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Cell-selective proteomics for biological discovery

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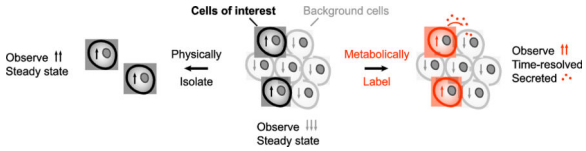
Abstract

Cells alter the proteome to respond to environmental and developmental cues. Global analysis of proteomic responses is of limited value in heterogeneous environments, where there is no "average" cell. Advances in sequencing, protein labeling, mass spectrometry, and data analysis have fueled recent progress in the investigation of specific subpopulations of cells in complex systems. Here we highlight recently developed chemical tools that enable cell-selective proteomic analysis of complex biological systems, from bacterial pathogens to whole animals.

Graphical Abstract



Keywords

cell-selective; proteome; TRAP; CTAP; BONCAT; SORT; OP-Puro; APEX; newly-synthesized; subcellular; click chemistry

Introduction

Cellular protein synthesis changes rapidly in response to internal and external cues in ways that vary from cell to cell. Global proteomic analyses of microbial communities, tissues and organisms have provided important insights into the behavior of such systems, but can obscure the diversity of responses characteristic of different cellular subpopulations (Figure 1). Cell-selective methods for the analysis of protein synthesis are being developed to resolve proteomic changes in space and time.

Cell-type-specific transcriptomics experiments have revealed mRNA expression patterns in a wide array of biological systems, but mRNA and protein levels are often dissonant [1].

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Moreover, some important elements of proteome dynamics, including posttranslational modification, degradation, and localization, cannot be addressed by mRNA measurements alone [2,3]. Until recently, changes in protein abundance in specific cells could be measured only in targeted, low-throughput experiments, but innovations in mass spectrometry and computational algorithms have facilitated the identification and quantification of thousands of proteins simultaneously from complex biological samples [4–6].

In this Opinion, we highlight recent developments in determining cell-type-specific proteomes and recommend experimental design strategies that are guided by the question at hand.

Cell-selective translatomics and ribosome profiling

Translatomic studies, which select for ribosome-associated transcripts, have yielded stronger correlations between transcript and protein abundances than experiments that measure steady-state mRNA levels [7]. Cell-type-specific studies have been enabled by translating ribosome affinity purification (TRAP), a method in which epitope-tagged ribosomes and their associated transcripts are captured, enriched and subjected to amplification and deep sequencing [8]. TRAP can be rendered cell-specific by placing expression of the tagged ribosome under control of a selective promoter.

More recently, Ingolia and Weissman have developed ribosome profiling, which identifies ribosome-protected mRNA footprints and allows investigators to determine ribosome occupancy with positional specificity. This information can be used to measure translation levels and locate non-canonical start sites [7]. Gonzalez *et al.* used TRAP to cell-selectively purify ribosome-bound transcripts, and employed ribosome profiling to identify the translatome of gliomas and to reveal decreased translation in glial progenitors compared to the tumor microenvironment [9]. Ribosome profiling is a powerful technique that we expect to find increasing use upon further development of cell-specific methods.

While translatomic studies provide greater depth of coverage than current proteomic measurements, ribosome binding does not ensure that a transcript is undergoing active translation [10].

Separating cells for steady-state proteomic analysis

The earliest strategies to determine cell-specific proteomes relied on separating and purifying the cells of interest prior to analysis. Cells can be sorted on the basis of expression of a transgene under control of a cell-specific promoter or by antibody staining of marker epitopes. These tools are well established and have been thoughtfully reviewed [10,11]. Physical methods have been used for years to isolate cell types from mammalian tissues for subsequent downstream analyses [12,13]. More recently these methods have been used to measure growth rates and elucidate proteomic signatures of *Salmonella* during murine infection [14].

Physical separations remain the best method for analyzing clinical specimens and genetically intractable organisms. However, imperfect separations and long sample

processing times can diminish selectivity and increase the likelihood of artifacts. Furthermore, such methods intrinsically yield steady-state proteomic information. In contrast, metabolic labeling strategies enable cell-specific proteomic analysis to be accomplished in time-resolved fashion.

