

Isolation of membrane-bound polysomal RNA

Maximilian Diehn

INTRODUCTION

Although DNA microarrays are most often used for genome-scale analyses of gene expression, they can in principle be used to compare any nucleic acid populations that can be physically separated. Thus, one important biological phenomenon that can be studied with DNA microarrays is the subcellular compartmentalization of mRNA. There are numerous documented examples in eukaryotes ranging from yeast to human, where the localization of RNA within a cell plays an important biological role in the functioning of that cell or organism. By serving as an assay for the presence or absence of thousands of transcripts, DNA microarrays can rapidly accelerate the identification of transcripts in various subcellular compartments. Given the biological importance of such subcellular mRNA distributions it is clear that studying them with DNA microarrays should lead to a deeper understanding of many cellular processes.

One of the most fundamental examples of differential subcellular localization of RNAs is the distinction between mRNAs that encode cytosolic or nuclear proteins and those that encode membrane-associated or secreted proteins. The proteins in the latter group are co-translationally inserted into the rough endoplasmic reticulum (rER) by ribosomes attached to the cytoplasmic surface of the rER while the first group is translated by ribosomes that are free in the cytosol of the cell. This spatial separation of protein synthesis creates distinct pools of mRNAs that can be separated based on the presence or absence of an association with cellular membranes. Once separated, the two fractions can be hybridized to DNA microarrays and subsequent analyses can yield two important types of information: 1) the subcellular localization of proteins encoded by the mRNAs can be inferred from the subcellular localization of their transcripts and 2) unexpected associations of mRNAs with cellular membranes can be identified and marked for further study.

There are a number of potential approaches for separating membrane-associated and free mRNA. One type of approach takes advantage of the relatively low density of cellular lipid structures by separating these from denser components by sedimentation equilibrium (Mechler 1987). A second set of methods uses differential detergent extractions coupled with sedimentation velocity centrifugation (Stoltenburg et al. 1995) to separate the two populations of mRNAs. It is also possible to isolate rER associated mRNAs via antibody-based purification protocols that are directed against components of the signal-peptide recognition machinery. The protocol described below employs sedimentation equilibrium centrifugation in a sucrose gradient. While the separation instructions are specific to this approach, the method of analysis that is described can be applied to any experimental approaches that sub-fractionate RNAs.

One important consideration in designing experiments studying the subcellular compartmentalization of RNA is the inclusion of control elements in the DNA microarrays to be used. In this case, elements representing transcripts

of known subcellular localization serve as controls for two important reasons. First, the success of the fractionation procedure can be assessed by examining the fluorescence ratio distribution for the genes of known subcellular localization. Secondly, the known genes allow calibration of the relationship between the measured fluorescence ratio and the likelihood that a given element encodes a protein with a given type of localization. In the case of experiments with membrane-associated polysomes, DNA microarrays should contain as many genes encoding proteins of known subcellular localization as possible. Minimally, approximately 500 of the array elements should be of this type.

MATERIALS

Important: To minimize risk of contamination of stock solutions with RNases use DEPC-treated water wherever water is needed.

Buffers and Solutions

Cycloheximide (10 millig/ml)

CAUTION: toxic; Store at -20°C .

Phosphate Buffered Saline (PBS)

Add cycloheximide to a final concentration of 10 microg/ml just before use.

Sodium Acetate (2 M)

Ethanol (95%)

Note: 200 proof ethanol can contain fluorescent contaminants and should never be used when processing samples that will be analyzed on DNA microarrays.

10 mM Tris-Cl, pH 7.4

Hypotonic-lysis buffer (10 mM KCl, 1.5 mM MgCl_2 , 10 mM Tris-Cl, pH 7.4)

Add cycloheximide to a final concentration of 10 microg/ml just before use.

Autoclave before use.

Gradient buffer (150 mM KCl, 5 mM MgCl_2 , 50 mM Tris-Cl, pH 7.4)

Autoclave before use.

