

Approaches for Monitoring Nuclear Translation

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Summary

The nuclear membrane is the defining feature of eukaryotes. It divides the cell into two functionally specialized compartments, and it is widely assumed that translation is restricted to only one: the cytoplasm. However, recent results suggest that some translation takes place in nuclei closely coupled to transcription. Various labeling techniques are described that enable nascent peptides to be labeled and then localized wherever they might be in the cell.

Key Words

Autoradiography; biotin; biotin-lys-tRNA; BODIPY-lys-tRNA; immunofluorescence; immunogold labeling; saponin; transcription; translation.

1. Introduction

The nuclear membrane is the defining feature of eukaryotes. It divides the cell into two functionally specialized compartments, and it has been widely assumed that translation is restricted to only one: the cytoplasm. However, recent results suggest that some translation takes place in nuclei (*1,2*). The evidence is of various types. First, the components required for translation can be found in nuclei. Second, although most nascent peptides are found in the cytoplasm, some can be found in nuclei. Different approaches were used to label and then localize the nascent peptides including incubating living cells with radiolabeled amino acids and then autoradiography, or incubating permeabilized cells (or nuclei) with biotin-lysine-tRNA (or BODIPY-lysine-tRNA) before immunolocalization using fluors and light microscopy (or gold particles and electron microscopy). Third, the production of the nascent peptides in the nucleus is closely coupled to transcription because inhibiting the nuclear transcription immediately inhibits the production of the nascent nuclear peptides. Fourth, the nascent nuclear peptides (and various components of the

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translation machinery) are found within a few nanometers of nascent nuclear RNA. Here, we describe various labeling techniques that enable nascent peptides to be labeled and then localized, whether they are in the nucleus or cytoplasm.

2. Materials

2.1. Growth Media

1. Dulbecco's modified Eagle medium (DMEM; Invitrogen, cat. no. 41966-029).
2. DMEM without leucine and MEM without leucine (these are no longer available; alternatives can be made by adding the appropriate supplements to DME/F12HAM without L-leucine, L-lysine, L-methionine, calcium chloride, magnesium sulfate, magnesium chloride from Sigma, cat. no. D9785).

2.2. Detergents Used for Permeabilization

1. Digitonin (Sigma, cat. no. D 1407).
2. Lysolecithin (Sigma, cat. no. L 4129).
3. Saponin (Sigma, cat. no. S-7900 or S-4521).
4. Triton X-100 (Pierce, cat. no. 28314).

2.3. Specialized Chemicals

1. Adenosine triphosphate (ATP), CTP, guanosine triphosphate (GTP), UTP (from Ultrapure NTP set Amersham International, cat. no. 27-2025-01) used for transcription reactions.
2. Biotin-lysine-tRNA from brewer's yeast (Roche, cat. no. 1 559 478).
3. BODIPY-lysine-tRNA (FluoroTect™ Green_{Lys}; Promega, cat. no. L5001).
4. Dextran (40, 70, 500 kDa) conjugated with FITC (Sigma, cat. no. FD-40S, FD-70S, FD-500S).
5. Diethylpyrocarbonate (Sigma, cat. no. D5758).
6. Fluorescein-12-ATP (New England Nuclear, cat. no. NEL439).
7. Gelatin (from cold water fish skin; Sigma, cat. no. G-7765).
8. Human placental ribonuclease inhibitor (Amersham International, cat. no. 799 025).
9. LR White (Agar Scientific, cat. no. 14380).
10. Paraformaldehyde (Electron Microscopy Sciences, cat. no. 15710-S).
11. Phenylmethanesulphonyl fluoride (Sigma, cat. no. P7626).
12. Phosphocreatine disodium salt (Sigma, cat. no. P7936).
13. Poly-L-lysine (Sigma, cat. no. P4707).
14. SYTO-16 (Molecular Probes, cat. no. S-7578).
15. TOTO-3 (Molecular Probes, cat. no. T-3604).
16. tRNA (Sigma; Type XI: from bovine liver, cat. no. R-4752).
17. Uranyl acetate (Agar Scientific, cat. no. R1260A).

2.4. Radiolabels and Amino Acid Mixtures

1. L-[4,5-³H]lysine (86 Ci/mmol; Amersham International, cat. no. TRK520).
2. Amino acids minus lysine (Roche, cat. no. 1 559 478).
3. L-[4,5-³H]leucine (147 Ci/mmol; Amersham International, cat. no. TRK510).

