

NPTEL VIDEO COURSE – PROTEOMICS

PROF. SANJEEVA SRIVASTAVA

HANDOUT

LECTURE-17

DIFFERENCE IN GEL ELECTROPHORESIS (CONTINUED)

DISCUSSION AND DATA ANALYSIS

Slide 1:

The previous lectures have covered gel based proteomic techniques and the performance of 2-DE. This lecture will discuss technologies that have emerged to overcome the limitations of this procedure.

Slide 2:

2-DE has various limitations, most of gel-to-gel artefacts and variations which emerge mainly from inconsistency in sample preparation, and then subsequent gel running itself, during the 1st and 2nd dimensions. The method of DIGE, which allows multiple complex protein samples to be run on the same gel, works towards eliminating these variables.

Slide 3:

This lecture (with the input of a guest speaker, Dr. Srinivas from Life Science Wipro GE Healthcare) will cover:

- A continuation of the discussion on gel-based proteomics
- DIGE: Advantages and disadvantages, data analysis using specialized software programs, data interpretation and derivation of meaningful biological information from such analyses.

Major advantages and disadvantages of 2-DE

- This is very user friendly process with low instrumentation costs.
- The complete proteome, with the differentially expressed or novel proteins can be investigated using this technique.

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- A number of user-friendly software programs are also available for data interpretation.
- The disadvantage is mainly a lack of reproducibility.

Suggestions to help overcome the limitations of traditional 2-DE technology

- One should have to prepare very good sample preparation so that the reproducibility issue will be overcome.
- Use of a new generation of 2-DE methods, 2DE-DIGE technology, where you can use up to 3 samples in a single strip. There will be an internal control as well as control and treatment samples. These 3 can run in a single strip so that these kind of reproducibility issues can be overcome as well as there is a very powerful scanner available in this technology, the Typhoon Trio which incorporates a laser based technology.

Video 1

The following video covers DIGE technology and involves the separation of 2 protein samples from different sources.

- In DIGE performed with Cyanine dyes, the 2 samples to be analysed and a pooled internal standard consisting of equal amounts of the 2 samples are added to 3 different eppendorf tubes and are labelled with Cy3 & Cy5 (samples) or Cy2 (pooled internal standard).
- After labelling, the samples and the pooled internal standards are mixed in a single tube and run on a single strip in the first dimension of isoelectric focussing.
- Following the completion of this procedure, the samples will be separated in the second dimension in an SDS-PAGE gel, as previously described.
- The gel with the samples separated in the 2 dimensions can be scanned in the Typhoon Trio plus scanner, which generates 3 images (one for each fluorescent dye) from a single gel.

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- Then this can be analysed with the help of DeCyder software, which analyses the gel completely using a program called 'DIA' (see <http://dige-proteomics.neuro.duke.edu/decyder.html>).

Video 2

DIGE following Cell surface labelling for study of membrane proteins

- In the cell surface labelling protocol, one labels the cells while they are still intact.
- Importantly, during the labelling process the dye will only have access to the cell surface proteins.
- After the labelling step, the cells are lysed.
- To verify cell surface-specific labelling, the labelled sample is fractionated into membrane and cytosolic proteins.
- A known fractionated sample is prepared in parallel for comparison. This fractionation analysis is not necessary, but is done just to show that a cell surface protocol is specific for cell surface proteins.
- The Standard Ettan DIGE labelling protocol (wherein the cells are lysed before labelling and in this way all cellular proteins are accessible for labelling) was performed in parallel to the surface labelling protocol. After the labelling step the samples are subjected to 2-DE.

Sample preparation

- Adherent cells are detached using a non-enzymatic procedure to avoid digestion of the cell surface proteins targeted in this protocol.
- A rubber policeman can be used to scrape the cells. Additionally trypsin-containing cell dissociation media is also an option.
- Count and divide the cell suspension into aliquots of 5-10 million cells per tube.
- The cells are then pelleted and washed in HBSS pH 7.4 to remove traces of cell culture media, as contamination from serum proteins and preparation components

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can interfere with labelling and detection. Cells growing in suspension are directly pelleted and washed before the labelling step.

Labelling with Cy dyes

- After the wash, the cell pellet is resuspended in 200 μL ice cold labelling buffer containing HBSS pH 8.5 in 1 M urea for optimal labelling conditions of cell surface proteins.
- Always check pH before labelling. In this case, 600 picomoles of Cy dye for 10 million CHO cells was used.
- The optimal ratio of Cy dye to cell number will vary depending on the cell type, since the exact protein concentration on the cell surface is not available. How to determine the optimal conditions for Cy dye labelling of proteins is described in the '2-DE principles and methods handbook'.

Incubation and quenching

- The cells are incubated with Cy dye DIGE fluorescent minimal dyes for 20 min on ice in the dark.
- After the labelling reaction, the unreacted dye is quenched by adding 20 μL of 10 mM lysine.

The labelled cells are now washed twice in cold HBSS pH 7.4 buffer to remove the excess Cy dye. There will, therefore, be no free dye left for unwanted intercellular labelling of proteins in the next step which is cell lysis.

Cell lysis

The proteins on the cells surface are now labelled and the cells are washed and are ready to be lysed. The pellet from the last washing step is resuspended in 150 μL cold lysis buffer containing 7 M urea, 2 M thiourea, 4% Chaps, 30 mM Tris, 5 mM Magnesium acetate, pH 8.5 and left on ice for atleast 1 hour with occasional vortexing.

Rehydration of IPG strips

The samples are now ready for 2-D gel separation. The first step in 2-DE is to prepare IPG strips for rehydration. Prepare the rehydration solution by adding IPG buffer

