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Exploring ribosome composition and newly synthesized proteins through proteomics and potential biomedical applications.

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Abstract

Introduction: Protein synthesis is the outcome of tightly regulated gene expression which is responsive to a variety of conditions. Efforts are ongoing to monitor individual stages of protein synthesis to ensure maximum efficiency and accuracy. Due to post-transcriptional regulation mechanisms, the correlation between transcriptome and proteome is higher than between transcriptome and proteome. However, the most accurate approach to assess the key modulators and final protein expression is directly by using proteomics.

Areas covered: This review covers various proteomic strategies that were used to better understand post-transcriptional regulation, specifically during and early after translation. The methods that identify both regulatory proteins associated with translational components and newly synthesized proteins are discussed.

Expert commentary: Emerging proteomic approaches make it possible to monitor protein dynamics in cells, tissues and whole animals. The ability to detect alteration in protein abundance soon after their synthesis enables earlier recognition of disease causing factors and candidates to prevent/rectify disease phenotype.

Keywords

post-transcriptional regulation; translation; ribosomes; newly synthesized proteins; dynamic proteomics; non-canonical amino-acid labeling; AHA; mass spectrometry; fluorescent imaging of synthesized proteins

1. Introduction

To assess gene expression, different methods are applied at various stages of the gene expression process. Addressing the dynamics of the cellular transcriptome (the set of all

mRNA molecules in one cell), RNA sequencing (RNA-Seq) [1] employs next-generation sequencing to analyze presence and quantity of RNA at any given time in a biological sample. Techniques incorporating RNA affinity tags [2–3] as well as RNA metabolic labeling approaches are used to study the rate of gene transcription and RNA processing [4]. For an identification/quantitation of the translome (all mRNA fragments that are translated in a particular time and cellular state), there are several techniques currently used, including polysome (polyribosome) profiling [5–7] and ribosome profiling (footprint) [8–9]. These methods can determine mRNA translation efficiency, provide insights into modulation of translation initiation, elongation and termination, and for ribosome profiling, a precise record of the position of ribosomes on the mRNA at the time at which translation was stopped [8]. However, due to multiple post-transcriptional regulatory mechanisms including various RNA-protein interactions [3,10], mRNA levels are not always reliable indicators of final proteins levels. In the literature, only a weak correlation between the respective abundances of mRNAs and proteomes has been reported, and this could be due to challenges arising from i) technical issues related to the technology and stringency used for quantification, and ii) biological factors contributing to this discrepancy, e.g. translation efficiency, protein turnover, modulation by regulatory proteins and/or regulatory RNAs [11–12]. Impairment in the regulation of protein synthesis can contribute to disease pathogenesis. Recently, microRNAs (miRNAs) were shown to be the key mediators in human diseases from cancer to cardiovascular diseases [13–14] as these small non-coding RNAs can bind and degrade the target mRNAs and inhibit translation [15–16].

Compared to the transcriptome, the analysis of the translome may reveal a more accurate information and correlation related to proteome composition and quality [17], although this is insensitive to variation in protein abundance due to potential degradation. As the polysome profiling technique can be combined with various downstream methods, the isolation step followed by proteomic analysis offers direct identification of the proteins comprising and associated with ribosomes and translational complexes as well as the specific proteins that are being synthesized. Identification and quantitation of the proteins early after their synthesis on the ribosomes is important from a clinical point of view, as the recognition of protein synthesis dysregulation at the early stage can indicate the factors causing the pathology and point to candidates to prevent and rectify the disease phenotype. However, to separate and distinguish low levels of newly synthesized proteins from the pre-existing pool of proteins early after translation is challenging and the development of the proteomic methods that can accomplish this goal is a crucial and ongoing process. The schema of gene expression in eukaryotes together with the examples of approaches used for mRNA/protein quantitation in translation/post-translational step for nuclear coded proteins that are parts of this review are shown in Figure 1.

In this review, we concentrate on the approaches and methodologies that were utilized to identify the translational components and proteins on ribosomes during translation. The major part is dedicated to proteomic approaches developed for the monitoring of dynamics of newly synthesized proteins shortly after they were translated. Since the polysome profiling technique allows mapping of proteins present in fractions separated according to polyribosome abundance, the combination of this technique with various labeling approaches that emerged recently for identification of newly synthesized proteins by

proteomics offers great potential for targeting the proteins in individual fractions. We have restricted our focus to the studies to mammalian cells, tissues and whole animals and included studies that compare mammalian cells with bacterial cells. Furthermore, we have included recent examples of newly synthesized protein visualization and imaging which can offer new approaches and already optimized techniques that can create a potential for protein identification and quantitation if modified for mass spectrometry analysis.

2. Strategy to characterize the composition of active ribosomes in regulated translation

Protein synthesis is a highly regulated process essential for proper function of cells and organisms. The ribosomes are responsible for protein synthesis and can be found in various eukaryotic cell locations such as cytosol, attached to endoplasmic reticulum, mitochondrial outer membrane, and/or nuclear envelope. A separate ribosomal machinery exists within mitochondria to translate a number of mitochondrial mRNA transcripts. The functional ribosome is formed by locking a small subunit onto a large one. It is created by complexes of ribonucleoproteins with about two-thirds of its mass composed of ribosomal RNA (rRNA) and one-third of ribosomal proteins. In eukaryotic translation, a single mRNA molecule can associate with multiple ribosomes simultaneously during translation; the number of ribosomes loaded on an mRNA depends on the length of the mRNA open reading frame and the rates of initiation, elongation, and termination of translation [18]. It was experimentally documented that ribosomes in cooperation with other translational components (tRNA, translational factors, *etc.*), can alter translational efficiencies of mRNA at the initiation and elongation steps and that translation velocity affects protein folding [19]. One of the methods applied for identification and characterization of the translome, polysome profiling [20–21], can analyze mRNAs and determine the translation efficiency based on the number of ribosomes bound. In this method, the analysis of mRNA associated with ribosomes/ribosomal subunits is performed by using ultracentrifugation with sucrose gradient (usually from 10% to 50% of sucrose). This size-based separation technique fractionates the mRNAs loaded with multiple ribosomes (polysomes) reflecting high-efficiency translation from mRNAs with few or single ribosomes (80S), and from non-translation mRNAs and ribosomal subunits (40S, 60S). After collection of the fractions, downstream analysis such as western blotting or RNA-Seq can be used to determine the particular transcripts or global transcriptome as well proteomic-based techniques can be applied for identification of both proteins associated with translational components and newly synthesized proteins. However, the polysome profiling method can be challenging due to rapid degradation of mRNA and various technical issues (e.g. sucrose gradient variability, apparatus tubing blocked by air bubbles, *etc.*). Nevertheless, this method is often used and the efforts are made to tailor it to specific biological samples and needs. For example, the polysome profiling protocol was modified for studying of central nervous system translome in mice with limited amounts of sample and application of myelin flotation in order to remove lipid rich components [22].

As post-transcriptional regulation defines the proteomes that drive various diseases (i.e. cardiovascular, neurodegenerative, *etc.*), the understanding of regulatory process that can

alter the relation between mRNA and proteins can contribute to better clinical intervention. And thus, identification of ribosome composition is important. Recently, proteomic studies of whole ribosomes were published, some of them incorporating polysome profiling technique in their analysis workflow [23–24]. By analyzing polysome profiling fractions and the ribosome protein composition, there are numerous associated non-ribosomal proteins present in addition to ribosomal proteins that fulfill various functions. For example, they can modulate ribosome activity [23]; control the stages during and immediately after translation [25]; act as chaperones that mediate the transfer of ribosomal proteins to their proper sites [26]; interact with newly synthesized polypeptide chains to ensure proper protein folding [27]. To detect non-ribosomal proteins that control ribosome activity, the composition of translationally active and inactive ribosomes was studied in HeLa cells by application of proteomics [23]. Cell lysate was layered on a sucrose gradient (10–50% sucrose) and centrifuged at 20,000 rpm for 9 hours at 4°C, and fractions were collected; inactive fractions (containing the 40S and 60S ribosomal subunits) were pooled and separated from the pool of active fractions (containing polysomes). The pools were concentrated using 100 kD molecular weight cut-off filters into Tris-HCl buffer consisting of 6.8% sucrose, digested with trypsin, desalted and analyzed by Thermo-Fisher LCQ ion trap mass spectrometer. This study identified glycogen synthase (GYS1) as a novel protein factor associated with active ribosomes. Phosphorylation of serine 640 site of GYS1 correlated with GYS1 association with translating ribosomes, and depletion of GYS1 from HeLa cells caused a loss of polysomes [23].

