Tissue and Fluid Proteomics

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Proteomics:

Protein profiles at the moment
Protein(s) involved in change of biological process (state 1 $\rightarrow$ 2)
Protein(s) functions in signaling pathways or regulatory cascades in the biological processes

These unique changes of proteins can serve as
- markers for early detection and predictive purposes
- novel targets for drug discovery and therapeutic intervention
Sample Sources for Proteomic Analysis

• Cell lines.
• Tissue sections.
• Body Fluids:
  – Blood and urine.
  – Fluids from secretion.
  – Fluids in interstitial spaces.

Fluids from Secretion

• Aqueous Humor
  – AH was collected by 27 G needle (150 µl) from patients w/ or w/o corneal rejection.
  – 2D gel w/ MS ID.

• Saliva
  – Whole saliva or major salivary gland secretions.
  – 2D gel w/o or w/ MS ID; LC-MSⁿ.
  – Proteome database for biomarkers of specific diseases.
Fluids from Secretion

- **Cerebrospinal fluids (CSF)**
  - Fluid surrounding the central nervous system.
  - Total vol ~140 ml, produced at 03-0.4 ml/min.
  - Samples were collected by lumbar puncture (10-12 ml).
  - 2D gel w/ MS ID; LC-MS^n
  - Studies of the path-physiological mechanism in front-temporal dementia, Alzheimer's disease

- **Synovial fluid**
  - A dynamic reservoir for proteins originating from serum, synovial tissue, and cartilage.
  - 2D gel / MS ID and LC^n-MS^n.
  - Study for biomarkers for Rheumatoid Arthritis.

- **Nipple aspiration fluid (NAF)/ Ductal lavage fluid**
  - NAF: breast ductal fluid collected by nipple aspiration.
  - Non-invasive way of sample collection.
  - NAF: sample vol: generally ~ 10-20 µl.
  - 2D gel, SELDI, and chromatography-MSMS.
  - Studies of the early diagnosis of breast cancer.

- **Bronchoalveolar lavage (BAL) fluids**
  - Obtained by washing the epithelial lining of lung with PBS.
  - 2D gel / MS ID and LC^n-MS^n.
  - Studies of fibrosing interstitial lung diseases, such as sarcoidosis, and allergic asthma.

### Protein Profiles of Bilateral Matched Paired NAFs by 2DE-Approach

#### Non-Cancerous Left Breast vs. Non-Cancerous Right Breast

<table>
<thead>
<tr>
<th>Spot Count</th>
<th>Left Breast</th>
<th>Right Breast</th>
</tr>
</thead>
<tbody>
<tr>
<td>1398 spots</td>
<td>3 spots found only in Left Breast</td>
<td>2 spots found only in Right Breast</td>
</tr>
</tbody>
</table>


#### Cancerous Breast vs. Non-Cancerous Breast

<table>
<thead>
<tr>
<th>Spot Count</th>
<th>Cancerous Breast</th>
<th>Non-Cancerous Breast</th>
</tr>
</thead>
<tbody>
<tr>
<td>1428 spots</td>
<td>202 spots found only in Cancerous Breast</td>
<td>54 spots found only in non-Cancerous Breast</td>
</tr>
</tbody>
</table>

Approaches for Sampling from Extra-cellular Space (Interstitial Fluids)

- Tissue perfusion (TIF)
- *In-vivo* sampling from interstitial space with capillary probes (CUF)

Fluids in Interstitial Spaces

Ex-vivo Interstitial fluid collection:

- Complexity of proteins in interstitial space.
- *ex-vivo* sampling technique.
- Difficulty in obtained samples from the same tissue at different disease stages.

In-vivo Sampling from Interstitial Space

**Microdialysis (MD)**
- Perfusion with saline solution at \( \mu l/min \)
- Microdialysate
- 200 – 600 µm (small molecules analysis)

**Ultrafiltration (UF)**
- Vacuum
- 200 – 600 µm

**Microdialysis vs Ultrafiltration for Proteomic Sampling**

**Advantage:**
- Sampling free drug or metabolites (non-protein-bound) in interstitial fluid at the site of interest.
- Excellent temporal resolution for PK studies from single animal.
- Real in-vivo sampling from live, freely-moving animals.

**Microdialysis:**
- diffusion-based technique
- poor recovery (for peptides and proteins)
- not suitable for long term in-vivo sampling.

**Capillary Ultrafiltration:**
- non-diffusion-based technique
- better & consistent recovery
- suitable for long term sampling (up to 6 month).
1. Polysulfone membrane: high MWCO
2. AN69 membrane: low MWCO

Capillary Ultrafiltration Probe

- Epoxy glue
- Teflon capillary (100 µm id)
- SS needle
- Semi-permeable hollow fiber
- Vacutainer

CUF Sampling from Animal Models

- *In-vivo* UF sampling from interstitial microenvironment in tumor masses at different developing stages.

