Introduction to Proteomics
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Lecture - 17
2D DIGE: Data Analysis

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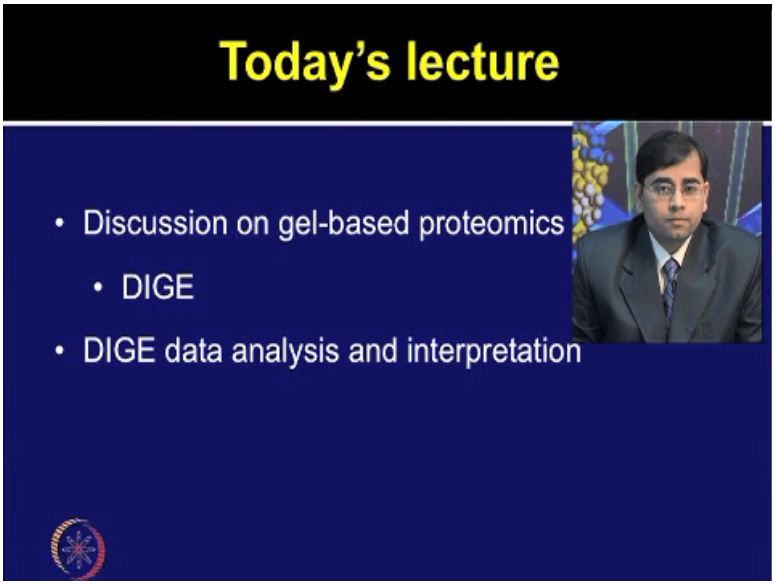

So in today's lecture we will continue our discussion on gel based proteomics certain advantages and challenges of performing DIGE experiments. How to analyze DIGE data by using few very specialized software and how to interpret that data, how to obtain some meaningful biological information from those analysis. So today I have invited a guest Dr. Srinivas from GE healthcare who is going to talk to us about DIGE technology.

And give us a demonstration on software to perform DIGE gel analysis.

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This is my great pleasure to introduce Dr. Srinivasarao from GE healthcare. He is an Application Specialist in the research product division of GE healthcare. Dr. Srinivas thank you for coming for discussion about 2D and DIGE technologies. So what are the major advantages and disadvantages you see by using 2D approach. "**Dr. Sanjeeva Srivastava – Dr. C. Srivivasarao conversation starts"** It is a very user friendly as well as very low instrumentation cost.

There are the complete proteomics the differentially expressed protein we can see exactly during this complete 2D electrophoresis technique as well in the normal protein we can able to isolate only from this technique. This we can identify very easily the software as well as very user friendly this is what we can do. There are some disadvantages. The reproducibility will be not there in most of the times.

This is a major disadvantage of this 2D electrophoresis and again software they are very user friendly still there is lot need to be developed so that we can use very effectively. "**Dr. Sanjeeva Srivastava – Dr. C. Srivivasarao conversation ends"** So as you rightly mentioned so two-dimensional electrophoresis is still is a very powerful tool to resolve thousands of protein on the gel. One can also analyze isoforms and post transition modifications including Phosphorylation and overall the process is very, very user friendly.

So still it is a one of the very powerful technologies currently available for proteome analysis. Now I also agree with your comment there is some reproducibility issues because users have to run the gels from the control and treatment separately and then there will be lot of manual

artifacts regardless of how meticulous they are doing experiment. So in that regard what is your suggestion like how user can overcome the limitation of traditional two dimensional electrophoresis technology.

"Dr. Sanjeeva Srivastava – Dr. C. Srivivasarao conversation starts". One should have to prepare a very good sample preparation so that the reproducibility thing will overcome. The second point is these days the new generation 2D electrophoresis system that is the DIGE technology is available where you can use a 3 samples in a single strip and we can co-migrate them in a single strip and there will be an internal control as well as control and treated.

These 3 you can run in a single strip so that these kind of reproducibility issues we can overcome as well as there is a very powerful scanner is available in this technology that is a typhoon trio where you can have this is the based on the laser based technology is completely so that you will have very efficient gel in your hand. "**Dr. Sanjeeva Srivastava – Dr. C. Srivivasarao conversation ends"**

As you mentioned that DIGE technology is one of the very powerful solutions to overcome several challenges which people face in running traditional 2D gels. So in that regard I think if you can elaborate little bit about DIGE technology so that will be useful to the students. "**Dr. Sanjeeva Srivastava – Dr. C. Srivivasarao conversation starts"** I have a small video regarding the DIGE technology.

I will show you that video so that everyone can able to easily understand what exactly DIGE technology. As we are talking about the 3 samples we can label with Cy2, Cy3, Cy5. Now we have 2 different samples from different source.

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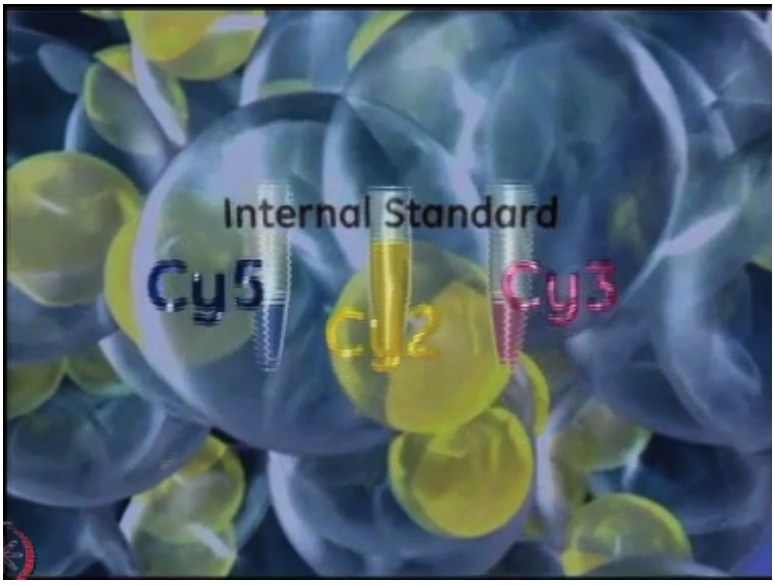

Now you are taking this 3 sample into three different kind of tubes and you are labeling with Cy3 dye as well as again another with Cy5 then you are taking those 2 samples pooling them and labeling with Cy2 then you are mixing those 3 into a single tube then you are running in your single strip. It will be analyzed in first dimension the IEF whereas in first dimension after analyzing IEF you will be placing it to second dimension where you are completely analyzed the second dimension.

