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# Affinity purification of ribosomes to access the translome

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## ABSTRACT

We describe ribosome affinity purification (RAP), a method that allows rapid purification of ribosomes and associated messages from the yeast *Saccharomyces cerevisiae*. The method relies on the expression of protein A tagged versions of the ribosomal protein Rpl16, which is used to efficiently recover endogenously formed ribosomes and polysomes from cellular extracts with IgG-coupled spherical microbeads. This approach can be applied to profile reactions of the translome, which refers to all messages associated with ribosomes, with those of the transcriptome using DNA microarrays. In addition, ribosomal proteins, their modifications, and/or other associated proteins can be mapped with mass spectrometry. Finally, application of this method in other organisms provides a valuable tool to decipher cell-type specific gene expression patterns.

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## 1. Introduction

The control of protein synthesis plays pivotal roles in diverse physiological processes such as development, neurogenesis, memory formation, and aging [1–3]. Misregulation of translation can cause problems for the cell to maintain proper cell function or to adapt to changing environmental conditions, and thus, can lead to a variety of human diseases [4,5]. Therefore, besides studying the mechanism of translation, deciphering the messages that undergo translational control is crucial to get insight into essential features of the gene expression regulatory program [6,7].

A reliable measure for translation of cellular mRNA is the degree of its association with ribosomes. Since the rate of initiation usually limits translation, most translational responses will alter the ribosome density on a given mRNA [8]. Actively translated mRNAs are typically bound by several ribosomes (polysomes) and can be separated from the small (40S) and the large (60S) ribosomal subunits and the 80S monosomes by sucrose gradient centrifugation (Fig. 1). In classical experiments, total RNA is isolated from different fractions of the polysomal gradient and assayed for the mRNA of interest by Northern blot analysis. Several laboratories have further extended this technique by using DNA microarray technology to perform genome-wide analysis of mRNAs in polysomes in yeast, *Drosophila* and mammals [6,7,9] (see also the report by Melamed et al. in this issue of *Methods*). Although sucrose density fractionation is recognized as the “gold” standard to monitor active translation, there are also some drawbacks: it requires special equipment (e.g. ultracentrifuge, gradient fractionation system)

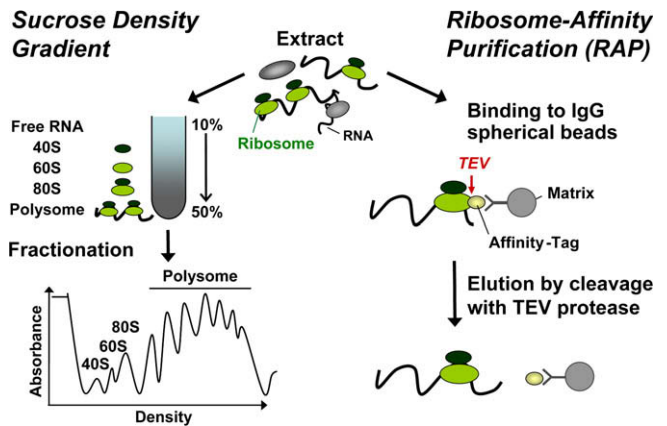
that may not be available in every laboratory; the procedure is time consuming and does not allow handling of many samples in parallel; and the samples are diluted in sucrose solution containing heparin, making more elaborate precipitation steps necessary to isolate RNA of sufficient quality to perform downstream experiments such as microarray analysis or reverse-transcription polymerase chain reactions (RT-PCR). Moreover, polysomal fractions may be contaminated by other high molecular weight complexes that are not an integral part of ribosomes such as lipid rafts, P-body components or pseudo-polysomes [10,11]. In this report, we therefore present a detailed protocol for an alternative approach, termed ribosome affinity purification (RAP), which allows rapid access to the cell’s translome without need for sucrose density fractionation. Besides the study of translational regulation upon changing environmental conditions, this method can also be applied to study cell-specific gene expression in complex tissues of model organisms [12–14].

