Review

The dynamic state of protein turnover: It's about time

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The continual destruction and renewal of proteins that maintain cellular homeostasis has been rigorously studied since the late 1930s. Experimental techniques for measuring protein turnover have evolved to measure the dynamic regulation of key proteins and now, entire proteomes. In the past decade, the proteomics field has aimed to discover how cells adjust their proteomes to execute numerous regulatory programs in response to specific cellular and environmental cues. By combining classical biochemical techniques with modern, high-throughput technologies, researchers have begun to reveal the synthesis and degradation mechanisms that shape protein turnover on a global scale. This review examines several recent developments in protein turnover research, emphasizing the combination of metabolic labeling and mass spectrometry.

New tools to measure protein turnover

Cells, whether bacteria, protozoa or neurons, live in a constant state of flux, yet achieve a state of dynamic equilibrium with their surroundings. Rather than existing as invariant scaffolds and indefatigable enzymes, cellular proteins are continually destroyed and renewed, even when total amounts remain constant [1–5]. Protein abundance can change, however, in response to external stimuli, developmental programs or the onset of disease states, eventually establishing a new equilibrium. Until recently, changes in cell states could only be measured in aggregate by considering all proteins [6], or in a highly targeted fashion, whereby specific proteins or the mRNA species that give rise to them are individually (and often painstakingly) assayed.

Omic techniques, including microarrays, next-generation DNA sequencing, high-throughput screening and mass spectrometry (MS), are ushering us from the golden age of molecular biology to the era of systems biology [7]. Biological understanding is increasingly communicated in the languages of mathematics, physics and computational modeling to precisely describe and predict higher-order, dynamic phenomena [8,9].

The changes in protein abundance that these technologies measure, however, are often understood to arise from increases or decreases in gene expression. Tools such as DNA microarrays, DNA sequencing and quantitative PCR (qPCR) are exquisitely sensitive but can only be used to describe half of the synthesis–degradation equation. It might be just as likely, and in many cases more likely, that altered proteolysis rates efficiently augment or diminish protein pools [10,11]. Thus, in the face of varying environmental stresses, cells continually adjust both synthesis and degradation to maintain protein homeostasis. This largely explains the often poor correlation found between transcript and protein abundance [12–16]. As cell biologists endeavor to understand how complex, dynamic regulatory networks function within cells, it will be crucial to directly measure not only the net magnitude and direction of protein abundance changes, but the concerted constructive and destructive mechanisms that produced these changes as well.

Protein turnover, the balance between synthesis and degradation, is one of many forms of regulation that are coordinated to achieve a unified cellular response. Accounting for protein turnover holds the potential to reveal crucial intersections of cellular regulation that so far have been invisible to chemical or genetic manipulations, and will thus be an essential component of systems-level models of cell behavior (Box 1). However, large-scale measurements of protein turnover rates have remained elusive. This review will focus on the burgeoning methods that hold the greatest promise for reaching this goal.

80 years of progress

Modern high-throughput methods for measuring protein turnover are built upon decades of small-scale studies. Degradation rates or, more conveniently, half-lives of specific proteins, have usually been measured by metabolically labeling cells in culture or in live animals with radioactive or isotopically enriched tracers. The first of these studies provided evidence for amino acid biosynthesis and recycling by using early mass spectrometers to quantify elemental ^{15}N from mice fed ^{15}N-labeled amino acids [17,18]. Later, following careful biochemical purification, scintillation counting-based detection demonstrated the differential turnover rates of subcellular compartments and isolated proteins from rats fed radioactive ^{14}C [19]. Two-dimensional electrophoresis (2DE) provided a marked improvement in throughput and was used to simultaneously measure synthesis and degradation rates for dozens to hundreds of distinct, yet unknown, proteins from radiolabeled [20,21] *Escherichia coli*. The introduction of antibody-based detection provided a more convenient way to assay the degradation of specific proteins, and has been used as follows: all proteins are metabolically labeled by culturing cells in "pulse" medium containing a radioactive element (e.g. ^{35}S) and then in chase medium containing the non-radioactive form (e.g. ^{32}S). The proteins of interest are

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0962-8924/$ – see front matter doi:10.1016/j.tcb.2011.02.002 Trends in Cell Biology, May 2011, Vol. 21, No. 5

Box 1. Correlating half-lives with protein function and regulation

Degron analysis. Half-life measurements of engineered proteins demonstrated that amino-terminal residues influence protein stability [81]. Furthermore, degron sequences such as those rich in proline, glutamic acid, serine and threonine (PEST) along with biophysical protein properties (e.g. length, molecular mass, isoelectric point and surface area) also affect protein stability [82–85]. Although these general principles have had limited or inconclusive predictive value [24,38,40], they will likely lend themselves to refinement with large-scale analyses.

Differentially regulated isoforms. A single gene can serve as the template for dozens of protein isoforms; understanding the differences in their properties is a major challenge in cell biology. Besides differences in localization, abundance and protein–protein interactions, isoforms may exhibit markedly different stabilities that might provide insights into their function and modes of regulation.

Co-regulated functional classes. Large turnover datasets can be mined for associations between protein turnover rates and gene ontology classes [24,38] or known protein networks to elucidate regulatory trends. Conversely, identifying similarly functioning proteins with uncorrelated half-lives could provide an avenue for discovering their specialized roles.

Protein complex dynamics. Subunits of multimeric proteins were observed, through targeted efforts, to be far less stable in their solitary state, as would be expected immediately following synthesis [86]. Whereas complex membership seems to convey stability to all components, it has been suggested that some subunits undergo targeted turnover regulation that controls the activity of the entire complex; by rapidly tuning the abundance of critical complex components, cells can shift the equilibrium between complex assembly and disassembly [87]. Combined with other proteomics techniques, turnover measurements can unravel the nature of protein complex regulation.