4. Amino acids minus leucine (available in the rabbit reticulocyte lysate system from Amersham International, cat. no. RPW3150).
5. L-[³⁵S]methionine (0.5 Ci/mmol; Amersham International, cat. no. SJ123).
6. Amino acids minus methionine (available in the rabbit reticulocyte lysate system from Amersham International, cat. no. RPW3150).

2.5. Enzymes, Proteins, and Inhibitors

1. Aminoacyl-tRNA synthetase (Sigma; from bakers yeast, cat. no. A-6302).
2. Bovine serum albumin (BSA), essentially free of fatty acids and γ -globulin (Sigma, cat. no. A-7030).
3. Creatine phosphokinase (Sigma, cat. no. C-3755).
4. DNase, RNase-free (Boehringer, cat. no. 1119915).
5. Human placental ribonuclease inhibitor (10 U/mL; Amersham International, cat. no. 27-0815-01).
6. Protease inhibitors cocktail for mammalian cells (Sigma, cat. no. P-8340).
7. Puromycin (Sigma, cat. no. P-7255).

2.6. Antibodies

1. Donkey anti-mouse IgG conjugated with Cy3 (Affinipure grade, Jackson ImmunoResearch, cat. no. 115-165-071).
2. Goat anti-mouse IgG conjugated with 5- or 10-nm gold-particles (British BioCell International, cat. nos. EM.GAM5 and EM.GAM10).
3. Mouse anti-biotin (Jackson ImmunoResearch, cat. no. 200-002-096).

2.7. Sundries

1. Coverslips, no. 1.5, glass, 16-mm diameter (Agar Scientific, cat. no. L4098-2).
2. Emulsion (dipping) film (Ilford K.5 supplied by Agar Scientific, cat. no. P9281).
3. Filter paper, Whatman no. 1 (Whatman International Ltd, cat. no. 1001 090).
4. Glass fiber discs (GF/C; Whatman International Ltd, cat. no. 1822 025).

2.8. Buffers

All buffers used up to fixation were ice cold unless stated otherwise. Where analysis of RNA is critical, RNase-free distilled H₂O should be used. This can be prepared by treated with diethylpyrocarbonate; add 0.5 mL of diethylpyrocarbonate to 500 mL of distilled H₂O, mix, stand at 37°C overnight, and autoclave.

1. Physiological buffer (PB): PB is 100 mM potassium acetate, 30 mM KCl, 10 mM Na₂HPO₄, 1 mM MgCl₂, 1 mM Na₂ATP (Sigma Grade I), 1 mM dithiothreitol, and 0.2 mM phenylmethylsulphonylfluoride (pH 7.4). As the acidity of ATP batches varies, 100 mM KH₂PO₄ (usually 1/100 vol) can be added to adjust the pH. When triphosphates were added to PB, extra MgCl₂ was added in an equimolar amount (*see Note 1*).
2. PB*: PB* is PB plus human placental ribonuclease inhibitor (10 U/mL).
3. PB-BSA and PB*-BSA: These contain 100 mg/mL BSA.

4. PBS+: This is PBS plus 1% BSA and 0.2% gelatin; where indicated the pH was adjusted to 8.0.
5. PB-diluted: This is 1 vol of PB mixed with 2 vol of distilled H₂O.

2.9. Translation Mixtures

Different mixtures are used, depending on whether intact or permeabilized cells are to be labeled, which tag is incorporated into the nascent peptides, and on which localization approach is used (as discussed in **Subheading 3.**).

2.9.1. Labeling Intact Cells

Cells are grown in media containing the appropriate radiolabeled amino acid, plus the other unlabeled amino acids (*see Subheading 2.4.*).

2.9.2. Labeling Isolated Nuclei or Permeabilized Cells

The translation mixture should contain the appropriate tagged precursor, the other amino acids, ATP, GTP, and an energy-regenerating system. Our basic translation mixture contains PB*-BSA (which contains ATP; *see steps 1 and 3 in Subheading 2.8.*), creatine phosphokinase (20 U/mL; *see Subheading 2.5.*), 2.5 mM phosphocreatine (*see Subheading 2.3.*), 0.25 mM GTP (*see Subheading 2.3.*), 0.5 mg/mL tRNA (*see Subheading 2.3.*), 200 U/mL aminoacyl-tRNA synthetase (*see Subheading 2.5.*), protease inhibitors for mammalian cells (*see Subheading 2.5.*), and the various supplements indicated. In addition, MgCl₂ is always added to ensure that it is in equimolar amounts to any nucleotide triphosphates present in the reaction. As nuclear translation is closely coupled to transcription, translation is improved by adding the 0.25 mM CTP and UTP required for transcription to this mixture. When synthetases were omitted the biotin incorporation falls by only 22%.