## 2. Description of the ribosome affinity purification (RAP) method

RAP is based on experiences with epitope-tagged RNA-binding proteins for the purification of ribonucleoprotein complexes (RNPs) to systematically analyze RNA targets with DNA microarrays (Fig. 1) [15–20]. In RAP, a tagged ribosomal protein (RP) of the large ribosomal subunit is used to capture fully assembled ribosomes on a matrix [12,21]. To increase the specificity of the reaction, we used an affinity-tag that can be cleaved-off from the ribosomal protein with a site-specific protease from the tobacco-etch virus (TEV). The eluate can then, on the one hand, be analyzed by mass spectrometry to identify ribosomal proteins, post-transla-

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**Fig. 1.** Experimental schemes to study the translome. (Left) “Classical” sucrose density fractionation. Cell-extracts are prepared in the presence of cycloheximide (a potent inhibitor of translational elongation) and separated by ultracentrifugation through a linear 10–50% sucrose density gradient. The gradient is then fractionated while continuously monitoring the absorbance at 254 nm allowing the separation of “free” RNA, the small (40S) and large (60S) ribosomal subunits, monosomes (80S), and polysomes. RNA is isolated from individual gradient fractions and pooled for subsequent microarray analysis. The relative position of a message in this profile is an indicator for its translational activity. (Right) RAP procedure. Affinity-tagged (e.g. Protein A) ribosomes are captured from extracts with IgG-coupled spherical microspheres (matrix) and released from the matrix with a site-specific protease from tobacco-etch virus (TEV). To profile the translome, RNAs associated with the ribosomes are analyzed with DNA microarrays.

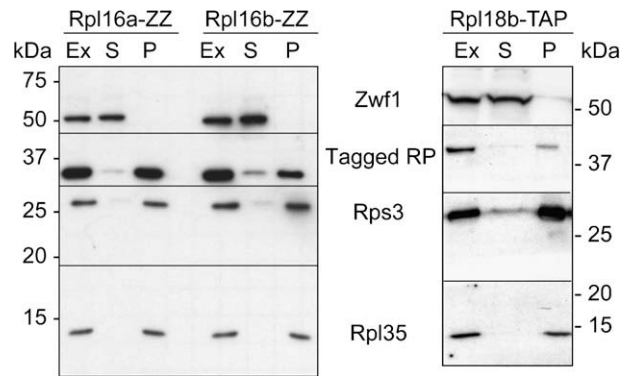
tional modifications, and additional ribosome-associated proteins (see also [11]). On the other hand, RNAs associated with ribosomes, which we further refer to as the translome, can be isolated from the purified ribosomes and quantitatively analyzed by quantitative reverse-transcription PCR (RT-qPCR), DNA microarrays, or may be sequenced. In this respect, we have successfully performed such an analysis and compared the relative changes of global transcript levels (corresponding to RNA isolated from the extract) with that of the translome (corresponding to messages associated with affinity-captured ribosomes) after application of diverse stresses to cells [22]. Besides previously known translationally regulated messages such as *GCN4*, which codes for a transcription factor that is increasingly expressed upon depletion of amino-acids from the medium, we identified, and in some cases verified, additional messages that likely undergo translational regulation, underlining the applicability of RAP to study translational regulation [22].

In the following, we highlight critical steps for establishing the procedure including the selection of a suitable tagged ribosomal protein and the choice of the matrix for affinity purification, and we provide a detailed protocol for RAP. Furthermore, we include a brief description of how to analyze the translome with yeast oligo microarrays.

## 2.1. Establishing RAP

### 2.1.1. Selection of tagged ribosomal proteins

The yeast ribosome consists of 78 RPs and four ribosomal RNAs (rRNAs) [23]. Fifty-nine of the 78 RPs are encoded by two paralogous genes (termed a- or b-copy). Therefore, a careful selection procedure is necessary to find a suitable RP for affinity purification. Most importantly, the fusion tag should not interfere with RP function, since this could affect the cell's physiology (i.e. cell growth) and/or promote specific stress programs that significantly alter the cells' translome. We evaluated six RPs that are preferentially located at the solvent accessible side of ribosomes [24]. This includes four proteins with paralogs (Rpl7, Rpl12, Rpl16, and Rpl18) and two non-duplicated proteins (Rpl25, Rpl30). To achieve



