Chapter 11

Metabolic Labeling in the Study of Mammalian Ribosomal RNA Synthesis

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Abstract

RNA metabolic labeling is a method of choice in the study of dynamic changes in the rate of gene transcription and RNA processing. It is particularly applicable to transcription of the ribosomal RNA genes and their processing products due to the very high levels of ribosomal RNA synthesis. Metabolic labeling can detect changes in ribosomal RNA transcription that occur within a few minutes as opposed to the still widely used RT-PCR or Northern blot procedures that measure RNA pool sizes and at best are able to detect changes occurring over several hours or several days. Here, we describe a metabolic labeling technique applicable to the measurement of ribosomal RNA synthesis and processing rates, as well as to the determination of RNA Polymerase I transcription elongation rates.

Key words Ribosomal RNA (rRNA), Precursor rRNA, rRNA synthesis rate, rRNA processing rate, RNA polymerase I (RPI or PolI), Transcription elongation, Metabolic labeling, Electrophoresis, Vacuum transfer of RNA, Fluorography, Scintillation counting, Phospho-imaging

1 Introduction

The four ribosomal RNAs (18S, 28S, 5.8S, and 5S) form the catalytic core of the ribosome, and as such, they account for 80% or more of the total RNA in mammalian cells. Their synthesis typically occupies some 30% of the total gene transcription in a proliferating cell and is regulated in a complex manner that is linked to cell growth and proliferation [1]. For many years, ribosomal RNA (rRNA) synthesis was considered as a slowly regulated housekeeping process, but metabolic RNA labeling revealed that in fact it responds within minutes to growth stimuli [2], and is directly regulated by mitogens and growth factors at the levels of transcription elongation and initiation competence [3, 4]. When applied to mammalian cells, methods such as Northern blot or RT-PCR analysis can generally not detect such rapid changes in synthesis rate due to the great abundance of cellular rRNA and the relatively slow processing rates. Quantitative RT-PCR, S1-protection, or Northern monitoring of the extreme 5′ of the 47S

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rRNA precursor has been widely used to measure rRNA synthesis, since this region of the rRNA is generally processed relatively rapidly, e.g. [4, 5]. However, the rRNA processing rate can vary widely and, more importantly, independently of 47S synthesis [6], making the interpretation of data from these techniques at best questionable and at worst fully misleading. Further, metabolic labeling can provide a direct measure of rRNA processing rates and pathways not available by other means.

Cellular synthesis of rRNA is often analyzed by culturing mammalian cells with a radiolabeled nucleoside such as [^{3}H] or [^{14}C]-uridine, or with [^{32}P] inorganic phosphate, and all have been extensively used for this purpose [2, 3, 7–9]. Given that the rRNA accounts for such a large fraction of total cellular transcription and this transcription is concentrated in a single precursor RNA and a small number of processing products, the newly synthesized rRNAs can be very simply analyzed by gel electrophoresis, e.g., Fig. 1a. Cells are usually labeled for periods from a few minutes to several hours dependent on the parameters of the experiment, and this will define the choice of radiolabel, [^{32}P] inorganic phosphate or [^{3}H]-uridine providing much greater sensitivity than [^{14}C]-uridine due to isotope half-life and disintegration energy. In the example in Fig. 1, labeling of mouse embryonic fibroblasts (MEFs) was performed for 3 h with [^{3}H]-uridine, and EtBr staining used in parallel to observe the steady-state rRNA pools. In this case, MEFs conditional for the gene for the essential factor UBF (UBTF) [10], which can be deleted using 4-hydroxy-tamoxifen (4HT), were labeled before and after gene deletion. In such a 3 h "pulse" labeling, the 47S precursor rRNA and its intermediate and final processing products are all visualized, and all are shown to be products of the rRNA genes since their labeling depends absolutely on the availability of UBF. So-called pulse-chase experiments can be used to follow the rRNA processing pathways and to determine the rates of processing [6, 9, 11], e.g., Fig. 2a. Since processing involves the 2′-O-methylation of the nascent 47S rRNA, it can also be analyzed using [^{3}H]- or [^{14}C]-methylmethionine as the methyl donor [6, 12–14]. Since, during pulse labeling times much shorter than the rRNA processing rates, the label accumulates almost exclusively in the 47S rRNA precursor, such labeling regimes can be used to follow the absolute level of rRNA synthesis, e.g., Fig. 3a. This approach has also been used to directly measure the time taken to complete transcription of a 47S rRNA precursor and hence to determine the average transcript elongation rate achieved by RNA Polymerase I (RPI or PolI) [3], e.g., Fig. 3b. Cells take up the radiolabel very rapidly such that any delay in incorporation into RNA can usually be neglected. However, the uptake rate can be readily determined using standard procedures, e.g. [3] or by measuring the incorporation rate into abundant short RNAs such as the 5S rRNA, e.g. [15].

