CHAPTER TEN

Genome-Wide Analysis of mRNA Polysomal Profiles with Spotted DNA Microarrays

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Contents

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Abstract

The sedimentation of an mRNA in sucrose gradients is highly affected by its ribosomal association. Sedimentation analysis has therefore become routine for studying changes in ribosomal association of mRNAs of interest. DNA microarray technology has been combined with sedimentation analysis to characterize changes in ribosomal association for thousands of mRNAs in parallel. Such analyses revealed mRNAs that are translationally regulated and have provided new insights into the translation process. In this chapter, we describe possible experimental designs for analyzing genome-wide changes in ribosomal association, and discuss some of their advantages and disadvantages. We then provide a detailed protocol for analysis of polysomal fractions using spotted DNA microarrays.

1. Introduction

Velocity sedimentation in sucrose gradients has been used for more than four decades to assess the translational status of an mRNA (Warner *et al.*, 1963). mRNAs are separated in a sucrose gradient by ultracentrifugation according to the number of ribosomes with which they are associated, and the distribution pattern of a specific mRNA can be determined by northern analysis. Two parameters that are related to translatability of a gene can be easily obtained from such analysis: (1) The percentage of transcripts that are associated with ribosomes (ribosomal occupancy). This parameter reports of the efficiency in which transcripts of a particular gene are recruited by the translation machinery. (2) The number of ribosomes with which the mRNA is associated (ribosomal density) (Arava *et al.*, 2003). The number of ribosomes on an mRNA reports on the overall balance between the steps of ribosome binding, progression along the coding region, and dissociation. Differences in these parameters among different mRNAs reflect differences in their translation efficiency. For example, low ribosomal occupancy for a particular mRNA (low percentages of association with ribosomes) may suggest a regulatory mechanism in which the mRNAs are stored in a nontranslating pool, awaiting a signal to be recruited by the translation machinery. Moreover, a change in one of these parameters for a particular gene upon change in growth condition or a mutation is indicative of translational regulation. For example, the GCN4 mRNA was shown to have increased ribosomal density under amino acid-deprivation conditions, which is an outcome of a translational regulation mechanism.

In the last few years, DNA microarrays have been utilized to perform simultaneous analysis of the translational status of thousands of mRNAs, thereby enabling a comprehensive view of translation efficiency and regulation. Analyses were performed on mRNAs isolated from various

organisms, under many different growth conditions or mutations, and at different resolutions (Arava *et al.*, 2003; Blais *et al.*, 2004; Branco-Price *et al.*, 2005; Johannes *et al.*, 1999; Kuhn *et al.*, 2001; MacKay *et al.*, 2004; Qin and Sarnow, 2004; Shenton *et al.*, 2006; Smirnova *et al.*, 2005). Such genome-wide analyses provided new insights to the process of translation in general and to the regulation of particular genes.

The data obtained has been derived from different experimental designs (namely, the number of collected fractions and the hybridization scheme), different microarray platforms (spotted versus Affymetrix arrays), and different data analysis schemes. In this chapter, we discuss various experimental designs for microarray analyses, provide detailed protocols for various experimental steps when using the spotted microarray platform, and discuss aspects of data analysis.

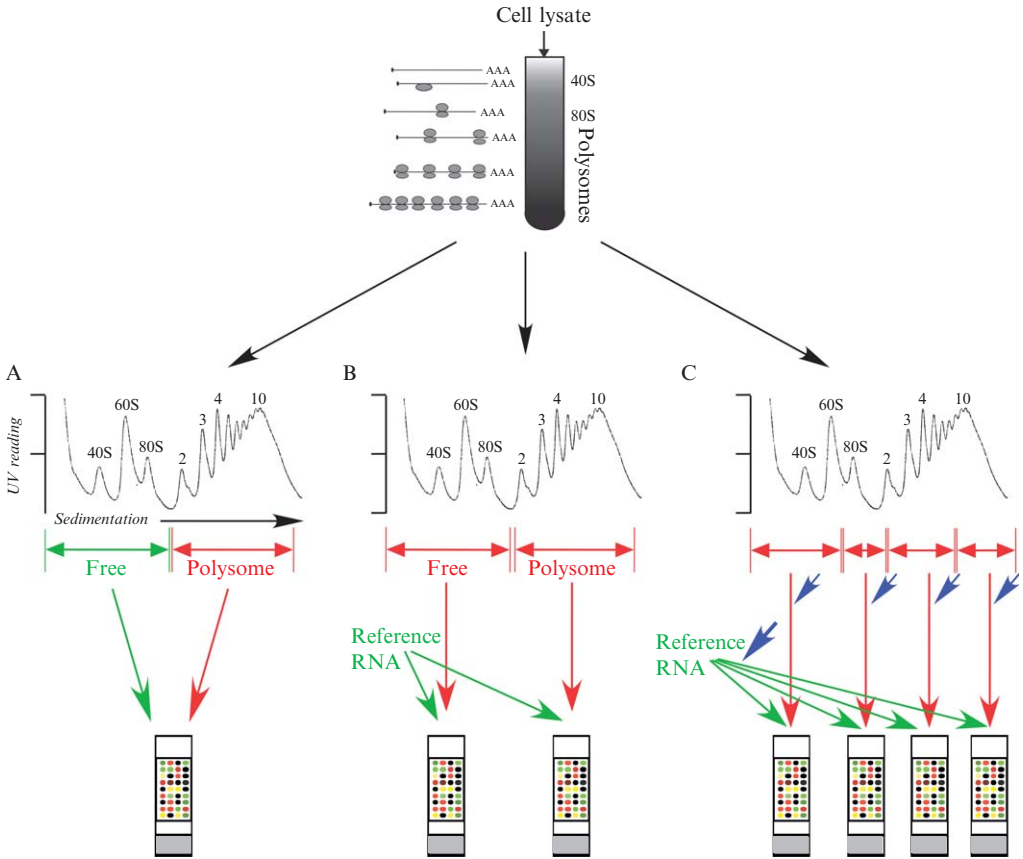
2. Experimental Designs

All experimental designs for studying the translational status of a cell share the general steps of separation of complexes on a sucrose gradient, fractionation of the gradient, RNA purification from the fractions, cDNA labeling, and hybridization to a DNA microarray. The variables that are most critical for data interpretation are the number of fractions into which the gradient is separated and the method by which experimental variations are corrected (''normalization'' method). In the following section, we discuss these issues in detail and provide simplified numerical examples to illustrate some points.