In another study, ribosomes from the liver, mammary gland and testis of rodents were investigated by using two-dimensional gel electrophoresis followed by mass spectrometry [24]. This study yielded in identification of 78 ribosomal proteins and confirmed ribosome protein heterogeneity including ribosomal proteins L10-like, L22-like1 and L39-like, although the degree of heterogeneity was far less than published for plant and yeast ribosomes.

As another example, polysome profile analysis was applied to study the distribution of ribosome proteins in an extract from HEK293 cells and using a sucrose gradient ranging from 7% to 47% of sucrose [28]. The proteins in separated sucrose gradient fractions were precipitated with trichloroacetic acid and washed with ice cold acetone. The resulted protein pellets were reconstituted and denatured in SDS sample buffer and analyzed by SDS-PAGE followed by Western blots. It was found that endogenous SIRT7 (NAD-dependent protein deacetylase sirtuin-7) co-fractionated with ribosomal proteins RPL11 and RPL5 in the monoribosome fraction and SIRT7 knockout caused a minor change in RPL11 distribution in polysome regions. SIRT7 interactions with proteins involved in ribosomal biogenesis were in accordance with a functional network analysis performed on SIRT7 interacting proteins after their identification in whole cell lysate by affinity purification mass spectrometry approach [29]. The findings that SIRT7 co-localized with ribosomal proteins and is associated with ribosome subunits implied regulatory role in protein translation.

More detailed information can be found in recently published articles: an investigation of RNA-protein interactions and their regulatory networks [30]; a review of approaches to

study the RNA-binding proteome [10]; and comparative study of a panel of mammalian cell lines to characterize the components that constitute actively translating ribosomes [31].

3. Strategy and methods to identify newly synthesized proteins by proteomics

The concentration of cellular proteins is determined by the rate of their synthesis and the rate of their degradation. Over many years, proteomics has primarily concentrated on evaluation of static proteomes by measurement of the changes in relative protein abundance across multiple biological samples often by using label-free methods. The move from analyzing the static proteomes to the examination of proteome dynamics has been a transformative accomplishment and it has enabled a deeper understanding of the regulation of protein abundance beyond mRNA gene expression. In order to quantify dynamic changes in protein levels and rates across the proteome during transport, synthesis or degradation within a defined time window, dynamic proteomics is required. Dynamic proteomics of newly synthesized proteins relies on metabolic labeling with labels that can be incorporated into proteins at the time of their synthesis. This involves exposing the cells to labeled compound (pulse) and then to the same compound in unlabeled form (chase) (pulse-chase analysis) followed by mass spectrometry identification.

To date, mass spectrometry-based dynamic proteomics has been performed to target newly synthesized proteins *in vitro* and more recently, *in vivo*. The attempts evolved from analysis of protein changes in various cells [32] to the alteration of newly synthesized proteomes in organs [33–34] and whole animals [35]. One of the major challenges accompanying this identification process is dealing with low abundances of newly synthesized proteins and their recognition and distinction from the pre-existing protein pool. Thus, various enrichment and isolation techniques have been incorporated into proteomic workflows to overcome this challenge.

3.1. Metabolic labeling of proteins

Stable isotope labeling is a technique that includes incorporation of isotope tags into proteins/peptides of one sample and after combining with unlabeled sample, the mass shift and magnitude of the signal ratio between labeled and unlabeled tryptic peptides in mass spectrometry spectra determines the changes in the expression levels. Among these, chemical labeling of intact proteins (e.g. isotope-coded affinity tag (ICAT) [36]) or peptides (isobaric tags for relative and absolute quantitation (iTRAQ) [37]) and metabolic labeling of proteins (^{15}N , ^{13}C , ^2H by using heavy water ($^2\text{H}_2\text{O}$) labeling, stable isotope labeling with amino acids in cell culture (SILAC), *etc.*) are commonly used [38]. Recently, metabolic labeling of proteins with non-canonical amino acids was introduced in which azide/alkyne amino acid analogs of methionine (Met) are incorporated [39]. The advantages/disadvantages of labeling techniques used in the analysis of the static and dynamic proteomes are included in Table 1.

Whereas *in vitro* chemical labeling can be accomplished relatively fast, *in vivo* metabolic labeling can enable the incorporation of isotopic labels in early stages of protein synthesis

but may require longer time windows for label incorporation when applied to whole animals. Moreover, introducing labels metabolically, i.e. at the cell level, diminishes the variations originating from parallel handling of labeled and unlabeled samples, since chemical labeling of proteins/peptides usually takes place after proteins are extracted from cells. On the other hand, chemical labeling can be used in biological samples that are not able to incorporate the isotope label metabolically. An early study to assess individual rates of protein synthesis and degradation in intact animal by using stable isotope-labelled amino acid in diet was published in 2005 [40]. In this study, 50% of the valine in chicken diet was replaced with [$^2\text{H}_8$] valine; chicken were fed for 120 hours and the proteins of skeletal muscle analyzed by in-gel tryptic digest followed by MALDI-TOF mass spectrometry. To measure protein dynamics, the labeling strategy incorporating deuterium ^2H from $^2\text{H}_2\text{O}$ was used for measurement of protein synthesis and dynamics in skeletal muscle of rodents and humans [41], for characterization of protein synthesis of various proteins of histocompatibility complex (MHC) [42], for proteome dynamics of mouse mitochondria [43] and for liver collagen turnover in humans revealing a high rate of collagen remodeling in advanced fibrosis [44]. Although not specifically used for detection of newly synthesized proteins but able to label whole animals, *in vivo* metabolic labeling using heavy isotope ^{15}N was described in several studies. For example, for quantification of *Drosophila* (fruit fly) proteomes [45], for preparation of ^{15}N -labeled protein standards from mice to compare unlabeled proteomes [46], for identification of variation in expression and turnover of dihydropyrimidinase-related proteins DPYSL3 and DPYSL5 during brain development by incorporation of ^{15}N into mice diet [47], and for determination of protein turnover rates on whole proteome scale by restricting all the nitrogen in the rodent diet to ^{15}N (spirulina algae) with reversion to normal ^{14}N diet with time points at days to years [48]. In addition, there have been various reviews published discussing both the strengths and the weaknesses of chemical and metabolic labeling of model organisms [49], the merits and difficulties of labeling methods for assessing protein turnover and dynamics [50–51], and a review article of stable isotopes and mass spectrometry in life sciences covering a time period from 1920 to 2016 [52]. Since SILAC and non-canonical amino acids labeling are discussed later in more details as the techniques of proteomic workflow used for metabolic labeling of newly synthesized proteins and their enrichment, identification and quantitation in the cells, tissues or whole animals, the brief basic description of each technique is included in following sections.

