the small amount of protein, equal amount of protein from all of these, you know, healthy individuals and disease individuals which you are talking in this context.

And then you can mix them together to make a pool and that pool could be labelled with a third

dye which is Cy2 dye. So now you for the gel 1, you have, you know, let us say healthy one H1 and then you have the disease one D1, plus you have internal standard. For the gel 2, you have H2 and you have D2, plus again internal standard and like that you can have HN to, you know, DN and plus internal standards. So each gel will have internal standard and it will have one set of healthy and diseased individual proteins.

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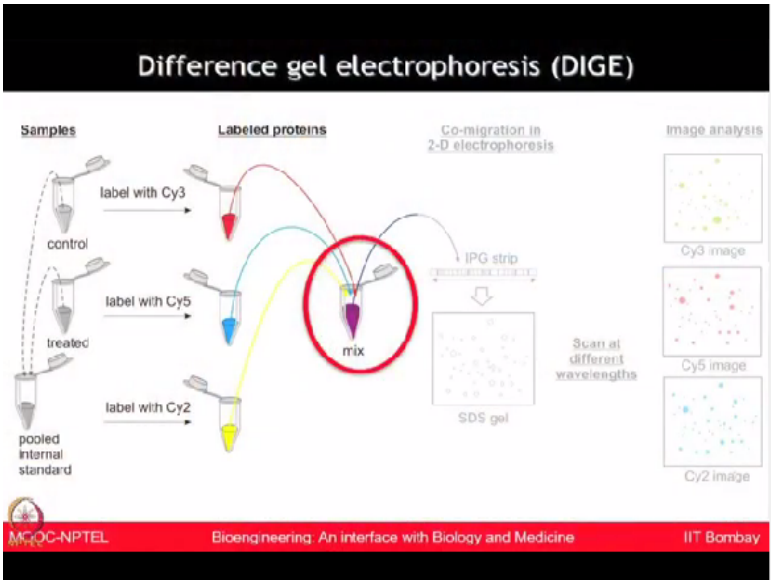

You are mixing them together. So let say H1+D1+internal standard, those 3 you have mixed together and now made a pool in a tube and from there onwards the process becomes exactly similar like what you have done in 2D electrophoresis.

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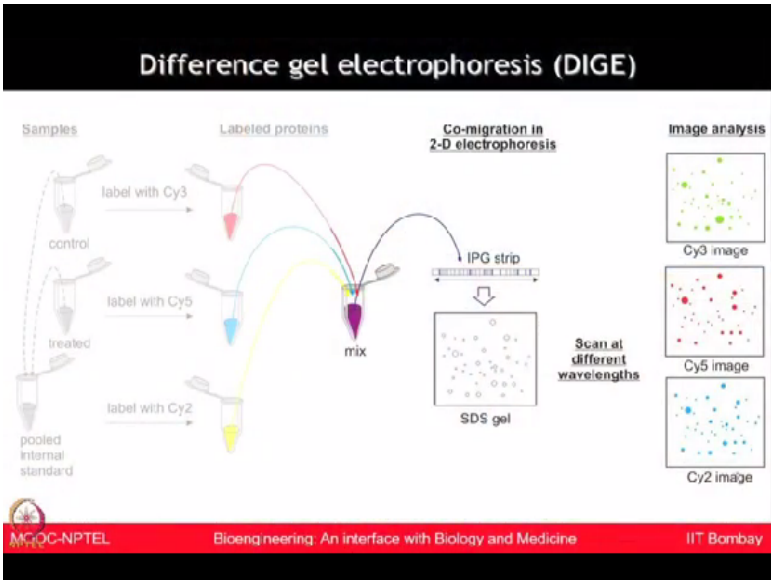

So now you can rehydrate this protein which is having all the 3 types of samples, your healthy individuals, disease individual and the pool internal standard and then it has all the those dyes from which you have labelled the proteins. Now you are doing the rehydration, you are doing isoelectric focussing and then subsequently, you are doing SDS page or second dimensional separation.

Once you have done that, then now you can scan this one gel which has, you know, both healthy and disease individual profile. Those 2 you can now separate on the single gel and you can scan them on different wavelength.

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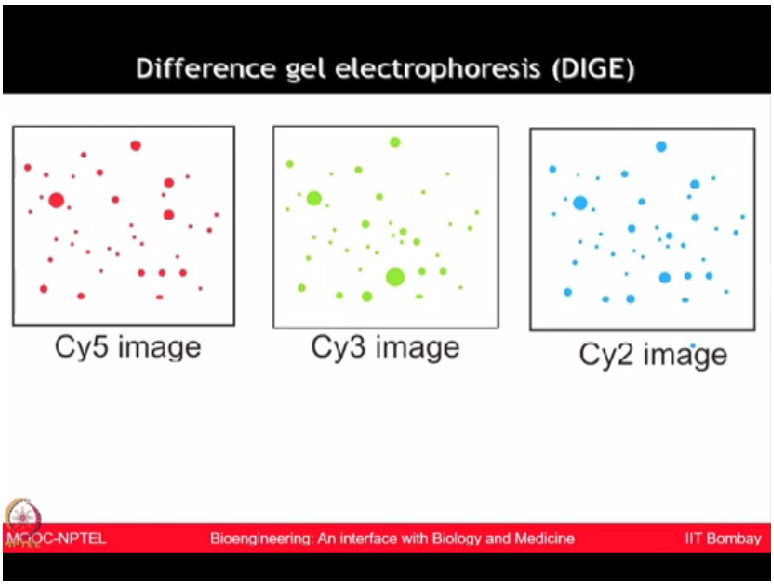

And now you can obtain different patterns of Cy2, Cy3 and Cy5 images which could be utilized for further quantitative analysis. So when you are aiming to do the labelling of the proteins, there are 2 broad strategies which people have used. One is the minimum labelling and other is saturation labelling. In minimum labelling, the epsilon amino group of the lysine is targeted for labelling.

These Cy dyes, especially Cy3 and Cy5, these are charged and size matched and there are no multiple labels which is required for, you know, these kind of technologies. It can label 3% of all the proteins. So therefore, this is one of very robust way of doing the protein labelling using DIGE technology especially Cy dyes. Alternatively, one could also label cysteines and that is

known as saturation labelling.

So those are again the charge neutral size matched, can be used in the context of, you know, the proteins which are having high propensity for the cysteine amino acids. So as I mentioned in the as comparison with the 2D electrophoresis, the DIGE technology is much superior for the qualitative purpose and that superiority comes mainly because of the internal standard which is being used and that will be used in all the different, you know, samples for analysis, could be used for the normalization and co-detection purpose.

