Proteomics: Principles and Techniques
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Lecture No. # 16
Two-dimensional Difference Gel Electrophoresis (2D DIGE)

Welcome come to the proteomics course, in previous lectures we talked about two dimensional electrophoresis work flow, how to perform two dimensional electrophoresis step by step, we discussed the protein extraction, we discussed about iso-electric focusing, equilibration, second dimension separation by using s d s page finally, we talked about different type of staining methods, which are available and how to scan an analyze those images, we then further talk about different advanced methods which are creative ways of overcoming limitations of gel based approaches.

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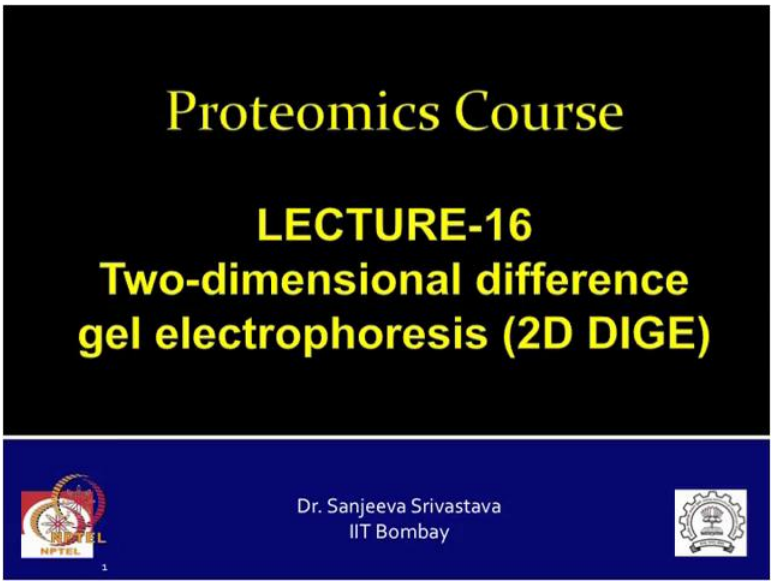

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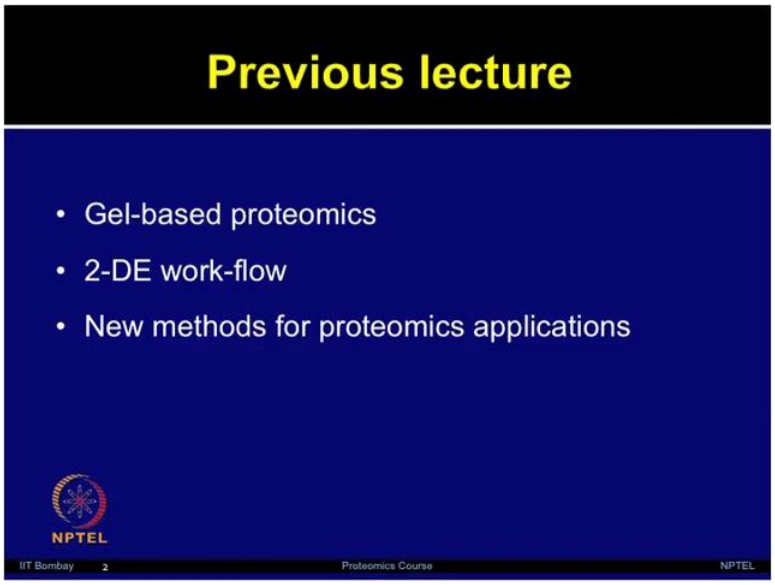

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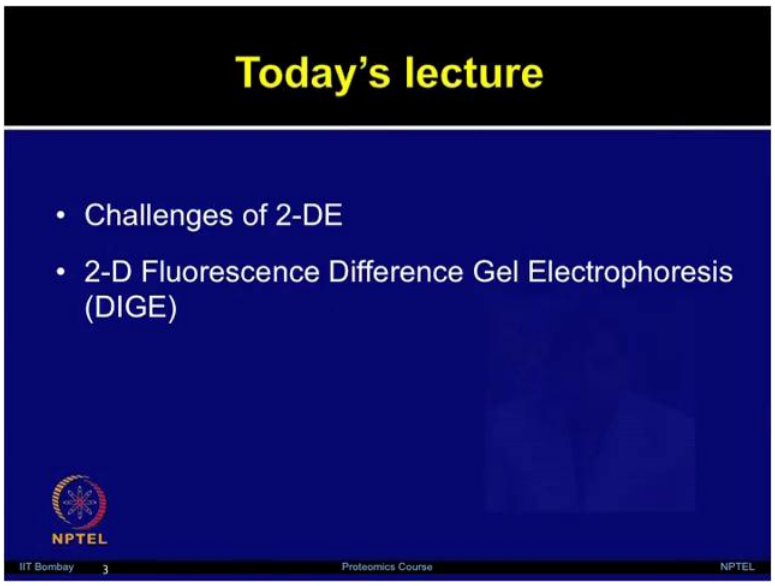

Now, in the same thing today we will talk about, another advanced method of gel based proteomics difference in gel electrophoresis or DIGE which is use for overcoming various limitations of, traditional two dimensional electrophoresis. So, the previous lectures focused on gel based proteomics, the 2 d e work flow and the new methods which are used for proteomics applications, in today's lecture we will talk about different

challenges which are associated with two dimensional electrophoresis and how to overcome those challenges, by using another advance method 2 d fluorescence difference gel electrophoresis or DIGE.

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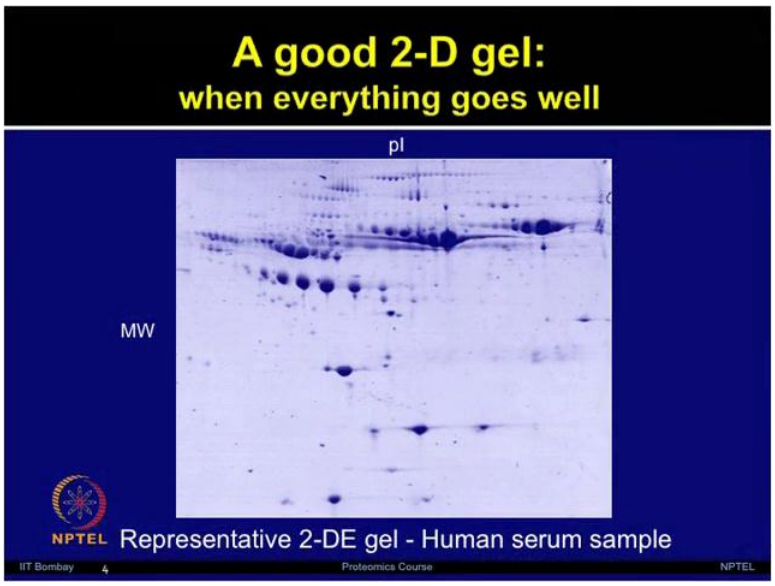

Now, in the previous lectures when we talked about, obtaining a good 2 d gel image then, you may realize or feel that at the end of that experiment all the 2 d gel image will look very good. So, I am showing you one 2 d gel, which is appearing very good and this is something when everything goes very well. So, if your protein separation and staining everything is fine then, at the end you should be able to see, when you got protein separation on the gel, this is a representative 2 d gel image shown with the human serum sample.

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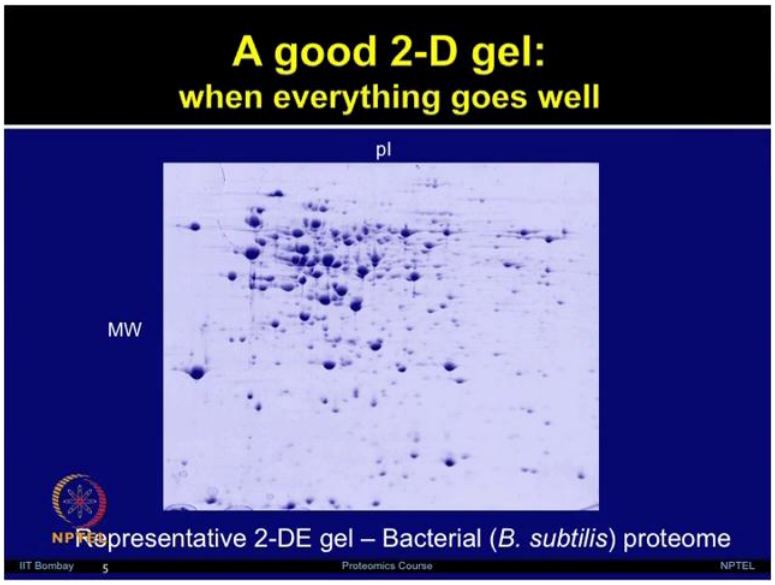

Now, I am showing you another good gel, obtained from bacterial sample the b subtilis, but often this is not the case, many times there are different type of issues, which could give rise to very bad images, these issues could be because of the sample preparation method because, of reagents involved because, of iso-electric focusing different type of parameters involved and finally, different type of staining methods being used.

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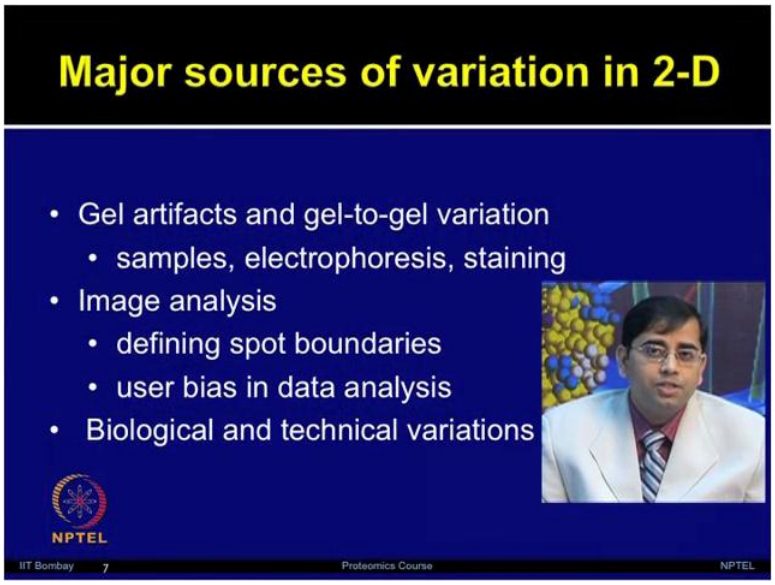

So, let us talk about some of these issues step by step. So, there are various challenges which are associated with two dimensional electrophoresis, mainly the gel artifacts are major limitations, how to compare two gels? How to minimize the variations often during the electrophoresis run, there is a lot of run to run variation, then you have different type of technical and biological variations, the image analysis that itself is a very challenging process.

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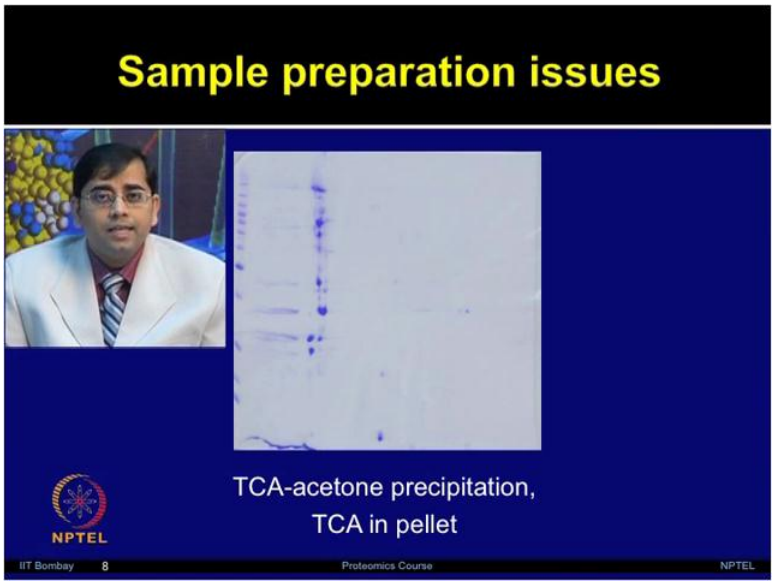

How to define the spot boundaries, how to extract the intensity information from those spots, often we use our bias in doing this type of data analysis. So, there are major sources of variations in two dimensional electrophoresis. I am showing you few problematic gels, how even the small mistakes or some issues inherent to your biological samples can give rise to very bad 2 d gels for example, I am showing you few sample preparation issues, in this gel as you can see the t c a acetone precipitation method was followed, but there was not good washing performed. So, t c a still remains in the pellet and one can obtain the pattern as shown in this gel.

