HANDOUT

LECTURE-19

APPLICATIONS OF 2-DE AND DIGE

Slide 1:

The workflow of 2-DE has been covered in detail from protein extraction to the final step of image analysis following SDS PAGE. Now, this same workflow is applied regardless of the type of biological question posed, however small modifications in individual steps may be introduced. This lecture will cover case studies of 2-DE applications.

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The lecture outline is as follows:

- Case studies (on conventional 2-DE) will be first discussed:
    - Sample analysis following Drug treatment of malaria caused by *Plasmodium falciparum*
    - Proteome analysis of SARS virus.
- A refreshment on DIGE methodology
- DIGE case study of serum proteome analysis of prostate cancer patients.

  The take home message from this lecture will convey that there are multiple applications that can be carried out using gel electrophoresis, and no matter if the technique is DIGE or 2-DE, the workflow remains almost the same and can be applied to a myriad of applications.

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The first case study aimed towards elucidating a proteome-based mechanism of action for the Novartis drug CoArtem or Riamet. This study was carried out by Makanga et al in 2005, published in the journal *Proteomics* New malaria cases result in over 1 million deaths worldwide due to lack of effective vaccines and widespread of resistance to anti-malarial drugs. The anti-malarial therapy of chloroquine and pyrimethamine have not been able to control the mortality rate because of the development of drug resistance. So therefore there is an urgent requirement for identifying new drug targets as well as understanding the course of action of these drugs by applying various types of high-throughput techniques.

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CoArtem is a combination of artemisinin-derived artemether with lumefantrine. These drugs are the drug of choice for all cases of non-severe malaria worldwide. The drug action of artemesinin is mediated specifically through its endoperoxide moiety. However more detailed mode of action of these drugs is still unknown.

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In Makanga et al 2005, the authors have described how two active components of CoArtem [artemether(ARM) and lumefantrine(LUM)] which are effective anti-malarials can be studied for the changes they individually exert on the proteome of the *P. falciparum* parasite, by 2-DE.The purpose of this study was to investigate the action of two active components of CoArtem; on human malarial parasite *P. falciparum* a Following the separation of the protein components of the proteome by 2-DE, They identified certain proteins which were commonly or uniquely altered by both or only one of these drugs respectively.

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Before looking at proteomic alterations, the authors determined the IC10, 50 and 90 values for both the drugs. In other words, the effect of these drugs on parasite growth over 24 hours was characterized. As shown by the growth curve in the slide, synchronized ring stage parasites were harvested over 24 hours period after the exposure to ARM and LUM. The parasite growth was determined by using Hypoxanthine uptake assay.

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After establishing culture conditions and optimal drug concentrations the authors looked for proteomic alterations. They first fractionated the *P. falciparum* proteome. During this process, the synchronized parasites were isolated from the host erythrocytes, washed and then solubilised in Tris buffer. The Tris soluble fraction was further subjected to extraction in the urea-based lysis buffer.

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Once the protein extraction was done, then the authors used IPG strip of pH range 3-10 for the first dimension separation of proteins. After IEF was done they equilibrated these IPG strips and then applied that on a 12.5% vertical SDS-PAGE gel. After second dimension separation based on molecular weight these gels were stained with silver or coomassie brilliant blue stains. After acquisition of images, image analysis was performed by using the PDQuest software. Using this workflow, the authors determined if there was a differential proteome response following drug treatment.

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Quantitative analysis of the protein expression levels following exposure to ARM and LUM were analysed and the differentially expressed proteins whose fold change reached statistical significance t were further analysed with mass spectrometry based analysis.

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The comparative analysis of 2-DE gels from untreated and drug treated protein fractions yielded findings of direct and distinct alterations in parasite proteome following ARM or LUM drug treatments. Some of the proteins altered were affected by both drugs, while others were differently modulated by the 2 drugs. Proteins such as membrane associated calcium binding proteins, aspartic proteinase, HSP60, 70 and 90 were up-regulated by both the drugs. On the other hand, proteins such as enolase, fructose biphosphate, aldolase and phosphoglycerate kinase were down-regulated by ARM treatment and up-regulated by LUM treatment.

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ARM treatment resulted in more than a 3-fold down-regulation of glycolytic enzymes such as enolase, fructose biphosphate aldolase, phosphoglycerate kinase and glyceraldehyde-3-phosphate-dehydrogenase. Interestingly, the expression of same enzymes were over 3-fold up-regulated due to the LUM treatment. However certain proteins such as stress response proteins like heat shock proteins were commonly up-regulated following either drug treatment, which could be reflective of a general stress response as opposed to a unique response to either drug.

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The major findings of this study, were that the authors successfully investigated the alterations of the parasite proteome induced by two components of CoArtem, artemether and lumefantrine. By using a proteomic approach they reported certain specific and non-specific effects of two anti-malarial drugs under pharmacologically relevant conditions. Modulation of certain proteins (including a membrane bound calcium binding protein which was up-regulated due to ARM and LUM treatment) were quiet interesting. The study also helped defined pharmacologically relevant concentrations and time of exposure for the two components of CoArtem.

