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Neutron-encoded mass signatures for multi-plexed proteome quantification

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Abstract

We describe a protein quantification method that exploits the subtle mass differences caused by neutron-binding energy variation in stable isotopes. These mass differences are synthetically encoded into amino acids and incorporated into yeast and mouse proteins with metabolic labeling; analysis with high mass resolution (>100,000) reveals the isotopologue-embedded peptide signals permitting quantification. We conclude neutron encoding will enable high levels of multi-plexing (> 10) with high dynamic range and accuracy.

Stable isotope incorporation with mass spectrometry (MS) analysis is the central technology for protein quantification.^{1,2} However the heavy isotopes are incorporated, metabolically^{3,4} or chemically,^{5–8} the aim is to differentially tag samples; the preferred spacing of ~ 4 Daltons (Da) limits isotopic cluster overlap. Unfortunately, this multi-Da spacing confines the quantitative capacity of stable isotope labeling by amino acids in cell culture (SILAC) to triplex comparisons for two reasons: (1) amino acid structures restrict the number of isotopes that can be added; and (2) spectral complexity increases as multiple isotopic clusters are introduced. Isobaric tagging provides up to 8-plexed analysis by concealing quantitative information in the MS1 scan and releasing it only upon tandem MS (MS/MS).^{9–11} It does, however, suffer from severe dynamic range compression and reduced

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Note: Supplementary information is available on the Nature Methods website.

AUTHOR CONTRIBUTIONS

A.S.H. and A.E.M. designed and performed research, analyzed data, and wrote the paper; D.J.B. performed theoretical calculations; M.S.W. analyzed data; E.R.S. designed research; D.J.P. and A.S. designed and prepared the murine model system; J.J.C. designed research and wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests.

Declaration: A.S.H. and J.J.C. are co-inventors on patent applications (US 13/660677), in part, related to the material presented here.

quantitative accuracy due to precursor interference.^{12,13} And, quantitative data can only be obtained for peptides that are selected for MS/MS—a serious problem during replicate analysis, particularly for protein post-translational modifications (PTMs), as there is high run-to-run variability in identifications (40–60%).¹⁴

A fortuitous discovery recently expanded the multi-plexing capacity of isobaric tandem mass tags (TMT) from six to eight: the concomitant swapping of a ^{12}C for a ^{13}C atom and a ^{15}N for a ^{14}N atom produces a new tag with a 6 mDa mass difference.^{10,11} This mass change results from the discrepancy in energetics of neutron binding between the isotopes, and can be distinguished with a mass resolution of 50,000 at m/z 130.¹⁵ This creative concept still relies upon MS/MS-based quantification, however, and does not resolve the accuracy and reproducibility issues of isobaric tagging. We reasoned that other elements, besides C and N, could encode neutron mass signatures. Indeed, mass defects can be induced with many elements and their isotopes, *e.g.*, $^{12}\text{C}/^{13}\text{C}$ (+3.3 mDa), $^1\text{H}/^2\text{H}$ (+6.3 mDa), $^{16}\text{O}/^{18}\text{O}$ (+4.2 mDa), $^{14}\text{N}/^{15}\text{N}$ (–3.0 mDa), and $^{32}\text{S}/^{34}\text{S}$ (–4.2 mDa), among others. We hypothesized that calculated incorporation of these isotopes into proteomes would generate a new MS1-centric quantification technology that combines the accuracy of SILAC with the multi-plexing capacity of isobaric tagging. We call this method neutron encoding (NeuCode).

A straightforward method of embedding neutron signatures is to use amino acids during protein synthesis (NeuCode SILAC). Consider a lysine molecule that is +8 Da; the 39 isotopologues of this amino acid span a mass range of 38.5 mDa, separated by ~ 1 mDa each (Fig. 1a). With infinite mass resolution, these 39 isotopologues would permit 39-plex NeuCode SILAC; not having access to infinite mass resolution, we calculated the minimum resolvable mass difference using current technology. With a library of 71,499 identified tandem mass spectra, we modeled the percentage of the peptidome that would be quantifiable (*i.e.*, separated at full width at 1% max, FWOM) when labeled at intervals of 12, 18, or 36 mDa, at resolutions ranging up to 10^6 (Fig. 1b). At a resolving power of 480,000, > 85% of identified peptides can be quantified (*i.e.*, resolved) when spaced 18 mDa apart. With 960,000 resolving power, achievable on both Fourier transform ion cyclotron resonance (FT-ICR) and Orbitrap MS systems, > 90% coverage with 12 mDa spacing could be achieved.^{16–18}

