PUNCH-P for global translatome profiling
Methodology, insights and comparison to other techniques

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Regulation of mRNA translation is a major modulator of gene expression, allowing cells to fine tune protein levels during growth and differentiation and in response to physiological signals and environmental changes. Mass-spectrometry and RNA-sequencing methods now enable global profiling of the translatome, but these still involve significant analytical and economical limitations. We developed a novel system-wide proteomic approach for direct monitoring of translation, termed PUromycin-associated Nascent CHain Proteomics (PUNCH-P), which is based on the recovery of ribosome-nascent chain complexes from cells or tissues followed by incorporation of biotinylated puromycin into newly-synthesized proteins. Biotinylated proteins are then purified by streptavidin and analyzed by mass-spectrometry. Here we present an overview of PUNCH-P, describe other methodologies for global translatome profiling (psILAC, BONCAT, TRAP/Ribo-tag, Ribo-seq) and provide conceptual comparisons between these methods. We also show how PUNCH-P data can be combined with mRNA measurements to determine relative translation efficiency for specific mRNAs.

Introduction

In recent years, regulation of mRNA translation has attracted increasing attention as an important determinant of gene expression in health and disease. Such regulation can result in different translation rates for mRNAs of similar abundance; therefore, estimates of steady-state mRNA levels by methods such as microarray or deep-sequencing (RNA-seq) may fail to capture critical aspects of gene expression. Along with advances in system-wide RNA and protein analysis platforms, several methods were developed to better understand the complex regulation of translation. While these methods were successfully employed to study many biological questions, a need still remained for a simple, low-cost technique that would allow direct measurement of system-wide protein synthesis in cells and whole tissues. For this purpose, we developed a novel approach termed PUromycin-associated Nascent CHain Proteomics (PUNCH-P), which directly monitors nascent polypeptide chains by mass-spectrometry without the need for prior metabolic labeling.

Overview of PUNCH-P

PUNCH-P is based on the isolation and identification of ribosome-associated nascent polypeptide chains (flowchart in Figure 1). It starts with cell lysis and recovery of intact ribosomes by ultracentrifugation, followed by incubation with biotin-puromycin to affinity label nascent chains. These polypeptides are then captured on streptavidin beads, washed rigorously and subjected to on-bead trypsin digestion and liquid-chromatography tandem mass-spectrometry (LC-MS/MS). To control for non-specific binding, similar amounts of isolated ribosomes are processed simultaneously, without the addition of biotin-puromycin. Proteins are identified and quantified using appropriate software e.g., MaxQuant, and specific binders are extracted using ANOVA or a student t test. These protein measurements represent the level of synthesis for each identified protein. For a detailed protocol, see Aviner et al., Nature Protocols (in press, 2014; DOI: 10.1038/nprot.2014.051).

The naturally-occurring antibiotic puromycin, a tyrosine-tRNA mimic, has been used to study protein synthesis by immunoblotting and immunofluorescence with anti-puromycin antibodies and biotin- or fluorophore-puromycin conjugates. Puromycin is catalytically incorporated by the ribosomal peptidyl-transferase center into the C-terminus of elongating nascent chains in a sequence-independent manner. This so called "puromycylation" reaction leads to translation termination and release of C-terminally truncated peptides bearing a single puromycin moiety. In PUNCH-P, we use biotin-puromycin to label newly-synthesized proteins because the biotin moiety allows efficient...
capture of puromycylated polypeptides, unlike currently available anti-puromycin antibodies. Compared with non-derivatized puromycin, biotin-puromycin is poorly incorporated into newly-synthesized proteins in cultured cells or whole cell lysates, possibly due to lower permeability through the plasma membrane, steric hindrance or lower affinity to the ribosome A-site. Nevertheless, both puromycin and biotin-puromycin are incorporated at similar efficiencies into newly-synthesized proteins following isolation of ribosome-nascent chain complexes by ultracentrifugation.