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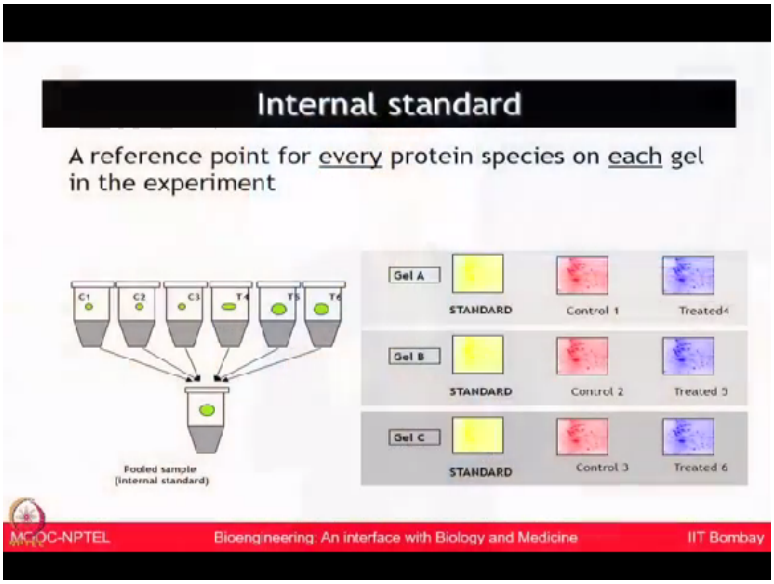

So internal standard is a reference point for every protein species which is present on each gel in the experiment and here it is shown that, you know, in which you need to make the internal standard when you are planning for experimental design. Also it is important for you to not have any bias when you are doing these kind of technologies, high throughput technologies, you need to make a robust plan and ensure that, you know, you do not have any bias.

So for example every sample if you are labelling for healthy individuals with Cy3 dye and every disease individuals with Cy5 dyes, there will be some bias. So you need to do the Cy swapping so that you can have few individuals of, you know, healthy population with Cy3 dye and few have been from Cy5 and vice versa for the disease population.

The internal standard which is the pool internal standard actually provides lot of benefits in this technology where every protein now present in any given population let us say there are 10 healthy and 10 disease individual, many proteins will be unique in those each individual. Everything will be actually present on each gel as a part of the Cy2 dye when you are, you know, getting the image obtained from the Cy2 dye.

It also decreases the gel to gel variation because now you can use this Cy2 image pattern from all the 10 gels to compare them one by one. Each sample can be now compared internally because of the same standard being used and this makes it much more robust and much more accurate for to provide the statistics for the each spot.

So by employing this kind of, you know, some changes, for example internal standard and use of the Cy dyes, the difference gel electrophoresis gel based technology has become much more accurate and become much more robust to provide the quantitative proteomic changes.

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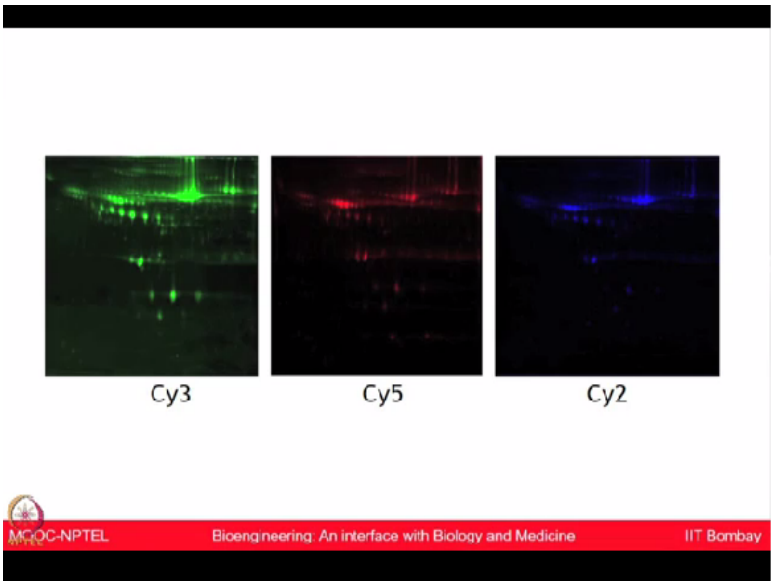

So here now you can see that, you know, the same gel is now scan a different wavelength and you can obtain a Cy3, Cy5 and Cy2 images of the same gel.

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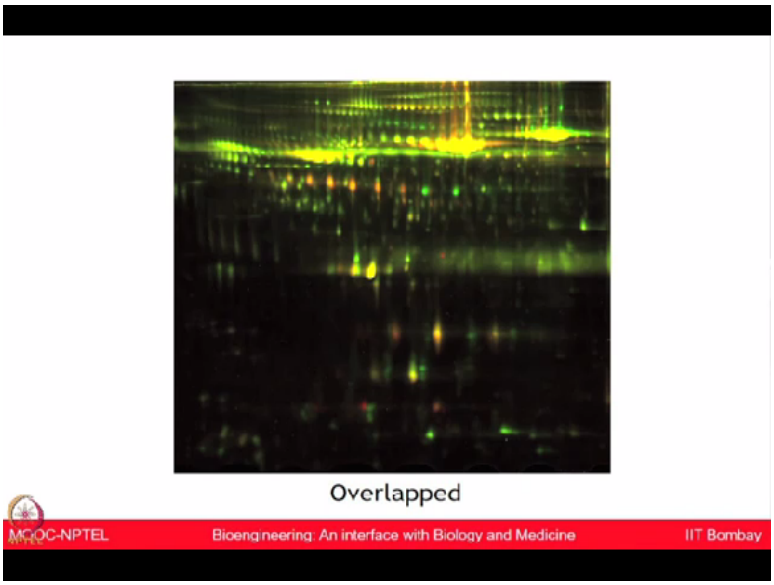

And now if you overlap them, you can see these kind of beautiful spots lighting up. This is one of the actual experimental gel obtained from one of the serum proteomics project. So now you can see there are some spots which are shown in the red colour, few spots are coming in the green colour, but most of the spots are yellow in colour. When it is yellow in colour, it means there is no change in the expression.

When it is red in colour, probably one of the condition, either healthy or diseased, is showing high expression of those proteins and the green means, you know, these proteins are highly prevalent in other conditions. So just by looking at these spots, one could obtain multiple information of the 2D dye gels.

For example, their isoelectric point, their molecular weight as well as their, you know, expression values, what kind of changes are happening. And of course, then you can go back and analyse the data in more detail and look at each spot why, what happens in the healthy individuals versus disease individual.