We tested NeuCode SILAC by growing yeast on normal “light” lysine (+0 Da) and on two +8 Da heavy lysine isotopologues: one with six ^{13}C atoms and two ^{15}N atoms (“heavy 1”, +8.0142 Da), and the other with eight ^2H (“heavy 2”, +8.0502 Da) (Supplementary Fig. 1). Peptides containing these lysine isotopologues differ in mass by 36 mDa, and are easily distinguished at resolving powers in excess of 200,000 (Fig. 1b and Supplementary Fig. 2). Traditional SILAC samples were prepared by combining the “light” and “heavy 1” labeled peptides in ratios of 1:1 and 1:5. NeuCode SILAC samples were similarly prepared, except with “heavy 1” and “heavy 2” labeled peptides. Samples from each method were loaded onto a capillary nano liquid chromatography (nLC) column and gradient-eluted into an ion trap-Orbitrap hybrid MS. For traditional SILAC, MS1 analyses were performed at a resolving power of 30,000, with the top 10 precursors selected for MS/MS analysis. For NeuCode SILAC, we implemented an additional 480,000 resolving power MS1 scan. The

high resolution spectrum distinguished the NeuCode SILAC pairs, decoding the embedded quantitative data. Consider a MS1 scan and the isotopic cluster of a selected precursor at mass-to-charge ratio (m/z) 827 (Fig. 1c). Here we plot the signal generated with either the typical 30,000 resolving power or the high resolution (480,000) quantification scan. The very close m/z spacing of the NeuCode SILAC partners is ideal for MS/MS scanning since both isotopologues are co-isolated, fragmented, and mass analyzed together to produce MS/MS spectra that are identical to non-multiplexed samples under normal resolution settings. Simply put, the encoded signatures are concealed, and spectral matching is unaffected. The high resolution scan does take ~1.6 seconds to complete, but, the system performs ion trap MS/MS analyses during that time, so that little effect on overhead is induced (16,974 vs. 18,074 MS/MS spectra, NeuCode SILAC vs. traditional SILAC).¹⁹ The NeuCode SILAC experiment produced considerably more unique peptide spectral matches (PSMs) than traditional SILAC: 3,078 vs. 2,401, respectively. In traditional SILAC, each peptide precursor appears at two distinct m/z values, causing a redundancy in peptide identifications and reduced sampling depth. NeuCode SILAC eliminates this problem because a single m/z peak encodes all quantitative information for that precursor, meaning redundant MS/MS scans on partner peaks are not acquired.

NeuCode SILAC posted 3,078 PSMs, 87% (2,693) of which were quantifiable (Fig. 2a and Supplementary Fig. 3). For traditional SILAC, 2,127 PSMs (89%) produced quantitative data. We conclude that NeuCode SILAC permits increased sampling depth, while maintaining comparable quantitative accuracy and precision. Multi-dimensional fractionation could ease this shortcoming of traditional SILAC. NeuCode SILAC peptide identifications were generated using the MS1 scans collected under low resolution settings (30,000, Fig. 1c). We plotted the distribution of mass error (parts per million, ppm) as a function of identification e-value (~significance) for both NeuCode SILAC and traditional SILAC for all identifications (1% false discovery rate (FDR), Supplementary Fig. 4). We note a very subtle decrease in mass accuracy for NeuCode SILAC, 3.5 vs. 2.5 ppm, but with comparable precision. This subtle increase in mass error stems from use of the low resolution (30K) MS1 scan for NeuCode, where the isotopologues are not resolved; however, it is not problematic, as database searching typically allows precursor mass error tolerances of ± 10 to ± 25 ppm. Using the mass values from the high resolution MS1 scan, where the isotopologues are resolved, completely eliminates this difference. Peptides bearing these lysine isotopologues have, on average, a 2.2 second chromatographic shift; however, our quantitative algorithm accommodates for this by detecting and quantifying MS1 pairs throughout a relatively wide retention time window (± 30 seconds). Further, the negligible effect of the chromatographic shift on performance is evinced by the accuracy of ratio calculations, which is similar to that of SILAC measurements.

Next, we benchmarked the NeuCode SILAC method against traditional SILAC by analyzing mouse C2C12 myoblasts and their differentiation to myotubes (Supplementary Fig. 5).^{3,20} NeuCode SILAC generated 5,747 quantifiable peptides, vs. 3,400 for traditional SILAC, translating to 45% more quantified proteins (1,458 vs. 1,031, Supplementary Table 1). NeuCode and traditional SILAC exhibit excellent correlation of measured protein

abundance (Fig. 2b). This observed correlation between the two methods is independent of label bias (Supplementary Fig. 6).

