Proteomics Course

LECTURE-24
Quantitative Proteomics:
Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC)

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Today's lecture

- MS-based quantitative proteomics
- *In vivo* labeling
- SILAC introduction
- SILAC experiment & work-flow
- SILAC merits and demerits

MS-based quantitative proteomics

MS-based quantitative proteomics

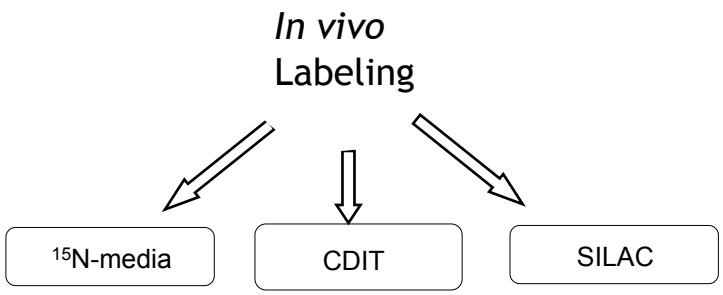
- Mass spectrometry is not quantitative by itself
  - Peptide ionization efficiency is unpredictable
- Differential stable isotope labeling to create a specific mass tag
- Tag can be recognized by MS and provides basis for quantification

MS-based quantitative proteomics

- Mass tags can be introduced into proteins
  - Metabolically, chemical means, enzymatically, or synthetic peptide standards
- Accurate quantitation in MS can be achieved by use of stable isotope-labeled standards

Quantitative Proteomics: *In vivo* labeling methods

In vivo labeling



Stable isotope labeling methods

- Stable isotope tagging methods use isotopic nuclei
- ^{2}H, ^{13}C, ^{15}N, ^{18}O
- Determines relative expression level of proteins in two samples

^{15}N-labeling

- Yeast or bacterial cultures grown in two separate media, one containing ^{15}N
- Cells are pooled together, proteins extracted
- Quantification using MS

^{15}N-labeling: disadvantages

- Different proteins incorporate unequal amount of stable isotopes therefore, labeled and unlabeled peptides exhibit variable mass shift in MS spectra
- Mammalian systems poorly incorporate stable isotope
- Difficult and expensive

Culture Derived Isotope Tags (CDIT)

- Cells cultured in stable isotope-enriched medium mixed with tissue samples
  - serves as an internal standard
  - synthetic unlabelled peptides used to quantify corresponding proteins labelled with stable isotope
- Protein extracted & separated, digested proteins are analyzed by MS
- Ratio between two isotopic distributions determined by MS

Stable isotope labeling by amino acids in cell culture (SILAC)

SILAC: an introduction

- Metabolic labeling strategy which uses stable isotope labeled amino acids in growth medium
- It depends on cellular protein synthesis to incorporate stable isotope-containing amino acids into whole proteome
- E.g. arginine or lysine containing six ^{13}C atoms

SILAC: an introduction (2)

- SILAC is done by incorporating stable isotope-labeled amino acids, such as L-Arginine containing six ^{13}C, through natural protein turnover and cell growth
- Cells are cultured in two separate media, “light” medium with amino acids of natural isotope abundance while the “heavy” medium contains the SILAC amino acid of choice

SILAC: an introduction (3)

- Labeled analog of amino acid supplied to cells
- Incorporated into all newly synthesized proteins
- After number of cell divisions, each instance of particular amino acid replaced by its isotope labeled analogue
- Relative protein abundance obtained by intensity of MS signals between light and heavy peptides

SILAC Experiment

SILAC: work-flow

1. Preparation of SILAC labeling medium

2. Adaptation of cells: from DMEM to SILAC labeling media

3. Differential treatment applied to the SILAC cells

4. Cell lysis and protein estimation

5. MS analyses and quantitation

Ref: Ong and Mann (2006) Nat Protoc 1(6), 2650-60

SILAC Experiment Workflow

1. Preparation of SILAC labeling medium

Preparation of SILAC labeling medium

- Two widely used media:
  - Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640
  - Arg, Lys, Met removed from standard formulations