Non-linear kinetics. Although synthesis and degradation rates are understood to follow linear kinetics under steady-state conditions, data collected across multiple time points might not support this assumption. Several groups have noted biphasic kinetics, whereby proteins demonstrate a rapid initial turnover rate, followed by a second, slower rate [41,88–90]. The defective ribosomal product (DRiP) hypothesis provides one explanation: many newly synthesized proteins are likely to be erroneously processed post-translation and are destined for rapid destruction, allowing properly formed, more stable proteins to gradually accumulate [91].

isolated at each time point by affinity reagents and turnover rates are determined by comparing the levels of radioactivity (Figure 1b).

Another classic method for studying protein degradation in eukaryotes involves the chemical inhibition of eEF2-mediated ribosome translocation with cycloheximide [22]. With the synthesis of nascent proteins blocked, degradation rates can be measured by Western blot, using antibodies targeted to the proteins of interest. The advantages of this method are that it does not require radiolabeling, and can assay specific proteins by Western blot more efficiently than the immunoprecipitations required by pulse–chase methods. However, the dependency on antibody-based detection limits the throughput of this method. Furthermore, cycloheximide-based inhibition induces substantial cellular stress, which can greatly skew stability measurements, particularly for longer-lived proteins [23].

Variations of these approaches continue to extend our understanding of protein dynamics because they require relatively common reagents and equipment and, thus, are accessible to a wide range of research laboratories. However, they represent compromises between sensitivity, specificity, accuracy, undesired perturbations and experimental ease. Despite their limits in throughput, classic techniques have taught us much about the relationships between synthesis, degradation and abundance, as well as the general principles of protein stability and turnover. Nevertheless, many questions remain unanswered (Box 2). Addressing these questions with truly comprehensive datasets will enable nuanced comparisons between numerous cell states, and more accurate predictions of how normal and diseased cells respond to various stimuli. Modern methods aim to more efficiently and accurately assess turnover rates and, at the same time, preserve the resolution of individual proteins. This Review explores how high-throughput technologies address the throughput deficits of older techniques, and evaluates the strengths and shortcomings of diverse modern proteomic approaches.

Large-scale measurements of protein turnover

By combining the principles of traditional, focused protein dynamics investigations with cutting-edge omic approaches, we can describe the qualities of an ideal global protein turnover experimental platform (Table 1). At present, no single methodology fulfills all of these requirements, but tremendous technical advances over the past five years indicate that proteome-wide turnover studies are within reach. Existing technologies either measure turnover rates directly or rank protein stabilities in a more relative fashion, and can be divided into two categories: those that require genomic tags and those that do not.

Tagging

One way to ensure global protein characterization is to tag proteins of interest with affinity or fluorescent tags. This was first demonstrated by measuring protein half-lives using more than 4000 tandem affinity protein (TAP)-tagged yeast strains. Protein synthesis was chemically inhibited with cycloheximide and protein stability was inferred for more than 3000 proteins from quantitative Western blot measurements of each tagged construct. Clustering analysis based on protein production rates, degradation rates and abundance led to the conclusion that yeast have two main patterns of protein turnover, one consisting of generally stable, highly abundant proteins involved in amino acid metabolism and protein biosynthesis, and another consisting of rapidly degraded, less abundant proteins that promote regulatory flexibility [24].

Box 2. Outstanding questions

- For any given protein, what governs the opposing forces of synthesis and degradation to achieve a desired abundance level?
- Which proteins are controlled by synthesis or degradation versus by sequestration or reversible post-translational modification, and why?
- Which proteins are degraded primarily by the proteasome versus the lysosome or other non-proteasomal means?
- How much do protein turnover rates vary with activation state, cell type, species, growth rate, etc.?
- Which complexes, molecular machines and organelles are turned over as a unit, and which have varying degrees of stability amongst their components?
- How much interchange occurs between a free monomer pool and multimeric complexes?