Here we report a fresh approach for protein quantification using stable isotopes. NeuCode exploits the subtle differences in neutron binding energy between isotopes.¹⁵ The approach effectively compresses isotopic information into a very narrow m/z space ($\sim 5\text{--}40$ mTh) so that it is easily concealed or revealed by varying mass resolution. Fourier transform (FT) MS systems offer ultra-high resolution ($> 1,000,000$) and will permit the use of NeuCode labeled peptides separated by as little as ~ 6 mDa.^{16–18} We envision synthesis of custom lysine isotopologues that offer four-plex quantification: $+8$ Da at 0, 12, 24, and 36 mDa spacings. Furthermore, such quad-plexed isotopologues could be generated with four, eight, or twelve additional neutrons. By combining these twelve isotopologues, NeuCode promises to facilitate 12-plex SILAC. Here, each peptide would be present in three isotopic clusters, just as in traditional three-plex SILAC; however, each cluster will reveal four distinct peaks upon high resolution analysis. We demonstrated this concept by combining duplex NeuCode SILAC with mass differential tags for relative and absolute quantification (mTRAQ) to achieve six-plexed MS1 quantification that retains the quantitative accuracy and precision of duplex NeuCode SILAC (Fig. 2c and Supplementary Fig. 7). Note that by using current commercial FT-MS mass resolution capability, NeuCode SILAC stands to easily deliver 9-plex quantification by easing lysine spacing to 18 mDa. Neither traditional SILAC nor NeuCode suffers from the pervasive problem of precursor interference that cripples quantitative accuracy in isobaric tagging, and, as mass resolution continues to improve, so will the multi-plexing capacity of NeuCode. Finally, it has not escaped our notice that these neutron mass signatures could be encoded in chemical tags for samples not amenable to metabolic labeling.

ONLINE METHODS

Theoretical calculations

First, a library of 71,499 yeast endoproteinase LysC derived peptides identified by mass spectrometry was composed. The theoretical full width at 1% max peak height (*FWOM*) for each library peptide across resolving powers (*R*) from 15 thousand to 1 million is calculated by:

$$FWOM = 2.57756788 \times \frac{m/z}{R \times \sqrt{\frac{400}{m/z}}}$$

where resolving power is defined as the minimum m/z difference that can be resolved at 400 m/z and the coefficient (2.57756788) is derived from Gaussian peak shape modeling. The m/z difference ($\Delta m/z$) for each theoretical isotope doublet assuming lysine isotopologue mass differences (ΔI) of 12, 18, and 36 mDa:

$$\Delta m/z = \frac{n \times \Delta I}{z}$$

where n is the number of lysines in the peptide sequence and z is the charge of the peptide. An isotopologue pair is only considered resolvable at the tested isotopologue mass difference and resolving power if $m/z > FWOM$.

Sample preparation

Saccharomyces cerevisiae strain BY4741 Lys1 was grown in defined, synthetic-complete (SC, Sunrise Science) drop out media supplemented with either “light” unlabeled L-lysine (+0 Da), “heavy 1” $^{13}\text{C}_6^{15}\text{N}_2$ -L-lysine (+8.0142 Da, Cambridge Isotopes), or “heavy 2” $^2\text{H}_8$ -L-lysine (+8.0502 Da, Cambridge Isotopes). Cells were allowed to propagate for a minimum of 10 doublings to ensure complete lysine incorporation. Upon reaching mid-log phase, the cells were harvested by centrifugation at $3,000 \times g$ for 3 minutes and washed three times with chilled ddH₂O. Cell pellets were re-suspended in 5mL lysis buffer (50mM Tris pH8, 8M urea, 75mM sodium chloride, 100mM sodium butyrate, 1mM sodium orthovanadate, protease and phosphatase inhibitor tablet), and total protein was extracted by glass bead milling (Retsch).

