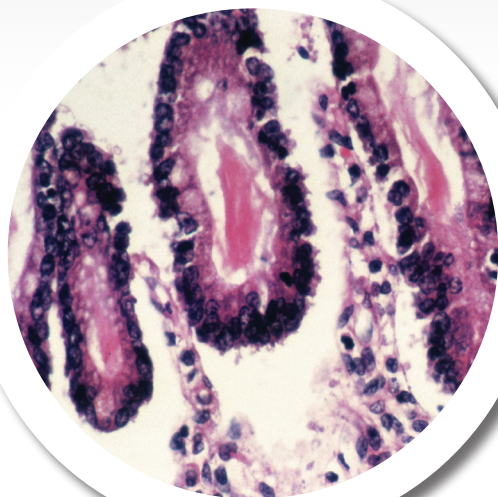




# Stable Isotope Labeling in Mammals with $^{15}\text{N}$ Spirulina

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Quantitative mass spectrometry has emerged as a powerful tool for biological research. Quantitative mass spectrometry typically utilizes proteins labeled with heavy stable isotopes, e.g.  $^{15}\text{N}$ ,  $^{18}\text{O}$ , or  $^{13}\text{C}$ . Labeled or “heavy” peptides maintain the same chemical characteristics as unlabeled or “light” peptides and co-elute into the mass spectrometer from liquid chromatography columns. In the mass spectrometer they are easily distinguished by their mass. Algorithms are then used to extract the light and heavy peptide ion chromatograms, which represent the peptide’s abundance. The light/heavy ratios are used to infer relative protein abundance. By mixing the same labeled protein standard with different unlabeled protein samples, changes in relative abundance can be determined between biological conditions.

Stable isotopes can be incorporated into peptides *in vitro* or *in vivo*. There are numerous covalent tags, such as ITRAQ, that react with specific amino acid side chains *in vitro*. A potential pitfall of these *in vitro* labeling techniques is the light and heavy samples are mixed after sample preparation and can introduce systematic errors in the quantitative analysis. Alternatively, metabolic labeling uses the cell’s own translational machinery to incorporate heavy isotopes into the entire proteome. Metabolic labeling allows for the light and heavy samples to be mixed prior to any sample preparation. Metabolic labeling is routinely performed in biological systems, such as bacteria, yeast, or mammalian cell culture, that grow rapidly and the nutritional source is easily manipulated.

To study animal models of disease, the technique Stable Isotope Labeling in Mammals (SILAM) was developed to introduce  $^{15}\text{N}$  comprehensively into an entire rodent. In the following, we discuss the rodent labeling process, experimental design, data analysis, and applications of SILAM.

## $^{15}\text{N}$ LABELING OF MAMMALIAN TISSUES

Both rats and mice have been successfully labeled with a diet containing spirulina (blue-green algae) enriched 98% with  $^{15}\text{N}$  from Cambridge Isotopes. Briefly, the dried  $^{15}\text{N}$  spirulina is mixed with a non-protein powder consisting of starch, sugar, essential vitamins

and other nutrients. This mixture is kneaded with water to form dough. The dough is then manually shaped into pellets in a similar size to normal rodent diet and dried in a food dehydrator. The only challenge with SILAM is that some tissues are difficult to label (low  $^{15}\text{N}$  enrichment <90%) and can result in less efficient and accurate quantitation of mass spectrometry data. Although the nitrogen source is the same for all tissues, the amino acid precursor pools are not. Tissues with slower protein turnover rates, such as brain and muscle, will take longer for the  $^{15}\text{N}$  labeled amino acids to equilibrate with the normal amino acid precursor pool. Initially, experiments were performed using a 1:3 (wt/wt) ratio of  $^{15}\text{N}$  spirulina and non-protein powder. In this study, a weaned rat was given this diet for six weeks. Many tissues were highly enriched (> 95%), but tissues with slow protein turnover were not. To obtain high enrichment of these tissues, the diet is fed to a weaned female rat during mating, pregnancy, and nursing of its pups. After weaning, the pups are given the  $^{15}\text{N}$  diet until postnatal day 45(p45). At this point, all the tissues have high  $^{15}\text{N}$  enrichment. Since mice are more prone to eating their young than rats, generational labeling should be avoided. We have achieved high  $^{15}\text{N}$  enrichment by increasing the  $^{15}\text{N}$  spirulina in the diet to a ratio of 1:2, and feeding the mice the  $^{15}\text{N}$  diet immediately after weaning for 10 weeks (unpublished data). Although not tested, the 1:2 ratio with the 10-week labeling time should also be sufficient for labeling rats. For a rat generational labeling experiment, 850g of  $^{15}\text{N}$  Spirulina (CIL Cat # NLM-8401) is required for a typical litter, and for a 10 week mouse labeling experiment, 100g of  $^{15}\text{N}$  Spirulina (CIL Cat # NLM-8401) is required for one mouse.