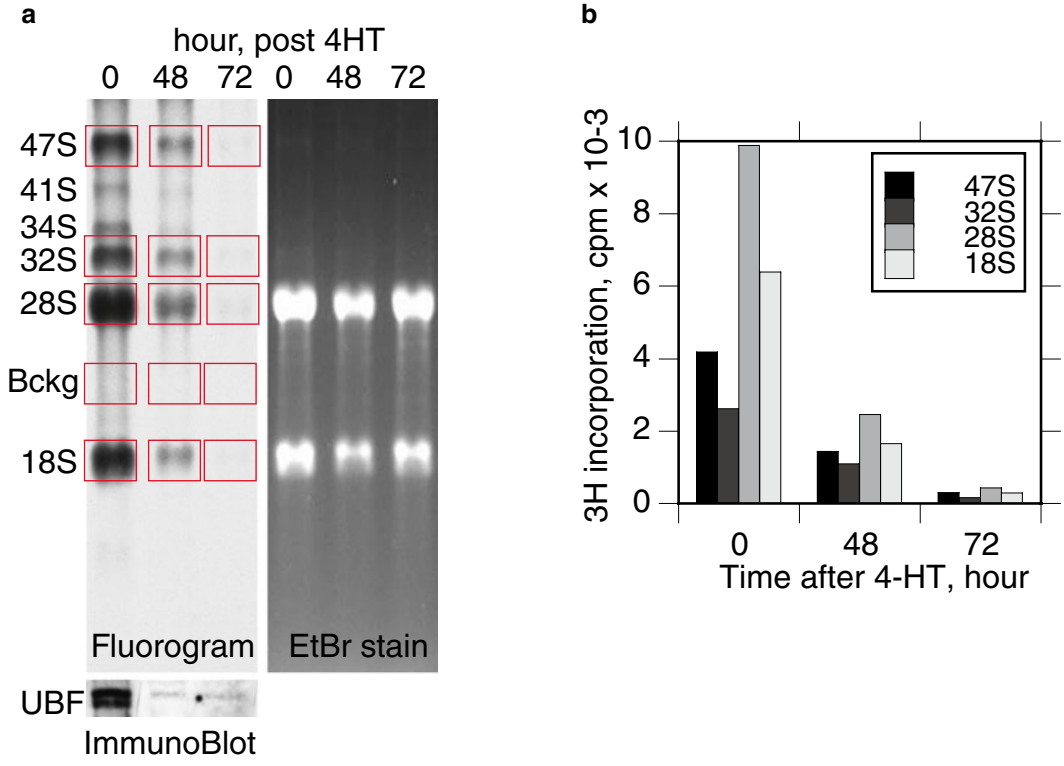

Fig. 1 Example of the pulse labeling of rRNA in mouse embryonic fibroblasts carrying homozygous conditional (Floxed) genes for the RPI factor UBF. (**a**) Electrophoretic analysis of a 3 h [^{3}H]-uridine labeling before and at 48 and 72 h after inducing the deletion of the UBF genes with 4-hydroxy-tamoxifen (4HT). *Left panel* shows the fluorograph and *right panel* the corresponding ethidium bromide stained gel. *Boxes* outlined in *red* indicate the areas cut from the fluorograph for scintillation counting. Immunoblot shows the corresponding levels of UBF. (**b**) Example of the raw counts per min (cpm) quantification data as determined by scintillation counting of the fluorograph in (**a**)