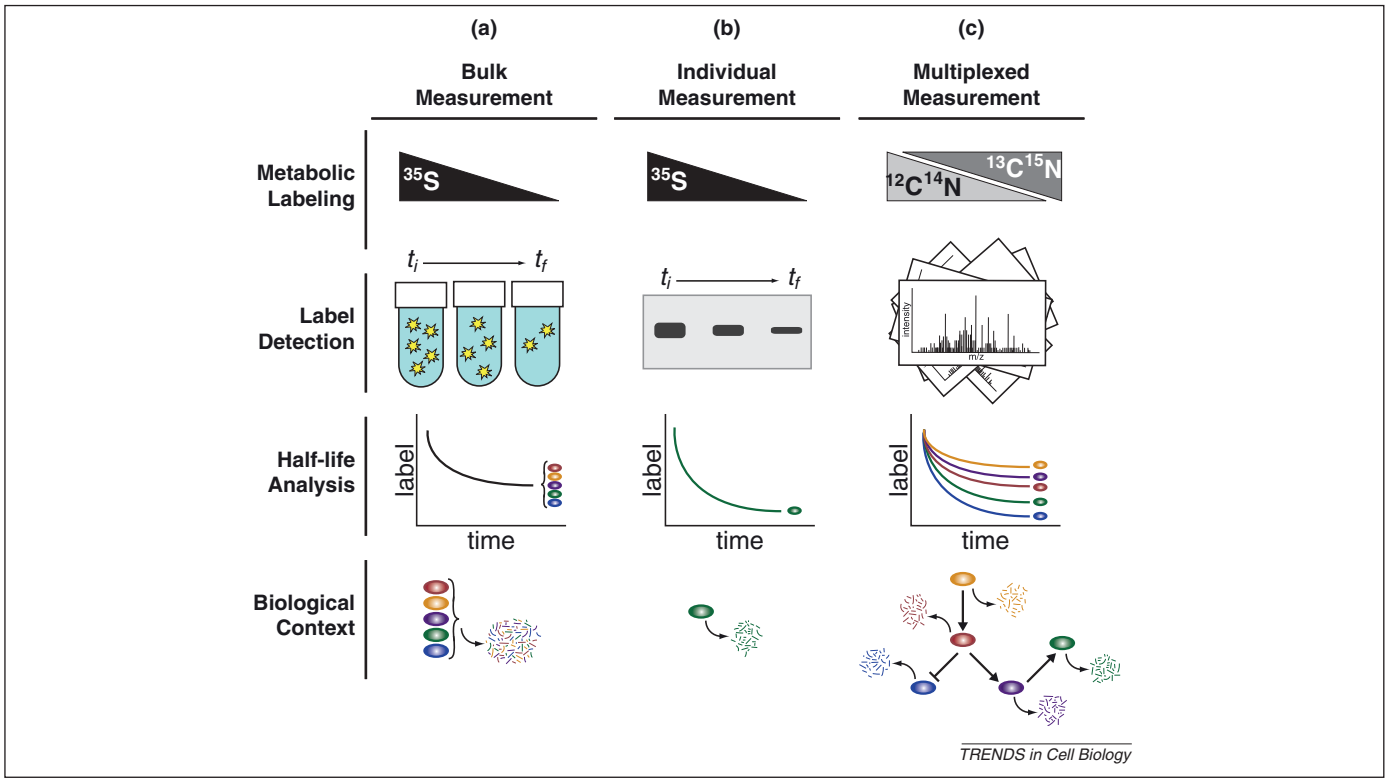

Figure 1. Measuring turnover of metabolically labeled proteins. **(a)** A classical experiment in which all proteins are labeled with a radioactive element (e.g. ^{35}S). The loss of radioactivity over time, measured by scintillation counting, corresponds to protein degradation. This measurement of bulk protein degradation reveals general characteristics about the overall degradation half-life of proteins in the cell. **(b)** All proteins are labeled with a radioactive element (e.g. ^{35}S). The half-life of a single protein of interest can be measured by coupling affinity purification with autoradiography, as depicted. Similarly, cycloheximide chase combined with western blot analysis can be used to measure the turnover of an individual protein. Results from these experimental platforms indicate degradation rates for only the assayed proteins. **(c)** All proteins exist in the natural $^{12}C^{14}N$ form. With the introduction of chronic labeling with $^{13}C^{15}N$, all newly synthesized proteins incorporate the stable, heavy isotopic label. Using mass spectrometry, the loss of $^{12}C^{14}N$ forms and the accumulation of $^{13}C^{15}N$ forms can yield information about degradation and synthesis of all detected proteins. Compared to methods for bulk or individual turnover measurement, methods relying on stable isotope tracers can determine half-lives in a high-throughput manner and thus, provide more insight into dynamic signaling networks.

This approach was extended with a multiplexed tagging strategy coupling flow cytometry with microarray analysis. This method, global protein stability (GPS) profiling, obviated the need for translation inhibition, while greatly improving throughput. GPS profiling was used to measure protein stabilities of nearly 8000 proteins in HEK293T cells. Ontological analysis suggested that proteins with short-to-moderate half-lives were enriched for cell-cycle

Table 1. Ideal criteria for large-scale protein turnover measurements

Criterion	TAP-tag	GPS	Bleach-chase	Ribosome profiling	Metabolic labeling and MS
Unbiased, global detection	++[a,b]	++[a,b,c]	++[a,b]	++[b,c,d]	+(++)[e]
Assay endogenous proteins	–	–	–	+++	+++
Unambiguous quantification, identification	++	+++	+++	+++	++
Simultaneous synthesis and degradation measurements	+[f]	++[g]	–	+[h]	+++
Minimal cell perturbation	–	+	+	+++	+++
Spatial resolution	–	+(++)[i]	+(++)[i]	–	++[j]
Single-cell resolution	–	+++	+++	–	–
Applicable to *in vivo* systems	–	–	–	+++	++[k]

[a]Not all tagged proteins will be expressed at detectable levels.

[b]Problematic distinction of post-transcriptionally modified forms.

[c]Global detection possible through addressable, microarray detection of expressed species.

[d]Global detection possible through exhaustive deep sequencing.

[e]Exhaustive proteome characterization possible, but not yet demonstrated in this context.

[f]Degradation only. Turnover calibrated with external data sources.

[g]Net sum of synthesis and degradation.

[h]Synthesis only. Turnover calibrated with external data sources.

[i]Subcellular localization possible if fluorescence is measured by microscopy, but has not been demonstrated.

[j]Can be evaluated through subcellular fractionation [96].

[k]Incomplete labeling in intact animals can yield sub-optimal data (Box 3 and Figure 2).

control proteins already known to be unstable, while long-lived proteins included structural proteins such as actins and tubulins [25].

Another method called bleach–chase allowed researchers to probe the dynamics of fluorescently tagged proteins in live H1229 cells. Differences in fluorescence decay between cells bleached with a brief pulse of light and non-bleached cells were used to extrapolate protein half-lives. Half-lives ranging from 0.75–22.5 h were measured for 100 YFP-tagged proteins. Furthermore, when cells were subjected to various forms of stress, an overall increase in half-life was observed in long-lived proteins [26].

Tagging studies demonstrated the power that large-scale turnover measurements have for discerning general principles relating protein stability with protein function. These strategies have allowed researchers to investigate the activities of specific protein classes [27] in a way that would be difficult with standard affinity-based protein assays. Nevertheless, caution should be exercised when interpreting these measurements quantitatively [24]. Any study involving ectopic expression of tagged proteins carries the risk of the tag disrupting structure, function, binding properties, localization or stability of the protein. It has been argued that such proteins need not retain native function as long as they can act as reliable reporters of endogenous proteins [28]; however, it is plausible that ectopic expression or overexpression could unpredictably alter synthesis or degradation. Although these responses might not change overall protein levels dramatically, they remain a central concern for turnover studies, because manipulating synthesis or degradation would render protein turnover measurements inaccurate. Finally, creating clonal libraries of tagged proteins represents a large investment in time and resources. It might not, therefore, be feasible to apply these strategies in a variety of cell lines, or in less genetically tractable systems, including primary cells or live animals.