2.1. Number of fractions into which the gradient is separated

2.1.1. Two fractions

The simplest experimental design includes separation of the entire gradient into two fractions: free mRNAs (free) and polysome-associated mRNAs (poly) (for simplicity of this discussion, mRNAs sedimenting as associated with one ribosomes will be included with the free fraction, although this might not be correct for all mRNAs). The labeling and hybridization of the free and polysome-associated fractions can be directed one against the other, where the polysome fraction is labeled with red fluorophore and the free with green fluorophore (Fig. 10.1A). Alternatively, the labeling and hybridization can be indirect, where the free and poly are each labeled in red and an unrelated RNA sample is labeled in green and serves as a common reference (Fig. 10.1B). The advantages and disadvantages of these schemes are described later and are relevant also for other experimental designs.


Cell lysate
AAA
AAA
AAA
AAA
AAA
AAA
40S
80S
Polysomes
A
B
C
UV reading
60S
40S
80S
2
3
4
10
Sedimentation
Free
Polysome
Reference
RNA

2.1.2. Direct comparison

When performing an experiment in which the polysomal fraction is labeled in red and the free fraction is labeled in green (also called a type I experiment), the resulting red-to-green ratios indicate the relative polysomal association of an mRNA. That is, genes with a high ratio are those that are highly associated with polysomes, and vice versa for low ratio. One can therefore compare the poly/free ratios of all genes at various growth conditions or treatments and determine which genes were most affected.

The main advantage of this experimental design is that both samples are compared on a single DNA microarray, and therefore errors that are due to the use of multiple microarrays, such as variation in microarray production, hybridization, and washing, are minimized. Time and money considerations are another important advantage of this design.

It should be stressed, however, that the obtained ratios from such experiments cannot be directly related to the actual distribution of an mRNA in free and polysomal fractions (i.e., a gene with a ratio of 1 does not necessarily have 50% of its molecules in free and 50% in polysomes). This is because the procedure that leads to the obtained ratios includes many steps, and each step may introduce a certain bias. Biases may arise during the steps of RNA purification, labeling, hybridization, and scanning, which results in skewed ratios. However, a reasonable assumption is that the bias is the same for all mRNAs within a sample, and therefore the ranking of the ratios is not affected by these biases. Namely, genes with higher ratios have higher polysomal association relative to other genes and therefore changes in their ranking are indicative of effects on their translation.

An inherent limitation of this design is that the information that it provides is in the form of a ratio (between the free and polysomes), and not the actual distribution in these fractions. This fact becomes a limitation when multiple treatments are compared, since the product of dividing one ratio by another does not necessarily reflect the *extent* of changes (see Example 10.1). A possible way to overcome this limitation is to extract

Figure 10.1 Experimental schemes for microarray analysis. All experimental schemes start with a separation step of the cell lysate by velocity sedimentation in a sucrose gradient (top scheme). Collection of the desired fractions is assisted by a continuous ultraviolet (UV) reading of the gradient (an example of such UV reading is shown in each section). This allows determination of the sedimentation position of the 40S, 60S, 80S, and polyribosomal complexes (2, 3, and more). Three general ways for fraction collection and analysis are presented (sections A, B, and C): (A) Collection of two fractions (free and polysomes) and direct comparison between them, with the free mRNA fraction labeled with green dye and the polysome fraction labeled with red dye. (B) Collection of two fractions and indirect comparison between them by utilizing an unfractionated reference RNA. (C) Collection of multiple fractions (four in this case), where each fraction is compared to an unfractionated reference sample. The blue arrows indicate the addition of spike-in RNA to each fraction and to the reference RNA.

Before treatment				After treatment			
(A) Microarray results							
gene	**Rb (P/F)**			**Ra (P/F)**		**Ra/Rb**	
a	0.25			0.02		0.08	
b	1.38			0.67		0.48	
c	24.00			3.55		0.15	
d	0.02			0.02		1	
(B) mRNA distribution							
gene	**Free**	**Polysome**	**Total**	**Free**	**Polysome**	**Total**	
a	80	20	100	98	2	100	
b	42	58	100	60	40	100	
c	4	96	100	22	78	100	
d	98	2	100	98	2	100	
gene		**Pb**			**Pa**		**Pa/Pb**
a		0.2			0.02		0.1
b		0.58			0.4		0.69
c		0.96			0.78		0.81
d		0.02			0.02		1

Example 10.1 Two fractions experiment, direct comparison. This theoretical example presents possible microarray results for four genes (*a–d*) from direct comparison hybridization (A). Two fractions (free [F] and polysomes [P]) from cells before or after a certain treatment were hybridized against each other and yielded the indicated ratios (Rb (Ratio before) and Ra (Ratio after)). To recapitulate the differential effects on genes *a–d*, a trivial way would be to divide the ratio after the treatment by the ratio before the treatment (Ra/Rb). From this example, one might conclude that gene *c* was more affected than gene *b*. However, this is not necessarily correct. A possible way to draw this erroneous conclusion is demonstrated in section (B). The distributions of transcripts of genes *a–d* in the free and polysome fractions, either before or after the treatment, are indicated. Calculating the polysome/free ratio for these genes will result in ratios identical to the ones in section (A) (the microarray results). Yet, for 3 of the genes (*a*, *b*, and *c*), 18 molecules had shifted from the polysome to the free fraction, and gene *d* did not change after the treatment (for simplicity, all genes have the same number of transcripts (100), which is indicated at the Total column). Thus, in terms of percent of molecules shifting out from polysomes, gene *b* was much more affected than gene *c*. This does not agree with what we would have concluded in section A from Ra/Rb. These effects are because the extent of change for a certain gene is dependent on its polysomal distribution before the treatment. Ratio of distribution that is close to one will result in apparent weaker effects, and the farthest that the ratio is from one, the stronger the apparent effects are. One way to better recapitulate the changes is to restore the values of polysomes before (Pb) and polysomes after (Pa) (this can be done by the formula [$P = R/(1 + R)$]), and then divide Pa by Pb. This manipulation is presented in the lower four rows. The column Pa/Pb recapitulates correctly the changes of each gene in the polysome fraction.

gene	Pb		Pa	Pa/Pb
a	0.2		0.02	0.1
b	0.58		0.4	0.69
c	0.96		0.78	0.81
d	0.02		0.02	1
gene	**Ra**	**Ra norm**	**Pa norm**	**Pa norm/Pb**
a	0.02	0.02	0.02	0.08
b	0.67	0.53	0.35	0.60
c	3.55	2.84	0.74	0.77
d	0.02	0.02	0.02	0.80

