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Competing interests statement
The authors declare no competing financial interests.

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INNOVATION

Functional and quantitative proteomics using SILAC

Matthias Mann

Abstract | Researchers in many biological areas now routinely characterize proteins by mass spectrometry. Among the many formats for quantitative proteomics, stable-isotope labelling by amino acids in cell culture (SILAC) has emerged as a simple and powerful one. SILAC removes false positives in protein-interaction studies, reveals large-scale kinetics of proteomes and — as a quantitative phosphoproteomics technology — directly uncovers important points in the signalling pathways that control cellular decisions.

Proteins are in direct control of almost all cellular processes, and post-genomic biology will not reach its potential until we have tools to study proteins on a large scale. Sadly, proteomics has lagged far behind DNA-based technologies, mainly because there are no protein-analysis methods similar to oligonucleotide hybridization, amplification and sequencing. This lag is diminishing rapidly, however, due to increasingly powerful mass spectrometry (MS)-based technologies[1]. Mass spectrometers can sequence thousands of peptides from complex mixtures in an automated manner[2]. The importance of quantitation in proteomics has recently become appreciated, and technologies such as isotope-encoded affinity tags (ICAT)[3] have generated widespread interest. Much of this interest is still focused on the determination of the relative levels of expression of, ideally, all proteins between two cell or tissue states (expression proteomics). Expression proteomics has also been the elusive goal of the two-dimensional gel electrophoresis community and has essentially the same principle at the protein level as microarrays have at the mRNA level (transcriptomics).

However, proteomics — unlike transcriptomics — is not limited to measuring whole cell or tissue expression levels. Here, I argue that the most important contributions of quantitative proteomics to biological understanding will come from its unique capability to determine changes in functionally relevant 'sub-proteomes'. Although many of the strategies described here can be, and sometimes have been, implemented with other quantitative-proteomics techniques, I also argue that stable-isotope labelling by amino acids in cell culture (SILAC) combined with sophisticated mass-spectrometric and bioinformatic technology is particularly well suited to reinvent or enhance biochemistry-based approaches. This argument will be made, in part, by describing successful applications of SILAC to a wide range of biological problems.

First, I explain the principles of SILAC and its advantages and limitations compared with other quantitative-proteomics strategies. SILAC has been used successfully for the determination of cholesterol-dependent lipid rafts and the elucidation of protein interactions, side-stepping the longstanding trade-off between assuring specificity of the measured interaction and preservation of weak interactions. I then provide some examples of the quantitation of changes in the phosphoproteome from the yeast pheromone pathway to stem-cell differentiation. Furthermore, SILAC has become a prime technology to add a time dimension to proteomics. Last, I describe the emerging use of SILAC to directly study protein synthesis and degradation. As we reach the conclusions and perspectives, I hope that the reader will appreciate how SILAC-based proteomics fundamentally transforms the questions we can ask using biochemical approaches. Indeed, the emergence of the SILAC strategy and the extraordinary progress in mass-spectrometric technology in just the past few years now offers exciting new strategies for advances in virtually all areas of biology.

Box 1 | **Advantages and limitations of SILAC**

Mass spectrometry is not inherently quantitative, as different molecules have different mass spectrometric responses. Stable-isotope analogues of the molecule to be quantified have therefore been used for many years for accurate quantitation in small-molecule mass spectrometry. To differentially quantify two proteomes, a stable isotope can be introduced in various ways, most commonly by chemical modification or by metabolic labelling[6]. Chemical labelling can be done on any proteome, including body fluids and biopsis material, whereas metabolic strategies require living cells. Chemical strategies involve a derivatization step that might not be complete, that might introduce side products, and that might limit the sensitivity of the analysis.

Another important difference between metabolic and chemical labelling is the labelling stage. In chemical labelling, the two proteomes to be compared have to be purified and fractionated separately and in precisely the same way to allow relative quantitation of the same fractions. By contrast, metabolic labelling allows mixing of labelled and unlabelled cells and therefore subsequent fractionation and purification steps will not introduce any errors in quantitation.

Metabolic labelling for quantitation was first introduced to proteomics by the Langen group in 1998 by feeding microbes a ^{15}N-substituted food source (described in REF. 55) and it was also used shortly afterwards for quantitation by the Chait group[56]. The stable-isotope labelling by amino acids in cell culture (SILAC) strategy has a number of advantages with respect to ^{15}N labelling. For instance, in SILAC, the expected mass differences are known before peptide identification, simplifying quantitation. Furthermore, mammalian cell lines are easily labelled by providing the SILAC amino acid instead of having to eliminate any unlabelled nitrogen source from the media and — because only one or two amino acids in a peptide are substituted and the degree of labelling is very high — quantitation is straightforward. SILAC does require adapting one's cell line to dialyzed serum, and further steps might have to be taken if cells don't adapt well. However, this step can be checked without labelled amino acids. The SILAC approach is not necessarily expensive, especially if amino acids are bought directly and in gram amounts from a supplier.