3. Methods

Various related strategies are available for localizing nascent peptides. All rely on tagging nascent peptides with a label, and then measuring the relative concentrations of tagged peptides in the nucleus and cytoplasm. The strategies differ in the type of tag (e.g., radiolabel, biotin, fluor) and localization approach (e.g., biochemical fractionation, autoradiography, immunolabeling coupled with light microscopy, immunogold labeling and electron microscopy). This gives a wide range of possible combinations and therefore only selected examples are given here.

3.1. Growth in a Radiolabeled Amino Acid, Cell Fractionation, and Scintillation Counting

Here, cells are grown in a radiolabeled amino acid and nuclei isolated; then, the amount of radiolabel in the whole cells and the isolated nuclei are compared.

3.1.1. Cell Growth

10^7 HeLa cells were grown in suspension in 50 mL of MEM without leucine for (5 min), pelleted, and regrown in 0.5 mL of the same medium with [^3H]leucine (147 Ci/mmol; 1 mCi/mL); after 10 s, 50 mL of ice-cold PBS was added and the cells pelleted. Essentially, the same procedure is used for attached cells growing in the appropriate medium, but centrifugation is not required.

3.1.2. Isolation of Nuclei

Many approaches are available for isolating nuclei from mammalian cells; almost all use hypotonic (i.e., unphysiological) buffers and must be adapted to the particular cell used. In our study (*I*), we isolated nuclei from HeLa cells as follows:

1. $4\text{--}20 \times 10^7$ cells are washed in PBS, resuspended in 10 mL PB*-diluted, and incubated (2 min; 37°C) to swell them.
2. 40 mL of PB*-diluted is added, and swollen cells are re-incubated (15 min) at 4°C.
3. The cells are broken using 10–15 strokes with a Dounce homogenization to release 95–99% nuclei (assayed by phase-contrast microscopy).
4. Triton X-100 is added to 0.25% (v/v).
5. After 5 min, spin nuclei (250 g; 5 min) through PB* with 10% glycerol, and resuspend pellet gently in PB*-BSA. The resulting nuclei can then be checked for ribosomal contamination (*see Note 2*) and for the integrity of the nuclear membrane (*see Note 3*).

3.2. Isolated Nuclei or Permeabilized Cells, “Run-On” Translation in Radiolabeled Precursors, and Scintillation Counting or Gel Electrophoresis

Here, permeabilized cells or nuclei isolated as in **Subheading 3.1.2.** are incubated in a “translation mixture” containing all the components required for translation (including a radiolabeled precursor), and the amount of radiolabel incorporated into peptides is measured by scintillation counting or autoradiography after gel electrophoresis.

3.2.1. Cell Permeabilization

Once nuclei have been isolated as in **Subheading 3.1.2.**—or cells permeabilized as described next—endogenous precursor pools are depleted by washing, and exogenous precursors must be added back to a concentration that gives the required rate of elongation. In the absence of a natural precursor, a modified precursor that is recognized as a substrate by the endogenous enzymes is then incorporated relatively efficiently. Cells can be permeabilized in many different ways, for example, using proteins (streptolysin O, α -toxin) or detergents (**3**), which include (rough concentrations indicated): Triton X-100 (0.02–0.05%),

digitonin (0.01–0.02%), lysolecithin (0.02–0.05%), and saponin (0.01–0.02%). We currently use saponin because it is sufficiently gentle that lysis is easily controlled; moreover, it preserves nuclear structure well (4). We also use this reagent in the presence of BSA to improve the structure further, but then higher concentrations of saponin must be used. Both saponin and BSA are mixtures of biomolecules that vary from batch to batch. Therefore, we routinely titrate saponin levels and choose those that give 95% lysis (assessed as below) with each batch of BSA.

1. Prepare a twofold dilution series of detergent in PB*-BSA.
2. Assess the level of permeabilization using trypan blue exclusion. Add 50:1 1% trypan blue in PB*-BSA to a coverslip; after 2 min, inspect by light microscopy; score the percentage of permeabilized, dark blue cells.
3. Choose the detergent concentration that permeabilizes >95% cells. If cells detach from coverslips during washing, use a lower concentration of detergent. Excess saponin also reduces translational activity as progressively more ribosomes are lost from the cytoplasm. It can also disrupt the integrity of the nuclear envelope (see **Note 3**).