Example 10.2 The effect of normalization. There are many steps in a microarray analysis, from cells' collection to obtaining the fluorescent signal, which are susceptible to experimental variation. These variations may lead to significant biases in certain samples. To allow comparison among different samples, it is critical to correct for such biases. Correction (or "normalization") is usually done by multiplying the microarray results by a constant value (k) that can be obtained by various means (see text). As a result of this multiplication, the ratios obtained from different microarrays are of similar range, and better comparisons can be made. When analyzing ratios between Polysomes and Free fractions, the step of normalization inserts some inaccuracy that should be noted. In this example, the values of Polysomes before (Pb) and after treatment (Pa) and their ratio (Pa/Pb) were taken from Example 10.1. To demonstrate the effect of normalization, the Ra values were multiplied by a (randomly chosen) constant (0.8) and presented in the "Ra norm" column (rounded to the second decimal). In reality, of course, the value will be different, yet its outcome is similar—multiplication of all ratios by the same factor. From the new Ra (Ra norm), the Pa was calculated based on the standard formula [P = R/(1 + R)] to yield Pa norm. Next, a new Pa/Pb (Pa norm/Pb) was calculated. Comparing the new Pa/Pb (Pa norm/Pb) values to the unnormalized (Pa/Pb) values reveals that although the overall trend is the same there are some differences. Specifically, some genes (*a* and *d*) seems to be more affected by the manipulation than others (gene *c*). The reason for this is that the normalization involves multiplication of the ratios by a constant (k). Considering the formula for deriving Pa norm [P = kR/(1 + kR)], the numerator and denominator are affected differently, depending on the initial Ratio and the normalization factor (k). Overall, since the general trend is kept, this is a good method to obtain first approximation of affected genes.

the underlying distributions in the two fractions from the experimental ratios, and from these values, determine the extent of changes (Example 10.1). This may provide more accurate results; yet, in such a case, one should be alert to the effects of normalization (see Example 10.2).

2.1.3. Indirect comparison

In an indirect comparison design, the free and polysome samples are labeled with a red fluorescent dye, and each is hybridized independently to a DNA microarray against an RNA sample that is labeled with green fluorescent dye (Fig. 10.1B). Because the green-labeled sample is the same for both samples, it serves as a common reference and the polysome-to-free ratio can

be obtained simply by dividing the ratio obtained from one microarray by the other (the common denominator cancels out, in such a case). The source of this RNA sample is not important because it serves only as a reference point: it can be an unfractionated RNA from the same cells or from other cells. The most important parameters for choosing this sample is that it will yield high-quality signals on the DNA microarray and that the same sample will be used in all analyses.

This experimental design (also called type II experiment) is more complex than the direct comparison design, because it requires twice the amount of DNA microarrays and an additional reference RNA sample. Thus, variation that is due to the DNA microarray production or its data acquisition might be a source for error. On the other hand, obtaining separate values for the free and poly fractions allows additional comparisons to be made. Specifically, one can compare each of these fractions before and after treatment and determine the relative changes for each gene in each fraction (thereby overcoming some of the limitations indicated in Example 10.1). This information helps in interpreting the biological significance of the results. In many cases, when multiple conditions are tested, type II experiments will provide the most consistent results since the use of a common reference to all samples allows correction for experimental variations.

It should be noted that a change in one fraction will not necessarily be reflected by a corresponding change in another fraction, even when the total amounts of mRNAs did not change. This is because the fold of change in a certain fraction is highly dependent on the initial amounts of RNA in this fraction, and these amounts are usually not similar in the two fractions. Therefore, a 10-fold decrease in polysomes will not necessarily be reflected by a 10-fold increase in the free fraction (see Example 10.3). Thus, a reciprocal effect in two fractions is not a prerequisite for assigning genes that were affected by a certain treatment.

2.1.4. Three or more fractions

When higher resolution is desired, more than two fractions need to be collected. Gradients can be divided to 3, 4, and even 25 fractions (Arava *et al.*, 2003; MacKay *et al.*, 2004), usually with the aim of identifying changes in the number of ribosomes associated with each mRNA. Experiments in which more than two fractions are analyzed can be performed only as type II experiments (indirect comparisons), in which each fraction is labeled in red fluorescence and hybridized to a microarray with an unfractionated RNA sample that is labeled in green (Fig. 10.1C). This experimental design leads to a set of ratios for each gene, which describes the relative abundance of its mRNA in each fraction. Ultimately, it would be desired to create from these ratios a distribution profile for each gene in the gradient, where a high ratio suggests a high abundance in this fraction and a low ratio suggests a low abundance. Such a distribution profile would provide a wealth of information

	Before treatment				After treatment		
gene	**F**	**P**	**Total**	**gene**	**F**	**P**	**Total**
a	80	20	100	*a*	98	2	100
b	42	58	100	*b*	60	40	100
c	4	96	100	*c*	22	78	100
d	98	2	100	*d*	98	2	100
	F/T	**P/T**			**F/T**	**P/T**	
a	0.80	0.200			0.98	0.020	
b	0.42	0.580			0.60	0.400	
c	0.04	0.960			0.22	0.780	
d	0.98	0.020			0.98	0.020	
			Fa/Fb	**Pa/Pb**			
		a	1.2	0.1			
		b	1.4	0.69			
		c	5.5	0.81			
		d	1	1			

Example 10.3 Indirect comparison. In this example, each fraction (free or polysome) was hybridized together with an unfractionated sample (total). The expected ratios for genes *a*–*d* are presented as F/T (free over total) and P/T (polysome over total). These ratios reflect the abundance of molecules in each fraction. Analysis of the changes in each fraction (Fa/Fb and Pa/Pb) reveals the genes that were most affected in each fraction. For example, gene *c* was induced 5.5-fold in the Free fraction, and gene *a* was repressed 10-fold in the polysome fraction. As can be clearly seen, there is no correspondence between the extent of change in the polysome and the change in the free fraction. Gene *a*, that was repressed 10 times in the polysome fraction, is increased by only 1.2 in the Free fraction. Gene *c*, on the other hand, was reduced by 0.81-fold in the polysome and increased by 5.5-fold in the Free. This lack of correspondence is due to the differences in the initial amounts of mRNAs of each gene in each fraction. Genes that had low amounts of mRNA in a certain fraction will show a relatively high effect and genes with high amounts will have a relatively small effects. Thus, genes that were strongly affected in one fraction will not necessarily appear affected in another fraction.

regarding the ribosomal association of each mRNA and its ribosome occupancy. Practically, however, experimental variations in the steps of RNA purification, labeling, hybridization, or scanning might skew the ratios in a particular fraction and therefore hamper the construction of an accurate distribution profile. For example, a better RNA purification of a certain fraction will lead to a higher labeling efficiency, and therefore higher ratios for this sample. The apparent high ratios may lead to an incorrect conclusion regarding the amounts of mRNAs in this fraction.

