
Elizabeth Yohannes, Ph.D. & Chao Yuan, Ph.D.  
Center for Proteomics & Bioinformatics  
Case Western Reserve University  
April 02, 2009
Section I

- 2D DIGE Workflow
- Example Center Project Using 2D-DIGE Platform

Section II

- 2D gel analysis of Myofilament proteins
Outline

1st part

- An overview protein profiling methods
- Traditional 2-D gel electrophoresis
- 2D DIGE for Quantitative Proteomics
- The three main features in 2-D DIGE
Protein Profiling Methods

Top down

Traditional 2-D gel Electrophoresis
2-D difference gel electrophoresis (2D DIGE)
Non - two gel separation methods
Protein and antibody arrays

Bottom up

Multi-dimensional protein identification technology (MudPIT)
Isotope-coded affinity tags
Accurate mass tag based protein profiling
Shotgun proteomics
Two dimensional (2D) gel Electrophoresis:

O’Farrel (J. Biol. Chem. 1975)

Principle:

1\textsuperscript{st} charge or Isoelectric point

2\textsuperscript{nd} size (SDS-PAGE)

Figure 1: Schematic diagram showing the different steps in the 2-D Electrophoresis
Limitations to Traditional 2D gel Electrophoresis

- **Gel to Gel variability**
  - Complex image analysis
  - System variation
    - Induced biological changes

- Time consuming, labor-intensive and expensive

- **Protein Visualization**

  **Coomassie Brilliant Blue**
  Detection limits ~ 1μg protein (~20 ng protein with colloidal coomassie blue-G)
  Variability from destaining, high background
  Poor linear response (1 order)

  **Silver Stain**
  Sensitivity: 1-5 ng protein
  poor linear response (1 order)
  less reproducible
Two-dimensional Difference gel Electrophoresis (2-D DIGE) Ünlü et al

Ünlü M, Morgan ME, Minden JS
Carnegie Mellon University

Figure 2: Schematic representation of the labeling reaction-formation of a covalent amide bond between the NHS ester group of CyDye DIGE fluor minimal dye and ε-amino group of lysine residue of a protein.
Two-dimensional Difference gel Electrophoresis (2-D DIGE) Ünlü et al

Figure 3: The original protocol of 2-D DIGE

Figure 4: An overlay of Cy3 and Cy5 images

Viswanathan, S, Ünlü, M et al. nature protocols 2006, 1, 1351-1358
Table 1: physical properties of cyanine dyes

<table>
<thead>
<tr>
<th>Dye</th>
<th>Cy2</th>
<th>Cy3</th>
<th>Cy5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>550.60</td>
<td>582.77</td>
<td>580.75</td>
</tr>
<tr>
<td>Mass added to Protein (Da)</td>
<td>434</td>
<td>466</td>
<td>464</td>
</tr>
<tr>
<td>Color of the fluorescence</td>
<td>Green</td>
<td>Orange</td>
<td>Far red</td>
</tr>
<tr>
<td>Absorbance max (nm)</td>
<td>489</td>
<td>550</td>
<td>649</td>
</tr>
<tr>
<td>Emission max (nm)</td>
<td>506</td>
<td>570</td>
<td>670</td>
</tr>
<tr>
<td>Excitation Filter (nm)</td>
<td>480 (30)</td>
<td>540 (25)</td>
<td>620 (30)</td>
</tr>
<tr>
<td>Emission filer (nm)</td>
<td>520 BP 40</td>
<td>580 BP 30</td>
<td>670 BP 30</td>
</tr>
</tbody>
</table>

Figure 5: Structure of the cyanine dyes
Table 1: physical properties of cyanine dyes

<table>
<thead>
<tr>
<th>Dye</th>
<th>Cy2</th>
<th>Cy3</th>
<th>Cy5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>550.60</td>
<td>582.77</td>
<td>580.75</td>
</tr>
<tr>
<td>Mass added to Protein (Da)</td>
<td>434</td>
<td>466</td>
<td>464</td>
</tr>
<tr>
<td>Color of the fluorescence</td>
<td>Green</td>
<td>Orange</td>
<td>Far red</td>
</tr>
<tr>
<td>Absorbance max (nm)</td>
<td>489</td>
<td>550</td>
<td>649</td>
</tr>
<tr>
<td>Emission max (nm)</td>
<td>506</td>
<td>570</td>
<td>670</td>
</tr>
<tr>
<td>Excitation Filter (nm)</td>
<td>480 (30)</td>
<td>540 (25)</td>
<td>620 (30)</td>
</tr>
<tr>
<td>Emission filter (nm)</td>
<td>520 BP 40</td>
<td>580 BP 30</td>
<td>670 BP 30</td>
</tr>
</tbody>
</table>