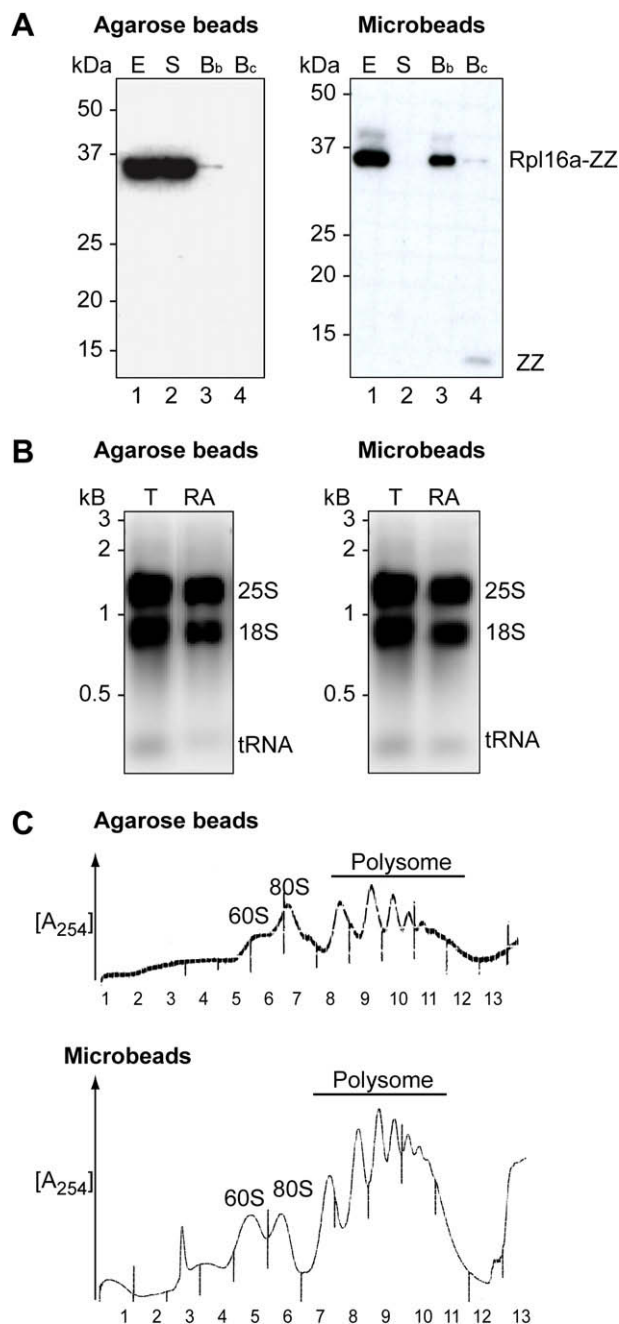
**Fig. 2.** Immunoblot analysis of sucrose cushions to evaluate incorporation of tagged RPs into ribosomes. Fractions of the extract (Ex), the supernatant (S) and the pellet containing ribosomes (P) were analyzed with specific antibodies detecting a cytoplasmic protein not expected to be present in the ribosome pellet (rabbit anti-Zwfl, Sigma; 1:5000), the tagged RP (peroxidase anti-peroxidase soluble complex [PAP], Sigma; 1:5000), and a protein of the small (rabbit anti-Rps3; 1:100,000) and of the large ribosomal subunit (rabbit anti-Rpl35; 1:20,000) [29]. The tagged RP under investigation is indicated on the top. The specific protein for analysis is indicated between the panels, molecular size markers are marked in kilodaltons (kDa) on the side.

stable expression of tagged RPs under the control of their endogenous promoters, we used the ZZ/TAP-tag integrated at the original chromosomal location by homologous recombination, more specifically, at the C-terminus of the corresponding open reading frame in BY4741 (*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*) wild-type yeast cells. The ZZ-tag, which comprises part of the tandem-affinity purification (TAP)-tag [25], contains two protein A IgG-binding units and a tobacco-etch virus (TEV) protease recognition sequence allowing elution of the bound material from the affinity resin [16].

