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Advances in quantitative proteomics via stable isotope tagging and mass spectrometry

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The high-throughput identification and accurate quantification of proteins are essential components of proteomic strategies for studying cellular functions and processes. Techniques that are largely based on stable isotope protein or peptide labeling and automated tandem mass spectrometry are increasingly being applied in quantitative proteomic studies. Over the past year, significant progress has been made toward improving and diversifying these technologies with respect to the methods for stable isotope labeling, process automation and data processing and analysis. Advances in stable isotope protein labeling and recent biological studies that used stable isotope based quantitative proteomics techniques are reviewed.

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Abbreviations

2DE	two-dimensional gel electrophoresis
CID	collision-induced dissociation
ICAT	isotope-coded affinity tag
MALDI	matrix-assisted laser desorption ionization
MS	mass spectrometry
MS/MS	tandem MS
RPLC	reverse-phase liquid chromatography
RP-μLC-MS	reverse-phase microcapillary liquid chromatography coupled on-line with MS

Introduction

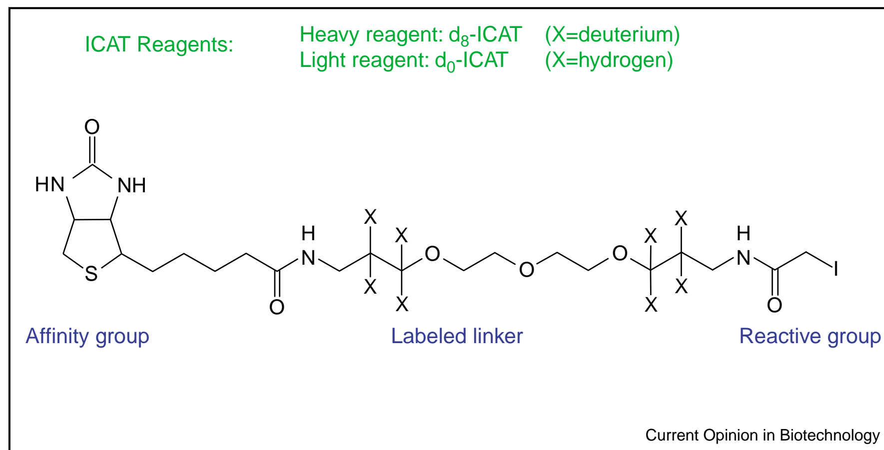
In addition to enumerating the proteins expressed in a species, the detection and quantification of differences in the protein profiles of cells, tissues or body fluids of different origins or states is increasingly being recognized as a key objective of proteomics research. The measurement of differential protein expression provides a more direct, more accurate and more versatile way to detect global changes in cellular dynamics in health and disease compared with the complementary and more mature technology of mRNA expression analysis [1]. Quantitative proteomics thus holds significant promise for the discovery of diagnostic or prognostic protein markers, for the detection of new therapeutic targets and as a powerful tool to further our understanding of basic biological processes and mechanisms. The realization of these expectations relies on the development of automated, robust and highly sensitive methods to identify and quantify proteins.

To date, three different approaches for quantitative protein profiling have been proposed. In the first and most mature approach, proteins are separated and quantified by two-dimensional gel electrophoresis (2DE), and identified by mass spectrometry (MS) [2]. The second approach, protein expression array analysis, is based on the generation of ordered arrays of reagents with high specificity for particular proteins. This method represents the linear translation of the mRNA expression array concept into proteomics and is at present, in spite of its enormous promise, least developed [3]. The third and currently most rapidly advancing approach is based on stable isotope labeling of proteins or peptides and automated tandem mass spectrometry (MS/MS). Chemically identical but mass-differentiated stable isotope tags are introduced into the proteins in two sample mixtures. The combined mixtures are then processed and analyzed by MS so that each analyte from one sample represents a quantitative standard for a chemically identical analyte from the other sample. The isotope tags can optionally contain an affinity tag so that the tagged analytes can be selectively isolated, thus simplifying the complexity of sample mixtures before mass-spectrometric analysis. This review summarizes recent advances in quantitative proteomics technology based on stable isotope tagging and MS/MS and discusses results from selected applications.