Amersham Biosciences (GE Healthcare)

Table 2: physical properties of cyanine dyes

<table>
<thead>
<tr>
<th>Dye</th>
<th>Cy2</th>
<th>Cy3</th>
<th>Cy5</th>
<th>Silver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity (ng)</td>
<td>1-5</td>
<td>1-5</td>
<td>1-5</td>
<td>1-5</td>
</tr>
<tr>
<td>Dynamic range</td>
<td>&gt; 3.6</td>
<td>&gt; 3.6</td>
<td>&gt; 3.6</td>
<td>1</td>
</tr>
</tbody>
</table>
Quantitative accuracy of 2D DIGE over traditional 2-DE

1. **Multiplexing.**

   Reduce
   - spot pattern variation
   - # of Gels
   - decrease $\$$

2. Internal standard sample.

3. Experimental Designs
Quantitative accuracy of 2D DIGE over traditional 2-DE

1. Multiplexing.

2. Internal standard sample.

### 3. Experimental Designs

Table 2: An example of experimental design for CyDye DIGE fluor minimal

<table>
<thead>
<tr>
<th>Gel number</th>
<th>Cy2</th>
<th>Cy3</th>
<th>Cy5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pooled standard</td>
<td>Control 1</td>
<td>Treated 1</td>
</tr>
<tr>
<td>2</td>
<td>Pooled standard</td>
<td>Treated 2</td>
<td>Control 2</td>
</tr>
<tr>
<td>3</td>
<td>Pooled standard</td>
<td>Control 3</td>
<td>Treated 3</td>
</tr>
<tr>
<td>4</td>
<td>Pooled standard</td>
<td>Treated 4</td>
<td>Control 4</td>
</tr>
</tbody>
</table>

Alban, A et al, Proteomics 2003, 3, 36-44
Quantitative accuracy of 2D DIGE over traditional 2-DE

1. Multiplexing.

2. Internal standard sample.
   
   Advantages
   
   3. Experimental Design
      
      ➢ Each sample within a gel can be normalized to internal standard
      
      ➢ Protein abundance can be measured as ratio (not volume)
      
      ➢ Accurate quantitation and spot statistics
      
      ➢ Separation of experimental from inherent biological variation
**Internal standard (reference) sample**

**Figure 6:** Comparison of gel electrophoresis with and without internal standard.
Figure 7: Scheme showing the different modules and image analysis workflow in the DeCyder software
Average Ratio = \frac{Cy3/Cy2}{Cy5/Cy2}

Figure 8: Scheme showing spot co-detection on images from a single gel in the DeCyder DIA module and protein difference ratios and statistics between gels in the DeCyder BVA module.
Quantitative accuracy of 2D DIGE over traditional 2-DE

1. Multiplexing.
2. Internal standards (reference) sample.
3. **Experimental Designs**

Table 3: Experimental design 1 (Total 8 gels)

<table>
<thead>
<tr>
<th>Gel</th>
<th>Cy3</th>
<th>Cy5</th>
<th>Cy2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C1_1W</td>
<td>D1_1W</td>
<td>Pooled sample (C1-4_1W + D1-4_1W + C1-4_2M + D1-4_2M)</td>
</tr>
<tr>
<td>2</td>
<td>D2_1W</td>
<td>C2_1W</td>
<td>Pooled sample (C1-4_1W + D1-4_1W + C1-4_2M + D1-4_2M)</td>
</tr>
<tr>
<td>3</td>
<td>C3_1W</td>
<td>D3_1W</td>
<td>Pooled sample (C1-4_1W + D1-4_1W + C1-4_2M + D1-4_2M)</td>
</tr>
<tr>
<td>4</td>
<td>D4_1W</td>
<td>C4_1W</td>
<td>Pooled sample (C1-4_1W + D1-4_1W + C1-4_2M + D1-4_2M)</td>
</tr>
<tr>
<td>5</td>
<td>C1_2M</td>
<td>D1_2M</td>
<td>Pooled sample (C1-4_1W + D1-4_1W + C1-4_2M + D1-4_2M)</td>
</tr>
<tr>
<td>6</td>
<td>D2_2M</td>
<td>C2_2M</td>
<td>Pooled sample (C1-4_1W + D1-4_1W + C1-4_2M + D1-4_2M)</td>
</tr>
<tr>
<td>7</td>
<td>C3_2M</td>
<td>D3_2M</td>
<td>Pooled sample (C1-4_1W + D1-4_1W + C1-4_2M + D1-4_2M)</td>
</tr>
<tr>
<td>8</td>
<td>D4_2M</td>
<td>C4_2M</td>
<td>Pooled sample (C1-4_1W + D1-4_1W + C1-4_2M + D1-4_2M)</td>
</tr>
</tbody>
</table>
Quantitative accuracy of 2D DIGE over traditional 2D