We first tested strains bearing tagged versions of the respective RP genes for growth defects. Six strains showed moderate to severe growth defects when cultured in rich or synthetic media (Rpl7a-TAP, Rpl12a-TAP, Rpl12b-TAP, Rpl16b-TAP, Rpl25-TAP, and Rpl30-TAP) and hence, were not further considered to be suitable for RAP (data not shown). Four strains had no growth defect (Rpl16a-ZZ, Rpl16a-TAP, Rpl16b-ZZ, and Rpl18b-TAP). We next investigated whether tagged RPs are efficiently incorporated into the ribosome which can be easily assessed by sedimentation of extracts through a sucrose cushion (details of the procedure are described below). We found that three tagged proteins Rpl16a-ZZ, Rpl16b-ZZ, and Rpl18b-TAP were efficiently (>80%) incorporated into ribosomes and hence, are valuable candidates for affinity purification of ribosomes (Fig. 2). On the other hand, Rpl16a-TAP as well as Rpl30-TAP and Rpl12a-TAP for which respective strains had slight growth defects, were only to about 50% incorporated into ribosomes (data not shown). The reason for the partial incorporation is not known but may be due to steric hindrance by the TAP-tag. Possibly, the use of a smaller tag than the relatively large TAP-tag (21 kDa) may be favored in these cases. For instance, Inada et al. used a FLAG-(His)<sub>6</sub> epitope-tagged Rpl25 which did not seriously compromise cell growth. However, the degree of incorporation of tagged-Rpl25p into actively translating ribosomes was not directly investigated [21].

### 2.1.2. Affinity purification – the matrix matters

Protein A-tagged ribosomes can be recovered from cell lysates by affinity selection on IgG-coupled beads. However, we observed that with regular IgG-coupled 4% agarose beads (Sigma) only a maximum of about 10–30% of the tagged ribosomes could be captured from the extract (Fig. 3A and B), with large polysomes being underrepresented in the purified fraction (Fig. 3C). Even an



**Fig. 3.** Evaluation of IgG coupled agarose versus spherical microbeads for RAP with ZZ-tagged Rpl16a. (A) Immunoblot analysis of the purification procedure applying IgG-coupled 4% agarose beads (left) or spherical microbeads (right) with PAP, which detects the ZZ-tag. Lanes: 1, (E) extract; 2, (S) supernatant after incubation of the extract with beads; 3, (B<sub>b</sub>) captured beads; 4, (B<sub>c</sub>) beads after incubation with TEV protease that cleaves the ZZ-fusion protein. Molecular size markers are indicated to the left. (B) Total RNA isolated from extract (T) and from affinity-purified ribosomes (RA) was separated on a 1% agarose gel and visualized with ethidium bromide. (Left) RNA isolated from ribosomes captured with agarose beads; (right) ribosomes captured with microbeads. The positions of the 25S, 18S rRNAs and tRNAs are indicated to the right. Molecular size markers are given in kilobases (kB) on the left. (C) Polysomal profile of affinity-purified ribosomes isolated from cells grown to mid-log phase in rich media applying agarose beads (top) or spherical microbeads (bottom). The positions of 60S ribosomal subunits, 80S monosomes, and polysomes are marked. Parts of the figure are adapted from [22].

increased amount of agarose beads relative to the extract did not significantly increase ribosome recovery. Agarose-IgG is a porous gel support, with an exclusion limit of 20,000 kDa for a 4% solution (<http://www.piercenet.com>). This could be refractory for the binding of polysomes with more than five ribosomes (4200 kDa each),

which may explain the low efficiency for the recovery of larger polysomes (>5 ribosomes) [21].

This limitation can be overcome by the application of spherical microbeads allowing capturing more than 95% of tagged ribosomes from extracts (Fig. 3A). Moreover, polysomal profiles of TEV-eluted ribosomes revealed the presence of 60S subunits, monosomes and polysomes, including large polysomes, whereas free 40S subunits were absent (Fig. 3C). The improved performance is also reflected by the five to 10 times greater recovery of RNA; we usually obtained about 50 µg of ribosome-associated RNAs with tagged Rpl16a from a 100 ml culture of yeast cells (OD<sub>600</sub> = 0.5). In contrast, we only recovered between 3 and 10 µg of RNA using IgG-coupled 4% agarose beads (Sigma). In all cases, less than 1 µg of RNA was recovered from an untagged wild-type control strain with both resins. Therefore, spherical microbeads are superior to porous agarose beads for the efficient purification of large RNP complexes such as polysomes. The following experimental procedure refers to a protocol applying spherical microbeads to isolate ribosomes.