Extracting intact ribosome complexes from cells or whole tissues prior to affinity labeling of nascent chains allows for efficient labeling without risking unwanted effects that may be induced by in vivo puromycin treatment. Such effects include cellular stress caused by accumulation of truncated or misfolded proteins, as well as targeted proteasomal-mediated degradation of puromycylated peptides. Instead of whole-cell metabolic labeling, intact ribosomes are first pelleted through a sucrose cushion to deplete translation factors and amino acids. Biotin-puromycin is then incorporated into nascent chains, labeling only one polypeptide per ribosome independent of the duration of labeling, because ribosomes following termination cannot re-start an additional round of translation without the soluble endogenous components of the translation machinery. As a result, the number of polypeptides labeled, captured and identified by MS is proportional to the amount of translating ribosomes recovered from the cells, thus requiring large amounts of starting material in order to obtain high coverage of the translatome. However, this unique in vitro labeling strategy confers high temporal precision because the resulting analysis does not represent a time-window, but rather a snapshot of translation at the specific time of harvesting. Thus, PUNCH-P is particularly powerful in studying biological questions that involve rapid or short-term modulation of translation, e.g., in response to physiological, environmental or pharmacological changes, in both cultured cells and whole tissues.

To capture labeled proteins and eliminate background binders, including the translation machinery itself, pull-down is performed with streptavidin beads under high stringency conditions with urea and SDS buffers and extensive washes. Because biotinylated proteins are difficult to elute under these conditions, we use on-bead trypsin digestion to prepare the peptides for MS analysis.

In contrast to common ribosome isolation protocols, we do not use cycloheximide to arrest elongating ribosomes prior to lysis and fractionation, as this hinders puromycin activity. In our hands, omitting cycloheximide from the ribosome isolation procedure had no effect on polysome size and stability in HeLa cells. However, cycloheximide can be replaced with emetine, which inhibits translation elongation but does not interfere with puromycin activity. Our data indicate that a 15 min emetine pretreatment of HeLa cells and inclusion of emetine in the lysis buffer do not affect the identity or relative quantity of proteins detected by PUNCH-P.

We applied PUNCH-P to study fluctuations in protein synthesis at selected time points throughout the cell cycle in HeLa cells, and found that out of almost 5,000 proteins identified, hundreds showed statistically significant variations in synthesis between different cell cycle stages. We further used PUNCH-P to identify and quantify over 4,000 newly-synthesized proteins in whole mice brains (unpublished results), confirming its applicability for analysis of tissue translatomes. Considering that the puromycin mechanism of action is highly conserved throughout prokaryotes and eukaryotes, we expect PUNCH-P to be applicable to any model system.

Translatome Profiling: Concepts and Comparison between Methodologies

Monitoring translation has been performed in the past decades by metabolic pulse-labeling of proteins with radioactive amino acids. These methods enabled examination of changes in overall or mRNA-specific translation efficiency, but did not provide a system-wide detailed view of the translatome. For high-resolution identification and quantification of newly-synthesized proteins, metabolic labeling is combined with MS analysis and the radioactive amino acids are replaced by amino acid analogs labeled with either stable isotopes or chemical tags. Translation can also be monitored by microarray analysis of ribosome-associated mRNA or deep sequencing of ribosome-protected mRNA fragments. Available methods for global translatome profiling are outlined in Figure 2 and discussed in greater detail in the following paragraphs.

Pulsed Stable Isotope Labeling by Amino acids in Cell culture (pSILAC)

pSILAC is based on pulse incubation of cell cultures with stable isotope labeled amino acids. Cells are then lysed and proteins are directly trypsinized and analyzed by MS, which quantifies the ratio of labeled (newly-synthesized) and unlabeled (pre-existing) proteins to provide a measure of protein synthesis. This method is successfully used to quantify long-term sustainable changes in protein synthesis, but cannot be used to detect rapid fluctuations because accurate quantification of SILAC pairs requires relatively long (> 10 h) pulses to achieve sufficient labeling. In addition, measured protein abundances reflect the net result of both protein synthesis and degradation.

Bio-Orthogonal/Quantitative Non-Canonical Amino acid Tagging (BONCAT and QuaNCAT)

These methods are based on pulse incubation of cell cultures with a modified methionine analog called azidohomoalanine (AHA), either alone or in combination with SILAC amino acids. Cells are then lysed and biotin or another affinity tag is added to newly-synthesized AHA-labeled proteins through click-chemistry reactions. Such tagged proteins are isolated using the appropriate affinity purification matrix, trypsinized and analyzed by MS. This method requires pre-depletion of the intracellular stores of endogenous methionine followed by supplementation of the AHA amino acid analog, both of which can result in cellular stress and potential alteration of translation patterns. The coverage of BONCAT and QuaNCAT has so far been limited to several hundreds of proteins.
Expression microarray or RNA-seq of ribosome-associated mRNA^{17,18}