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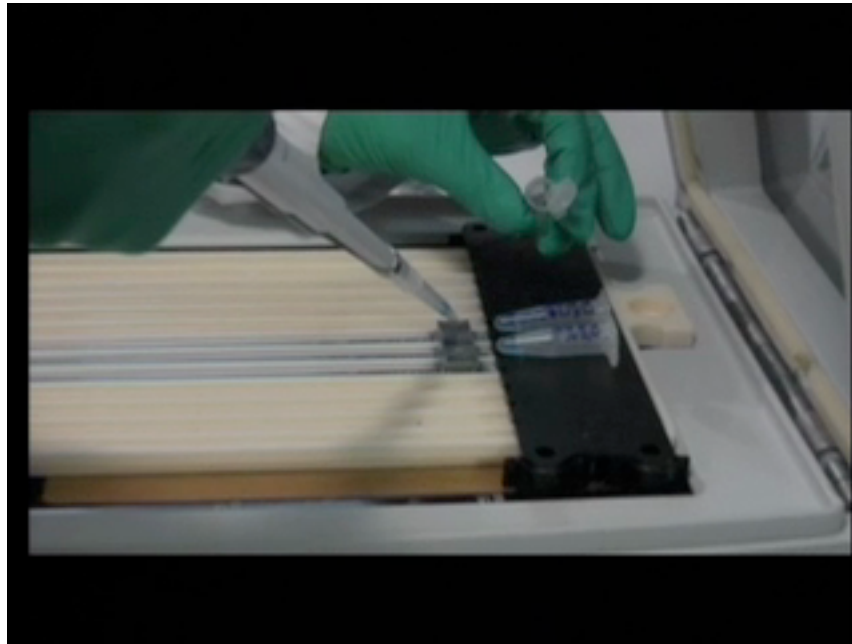
corresponding to the pH in the strip used and add the solution in the lanes of the rehydration tray. Remove the protective film of the IPG strip and place the strip carefully with dried gel facing down in the rehydration tray containing the rehydration solution. Close the lid of the rehydration box and rehydrate the strips overnight.



Placing the IPG strip in the rehydration tray

Isoelectric focusing (IEF)

- In the first dimension IEF the proteins are separated according to their pI. The rehydrated strip is placed in the manifold and the electrode is mounted on top. 50 μ l of each sample is applied using sample application caps. The non-fractionated samples can be directly applied or alternately, the samples can be fractionated into membrane and cytosolic samples prior to application.
- The lid is then closed to protect the fluorescent samples from the light. The instrument is programmed according to the recommendation and run overnight.



Application of samples on the rehydrated IPG strips

Gel assembly

- Large 12% lamli gels can be cast using a dual 12 gel caster.
- The displacing solution is added to avoid polymerized acrylamide gel in the tubing.
- The gels are allowed to polymerize overnight at room temperature prior to use.

Equilibration of IPG strip

- After the IEF, the strips are removed and equilibrated in SDS-containing buffer in two steps using DTT to reduce the disulfide bonds in cysteine residues followed by alkylation by IAA to avoid modification by acrylamide.
- The IPG strips are dipped in running buffer and carefully placed on the top of the large 2-D gels. Avoid trapping air between the strip and the gel. Seal by adding melted 2% agarose solution with bromophenol blue on top. The gels are now ready for second dimension SDS-PAGE separation.

Placing the equilibrated IPG strip onto a 2-D gel

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SDS-PAGE

- In the second dimension SDS-PAGE the proteins will be separated according to the molecular weight and this is performed using the Ettan Dal6 system.
- Fill the electrophoresis chamber with anodic running buffer, insert the gels and fill the top compartment with cathodic running buffer.
- Program the power supply according to the recommendations and run the second dimension protected from light for 4-5 hours or until the dye front reaches the bottom of the gel.

Filling the top compartment with cathodic buffer

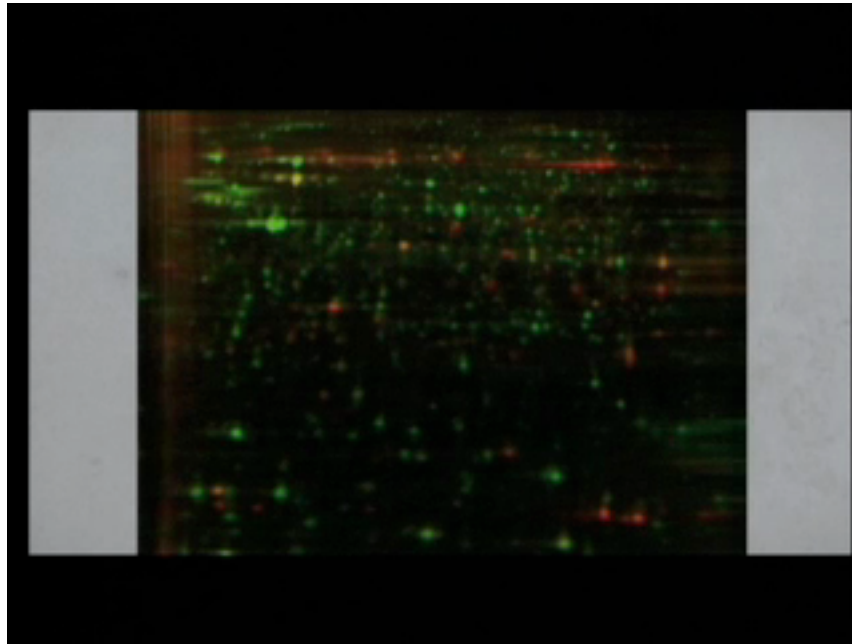
Gel scanning

After the second dimension electrophoresis, the gel cassettes are placed using the grippers in the typhoon imager. 2 gels and 3 channels can be scanned simultaneously.

Scanning of the processed gel

Results: DIGE gels

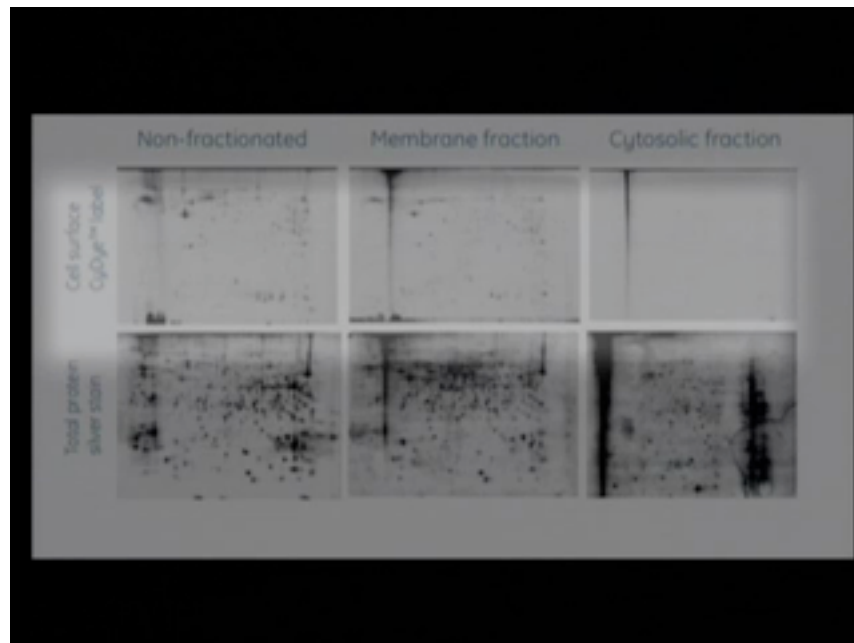
The result from this 2-D gel shows high resolution of membrane proteins in the sample. Even if there are some known restrictions for hydrophobic proteins to be detected in a 2-D gel, the results show many new cell surface label spots shown here in red that are not detected using the standard labelling protocol shown here in green.