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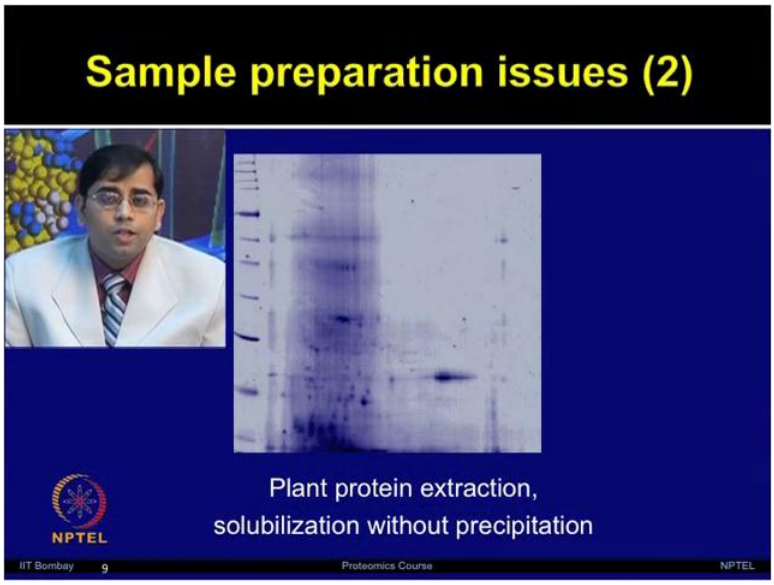

Now, if you are performing the plant protein extraction, but the solubilization is not sufficient without precipitation, one can see this type of streaking and an uneven gel pattern, now if you have samples which are having proteins in the abundance such as crude serum sample.

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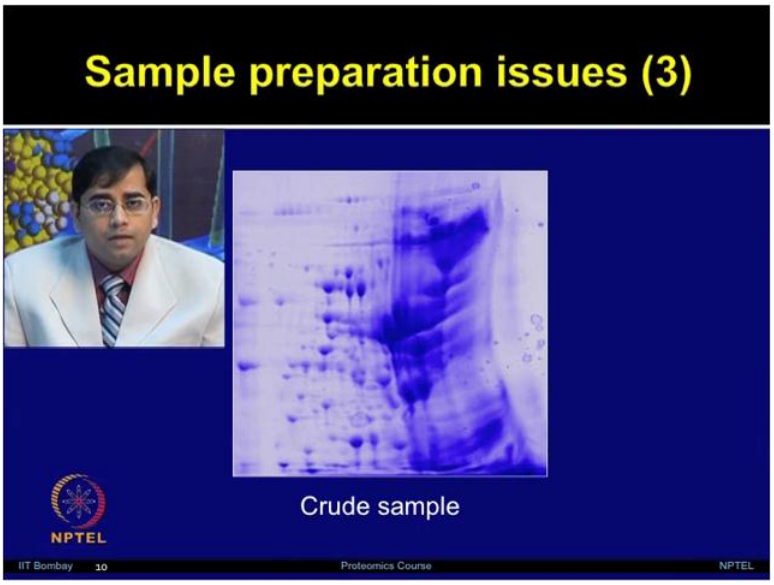

So, these samples will show you different interference in the focusing strip because of large salt or other interfering components present and then you can see this type of bad gels because of the either salt or different type of abundant proteins present in the mixture.

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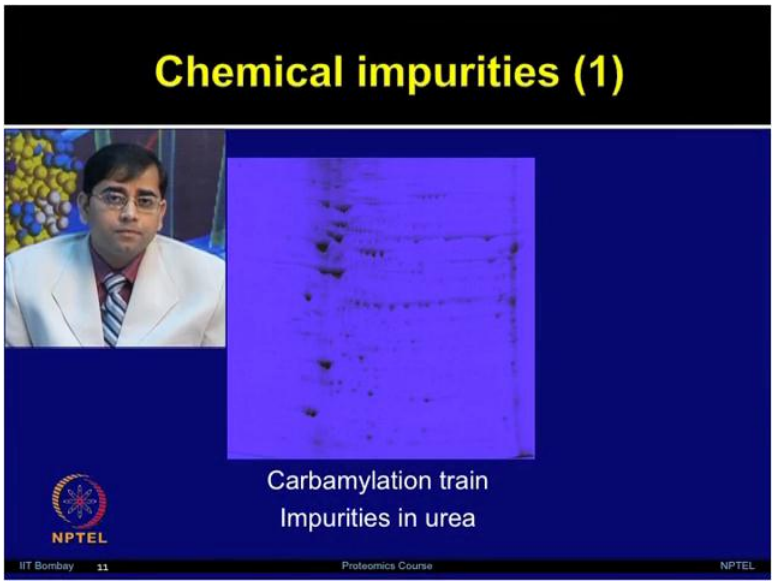

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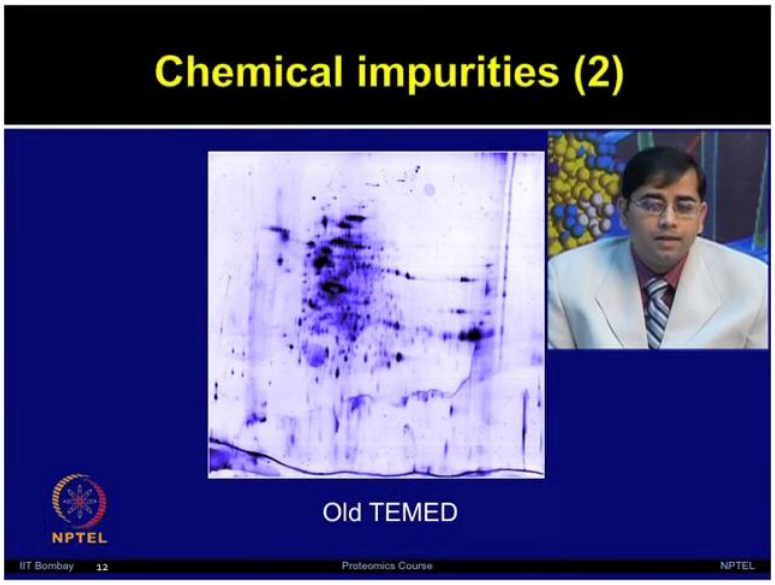

Now, the sample variation is one problem often the chemical impurities or the chemical ingredients can also give rise to different type of bad gels such as, the chemical impurities shown in this gel where, the urea is very impure and one can see the carbamylation train as shown in this gel, if the timid is very old, often very small amount of timid is used in making the second dimension gel s d s page.

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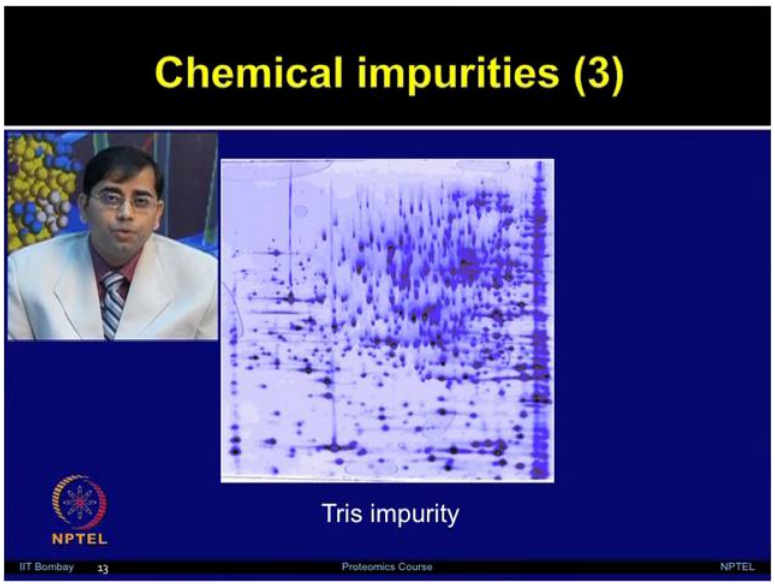

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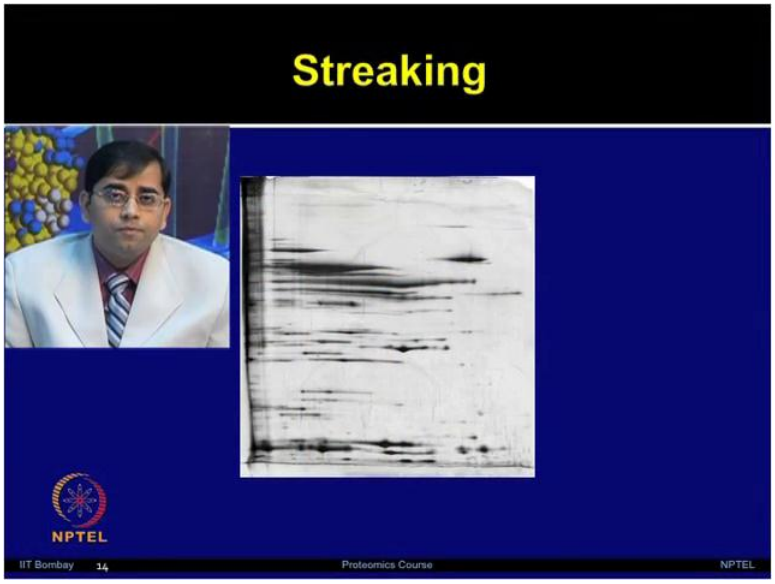

So, people often use that bottle for very long time and timid is very old, you can see some back pattern on the gel, something similar to, as shown in this image. Now, if your chemical such as stress, is not very good quality it is impure again, that will show some multipacks in the gel. Streaking is very often seen because, of different type of problems which could be associated whether, it is coming from the sample preparation or because of different type interfering components, often we have nucleic acid or carbohydrates is still present in the mixture, the extraction method was not sufficient to eliminate all of those impurities, many times salts and different other interfering components are also present which interfere in the i f process and one can see the streaking pattern, after look at this staining of the gels.

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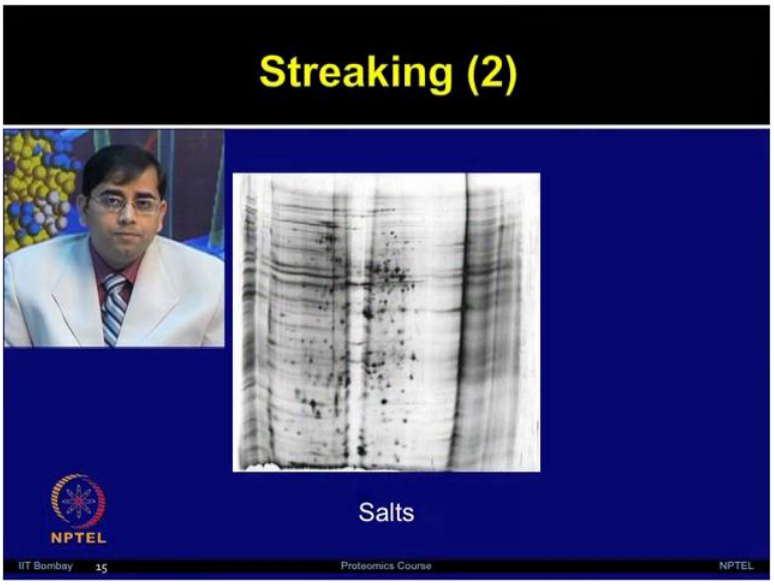

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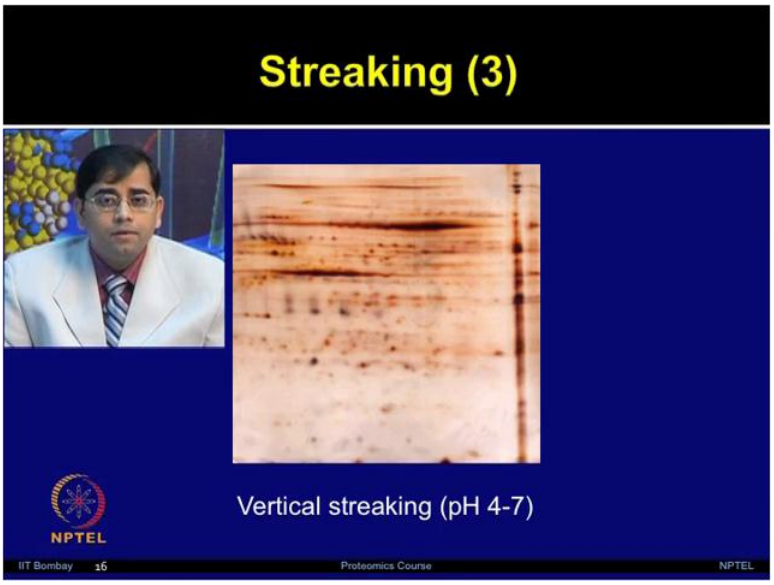

So, as shown in this gel the presence of salt, can be interfering and may result into vertical streaking as shown in this gel if you are using narrow pH gradient strip such as, 4 to 7 pH strip then, the vertical streaking can appear because, all the proteins beyond 7 p I will extract together in the region and one can see the vertical streaking.