3.1.1. SILAC—One of the widespread metabolic labeling techniques is stable isotope labeling with amino acids in cell culture (SILAC) [53–54] that was reviewed recently [55–56] and allows for simultaneous quantitative analysis of multiple cell samples (the number depends on the choice of label) under various conditions. Importantly, it can be used in pulse-chase experiments in cell cultures to label the newly synthesized proteins and differentiate them from non-labeled (static) proteins by mass spectrometry. It is based on metabolic incorporation of selected amino acids labeled with heavy isotopes into the proteins in cell culture during cell growth, with arginine ($^{13}\text{C}_6$, $^{15}\text{N}_4$), lysine ($^{13}\text{C}_6$) and tyrosine ($^{13}\text{C}_9$) being the most commonly applied. After several cell divisions, isotope-labeled amino acids are fully incorporated into recently synthesized proteins. Commonly, two differentially labeled populations of cells are compared, one containing natural (light)

amino acids and another with isotope (heavy) amino acid labeling. The samples with light and heavy labeled proteins are mixed together and differences in protein quantities analyzed by mass spectrometry. Heavy isotope labeled amino acids in proteins results in mass shift in mass spectrum of the corresponding peptides (e.g. 6 Da for single Lys) and ratio in spectrum peak intensities or spectral counting indicates the differences in relative protein abundances. One of the advantages of this approach is that there is a minimum experimental variation as the label is incorporated at the amino acid level and thus, analysis of mass spectra is straightforward and highly precise. Although SILAC was developed and used widely in cell cultures for quantitative proteomics of both primary cells [57] and cell lines [58], recent studies have used SILAC for *in vitro* cell-based organoids [59] and also *in vivo* [60]. An organoid is derived from induced pluripotent stem cells of a specific donor and can be considered a near-physiological 3D model that closely approximates the genetic and functional characteristics of the stem cell donor. Organoids derived from a variety of induced pluripotent stem cell donors can address individual human population variability, making it useful for applications in precision medicine [59,61–62]. The complete and almost complete labeling (96.2%) by SILAC *in vivo* was reported for mice [63] and flies [60], respectively, and partial SILAC labeling was accomplished for chickens [40,64] and newts [65]. In these experiments, various heavy isotopes of amino acids were added to animal diets with various feeding time spans (e.g. $^2\text{H}_8$ valine, $^{13}\text{C}_6$ lysine and $(^{13}\text{C}_6 \text{ }^{15}\text{N}_2)$ lysine; 5 days to 4 animal generations). SILAC stable-labeled mouse ($^{13}\text{C}_6$ lysine in diet) [19] can be used as a “spike-in” internal standard for experiments with various organ cells/tissues and allowed for study of pathology models and knockout mice [63]. Recently, this approach was used for global protein quantitation of mouse heart tissue [66].

3.1.2. Non-canonical amino acid labeling—To identify and enrich for low abundance newly synthesized proteins from the background of the pre-existent proteome pool, the methodology called biorthogonal non-canonical amino-acid tagging (BONCAT) [39,67] was developed. It includes the incorporation of non-canonical azidohomoalanine (AHA) [39], azide analog of Met, into proteins; this is followed by copper-catalyzed azide-alkyne ligation (Click-iTTM reaction) between azide-labeled proteins and alkyne-bearing affinity tag (e.g. biotin-alkyne) enabling affinity purification. The enrichment at the label level is not provided with SILAC labeling and so lower abundance proteins can be challenging to detect. After affinity purification, the AHA-labeled proteins undergo enzymatic digestion and identification by mass spectrometry. In these particular studies [39,67], a biotin-FLAG-alkyne tag was used with an amino acid sequence (-GGADYKDDDDK-) which enabled cleavage by trypsin directly from the affinity avidin resin without need for peptide elution. For mass spectrometry identification, a complete tag cleavage and one miscleavage after trypsinization resulted in the mass gain of tagged AHA over Met, 107 AMU and 695.3 AMU (atomic mass unit), respectively. It was demonstrated that AHA is an effective replacement for Met, it is accepted by the methionyl-tRNA synthetase and incorporates into proteins *in vivo*, azide-bearing AHA is not toxic (at least in the short term), and does not alter rates of protein degradation [31,67–68]. In addition, the biorthogonal labeling to detect newly synthesized proteins can be done using an alkyne-containing non-canonical amino acid analog of Met (e.g. homopropargylglycine (HPG)) followed by Click-iTTM chemistry with an azide-bearing affinity tag (e.g. biotin-azide) [69].

However, one of the disadvantages is that the cell affinity for Met over AHA/HPG is about 500 fold [70] and although this drawback can be overcome by AHA/HPG labeling of cells in Met-depleted medium *in vitro*, labeling *in vivo* can be rather challenging. While SILAC-based methods rely on the full incorporation of heavy isotope amino acid into proteins that can be only accomplished during several cell divisions and thus a certain time window is required, metabolic AHA labeling was shown to be effective after relatively short periods of time. As well, AHA labeling presents a promising tool for analysis of post-mitotic cells.

3.1.3. Examples of *in vitro* AHA/HPG labeling—Peripheral blood mononuclear cells (PBMC) purified from whole blood of stroke patients and control individuals were used for identification and quantitation of the differences in both PBMC newly synthesized proteins and the total quantifiable proteome. The newly synthesized proteins were labeled in purified PBMC cultures with 50 μ M AHA for 2 hours, lysed, followed by Click-iT™ reaction and analyzed by mass spectrometry. The differences were identified between stroke and control patients in PBMC newly synthesized proteome as well as in PBMC total proteome and it was suggested that the newly synthesized proteome in PBMC can serve as a pool for biomarker discovery in human stroke studies [71].

The other approach was used for profiling of new proteome synthesis during starvation-mediated autophagy by using a BONCAT method including AHA labeling combined with mass spectrometry iTRAQ technique. The authors identified 711 proteins that met their pre-defined criteria for newly synthesized proteins. By using bioinformatic analysis, the relation of proteins to pro-survival outcome of autophagy was identified [72].

Recently, the nitrilase-activatable non-canonical amino acid tagging (NANCAT) strategy was described [73] in which nitrile precursors of non-canonical amino acids (α -carboxylic acid is replaced with a nitrile group) can be enzymatically converted to AHA or HPG by an exogenous nitrilase. As the nitrile group is abiotic in animals and highly stable and inert under physiological conditions and various nitrilases have been well characterized as biocatalysts the authors opted for examination of a nitrilase-nitrile as the enzyme-precursor pair. The chemically synthesized nitrile-substituted precursor of AHA ((S)-2-amino-4-azidobutanenitrile (Abn)), nitrile-substituted precursor of HPG ((S)-2-amino-hex-5-ynenitrile (Ahn)) and the nitrilase from *Rhodococcus rhodochrous* (RrNIT) that exhibited high enzyme activity were used to validate NANCAT strategy in *E. coli* and HeLa cells. After incubation of *E. coli* strain constitutively expressing RrNIT (RrNIT⁺) and wild type *E. coli* (RrNIT⁻) with 500 μ M Abn for 3 hours, cell lysates underwent Click-iT™ reaction with alkyne-Cy5 and the proteins were visualized by in-gel fluorescence scanning as well as analyzed by using confocal fluorescence microscopy. Abn showed effective labeling of proteins in RrNIT⁺ cells (similar to AHA labeling) with no labeling shown in RrNIT⁻ cells, and the labeling in RrNIT⁺ cells was abrogated with 1 mM Met. In similar manner, Ahn resulted in selective incorporation of HPG into newly synthesized proteins in RrNIT⁺ cells by using Click-iT™ reaction with azide-containing tags. A similar experimental procedure applied to HeLa cells expressing EGFP-RrNIT (RrNIT⁺) demonstrated selective labeling by AHA generated from Abn that was abolished with Met treatment and no labeling was found in wild type RrNIT⁻ HeLa cells. The labeling with Abn in RrNIT⁺ HeLa cells was concentration and incubation time dependent. The proteomic analysis of RrNIT⁺ HeLa cells

treated with Abn was further performed and evaluated. After cell lysis and Click-iT™ reaction of resulted lysates with photocleavable alkyne-biotin on streptavidin beads, the enriched proteins were on-bead trypsin digested to remove the peptides with no azide labeling. The modified peptides on the beads were subjected to UV cleavage and analyzed by mass spectrometry. This yielded in detection of 616 unique Abn modified Met sites on 584 peptides corresponding to 400 newly synthesized proteins. In conclusion, the NANCAT was shown to be a strategy for labeling and identification of newly synthesized proteins in both bacterial and mammalian cells.