3.2.2. A Basic "Translation Mixture" for "Run-On" Translation

The translation reaction involves incubating nuclei or permeabilized cells with the appropriate labeled precursor, the other amino acids, ATP, GTP, and an energy-regenerating system. Our basic translation mixture contains PB*-BSA (which contains ATP), creatine phosphokinase (20 U/mL), 2.5 mM phosphocreatine, 0.25 mM GTP, 0.5 mg/mL tRNA, 200 U/mL aminoacyl-tRNA synthetase, protease inhibitors for mammalian cells, and the various supplements indicated. In addition, MgCl₂ is always added to ensure that it is in equimolar amounts to any nucleotide triphosphates present in the reaction. Because nuclear translation is closely coupled with transcription, translation is improved by adding the 0.25 mM CTP and UTP required for transcription to this mixture. When synthetases were omitted the biotin incorporation falls by only 22%.

3.2.3. Translation Reaction Using Isolated Nuclei or Permeabilized Cells

Isolated nuclei or permeabilized cells are incubated in the basic translation mixture (**Subheading 3.2.2.**) supplemented with the appropriate labels as required; at the end of the reaction, samples are denatured, nascent peptides precipitated on to filter discs, washed thoroughly to remove unincorporated label, and the amount of radiolabel measured by scintillation counting (5) or by autoradiography after resolving nascent peptides by gel electrophoresis (1).

1. 2X Concentrates of permeabilized cells (8–50 × 10⁶/mL) or nuclei (4–20 × 10⁷/mL) in suspension and the basic translation "mixture" (plus any supplements and

inhibitors as required) are preincubated separately (3 min, 27.5°C). In general, the supplements include one radiolabeled amino acid, plus all the other unlabeled amino acids. Examples of such supplements include the following: (1) 5 μM L-[4,5- ^3H]lysine (86 Ci/mmol) + 50 μM amino acids minus lysine, (2) 5 μM L-[4,5- ^3H]leucine (147 Ci/mmol) + 50 μM amino acids minus leucine, (3) 1 μM biotin-lysine-tRNA from brewer's yeast + 5 μM L-[4,5- ^3H]leucine (147 Ci/mmol) + 50 μM amino acids minus lysine, and (iv) 200 $\mu\text{Ci/mL}$ L-[^{35}S]methionine (0.5 Ci/mmol) + 50 μM amino acids minus methionine.

2. The two are mixed, and incubated together (27.5°C) for the appropriate time.
3. For scintillation counting, reactions are stopped by transferring 100- μL samples to 350 μL 2% sodium dodecyl sulfate plus 50 μL 5 M NaOH. After incubation at 37°C for at least 30 min, 100 μL of this mixture is spotted on to glass fiber discs, the discs washed successively in 5% trichloroacetic acid (10 changes), ethanol (2 changes), and ether dried and their radioactivity estimated by scintillation counting.
4. For gel electrophoresis and autoradiography, the transcription reaction can contain [^{35}S]methionine. Then, nascent [^{35}S]peptides in 250- μL reaction mixture are removed and added to 10 mL of PB* to stop the reaction. After pelleting, cells or nuclei are rewashed in 10 mL of PB* to remove most unincorporated label. Now, DNA in the pellet of cells or nuclei is removed. Cells or nuclei are resuspended in 150 μL of PB*-diluted plus 1 mM MgCl_2 , 1 mM dithiothreitol, 25 U/mL human placental ribonuclease inhibitor, 100 U/mL RNase-free DNase, and protease inhibitors; after incubation (37°C, 10 min) the digestion is stopped by adding 100- μL sample buffer used for electrophoresis. [^{35}S]proteins from 10^5 cells or 3×10^5 nuclei are run on a 10–20% gradient polyacrylamide gel and an autoradiograph of the gel prepared (4).

3.3. Incorporation of Radiolabels Assessed by Autoradiography

Autoradiography can be used to detect sites of translation after incubating cells with radiolabeled amino acids (e.g., [^3H]leucine). This approach has some disadvantages:

1. It is rather specialized and technically demanding.
2. Detection of translation sites, rather than distant sites where the translation products might accumulate subsequently, requires very short pulses as the translation rate is so rapid (peptides are extended by approx 5 residues/s in vivo). Because the cellular pools of unlabeled amino acids are relatively high, little radiolabel is incorporated during short pulses, necessitating lengthy autoradiographic exposures. This problem can be mitigated by the use of isolated nuclei or permeabilized cells, where cellular pools are washed away (**Subheadings 3.1.2.** and **3.2.1.**).
3. The path-length of the β -particles emitted by ^3H is so long that autoradiographic grains often lie hundreds of nanometers away from the incorporation site.
4. Labeling other antigens is technically difficult.