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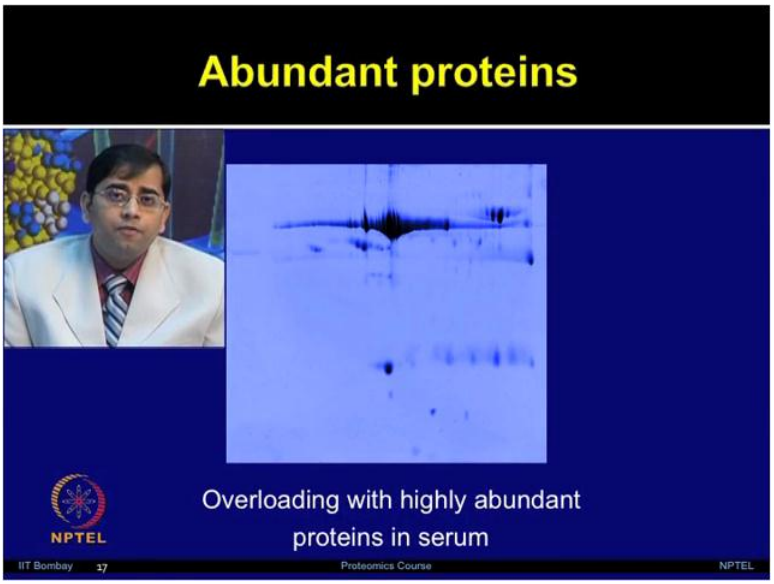

Now, abundant proteins, as I briefly talk last time some time crude samples to contain highly abundant proteins and salts. So, abundant proteins are one of the major interfering components which can be easily seen on the gel, if you have serum sample there are many interfering components present in the serum including some abundant proteins such as serum albumin.

Now, in the plants one can expect Rubisco, as the one of the very large protein present in the plant leafs. So, these abundant proteins they mask many smaller protein and create problem in doing the two dimensional electrophoresis because, of i f process as well as stain on the gel, you can see different type of streaking as well as different in the whole region is masked because of the abundance of that protein.

So, in this gel it is shown that, the human serum protein contains, high abundant proteins which is visible in this area, now there are various ways one can overcome these limitations and as in the previous class of the protein extraction and sample preparation we have talked about how to overcome these limitations, how to remove the highly abundant proteins. So, please refer to that lecture and one can overcome this limitation.

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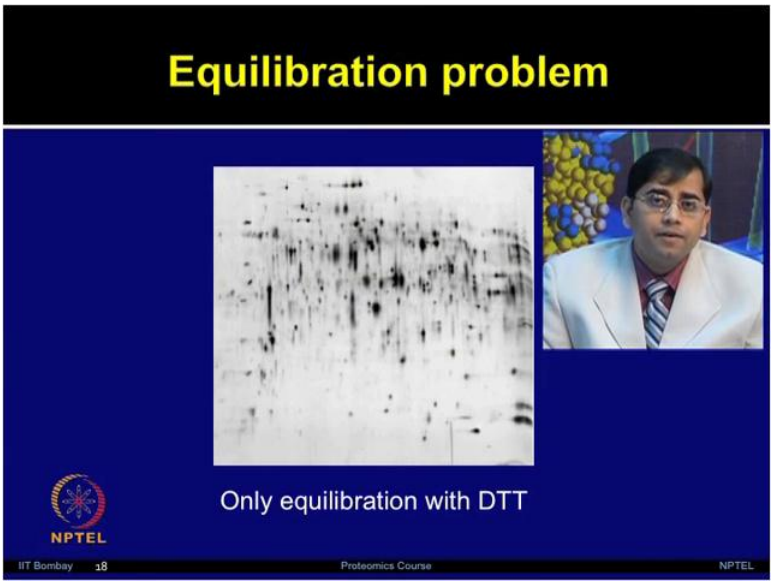

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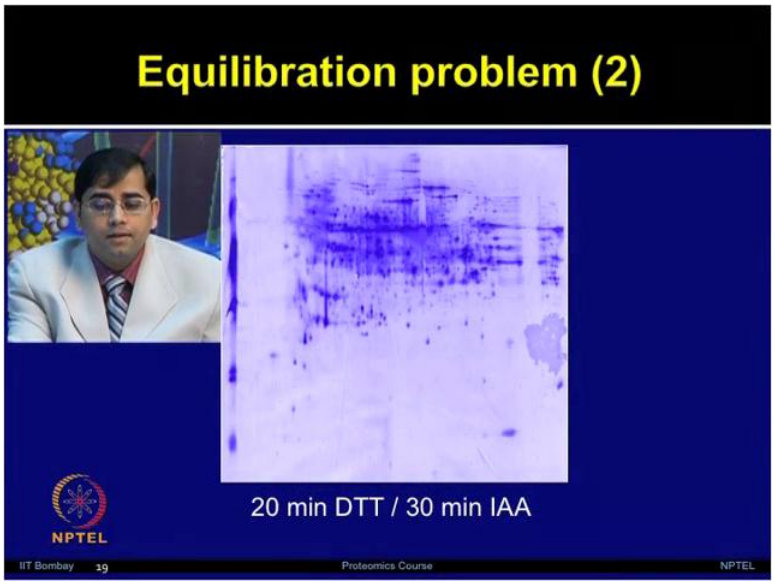

Now, after protein extraction and if process is done, one needs to equilibrate this strip, equilibration process itself can create some problems for example, if you have perform only first equilibration with d t t and forgot to do the second dimension, the second equilibration prior to doing the second dimension separation then, you can see some pattern similar to as shown here that, only equilibration with the d t t, many times if you think that by increasing the time of doing equilibration probably the gel quality may appear better, but that is not the case in this gel it is shown that, by increasing the equilibration time to 20 minutes of d t t and 30 minutes of iodo acetamide, the gel pattern looks very problematic and that is because of the equilibration excess.

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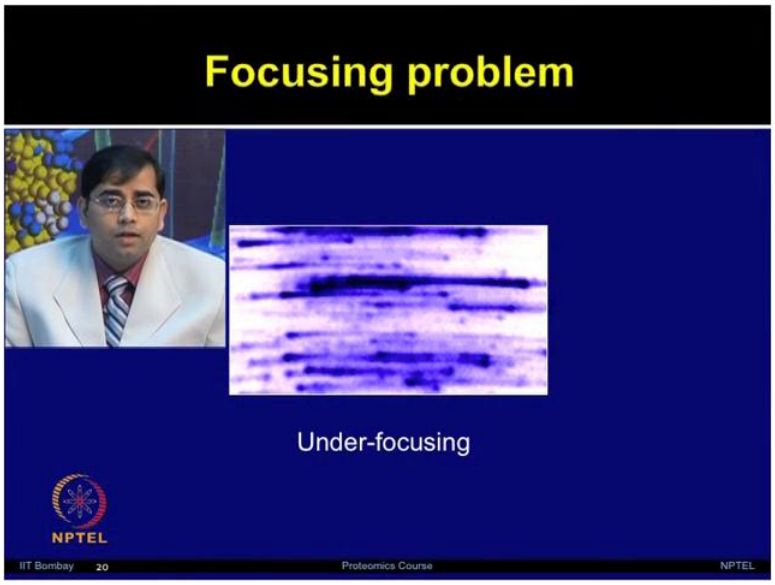

Now, if process is very crucial many times during the i f run itself, one can monitor the problems, if you have a software which is showing you how the run is progressing, often if your port setting is not correct, it is insufficient for the complete focusing, it may result into the under focusing as shown, in this image here for the under focusing.

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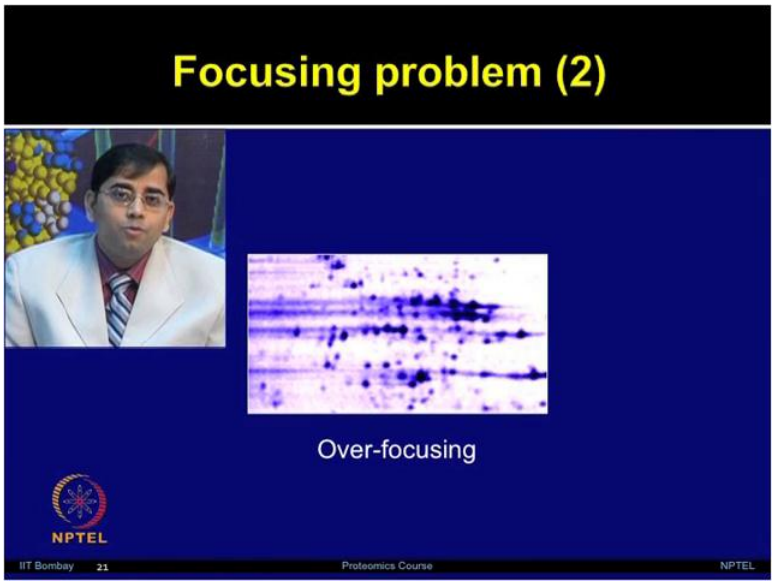

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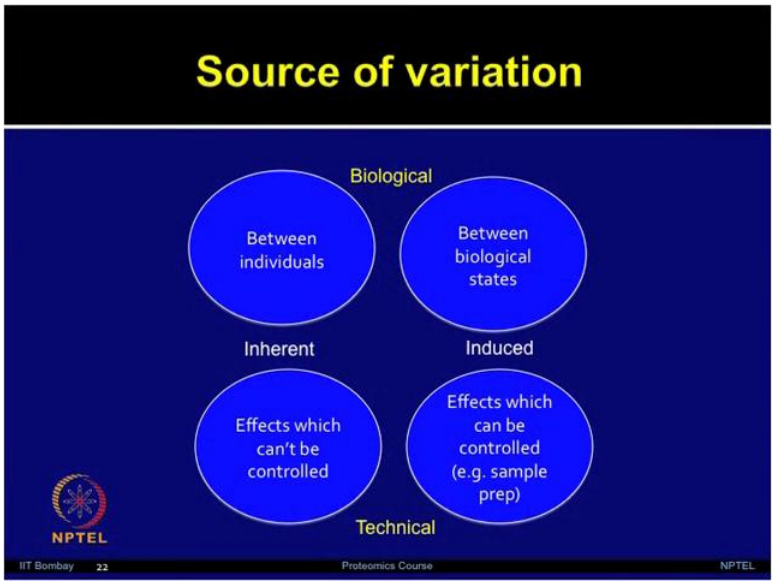

Now, if you have too much voltage and the overall volt hour is very high then, it may result into over focusing. So, optimizing a focusing protocol, for the appropriate duration and over all volt hour is very important. So, there are different type of source of variations can be introduced, from the biological as well as, technically there are different biological variations, on which there is no control, if you are doing some clinical studies then, even if you have done proper age and gender match is still there would be some variations due to each individuals uniqueness. So, those biological variations are very tough to eliminate.

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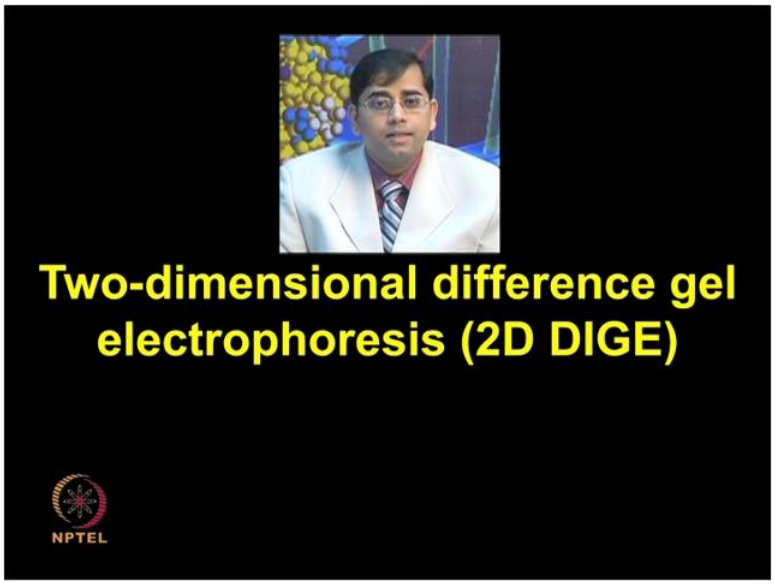

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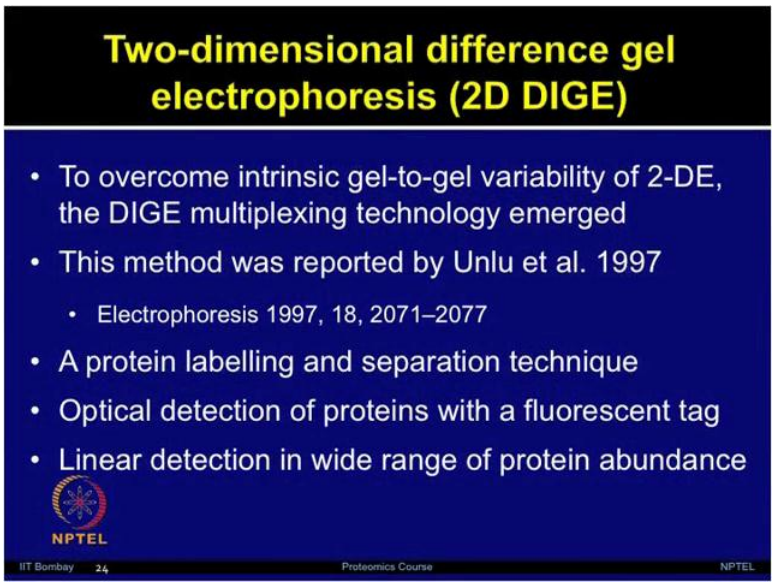

But, there are lots technical variations which can be improved during the sample preparation, during the i f process, during equilibration steps. So, all of those steps may result into large variations. So, in two dimensional electrophoresis, when you are separating your control and treatment gels, on two different gels and you have so many variations coming from both biological and technical variants then, over all the analysis

and the confidence in the data becomes very questionable, to overcome those limitations the new approach the advanced electrophoresis method two dimensional difference gel electrophoresis or 2 d DIGE have immerse. The 2 d DIGE method was basically aim to overcome the gel to gel variability, which was observed in the two dimensional electrophoresis and it allowed the multiplexing samples.