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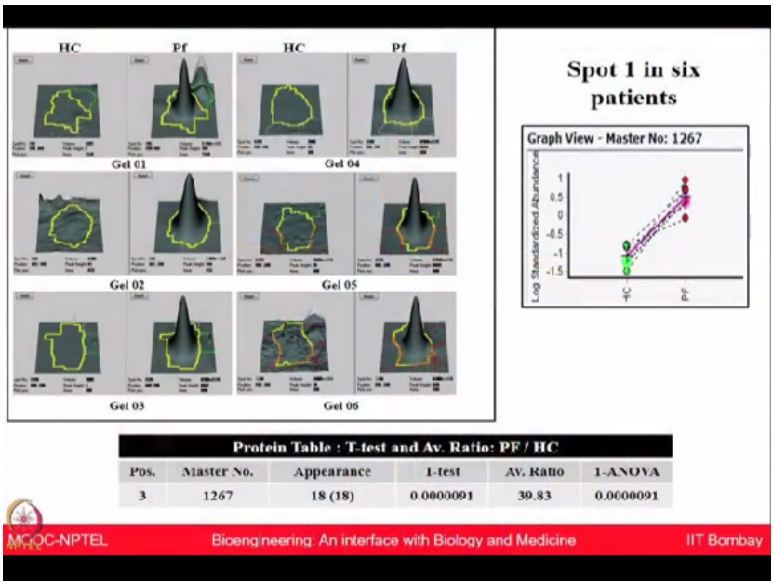

And if you can see sometime these kind of, you know, 3D spot views which shows here that, you know, some of the spots are shown quite, you know, highly expressed in a disease condition. That looks pretty, you know, interesting because in many gels, as you can see here in all the 6 gels, this one particular protein is highly (()) (10:05) in the 3-dimensional views when we visualize and that shows kind of, you know, in all the patients are expressing this protein in very high abundance.

And now this is something which is very interesting protein for you to identify and take it forward for your research purpose. So DIGE technology, what kind of advantage it provides. You are avoiding the source variations. You are avoiding gel to gel variations because now you are adding the internal standards. You are adding very sensitive cyanine dyes and by, you know, doing the dye swapping, you can actually eliminate those, you know, biases of the comparison.

So by using these internal standards and the dye swapping method, now you can achieve very high reproducibility and you can achieve high degree of automation and you can use the co-detection process to ensure that now your gels are analysed properly without any bias. So in the conventional technologies, there is, you know, as I said, it can be user bias, may have in their traditional 2D electrophoresis.

Whereas in the DIGE technology, the softwares are very robust and they use the Cy2 image for

doing the normalization and therefore, it is in a much more robust method and there is no user interface involved to do any bias here. Lot of, you know, variability which are part of the experiment of biological samples, that of course we cannot avoid which is biological variation.

But our technical variations and analysis based variations, they have been quite a bit eliminated at the level of, you know, the DIGE technology and the software which have been, you know, come forward to analysis this kind of data and very robust statistics which can be utilized to now identify these spots of interest which could be now taken forward with much higher confidence.

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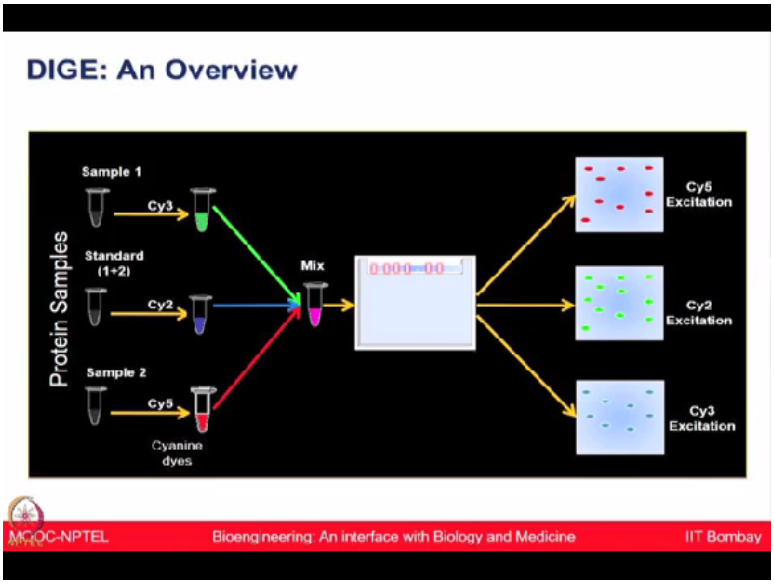

So again, you know, summing up on the DIGE technology, we can see the overview of the technology. We have, you know, we are comparing a test and the control conditions. We have labelled the sample with Cy3 Cy5 dye. We have made internal standard which is now which they labelled with Cy2 dye, mixed all the 3 samples, separate on one gel and now you can scan on different wavelength to obtain different colour pattern of these dyes.

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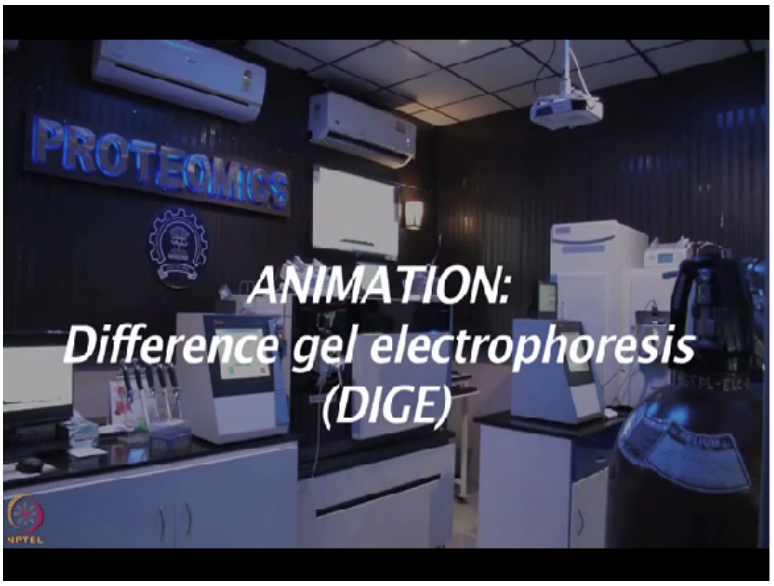

Let me explain you this in more detail in the following animation.

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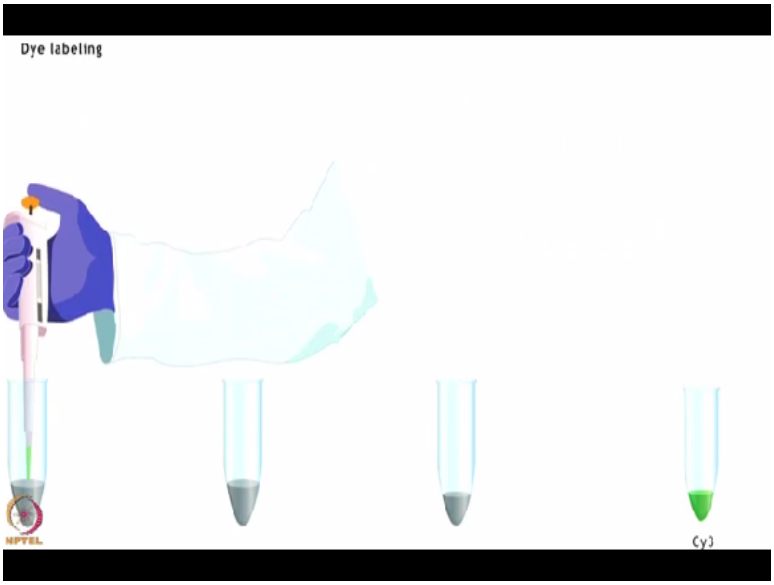

Each protein sample as well as the internal standard is labelled with a differently fluorescing cyanine dye which allows all protein samples to be simultaneously run on a single gel. The dye binds covalently to the E-amino group of lysine residues in proteins. The labelled protein samples are mixed and run on a single 2DE gel. Separation takes place on the basis of isoelectric points of the proteins in 1-dimension and based on molecular weight of the proteins in the second dimension with the smaller proteins migrating further along the channel.