2-DE gel image

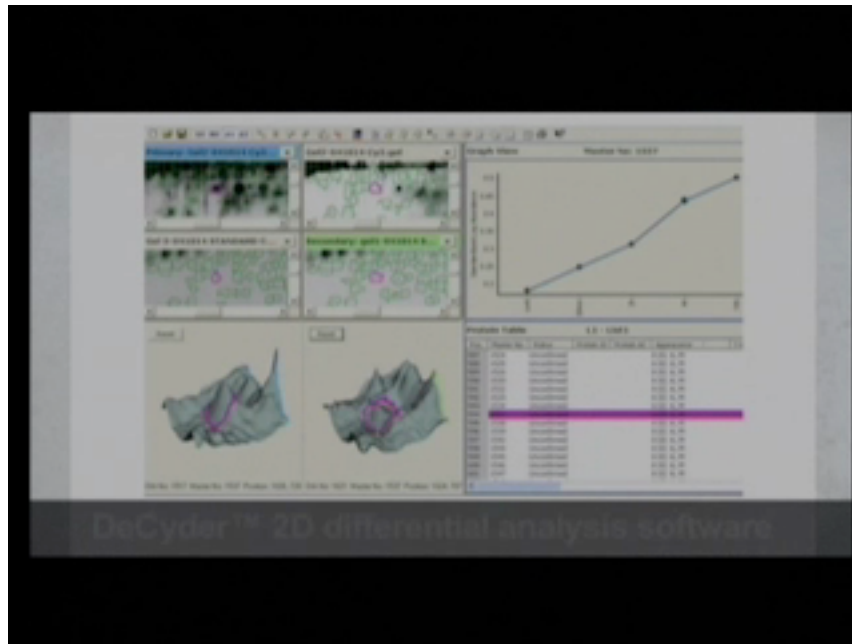
Analysis

- These results show that the cell surface labelling protocol is highly specific for labelling cell surface proteins.
- Since the cell-surface proteins are exclusively labelled, they are easily visualized and attenuation by abundant cytosolic proteins is avoided.
- The fluorescent gels with known fractionated membrane fractions or cytosolic fractions are shown on top. Below are the images of the same gels co-stained with silver. The results demonstrate no fluorescent labelling of cytosolic proteins (as labelling was done prior to cell lysis) but the silver staining shows that there are proteins in the gels.
- The results also show similar map patterns for known fractionated and membrane fractions demonstrating that there no need for fractionation prior to 2-DE with the cell surface labelling technique, which makes this protocol both simple and convenient. Cy2, Cy3 and Cy5 show similar labelling patterns and all compatible with the cell surface protocol.



Analysis using DeCyder

- A DIGE experiment was performed using all 3 Cy-dye DIGE fluor minimal dyes for studying different expression of cell surface proteins in CHO cells following serum starvation of varying time periods.
- Cy2-labelled cell surface samples from the different conditions in the experiment are pooled and used as an internal standard.
- The differential changes of several cell surface proteins could be followed during the starvation period.
- Over 18 novel cell surface proteins were discovered during the cell surface protocol, that were not detected with the standard Ettan DIGE protocol.
- To determine their identity using a protein preparative gel, it was necessary to spike with the cell surface protein to facilitate matching back with the analytical data set.



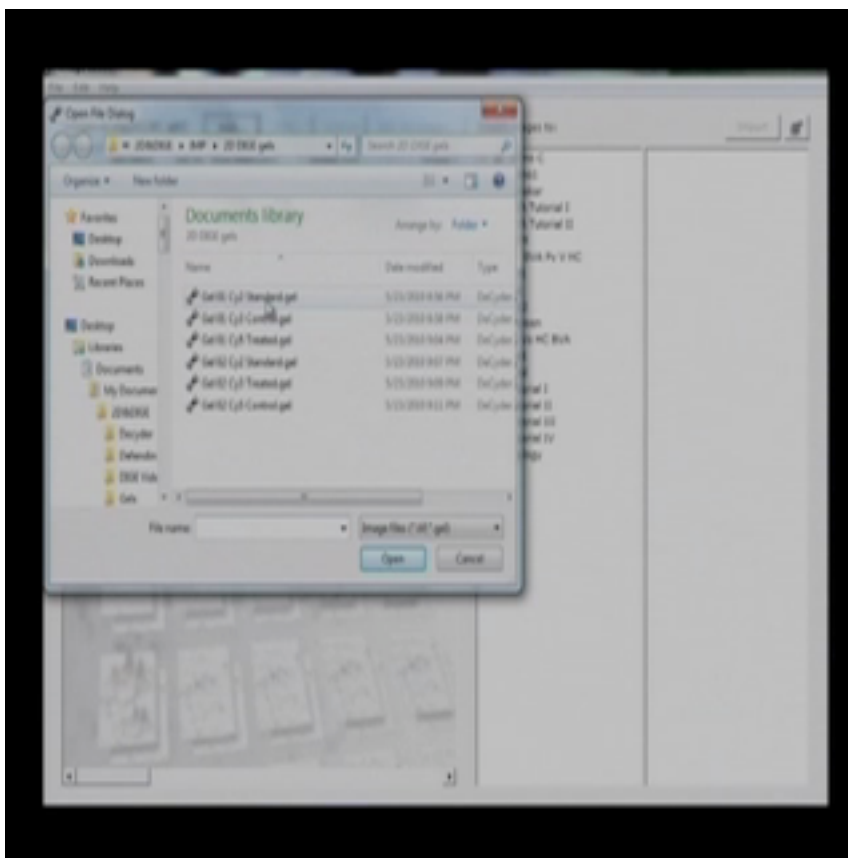
A brief overview of DeCyder software used to analyse DIGE gels.