Metabolic labeling: trade-offs between sensitivity and perturbation

Metabolic labeling methods are temporally resolved and use an arsenal of amino acid isotopologs, non-canonical amino acids, and analogs of protein synthesis inhibitors (Figure 2). Each of these strategies can be placed under control of cell-specific genetic elements to afford cellular resolution. The choice of promoter(s) is key for these systems, and the degree of protein labeling needs to be weighed against the possibility of perturbing the system. Results should be validated via independent assays because labels may affect protein expression, stability, and/or function.

Cell-type-specific labeling using amino acid precursors (CTAP)

Stable isotope labeling by amino acids in cell culture (SILAC) relies on the incorporation of isotopically labeled amino acids into proteins. To make SILAC cell-selective, Gauthier *et al.* introduced cell-type-specific labeling using amino acid precursors (CTAP), a method that exploits the fact that lysine is an essential amino acid in mammalian cells [15]. Cell-selective expression of biosynthetic enzymes allows L-lysine isotopologs to be synthesized *in situ* starting from isotope-labeled precursors. Only minor differences in gene expression resulted from feeding the heavy precursor to cells expressing the biosynthetic machinery versus supplementing cells directly with L-lysine.

In principle, both exchange of L-lysine between cells and extracellular processing of the precursor can compromise the cell-specificity of the CTAP method. When Lavis and coworkers employed an analogous strategy to unmask fluorophores in targeted cells, they noted that the unmasked small molecule diffused through gap junctions. This effect can be exploited to study cell-cell connectivity, but would confound cell-specific protein labeling if the small molecule were to diffuse to cells lacking the decaging enzyme [16]. To address these concerns, Tape *et al.* optimized CTAP for eukaryotic cell types and achieved ~90% cell-specific labeling in ten-day co-cultures [17]. Using their optimized method, Tape *et al.* combined CTAP with phosphoproteomics to study heterocellular $KRAS^{G12D}$ signaling in pancreatic ductal adenocarcinoma cells [18]. By restricting their proteomic analysis to cells that expressed $KRAS^{G12D}$, the authors showed that the oncogene regulates AKT through reciprocal signaling – not through the accepted cell-autonomous pathway.

Bio-orthogonal Non Canonical Amino acid Tagging (BONCAT)

CTAP is most suitable for cell-specific experiments conducted in culture on timescales of 3–7 days [19]. For studies that require better time resolution, the bio-orthogonal non-canonical amino acid tagging (BONCAT) method, introduced by Dieterich and coworkers, offers a good alternative [20,21]. In its original form, BONCAT exploits the capacity of the endogenous aminoacyl-tRNA synthetases to charge non-canonical amino acids (ncAAs) to their cognate tRNAs for incorporation into proteins. ncAAs bearing bio-orthogonal chemical

handles, often azides or alkynes, enable conjugation to affinity tags and separation of tagged proteins from the rest of the protein pool. The methionine surrogates azidohomoalanine (Aha) and homopropargylglycine (Hpg) have been used to probe proteome dynamics in bacterial [22–26] and mammalian [27] systems, and notably, to enrich and quantify secreted proteins [28]. Depletion of cellular methionine is not necessary for Aha labeling; Bagert *et al*. showed that a 30:1 ratio of Aha to Met yielded excellent protein labeling while minimizing perturbations that might be expected to arise from methionine starvation [29]. Other studies have shown that ncAA labeling for periods of up to two days do not perturb embryonic growth in live mice [30]. In designing a BONCAT experiment, the investigator should choose concentrations of the ncAA label and its natural counterpart that reflect the relative rates of activation of the amino acids by the cognate synthetase.

In 2009, Ngo and coworkers developed a cell-selective version of BONCAT by engineering an *E. coli* methionyl-tRNA synthetase (*Ec*MetRS) variant that activates azidonorleucine (Anl). Because Anl is a poor substrate for wild-type *Ec*MetRS, labeling is essentially restricted to cells that express the mutant synthetase. In the first example of the cell-specific BONCAT method, Ngo *et al*. reported specific labeling of *E. coli* cells co-cultured with murine alveolar macrophages [31]. Grammel *et al*. expanded on this method by enriching for proteins synthesized during *Salmonella typhimurium* infection [32], and Mahdavi and coworkers used BONCAT to determine the order in which *Yersinia enterocolitica* effector proteins are injected into HeLa cells in the course of infection [33].