The gel containing all the protein samples can be viewed by illuminating it alternately with

exciting wavelengths corresponding to the various cyanine dyes. View of a superimposed DIGE gel depicting all protein spots of multiple samples. Information on molecular weight and pI of proteins can be obtained from these spots.

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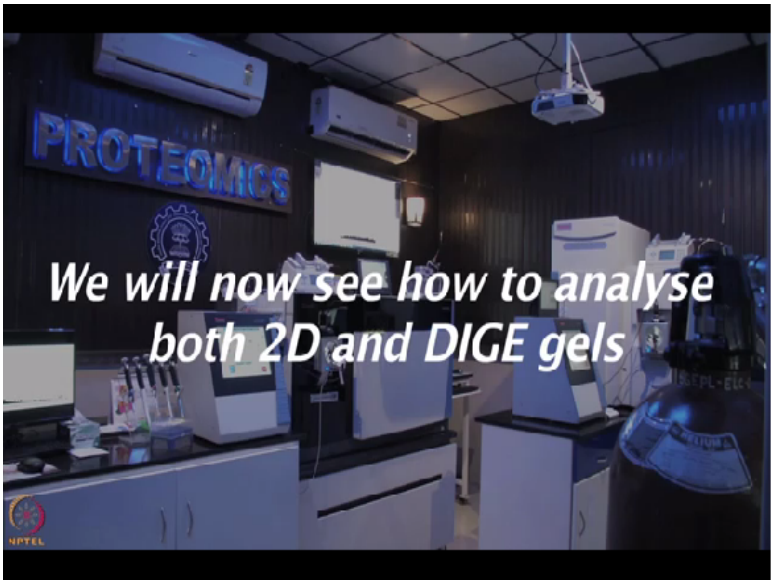

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It is possible to load either a single or multiple gel images simultaneously. This is done by means of the load option in the file menu. The saved gel images must be chosen which are then displayed on the software. Several tools are available for analysis of the gels. It is possible to crop the gels by selecting a specific region that is to be studied and then selecting the crop gels function.

Cropping gels helps in selection of regions with high spot density or in trimming of regions with high background staining of no spots. Specific selected regions of the gel can be zoomed into for viewing the spots more closely and for comparison of spots between 2 gels. This is particularly useful for gels having large number of spots. Overlaying of images is a particular useful tool for comparison of 2 gels.

The gels are overplayed such that they appear merged and spots that coincide will overlap with each other. This is extremely helpful while comparing clinical samples of control and treatment providing clear indication of the proteins that are differentially expressed. The spots on the gels can be displayed as 3 dimensional graphs. Either the entire gel can be chosen or a particular region can be selected for this representation.

The peaks obtained in the graphical representation are directly related to the spot intensity. Every spot on the gel can be detected by selecting the detect spots option. Parameters such as smoothness, saliency and minimum area must be suitably adjusted for maximum clarity. Once this is done, each spot will either be encircled or marked with a cross depending on the settings along with the spot numbers.

The software facilitates interpretation of the gel images by matching 2 different gel images obtained. The matching spots are marked and any variations in the spot position are indicated by blue lines in one of the gels. This provides an understanding about the reproducibility across gels. Information regarding the various physical parameters of each spot can be obtained via the spot table.

This table provides the spot number, intensity, area and volume of spots as well as the saliency of spots. These parameters help in judging the quality of a gel. In addition to physical parameters, several statistical parameters can also be computed from each spot on the gel such as central tendency, mean, median, dispersion, coefficient of variation, standard deviation, etc. Scatter plots and histograms can also be plotted for clear data analysis.

These provide information regarding intra and inter gel variations. It is also possible to specifically compare a particular selected spot across gels. When the gel is run with molecular weight markers, the weight of unknown proteins can be estimated from these. It is also possible to estimate the pI of the proteins. These parameters in addition to other physical and statistical parameters can be obtained for each spot.

It is also possible to specifically compare a particular selected spot across gels. When the gel is run with molecular weight markers, the weight of unknown proteins can be estimated from these. It is also possible to estimate the pI of the proteins. These parameters in addition to other physical and statistical parameters can be obtained for each spot. Software for difference in gel electrophoresis analysis varies with respect to certain features compared to normal 2DE analysis.

It can compare 3 gels simultaneously. Of which one is typically pooled internal standard containing all spots. Any changes implemented in one gel such as cropping, spot selection, etc., will be implemented across all 3 gels in DIGE. Other features and tools for DIGE analysis are seen as those used for 2DE analysis. Physical and statistical parameters of all spots of the gels can be determined through their corresponding reports. Three dimensional graphical representations provide information regarding intensity of spots on the gel.

Summing up the entire gel based proteomics which we started in the last lecture where we can see that, you know, the SDS page and 2D electrophoresis, they are very powerful technologies. However, when it comes for the differential proteomics or quantitative proteomics. 2D electrophoresis has certain limitation which has been actually quite a bit overcome by using the DIGE technology which is one of the multiplexing platform which reduces the lot of intrinsic variability which we have encountered in the 2D gel based workflow and that is kind of a way forward to do gel based quantitative proteomics.

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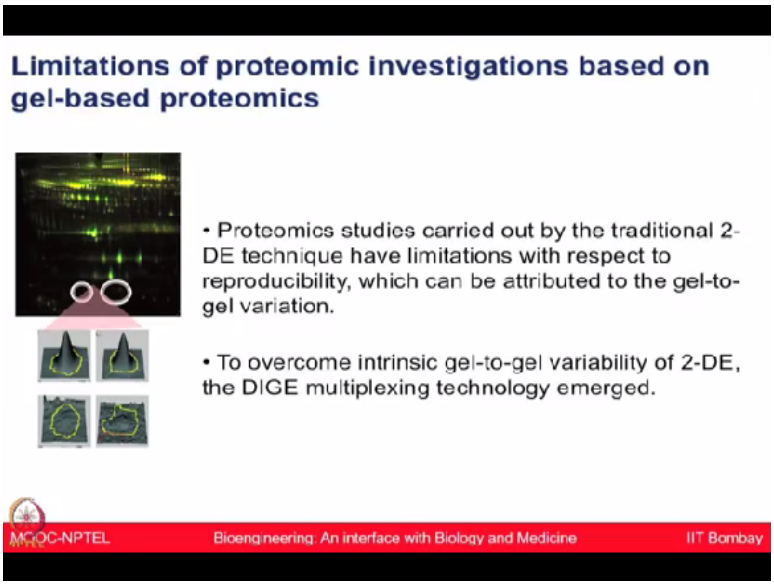

Let us now move on to think about the protein purification because, you know, the protein purification becomes very essential to study the structural and the functional proteomics study and for all kind of functional annotation, it is very crucial for you to verify a given protein which you want to further understand.