Current status and applications of quantitative proteomics using stable isotope tags and mass spectrometry

The prototypical method to generate quantitative protein profiles based on stable isotope affinity tagging and MS is the isotope-coded affinity tag (ICAT) reagent method [4]. The reagents (Figure 1) consist of a cysteine-reactive group, a linker that contains either heavy or light isotopes, and a biotin affinity tag. The ICAT reagent method (Figure 2) involves *in vitro* derivatization of cysteine residues with the isotopically heavy or light form of the reagent, respectively, then enzymatic digestion of the combined sample and isolation and mass spectrometric analysis of labeled peptides [4]. In the process the complexity of the peptide sample mixture is significantly reduced and each peptide is isotopically tagged to facilitate quantitative mass spectrometric measurements.

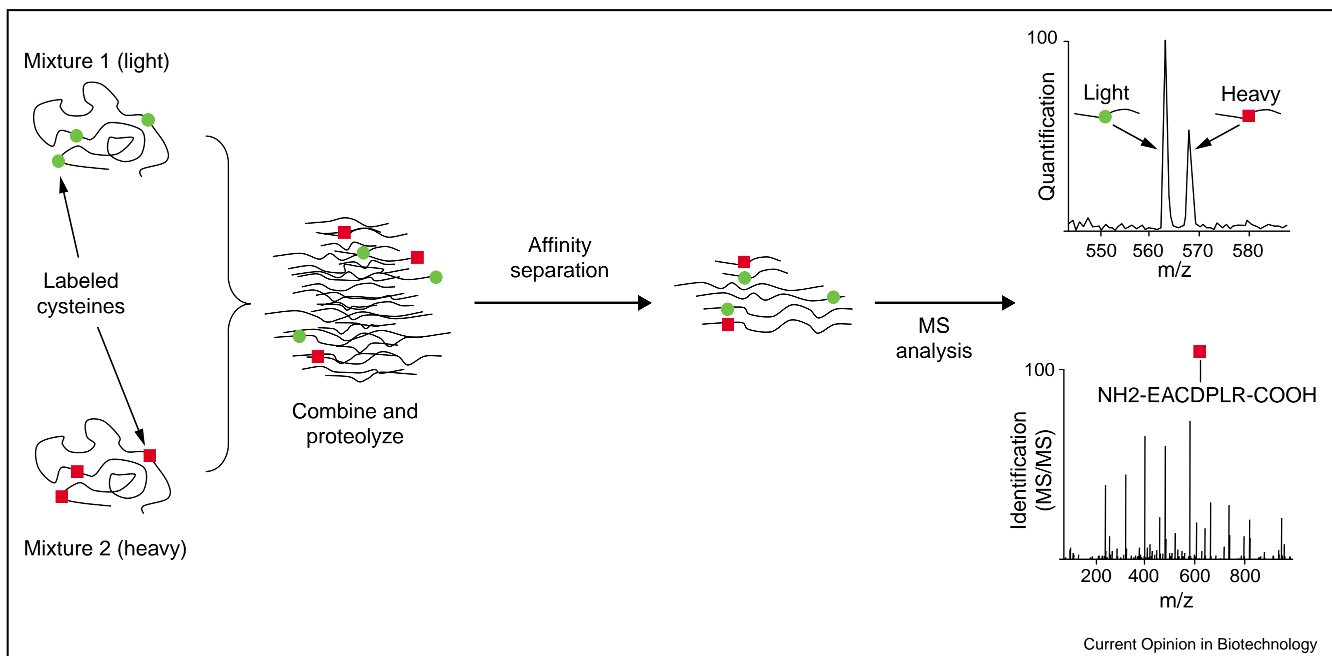
Figure 1



The structure of ICAT reagents. ICAT reagents comprise a cysteine-reactive group, a linker containing either heavy or light isotopes and a binding affinity tag.

