Protein expression profiling and biomarker discovery using stable isotopically labeled amino acids in cell culture (SILAC)

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Different proteomes yield different phenotypes...

Genetically identical...Phenotypically different

Phenotypic differentiation occurs at the protein expression level
Genomics vs. Proteomics

Genome - Static

Proteome - Dynamic

Need quantification of protein level
Comparative Quantitation of Differential Expression

Healthy cells
Control cells
Stem cells

Phenotype A

Phenotype B

Disease cells
Drug-treated cells
Differentiated cells

What proteins are differentially expressed (i.e. abundance)?

Most proteins’ abundance is unaffected

up or down regulated

The change in abundance of these proteins is a) diagnostic and b) a very few of them may be causal for the disease and are c) therapeutic targets (BIOMARKERS AND/OR DRUG TARGETS!)

Finding a Small number of responding elements in a massive background
Mass Spectrometry &

Stable Isotope labeling by amino acids in cell culture
Life Technologies Proprietary & Confidential

Quantitate relative protein levels by measuring peak ratios

Cell pool 1

Combine

Cell pool 2

Extract, purify, separate

Excise protein bands, digest extract peptides

Mass

Analyze by MS

Identify proteins

Protein A

Protein B

Protein C

Metabolic Labeling with e.g. $^{15}$N, $^{13}$C

In vivo, SILAC vs. in vitro, 2D-DIGE labeling

**DIGE** (chemical labeling)

(Differential in-gel electrophoresis)

<table>
<thead>
<tr>
<th>Proteome</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cy5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2D-GE

Two-color imaging

**SILAC** (metabolic labeling)

<table>
<thead>
<tr>
<th>State A</th>
<th>State B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu-d0</td>
<td>Leu-d3</td>
</tr>
</tbody>
</table>

Optional protein purification

Combine and digest with trypsin

Quantitation by MS

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**Performance**

<table>
<thead>
<tr>
<th></th>
<th>DIGE</th>
<th>SILAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Easy to use</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Labeling efficiency</td>
<td>5%</td>
<td>&gt;97%</td>
</tr>
<tr>
<td>Sample Species</td>
<td>Heterogeneous</td>
<td>Homogeneous</td>
</tr>
<tr>
<td>Dynamic range for quantitation</td>
<td>Poor</td>
<td>40 fold over a range of 10^5 express</td>
</tr>
<tr>
<td>Accuracy, esp. w/ overlapping proteins</td>
<td>Poor</td>
<td>Excellent</td>
</tr>
<tr>
<td>Detection of low abundant proteins</td>
<td>Poor</td>
<td>Good</td>
</tr>
<tr>
<td>Flexibility in Workflow</td>
<td>Poor</td>
<td>Excellent</td>
</tr>
<tr>
<td>Sampling bias</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Membrane proteins, acidic and basis proteins</td>
<td>Poor</td>
<td>Good</td>
</tr>
</tbody>
</table>
SILAC Workflow

Stable Isotopically Labeled Amino acids in cell Culture

Identical cell cultures

Grow for 6 doublings to ensure 100% incorporation

Differential treatments

Mix cells 1:1, Lysis and optional subcellular fractionation

Fractionate proteins by 1D PAGE

Lys and Arg

Untreated control

13C6-Lys and/or 13C6,15N4-Arg

Apply stimulus

Mix + Lyse

Digest with Trypsin

1D PAGE

Submit for MS analysis

Determine differences in protein expression by measuring relative MS intensities of light vs. heavy
Stable Isotope labeling by amino acids in cell culture (SILAC)
(An ideal quantitative proteomic approach for cell-based analysis)

Cells are mixed at 1:1 ratio

Light Lys and Arg (-) or (+) stimulus

Heavy Lys and Arg (+) or (-) stimulus

Culture media

Cell lysate

Media are concentrated using Amicon filter with MW 5K cutoff

Affinity Enrichment
- Glycoprotein
- Membrane protein
- Phosphoprotein

Identify growth factors or differentiation factors...

tryptic digest and LC-MS/MS analysis

Identify cell surface markers
Study transcriptional regulation and signal transduction......