Cell-selective BONCAT has now been extended to proteomic analysis in live animals, highlighting its potential utility in creating cell-specific proteomic “atlases”. In 2015 we reported a mutant phenylalanyl-tRNA synthetase (PheRS) that enables the use of *p*-azidophenylalanine (Azf) as a BONCAT probe in *Caenorhabditis elegans* [34]. Combining cell-selective BONCAT with stable isotope labeling, we used the *myo-2* promoter to direct expression of the mutant synthetase to the 20 pharyngeal muscle cells of the worm. We were able to quantify 2270 proteins by this method, and to verify the pharyngeal expression patterns of several previously uncharacterized proteins.

Dieterich and coworkers have adapted cell-selective BONCAT labeling to *Drosophila melanogaster* through controlled expression of the *Dm*MetRS L262G mutant [35]. Chronic administration of Anl in developing flies expressing the mutant synthetase caused slight impairments in larval growth and behavior, but shorter (48 h) labeling times led to no noticeable defects. Importantly, administration of the amino acid in flies that did not express the mutant MetRS caused no discernible effect. Using this strategy, Niehues *et al*. measured reduced neuronal protein synthesis rates in a *Drosophila* model of Charcot-Marie-Tooth (CMT) neuropathy. Mahdavi *et al.* and Muller *et al.* have employed the analogous (L274G) mouse synthetase in mammalian cell culture and in a neuronglia co-culture system, respectively [36,37]. The latter experiments enabled the investigators to monitor changes in the astrocytic proteome in response to treatment with brain-derived neurotrophic factor (BDNF).

Split synthetases have been developed to enable cell-selective analysis of systems in which no single promoter restricts expression of the mutant enzyme to the cells of interest [38].

Notably, all amino acids and enrichment media needed for BONCAT experiments are commercially available.

Stochastic Orthogonal Recoding of Translation (SORT)

Chin and coworkers have developed a residue-specific ncAA-labeling technology termed stochastic orthogonal recoding of translation (SORT), which – like BONCAT – allows chemoselective modification and enrichment of newly synthesized cellular proteins. SORT relies on expression of a pyrrolysyl-tRNA synthetase and its cognate tRNA [39,40]. Using this method, Elliott *et al.* cell-selectively labeled and identified proteins made during different stages of larval growth in *Drosophila*. Importantly, SORT allows the anticodon of the cognate tRNA to be changed to direct the ncAA to different sets of codons in the labeled proteins. Elliott *et al.* have characterized the enrichment process and found that tagging at different codons leads to the enrichment of overlapping, but distinct sets of proteins [41]. The authors noted that simultaneous expression of multiple tRNAs (i.e., tRNA-Ala, -Ser and -Met) increases labeling efficiency. Furthermore, Elliott *et al.* found that enrichment after tagging improves detection of low-abundance proteins.

Cell-selective O-propargyl-puromycin (OP-Puro) labeling

The *O*-propargyl-puromycin (OP-Puro) method also incorporates “clickable” handles into nascent proteins [42]. Cohen and coworkers recently achieved cell-targeted OP-puromycin labeling by using a phenylacetyl-caged analog that is uncaged by cell-selective expression of penicillin G acylase (PGA) [43]. The OP-puro method is the fastest of the metabolic labeling methods and the best suited for studies requiring ultra-short labeling times [44]. Prolonged labeling with OP-puro would be expected to perturb cellular behavior through inhibition of global translation. Furthermore, premature truncation renders this method ineffective for the identification of secreted proteins.

Spatially restricted & subcellular proteomics

Ting and coworkers first used a mutant ascorbate peroxidase (APEX) to selectively tag proteins localized to the mitochondrial matrix [45,46]. Unlike the cell-selective metabolic labeling methods just described, this method labels all proteins, including pre-existing proteins, within a subcellular volume. Chen *et al.* used this elegant strategy to characterize multiple cell types in *Drosophila*, including the mitochondrial matrix of muscle tissue [47]. The Weissman laboratory has combined the APEX labeling method with ribosome profiling to characterize localized protein synthesis in yeast [48,49]; extension of their method to cell-selective analysis is readily imagined.

Choosing a cell-selective proteomic method

The choice of a cell-selective method of proteomic analysis should reflect careful consideration of the advantages and disadvantages of each of the available approaches (Table 1).