1. Multiplexing.
2. Internal standards (reference) sample.
3. **Experimental Designs**

Table 4: Experimental design 2 (Total 20 gels)

<table>
<thead>
<tr>
<th>Gel</th>
<th>Cy3</th>
<th>Cy5</th>
<th>Cy2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schizophrenia white mater (10 samples)</td>
<td>Gels 1-5</td>
<td>Schizophrenia</td>
<td>Schizophrenia</td>
</tr>
<tr>
<td>Control white mater (10 samples)</td>
<td>Gels 6-10</td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>Schizophrenia grey mater (10 samples)</td>
<td>Gels 11-15</td>
<td>Schizophrenia</td>
<td>Schizophrenia</td>
</tr>
<tr>
<td>Control grey mater (10 samples)</td>
<td>Gels 16-20</td>
<td>Control</td>
<td>Control</td>
</tr>
</tbody>
</table>
Proper sample size is Important

If too large

unnecessary involvement of extra subjects (samples)

Increase $$$$$$

If too small:

Unable to detect important differences

If not done properly: Fail to get funding, publication

\[
    n = \frac{2(Z_{\alpha/2} + Z_{\beta})^2 \sigma^2}{(\mu_1 - \mu_2)^2}
\]

http://hedwig.mgh.harvard.edu/sample_size/js/js_parallel_quant.html
Figure 9: Relationship between power and number of replicates in detecting various fold changes when the variance encompasses 75% of the spots.
**Limitations to 2-D DIGE**

2D DIGE does not represent true global technique.

- Very acidic or basic proteins
- Excessively large or small proteins
- Membrane proteins
- Low abundant proteins

  Dynamic range 2-D DIGE $10^3$-$10^4$

  Single cell or individual cell type: low abundant (100 copies), most abundant proteins ($10^6$)
Target identification for Diabetes Mellitus Associated Urogenetal Dysfunction.

In collaboration with Dr. Kelvin Davies (Albert Einstein College of Medicine) & Dr. George Christ (Wake Forest University School of Medicine)
Outline

I. Introduction

Diabetes and its complications

II. Objectives

II. Proteomics Approach

2D-DIGE/MS

MetaCore™, Validation Western blotting

IV. Summary of results

V. Acknowledgement
Diabetes mellitus

Diabetes mellitus:
- Group of metabolic diseases
- Hyperglycemia
  Diabetes mellitus means “sweet urine”

Type I (= insulin-dependent diabetes = juvenile onset diabetes)
- Caused by destruction of the B cells
- Generally appears in childhood
- Absolutely dependent on insulin replacement

Type II (= insulin-independent diabetes = adult onset diabetes)
- Caused by target cell resistance to insulin (InsR decreased, signaling defect)
  - Obesity appears to reduce the number of insulin receptors
  - Mostly appears in obese individuals
  - Can be treated with oral hypoglycemic drugs
Diabetes mellitus

Pancreas:
- Islets of Langerhans: site of hormone production
  - $\alpha$ (alpha) cells - produce Glucagon
  - $\beta$ (beta) cells - produce Insulin
  - $\delta$ (delta) cells - produce Somatostatin

Insulin and Glucagon are the major regulators of blood glucose

Figure 1. Anatomy of the middle digestive tract. Source: © MedicineNet, Inc.
Diabetes mellitus

Diabetes mellitus:
- **Group of metabolic diseases**
- **Hyperglycemia**  
  Diabetes mellitus means “sweet urine”

**Type I (insulin-dependent diabetes = juvenile onset diabetes)**
- Caused by destruction of the B cells
- Generally appears in childhood
- Absolutely dependent on insulin replacement

**Type II (insulin-independent diabetes = adult onset diabetes)**
- Caused by target cell resistance to insulin (InsR decreased, signaling defect)
- Obesity appears to reduce the number of insulin receptors
- Mostly appears in obese individuals
- Can be treated with oral hypoglycemic drugs
Diabetes mellitus

Complications:

• **Short-term**
  - Hyperglycemia, (hypoglycemia)
  - Ketoacidosis

• **Long-term**
  - Disruptions in blood flow => Cardiovascular complications => Amputations
  - Retinopathy - blindness
  - Nephropathy - primary cause of morbidity and mortality
  - Neuropathy - nerve damage
  - **Bladder dysfunction** (Yohannes *et al* MCP 2008, 7:1270-85)
  - **Erectile dysfunction** (impotence)
Erectile dysfunction (ED)

Risk Factors:
- psychological => Stress,
- damages => nerves, blood vesicles, and/or smooth muscles.

