An experimental correction for arginine-to-proline conversion artifacts in SILAC-based quantitative proteomics

To the editor: Stable isotope labeling by amino acids in cell culture (SILAC)[1] with heavy arginine and lysine is one of the most widely used methods for mass spectrometry–based quantitative proteomic analysis of cells[2]. But its accuracy is compromised by the metabolic conversion of arginine to proline in eukaryotes (**Supplementary Fig. 1** online). As a result, [$^{13}C_6$]-arginine and [$^{13}C_6$,$^{15}N_4$]-arginine become [$^{13}C_5$]-proline and [$^{13}C_5$,$^{15}N_1$]-proline, respectively. This results in the generation of multiple satellite peaks for all proline-containing tryptic peptides in the labeled state (**Supplementary Fig. 2** online), which hampers accurate quantitation[3]. A widely used simple solution is to empirically reduce the arginine concentration to minimize conversion to proline[4]; however, this is not applicable to every cell type as it affects cell behavior and reduces growth rates, especially of fast-growing cells[5] (**Supplementary Table 1** and **Supplementary Discussion** online). Alternative solutions include manual or mathematical corrections for all proline-containing peptides[6], adding the contribution from the heavy proline to the heavy arginine peak[4]. This, however, can substantially reduce accuracy, particularly for low-intensity ion signals, and is laborious for large data sets.

Here we describe a simple and effective adjustment of the existing SILAC method, which results in highly accurate quantitation and may be useful for cell types in which arginine starvation is not a viable option. The principle is based on using [$^{15}N_4$]-arginine in combination with normal lysine in the light condition and [$^{13}C_6$,$^{15}N_4$]-arginine in combination with [$^{13}C_6$,$^{15}N_2$]-lysine in the heavy condition. Heavy proline will be formed at the same rate under both conditions (that is, [$^{15}N_1$]-proline and [$^{13}C_5$,$^{15}N_1$]-proline, respectively), thus providing an internal correction for arginine conversion (**Supplementary Fig. 3** online).

Because we are interested specifically in proteins that convey self-renewal in human embryonic stem cells (hESCs)[7], which are sensitive to arginine starvation, we used hESCs to compare a conventional SILAC-based experiment with our modified approach