Light Medium

Naturally abundant isotopic forms of amino acids

Heavy Medium

Same medium lacking desired amino acids (e.g. Arg and Lys) and substituted as heavy isotopic forms

Stable isotope-labeled amino acids

L-Lysine: ($^{13}C_6$)
- $^{13}C_6$ L-Lysine is a stable isotope of $^{12}C_6$ L-Lysine
- It is 6 Da heavier than $^{12}C_6$ L-Lysine

L- Arginine: ($^{13}C_6$)
- $^{13}C_6$ L- Arginine is a stable isotope of $^{12}C_6$ L- Arginine
- It is 6 Da heavier than $^{12}C_6$ L- Arginine

Preparation of SILAC labeling medium (2)

- Prepare the medium by adding supplements (serum, antibiotics)
- 10% dialyzed fetal bovine serum
- 1% antibiotics (penicillin and streptomycin) and glutamine

SILAC Experiment Workflow

2. Adaptation of cells: from DMEM to SILAC labeling media

Adaptation of cells: from DMEM to SILAC labeling media

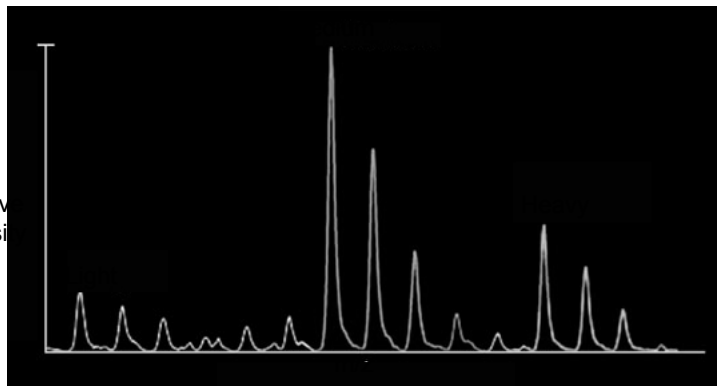
- Cells grown in DMEM medium formulation should be split into two culture dishes, containing light and heavy SILAC medium
- Take 10–15% of cells from the original culture and allow it for doubling
- Subculture cells in respective SILAC medium and allow it for at least five cell doublings

Adaptation of Cells: from DMEM to SILAC labeling media

- At the end of adaptation phase, lyse an aliquot of cells by adding 6M urea, 2M thiourea and extract protein
- Reduce disulfide bonds by adding 1mM DTT
- Alkylate cysteines by adding 5mM iodoacetamide
- Add trypsin (enzyme : substrate 1:100) and incubate at 37^0C for overnight
- Analyze the sample by LC–MS
- Check for full incorporation of SILAC amino acid

SILAC adaptations

Relativ
Intensi



Ref: Harsha et al. Nature Protocols 3, 505 - 516 (2008)

SILAC experiment workflow

3. Differential treatment applied to the SILAC cells

Differential treatment applied to the SILAC cells

- After complete incorporation of SILAC labels, differential treatment can be applied
- Treatments e.g. an external stimuli exposure, drug, growth factor, immunoprecipitation, comparison of differentiated and undifferentiated cells
- A reverse labeling should be performed to check for reproducibility in fold change

Check Arg-to-Pro conversion

- Manually adjust experimental conditions
- Reduce Arg concentration or Add Pro to medium
- Software designs can be used that count for conversion

SILAC Experiment Workflow

4. Cell lysis and protein estimation

Cell lysis and protein estimation

- Cell harvesting can be performed using any standard protocol in tissue culture
- Obtain a small aliquot of each cell lysate and estimate protein concentration
- After protein concentration determination, protein concentration should be normalized in “heavy” and “light” lysates

