Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics

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Accurate quantification of protein expression in biological systems is an increasingly important part of proteomics research. Incorporation of differential stable isotopes in samples for relative protein quantification has been widely used. Stable isotope incorporation at the peptide level using dimethyl labeling is a reliable, cost-effective and undemanding procedure that can be easily automated and applied in high-throughput proteomics experiments. Although alternative multiplex quantitative proteomics approaches introduce isotope labels at the organism level ('stable isotope labeling by amino acids in cell culture' (SILAC)) or enable the simultaneous analysis of eight samples (isobaric tagging for relative and absolute quantification (iTRAQ)), stable isotope dimethyl labeling is advantageous in that it uses inexpensive reagents and is applicable to virtually any sample. We describe in-solution, online and on-column protocols for stable isotope dimethyl labeling of sample amounts ranging from sub-micrograms to milligrams. The labeling steps take approximately 60–90 min, whereas the full protocol including digestion and (two-dimensional) liquid chromatography-mass spectrometry takes approximately 1.5–3 days to complete.

INTRODUCTION

Quantitative proteomics by differential isotopic labeling

The quantitative analysis of proteomes is an increasingly important aspect of MS-based proteomics. The most commonly used methods for comparing and accurately quantifying protein levels rely on the use of differential isotopic labeling[1]. Proteins or peptides from different samples are labeled using compounds with near identical chemical properties yet each containing a unique stable isotope composition resulting in different masses. This way, the different samples can be combined and still be distinguished in a single MS analysis. Protein quantification can be achieved by comparing their relative ion abundances[2]. Isotope incorporation can be performed at the protein or peptide level using, for example, ^{2}H, ^{13}C, ^{15}N or ^{18}O, as heavy isotopes[3].

Metabolic and chemical labeling are two common strategies used for introducing heavy isotopes into samples. Metabolic labeling of proteins is achieved by supplying heavy isotopes to an organism or cell culture in a way that they are exclusively incorporated into newly synthesized proteins[3]. A popular protocol for isotopic labeling of proteins in eukaryotic cells is SILAC, where cells are grown in culture media lacking essential (auxotrophic) amino acid(s)[4]. These amino acids are then supplied in either their natural form or in a stable isotope form to cause *in vivo* incorporation of the labeled amino acid(s). The differentially labeled samples are mixed and digested with a protease (most often trypsin) to obtain samples that can be readily analyzed by the mass spectrometer[5].

Alternatively, the stable isotopes can be introduced by chemical labeling at the protein or peptide level with isotopomeric tags[1]. This method is particularly suited for tissue samples derived from animals or humans where metabolic incorporation is difficult. A well-known example of labeling at the protein level is the isotope-coded affinity tag method, which derivatizes cysteine residues[6]. Labeling at the peptide level is often carried out by allowing the free amines in peptides to react with, for instance *N*-hydroxysuccinimide esters[7,8] that contain either natural or heavy stable isotopes. Isotope labeling of more than two samples with compounds containing unique masses (multiplex labeling) can be more advantageous as it allows for the comparison of multiple samples (e.g., conditions or time points) in a single experiment. Among others, combinations of multiplexing have been reported utilizing SILAC[9,10] and iTRAQ[11,12].

Stable isotope dimethyl labeling

Here, we describe a very straightforward, fast and inexpensive alternative multiplex quantitative proteomics method based on chemical labeling. Samples are digested with proteases such as trypsin. The derived peptides of the different samples are then labeled with isotopomeric dimethyl labels. The labeled samples are mixed and simultaneously analyzed by LC-MS whereby the mass difference of the dimethyl labels is used to compare the peptide abundance in the different samples. The protocols presented here use three differential stable isotopes and are based on a method described earlier by Hsu *et al.*[13] that was further optimized and automated[14,15]. In this method, all primary amines (the N terminus and the side chain of lysine residues) in a peptide mixture are converted to dimethylamines. The only exception is in the rare occurrence of an N-terminal proline. In this instance, a monomethylamine is formed[16]. By using combinations of several isotopomers of formaldehyde and cyanoborohydride, peptide triplets can be obtained that differ in mass by a minimum of 4 Da between the different samples[14]. Although other combinations of ^{13}C- and D-isotopomers of formaldehyde and cyanoborohydride can be used to obtain a larger number of different labels (see also **Table 1**), the resulting peptides would differ in mass by only 2 Da between labeled peptides[17]. This can lead to significant difficulties in the interpretation and especially quantification of the data obtained as the isotope envelopes of the differentially labeled tryptic peptides might overlap. Stable isotope dimethyl labeling has also

TABLE 1 | Selected applications of stable isotope dimethyl labeling.

Sample type	Dimethyl labels	Analysis	Reference
Immortalized E7 cells	2× CH_3, 2× CHD_2	LC-MS	13
E. coli	2× CH_3, 2× $^{13}CHD_2$	LC-MALDI, LC-MS	35,36
Human carcinoma cells	2× CH_3, 2× $^{13}CHD_2$	SCX, LC-MALDI	22
Bovine milk	2× CH_3, 2× $^{13}CHD_2$	MALDI	37
Hemoglobin	2× CH_3, 2× CH_2D, 2× CHD_2, 2× CD_3	LC-MS	17
Rat uteri	2× CH_3, 2× CHD_2	IMAC, MALDI, LC-MS	27
Rat brain	2× CH_3, 2× CD_3	SCX, avidin chromatography, LC-MS	38
Human urine	2× CH_3, 2× $^{13}CH_3$ or 2× CHD_2	HILIC, LC-MS	39
Mouse macrophage-like cells	2× CH_3, 2× CHD_2	LC-MS	40
Bovine retina	2× CH_3, 2× CHD_2	LC-MS	21
Murine erythro leukemia cells	2× CH_3, 2× CHD_2, 2× $^{13}CD_3$	HILIC, LC-MS	14
Rat skeletal muscle	2× CH_3, 2× CHD_2, 2× $^{13}CD_3$	SCX, LC-MS	14
Bovine proteasome	2× CH_3, 2× CHD_2 (online labeling)	Online labeling, LC-MS	15
Zebrafish embryos	2× CH_3, 2× CHD_2	SCX, online-TiO_2, LC-MS	20
Yeast exosome	2× CH_3, 2× CHD_2	Online-TiO_2, LC-MS	23
Rat testis and lung	2× CH_3, 2× CHD_2	SCX, LC-MS	41

HILIC, hydrophilic interaction chromatography; IMAC, immobilized metal affinity chromatography; LC, liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; SCX, strong cation exchange.

been performed on intact proteins[18,19], but this procedure places constraints on the choice of proteases as trypsin and Lys-C no longer cleave modified lysine residues.