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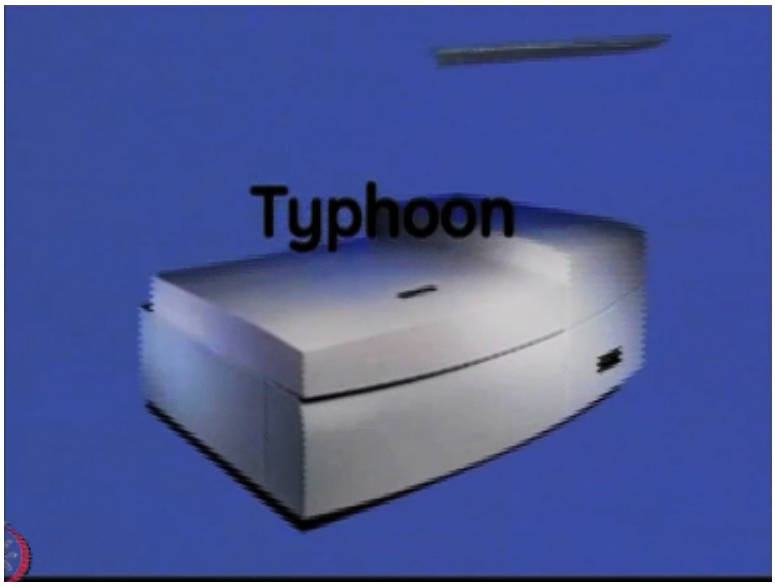

Now this gel can be scanned in the typhoon with Trio Plus where you will have 3 images from a single gel.

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Then this can be analyzed with the help of Decyder software then where the complete analysis can be done with the help of Decyder.

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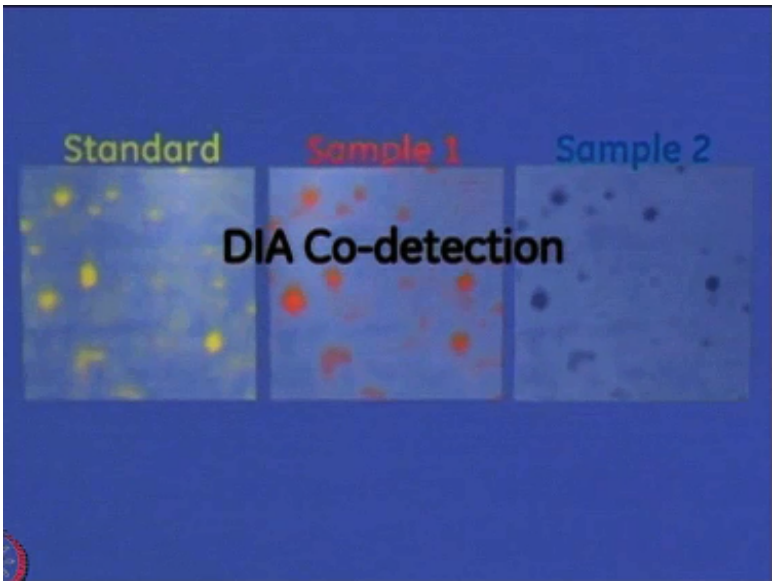

Then in DIA there will be a co-detection.

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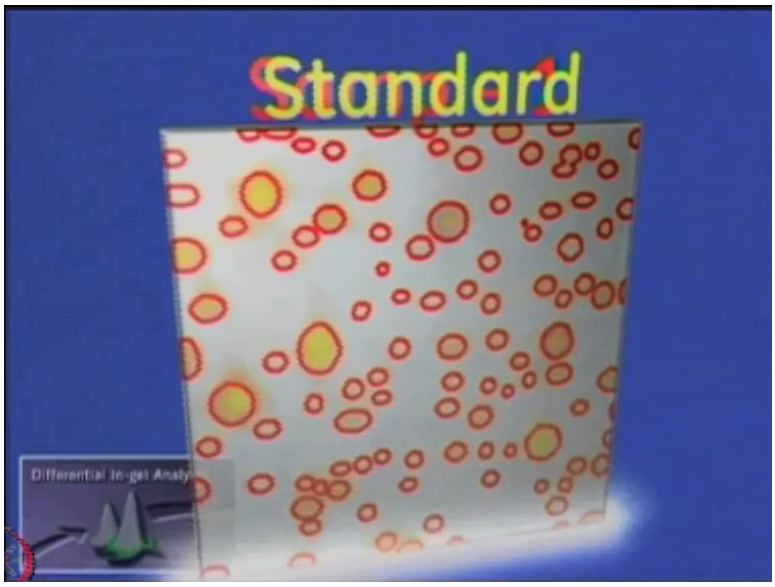

The co-detection nothing. but there is a standard gel which is labeled with Cy2 from the same area where expanding remains 2 images also so that the artifacts can be minimized.

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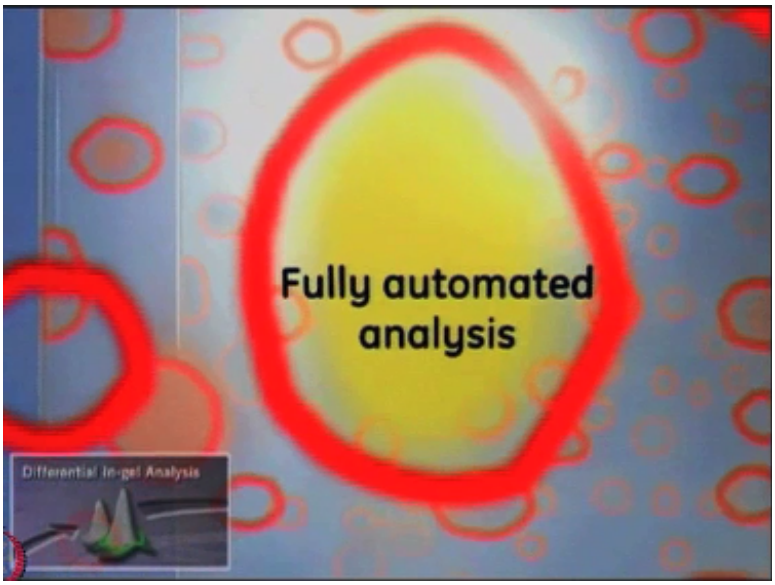
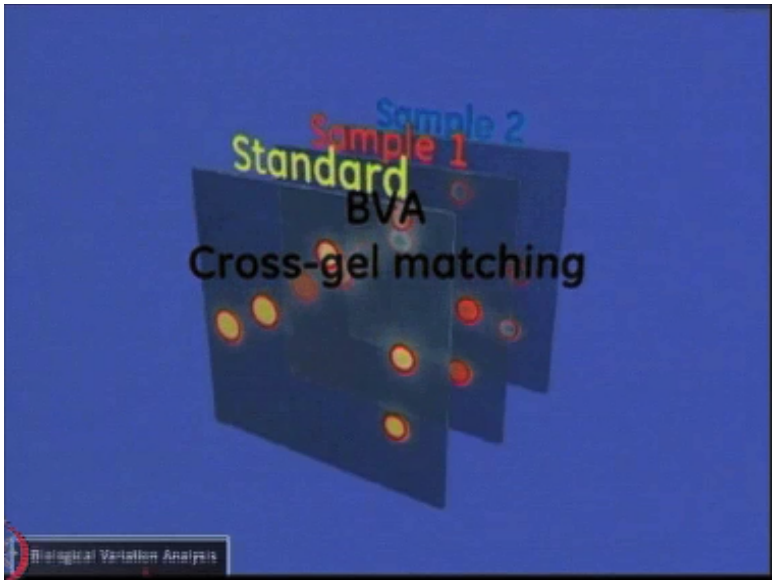