Men with Diabetes
- 75% (10 to 15 years earlier)
- worst DSHRQL => Depression, loss of self-esteem, & poor self-image

- Less responsive to pharmacological therapies

- Progressive disease
- No systematic study (Initiation, development and progression)
Objectives

- To delineate the proteome changes: during the initiation, and development of diabetes-related ED.
  - Using 2D-DIGE/MS platform

- To probe protein-protein interaction networks: predict the possible pathways => activated or deactivated

- To identify transcriptional factors and relatively low-abundant proteins: Not identified by 2D-DIGE/MS => further analyzed the 2D-DIGE data => MetaCore™ pathway analysis tools.

- To validate 2D-DIGE/MS and MetaCore™ pathway for specific expression changes by western blotting.
Experimental

Animal Model and induction of diabetes:

<table>
<thead>
<tr>
<th>Animal Models (8-10 weeks F-344 Rat)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diabetes</strong></td>
</tr>
<tr>
<td>35 mg/kg body weight STZ</td>
</tr>
<tr>
<td>&gt; 300 mg/dl</td>
</tr>
</tbody>
</table>

Erectile responses:

Intracavernosal pressure (ICP) in response to cavernous nerve stimulant => For both 1 week and 2 months diabetes and age much controls
Figure 3. Erectile function in non-diabetic rats compared to rats with one week and 2 months of diabetes. The values are statistically significant (*= P<0.05 compared to non-diabetic).
Tissue collection:
- sacrificed
- penile dissected
- corpora cavernous smooth muscle (nitrogen flash frozen and stored at -80°C)

=> 2D-DIGE/MS, Verification

Figure 4. The transverse section of penile. Tom F. Lue, The new England Journal of Medicine 324, 1802, 2007
Experimental

Test hypothesis:

- Time or treatment significantly changes the dependent variable (Protein expression)
- There are interaction effects between the two factors (time and treatment)

Table 1. Experimental design.

<table>
<thead>
<tr>
<th>Gel</th>
<th>Cy3</th>
<th>Cy5</th>
<th>Cy2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C1_1W</td>
<td>D1_1W</td>
<td>Pooled sample (C1-4_1W + D1-4_1W + C1-4_2M +D1-4_2M)</td>
</tr>
<tr>
<td>2</td>
<td>D2_1W</td>
<td>C2_1W</td>
<td>Pooled sample (C1-4_1W + D1-4_1W + C1-4_2M +D1-4_2M)</td>
</tr>
<tr>
<td>3</td>
<td>C3_1W</td>
<td>D3_1W</td>
<td>Pooled sample (C1-4_1W + D1-4_1W + C1-4_2M +D1-4_2M)</td>
</tr>
<tr>
<td>4</td>
<td>D4_1W</td>
<td>C4_1W</td>
<td>Pooled sample (C1-4_1W + D1-4_1W + C1-4_2M +D1-4_2M)</td>
</tr>
<tr>
<td>5</td>
<td>C1_2M</td>
<td>D1_2M</td>
<td>Pooled sample (C1-4_1W + D1-4_1W + C1-4_2M +D1-4_2M)</td>
</tr>
<tr>
<td>6</td>
<td>D2_2M</td>
<td>C2_2M</td>
<td>Pooled sample (C1-4_1W + D1-4_1W + C1-4_2M +D1-4_2M)</td>
</tr>
<tr>
<td>7</td>
<td>C3_2M</td>
<td>D3_2M</td>
<td>Pooled sample (C1-4_1W + D1-4_1W + C1-4_2M +D1-4_2M)</td>
</tr>
<tr>
<td>8</td>
<td>D4_2M</td>
<td>C4_2M</td>
<td>Pooled sample (C1-4_1W + D1-4_1W + C1-4_2M +D1-4_2M)</td>
</tr>
</tbody>
</table>

Control_1week (C_1W), Diabetes_1week (D_1W, Control_8-10_weeks (C_2M), Diabetes_8-10_weeks (D_2M)
Figure 5. An overview of the different steps involved in the 2D-DIGE:

1. MIX of cy2, cy3, and cy5
2. 2D E (two-dimensional electrophoresis)
3. 3-mode scanning with Typhoon
4. DeCyder 2-D Software
5. Pick list
6. Excised gel plug
7. In-gel Digest
8. Protein ID/MALDI or LC-MS => 57
**Statistical outcome**