Physical sorting methods allow straightforward characterization of the steady-state proteome of the cell type of interest. However, removing cells from their natural environments prior to

analysis raises concerns about artifacts, leads to limited temporal information, and sacrifices information about secreted proteins.

Ribosome profiling, when combined with cell-selective TRAP, provides significantly higher coverage of the gene expression profile than any direct proteomic measurement. But ribosome profiling is not a perfect proxy for protein synthesis and yields no information regarding protein secretion [50]. Moreover, only direct proteomic methods allow detection of post-translational modifications.

CTAP simplifies quantitative proteomic measurements for samples of relatively low complexity, but enrichment-based strategies (i.e., BONCAT, SORT or OP-Puro) are likely to be superior for short labeling times or for analysis of rare cells in complex tissues. Only APEX yields snapshots of the steady-state proteome with sub-cellular resolution. All cell-selective, enrichment-based experiments require the use of genetically tractable organisms.

Optimization of enrichment-based strategies requires careful consideration of alternative purification chemistries. Attachment to the resin used for purification can be accomplished either by direct covalent ligation or by a two-step process of affinity-tagging (e.g., with biotin reagents) and non-covalent binding (e.g., to streptavidin resins). Following appropriate washing steps, samples can be released from the resin by competitive binding, by proteolysis, or by selective cleavage of the affinity reagent. APEX appends biotin to surrounding molecules, so streptavidin-based resins are used to enrich for labeled proteins [46]. OP-Puro requires an azide-based affinity handle or resin for enrichment [43]. SORT uses cyclopropene labels and tetrazine linkers in a ligation reaction reported to be 100 to 1000 times faster than the strain-promoted azide-alkyne cycloaddition [41]. BONCAT labels with either alkynes or azides, and enriches with complementary azide or alkyne reagents. A special consideration arises in the analysis of lysates labeled with azides: Free thiols, which are known to react with cyclooctynes, must be blocked with capping reagents such as iodoacetamide or N-ethylmaleimide to avoid high background [34]. Many azide and alkyne resins and linkers are commercially available, and tetrazine-based reagents are beginning to appear on the market.

If the investigator wishes to identify the sites at which protein labeling has occurred, linkers with cleavable moieties can be used [51]. For many experiments, though, identification of labeling sites is not necessary, and on-bead digestion of enriched proteins is often simpler and more straightforward. In our hands, directly conjugating azide-labeled lysates to cyclooctyne resins has allowed us to identify larger numbers of relevant proteins [34]. Because enrichments are never perfect, running mock enrichments of unlabeled sample along with labeled samples provides a useful indication of background reactivity and non-specific protein contamination. Samples with abundant contaminating biopolymers such as pectin, serum proteins, or mucin may need an additional step to remove or degrade these contaminants and facilitate successful enrichment.

Conclusions and future outlook

Recent years have witnessed the introduction of powerful techniques that allow investigators to monitor protein synthesis with unprecedented resolution in space and time. Cell-specific proteomic analyses will play a key role in the identification of the mechanisms that govern cell specialization and that allow complex organisms to respond to changing environments.

Acknowledgments

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Highlights

- Cell-selective proteomics is important in complex, heterocellular environments
- Innovative chemical tools enable unbiased cell-type-specific interrogation of translation
- Labeling methods including TRAP, CTAP, BONCAT, SORT, OP-Puro and APEX have been developed for cell-selective analysis
- Sequencing and mass spectrometry-based strategies complement one other in the study of protein synthesis
- The strengths and limitations of each analytical method must be considered carefully in the context of the biological question to be addressed

