HANDOUT

LECTURE-18

APPLICATIONS OF TWO DIMENSIONAL ELECTROPHORESIS

Slide 1:

Following the in depth discussion of 2-DE and DIGE and their workflows, this lecture will now cover the applications of these techniques and convey an understanding how they might be correctly applied to answer certain biological questions.

Slide 2:

This lecture will first provide an overview of the 2-DE technique, followed by the discussion of 2 case studies, in both of which 2-DE has been applied for differential proteomic analysis, wherein changes in the proteome induced by a disease, a chemical or a drug treatment are examined.

Slide 3:

Starting with the overview of previously discussed techniques, this slide is a quick visual recap of gel-based proteomics including SDS-PAGE, 2-DE-DIGE and gel analysis to obtain meaningful biological information from a large number of gel data, which constitutes the most challenging aspect of the process.

Slide 4:

The first step of the 2-DE process is protein extraction from any given sample. This step is the one that needs to be specifically tailored for any given experimental question/condition/biological sample. Obtaining a good quality extract, ie, one that has a minimal amount of interfering components, and which retains a high enough concentration of proteins to be separated, is key. Following preparation and determination of protein concentration, the sample is then separated by iso-electric focussing in the first dimension,

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After the first dimension separation, equilibration is performed to prepare the samples for protein separation on the basis of mass in the second dimension. In this process, proteins are denatured and disulphide bonds are broken and then alkylated to prevent reformation, by using the compounds DTT and IAA, as previously discussed. After

equilibration is completed in 2 steps, the sample is run on a SDS-PAGE gel in the second dimension.

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This is a representative 2-DE gel image which shows that a large number of proteins have been separated. Immediately by looking at a given protein spot one can obtain 2 pieces of information, what is its molecular weight and what is its charge, reflected in its isoelectric point. Then, after comparison of the gels from treatment and sample conditions, one can know which proteins are differentially expressed.

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As discussed in much detail , data analysis is a very crucial aspect of the 2-DE workflow. A large number of gels can be quickly run, but analysing them to find proteins that reach statistical significance in their differential expression is a far longer and challenging process.

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The first case study (Ray et al) that emerges from Dr. Sanjeeva Shrivastava's group, examines the serum proteome analysis of vivax malaria, providing insight into disease pathogenesis and the host immune response.

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As seen above, Asia and Africa are most affected due to malaria. Asia is suffering from challenges of drug resistant strains of malaria and this disease remains the single largest cause of mortality in Africa. However, Asia and Africa are not the only continents affected; Malaria is an epidemic in 103 countries around the world. Over 300 million cases, and 1.1-2.7 million deaths are reported annually These statistics provide an indication on the seriousness of this problem. *Plasmodium vivax* and *P. falciparum* account for 95% malarial cases worldwide.

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So the estimated clinical cases of vivax malaria range from 70-390 million excluding those from Africa. *P. vivax* accounts for more than 50% of all the malarial cases worldwide. But the morbidity associated with this disease and the spectrum of infection still remains neglected. Nearly 2.6 billion people around the globe are at the risk of being infected by *P. vivax* infection. The risk for infection with *P. vivax* is slightly higher than that of being infected by *P. falciparum*. Although *P. vivax* infection is historically regarded as benign, recently severe and fatal incidences of vivax malaria have been reported from different regions of the world=. By studying the complex life cycle of the

malarial parasite, insight on how *P. falciparum* and *P. vivax* behave in humans , as well as proteomic knowledge of the immune response directed against them can be obtained.

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Above is the list of known malarial parasites of the *Plasmodium* species, and the kind of infections they induce.*P. vivax* causes benign tertian malaria, *P. falciparum* causes malignant tertian malaria, *P. ovale* causes tertian malaria and *P. malariae* causes quartan malaria. In addition to these *P. knowlesi* can also cause an acute and severe illness but mortality rates are quite low for this species. Among the different species of *Plasmodium*, *P. vivax* and *P. falciparum* are the prime targets for investigation because as discussed earlier, they together are responsible for over 95% al all malaria cases worldwide.