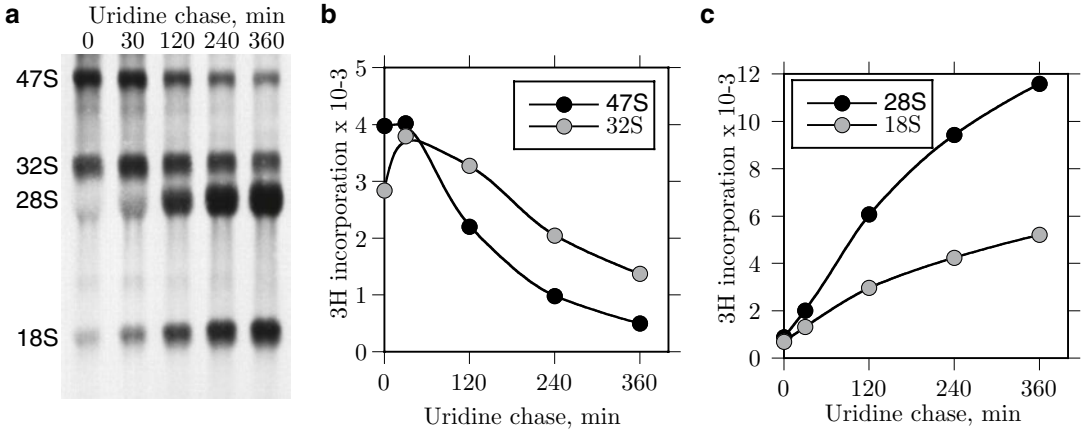

Fig. 2 Example of a pulse-chase experiment. Cells were labeled with [^{3}H]-uridine for 1 h before chasing with unlabeled uridine. (**a**) Fluorograph of RNA samples taken at 0–360 min after start of chase. (**b**) and (**c**) show the raw quantitation data for the 47, 32, 28, and 18S rRNAs. Note the 32S initially accumulates before decaying while the 28 and 18S rRNAs continue to accumulate throughout