- Continuous UF sampling from a freely-moving mouse model with chemical-induced Allergic Contact Dermatitis.
Regressive Skin Tumor Model (C2240)

C3H/HeN Mice

Nude mice

Tumor volume (mm$^3$)

0 50 100 150 200 250 300 350 400 450 500 550 600

weeks

Tumor volume (mm$^3$)

0 1000 2000 3000 4000 5000

Dynamic interaction between Tumor and Host cells

UAB Center of Skin Diseases

CUF Sampling from Regressive Tumor Model

C3H/HeN Mice

1. Measure tumor size
2. Implant CUF probe in tumor masses for IF collection.

- Tumors grew on WK 1, but tumor masses decreased after WK2.
- Interstitial fluid in tumor was collected by a high MWCO probes for 3 hours.

Protein ID through PROWL & SWISS-PROT database

Nano-LC-qTOF MS for peptide sequencing

~ 2–5 µl of IF collected
Secretomes from Regressive Skin Tumors

Tumors progress at 1st week
1. **S100A4** (Metastasis-associated calcium binding protein)
2. **Thymosin β4**
3. **Thymosin β10**
4. **Profilin 1** (dendritic exosomes)
5. **beta 1-globin**
6. **Hemoglobin β2**

Tumors regress at 3rd week
1. **Fetuin-A** (α-2HS-glycoprotein)
2. **Apolipoprotein A-1**
3. **Alpha-antitrypsin**
4. **Contrapsin** (Trypsin inhibitor)
5. **beta 1-globin**
6. **Hemoglobin β2**

**All are secretory proteins**

**Underline**: tumor associated proteins

CUF can be used to sample clean interstitial fluid *in-vivo* from animals at different physiological / disease stages

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**Allergic Contact Dermatitis**

1st contact

1. **Hapten**
2. **Poison Ivy**

2nd contact

1. **Lymph node**
2. **Langerhans cell**
3. **antigen-specific T cells**
4. **Activated T cells**

**SKIN**

- **No dermatitis**
  - (Induction Phase)

- **Inflammatory response**
  - (Elicitation Phase)

- **Dermatitis**
Dynamic CUF Sampling from ACD Model

Animal: C3H/HeN mouse
Hapten: DNFB (dinitrofluorobenzene)
control/control, control/DNFB, DNFB/DNFB

Day 1 + 2
Induction:
0.5 % DNFB (in 20 % olive oil in acetone)
- 25 µl on shaved abdominal skin and footpads

Day 6 (the day before elicitation)
UF probe implantation:
CUF probe was implanted subcutaneously in ear
and housed in a freely-moving system with access
to water and food
- collect interstitial fluid (IF) samples 12 hours
before elicitation

Day 7
Elicitation:
0.2 % DNFB (in 20 % olive oil in acetone)
- 10 µl on both side of each ear
- IF samples were collected continuously into 24 hours
intervals for 3 days

1. Ear thickness were measured daily before and after DNFB elicitation.
2. UF samples were processed with 2DE cleanup kit and analyzed with 75 µl protein load on 3-10 IEF/12.5% SDS gel / Sypro staining.

Ear Swelling of ACD Model

<table>
<thead>
<tr>
<th></th>
<th>CC-1</th>
<th>CD-1</th>
<th>CD-2</th>
<th>DD-1</th>
<th>DD-2</th>
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<tbody>
<tr>
<td>24 hrs after elicitation</td>
<td>0/1</td>
<td>0/2</td>
<td>0/2</td>
<td>4/-</td>
<td>7/10</td>
</tr>
<tr>
<td>48 hrs after elicitation</td>
<td>0/2</td>
<td>0/0</td>
<td>1/2</td>
<td>7/-</td>
<td>5/5</td>
</tr>
</tbody>
</table>

1. Left ear / Right ear (with Probes), unit: 0.01 mm.

UAB Center of Skin Diseases, ABRF 2006
2DE Analysis of IFs from different ACD Stages

Protein ID of IFs from different ACD Stages

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mass</th>
<th>MS</th>
<th>MOWSE</th>
</tr>
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<tbody>
<tr>
<td>Cp protein</td>
<td>121074</td>
<td>MALDI</td>
<td>126</td>
</tr>
<tr>
<td>Gsn protein</td>
<td>80712</td>
<td>MALDI</td>
<td>114</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>90723</td>
<td>MALDI</td>
<td>88</td>
</tr>
<tr>
<td>transferrin</td>
<td>76628</td>
<td>MALDI</td>
<td>140</td>
</tr>
<tr>
<td>albumin 1</td>
<td>68678</td>
<td>MALDI</td>
<td>167</td>
</tr>
<tr>
<td>vitamin D-binding protein</td>
<td>53051</td>
<td>MALDI</td>
<td>82</td>
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<tr>
<td>kininogen 1</td>
<td>47868</td>
<td>MALDI</td>
<td>74</td>
</tr>
<tr>
<td>Serpina1a protein</td>
<td>45593</td>
<td>MALDI</td>
<td>103</td>
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<td>apolipoprotein A-IV</td>
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<td>MALDI</td>
<td>118</td>
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<tr>
<td>trophoblast specific protein beta</td>
<td>13802</td>
<td>MALDI</td>
<td>66</td>
</tr>
<tr>
<td>vitamin D-binding protein</td>
<td>53085</td>
<td>Q-TOF</td>
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<tr>
<td>Calgranulin B</td>
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* This protein list only represents spots observed in most of 9 gels. Detail analysis of differences between gels is not shown.
CUF for Proteomics Analysis of Interstitial Microenvironments