Quantitation is based on the relative intensity ratio of the isotopic peptide pairs in MS spectrum

growth factor stimulation

Drug treatment

Cell differentiation
Advantages of SILAC

1. **Simplicity** – labeling by metabolic machinery of cells.

2. **Homogeneity** – 100% labeling efficiency on multiple sites per protein.
   This can not be achieved w/o serious loss of fidelity using other methods.

3. **Comparability** – Proteins and peptides with light or heavy a.a. are enzymatically & chemically identical so they have the same activities and co-migrate in any separation methods

4. **Unequivocal quantification** – mixing during the earliest steps of analysis eliminates bias due to unequal sampling, migration or detector response

*Identify unique markers for stem and differentiated cells*
What is the need for metabolic labeling?

Stable Isotopically Labeled Amino acids in cell Culture

100% incorporation, for high and low abundance components
Stable isotopic labeling

Most elements have naturally occurring **stable** isotopic variants...\(^{13}\text{C}, \ ^{15}\text{N}\)

MALDI-TOF-MS of Calmodulin tryptic fragment

Each peak is chemically indistinguishable...different only by isotopic mass (i.e. differ by # of neutrons)
Stable isotopic labeling

There are several methods that allow isotopic enrichment....

...such that mass shifts can be induced

$4 \times ^{15}\text{N} = 4\text{Da}$
Stable isotopic labeling

Thus, just like the DIGE experiment...

Proteome A → Enrich with one isotopic variant
Proteome B → Enrich with another isotopic variant

Mix → Digest

Relative intensities ARE proportional to sample component abundances...

MS CAN DO RELATIVE QUANT!
SILAC versatility

Stable Isotopically Labeled Amino acids in cell Culture

Drugs:
*Pharmacology, toxicology, immunology*

Environmental stimulation:
*Media components, ischemia, radiation*

Gene manipulations:
*GOF or KO’s*

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**Diagram:**
- **Lys and Arg**
- **$^{13}$C$_6$-Lys and/or $^{13}$C$_6$, $^{15}$N$_4$ -Arg**

**Steps:**
1. Untreated control
2. Apply stimulus
3. Mix + Lyse
Why fractionate?

Stable Isotopically Labeled Amino acids in cell Culture

- Lys and Arg
- Untreated control
- Apply stimulus
- Mix + Lyse
- 1D PAGE

Separate high and low abundance components
Why fractionate?

Stable Isotopically Labeled Amino acids in cell Culture

Separate high and low abundance components

Fractionation reduces the complexity of a sample, aiding in identification and quantitation

Fractionation reduces mutual suppression effects
SILAC workflow

Stable Isotopically Labeled Amino acids in cell Culture

Identical cell cultures

Grow for 6 doublings to ensure 100% incorporation

Differential treatments

Mix cells 1:1, Lysis and optional subcellular fractionation

Fractionate proteins by 1D PAGE

Lys and Arg

Untreated control

Apply stimulus

Mix + Lyse

1D PAGE

Submit for MS analysis
Proteomics ID workflow

1) Measure m/z of eluting components (MS1)
2) Select for fragmentation in (MS2)

Are there matches?