- In first stage the gels are uploaded in to the software, run on the Oracle database. Therein, once the gels are added into the software, the gels to be uploaded can be

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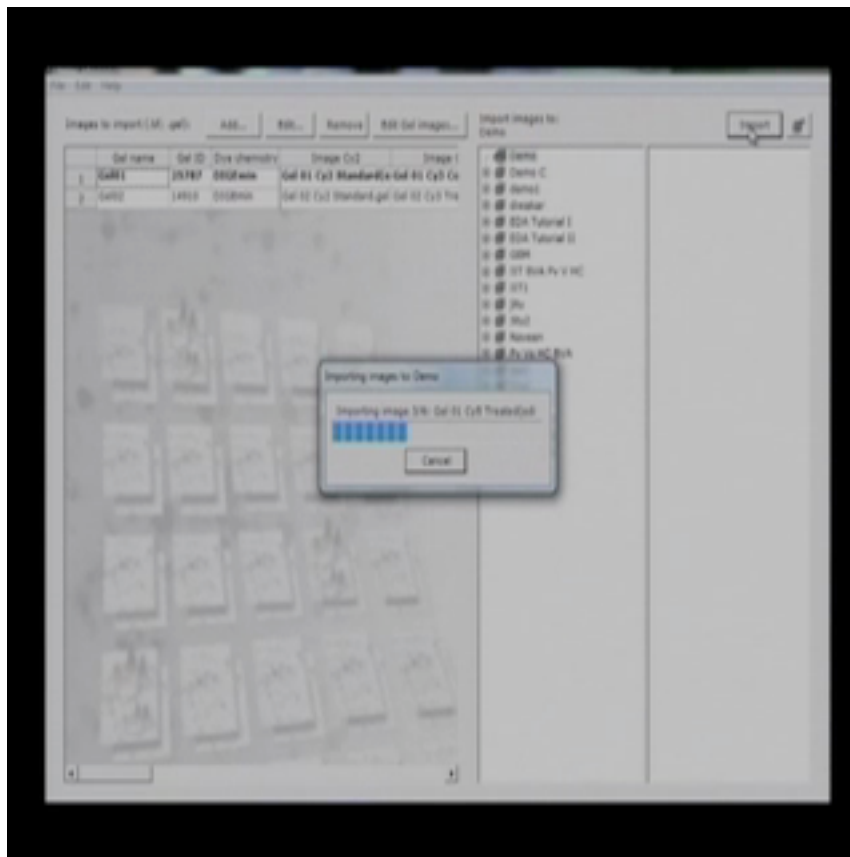
selected, and then edited by cropping, etc.



- Once the overlay of 3 gel image is generated, it can be suitably cropped, removing the regions that are not the area of interest.
- Once such editing is done, it is saved as a new 'project', which in this example, is called 'Demo'. The gel images can be added to the project by using the 'Import function' In this case, the 2 gels, scanned at 3 wavelengths(for the Cy2, Cy3 and Cy5 dyes), produce 3 images each, to give a total of 6 images. See below a screenshot of the image editor and image loader.

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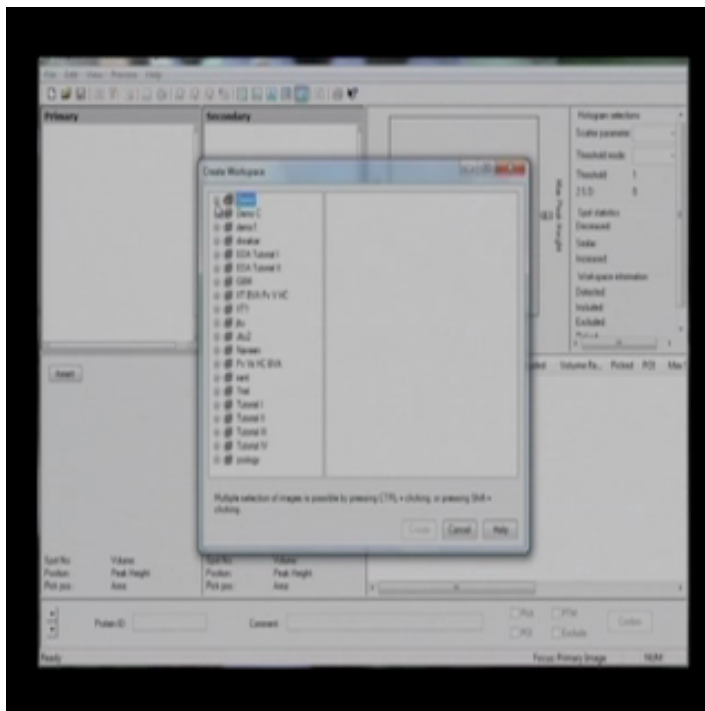


- At the main DeCyder screen, click on Differential In-gel Analysis or DIA.

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- In this interface, a new project can be created using the 'create workspace' option. That will take you to the place wherein the gel saved in the database can be

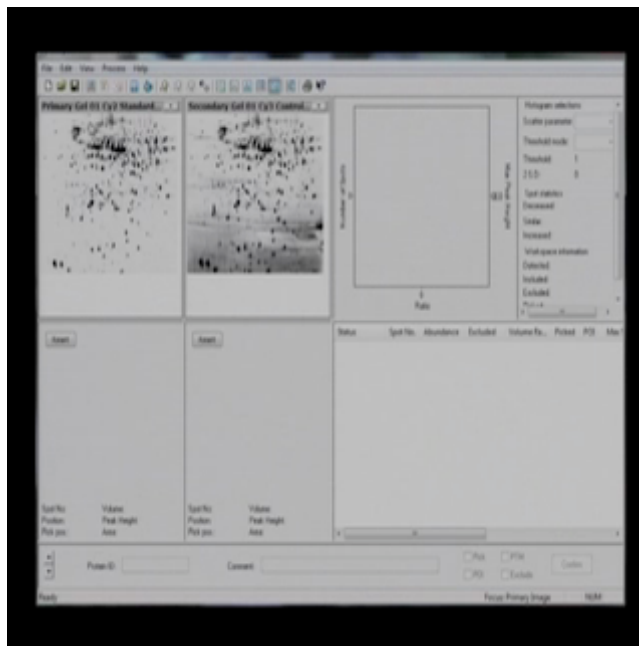


accessed.