Various ''normalization'' protocols have been developed to correct for such biases in microarray experiments (Quackenbush, 2002). Most of them are based on the assumption that the mRNA levels of most genes, or of a

(A) mRNAs in each fraction

	Before treatment			After treatment		
	Free	**Mono**	**Poly**	**Free**	**Mono**	**Poly**
a	78	2	20	78	20	2
b	2	2	96	2	20	78
c	6	2	92	2	22	76
d	96	2	2	96	2	2

(B) Theoretical

(i) Ratio

	Before treatment			After treatment		
	F/T	**M/T**	**P/T**	**F/T**	**M/T**	**P/T**
a	0.78	0.02	0.20	0.78	0.20	0.02
b	0.02	0.02	0.96	0.02	0.20	0.78
c	0.06	0.02	0.92	0.02	0.22	0.76
d	0.96	0.02	0.02	0.96	0.02	0.02

(ii) Percent

	Free	**Mono**	**Poly**	**Free**	**Mono**	**Poly**
a	78	2	20	78	20	2
b	2	2	96	2	20	78
c	6	2	92	2	22	76
d	96	2	2	96	2	2

(iii) Percent change

	Free	**Mono**	**Poly**
a	0	18	−18
b	0	18	−18
c	−4	20	−16
d	0	0	0

(C) Normalized

(i) Ratio (sum of ratios = 1)

	Before treatment			After treatment		
	F/T	**M/T**	**P/T**	**F/T**	**M/T**	**P/T**
a	0.43	0.25	0.10	0.44	0.31	0.01
b	0.01	0.25	0.46	0.01	0.31	0.49
c	0.03	0.25	0.44	0.01	0.34	0.48
d	0.53	0.25	0.01	0.54	0.03	0.01
sum	1.00	1.00	1.00	1.00	1.00	1.00
norm. fac	0.55	12.50	0.48	0.56	1.56	0.63

(ii) Percent

	Free	**Mono**	**Poly**	**Free**	**Mono**	**Poly**
a	55.4	32.3	12.3	57.4	40.9	1.7
b	1.5	34.8	63.7	1.4	38.2	60.4
c	4.6	34.7	60.8	1.3	41.1	57.5
d	67.0	31.8	1.2	92.5	5.4	2.2

(iii) Percent change

	Free	**Mono**	**Poly**
a	2.0	8.6	−10.6
b	−0.2	3.4	−3.3
c	−3.2	6.4	−3.2
d	25.4	−26.4	1.0

Example 10.4 Effect of normalization on multiple fractions. The example herein presents the effect of using a "standard" normalization procedure, which is based on the assumption that all samples have the same amounts of mRNA. In this example, the gradient was divided into 3 fractions (free, monosome, and polysome). (A) The numbers of molecules in each fraction are presented for 4 genes (*a*–*d*) either before or after a treatment. Note that for simplicity the total amounts of mRNAs for each gene did not change. (B) The theoretical ratios from hybridization of each fraction are presented in (i), and transformed into percent in (ii). The percent change upon treatment is shown in (iii). As can be easily seen, the changes for each gene in percentages exactly reflect the changes in terms of mRNA copies. Note that gene *d* did not change its distribution upon treatment. (C) (i) The data from (B) was normalized such that the sum of ratios of all genes will be 1. The assumption behind this normalization is that deviation of certain fractions from this value (e.g., the monosome fraction) is due to experimental variation, such as mRNA losses or labeling efficiency. The factor by which each value was multiplied is indicated below each column (norm. factor). (ii) The percentages in each fraction after the normalization. (iii) The changes in distribution for each gene. As can be clearly seen, there is no relation between the actual changes and the calculated changes (compare B (iii) and C (iii)). This is most easily seen for gene *d*, which did not change at all, yet the normalized percentages suggest a shift of 26.4% out of the Mono (25.4% to the free fraction and 1% to the Poly fraction).

few specific mRNAs (“housekeeping” genes), are similar among the samples. This assumption is incorrect in the case of polysomal separation, because it is well established that mRNA levels vary significantly among fractions of a sucrose gradient and therefore using such a method will lead to incorrect results (see Example 10.4). A more reliable normalization procedure is based on the inclusion of exogenous, *in vitro* transcribed mRNA (van de Peppel *et al.*, 2003; Yang, 2006) (see Section 2.2).

2.2. Inclusion of exogenous RNA (spike-in controls)

One method to overcome some of the limitations that arise when using multiple fractions is to introduce into each fraction, immediately at its collection, known amounts of *in vitro* transcribed mRNAs (“spike-in”), which have corresponding spots on the DNA microarray (Arava *et al.*, 2003; van de Peppel *et al.*, 2003; Yang, 2006). These mRNAs should be with minimal homology to the tested mRNAs and are usually taken from an unrelated organism. Since they are introduced to each fraction immediately at its collection, they will be subjected to all of the remaining experimental steps, including RNA purification, labeling, hybridization, and scanning. Therefore, any variation in their signal between fractions is due to variations in the experimental steps following collection. Thus, a normalization factor can be derived based on the variation in their signal, by which the signals for the rest of the mRNAs are corrected. The introduction of equal amounts of external RNA has the advantage that no prior assumptions regarding the levels of mRNAs in each fraction need to be done. Following normalization for experimental variation, the obtained ratios for a gene represent its distribution within the gradient. From these ratios, one can determine the fraction in which a specific mRNA is most abundant (peak fraction) or the percent of molecules that are free of ribosomes.

While the use of spike-in RNA introduces many advantages to quantitative polysomal analysis, a few points should be noted: (1) It is important to use a pool of several RNAs, each of them present at different amounts, in order to minimize biases that are due to sequence, length, or expression levels. (2) The RNAs must be of good quality and introduced at exact amounts into each fraction. Small variations in pipetting these RNA will affect the resulting normalization factor. (3) Each DNA microarray should include many spots that correspond to these RNAs that are positioned at different regions on the microarray. These spots should yield strong signals following hybridization with no cross hybridization with mRNAs from the tested sample. Any error in the signals of these spots will affect the entire microarray and thus the entire data set, and therefore may influence the conclusions from the experiment.

2.3. Analysis of changes at the transcriptome level

All experimental designs described previously are focused on determining changes in ribosome association and are refractory to changes in the steady-state mRNA levels. To derive correct conclusions regarding the effects on translation, an analysis of changes in steady-state mRNA levels needs to be performed. This is not only because it will allow better understanding of cellular processes, but because changes in steady state are expected to differentially affect polysomal fractions. It is likely that the fraction of mRNAs free of ribosomes will be more affected by changes in mRNA levels, compared to fractions of mRNAs associated with ribosomes. Because of this differential effect, a decrease in mRNA levels in the fraction free of ribosomes can be interpreted as a shift to the polysomal fractions (increase in translation) or degradation of mRNA (decrease or no change in translation). Analysis of steady state mRNA levels may help distinguish between these options.