This method was first reported by unlu et al in 1997 and this method finally, became very often and now thousands of laboratories worldwide are using this technology to overcome the gel to gel variations, encountered by traditional or conventional two dimensional electrophoresis. So, this method the DIGE method involves protein labeling and separation techniques, the optical detection of the proteins is done by using the fluorescent tags and this method performs the linear detection in the wide range of the protein abundant. So, over all one can expect a very sensitive method, for giving rise to very large dynamic range of the protein detection by using fluorescent tags and this method is giving the multiplexing and separating proteins on one gel. So, it is expected that it will avoid lot of difficulties which are encountered in the traditional 2 d gels.

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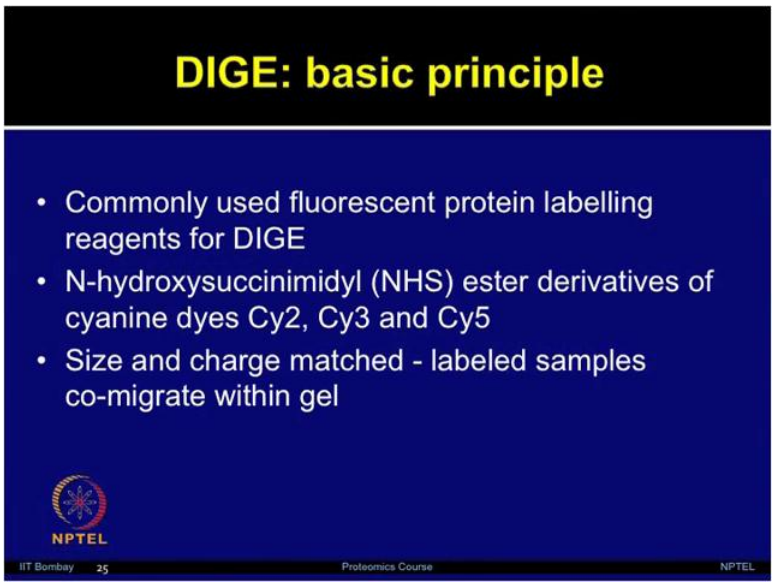

So, let us talk briefly about basic principle involved in DIGE technology. So, we will discuss, how to do the protein labeling then, what is the need for doing the internal

standards. So, let us talk this step by step, as we discussed this is commonly used fluorescent protein labeling reagent for the DIGE, the n-hydroxysuccinimide or n h s ester derivatives of cyanine dyes such as cy 2 cy 3 and cy 5 are used in this method, the cy 3 and cy 5 are used to label your control and your treatments, one should always try to do the dyes swapping. So, that there is no dye bias. So, if in one sample you are adding the cy 3 in control and cy 5 in treatment in other sample you should do the vice versa. So, for cy 2 remains a consistent, for doing the internal standard.

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Now, for cy 3 and cy 5, these dyes the size and charge is matched. So, the label samples can co-migrate within the gel, now I will talk about the labeling methods used in dyes technology. So, when we are talking about two dimensional electrophoresis work flow in the last class, we had looked at different type of staining methods currently available such as, co-massie, silver staining sypro ruby and in that context, I briefly described about cyanine dyes.

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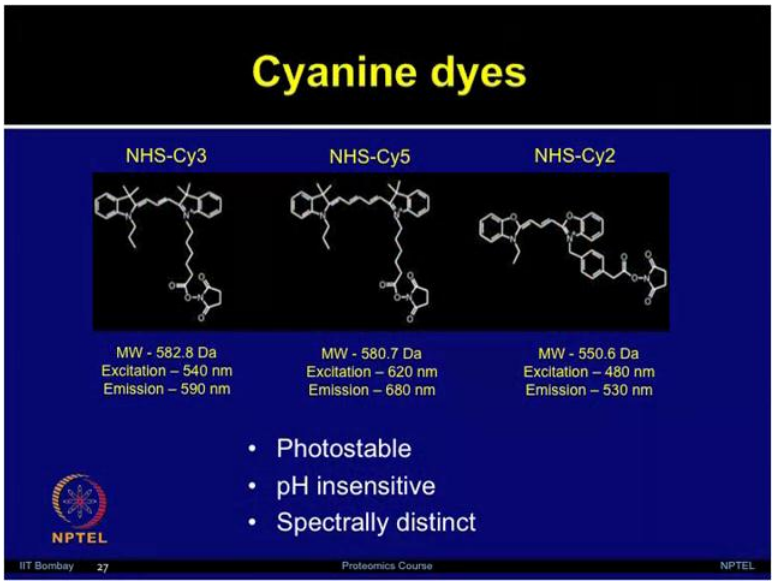

So, in the dyes technology, cyanine dyeser is used, there are three different cy dyes, as I mentioned the cy 3 cy 5 and cy 2, that citation and emission wave length of these three are defined the cy 3 it citation wave length is at 540 nanometers, emission wave length at 590 nanometers, the cy 5 a citation at 620 nanometers and emission at 600 and 80 nanometers, the cy 2 citation at 400 and 80 nanometers and emission at 530 nanometers.

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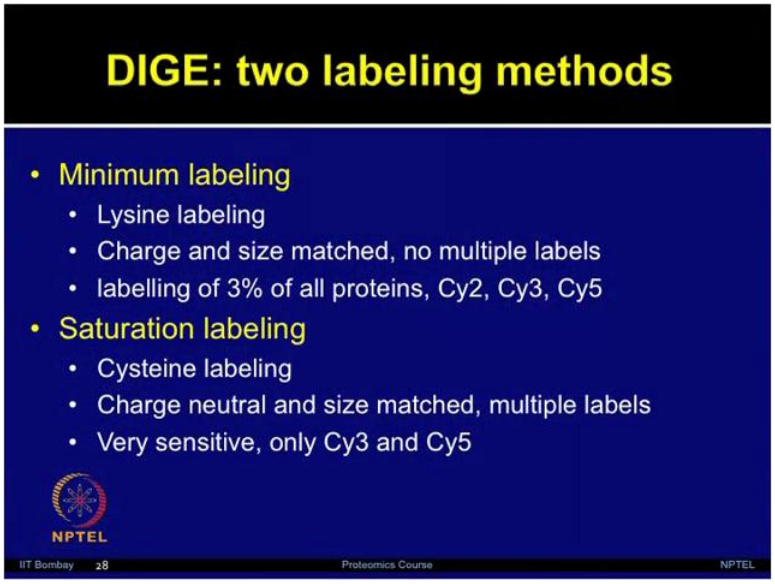

So, these dyes are photo stable, there p h insensitive and spectrally district, by using these dyes, the dyes method can be used. Now, let us talk about different type of labeling methods, in dyes there are two different type of labeling methods which are possible, one is most commonly used, the lysine labeling or other is cysteine labeling, lysine labeling method also known as the minimum labeling method, where the charge and size of dyes are matched, there are no multiple labels and it is known as minimum labeling because, it is only labeling the 3 percent of all the proteins, I will give you the reason, why we are aiming for very small amount of proteome to be labeled and that will be covered in the next slide.

The saturation labeling is done by using cysteine tagging. So, amino acid cysteine is target to label, it is charge neutral and size matched multiple labels are used, it is very sensitive and use only for the cy 3 and cy 5 labeling. So, saturation labeling is not so widely used, but this is another method, one can use for labeling the proteins and it can be use in DIGE methodology.

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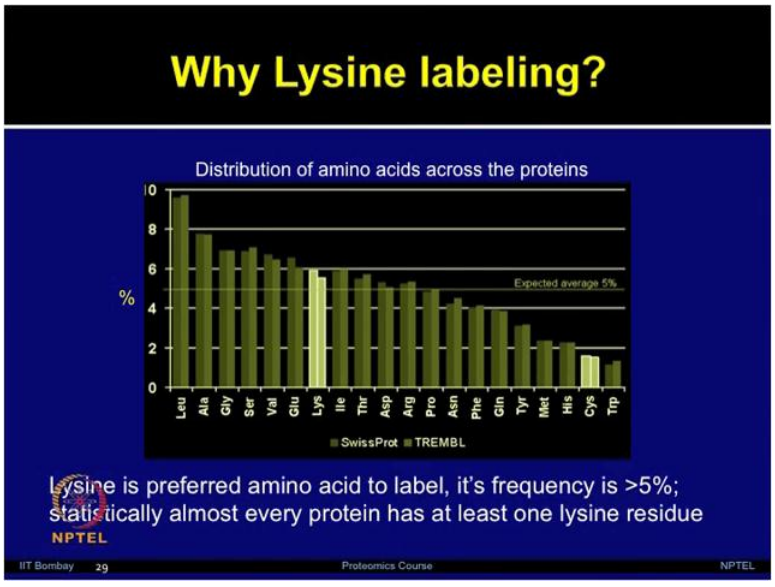

So, let us talk about the popular method of targeting lysine. So, why to label only lysine amino acid, lysine is preferred amino acid as I have shown you in this graph, here the distribution of amino acids across the proteins and there percentage distribution. So,

lysine is proffered amino acid to label, its frequency is greater than 5 percent.

So, statistically almost every protein has at least one lysine residue. So, there is good probability that, your protein will have lysine residue and it will get labeled, if you are targeting the lysine, but if that is not the case, one can always try the saturation labeling of cysteines.

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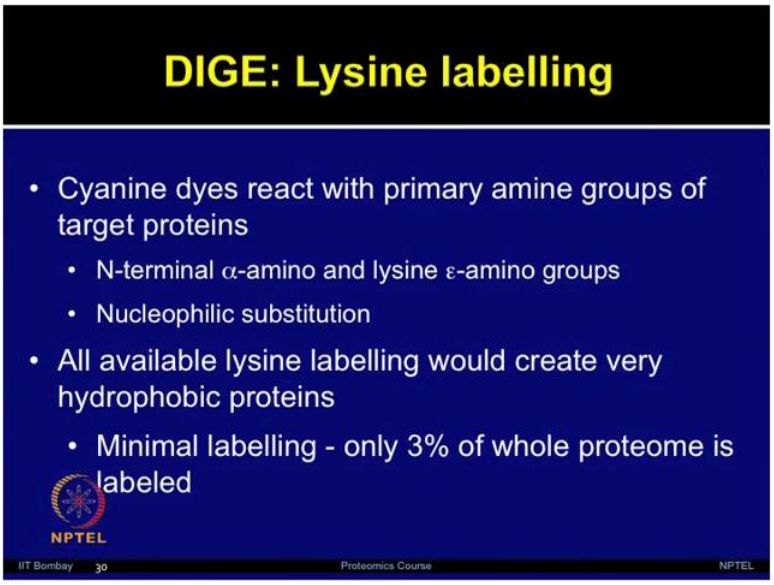

Now, let us talk in more detail about, the minimal labeling or lysine labeling. So, cy dyes they react with the primary amino group of target proteins and the end terminal alpha amino group at the lysine epsilon amino groups, they are labeled by the process of nucleophilic substitution. So, all the available lysine labeling would create a very hydrophobic environment of the proteins, which was a case observed by the original paper derived by unlu et al . So, to avoid that hydrophobic protein atmosphere, it is recommended to do the minimal labeling of only 3 percent of the whole proteome. So, that overall it is not very hydrophobic.