The suitability of stable isotope dimethyl labeling for quantitative MS has been demonstrated in several studies (see **Table 1**). For example, it has been used to compare the phospho-proteome of wild-type zebrafish embryos and zebrafish embryos with morpholino-mediated knockdown of the Fyn/Yes kinases[20]. Enrichment of differentially labeled phosphopeptides by online TiO_2 chromatography enabled the detection and quantification of changes in protein phosphorylation of gastrulation cell movement regulators. Stable isotope dimethyl labeling was also used to determine proteome differences between different parts of the rod outer segment of photoreceptor cells in the bovine retina[21]. Differences detected with isotope labeling were in close agreement with those detected by traditional western blotting, whereas many more proteins could be quantified with the dimethylation approach. In another study, protein expression differences were quantified between E-cadherin deficient and transfected human carcinoma cells[22]. As expected, differences were found in the expression of proteins involved in cell–cell and cell–matrix adhesion and some were validated by immunoblotting and immunofluorescence assays. The method has furthermore been used to unravel differences in constituency between highly related protein complexes, such as tissue-specific bovine proteasomes and the yeast nuclear and cytoplasmic exosome protein complex[15,23].

In **Table 2**, the advantages and disadvantages of stable isotope dimethyl labeling are weighed against multiplexed SILAC and iTRAQ. iTRAQ is now capable of simultaneously analyzing eight samples without increasing MS complexity[12] and should therefore decrease the time needed for LC-MS analysis. However, the method is limited to mass spectrometers capable of efficiently detecting ions

TABLE 2 | Comparison of multiplexed stable isotope dimethyl labeling with iTRAQ and SILAC.

	Strengths	Weaknesses
Stable isotope dimethyl labeling	Cheap reagents Quick reaction Can be automated by performing reaction online with LC-MS Applicable to any sample (animal/human tissue samples) Capable of labeling of sub-micrograms to milligrams of sample	Introduction of isotope label at peptide level Small isotope effect in LC separation
iTRAQ	Labeling of up to eight different samples No increased complexity at MS level Applicable to any sample (animal/human tissue samples)	Introduction of isotope label at peptide level Reagents are chemically not very stable Expensive reagent Choice of mass spectrometer is limited to those capable of measuring at low *m/z* Peptide quantification is based on a single tandem mass spectrum
SILAC	Introduction of isotope label at the cell or organism level No limits to amount of sample to be labeled	Not applicable to human samples Expensive to culture and reach full incorporation Labeled arginine might convert to proline

LC, liquid chromatography; MS, mass spectrometry.

that are present at a relatively low *m/z* and peptide quantification is based on a single fragmentation mass spectrum. SILAC has the advantage of introducing isotope labels as early as at the organism level and does not pose limits to the amount of sample to be labeled. Nevertheless, SILAC is limited in sample applicability as, for example, human tissue cannot be SILAC labeled and the method may be hampered by *in vitro* conversion of labeled arginine to proline[24]. Stable isotope dimethyl labeling is advantageous in that it can be used to label virtually any biological sample, with no restriction as to the choice of protease, whereas using inexpensive reagents. We have calculated that a triplex stable isotope labeling experiment of three times 25 µg of sample costs less than €0.10 for the labeling reagents. This, and the fact that the labeling reaction is quick and the reagents relatively stable, gives freedom to automate the method by performing the reaction online with LC-MS and/or to scale up to milligram amounts of sample. On the downside, stable isotope dimethyl labeling is performed in one of the final steps of a proteomics procedure and thus is more prone to variation introduced in the earlier steps of sample preparation.

Experimental design

Stable isotope dimethyl labeling is based on the reaction of peptide primary amines with formaldehyde to generate a Schiff base that is rapidly reduced by the addition of cyanoborohydride to the mixture. These reactions occur optimally between pH 5 and 8.5. As can be seen in **Figure 1**, the combination of regular formaldehyde and cyanoborohydride generates a mass increase of 28 Da per primary amine on a peptide (light label). Using deuterated formaldehyde generates a mass increase of 32 Da per primary amine (intermediate label)[13]. The third label with a mass increase of 36 Da can be achieved through combining deuterated and ^{13}C-labeled formaldehyde with cyanoborodeuteride (heavy label)[14]. These stable isotope dimethyl labels can be employed to differentially label three different samples to allow simultaneous quantitative LC-MS analysis. After digestion of the samples, peptides of the different samples are separately labeled with either of the dimethyl isotopomers. The labeled samples can then be mixed as the different isotopes do not affect the behavior of the labeled peptides in LC-MS. In MS, the different stable isotopically labeled peptides can be recognized by the known mass difference between them. Finally, quantification can be performed by comparing the signal intensity of the differentially labeled peptides.

As the stable isotope dimethyl labeling is performed at the peptide level, the method is not subject to restrictions on the origin of the biological sample. Once a protein sample is digested to peptides it can be dimethyl labeled and quantified by LC-MS. This includes not only cultured cells but also animal and human tissue samples. Stable isotope dimethyl labeling can be performed in up to 8 M urea and also after in-gel digestion. Adding an excess of labeling reagent is not a problem as no significant side reactions have been observed when labeling for up to 1 h.