Results from a series of applications of the method have illustrated its versatility, documented current technical limitations and showed new uses for quantitative proteomic analyses. The applications can be broadly grouped into three classes, as detailed below. In the first, quantitative proteomics was used to ask whether the analysis of perturbation-induced changes measured at the mRNA and protein levels provide redundant or complementary information. In the second class, quantitative proteomics was used to gain new insights into specific cellular

Figure 2



The ICAT reagent strategy for quantifying differential proteins. Two protein mixtures representing two different cell states are treated with the isotopically light (green) or heavy (red) ICAT reagents, respectively. The labeled protein mixtures are then combined and proteolyzed; tagged peptides are selectively isolated and analyzed by MS. The relative abundance is determined by the ratio of signal intensities of the tagged peptide pairs. Every other scan is devoted to fragmenting a peptide. The CID spectra are recorded and searched against large protein sequence databases to identify the protein. Therefore, in a single operation, the relative abundance and sequence of a peptide are determined.

mechanisms (e.g. specific signaling mechanisms). In the third class, applications that use quantitative proteomics for purposes that go beyond simple protein profiling were explored.

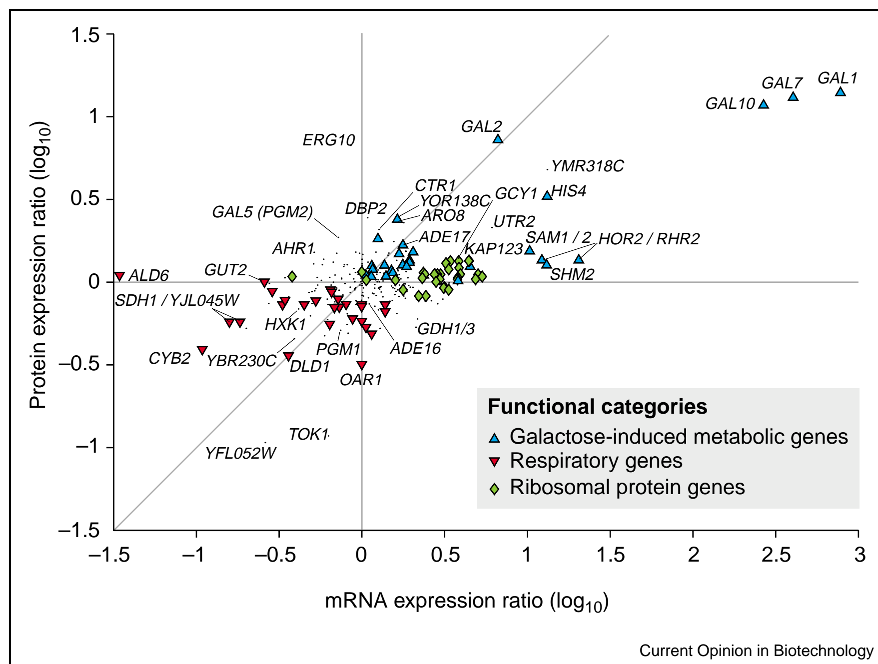
Are the measurement of the cellular response to external perturbations at the mRNA and protein level redundant or complementary?

A main objective of the emerging 'Systems Biology' approach is to use different types of systematic and quantitative measurements to dissect the complex systems and networks that underlay cell physiology [5]. To date, the types of data most commonly acquired in this context are mRNA expression profiles and protein expression profiles. Several studies examined the important question of whether the information obtained from studying the responses of a system to external perturbations measured at the mRNA and protein level is redundant or complementary.

In the first such study the responses of cells of the yeast *Saccharomyces cerevisiae* to perturbations along the extensively studied galactose utilization pathway were analyzed using full-genome cDNA microarrays and quantitative proteomics via the ICAT reagent method [6••]. The protein-abundance ratios of 289 proteins were determined for wild-type cells grown in the presence (+gal) or absence (−gal) of 2% galactose and related to the corresponding mRNA expression ratios. The results, reproduced in Figure 3, indicate a loose positive correlation between the two types of data. More interestingly, the data also suggested that the expression of 15 proteins involved post-transcriptional regulation and that groups of functionally related genes showed similar expression patterns. This is exemplified by the observation that the mRNA levels of several ribosomal proteins increased three- to five-fold in response to galactose addition, whereas the corresponding protein abundances remained unchanged.

A similar study investigated the effects of carbon-source perturbation on steady-state gene expression in *S. cerevisiae*. The cells were grown in galactose or ethanol and the induced changes in mRNA and protein profiles were measured and correlated [7]. For genes involved in glycolysis, the response measured at the mRNA and protein levels correlated well, whereas for mitochondrial proteins involved in energy metabolism and for proteins involved in protein synthesis, significant discrepancies in expression ratios were observed. Using 2DE–MS and gene expression arrays, respectively, to study the activation of human neutrophils by lipopolysaccharide at the protein and mRNA levels, Fessler and co-workers [8•] also found generally poor correlation between the datasets. Collectively, these results emphasize the importance of integrated mRNA and protein expression measurements for understanding biological systems.