Then this is fully automated analysis. There will be very less manual interference would be there in this analysis.



And after finishing of DIA we can go to BVA.

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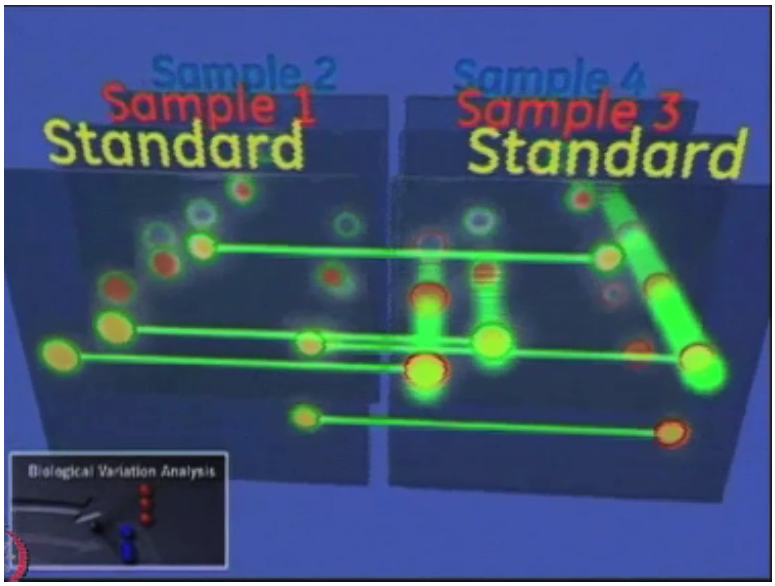

In BVA we can compare all DIAs together like as you can see in this video first of all it matches between the standard gel and from standard gel to again the corresponding DIA. It matches each and individual spot and it will give you the final data. Then these BVAs can be analyzed in the further in EDA that is Extended Data Analysis. Here you will have lot of stringent statistical data that is differential expression analysis, pattern analysis, discriminant analysis and PCA analysis. "**Dr. Sanjeeva Srivastava – Dr. C. Srivivasarao conversation ends"**

Dr. Srinivas it was very useful to get the glimpse of the overall process in DIGE technology, would you like to elaborate or demonstrate some more detail about steps involved in doing

the labeling of this technology? "**Dr. Sanjeeva Srivastava – Dr. C. Srivivasarao conversation starts"**. Yes there is another video where you can see now this is the second video which you can see more elaboratively this is particularly protocol which is developed for membrane protein analysis.

Now we can see this video. "**Dr. Sanjeeva Srivastava – Dr. C. Srivivasarao conversation ends"**

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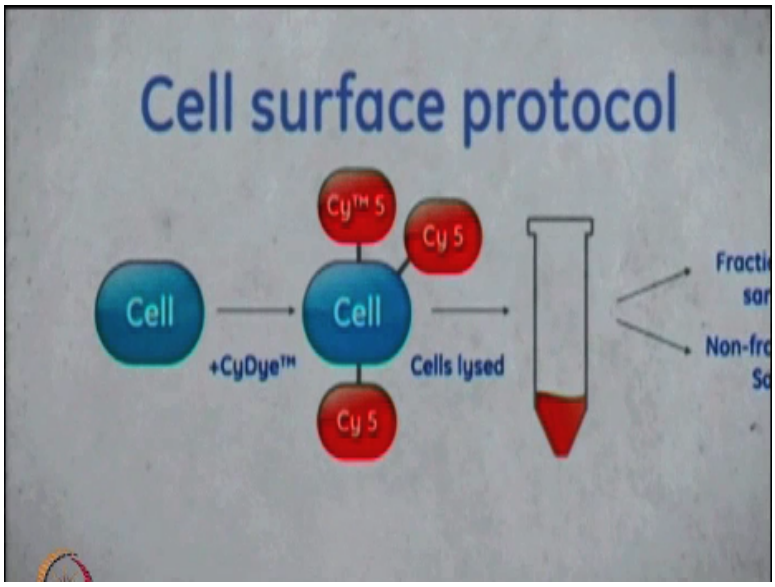

"**Dr. Sanjeeva Srivastava – Dr. C. Srivivasarao conversation starts"** In the cell surface labeling protocol seen on top you label the cells while they are still intact. During the labeling process, the dye will only have access to the cell surface proteins. After the labeling step the cells are lysed to verify cell surface specific labeling the label sample was fractionated into membrane and cytosolic proteins.

A non fractionated sample was prepared in parallel for comparison. This fractionation analysis is not necessary, but was done here just to show that the cell surface protocol is specific for cell surface proteins.