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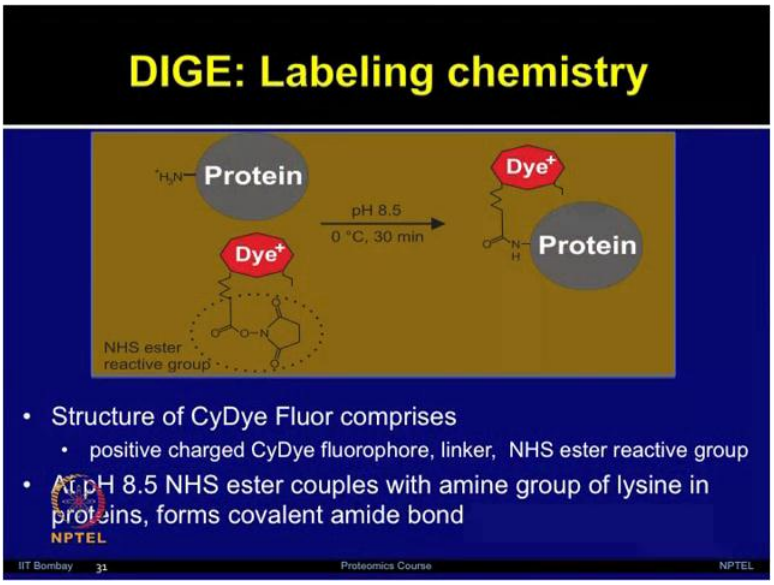

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Now, in the slide I will describe you the labeling chemistry. So, as you can see in this structure of cy dye Fluor comprised of a positive charge cy dye flourophore, a linker and NHS ester reactive group. So, at p h 8.5 the NHS ester couples with amino group of lysine in the proteins and it forms a covalent bond, the amino group of lysine residues are positively charged at neutral or acidic iso-electric points, dye is also positively charged.

So, the net p I will be unchanged therefore, the cy 3 and cy 5 these are not going to alter any mass as well as charge overall and they are very well respectively resolved.

So, people prefer doing lysine labeling, it is not going to alter overall p i values, it is not going to alter overall mass because, both cy 3 and cy 5 will carry the same mass of these labels and there is a good probability, that your protein will have lysine residues and it will get labeled because of the cyanine dyes.

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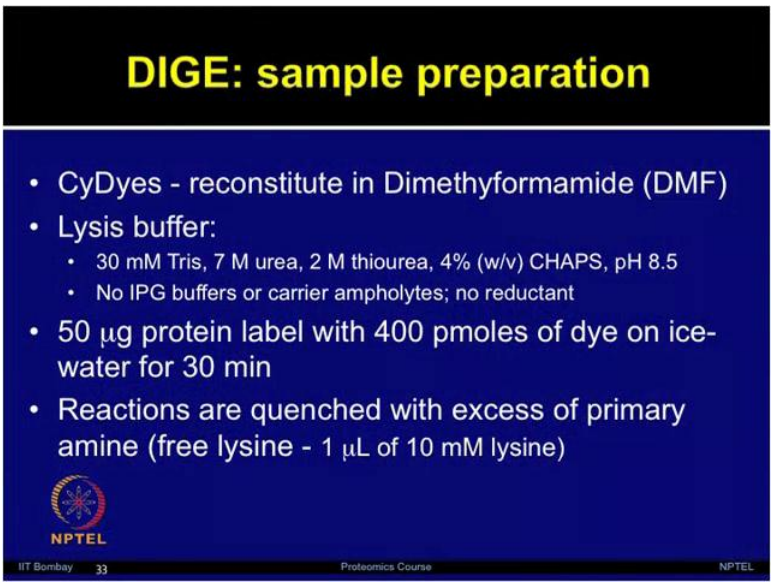

So, how to prepare the sample to perform the DIGE experiment? First of all you need to reconstitute your dye in dimethyformamide or d m f, the Lysis buffer that is, in which your protein sample is going to be dissolved, consist of 30 mille Molar Tris, 7 molar urea 2 molar of thiourea, 4 percent of chaps and you have to adjust the p h at 8.5, please make sure there is no i p g buffer or carrier ampholyte and there is no reductant added in this lysis buffer, since you want to perform the minimal labeling, you need to add only 400 pico mole of cy dyes, in a small amount of 50 microgram of proteins and during that process the reaction should be performed on ice water for half an hour, if you go back and remember about our pervious discussion to visualize a gel, with co-massive stain, you need more protein, even from silver another stains, you need good amount of protein, but this fluorescence dyes are very sensitive are very sensitive. So, all you are

looking for a very small amount of protein, 50 microgram protein quantities sufficient for performing this experiment.

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Now, if this reaction of half an hour is done, after that reaction should be quenched with access of primary amine the free lysine by adding 1 microlitre of 10 milllimolar of lysine. So, then reaction will be stopped and you can proceed with the next step. So, while you are doing this reconstitution of dyes and labeling your protein samples with the cy 3 and cy 5 dyes, now let us think about our cy 2 dye and the internal standard.

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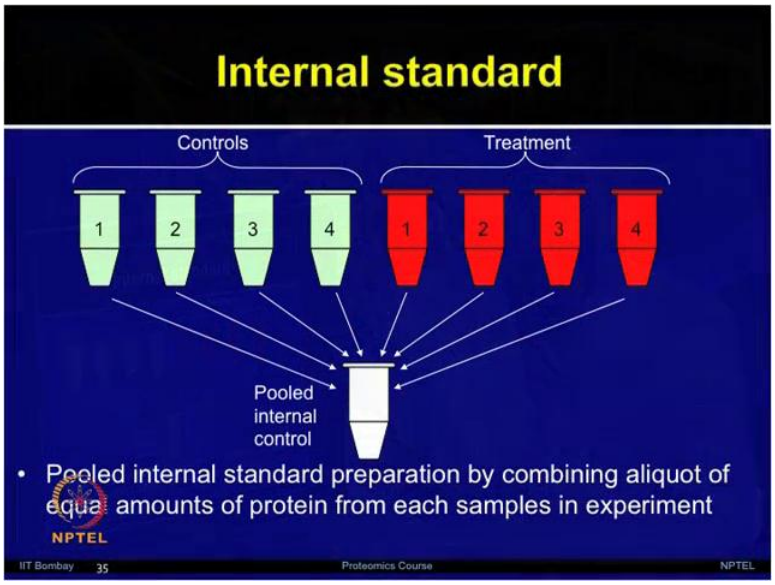

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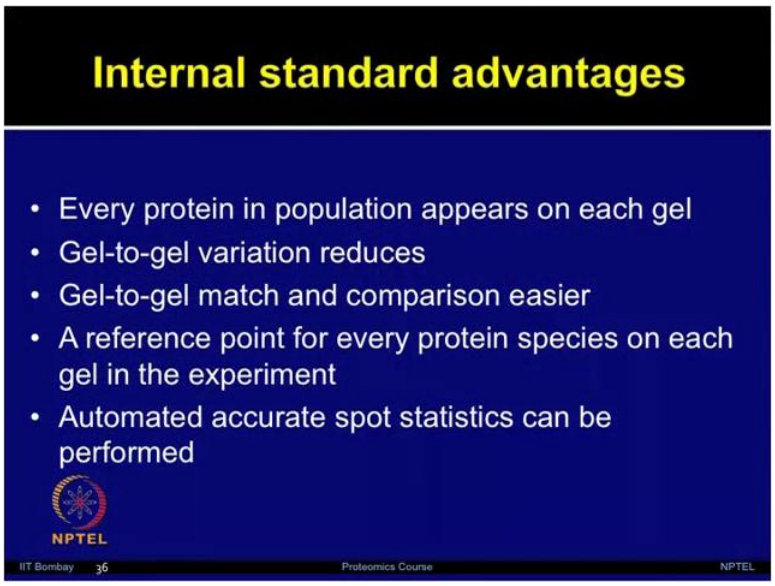

So, I am taking a very simple case here, where you have four healthy controls and 4 patients suffering from a disease. So, to represent all the proteins, which are present in each of these samples in healthy individuals and diseased patients, first you need to make an internal pool which is representative of this entire 8 sample. So, you can take 25 microlitre from each of these 8 samples and make it to 200 microlitre of one pooled

sample which is your internal pooled standard. So, this pooled standard preparation can be performed by combining the aliquots of equal amount of protein, from each samples in the given experiment, now the cy to dye you are going to label up with this pooled sample. So, that can be used as a internal standard. This, internal standard has many advantages and this is actually one of the major success, that how 2-d limitations have been overcome, since every protein is present in the internal pooled population. So, that is going to be appearing on your gel.

So, there is a reference point for each already present on your gel, whether it is from any patient or any controls. So, the gel to gel variation will be reduced, now gel to gel matching and comparison will be much efficient and easier, now this internal standard is creating a reference point for every protein on each gel in the experiment, later on when software performs some data analysis, this results into accurate spot statistics and more meaningful biological data can be interpreted from this analysis.

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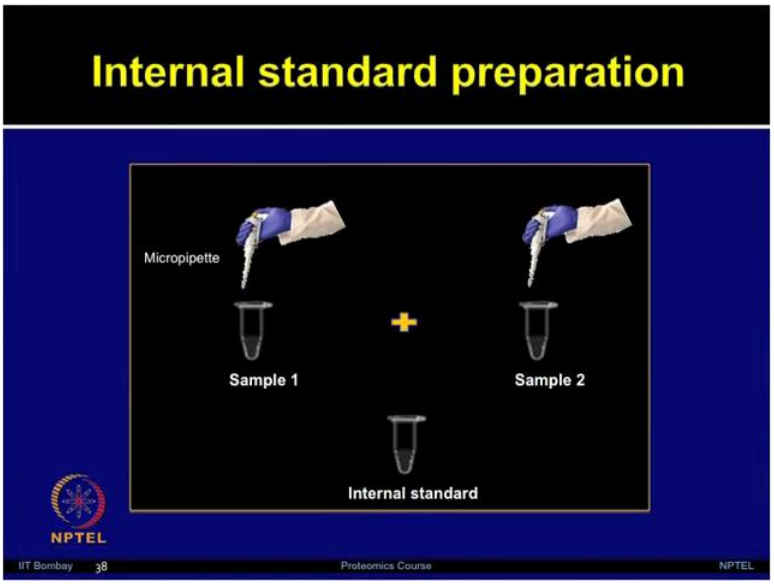

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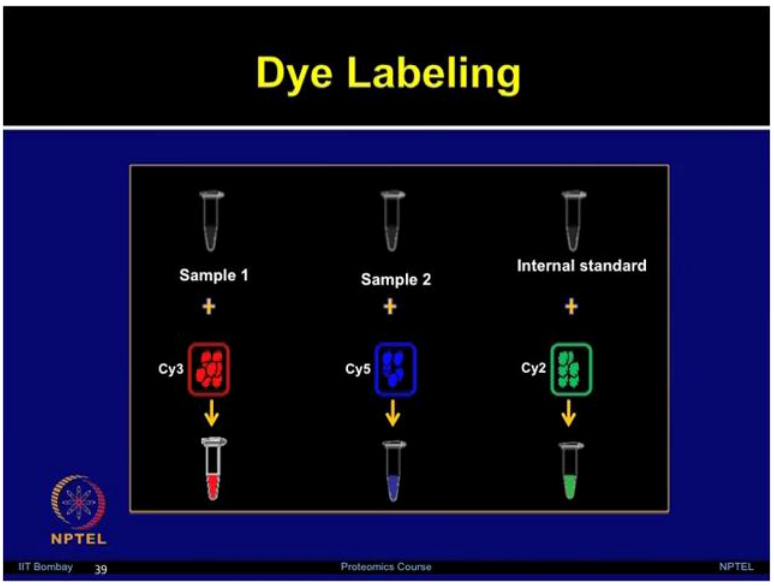

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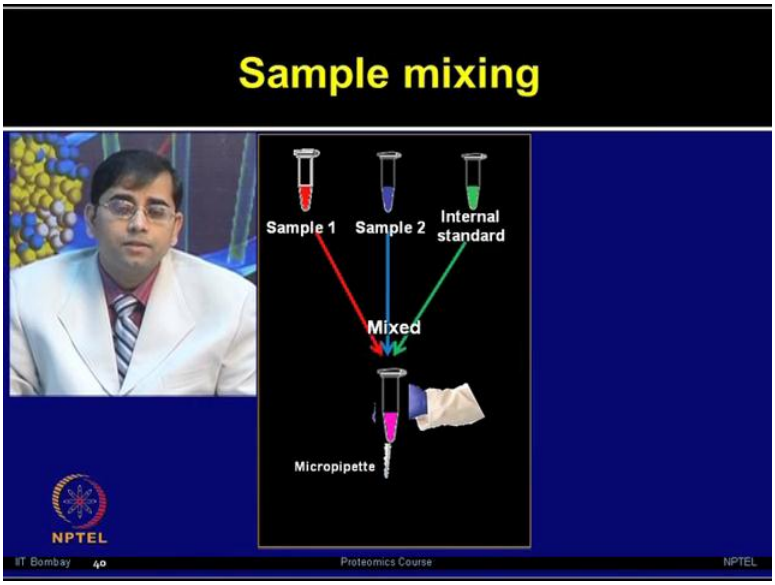