C2C12 cells were grown in DMEM lysine, arginine dropout culture media (Cambridge Isotopes) supplemented with 10% dialyzed FBS, antibiotics, 100 mg/L unlabeled L-arginine, and 100 mg/L of “light” unlabeled L-lysine, “heavy 1” $^{13}\text{C}_6^{15}\text{N}_2$ -L-lysine, or “heavy 2” $^2\text{H}_8$ -L-lysine for six passages. 1.3×10^6 cells from these plates were seeded onto fresh plates with the same media type and allowed to grow for two days. Cells grown with “light” and “heavy 2” lysine were harvested, and the cells grown with “heavy 1” lysine were re-fed with DMEM supplemented with 2% dialyzed FBS, then allowed to differentiate for five additional days before harvesting. Cells were pelleted, and washed with ice-cold PBS. The cell pellets were resuspended in 8 M urea, 50 mM Tris pH 8.0, 5 mM CaCl₂, and protease inhibitors (Roche). Cells were lysed by sonication. For the label flipping experiment, cells were grown exactly as above, except “light” - undifferentiated and “heavy 2” - differentiated cells were harvested were for the SILAC experiment and “heavy 1” - undifferentiated and “heavy 2” - differentiated cells were harvested for the NeuCode experiment.

Lysate protein concentration was measured by BCA (Pierce). Protein was reduced by addition of 5 mM dithiothreitol and incubation for 30 minutes at ambient temperature. Free thiols were alkylated by addition of 15 mM iodoacetamide and incubated in the dark, at ambient temperature, for 30 minutes, followed by quenching with 5 mM dithiothreitol. Urea concentration was diluted to 4 M with 50 mM Tris pH 8.0. Proteolytic digestion was performed by addition of LysC (Wako), 1:50 enzyme to protein ratio, and incubated at ambient temperature for 16 hours. The digest reaction was quenched by addition of TFA and desalted with a tC18 Sep-Pak (Waters).

SILAC yeast known ratios were prepared by mixing “light” = +0 Da and “heavy 1” = +8 Da labeled peptides in ratios 1:1 and 1:5 by mass. NeuCode SILAC ratios were prepared exactly the same, except “heavy 1” = +8.0142 Da and “heavy 2” = +8.0502 Da.

C2C12 mouse peptides were mixed 1:1 by mass. For the SILAC comparison, “light” peptides from myoblasts were combined with “heavy 1” peptides from myotubes. The

NeuCode SILAC comparison combined “heavy 1” myotube peptides with “heavy 2” myoblast peptides.

6-plex samples were prepared by labeling each NeuCode SILAC yeast peptide with three mTRAQ tags (AB SCIEX), according to the manufacturer’s protocol, except that hydroxylamine was added to quench the labeling reaction after 2 hours. These peptides were mixed in the ratio 10:10:5:5:1:1 by mass.

LC-MS/MS

Each sample was injected onto a 75 μm capillary packed with 30 cm of 5 μm Magic C18 (Michrome) particles in mobile phase A (0.2% formic acid in water). Peptides were gradient-eluted with mobile phase B (0.2% formic acid in acetonitrile). The known ratios and 6-plex experiments employed a 60 minute gradient, while the mouse experiment used a 300 minute gradient. Eluted peptides were analyzed by an Orbitrap Elite mass spectrometer (Thermo Scientific). A survey scan was performed by the Orbitrap at 30,000 resolving power to identify precursors to sample for data dependent top-10 ion trap CAD tandem mass spectrometry. NeuCode SILAC analysis had an additional quantitative 480,000 resolving power scan immediately following the survey scan. The 30,000 resolving power scan assigned precursor charge state more often than the 480,000 resolving power scan and thus was used to trigger MS/MS events. The traditional SILAC sample mixed in a 1:1 ratio was additionally analyzed using 60,000 or 120,000 resolution for survey scans; increased resolving power did not lead to significant improvements in unique peptide sequences identified, quantitative accuracy, nor precision. The 480,000 resolving power scan is available on Orbitrap Elite systems equipped with the developer’s kit. Preview mode was enabled, and precursors with unknown charge, or charge = +1, were excluded from MS/MS sampling. MS1 and MS/MS target ion accumulation values were set to 1×10^6 and 4×10^4 , respectively. Dynamic exclusion was set to 30 seconds for $-20.55\text{ }m/z$ and $+2.55\text{ }m/z$ of selected precursors.

