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In Situ Immunofluorescence Analysis

Immunofluorescence Microscopy

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1. Introduction

Immunofluorescence is one of the most widely used techniques to study the localization of transcription factors, proteins, and structural components of nuclear architecture and cytoarchitecture. High-resolution *in situ* immunofluorescence approaches permit assessment of functional interrelationships between nuclear structure and gene expression that are linked to the intranuclear compartmentalization of nucleic acids and regulatory proteins (an example is shown in **Fig. 1**). The success of this method is dependent on the quality and specificity of the antibodies and the relative stability of antigens. Generally, the overall scheme for localization of cellular proteins involves fixation and permeabilization of cells for antibody accessibility, blocking, and staining with specific antibodies before microscopic examination. To reveal the subcellular and subnuclear macromolecular complexes that comprise and govern activation of the regulatory machinery for gene expression, cells can be subjected to selective extractions before immunodetection as described below.

2. Materials

1. Sterile glass cover slips (Fisher) coated with 0.5% gelatin (Life Technologies).
2. Cytoskeleton (CSK) buffer: (10× stock solution): 1 *M* NaCl, 100 m*M* PIPES, pH 6.8, 30 m*M* $MgCl_2$, 10 m*M* ethylenebis (oxyethylenenitrilo) tetraacetic acid (EGTA), 5% Triton X-100. (1× working solution): Freshly prepare 100 mL of 1× CSK buffer by dissolving 10.27 g sucrose in 77.6 mL of double-distilled water.

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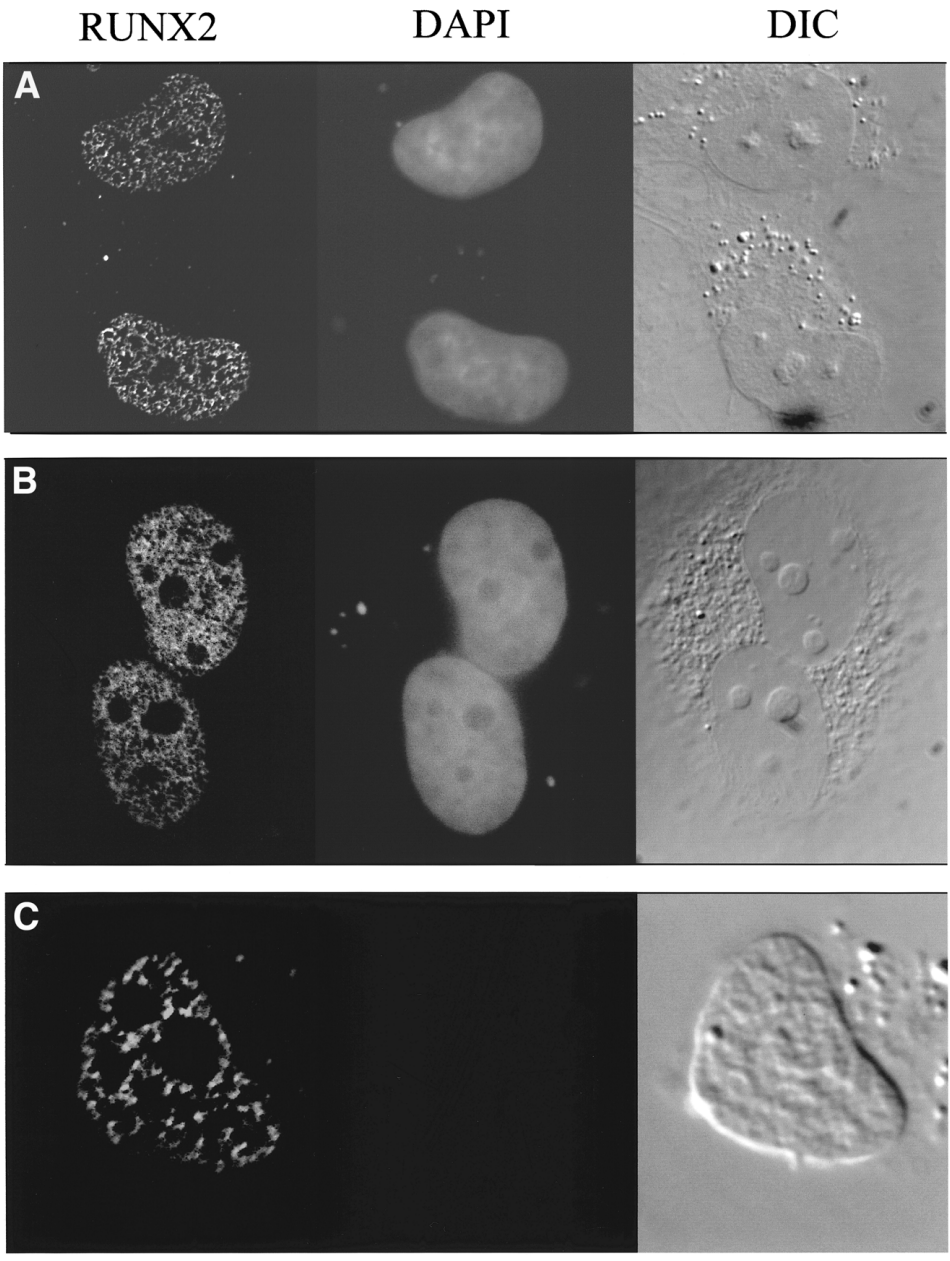
RUNX2
DAPI
DIC
A
B
C