Analysis of steady-state mRNA levels is performed by extracting total RNA from cells and labeling the RNA without a separation step on a sucrose gradient. Following labeling, samples are hybridized to a microarray and their signals are quantified. As has been indicated, most experimental steps might introduce certain errors that will be different from one sample to another. Therefore, the resulting signals do not reflect the exact amounts of mRNA in the cell, and thus do not provide the absolute changes in mRNA levels but only changes relative to other genes. For example, a two-fold increase for a gene means that it increased by two-fold relative to the change of all other transcripts. If the levels of all other transcripts were actually reduced, then the relative two-fold increase might actually be a decrease in the absolute levels of the transcripts of that gene. The fact that the changes are not absolute, but relative to other genes, should be taken into consideration when the data (from both the total and fractionated RNA analyses) are interpreted.

3. Methods

There are numerous protocols for polysomal gradients preparations that differ mainly at the step for harvesting the cells, and the gradient composition and separation times. The protocol presented later was optimized for isolation of polysomal mRNA from the yeast *Saccharomyces cerevisiae*, yet many steps will be similar to other eukaryotes and the procedure can easily be modified for other organisms. We will use this protocol as a template on which we will indicate and highlight points that are critical for the microarray analysis. Generally, the RNA isolated by this protocol can be used for analysis by DNA microarray, Northern blot, or RT-PCR.

3.1. Gradient preparation

We typically use 11 ml of 10 to 50% linear sucrose gradients, onto which 1 ml of cells lysate is laid. The gradients can be made by a gradient maker just before use or be prepared by hand a day before using any of several methods. The protocol that is presented here is very simple and reproducible and can be used to make multiple gradients simultaneously. The gradients are poured 12 to 24 h before use and allowed to equilibrate at 4°. Alternatively, immediately after pouring, the gradients can be stored at −80° indefinitely. The gradients can be thawed at 4° 12 to 24 h before use with no change in quality.

For 1 gradient of 11 ml:

1. Prepare 3-ml mixes of 10, 20, 30, 40, and 50% sucrose solutions in a gradient buffer containing 20 m*M* Tris-HCl (pH 7.4), 140 m*M* KCl, 5 m*M* $MgCl_2$, 0.5 m*M* DTT, 0.1 mg/ml Cyclohexamide (C-7698, SIGMA), and 0.5 mg/ml Heparin (H-3393, SIGMA). For more than one gradient, increase the volume of the solutions proportionally.
2. Use a long Pasteur pipette to layer 2.2 ml of the 10% sucrose solution in a Beckman polyallomer tube (14 × 89 mm, #331372). Then, underlay 2.2 ml of the 20% sucrose solution by inserting the tip of the pipette to the bottom of the tube and slowly pipetting the 20% sucrose solution under the 10% solution. Underlay the 30, 40, and 50% sucrose solutions in the same manner. Cover the tube with aluminum foil and store overnight at 4° to establish a linear gradient.

3.2. Cell lysis

The presented protocol is for yeast cells grown in rich media and collected at the logarithmic phase of growth. It is designed for a liquid culture of 50 to 100 ml ranging from an OD_{600} of 0.4 to 0.8. The necessary amount of cells will vary with respect to the aims of the experiment and the number of fractions to be analyzed. Under certain growth conditions or for certain mutations, higher amounts of cells might be needed. It is therefore advised to perform a preliminary experiment for polysomal separation, and to measure the amount of RNA in each fraction. For analysis in yeast, we found that 15 to 50 μg provides a good signal on the DNA microarray and therefore the amount of cells to be grown should yield at least that amount.

1. Grow a 50 to 100 ml culture to an OD_{600} 0.4 to 0.8.
2. Add cyclohexamide to a final concentration of 0.1 mg/ml (stock solution 10 mg/ml in water) in order to arrest the elongation step of translation.
3. Transfer the culture into an ice-cold 50-ml tube and immediately spin down the cells at 4000 rpm for 4 min at 4°.

Quick pelleting of the cells and removal of the rich medium assists in halting

the translation process. Delays in this step led, in some cases, to an increased 80S peak, presumably due to recruitment of free mRNA molecules after addition of the cyclohexamide.

4. Discard the supernatant, resuspend the cell pellet in 4 ml lysis buffer (20 m*M* Tris-HCL (pH 7.4), 140 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.5 m*M* DTT, 0.1 mg/ml Cyclohexamide, 1 mg/ml Heparin, 1% Triton X-100), and pellet the cells at 4000 rpm for 4 min at 4°. Repeat this step one more time to get rid of residual culture media.

   Heparin inhibits many enzymes, including all RNases, therefore, it is critical for this step. It also inhibits the labeling step; therefore it needs to be removed later (by a LiCl precipitation). We did not find any comparable alternative (in efficiency and cost) to heparin. The composition and concentrations of other ingredients can be changed depending on the cell type or experiment.
5. Discard the supernatant, resuspend the pellet in 400 μl lysis buffer, and transfer to a screw-capped microfuge tube.
6. Add 1.5 ml of chilled glass beads (0.45–0.55 mm) and vortex vigorously in a bead beater by two pulses of 90 s at maximum level to achieve complete lysis.
7. To recover the lysate, puncture the bottom of the screw-capped tube with a flame-heated needle and place it on top of a 15-ml conical tube. Use the cylinder of a 5-ml syringe as an adaptor between the screw-capped tube and the 15-ml tube.
8. Spin down the assembly composed of the lysate-containing tube, the adaptor, and the 15-ml conical tube at 4000 rpm for 1 min at 4°.
9. Transfer the beads-free lysate from the 15-ml tube (supernatant and pellet) into a new cold microfuge tube.
10. Spin at 9500 rpm (~8200*g*) for 5 min at 4° and transfer the supernatant into a new cold microfuge tube. This step removes most of the cell debris, leaving a cleared lysate.
11. Bring the lysate to a final volume of 0.8 ml with lysis buffer.
12. Carefully load the lysate onto a sucrose gradient and insert the gradient into a cool SW41 rotor bucket. The centrifuge tube should be filled almost to the top to avoid problems with the tube collapsing during ultracentrifugation.
13. Centrifuge at 35000 rpm for 160 min at 4°.

    A good separation of up to ~8 to 10 ribosomes was obtained with these centrifugation parameters. Although different centrifugation times might allow better resolution for larger complexes, we did not find much improvement.