3.3.1. Whole Cells

Procedures for labeling translation sites require various manipulations and are applied with difficulty to cells free in suspension; therefore, an approach involving cells attached to coverslips is described.

1. Wash glass coverslips in 70% ethanol for 15 min, rinse twice with distilled H₂O, drain, blot dry with Whatman no. 1 filter paper and heat sterilize (180°C dry heat for 12 h). If the cells tend to detach from the coverslip during subsequent manipulation, coverslips can be coated with gelatin or poly-L-lysine before plating to improve cell attachment.
2. Place dry coverslips in tissue-culture dishes.
3. Seed cells in the dish and allow to adhere. Best results are obtained with well-spread cells, covering 30–50% of the coverslip during labeling.
4. Grow cells in DMEM overnight.
5. Substitute the media for DMEM without leucine, incubate for at least 1 h and then add 50 μ M L-[4,5-³H]leucine (147 Ci/mmol) and incubate for 2 min or longer.
6. Fix in 4% paraformaldehyde in 250 mM HEPES pH 7.4, 20 min at 4°C.
7. Wash in 5% TCA, rewash in H₂O, and dry.
8. Cover with emulsion (dipping) film, expose for 3 d, and develop.
9. Count grain numbers over nucleus and cytoplasm.

3.3.2. Permeabilized Cells

1. Incubate permeabilized cells (prepared as described in **Subheading 3.2.1.**) with translation mixture supplemented with [³H]lysine (100 μ Ci/mL; 86 Ci/mmol) + 50 μ M amino acids minus lysine.
2. Fix cells in 4% paraformaldehyde in 250 mM HEPES, pH 7.4; 20 min at 4°C.
3. Wash in 5% TCA, rewash in water, and dry.
4. Prepare autoradiographs as in **Subheading 3.3.1.**

3.4. Incorporation of Biotin-Lysine-tRNA Assessed by Immunofluorescence

The immediate precursor—biotin-lysine-tRNA^{Lys}—is accepted by the translation machinery and incorporated into the growing peptide chain (6). Therefore, translation sites can be localized with high resolution by allowing permeabilized cells to elongate nascent peptides by only a few residues in the presence of biotin-lysine-tRNA^{Lys} and then immunolabeling the resulting biotin peptides with fluors (1).

1. Cells on coverslips are permeabilized as in **Subheading 3.2.1.**
2. Permeabilized cells are incubated in the basic translation mixture supplemented with 1 μ M biotin-lysine-tRNA from brewer's yeast + 50 μ M amino acids minus lysine.
3. Samples are fixed (20 min, 4°C) in 4% paraformaldehyde in 250 mM HEPES, pH 7.4.

4. Biotin-peptides are now indirectly immunolabeled with a fluor (Cy3 is chosen as an example). Coverslips are incubated (120 min; 20°C) with anti-biotin (5 µg/mL diluted in PBS+), washed in PBS, incubated with donkey anti-mouse IgG conjugated with Cy3 (0.5 µg/mL), and rewashed.
5. Nucleic acids are counterstained with 20 µM TOTO-3, and the sample is mounted in Vectashield.
6. Images are collected using a confocal microscope (4).
7. The extent of nuclear translation can be estimated by quantitative analysis of the resulting images. Intensities are measured (e.g., using EasiVision or Metamorph software) over the cytoplasm, nucleoplasm, and a cell-free area of the slide (for measurement of background), and data exported to Excel (Microsoft) for background subtraction and analysis. Average intensities (confocal sections) of nucleoplasm and cytoplasm abutting the nucleus are determined, and multiplied by the volume fraction of the two compartments (i.e., 400 and 673 µm³ in the case described in **ref. 7**) to obtain relative contents.

3.5. Using Permeabilized Cells and BODIPY-Lysine-tRNA

BODIPY-lysine-tRNA^{Lys} also is accepted by the translation machinery and incorporated into the growing peptide chain (8). Therefore, translation sites can be localized more directly by allowing permeabilized cells to elongate nascent peptides by only a few residues in the presence of this immediate precursor and then viewing the resulting BODIPY-peptides (**Fig. 1; ref. 1**).