3.1.4. Examples of *in vivo* AHA/HPG labeling—For the most part, analysis of newly synthesized proteins and their identification by mass spectrometry have been applied to cultured cells *in vitro*. As mentioned previously, AHA labeling *in vitro* can be accomplished relatively easily in cell cultures with Met-free media but *in vivo*, with endogenous Met present and the high affinity of cells for Met over AHA, labeling poses a major challenge. In 2014, AHA labeling followed by Click-iT™ chemistry with biotin tag was used to study newly synthesized proteins in HEK 293T cells *in vitro* and in mammals *in vivo*, specifically in rat neuronal tissues (retinas and optic nerves) [35]. To improve the detection of the biotin-modified peptides by increasing labeled/nonlabeled peptide ratio, a new strategy (Direct Detection of Biotin-containing Tags; DiDBiT) was applied in which AHA-biotin modified proteins after Click-iT™ reaction were digested prior to the enrichment with neutravidin beads and resulting affinity enriched AHA-biotin peptides eluted by boiling in TFA/FA/acetonitrile and analyzed by mass spectrometry [35]. This strategy increased the yield of enriched AHA-biotin peptides in mass spectrometer and resulted in increased detection sensitivity that is crucial for identification of low abundance proteins. The mass shift in MS spectrum resulting from AHA-biotin-alkyne label detected the peptide that originated in newly synthesized protein. The method was applied to study the efficiency of AHA incorporation into newly synthesized proteins, by labeling HEK 293T cells by AHA in Met-depleted medium for 1 hour, and by intraocular administration of AHA into adult rat eyes in a dose and time-dependent manner. The optimum concentration of 1.4 mg/kg AHA was intravitreally injected into rat eyes, i) a single injection followed by harvesting the retina and optic nerve after 3 hours, and ii) two injections (spaced 20 hours apart) with retina and optic nerve harvested 24 hours after first injection. By analysis of neuronal tissue lysates of i) and ii), 1042 and 2452 AHA-biotin labeled peptides were identified that corresponded to 618 and 1149 proteins, respectively. 78% of proteins identified after the “a single injection/3 hours AHA labeling” were found in the “two injections/24 hours AHA labeling” as well with additional 58% of proteins detected in the “two injections/24 hours AHA labeling” experiment. Quantitative analysis showed that 80% of proteins in the “a single injection/3 hours AHA labeling” experiment was detected mostly by one spectral count with increase in abundance of AHA-biotin labeled proteins in experiment with the “two injections/24 hours AHA labeling”. As the authors suggested, one spectral count proteins are very low abundance proteins and the longer time window during longer exposure to AHA allows for newly synthesized proteins to accumulate. As shown in this paper, 20-fold increase in detection of newly synthesized peptides was reached in comparison to the studies where biotin tag was enriched on the proteins instead of the peptides. Thus, it can be concluded that in early stages of their synthesis, many proteins detected by this new strategy would

otherwise remain below the detection limit [35]. Moreover, this study demonstrated that optimized AHA concentration can overcome the competition with endogenous Met *in vivo* and can successfully label the newly synthesized proteins in intact animals. The applied strategy allowed a mass spectrometry detection of low abundance proteins within a short time window of 3 hours after synthesis and thus, has a potential for *in vivo* applications and investigation of the proteome dynamics in intact animals [35].

This work was further extended, by incorporating AHA into the whole mouse proteome in order to analyze the quantitative alteration of newly synthesized proteins in animal models of disease at different discrete time points [33]. This strategy, called “Pulse AHA labeling in mammals” (PALM) involved the creation of a special diet in which Met was replaced with AHA (2g/kg), and other amino acids in standard diet were altered to compensate for the reduced Met (PALM diet). The animals were fed for 2, 4 and 6 days to test the minimum time needed for AHA distribution and incorporation. After Click-iT™ reaction with biotin-alkyne using the tissue homogenates (brain, heart, liver and lung) dissected from mice, the immunoblots with streptavidin showed that AHA was incorporated in all tissue types after 2 days with an increase over 4 to 6 days. Analysis of the brain tissue homogenate further showed AHA-labeled proteins in multiple subcellular fractions (mitochondrial, nuclear and synaptosomal) after sucrose gradient fractionation. The DiDBiT strategy followed by mass spectrometry was applied to mouse brain tissue to identify newly synthesized proteins after 2, 4 and 6 days on the PALM diet; it yielded 642, 8642 and 6810 AHA-labeled peptides corresponding to 425, 2951 and 2811 proteins, respectively, and 842, 875 and 1037 unmodified peptides corresponding to 397, 441 and 482 proteins, respectively. The power of neutravidin enrichment was demonstrated, as brain tissue from mouse on the PALM diet for 6 days subjected to identical conditions without neutravidin enrichment resulted in only 44 AHA-labeled peptides from 30 proteins and 22801 unmodified peptides from 4028 proteins. As well, 95% of the AHA-labeled proteins after 2 days of PALM diet were found at 4 and 6 days as well. Moreover, 38% of identified proteins were only found in the experiment with neutravidin enrichment [33]. To quantify the changes of newly synthesized proteomes between different samples using the PALM strategy, liver tissues of both wild type (WT) and liver kinase B1 (LKB1) knockout (KO) mouse (KO LKB1) were compared. The AHA-labeled peptides in homogenates of WT and KO LKB1 liver tissues were subjected to Click-iT™ reaction with heavy stable isotope of biotin-alkyne ($C_{13}H_{24}N_3O_3S$ - $^{13}C_3^{15}N$) and light unlabeled biotin-alkyne ($C_{16}H_{24}N_4O_3S$), respectively, then combined at a 1:1 (w/w) ratio and processed. To evaluate the reproducibility of the process, two biological replicates of both WT and KO LKB1 livers were examined with one biological replicate for each tissue prepared three times independently. This quantitation approach was reproducible for both technical and biological replicates as examination of the heavy/light AHA peptide ratios were highly correlated ($r > 0.7$ for the three technical replicates). The quantitative analysis of the newly synthesized proteomes between WT and KO LKB1 livers showed a significant increase in proteins involved in gluconeogenesis (e.g. phosphoenolpyruvate carboxykinase, PEPCK) and a decrease enzymes involved in fatty acid metabolism in KO LKB1 livers. It was concluded: i) 4 to 6 days on PALM diet were sufficient for detection of newly synthesized proteins by mass spectrometry; ii) application of heavy biotin-alkyne for quantitation of the changes in newly synthesized proteins is accurate and reproducible; iii)

short labeling time has a potential to identify early regulators and alteration related to pathological states, and iv) enrichment is crucial for proteins that are present at low concentrations and would not be detected if whole proteome analysis was performed [33].

AHA and HPG labeling via intraperitoneal injection was recently used to characterize the proteome in developing mice [34]. First, it was demonstrated that in juvenile mice (25–35 days old), AHA and HPG incorporated into all tissues under investigation (heart, lung, brain, skeletal muscle and kidney) in dosage-dependent increase (0.025, 0.05 and 0.1 mg/g of AHA or HPG per day; 2 days of administration). Secondly, AHA and PBS (control) were injected intraperitoneally into pregnant dams during period of two days (0.1 mg/g per day; E10.5-E12.5 embryos) to identify the newly synthesized proteins during embryonic development. The AHA –labeled proteins in embryo lysates were labeled with fluorophore-conjugated alkyne, separated by SDS-PAGE and the two high fluorescence signal bands analyzed (42 and 48 kDa) using LC-MS/MS (AB Sciex Triple TOF 5600). The protein list was compared to the proteins present in the equivalent gel bands from the control samples. In this study, no substantial alteration in total protein expression due to AHA labeling was observed. 89 % and 88 % out of 246 uniquely identified proteins were detected in AHA-labeled and control samples for bands 42 kDa and 48 kDa, respectively. Only 10% and 5.7% of those overlapped proteins in these gel bands, respectively, displayed significantly different expression ($p < 0.05$). Of note, there were no physical or behavioral differences between mice from AHA-labeled and control non-labeled litters after 6 months. The approach suggest that AHA can be used for in vivo experiments in order to characterize and quantify protein synthesis and turnover.