Figure 3



Scatter plot of protein expression ratios versus mRNA expression ratios. Ratios of protein expression for 289 proteins were determined using the ICAT technique. The values are plotted against the corresponding mRNA expression ratios measured by full-genome microarrays. Many metabolic (blue triangles) or ribosomal (green diamonds) proteins were elevated in wild type + gal, while those involved in respiration (red triangles) had reduced expression levels. Names of genes that were indistinguishable in both mRNA and protein expression are separated by a slash (from [6••]).

Insights into specific signaling mechanisms

Specific signaling pathways have traditionally been studied at the protein level using antibody-based approaches including western blots, enzyme-linked immunosorbent assays, immunoprecipitation of signaling complexes and immunohistochemistry. Although these methods are extremely sensitive and specific, the reliance on high quality antibodies precludes quick and high-throughput analyses and limits the generality of the approach. Furthermore, an antibody that binds a native protein may not be useful for detecting the denatured protein on a western blot and antibody-based methods necessarily anticipate the events that are being assayed and are blind to unexpected events. The ability of quantitative proteomics technologies to compare the expression levels of hundreds-to-thousands of proteins in cells of different states of activation promises new insights into the mechanism of specific signaling pathways and, more importantly, into the interconnection between different signaling systems.

These expectations were realized by a study in which the ICAT reagent method was applied to investigate the function of the Myc oncoprotein [9•]. By comparing the global pattern of protein expression between *myc*-null and *myc*-plus cells, groups of functionally diverse proteins were identified as being regulated by the presence or absence of Myc. These included those predicted to lead to increased cell growth, reduced focal adhesions, as well as altered morphology. A novel cytoskeletal function of Myc was revealed using this approach, indicating the feasibility of quantitative whole-proteome analysis in mammalian cells to understand the molecular functions of pleiotropic regulators such as Myc. Using the same experimental strategy, Han *et al.* [10] identified an unprecedented number of microsomal proteins and determined their quantitative response to the pharmacologically induced differentiation in human myeloid leukemia (HL-60) cells; Smith and co-workers [11] identified and quantified changes in protein expression between control and camptothecin-treated mouse cortical neurons; and Baliga and co-workers [12] studied the regulatory networks underlying the shift of the extremely halophilic Halobacterium from aerobic to anaerobic phototrophic energy production. These studies indicate that quantitative proteomics can provide new insights into the molecular mechanisms that control diverse physiological functions in prokaryotic or eukaryotic cells.

Beyond profiling

Most cellular functions are not performed by individual proteins but rather by complexes of interacting proteins. It is therefore an important component of quantitative proteomics to identify specifically interacting proteins and to determine differences in the composition of protein complexes isolated from different cellular sources.

A generic strategy to characterize partially purified macromolecular complexes using the ICAT reagent method has been presented (JA Ranish *et al.*, unpublished results). The principle of the method is schematically illustrated in Figure 4. By comparing the relative abundance of peptides from a control sample that does not contain the target complex and a sample that contains the target complex within a matrix of proteins unrelated to the complex, the specific complex components can be identified on the basis of the enrichment factor observed between the two samples. The method was applied to study the large RNA polymerase II pre-initiation complex from yeast cells that was purified from nuclear extracts by a single-step promoter DNA affinity procedure. In a single experiment, 45 of the 49 known components of the complex were identified within a background of 206 contaminating proteins, illustrating the power of quantitative proteomics technology for the characterization of protein complexes.

Stable isotope labeling in combination with the β-elimination of *O*-phosphate was also proposed for the quantitative analysis of phosphopeptides [13]. The study addressed an issue of central importance for proteomics — the comprehensive and quantitative analysis of cellular protein phosphorylation profiles. The study also illustrated the remaining substantial challenges facing systematic protein phosphorylation analysis, including the diverse chemical nature of the phosphate–ester bonds occurring on proteins, difficulties in detecting phosphorylation events in low abundance proteins, and the potential of other groups to undergo β-elimination, thus complicating the interpretation of mass spectra obtained from such experiments.