Non-tagging

Addressing the role of protein turnover on the proteome level ideally utilizes large-scale analytical methods that minimally perturb the biological system (Table 1). One way to measure the steady-state dynamics of endogenous proteins is to focus solely on their synthesis. Ribosome density mapping (RDM) was developed to study protein synthesis rates by measuring the spatial distribution of ribosomes on transcripts. RDM extended polysome profiling, a microarray-based approach for ranking mRNA translation rates to include a step in which gene-specific antisense oligonucleotides were annealed to transcripts, creating RNase H-sensitive regions [29,30]. The resulting fragments were separated by a second sucrose gradient to provide better resolution of ribosome occupancy and density across specific transcripts, as measured by Northern blots. These studies laid the foundation for a deep sequencing strategy for measuring protein translation [31]. After polysomes were recovered from cycloheximide-treated yeast, they were digested with RNase I to produce short (28 nt) ribosome-protected mRNA fragments. Parallel sequencing of DNA reverse-transcribed from these fragments delivered extremely precise mapping of ribosome positions such that individual codons could be ascertained. The ratio of ribosome density to overall mRNA transcript abundance provided a quantitative measure of protein synthesis, which was correlated more favorably with protein abundance than mRNA transcript levels alone [31]. Although the synthesis assays described here do not explicitly measure protein levels, data from complementary methods, most notably MS as discussed below, stand to complement these studies.

Experiments using GPS and ribosome profiling methods captured cell states at a single moment in time, rather than measuring the changes of experimental variables over time. Thus, these methods measure only the relative extents of protein degradation or synthesis. TAP-tagging and bleach–chase strategies included time series, but several experimental variables including treatment with cycloheximide (TAP-tag), artificial protein expression and imprecise quantification might limit the reported protein half-lives from being extrapolated to other biological systems [24,26]. Instead, the experimental data should be viewed as relative rankings of protein stability that can help identify large-scale regulation of protein dynamics.

More quantitative, global measurements of synthesis and degradation rates will allow researchers to make increasingly accurate and predictive models of cellular responses. Given their capacity for yielding true rate constants from endogenously expressed proteins, both *in vitro* and *in vivo*, MS-based methods appear to have the potential for satisfying most of the conditions given in Table 1. We now focus on MS-based methods for studying protein turnover, and outline several points to consider when conducting these experiments and interpreting results.

Direct measurements of protein turnover by mass spectrometry (MS)

Recent advances in MS present an opportunity to study native protein turnover on a truly comprehensive scale [32–34]. Where radiolabeling and Western blot-based studies require painstaking techniques to isolate, identify and quantify a protein of interest, now thousands of endogenously expressed proteins can be simultaneously measured from a single biological sample (Figure 1). Protein turnover analysis by MS has been accomplished by adapting classic pulse–chase or chronic labeling protocols to stable isotope labeling by amino acids in cell culture (SILAC) workflows [32,35–42].

Typically, SILAC has been used in large-scale, MS-based proteomics investigations to make quantitative, binary comparisons between light (containing only naturally occurring isotopes) and heavy (isotopically enriched) samples. Proteins labeled in such a manner are chemically identical to naturally occurring forms but exhibit a defined mass shift that can be detected by MS. Following mixing, light to heavy ratios of specific peptides can be recorded and used to infer the relative abundance of their respective proteins (Figure 2a) [35,36].

Steady-state protein turnover is measured not by mixing two labeled samples, but by introducing isotopically enriched labels in pulse–chase or chronic labeling contexts.