Translated genomic database

*In-silico* trypsin digest

In-silico MS2 fragmentation

excise

Trypsin

Proteolysis &

peptide extraction

MS
Protein Identification

(MATRIX) Mascot Search Results

Pepptide View

MS/MS Fragmentation of VLQLNDNTALTASYGVFR
Found in gi|5458832, oxygen regulated protein precursor, oxygen regulated protein (150kD) [Homo sapiens]

Match to Query 183: 2100 128848 from(1051.671700,2+)
Sum of 8 scans in range 979 (m/z=2054.96, f=2, i=125) to 992 (m/z=2085.29, f=3, i=137) [INR:803MarshallSILACIMP14/16/18exp1band9 raw]
From data file C:\DOCTME\1licing24\LOCALSE--INTemp\Dna10DF tmp

Click mouse within plot area to zoom in by factor of two about that point
Or, Plot from [100] to [1700] Da
Exquisite Precision

Differential Expression of Vimentin

Relative abundance

Normal    Cancer

51%

8% CV
N=9

Vimentin (gi|418249)

Exquisite Precision

Relative abundance

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8% CV
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Vimentin (gi|418249)
Reproducibility from one experiment to another

gi|418249 Vimentin

gi|5453832 oxygen regulated protein precursor

10% CV
What can you do with SILAC™?

**SILAC™ Kits**

Differential expression of proteins and identification of disease biomarkers

Cell signaling dynamics

Analysis of yeast pheromone signaling pathway

Identification of methylation sites

Identification of protease substrates

Study of protein complexes/protein interactions

Study of secreted growth factors

Analysis of signaling pathways and effect of pharmacological inhibitors

Subcellular proteomics and organogenesis, and dynamics
SILAC and Biomarkers in Breast Cancer

Breast Cancer Primary cells

- **Heavy:** U^{13}C_{6} Lys U^{13}C_{6} Arg
- **Light:** Lys + Arg

Combine at 1:1 ratio

**12-100 proteins/band**
- ID’d. & quant 1600+ gene products which group into 997 protein families
- 830 are membrane proteins
- 100 unknown
- 67 histones, HSP, actin, etc.

All heavy peptides = up regulated
All light peptides = down regulated

One protocol uncovers OVER 40 membrane proteins w/expression changes of 5 fold → HPT for biomarkers
Human Breast Cancer Validation

IHC Analysis performed on panels of several different tissues

Validation Ex: OSF-2 is up-regulated in breast carcinomas

Validation Ex: CD13 is down-regulated in breast carcinomas

Tissue Staining Confirms SILAC Defined Biomarkers are Breast Specific

Liang, Xiquan; et al., Proteomics (2006) 6, 4554-4564.
Malignant cells secrete proteins to support invasive behavior

**Figure 5A**  
*gi|480007 osteoblast-specific factor 2*

**gi|27769056 SERPINE2 protein**

**Functional category**
- Matrix and cytoskeleton
- Metabolism
- Signal transduction
- Protease, metalloprotease, or protease inhibitor
- Growth factor, cytokine, chemokine
- Nucleic proteins
- Heat shock
- Ribosomal
- Other

380 proteins, 94% are secreted according to literature or database

**Extracellular matrix proteins upregulated**
Protease inhibitors downregulated
SILAC and beyond
SILAC™ kits are available for most cell lines including ESC.
SILAC for Biomarker Discovery
Biomarker Validation: Profiling by NCode™ miRNA Microarray and SILAC™

- Differential expression of secreted protein was measured by SILAC in normal and malignant breast cells
- Expressed miRNA’s were isolated using PureLink NAP purification kit and profiled on NCode microarrays (normal vs. malignant breast cells)
- In many cases, putative miRNA binding sites were found in the 3’ UTRs of mRNAs coding the differentially expressed proteins.
- Patient samples – triple replicates with cells from one patient
Inverse correlation between miRNA and protein expression

- Differentially expressed miRNAs and proteins
- Malignant vs. normal breast epithelial cells
- miRanda (Sloan-Kettering) + TargetScan (MIT)
- 3’UTR seeds
- Validation next steps

<table>
<thead>
<tr>
<th>miRNA</th>
<th>miRNA fold change</th>
<th>protein fold change</th>
<th>protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-195</td>
<td>6.2</td>
<td>-13.0</td>
<td>Plasma protease (C1) inhibitor precursor</td>
</tr>
<tr>
<td>hsa-miR-17-5p</td>
<td>3.7</td>
<td>-18.0</td>
<td>Laminin A3</td>
</tr>
<tr>
<td>hsa-miR-30b</td>
<td>3.6</td>
<td>-11.7</td>
<td>Serpine 2</td>
</tr>
</tbody>
</table>
SILAC, RNAi and pathways