Rather than analyzing global protein profiles, in what they termed the ‘mass western method’, Arnott and co-workers used quantitative proteomics technology based on stable isotope tagging to selectively detect the presence of the cell-surface proteins prostate stem cell antigen (PSCA) and ErbB2 and to determine their relative abundance in different cell lines [14•]. This method obviates the need for gel electrophoresis or other initial purification steps.

These examples illustrate that proteomics technologies based on stable isotope labeling and quantitative MS are being used in creative experiments to measure biologically informative properties of the proteome that are beyond the sequence and quantity of expressed polypeptides.

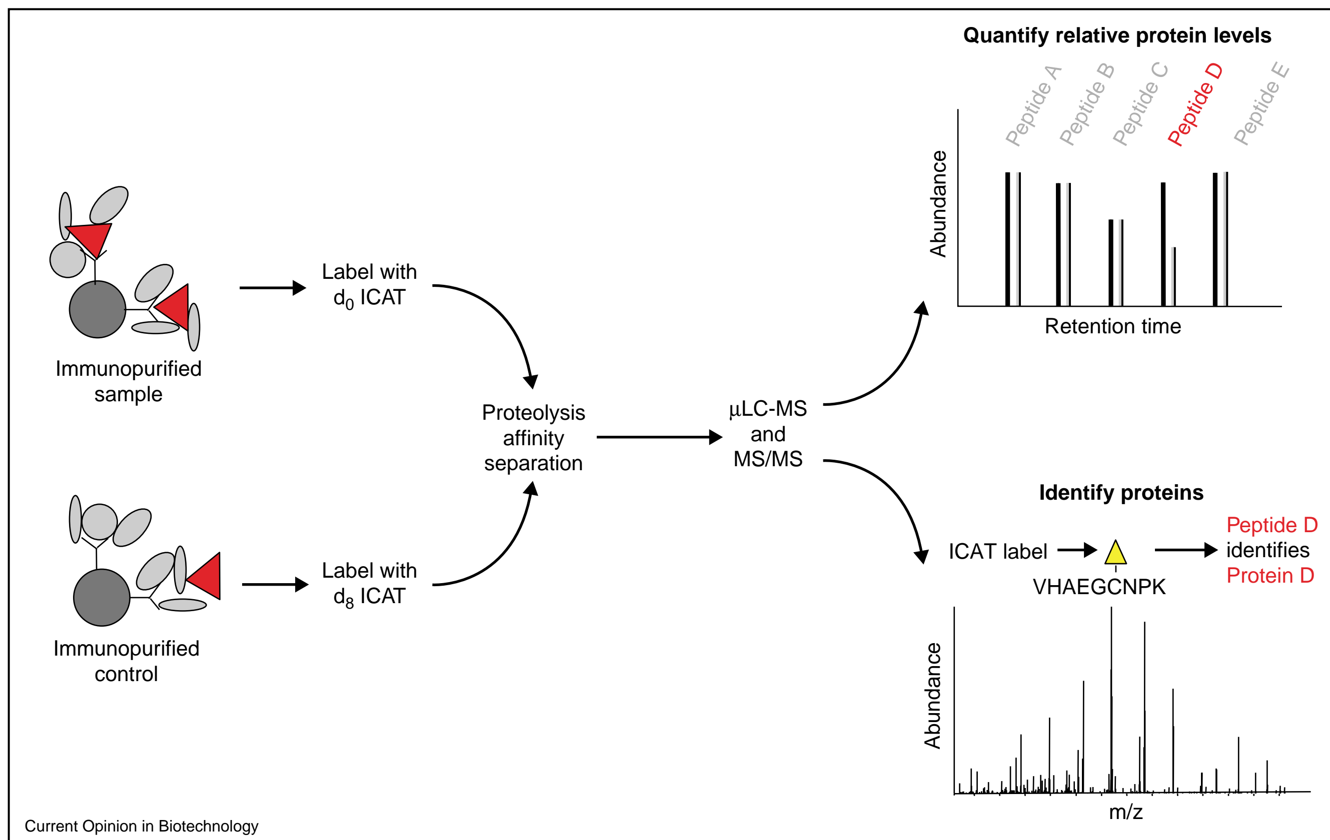
Technical advances

Chemistry

New isotope-coded affinity-tagged reagents

Early applications of the ICAT reagent method identified several drawbacks of the first-generation reagents. First,