Fig. 1. *In situ* immunofluorescence detection of transcription factors at intranuclear sites. Runx/Cbfa/AML transcription factors provide an example of regulatory proteins that can be detected *in situ*. HeLa cells grown on gelatin-coated cover slips were transiently transfected with 0.5 µg of Runx2 expression plasmid, using "SuperFect" reagent (Qiagen Inc, CA). Cells were processed 20 h later for *in situ* detection of Run µ2 in intact cells **(A)** or after removal of cytoskeletal component **(B)** or in nuclear matrix preparations **(C)**. Run µ2 proteins were detected with a rabbit polyclonal Run µ2 antibody and an fluorescein isothiocyanate-conjugated antirabbit secondary antibody. DAPI detects deoxyribonucleic acid (DNA) in nuclei of whole cells and CSK extracted cells but not in NMIF preparations because DNA has been digested and extracted. Differential interference contrast microscopy shows a bright field image of cells. The punctate, non-nucleolar distribution of Run µ2 protein is preserved throughout the extraction procedure. Original magnification × 63.

Add 10 mL of 10× stock CSK buffer, (Sigma), 0.8 mL of ribonucleoside–vanadyl complex (RVC) (New England Biolabs) and 0.2 mL of 400 m*M* 4-[2-aminoethyl] benzenesulfonyl fluoride (AEBSF).

3. Digestion buffer (DB): (10× Stock Solution): 0.5 *M* NaCl, 100 m*M* PIPES, pH 6.8, 30 m*M* $MgCl_2$, 10 m*M* EGTA 5% Triton X-100. Freshly prepare 1× DB as described above for 1× CSK buffer except for using 10× DB instead of 10× CSK buffer.
4. Phosphate-buffered saline (PBS): 9.1 m*M* dibasic sodium phosphate, 1.7 m*M* monobasic sodium phosphate, and 150 m*M* NaCl. Adjust pH to 7.4 with NaOH.
5. Fixatives: 3.7% formaldehyde in PBS (WC fixative), or in 1× CSK buffer (CSK fixative), or in 1× DB (nuclear matrin intermediate filament [NMIF] fixative). All fixatives should be freshly prepared.
6. Stop solution: 250 m*M* ammonium sulfate in 1× DB. (Add 1 volume of 2 *M* ammonium sulfate to 8 volume of 1× DB).
7. Permeabilizing solution: 0.25% Triton X-100 in PBS.
8. PBSA: 0.5% bovine serum albumin (BSA) in PBS. **Note:** Filter sterilize all solutions before use.

3. Methods

3.1. Whole Cell (WC) Preparation

Note: This method is for adherent cells. Biochemical sub cellular fractionation can be performed as described in **Fig. 2.**

1. Plate cells at a density of 0.5×10^6 cells per well and incubate in humidified incubator at 37°C.
2. After 24 h, wash cells twice with ice-cold PBS.

Biochemical Fractionation

Two 100mm plates per construct

Scrape cells in 1ml of 1XPBS containing protease inhibitor cocktail

Harvest cells in 300µl of direct lysis buffer; collect cell slurry, boil for 5 min. (**"WC" fraction**)

Collect cells by centrifugation at 14,000rpm for 15 seconds

Extract cell pellet with 150µl of CSK buffer for 10 minutes on ice

Spin samples at 14,000rpm for 10 minutes and transfer the supernatant to a clean tube (**"CSK fraction"**)

Repeat CSK extraction as above, pool the supernatant with previous one

Resuspend pellet in 150µl Digestion buffer containing 400U/ml of DNaseI for 25 minutes at room temperature

Spin samples at 14,000rpm for 10 minutes, collect supernatant (**"Nuclease fraction"**)

Repeat extraction with Digestion buffer as above, add ammonium sulfate to obtain a final concentration of 250mM, extract for additional 5 minutes

Spin for 10 min. at 14,000rpm, collect and pool supernatant with nuclease fraction

Resuspend pellet in 300µl of Direct lysis buffer; boil for 5 minutes (**"NM-IF"**)

