

Nucleolar Isolation Protocol

We recommend that you first download and read this page as a [PDF file](#). Using that as your guide, you can then follow the protocol below and view a Quicktime movie demonstrating the key steps. We have also included higher resolution stills which provide close-ups of certain steps of the protocol.

Special Feature: Complete nucleolar isolation protocol in one Quicktime movie	
Click here to view at high resolution (25.8 Mb) <i>Warning: this can take a long time to load, depending on the speed of your connection. Please be patient!</i>	Click here to view at low resolution (9.2 Mb) <i>This movie loads more quickly than the high resolution version, but some details are difficult to resolve.</i>
The movie stars Dr. Yun Wah Lam , who optimized the original protocol.	

Buffers and solutions

(All solutions are supplemented with Complete Protease inhibitor tablet (Roche, Cat no: 1-873-580) at the final concentration of 1 tablet/50ml):

PBS

Buffer A: 10 mM Hepes, pH 7.9, 10mM KCl, 1.5mM MgCl₂, 0.5mM DTT

S1 solution: 0.25 M Sucrose, 10 mM MgCl₂

S2 solution: 0.35 M Sucrose, 0.5 mM MgCl₂

S3 solution: 0.88 M Sucrose, 0.5 mM MgCl₂

See "Notes" below on making stock sucrose solution.

Procedure

Note: Nucleoli were prepared from HeLa cells using a variation on a method described by Muramatsu and co-workers in 1963 (*Muramatsu M, Smetana, K., and Busch, H.: Quantitative aspects of isolation of nucleoli of the Walker carcinosarcoma and liver of the rat. Cancer Res. 1963; 25:693-697*).

1. Seed HeLa cells (ATCC number: CCL-2) on to 10x14 cm Petri dishes and culture at 37°C in 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM) containing 4mM L-glutamate, 4.5 mg/ml glucose and 0.11 mg/ml sodium pyruvate (Invitrogen UK, Cat no: 41966-029), supplemented with 100 U/ml Penicillin and 100 µg/ml Streptomycin (1% v/v Penicillin/Streptomycin solution, Invitrogen UK, Cat no: 15140-122) until >90% confluence (approx. 107 cells per dish). This number of HeLa cells consistently provides nucleoli with excellent yield and purity. It is possible to scale down the preparation, although purity of the isolated nucleoli may suffer. Make sure you monitor every step using a phase contrast microscope (see below). 1 hour before nucleolar isolation, replace with fresh, pre-warmed medium.

2. Harvest cells by trypsinization. Rinse each dish 3X with pre-warmed PBS, and on removal of the last rinse, add 2 ml of trypsin-EDTA solution (Invitrogen UK, Cat no: 25300-054) per dish. Swirl the dishes to make sure the trypsin-EDTA is evenly distributed, and return the dishes to the incubator for about 5 min. Check under a phase contrast microscope that all the cells are detached. Prolong incubation if needed. Add into each dish 8 ml of pre-warmed medium, pipette up and down so that all the cells are collected as a single-cell suspension. Pool all the harvested cells into 2x 50ml Falcon tubes. For some strains of HeLa cells, it is also possible to

harvest the cells by scraping them in 5 ml ice-cooled PBS per dish. Since scraping may lead to impure nucleolar preparation in some HeLa strains, it is not recommended as the method of first choice.

3. Wash 3 X with ice-cold PBS at 218 g (1000 rpm, Beckman GS-6 centrifuge, GH-3.8 rotor) at 4°C.

4. After the final PBS wash, resuspend the cells in 5ml of Buffer A and incubate the cells on ice for 5 min. Put a small drop of the cell suspension on a glass slide and check under a phase contrast microscope, such as a Zeiss Axiovert 25, using a 20X objective. The cells should be swollen, but not burst (Fig 1). Nucleoli of cultured mammalian cells disassemble at 37°C in hypotonic conditions (Zatsepina et al, 1997). It is therefore imperative to keep the cell suspension on ice during this step.