3.1.5. Simultaneous SILAC and AHA labeling approach—A recently developed approach employs two metabolic labeling methods, pulsed AHA for enrichment/isolation and pulsed SILAC applied for quantitation of changes in both newly synthesized proteins and cell secretomes via mass spectrometry [32,74]. The advantage of this approach, although technically more complex, is that cells can be grown and compared under different conditions in a culture medium that contains the usual concentration of serum proteins to support cell viability and thus avoiding any possible cell alteration/death and/or secretome changes if serum-free medium is used. Since low abundance newly synthesized proteins are labeled by AHA and enriched further by Click-iT™ reaction, the need for pre-fractionation to simplify the complex protein mixture is not necessary. To evaluate the feasibility of this approach, the combination of AHA and SILAC with Click-iT™ chemistry enrichment was compared to SILAC technique alone [32]. Human PC3 and WPMY-1 cells were AHA and SILAC labeled in cultures containing 10% bovine serum for 24 hours. The conditioned media from PC3 (prostatic adenocarcinoma) and WPMY-1 (myofibroblast stromal) cells with heavy and intermediate SILAC labels (heavy $^{13}\text{C}_6$ $^{15}\text{N}_4$ -Arg and ^{13}C $^{15}\text{N}_2$ -Lys; intermediate $^{13}\text{C}_6$ -Arg and d_4 -Lys) were mixed together, divided into two halves and proceeded with and without Click-iT™ reaction enrichment, respectively. A total of 1136 proteins were detected in conditioned media from PC3 and WPMY-1 cells, including 74 ligands (33 cytokines and/or growth factors), 65 receptors, 62 proteases and 211 other enzymes. Out of total 1136 proteins, i) 684 proteins were quantified in the enriched sample with 601 differentially secreted between PC3 and WPMY-1 cells (false discovery rate, FDR,

of 1%) and with high correlation for two biological replicates (0.961); only 22 proteins were quantified in the sample without enrichment with low correlation for biological replicates (0.025), ii) 395 proteins could be considered as signal/secreted based on UniProt database or SignalP prediction, and iii) 459 proteins originated in PC3 cells with 325 (71%) known to be secretory. In the same study, the simultaneous AHA and SILAC approach was applied in order to access the secretory profiles of both primary mouse hepatocytes and hepatoma cell lines Hepa1–6 and Hepa1c1 cells after media collection at time points of 4 hours and 24 hours. It was concluded that this approach is applicable to primary cells as well as cell lines and although secretomes of Hepa1–6 and Hepa1c1 were highly similar, they differed considerably from primary hepatocytes. In addition, the authors examined the ability of the described approach to detect changes in newly synthesized proteins at both shortly after stimulation and secreted later [63]. Specifically, mouse macrophages (RAW 264.7), which are known to poorly tolerate serum-free media, were labeled by AHA and SILAC labels for duration of 2 hours at time points 0, 6 and 17 hours after stimulation by lipopolysaccharide (LPS). A total of 500 proteins were identified and quantified at short labeling time (2 hours), many of them recognized as signaling proteins. Related to the LPS stimulus, 97 proteins were differentially secreted after LPS treatment in at least one of the time points with about 90% known to be secreted (based on UniProt database and SignalP prediction). Several growth factors (e.g. TGF beta 1 and VEGFA) were detected only immediately after LPS stimulation and the secretion of the set of cytokines was found to be elevated at each time point, starting from 0–2 hours after LPS stimulation with peak secretion in 6–8 hours (e.g. TNF and CCL2). Several cytokines and receptors (e.g. CCL9 and NRP2) secretions were delayed as they were secreted at 6–8 hours with a slight decrease at 17–19 hours. The secretion of 28 proteins were found to be inhibited over time, which included the factors associated with signaling and extracellular matrix remodeling (e.g. LFNG and DAG1). In conclusion, it was demonstrated that (AHA and SILAC) approach can be applied for monitoring of the temporal profiles of newly synthesized proteins, for the changes that occur in early stages after various stimuli, and the method was suggested to be suitable for examination of alternative secretion mechanisms such as exosomes [32]. The study was further extended to the characterization of proteome changes on rapid time scale during RAW 264.7 macrophage activation (3 hours after LPS stimulation), in a recent publication by the same authors [75]. They were able to determine the expression patterns of transcripts, proteins, newly synthesized proteins and secreted proteins.

Combining the strengths of both BONCAT and SILAC, an approach called quantitative non-canonical amino acid tagging (QuaNCAT) was used to monitor the alteration in protein synthesis of isolated human CD4 T cells (hCD4⁺) subjected to stimulation by phorbol 12-myristate 13-acetate (PMA) and ionomycin (the drugs eliciting cell proliferation and differentiation) [76]. The hCD4⁺ cells were cultured with 1 mM AHA and either heavy [¹³C₆, ¹⁵N₄]-L-Arg and [¹³C₆, ¹⁵N₂]-L-Lys or medium [¹³C₆]-L-Arg and [²H₄]-L-Lys (control cells) for 2 and 4 hours. The cells with heavy SILAC label were stimulated by 100 nM PMA and 1.4 μM ionomycin. The stimulated and control hCD4⁺ cells were mixed in a 1:1 ratio and after Click-iT™ reaction of AHA-labeled proteins with alkyne-containing biotinylated cleavable linker, biotin-tagged proteins were enriched by using NeutrAvidin beads, eluted from beads by DTT, trypsin digested and analyzed by LC-MS/MS. A total of

410 and 550 proteins were identified at 2 hours and 4 hours of stimulation, respectively. 107 proteins were differentially expressed ($p < 0.05$) with 93 proteins up-regulated after stimulation. 56 proteins were identified to be differentially expressed at 2 hours; of those, 33 proteins were still detected at 4 hours, and 50 proteins were only differentially expressed after 4 hours of drug stimulation. Fold changes ranged from 1.6 to 589 fold. Many proteins related to transcriptional regulation were identified as well as cytokines, various protein chaperones, activation surface markers and proteins involved in vesicle transport [76].

The brain-derived neurotrophic factor (BDNF)-induced changes in newly synthesized protein levels were examined in HEK-293 cells overexpressing tropomyosin-related kinase B (TrkB) after 2 hours of BDNF stimulation [77]. To detect changes in this short period of time, two proteomic approaches were compared in parallel, BONLAC, i.e. BONCAT in combination with pulsed SILAC, and a second approach of pulsed SILAC only, without enrichment of AHA-labeled proteins. For both approaches, HEK-293 cells were incubated for 2 hours in two different AHA-SILAC media each containing AHA (1 mM) and one of the two sets of heavy isotope labeled Arg and Lys (either [d₄-Lys and ¹³C₆-Arg] or [¹³C₆¹⁵N₂-Lys and ¹³C₆¹⁵N₄-Arg]), with one set as a control. The two differently SILAC labeled cell populations (control and with 25 ng/mL of BDNF treatment) after lysis were mixed at a 1:1 ratio. For the BONLAC approach, cells were lysed, the newly synthesized proteins containing AHA/SILAC labels were enriched by Click-iTTM reaction with biotin-alkyne agarose beads, on-resin trypsin digested and desalted. For SILAC-only approach, the cells were lysed, trypsin digested and desalted. The tryptic peptides from both BONLAC and SILAC techniques (approximately the same 10 µg of peptides for each technique were compared) were separated by hydrophilic interaction liquid chromatography (HILIC) into twelve fractions and further analyzed by LC/MS for protein identification and quantitation. Each BONLAC and SILAC were repeated in two biological replicates with SILAC labels reversed between replicates and with two LC/MS replicates for each biological one. This work showed that i) SILAC samples had very low SILAC label incorporation (1.7%) compare to BONLAC samples (76%) showing the strength and necessity of the enrichment step; ii) BONLAC and SILAC experiments resulted in identification of 7474 and 7144 proteins, respectively, with high overlap between them (6389 proteins) suggesting that the newly synthesized proteome was similar to the steady-state proteome; iii) from total 8229 proteins, 1085 and 722 proteins were identified in only in BONLAC and SILAC samples, respectively; and iv) 53 proteins were significantly altered by BDNF treatment by using the BONLAT strategy with 24 proteins having transcription regulator activity, and 16 proteins recognized as transcription factors. Interestingly, most of the identified proteins were not previously connected to BDNF signaling pathway [77].

Another study that examined the potential artifacts in protein quantification that might be caused by AHA labeling was published recently [78]. In this paper, it was shown that although complete replacement of Met by AHA during labeling of HeLa cells after 24 hours had no significant effect on protein identification, it did alter protein abundances. For the studies where the accurate detection of protein abundances is crucial, the author recommended an AHA labeling approach that used 30:1 ratio of AHA to Met (AHA^{30:1}) that allows sufficient enrichment of newly synthesized proteins by Click-iTTM reaction but reduces the perturbations in protein abundance. By using the simultaneous BONCAT and

SILAC approach (in triplicate experiments), the authors quantified 1931 and 1529 proteins synthesized by HeLa cells labeled by AHA and AHA^{30:1} within 4 hours, and 1484 and 416 proteins synthesized by HeLa cells labeled by AHA and AHA^{30:1} within 30 min, respectively. In addition, the comparison of this (BONCAT and SILAC) approach for similar experiments performed by SILAC method only, 589 and 9 newly synthesized proteins were quantified within 4 hours and 30 min by using SILAC labeling, respectively. Thus, full AHA labeling increased affinity enrichment and resulted in detection of a higher number of proteins, which was especially important when short labeling times were used.