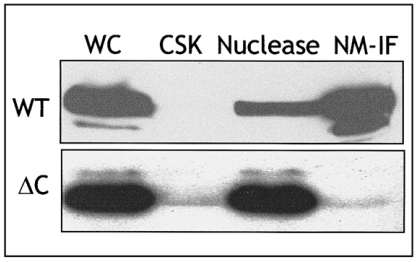

Fig. 2. Protocol overview for biochemical detection of regulatory molecules in subcellular compartments. A stepwise schematic diagram indicates the procedures required for detection of regulatory proteins in subcellular and subnuclear components of cell by biochemical fractionation. HeLa cells were transiently transfected with HA-tagged RUNμ2 expression constructs for wild type (1-528 amino-acids) and C-terminal deletion mutant ΔC (1-376 amino-acids). Cell pellets were subjected to extraction buffers and different fractions were collected as indicated. Equal volumes (30 μL) of all fractions were separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were immobilized onto a polyvinylidene difluoride membrane (Millipore) and probed with monoclonal anti-HA antibody. The full-length protein (WT) is resistant to high salt extraction and is tightly bound with the nuclear matrix. However the mutant protein (ΔC) is not associated with the nuclear matrix and is released into the nuclease fraction.

3. Fix the WC preparation on ice for 10 min (typically two wells of a six-well plate) by adding 2 mL of WC fixative per well.
4. Wash cells once with PBS.
5. To facilitate antibody staining of WC preparations, permeabilize WC preparations with 1 mL of permeabilizing solution on ice for 20 min.
6. Aspirate permeabilizing solution and wash twice with PBS.
7. Add 1 mL of PBSA in the wells.

3.2. Cytoskeleton (CSK) Preparation

If further subcellular fractionation is required, we recommend extraction be performed first followed by fixation and antibody labeling as described below.

1. Wash cells twice with ice-cold PBS.
2. Add 1 mL of 1× CSK buffer per well and incubate plates on ice for 15 min while swirling plates every 2–3 min.
3. Aspirate CSK buffer and add fresh 1 mL of 1× CSK buffer and incubate plates on ice for additional 15 min while swirling plates every 2–3 min.
4. Wash wells for CSK preparation (typically two wells of six well plates) once with ice-cold PBS and fix cells by adding 2 mL of CSK fixative per well.
5. Aspirate CSK fixative after 10 min and wash twice with PBS.
6. Add 1 mL of PBSA to the wells.

3.3. NMIF Preparation

1. Follow **steps 1–3** of **Subheading 3.1.2.**
2. Wash cells once with ice-cold PBS.
3. Prepare 1 mL of DB by adding 400 U of RNase free DNase I (Roche) to 1× DB.

4. Flattened parafilm on the covers of plates and dispense 20 µL drop of DB containing RNase free DNase I on the covers of respective plates. (This step is to conserve the amount of DNase I; otherwise, add 1 mL of DB containing RNase free DNase I to each well.)
5. Carefully place the cover slips for digestion of the chromatin with DNase I so that cells will face DB containing DNase I.
6. Incubate cells for 20 min at room temperature (20–22°C). In case the digestion with DNase I is partial (different degrees of low intensity signals will be detected with DAPI stain), we recommend carrying out first digestion at 28°C and second at room temperature. Place cover slips back in their respective wells, wipe the DB from the covers and repeat the DNase I digestion.
7. Place cover slips back in their respective wells. Add 1mL stop solution to the wells and incubate plates on ice for 10 min to stop the activity of DNase I.
8. Wash once with ice-cold PBS and fix NMIF preparations in 2 mL of NMIF fixative on ice for 10 min.
9. Aspirate fixative and wash twice with PBS.
10. Add 1 mL of PBSA.

3.4. Immunostaining of the Samples

1. Dilute antibody in PBSA to an appropriate dilution. We recommend several dilutions to be tested as quality and specificity of antibodies vary among suppliers and lots. While immunolabeling cells for two proteins, caution must be practiced to assure that the antibodies used are raised in different species (e.g., mouse vs rabbit). If raised in same species, they must be of different isotypes (e.g., IgG vs IgM).
2. Dispense a 20-µL drop of antibody dilution for each well on parafilm already flattened on the lids of plates. Carefully place cover slip on the drop so that the cells are in direct contact with the antibody. Avoid creating air bubble by gently placing the cover slips from one edge on the antibody. Incubate for 1 h at 37°C.
3. Place cover slips back in respective wells with cells facing upward and wash four times with ice-cold PBSA.
4. Stain cells with appropriate secondary antibodies conjugated with fluorochromes (e.g., Texas Red or fluorescein isothiocyanate) for 1 h at 37°C.
5. Place cover slips back in their respective wells and wash four times with ice-cold PBSA.
6. Stain cells with DAPI (0.5 µg of DAPI in 0.1% Triton × 100-PBSA) for 5 min on ice.
7. Wash once with 0.1% Triton × 100-PBSA followed by two washes with PBS. Leave cells in last wash on ice to avoid descication.
8. Immediately mount cover slips in antifade mounting medium (e.g., VectaShield) and air dry excess of mounting medium for 10–15 min. Seal cover slips and store at –20°C in the dark.

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