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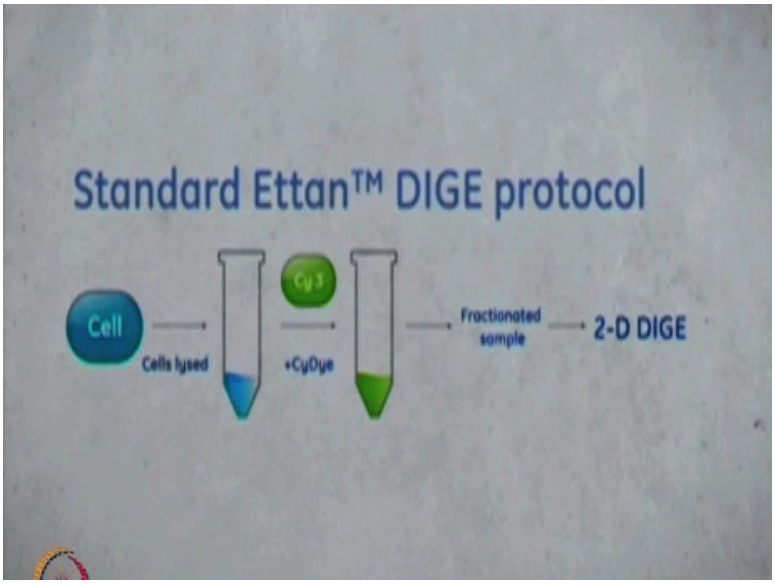

We also perform the standard Ettan DIGE labeling protocol seen below. The cells are lysed before labeling and in this way all cellular proteins are accessible for labeling. After the labeling steps the samples are subjected to 2D electrophoresis. Adherent cells are detached using a non enzymatic procedure to avoid digestion of the cell surface proteins targeted in this protocol.

We used to rather (()) (10:15) but using enzyme free cell dissociation media is also an option.

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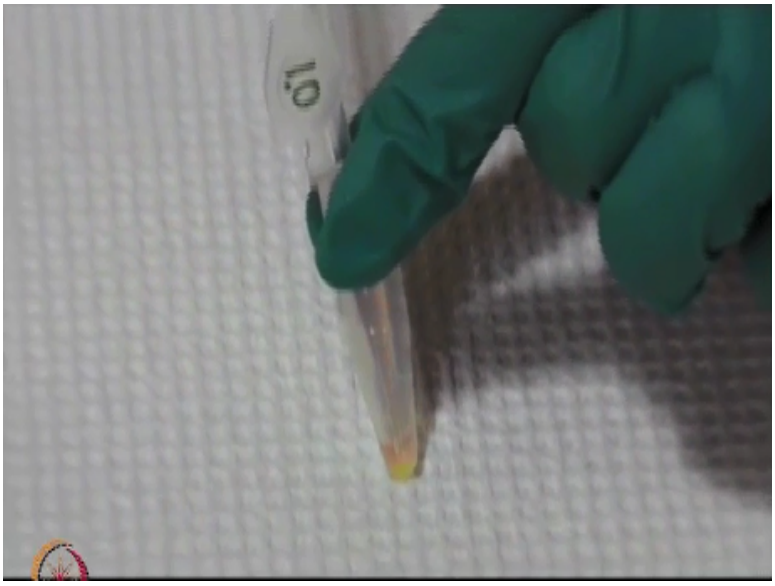

Count and divide the cell suspension into aliquots of 5 to 10 million cells per tube.

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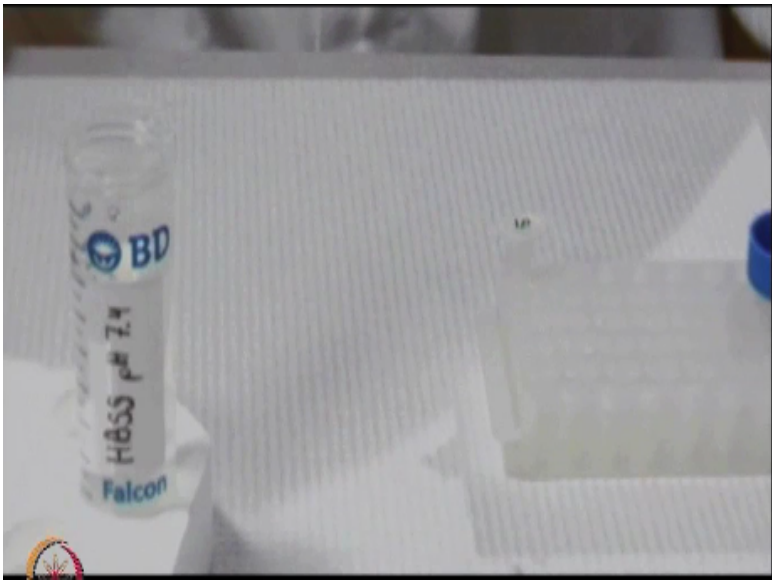

The cells are then palliated and washed in a HBSS pH 7.4 to remove traces of cell culture media. Contamination from serum proteins and fresh and components can interfere with labeling and detection. Cells growing in suspension are directly palliated and washed before the labeling steps.

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After the wash the cell pallet is re-suspended in 200 micro liter ice cool labeling buffer containing HBSS pH 8.5 and one molar Urea for optimal labeling conditions of cell surface proteins. Always check pH before labeling. We used 600 picomole Cy dye for 10 million CHO cells. The optimal ratio of Cydye to cell number will vary depending on the cell type. Since we do not know the exact protein concentration on the cell surface.

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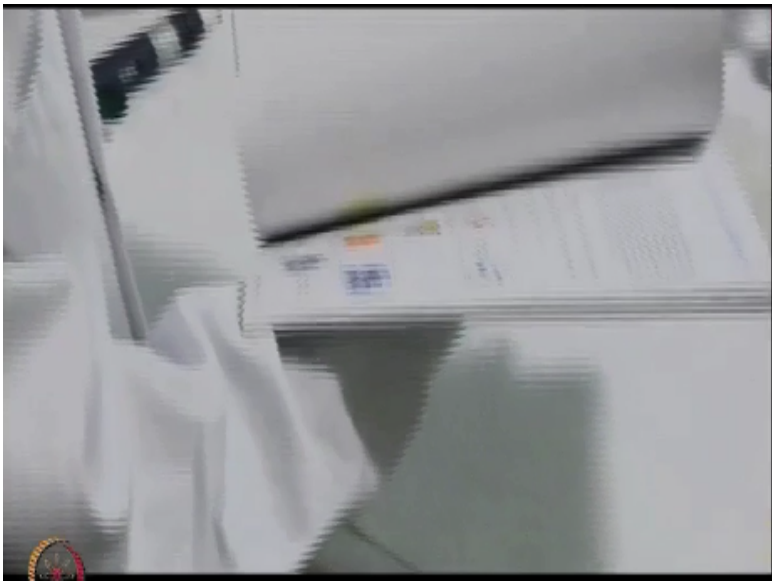

How to determine the optimal conditions for Cydye labeling of proteins is described in the 2D electrophoresis principles and method handbook.