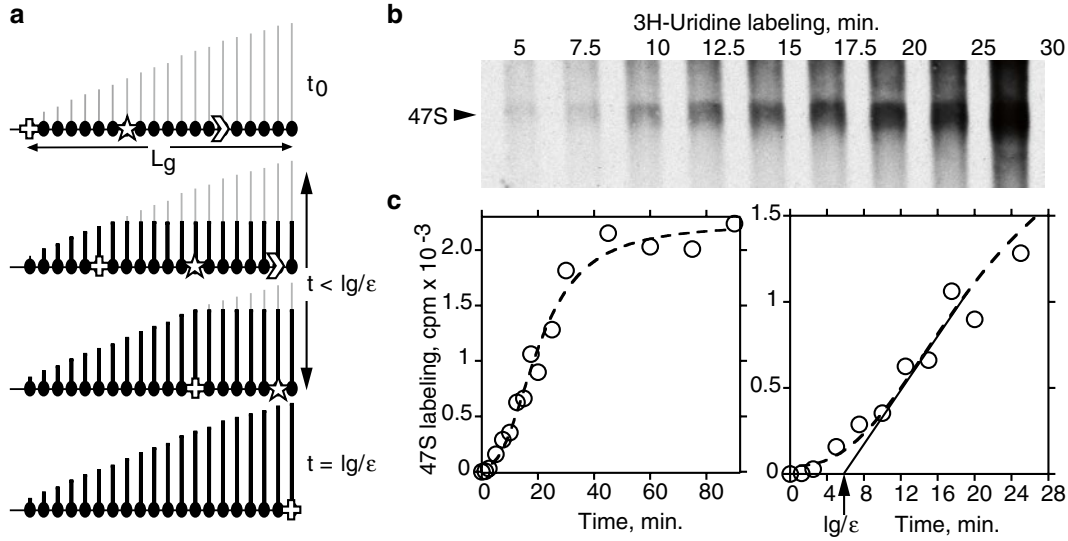

Fig. 3 Example of a series of short labeling pulses to analyze RPI elongation rate. (**a**) Diagrammatic demonstration of label incorporation at times shorter than the time required for RPI to complete a full transcript. Symbols indicate the advance of the transcription complexes. (**b**) A typical fluorograph of 47S rRNA labeling time course. (**c**) *Left*, plot of raw incorporation data from 0 to 90 min of pulse labeling, showing that 47S processing becomes apparent after about 30 min. *Right*, expanded plot of the early time points showing the transition from the nonlinear to linear labeling kinetics, and the extrapolation of the linear domain to determine elongation time. *See* Subheading 3.8 for more details

Here, we describe the procedures to metabolic label and analyze rRNA from mammalian tissues culture, using the NIH3T3 monolayer cells as typical example. The basic procedure uses [^{3}H]-uridine labeling, followed by isolation of total RNA, its fractionation by denaturing formaldehyde agarose gel electrophoresis [16–18], imaging by fluorography, and quantitation by scintillation counting or phosphoimaging. The procedure can be adapted to the use of [^{14}C]-uridine and [^{3}H]- or [^{14}C]-methylmethionine.

2 Materials

Prepare all solutions using single distilled or Milli-Q™ equivalent water and analytical grade reagents. All solutions should be RNase free. Where appropriate, stock solutions and water should be autoclaved for 45 min at 120 °C. Other reagents should be taken fresh from a new bottle and transferred, preferably by pouring not pipetting, into an RNase free, autoclaved, tube. Follow all local waste disposal regulations for radioactivity and toxic compounds.

2.1 Materials for Metabolic Labeling

1. DMEM or other appropriate medium for cell culture. When labeling with [^{3}H]-methylmethionine, use medium without methionine and rinse cells in this medium before beginning the labeling.

2. [5,6-^{3}H|-uridine, [^{14}C|-uridine, or [^{3}H]-methyl-methionine (PerkinElmer, Waltham, Massachusetts, United States).
3. For pulse-chase experiments, prepare a solution of 1 mM uridine or 1 mM methionine (Sigma-Aldrich, St. Louis, Missouri, United States).

2.2 Materials for RNA Extraction

1. Trizol™ Reagent (Life Technologies, Carlsbad, California, United States) or QIAzol Lysis Reagent (QIAGEN, Hilden, Germany). Or alternatively, follow the original guanidinium thiocyanate–phenol–chloroform extraction procedure [19].
2. Chloroform:isoamyl alcohol 24:1 v/v.
3. Isopropanol.
4. 70% v/v Ethanol in water, keep at −20 °C.
5. Formamide (Sigma-Aldrich, St. Louis, Missouri, United States).