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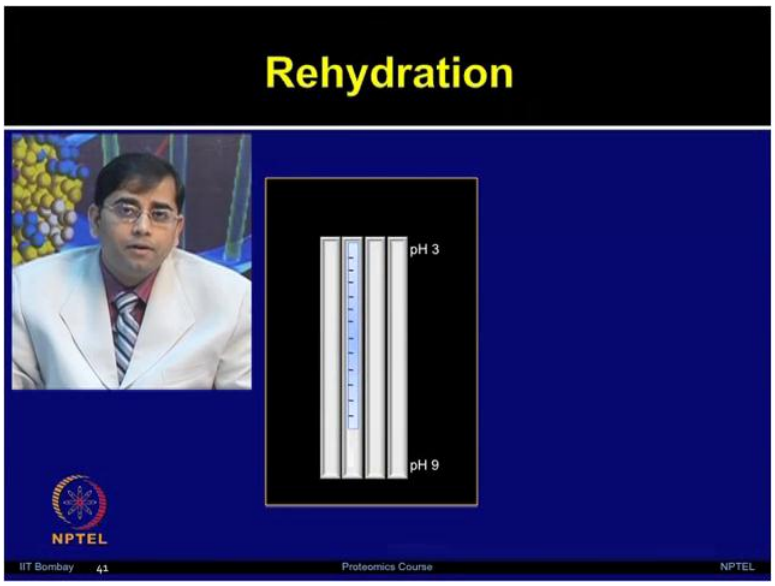

So, now we have talked about the DIGE principle how to label the samples with different cy dyes, why there is a need for making the internal standard and how to label the internal standard with the cy to dyes. So, let us go to this step by step procedure of DIGE method. So, very first thing is you have extracted your protein samples and you need to make the internal standards, now your control sample, treatment sample and internal

standards, should be labeled with different cyanine dyes, internal standard is always fixed with cy 2 dyes, but your control and treatment should be dye swapped. So, sometime it should be, cy 3 and then few sample should be cy 5 and vice versa. Once labeling is done then, sample should be mixed. So, all the three samples cy 3, cy 5 and cy 2, all three labeled sample should be mixed in one combined tube, after that process is done, you can take that sample and do the rehydration, we have talked about rehydration step and the need for it in the previous lecture in the 2-d work flow.

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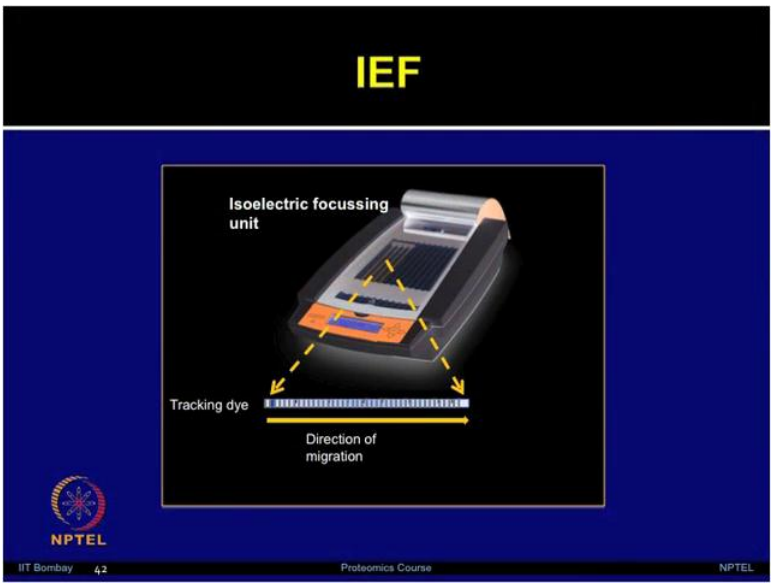

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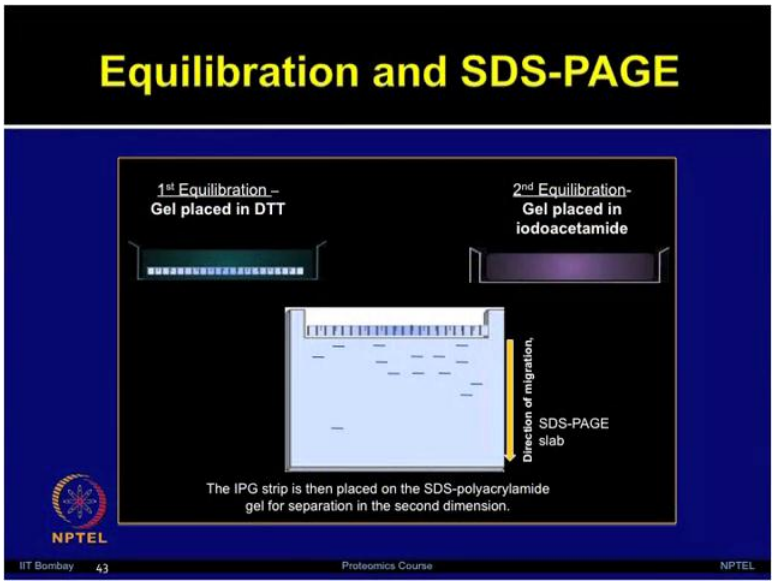

Now, this rehydrated strip, can be focused in an iso-electric focusing instrument, it is good idea to avoid any light exposure, during the rehydration, dye reconstitution process as well as during the iso-electric focusing run is in progress. Once, i f is done again you have to do the equilibration, first equilibration and second equilibration followed by s d s page. So, overall step as you can see, it is exactly same what one is doing in the two dimensional electrophoresis only variation here is, that all in a control and treatment samples have been mixed together and now they are separated on one i p g strip, now this strip is placed on one s d s page gel and all the proteins will be separated in one gel itself whether, it is coming from the control or treatments.

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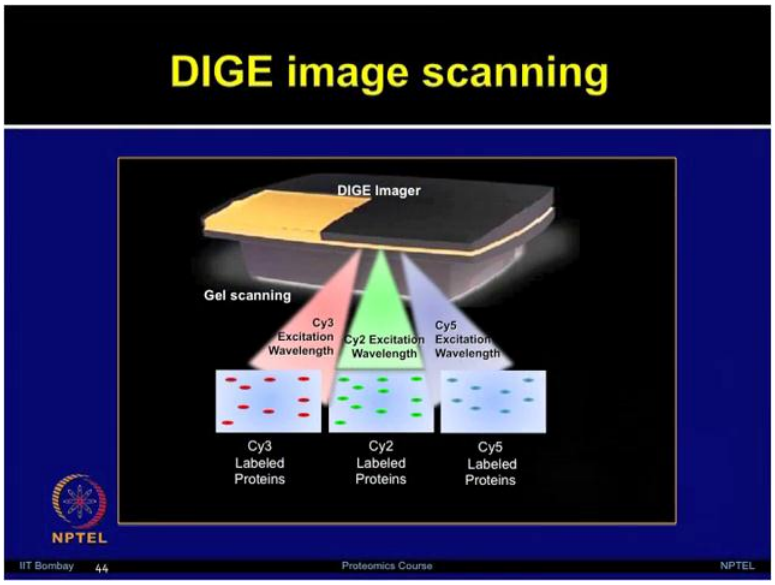

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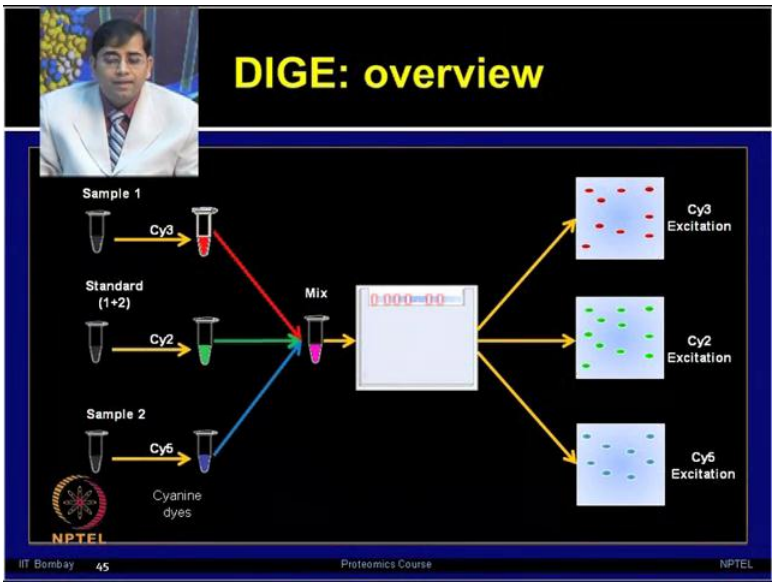

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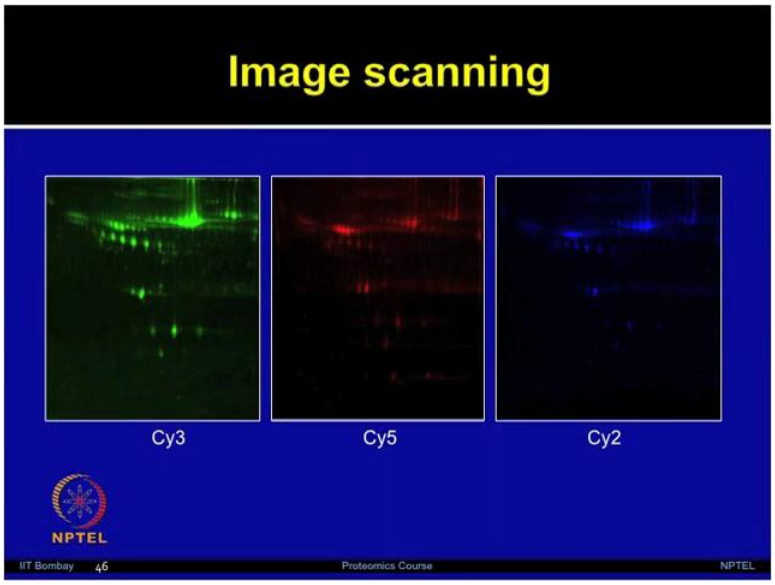

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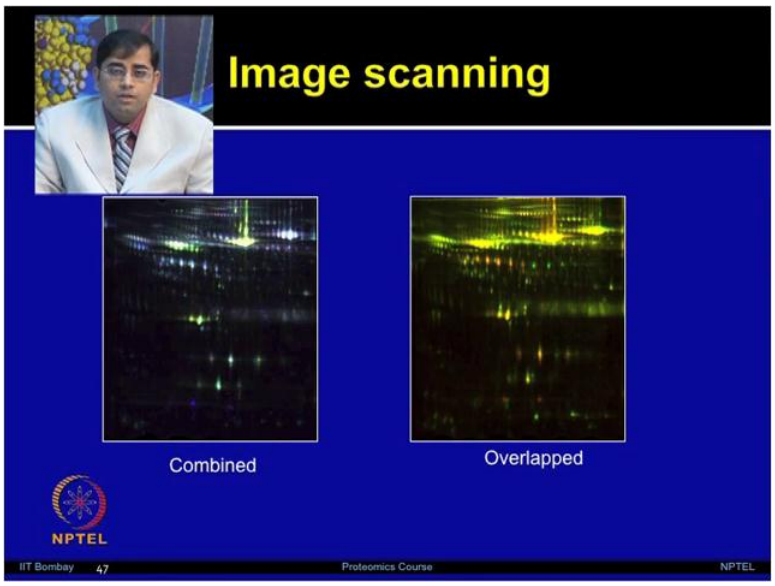

Now, this one gel can be stand by using fluorescence scanner, such as one shown here in the DIGE imager and cy 3 cy 5 and cy 2 patterns can be obtained. So, these slide gives you an overview of the whole DIGE process, where one needs to mix cy 3 cy 5 and cy 2 dyes in one tube, resolve those on one gel on s d s page and then scan that one gel, with different excitation and emission wavelengths to obtain three gel images derive from the

same gel, here few representative gels are shown which are obtained from the DIGE experiments for cy 3 cy 5 and cy 2, software also generates the combined image obtained from mixing all the dyes and the overlapped image, which shows you the abundance of certain proteins, the red and the green are indicative here, in overlapped image, the red and green are indicative of over and under expression or up regulation and down regulation and the white and yellowish color is representing that, there is no change.