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The cells are incubated with Cydye DIGE flour minimal DIGE for 20 minutes on ice in the dark. After the labeling reaction the un-reacted dye is quenched by adding 20 micro liters of 10 millimolar lysine. The labeled cells are now washed twice in cold HBSS pH 7.4 buffer to remove the excess Cydye. There will therefore be no free dye left for unwanted intercellular labeling of proteins in the next steps which is cell lysis.

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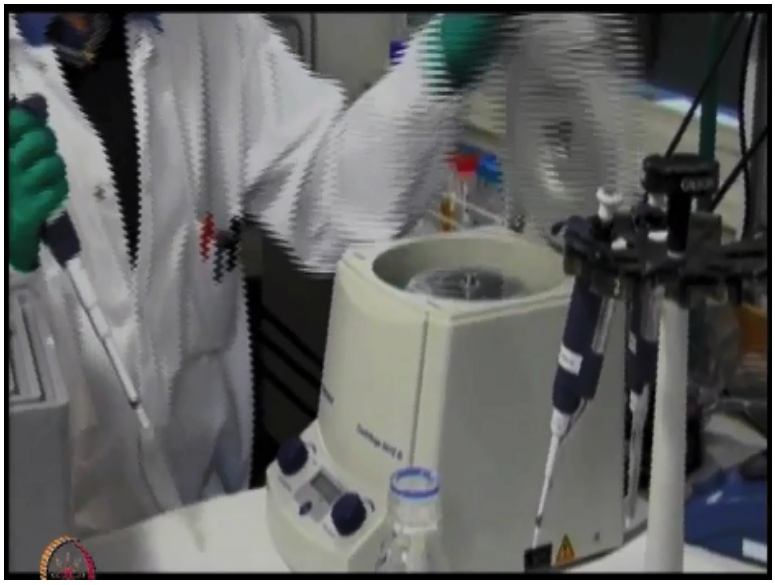

The proteins on the cell surface are now labeled and the cells are washed and ready to be lysed. The pallet from the last washing step is re-suspended in 150 micro liter cold lysis buffer containing 7 molar Urea, 2 molar thiourea, 4% of CHAPS, 30 millimolar Tris, 5 millimolar magnesium acetate pH 8.5 and let on ice for atleast 1 hour with occasional vortexing.

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The sample are now ready for 2D gel separation. The first step in 2D electrophoresis is to prepare IPG strips for rehydration.

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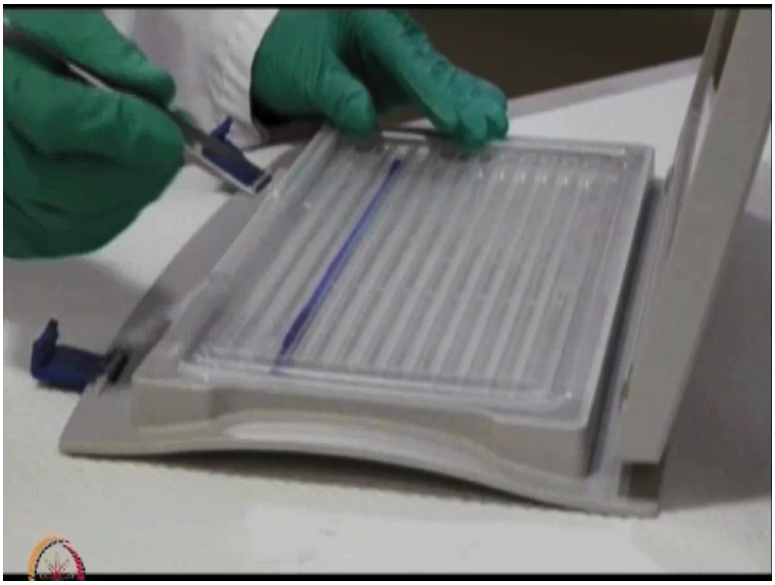

Prepare this strip rehydration solution by adding IPG buffer corresponding to the pH interval of the strips used and add the solution in the lens of the rehydration tray. Remove the protected film of the IPG strip and place the strips carefully with a dried gel facing down in the rehydration tray containing the rehydration solution. Close the lid of the IPG box and rehydrate the strips overnight.

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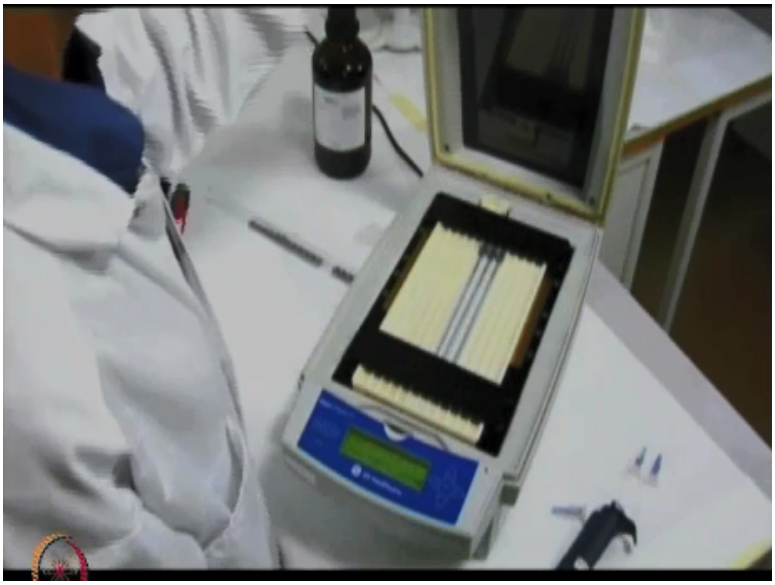

In the first dimension Isoelectric focusing the proteins are separated according to the PI. This is performed using the IPG for free. The rehydrated strips is placed in the manifold and the electrode is mounted on top. 50 microgram from each samples were applied using sample application caps. We have either directly applied non fractionated samples without prior fractionation old fractionated the samples into membrane and cytosolic fraction before they were applied.

The lid is closed to protect the fluorescent samples from light. The instrument was programmed according to recommendation and run overnight.

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Large 12%. Laemmli gels were caused using (()) (15:20) gel caster. This dysplating solution was added to avoid polymerized acrylamide gel in the tubings. The gels were allowed to polymerize using overnight at room temperature prior to use.