SILAC Experiment Workflow

5. MS analyses and quantitation

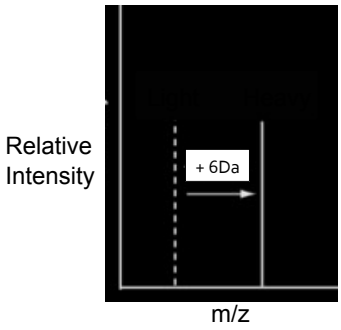
MS analyses and quantitation

- DTT, IAA and trypsin treatment should be performed
- Combine digested mixture and desalt peptides through C^{18} column
- Using raw MS data files, extract sequence-specific MS/MS peak list
- Identify peptides and proteins using database search software

Quantitation and abundance ratio

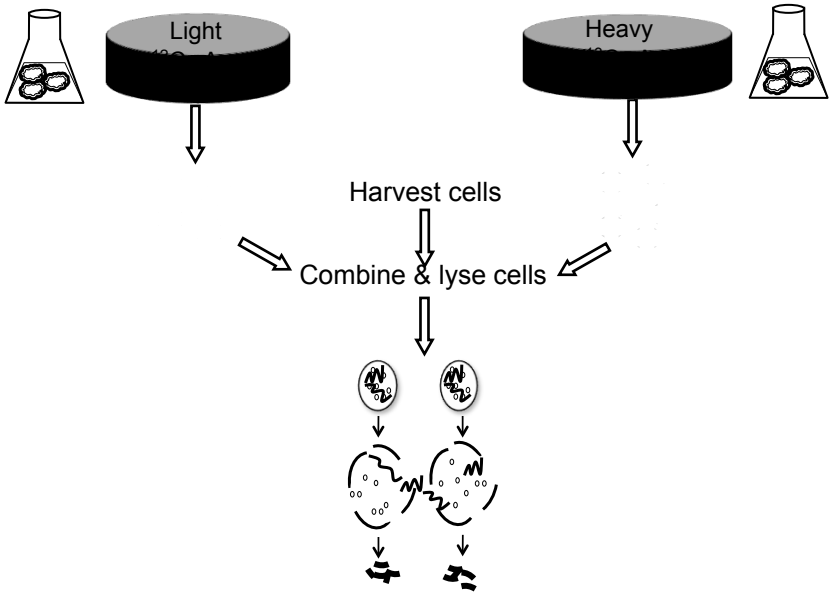
- Quantitation of SILAC-labeled peptide pairs – light and heavy peptide pairs
- Peptides containing heavy Arg are heavier than normal ones (6Da)
- Fold abundance ratio determine in two ways:
  - Ratio of intensities of each peptide from individual MS spectrum
  - The ion chromatograms of “light” and “heavy” peptides eluted from reversed-phase column, and then determining ratio of areas under curves

SILAC MS data

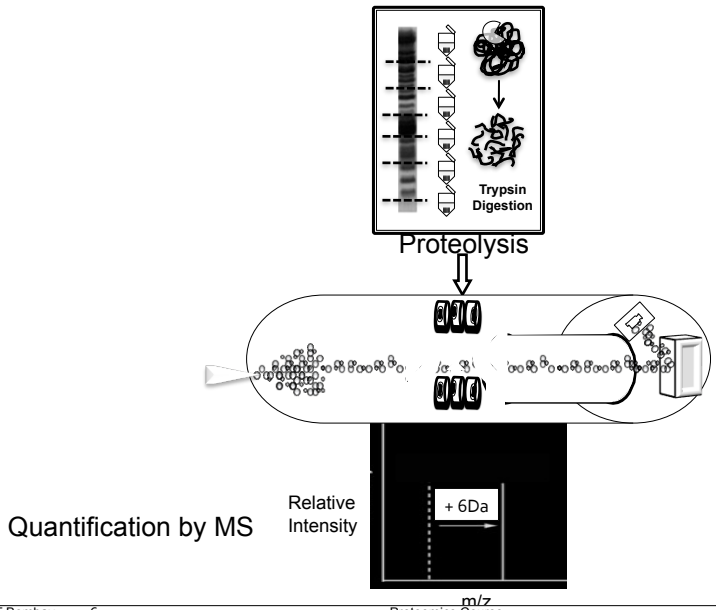


Mass separation between the pair is 6 Da

SILAC: An overview of protocol



SILAC: An overview of protocol



Quantification by MS

Stable isotope labeling by amino acids in cell culture (SILAC):
Animations

SILAC: Advantages, disadvantages
Comparison with other methods

SILAC advantages

- No chemical difference between labeled and natural amino acid isotopes
- Cells behave exactly like control cell population grown in presence of normal amino acid
- Efficient and reproducible
- Incorporation of isotope label is 100%