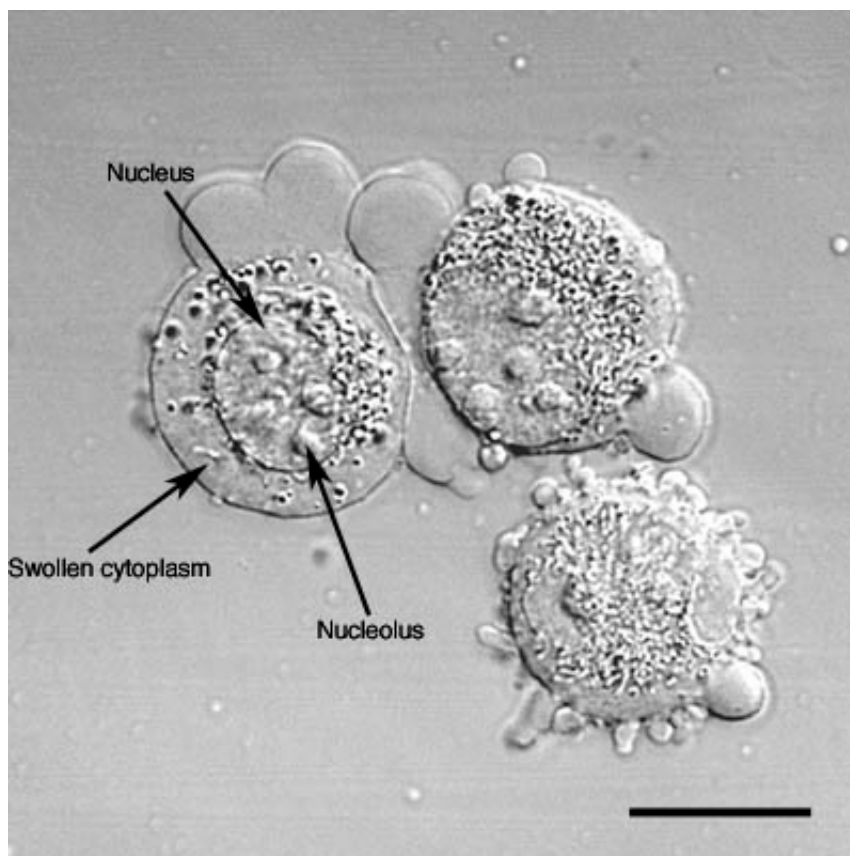
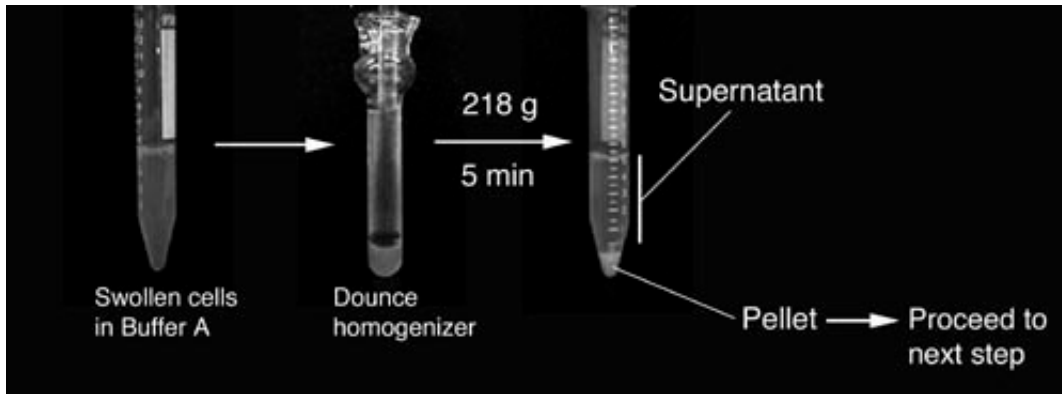


Figure 1: HeLa cells after step 4. Note the swollen cytoplasm and prominent nucleoli. Bar: 10µm.

5. Transfer the cell suspension to a pre-cooled 7 ml Dounce tissue homogenizer (Wheaton Scientific Product Cat no: 357542). Homogenize 10 times using a tight pestle ("A" specification: 0.0010" - 0.0030" clearance), while keeping the homogenizer on ice. The number of strokes needed depends on the cell type used (see "Notes"). It is therefore necessary to check the homogenized cells under a phase contrast microscope after every 10 strokes. Stop when >90% of the cells are burst, leaving intact nuclei, with various amounts of cytoplasmic material attached. In most cases, the presence of this cytoplasmic contamination does not affect the final purity of the isolated nucleoli (Figure 2).