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The above slide gives the overview of the workflow followed to investigate the host response against *P. vivax* in human serum. Serum samples were obtained, proteins were extracted and the protein samples were compared between healthy and malaria patients by 2DE-DIGE. A large number of proteins were separated and those proteins that were significantly differentially expressed in malaria patients as compared to the healthy controls were identified and subjected to mass spectrometry analysis. Following further validation of results, we studied the biological pathways the targets were involved in, and how this might affect disease.

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The following is an overview of all the steps involved in such a project.

1. Protein extraction from human serum

Carefully withdraw intravenous blood into a vaccutainer tube and store the tube on ice to allow the blood to coagulate. Centrifuge the contents to separate the coagulated blood cells and clotting factors from the serum which forms a clear supernatant. Collect the serum in a fresh tube and then sonicate the serum proteins to break down any large protein complexes. Serum contains several proteins in a wide range of concentrations of which albumin and immunoglobulin G are the most abundant. Presence of such high abundance proteins can interfere with experimental analysis while trying to detect other proteins present at lower concentrations. It is therefore preferred to remove these high abundance proteins before electrophoresis. This is done with the help of commercially available depletion columns, which bind only the high abundance proteins on to their matrix through affinity interactions. Once the serum has been processed using a

depletion column, precipitate out the remaining proteins using trichloroacetic acid and acetone.

Sonication

2. Quantification of proteins

Prepare the Bradford dye reagent and label the tubes suitably for standard and test samples. Add the standard and sample solutions to their respective tubes. Then add the Bradford colour reagent to the tubes and mix thoroughly. An electron transfer reaction between the red form of the Coomassie dye and the protein disrupts the native protein structure and establishes several non-covalent interactions between the dye and protein. The transfer of electrons also converts the dye to its blue form thereby giving the solution a blue colour. Adjust the zero reading on the UV spectrophotometer using a blank solution and then measure the absorbance of all standard and unknown protein samples at 595 nm. Protein concentration of the unknown can be determined from the standard plot based on the absorbance value obtained.

3. **Isoelectric focusing**

This process involves two major steps, rehydration of the IPG strips and focusing of the rehydrated strips.

IPG strip rehydration

Clean all the apparatus thoroughly before use to avoid any contamination. Remove the IPG strip from its cover and place it carefully in a well of the rehydration tray. Then add the reconstituted protein sample on to the strip. After around ten minutes, pour some mineral oil over the strip to prevent the strip from drying up. Cover the tray and leave it overnight to allow rehydration to occur.

Preparing the rehydratedIPG strip for isoelectric focusing

Isoelectric focusing

Cut the paper wicks required for focusing to a suitable length and wet them with a small amount of water before use. Initialize the instrument and clean the surface well with a dust free cloth. Place the focusing tray on the instrument and ensure that it is properly balanced. Carefully remove the IPG strip from the rehydration tray and drain out any excess oil by blotting it on a tissue paper. Place the strip in the focusing tray and immerse it with mineral oil. Then place the two wicks at either end of the IPG strip

followed by an electrode at each end. Fill all the adjacent wells with mineral oil to ensure uniform current flow. Then input the desired protocol on the instrument software along with details of strip length, pH range and number of strips and start the focusing process. The voltage-time curve will appear based on the protocol that has been set. Protein separation occurs on the basis of net charge on the protein. Proteins will migrate along the strip and come to rest at a point when their net charge becomes zero, known as their isoelectric point.