SILAC advantages (2)

- Samples mixed before processing, sample handling error minimized
- It can compare up to 5 states in one experiment
- Applications for quantification of each protein

SILAC disadvantages

- SILAC only applicable for cultured cells
- It can not be used for tissue and body fluid
- Culture process is time consuming
- Limited heavy forms of amino acids available
- Limited number of cellular states can be directly compared

SILAC disadvantages (2)

- Metabolic conversion of Arginine to Proline results in tryptic peptides containing heavy Proline
- Experimental or bioinformatics solutions can be used to minimize interference from incompletely labeled peptides

Labeling: SILAC vs. Radioactivity

- SILAC seeks to replace the labeled amino acid
  - Unlike, radioactive labeling that uses ^{32}P or ^{35}S-Met
- SILAC ensures full incorporation of labeled amino acid; however, small percentage of unlabeled amino acid can be detected
  - Radioactivity, full incorporation is not necessary
- SILAC uses MS readout
  - radioactivity detection with scintillation counters

SILAC Applications

SILAC Applications

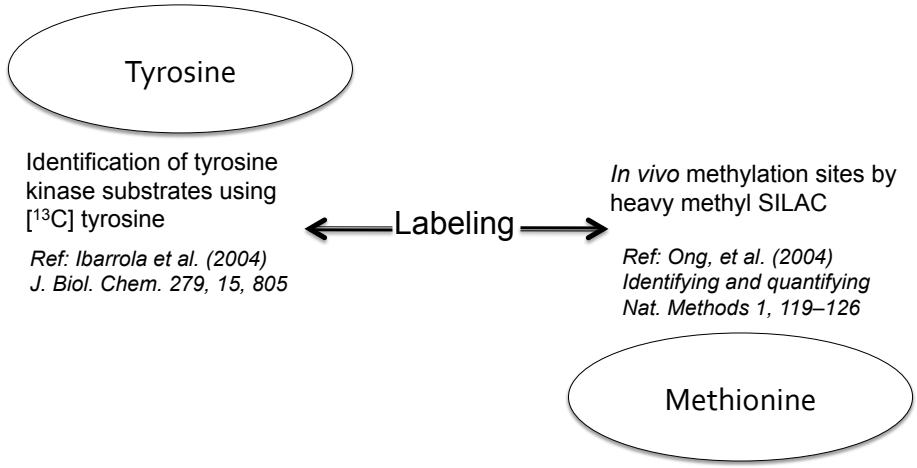
- Any cell lines can be used for SILAC analysis
  - HeLa, C127, HEK293 etc.
  - Media formulation and growth optimization required for each cell line
- Cell signaling
- Study induced protein complex
- Temporal dynamics
- Identification of kinase substrate
- Differential membrane proteomics

SILAC: applications

Relative quantitation of changes in protein expression during the time course of myoblast differentiation in mouse C2C12 cells

- SILAC application was first demonstrated in this study
- *Ref: Ong et al. (2002) Mol. Cell Proteomics 1, 376–386.*

SILAC: unique metabolic-labeling strategies



SILAC: global protein profiling

Global protein expression profiling

- Analyzed expression levels of > 440 proteins in microsomal fractions of prostate cancer cells with varying metastatic potential
- *Ref: Everley et al. (2004) Mol. Cell Proteomics 3, 729–735.*
- Investigated early stage of apoptosis by inducing the p53 up-regulated modulator of apoptosis
- *Ref: Gu et al. (2004) J. Proteome Res. 3, 1191–1200.*