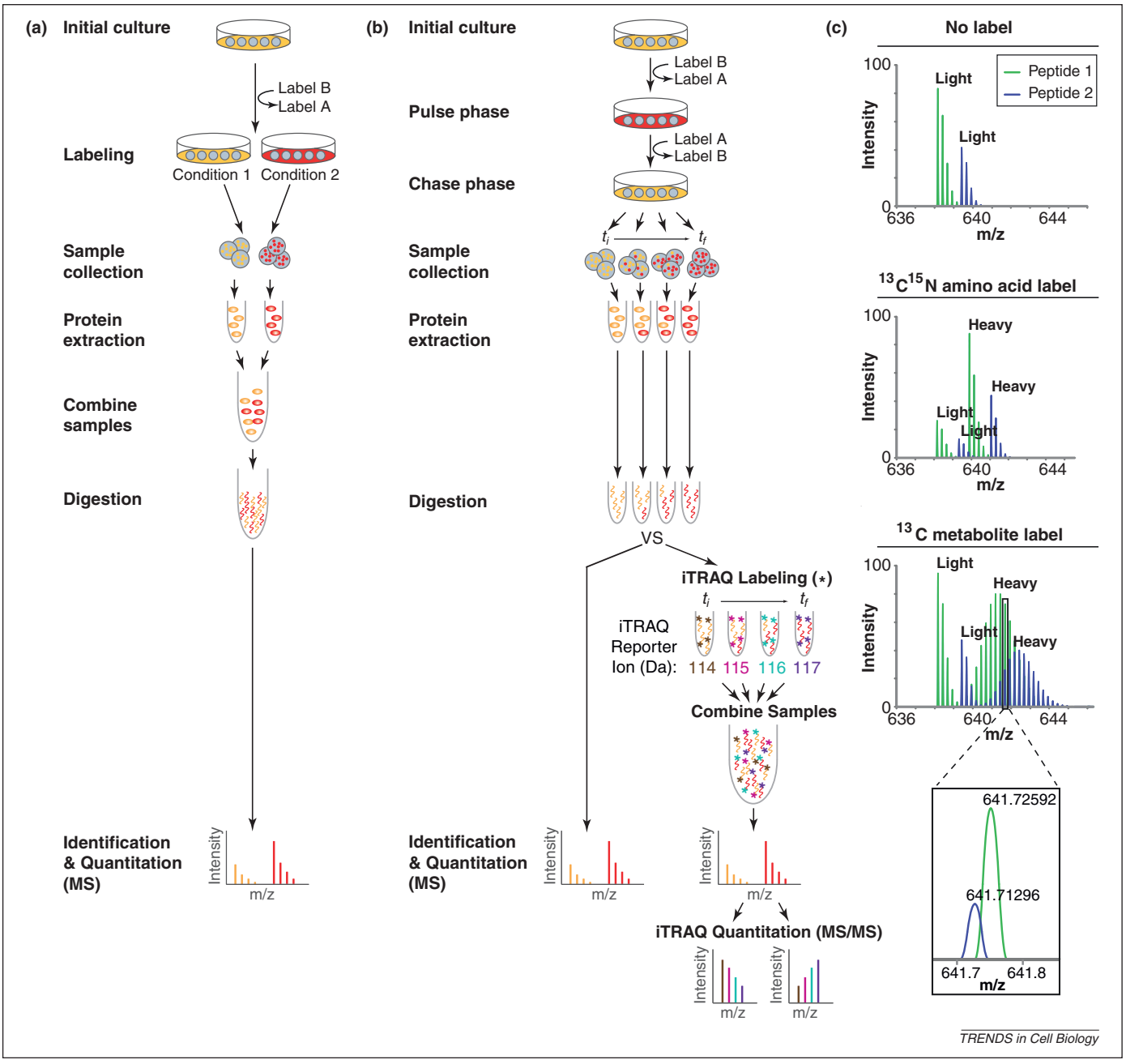

Figure 2. Comparison between SILAC and dynamic labeling methodologies. **(a)** Workflow of a typical SILAC experiment [36]. Cells are divided into two populations, which are then completely labeled with stable isotope labels A (yellow) and B (red). The two labeled cell populations are treated under different conditions. Protein extracts of the two samples are combined and digested enzymatically for analysis by mass spectrometry. The relative abundance of proteins can be inferred from the ratios of peak intensities in the mass spectra. **(b)** Dynamic labeling strategies adopt the use of SILAC in the context of a pulse-chase labeling experiment [32,37–39,41]. Cells are pulsed with label B and subsequently chased with label A. Samples are collected during the chase then protein extracts are made and digested for analysis by mass spectrometry; in the case of experiments performed under non-steady-state conditions, peptide samples from each time point are chemically labeled with iTRAQ reagents (denoted by an asterisk) then combined [40]. Quantitative reporter fragment ions of masses in the range 114–117 Da (brown, pink, teal and purple), derived from the iTRAQ tag in MS/MS spectra are used to determine the contribution of each time point to the metabolic labeling ratio determined by MS. (c) The introduction of a metabolic isotopic label necessarily complicates spectra as shown in the top and middle panels. The use of isotopically enriched metabolites such as glucose or salts further expands the number of isotopic peaks, which obscure the detection of multiple peptides (peptide 1, green; peptide 2, blue) occupying the same *m/z* range as that shown in the lower panel [37,39]. Interpreting such spectra requires a high-resolution mass spectrometer to distinguish co-eluting peptides (inset).

Monitoring the change in relative abundance of heavy and light protein variants over time allows protein dynamics to be tracked (Figure 2b). In one example, A549 cells were labeled with [$^{13}C_6$]arginine and chased with [$^{12}C_6$]arginine to measure the half-lives of more than 500 proteins [38]. This approach, with various modifications, has been applied to numerous model systems, ranging from single-cell organisms to mice, with minimal unintended perturbation [32,37–42].

Important considerations

Successful turnover measurements are highly dependent on several factors, including the selection of a suitable metabolic tracer and downstream sample processing (Figure 2c; Box 3). Workflows should be designed to best suit the model system and available instrumentation. Several of the most salient considerations include metabolic label choice (Box 3), steady-state conditions, basal labeling state and instrumentation.

Box 3. Considerations for metabolic labeling.

Criteria for ideal labeling with amino acids:

Cells are auxotrophic for the desired amino acid. Ensures the labeling pool will not be contaminated by endogenously synthesized amino acids.

The amino acid is highly abundant. Generally allows for at least one heavy amino acid per peptide.

The amino acid is metabolically isolated. Prevents labeled atoms from being transferred to unexpected amino acids. Arginine, for example, produced by the ornithine cycle, is a metabolic precursor for proline. Thus, heavy atoms from labeled arginine might become reincorporated into proline, adding an unintentional level of sample complexity while diminishing quantification accuracy [92,93].

The heavy and light labels have a mass difference of at least 4 Da. For optimal distinction between isotopic envelopes, the most abundant heavy isotopic peaks (usually the monoisotopic or second isotopic peak) should be readily distinguishable from higher isotopic peaks of the light distribution (Figure 2c).

Minimal increase in mass spectrum complexity. A typical peptide will be observed in three to six isotopic forms, due to the natural abundance of ^{13}C in biological material. Typical SILAC-style labeling with an enriched amino acid will double the number of isotopic peaks per peptide, whereas incorporating enriched atoms (^{13}C or ^{15}N) into all amino acids will further expand the number of isotopic peaks (Figure 2c). Increased numbers of isotopic species diminish the ability to distinguish and measure multiple peptides that occupy similar space in the mass spectrum.

Pair amino acid label with a compatible protease. Commonly used labels and enzymes are lysine and arginine with trypsin, and lysine with endoproteinase Lys-C; these pairings ensure that all proteolytic peptides contain at least one labeled residue, with the exception of proteins' C-terminal peptides (for carboxyl proteases).

