Note

Labeling of Polyethylenimine with Fluorescent Dye to Image Nucleus, Nucleolus, and Chromosomes in Digitonin-Permeabilized HeLa Cells

Masayuki Saito1 and Hisato Saitoh1,2,3,†

1Department of Biological Sciences, Graduate School of Science and Technology, Kumamoto University, Kumamoto 860-8555, Japan
2Department of New Frontier Sciences, Graduate School of Science and Technology, Kumamoto University, Kumamoto 860-8555, Japan
3Global Centers of Excellence (COE) Program, Global Initiative Center for Pulsed Power Engineering, Kumamoto University, Kumamoto 860-8555, Japan

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The visualization of nuclear architecture, which changes dynamically depending on the physiological and the pathological situation, remains an important challenge. Here we report that exposure of fluoresceinisothiocyanate-labeled polyethylenimine (FITC-PEI) to digitonin-permeabilized cervical cancer HeLa cells enable rapid detection of the morphology of the nuclear rim, the nucleolus, and mitotic chromosomes. This simple detection strategy can aid in scientific investigation for both basic research and diagnostic purposes.

Key words: polyethylenimine (PEI); nucleus; nucleolus; chromosome

Nuclear, nucleolar, and chromosomal architectures change dynamically depending on the physiological and the pathological situation.1–6) For instance, cells undergoing cell cycle progression display complete alteration, and alteration of nuclear and/or chromosome structures may be sufficiently characteristic of a given tumor type and stage for use in cancer diagnosis. Indentations, undulations, nuclear shape fold, enlarged nucleolus, and aberrant nucleolus and chromosomal numbers are often associated with particular types of cancer and several other human disorders.1–6)

Despite the importance of the visualization of such structural alteration to both basic research and diagnosis, practical staining methods are limited. Indirect immunofluorescence is a conventional fluorescence-based method used to visualize physiological and pathological changes in nuclear and chromosomal architectures. This method requires both selection of primary antibodies that bind specifically to antigens and fluorescent-labeled secondary antibodies and the purchase of these antibodies, making it expensive. Moreover, multiple incubation of antibodies with cells followed by washing to visualize the signals is time-consuming.

A large number of natural and synthetic polymers have been synthesized and characterized with respect to their interaction with nucleic acids, such as DNA and RNA. Among these are polyethylenimines (PEIs), cationic macromolecules that are inexpensive and widely used in DNA/RNA gene delivery experiments on higher eukaryotic cells.7–11) Here we describe our examination of the exposure of fluoresceinisothiocyanate (FITC)-labeled PEI to digitonin-permeabilized cultured human cells, including cervical cancer HeLa cells. The FITC signals were found not to be distributed randomly in permeabilized HeLa cells, but instead were concentrated around the interphase nucleus, at the nucleolus, and on the mitotic chromosomes. Based on this, we describe a novel method for the visualization of nuclear and chromosomal architectures, one that shows diagnostic potential.

The details of the experimental procedures and results are as follows: To prepare fluorescent labeled PEI, 50% PEI (Mn about 60,000, Mw 750,000) in H2O (Sigma-Aldrich, St. Louis, MO) was diluted by PBS to 20 mg/mL. FITC (Pierce, Rockford, IL) was dissolved in DMSO to a final concentration of 10 mM. The PEI solution was mixed with FITC for 2.5 h at 18 °C to a final concentration of 0.1 mM FITC. The reaction mixture was dialyzed (MWCO 3,500) against 1 liter of ice-cold PBS to remove any FITC not linked to PEI. After measurement of the final volume of the solution containing PEI labeled with FITC, the concentration of FITC-PEI was calculated to be 10 mg/mL. Although, those used throughout this study were FITC-PEI molecules prepared as described above, fluorescent-labeled PEIs such as jet-PEI™-FluoF (Polyplus-transfection SA, Illkirch, France) are commercially available, but because neither the concentration nor the amount of commercially available fluorescent-labeled PEI was sufficient to carry out the detection method we describe below, we do not recommend use of this reagent in applying the method.