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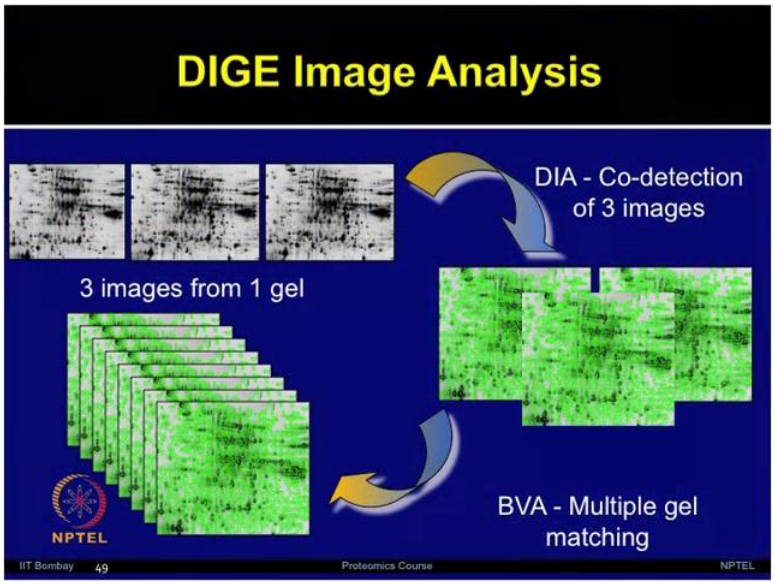

So, DIGE image analysis, although it is very software driven process, it is much more automated as compared to 2-d analysis, but it requires a lot of tools which can be used for good interpretation. So, from one gel you have obtained three images now and those can be further analyzed by using d i a, in the co detection of three images. So, I am talking about a software decider, which takes these images in account and make one image pool from it, by the process of d i a, now these images the multiple d i a's can be matched in the b v a set.

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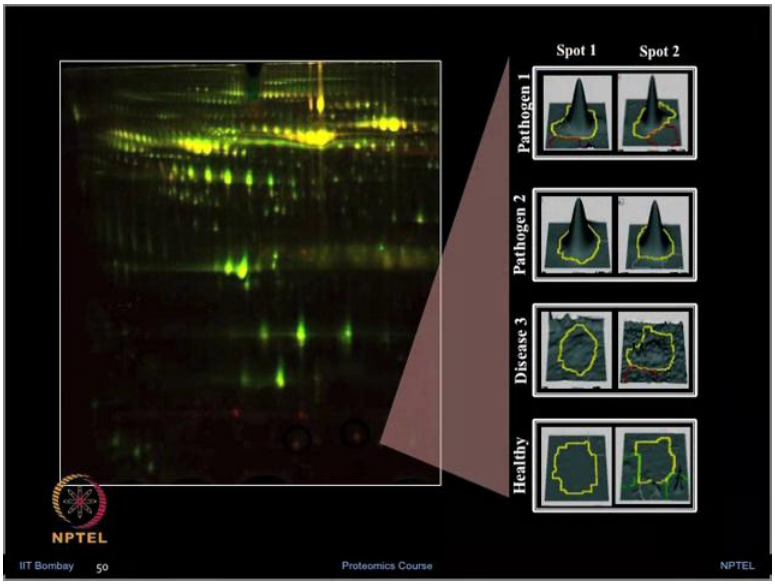

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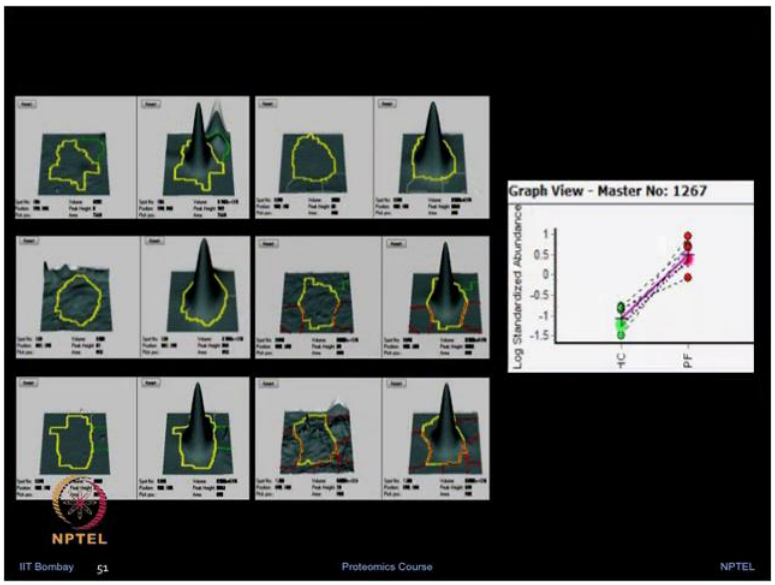

So, these b v s can be further used for obtaining statistical information about these protein changes whether, those are statistical significant or not. So, I am showing a representative gel here, where one spot is highlighted. In fact, two spots are highlighted shown in disease and healthy controls. So, one can look for the abundance of those proteins in the three dimensional views and as shown here, if these proteins are showing

higher abundance, those are visible in different treatments whereas, there is no protein change happening in the healthy control, now one need to look at the same trend of these three dimensional views, across all the patients. So, for example, I have selected here the group of six patients and six healthy controls.

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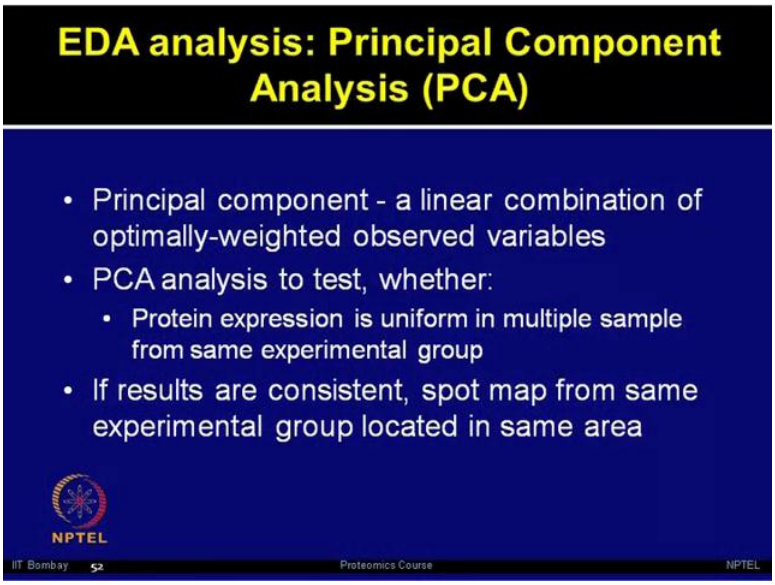

Now, this particular protein is showing up regulation, in all the six patients as you can see here and there was no change in this protein level, in all the healthy individuals. So, by looking at the data carefully, patient wise and reproducibility pattern one can be convinced that this protein is having some biological relevance and then need to be further identified and characterized. So, after doing the b v analysis further statistical analysis of different classes can be performed by using e d a or extended data analysis.

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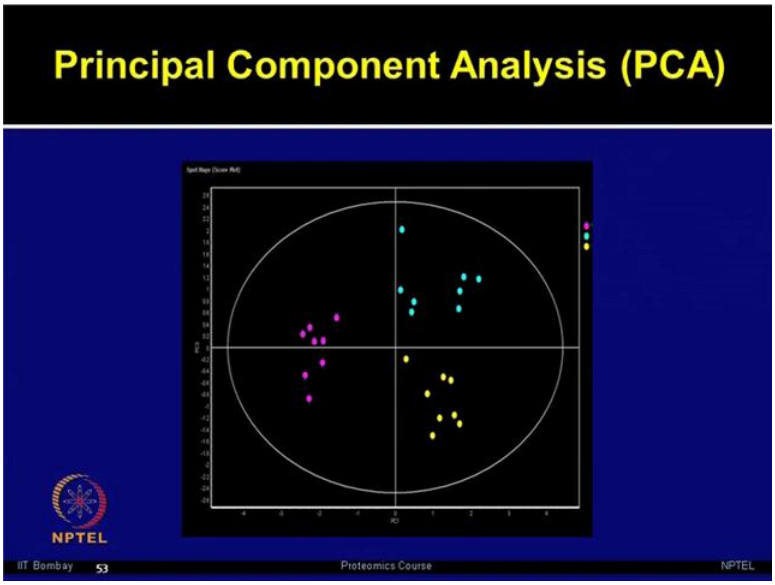

Now, e d a has different type of tools in the software one of the most commonly used is principal component analysis or p c a. The principal component is a linear combination of the optimally weighted observed variables; now p c a analysis is used to test whether, in your protein samples in different patients and controls, the protein expression is uniform across, all the multiple samples derived from the same experimental group. So, a few results are consistent across the whole population, the same protein pattern is consistent in all the controls and in all the treatments, then this spot map from the same experimental group, will be located in the same region, to clarify this point I will show you this image here, if you have one control and two treatments and by looking at the significant proteins, a software can use this tool principal component analysis tool and give rise to the pattern of these healthy and different type of treatment patients and if the grouping is together it means it is highly significant, as you can see here, the red dots are showing the controls and blue and yellowish dots are showing two different type of treatment groups.

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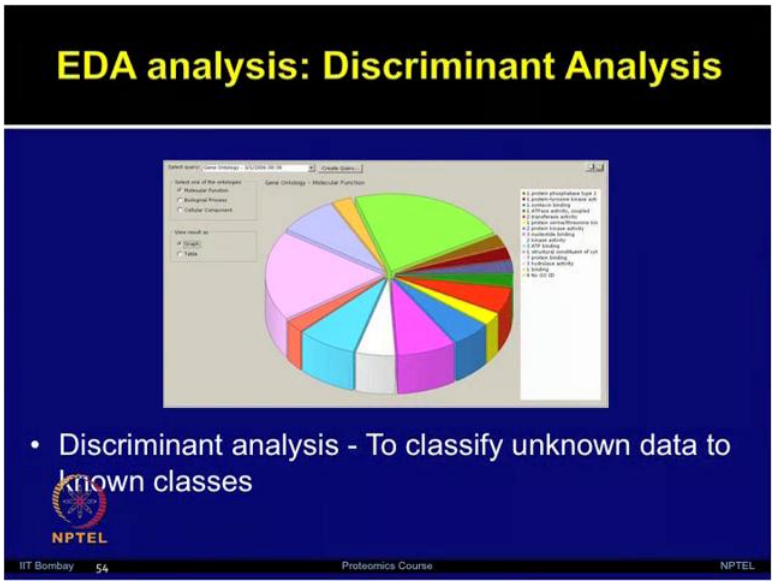

So, now these three groups are clustered quite closely, there will be some heterogeneity because of the different clinical samples and patients biological variations involved, but overall it shows that data is quite statistically significant, in the experimental groups. Now, by using the software, one can obtain further information about different type of discriminate and classes. So, the discriminate analysis is used to classify the uniform data for the known classes, one can initially take all the proteins which are significant and then try to group those based on the known classes, the hierarchical clustering is another method which is giving very useful information for example, in this data we tried to look at one control and two different type of treatment groups together.

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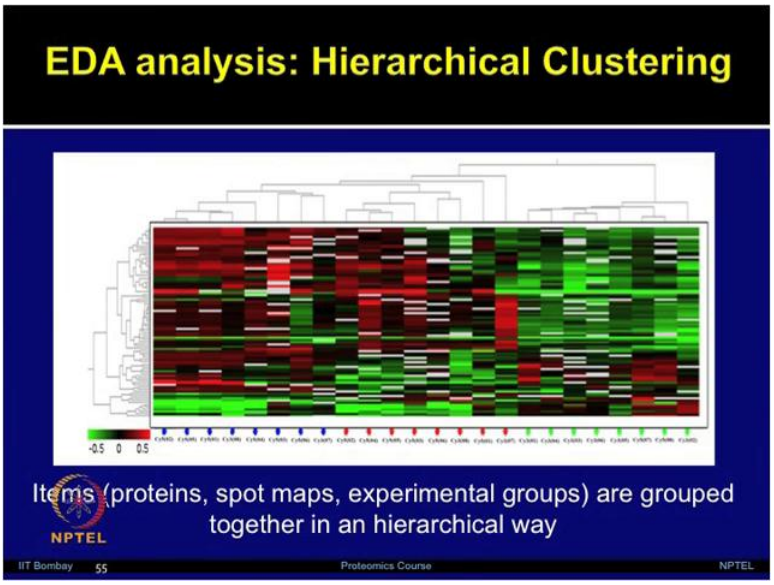

Now, on the extreme right side all the green shades are from the healthy controls and then, there is two more population from the patients group, which is showing many proteins in the red region, it means it has higher protein abundance. So, this clustering is showing that the control is belonging to one population and these two treatment groups are derived from same population, but even within that population, they have certain variation. So, these items whether proteins spot maps or experimental groups can be grouped together, in the hierarchical way by using this clustering.

