Chapter 2

Lysis Gradient Centrifugation: A Flexible Method for the Isolation of Nuclei from Primary Cells

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Abstract

The isolation of nuclei from eukaryotic cells is essential for studying the composition and the dynamic changes of the nuclear proteome to gain insight into the mechanisms of gene expression and cell signalling. Primary cells are particularly challenging for standard nuclear isolation protocols due to low protein content, sample degradation, or nuclear clumping. Here, we describe a rapid and flexible protocol for the isolation of clean and intact nuclei, which results in the recovery of 90–95 % highly pure nuclei. The method, called lysis gradient centrifugation (LGC), is based on an iso-osmolar discontinuous iodixanol-based density gradient including a detergent-containing lysis layer. A single low g-force centrifugation step enables mild cell lysis and prevents extensive contact of the nuclei with the cytoplasmic environment. This fast method shows high reproducibility due to the relatively little cell manipulation required by the investigator. Further advantages are the low amount of starting material required, easy parallel processing of multiple samples, and isolation of nuclei and cytoplasm at the same time from the same sample.

Key words Nuclear isolation, Mild lysis, Fractionation, Single step, Discontinuous density gradient

1 Introduction

The spatial separation of the nuclear and the cytoplasmic compartment plays an essential role in the regulation of gene expression in all immune cells [1]. Gaining information about the composition and the dynamic changes in the nuclear proteome is important for understanding the mechanisms of gene regulation and cell signaling [2–4]. A number of protocols aim to isolate pure and intact nuclei from eukaryotic cells for further analysis by western blots, electrophoretic mobility shift assays (EMSA), or mass spectrometry [3–8]. The most important and initial step of frequently employed nuclear isolation protocols is the disruption of the cytoplasmic membrane, while the nuclear membrane should remain intact. This is usually achieved by induction of cell swelling through

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the incubation of the cells in a hypotonic buffer for a tightly controlled period of time followed by mechanical or chemical disruption of the cell membrane [9]. NP-40 is a nonionic non-denaturing detergent and was for a long time the most frequent detergent used in nuclear isolation protocols. It was either directly included into the hypotonic lysis buffer or added after cell swelling [10, 11]. As NP-40 is no longer commercially available, it was replaced by the chemically indistinguishable IGEPAL CA-630. Mechanical stress is often used to aid plasma membrane disruption and is introduced either by defined vortexing times or by passing the cells through a syringe. The crude lysates containing the nuclei are then usually washed by pelleting the nuclei by centrifugation. Alternatively, Graham and coworkers developed a method to purify the nuclei from the crude lysate based on a discontinuous density gradient formed by iodixanol followed by centrifugation and nuclear banding [9]. This method has been used frequently to study nuclear proteins [12–14].

From a biochemical point of view, the purification of nuclei from mammalian cells is associated with many technical problems [11]. Temperature control cannot be assured easily at any time of the nuclear isolation procedure during mechanical disruption methods, and moreover the nuclei are in extensive contact with the cytoplasmic environment containing lysis-activated proteases that lead to partial degradation and modification of the samples despite the presence of protease inhibitors [15, 16]. Repeated washing steps, necessary to remove nucleus-associated membrane systems and cytoskeletal components, may result in nuclear leakage due to overexposure to detergent and mechanical stress. Therefore, nuclear leakage results not only in loss of target proteins but also in clumping of the nuclei due to released chromosomal DNA [17]. Clumping leads to a low recovery of nuclei, explaining the large amount of starting material required by standard fractionation procedures. Many protocols are optimized for distinct cell types as the nuclear density and stability show profound variations among cell types and stimulation procedures [18, 19]. All of these caveats are true for cell lines and primary cells; however, it is particularly difficult to get reliable and clean nuclear preparations from primary cells which are available only in a limited amount and have low protein content [20].

2 Materials

2.1 Cells

This method has been used successfully for:

Primary cells: CD14+ monocytes; macrophages (MΦ); T lymphocytes.

Cell lines: JE-6 (Jurkat cells, an immortalized line of T lymphocytes); RAJI (a lymphoblastoid cell line derived from a Burkitt

lymphoma); MEF (murine embryonic fibroblasts); THP-1 cells (a promonocytic cell line derived from a human acute lymphocytic leukemia patient) (*see* **Note 1**).