Since labeling of PEI with FITC was expected to neutralize the PEI positive charges, it was thought possible that FITC-PEI might lose its DNA-binding activity. To determine whether this is the case, we performed a DNA mobility shift assay using λDNA (Promega, Madison, WI).9) Briefly, 1 µg of λDNA was

† To whom correspondence should be addressed. Tel/Fax: +81-96-342-3450; E-mail: hisa@kumamoto-u.ac.jp

Abbreviations: PEI, polyethylenimine; FITC, fluoresceinisothiocyanate; PBS, phosphate buffered saline; DMSO, dimethyl sulfoxide; DAPI, 4’,6-diamidino-2-phenylindole; PI, propidium iodide
digested with HindIII and EcoRI, and then the appropriate amount of non-labeled or FITC-labeled PEI stock solution was supplemented with PBS to 20 μL. As the control, a mixture containing no DNA with 0.1 μg of non-labeled PEI or FITC-PEI was subjected to gel electrophoresis (lanes 9 and 18).

Fig. 1. Gel Mobility Shift Assay for PEI-DNA Complexes. HindIII-EcoRI-digested λDNA (0.1 μg) was incubated for 5 min at room temperature with 0, 10, 20, 50, 60, 70, 80, and 100 ng of non-labeled PEI (left panel, lanes 1–9) or FITC-labeled PEI (right panel, lanes 10–18) in PBS followed by agarose gel electrophoresis. The gel was stained with SYBR green to visualize the DNAs. As the control, a mixture containing no DNA with 0.1 μg of non-labeled PEI or FITC-PEI was subjected to gel electrophoresis (lanes 9 and 18).

Next we incubated FITC-labeled PEI with permeabilized HeLa cell, and assessed the distribution of PEI in the cells, as illustrated in Fig. 2A. HeLa cells were grown on a coverslip in Dulbecco’s Modified Eagle’s Medium (containing 10% fetal bovine serum and antibiotics) at 37 °C in a 5% CO₂ incubator. The coverslip was rinsed briefly with ice-cold PBS, and the cells were then permeabilized for 5 min on ice with PBS plus 500 μg/mL of digitonin (Merck-Calbiochem, Darmstadt, Germany).

After digitonin permeabilization, the cells were rinsed twice with ice-cold PBS. Appropriate amounts of FITC-PEI were added to 1 mL of PBS, to a final PEI concentration 500 μg/mL. This mixture was then applied to the digitonin-permeabilized cells, followed by incubation for 10 min at 18 °C. The cells were washed once for 5 min with ice-cold PBS, and then fixed with 4% paraformaldehyde in PBS for 5 min at room temperature. After fixation, the cells were rinsed once with PBS, and a coverslip was applied using a mounting medium (2.5% 1,4-diazabicyclo[2.2.2] octane, DABCO, in 80% glycerol). Samples were analyzed with a DP72 microscope (Olympus, Tokyo). Fluorescent images were obtained using a U-MNIBA3 fluorescent mirror unit (excitation filter, 470–495 nm; emission filter, 510–550 nm). As shown in Fig. 2B, the HeLa cells displayed a non-uniform distribution of FITC signals in both interphase and mitotic cells. Both cell types accumulated FITC signals within min and they reached the maximum level at 10 min, suggesting that PEI targeted to these cell types with very rapid kinetics. Interphase cells clearly displayed enhanced FITC signals at the nuclear rim and one or more spots in the nucleus. These spots, we suggest, represent the nucleolus, since they can be merged with dark spots in the nucleus using a phase-contrast microscope, which is indicative of the nucleolus. To confirm this, we performed indirect-immunofluorescence analysis using antibodies specific to one of the nucleolus marker proteins, fibrillarin (Abcam, Cambridge, MA). As shown in Fig. 2C, the FITC-PEI-positive spots in the nucleus were co-stained with the antibody, indicating that the spots detected by FITC-PEI in the nucleus represented the nucleolus.

When interphase cells were observed at high magnification, FITC signals also appeared in the cytoplasm as several small intense spots, indicating the existence of specific regions with higher affinity to PEI in the cytoplasm (Fig. 2B, inset). In contrast to the interphase cells, mitotic chromosomes in the cells undergoing mitosis displayed FITC-PEI in a relatively uniform manner (Fig. 2B, arrows), suggesting that PEI can be efficiently targeted to and maintained on the entire region of each condensed chromosome. As a control, FITC and PEI were individually incubated with the permeabilized cells. Neither of them emitted fluorescent signals (data not shown). Taken together, our results indicate previous unnoted PEI behavior in permeabilized HeLa cells. PEI accumulates rapidly at the nuclear rim and in the nucleolus in interphase cells, and also has the ability to localize on mitotic chromosomes.