As both the peptide N termini and lysine residues are labeled, the protocol is compatible with the peptide products of virtually any protease, e.g., trypsin, Lys-C, Lys-N, Arg-C and V8 (see ref. 14). Typically, for proteomics experiments the enzyme trypsin is used, which cleaves C-terminal of lysine and arginine residues[25]. When labeling tryptic peptides with the method described here, most peptides will differ in mass by either 4 Da (when cleaved after an arginine residue, only the N terminus is labeled) or

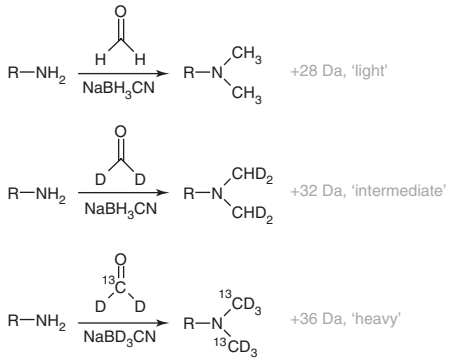

Figure 1 | Labeling schemes of triplex stable isotope dimethyl labeling. R: remainder of the peptide. Figure reprinted with permission from ref. 14.

8 Da (when cleaved after a lysine residue, both the N terminus and the lysine residue are labeled) between the light and intermediate and between the intermediate and heavy label. Differential labeling of peptides resulting from digestion with Lys-C or Lys-N (cleaving respectively C- and N-terminal of lysine residues) will result in mass differences of mainly 8 Da (both the N terminus and the lysine residue are labeled), whereas differentially labeling peptide products from Arg-C and V8 will result in varying mass differences as the number of lysine residues per peptides varies.

Here, we present three different protocols for dimethylation of peptide samples: in-solution, online and on-column. In **Figure 2**,

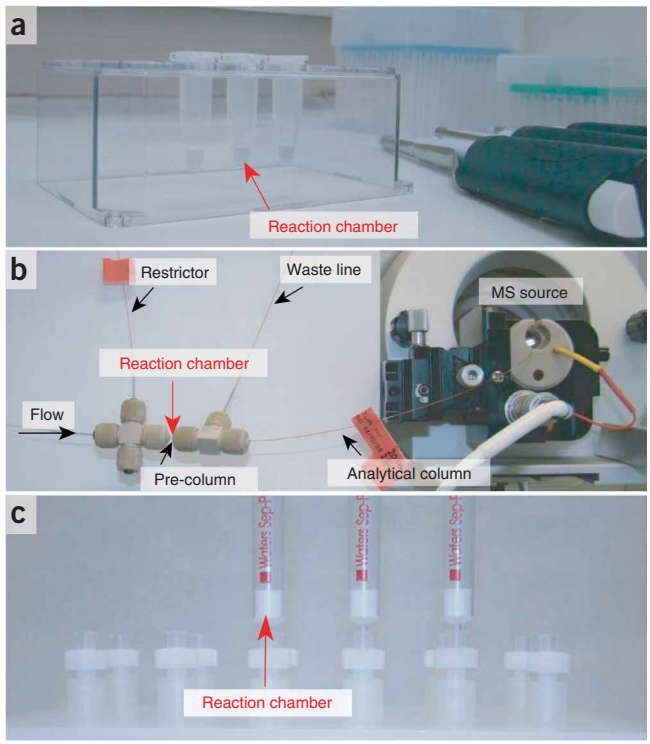

Figure 2 | Pictures of various experimental set-ups for stable isotope dimethyl labeling. Stable isotope dimethyl labeling can be performed (**a**) in-solution, (**b**) online with LC-MS or (**c**) on-column using SepPak columns.

the different experimental set-ups are shown and the labeling reaction chamber is indicated. In the first protocol, the peptides are labeled in-solution[13,14], a method that is easily scalable but might be prone to contaminating and interfering components remaining after sample preparation. These components might include primary amine-containing molecules such as ammonium bicarbonate or Tris. As formaldehyde will also react with these amines, these compounds affect the labeling efficiency. This can be circumvented by desalting the peptide sample before labeling or performing the digestion in buffers without primary amines (e.g., triethyl ammonium bicarbonate (TEAB)). The in-solution stable isotope dimethyl labeling protocol described here has been optimized for sample amounts of up to 25 μg. For larger amounts, the protocol can be scaled up by increasing the reagent volume and/or concentration. The second protocol is a largely automated procedure, where the labeling occurs on a C18 trapping column and is performed online with an LC-MS system allowing immediate MS analysis of the labeled samples[15]. All labeling reagents should be directed to waste rather than injected into the mass spectrometer, for example by using a vented column LC system with C18 trapping column[26]. As labeling reagents have a pH of around 8, the trapping column should be packed with material that is compatible with these pH conditions. In this protocol, Aqua C18 (Phenomenex) is used. Furthermore, the capacity of the (pre-)column should be significantly (>2 times) higher than the total amount of sample loaded to ensure no sample is lost. The online stable isotope labeling protocol has been optimized for sample amounts of up to 1 μg using a pre-column with a size of 100 μm × 20 mm and packed with 5 μm C18 material. The final protocol describes an adaptation of this method in which larger C18 solid phase extraction columns are used to perform the same solid phase labeling but for allowing larger quantities of peptides. The protocol has been optimized for sample amounts of 1–5 mg using a 200 mg, 3cc C18 SepPak column. For different amounts of peptide material, the protocol can be scaled up or down by proportionally adjusting reagent volume and/or concentration and using SepPak columns with a different capacity. The binding capacity of the SepPak column should be significantly (>2 times) higher than the total amount of sample loaded to prevent sample loss. In **Table 3**, the advantages and disadvantages for each of the three methods are listed. In-solution stable isotope dimethyl labeling is most suited for experiments where more than only a few samples have to be labeled, as labeling can be performed in parallel. Online stable isotope labeling is the optimal method for the labeling of small quantities (≪1 μg) of sample, because sample loss is diminished by combining sample clean-up and labeling and by performing LC-MS analysis directly after labeling. Finally, the on-column stable isotope labeling method is most suited for larger (up to milligrams) sample amounts, as sample clean-up and labeling steps are combined and the quenching step is avoided.

Complex samples that were differentially stable isotope dimethyl labeled by the in-solution or on-column protocol may be separated by a 2D LC approach[14]. This way, more peptides can be identified and quantified as the total MS time is increased and peptides are better resolved. The means of fractionation in the first dimension of the 2D-LC-MS method should preferably not be based on hydrophilicity/hydrophobicity. As the hydrophilicity of deuterium is slightly higher than hydrogen, a partial separation of differentially labeled peptides might occur. This might lead to a differential separation over consecutive fractions of differentially labeled peptides. Low-pH strong cation exchange (SCX) is an appropriate choice for 1D fractionation, as separation is based on peptide charge[14]. Also enrichment of phosphopeptides can be performed by TiO_2 (see ref. 20) or immobilized metal affinity chromatography (IMAC)[27] chromatography prior to LC-MS analysis.