2.2 Lysis Gradient Preparation

1. Visipaque™ 320 (65.2 % Iodixanol; GE Healthcare).
2. Phosphate-buffered saline solution (PBS): 210.0 mg/L KH_2PO_4, 9,000 mg/L NaCl, 726.0 mg/L Na_2HPO_4-$7H_2O$ (Lonza).
3. Protease inhibitor cocktail: 10× solution made by dissolving one tablet of cOmplete EDTA-free (Roche) in 2 mL of PBS.
4. Coomassie Brilliant Blue R250 (Bio-Rad).
5. Crystal Violet (Sigma-Aldrich).
6. IGEPAL CA-630 (Sigma).
7. Polystyrene tubes: 8 mL, 13 × 100 mm (BD Biosciences).
8. Crushed ice.

2.3 Nuclear Gradient Centrifugation

1. Standard refrigerated laboratory centrifuge (e.g., Rotanta 460RS, Hettich) equipped with a swinging bucket rotor.
2. Vacuum-attached glass pipette.

2.4 Western Blots

1. Denaturing SDS-sample buffer (4×): 250 mM Tris–HCl, pH 6.8, 40 % glycerol, 8 % SDS, 400 mM dithiothreitol, and 0.04 % (w/v) bromophenol blue.
2. Nitrocellulose membranes: Protran, Whatman.
3. PBS-T: 0.05 % Tween-20 in PBS.
4. Blocking solution: 4 % dry milk (Bio-Rad) in PBS-T.
5. Primary antibodies: Rabbit anti-GAPDH mAb (Cell Signaling) 1:1,000 in PBS-T + 0.02 % NaN_3; rabbit anti-ABTF-IID polyclonal (Santa Cruz) 1:500 in PBS-T + 0.02 % NaN_3.
6. Secondary antibody: ECL donkey anti-rabbit IgG, HRP-linked whole Ab [GE Healthcare): 1:3,000 in PBS-T + 4 % dry milk.
7. HRP Substrate: Immobilon Western Chemiluminescent (Millipore).
8. Chemiluminescence imager.

3 Methods

Our method is based on an iodixanol-based gradient [21] where we included a lysis layer containing the detergent IGEPAL CA-630 [22]. The incorporation of a lysis layer allows the isolation of intact nuclei from living cells in a single centrifugation step, and therefore minimizes the exposure of the nuclei to cytoplasmic protease activity. The gradient for LGC is built in such a way that the whole suspension of living cells can be directly loaded onto the iodixanol-based

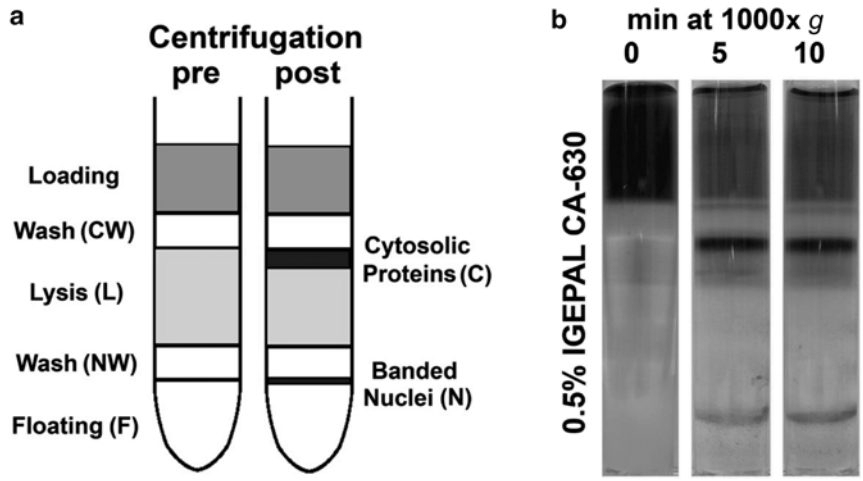

Fig. 1 (**a**) Principle of the method. The living cells are applied on top of the gradient in the original culture medium. During 10 min of low gravity centrifugation in a swinging bucket rotor, the cells pass through different functional layers including the cell wash layer (CW) and the lysis layer (L), at the beginning of which the cytoplasmic membrane is disrupted. Cytoplasmic proteins (C) remain on top of the lysis layer. Nuclei continue to pass through the nuclei wash layer (NW) and form a band on top of the floating layer (N). (**b**) A nuclear gradient purification performed with 2×10^6 THP-1 cells. The cells were centrifuged for the indicated times at $1{,}000 \times g$

gradient in its original culture medium (Fig. 1). The discontinuous iso-osmolar lysis gradient allows a *g*-force driven discrimination between unlysed and lysed cells and clean and ER-contaminated nuclei during centrifugation. A band of pure nuclei accumulates at the lowest interface, while nuclei contaminated with other cellular structures are trapped at upper layers due to their lower density (*see* **Note 2**). The cells can be stained with Crystal Violet and Coomassie Blue in order to visualize the bands occurring in the gradient and therefore enables easy harvesting of the nuclei.