Finally, a study employing the combined (AHA and SILAC) approach was recently published on TC32 human Ewing sarcoma cells that were labeled with light/heavy isotopes of Arg and Lys and co-translationally pulsed with AHA [79]. By using Click-iTTM reaction with biotin-alkyne tag, the newly synthesized proteins that are translationally regulated by Y-box binding protein-1 (YB-1; conserved translational regulator implicated in cancer progression) were identified and quantified after brief (45 min) labelling time.

4. Methods for imaging/visualization of newly synthesized proteins

To study the changes in newly synthesized proteomes in cells, tissues and whole organisms, the labeling methods that can be used for imaging of protein synthesis in spatial and temporal manner, are sensitive, work relatively fast and enable labeling in complex biological samples, are in high demand. Some of the strategies developed over years have relied on incorporation of modified Met surrogate bearing azide- and alkyne- groups into proteins as discussed above (e.g. AHA, HPG and azidonorleucine [80]) but in this particular instance, the incorporation of the unnatural amino acid is followed by Click-iTTM reaction with corresponding fluorophores. Other approaches were based on various fluorescent conjugates of puromycin that formed an amide bond between the primary amine group of puromycin and the C-terminus of the newly synthesized polypeptide [81]. Chemical strategies and methods optimized for tagging and fluorophore-labeling of subsets of proteomes in both bacterial and mammalian cells were reviewed in 2011 [82]. Even though the ultimate goal of these studies is visualization and imaging of newly synthesized proteins, some of them can offer new approaches that can allow for the possibility of further protein identification and quantitation by mass spectrometry analysis and thus, we have included several examples here as well.

Newly synthesized proteins in Rat-1 fibroblasts were metabolically labeled with AHA and HPG and then visualize using modified lissamine rhodamine (LR), 7-dimethylaminocoumarin (DMAC) and Bodipy-630 (BDPY) after Click-iTTM reaction with AHA and HPG labeled proteins [83]. Except for BDPY, both DMAC-alkyne (10 μ M) and DMAC-azide (50 μ M) as well as LR-azide (50 μ M) exhibited selective labeling of newly synthesized proteins inside the cells (cytoplasm, nucleus and nucleoli) and showed consistent staining among different cells in a population. The cells incubated with AHA (1 mM) and HPG (1 mM) for 5 hours resulted in fluorescence 3- to 7-fold higher compared to control cells incubated with Met (1 mM). To label two distinct populations of proteins, this approach was further extended by using metabolic labeling with sequential additions of two amino acid analogs. Rat-1 fibroblasts were incubated for 3 hours in media containing the

first amino acid (either AHA, HPG or Met) followed by second labeling pulse for 2 hours with one of the three amino acids as the second amino acid in the same sample. The cells were fixed with a 3.7% paraformaldehyde solution and the Click-iT™ reaction was performed with LR-azide for 1 hour followed by DMAC-alkyne for additional 1 hour. The confocal fluorescence microscopy validated by flow cytometry showed bright two-color images by LR and DMAC labeling for cells incubated sequentially with AHA and HPG. The reverse addition order of the dyes (first DMAC-alkyne followed by LR-azide) resulted in similar results and this two-color imaging of temporally defined protein populations was shown to work in the rat exocrine cell line AR42J as well.

To demonstrate selective labeling of newly synthesized proteins in a mixture of different cells, this study was carried out with bacterial and mammalian cells [80]. Murine alveolar macrophages were incubated with 2 mM of azidonorleucine (Met analog) and simultaneously infected (co-incubated) with *E. coli* cells that expressed either NLL-MetRS (mutant methionyl-tRNA synthetase) or green fluorescent protein (control) for 35 min at 37°C. The cells containing mutant MetRS were able to incorporate azidonorleucine as a replacement for Met in protein synthesis while the cells containing only the normal Met tRNA synthetase lacked this ability. After fixing the cells, the cells were subjected to Click-iT™ reaction with TAMRA-alkyne. In both NLL-MetRS and control experiments, bacteria cells were bound and internalized by macrophages (confocal microscopy and 3D analysis) with strong TAMRA fluorescent signal for macrophage-associated bacterial cells expressing NLL-MetRS but no conjugation was found to TAMRA-alkyne above background for control sample [80]. In conclusion, it was confirmed and shown that in co-culture, that only the mutant cell types containing NLL-MetRS can synthesize the proteins that can incorporate azidonorleucine and thus, can be labeled and imaged with fluorophore-alkyne, and thus distinguished from the proteins of cells that could only incorporate Met.

Likewise, AHA and HPG labeling has been used for imaging of newly synthesized proteins in rat hippocampal neurons [84]. Two fluorescent alkyne tags, TexasRed-PEO₂-alkyne (TRA) and 5'-carboxyfluorescein-PEO₈-azide (FLA) were synthesized for ligation to AHA-labeled or HPG-labeled proteins using Click-iT™ reaction that allowed the visualization of newly synthesized neuronal proteins by fluorescence microscopy. For *in situ* visualization of newly synthesized proteins, the incubation of primary hippocampal neurons in culture with either AHA or HPG for 1 hour yielded in high fluorescent signals in somata and dendrites that were comparable for both AHA and HPG labeling. When neurons were co-incubated with anisomycin (protein synthesis inhibitor), the fluorescence was greatly decreased confirming the specificity of the labeling procedure for newly synthesized proteins. It was found that fluorescent labeling did not interfere with immunolabeling and thus, fluorescent labeling can be followed by conventional immunocytochemistry analysis. The method of fluorescent tagging with AHA labeling for 4 hours was tested in organotypic hippocampal slices of intact tissue (150 µm in thickness) and resulted in visualization of newly synthesized proteins throughout the entire slice; specificity was confirmed by loss of the fluorescent signal with co-application of 40 µM anisomycin during AHA labeling. Further, the pulse-chase experiments were carried out with sequential application of AHA and HPG for visualization of two distinct proteomes synthesized in two sequential periods of time. The best strategy for labeling of neurons during two intervals of protein synthesis was

determined as labeling first with HPG for 1 hour then washed the cells and followed with AHA labeling step for additional 1 hour. After that, the cells were exposed overnight first to TRA tag, rinsed with PBS and then subjected to FLA tag overnight. The use of HPG prior AHA labeling and application of TRA tag prior to FLA tag resulted in the highest and most consistent fluorescent signal. By examination of the sensitivity of this labeling approach, it was found that newly synthesized proteins can be visible only after 10 min of AHA/HPG exposure in neuronal somata, after 20 min in proximal dendrites and after 2 hours in distal dendritic segments with AHA incorporating rates higher compared to HPG for early time points (up to 30 min). As well, the dynamics of newly synthesized proteins in the membranes of living neurons was investigated in the same study, by using single-particle tracking and fluorescent quantum dots (QDs) coupled to membrane proteins labeled by AHA [84]. For these experiments, neurons labeled by AHA for up to 4 hours were subjected to incubation with membrane impermeant reagent difluorinated cyclooctyne (DIFO)-biotin derivative followed by tagging with streptavidin-conjugated QDs. The newly synthesized proteins associated with QDs were detected on both neurites and somata and their movements were characterized showing diffusion coefficient values spreading over 5 orders of magnitude and reflecting the heterogeneity of the proteins.