Figure 4



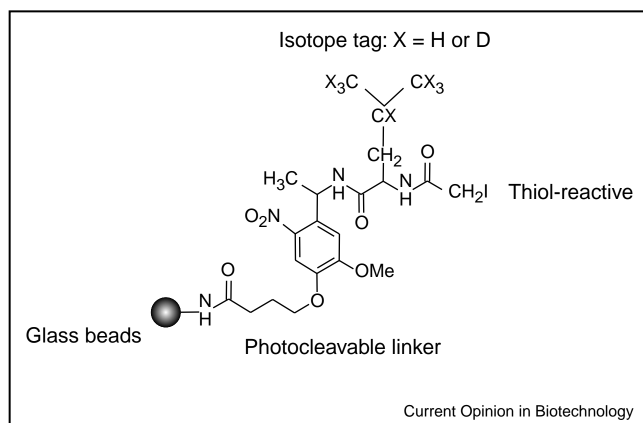
Quantitative proteomics approach for the analysis of affinity-purified macromolecular complexes. To distinguish specific complex components from copurifying proteins, a control purification is performed in which the complex of interest is not enriched. Proteins purified by immunoprecipitation from sample and control are labeled with either the isotopically heavy or light version of the ICAT reagent, and combined. After proteolysis, sample complexity is reduced in three sequential chromatographic steps, and the peptides are analyzed by ESI-MS/MS to obtain their identity and quantity. The relative quantification can then be used to distinguish specific complex components from copurifying proteins.

the combined biotin–isotope tag is relatively large and bulky. As the tag remains on each peptide during MS analysis it is also subject to fragmentation under collision-induced dissociation (CID) conditions and the resulting tag-specific fragment ions can complicate the manual or computer-assisted interpretation of tandem mass spectra of labeled peptides. Second, the eight deuterium atoms that were used as the mass-tags in the original ICAT reagents caused an isotope effect that was sufficient to partially resolve isotopic peptide pairs by high-resolution reverse-phase liquid chromatography (RPLC) [15•]. Therefore, accurate quantification requires the calculation of the abundance ratios on the basis of the integrated peak areas of the heavy and light forms of each peptide, complicating automated data analysis [10].

To address these issues, several second-generation chemistries for cysteine-specific isotope tagging have been developed. Zhou and co-workers [16•] described a method that uses solid-phase capture-and-release to introduce stable isotope tags at cysteine residues and to isolate the tagged peptides. The solid-phase resin was prepared by modifying the surface of controlled pore glass beads with a chemical structure consisting of a photo-cleavable linker, an isotope-tag transfer group and a specific reactive group (Figure 5). Using Fmoc peptide synthesis chemistry two types of beads were synthesized with surface modifications that were chemically identical but differed in the isotopic composition of the tag.

Compared with the solution-phase ICAT reagent method, the solid-phase tag transfer method has several advantages. First, as the isolation of cysteine-containing peptides and incorporation of stable isotopes are achieved in a single step, the solid-phase method is faster and simpler, requires less manual input and is easier to automate. Second, stringent wash conditions are compatible with the covalent capture of peptides, resulting in the recovery of a sample mixture highly enriched for cysteine-containing peptides. Third, no additional steps are needed to remove reagents. Fourth, the minimal number of sample handling steps increases the sensitivity

Figure 5



Structure of reagents for solid-phase isotope tag transfer.

of the method. A side-by-side comparison of solution and solid-phase tagging approaches to the detection of protein expression changes in *S. cerevisiae* demonstrated that the solid-phase ICAT method identified more proteins than the solution-phase method from comparable amounts of protein. Finally, the mass-tag on the cysteine residue is small (170 Da total for the d_0-leucine tag) and undergoes minimal fragmentation, facilitating the interpretation of CID spectra of tagged peptides.

A similar solid-phase approach using an acid-labile resin has also been described [17•] and Applied Biosystems, Inc. (Foster City, CA) recently introduced a new version of ICAT reagents that contain an acid-cleavable linker between the isotope tag and the biotin group [18•]. Peptides labeled with the second-generation ICAT reagents are subjected to the same separation procedures as those labeled with the original version of the reagent, except that as an additional step the biotin group is cleaved off before MS analysis of the tagged peptides. The isotope tag introduced with this reagent consists of ^{13}C instead of deuterium and is relatively small. As expected [15•] the peptides labeled with the $^{13}C_6$ or $^{12}C_6$ isotope tag have virtually the same retention time during RPLC.