3.1 Preparation of the Lysis Gradient

1. Prepare iodixanol dilutions up to 40 % (w/v) by diluting Visipaque 320 in PBS (*see* **Note 3**). The volume of Visipaque and PBS for the different layers of the gradient can be found in Table 1. Add one-tenth volume of 10× protease inhibitor solution to the cell lysis layer and nuclei wash layer immediately before use.
2. Add Coomassie Brilliant Blue to the lysis layer to a final concentration of 4 μg/mL (*see* **Note 4**).
3. Add Crystal Violet to the cell suspension and lysis layer to a final concentration of 5 μg/mL (*see* **Note 4**).
4. Add IGEPAL CA-630 to the lysis layer to a final concentration of 0.5 % (v/v).
5. Prepare lysis gradients in 8 mL polystyrene tubes suitable for 2×10^5–5×10^6 cells. The gradients are set up by sequentially overlaying 1 mL of floating layer with 0.5 mL of nuclei wash

Table 1
Composition of LGC gradients for isolating nuclei from different cell types

Iodixanol [% (w/v)]	Density [g/cm³]	PBS [mL]	Visipaque [mL]	Primary cells			Cell lines			
				CD14+	MΦ	T-Cells	THP-1	JE-6	RAJI	MEF
0	1.016	10.0	0.0							
5	1.051	9.2[a]	0.8	CW	CW	CW	CW	CW	CW	CW
10	1.071	8.5[a]	1.5	L	L	L	L	L	L	L
15	1.100	7.7	2.3							
20	1.129	6.9	3.1		NW					NW
25	1.155	6.2	3.8	NW		NW	NW	NW	NW	
30	1.178	5.4	4.6							
35	1.199	4.6	5.4	F	F	F	F	F	F	F
40	1.234	3.9	6.1							
65.2	1.372	0.0	10.0							

CW cell wash layer, *L* lysis layer, *NW* nucleus wash layer, *F* floating layer
[a]Total volume; includes 1 mL of 10× protease inhibitor solution added immediately before use

layer, 1 mL of lysis layer, and 0.5 mL of cell wash layer. All these steps are carried out on ice (*see* **Note 5**).

6. Stain the cooled cell suspension with 5 µg/mL Crystal Violet (*see* **Note 4**).
7. Apply the cooled cell suspension in up to 1 mL of the original culture medium immediately before lysis gradient centrifugation.

3.2 Nuclear Gradient Centrifugation and Harvesting of Fractions

1. After setting up the lysis gradient and adding the cell suspension, centrifuge at 1,000 × *g* for 10 min at 4 °C in a refrigerated centrifuge equipped with a swinging bucket rotor.
2. After centrifugation, place the tubes carefully on ice.
3. Gently remove the culture medium as well as the upper part of the cell wash layer using a vacuum-attached glass pipette.
4. The upper 500 µL of the lysis layer are collected with a 1 mL Gilson pipette for the analysis of cytoplasmic fractions.
5. The residual lysis layer and the upper half of the nuclei wash layer are removed with a vacuum-attached glass pipette.
6. Collect the light blue band of nuclei (150 µL) between the nuclei wash layer and the floating layer (*see* **Note 6**).

7. For western blot analysis, lyse the nuclei directly by adding 50 µL of 4× reducing SDS-PAGE sample buffer followed by denaturation for 5 min at 95 °C (*see* **Note 7**).

3.3 Western Blots

1. Lyse the harvested fractions by adding one volume of 4× reducing SDS-PAGE sample buffer to three volumes of the fractions.
2. Denature proteins at 95 °C for 5 min.
3. Cool the samples to 10 °C and centrifuge in a microcentrifuge for 2 min at 25,000 × *g*.
4. Subject the supernatants to SDS-PAGE and blot the gels onto nitrocellulose membranes.
5. Incubate the membranes in blocking solution for 1 h at room temperature.
6. Incubate the membranes with primary antibodies at 4 °C overnight. In order to confirm the purity of the nuclear and cytoplasmic fractions, make use of antibodies against GAPDH and TF-IID: GAPDH should only be seen in the cytoplasmic fractions and the transcription factor TF-IID should only be present in the nuclear fraction (Fig. 2).
7. Wash the membranes 3× with PBS-T and add the horseradish peroxidase-labeled secondary antibody for 1 h at room temperature.