The techniques that used fluorescent puromycin conjugates [81] and anti-puromycin immunofluorescence [85] to detect and monitor protein synthesis were used in the past. However, the method published in 2012, is based on an alkyne analog of puromycin, O-propargyl-puromycin (OP-puro), that inhibits protein synthesis in cells and covalently binds to the C-terminus of newly synthesized polypeptide chains [86]. The polypeptide-OP-puro conjugates can undergo the Click-iT™ reaction with fluorescent-azide or biotin-azide for fluorescent microscopy visualization/imaging or isolation by affinity enrichment. Since OP-puro can be used in the presence of Met, it is suitable for *in vivo* applications. Compared to [81,85], the cell permeable OP-puro exhibits fluorescent staining with a high signal-to-noise ratio of about 24 (NIH 3T3 cells with 50 µM OP-puro incubation time of 1 hour and stained with Alexa568-azide), enabling high visualization sensitivity of protein synthesis. It was shown that the subcellular pattern of newly synthesized proteins by using OP-puro is consistent with the pattern obtained with HPG labeling (nuclear and nucleolar) [39]. The OP-puro incorporation required functional ribosomes and the labeling (50 µM OP-puro, 1 hour incubation) was abrogated in cells treated with cycloheximide (50 µg/ml) as protein translation was blocked. This method was applied to whole animals and exhibited a high sensitivity visualization of protein synthesis patterns in large tissue explants from various organs. Particularly, the 100 µl of 20 mM OP-puro solution were injected into peritoneal cavity of 3-week-old mouse, various organs (small intestine, liver, kidney and spleen) were harvested after 1 hour of OP-puro incorporation, fixed in formalin and Click-iT™ reacted with 20 µM tetramethylrhodamine (TMR)-azide, either in paraffin fixed slices or in whole mount. The level of protein synthesis varied among organ tissues. For example, in the small intestine, the intense OP-puro labeling was located in secretory vesicles in Paneth cells (shown in both the tissue slices and in whole mount), while in liver, high levels of protein synthesis were found in hepatocytes. As highlighted by the authors, using OP-puro has major advantages over application of Met analogs (e.g. AHA and HPG) in metabolic labeling, i) it does not require Met-free medium, ii) one molecule of OP-puro per newly

synthesized polypeptide chain is incorporated, iii) labeling by OP-puro does not depend on presence of Met in proteins, and iv) OP-puro covalently conjugates with newly synthesized proteins directly without any prior processing. Although not employed in this study, newly synthesized proteins labeled by OP-puro can be suitable for further identification by mass spectrometry.

SIRT7 down-regulation is hypothesized to contribute to an antitumor effect [87] partly via the inhibition of protein synthesis. To gain better understanding of this mechanism, the HPG labeling of HeLa cells was used to assess the effect of protein SIRT7 on protein synthesis [28]. Cells were grown in in Met- and cysteine-free medium for 1 hour and subjected to HPG labeling for 2 hours. After pulse HPG labeling, the cells were fixated in 80% ethanol for 15 min on ice and Click-iT™ reaction was performed with fluorescent Alexa 488-azide to acquire HPG incorporation profiles by analyzing large cell populations (n = 10,000) by flow cytometry. The measurements of HPG incorporation into cells treated with siRNA targeting SIRT7 for 3 days revealed that after SIRT7 knockdown, protein synthesis activity was significantly suppressed relatively to control siRNA or untreated cells [28]. Furthermore, SIRT7 knockdown preferentially reduced protein synthesis over RNA synthesis which was measured simultaneously. Overexpression of SIRT7 did not result in an increased rate of protein synthesis, implying that the overexpression of SIRT7 itself is not sufficient to change protein synthesis. Since SIRT7 is an emerging target for anticancer therapy, the results of this study contributed to the knowledge about SIRT7 antitumor effect. Summary of proteomic approaches/techniques for the identification and quantification of newly synthesized proteins which are covered in this review is given in Table 2.

5. Conclusion

Although proteomics is the most accurate way to assess and quantify protein levels and monitor protein dynamics as compared to estimation based on mRNA analysis, the understanding of the molecular changes that occur at various steps of gene expression and regulation is important. The cellular state and various clinical outcomes are the results of various components, their interplay, crosstalk and complex interconnected mechanisms. Thus, gathering the conclusions and results from integrated analyses of transcriptome, translome, ribonome and proteome would be ideal for a more comprehensive understanding of global gene regulation. New efficient and often high-throughput methods and approaches that are emerging in each of these –omics fields contribute to this effort. These approaches have been able to begin to address the questions related to gene/protein dysregulation, monitor the dynamic changes of RNAs/proteins, quantify the protein levels with low experimental errors and high accuracy and target the candidates for proper clinical intervention and treatment. An example is the progress in labeling methods and combined approaches discussed in this review that allows identification/quantitation of newly synthesized proteins as well as characterization and monitoring of protein synthesis dynamics. Currently, the results obtained by direct analysis of proteomes by proteomics complement the data acquired for DNAs/RNAs from deep-sequenced DNAs/RNAs and methods such as polysome and ribosome profiling. The relevance of analyzing newly synthesized proteins to medicine is gradually increasing as the ability to detect dysregulated

synthesis of particular proteins in specific disease creates a platform for the application of proper therapeutic agents.

Expert commentary

Proteomic characterization of post-transcriptional regulation with direct identification of proteins that are associated with translational components/complexes and interconnected in multiple interaction mechanisms is an ultimate goal for understanding protein synthesis and its modulation. This objective has been hampered by the previous lack of methods that are directly able to label and enrich for newly synthesized proteins and to differentiate them from the bulk of static proteome but fortunately, a number of new efficient methods have emerged over past few years. Thus, non-canonical amino acids were used to metabolically label the newly synthesized proteins in various cells (HEK 293, PBMC, HeLa) *in vitro*, and in whole animals (mouse, rat, chicken), via injection or digestion of specifically modified diet. The application of combined metabolic labeling approaches that involve non-canonical amino acids and SILAC simultaneously allowed not only for enrichment and distinction of the newly synthesized proteins from pre-existing proteome but also enabled quantitation of these proteins, monitoring protein synthesis dynamics and detection of the changes in protein expression that occur at early stages under various stimuli and treatments (lipopolysaccharide, brain-derived neurotrophic factor, phorbol 12-myristate 13-acetate and ionomycin). As the enrichment is not provided by SILAC labeling, the combination with non-canonical amino acid labels allows detection of low abundance proteins otherwise not possible to identify by analysis of whole proteome. Moreover, affinity enrichment of AHA-biotin peptides shown 20-fold increase in detection of newly synthesized proteins by mass spectrometry as compared to the experiments where biotin tag was enriched on proteins. As well, it was shown that competition with endogenous Met *in vivo* can be overcome by using optimized AHA concentration and that the proteins can be detected in short time window after synthesis in intact animals.

The application of these methods that are specifically focused on characterization of newly synthesized proteins enables the identification of new mechanisms of post-transcriptional regulation in various diseases, which may reveal novel therapeutic targets. As these methods are developed for application in animal models as well as in humans, they must also be evaluated for accuracy, reproducibility and safety. A good example is a work carried out to determine plasma proteome dynamics using *in vivo* administration of deuterium oxide ($^2\text{H}_2\text{O}$) to human individuals (n=10). The proteins that were enriched with deuterium were subsequently quantified by mass spectrometry [88]. In this study, the protocol was developed to confirm the safety, efficiency and reproducibility of $^2\text{H}_2\text{O}$ intake for 14 days and also established endogenous turnover rates of 542 human proteins in plasma. In this instance, the approach was used for quantification of protein turnover; it represents a novel and efficient approach to investigate the plasma protein dynamics in human diseases with potential clinical applicability [88].

The other aspect that has empowered the progress was the development and the availability of efficient mass spectrometry instrumentation. State-of-the-art mass spectrometry instruments currently feature high resolution, sensitivity and accuracy allowing for the

identification and quantitation of thousands of proteins or for selective targeting of particular proteins of interest. Moreover, there are continual advances in the development of software and search engines able to process large numbers of generated spectra as well as to target particular protein modifications. There is ongoing progress in biostatistical methodology and the building of custom-made protein/peptide libraries necessary for accurate interpretation of mass spectrometry datasets.

Five-year view

The methods and approaches that have emerged recently render mass spectrometry-based proteomics a powerful tool that can contribute to the high priority effort to translate the knowledge acquired at the laboratory bench to the clinic. The availability and application of these methods make it possible to detect alterations in protein levels related to disease states, and recognition and identification of the relevant post-transcriptional mechanisms that regulate these changes. The new understanding and insight into pathologies that we are able to assess today was not possible a few years back due to methodological limitations. For example, the transition from static to dynamic proteomics with various techniques applied simultaneously, or complementary, with novel protein labeling methods and advanced instrumentation, software and bioinformatic tools have made it possible to monitor and characterize protein synthesis dynamics. This knowledge is tightly related to the ability to target post-transcriptional aspects of protein synthesis and to control protein concentration levels by applying new therapeutic agents and drugs. The use of dynamic proteomics allows for insights into the mechanism of drug action and target engagement and can reveal novel therapeutic targets. Thus, the successful application of proteomics to the clinic will equip the clinicians with powerful new tools for early diagnosis and effective disease treatment.