SILAC™ Protein Identification and Quantitation Kits

1. Quantitate Knock Down (KD)
   - Control (normal amino acids)
     - Arginine
     - Lysine
   - Mock RNAi (Labeled Lysine)
     - Arginine
     - Lysine $^{13}$C$_6$
   - Test RNAi (Labeled Arginine)
     - Arginine $^{13}$C$_6^{15}$N$_4$
     - Lysine

Mix cells 1:1:1

Fractionate, MS analysis

2. Assess Off Target Effects (OTE)
   - 10 Da
     - KD
     - m/Z
   - 10 Da
     - No KD
     - m/Z
   - 6 Da
     - No OTE
     - m/Z
   - 6 Da
     - OTE
     - m/Z

3. Identify affected proteins

4. Map to pathway

* Could be upregulated as well
SILAC, RNAi and pathways

Arg and Lys
No treatment

*Lys
(-) shRNA

*Arg
p53 shRNA

Mix + Lyse

1D PAGE

Excise bands
+

Proteolyze

LC/MS

10 Da p53 shRNA

6 Da (-) shRNA
RNA interference (RNAi) technology exploits the natural ability of eukaryotic cells to degrade foreign RNA particles to knockdown, or inhibit, expression of specific genes.

1) Knockdown specific gene
2) Biochemically confirm KO, PCR
3) Assess phenotype

Artwork by Christian Kroun Damgaard.
SILAC and RNAi Cellular Phenotyping

But what about partial KO’s?

What about ambiguous phenotypes?

For knockdown products or pathways that lack an assay, how does one quantitate the phenotype and correlate to the extent of KO?
In particular, how does one assess how the KO of one protein upstream affects the expression of many proteins downstream?
SILAC assay for shRNA off-target effects

If I knock-down the non-essential gene, Lamin A/C, I should see no changes in expression across the proteome.

How specific is my shRNA construct?

T-Rex™ CHO

Arg Arg

Untreated control

Lamin A/C shRNA

Mix + Lyse

Trypsin proteolysis

Cut into 6 pieces

LCMS

NL pH 3-10

Harvest at 3 and 5 days

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Trypsin proteolysis

Cut into 6 pieces

LCMS

NL pH 3-10

Harvest at 3 and 5 days
SILAC assay for shRNA off target effects

• 146 proteins identified so far

• 0 proteins had a change in abundance greater than 5% (except for Lamin A/C)
Can I label animals?

**Metabolic Labeling of Mammalian Organisms with Stable Isotopes for Quantitative Proteomic Analysis**

Christine C. Wu,†,‡ Michael J. MacCoss,†,‡ Kathryn E. Howell,§ Dwight E. Matthews,‖ and John R. Yates, III*,†

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Short List of SILAC References


8. Liang, Xiquan; Zhao, Jenson; Hajivandi, Mahbod; Wu, Rina; Tao, Janet; Amshey, Joseph W.; Pope, R. Marshall, Quantification of membrane and membrane-bound proteins between normal and malignant breast cells isolated from the same patient with primary breast carcinoma, J.Proteome Res. (2006) 5, 2632-2641.

9. Liang, Xiquan; Hajivandi, Mahbod; Veach, Darren; Wisniewski, David; Clarkson, Bayard; Resh, Marilyn D.; Pope, R. Marshall, Quantification of change in phosphorylation of BCR-ABL kinase and its substrates in response to imatinib treatment in human chronic myelogenous leukemia cells, Proteomics (2006) 6, 4554-4564.


More references may be found at:

http://www.silac.org/publications

http://www.pil.sdu.dk/silac_bibliography.htm

http://www.pil.sdu.dk/Publications.htm