Data analysis

MS raw files were converted to searchable text files and searched against a target-decoy²¹ database (*Saccharomyces* Genome Database (yeast), www.yeastgenome.org, February 3, 2011; UniProt (mouse), www.uniprot.org, October 1, 2011) using the Open Source Mass Spectrometry Search Algorithm (OMSSA).²² For all samples, methionine oxidation and cysteine carbamidomethylation were searched as variable and fixed modifications, respectively. SILAC samples were searched independently with an unmodified lysine and +8.014199 Da fixed modification, and later combined during false discovery rate filtering. NeuCode SILAC samples were searched with a single fixed modification representing the average mass increase of the $^{13}\text{C}_6^{15}\text{N}_2$ and $^2\text{H}_8$ isotopologues (+8.0322 Da) compared to unmodified lysine. Precursor mass tolerance was defined as 100 ppm and fragment ion mass tolerance was set to 0.5 Da. This relatively wide precursor mass tolerance was used to account for the mass difference observed between isotopologues. Search results were filtered to 1% FDR based on E-values. Peptides were grouped into proteins and filtered to 1% FDR according to rules previously described.^{23,24}

Quantification

Following database searching, the FDR-filtered list of peptide-spectral matches was first utilized to calculate the systematic precursor mass error associated with the data set. After adjusting precursor masses for this error, every high-resolution MS1 scan within ± 30 seconds of all PSMs identifying a unique peptide sequence was inspected; this retention time window ensured that quantitative data was extracted throughout the entirety of a peptide's elution and accommodates any chromatographic shifts between isotopologues due to deuterium incorporation. In each MS1 scan (480,000 resolution for NeuCode; 30,000 resolution for traditional) quantitative pairs were isolated for the mono and first two isotopes of the isotopic cluster. If at least two peaks, with signal-to-noise greater than 3, were found within the specified tolerance (± 5 ppm for NeuCode SILAC; ± 10 ppm for SILAC), a SILAC pair was created. Any peaks below the noise level simply contributed a noise-based intensity to the appropriate missing channel. Peaks exhibiting possible peak coalescence, as determined by de-normalizing intensity by injection time, were excluded from quantification. The intensities for each channel were summed across the peptide's elution profile so that chromatographic shifts between isotopologues did not impair quantification. To eliminate the noise-capped peaks on the fringes of a peptide's elution profile compressing the quantitative ratio towards 1:1, peaks with intensities below $1/2e$ the maximum intensity were discarded (e refers to the mathematical constant); this peak filtering threshold is approximately the 1.75th standard deviation of the standard normal distribution (the assumed shape of a peptide's elution profile); the distribution area covered by ± 1.75 standard deviations is $> 90\%$. Peptides were required to have a minimum of 3 ratio-providing pairs (i.e., quantified across at least 3 MS1 scans) to be eligible for quantification. Protein quantification was accomplished by averaging the ratios of all corresponding peptides. The resulting protein ratios were normalized to a median fold-change around 1 to account for unequal mixing. This algorithm was utilized to quantify both traditional and NeuCode SILAC data sets.

To perform manual analysis of NeuCode SILAC peptides, zoom in on the precursor in the MS raw file, look for properly-spaced isotopologue peaks, and record their intensities. Ion chromatograms can be extracted by centering a 10 ppm window on the observed m/z (i.e., peak $m/z \pm 5$ ppm) and integrating the peak areas.

To adapt existing quantification algorithms to analyze NeuCode data, set the lysine label masses to +8.0142 Da and +8.0502 Da and search for peaks within a ± 5 ppm tolerance of theoretical precursor masses that have been adjusted for systematic error.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. NeuCode feasibility and scan sequence

(a) Theoretical mass calculations of the 39 isotopologues for a +8 Da lysine amino acid. Shown in solid black are the isotopologues used for the experiments presented here. (b) Theoretical calculation depicting the percentage of peptides that are resolved (FWOM) when spaced 12, 18, or 36 mDa for resolving powers 15 thousand to 1 million. (c) MS1 scan collected with 30,000 resolving power from an nLC-MS/MS analysis of yeast LysC peptides and inset of a selected precursor having m/z at 827 (black trace). The signal recorded in a subsequent high resolution MS1 scan (480,000 resolving power) is shown in red – only at this high resolution is the quantitative data revealed. Presented below the MS1 scan is an MS/MS spectrum following CAD and ion trap m/z analysis of the neutron encoded SILAC pair. The inset displays that the SILAC pair is concealed at typical resolution.

Figure 2. NeuCode SILAC quantitative results

(a) Boxplots showing the measured (box and whiskers) and true (dashed lines) values for both methods at mixing ratios of 1:1 and 1:5. Boxplots demarcate the median (stripe), the 25th to 75th percentile (interquartile range, box), 1.5 times the interquartile range (whiskers), and outliers (open circles) for both SILAC (black) and NeuCode SILAC (red). (b) NeuCode SILAC and SILAC demonstrate a strong correlation for quantifying protein changes during the myogenic differentiation of mouse-derived C2C12 myoblasts ($R^2 = 0.78$). (c) The

combination of NeuCode SILAC with mTRAQ labeling affords six channels of MS1-centric quantification.

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