These  $^{15}\text{N}$  labeling protocols results in an average  $^{15}\text{N}$  enrichment of at least 95% in all tissues tested. The percent enrichment of the  $^{15}\text{N}$  tissues can be predicted from the isotopic distribution of the digested peptides using the algorithm Census.

## EXPERIMENTAL DESIGN

When designing SILAM experiments, it is important to remember the  $^{15}\text{N}$  tissues are used only as internal standards. To compare two samples, the  $^{15}\text{N}$  tissue is mixed with the two samples separately, and then the two  $^{14}\text{N}/^{15}\text{N}$  mixtures are analyzed. Since the  $^{15}\text{N}$  tissues are only used as internal standards, this analysis eliminates problems from any potential isotopic effects of the  $^{15}\text{N}$  (although we have not observed any adverse biological effects) and corrects for systematic errors that may occur in an experiment. Furthermore, with this experimental design, the  $^{15}\text{N}$  internal standard does not need to be identical to the unlabeled samples. For example, we examined differences between the nuclear phosphoproteome of liver and brain tissue using  $^{15}\text{N}$  labeled liver as the internal standard (**Figure 1**). In this study,  $^{15}\text{N}$  labeled liver homogenate was mixed 1:1 with  $^{14}\text{N}$  liver and  $^{14}\text{N}$  brain homogenates separately. The nuclei were isolated from the  $^{14}\text{N}/^{15}\text{N}$  mixtures and then, digested to peptides. The phosphopeptides were enriched using iron metal affinity chromatography (IMAC) and then, the resulting enriched samples were analyzed by Multidimensional Protein Identification Technology (MudPIT). The labeled and unlabeled peptides were

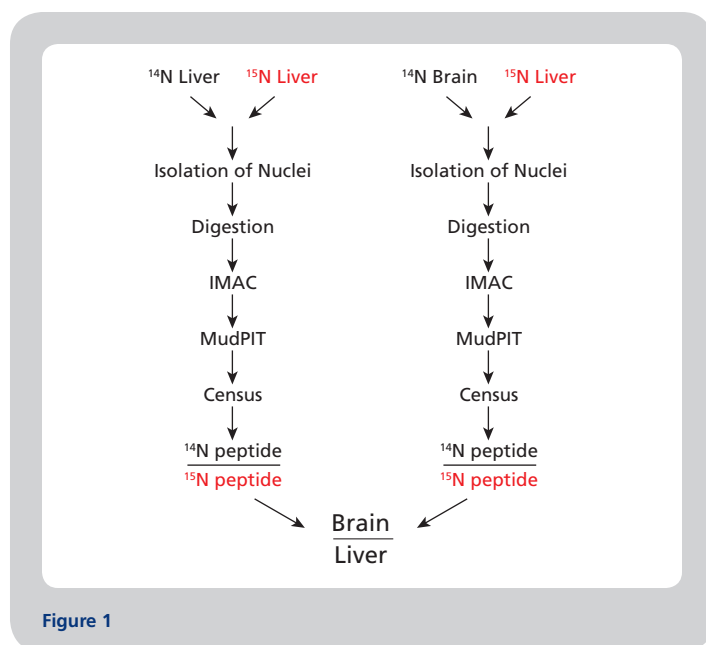


Figure 1

identified by SEQUEST, and quantitative data was calculated by using the Census software.

## DATA ANALYSIS

The  $^{14}\text{N}/^{15}\text{N}$  mass spectrometry data can be quantitated using the algorithm Census. An example of the Census output for the phosphorylated peptide, NLAKPGVTSTpSDSEEDDDQEGEK, from the aforementioned liver/brain study is shown in **Figure 2**. First, the elemental compositions and corresponding isotopic distributions for both the unlabeled and labeled peptides are calculated, and this information is then used to determine the appropriate  $m/z$  range for both the  $^{14}\text{N}$  and  $^{15}\text{N}$  precursor peptides from which to extract the ion chromatograms. Thus, only the identification of the  $^{14}\text{N}$  or the  $^{15}\text{N}$  peptide is necessary to generate a  $^{14}\text{N}/^{15}\text{N}$  ratio. Census then calculates the peptide ion intensity ratios for each pair of extracted  $^{14}\text{N}/^{15}\text{N}$  ion chromatograms by employing a linear least-squares correlation, which is used to calculate the ratio (i.e., slope of the line) and closeness of fit (i.e., correlation coefficient( $r$ )) between the data points of the  $^{14}\text{N}$  and  $^{15}\text{N}$  ion chromatograms. Census allows users to filter peptide ratio measurements based on the correlation coefficient (values between zero and one). It is recommended accepting only peptide ratios with correlation values greater than 0.5 for accurate quantitation. In addition, Census provides an automated method for detecting and removing statistical outliers using the Grubbs test.