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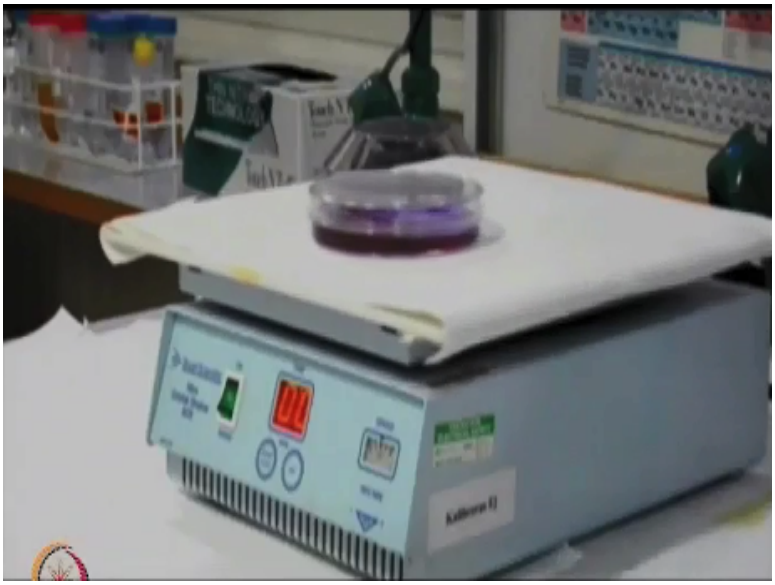

After the Isoelectric focusing the strips are removed and equilibrated in SDS containing buffer in 2 steps using DTT to reduce the disulfide bond of cysteine residues followed by articulation with Iodoacetamide to avoid modification by acrylamide.

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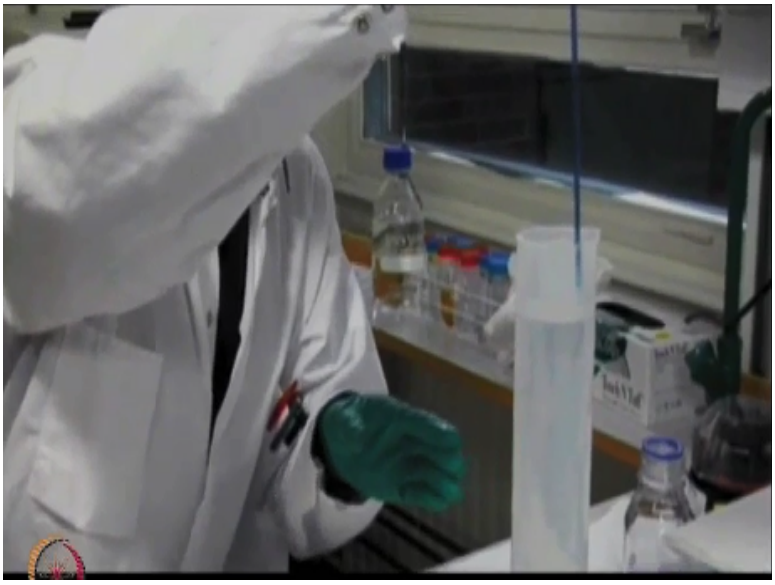

The IPG strips are dipped in running buffer.

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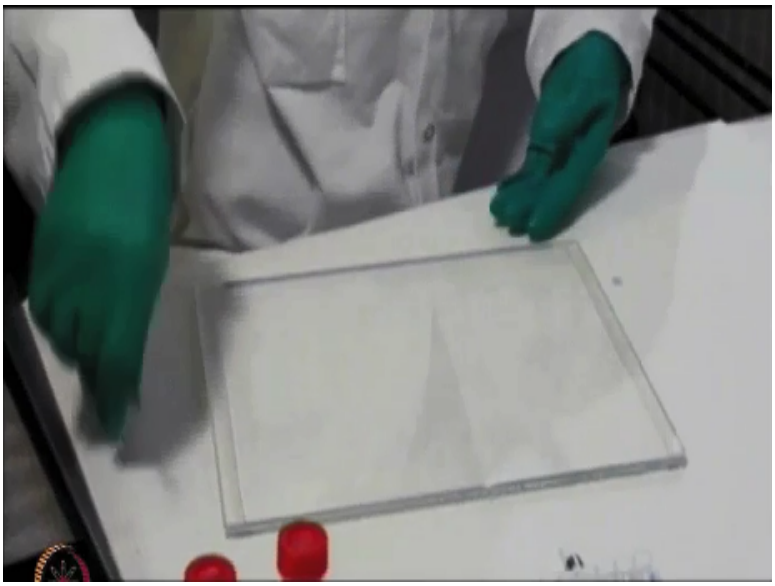

And carefully placed on top of the loge 2D gels. Avoiding trapping air between the strips and the gel.

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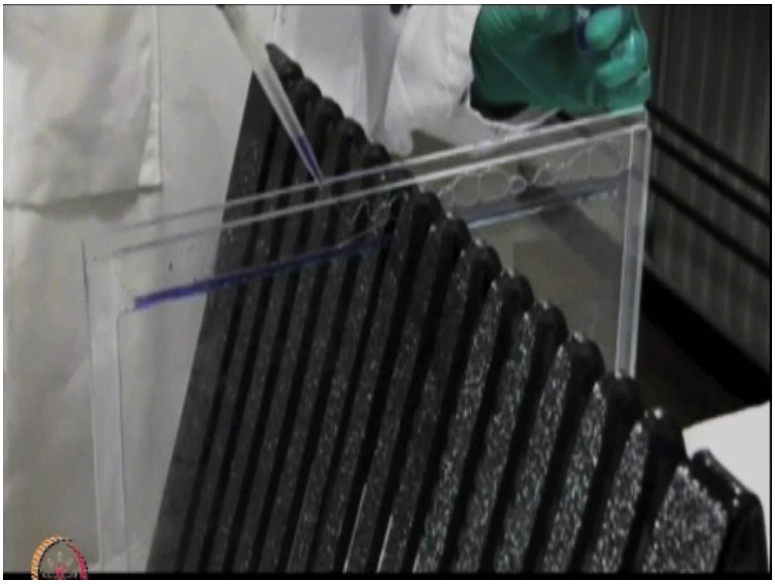

Seeing by adding melted 2% agro solution with Bromophenol blue on top. The gels are now ready for second dimension SDS PAGE separation.

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In the second dimension SDS PAGE the proteins will be separated according to molecular weight and this is performed with Ettan DALT 6-system. Filled electrophoresis chamber with anodic running buffer insert the gel and fill the top compartment with cathodic running buffer. Program the power supply according to recommendations and run the second dimension protected from light for approximately 4 to 5 hours or until the dye front reaches the bottom of the gel.