2.5 M sucrose gradient buffer

This solution is extremely viscous and needs to be made carefully. Add 855.75 g sucrose in approximately 150 g increments to 250 ml 65°C Gradient buffer in a 2 L beaker. Stir on a hot plate using an appropriately sized stir bar and keep the temperature of the solution near 65°C . If the solution becomes too viscous, add a small amount of 65°C gradient buffer. Once all of the sucrose has been added, adjust the final volume to 1 L using 65°C Gradient buffer. It is possible although not necessary to measure the concentration of sucrose in the solution using a refractometer and to adjust the solution accordingly. Each gradient requires 15 ml of the 2.5 M sucrose buffer. Just before use in a gradient, add cycloheximide to a final concentration of 10 microg/ml.

1.95 M sucrose and 1.3 M sucrose gradient buffers

Make these solutions by appropriate dilution of the 2.5 M sucrose stock with gradient buffer. Each gradient requires 13 ml of 1.95 M sucrose buffer and 6 ml of 1.3 M sucrose buffer. Just before use in a gradient, add cycloheximide to a final concentration of 10 microg/ml.

Trizol-LS[®] (Life Technologies, Inc. Cat. No. 10296)

Special Equipment

Barrier tips for micropipettes

Ball bearing homogenizer or 5-10 ml glass dounce homogenizer

2 ml microcentrifuge tubes

Ultra-clear 25x89mm centrifuge tubes (Beckman Instruments, Inc. Cat. No. 344058)

SW-28 ultracentrifuge rotor

Ultracentrifuge

18-gauge, 1 inch needles

50 ml Oak Ridge centrifuge tubes (Nalge Nunc International)

Roller bottles or spinner flasks for tissue culture

Spectrophotometer for OD 260 nm measurements

Hemocytometer

PROTOCOL STEPS

Preparation of tissue culture cells

1. Grow up tissue culture cells using conditions appropriate for the cells of interest. Since a large number of cells is required for each gradient (5×10^8), it is advisable to grow cells in roller bottles or spinner flask. Keep cells near a concentration of 5×10^5 cells per ml since overcrowding may lead to down-regulation of protein synthesis and therefore lower yields of polysomal mRNA.
2. Add cycloheximide to a final concentration of 10 microg/ml to the cell culture and return to incubator or warm room. Incubate cells at 37°C for 5-10 minutes and proceed with pelleting procedure. If cells were grown on plastic petri dishes, remove media, add a small volume of ice cold PBS and scrape from dish using a rubber policeman. Transfer to 250 ml centrifuge tube and proceed as with suspension cells.
3. Pellet cells in 250 ml centrifuge tubes for 10 minutes at $1000g$ (4°C). From this point on all steps should be performed at 4°C or on ice.
4. Wash cells 2 times with 125 ml of ice cold PBS supplemented with 10 microg/ml cycloheximide. After the last wash, resuspend each pellet in 10 ml ice cold PBS containing 10 microg/ml cycloheximide.
5. Pool all cells into one 250 ml centrifuge tube and count cells using a hemacytometer. Aliquot the equivalent of 5×10^8 cells to a 15 or 50 ml conical. Pellet cells as before. Note: It is possible to stop here and freeze the cell pellet on liquid nitrogen. Store pellets at -80°C . Cell pellets can be used for up to at least 6 months after freezing.

Hypotonic Lysis and Gradient Construction

6. Resuspend cell pellet to 2.5×10^8 cells/ml using 2 ml of ice cold hypotonic lysis buffer. Allow cells to swell on ice for 5-10 minutes. Note: It may be necessary or desirable to include RNase inhibitors in the lysis buffer and subsequent gradient fractions. Since cell lines and tissues vary in the amount of RNase activity this decision must be made on a case-by-case basis.