1. Cells on coverslips are permeabilized as in **Subheading 3.2.1**.
2. Permeabilized cells are incubated in translation mixture supplemented with a 1/10 dilution of BODIPY-lysine-tRNA (FluoroTect™ Green_{Lys}) + 50 µM amino acids minus lysine.
3. Samples can now be viewed directly, or fixed (20 min; 4°C) in 4% paraformaldehyde in 250 mM HEPES, pH 7.4, and analyzed as in **Subheading 3.4**.

3.6. Incorporation of Biotin-Lysine-tRNA Assessed by Electron Microscopy

Biotin (**Subheading 3.4**) can also be localized after immunogold labeling using the electron microscope (1).

1. Permeabilized cells are allowed to extend nascent peptides in biotin-lysine-tRNA and fixed, as in **Subheading 3.2.1**.
2. Samples are embedded in LR White and ultrathin sections cut.
3. Sections are incubated with PBS+ for 30 min.
4. Biotin-peptides are indirectly immunolabeled with mouse anti-biotin (10 µg/mL) in PBS+ for 2 h at 20°C.
5. Wash in PBS and immunolabel with antibodies conjugated with gold-particles (e.g., goat anti-mouse IgG conjugated with 5- or 10-nm particles; 1:25 dilution).
6. After contrasting with uranyl acetate, digital images are collected on an electron microscope (7).

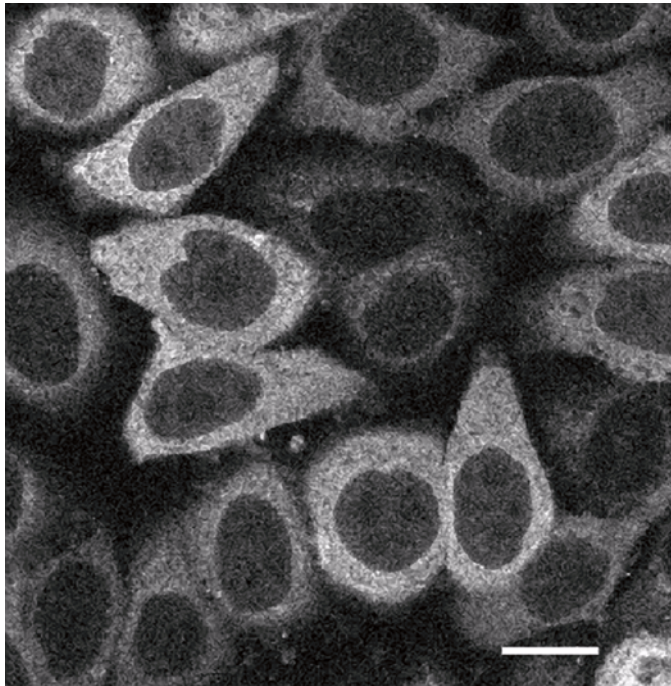


Fig. 1. Translation sites labeled with BODIPY. HeLa cells were permeabilized and allowed to extend nascent polypeptides by approx 20 residues in the presence of BODIPY-lysine-tRNA; after fixation in paraformaldehyde, cells were imaged using a confocal microscope (*I*). Nascent BODIPY-peptides are found mainly in the cytoplasm, but some are also found in nuclei. Bar: 10 μm .

4. Notes

1. The concentration of divalent cation must be carefully controlled. As little as 0.5 mM free Mg^{2+} causes the visible (by EM) collapse or aggregation of chromatin. Therefore, we use an equimolar Mg/ATP combination so that there is very little free Mg^{2+} ; this preserves chromatin structure while supporting the action of Mg-dependent enzymes.
2. The extent of extranuclear ribosomal contamination can be determined (after fixation and staining with osmium) by electron microscopy of Epon sections and the application of standard stereological procedures (7). For example, nuclei isolated by the procedure described in **Subheading 3.1.2.** are associated with fewer than 5% extranuclear ribosomes seen in whole cells.
3. The integrity of the nuclear membrane in isolated nuclei can be monitored by incubation with a dextran conjugated with fluorescein that is too large to pass through the nuclear pore. For example, 96% nuclei isolated by the procedure described in **Subheading 3.1.2.** excluded a 500-kDa dextran conjugated with

FITC; this makes it unlikely that the nuclear envelope is sufficiently disrupted to allow larger cytoplasmic ribosomes to enter. In addition, 94% nuclei prepared similarly but that were not washed with Triton also excluded a 70-kDa dextran conjugated with FITC—but not fluorescein-12-ATP, which was used as a positive control.

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