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After the second dimension electrophoresis the gel cassettes are placed by using the gripper in the typhoon fluorescent imager. Two gels and 3 channels can be scanned simultaneously.

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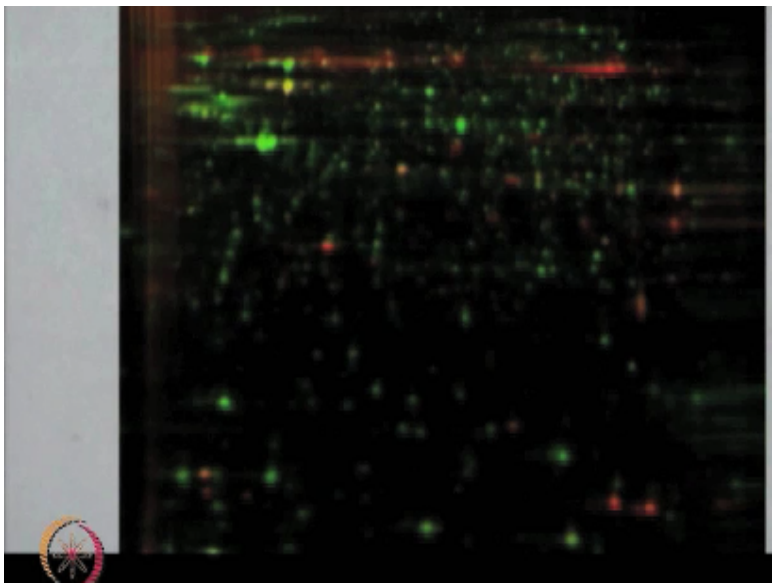

The result from this 2D gel shows high resolution of membrane proteins in the sample even if there are some known restriction for hydrophobic proteins to be detected in a 2D gel. The result (()) (18:50) cell surface label spot shown here in red that are not detected using the standard labeling protocol shown here in green.

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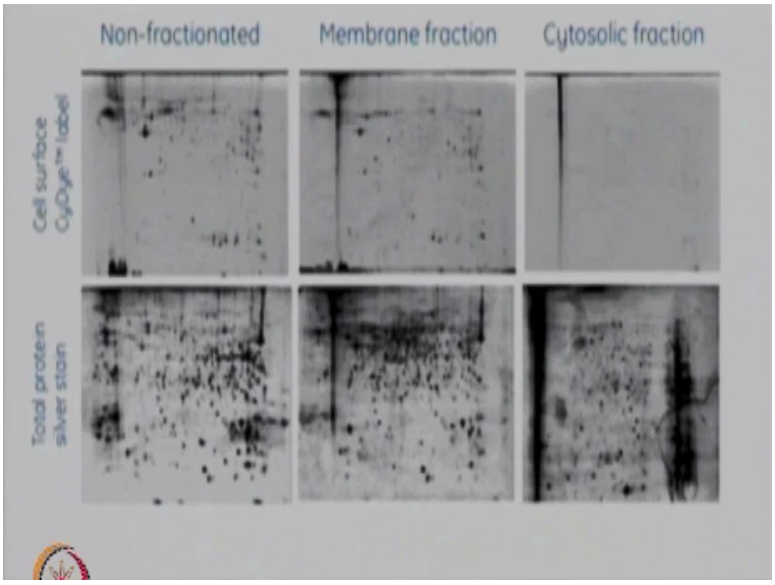

This result show that the cells surface labeling protocol is highly specific for labeling cell surface proteins. Since cell surface proteins are exclusively labeled they are more easily visualized and attenuation by abundance cytosolic proteins is avoided. The fluorescent image of gels with non-fractioned membrane fraction or cytosolic fraction is shown on top. Below is an image of the same gel co-stain with silver.

The result shown no fluorescent labeling of cytosolic proteins but a sliver staining shows that they are all proteins in the gels. The results also show similar spot map pattern from non-fractionated and membrane fractions demonstrating that there is no need for fractionation prior to 2D electrophoresis which makes this protocol both simple and convenient.

Cy2, Cy3 and Cy5 show similar labeling patterns and are all compatible with the cell surface protocol.

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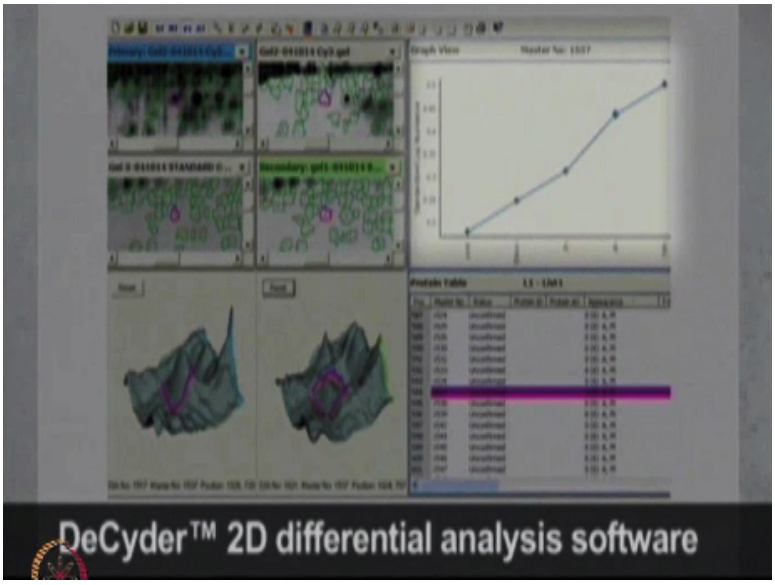

The DIGE experiment was performed using all 3 sides Cydye DIGE flour minimal DIGE for studying differential expression of cell surface proteins in CHO cells of the serum starvation for different length of time. Cy2 cell surface samples from old samples in the experiment are pooled and used as an internal standard. The differential changes of several cell surface proteins could be fall of during the starvation period.