7. Dounce homogenize cells with 10 strokes in a dounce homogenizer or alternatively with 10 passes through a ball bearing homogenizer. If desired, freeze a small aliquot of the lysate at -80°C for later analysis as total cellular RNA.
8. Centrifuge lysate for 2 minutes at $2000g$ (4°C) to pellet nuclei and unlysed cells. The pellet will be very soft and of varying size depending on the cell type. Remove supernatant and adjust volume to 2 ml using ice cold hypotonic-lysis buffer.
9. Add 2ml of lysate to 11 ml of 2.5M sucrose gradient buffer in a 50 ml conical and mix well by vortexing.
10. Construct sucrose step gradient in Ultra-Clear 25x89 mm centrifuge tubes (Beckman) for an SW-28 ultracentrifuge rotor. First place 4 ml 2.5M sucrose gradient buffer in bottom of tube. Next, carefully layer the 13 ml containing the lysate from the previous step onto the 2.5M sucrose cushion. Finally, successively layer 13ml 1.95 M sucrose gradient buffer and 6ml 1.3M sucrose gradient buffer onto the gradient (see Figure 1.)
11. Centrifuge for at least 5 hours at $90,000g$ in an ultracentrifuge. Note: It is critical to balance tubes to be spun in an ultracentrifuge. If an even number of gradients is being prepared, balance them on a scale using 1.3 M sucrose gradient buffer. If an odd number of gradients is being set up, prepare a mock gradient using 2 ml of water instead of lysate in step #9. After the spin, small membrane particles should be visible at the interface of the 1.95 M sucrose and 1.3M sucrose sections.
12. Harvest gradients by puncturing the bottom of the centrifuge tubes with an 18-gauge needle and collecting 1.5 ml fractions in 2 ml microcentrifuge tubes. Alternatively, to minimize the risk of contamination with free RNA, the membrane fraction can be isolated by successively removing 10 ml from the top of the tube using a P1000 pipetman. The remainder of the gradient is then harvested by the puncture method. Note: To hold the ultracentrifuge tubes during the harvesting of the gradients carefully cut a polypropylene 50 ml conical in half using a sharp knife or razor blade and discard the top half of the tube. Next cut off the very tip of the conical section remaining on the bottom of the tube. This device can be suspended over the bench using a standard clamp and ring stand and has the right diameter to snugly fit an ultracentrifuge tube. To collect the fractions just manually slide a microcentrifuge tube rack containing open 2 ml microcentrifuge tubes under the punctured gradient tube.
13. Measure the absorbance of each fraction at 260 nm using a spectrophotometer to determine presence of nucleic acid (see Figure 2). Free ribosomes and free mRNA will be present in the load zone while membrane-associated ribosomes and mRNA will be at the interface between the 1.95 M and 1.3 M sucrose steps.

RNA Isolation and Array Hybridization

14. Separately pool load zone fractions (Free RNA) and 1.95 M/1.3 M interface fractions (Membrane-associated RNA) based on the OD 260 nm measurements and isolate total RNA from each with Trizol-LS[®] Reagent. Use 3 parts Trizol-LS[®] to 1 part sucrose solution and perform

- procedure in 50 ml Oak Ridge centrifuge tubes. Follow the protocol supplied by the manufacturer and include the modified isopropanol precipitation for proteoglycan and polysaccharide contamination.
15. Resuspend the final RNA pellet in 400 microl of 10 milliM Tris-HCl, pH 7.4 and perform an additional ethanol precipitation by adding 60 microl of 2 M Sodium Acetate and 1 ml of 95% ethanol to the RNA in a microcentrifuge tube. Perform one wash using 500 microl of 70 % ethanol and resuspend pellet in 40 to 200 microl of water. Yields of RNA in the two fractions can vary significantly depending on the cell line or tissue in question. The membrane-associated RNA yield can range from 10 to a few hundred microg while the free RNA yield is generally 10 to 150 times larger. RNA can be stored at -80°C for up to at least one year.
 16. Perform array hybridization as described in Chapter ?. If you are using a DNA microarray, label the membrane-associated RNA with Cy5 and the free RNA with Cy3 and hybridize them to the same array. In general, a hybridization using 30 to 50 microg of total RNA as input will work well on a DNA microarray. If less than this amount is recovered from the gradient in the membrane fraction, it is desirable to amplify the two fractions using an in vitro transcription based amplification strategy (Chapter ?; Wang et al. 2000).