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Key issues

- Only a weak correlation between mRNA abundances and final protein levels has been reported in the literature due to multiple regulatory mechanisms during post-transcriptional steps, biological factors and technical issues.
- Methods are needed to quantify dynamic alteration in protein levels and rates across the proteome during transport, synthesis or degradation in a defined time window.
- The move from analyzing the static proteome to the examination of dynamic proteome has set the stage for therapeutic control of protein synthesis other than based solely on mRNA perturbation.
- Metabolic labeling approaches have emerged that enable the incorporation of the labels into proteins at the time of their synthesis.
- New proteomic approaches make it possible to distinguish low abundance newly synthesized proteins from pre-existing protein pools.
- Dynamic proteomics using pulse-chase metabolic labeling and mass spectrometry has allowed identification and characterization of newly synthesized proteins *in vitro* and *in vivo*, in cells and whole animals as early as 3 hours after their synthesis.
- Polysome profiling technique followed by proteomic analysis now offers the direct identification of the proteins associated with translational components from isolated polysome, monosome and ribosome subunit fractions as well as the specific proteins that are being synthesized.
- Identification and quantitation of the proteins early after their synthesis on the ribosomes is important, as the recognition of protein synthesis dysregulation at the early stage can indicate the factors contributing to pathology and can reveal new therapeutic targets.

Figure 1.

Nuclear encoded protein expression.

Transcription – information from DNA template in nucleus is transcribed to RNA (pre-mRNA; RNA transcript) that is further processed into functional messenger mRNA by capping, splicing (introns removed and exons ligated) and polyadenylation. Mature mRNA containing codons is exported into cytoplasm via nuclear pore complex. Various RNA-binding proteins, RNA-associated proteins (spliceosome, small nuclear snRNPs), splicing and transcription factors and accessory proteins regulate this step. In cytoplasm, mRNA becomes associated with multiple ribosomes forming polysomes. The amino acids (aa_s) present in cytoplasm are activated, attached to proper transfer tRNAs containing anti-codons in the presence of aminoacyl-tRNA-synthetase (tRNAs present in cytoplasm are "charged") and transported to mRNA on ribosome. At the beginning of protein synthesis, the ribosome is positioned over a start codon of mRNA and polypeptide synthesis takes place. The amino acids are transferred to growing peptide chain as mRNA moves through the ribosome and empty ("uncharged") tRNAs return to cytoplasm. After polypeptide is completed, it is released to cytoplasm, subjected to post-translational processing into active protein (folding, splicing, post-translationally modified...) and ribosome subunits separate. During this post-transcriptional steps, many regulatory RNAs, RNA-binding and associated proteins are present as well as various translation factors and chaperones. The methods and approaches for identification/quantitation mRNA/proteins are described in detail throughout the text.

Table 1.

Pros and cons of labeling techniques used in the analysis of the static and dynamic proteomes.

Labeling method	
	Static proteomics
Label-free	
spectral counting total ion current (TIC)	<u>Pros</u> Any sample, no labeling needed
	<u>Cons</u> Lower accuracy for low abundance proteins
Chemical	
ICAT isotopic tags	<u>Pros</u> Fast, any sample Multiple samples analyzed simultaneously by MS Can be used on samples not able to incorporate label metabolically (e.g. tissue)
	<u>Cons</u> Intact protein incorporation (medium variation) Applicable only <i>in vitro</i> Experimental variation due to sample parallel processing Limited incorporation (requires free Cys for labeling)
iTRAQ isobaric tags	<u>Pros</u> Fast, any sample Multiple samples analyzed simultaneously by MS Can be used on samples not able to incorporate label metabolically (e.g. tissue) Full incorporation (requires Lys)
	<u>Cons</u> Peptides incorporation (can have high variation) Applicable only <i>in vitro</i> Experimental variation due to sample parallel processing
	Dynamic proteomics
Metabolic	
^{15}N , ^{13}C isotopic atoms	<u>Pros</u> Applicable <i>in vitro</i> and/or <i>in vivo</i> N (C) atoms allowing the full proteome to be labeled
	<u>Cons</u> Longer time for full incorporation <i>in vivo</i>
SILAC isotopic amino acids	<u>Pros</u> Applicable <i>in vitro</i> and/or <i>in vivo</i> Minimum variation (labeling at cell/organism levels) Pulse-chase analysis of newly synthesized proteins Used for quantitative analysis
	<u>Cons</u> Longer time for full incorporation (several cell divisions) Labeling does not provide enrichment of proteins
non-canonical amino acids (AHA, HPG; azide/alkyne analogs of Met)	<u>Pros</u> Applicable <i>in vitro</i> and/or <i>in vivo</i> Enrichment by carrying out Click-iT™ reaction Minimum variation (labeling at cell/organism levels) Fast incorporation at early stages of protein synthesis Pulse-chase analysis of newly synthesized proteins Applicable to cells not undergoing further cell division
	<u>Cons</u> 500-fold affinity of cells for Met over AHA/HPG

Table 2.

Summary of proteomic approaches/techniques for the identification and quantification of newly synthesized proteins/proteome (NSP) which are covered in this review.

labeling/strategy	cells(organism)/setting	findings	Ref.
AHA/BONCAT	human HEK 293/ <i>in vitro</i>	NSP synthesized in 2-hour window were identified; technology is feasible for temporal/spatial characterization of NSP.	[39]
AHA	human PBMC/ <i>in vitro</i>	NSP in PBMC can serve as pool for biomarker discovery in human stroke.	[71]
AHA, iTRAQ/BONCAT	<i>in vitro</i>	Relation of NSP to pro-survival outcome of starvation-mediated autophagy was established.	[72]
AHA, HPG/NANCAT	HeLa, <i>E.coli</i> / <i>in vitro</i>	Nitrilase activated AHA labeling yielded in 616 modified Met sites corresponding to 400 NSP.	[73]
AHA/DiDBiT	human HEK 293T/ <i>in vitro</i> rat eyes administration/ <i>in vivo</i>	20-fold increase in NSP detection due to enrichment of peptides as opposite to enrichment of proteins; NSP identified within 3 hours after synthesis.	[35]
AHA in diet/PALM	mouse/ <i>whole animal</i>	4 to 6 days on diet is sufficient for NSP identification in mice implying that recognition of early regulators/alteration related to diseases is feasible.	[33]
AHA, HPG	mouse intraperitoneal injection/ <i>in vivo</i>	AHA/HPG was incorporated into multiple tissue types; AHA labeling has no substantial effect on protein expression and on embryos of AHA-labeled mice	[34]
AHA and SILAC	human PC3, WPMY-1/ <i>in vitro</i>	NSP identification after affinity enrichment from secretomes containing 10% serum.	[32]
AHA and SILAC; LPS treatment	mouse RAW 264.7 macrophages/ <i>in vitro</i>	LPS-induced macrophage activation yielded in identification of NSP within first 3 hours.	[75]
AHA and SILAC PMA and ionomycin treatment	human CD4 T cells/ <i>in vitro</i>	107 proteins differentially expressed were identified after 2 hours and 4 hours of treatment	[76]
AHA and SILAC/BONLAT (BONCAT and SILAC); BDNF treatment	human HEK 293/ <i>in vitro</i>	53 proteins altered by BDNF treatment of HEK 293 cells were identified; proteins connected to BDNF pathway were newly recognized.	[77]
AHA, AHA ^{30:1} and SILAC	HeLa/ <i>in vitro</i>	AHA and AHA ^{30:1} labeling yielded in 1931 and 1529 NSP quantified within 4 hours, and 1484 and 416 NSP within 30 min of labeling, respectively	[78]
AHA and SILAC	human TC32/ <i>in vitro</i>	NSP regulated by YB-1 in translation were quantified after 45 min labeling of TC32 cells.	[79]