3.3. Fractions collection

Fractions from a sucrose gradient can be separated either according to the complexes that they contain or to a fixed volume. When the gradient is separated to a small number of fractions (e.g., free mRNA, monosomes, and

polysomes), it is feasible to separate the fractions according to the complexes they contain. This ensures that equivalent fractions from different gradients will contain mRNA molecules associated with the same complexes. Thus, even if there are variations in gradient volumes or in complexes sedimentation, the analyzed fractions are the same. Collecting in this manner obviously necessitates knowing the sedimentation position of each complex; therefore, the use of a continuous ultraviolet (UV) detector, such as ISCO UA6, is highly recommended. When many fractions are necessary, it is advisable to separate the gradient into fractions of a constant volume, and thereby minimize the errors caused by inaccuracy in complexes identification. In some cases, a combination of the two methods will work well, for example, collecting the fractions of free mRNA and monosomes according to the complexes, and then collecting the rest of the gradient at fixed volumes. The fact that these fractions have different amounts of mRNAs does not pose any conceptual problem since these differences are eliminated or corrected by the normalization procedure. It might, however, introduce a technical problem in having the optimal amount of RNA for the labeling step.

3.4. Adding spike-in controls to fractions

Spike-in controls are *in vitro* transcribed RNAs that are added to each fraction at the time of collection. They are necessary in order to correct for differences in mRNA levels in the various fractions from the gradient. These controls should have minimal cross-reactivity with the tested genome and should be added at known (and usually equal) amounts to the tested fractions. The exact amount to be added should be such that their signals will be neither too low nor too high relative to the rest of the mRNA. This might not be trivial because some polysomal fractions contain high amounts of mRNAs while others contain low amounts, and therefore their signals on the DNA microarray will differ significantly. Although it is not optimal, it is possible, in such cases, to introduce into each fraction different amounts of the *in vitro* transcribed RNAs. Since they are added at known amounts, their signals on the DNA microarray should be relative to the amounts added, and if not, a correction factor (''normalization faction'') should be imputed. It is recommended to use more than one type of RNA and to spike the different RNAs at different amounts, in order to have a better coverage of sequences and expression levels.

We routinely use a mix of five mRNAs that are derived from the *lys* (ATCC no. 87482), *trp* (ATCC no. 87485), *dap* (ATCC no. 87486), *thr* (ATCC no. 87484), and *phe* (ATCC no. 87483) clones from the bacterium *Bacillus subtilis* cloned into a vector that contains a stretch of As. These RNAs are generated by *in vitro* transcription using a T3 *in vitro* transcription kit (e.g., MEGAscript from Ambion) of the linearized DNA template with the appropriate restriction enzyme.

Table 10.1 Spike-in mix stock solution

ATCC clone	Clone name	Concentration in mix
87482	lys	80 pg/μl
87485	trp	160 pg/μl
87486	dap	200 pg/μl
87484	thr	240 pg/μl
87483	phe	320 pg/μl

PCR products, corresponding to these clones, should be present at multiple sites on the DNA microarray. We routinely put at least 20 spots for each clone.

Currently, most commercially available DNA microarrays include spots that correspond to *in vitro* transcribed RNA from an unrelated organism (Yang, 2006). These RNAs can be either purchased as a ready-to-use mix or synthesized *in vitro*.

1. Prepare a mix of the five different mRNA spikes at the concentrations indicated in Table 10.1. Store at −80°.
2. Thaw the mix on ice just before use and add the appropriate volume to each fraction immediately after collection.

   The amount of mix to add depends on the experimental setting and the number of fractions collected. We typically add 70 μl of spike-in mix into an entire sucrose gradient, where each fraction receives the relative share from that amount. For example, 35 μl of the spike-in mix will be added to each fraction of a gradient that was divided into two, and 7 μl of this mix will be added to each fraction of a gradient that was divided into 10 fractions. The added amounts should consider losses during purification steps and that a minimum of 0.2 ng of each spike is needed to yield sufficient signal in the microarray hybridization.

3.5. RNA extraction from fractionated gradients

The following protocol is adapted to precipitation of RNA from a gradient that was fractionated to four fractions: free mRNA, monosome, low polysome, and high polysome. Most of the procedure is aimed at removing proteins (e.g., RNases) or reagents that will inhibit the labeling step (such as heparin). It can be used to purify RNA from sucrose gradients of any organism.

1. Collect fractions into 50-ml Oak-ridge tubes.
2. To each fraction, add 1 volume of 8 *M* guanidium-HCl and 2 volumes of 100% ethanol.
3. Add 17.5 μl of spike-in mix to each fraction (regardless of the fraction's volume). Mix the samples thoroughly and store for overnight at −20°.
4. Centrifuge at >10,000 rpm for 30 min at 4°.

5. Discard supernatant, add 5 ml ice-cold 75% ethanol, and centrifuge at >10,000 rpm for 20 min at 4°.
6. Resuspend pellets in 400 μl TE (pH 8.0). Let the samples stand for few minutes at room temperature to allow efficient dissolving.

   Heparin is found in high amounts in the first fractions (remnants of the lysis buffer); therefore, make sure that these fractions are dissolved well.
7. Transfer to a microfuge tube and add 0.1 volumes of 3 *M* sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol.
8. Incubate for at least 1 h at −20° and spin at 14,000 rpm for 30 min at 4°.
9. Discard the supernatant and resuspend the pellet in 650 μl RNase-free water. Add an equal volume of water-saturated phenol:chloroform (5:1), pH 5.2. Vortex vigorously and spin at top speed for 5 min at room temperature. Take 500 μl of the aqueous phase into a new microtube tube.

   This step removes any residual proteins. It is important to avoid contamination of phenol in the extracted phase because this will affect the reverse transcriptase reaction. It is also important to avoid the interphase, which is enriched with DNA.
10. Bring to 1 ml with RNase-free water, add lithium chloride to a final concentration of 1.5 *M* (175 μl from 10 *M* stock), and incubate overnight at −20°. Thaw on ice and centrifuge at top speed for 20 min at 4°.

    The LiCl precipitation is necessary to remove any residual heparin, which may interfere with the labeling reaction. Some vendors (e.g., Ambion and Qiagen) sell RNA purification columns that should remove heparin. We have not tested any of these yet.
11. Carefully discard the supernatant, add 200 μl of 75% ethanol, and centrifuge at top speed for 20 min at 4°. Discard supernatant and resuspend in 150 μl RNase-free water.

    The pellet at this step is unstable and hardly visible. Carefully decant the supernatant and spin again if the pellet becomes unstable.
12. Precipitate again with sodium acetate as in steps 8 and 9.
13. Spin down at top speed for 20 min at 4°. Wash with 75% ethanol and air dry.
14. Resuspend pellet in 25 μl RNase-free water and store at −80°.

3.6. Preparation of a reference sample

There are two sources of RNA that can be used as a reference sample (the green-labeled sample): RNA from cells that are not related to the experiment or unfractionated RNA from the same cells under the same treatment. It is preferable to take an unrelated RNA sample that will be used in all future experiments, thereby serving as a common reference for all conditions and treatments. The most important parameter for this sample is that it yield a strong and reliable signal for as many genes as possible on

the microarray. The optimal sample will yield signals for all genes on the microarray and therefore it will be possible to assign red-to-green ratio to all genes. To minimize variations, the reference sample should be extracted at large amounts so the same sample could be used in all experiments.