Post Hybridization Analysis

While these experiments can be analyzed using standard supervised and unsupervised methods, these data also lend themselves to specialized analyses that allow the generation of "confidence estimates" for the likelihood that a given gene encodes a membrane-associated or free polypeptide (Diehn et al. 2000.) The first step in such analyses is the identification of genes included on the array that encode proteins that have empirically documented subcellular localization data. Depending on the organism in question, there are various publicly accessible databases that curate and provide these data. Among these are SWISS-PROT (<http://www.expasy.ch/sprot/>), the Proteome databases (<http://www.proteome.com/databases/index.html>), and resources that synthesize data from multiple sources such as NCBI's LocusLink (<http://www.ncbi.nlm.nih.gov/LocusLink/>) and Stanford's SOURCE (<http://genome-www.stanford.edu/source/>). Using one or a combination of these databases, it is straightforward to generate a list of elements on the array that encode known membrane-associated (i.e. secreted, plasma membrane, nuclear membrane, vesicular, Golgi, or ER-associated) or free (cytoplasmic or nuclear) polypeptides.

The array elements with known subcellular localization can then be used to generate a calibration curve relating Cy5/Cy3 ratio to the fraction of membrane-associated genes at that region of ratio space (Figure 3A.) First, an appropriate window size (N) for the average calculation needs to be chosen. A window size of 75-200 elements works well, but it may need to be smaller if only a small number of control elements were included on the microarray. Next, the fraction of membrane-associated proteins for N adjacent genes in Cy5/Cy3 ratio space is computed and plotted as a function of the central gene in the window. The N gene window is then moved by one gene on the Cy5/Cy3 axis and the fraction is re-calculated. This process is reiterated until the end of the Cy5/Cy3

distribution is reached. The resulting distribution should look roughly like the example show in Figure 3B. By fitting a low degree polynomial function to the distribution, an equation can be generated which allows direct conversion of Cy5/Cy3 ratios to the fraction of membrane-association proteins and hence an estimation of the likelihood that an unknown gene with this Cy5/Cy3 ratio encodes a membrane-associated protein.

In addition to analyzing single experiments in this fashion, data from multiple experiments can further refine the presumptive subcellular localization of the protein products of mRNAs. For example, after analyzing membrane-associated mRNAs for multiple conditions or in different types of tissues, a list of potential membrane-associated proteins can be generated by including all genes which were present in the greater than 90% membrane-associated region on at least one array or in the greater than 80% region on at least two arrays. The exact place at which to draw the cutoff should be tailored to the specific goals of the application in question.

FIGURE LEGENDS

Figure 1. Schematic of sucrose gradient construction and RNA isolation.

Figure 2. OD 260 nm profile of sucrose gradient fractions. In this example, MOLT 4 T cells were fractionated as described. The curve shows the OD 260 nm measurements for each of the 1.5 ml fractions that were isolated using the puncture method, numbered in ascending order from the bottom of the tube to the top. Note the two peaks separated by a large region of low OD 260 nm fractions. The right peak corresponds to the membrane-fractions while the left peak makes up the free fractions. In this case fractions 1 through 10 were pooled as the free fraction and 17 through 22 were pooled as the membrane fraction.

Figure 3. Generation of calibration curves for relating fluorescence ratio to the fraction of mRNAs encoding membrane-associated proteins. (A) Schematic of moving average algorithm for a hypothetical experiment containing 10 elements representing genes encoding protein of known subcellular localization. The first two iterations of the moving average calculation are shown. (B) Actual calibration curve from an experiment using MOLT 4 T cells. The microarrays used contained over 20,000 elements, of which approximately 1000 had annotated subcellular localization data in Swiss-Prot. The window size was 150 elements. The horizontal red line represents the overall fraction of mRNAs encoding membrane-associated proteins in the set of genes of known subcellular localization.

REFERENCES

- Diehn M, Eisen M.B., Botstein D, and Brown P.O. 2000. Large-scale identification of secreted and membrane-associated gene products using DNA microarrays. *Nat Genet* **25**: 58-62.
- Mechler B.M. 1987. Isolation of messenger RNA from membrane-bound polysomes. *Methods in Enzymology* **152**: 241-248.
- Stoltenburg R., Wartmann T., Kunze I., and Kunze G. Reliable method to prepare RNA from free and membrane-bound polysomes from different yeast species. *Biotechniques* **18**: 564-568.

Wang E., Miller L.D., Ohnmacht G.A., Liu E.T., and Marincola F.M. High-fidelity mRNA amplification for gene profiling. *Nat. Biotechnol.* **18**: 457-459.