The isoelectric focusing program

4. Equilibration of IPG strips

Place the thawed IPG strips in the tray and over it, pour the first equilibration solution consisting of urea, tris-HCl of pH 8.8, glycerol, SDS, bromophenol blue along with dithiothreitol(DTT). Place the assembly on a mechanical shaker for 10 minutes. The dithiothreitol enables cleavage of all disulphide bonds present in the proteins on the IPG strip. Next, add the second equilibration solution to another well of the tray. This solution has the same composition as the first except that the dithiothreitol is replaced by iodoacetamide. Transfer the IPG strip into this solution after draining off any excess liquid from the first. Again place the assembly on the shaker for 10 minutes. Iodoacetamide prevents re-formation of the disulphide bonds by binding to the free sulphydryl groups that were obtained after DTT treatment.

5. Protein separation by SDS-PAGE

SDS-PAGE, which constitutes the second dimension of two dimensional gel electrophoresis, involves assembly of the gel apparatus, gel casting, equilibration of the IPG strip followed by placement of the IPG strip on the gel and protein separation.

Assembly of gel apparatus & gel casting

Clean all the components of the gel assembly thoroughly with water and dry them with paper towels. Carefully arrange the glass plates in the gel casting assembly interspersed with separator sheets, depending on the number of gels to be run. Prepare the gel casting solution consisting of Acrylamide-bis-acrylamide, Tris-chloride, SDS, APS and TEMED and pour it with the help of a funnel into the central channel of the

casting assembly. Ensure that the solution spreads evenly such that the top edge of the gel is uniform. Allow the solution to stand for polymerization to occur and spray a solution of SDS across the top to ensure that the gel does not become dry.

6. **Gel staining and destaining**

This process involves removal of the gel from the electrophoresis assembly followed by treatment with a fixing solution, staining solution and finally the destaining solution.

Gel removal and protein fixation

Soak the gel present between the glass plates in fresh distilled water to remove any bound SDS. Pour the fixing solution containing methanol and acetic acid into another tray. Open the glass plates and carefully remove the IPG strip from the gel. Then transfer the gel into the fixing solution by gently sliding it off from the glass plate. Place the tray on a mechanical shaker for an hour to ensure that the protein spots get fixed on to the gel thereby minimizing any diffusion.

Gel staining

Transfer the gel carefully into another tray containing the Coomassie blue staining solution and place it on the shaker to ensure uniform contact of the gel with the solution. The negatively charged Coomassie dye interacts with protein through ionic and other non-covalent interactions, thereby staining them with a blue color. Add some more staining solution such that the gel is immersed completely and leave it overnight for around 8-10 hours.

De-staining

Drain out the staining solution from the tray and pour the de-staining solution consisting of methanol and acetic acid into the tray. Place the tray on the shaker for around 6-8 hours until the background stain of the gel is completely removed and the spots are clearly visible.

7. Gel data analysis

Load the control and treated gel images to be compared and crop the gel such that majority of the spots get selected. Use these cropped gel images for further analysis.

Detect specific spots on the gels by setting defined values for parameters such as smoothness, saliency and minimum spot area. Spots on the gels can also be deleted or selected manually.

Compare the gels with each other by setting up a landmark spot on one gel which acts as a reference point for comparison of all other spots. The software returns the number of matched spots, indicated by red crosses.

Various information regarding the spots such as their area, volume, intensity etc can be obtained from the spot table. Statistical parameters such as standard deviation, central tendency etc can also be calculated using the software. A histogram can be viewed to get a better understanding of the spot intensities. The spots can also be viewed as three dimensional graphical representations with peak heights corresponding to the spot intensities.