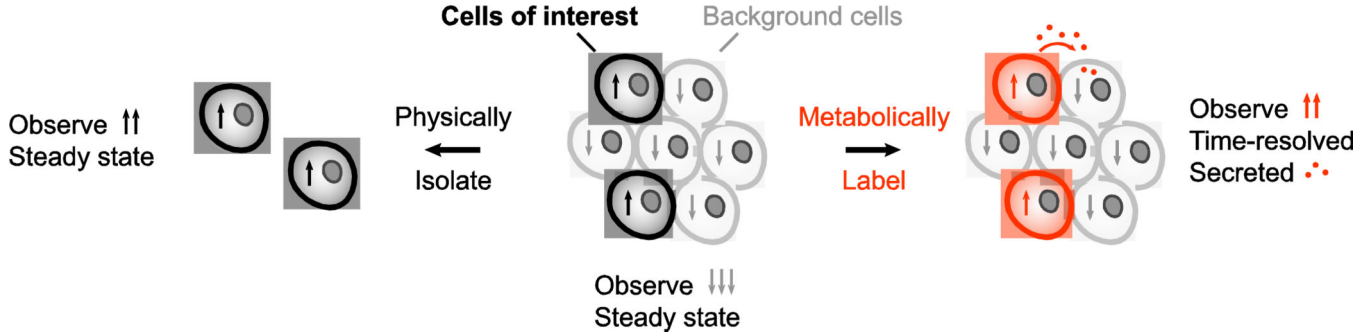

Figure 1.

The importance of cell-type-specific proteomics. Bulk measurements of complex tissues can obscure proteomic changes that occur in specific sub-populations of cells. A protein that is highly expressed (up arrows) in the cells of interest might be detected at low abundance overall due to low expression (down arrows) in background cells. Cells of interest must be physically isolated or tagged to measure the cell-specific proteome. Physical isolation measures steady-state levels of intracellular proteins, whereas labeling methods can be time-resolved and used to identify secreted proteins.