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The next case study is the Plasma proteome analysis of severe acute respiratory syndrome (SARS), studied by Chen *et al*. in 2004.

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The purpose of this study was to perform a comprehensive proteome analysis of plasma from SARS virus-infected individuals and its compare this with healthy non-infected counterparts. In this study authors employed conventional 2-DE, analysed the images from diseased and control samples and identified some differentially expressed proteins by using mass spectrometry techniques including MALDI-TOF/TOF and LC-MS/MS. Finally the proteins identified by this approach were validated from other techniques such as western blot.

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The SARS outbreak occurred in 2002-03 and thousands of deaths were reported in many countries around the globe. The genome sequence of SARS coronavirus was already known as was the structure of the main protease and surface receptors. However the pathogenesis of SARS was not clearly understood. Since serum or plasma provide valuable samples to identify targets for diagnostic, prognostic and therapeutic purposes, the authors used the plasma samples for analysis of proteomic alterations following SARS development.

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For the plasma proteomic analysis, the authors explored the possible pathogenic mechanism of progression of SARS by analysing plasma protein composition of 22 different plasma samples which were obtained from 4 SARS patients and 6 healthy controls. Theplasma proteome was analysed using 2-DE on 4-7 pH IPG strips, followed by SDS-PAGE and gel staining using the sensitive stain SYPRO Ruby. Differentially expressed proteins were subjected to in-gel trypsin digestion followed by mass spectrometry.

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The comparative proteomic analysis of these 2-D gels revealed that 38 protein spots were differentially expressed over 2-fold. Among these, 35 proteins which were up-regulated and 3 were down-regulated.

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The 38 proteins of interest were excised from the gels and subjected to MALDI-TOF mass spectrometry for analysis. Authors also employed liquid chromatography tandem MS-MS system for analysis of these spots. =The data obtained from this was analysed using the Mascot search engine.

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After the identity of these proteins was established, seven proteins which were not detectable in the healthy controls and only observed in the SARS patients were identified. These included glutathione peroxidase, PrxII, retinol binding protein, vitamin D binding protein and serum amyloid A proteins. 8 proteins over-expressed in SARS patients included pigment epithelium derived factor, 2-HS glycoprotein, complement factor H-related protein and leucine-rich Alpha-2 glycoprotein. For complete list of the proteins identified you can refer to the original study (PMID:15572443)

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After the identity of these proteins was established, then western blot analysis was used to validate a few targets.

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Among the identified proteins, peroxiredoxin II (PrxII) was signled out for further study. Intracellular protein PrxII (secreted by T cells) was exclusively found in the plasma of SARS patients but was absent in the healthy individuals. Western blot performed under both reducing and non-reducing conditions as revealed that PrxII is present as monomeric form at 22 kDa under reducing conditions while is present in the dimeric form at 44 kDa under non-reducing conditions. The authors also found that 4 out of 20 SARS probable cases and 4 of the 20 suspected cases showed higher up-regulation of plasma PrxII.

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Among various proteins of interest identified in this study, peroxiredoxin II appeared notable and was further validated by independent techniques. The level of plasma PrxII in patients with SARS was significantly high, indicating that it was could be ased as a biomarker toidentify SARS infection.

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This study also showed that several of plasma proteins in SARS patient plasma were shown to be The APPs found in the SARS patients suggested that the liver health status in these patients may be affected their defence against the SARS virus. Overall, this study has helped reveal an initial profile of alterations in plasma proteins of SARS patients, and the identification of PrxII as a potential candidate for a SARS biomarker.

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2-DE is well suited to separate and analyse proteins as well as their isoforms. In parallel, the development of of the use of fluorescent tags to label proteins enhanced

the dynamic range for their detection. In 1997 Ünlü and colleagues combined these two techniques of 2-DE and fluorescent tagging to develop the method of DIGE. The following is a recap of the DIGE technique.

Animation

DIGE

DIGE is an extremely valuable tool for analysis of large number of samples simultaneously without having to overcome the problem of gel-to-gel variations. In DIGE, controls and test samples can be differentially labelled by using cyanine dyes then run on the single gel. The pooled internal standard for DIGE is prepared by mixing equal amounts of all the samples that need to be run on the gel and this prevents the problem of gel-to-gel variations.

From the same gel 3 different images can be obtained for Cy2, Cy3 and Cy5. Thereby eliminating or minimizing gel variations and artefacts that can confound spot analysis. issues.

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To recap, the aim for the development of difference in gel electrophoresis is to overcome the inherently poor reproducibility of conventional 2-DE. So DIGE is a sensitive technique, able to detect less 1 fM of proteins and moreover enable the linear detection of very broad dynamic range of proteins.