Our choice of digitonin for permeabilization in this protocol rather than non-ionic detergents such as Triton X-100, NP-40, or Tween-20 was due to the following: Digitonin is a widely used detergent-like molecule that preferentially extracts cholesterol from membranes, and selectively permeabilizes only a few membrane components of mammalian cells, e.g., the plasma membrane but not the endoplasmic reticulum or the nuclear membrane. This mild permeabilization of cellular membranes is often preferred for better preservation of cellular architecture, and makes digitonin a good choice for this method. Indeed, HeLa cell morphology and its intracellular structure appeared to be preserved after exposure of the cells to digitonin under our experimental conditions (Fig. 2B). In contrast, exposure of HeLa cells to Triton-X100 instead of digitonin often resulted in damaged cells. Many HeLa cells were detached from the culture dish, and large amounts of cellular proteins appeared to be released from the cells (data not shown), confirming that digitonin is for less prone than non-ionic detergents like Triton X-100 to extract membrane proteins from cells. Furthermore exposure of FITC-PEI to cells fixed with paraformaldehyde without digitonin permeabilization resulted in diffused accumulation of fluorescent signals without a substantial concentration at the nuclear rim and the nucleolus (data not shown). This argues that order of digitonin treatment and fixation in the procedure is critical to detect specific nuclear PEI signals.

Next, to test whether the observed PEI subcellular localization is specific to HeLa cells or rather is common to various human cell types, four different cultured human cell lines, embryonic kidney (HEK) 293, breast...
adenocarcinoma MCF7, neuroblastoma SH-SY5Y, and osteosarcoma U-2 OS, were cultured and exposed to digitonin, followed by incubation with FITC-PEI. After washing and fixing the cells, we detected fluorescence signals under the fluorescent microscope as described above. As shown in Fig. 3A, FITC-PEI was distributed in HEK293, MCF7, SH-SY5Y and U-2 OS cells in a pattern similar to that observed in HeLa cells. Interphase cells clearly displayed enhanced FITC signals at the nuclear rim and at one or more nucleolar spots in the nucleus. These results suggest that the subcellular localization of PEI shows a similar pattern among various human cell types when exposed to permeabilized cells.

Finally, we compared this fluorescent-labeled PEI method with standard nuclear/nucleolus staining methods using 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO), propidium iodide (PI; Invitrogen, Carlsbad, CA), and a commercially available nucleolus detection kit (Nucleolar-ID Green Detection Kit; Enzo Life Sciences, Farmingdale, NY). As shown in Fig. 3B, DAPI and PI visualized nuclear structures as effectively as the method that utilizes FITC-PEI. However, when we discriminated among staining patterns the PEI, DAPI, and PI-staining methods, we noted several features unique to FITC-PEI detection. In particular, FITC-PEI preferentially accumulated at the nucleolus and to a lesser extent in the nucleoplasmic region, whereas DAPI strongly stained chromatin regions in the nucleoplasm and poorly visualized the nucleolus, providing reversed contrast with respect to the nucleolar image. Comparing FITC-PEI staining with PI staining, we found that PI stained the nucleolus as well as the cytoplasmic region more strongly than PEI did, probably due to high-affinity binding of PI to RNA molecules, and noticed that PI did not clearly visualize mitotic chromosomes in the cells undergoing mitosis. The Nucleolar-ID detection kit stained the nucleolus clearly only in living HeLa cells (Fig. 3B). The fixed and permeabilized HeLa cells were barely stained by this
detection kit (data not shown), suggesting an advantage of the PEI method when researchers must obtain images of both the nucleus and the nucleolus in permeabilized cells. Although the molecular factors governing PEI localization in permeabilized cells and discriminating its staining pattern from that obtained by other conventional staining methods awaits further elucidation, these results emphasize the potential of a method that utilizes fluorescent labeled PEI to visualize nuclear morphological changes which has not been visualized by previously established detection protocols.

In sum, our results indicate that FITC-labeled PEI efficiently penetrates the nucleus and accumulates around the nuclear rim, in the nucleolus, and on mitotic chromosomes in digitonin-permeabilized HeLa cells. While fluorescent-labeled PEIs have been used as a trace-marker for DNA/RNA transfection efficiency and for trafficking of DNA/RNA-PEI-complex in transfected cells, their application in cell-staining to evaluate the physiological and pathological states of the cell nucleus, nucleolus, and chromosomes has not been reported to our knowledge. We anticipate that this method to be a new type of fluorescent-base evaluation of the physiological states of cells. In addition, given that PEI is inexpensive and obtainable commercially, in contrast with antibodies, and given the apparent rapid kinetics of PEI targeting in permeabilized human cells, both expense and time can be saved by our method in comparison with conventional indirect-immunofluorescence assays.

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