<table>
<thead>
<tr>
<th>Treatment (Diabetes)</th>
<th>Time</th>
<th>Both</th>
<th>Interaction effect</th>
</tr>
</thead>
<tbody>
<tr>
<td># of spots</td>
<td>51</td>
<td>19</td>
<td>100</td>
</tr>
</tbody>
</table>

Protein: 114

Average ratio: -2.69

Student's t-test:

One-way ANOVA: 4.68E-6

Two-way ANOVA: 2.78E-6 6.68E-4 Time treated 0.0105 Interaction
Figure 6. Principal component analysis (PCA) of the proteins mediated by STZ-induced diabetes. The protein expression profiles of experimental groups were visualized in two-dimensional Euclidian space. The PCA, distinctly clustered the 15 individual samples into four experimental groups (C-1W = 1 week control, D-1W = 1 week diabetes, C-2M = two months control, and D-2M = two months diabetes).
Figure 7: Differential abundance spot. Principal component analysis of 108 spot features that have statistical significant (ANOVA p<0.05) changes in abundance and are present in all gels (A). The black rectangle on the gel image shows the region on the gels where the potential outlier spots (115 and 116) were situated. Two magnified views of this region showing control and diabetes images (B). 3D images of spot feature 115 showing the 3 fold decreases at one week time point and 7.5 fold decreases at two months time point in abundance (C1 and C2 respectively). Graphical representation of the standardized log abundance data obtained for spot feature 115 (D).
Figure 8: 2-D gel image, showing the pick location of differential expressed proteins.
Table 2: Protein profile in an STZ-induced rat corpora smooth muscle

<table>
<thead>
<tr>
<th>Pos.</th>
<th>Gene name</th>
<th>Protein identities</th>
<th>Accession number</th>
<th>MW (KDa), pI&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt;(Average ratio)&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2/1</td>
</tr>
<tr>
<td>6</td>
<td>Apoa1</td>
<td>Apolipoprotein A-I precursor</td>
<td>P04639</td>
<td>30.1, 5.5</td>
<td>2.89</td>
</tr>
<tr>
<td>7</td>
<td>Apoa4</td>
<td>Apolipoprotein A-IV</td>
<td>P02651</td>
<td>44.5, 4.98</td>
<td>2.24</td>
</tr>
<tr>
<td>8</td>
<td>Apoe</td>
<td>Apolipoprotein E precursor</td>
<td>P02650</td>
<td>35.8, 5.2</td>
<td>-1.97</td>
</tr>
<tr>
<td>17</td>
<td>Col14a1</td>
<td>Collagen, type XIV, alpha 1</td>
<td>gi</td>
<td>109480777</td>
<td>193.22, 4.9</td>
</tr>
<tr>
<td>18</td>
<td>Col1a1</td>
<td>Collagen, type 1, alpha 1</td>
<td>P02454</td>
<td>137.86, 5.7</td>
<td>-1.68</td>
</tr>
<tr>
<td>19</td>
<td>Col1a2</td>
<td>Collagen, type 1, alpha 2</td>
<td>P02466</td>
<td>129.48, 9.6</td>
<td>-1.96</td>
</tr>
<tr>
<td>20</td>
<td>Col6a2</td>
<td>Collagen, type 6, alpha 2</td>
<td>Q5EB88</td>
<td>109.5, 6.2</td>
<td>-1.52</td>
</tr>
<tr>
<td>51</td>
<td>Hsp47</td>
<td>heat shock protein</td>
<td>P29457</td>
<td>46.6, 9.2</td>
<td>-2.94</td>
</tr>
<tr>
<td>36</td>
<td>Igc</td>
<td>anti-NGF antibody light-chain</td>
<td>gi</td>
<td>4096754</td>
<td>23.80, 5.9</td>
</tr>
</tbody>
</table>

Spot 155 (*Igc*)

Average ratio: $2:1 = 1.86$, $4:3 = 2.02$

One way ANOVA: $3.68\times10^{-5}$

Two way ANOVA $1.27\times10^{-5}$

Time

$1.95\times10^{-3}$ Treated

$0.2$ Interaction

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>0.013</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>$2.88\times10^{-4}$</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>$3.73\times10^{-5}$</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>0.0592</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>$3.02\times10^{-3}$</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>$2.32\times10^{-4}$</td>
</tr>
</tbody>
</table>

2-D DIGE expression profile of *Igc*
Diabetic rat models => ↓ in Tissue NGF level in the bladder and Lumbosacral dorsal root ganglia (DRG) <=> the progression of diabetic cystopathy

FIG. 9. NGF levels measured by enzyme-linked immunosorbent assay in the bladder (A) and L6 DRG (B) of normal rats (n = 12), untreated diabetic rats 12 weeks after STZ injection (DM12W), diabetic rats with SHZ (HSV-1 without NGF) injection (SHZ, n = 6), and diabetic rats with SLN (HSV-1 with NGF) injection (SLN, n = 8). Virus vectors were injected 8 weeks after diabetes induction, and NGF levels were measured 4 weeks after virus injection. Note that reduced NGF levels in the bladder and L6 DRG were significantly elevated in diabetic rats with SLN injection compared with untreated diabetic rats and diabetic rats with SHZ injection (bladder: P < 0.01; L6 DRG: P < 0.05). *P < 0.05, **P < 0.01, prot., protein.