## 2.2. RAP: experimental procedures

The detailed method and suggestions described here have been tested for Rpl16a/b-ZZ. However, we believe that the same procedure is also applicable to other RPs.

### 2.2.1. Coupling IgG to microbeads for affinity purification

Rabbit IgGs (Sigma) are coupled to Carboxyl (COOH) microbeads (1 micron, Polysciences, Inc.) with the PolyLink Protein Coupling Kit for COOH microspheres (Polysciences) generally following the manufacturer's instructions. Five-hundred microliters of the provided 2.5% solids (w/v) aqueous suspension of microparticles (equivalent to 12.5 mg) are transferred into a 1.5 ml polypropylene microcentrifuge tube and pelleted via centrifugation (1000g, 10 min, room temperature [RT]). The microparticle pellet is resuspended in 400 µl of PolyLink Coupling buffer by vortexing, pelleted again (1000g, 10 min, RT), and then resuspended in 170 µl of PolyLink Coupling buffer. EDAC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide, hydrochloride) solution (200 mg/ml) is prepared just before use by dissolving 5 mg EDAC in 25 µl PolyLink Coupling buffer. Twenty microliters of the EDAC solution is added to the beads, which are then briefly vortexed at full-speed. Three-hundred micrograms of rabbit IgG (60 µl of 5 mg/ml) is finally added to the solution and briefly vortexed. Please note that it is very important that the beads are completely redissolved before adding EDAC-IgG to the solution since this may otherwise lead to bead aggregation resulting in increased unspecific interactions during affinity purification. The beads are then incubated for one hour at RT on an Eppendorf Thermomixer Compact while shaking at 850 rpm. We repeatedly observed that 'end-over-end' mixing does not allow maximal coupling efficiency, probably due to self-aggregation of the beads and is therefore not recommended. After coupling, the beads are pelleted by centrifugation (1000g, 10 min, RT), and washed at least twice with 400 µl PolyLink Wash/Storage buffer. The supernatants are kept and pooled to determine the coupling efficiency. Therefore, the protein concentration/amount of the unbound material is measured with a Protein Assay Kit (e.g. Bradford) and subtracted from the amount of IgG in the starting material (300 µg). Normally, 60–80% of the IgG is coupled to the beads. The coupled microbeads are then stored in 400 µl PolyLink Wash/Storage buffer at 4 °C. We suggest usage of the coupled beads within one month's time.

### 2.2.2. Yeast extract preparation and sucrose cushions

The protocol is optimized for 100 ml yeast cultures grown to mid-log phase (OD<sub>600</sub> = 0.5) at 30 °C in Synthetic-Complete-Dextrose (SCD) or Yeast-Peptide-Dextrose (YPD) medium while

shaking at 250 rpm. Cycloheximide (0.1 mg/ml) (Sigma) is added 1 min prior to harvest of cells to block translational elongation [26]. Cells are collected by vacuum filtration (HVPL type filters [0.45  $\mu$ m]; Millipore), briefly washed on filters with 10 ml ice-cold buffer A (20 mM Tris–HCl [pH 8.0], 140 mM KCl, 2 mM MgCl<sub>2</sub>, 1% Triton X-100, 0.2 mg/ml heparin [Sigma], 0.1 mg/ml cycloheximide [Sigma]), then cells are scraped from filters and put into 50 ml Falcon tubes and flash-frozen in liquid nitrogen. We prefer to harvest cells by vacuum filtration rather than centrifugation which would take significantly longer for recovery and washing of cells (centrifugation for 10 min at 3000g). Especially for gene expression profiling studies, cells should be collected as quickly as possible to minimize potential secondary effects introduced by cell collection, temperature changes etc. Moreover, to measure relative changes of the translome upon changing environmental conditions, an untreated or solvent-only treated control sample should be run in parallel and processed on the same day. Cells can be kept frozen at  $-80^{\circ}\text{C}$  for several months.