So there are several chromatographic purification technologies have been developed which aimed to utilize, you know, some properties of these proteins whether it is size, their shape, their charge or the chemical, specificity and looking at those properties, now these proteins could be separated in the chromatography.

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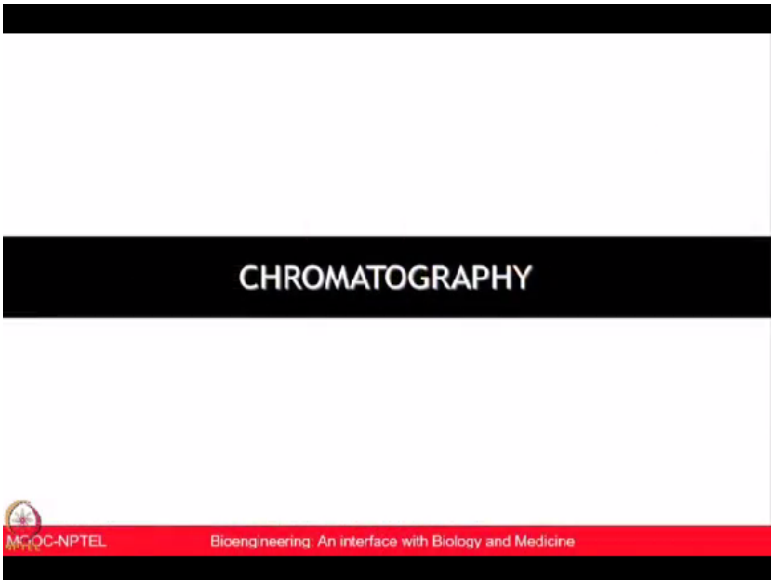

So let us talk about what is chromatography? Chromatography broadly is a collection of lab techniques which carry out the separation of complex mixtures by making use of the inherent properties of the component of the mixture. It involves the differential partitioning of molecules between a suitable a stationary phase and mobile phase. The most commonly used chromatography techniques include the gel filtration technology, ion exchange chromatography, affinity chromatography, etc.

Let us briefly talk about each of these techniques in some detail. Start with gel filtration chromatography.

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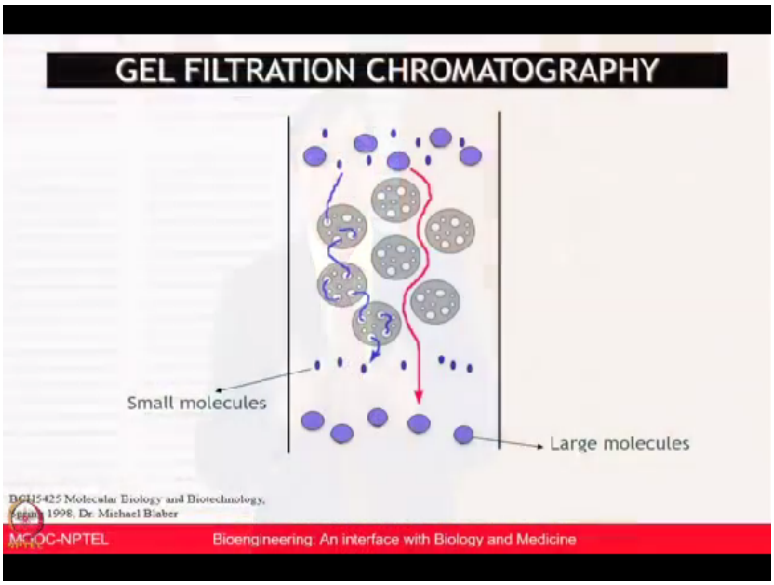

So in gel filtration chromatography, the columns are composed of the porous beads which are made from polyacrylamide, dextran, or agarose. In this technology, in the size exclusion chromatography, proteins are separated according their size. Now the small molecules like salt, they are retained longer in these porous mattresses or the beads and then larger protein molecules, they will elute first.

So now looking at their size, you can elute the proteins which are large in size first and then the small proteins are retained, they will travel longer in the beads and then eventually they will come out of these columns. So looking at their size, now you can separate proteins using gel filtration chromatography.

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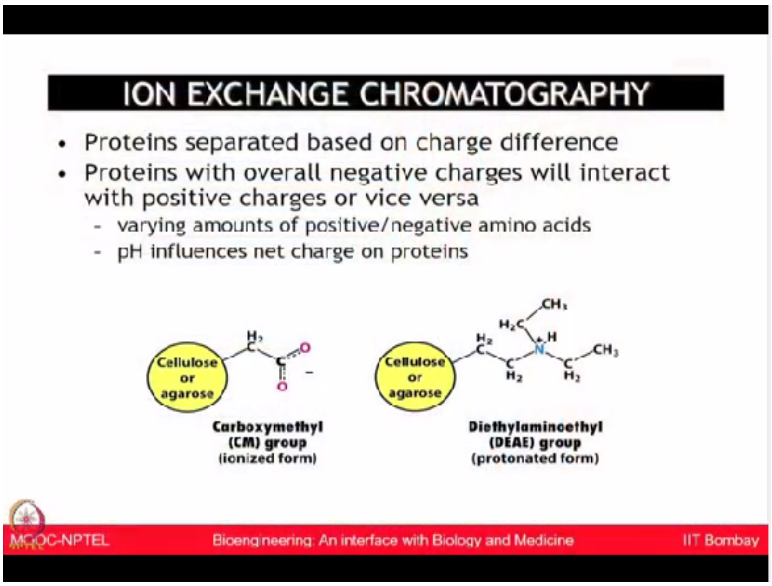

Let us now talk about ion exchange chromatography. Here the proteins are separated based on what are the charge differences in the overall protein structure is. So proteins with overall negative charges will interact with the positive charges or vice versa. So accordingly you can select the beads for example carboxymethyl group or you can choose the diethylaminoethyl DEAE group.

So these kind of, you know, beads could be chosen which now can use the properties of positive or negative charge of amino acids and use that to separate proteins in ion exchange chromatography.