FIG. 10. Micturition patterns in a metabolic cage study to evaluate the efficacy of HSV vector-mediated NGF delivery to the bladder. A: Representative traces of voided urine volume plotted against time in normal rats (upper trace) and diabetic rats (middle trace: diabetic rats with SHZ control vector injection; lower trace: diabetic rats injected with SLN). B: Averaged voided volume per micturition (normal rats: n = 9; SHZ: n = 10; SLN: n = 11). **P < 0.01, *P < 0.05.

Figure 11: Protein networks associated with differential expressed proteins in response to STZ-induced diabetes.
Verification of 2D-DIGE/MS & MetaCoreTM Results

Figure 12: A) Conformational immunoblots for Hsp47. B) 2-D DIGE expression profile of Hsp47
Verification of 2D-DIGE/MS & MetaCoreTM Results

Figure 13: Conformational immunoblots for p53 and HDAC1, that were hypothetically identified by network analysis. Each lane is loaded with a sample from independent biological replicate (n = 2/experimental group).
Summary

STZ-ID significantly altered protein expression in corpora smooth muscle.

- Decreased the expression of different isoforms of collages, which are precursor to fibrils forming collagen type 1, hsp47 that assists and mediates the proper folding of procollagen, type I, alpha 1 and procollagen, type I, alpha 2 and proteins involved in muscle remodeling (eg. LIM protein).

- Increased the proteins involved in oxidative stress (eg. Glutathione peroxidase 3), protein that neutralize the biological activity of nerve growth factor (eg. Anti-NGF), and proteins involved in inflammatory response (eg Fga, Fgb, Fgg, ApoA1, ApoA4, C3, and C5) proteins that suppresses and induced apoptosis (HSCO & p53 respectively).

- Our study reports novel proteins that may contribute to diabetic-dependant development ED.

- Used to develop novel diagnostic, preventative or therapeutic strategies.

- also provide hypotheses that can be tested by future studies.
Acknowledgement

- Mark Chance (Mentor)
- Kelvin Davies (Collaborator)
- Jinsook Chang (Collaborator)

- Janna Kiselar (LC-MS)
- Serguei Ilchenko (LC-MS)
- Giri Gokulrangan (LC-MS)
  & Katy Lundberg (MALDI)

- Hong Zhao (2-D DIGE)

Thanks Everybody in the Center
2D Gel Analysis of Myofilament Proteins

Chao Yuan
Myofilament Proteins

• Myofilament proteins are responsible for muscle contraction and relaxation.

• In heart, this process is Ca\(^{2+}\) regulated and modulated by phosphorylation of key regulatory myofilament proteins.
Schematic Diagram of Myofilament Proteins

Phosphorylatable proteins are indicated in red
Outline

• Standard 2D protocol
• Special 2D protocols for
  – Phosphoproteins
  – Large proteins
  – Basic proteins
  – Proteins with close pI points
  – Proteins with close molecular weights (MW)
Standard 2D Protocol-IEF-Material

• IEF buffer
  – Urea/thiourea (non-ionic detergent)
  – CHAPS (zwitterionic detergent)
  – Ampholyte (Prevent protein precipitation)
  – DTT (Prevent protein oxidation)
  – BPB (IEF Progression Indicator)

• Maximum detergent strength/minimum ionic strength (no ionic detergents, no salt)
Standard 2D Protocol-IEF-Protocol

- 50 volts overnight (Rehydration)
- 50-250 volts in 15 min
- 250-10K volts in 3 hours (for 24 cm strips)
- 10K volts for 40K volt/hours
- Current limit: 50 mA/Gel
- Total time: 22~30 hours (24 cm strips)

↑ Voltage  ↓ Current  ↑ Resistance  ↓ Conductivity
Standard 2D Protocol-2$^{\text{nd}}$ Dimension

- Equilibration buffer 1
  - Urea (6M)
  - Glycerol (30%)
  - SDS (2%)