To correctly determine the mass differences between stable isotopes, stable isotope dimethyl-labeled samples should be analyzed by a mass spectrometer that is capable of determining peptide precursor charge states and fully resolving all peaks. In practice, this means using high-resolution ($R > \sim 10{,}000$) instruments such as those based on time-of-flight, Fourier Transform Orbitrap or Fourier Transform Ion Cyclotron Resonance. In these instruments, the *m/z* of the labeled peptides is measured with this high resolution and peptide ions are selected for fragmentation (tandem MS or MS/MS) to determine the amino-acid sequence. After the LC-MS run, these tandem MS spectra are matched against theoretical spectra obtained from a protein sequence database using algorithms such as Mascot[28], SEQUEST[29], Phenyx[30] or X! Tandem[31]. Each of these algorithms is capable of identifying differentially stable isotope dimethyl-labeled peptides. An advantage of

TABLE 3 | Considerations for an informed selection of the appropriate stable isotope dimethyl labeling protocol.

(A) In-solution	(B) Online	(C) On-column
1 μg to milligrams of sample	Up to 1 μg of sample	Sub-micrograms to milligrams of sample
(+) Several samples can be labeled in parallel	(+) Automated	(+) Sample clean-up and labeling combined diminishes sample loss
(+) No SPE equipment required	(+) Analysis directly after labeling diminishes sample loss	(+) Quenching step is avoided
(+) No changes to LC-MS protocols	(+) Quenching step is avoided	(+) No changes to LC-MS protocols
(+) Labeling efficiency and sample amounts can be checked before mixing differentially labeled samples	(−) Requires slight adaptation of LC-MS protocols	(+) Labeling efficiency and sample amounts can be checked before mixing differentially labeled samples
(+) 2D-LC-MS possible	(−) Increases LC-MS run time	(+) 2D-LC-MS possible
(−) Multiple sample handling steps and clean-up, when required, might increase sample loss		(−) Requires SPE system and cartridges
(−) Is susceptible to interfering components with primary amines in the sample		(−) Time consuming when many samples are labeled
(−) Quenching step is exothermic reaction and might cause frothing or heating of the sample		

(+) indicates positive attribute of protocol; (−) indicates negative attribute of protocol.

Mascot is its special quantification modes in which the N-terminal and lysine dimethylation can be set as an interconnected modification (i.e., when the peptide is dimethyl labeled on a lysine residue, the N terminus is also expected to be labeled with the same isotopomeric dimethyl label and vice versa) to lower the number of false-positive identifications of peptides with different isotope labels. Quantification is achieved by comparing the intensities of the labeled peptides at MS level. Bioinformatically, stable isotope dimethylation is a relatively straightforward modification for quantitative proteomics. Quantification can be performed by integrating extracted ion chromatograms of the differentially labeled peptides. This can be carried out manually or, for larger data sets, by using commercial software packages such as Mascot Distiller and Spectrum Mill or open source software such as MSQuant (http://msquant.sourceforge.net/) and StatQuant (https://bioinformatics.chem.uu.nl/supplementary/StatQuant/).

MATERIALS

REAGENTS

- Acetic acid (Merck, cat. no. 1.00063)
- Acetonitrile (ACN) (Biosolve, cat. no. 75-05-8)
- Ammonia solution (25% (vol/vol), Merck, cat. no. 1.05432)
- Formaldehyde (CH_2O) (37% (vol/vol), Sigma, cat. no. 252549) ! CAUTION Formaldehyde solutions and formaldehyde vapors are toxic, prepare solutions in a fume hood.
- Formaldehyde (CD_2O) (20%, 98% D, Isotec, cat. no. 492620) ! CAUTION Formaldehyde solutions and formaldehyde vapors are toxic, prepare solutions in a fume hood.
- Formaldehyde ($^{13}CD_2O$) (20%, 99% ^{13}C, 98% D, Isotec, cat. no. 596388) ! CAUTION Formaldehyde solutions and formaldehyde vapors are toxic, prepare solutions in a fume hood.
- Formic acid (Merck, cat. no. 1.00264)
- Sodium cyanoborohydride ($NaBH_3CN$) (Fluka, cat. no. 71435)
- Sodium cyanoborodeuteride ($NaBD_3CN$) (96% D, Isotec, cat. no. 190020)
- Sodium dihydrogen phosphate (NaH_2PO_4) (Merck, cat. no. 1.06346)
- Di-sodium hydrogen phosphate (Na_2HPO_4) (Merck, cat. no. 1.06580)
- TEAB (Sigma, cat. no. T7408)

EQUIPMENT

- Bench top test tube mixer
- Centrifuge
- LTQ Orbitrap mass spectrometer (Thermo Fisher) equipped with a nanoHPLC system (Agilent)
- Milli-Q purification system (Millipore)
- SepPak C18 cartridges (Waters)
- Vacuum centrifuge
- Vortex
- Visiprep DL Vacuum manifold system (Supelco)

REAGENT SETUP

See **Table 4** for the correct isotope combinations of formaldehyde and cyanoborohydride to generate the light, intermediate and heavy dimethyl labels.

In-solution labeling reagents Per sample/label: 100 µl of 100 mM TEAB (pH ~8), 4 µl of 4% (vol/vol) formaldehyde (CH_2O, CD_2O or $^{13}CD_2O$), 4 µl of 0.6 M cyanoborohydride ($NaBH_3CN$ or $NaBD_3CN$), 16 µl of 1% (vol/vol) ammonia and 8 µl of 5% (vol/vol) formic acid in water. ! CAUTION Formaldehyde solutions and formaldehyde vapors are toxic, prepare solutions in a fume hood. ▲ CRITICAL Cyanoborohydride ($NaBH_3CN$ and $NaBD_3CN$) solutions should be kept at 4 °C and not stored longer than 24 h to ensure labeling efficiency[15].