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Sample Collection for Clinical Studies:In clinical studies, getting the right type of clinical samples, with suitable controls selected with optimal inclusion and exclusion criteria is both crucial and challenging. This process also requires the approval from an Ethics committee, as well as consent from the patients and controls themselves. In addition to the age, gender, physiological status, health histories, as well as behavioural habits such as alcohol, cigarette or drug use are determined. All of this information is carefully tabulated and is very useful , pto help analyse while performing proteomic analyses. Moreover, the selection of the healthy control becomes critical because you want to compare healthy with the disease samples. A very large number of subjects in both groups is optimal as it helps eliminate individuals affeceted by confounding factors, such as a 'healthy' individual who is suffering from a as yet undetected disease. Suppose 200 cases and 200 controls are enrolled in the study. Running individual gels for all the patients and controls, in replicate can be very challenging and tedious. There remains discussion as to whether pooling patient groups is possible. For example, when analysing 200 samples then you make pool of 10 patients then you have 20 samples. But it is dependant on the investigator and the biological question posed. Overall, the take home message is that choosing of subjects, sample collection and proper storage are all crucial to the success of a study.

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See above a depiction of the tabulation of information of P.vivax infected patients and healthy controls enrolled in the study. Such knowledge of the patients can be crucial during the data interpretation at the end of the study.

Slide 16:

The first step in the workflow is collection the blood sample, following separation of the serum and storage in small aliquots at -80°C. The above slide details thevarious steps involved in this process. Ideally 5 mL of blood is sufficient for getting enough serum to perform such proteomic investigations.

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Collect 5 mL blood and keep on ice till serum isolation. Allow it to clot for an hour and then centrifuge at 20°C for 10 min followed by separation of the supernatant, the serum, immediately. Collected serum can be stored at -20°C(shortterm) or -80°C(for longterm storage).

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Serum presents the challenge of the presence of abundant proteins, which can obscure detection of more rare proteins of interest. Such abundant proteins (which include albumin and IgG) are removed by the use various standard columns available from various commercial manufacturers Furthermore, there are columns available which can deplete almost 14 other abundant proteins in the serum.

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What type of serum preparation produces the ideal sample for proteomic analysis? To address this question, we used several approaches. The use of crude serum as the sample in 2-DE produced a lot of interference because of salt, in the first step of IEF itself. Desalting,alone or in combination with sonification was tried, as was the use of columns to remove abundant proteins as an alternative to the step of abundant protein depletion by commericial colums(which is quite expensive in large scale clinical studies)

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The sample was processed by the 4 alternate routes as detailed above and then run in the 2DE workflow.

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When serum is the sample source, ,IEF becomes tricky because of the high salt concentration in it. .If the salt removal is not effective, the IEF run may not be very smooth. The progress of the IEF run can be monitored using software programs.

Slide 22:

Various types of sample preps: crude serum alone, sonicated or desalted, desalted alone, depleted and desalted were run and the results compared for efficacy of overall proteome coverage.

Slide 23:

Different sized gels were also evaluated. As expected, small gels of 7 cm size could not provide a very large number of resolved proteins. But such gels can be used for the stain digestion process. As seen above, sonification and desalting of serum gave more superior results as opposed to desalted or crude serum alone.

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The same run was tried on a larger 11 cm strip. On reconfirmining earlier findings very large strips, 24 cm and sonicated desalted samples were used for the clinical studies.

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In general, 40 µL of the serum was precipitated with 4 volumes of ice cold acetone containing 10% of TCA, incubated at -20°C, centrifuged and then the precipitate obtained was washed with 1 mL of ice cold acetone for 15 min on ice and then re-centrifuged. The acetone containing supernatant was removed and then pellet was dried in the lysis buffer.

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From examining replicate gels, desalted, sonicated and depleted samples, not surprisingly, gave us largest number of spots on the gel as compared to crude alone or desalted alone or desalted and sonicated combinations. Such pilot runs were performed on small strips to standardize the procedure. In the end, for the final

experiment the desaltation, sonification and depletion method was used to process all samples.

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After processing the samples, the extraction method had to decided.The protein extraction protocol was next optimized. The above listed choices (crude serum usage, TCA-acetone precipitation, sonicated desalted serum, trizol extraction method as well as abundant protein removal and TCA-Acetone precipitation) were available.

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The effect of acetone or TCA-Acetone precipitation was compared. As seen above, overall more number spots were resolved on the TCA-Acetone gel. So the modified procedure of TCA-Acetone precipitation was finally applied to depleted, desalted and sonicated serum.