6. Centrifuge the homogenized cells at 218g (1000 rpm, Beckman GS-6 centrifuge, GH-3.8 rotor) for 5 min at 4°C. The pellet contains enriched, but not highly pure, nuclei.

7. Resuspend the pellet with 3 ml S1 solution (Figure 3). The pellet should be resuspended readily by pipetting up and down. A pellet that cannot be resuspended contains lysed nuclei and should be discarded. Layer the resuspended pellet over 3 ml of S2 solution. Take care to keep the two layers cleanly separated. Centrifuge at 1430g (2500 rpm, Beckman GS-6 centrifuge, GH-3.8 rotor) for 5 min at 4°C. This step results in a cleaner nuclear pellet (Figure 3). Resuspend the pellet with 3 ml of S2 solution by pipetting up and down.

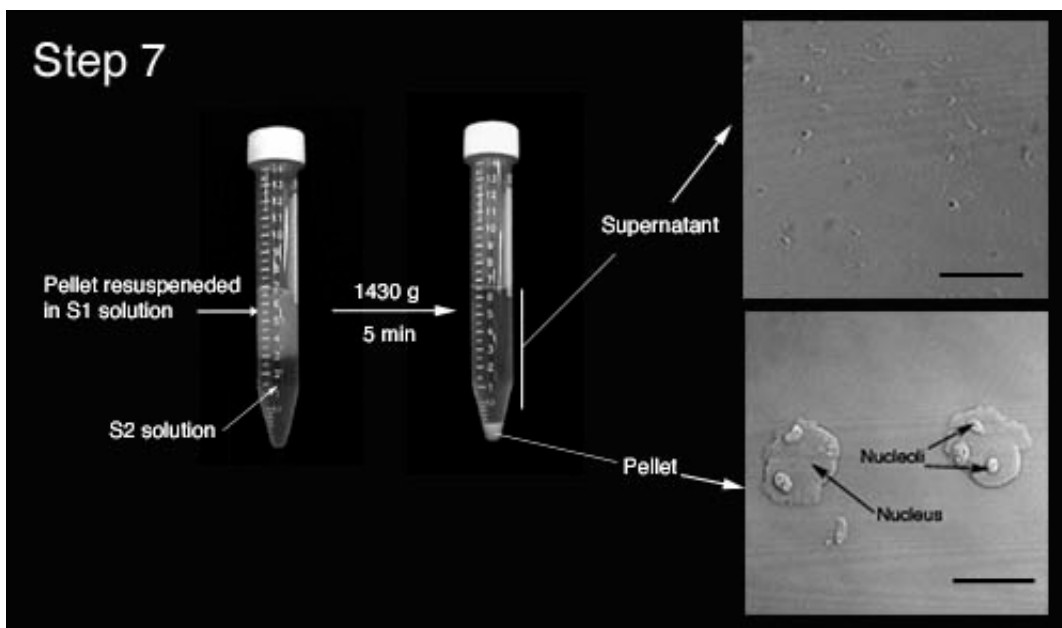


Figure 3. Step 7 of the procedure. Note the clear boundary between S1 and S2 layers before centrifugation. Insets show the DIC images of the supernatant and pellet. Note the prominent nucleoli inside the nuclei in the pellet. Bars: 10 μ m.

8. Sonicate the nuclear suspension for 6 x 10 second bursts (with 10 second intervals between each burst) using a Misonix XL 2020 sonicator fitted with a microtip probe and set at power setting 5 (Figure 4 Left). Check the sonicated nuclei under a phase contrast microscope. There should be virtually no intact cells and the nucleoli should be readily observed as dense, refractile bodies (Figure 4 right). The optimal sonication time depends on the cell type used. If you attempt to isolate nucleoli from a cell type from the first time, it is necessary to check the sonicated material under a microscope after every 10 seconds of sonication. Over-sonication leads to destruction of nucleoli.