The following protocol is one of the versions of the "hot phenol" method and it is scaled for 100 to 150 ml of mid-log yeast culture (OD_{600}- 0.5–0.7). For different cell amounts, simply adjust the solution volumes.

1. Grow cells to mid-log phase.
2. Pellet cells at 3500*g* at room temperature for 3 min.
3. Discard the supernatant, wash the cells in sterile water, and pellet again as in step 2 to get rid of residual media. Following removal of supernatant, one can freeze and store the pellet at −80°, or continue directly to step 4.
4. Resuspend the pellet in 4 ml lysis buffer (10 m*M* Tris-HCl pH 7.5, 10 m*M* EDTA, 0.5% SDS). Place in a phenol-resistant tube (e.g., Sarstedt 13 ml, cat # 55.518).
5. Add 4 ml acid phenol (water saturated, low pH [e.g., Sigma P4682]). Vortex well.
6. Incubate at 65° for 1 h with occasional vigorous vortexing.
7. Place on ice for 10 min. Centrifuge at 4° for 10 min at top speed.
8. Transfer the upper aqueous phase into a new tube. Be careful not to take the interphase because this will lead to a contamination with DNA and/or phenol.
9. Add equal amount of acid equilibrated phenol:chloroform 5:1 (Sigma P1944), vortex well, and centrifuge again at room temperature for 5 min at top speed.
10. Transfer the upper aqueous layer into a new tube. Add equal amount of chloroform, vortex well, and centrifuge again at room temperature for 5 min at top speed.
11. Transfer the upper aqueous layer into a new tube. Precipitate the RNA by adding 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2 volumes of 100% ethanol. Incubate overnight at −20°.
12. Centrifuge at 4° for 20 min at top speed.
13. Remove supernatant, add 1 ml of 75% ethanol, and centrifuge again at 4° for 20 min at top speed. Repeat this step and air-dry the sample.
14. The typical yield of this protocol is 1 to 2 mg RNA. Resuspend the RNA pellet to a final concentration of 5 to 10 $\mu g/\mu l$ in nuclease-free water and divide into aliquots. Store at −80°.

3.7. Reverse transcription and amino–allyl coupling

Labeling of the RNA sample with a fluorescent nucleotide can be done either directly or indirectly. In a direct labeling, one of the nucleotides (usually dUTP or dCTP) has a fluorescent moiety (either Cy3 or Cy5)

linked to its base group. Therefore, during the reverse transcription step, the cDNA becomes labeled. In an indirect labeling, one of these nucleotides contains an amino–allyl group linked to its base group. An additional coupling step is therefore necessary, in which the fluorescent dye is coupled. We use the latter method, since it is less expensive, it is more consistent with different dyes, and it should yield longer cDNAs due to low structural hindrance by the allyl moiety.

3.7.1. cDNA synthesis

For one reaction:

For multiple samples, simply multiply everything by the necessary factor. The reference sample should be treated as one pool during the entire labeling process and split only prior to the hybridization step.

1. Prepare the following mixture:

   5 μg Oligo dT (T_{20}VN [V = any nucleotide except T])
   15 to 50 μg RNA of interest

   Exact amount depends on the mRNA "richness" of the fraction. The exact amount of RNA is not critical because the spike-in RNA will correct for any variations. For example, taking twice the amount of RNA from one fraction will be associated with taking twice the amounts of its spiked-in RNA and therefore the resulting signals will be corrected accordingly.
   Nuclease-free water to 15.5 μl

   Incubate 10 min at 70° and transfer to ice for 10 min for annealing of the Oligo-dT to the mRNAs.

2. Prepare a reverse transcription reaction mix (based on Promega ImProm-II reverse transcription system).

   6.0 μl 5× reaction buffer
   4.0 μl 25 m*M* $MgCl_2$
   1.2 μl 25× Amino–allyl mix (12.5 m*M* dATP, 12.5 m*M* dGTP, 12.5 m*M* dCTP, 5 m*M* dTTP, 7.5 m*M* amino–allyl dUTP (Ambion #8439))
   0.3 μl Nuclease-free water.
   3.0 μl Reverse transcriptase

3. Incubate at 42° for 2 h.
4. Add 10 μl of 1 N NaOH, 10 μl of 0.5 M EDTA, and incubate at 65° for 15 min to degrade the RNA following the cDNA synthesis.
5. Add 25 μl of 1 M HEPES (pH 7.0) and bring the total volume to 100 μl by adding 25 μl of nuclease-free water. Precipitate the cDNA by adding 10 μl of 3 *M* sodium acetate (pH 5.2) and 275 μl of 100% ethanol.
6. Wash once with 70% ethanol, air dry, and resuspend in 9 μl of nuclease-free water. The samples can be stored at −80° for at least a month.

3.7.2. Fluorescent labeling

1. Add 1 μl of sodium bicarbonate 1 *M* (pH 9.0) to the amino–allyl labeled cDNA solution.
2. Add 1 μl of Cy3 or Cy5 dyes (Amersham cat. #336219 or 335176, respectively). Cy dyes should be suspended in 12 μl DMSO and divided into aliquots of 1 μl.

   An aliquot can be used immediately to label one cDNA sample or be completely dried out and stored at 4° in desiccator for later use. It is important to minimize the exposure of the dyes or labeled samples to light.
3. Incubate at room temperature for 1 h to allow coupling of the dye to the amino–allyl groups.
4. Purify the Cy-labeled cDNA using a DNA clean and concentrator kit (Zymo Research), according to the kit protocol. Elute the DNA with Nuclease-free water in a final volume of 5 μl.

3.8. Microarray slides preparation

The DNA microarrays are spotted on glass slides coated with amino-silane (Corning GAPS II). They should be ready for hybridization immediately when the labeled cDNA is ready. Thus, while the dyes are coupling to the cDNA (step 3 in the previous section), it is recommended to start the following process.

1. The slides are routinely stored in dark under desiccation. Handle all slides with powder-free gloves. Before use, mark the array boundaries with a diamond pen on the back of the slide since the arrays will not be visible after processing.
2. UV cross-link the slides at 300 mJ.
3. Put the slides in a metal slide rack (Shandon cat. #113) and submerge the rack in 0.1% SDS solution. Keep the tops of the slides under the level of solution and shake carefully for 30 s.
4. Dip the slides in DDW heated to 95° and incubate for 3 min.
5. Transfer the slides into a beaker with 70% ethanol and shake for 2 min.
6. Transfer the slides from the wet rack into a new, dry rack. While doing so, remove excess solution by striking gently and swiftly the bottom of each slide on a dried^{TM} Kimwipe.
7. Centrifuge the rack at 500 rpm for 5 min at room temperature for complete drying.
8. Prehybridize the slides in a coupling jar containing preheated and filtered prehybridization buffer (1% BSA (A7906 sigma), 5 × SSC, and 1% SDS) and incubate at 42° for 1 h.
9. Transfer the slides into a clean rack and soak in DDW for few seconds.
10. Transfer the slides into a dry rack and dry, as in steps 6 and 7. The slides are ready for hybridization.