- From the database, any particular project can be selected and from there, in this example, the saved gel number 1 is selected, from all the gels uploaded. Herein, one can see the gels uploaded, that are now ready for processing.
- During the processing, the gels have to be suitably named (refer this website for instructions: <http://dige-proteomics.neuro.duke.edu/decyder.html>)

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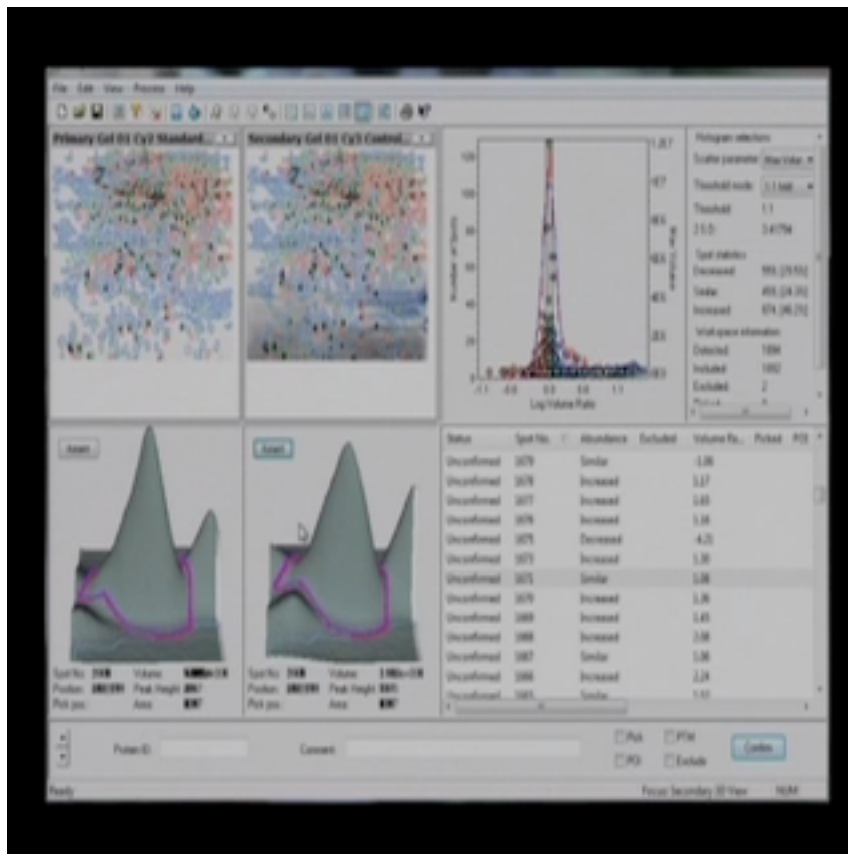
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- The next step is codetection. This uses information from all 3 channels and creates a geometrically identical spot boundary for a spot across all the channels. From the 3 channels (of Cy2, Cy3 and Cy5) it creates a particular volume and the same area can be applicable for the remaining 2 gels.
- In DIA, each image is co-detected with its internal control producing 2 images pairs. The ratio of standard sample is calculated for each protein in each image. In the image, one can see that a number of spots have been detected in which there are some red colour spots in the test sample which are down regulated compared with control. And the blue colour spots where you can see have been up regulated when compared with control. In between there are some other blue coloured spots. These all are similarly regulated. This example demonstrates the results obtained from the DIA interface. Each individual spot can be examined in the 3D view. It is then determined whether it is a real spot or background. If it is background it will be removed from the 'protein table'. The same procedure has to be repeated for every single spot, to generate accurate data.

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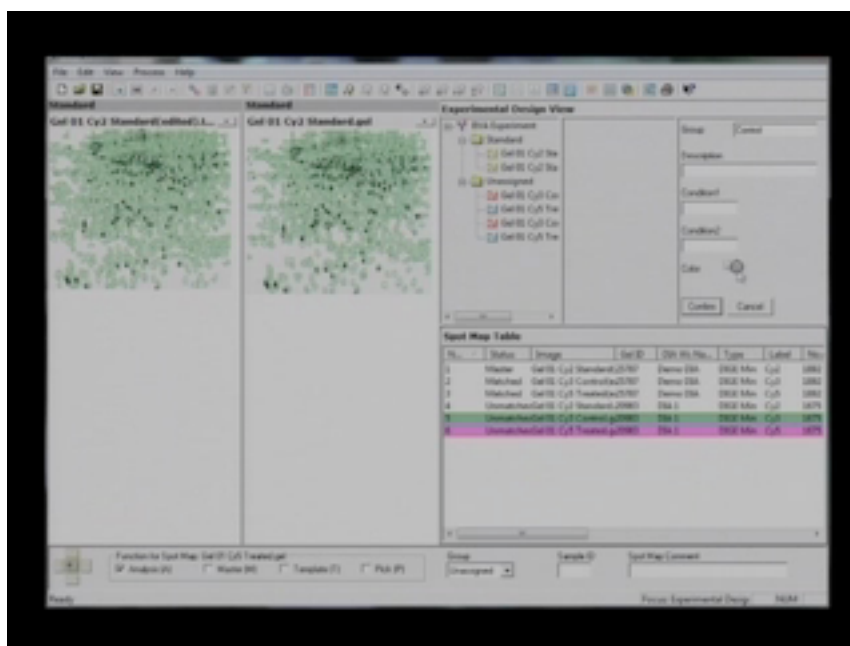


- Now, we are done with DIA. The next step is the BVA(biological variation analysis). One of the internal standard images (usually the one with the most spots) is selected as a 'master image' and all internal standard images are matched to this, generating the standard spot ratio for each protein. Each sample then compared giving a T-test value, fold-change value and an ANOVA value. To create a BVA workspace, please refer to this website (<http://dige-proteomics.neuro.duke.edu/decyder.html>). Note that a minimum of two DIAs are required for BVA. After selecting DIAs click on "create BVA" and a new BVA is created. From here all Cy2 gels automatically go to a standard folder and the rest of the gels remain in the unassigned folder, wherein they are assigned into different folders based on the groups (such as control or treated).

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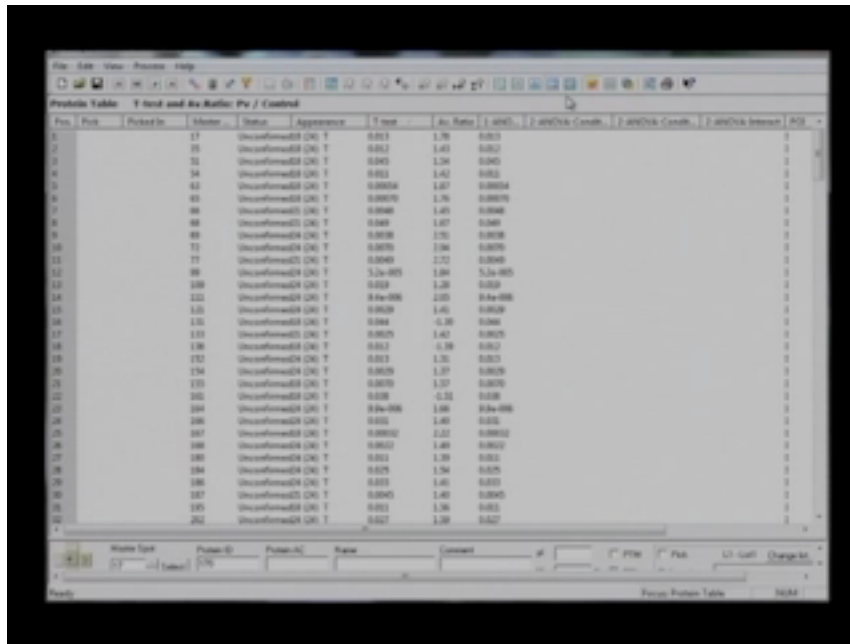
- After the images have been created, and shifting the control image to control folder and treated gel image to the treated folder, all the gels should be 'matched'. As we discussed out of all gels, one gel has been selected as a master image and the remaining gels will be compared with this, to generate the 'comparison data'.