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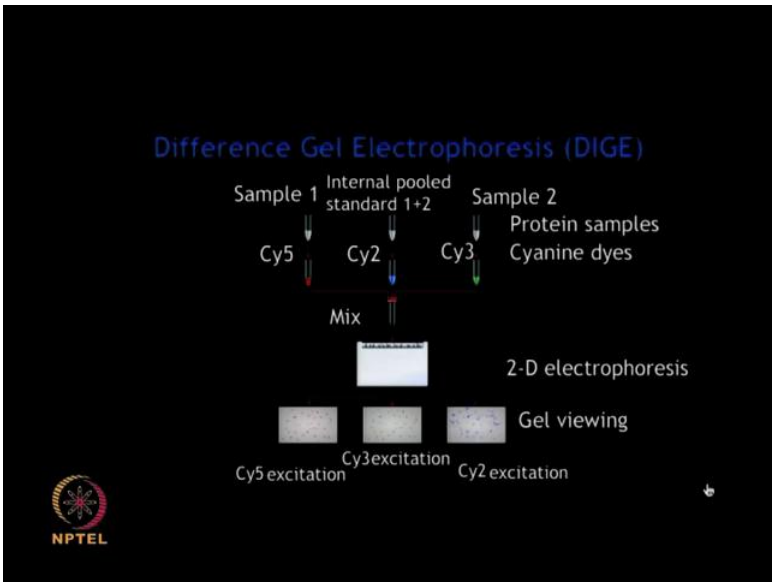

So, now I will describe you the animation process, how to perform DIGE experiment and you will be able to recall different concepts, which we have discussed. So, far difference gel electrophoresis DIGE, the pooled internal standard for DIGE is prepared by mixing the equal amount of all the samples that are being run in a given experiment, the internal standard prevents problem of gel to gel variations, now let us talk about dye labeling

each protein sample as well as, internal standard is labeled with a differentially fluorescing cyanine dye, which allows all protein samples to be simultaneously run on a single gel, the dye by means covalently to the epsilon amino group of lysine residues in protein, these samples should be mixed together and labeled proteins can then be run on single 2-d e gel, the separation takes place on the basis of iso-electric focusing, in first dimension followed by the molecular weight separation, in the second dimension, the gels containing all the protein samples, can be viewed by eliminating it alternately with excitation wavelengths, corresponding to cy 2, cy 3 and cy 5 dyes.

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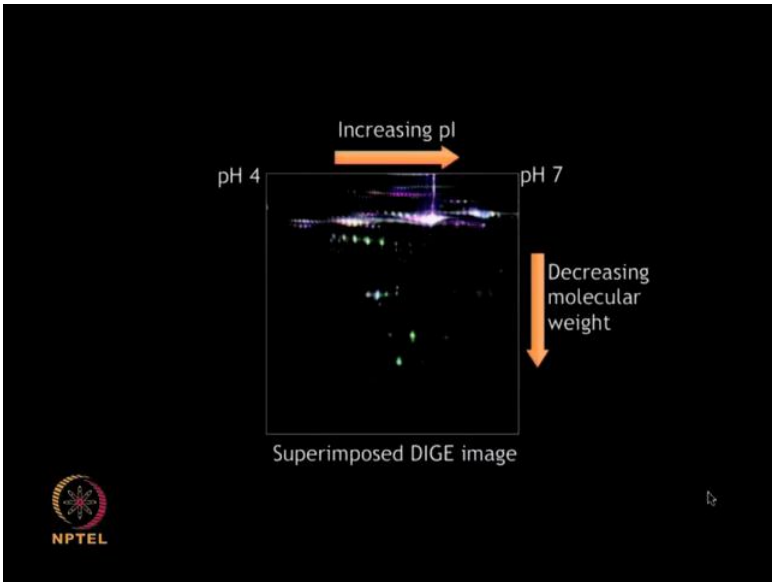

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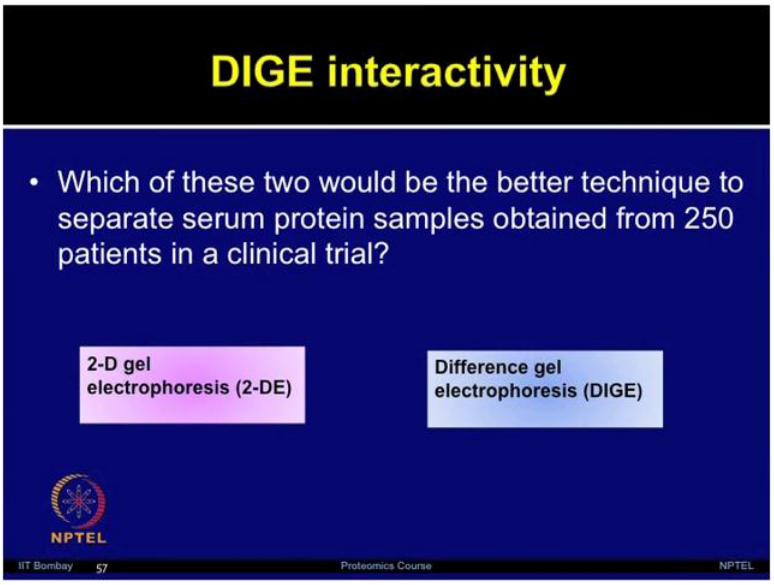

Now, the superimposed 2-d DIGE image, can be viewed, in formation for the molecular weight and iso-electric point can be obtained from the gel, but most importantly by looking at the gel pattern, if you are doing the abundance proteomics comparing the expression of two samples, different protein expression then, that pattern can be immediately observed on the gel, with the red and green patterns, if there is no change it will appear as white. So, after knowing the concepts and after looking at the animation, now you should be very confident in overall concepts related to the DIGE experiments.

So, let me give you a very simple problem, if you want to perform some gel-based proteomics approach and you had been given 250 patients from a clinical trial. So, which of the two methods will be useful? Whether, you would like to use 2-d electrophoresis or difference electrophoresis DIGE. So, which of these two methods would be better technique to separate serum protein samples obtained from 200 patients, in a given clinical trial. So, let me give you this interactivity and ask your answer. So, if your answer is 2 d electrophoresis, that is not correct, you need some method which can minimize the variations across large population. So, you need difference gel electrophoresis. So, let me describe you this concept, in the animation in the following slides.

So, two dimensional electrophoresis, although it is a very useful technique, but may not be best option in this case, for analysis of serum proteins obtained from large number of patients, as it would involve running several individual gels, which would be a very time consuming process also there will be variations across the gels, would make comparison a very tedious whereas, the DIGE can be extremely viable tool, for the analysis of large number of samples, simultaneously without having to overcome the problem of gel to gel variations, the control at this samples can be differentiable by using cy dyes and run on a single gel finally, you can scan the gels by using a DIGE imager.

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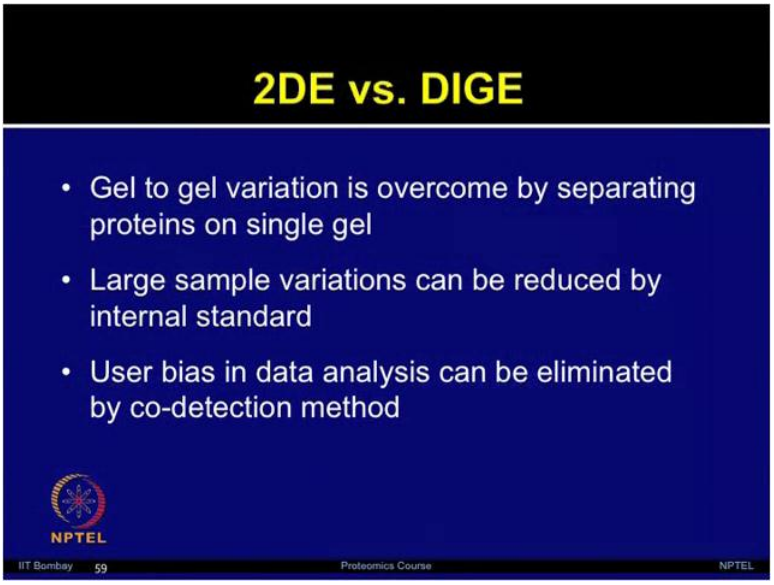

So, now you have a good clarity, that what are the disadvantages of two dimensional electrophoresis and advantages of DIGE. So, let us summaries our concepts of 2 d e and DIGE. So, in 2 d e there is gel to gel variations, which can be overcome in DIGE method by separating the proteins on one gel, in two dimensional electrophoresis, there is large sample variations whereas, in DIGE this variations can be reduced, by making an internal pool or internal standard, which is going to give the reference point for all the proteins.

Now, in two dimensional electrophoresis, often in the data analysis there is user bias, how to define the spot boundaries and obtain the statistical information whereas, in

DIGE there is a co-detection method, involved by the software analysis, which eliminates lot of user bias of performing this analysis. So, overall if you compare 2 d e and DIGE, there is definitely more merit to perform DIGE experiments, but not to say that, before you want to move towards 2 d DIGE approach, first you need to ensure that your sample is good, your technique is working fine and overall you can obtain a very good gel. So, first you need to perform traditional or conventional 2 d gel, stain with co-massive or silver stain, to see whether everything is looking good, you are able to obtain a very good image and then you can apply the same protein extract to perform the DIGE experiment that will be very cost effective.

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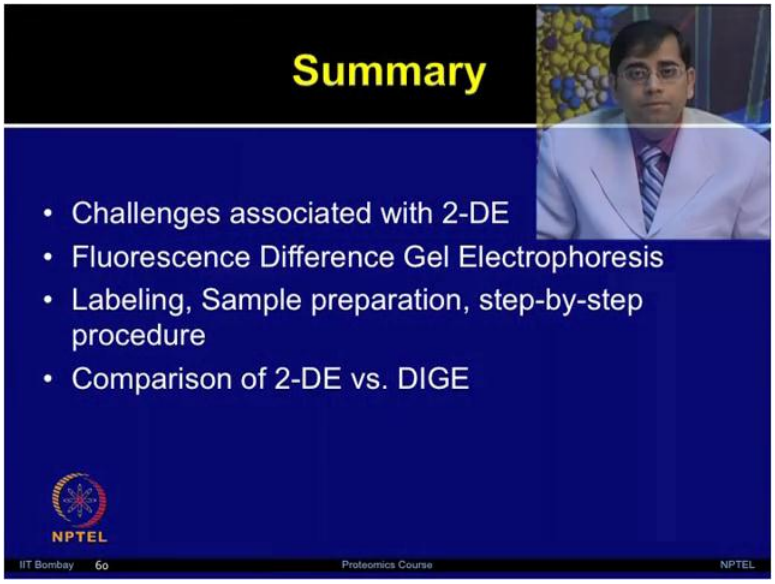

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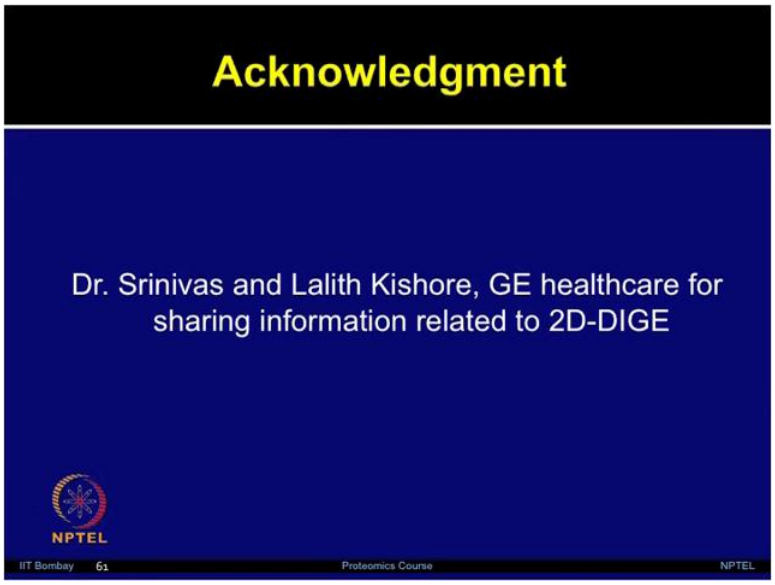

So, in summary in today's lecture we talked about different challenges, which are associated with two dimensional electrophoresis, then we looked at DIGE workflow different concepts involved as well as, how to do the labeling of samples, how to make internal standards and then we have seen the steps in animation. We finally, compared 2 d e method and DIGE method and I gave you an interactivity option to perform experiment by using 2 d e or DIGE and you gave the right answer of doing the experiment with DIGE method. So, overall from this whole experiment, you should be able to conclude that, DIGE is better way of separating proteins and it can still provide you very useful quantitative information, in the gel based proteomics. Thank you.