2.3 Materials for Formaldehyde Gel Electro-phoresis

1. Horizon 11.14 (11 × 14 cm) horizontal agarose gel electrophoresis chamber (Apogee Designs, Ltd. Baltimore, MD United States) or similar apparatus.
2. Gel comb to form loading slots for up to 40 µL samples, for example, 0.5 cm wide × 0.2 cm thick.
3. RNaseZap® (Ambion Thermo Fisher Scientific) or the cheaper Windex® (SC Johnson, United States).
4. Agarose (e.g., UltraPure™ Agarose, Life Technologies, Carlsbad, California, United States) (*see* **Note 1**).
5. Formaldehyde, 37% w/w (Sigma-Aldrich, St. Louis, Missouri, United States).
6. Ethidium bromide (Sigma-Aldrich, St. Louis, Missouri, United States), 10 mg/mL stock solution.
7. 10× MOPS solution: 0.2 M MOPS (3-*N*-morpholino propanesulfonic acid) (Sigma-Aldrich, St. Louis, Missouri, United States), 0.05 M sodium acetate, 0.01 M Na_2EDTA. Adjust pH to 7.2 with 10 N NaOH at 20 °C.
8. 1× electrophoresis buffer: 1× MOPS.
9. 10× RNA loading buffer without dyes: 50% v/v glycerol, 10 mM EDTA pH 8.0.
10. 1× RNA loading buffer for denaturing formaldehyde gels. Prepare sufficient volume for all samples immediately before use. Each sample requires: 18 µL formamide, 6 µL 37% w/w formaldehyde, 1 µL 1.6 mg/mL ethidium bromide, 3.6 µL 10× MOPS, 4 µL 10× RNA loading buffer without dye (*see* **Note 2**).

2.4 Materials for Fluorescent Imaging of RNA Fractionation

1. 250 or 300 nm UV transilluminator and gel documentation camera suitable for ethidium bromide fluorescence.
2. If ethidium bromide is not included in the RNA loading buffer, a suitable container in which to stain the gel is necessary.

2.5 Materials for the RNA Transfer

1. Biodyne membrane (Pall Corporation, Port Washington, NY, USA).
2. 20× SSC (Standard Saline Citrate): 175 g/l NaCl, 88 g/l sodium citrate.
3. Whatman® 3MM paper.
4. UV cross-linker: Hoefer UVC 500 or similar.

2.6 Materials for Fluorography

1. EN^{3}HANCE™ Spray Surface Autoradiography Enhancer (PerkinElmer, Waltham, Massachusetts, United States). Use only in a chemical hood (*see* **Note 3**).
2. Radioactive ink: Dilute any nontoxic [14C]-radiolabeled molecule, e.g., [14C]-uridine in commercial black writing ink to give 2–3 μCi/mL final concentration (*see* **Note 4**).
3. X-Ray Film Cassette with two sheets of Whatman 3MM paper for protection. The use of EN^{3}HANCE™ Spray will contaminate the cassette, so it is best to dedicate one to this procedure.
4. Biomax XAR film (Carestream Health, Inc., Rochester, NY, United States).
5. Biofreezer for autoradiography exposures, −70 °C or lower.

2.7 Materials for Quantitative Analysis

1. Light-box, a visible light transilluminator.
2. Saran Premium Wrap (SC Johnson, United States) (*see* **Note 5**).
3. Ballpoint pen.
4. Scissors.
5. Glass scintillation vials with caps.
6. Scintillation liquid, e.g., Scintiverse (Thermo Fisher Scientific, Waltham, MA, United States) or similar.
7. Liquid scintillation counter.
8. Computer with ImageJ (National Institutes of Health, United States) and Excel (Microsoft, Redmond, Washington, United States) or similar image analysis and graphing software.

3 Methods

3.1 Pulse Labeling

1. Plating of monolayer cells. Typically, 0.8×10^6 NIH3T3 were plated in 60 mm petris the day before labeling. For other cell lines the cell number may need to be varied according to the cell growth and cell size. Cells should be not more than 60–80% confluent at the time of labeling since most cells will down-regulate ribosomal transcription at higher confluence. At this stage it is important to decide if it is intended to accurately quantify labeling. If this is the case, cells should be plated in triplicate.

2. Prepare a 10× solution of radiolabel in tissue culture medium to give 10 μCi [^{3}H]-uridine per 100 μL medium, keeping in mind the total number of petris to be labeled including triplicates. (*See* **Note 6** for other radiolabels and very short pulse labeling.)
3. Adjust the medium volume in each culture to exactly 2 mL (*see* **Note 7**).
4. Add 100 μL of the 10× radiolabel solution to each culture mixing well but with care.
5. Time of labeling will vary depending on the experiment.