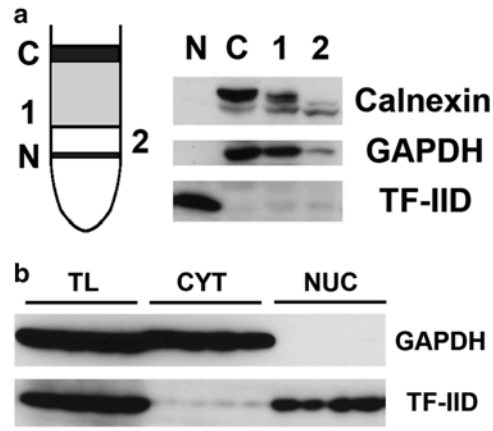

Fig. 2 Purity and integrity of LGC-isolated nuclei. (**a**) 10^6 THP-1 cells were subjected to LGC followed by taking aliquots of proteins for western blots from the cytoplasmic fraction (C), the lower interface of lysis layer (*1*), the central nuclear washing layer (*2*), and the nuclear band (N). Equal volumes of the indicated layers were western blotted and immunolabeled for Calnexin, GAPDH, and TF-IID. (**b**) Aliquots of nuclear (NUC) and cytoplasmic (CYT) fractions obtained by LGC from 10^6 THP-1 cells per treatment condition were investigated by western blotting for GAPDH and TF-IID. Total lysates (TL) in the same treatment conditions are shown as control

8. Wash the membranes 3× with PBS-T and detect antibody-labeled proteins with Immobilon Western Chemiluminescent HRP Substrate.
9. Detect the signals using a chemiluminescence imager.

4 Notes

1. This method can be used for the isolation of nuclei from virtually any cell type by just optimizing the densities of the different functional layers. It is also suitable for isolating nuclei from only a limited amount of cells; for example we have isolated nuclei from 5×10^5 primary macrophages and successfully performed western blotting. After an initial trypsinization step, adherent cells can also be processed with this protocol.
2. In principle, LGC can be adapted for ultracentrifugation to isolate cell organelles such as mitochondria, lysosomes, or peroxisomes requiring only a layer with the correct density to be present in the gradient [23, 24].
3. Visipaque 320 containing iodixanol is widely used as an intravenous contrast agent in clinical radiology, and the amount discarded from single-use containers can reach up to 50 % of the pack volume. The remaining Visipaque 320 provides a cheap and easily available source of iodixanol with the major advantage of iso-osmolarity, in contrast to OptiPrep which is a 60 % aqueous solution of Iodixanol whose iso-osmolarity after dilution cannot be controlled as precisely as with Visipaque [11].
4. If you want to carry out more sensitive methods than western blot after the LGC, it is recommended to avoid the use of Coomassie Brilliant Blue and Crystal Violet. The gradient can also be carried out without the addition of these dyes, which might interfere with more sensitive assays.
5. To minimize mixture of the layers when preparing the lysis gradient, the solutions should flow down the tube wall slowly and tubes should be placed on ice before the addition of the next layer. By omitting the lowest floating layer, the nuclei can be easily pelleted although this might influence their purity due to proteins sticking to the upper portion of the tubes.
6. Do not penetrate the wash layers with the pipette in order to harvest the nuclei, as proteins from the wash layer might stick to the pipette tip and contaminate the nuclei. It is highly recommended to suck off the medium and the upper half of the cell wash layer with a vacuum-attached glass pipette before harvesting the lysis layer for analysis of the cytoplasmic proteins. Subsequently, the residual layers and the upper half of the nuclei

wash layer should again be removed with the glass pipette before harvesting the nuclei.

7. The protein concentration of the banded nuclei collected in 150 μL is usually 0.25–0.5 mg/mL depending on the cell type. In order to measure the protein concentration or obtain a higher concentration in the sample the banded nuclei can be collected, diluted with 600 μL of PBS, pelleted by centrifugation at 1,000 × *g*, followed by protein extraction in a reduced volume of the desired extraction buffer.

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