Principles of SILAC

SILAC was first described in the literature only four years ago[4,5], later than many other schemes for quantitative proteomics (REF. 6; see also reviews[7–11] for different aspects of the field). It is conceptually and experimentally straightforward: it involves growing two populations of cells, one in a medium that contains a 'light' (normal) amino acid and the other in a medium that contains a 'heavy' amino acid. The heavy amino acid can contain ^{2}H instead of H, ^{13}C instead of ^{12}C, or ^{15}N instead of ^{14}N. Incorporation of the heavy amino acid into a peptide leads to a known mass shift compared with the peptide that contains the light version of the amino acid (for example, 6 Da in the case of $^{13}C_6$-Arg), but to no other chemical changes.

In some respects, SILAC is similar to other metabolic labelling techniques that have been used for decades in biological research (for advantages and limitations of SILAC compared with other quantitative-proteomics technologies, see BOX 1). For example, in pulse-chase experiments, a radioactive form of an amino acid is added to a medium with cells for a certain length of time and the radioactivity that is incorporated into the newly synthesized proteins is measured. However, in contrast to pulse-chase experiments, the isotopes of SILAC amino acids are stable — no radioactivity is involved. Furthermore, the objective in SILAC is to distinguish two proteomes by the molecular weight of the light or heavy amino acid that is used during the growth of the two cell populations. This requires complete labelling — the SILAC amino acid in all proteins should be replaced — and this is achieved after five cell doublings, even for proteins with no significant turnover.

To ensure that cells only incorporate the added, labelled amino acid into their proteome, essential amino acids are chosen and cells are grown with dialyzed serum, when appropriate. Some cell types require low molecular mass growth factors, which are not present in dialyzed serum. In these cases, exogenous growth factors or serum dialyzed with a low molecular mass cut-off can be used. Alternatively, the use of a small amount of undialyzed serum — not sufficient to significantly influence quantitation — has proven useful[12]. Early concerns that the isotope labels would be scrambled through metabolic cycles that involve different amino acids have proven unfounded. An exception is Arg, which is converted to Pro by some cell types when present at high levels in the media[13]. Peptides that contain both Arg and Pro can be quantified separately, or Arg in the media can be titrated down so that Pro conversion becomes negligible and cells can still grow normally.

In a straightforward expression-proteomics experiment, one cell population is labelled with a light amino acid (population A) and another cell population is labelled with a heavy amino acid (population B). Then the cells are mixed and their proteomes are extracted and measured by MS. Each peptide appears as a pair in the mass spectra — the peptide with lower mass contains the light amino acid and originates from population A, and the peptide with higher mass contains the heavy amino acid and originates from population B. If the SILAC peptide pair appears in a one-to-one ratio then there is no difference in the abundance of this protein between the proteomes. A higher peak intensity from the peptide that contains the heavy amino acid indicates that the protein was more abundant in population B. Because the light and heavy amino acids are chemically identical, except for their mass difference, the ratio of peak intensities in the mass spectrometer directly yields the ratio of the proteins in population A versus population B.