Assumptions about metabolic labeling:

Negligible delay in amino acid incorporation. For most systems, the time taken for an amino acid to be imported into a cell, charged to an appropriate tRNA and displace the pre-existing charged tRNA pool is not well characterized. If this process takes several minutes, it could compromise the ability to measure turnover rates of short-lived proteins accurately.

Minimal amino acid recycling. If the charged tRNA pool contains a substantial proportion of recycled amino acids, transitions between labeled states will be blurred. The extent of recycling can be estimated using peptides containing multiple labeled residues [50], and used as a corrective factor.

Kinetic trends for protein synthesis and degradation. Synthesis tends to be independent of protein concentration and follows zero-order kinetics:

$$S_t = s\Delta t \quad [1]$$

where S is the protein synthesized over the time period given by Δt, and s is the protein synthesis rate.

Degradation rates are dependent on protein concentration and tend to adhere to first-order kinetics:

$$D_t = D_0 e^{d\Delta t} \quad [2]$$

where D is the protein degraded over the time period given by Δt and d is the protein degradation rate [94]. A more detailed review of modeling protein kinetics is given in reference [95].

Steady-state versus non-steady-state

In a given cell, expression programs and degradation pathways are continually tuned to maintain a dynamic equilibrium. Decades of turnover research have primarily focused on steady-state systems, particularly in cells undergoing steady-state, asynchronous log-phase growth. Compared to non-dividing cells, growing cells incorporate relatively large amounts of label during the course of an experiment, making it possible to quantify very large changes, which can improve measurement accuracy. Unlike differentiating or perturbed systems, steady-state cells provide a simplified analysis paradigm in which it can be assumed that overall synthesis and degradation rates are balanced, cellular protein concentrations are constant and any net changes in protein abundance can be attributed entirely to cell growth. Measured growth rates can therefore be used, along with measurements of flux—the sum of basal protein turnover and synthesis due to growth—to calculate protein degradation rates. Turnover studies performed under steady-state growth conditions that do not take cellular growth into account, however, describe only the overall flux of protein synthesis and degradation [40]. Conversely, for non-dividing, steady-state cells such as quiescent cells, no such transformation is necessary. In these cases, however, without cell division, it might not be possible to achieve sufficient, quantifiable label incorporation during the course of the experiment. The question of whether protein stability measured under steady-state growth is comparable with that measured during quiescence remains unanswered.

When cells undergo differentiation or experimental manipulation, and do not conform to steady-state conditions, constant cellular protein concentrations can no longer be assumed. Thus, changes in protein abundance must be considered when measuring protein turnover. One strategy for accomplishing this was demonstrated recently using the multicellular, continually differentiating bacterium *Streptomyces coelicolor*, which was completely labeled with heavy amino acids before being returned to the standard medium. To simultaneously measure changes in protein levels across each of four time points, lysates prepared from each sample were digested with trypsin and labeled with one of four isotopic variants of the iTRAQ chemical label (Figure 2b) [43]. All four chemically labeled samples were then combined, and subjected to liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. Light to heavy ratios were measured from intact arginine-containing peptides and the proportion of the light or heavy isotopic distributions contributed by each time point was determined from quantitative reporter ions in the MS/MS fragmentation spectrum (Figure 2b). These data were sufficient to simultaneously determine the amount of heavy-labeled protein that had degraded by the indicated time point, as well as the amount of light-labeled protein that had been newly synthesized. As expected, abundance-corrected turnover rates were deemed more reflective of true protein dynamics, although these conclusions were drawn from a modest number of protein measurements ($n < 70$) [40]. This report demonstrated the feasibility of measuring complex protein dynamics without the need to rely on assumptions of constant protein levels. Applying this multi-tagging approach to other systems, both steady-state and non-steady-state, might provide answers to questions regarding the protein dynamics of growing versus quiescent cells.

Basal labeling state

Considering traditional pulse–chase experiments, cells are first metabolically labeled with a tracer isotope, the loss of which is monitored after they are returned to the standard

(non-radioactive) medium. This strategy is effective because it does not require complete labeling of a target protein to monitor its decay. In contrast, accurate quantification by MS requires that the relative abundances of isotopically distinct species are compared. Consequently, it can be advantageous to begin the time course with cells containing only one isotopic species to the greatest extent possible. Modern mass spectrometers can measure ion mass with high precision and accuracy (0.5–5 ppm) but have limitations in measuring ion intensity (2%–30%) [44,45]. Thus, it is preferable to design experimental conditions that play to the strengths of the mass spectrometer, measuring slight differences in mass rather than small differences in ion intensity. Complete isotopic labeling increases the possible dynamic range between the present and incoming isotopes, reducing errors introduced by measuring minute differences between the two conditions.

Quantifying the transition from one labeled state to another depends on observing the destruction of pre-existing proteins and the incorporation of the new label into nascent proteins. Unlike autoradiography and scintillation counting, MS measures both isotopically labeled and unlabeled atoms. The loss of a pulse-labeled peptide can thus be recorded regardless of its heavy or light origin. Thus, the choice of starting conditions (100% heavy or 100% light) is arbitrary if the cells in question meet ideal labeling criteria (Box 3). Turnover rates have been reported from chronic labeling experiments from which the initial pulse phase has been omitted [39,41]. In addition to being simpler and more cost-efficient, monitoring cells as they transition from their natural state to a heavy-labeled state is advantageous because, at the beginning of the time course, proteins necessarily exist with 100% naturally occurring atoms. In this case, the extent of label incorporation during the experimental time course is limited only by the label purity and protein turnover rate. Both labeling strategies appear to produce data of a similar quality, although it remains to be directly determined if turnover rates are equivalent.