• We have evaluated the use of Capillary Ultrafiltration for proteomic study in interstitial microenvironments by providing both in-vivo and dynamic sampling.

• Challenges in analyzing CUF samples by 2DE:
  – **Salty matrix**: may not be a problem; desalt cleanup may lose proteins.
  – **Albumin**: albumin depletion assay: insufficient, protein loss.
  – **Sample size**: increase collection area (multiple probes or longer probes) and longer collection time (lost of temporal resolution).
  – **Quantitative analysis**: DIGE will help.

2DE Analysis of CUFs

![2DE Analysis of CUFs](image)
2DE Analysis of CUFs from different CD Stages

-12 ~ 0 hr before induction of CDs

0 ~ 12 hr after induction of CDs

24 ~ 48 hr after induction of CDs

60 µg, IEF: 4-7, 10-20 %% SDS PAGE, Sypro Ruby staining

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<td>44545</td>
<td>MALDI</td>
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<td>12909</td>
<td>Q-TOF</td>
<td>167</td>
<td></td>
</tr>
<tr>
<td>transthyretin</td>
<td>15766</td>
<td>MALDI</td>
<td>103</td>
<td>4</td>
</tr>
<tr>
<td>Apolipoprotein A-I, precursor</td>
<td>30358</td>
<td>MALDI</td>
<td>96</td>
<td>2</td>
</tr>
<tr>
<td>Apolipoprotein A-I</td>
<td>23008</td>
<td>MALDI</td>
<td>131</td>
<td>3</td>
</tr>
<tr>
<td>serine (or cysteine) proteinase inhibitor, clade A, member 1d</td>
<td>45969</td>
<td>MALDI</td>
<td>89</td>
<td>1</td>
</tr>
<tr>
<td>Serum amyloid P-component precursor</td>
<td>26230</td>
<td>MALDI</td>
<td>130</td>
<td>6</td>
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<tr>
<td>complement component c3d</td>
<td>33442</td>
<td>MALDI</td>
<td>74</td>
<td>5</td>
</tr>
</tbody>
</table>
**Sample Prep for Proteomic Analysis of IFs**

IFs (CUF or EIF) → Multiple Affinity Removal Spin Cartridge (remove mouse albumin, IgG, and transferrin) → retained high abundant proteins → Low abundant protein → Microcon YM-10 (MWCO: 10 kda) → Concentration and buffer exchange For 2DE analysis or MS analysis

**2DE Analysis of CUFs from different CD Stages**

Pooled BK CUF collected -12-0 hr before treatment

CC CUF collected 0-24 hrs after treatment

CC CUF collected 24-48 hrs after treatment
2DE Analysis of CUFs from different CD Stages

CD CUF collected 0-24 hrs after DUFB treatment

ACD (DD) CUF collected 0-24 hrs after treatment

2DE Analysis of TIFs from TPA or DNFB Irritated Ear

Male C3H/HeN mice

Irrigation induced by painting 1 % DNFB or 20 nmol TPA on ears

6 hours

Harvest dorsal ear skin, let the ear skin float on PBS with PI (1ml/ear), and incubate at 37 C for 1 ½ hour

Collect supernatant as TIF

PPT with 2D cleanup kit

Dissolved in IEF buffer for 2DE analysis

60 ug

3-11NL + 10-20% gel
2DE Analysis of TIFs Collected from TPA- Irritated or DNFB-CHS Ear

** TIF collected at 24 hrs after treatment

Quantitative Proteomics

- Gel based approach:
  - Difference gel electrophoresis (DIGE)
**DIGE of TIF samples**

- 10 µg Cy dye labeled samples
- Limited cy dye labeling (0.5%)
- 30 mg/gel
- IEF: 3-11 NL; 10-20% SDS PAGE

**Summary**

- The use of Capillary Ultrafiltration for proteomic study in interstitial microenvironments by providing both in-vivo and dynamic sampling.

- Challenges in analyzing UF samples by 2DE:
  - **Salty matrix:** may not be a problem; desalt cleanup may lose proteins.
  - **Albumin:** depletion kit from Sigma did not work well.
  - **Sample size:** increase collection area (multiple probes) and longer collection time (lost of temporal resolution).
  - **One shot deal:** DIGE will help.

- Other Multi-dimentional approaches may be more suitable for analysis of IF samples.