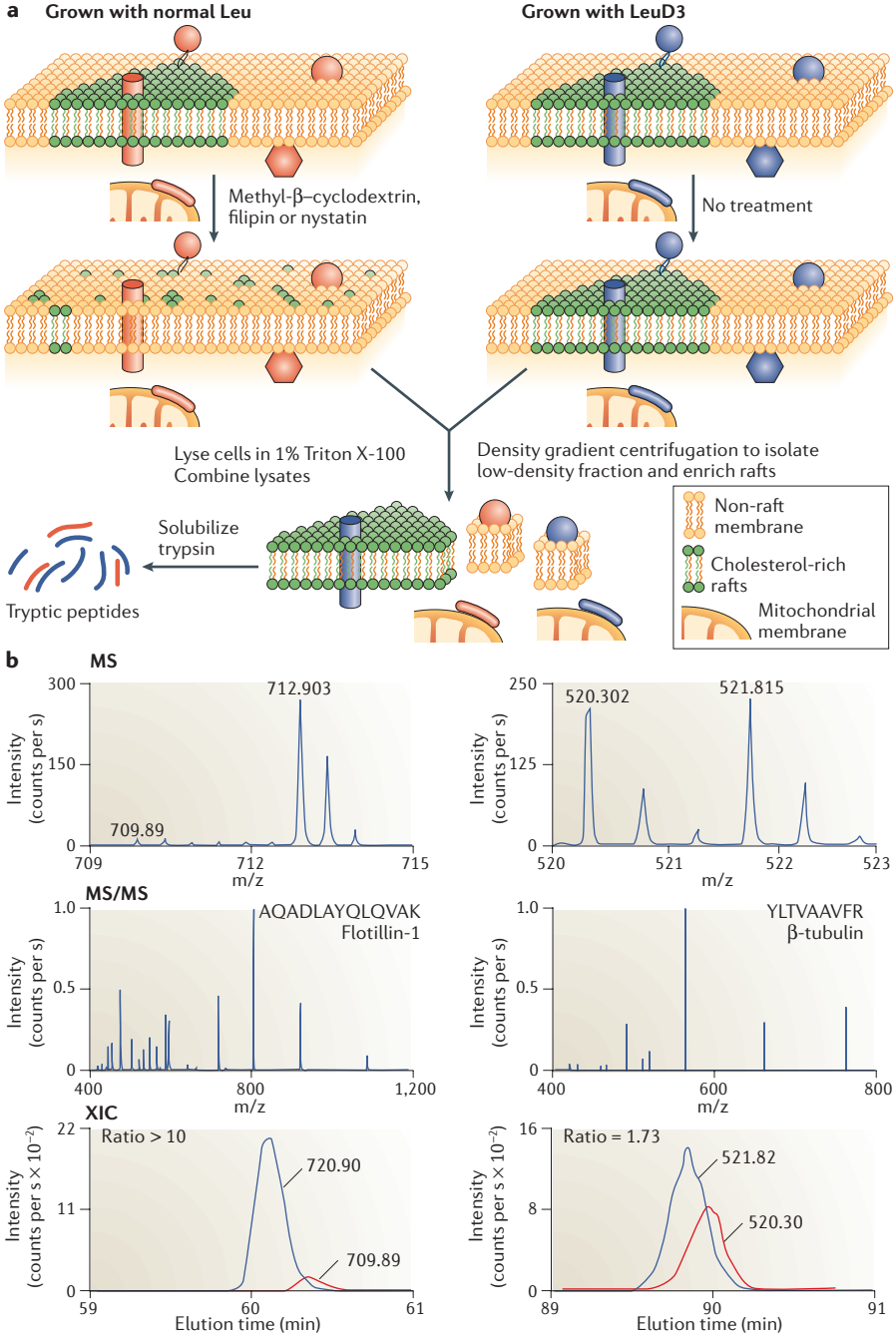
Accuracy of quantitation depends on the abundance and signal-to-noise ratio of the peptide pair, and can be as good as a few percent[14]. Usually, ratios of 1.3–2.0-fold have been used as cut-offs for both statistical and biological significance. For example, Everly *et al.* used SILAC to compare microsomal fractions of a more metastatic versus a less metastatic prostate-cancer cell line[15,16]. A total of 1,395 proteins were quantified, and 20% of these were differentially expressed by more than 3-fold between the 2 cell lines.

Box 2 | **Principle of a functional SILAC experiment, exemplified by the lipid-raft proteome**

Lipid rafts are areas of the plasma membrane with specific lipid and protein composition that are thought to organize signalling events[57]; however, the concept of lipid rafts is controversial[58], partly because rafts cannot be directly visualized or purified completely. To investigate the existence of lipid rafts (panel **a**), we used a deuterated-Leu-labelled (LeuD3) cell population as a control and a normal Leu-labelled population that was treated with cholesterol-disrupting agents[59]. We then combined both cell populations and enriched for detergent-resistant lipid rafts in the standard way. Lipid rafts are destroyed in the treated, but not the untreated, stable-isotope labelling by amino acids in cell culture (SILAC)-labelled cell populations, and cells are combined.

The actual mass spectrometry (MS) data for peptides from two different proteins is shown in panel **b**. The SILAC peptide pair at mass-to-charge ratio (m/z) 709.89 and 712.903 had higher intensity in the heavy-amino-acid form. Peptide sequencing (MS/MS; middle) identified the peptide as originating from flotillin-1, and the extracted ion current (XIC; peptide ion signal as a function of elution time; bottom) indicates the total signal per peptide (see REF. 60 for an introduction to peptide sequencing). Clearly the integrated signal of the light form is much less than that of the heavy form, indicating that the cholesterol-disrupting drug removed flotillin-1 from the preparation. By contrast, a peptide from β-tubulin (SILAC peaks at m/z 520.302 and 521.815) is present even when rafts are destroyed (ratio 1.73, which is not statistically significant in this experiment) and this protein is therefore not a genuine member of lipid rafts.

MS analysis identified 703 proteins, but only 241 of these had statistically significant fold changes, indicating that they were depleted by removal of cholesterol. By definition, these 241 proteins are members of cholesterol-dependent lipid rafts. Figure redrawn with permission from REF. 59 © (2003) National Academy of Sciences, USA.