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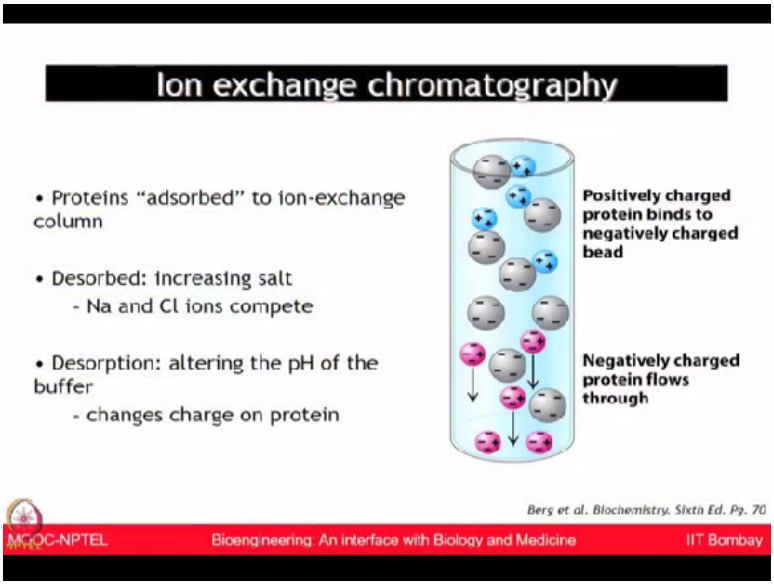

Now in ion exchange chromatography, the proteins are first adsorbed to the ion exchange column and then, you know, after they are bound with these beads, now you want to sort of desorb them by increasing the salt concentration. So therefore, now the sodium and chloride ions, they compete and then by altering the pH of the buffer, you can change the charge on the protein. So therefore, you can elute the proteins based on their positive or negative charge.

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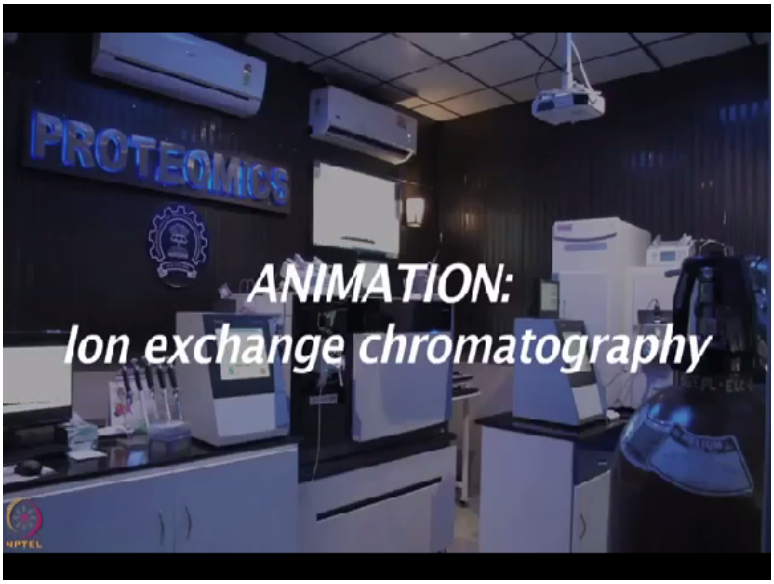

Let me explain you this in more detail in the following animation. (Video Starts 25:20 - Video Ends 30:21)

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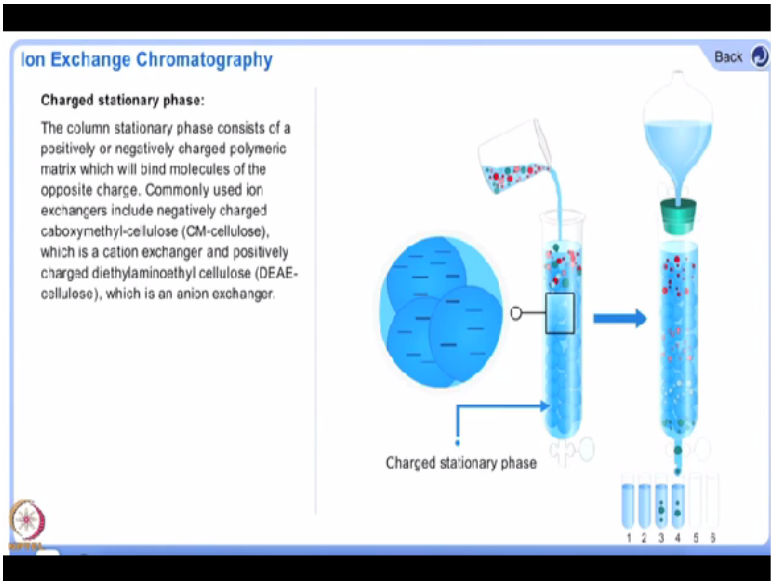

Charged stationary phase. The column stationary phase consists of a positively or negatively charged polymeric matrix which will bind molecules of the opposite charge. Commonly used ion exchanges include negatively charged caboxymethyl-cellulose, CM-cellulose which is a cation exchanger and positively charged diethylaminoethyl cellulose, DEAE-cellulose which is an anion exchanger.

Protein mixture. The unpurified protein mixture which consists of proteins of different net charges are loaded on to the column. The proteins having charges opposite to that of the stationary matrix will bind to it while the remaining proteins will be eluted from the column. Mobile phase. Following sample application, the proteins are eluted out of the column by means of a suitable mobile phase that carries the protein out with it.

For ion exchange chromatography, buffer systems of suitable pH are used which will remove the unbound proteins. The buffer is then changed such that the charge of the bound proteins is modified and they are also eluted out of the column. Effluent sample fractions. The solution leaving the column is collected in suitably sized fractions for further analysis. The unbound proteins having same charge as the column matrix will be eluted out in the initial fractions while the bound proteins will be eluted later upon changing the buffer system.

The column is packed with the suitable cation or anion exchange resin depending upon the

charge of the protein that needs to be bound to the column and purified. Here we represent purification of positively charged molecules using a cation exchanger. The cation cup exchange column is then loaded with impure protein mixture consisting of various positively and negatively charged proteins.

The column is eluted with a buffer solution of suitable pH such that the negatively charged molecules are removed from the column while positively charged molecules remain bound to the ion exchange resin. The buffer solution is then changed such that the net pH of the protein of interest is modified and it no longer binds the ion exchange resin. Therefore, the bound protein also gets eluted out of the column in this manner.

Fractions of appropriate size must be collected and analysed for their protein content. The negatively charged proteins which get eluted first will be present in the initial fractions while the positively charged proteins that bind to the column are eluted in the later fractions. The fractions are then analysed for their protein content using UV visible spectrophotometer at 280 nanometers. A graph of eluent volume versus protein concentration is then plotted with each (()) (30:08) being proportional to the concentration of protein.