Instrumentation

Numerous instrumentation configurations have proven effective for protein characterization, but the coupling of high-performance liquid chromatography (HPLC) with ion trap tandem mass spectrometers has been most widely applied in large-scale proteome studies [46]. The combined LC-MS/MS system can rapidly acquire tens of thousands of MS/MS spectra from complex peptide mixtures in a single analysis. The spectra can then be interpreted to reveal peptide sequences and, by inference, the proteins that gave rise to observed peptides. High mass accuracy and precision greatly increase the confidence in peptide and protein identifications and make accurate quantification possible. The largest breakthroughs in global quantitative proteomics have come from instrumentation delivering exceptionally high mass accuracy (< 5 ppm), resolution (30,000–100,000), speed (>5 Hz) and sensitivity (<1 fmol) to measure far more ions from complex mixtures than ever before. The latest generation of triple quadrupole time-of-flight (Q-TOF) mass analyzers are joining the ranks of hybrid linear ion trap/Fourier transform instruments [47–49] as the vanguard devices that are allowing scientists to characterize complex proteomes, surpassing what was impossible even five years ago. Use of lower-throughput or lower-resolution, older instrumentation can be viewed as but one factor that diminished the depth of earlier turnover studies (Table 2).

Obstacles

With few exceptions, most MS-based protein turnover studies have been able to successfully measure rates for dozens to hundreds of proteins, numbers that pale in comparison to several recent proteomics efforts that achieved nearly complete proteome coverage [32–34,38–40,50,51]. To reach the full potential of MS-based proteome turnover studies, several obstacles must be addressed. Some stem from inherent difficulties with global mass spectrometry experiments while others are more specific consequences of chronic metabolic labeling time course experiments (Figure 2). The most critical obstacles include sample complexity, data analysis and validation.

Sample complexity

Discovery-oriented strategies relying on shotgun sequencing by LC-MS/MS are concentration-dependent and are therefore biased to sampling abundant species. Consequently, the problems of proteome complexity—distinguishing the vast number of unique protein species present in a biological sample—and dynamic range—measuring rare proteins in the presence of highly abundant species—are major hurdles for current MS-based methods. These problems are magnified several-fold by the necessary use of proteolytic enzymes, which cleave proteins into many peptide fragments amenable to LC-MS/MS. Metabolic labeling with stable isotopes further increases the number of distinct peptide forms with which the mass spectrometer must contend (Box 3; Figure 2c). Multiple fractionation dimensions can mitigate problems stemming from complexity and dynamic range by limiting the number of species simultaneously entering the mass spectrometer while separating abundant peptides from the rare peptides they might mask [34,52–54]. Such fractionation permits near-complete proteome coverage in certain situations, but requires larger amounts of input material and places a substantial burden on downstream instrument and data analysis throughput. Thus, researchers must strike a balance between available biological material, depth of proteome coverage and time spent analyzing many fractions.

Data analysis

Typical quantitative MS-based proteomics experiments pose numerous bioinformatic challenges: search engine speed and accuracy [55–58], rejection of false positives [59,60], protein inference [61,62], accurate quantification [63], data organization, candidate prioritization, and meta analysis integrating complementary data sources [64–66]. Protein turnover experiments extend these challenges by virtue of their time course nature and the expectation that measured synthesis and degradation obey well defined kinetics.

Assuming linear synthesis rates and exponential degradation rates, theoretically, only two data points are

Table 2. Surmounting obstacles to large-scale protein turnover studies by mass spectrometry

	Issue	Description	Recent or impending solution
1	Tendency to measure abundant proteins	The vast proteome dynamic range and complexity prevents low-abundance species from being consistently measured, particularly when in the presence of highly abundant species	• Automated multi-dimensional separations [a] • Fast, modern mass spectrometers [b] • High-resolution mass spectrometers [c]
2	Need for extensive fractionation	Each additional subfraction to be analyzed creates a multiplicative factor for the amount of mass spectrometer runs and overall amount of data to be analyzed	• Fast, modern mass spectrometers [b] • Automated instrumentation [d] • Automated data analysis platforms [e]
3	Multiple time points	Each additional time point creates a multiplicative factor for the number of samples to be analyzed. The stochastic nature of shotgun proteomics experiments and imprecise fractionation may prevent peptides from being observed across all time points	• Fast, modern mass spectrometers [b] • High resolution mass spectrometers [c] • Automated data analysis platforms [e] • Targeted proteomic analyses of pre-defined peptides (MRM analysis) [f]
4	Extra isotopic envelopes	One or more isotopically enriched labels extend a peptide mass ''footprint,'' making it increasingly likely for multiple species to overlap in an irreconcilable fashion (Figure 2c). This is an even greater problem when labeling with isotopically enriched metabolites rather than amino acids	• Automated multi-dimensional separations [a] • High resolution mass spectrometers [c]
5	Very large raw data sets	Large data sets can exceed the capacity of desktop computational solutions with respect to data storage, spectrum interpretation and evaluation, peptide quantification, and higher order, holistic analyses	• Automated data analysis platforms [e]

[a]See [53,54].

[b]Several mass spectrometers introduced since 2004 deliver scanning speeds of 10 Hz and faster [47–49]. This enhances overall throughput, and can characterize individual samples more fully.

[c]Hybrid (e.g. LTQ-Orbitrap) and autonomous (e.g. Q-TOF) mass spectrometers generate high-resolution (>30,000) mass measurements, enabling more isotopically labeled peptides to be distinguished from each other, and quantified from increasingly complex spectra.

[d]Automation platforms are continually improving the throughput with which they prepare and deliver samples to mass spectrometers[97].

[e]Multiple software solutions operating on increasingly powerful computer systems have been introduced to automate the interpretation of raw mass spectrometry data, select accurately identified and quantified peptides, and organize the results [64–66]. Further collaboration between informaticians and experimental biologists is needed, and stand to increase the power of these tools.