Before comparing the  $^{14}\text{N}/^{15}\text{N}$  ratios between biological conditions, the data must be normalized. Since the  $^{14}\text{N}$  and  $^{15}\text{N}$  tissue samples are mixed at a 1:1 ratio, the median of all the  $^{14}\text{N}/^{15}\text{N}$  peptide ratios within an analysis should be one and if not it is assumed the deviation is due to human error, such as pipetting. Census is able to shift and thus correct the median  $^{14}\text{N}/^{15}\text{N}$  ratio in order to normalize the data.

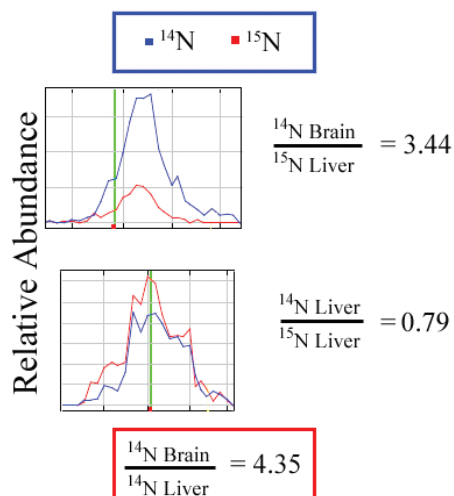


Figure 2

It is possible that the difference between a  $^{14}\text{N}$  and corresponding  $^{15}\text{N}$  peptide is larger than the dynamic range of the mass spectrometer, and this situation is termed a singleton peptide. As a consequence, a singleton peptide is penalized with a low  $r$  value and will be filtered out of the dataset. To address this limitation, Census uses a specific linear discriminant analysis to detect singleton peptides.

Census is available from the authors for individual use and evaluation through an Institutional Software Transfer Agreement (for details, see <http://fields.scripps.edu/census>).

### SILAM IN THE LITERATURE

In one report, isolated synapses were quantitated at four developmental time points. For this analysis, the  $^{14}\text{N}$  and  $^{15}\text{N}$  brain tissue homogenates were mixed prior to the isolation of the synapses. The labeled brain tissue from a p45 rat was employed to quantitate unlabeled brain tissue from rats at p1, p10, p20 and p45 highlighting that accurate and efficient quantitation can be obtained even when the labeled and unlabeled samples are not identical. In this study, the protein expression level of 1138 proteins in four developmental time points were quantitated, and 196 protein alterations were determined to be statistically significant. Over 50% of the developmental changes observed had been previously reported using other protein quantification techniques, and potential novel regulators of neurodevelopment were identified.

In a second report, the phosphoproteome was quantitated from isolated nuclei during brain development. Again, the  $^{14}\text{N}$  and  $^{15}\text{N}$  tissues were mixed prior to the isolation of the organelle. Although phosphorylation is a reversible and labile modification, a labeled p45 brain successfully quantitated the phosphoproteome at different developmental timepoints. Using IMAC to enrich phosphopeptides from the  $^{14}\text{N}/^{15}\text{N}$  mixtures, 705 and 1477 phosphopeptides were

quantitated from the p1 and p45 brains, respectively. It has been widely observed that different phosphorylation sites on the same protein can be differentially regulated. In this study, differential regulation of phosphorylation sites of methyl-CpG-binding protein 2 (MeCP2) were observed during development. Loss of function mutations in MeCP2 can cause Rett syndrome, a neurodevelopmental disorder, but cellular mechanisms linking the mutations to the phenotype are poorly understood. In a subsequent study, the differential regulation of the phosphorylation sites of MeCP2 identified by SILAM were demonstrated to be crucial for the regulation of transcription by MeCP2.

### RELATED PRODUCTS

NLM-8401	Spirulina (U- $^{15}\text{N}$ , 98%)
MLK-LYS-C	Mouse Feed Labeling Kit (includes 1 kg of L-Lysine $^{13}\text{C}_6$ Feed / 1kg of unlabeled feed)
CLM-2247-H	L-Lysine: 2HCL (U- $^{13}\text{C}_6$ , 99%)
CNLM-291-H	L-Lysine: 2HCL (U- $^{13}\text{C}_6$ , 99%; $^{15}\text{N}_2$ , 99%)
CM-2265-H	L-Arginine: HCL (U- $^{13}\text{C}_6$ , 99%)
CNLM-539-H	L-Arginine: HCL (U- $^{13}\text{C}_6$ , 99%; $^{15}\text{N}_4$ , 99%)
CLM-2262-H	L-Leucine (U- $^{13}\text{C}_6$ , 99%)
CNLM-281-H	L-Leucine (U- $^{13}\text{C}_6$ , 99%; $^{15}\text{N}$ , 99%)
CLM-2248-H	L-Isoleucine (U- $^{13}\text{C}_6$ , 99%)
CNLM-561-H	L-Isoleucine (U- $^{13}\text{C}_6$ , 99%; $^{15}\text{N}$ , 99%)

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