To prepare extracts, the frozen cells are thawed on ice and resuspended in 1 ml buffer B (buffer A plus 0.5 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonylfluoride [PMSF], 0.5  $\mu$ g/ml Leupeptin, 0.2  $\mu$ g/ml Pepstatin, 20 U/ml DNase I [Sigma], 100 U/ml RNase Out [Invitrogen]) in a glass Corex tube, then the cells are broken mechanically with 1/3 volume of 0.5 mm glass beads by vortexing four times at full speed for 20 s followed by 90 s cooling steps on ice in between. These and further steps should be performed in a cold room at  $4^{\circ}\text{C}$  to minimize alterations in temperature. After cell lysis, crude extracts are cleared by three subsequent centrifugation steps (2600g, 8600g, and 13,400g; each for 5 min at  $4^{\circ}\text{C}$ ) in a microcentrifuge and brought to 1 ml with buffer B. The extract ( $\sim 7.5$  mg/ml of total protein) can be frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for several months. However, we propose to proceed with RAP the same day – freezing of cell extract before affinity-isolation increases the potential of mRNA degradation.

To rapidly analyze the incorporation of tagged RPs into ribosomes, 0.5 ml of extract is loaded on top of a 2 ml 0.5 M sucrose (Sigma S089) cushion prepared in buffer B and provided in a thick-walled TLA-100.4 centrifuge tube (Beckman). The sample is centrifuged for 45 min at 100,000g at  $4^{\circ}\text{C}$  in a TLX tabletop centrifuge (Beckman). A fraction of the pellet and the supernatant is run on a SDS–polyacrylamide gel and tested for the presence of ribosomal and cytoplasmic control proteins by immunoblot analysis with specific antibodies (Fig. 2).

### 2.2.3. Affinity purification of ribosomes

Four aliquots, each containing 500  $\mu$ l of a 2.5% solution (w/v) of IgG coupled microbeads, are required for RAP from 750  $\mu$ l cell-free extract ( $\sim 5$ – $6$  mg of total protein). The beads are blocked twice for 10 min with 1 ml of buffer A supplemented with 0.4 mg/ml heparin (Sigma), 0.1 mg/ml *Escherichia coli* tRNA (Roche), and 1% BSA to prevent unspecific binding of proteins/RNA to the microbeads. Thereby, the beads are shaken at 850 rpm in an Eppendorf Thermomixer during the blocking steps and collected by centrifugation for 10 min, 1000g at RT. The beads are further washed once with buffer A for 10 min, pooled in a 2 ml microcentrifuge tube, and finally collected by centrifugation (10 min, 1000g, RT). Seven hundred and fifty microliters of cell-free extract ( $\sim 5.5$  mg of total protein) is incubated with the beads for 2 h at  $4^{\circ}\text{C}$  while shaking on a Thermomixer at 850 rpm. The beads are then collected by centrifugation (2 min, 3300g,  $4^{\circ}\text{C}$ ) and washed four times for 15 min in buffer C (20 mM Tris–HCl [pH 8.0], 140 mM KCl, 2 mM MgCl<sub>2</sub>, 5% glycerol, 0.5 mM DTT, 40 U/ml RNase Out [Invitrogen]). Ribosomes are released from beads by incubation with 750  $\mu$ l buffer C supplemented with 0.8 U/ $\mu$ l TEV protease (Invitrogen) for 2 h at  $15^{\circ}\text{C}$  (Thermomixer, 750 rpm). Beads are pelleted by centrifugation (5 min, 13,400g,  $4^{\circ}\text{C}$ ), and the supernatant containing purified

ribosomes is collected. To isolate RNA, the volume of the eluted material is reduced to 200  $\mu$ l with a Microcon column YM-30 (Millipore). RNA is isolated from cell-free extracts (total RNA) and from affinity-purified ribosomes with RNeasy Mini columns following the manufacturer's instructions for RNA clean-up including on-column DNase I digestion (Qiagen).