[f]Scheduled identification of pre-defined peptide sets serves to standardize the manner in which proteins can be identified and quantified across multiple analyses [78–80].

necessary to calculate rate constants. However, since peptides might not be confidently identified and quantified across each time point, multiple time points increase the likelihood of obtaining sufficiently informative measurements. Multiple time points also have a major advantage, in that they serve as replicate samplings of a complex peptide mixture, which has been shown to increase the overall number of identifiable peptides and proteins and the confidence in those identified multiple times [67–69]. Additional time points also decrease the influence of outliers on rate calculations, thus making them more robust. Even with outlier rejection, however, time series for some peptides might not strictly adhere to expected kinetics [41]. Some studies attributed such deviations to measurement error and, therefore, dismissed rates calculated from peptides failing to meet minimal criteria (e.g. confident identification and quantification in $\geq$3 time points, $R^2 \geq 0.85$) [40]. Including constraints such as these to achieve high-confidence rate constants has been shown to reduce the number of reported proteins by nearly half [40]. Thus, there can be a large gap between the number of proteins confidently identified in an analysis and those with accurately measured turnover rates.

For high-throughput MS to meet its potential for sensitive proteome characterization, data analysis methods must improve to match modern instrument sensitivity. For the most part, assembling each computational element—peptide and protein identification, false positive discrimination, quantification and rate measurement—has been done with in-house software. As workflows are optimized, we presume software tools dedicated to turnover analysis will converge on a robust computational solution and become widely available.

Validation

Almost uniformly, authors of large-scale protein turnover studies caution that measured rates should not be taken as absolute measurements, but rather as general guidelines for the stability of characterized proteins [24,38,40,70,71]. Such assertions raise questions of whether presented data are accurate, and to what extent reported protein half-lives can be extrapolated to other experimental systems. Turnover rate diversity across proteins and between different experiments is consistent with a high degree of regulation in which protein stabilities are tuned to a cell's needs at a precise moment, perhaps by highly specialized, organism-, tissue- and cell type-specific mechanisms. Variability can indicate large measurement error rates, or inherently noisy biological systems. Without validating select turnover rates with orthogonal approaches, it is difficult to evaluate whether any discrepancy between studies is attributable to measurement error or fundamental differences of the diverse biological systems examined. Wide acceptance and utility of large-scale protein stability data will therefore require careful validation by complementary approaches and replicate studies.

So far, most large-scale turnover studies have not undertaken such validation. Consequently, it is difficult to reconcile results from the various turnover studies completed to date. The publications examined here have all investigated different model systems with varied experimental conditions. Moreover, the MS-based studies have been limited in

the extent of proteome coverage and often biased towards highly abundant proteins. Further optimization of these methods is needed for a clearer understanding of proteome turnover and a more thorough analysis of the biochemical and/or functional characteristics that might contribute to protein stability.

Outlook

The concerted measurement of protein synthesis and degradation and the consequent regulation of protein abundance reveals a missing dimension of proteomics [32]. Particularly, the balance between protein synthesis, generally assumed to be concentration-independent (zero-order), and protein degradation, which has largely been shown to be concentration-dependent (first-order), has profound implications for the mode of a protein's regulation. For example, achieving a rapid increase in the abundance of a particular protein might not be possible if the cell must first activate its transcription, process its mRNAs, initiate its translation and properly fold the protein. Such a protein could be regulated more rapidly if its basal synthesis rate was high but was balanced by a high rate of proteolysis to maintain low levels. Reducing its degradation rate, even slightly, could rapidly and dramatically increase its abundance. One might question whether a rapid synthesis and degradation of a protein is energetically favorable. Constantly expressing and destroying low-abundance, short-lived proteins would clearly be costly; however, this manner of regulation might be more efficient for regulating keystone components of molecular machines or transcriptional networks whose activities mobilize a much more substantial commitment of cellular resources. Considering essential homeostasis-preserving proteins, regulation by post-translational modification (PTM) might be more appropriate to achieve the necessary changes in function. The acetylation, methylation, phosphorylation, ubiquitylation and/or ADP-ribosylation of histone tails provide just one obvious example [72,73]. Thus, the large-scale analysis of protein turnover combined with further investigation of PTMs can be used to pinpoint key regulators of cellular responsiveness, and help formulate hypotheses for elucidating regulatory networks.

Dominant modes of regulation should come into sharper focus by extending the large-scale studies cited here to other stimuli, biological states and organisms. In the coming years, curated databases of protein half-lives might be integrated with other genome-scale data, including transcript abundance, and post-translational modifications to improve predictions made using omic scale data.

MS-based metabolic labeling studies hold great promise for unprecedented measurements of protein turnover, but this technology alone cannot fulfill all the criteria laid out in Table 1. Discovering which proteins exist as spatially distinct subpopulations with half-lives that correlate with their localization will likely require detailed, time-resolved microscopic analysis. Bleach–chase experiments, for example, offer an avenue for revealing the intricacies of protein dynamics in live cells [26]. We envision the GPS approach, with the addition of cycloheximide or repressible promoters, could also be adapted to image-based protein detection to measure protein turnover with subcellular resolution [25]. Multiplexed microscopic analyses have been improving in their throughput, resolution and computational sophistication [74–77], suggesting a practicable experimental design is within reach. Another shortcoming of the shotgun MS approach described here is its inability to robustly track all natively expressed proteins of interest regardless of abundance or sample complexity. The multiple-reaction-monitoring (MRM) MS approach (Table 2) holds great promise. This method exploits the selective power of triple quadrupole mass spectrometers to hone in on specific peptides of interest. The combination of pre-defined HPLC retention times and fragmentation properties can uniquely and sensitively identify small amounts of peptide, even in the presence of much more abundant, co-eluting species [78–80]. By focusing analyses on proteotypic peptides, half-lives for hundreds to thousands of specific proteins should be obtainable in a single analysis. Experimental designs that capitalize on the complementary strengths of the approaches described here will likely yield the most impactful insights into the dynamic state of protein turnover.

Acknowledgments

J.E. is a Damon Runyon-Rachleff Innovation Awardee supported, in part, by the Damon Runyon Cancer Research Foundation (DRR-13-11). I.H. is supported by a National Science Foundation Graduate Research Fellowship.

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