- The next step is the calculation of statistical tests, including independent T-tests, average ratio, student-T-test, one-way ANOVA in between different groups to determine if any statistically significant differences exist between the test and control groups.

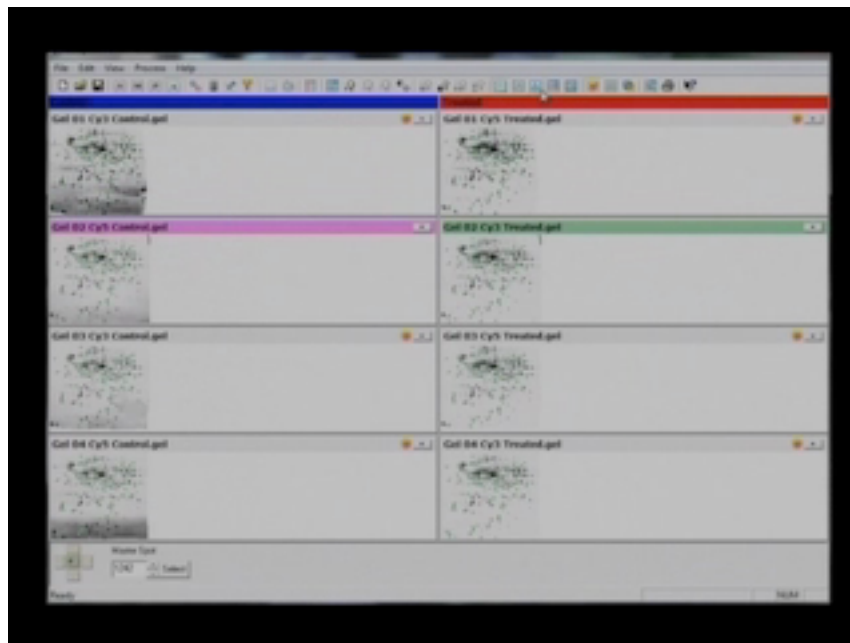
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Protein ID	Name	Status	Appearance	T score	Au. Ratio	Log2(Ratio)
17	Uncharacterized (AU)	T	0.013	1.76	0.013	
18	Uncharacterized (AU)	T	0.013	1.63	0.013	
19	Uncharacterized (AU)	T	0.043	1.04	0.043	
20	Uncharacterized (AU)	T	0.021	1.62	0.021	
21	Uncharacterized (AU)	T	0.00014	1.07	0.00014	
22	Uncharacterized (AU)	T	0.00019	1.76	0.00019	
23	Uncharacterized (AU)	T	0.040	1.01	0.040	
24	Uncharacterized (AU)	T	0.048	1.07	0.048	
25	Uncharacterized (AU)	T	0.010	1.71	0.010	
26	Uncharacterized (AU)	T	0.070	1.04	0.070	
27	Uncharacterized (AU)	T	0.040	1.12	0.040	
28	Uncharacterized (AU)	T	5.2e-005	1.04	5.2e-005	
29	Uncharacterized (AU)	T	0.029	1.28	0.029	
30	Uncharacterized (AU)	T	8.4e-006	1.03	8.4e-006	
31	Uncharacterized (AU)	T	0.028	1.62	0.028	
32	Uncharacterized (AU)	T	0.04	-0.28	0.04	
33	Uncharacterized (AU)	T	0.0070	1.63	0.0070	
34	Uncharacterized (AU)	T	0.012	-0.38	0.012	
35	Uncharacterized (AU)	T	0.013	1.11	0.013	
36	Uncharacterized (AU)	T	0.010	1.07	0.010	
37	Uncharacterized (AU)	T	0.010	1.07	0.010	
38	Uncharacterized (AU)	T	0.010	-0.32	0.010	
39	Uncharacterized (AU)	T	8.2e-006	1.04	8.2e-006	
40	Uncharacterized (AU)	T	0.01	1.62	0.01	
41	Uncharacterized (AU)	T	0.00012	1.02	0.00012	
42	Uncharacterized (AU)	T	0.0012	1.48	0.0012	
43	Uncharacterized (AU)	T	0.012	1.70	0.012	
44	Uncharacterized (AU)	T	0.015	1.04	0.015	
45	Uncharacterized (AU)	T	0.011	1.41	0.011	
46	Uncharacterized (AU)	T	0.040	1.48	0.040	
47	Uncharacterized (AU)	T	0.012	1.36	0.012	
48	Uncharacterized (AU)	T	0.027	1.39	0.027	

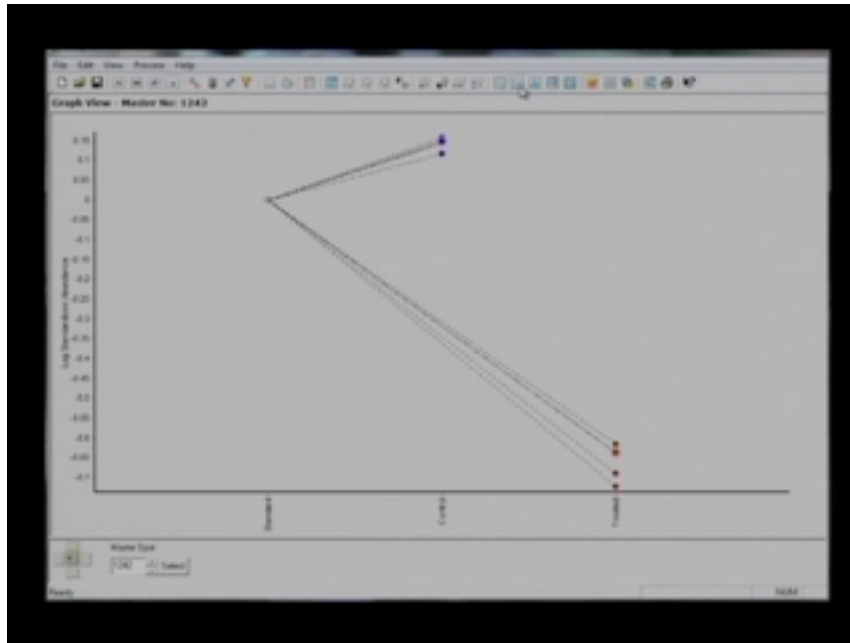
- After analysing the statistical data, complete results are produced as shown in the table above, in different formats such as the table view and the histogram view. The histogram view demonstrates how the protein is behaving under the control and treatment conditions.