### 2.3. Oligo microarray analysis of the translome

The RNA isolated from ribosomes (translatome) can be directly used for DNA microarray analysis to determine relative changes of the translome upon changing conditions. Although diverse array formats are available, here we briefly describe the dual-color hybridization to yeast oligo microarrays containing long oligonucleotides (70 mers). In our case, these arrays contain 10,944 oligo probes from the Array-Ready Oligo Set Version 1.1 (Operon) representing 6388 *S. cerevisiae* ORFs, and the Yeast Brown Lab Oligo Extension Version (YBOX vers. 1.0) with 3456 probes to detect annotated noncoding RNAs, ribosomal RNA precursors, introns, exon–intron and exon–exon junctions, other sequences predicted to be expressed, additional probes for genes with high cross-hybridization potential and controls for array quality measurements and normalization. Details of oligonucleotide selection and probe sequences are available from the Operon website ([www.operon.com](http://www.operon.com)). The probes were printed on epoxy coated glass slides (Nexterion slide E; Schott) at the Center for Integrative Genomics, University of Lausanne, Switzerland. The slides are stored in the dark under desiccation and handled with powder-free gloves.

Before use, the oligo arrays are blocked in  $5 \times \text{SSC}$ , 0.1 mg/ml BSA, 0.1% SDS for 1 h at  $42^{\circ}\text{C}$ , and subsequently washed three times in  $0.1 \times \text{SSC}$  for five minutes at RT, rinsed in water for 30 s and dried by centrifugation for 2 min at 500g. The slides should then be used the same day.

Microarray analysis is performed by competitive hybridization of Cy3 and Cy5 fluorescently labeled cDNA. Depending on the biological question individual array designs have to be conceptualized (see Melamed et al. in this issue of *Methods*). For example, to get a measurement for the selective enrichment of each transcript in the translome compared to the global transcript levels (transcriptome), cDNAs derived from total RNA from extract is labeled with Cy3, whereas the ones derived from the ribosome-associated RNAs are labeled with Cy5. The ratio of the red (Cy5) to the green (Cy3) fluorescence signals at each arrayed features represents then the relative enrichment to all features represented on the array.

To prepare cDNA from the RNA samples, total RNA (5–15  $\mu$ g) is reverse transcribed with SuperScript™ II Reverse Transcriptase (Invitrogen) in the presence of 2.5 mM aminoallyl-dUTP (Sigma), 2.5 mM dTTP and each 5 mM dATP, dGTP, and dCTP, respectively, with a 1:1 mixture of dT20 V and random nonamer (N9) primers (5  $\mu$ g of each, Sigma) for 2 h at  $42^{\circ}\text{C}$ . After first strand cDNA synthesis, RNA is hydrolyzed with 0.1 M NaOH and 0.1 M EDTA at  $65^{\circ}\text{C}$  for 15 min, and samples are neutralized with 0.35 M Hepes–KOH (pH 8.0). To remove non-incorporated nucleotides, the reaction mix is centrifuged three times in a Microcon-YM30 filter (Millipore) filled with 450  $\mu$ l distilled water. Amino-allyl containing cDNA is eluted with 100 mM NaHCO<sub>3</sub> (pH 9.0) and covalently linked to either fluorescent Cy3 or Cy5 NHS-monoester (GE Healthcare). Unincorporated dyes in each sample are removed with the QIA-quick PCR Purification Kit (Qiagen). Cy3 and Cy5 labeled samples are combined in standard formamide based hybridization buffer (Ocimum Biosolution Hybridization Solution) supplemented with 1 mg/ml polyadenosines (Sigma) in a final volume of 20  $\mu$ l. The sample is incubated at  $80^{\circ}\text{C}$  for 10 min, briefly spun in a microcentrifuge and hybrid-

ized at 42 °C for 12–16 h inside a sealed hybridization chamber (Corning) in a water bath using a glass coverslip to contain the probe on the microarray. Arrays are successively washed in three buffer chambers filled with 2 × SSC, 0.2% SDS; 2 × SSC; and 0.2 × SSC. The first wash is performed at 42 °C and the subsequent two washes are performed at room temperature for 12 min each. After briefly rinsing in ethanol, the microarrays are dried by centrifugation for 2 min at 500g and subsequently scanned with an Axon Instruments Scanner 4200A (Molecular Devices). Scanning parameters are adjusted to achieve similar fluorescent intensities in both channels. Data are collected with GenePix Pro 5.1 (Molecular Devices) and spots with abnormal morphology are excluded from further analysis. Array data can be exported into Acuity 4.0 (Molecular Devices) and normalized to the mean of ratio of medians = 1 excluding the signals from various control features. Alternatively, arrays can be normalized to RNA probes that were added to initial RNA samples as an internal standard.