3.2 Pulse-Chase Labeling

1. Follow **steps 1–4** of Subheading 3.1.
2. After labeling for 0.5–1 h, change the medium for fresh medium containing 0.5 mM unlabeled uridine and continue culturing for 1–6 h. (For [^{3}H]-methylmethionine labeling *see* **Note 6**.)

3.3 RNA Extraction

1. Remove culture medium containing the radiolabel. (Store and dispose of contaminated solutions and plastics according to local regulations.)
2. Lyse the cells directly with 1 mL of Trizol™ or equivalent. Homogenize and reduce viscosity by pipetting "up and down." Transfer into 2 mL microcentrifuge tube.
3. Add 200 μL chloroform, close tube, and mix thoroughly by manual shaking, not vortexing, for about 15 s.
4. Centrifuge at 10,000 × *g* for 15 min at 4 °C.
5. Remove 600 μL of the upper aqueous phase, avoiding the interphase, and transfer into 1.5 mL microcentrifuge tube.
6. Add 600 μL of isopropanol and mix well by inversion. Leave 15 min at room temperature.
7. Centrifuge at 10,000 × *g* for 10 min at 4 °C.
8. Remove supernatant as completely as possible (*see* **Note 8**).
9. Add 1 mL of 70 % v/v ethanol and centrifuge at 10,000 × *g* for 5 min at 4 °C.
10. Completely remove supernatant and dissolve the RNA pellet in 40–60 μL of formamide. Leave for 30 min at room temperature to allow RNA to dissolve and finally vortex to ensure solution is homogeneous. If necessary, the solution can be heated to 50 °C for 3 min to help dissolution.
11. To determine the final concentration of the RNA sample, dilute a few microliters of the solution around 40 times in water and determine the optical absorbance at 260 nm in a spectrophotometer using a 1 cm quartz microcuvette or with a NanoDrop® or similar apparatus. $1\,AU_{260\,nm}{}^{1\,cm} = 40$ μg/mL.

3.4 Denaturing Agarose Gel Electrophoresis

1. The electrophoresis chamber should be clean, but there is no need for it to be RNase-free. The gel tray and comb should be clean and free from dried agarose residue. It should be sprayed with RNaseZap® (Ambion Thermo Fisher Scientific), or the cheaper Windex® (SC Johnson, United States), then rinsed well with RNase-free water (*see* **Note 9**).
2. Place the electrophoresis apparatus in a chemical hood.
3. A 1% w/v agarose gel is ideal to fractionate the 47S to 18S ribosomal RNAs. For an 11 × 14 cm tray, weigh 1.2 g of agarose into an Erlenmeyer flask and add 85 mL of water. Boil until the agarose is fully dissolved, then quickly cool it to about 65 °C.

   The following steps should be performed in a chemical hood (*see* **Note 10**).
4. Add 35 mL of a freshly prepared mix of 37% w/w formaldehyde (23 mL) and 10× MOPS buffer (12 mL). Mix rapidly then pour into gel tray. Leave to polymerize for 1 h before submersing in 1× MOPS electrophoresis buffer.
5. Add 36 μL of RNA loading buffer to 1–10 μL of each RNA sample (2–4 μg) and heat for 3 min at 65 °C. Load samples immediately into gel pockets.
6. Electrophorese samples at 2 V/cm for 16–18 h.

3.5 Fluorescent Imaging of Gel and Transfer of RNA to Membrane

1. If ethidium bromide was included in the RNA loading buffer, the RNA fractionation can be imaged immediately following electrophoresis using the UV transilluminator and gel documentation system, e.g., *see* Fig 1a. If not, the gel must first be stained in electrophoresis buffer plus 0.5 μg/mL ethidium bromide. Take care to obtain a series of unsaturated exposures, as these will be used to correct the labeling data for any small variability in RNA loadings.
2. Cut a suitable sized sheet of Biodyne B membrane and soak in 2× SSC.
3. RNA is transferred to the membrane most effectively using a vacuum blotting system. We use a Biometra Vacu-Blot (Biometra GmbH, Göttingen, Germany) and perform transfer using 2× SSC for 1.5 h at a pressure of 200 mbar/cm^2.
4. If necessary, the RNA can be cross-linked to the membrane by UV irradiation at 70 J/cm^2.