In this method, cells are lysed following cycloheximide pre-treatment to arrest nascent chain elongation, followed by polysome separation on a sucrose gradient. Polysome-associated mRNA is then extracted, reverse-transcribed and hybridized to microarrays or analyzed by RNA-seq. Other versions of this method use cell or tissue extracts and tag recombinant ribosomal proteins, such as eGFP-Rpl10a in the Translating Ribosome Affinity Purification technique (TRAP)^{19} or HA-Rpl22 in the Ribo-tag technique.^{20} These are used to selectively pull-down ribosomes from cultured cells and specific tissues, followed by microarray analysis of the co-eluted mRNAs.

Ribosome profiling (also termed Ribo-seq)^{21,22}

This protocol involves pre-treatment of cultured cells to arrest nascent chain elongation, followed by lysis and nuclease digestion to degrade all mRNA molecules that are not protected by ribosomes. The remaining undegraded RNA fragments are isolated by a series of purification steps, including PAGE fragment size selection and rRNA subtractive hybridization to remove rRNA fragments, followed by reverse transcription and deep-sequencing analysis. Ribo-seq is a powerful technique that provides exact positional information of ribosome binding along mRNAs and therefore offers the unique possibility to investigate translation at a sub-codon resolution. It is used to measure translation efficiency, identify translation initiation and translation pause sites and map novel open reading frames^{23,24}; as such, it is currently the only method that allows global investigation of this so-called “hidden translatome.”

A comprehensive comparison between the different methods is presented in Tables 1–3. Table 1 summarizes the fundamental differences between the methods based on type of readout (protein or RNA) and mode of monitoring. In principle, RNA-based methods do not require in vivo labeling and generate a snapshot prediction of protein synthesis, based on the steady-state amount of ribosome-bound mRNA molecules at a specific point of time. Because these methods do not require in vivo labeling, they can also be used to analyze translation in whole tissues. However, the assumption that association of mRNA with ribosomes is necessarily a reliable indicator of active translation could be misleading, as inhibition of protein synthesis does not always correlate with a proportional decrease in the number of ribosomes associated with the encoding mRNA.^{25-29}

In contrast, protein-based methods (except PUNCH-P) require in vivo labeling and measure accumulation of translated proteins over the labeling period. However, the coverage of these methods depends heavily on relatively long labeling durations, leading to identification of cumulative changes in protein synthesis and degradation and precluding measurements of rapid or short-term changes in translation. In addition, BONCAT may be susceptible to bias introduced by the pre-depletion of methionine and supplementation of amino acid analogs. In comparison, PUNCH-P directly measures newly-synthesized proteins but does not require in vivo labeling, thus generating a snapshot of translation similar to RNA-based methods. This is a unique attribute of PUNCH-P, because pSILAC or BONCAT can only be used to label proteins in intact cells and not following extraction of ribosome-nascent chain complexes. Nevertheless, additional research is required to understand whether proteins whose synthesis is stalled post-initiation may be over-represented in PUNCH-P.

Table 2 summarizes points related to resolution, coverage and starting material. In PUNCH-P, since labeling is performed in vitro, coverage is limited by the duration of labeling but the amount of translating ribosomes. As a result, in order to achieve high proteomic coverage, large amounts of starting material are necessary. In terms of coverage, PUNCH-P outperforms BONCAT and pSILAC, but cannot compete with the resolution and coverage of deep-sequencing. In addition, PUNCH-P does not provide single-nucleotide resolution, which allows Ribo-seq users to detect mRNAs translated at very low levels and identify non-canonical ORFs or alternative translation initiation sites. However, this higher resolution of Ribo-seq may also render it more susceptible to small differences in sample harvesting and processing, particularly when analyzing whole tissues, where the delivery of cycloheximide or other similar drugs can be non-homogenous and incomplete. Because MS calculates protein levels based on the relative abundance of different peptides for the same proteins, PUNCH-P is less likely to be affected by such differences.

Table 3 summarizes points related to experimental complexity, data analysis and cost. Of all methods described, Ribo-seq is the most technically challenging to perform and analyze and is associated with the highest costs. PUNCH-P involves simple experimental setup and data analysis with short turnaround times at a fraction of the Ribo-seq cost, allowing for analysis of larger sets of samples.