Online labeling reagents Per sample/label: 500 µl of 50 mM sodium phosphate buffer pH 7.5 (prepared by mixing 1 ml of 50 mM NaH_2PO_4 with 3.5 ml of 50 mM Na_2HPO_4) is mixed with 5 µl of 4% (vol/vol) formaldehyde in water (CH_2O, CD_2O or $^{13}CD_2O$) and 5 µl of 0.6 M cyanoborohydride in water ($NaBH_3CN$ or $NaBD_3CN$). ! CAUTION Formaldehyde solutions and formaldehyde vapors are toxic, prepare solutions in a fume hood. ▲ CRITICAL Labeling reagent mixtures should be kept at 4 °C and not stored longer than 24 h to ensure labeling efficiency.

On-column labeling reagents Per sample/label: 4.5 ml of 50 mM sodium phosphate buffer pH 7.5 (prepared by mixing 1 ml of 50 mM NaH_2PO_4 with 3.5 ml of 50 mM Na_2HPO_4) is mixed with 250 µl of 4% (vol/vol) formaldehyde in water (CH_2O, CD_2O or $^{13}CD_2O$) and 250 µl of 0.6 M cyanoborohydride in water ($NaBH_3CN$ or $NaBD_3CN$). ! CAUTION Formaldehyde solutions and formaldehyde vapors are toxic, prepare solutions in a fume hood. ▲ CRITICAL Labeling reagent mixtures should be kept at 4 °C and not stored longer than 24 h to ensure labeling efficiency.

HPLC solvents Reversed phase (RP): solvent A, 0.6% (vol/vol) acetic acid; solvent B, 0.6% (vol/vol) acetic acid and 80% (vol/vol) ACN.

EQUIPMENT SETUP

LC-MS/MS equipment The Agilent 1100 HPLC system connected to the LTQ-Orbitrap mass spectrometer is equipped with a 100 µm × 20 mm Aqua C18 (Phenomenex) pre-column and a 50 µm × 250 mm Reprosil C18 (Dr Maisch) analytical column according to a vented column setup[26]. Trapping and all labeling steps are performed at 5 µl min^{-1}, whereas gradient elution is performed at ~100 nl min^{-1}.

PROCEDURE

Sample preparation ● TIMING ~ 1 d

1| Determine and adjust the protein amounts of the three samples to be differentially labeled by, for example, a Bradford assay[32] or using a 2D Quant Kit (Amersham Biosciences). The in-solution protocol is optimized for sample amounts of up to 25 µg per label, the online protocol is suited for sample amounts of <1 µg per label and the on-column protocol is suited for 1–5 mg of sample per label.

2| Perform protein digestion as described previously[25].

3| Dry the samples by vacuum centrifugation (1500 r.p.m., room temperature) for 30 min.

TABLE 4 | Mass shifts per modified moiety resulting from the combination of isotope-labeled formaldehyde and cyanoborohydride to create dimethyl labels.

Label	Light	Intermediate	Heavy
Mass increase per label	+28.0313 Da	+32.0564 Da	+36.0757 Da
Formaldehyde isotope	CH_2O	CD_2O	$^{13}CD_2O$
Cyanoborohydride isotope	$NaBH_3CN$	$NaBH_3CN$	$NaBD_3CN$

4| Prepare the labeling reagents as described in the reagent setup. See **Table 4** for the correct isotope combinations of formaldehyde and cyanoborohydride to generate the light, intermediate and heavy dimethyl labels.
▲ CRITICAL STEP When comparing only two samples, use the light and intermediate stable isotope dimethyl labels.

5| Perform one of the following stable isotope dimethyl labeling protocols: (A) in-solution, (B) online or (C) on-column stable isotope dimethyl labeling (**Fig. 2**). See **Table 3** for selecting the appropriate protocol.

(A) In-solution stable isotope dimethyl labeling ● TIMING ~**80 min**

(i) Reconstitute the digested samples (<25 µg) from Step 3 in 100 µl of 100 mM TEAB. The three samples are differentially isotope labeled in parallel in three different tubes.
▲ CRITICAL STEP The sample should be free of any primary amine-containing molecules that formaldehyde can react with, such as ammonium bicarbonate or Tris, other than the peptides to achieve maximal labeling efficiency.
▲ CRITICAL STEP The sample solution should have a pH between 5 and 8.5.

(ii) Add 4 µl of 4% (vol/vol) CH_2O, CD_2O or $^{13}CD_2O$ to the sample to be labeled with light, intermediate and heavy dimethyl, respectively (see **Table 4**).
! CAUTION Formaldehyde solutions and formaldehyde vapors are toxic, prepare solutions and perform Steps 5A(ii)–(ix) in a fume hood.

(iii) Mix briefly and spin the solution down.

(iv) Add 4 µl of 0.6 M $NaBH_3CN$ to the samples to be light and intermediate labeled and add 4 µl of 0.6 M $NaBD_3CN$ to the sample to be heavy labeled (see **Table 4**).

(v) Incubate in a fume hood for 1 h at room temperature (15–22 °C) while mixing using a bench top test tube mixer.
▲ CRITICAL STEP When performing the protocol for the first time or with a new sample, it is advised to check the labeling efficiency and sample amount by measuring a fraction of the sample by LC-MS before quenching the reaction and mixing the differentially labeled samples. When non-labeled peptides are found after performing a database search with these LC-MS results, Step 5A(ii)–(v) can be repeated.
? TROUBLESHOOTING

(vi) Quench the labeling reaction by adding 16 µl of 1% (vol/vol) ammonia solution.

(vii) Mix briefly and spin the solution down.

(viii) Add 8 µl of formic acid to further quench the reaction and to acidify the sample for consecutive LC-MS analysis.
! CAUTION The addition of ammonia solution and formic acid may result in the exothermic formation of small amounts of HCN and H_2. Perform Step 5A(vi–viii) in a fume hood and on ice to prevent frothing and/or heating of the sample.
? TROUBLESHOOTING

(ix) Mix the differentially labeled samples.

(x) Analyze the differentially labeled sample by (2D-)LC-MS[33] (continue with Step 6) or dry the sample by vacuum centrifugation as described in Step 3.
■ PAUSE POINT At this point the labeled sample can be stored at −20 °C for several weeks.

(B) Online stable isotope dimethyl labeling ● TIMING ~**30 min per label plus an LC-MS run**

(i) Reconstitute the three samples (<1 µg) prepared in Step 3 in 5 µl of 5% (vol/vol) formic acid.