Gene-ontology analysis revealed that 10% of the most highly downregulated proteins in the more metastatic cell line are involved in cell adhesion. Similarly, Gronborg *et al.* used SILAC to compare secreted proteins from pancreatic-cancer-derived cells with non-neoplastic pancreatic cells and found a number of differentially expressed proteins[17].

In contrast to expression proteomics, which aims to quantify the complete proteome, in a 'functional' SILAC experiment one cell population functions as a control, whereas the other one is perturbed in some way and a sub-proteome of interest is isolated (BOX 2). The SILAC approach itself is straightforward, but identifying the most elegant perturbation and control might require considerable ingenuity.

Eliminating false-positive interactions

One of the most spectacular applications of proteomics has been the elucidation of protein-interaction networks. Specifically, two large-scale studies in yeast used immunoprecipitation followed by MS, and these were influential in promoting the view that most proteins exist as parts of multiprotein complexes rather than as solitary factors[18,19]. Unfortunately, these studies are extremely difficult to control for false-positive interactions[20,21]. The problem is that MS-based proteomics is now so sensitive that any pull-down assay, regardless of bait, will result in a large number of identified background proteins. More specific and stringent purification, such as

with the tandem affinity purification (TAP) tag[22], is an improvement but not a panacea, as low-affinity interactions tend to be lost.

Both the Aebersold group, using ICAT, and my group, using SILAC, showed that quantitative proteomics can completely overcome the problem of nonspecifically interacting proteins[5,23,24]. The basic strategy is explained in FIG. 1 using a phosphodependent protein–protein interaction that involves a bacterial protein, which is injected into a human host cell in the course of bacterial infection. Following the synthesis of both non-phosphorylated and phosphorylated forms of the peptide that contains the phosphorylation site of the bacterial protein, the non-phosphorylated and phosphorylated peptides were incubated with light- and heavy-amino-acid-containing cell lysates, respectively. After gentle washing to preserve weak interactions, bound proteins were eluted and mixed. The vast majority of the proteins that were identified in the mixed eluates were present in a one-to-one ratio, which indicates that they were binding nonspecifically to the beads or to the peptide. Proteins that bind specifically as a result of phosphorylation are easily distinguishable because of their statistically significant ratios. As a further control, the phosphorylated peptide can be incubated with the heavy-amino-acid-containing lysate and the non-phosphorylated peptide with the light-amino-acid-containing lysate. In this 'cross-over' experiment, all SILAC-peptide ratios should be inverted.

We scaled up this technology to identify the interactome of all potentially Tyr-phosphorylated residues of the ERBB-receptor family. Our findings recapitulated many of the interactions that were identified over the past 15 years. Furthermore, some new interactions, which implicate epidermal growth factor receptor (EGFR) and ERBB4 in more diverse signalling roles than ERBB2 and ERBB3, were also identified[25]. All interactors with a statistically significant SILAC ratio had phosphotyrosine-binding domains. This finding, compared with data already available in the literature, indicated that the screen did not result in any false-positive interactions.

Identifying binding partners of PP1. The principle outlined above can be used with any protein interactions as long as bait and control are available and specific interacting proteins are expressed in sufficient amounts for detection by MS. Trinkle-Mulcahy *et al.* tagged isoforms of protein phosphatase-1 (PP1) with green fluorescent protein (GFP) and used SILAC to identify and distinguish their binding partners[26]. Three cell states were labelled for pull-down with control–GFP, PP1α–GFP and PP1γ–GFP. Out of the hundreds of the proteins that were identified with anti-GFP antibody immunoprecipitation, the SILAC ratios showed that some proteins bind to all PP1 isoforms, whereas others bind specifically to PP1α or PP1γ. This study also identified a new protein, Repo-Man, which binds to PP1γ, targets PP1 to chromatin and seems to have important functions in the cell cycle[27].

SILAC is currently used in a similar manner to probe protein interactions of immobilized drugs. In our laboratory we have also used SILAC to identify transcription factors and cofactors by incubating double-stranded synthetic DNA with nuclear extract. Any protein that preferentially binds to the wild-type DNA compared to a point-mutated sequence is a candidate sequence-specific DNA-binding protein. Also, SILAC can be combined with small interfering (si)RNA knockdown of the antigen of interest in an immunoprecipitation. By comparison of eluates of the antibody from cells with and without siRNA treatment, crossreactivity of the antibody is eliminated and true interactors of the endogenous protein are obtained[28].

SILAC for quantitative phosphoproteomics

MS can directly measure and sequence endogenous phosphopeptides without the need to generate special reagents, so it has therefore become a method of choice for determining phosphorylation sites (reviewed in REFS 29–31). Improvements in phosphoproteomics technology now allow sequencing of hundreds or even thousands of phosphorylation sites in a single experiment[32]. However, it has rapidly become clear that a functional filter is needed to extract biological insights from such experiments.