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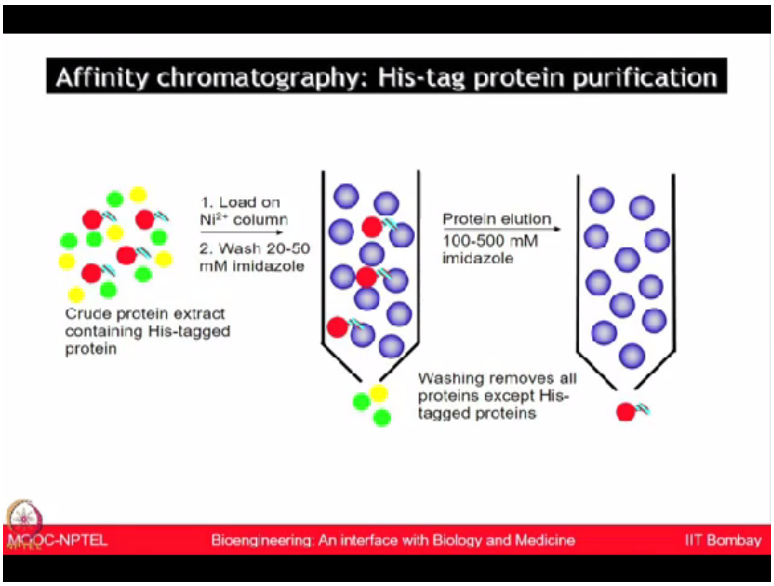

Then it comes to the affinity chromatography which is based on affinity of the protein to other molecules. It could be different substrates or products or cofactors, antibodies, or metal. These

matrix beads, they are chemically coupled to the ligands and now the proteins are actually bound through some specific interactions. So only the protein which, you know, the property which you want to utilize for protein separation, only those proteins are going to get bound and rest of the proteins will not bind and they will get eluted out.

So proteins can be then desorbed by excess ligand in the solution forms. Thinking about the DNA technologies and the genetic engineering method which we have, you know, discussed in the past, when you are doing the DNA cloning, at that time you can introduce some histidine tag in the protein and let us say that property could visualize for the affinity chromatography for the histidine tag based protein purification.

So now each protein which is formed from that gene, can take the histidine tagged based protein and now if you have a column which is having nickel NTA beads or having nickel in the column, now it is going to bind with the histidine tag with the (()) (31:34) chemistry and now only those proteins which are, you know, the protein of your interest where you have added histidine tag, is only going to get bind to the affinity column.

Then all rest of the proteins can be removed and they will all be in dilute form. So now you are going to add imidazole in the increasing salt concentration of 100 to 500 millimolar. Initially, you know, some of the weakly bound and non-specific proteins, they will get eluted out and then as you increase the salt concentration, only those proteins which are tightly bound which is protein of your interest is going to get eluted out.

So here the, in the affinity chromatography, your intention is to look for specific interactions and look for high degree of protein purification. Unlike the gel filtration technology, you are not limited with the overall sample volumes. You are not using very long columns here. And easily you are able to achieve in a much higher purity, so people use this particular type of technique specially to look into the higher protein purification.

Especially if you want to characterize the proteins further. So in summary in the last couple of lectures, we have been discussing about the various protein technologies.

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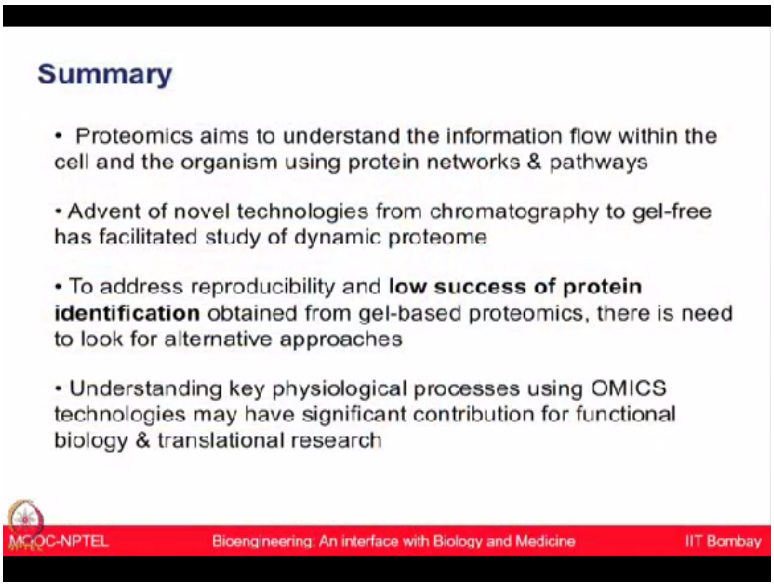

And today, we essentially looked into how the advancement of 2D electrophoresis has resulted into technology like, you know, difference in the electrophoresis which could be utilized for the quantitative proteomics based experiments. Then we talked about, you know, it is so crucial to purify the proteins and different type of chromatography techniques have been employed which looks into the properties like Cy's, like their charge and their affinities to the different substrates.

And these could be utilized by different chromatography methods to purify these proteins which could be further used for studying their protein structure and function. We will continue what some of the proteins technologies in the next lecture and then we will try to conclude how knowing the protein basics and these technologies can be so useful for various type of ongoing research in the area of bioengineering. Thank you very much.

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So in the last lab demonstration session, you have seen how 2D electrophoresis can be performed. Let us now see some of the minor changes which happens while performing difference gel electrophoresis. So let us start the laboratory demonstration session now for DIGE where we will only show you the key steps which are different in performing DIGE as compared to the 2D workflow and then rest of the workflow remains similar for 2D DIGE base experiments. (Video Starts 34:11 - Video Ends 39:05)

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So when we run a 2D gel, we normally run 2 to 3 gels. One represents a control gel. Another is a disease gel or different treated sample and then we compare the 2 different gels which sometimes leads to some reproducibility issues because the same spot will not be present at the exact

location in the 2, 3 different gels. So then we have an advance technique which is DIGE which overcomes these gel to gel reproducibility issues.

As we have to learn only one gel for 2, 3 different samples. So in case you have 2 samples. One is your control and another is your treated sample, you can mix them with Cy dyes, you can pool them and that common pool you can run on a one single DIGE gel. So we have 3 different dyes, Cy dyes, Cy2. So normally what we do suppose in case of 2 samples we have and we want to label 50 mg of each with the dye.

So we will take 50 mg of a sample and then we will add Cy3 for example in one case. The next sample we have a treated sample. We will again take 50 mg of our sample and we will add a Cy5 dye and then what we will do is, we will take 25 mg from each, the control sample as well as the treated sample. We will make a pool and then we will use Cy2 which is our internal standard.

This will make sure that issues between different samples are minimized where we use… So any variation is minimized by the use of internal standard. So we will have 3 samples, one control 1 treated and another sample is a pool of the 2 labelled with Cy2. Then what we do is? So we pool all those 3 samples. So these Cy dyes, they are light sensitive. So we have a pooled sample already prepared.

So this is our pooled sample. We have put aluminium foil and it has all 3 samples. So then the rest of the steps are exactly same as what we do in 2D. We will first rehydrate the strip using our sample. But the only thing is that we have to maintain dark conditions because these samples are light sensitive. We will carry out rehydration, we will carry out isoelectric focusing, SDS page but then later, we do not have any staining or destaining procedures.