For fluorographic imaging continue with Subheading 3.6, for phosphor-imaging skip Subheading 3.6 and go directly to Subheading 3.7.

3.6 Fluorographic Imaging and Quantitation by Scintillation Counting

The treatment of membrane with EN^{3}HANCE™, its subsequent manipulation, and the handling of scintillation liquid should all be performed in a chemical hood (*see* **Note 11**).

1. Label the corners of the membrane with a coded series of radioactive ink dots. Allow to dry thoroughly in a chemical hood on paper towels.
2. Still working in a chemical hood, spray the membrane with EN^{3}HANCE™ from a distance of 20–30 cm, moving evenly from aside. This treatment can be repeated a second time but should not be excessive. Allow membrane to dry in chemical hood for 10–15 min.
3. Place membrane in radiography cassette between Whatman 3MM paper. Place a sheet of Biomax XAR film directly in contact with the membrane. Do not interpose a layer of plastic film as this will negatively affect exposure. Close cassette and expose at −70 °C for 1 to several days depending on the expected signal. Typical fluorographic autoradiographs of pulse labeling experiments are shown in Figs. 1a and 3b and of a pulse-chase experiment in Fig. 2a.
4. After satisfactory fluorographic exposures have been obtained, place an appropriate fluorograph on a light-box, cover it with transparent Saran Wrap and place the membrane on top so that radioactive dots align. Hold membrane firmly in place and with a ballpoint pen mark each RNA band and one or more suitable unlabeled areas for estimates of background levels, *see* Fig. 1a. It is advisable not to use a ruler as it may stick to the membrane.
5. Remove membrane and, using scissors, cut out each RNA band and background area. Place each membrane fragment into a glass scintillation vial and add 10 mL of scintillation liquid. Count each vial in a liquid scintillation counter using an energy window suitable for tritium. To ensure an accurate count, inverse the membrane fragment in each vial and recount. If necessary, repeat this process to avoid counting errors (*see* **Note 12**).
6. To correct for any small variability in RNA loading, the digital ethidium bromide staining images from Subheading 3.5, **step 1** are used. The integrated 28S or 18S rRNA band densities determined from one or several unsaturated images should be equal across the gel if there is no error in gel loading. Small relative variations in 28 or 18S levels can be used to correct the radiolabel counts for each gel track.
7. Enter triplicate counts for each RNA species and track into an Excel or similar worksheet. Correct counts from each track for RNA loading variations and establish mean and standard error of

triplicates, e.g., *see* Fig. 1b. Normalization to one condition allows easy comparison of RNA synthesis and/or processing rates under various cell cultures and labeling regimes, e.g., *see* Fig. 1b.

3.7 Phosphor-Imaging

It is possible to replace the fluorography and quantitation procedures by direct exposure of the transfer membrane to storage phosphor screens, assuming suitable equipment for their scanning is available. The approach requires the use of special tritium-sensitive phosphor screens that lack a protective layer that would otherwise greatly reduce sensitivity of the approach. One disadvantage is that these screens are expensive and easily contaminated. To the best of our knowledge, a direct comparison of the detection sensitivities of fluorography and phosphor-imaging for tritium has not been published, but phophor-imaging has been applied to follow rRNA processing in yeast [15].