Mapping yeast pheromone responses. SILAC is ideally suited to distinguish the phosphorylation events that are associated with a particular stimulus from a vast excess of basal phosphorylation sites. As all peptides need to be quantifiable, both Arg and Lys are usually labelled[33], and trypsin is used as a protease. Gruhler *et al.* labelled a yeast strain by SILAC and exposed one population to mating factor and enriched phosphopeptides from total cell lysate[34]. Sequencing and quantitation of more than 700 phosphopeptides showed a 2-fold change in 139 of these peptides upon pheromone stimulation. These phosphorylation sites mapped to proteins in the entire mating-signalling pathway from receptors to transcription factors.

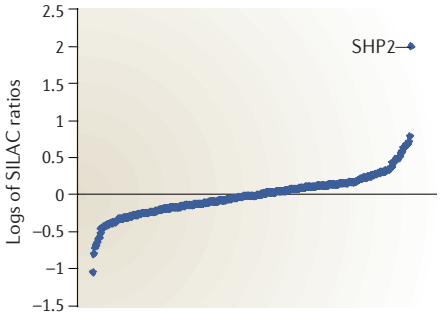


Figure 1 | **Interaction of a bacterial phosphoprotein with a human host protein.** An immobilized synthetic phosphopeptide that corresponds to the phosphorylation site of the bacterial protein CagA was incubated with heavy stable-isotope labelling by amino acids in cell culture (SILAC)-labelled mammalian cell extracts. As a control, the peptide that lacks the phosphogroup was incubated with the light-SILAC-labelled cell extract. Eluates from phosphopeptide and non-phosphopeptide baits were mixed and analysed by liquid chromatography–tandem mass spectrometry. The proteins that were identified are aligned according to the logs of their SILAC ratios. More than 150 proteins were identified with ratios close to one to one, which shows that these proteins bind equally well to the peptide, regardless of the presence of the phosphogroup. Only SHP2 (Src-homology-2 domain-containing protein tyrosine phosphatase-2) has a statistically significant ratio, which indicates that SHP2 binds specifically to the phosphopeptide. These data confirm a recently described interaction[61]. Figure courtesy of M. Selbach, Max-Planck Institute for Biochemistry, Martinsried, Germany.

Examining stem-cell differentiation. Quantitative phosphoproteomics can also directly identify factors that control cellular decisions. We were interested in understanding how growth factors influence stem-cell differentiation, particularly the differentiation of adult mesenchymal stem cells to bone-forming cells[35]. We found that two related growth factors, EGF and platelet-derived growth factor (PDGF), induced Tyr phosphorylation; however, only EGF strongly enhanced differentiation to bone-forming cells.

To determine the cause of this difference, we SILAC-labelled three cell populations and exposed them to no stimulus, EGF stimulation or PDGF stimulation (FIG. 2). We then enriched the Tyr phosphoproteome with anti-phosphotyrosine antibody. As expected, most of the Tyr phosphoproteome was regulated by both EGF and PDGF, albeit often to different degrees. However, there were also a few proteins that were only activated by one of these growth factors. In particular, phosphatidylinositol 3-kinase (PI3K)