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As seen in the slide above, the controls and treatment samples are labelled with 2 different dyes Cy3 and Cy5. But a small aliquot of both of the samples is mixed together to make an internal pool. That internal pool is labelled with another dye Cy2. Now all these protein samples are mixed together in one tube which contains both control and treatment as well as the reference proteins from both that form the internal pool. All these protein mixtures are separated in the first dimension on the same strip, which is then separated in the second dimension based on molecular weight. Then the same gel is scanned with the 3 different excitation wavelengths to obtain images from Cy2, Cy3 and Cy5. This technique eliminates the gel-to-gel variations arising from differential acrylamide polymerization as well as electrical, pH and thermal fluctuations in different gels that are inherent liabilities of the conventional 2-DE technique.

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S DIGE provides very uniform staining from gel-to-gel and shows high sensitivity and linear dynamic range of detection for the expression profiling of complex biological samples, providing the potential for comprehensive proteome coverage and making it an excellent platform for performing comparative or differential proteomic analysis

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The 3 images obtained for a single gel can be analysed using various programs such as the DeCyder software. Analysis of 3D views and other representations of your data helps provide statistical information which will help decide if a candidate spot represents a protein of interest which should be further characterized.

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Once such spots are analysed and excised from the gels, mass spectrometry can be performed, and the spectra obtained from here can be further analysed using various bioinformatics tools such as the Mascot program.

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Applications of 2D-DIGE can be found in virtually all research areas. With publications in the areas of cell signalling, developmental biology, plant proteomic analysis, neurosciences, clinical studies and different types of diseases including cancer.

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The next case study presented examines2D-DIGE as a strategy to identify serum markers for the progression of prostate cancer. This was studied by Byrne *et al.* in 2009.

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In this study, the authors investigated biomarkers for the identification of early prostate cancer. by using 2D-DIGE. Prostate cancer is a significant problem in older male population. The prostate cancer screening relies heavily on testing for the prostate specific antigen or PSAin serum. While PSA is a very sensitive marker, but there is a lot of discussion on the reliability and specificity of PSA as a prostate cancer biomarker as it is found to be elevated in other conditions such as benign prostatic hyperplasia or prostitis, leading to overtreatment of patients. Therefore, a need for other serum biomarkers to improve the efficacy of early screening for prostate cancer exists.

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As previously discussed, abundant proteins in biological samples can cause various artefacts and can confound the detection of rarer, less abundant proteins. In serum, both serum albumin and immunoglobulins represent such 'abundant proteins' which need to be removed prior to gel running. In this case, the authors used a multiple affinity removal system from Agilent technologies, which helped eliminate albumin, IgG, anti-trypsin, IgA, transferin and haptoglobin.

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The differential proteomic analysis was performed in two different cohorts of histologically confirmed prostate cancer with different disease grading based on Gleason grading system for prognostic evaluation of prostate cancer. Depleted serum samples obtained from patients with Gleason score 5 and Gleason score 7 were used for comparison and further analysis. These samples were first labelled with Cy3 and Cy5 and also simultaneously pooled to create the internal reference which was labelled with Cy2 dye. These samples were then mixed.

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The depleted cancer serum from first cohort of Gleason score 5 and second cohort of Gleason score 7 were mixed and separated in the first dimension and followed by proteins separation in the second dimension.

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Following analysis of the DIGE images, 63 protein spots were differentially expressed between the Gleason score 5 and Gleason score 7 cohorts. Difference in expression of 13 of these proteins reached statistical significance.

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After 2D-DIGE image analysis, the authors excised those spots and used mass spectrometry to identify those proteins, using the MS-MS technique Data generated from these experiments was analysed using Bioworks browser by using sequest program.

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The authors also tried the analysis of these DIGE gels by using two different software packages; the DeCyder and Progenesis, to generate more confidence in their findings prior to mass spectrometry. The proteins that reached statistical significance in their differential expression by both methods were then further analysed. Downregulated proteins included pigment epithelium derived factor (PEDF) and Ficolin 3 while

upregulated proteins included Zinc-Alpha 2-glycoportein (ZAG) Apolipoprotein A-II. Importantly, the same trend was observed for each of these proteins regardless of the software used.

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For further validation, the authors employed various techniques including western blots, ELISA and immunohistochemistry. PEDF and ZAG were further examined by the ELISA technique, which then validated their DIGE-findings. S

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Authors also employed immunohistochemistry or IHC for validating PEDF and ZAG to increase the confidence that the proteins identified from proteomic profiling represent relavent biomarkers.

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This study demonstrated that serum markers which are reflective of the pathological grade and stage could be beneficial for the identification of appropriate treatment strategies. Authors confirmed that differential expression of PEDF and ZAG can be performed using various validation techniques such as western blot, ELISA and immunohistochemistry. Based on their studies and follow up experiments they concluded that PEDF a potential marker of early stage prostate cancer. However more studies and follow-ups are required on a much larger number of patients before it can be established as a validated biomarker for routine clinical practice.

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To summarize, this lecture covered application case studies, both both 2-DE and DIGE. The one thing to be noted is that all the middle steps in the entire workflow of these 2 techniques are very similar while the very first sample preparation step that is unique to various applications.In other words, creativity and good experimental design play a major role in the first step. Once the sample is suitably prepared, then the workflow of all the steps we talked about earlier for 2-DE and DIGE that remains very similar.