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Image view

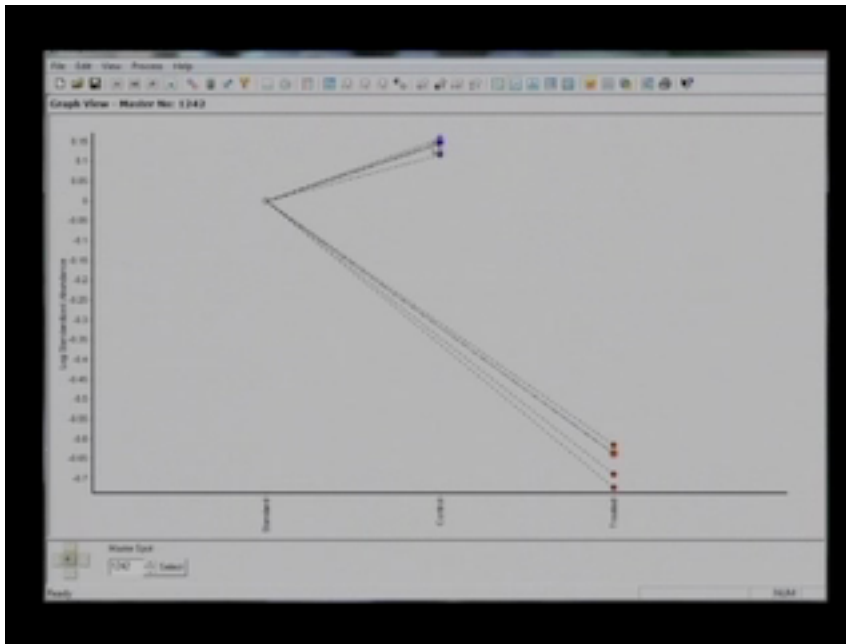


Graph view

- The standard, which is extrapolated from the internal control or the 'master' image, is considered to be at the 'zero' position. The positions of both the control and treatment in reference to the position of the standard are used to determine whether they are up or down-regulated. In this example, we can see that the control (in blue) is upregulated compared to the standard while the treatment (in red) is downregulated.

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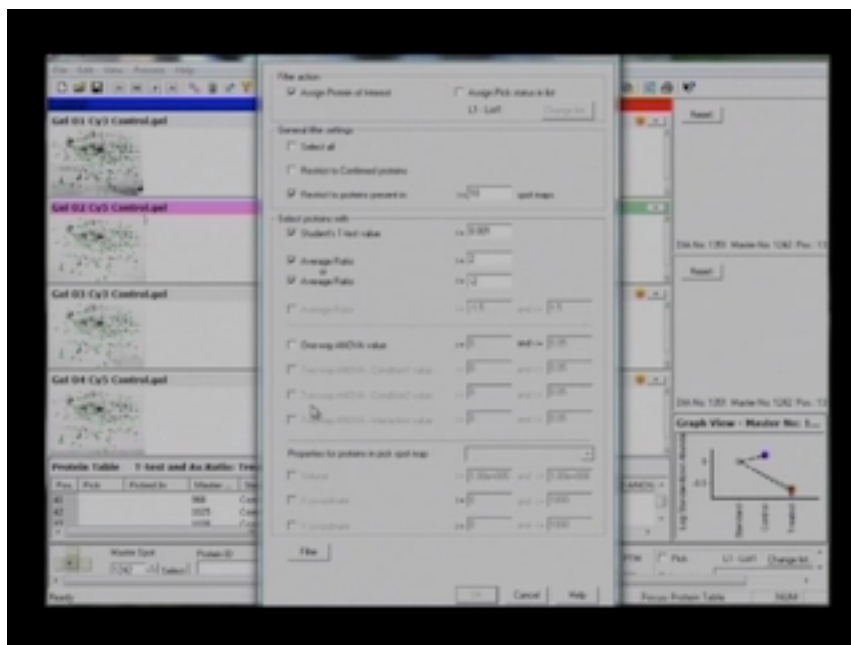
Up-regulation of controls and down-regulation of treated

- In the figure below, which is a snapshot of the BVA workspace, four views at a time are generated. A spot or protein table (shown on the bottom left) lists multiple parameters for every individual spot, including student t-test, average ratio and one-way ANOVA value. Only a very limited number of protein spots on the gel will reach statistical significance when judged by these parameters. The ones that pass

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become proteins of interest and are assigned to a pick-list and given to the spot



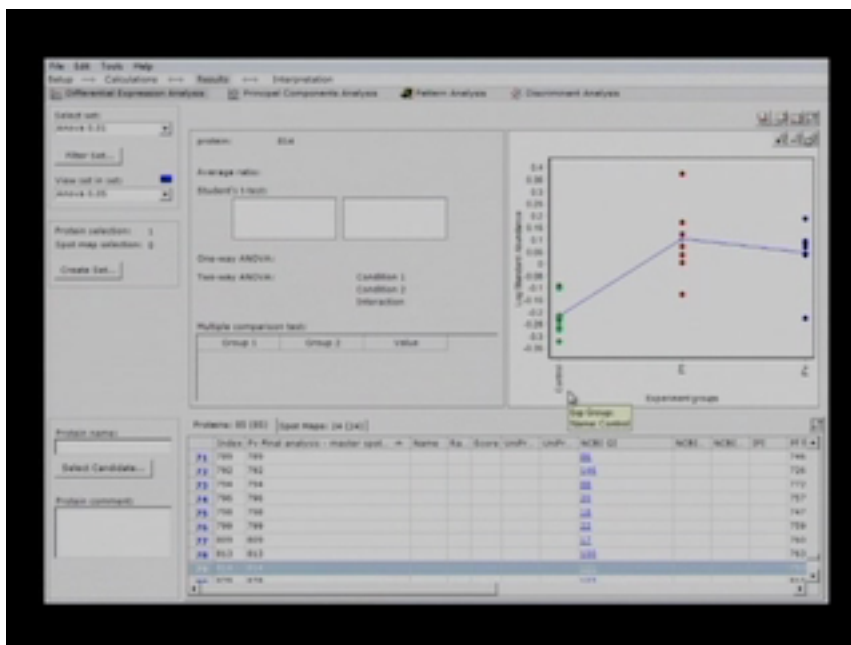
picker.