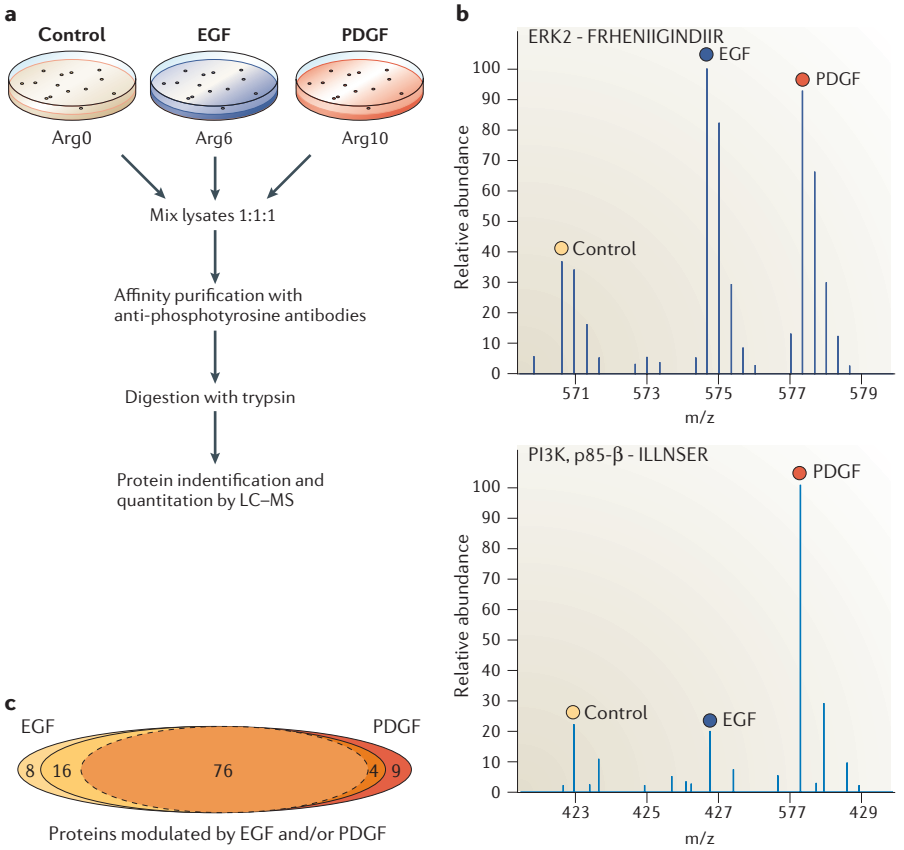


Figure 2 | **Systems biology of stem-cell differentiation. a** | Mesenchymal adult stem cells differentiated to bone cells upon epidermal growth factor (EGF), but not platelet-derived growth factor (PDGF), treatment. To determine the proteins that control the differentiation process, stable-isotope labelling by amino acids in cell culture (SILAC; using Arg0, Arg6 and Arg10) was used to analyse three cell populations; EGF-treated cells, PDGF-treated cells and control cells that were untreated. Subsequently, cells were mixed and the phosphotyrosine proteome was enriched using an anti-phosphotyrosine antibody. **b** | The heavy-amino-acid SILAC forms of a peptide from mitogen-activated protein kinase-2 (MAPK2, also known as ERK2) from the EGF- and PDGF-stimulated populations are more intense than the control, which indicates that ERK2 is stimulated by both growth factors. However, a peptide from the p85β subunit of the phosphatidylinositol 3-kinase (PI3K) is activated over the control (present in the Tyr phosphoproteome) only in the population that is stimulated by PDGF. This finding indicates that PI3K might be a possible control point for the differentiation to bone cells. **c** | Evaluation of the entire data set showed that 113 proteins are modulated by EGF and/or PDGF (entire oval); 76 proteins are activated similarly by EGF and PDGF; 16 are more activated by EGF and 4 are more activated by PDGF (up to threefold); 8 proteins are only activated by EGF, and 9 are only activated by PDGF. LC–MS, liquid chromatography–mass spectrometry; m/z, mass-to-charge ratio. Adapted with permission from REF. 35 © (2005) American Association for the Advancement of Science.

was only activated by PDGF and was therefore a candidate for the differential effects. Indeed, the PI3K inhibitor wortmannin caused PDGF stimulation to be as potent in bone-cell differentiation as EGF *in vitro* and *in vivo*. This finding, which might also be of clinical interest, shows how quantitative phosphoproteomics can help to pinpoint sites of cellular decision making.

The Tyr phosphoproteome has also been studied in cancer cells. ERBB2 is overexpressed in a subset of breast cancers and phosphotyrosine signalling downstream of this receptor has been studied by two groups. Zhang *et al.* used ligand stimulated versus unstimulated cells in the SILAC experiment[36]. Bose *et al.* quantified the Tyr phosphoproteome of untreated and phosphatase-inhibitor treated ERBB2-overexpressing cells and used network modelling to account for the changes in the phosphoproteome[37].

Adding the time dimension to proteomics

So far, most proteome experiments characterize a static time point or an on–off situation. To incorporate kinetics into organellar proteomics, three different cell populations were labelled, treated with an inhibitor of transcription and harvested at three different time points[38]. Cells were subsequently mixed and their nucleoli were isolated. In response to inhibition of transcription, some proteins leave the nucleoli, some remain unchanged and some are recruited to the nucleoli. Previously, such changes had to be visualized in a candidate-based approach using GFP-fusion proteins. By contrast, SILAC directly measures changes in endogenous proteins in the entire nucleolar proteome. A peptide from a protein recruited to the nucleolus would show a triplet with increasing intensities, because more of the protein is present in the nucleolar preparations from later time points (FIG. 3). By repeating the experiment several times with a common time point, up to nine point kinetics were obtained. Interacting proteins left the nucleolus with similar time profiles. These time profiles also helped to group the nucleolar proteome into interacting modules by machine learning, which is an area of artificial intelligence that allows the computer to uncover patterns and classify data accordingly[39].

Once the experiment is set up, SILAC time courses of a proteome can be obtained rapidly. Use of different inhibitors of transcription showed trafficking of different protein populations from or to the nucleolus. For example, inhibition of the proteasome resulted in largely opposite kinetics compared to a specific inhibitor of polymerase II transcription[38]. Such findings can be followed up by further experiments in conjunction with single-cell fluorescence microscopy. The combination of modern microscopy techniques with SILAC is especially powerful because SILAC is unbiased, comprehensive and measures endogenous proteins, whereas biochemical approaches average over large cell populations. On the other hand, microscopy supplies detailed spatial, temporal and interaction information at the single-cell level, ideally complementing quantitative proteomics.