- Equilibration buffer 2
  - DTT (1%)
  - IAA (2.5%)
2DE of Myofilament Proteins

MW

pI (3 to 11, 18 cm)

MW

MHC 220KDa

MyBP-C

Actin TnT

Tm

MLC1

MLC2

TnI (pI 9.5)
2D-DIGE

WT Mouse Heart

PKC TG Heart

Cy2

Cy5

Mix/2D Gel

Imaging & Analysis
2D-DIGE of Mouse Heart Proteins

Green: control
Red: PKC TG

MyBP-C
Quantification

MyBP-C total protein change: 1.14%
Co-detection of Phospho- & total-Protein

1. Label Proteins with Cy2/Cy5
2. 2D-Gel analysis
3. ProQ Diamond Stain
4. Total Protein: Cy2/Cy5
   Phospho-Protein: ProQ
Co-detection of Phospho- & total-Protein

Green: WT
Red: TG
Blue: ProQ
Estimation of Phosphorylation Degree (♯) for each spot

ProQ/(Cy2+Cy5); Compare with Spot 3
Confirmation of Phosphorylation by Western

PVDF
- Neonatal
- Adult

Western
- Phospho-PKC motif Antibody
Novel Phosphorylation Sites

<table>
<thead>
<tr>
<th>I (PKA)</th>
<th>II (CamK)</th>
<th>II*</th>
<th>III (PKA/PKC)</th>
<th>III*</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOG</td>
<td>ELHITDAQPTSAGGYRCEVSTKDKFSCNFNLTVHEAVGPGDLDLSAFRRTSLAGGRR 286</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HUMAN</td>
<td>ELHITDAQPAGTGYRCEVSTKDKECSNFNLTVHEAMGTDLDLLSAFRRTSLAGGRR 282</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOUSE</td>
<td>ELHITDAQTSTSAGGYRCEVSTKDKFSCNFNLTVHEAIGSGDLDDLRSAFRRTSLAGGRR 280</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAT</td>
<td>ELHITDAQATASAGGYRCEVSTKDKFSCNFNLTVHEAIGSGDLDDLRSAFRRTSLAGGRR 293</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHICKEN</td>
<td>EME11EANMFTAGGYRCEVSTKDKESSNFLTVNEAPVSHEMD1KRAAFRTSLAGGRR 271</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FROG</td>
<td>EQIIGAKTTYAGGYRCEVSSKDKFSCNFNLAVHEAASSGEVTDRAAFRTSLVGAAKR 287</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| DOG     | TS--------DSHEDAGTLDFSSLKKRDSSFR--------RDSKLEAPAEEDVWEILRQAPPSEY 335 |
| HUMAN   | TS--------DSHEDTGLDFSSLKKRDSSFR--------PRDSKLEAPAEEDVWEILRQAPPSEY 333 |
| MOUSE   | TS--------DSHEDAGTDPDFSSLKKRDSSFR--------RDSKLEAPAEEDVWEILRQAPPSEY 329 |
| RAT     | TS--------DSHEDAGTLDFSSLKKRDSSFR--------RDSKLEAPAEEDVWEILRQAPPSEY 342 |
| CHICKEN | MTSAFLSTEGRGELNSALLKKRDSFLRTANRGDKGKSQSPOPVDVWEILKKAPPSEY 331 |
| FROG    | RVSTIAFSGDGETAGELDPSALLKKRDSFLRSEPNREPKQGTPPDVWEILKKAPPSEY 347 |
Distribution of Identified Phosphorylation Sites

pH 6

II II* III* IV
II II* III*
II II* III*
II I
II II
II II
II -
II -

6.7

Pro-Q
MyBP-C has 5 unphosphorylated spots

Before Commassie

Myomesin

After Alkaline Phosphotase treatment

After

Some sites are resistant to AP treatment

Comassie

Pro-Q
Special 2D Protocol for MyBP-C

Standard Protocol

Special Protocol

150 KDa
Special Protocol for MyBP-C

- Sequential IEF: After 1\textsuperscript{st} IEF, excise gel region corresponding to MyBP-C, and perform a 2\textsuperscript{nd} IEF at a higher than normal voltage.
- Use 5\% SDS, instead of 2\% SDS, in equilibration buffer.
- Use 4-12\% gradient SDS-PAGE gel.
Detection of Phosphorylated Myofilament Proteins with ProQ

<table>
<thead>
<tr>
<th>MW</th>
<th>200</th>
<th>116</th>
<th>pI</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tm</td>
<td>MLC2</td>
<td>TnT</td>
<td>MyBP-C</td>
<td>ProQ/WT/PKC</td>
</tr>
</tbody>
</table>