(ii) Load the first sample onto the pre-column. All loading, flushing and washing steps are performed at 5 µl min^{-1}.
▲ CRITICAL STEP The RP pre-column packing material should be capable of withstanding pH conditions of up to pH 8.5. In this protocol, Aqua C18 (Phenomenex) is used.

(iii) Flush the pre-column with 40 µl of light labeling reagent.

(iv) Wash the pre-column with 5 µl of 5% (vol/vol) formic acid and 50 µl of RP solvent A.

(v) Load the second sample onto the pre-column.

(vi) Flush the pre-column with 40 µl of intermediate labeling reagent.

(vii) Wash the pre-column with 5 µl of 5% (vol/vol) formic acid and 50 µl of RP solvent A.

(viii) Load the third sample onto the pre-column.

(ix) Flush the pre-column with 40 µl of heavy labeling reagent.

(x) Wash the pre-column with 40 µl of 5% (vol/vol) formic acid.
▲ CRITICAL STEP When comparing only two samples omit Step 5B(vii)–(ix).

(xi) Switch the LC-MS system to the analytical position and start a gradient to perform regular nanoLC-MS analysis[15].
? TROUBLESHOOTING

(C) On-column stable isotope dimethyl labeling ● TIMING ~ **1 h**

(i) Reconstitute the samples (1–5 mg) from Step 3 in 1 ml of 5% formic acid.
! CAUTION Formaldehyde solutions and formaldehyde vapors are toxic, place the vacuum manifold in a fumehood.
▲ CRITICAL STEP The vacuum manifold solvent liners should be inert and disposable to prevent carry-over between experiments.

(ii) Wash three SepPak columns with 2 ml of ACN.

(iii) Condition the SepPak columns twice with 2 ml of RP solvent A.
(iv) Load each of the three samples on a separate SepPak column.
(v) Wash the SepPak columns with 2 ml of RP solvent A.
(vi) Flush each of the SepPak columns five times with 1 ml of the respective labeling reagent (light, intermediate or heavy).
▲ CRITICAL STEP To allow for complete labeling, make sure that Step 5C(vi) takes at least 10 min.
(vii) Wash the SepPak columns with 2 ml of RP solvent A.
(viii) Elute and collect the labeled samples from the SepPak columns with 500 µl of RP solvent B.
▲ CRITICAL STEP When performing the protocol for the first time or with a new sample, it is advised to check the labeling efficiency and sample amounts by measuring a fraction of the sample by LC-MS before mixing differentially labeled samples. When non-labeled peptides are found after performing a database search with these LC-MS results, the sample can be dried by vacuum centrifugation for 1 h as described in Step 3 and Step 5C(i)–(viii) can be repeated.
? TROUBLESHOOTING
(ix) Mix the differentially labeled samples.
(x) Analyze the differentially labeled sample by (2D-)LC-MS[33] (continue with Step 6) or dry the sample by vacuum centrifugation for 1 h as described in Step 3.
■ PAUSE POINT At this point, the labeled sample can be stored at −20 °C for several weeks.

LC-MS analysis ● TIMING ~1–30 h (depending on whether additional separation methods were applied)

6| Reconstitute the differentially labeled samples from Step 5A(x) or 5C(x) in 10 µl of RP solvent A.

7| Inject the sample onto the nanoLC-MS/MS system. See EQUIPMENT SETUP for details.

Data analysis ● TIMING 1 d to several weeks

8| Search the tandem mass spectra against an appropriate protein sequence database. Set the different dimethyl isotope labels as variable modifications on the peptide N termini and lysine residues. The monoisotopic mass increment of the light, intermediate and heavy dimethyl label is 28.0313, 32.0564 and 36.0757 Da, respectively.
? TROUBLESHOOTING

9| Carry out the relative quantification of differentially labeled peptides and statistical analysis.
▲ CRITICAL STEP Ensure that the whole peak area of the eluting peptide triplet is extracted to minimize quantification errors caused by isotope effects on retention time.

● TIMING
Steps 1–4, sample preparation: ~ 1 d
Step 5A, in-solution stable isotope dimethyl labeling: ~80 min, several samples can be labeled in parallel
Step 5B, online stable isotope dimethyl labeling: ~30 min per sample plus an LC-MS run time
Step 5C, on-column stable isotope dimethyl labeling: ~1 h
Steps 6–8, LC-MS analysis: approximately 1–30 h (depending on whether additional separation methods were applied)
Steps 9 and 10, data analysis: 1 d to several weeks, depending on the scale of the experiment and the software package used

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 5**.

TABLE 5 | Troubleshooting table.

Steps	Problem	Possible reason	Solution
5A(v), 5B(xi), 5C(viii) or 8	Some peptides are partially labeled. Particularly, the N terminus is not labeled	A contaminating primary amine in the solution quenches the reaction	Make sure the sample does not contain primary amines such as ammonium bicarbonate or Tris by desalting the sample solution[42] or performing the digestion in TEAB buffers[11]
		Not enough reagent	Increase the volume or concentration of the labeling reagents used

(continued)

TABLE 5 | Troubleshooting table (continued).