Another area in which the time dimension is of great interest is in signalling cascades. Such kinetics are difficult to obtain in a global and unbiased way. Using SILAC and a time-course experiment similar to the one described for the nucleolus experiment, Blagoev *et al.* mapped changes in the phosphotyrosine proteome as a result of EGF stimulation. Activation profiles were obtained covering all classes of Tyr phosphorylated effectors[40]. Recently, we have extended this approach to quantitatively measure thousands of Ser/Thr/Tyr phosphorylation sites, which yielded a first

overall map of early cellular information processing in response to stimuli, from receptor to transcription factor[41].

Studies of protein turnover

All of the examples discussed so far entail complete labelling of each cell population. However, as pioneered by Beynon and Gaskel[42,43], SILAC can also directly measure protein turnover. The technique consists of switching the SILAC amino acid in the media at a particular time point from light to heavy (or from heavy to light). From this time point on, all newly synthesized proteins incorporate the heavy SILAC amino acid and are easily distinguishable from the pre-existing proteome. In principle, it is straightforward to measure turnover of proteins in any cellular compartment.

In a clever application of this principle, Admon and co-workers studied major histocompatibility complex (MHC) peptides with the SILAC technique[44]. MHC class I peptides are generally derived from cellular proteins and are presented on the cell surface for inspection by the immune system. The MHC peptidome is thought to contain populations with different turnover times: long-lived proteins that are degraded at the end of their useful life, short-lived proteins that are mainly regulated by degradation, and also newly synthesized proteins that fail to pass the protein-quality-control machinery and are immediately degraded[45].

Indeed, the SILAC data showed that protein classes with different turnover rates were present. Some proteins were degraded within minutes of ribosomal synthesis and others persisted for days or longer. Intriguingly, this analysis found limited correlation between the proteome and the MHC peptidome, highlighting one of the reasons that mRNA levels and protein levels are sometimes poorly linked. In another recent study of the MHC peptidome, Meiring *et al.* used SILAC to recognize and sequence virus-derived MHC peptides after infection of a cell line[46]. Direct measurement of protein synthesis and degradation opens up an exciting new window for cell biology and proteomics.

Conclusions

As shown above, SILAC in combination with existing cell biological and biochemical approaches can be used to ask profound questions, which are limited only by the imagination of the researcher. However, the impact of SILAC on the biological community is currently restricted by a lack of access to the technology. Although SILAC labelling is easy for any laboratory that uses cell culture, the MS technology that is required is still beyond the capabilities of most groups.