3.9. Hybridization

1. Add 5 μl of each of the Cy-labeled samples into 20 μl hybridization buffer (4.5 × SSC, 75% formamide, and 0.15% SDS).
2. Add 3 μl (30 μg) poly(A)-RNA (Sigma P-9403).
3. Mix, microfuge briefly, and incubate at 95° for 3 min.
4. Microfuge briefly and add 3 μl of 10% BSA.
5. Mix and spin down at top speed for 1 min.
6. Place a microarray slide in a hybridization chamber and pipette 25 μl of the labeled probe mixture on the slide surface near one end of the microarray print area.

   Hybridization volumes will vary depending on the print size. The volumes herein are for 32 blocks, and should be adjusted for 16 or 48 blocks.
7. Grasp one end of a dust-free 22 mm × 40 mm microscope glass coverslip with forceps. Lower one end near the cDNA probe until it touches the surface outside the printed area and slowly lower the opposite end of the coverslip onto the slide. The solution will spread across the entire print area beneath the coverslip. Use a yellow tip to carefully adjust the position of the coverslip over the printed area. Large air bubbles can be moved away from the hybridization area by a gentle tapping on the coverslip with a yellow tip. Small air bubbles will be released during hybridization.
8. Add 10 μl of sterile water to the wells in both sides of the hybridization chamber in order to keep a moist environment.
9. Close the hybridization chamber and place in a water bath that has been preheated to 42°. Incubate for at least 6 h.

3.10. Washing

1. Remove one slide from the hybridization chamber at a time. The unsealing of the chamber should be done horizontally to prevent sliding of the coverslip.
2. Quickly transfer the slide (without removing the coverslip) into a rack and submerge it in Wash buffer 1 (2 × SSC and 0.05% SDS). Similarly, transfer each of the other slides into the soaked rack. Use needle to gently assist with the falling of the coverslips from the hybridization areas of the slides. Be careful not to touch any of the printed area. Shake the slides for 5 min at room temperature.
3. Transfer the rack into Wash buffer 2 (1 × SSC). Absorb excess Wash buffer 1 with a paper towel before putting the rack in Wash buffer 2. Shake the slides for 5 min at room temperature.
4. Transfer the rack into Wash buffer 3 (0.1 × SSC). Shake the slides for 5 min at room temperature.

5. Spin dry at 500 rpm for 5 min at room temperature. The slides are ready for scanning. Store the dried slides in dark box until scanning.

4. Data Acquisition and Analysis

4.1. Microarray scanning

Several types of microarray scanners exist. Generally, all utilize lasers for the excitation of fluorochromes and photomultiplier tubes (PMT) to capture their fluorescence emission. Basically, increasing the laser power and/or the PMT voltage will produce higher signal intensities. For a large range of intensities, the emitted light is proportional to the number of fluorochromes in the spot. However, due to the PMT detection system limitations, extreme signals (low and high) deviate from this linearity (Lyng *et al.*, 2004). Therefore, it is recommended to perform two scans for each slide. The first scan should be at high PMT voltage to increase the intensities of the low signals to the level of linearity, thus allowing the high signals to be saturated. The second scan should be done at low PMT voltage in order to minimize the saturated spots. Subsequently, the data for the spots that were saturated in the first scan can be recovered from the second scan. It is important to ensure that the scans provide reliable signals to the spike in RNA (not too low and not saturated), since their signals will be used to normalize the signals of all other spots.

We utilize the GenePix 4000B scanner, which allows simultaneous detection of two dyes (Cy3 and Cy5). The following parameters are used for scanning:

Laser power—100% (we never change this parameter)
PMT gain—usually from 500 to 800, depending on signal intensity
Pixel size—10 μm
Lines to average—2
Focus position—10 μm

4.2. Creating a reliable dataset

Following scanning, a "gridding" step is performed in which each spot is cataloged and its signal quantified. Importantly, during this step, spots of low quality or with unreliable signals are flagged out. This step is performed automatically by gridding software, which utilizes various parameters, including differences between the spot and its background, the spot diameter and circularity, and the homogeneity of the signal in the spots in order to create a grid for all spots. Many software applications are available for this purpose (e.g., GeneSpring, Spotfire, and ImaGene), some of which are freely available (Scanalyze and Spotfinder). We use the software "GenePix" from Molecular

Devices. Although the gridding step is done automatically, it is highly recommended to go over the analysis performed by the program and correct misjudgment events. This step ensures that the subsequent statistical analysis will be performed with high-quality spots and with correct quantification.

The stringency of the filtration criteria will, of course, determine the number of spots to be analyzed and the reliability of the results. When possible, it is recommended to apply the same parameters to each slide in order to keep a common standard among fractions. Yet, the number of spots that pass the filtration criteria may differ greatly from slide to slide. This could be the outcome of low amounts of mRNA in certain fractions (e.g., free and monosome populations) or due to bad hybridizations. Because achieving signals from all fractions is crucial for determining the polysomal distribution of all mRNA, losing information for one fraction may damage the entire analysis. Therefore, it is sometimes necessary to reduce the stringency of filtration at the cost of less accurate data.

Beyond the standard features of selection criteria employed by the program, we typically add the following parameters to achieve reliable data:

1. At least 80% of a spot's pixels should have intensities more than two standard deviations above the background intensity for that spot, at each wavelength.
2. At least 80% of feature pixels at each wavelength are not saturated.
3. The diameter of the spot is greater than 55% of the feature size.
4. The coefficient of the regression line (R^2) is greater than 0.6.

4.3. Data verification

The methods for genome-wide analysis of ribosomal association are conceptually different from those for analysis of a single mRNA (e.g., northern analysis). While in assays of a single gene by northern analysis it is simple to compare the distribution of an mRNA in different fractions, in a genome-wide assay, the mRNAs are usually compared relative to others within the fraction. Because of that, it is important to analyze by northern analysis several mRNAs that appeared to be affected to different extents, and to compare the relative effects among this group between the microarrays and the northern analysis.

In experiments where spike-in controls are added, validation is simpler because one can perform a northern analysis for one of the spiked-in RNAs and use their signals to normalize any differences between fractions. This allows direct comparison between northern blot and microarray results.

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