Steps	Problem	Possible reason	Solution
		Labeling/incubation time was too short	Make sure that the incubation (Step 5A(v)) in the in-solution protocol takes at least 30 min and in the on-column protocol the flushing with labeling reagent takes at least 10 min
		pH was not correct	Make sure that the pH of the reaction mixture is between 5 and 8.5
		Labeling reagent has expired	Cyanoborohydride solutions should be kept at 4 °C and not stored longer than 24 h to ensure labeling efficiency
5A(viii)	The quenching step in the in-solution labeling protocol results in extensive frothing	The concentrations of cyanoborohydride, ammonia and formic acid are too high	Perform the quenching step on ice with pre-chilled reagents or use the on-column protocol where the quenching step is not required
5B(xi)	Some peptides have different isotope labels after online dimethyl labeling	Partially labeled peptides are further labeled in the consecutive labeling step	Make sure that peptides are fully labeled before moving on to the next labeling step (see above)
	The input sample amounts for online labeling are 1:1:1, however, the output is not	The column is overloaded	Make sure that the capacity of the column is more than two times above the total amount of peptide loaded by decreasing the amount of sample or increasing the size of the column
	Polymers are visible in LC-MS chromatogram after online labeling	The LC system is not clean (cyanoborohydride can react with adventitious materials including polymers that are present in the LC system)	Perform a BSA or mock online labeling before running a real sample to remove polymers from the system. Small amounts of polymers will, however, not affect the analysis
		The sample contains polymers	Make sure to avoid polymer-based compounds such as detergents
5B(xi) or 8	The average ratio of all quantified peptides is not 1	The sample input was not 1:1:1	Adjust the amounts of sample when mixing differentially labeled samples or normalize the data to correct for the difference in the amount of sample input
		One of the samples was not fully labeled	Check the labeling efficiency after the labeling step (Step 5A(v) or 5C(viii)) and repeat the labeling procedure if a sample was not fully labeled
		A biological reason	When a significant percentage of lower abundant proteins is changed in a data set, the average ratio might be flawed. Check if higher abundant proteins that are expected to be unchanged (e.g., housekeeping proteins) have a ratio of 1

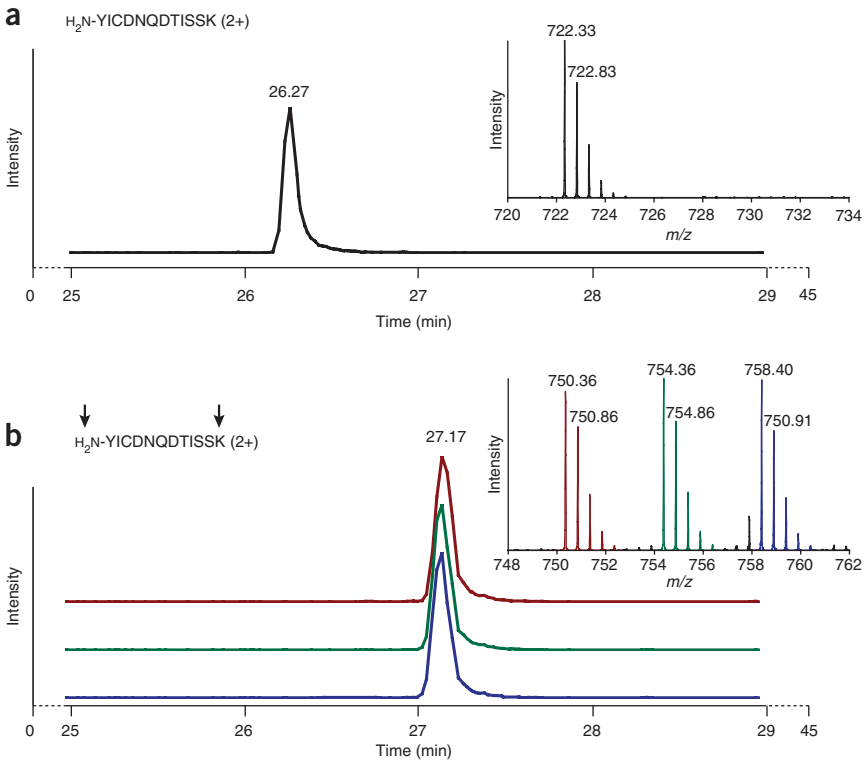

Figure 3 | Extracted ion chromatograms and mass spectra of the BSA peptide YICDNQDTISSK. Before (**a**) and after (**b**) triplex stable isotope dimethyl labeling. Light (red line), intermediate (green line) and heavy labeled (blue line) peptides shifted respectively by 28.03, 32.06 and 36.08 *m/z*, corresponding to 56.06, 64.11 and 72.15 Da as the peptide ion is doubly charged. Upon labeling of the peptide, its retention time on the C18 column is increased. Arrows indicate the sites of dimethylation, the insets show the recorded mass spectrum at the indicated retention times using the same color coding for the labeled peptides.

ANTICIPATED RESULTS

To illustrate the LC-MS outcome of stable isotope dimethyl labeling, a BSA digest was dimethyl labeled in a 1:1:1 light/intermediate/heavy ratio. **Figure 3** shows the extracted ion chromatograms and mass spectra of non-labeled and triplex differentially stable isotope dimethyl-labeled BSA peptide YICDNQDTISSK. An *m/z* shift compared with the non-labeled peptide (*m/z* 722.33) of, respectively, 28.03, 32.06 and 36.08 is found for the light, intermediate and heavy labeled peptide ions, respectively. As the peptide ion is doubly charged, these *m/z* shifts correspond to mass increments of respectively 56.06, 64.11 and 72.15 Da. This is consistent with the fact that the peptide has two sites available to be labeled, the N terminus and the lysine residue, as indicated by the arrows in **Figure 3b**. Complete labeling was obtained as no peaks at the *m/z* corresponding to unlabeled peptides could be observed in the dimethyl-labeled sample. Upon labeling of the peptide, a shift in retention time can also be observed. The addition of two dimethyl labels makes the peptide more hydrophobic, thereby increasing its retention on the C18 column (**Fig. 3b**). In some cases, depending on the LC setup and RP column used, a slight separation can be observed between the differentially labeled species of the same peptide. As deuterium is slightly more hydrophilic than hydrogen, the heavy and intermediate labeled peptides can elute slightly before the equivalent light labeled peptide. For this reason, quantification should be based on the integration of the entire extracted ion peaks of each of the three *m/z* values for a peptide triplet[1].