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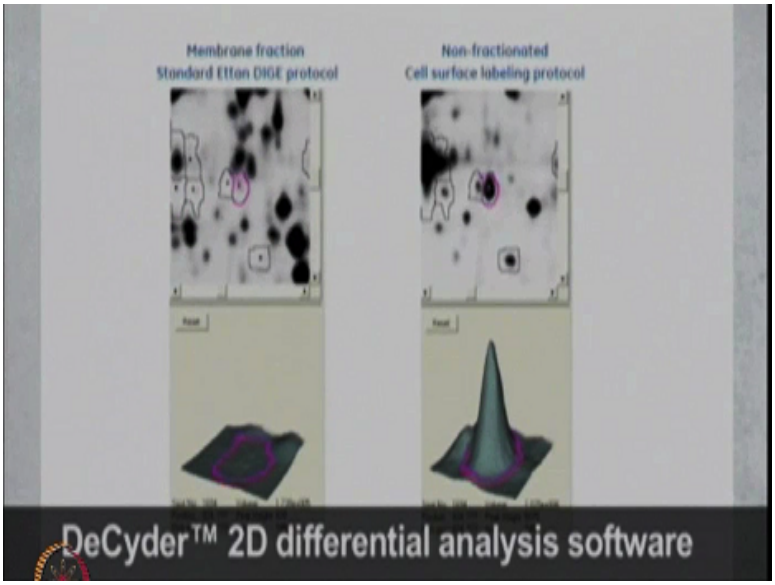

Over 18 overall membrane proteins were detected using the cell surface protocol that were not detected with the standard Ettan DIGE protocol. For finding their identity of the proteins in the preoperative gel it was necessary to spike with the cell surface sample to facilitate matching back to the spots on the analytical data set. "**Dr. Sanjeeva Srivastava – Dr. C. Srivivasarao conversation ends".**

So Dr. Srinivas it was very useful to see all this steps which are involved in doing this DIGE technology. There is that discussion going on in the proteomics field that due to advancement in Mass spectrometry and microarrays. How do you foresee the future of two-dimensional electrophoresis and DIGE technology, do you think it is going to be simultaneously working along with mass-spec and microarrays or do you think it is going to be behind because more advanced approaches having emerged. So what is your suggestion on that?

"**Dr. Sanjeeva Srivastava – Dr. C. Srivivasarao conversation starts"** Yes of the MS and microarrays developed differently. They have their own advantages as well as disadvantages like disadvantages like very high instrumentation cost and high protein requirement as well as technical variance would be there that is why still 2D DIGE will be the core technology to analyze proteomics one can easily analyze. "**Dr. Sanjeeva Srivastava – Dr. C. Srivivasarao conversation ends"**

So you are still convinced that by using 2D DIGE approach that will still remain one of the core proteomics technology for proteomics analysis and it will be used simultaneously along with mass spectrometry and microarrays. Yes. So at the end I would like to request you to give a brief overview of the Decyder software which is being used for analysis of DIGE gel.
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"**Dr. Sanjeeva Srivastava – Dr. C. Srivivasarao conversation starts"** Decyder is the software to analyze DIGE gels where we can analyze DIGE gels in different stages. In first stage, we have to upload or gels into our software. This basically works on the oracle database where we are incorporating our gels into database there you can add these gels into

software in this way and you can select what are the gels you want to upload.

Once we have upload then you can able to edit these gels here itself like you want to crop or edit anything or any sort of things you can do here. It will allow you to edit these images.

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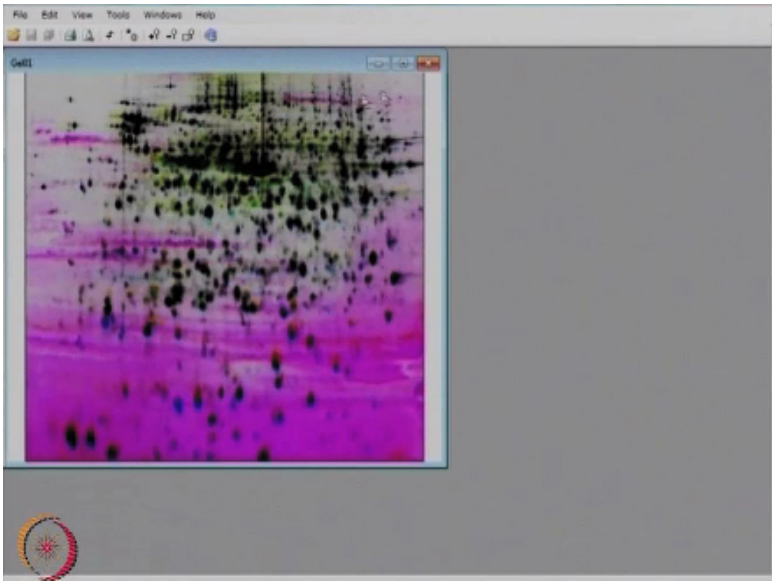

So now you can see this is the overlay of 3 images. Now you can crop all these images like what are the portion you want to take if you have to remove any area which is not your interest of then you can directly crop those images once you directly editing you can save it yes save it and close it. This is what you can edit these images then you have to create a new project. Suppose your project name would be according to your project name.

This is demo now we have the project name of demo. Now you can able to import these 2 gel images into a new project where we created just click on import. Now these gels will be imported into this particular project.

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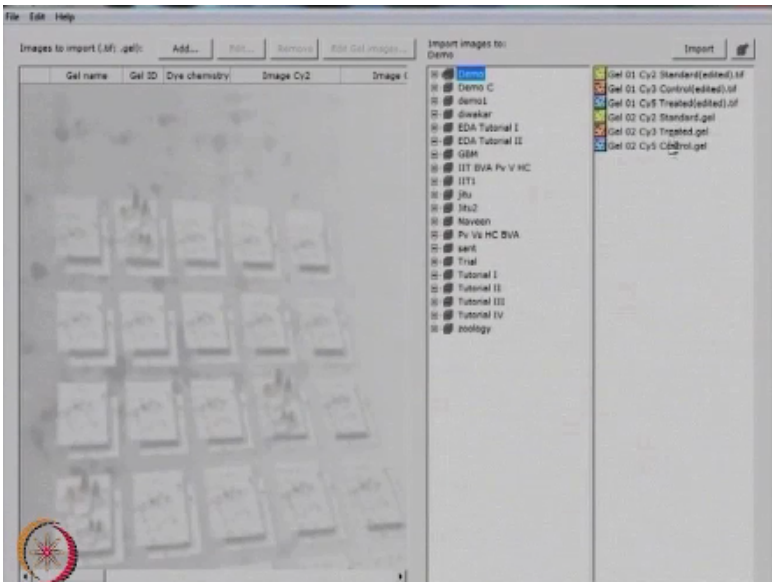

Now here you can see all 6 images though these are at the basically 2 gels. We think these 2 gels we have Cy2, Cy3, Cy5 and total 6 gel images. This is the image editor and image loader. "**Dr. Sanjeeva Srivastava – Dr. C. Srivivasarao conversation ends"**