**PUNCH-P Performance Relative to Other Methods**

To determine how PUNCH-P performs relative to other techniques for global translatome profiling, we compared it to one protein- and one RNA-based method (10 h-pSILAC and Ribo-seq,^{23} respectively) and found that all three methods correlate similarly to overall protein abundance obtained from a whole-proteome analysis of cycling HeLa cells \(r = 0.41, 0.42\) and 0.42, respectively), supporting prior observations that protein abundance is predominantly controlled at the level of translation.^{5,30} For this comparison, we used a previously-published deep-proteome data set generated by another laboratory,^{31} to avoid a specific bias against Ribo-seq due to inter-laboratory variability. When PUNCH-P measurements were compared with a steady-state proteome analyzed in our laboratory under the same conditions, the correlation increased up to \(r = 0.62\) (Supplementary Figure 1 and Supplementary Table 1). Nevertheless, the correlation between PUNCH-P and pSILAC was significantly higher than the correlation between Ribo-seq and either PUNCH-P or pSILAC \(r = 0.61\) compared with 0.37 and 0.43, respectively),^5 possibly representing inherent technical differences between protein and RNA detection platforms. Because the translatome data sets used here were generated by different laboratories under slightly different conditions, leading to high inter-laboratory
variability, a detailed comparison of the methods and determination of translation rates of individual proteins in each method cannot be performed. Future PUNCH-P, Ribo-seq and pSILAC experiments performed in parallel under the exact same conditions will allow for a more accurate comparison and better understanding of possible method-specific biases.

PUNCH-P Offers Insights into Translation Regulation

The main application of PUNCH-P is global measurement of gene expression at the level of protein synthesis. However, it can also be combined with mRNA microarray or RNA-seq analysis of mRNA abundance to detect differences in translation efficiency of specific mRNAs, or with MS analysis of steady-state proteome to quantify protein stability.

To demonstrate that PUNCH-P can be used to estimate translation regulation of specific transcripts, we compared the protein measurements generated by PUNCH-P analysis of HeLa cells synchronized to G1, S and G2/M phases of the cell cycle5 with a published microarray data set of mRNA expression levels from similarly synchronized HeLa cells32 (Supplementary Table 2). The double-thymidine block protocol that was used for synchronization in both experiments establishes a common baseline by arresting cells at the G1/S boundary and allowing them to progress into S, G2/M and G1 phases without further interventions. The choice of synchronization protocol is particularly important for studies of translation activity, because the use of drugs that arrest cells in mitosis e.g., nocodazole has been shown to cause significant polysome destabilization and translation inhibition.28,29

Table 1. Comparison of readout type and monitoring mode

| Measures                     | Mode of monitoring | Labeling                      | Applicable for tissues |
|------------------------------|--------------------|-------------------------------|------------------------|
| PUNCH-P                      | Protein (MS)       | In vitro (post-lysis)         | Biotin-puromycin       | Short + |
| pSILAC                       | Protein (MS)       | In culture                   | SILAC amino acids (e.g., Lys8/Arg10) | Long - |
| BONCAT/QuNCAT                | Protein (MS)       | In culture                   | Methionine homolog (AHA) | Medium - |
| Polysome-bound mRNA analysis | RNA (Microarray)   | In vitro (post-lysis)         | Fluorophore dyes (cy3, cy5) | Medium + |
| Ribo-seq                     | RNA (Deep-seq)     | -                             | No labeling            | - + |

*Snapshot – translation at a specific point of time; Cumulative – translation over the period of labeling; Short labeling – 15 min; medium – 30 min to 2 h; long – over 10 h for reproducible results; "One study in zebrafish has shown that BONCAT can also be used for whole-organism analysis of simple eukaryotes."§ Can also be combined with deep-sequencing
To determine the translation efficiency for each mRNA-protein pair, i.e., how much protein is being made per mRNA at a specific time point, we calculated the ratio of mRNA translation (PUNCH-P) to steady-state mRNA level for each transcript at each cell cycle stage. The higher the ratio, the more protein is being synthesized from the same mRNA due to translational control mechanisms such as preferential recruitment to ribosomes, non-classical translation initiation or better codon adaptation. This relative translation efficiency score allows for the statistical testing of variations in translation efficiency of specific mRNAs.

Figure 2. Comparison of methods for global profiling of the translatome, illustrating the major steps in each method.