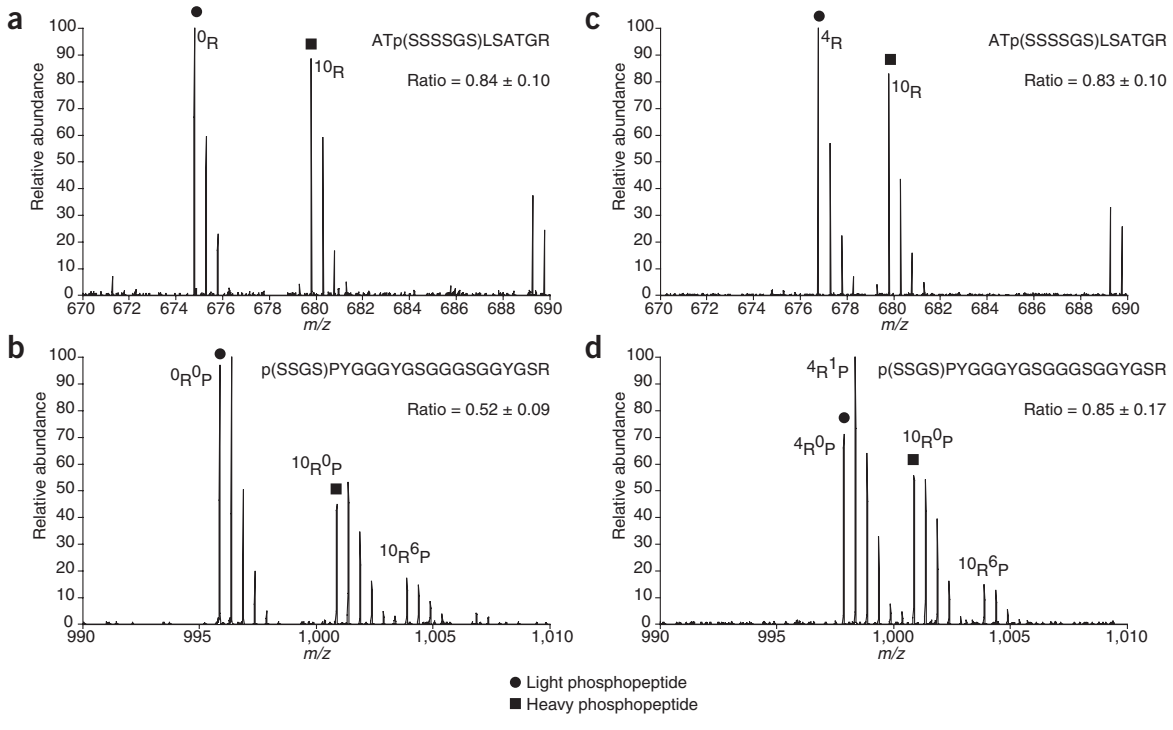

Figure 1 | Comparison of conventional SILAC labeling with our approach, tolerant to arginine conversion. (**a**–**d**) Although both strategies use [$^{13}C_6$-$^{15}N_4$]-arginine (^{10}R) and [$^{13}C_6$,$^{15}N_2$]-lysine (^{8}K) to create the 'heavy' sample, they differ in the fact that [$^{15}N_4$]-arginine (^{4}R) instead of unlabeled arginine (^{0}R) is used in the conversion-tolerant approach. Abundance ratios were calculated from spectral intensities of the mono-isotopic peaks of light and heavy phosphopeptide pairs obtained by conventional labeling (**a**,**b**) and conversion-tolerant labeling (**c**,**d**). The peptide pairs shown contain either no (**a**,**c**) or one (**b**,**d**) proline. ^{0}P, ^{1}P and ^{6}P indicate the nominal mass increase of proline as a result of conversion from arginine.

(**Supplementary Methods** online). The conventional experiment showed a typical arginine-to-proline conversion rate of ~30-40% of all proline-containing peptides (**Fig. 1a**,**b**), comprising about half of the identified peptides (**Supplementary Discussion**). When we grew hESCs in the conversion-tolerant light medium, the ratio between light and heavy peptides without proline was unaffected (**Fig. 1c**,**d**), but progressively changed with increasing numbers of prolines in the peptide (**Fig. 1** and **Supplementary Fig. 4** online). When we grew hESCs in the conversion-tolerant light medium, the first monoisotopic peak in the spectrum of the light peptide (containing [$^{15}N_4$]-arginine and [$^{15}N_1$]-proline) was reduced by ~30% owing to the presence of [$^{15}N_4$]-arginine-derived [$^{15}N_1$]-proline (**Fig. 1d**). This was manifested as an increase in the height of the second peak in the same spectrum (containing [$^{15}N_4$]-arginine and [$^{15}N_1$]-proline). As expected, the first peak in the peptide spectrum of the heavy peptide (containing [$^{13}C_6$,$^{15}N_4$]-arginine and [$^{13}C_5$,$^{15}N_1$]-proline) was similarly reduced upon conversion of [$^{13}C_6$,$^{15}N_4$]-arginine to [$^{13}C_5$,$^{15}N_1$]-proline (**Fig. 1d**). Notably, the same ratio measured for peptides from the light and heavy samples was consistent (**Fig. 1c**), also for peptides containing more than one proline (**Supplementary Fig. 4**). Therefore, substitution of normal arginine by [$^{15}N_4$]-arginine in the light condition allowed accurate quantitation of peptide ratios.