ProQ/Cy2/5

Cy2/Cy5

Q/Cy2/5

MyBP-C

Tm

MLC2

TnT

TnI
Special Protocol for TnI (pI 9.5)
Special Protocol for TnI

• Horizontal streaking of basic proteins is due to protein (cystein) oxidation.
• Cysteinyl oxidation is due to lack of DTT.
• DTT can be depleted during IEF, especially from basic end, because it is a weak acid.
• A so called “destreak reagent” (HED) did not improve 2D gel resolution of TnI.
Special Protocol for TnI

• Perform IEF as usual.
• Shortly before the end of IEF, add DTT (3%~5%) soaked paper wick to the basic end of the gel.
• Continue IEF for 15~20 minutes.
### 2DE of Proteins with Close pI Values

#### Relative Focusing Power of IPG Strip

<table>
<thead>
<tr>
<th>pH</th>
<th>3 cm</th>
<th>4 cm</th>
<th>5 cm</th>
<th>6 cm</th>
<th>7 cm</th>
<th>8 cm</th>
<th>9 cm</th>
<th>10 cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-10</td>
<td>1x</td>
<td>1.6x</td>
<td>2.4x</td>
<td>2.6x</td>
<td>3.4x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-10 nonlinear (NL)</td>
<td>1x</td>
<td>1.6x</td>
<td>2.4x</td>
<td>2.6x</td>
<td>3.4x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Narrow Range</td>
<td>3-6</td>
<td>2.3x</td>
<td>3.7x</td>
<td>5.7x</td>
<td>6.0x</td>
<td>8.0x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-8</td>
<td>2.3x</td>
<td>3.7x</td>
<td>5.7x</td>
<td>6.0x</td>
<td>8.0x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-10</td>
<td>2.3x</td>
<td>3.7x</td>
<td>5.7x</td>
<td>6.0x</td>
<td>8.0x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-7</td>
<td>2.3x</td>
<td>3.7x</td>
<td>5.7x</td>
<td>6.0x</td>
<td>8.0x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micro Range</td>
<td>3.9-5.1</td>
<td>5.8x</td>
<td>9.2x</td>
<td>14.2x</td>
<td>15.0x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.7-5.9</td>
<td>5.8x</td>
<td>9.2x</td>
<td>14.2x</td>
<td>15.0x</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.5-6.7</td>
<td>5.8x</td>
<td>9.2x</td>
<td>14.2x</td>
<td>15.0x</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.3-8.3</td>
<td>3.5x</td>
<td>5.5x</td>
<td>8.5x</td>
<td>9.0x</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ReadyStrip IEF Buffer:
- 3-10
- 3.9-5.1
- 4.7-5.9
- 5.5-6.7
- 6.3-8.3
2DE of Proteins with Close pI Values

24 cm, 3-10

18 cm, 4.5-5.5
1DE of Proteins with Close MW

• Standard 1D Gel Recipe
  – 10~12% gel
  – 29:1 (acrylamide:bisacrylamide)

• TnT isoforms (MW; 40 Kda; Difference: 3 aa)
  – 14% gel
  – 200:1 (acrylamide:bisacrylamide)
  – 5% Glycerol

• α- and β-MHC (MW 220 KDa; Difference: 0.4 KDa)
  – 6% gel
  – 50:1 (acrylamide: DATD)
  – 10% Glycerol
Summary

• Overview of 2D Gel and DIGE
• 2DE of Phosphorylation
• 2DE of large proteins
• 2DE of basic proteins
• 2DE of proteins with close pI and MW values
Acknowledgements/Publications


The Third Quantitative Proteomic Approach-Antibody Array

Sample1

Cy3

Sample2

Cy5

CloneTech Ab Array 500
Ab Array-Preliminary Results (n=1)

Cy3: PGDH-- Crypt, Cy5: PGDH++ Crypt
Reverse labeling was also performed

Yellow means no change, red or green means protein changes.
Functional Categories

- Monoclonal antibodies detect proteins across five major functional categories
- One antibody can be present in more than one category
- Results can be sorted by functional category

<table>
<thead>
<tr>
<th>Ab Name</th>
<th>Apoptosis</th>
<th>Cancer</th>
<th>Neuroscience</th>
<th>Cell Cycle</th>
<th>Protein Kinases</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNX2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Arrestin1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rift</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fas (CD95)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rag-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IAF II 70</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p21 (Cip1/WAF1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XPD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRB14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRP1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPTPα</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Three Proteomic Approaches

2D Gel  Mass Spectrometry  Antibody Array
Up Coming Workshops

- Quantification
- Structure
- Interaction
- PTM
- Bioinformatics