3.8 Determination of Transcription Elongation Rates

Direct determination of RPI transcription elongation rates has been successfully achieved in both growth-stimulated and growth-inhibited NIH3T3 cells using a series of labeling pulses of increasing length. These data revealed that the transcription elongation rate of RPI is growth regulated [3]. During radiolabeling, initially existing nascent transcripts will incorporate radiolabel such that only a short 3′-terminal segment of newly completed 47S rRNAs will be labeled, Fig. 3a. But as labeling continues the labeled segment of each completed transcript will increase in length, until by the time taken for a polymerase to traverse the whole 47S rRNA region, label will be incorporated throughout. Subsequently, all transcripts will be fully labeled and radiolabel in the 47S rRNA fraction will accumulate linearly until processing becomes significant. If we assume the cellular uptake of radiolabel is rapid compared with its incorporation into rRNA and processing is negligible during the time taken to synthesize the full-length precursor transcript, then the incorporation I of radiolabel into full length 47S rRNAs is described by;

$$I \propto \int_{t=0}^{t=l_g/\epsilon} n_g \rho_p \varepsilon t dt$$

Thus;

$$I \propto \left[n_g \rho_p \varepsilon t^2 \right]_{t=0}^{t<l_g/\epsilon}$$

Where;

I = incorporation of radiolabel into 47S rRNA,
l_g = gene length in base pairs,
t = time of labeling, minutes,
n_g = number of active genes,
ρ_p = density of polymerases, molecules/bp, and
ε = transcription elongation rate, nucleotides/min.

Simply put, I will vary as t^2 until the first full-length transcript is completed from initiation to termination, and subsequently I will vary linearly with t. By measuring radiolabel incorporation into 47S rRNA at increasing times (Fig. 3b), it is then possible to determine the mean time taken by RPI to synthesize a complete transcript. This elongation time l_g/ε can be read off from the plot as shown in Fig. 3c, and the elongation rate ε calculated from this and from the known length of the gene l_g.

4 Notes

1. Many sources of agarose have been tried and most found to function well.
2. The inclusion of ethidium bromide in the loading buffer is optional, but aids loading of gel and avoids the need for staining after electrophoresis. The ethidium bromide is cross-linked to the RNA during sample heating. Ethidium bromide should not be included in the electrophoresis buffer.
3. Early protocols used 2,5-Diphenyloxazole (PPO) or liquid EN^{3}HANCE™ as scintillant for fluorography [20]. In this case, the gel is not transferred to a membrane but treated directly and then vacuum dried.
4. An autoradiography phosphorescent marker pen can be used in place of the ^{14}C-ink.
5. Other makes of plastic wrap can be used but may be partially permeable to EN^{3}HANCE™.
6. Using the present protocol, after 30 min labeling only the 47S will be detected. To detect ribosomal RNA processing products, a pulse labeling of 2–3 h will be required. When using pulse labeling times of less than 30 min cells should be labeled by the addition of 50 μCi [^{3}H]-uridine in 100 μl medium. For [^{3}H]-methylmethionine labeling cells are washed twice with methionine-free medium and finally 2 mL of methionine-free medium containing 10 μCi of radiolabel is added. Typically cells are labeled for 1 h and either RNAs analyzed directly or after chasing for 1–6 h in fresh medium containing 0.5 mM methionine.
7. If a quantitative measurement of ribosomal RNA synthesis is required, it is preferable not to change the culture medium or to add fresh medium before labeling as this can significantly affect synthesis and hence radiolabel incorporation.
8. Leaving traces of the supernatant can cause migration problems during electrophoresis due to the carryover of guanidinium.
9. RNaseZap® or Windex® solution is strongly alkaline and will affect electrophoresis if not removed thoroughly.

10. Formaldehyde vapor is highly toxic and is especially strongly released from the hot agarose solution.
11. Due to the volatile and toxic solutions, treatment of membranes or gels with EN^{3}HANCE™ or with 2,5-Diphenyloxazole (PPO), their subsequent handling and preparation for scintillation counting should be performed in a chemical hood.
12. The membrane fragment will not be completely transparent and may in some cases affect the efficiency of counting. The potential error cannot be corrected using either external or internal standards, hence it is important to ensure the counts are fully reproducible.

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