Table 2. Comparison of resolution, coverage and starting material

| Method                        | Resolution          | Coverage                          | Coverage proportional to | Starting material | Ref. |
|-------------------------------|---------------------|-----------------------------------|--------------------------|------------------|-----|
| PUNCH-P                       | Single-peptide      | ~5000 newly-synthesized proteins | Amount of starting material | Large            | 5   |
| pSILAC                        | Single-peptide      | ~2000 newly-synthesized proteins | Duration of labeling     | Small            | 5,9,10 |
| BONCAT/QuaNCAT                | Single-peptide      | Up to ~600 newly-synthesized proteins | Amount of starting material and duration of labeling | Large            | 11-14,39 |
| Polysome-bound mRNA analysis  | Whole-mRNA          | Whole ribosome-associated exome   | No. of probes on microarray | Varies           | 17-20 |
| Ribo-seq                      | Single-nucleotide   | Whole translatome; can predict novel ORFs and alternative initiation sites | No. of sequencing runs per sample | Small            | 21-24,37,40 |

*Large – 25 x 10⁶ cultured cells for PUNCH-P, or up to 90 x 10⁶ for BONCAT; Small – 0.5–5x10⁶ cells; Varies – small amounts for sucrose gradient fractionation and large amounts for ribosome IP; In a single MS run.
Supplementary Table 3 shows a summary of translation efficiency scores for each gene identified by both PUNCH-P and mRNA microarray analyses, at each cell cycle phase. ANOVA analysis of the differences in translation efficiency found that the translation efficiency of 674 mRNAs varies along the cell cycle (FDR = 0.01; Supplementary Table 3). Hierarchical clustering of these fluctuating mRNA-protein pairs shows clear segregation into several distinct clusters, representing variation in translation efficiency between G1, S and M phases of the cell cycle (Fig. 3). In the majority of cases, elevation in translation efficiency is associated with an increase in steady-state mRNA levels; however, these are not proportional, and translation efficiency often increases more significantly.

Among mRNAs whose translation efficiency was found to be elevated during mitosis are known mitotic regulators e.g., Aurora Kinase A and B (AURKA, AURKB), Protein regulator of cytokinesis 1 (PRC1), Cell division cycle protein 20 (CDC20), Mitotic checkpoint kinase BUB1 β (BUB1B) and Protein spindly (CCDC99). Importantly, this list also includes several examples of proteins known to be translationally upregulated during mitosis due to the presence of an internal ribosome entry site (IRES) or other cis-acting elements, e.g., Cyclin-dependent kinase 1 (CDK1), Centrosomal proteins E and F (CENPE, CENPF), DNA topoisomerase 2 (TOP2A) and Polo-like kinase-1 (PLK1). Conversely, mRNAs whose translation efficiency was elevated during S-phase include cell-cycle regulators known to peak at the entry to or during S-phase e.g., Cyclin dependent kinase-2 (CDK2), Cyclin-dependent kinase inhibitor 1 (CDKN1A or p21) and Nuclear factor NF-kappa-B (NFKB1/B2). Other mRNAs with known S-phase activity include several histones, Topoisomerase 1 (TOP1), and Rapamycin-insensitive companion of mTOR (RICTOR), which was recently reported to be translationally upregulated in S-phase. Taken together, these findings suggest that mRNAs encoding for proteins that share cell cycle-specific functions may be co-regulated at the level of translation, thus allowing fine-tuning of gene expression during the dynamic cell cycle progression. These results also confirm that PUNCH-P can be used not only to profile the global translatome but also to determine, in combination with transcriptome data, mRNA-specific translation efficiency.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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|                      | Protocol complexity | Min. duration of experiment | Data analysis | Cost |
|----------------------|--------------------|-----------------------------|---------------|------|
| PUNCH-P              | Medium             | 2 d                         | t test/ANOVA  | Low  |
| psILAC               | Low                | 2 d                         | t test/ANOVA  | Low  |
| BONCAT/QuaNCAT      | Medium             | 2 d                         | t test/ANOVA  | Low  |
| Polysome-bound mRNA analysis | Medium         | 3 d                         | Normalization, t test/ANOVA | Medium |
| Ribo-seq             | High               | 9 d                         | Complex       | High |

*Low complexity: requires basic cell culture and biochemical skills; Medium: requires additional ribosome isolation or click-chemistry techniques; High: requires expert biochemical and RNA handling skills and advanced knowledge in bioinformatics.*
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