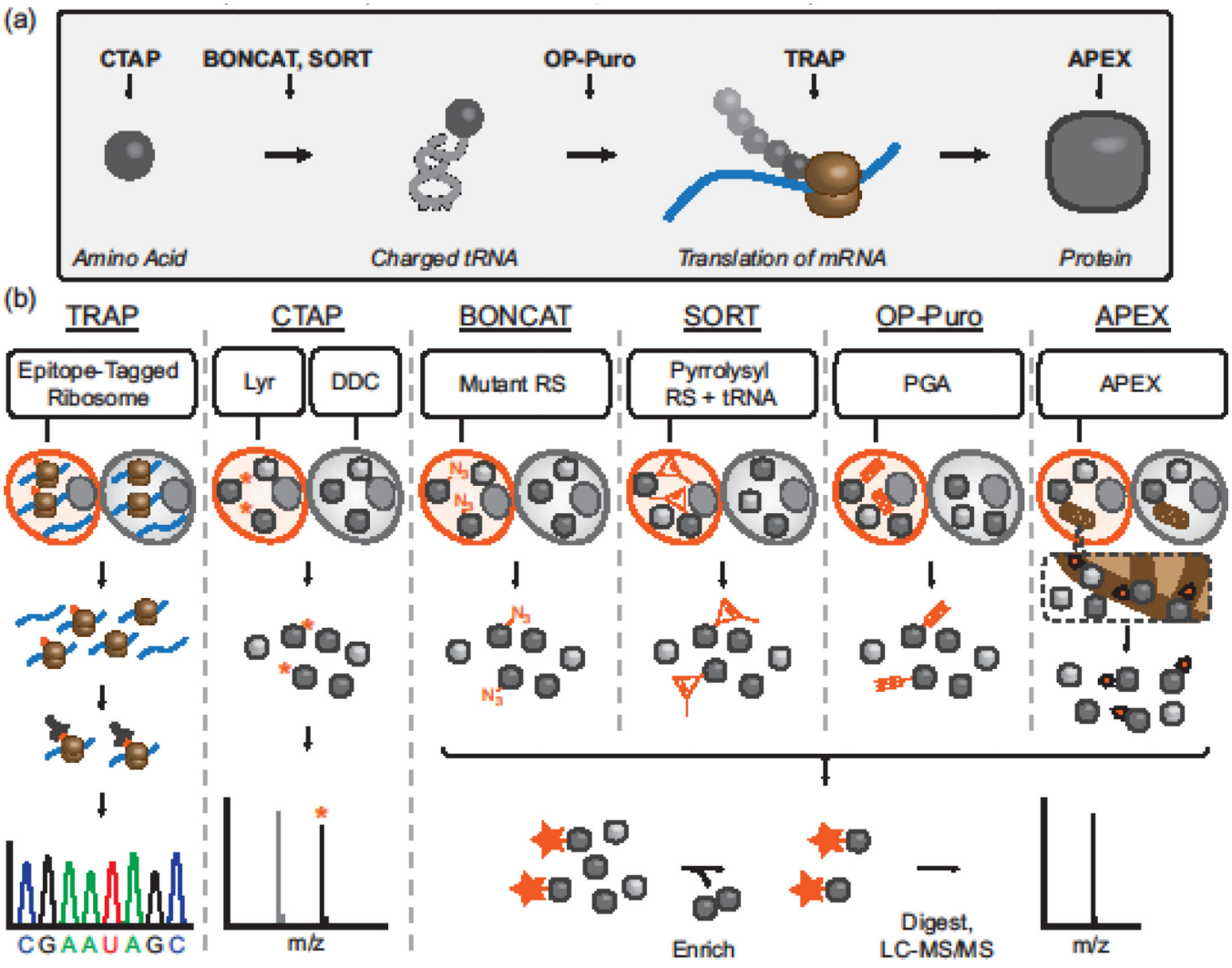

Figure 2.

Labeling strategies for cell-selective proteomics. a) The process by which amino acids are incorporated into proteins, and the step exploited by each of the labeling methods discussed in this Opinion. b) Schematic of each technique.

Translating ribosome affinity purification: TRAP; Cell type-specific labeling using amino acid precursors: CTAP; Bio-orthogonal non-canonical amino acid tagging: BONCAT; Stochastic orthogonal recoding of translation: SORT; *O*-propargyl puromycin: OP-Puro; ascorbate peroxidase: APEX; Lysine racemase:Lyr; diaminopimelate decarboxylase: DDC; aminoacyl-tRNA synthetase: RS; penicillin-G-acylase: PGA.

Table

Advantages and disadvantages of cell-specific proteomic methods.

	Cell-Specific Method	Biomolecule identified	Organisms demonstrated in?	Temporal Resolution	Secreted?	PTM?	Advantages	Disadvantages	References
Translatomics	TRAP	mRNA	Prokaryotes, eukaryotes	Snapshot of translation	No	No	High sequence coverage, able to combine with ribosome profiling	Requires expression of tagged ribosome, miss translational control	[7–11]
Cell Separation	Manual	mRNA, Protein	Prokaryotes, eukaryotes, clinical samples	Steady-state proteome	No	Yes	Straightforward, inexpensive	Possible artifacts from sample preparation, time and labor intensive	[3], [12–13]
	FACS	mRNA, Protein	Prokaryotes, eukaryotes, clinical samples	Steady-state proteome	No	Yes	High-throughput	Requires dissociation of cells, need expression of transgene or recognizable epitope, need specialized equipment	[14]
Metabolic	CTAP	Protein	Cell culture	Up to 10 days continuous cell culture	Yes	Yes	Quantitative, compatible with long-term cell culture	Requires expression of Lyr/DDC, cells must be auxotrophic for lysine, restricted to cell culture	[15], [17–19]
	BONCAT	Protein	Prokaryotes, eukaryotes, cell culture	Newly synthesized proteins in minutes (prokaryotic, cell culture) to days (whole animal)	Yes	Yes	Commercially available reagents, high degree of temporal resolution	Requires expression of synthetase; only Met/Phe residue replacement available currently, requires delivery of the ncAA	[20–38], [44]
	SORT	Protein	Eukaryotes, cell culture	Newly synthesized proteins in minutes (cell culture) to days (whole animal)	Yes	Yes	Easy to change the residue of non-canonical amino acid incorporation, high degree of temporal resolution	Requires expression of synthetase/tRNA pair, reagents not currently commercially available, requires delivery of the ncAA	[39–41]
	OP-Puro	Protein	Cell culture	Newly synthesized proteins in minutes (cell culture)	No	No	Not residue-dependent, highest degree of temporal resolution	Requires expression of PGA, reagents not currently commercially available	[42–44]
Spatial	APEX	mRNA, Protein	Eukaryotes, cell culture	Subcellular, steady-stateproteome	No	Yes	High degree of spatial resolution (subcellular)	Requires expression of APEX/HRP, no temporal resolution, requires delivery of peroxide + biotin-phenol reagent, possible toxicity of peroxide over longer timescales,	[45–49]

Cell-Specific Method	Biomolecule identified	Organisms demonstrated in?	Temporal Resolution	Secreted?	PTM?	Advantages	Disadvantages	References
							needs oxidative environment	