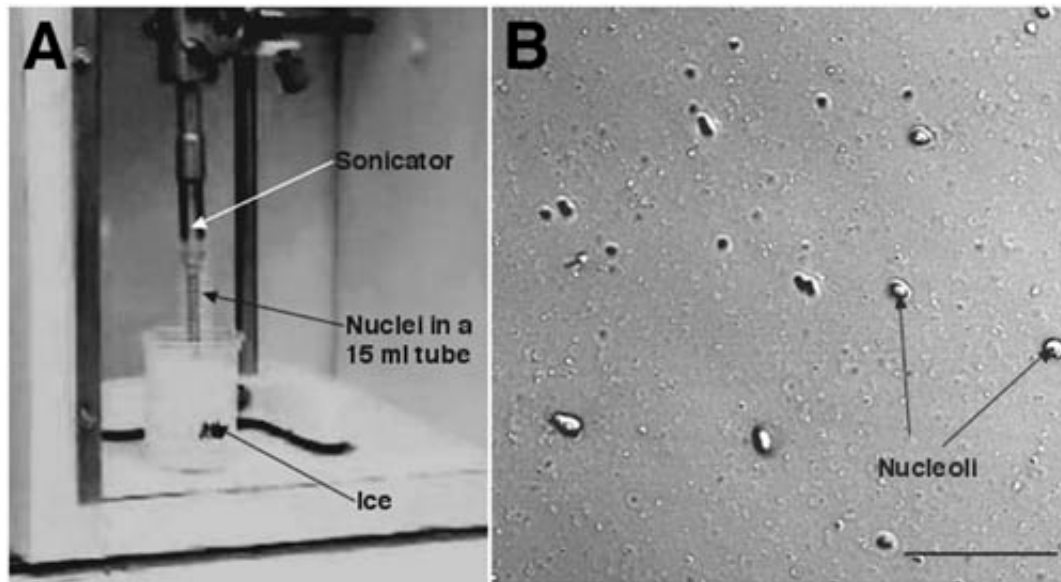


Figure 4. Left. Setup for sonication. Right. DIC image of sonicated nuclei. Note the presence of prominent nucleoli. Bar: 10 μ m.

9. Layer the sonicated sample over 3 ml of S3 solution and centrifuge at 3000g (3500 rpm, Beckman GS-6 centrifuge, GH-3.8 rotor) for 10 min at 4°C (Figure 5). The pellet contains the nucleoli, whilst the supernatant can be retained as the “nucleoplasmic fraction” (Figure 5).

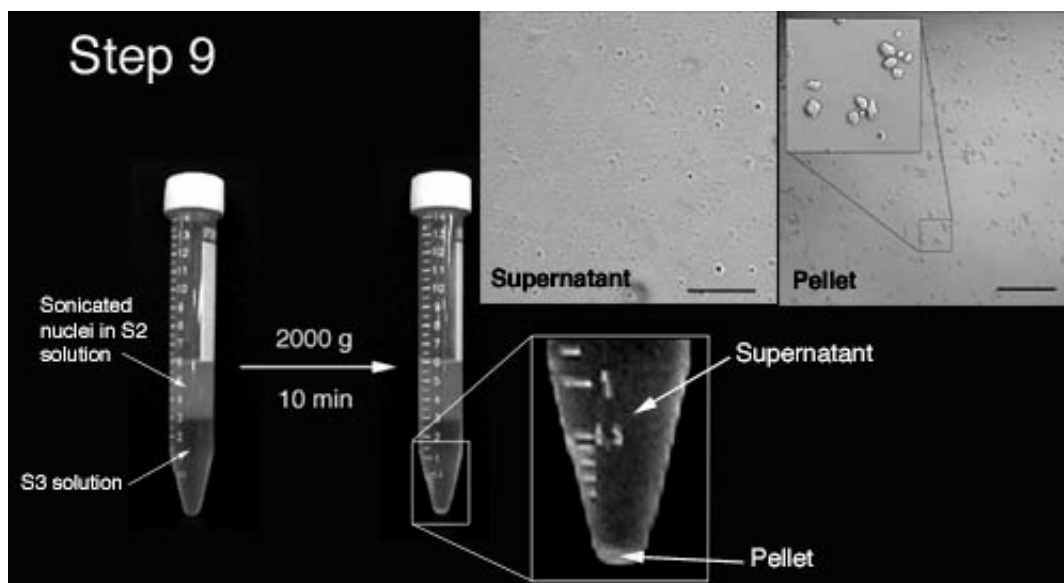


Figure 5: Step 9 of the procedure. Note the clear boundary between S2 and S3 layers before and after centrifugation. The pellet should be small but visible. Insets show DIC images of the supernatant and pellet. The pellet should contain purified nucleoli. Bars: Left inset: 10 μ m, right inset: 20 μ m.