- The BVA is used can be used to present data in a number of user friendly formats, without much manual interference and hence is very useful in the analysis of DIGE gels.
- The EDA allows for comparison of 2 BVAs, to generate various statistical data such as majorly differential expression, PCA and discriminant analysis.
- Importantly, The EDA interface allows the comparison of 2 or more BVAs. In other words, the expression of any particular protein can be compared between different experimental groups or disease states (for example, Dengue Fever, *P. vivax* malaria and *P. falciparum* Malaria) and between the controls groups in these studies. Additionally, this allows for differential expression analysis of proteins along multiple

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disease states, while using a single control group as the healthy comparator.

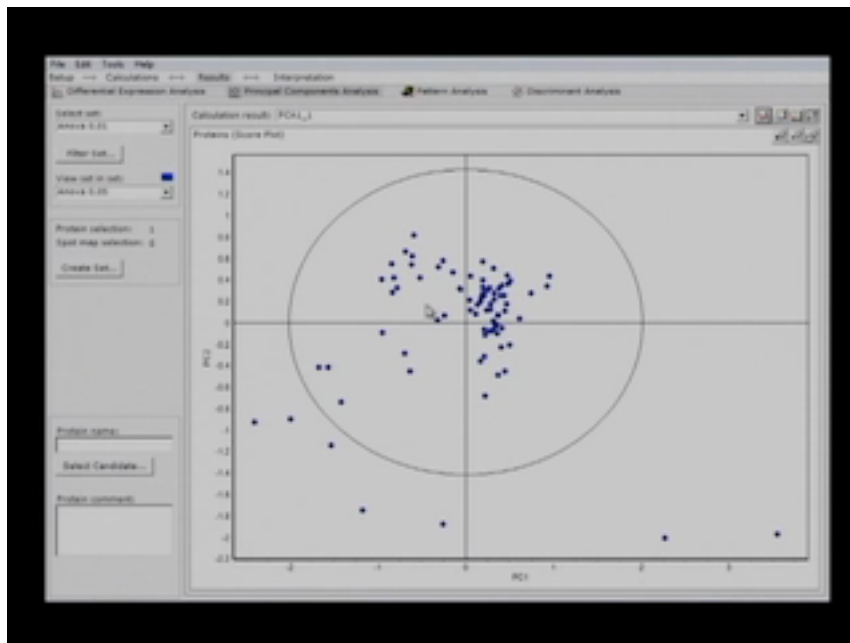


Differential expression analysis

- In the provided figure, is the data being analysed spot-by-spot. Even the index number shows that there is a master gel. From that master gel, you can exactly see this number. This is what we are seeing for each individual spot. Here you can see result as well as you can see principle component analysis for this data. Here you can see 89 spots. Out of these 89 proteins as you can see many proteins are present inside the circle and some proteins which are outliers are present out of the circle. In the circle there are about 95% statistically significant proteins and the outliers are non-reproducible spots or they might be highly up-regulated or highly down-regulated. These can be used as markers as well. Then we have to go back to our BVA data and check the data for the regulation of the protein. Then we can identify the protein and use for further analysis.

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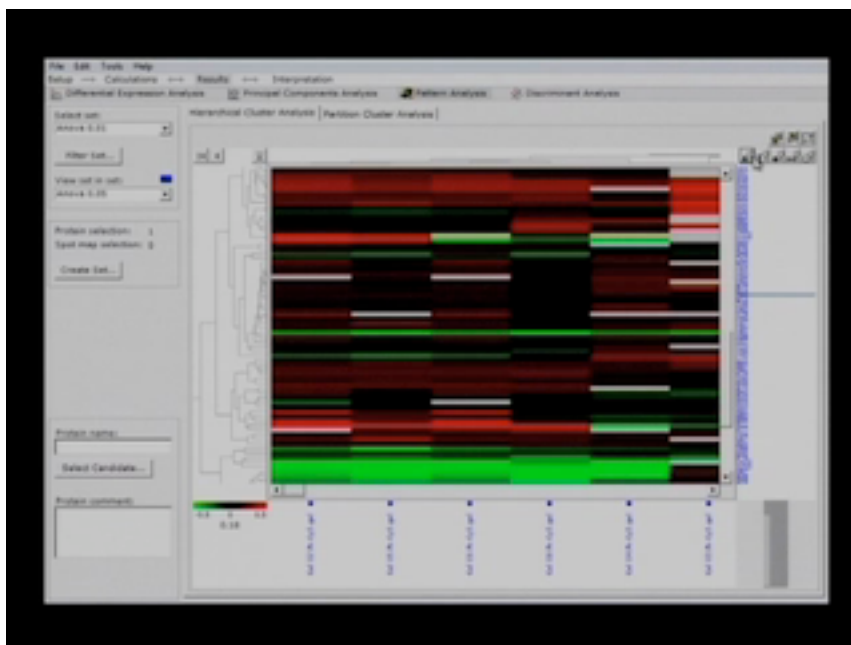
Principle component analysis

- This is a powerful statistical parameter and by using it you can identify some outliers which can be the potential discriminators between the control and treatment. And once you identify those proteins then you can go back to your original data in BVA and obtain the analysis for any given protein.
- Pattern analysis. You can see the whole proteome and individual proteins differ in expression. The below figure is the heat map made from the total of 82 proteins which we took into consideration. The green area is completely down-regulated area and red area is up-regulated area while the black sections represent similarly regulated proteins. This kind of representation allows the entire data set to be

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summed up at a glance.



Slide 4:

In this lecture and discussion have hopefully provided an introduction to data analysis using the DeCyder Software, with a preliminary idea of how biological information can be extrapolated from the raw data generated during DIGE.

In summary, so far these lectures have discussed gel-based techniques, we have talked about basic separation using SDS-PAGE, BN-PAGE and then how to perform IEF and by combining those two methods how to perform 2-DE. The workflow for performing 2-DE different challenges involved and a few creative ways of performing non-gel-based proteomics including OFFGEL fractionation methods have also been covered, followed by an in-depth discussion on DIGE.

While this knowledge forms a starting point for performing such experiments, it should be kept in mind that each experiment, each sample, each biological question, brings its own unique challenges and depending upon those conditions and the sample types the

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methods need to be optimized. There no one technology which can answer all of your questions but it's a good idea to know the different methods available for use.