In **Figure 4**, typical MS results for four triplex stable isotope dimethyl-labeled peptides are shown to illustrate typical *m/z* shifts between differentially labeled peptides. The exact theoretical mass difference between the intermediate and light labels is 4.0251 Da and between heavy and intermediate is 4.0192 Da. As peptide LGEYGFQNALIVR has only one site that can be dimethyl labeled, the 2+ and 3+ peptide ion *m/z* differences are 2.01 and 1.34, respectively. Peptide TCVADESHAGCEK is dimethyl labeled on both the N terminus and the lysine

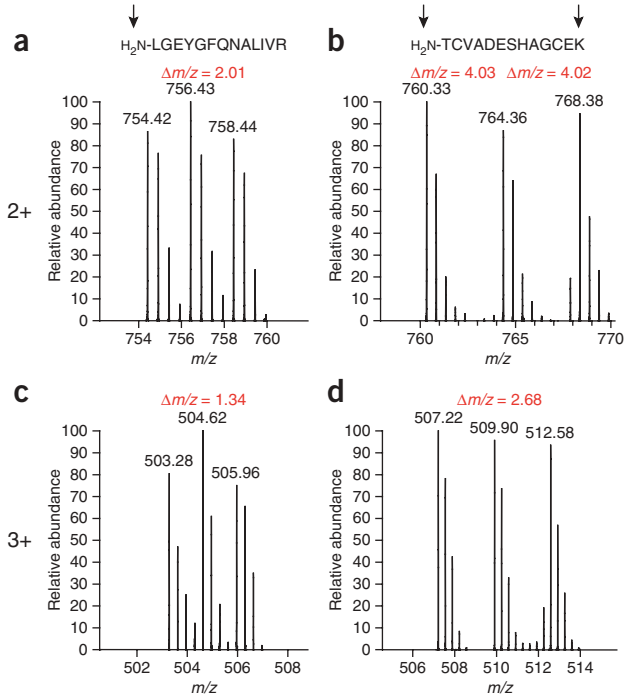

Figure 4 | MS triplets of doubly and triply charged triplex stable isotope dimethyl-labeled BSA peptides LGEYGFQNALIVR and TCVADESHAGCEK. Peptide LGEYGFQNALIVR is dimethyl labeled on one site showing *m/z* differences of, respectively, 2.01 and 1.34 for the (**a**) doubly and (**c**) triply charged peptide ion. Peptide TCVADESHAGCEK is dimethyl labeled on two sites showing *m/z* differences of 4.03 (and 4.02) and 2.68 for, respectively, the (**b**) doubly and (**d**) triply charged peptide ions. Arrows indicate the sites of dimethylation.

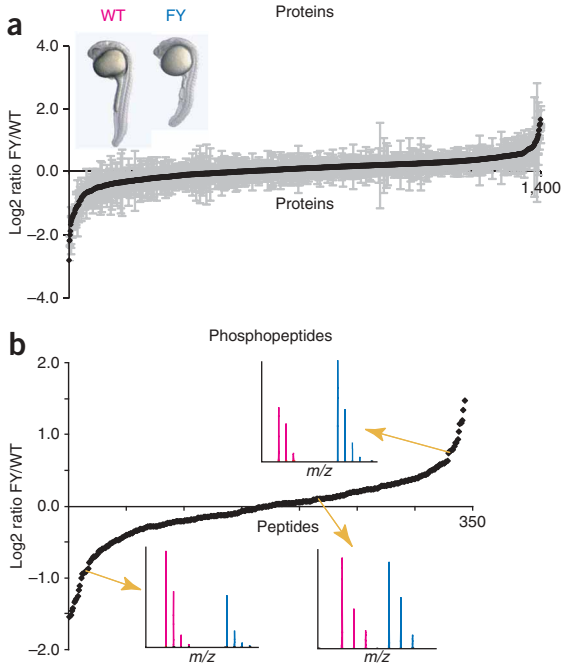

Figure 5 | Differential protein expression and phosphorylation after morpholino-mediated Fyn/Yes knockdown in zebrafish embryos. Around 1,400 proteins and 350 specific phosphosites were identified and quantified using duplex stable isotope dimethyl labeling. Protein expression ratios in Log2 (± standard deviation) of proteins, based on the FY/WT ratio of at least two different peptides (**a**). Abundance ratios of phosphopeptides enriched by TiO_2 (**b**). The insets display mass spectra of indicated differentially labeled peptides with the isotope envelope of the light dimethyl-labeled peptide in pink (FY) and the intermediate labeled peptide in blue (WT). WT: wild type; FY: Fyn/Yes knockdown. Adapted from Lemeer *et al.*[20].

residue. The *m/z* of the intermediate-labeled 2+ peptide ion is therefore 4.03 higher than the light-labeled peptide ion and the *m/z* of the heavy-labeled 2+ peptide ion is another 4.02 higher. The *m/z* difference between the 3+ peptide ions is 2.68.

In **Figure 5**, the results of a moderate scale (phospho)proteomics study using duplex stable isotope dimethyl labeling are shown[20]. The role of Fyn and Yes kinases in the embryonic development of zebrafish was studied by comparing 30 wild-type- and 30 morpholino-mediated Fyn/Yes knockdown zebrafish embryos (corresponding to ~3 µg protein per embryo). After cell lysis and protease digestion, the peptides were differentially stable isotope dimethyl labeled, mixed and separated by SCX followed by online TiO_2-LC-MS/MS (LTQ-Orbitrap)[20,34]. In total, 1,400 proteins were identified with at least two peptides (**Fig. 5a**). Observed protein expression differences ranged between seven times lower and three times higher abundance in the Fyn/Yes knockdown embryo, whereas the expression of the majority of proteins was unchanged. Some proteins that are known to be involved in gastrulation cell movements were found to be differentially expressed. As knockdown of the kinases Fyn and Yes likely affects the *in vivo* phosphorylation state of the zebrafish proteome, online TiO_2-enrichment of phosphopeptides was performed to allow the focus on differential phosphorylation. This way, phosphorylation differences were found on known and novel potential regulators of gastrulation cell movement (**Fig. 5b**). Stable isotope dimethyl labeling thus shows to be applicable in the analysis of low amounts of complex zebrafish embryo tissue and implementation of phosphopeptide enrichment with TiO_2 is feasible. In **Figure 5a**, some proteins show relatively large standard deviations. Often, in these instances differences were found in phosphopeptide abundance, whereas the total protein expression was unaltered, suggesting changes in *in vivo* phosphorylation state upon knockdown of Fyn and Yes. These outstanding phosphopeptides can explain the larger than average standard deviation as the protein ratio is biased toward phosphopeptides as a result of the phosphopeptide enrichment. In general, outliers may be an indication that changes have arisen on protein modification level rather than expression level. This example and others (see **Table 1**) clearly demonstrate that stable isotope dimethyl labeling is an outstanding, robust and cost-effective alternative method for quantitative (phospho-) proteomics.

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