10. Resuspend the nucleoli with 0.5 ml of S2 solution, followed by centrifugation at 1430 (2500 rpm, Beckman GS-6 centrifuge, GH-3.8 rotor) for 5 min at 4°C. The pellet contains highly purified nucleoli. Check under a phase contrast microscope to ensure this preparation contains only highly purified nucleoli without any other material (Fig 5). The nucleoli can be resuspended in 0.5ml of S2 solution and stored at -80oC.

Notes

(1) Making 2.55M sucrose stock

Here is a protocol for preparing a sucrose stock solution (Cline and Ryel, 1971) suitable for the nucleolar isolation protocol. The resulting solution is 2.55M, or 66% by weight. Its density is 1.3224g/cm³ at 20°C, and refractive index is 1.4558. The stock solution is stable indefinitely at 4°C. This procedure can be carried out at RT. There is no need to heat up the solution to help dissolving the sucrose. Heating up an incompletely dissolved sucrose solution can lead to charring of sucrose and affect the quality of the sucrose solution.

1. Weigh out 1710 g sucrose (BDH). Keep it aside in a clean container.
2. Put exactly 900ml water and a magnetic bar in a 5 litre beaker. Put the beaker on a stirrer and start stirring.
3. Add 1/3 of the sucrose into the beaker. Make sure the magnetic bar is rotating freely. Stir for 1 hour.
4. Add another 1/3 of the sucrose into the solution. Again make sure the rotation of the stir bar is not impaired. Stir for another 1 hour.
5. Add the remaining sucrose. Stir for another 1 hour, or until all the sucrose has gone into solution. The final volume should be exactly 2 litres.

(2) Sonication

We use a Misonix 2020 sonicator fitted with a microtip at power setting 5. To ensure reproducible sonication these points should be followed:

- It is necessary to tune the sonicator every time after you change the probe. Follow the manufacturer's manual for the tuning procedure.
- Sonication produces intense and localized heat in your solution. If you are concerned about the heating, the correct way to reduce heating is to shorten the sonication time and to increase the intermission between bursts. Keeping the tube on ice or performing the sonication in the cold room is helpful, but is not the most effective way of heat control.
- If the probe is too close to the liquid surface, it produces a foam and reduces the efficiency of sonication. Make sure the probe is well submerged in the solution, about 5mm above the bottom of the tube. Do not, however, touch the bottom or the wall of the tube with the probe.
- Sonicator probe that has been used repeatedly develops pits on its end. The sonication efficiency gradually decreases as time goes on. Therefore, the sonication time recommended here can only be used as guideline. Always monitor the outcome of sonication using a phase contrast microscope. You may need to adjust the sonication time to maintain the efficiency especially if the probe is getting old. Change the probe when the efficiency is noticeably down.

(3) Analysis of the isolated nucleoli

- To immunolabel the purified nucleoli, spot about 5 µl of the nucleolar suspension on to a polylysine-coated slide (BDH Cat no: 406/0178/00), and air dry the spot. Rehydrate the slide in PBS for 5 min before carrying out a standard immunostaining procedure.
- To separate nucleolar proteins on a gel, either resuspend directly in Laemmli SDS sample buffer or in your preferred buffer. The high concentration of nucleic acid in the isolated nucleoli makes the lysed sample very viscous. The sample can be clarified by passing through a QIAshredder spin column (Qiagen Cat no: 79654). Nucleoli can also be extracted with RIPA buffer (150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, COMPLETE protease inhibitor cocktail). Immunoprecipitations can be performed from nucleolar lysates prepared in RIPA buffer.

(4) Adapting nucleolar isolation protocol to use with other cell types

The above protocol can readily be adapted to other cell types. Apart from HeLa cells, we have used this protocol, with minor modifications, to isolated nucleoli from MCF-7 (human breast epithelium), WI-38 (human fibroblast), IMR-32 (human neuroblastoma), HL60 (human promyelocytic leukemia) and plant Arabidopsis thaliana cells. When adapting the protocol to a different cell type, make sure you control each step by carefully checking the products after each step under a phase contrast microscope. For example, different cell types may require a different homogenization (step 4) and/or sonication strength (step 7). The concentration of MgCl₂ also appears crucial to the purity of the isolated nucleoli. If the isolated nucleoli are not pure enough, try lowering the concentration of MgCl₂ in the S2 and S3 solutions. If the yield is poor, or if the nucleoli look fragmented, use more MgCl₂.