These Cy dyes are fluorescent labelled and then we can analyse using the DeCyder software. So I will take this sample for rehydration. I will add my strip. I will put a cover and then oil and then I will also put a cloth so that it remains in dark condition and no light falls on it. So the rest of the steps, SDS page, equilibration and separation in 2D are same. Then we come to scanning of a 2D DIGE gel.

So this is the representative image of a DIGE gel. You can see there are multiple spots and definitely the Cy dyes are more sensitive than Coomassie stain. So as compared to a 2D gel, more number of spots here. So what we do usually, we scan the gel at 3 different wavelengths. So you can see in this particular image the gel has been scanned only at Cy2. In the next image, it has been scanned at Cy3.

The next at only Cy5. So you may find some spots which are there only in the image which is scanned at Cy3 wavelength. So it means there are these proteins are only present in the control samples or you may see some spots are brighter in one of the gels, vaguely a Cy5 scanned gel image. So that means that particular spot, may be that protein abundance values are more in treated sample or another sample.

So this is how the 2D DIGE principle work and it is a very important gel based proteomics approach and has definitely more advantages than the regular 2D approach but 2D approach again, a lot of people use it for other purposes. So both the techniques, they have their own merits and demerits but they are very important gel based proteomic approaches.

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So we have learnt various techniques for performing gel based proteomics. Now let us move on to chromatography based lab demonstration. You will see how specific proteins can be purified

using chromatography. So let us start the laboratory demonstration session for chromatography now. (Video Starts 39:28 - Video Ends 44:45)

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Hello. So today we are going to discuss about protein purification. Protein purification is generally use for to isolate a single protein or few proteins from our complex mixture of proteins and we are exploiting different properties of the protein such as the hydrophobicity of the protein, the affinity of the protein. Like here you can see this is a one column. There is Superdex 200 column which is used for the gel purification chromatography.

So here we can separate the protein on the basis of their sizes. So the higher proteins which are in the higher molecular weight, so they will elute first and the proteins which are in the smaller molecular weight, so they elute slowly. Once we put the sample so the small protein will pass into the pour of the base and then experience more resistance, more friction, so that is why they move very slowly.

So here we can separate the protein on the basis of their molecular sizes. These are another columns where the resins are there and the nickel NTA is there. Here we are looking the properties, the affinity properties of the protein. So like the proteins which are having a his-tag, so the his-tag can bind to the nickel NTA column so the interested protein which is having a his-tag, they can bind to the column and other proteins will come out.

Then we put a solution like imidazole solution which can compete with his proteins and can bind to the nickel NTA column. So in this way, our interested protein will come up and then imidazole will bind out. So this is FPLC system, fast protein liquid chromatography where we can use different chromatography techniques and then we can separate the protein by this system.

Here you can see there are some basic component of like FPLC system. There are 2 pumps. So this is pump A and this is other pump B. So each pump having a different buffer. So once we pass the buffer, the passed buffer will come from here and then will go there and these are the mixture of the buffer where the buffer will be mixed. So here is the injection valve. So here we can put our, suppose these are like, this has a very small amount of sample.

We can take the sample and we can put here in the injector valve. Once we put the sample here and these are the loops. So the capacity of this loop is 500 microliter. Now this is having a more volume of sample, we can use 10 ml or 20 ml of loop. The sample will pass from the injector valve and it will go through the, this column, there is a affinity column and now these are flow cell.

Each can measure the conductivity pH of the sample. And the UV detector. So once the sample will pass, so we can measure the, give you the exact protein is coming or not coming. We can measure the pH, we are changing the buffer, the pH of the buffer is changing or not and the conductivity if we are using a different amount of salt. So from here one sample will pass through, then it will go to the fraction collector.

So this is the tube which is coming from the (()) (42:37) flow cell and it will go to the fraction collector. Here 280, we have to collect this sample, so it will collect the sample in the tube. So this FPLC system, we can control by using Unicorn software. So once we click on the software, then it will open the system control, method editor and the Unicorn manager. In the evaluation, so like once you run your samples and then you have to evaluate your protein, so you can evaluate here.

So here I am just going to like system control, how you can control the FPLC system. So here you can go in manual. You can set the flow rate. So once you put here 5, flow rate 5 ml/m, just insert and then execute. So here you can see the changes 5 ml/m. Now it has been started. Now we are using 2 buffers. Buffer A and Buffer B. So now it is here to increase the salt concentration, then we have to increase the gradient.

So go in manual, click on pump. Then here you can see the gradient. So if you want to achieve the gradient 80%, we click 80% gradient. In the 80% gradient, the time that will take for. If we want to achieve 80% gradient in 5 minutes, so we will take here 5 minutes and then just insert and then execute. Now here once you setup the parameters, the protein is eluting. So it is measuring 280, the program at 280 protein, so here you can just leave it going.

At this state of protein is coming out of the column. So once you run the sample and your protein will come out, so here like it will get this type of heap. So at 280, it will absorb and then it will give the intense move. So here you can see this is protein, your protein eluting out and here like at what fraction. So you can elute those fraction and then can kind of store and this will be your purified protein. Thank you.

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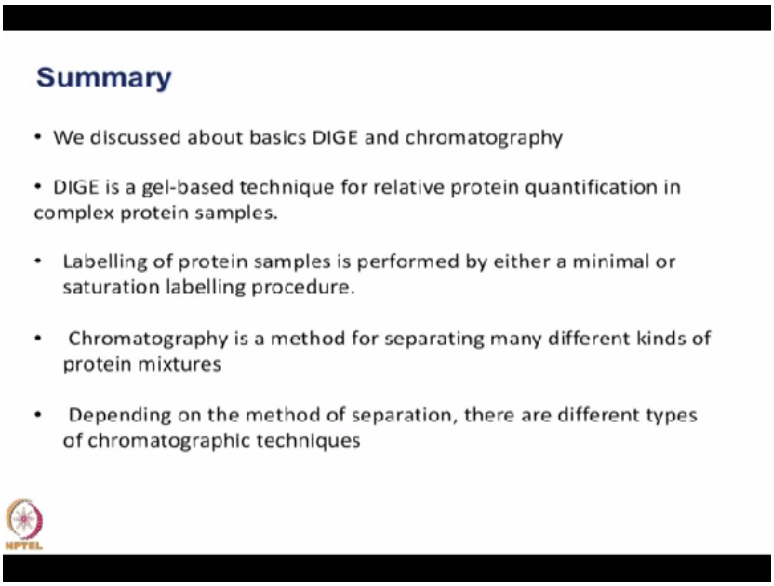

I hope in today's lab demonstration sessions on DIGE and chromatography, you got better understanding of how theoretical concepts which we have been discussing, how in the laboratory

settings, they could be used. So I hope this lab demonstration session was useful for you and to help you to read the chapters, read the papers with much more confidence. Thank you.

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