We tested several alternative strategies of labeling these hESCs, which are notoriously sensitive to environmental changes, for accurate SILAC-based quantitation without success (**Supplementary Discussion**). Substitution of normal arginine by [$^{15}N_4$]-arginine in the light condition provides an efficient means to quantify peptide ratios, even in cells with high conversion rates and in data sets of increased complexity because of partial conversion of arginine. We believe that these advantages outweigh the costs of using additional isotope labels and that this method extends the applicability of SILAC to cells that would otherwise be inaccessible for proper protein quantitation.

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

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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New lasers for flow cytometry: filling the gaps

To the editor: Flow cytometers rely almost exclusively on lasers for excitation of fluorescent probes. Although lasers are excellent excitation sources, their discrete wavelengths limit the visible light that is available for fluorophore excitation. Even the most modern cytometers typically provide no more than four laser wavelengths, with the traditional blue-green (488 nm) and red (633–640 nm) wavelengths being the most common. Even with multilaser instruments, coverage of the spectrum is incomplete, with gaps that exclude many useful fluorophores. This is largely due to the limited selection of wavelengths available with existing laser technology.

Two advances can eliminate these gaps. First, new diode-pumped solid state lasers provide a variety of discrete laser lines applicable for flow cytometry. Green (532 nm) and yellow-green (561 nm)

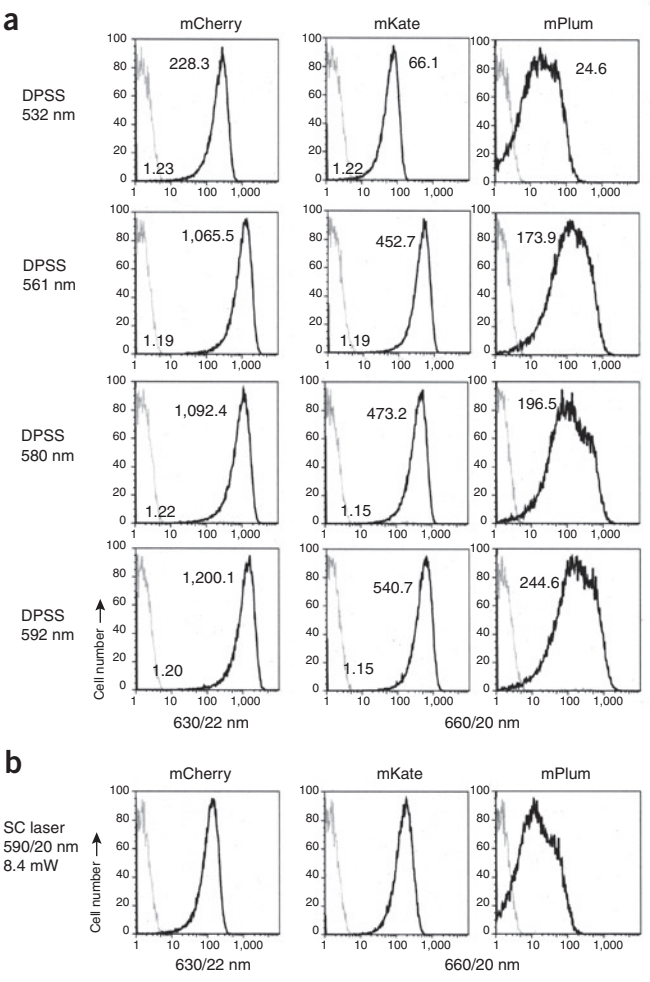

Figure 1 | Excitation with DPSS and supercontinuum laser sources. (**a**) Flow cytometric analysis of mCherry, mKate or mPlum expression in *Escherichia coli* using DPSS 532, 561, 580 or 592 nm lasers, all emitting at 50 mW. Untransfected (gray) and RFP expressing cells (black) are shown in each histogram, with mean fluorescence intensities given for each peak. (**b**) Flow cytometric analysis of mCherry, mKate or mPlum expression using a supercontinuum white laser source, with an interposed 590/20 nm bandpass filter.