Isotope tagging with chemical specificities different from sulfhydryl groups

Thiol-specific reagents have the advantage that they selectively isolate cysteine-containing peptides from a complex sample mixture and therefore reduce the complexity of the sample to be analyzed by MS. The reagents have the disadvantage that proteins that do not contain any cysteines are transparent to the method and that generally relatively low sequence coverage of the identified proteins is achieved. In an attempt to isotopically label every peptide in a protein digest, N-terminal labeling protocols have been described [19,20]. Although in this approach virtually every peptide is quantified, the enormous complexity of the peptide mixtures generated by the digestion of complex protein mixtures makes N-terminal peptide tagging challenging for proteome-wide analyses. Several other approaches have been investigated to selectively isolate proteins or peptides that contain a specific functional group [21,22]. Generally, these methods are based on the use of affinity enrichment protocols. They include the selection of histidine-containing peptides via immobilized metal affinity chromatography (IMAC) on Cu(II) columns [22], and the isolation of phosphorylated peptides [13,23–25] and glycosylated peptides [26]. It can be expected that in the near future additional selective chemistries will be developed.

Enzyme-catalyzed ^{18}O and metabolic stable isotope labeling

Besides isotopic tagging of peptides using chemical reagents, the venerable method of using proteases to introduce ^{18}O from $H_2{}^{18}O$ into carboxyl groups during proteolysis was re-evaluated and successfully applied [27,28]. Although this is a simple and useful isotopic labeling method, it is by no means without pitfalls. The small mass difference between the light and heavy forms of a peptide complicates quantitative analysis, especially in low resolution and electrospray ionization (ESI) mass spectrometers, the exchange can be inhibited by urea and the rate of exchange can be structure specific.

Apart from chemical and enzymatic methods, metabolic protein labeling has also been used to introduce stable tags into proteins for quantitative proteomics. Whereas in the past metabolic stable isotope labeling was achieved by using culture media containing salts that were isotopically enriched [29–31], or depleted [31,32], more recently proteins were also labeled via the metabolic incorporation of 'heavy' amino acids [33•,34–36]. These advances provide the opportunity to also perform metabolic labeling in cultured mammalian cells. The labeling of proteins in whole higher organisms remains, however, beyond the reach of metabolic stable isotope labeling methods.

Non-isotopic reagents

A method that is conceptually analogous to the ICAT reagent labeling method but does not make use of stable isotopes was described by Cagney and Emili [37]. The method, termed mass-coded abundance tagging (MCAT), relies on the selective and quantitative guanidination of the ε-amino group of C-terminal lysine residues of tryptic peptides and thus the transformation of lysines into homoarginine. After differential modification of tryptic peptides contained in one sample and the combination of the tagged peptides with the untagged peptides in a second sample, the relative abundance of each peptide contained in the two samples can be estimated by mea-

suring the relative signal intensities of each sister peptide pair in full-scan MS mode. The relatively large mass difference (42 Da) between unmodified and modified peptides and the potential for partial modification of the α-amino groups during the guanidination reaction potentially complicate the MCAT approach for the analysis of very complex mixtures.