SILAC: protein-protein interactions

Functional assays to study protein–protein interaction

- Differential labeling of proteins in EGF-stimulated versus unstimulated cells
- *Ref: Blagoev et al. (2003). Nat. Biotechnol. 21, 315–318*
- Quantification of proteins interacting in an attachment-dependent manner with focal adhesion proteins
- *Ref: de Hoog, C. L., Foster, L. J., and Mann, M. (2004) Cell 117, 649–662*

SILAC: functional analysis

Identification of proteins enriched in specific cellular structures

- First functional proteomic analysis of rafts
- Specific detection of proteins depleted from rafts by cholesterol-disrupting drugs
- *Ref: Foster, et al. (2003). Proc. Natl. Acad. Sci. USA 100, 5813–5818.*

SILAC: comparison of cellular state

Multiplexed analysis to compare cellular states

- Quantitative analysis of the proteome of human nucleoli
- *Ref: Andersen et al. (2005). Nature 433, 77–83*
- Temporal analysis of phosphotyrosine-dependent signaling networks to compare proteome of three cell populations
- *Ref: Blagoev et al. (2004). Nat. Biotechnol. 22, 1139–1145*
- Analysis of divergent growth factors in mesenchymal stem cell differentiation
- *Ref: Kratchmarova et al. (2005). Science 308, 1472–1477*

SILAC: protein turnover study

Studying protein turnover

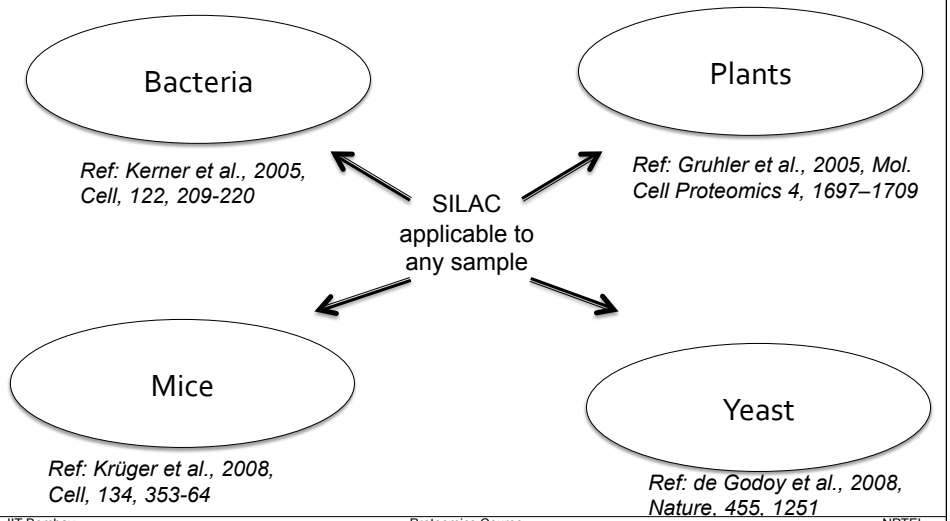
- Rate of breakdown of individual proteins by analysis of mass shifts in tryptic fragments
- Analysis of abundant proteins in glucose-limited yeast cells grown in aerobic chemostat culture at steady state
- *Ref: Pratt et al. (2002). Mol. Cell Proteomics 1, 579–591*

SILAC: Posttranslational modifications

Identification and
quantitation of protein posttranslational modifications

- Identification and quantitation of phosphorylation sites
- *Ref: Ibarrola et al. (2003) Anal. Chem. 75, 6043–6049*
- Phosphorylation profiling of the ERK/p90 ribosomal S6 kinase-signaling
- *Ref: Ballif, et al. (2005) Proc. Natl. Acad. Sci. USA 102, 667–672*

SILAC: success in different organisms



SILAC applications
Animations

Summary

- Quantitative proteomics, *In vivo* labeling
- SILAC experiment & work-flow
- SILAC merits and demerits

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