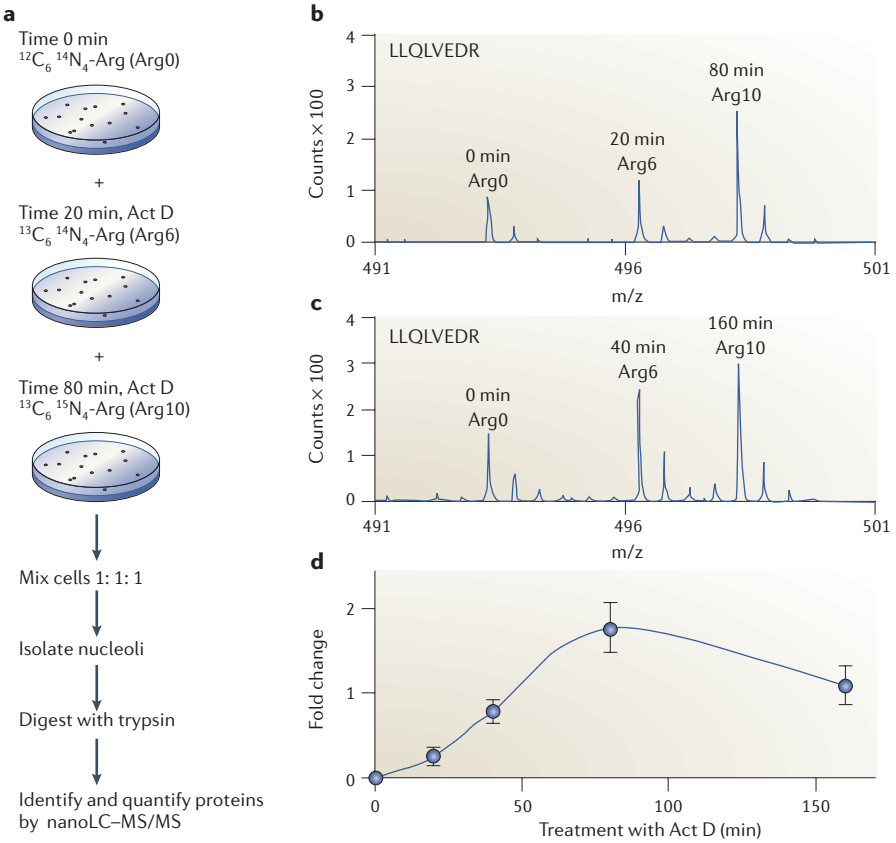


Figure 3 | **Nucleolar proteome dynamics. a** | Three Arg-stable-isotope labelling by amino acids in cell culture (SILAC)-labelled cell states (Arg0, Arg6 and Arg10) were treated for various times with actinomycin D (Act D), an inhibitor of transcription, and nucleoli were isolated. **b** | Mass spectrum of a peptide from the nucleolar protein p68 in three SILAC-labelled forms. The intensity of the peptide is highest in the peptide derived from the nucleoli that have been isolated after 80 minutes of transcription inhibition, indicating that upon inhibition of transcription, the protein is recruited to the nucleolus. **c** | Mass spectrum for the same peptide after inhibition for different lengths of time. **d** | Combination of the data from panels **b** and **c** leads to a kinetic curve of recruitment of p68 to nucleoli. nanoLC–MS, nano liquid chromatography–tandem mass spectrometry; m/z, mass-to-charge ratio. Reproduced with permission from REF. 38 © (2005) MacMillan Magazines, Ltd.

Fortunately, new mass spectrometers such as a linear ion trap–orbitrap combination now allow very high performance in a compact and robust format[47,48]. One of the factors that contributed to the rapid acceptance of the SILAC technology was the availability of an open-source programme, MSQuant, for interpreting results[24]. However, because of the large amount of data involved, interpretation of results is still a limiting factor. Further software developments, using concepts from MS algorithms for biomarker development (see REF. 49 as an example) will surely advance the field. Further automation will also help in better defining statistical validity in quantitative-proteomics projects.

Perspectives

In the future, one could imagine researchers using SILAC in two modes. In the exploratory mode, experiments will be planned, executed and analysed rapidly to generate hypotheses and ideas for further experimental refinement. In the exploratory mode, statistical validity is not the primary concern, but rather the objective is the discovery of a biological effect. In the validation mode, experiments will be performed several times (analytical replicates) and perhaps with different cell populations (biological replicates). Analysis of these experiments should ideally also be automated so that they result in a list of proteins with statistically validated ratios.

At the other extreme, a main application area of SILAC might be the quantitative analysis of modifications of particular purified proteins. A protein of interest can be

enriched from differentially-treated SILAC-labelled cells and analysed for changes in processing or for other modifications induced by the treatments. As an example, histone methylation can be quantified by using labelled Met, which is the sole methyl donor of the cell (heavy methyl SILAC, see REF. 50). Illustrating the biological insights that can be obtained rapidly with such approaches, Timmer and co-workers expressed a tagged potassium channel, stimulated a SILAC population with different treatments and found regulated phosphorylation sites[51]. Similar experiments can be done by any laboratory with access to MS equipment and results can be analysed manually.

Will SILAC always be restricted to cell-culture experiments? Microorganisms can already be labelled if one uses auxotroph strains, and Nirmalan *et al.* showed that the malaria parasite can be labelled with Ile, an amino acid that is not found in haemoglobin, the preferential source of amino acids for the parasite as it grows in red blood cells[52]. For metazoans, Ishihama *et al.* have developed culture-derived isotope tags (CDITs)[53]; the authors used SILAC labelling for a neuronal cell line, which was then used as an internal standard to quantify brain sections. Others have metabolically labelled organisms as large as rats[54] and we have labelled mice with SILAC amino acids with a defined amino-acid diet.

It is a fair bet that SILAC kits by various companies will arrive on the market in the next few years. With better software and more accessible MS, SILAC might become a routine assay for the next generation of biochemists and cell biologists. Through its capability to quantify both the proteome and its modifications in response to stimuli and perturbations, SILAC might also become an important foundation for systems biology.

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doi:10.1038/nrm2067

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Acknowledgement
I thank S.-E. Ong, L. Foster, B. Blagoev, J. Andersen and members of the Department for Proteomics and Signal Transduction for critical discussion of this manuscript. This work was supported by the Danish National Research Foundation and the Max-Planck Society.

Competing interests statement
The author declares no competing financial interests.

FURTHER INFORMATION
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Pandey laboratory SILAC pages at John Hopkins University: http://www.silac.org
Open source program MSQuant, which allows quantitation of SILAC data: http://msquant.sourceforge.com
Phosphorylation-sites database (Phosida) at the Max-Planck Institute for Biochemistry: http://www.phosida.com
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