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The procedure was initially standardized on small gels and then applied to the large gel of 24 cm.On comparing crude versus depleted response on the large gel, as expected, the depleted serum sample showed more spots as compared to the crude serum samples.

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Different staining methods of coomassie blue staining or silver staining were also compared. While the silver staining technique yielded more spots. it also provided far less uniformity than the coomasie stain. Hence, colloidal coomassie and biosafe coomassie staining was finally used.

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The above slide gives you an overview of various parameters which we investigated and the final number of spots obtained. This provides an idea of all the choices available to standardize your experiment to get the best possible results.

Slide 32:

Following comparative analysis and standardizing the protocol for sample preparation and protein extraction, a comparative study of vivax serum vs. healthy controls was performed.

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This is one of the gel images demonstrating expected results on a 24 cm large 2-DE gel.

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Shown above are the representative gel images for healthy control and *P.vivax* infected samples.

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After separating the protein samples from these 2-DE gels from various patients and control sera, the most challenging task of data analysis remains. So manual and automated matchingsteps are performed to determine the protein spots significantly modulated on account of infection with *P. vivax*.

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Shown above are only a few patient samples from the whole group. However, the spots of interest, present in patient samples but not in healthy control sera, werewere observed in in all the patientsand were hence picked for analysis by mass spectrometry.

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The spots of interest were also validated by examining the 3-D view, to ensure that they were not artefacts.

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After completing the analysis, a large number of proteins which were differentially expressed were obtained. As seen in the graph above, the fold change in expression has a very wide range. Many proteins show a small change in expression between 1 to 1.5 fold and there are few proteins which are more than 10 fold up- or down-regulatated. Spots whose fold change between the 2 groups was statistically significant were further analysed using mass spectrometry techniques.

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So this slide gives you an overview of tandem mass spectrometry followed by how LC-MS/MS data can be analysed using Mascot search engine. Different types of mass

spectrometry techniques are available including MALDI-TOF/TOF and LC-MS based approaches and we will talk about these techniques in greater detail in the next module of mass spectrometry.

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Just to give you an overview, mass spectrometry analysis requires both standard proteins to ensure the validity of spectra obtained as well as a number of additional quality control checks

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The details of mass spectrometry will be covered in the next module.

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This slide shows the results of the MS analysis of the 2-DE experiment detailed above. . Several proteins in the host human were differentially expressed due to the *P. vivax* infection. Downregulated proteins included haptoglobin, apolipoprotein A-1, serum albumin precursor and clusterin precursor while serum amayloid A, ceruloplasmin precursor, leucine rich alpha-2-glycoprotein precursor, alpha-1-antitrypsin precursor were up-regulated.

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The conclusions/findings from this case study were as follows:

- Identification of a few differentially regulated proteins including ceruloplasmin, haemopexin, alpha-antichimotrypsin, alpha-1-antitrypsin not previously reported in the vivax malaria pathogenesis.
- A very important role of some proteins such as serum amyloid A and P, haploglobin, apolipoprotein A-1 and E were established in *Pvivax*-induced malaria. This study enhanced the understanding of disease pathogenesis and provided few potential targets for further investigation.

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This lecture illustrated how the 2-DE technique can be applied for various biological questions. Another case study, which deals with the effect of an anti-malarial drug on *P. falciparum* proteome will be discussed in the next class. However, this lecture has hopefully provided an idea of how optimizing experimental conditions, especially during the sample preparation step, can maximize the number of protein spots identified in the biological sample. If the overall proteome coverage can be increased, over 1500-2000 proteins can be reproducibly separated on the gel, providing a good chance of identifying various potential drug targets orbiomarkers. The discussion on applications of gel electrophoresis will also be expanded to cover 2DE-DIGE, as this technique affords greater sensitivity and hence greater proteome coverage.