### 3. Concluding remarks

RAP allows rapid access to purified ribosomes devoid of contaminations by lipid rafts, P-body components or pseudo-polyosomes, which may be present in classical polysomal gradients [10,11]. The purified ribosomes can be obtained in a relatively concentrated solution without sucrose allowing easy processing for mass-spectrometric analysis. Moreover, RNA can be easily isolated for further analysis with DNA microarrays or RT-qPCR. However, we wish to note that this procedure simply defines the set of messages that are associated with ribosomes. It cannot distinguish if an mRNA is bound by one or several ribosomes and hence, it does not indicate the extent to which the messages are actively translated. For instance, directly comparing the relative expression of messages derived from extracts to messages derived from purified ribosomes indicates the degree of enrichment of each message in the translome. Since it is generally believed that translation is mainly regulated at the initiation step, enrichment of messages in the translome somewhat reflects the degree of translational initiation and therefore provides an estimate for the initiation activity of messages compared to all the messages that are expressed in a particular cell/tissue. In future, the combination of RAP with a recently published ribosome profiling method, which is based on deep sequencing of ribosome protected mRNA fragments, may allow to determine binding sites as well as numbers of tagged ribosomes on particular messages and hence, provide an estimate for translational efficiency [27].

Besides the study of translational regulation, RAP can also be applied to test for differential association of messages with paralogous RPs, which cannot be achieved by classical sucrose density fractionation. This may become of interest in light of recent evidence suggesting a model in which functionally distinct ribosomes formed by paralogous RPs may exist in cells that may even differentially regulate specific messages under certain conditions [28]. Finally, it has recently been reported that tagged Rpl10a in mammalian cells was used to measure neuronal cell-type specific expression in the central nervous system [13,14]. Likewise application in other model organisms may provide a formidable tool to decipher cell-specific gene expression programs.

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### Appendix A. Reagents and equipment (alphabetical order)

AcTEV protease, Invitrogen, Cat. No. 12575-015 (1000 U) or 12575-023 (10,000 U).  
 Aminoallyl-dUTP, Sigma, Cat. No. A0410.  
 Cycloheximide, Sigma, Cat. No. C4859.  
 Cy3/Cy5 NHS-monoester, GE Healthcare, Cat. No. 23001/25001.  
 Formamide based hybridization buffer, Ocimum Biosolutions, Cat. No. 1180-000010.  
 Heparin sodium salt, Sigma, Cat. No. H3400.  
 Hybridization chambers, Corning, Cat. No. 2551.  
 Microcon Centrifugal Filter Devices YM-30, Millipore, Cat. No. 42410.  
 Nexterion slide E, Schott, Cat. No. 1066643.  
 Polybead carboxylated 0.75–1 micron, Polysciences Inc, Cat. No. 07759.  
 PolyLink Protein Coupling Kit for COOH Microspheres, Polysciences Inc., Cat. No. 24350-1.  
 PAP, Sigma, Cat. No. P1291.  
 QIA-quick PCR Purification Kit, Qiagen, Cat. No. 28104.  
 Rabbit IgG, purified, Sigma, Cat. No. I5006.  
 RNase Out, Invitrogen, Cat. No. 10777-019.  
 RNeasy Mini Kit, Qiagen, Cat. No. 74104.  
 RNase-Free DNase Set, Qiagen, Cat. No. 79254.  
 SuperScript™ II Reverse Transcriptase, Invitrogen, Cat. No. 18064-014.  
 Themomixer compact, Eppendorf, Cat. No. 5350 000.013.  
 tRNA from *E. coli*, Roche, Cat. No. 10 109 550 001.

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