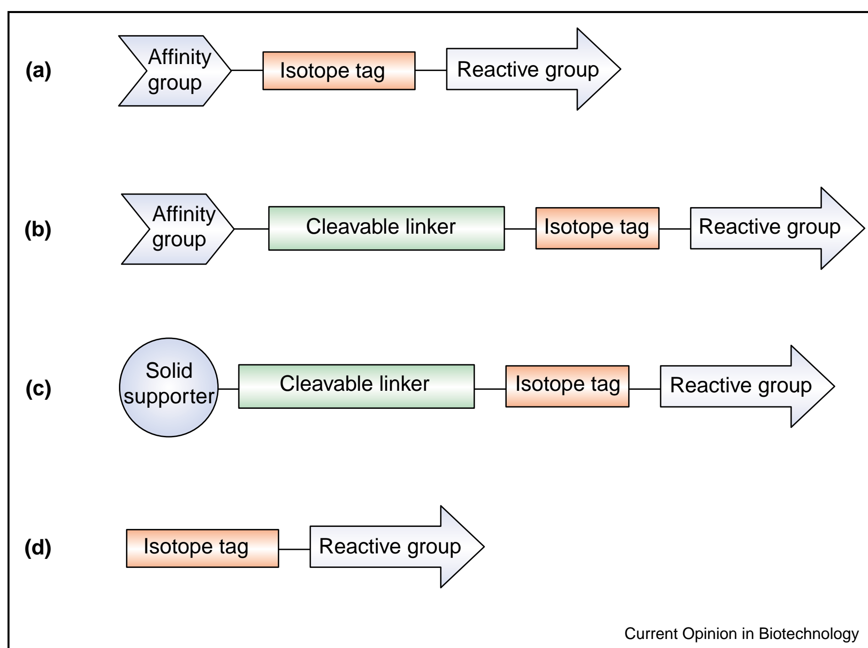
Data acquisition and instrumentation

To date, most analyses based on the ICAT reagents or related methods have been carried out using reverse-phase microcapillary liquid chromatography coupled on-line with MS (RP-μLC MS) and RP-μLC MS/MS. In such experiments, peptides at specific mass-to-charge (m/z) values are selected for CID using a data-dependent software routine with dynamic exclusion and are identified by searching the resulting CID spectra against a protein sequence database. The abundance ratio is then determined for each identified peptide. Usually a routine of alternating MS and MS/MS scans is used to collect both qualitative and quantitative data in the same experiment.

Further developments have been made on improving peptide identification and accurate quantification. One such case is the establishment of an empirical statistical model to estimate the accuracy of peptide identifications assigned to CID spectra made by database search applications [38]. The model computes probabilities that peptide assignments to spectra are correct on the basis of database search scores, providing a particularly useful criterion for distinguishing correctly from incorrectly assigned peptides. It also has become apparent that the fact that the analytes contain isotopic signatures can be used to adapt the MS strategies for their analysis. An early example of such an adaptation is the development of instrument control protocols in a matrix-assisted laser desorption ionization ESI electrospray ionization (MALDI) quadrupole time-of-flight MS (QqTOF) instrument that automatically selects precursor ions on the basis of abundance ratio of detected, isotopically labeled peptide pairs [39]. The rationale for the method is the observation that in most proteomic experiments that compare perturbed cellular states, the abundance of the majority of the detected proteins remains unchanged. The described protocol therefore focuses the mass spectrometer on those peptides that show a difference in expression, that is, the biologically most informative species.

The use of MALDI-MS/MS offers the significant advantage that the temporal constraints of on-line detection that are typically encountered with ESI-MS/MS are eliminated as the peptides separated by μLC are deposited on a MALDI sample plate before MS analysis. It can be expected that the absence of this temporal constraint will catalyze the development of further MS and MS/MS

Figure 6



Summary of available isotope tagging reagents. **(a)** Classical ICAT reagents that contain an affinity group, an isotope linker, and a reactive group. **(b)** Cleavable ICAT reagents that have an additional cleavable linker between affinity group and isotope tag; both (a) and (b) reagents are suitable for solution-phase derivation of cysteine residues on proteins. **(c)** Reagents for stable isotope tag transfer via solid-phase capture and release. **(d)** Reagents that introduce a stable isotope tag on peptides.

protocols that focus on selected proteins of great biological significance, a trend that will surely be accelerated by the availability of new types of MALDI MS/MS instruments such as the MALDI-TOF-TOF (MALDI tandem time-of-flight) [40–43].

Conclusions

With steady advances in chemistry (summarized in Figure 6), instrumentation and reference tools for the generation of stable isotope tagged polypeptides and their analyses, quantitative proteomics is poised to realize its immense potential for basic and clinical research. Fully robotized, high-throughput platforms, robust isotope tagging chemistries with different selectivities, improved transparent, probability-based data analysis tools and optimized interfaces between the analytical platforms and the biological specimens remain significant challenges facing the proteomics field. It can be expected that current and emerging proteomic approaches will find applications to various biological systems [44,45] and that the coming years will demonstrate their enormous utility for drug discovery, disease prevention and diagnosis and basic biological research.

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