Immunonucleochemistry: a new method for in situ detection of antigens in the nucleus of cells in culture

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Abstract The advancement of immunocytochemistry (ICC) allows one to observe detailed spatial distribution of cellular antigens, but, with some limitations. Using conventional ICC, it is difficult to distinguish the nuclear localization from cytoplasm, as two large subcellular compartments overlap on the z-axis. In this study, we have investigated whether in situ immunostaining of ‘naked’ nuclei could provide an unambiguous method for detection of nuclear antigens. We have designed a protocol that efficiently lyses plasmalemma, while keeping the nuclear envelope intact. The optimal condition for lysing the plasmalemma was 0.5% Nonidet P-40 for 5 min in both neuronal and non-neuronal cultured cells. Using

this protocol, we could unambiguously isolate nuclear from cytoplasmic ICC signals. Since the present protocol has been designed for immunostaining of ‘naked’ nuclei from cultured or isolated cells, we have coined a new term to refer to this procedure as ‘immunonucleochemistry’ (‘INC’ for abbreviation).

Keywords Hippocampal neuron · Immunocytochemistry · Immunonucleochemistry · NP-40 · Nucleus

Abbreviations

NAGK	N-acetylglucosamine kinase
DIV	Day in vitro
HEK	Human embryonic kidney
ICC	Immunocytochemistry
IHC	Immunohistochemistry
INC	Immunonucleochemistry

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Introduction

A method for in situ examination of cellular phenomena is essential for the study of the spatio-temporal expression of specific antigens. Albert H. Coons and his colleagues published landmark papers describing the identification of tissue antigens using a direct fluorescence protocol (Coons et al. 1941, 1942). Since then, for this purpose, various

immunohistochemistry (IHC) techniques have been developed that include the use of enzyme labels such as peroxidase (Avrameas and Uriel 1966; Nakane and Pierce 1967) and alkaline phosphatase (Mason and Sammons 1978). Introduction of the colloidal gold label (Faulk and Taylor 1971) has further enabled identification of antigens at the electron microscopy level. In contrast to IHC, which is performed on tissue sections, immunocytochemistry (ICC) is performed on samples of intact cells that have had their surrounding extracellular matrix removed. This includes cells grown in culture, or taken from a smear.

An advantage of ICC is that it allows one to observe a more detailed spatiotemporal expression of cell antigens. However, the subcellular localization of antigens is ambiguous when the two compartments overlap on the *z*-axis. Among all compartments, the nuclei can be the most problematic, because the two relatively large compartments, nucleoplasm and cytoplasm, overlap. Theoretically, the optical dissection by confocal microscopy can differentiate the two compartments of ICC samples. However, delicate signals such as those associated with the nuclear envelope can be obscured by overlying cytoplasm (our observation). To potentially circumvent this problem, one can use methods developed to isolate the nuclear fractions, and then carry out Western blot analysis (Garrido-García et al. 2009; McEwen et al. 1980). However, cross contamination between the cytoplasm and nuclear fractions is inevitable due to problems including incomplete lysis of the plasmalemma and lysis of the nuclear envelope.

The goal of this study was to test a novel direct method for nuclear localization by immunostaining nuclei in situ after lysing the plasmalemma and washing out the cytoplasm components. After wash out of the cytoplasmic components, the nuclei were fixed and immunostained. This protocol efficiently removed the components of the cytoplasm, and resulted in the improved visualization of the nuclear morphology and more specific detection of nuclear antigens even by fluorescence microscopy, which is much more rapidly performed with less expensive equipment than required for confocal microscopy. Since the present method is designed for immunostaining ‘naked’ nuclei of cultured or isolated cells, we coined a new term ‘immunonucleochemistry’ (‘INC’) for this method.

Materials and methods

Antibodies

The following antibodies were used at the indicated dilutions: chicken polyclonal N-acetylglucosamine kinase (α NAGK) (1:1,000; GenWay Biotech, Inc., San Diego, CA, USA); monoclonal (MAb) tubulin α -subunit (α Tubulin) (1:2,000; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA); rabbit polyclonal phospho-H2AX (1:500; Upstate Biotechnology Inc., now a part of Millipore, Billerica, MA, USA); rabbit polyclonal nucleolin antibody (1:2,000; Sigma, St. Louis, MO, USA).

Cell cultures

Dissociated hippocampal cells from Sprague–Dawley rats removed on embryonic day 18 (E18) were plated onto 12-mm diameter polylysine/laminin-coated glass coverslips at a density of ~ 150 neurons/mm², as described by Brewer et al. (1993), and grown in Neurobasal medium supplemented with B27, as described by Goslin et al. (1998) and Moon et al. (2007). Human embryonic kidney 293T (HEK293T) cells were grown on polylysine-coated glass coverslips in MEM, supplemented with 10% heat-inactivated horse serum. Human colon adenocarcinoma cell line (HT-29), murine hepatoma cell line (Hepa1c1c7) and human breast cancer cell line (MDA-MB-231) were obtained from the Korean Cell Line Bank (Seoul, Korea). HT-29 and MDA-MB-231 cells were grown in 5% CO₂ at 37°C in a RPMI 1640 medium supplemented with 10% FBS (fetal bovine serum), penicillin/streptomycin, while Hepa1c1c7 cells were cultured in α -MEM medium, supplemented with 10% fetal bovine serum under the same conditions.

Lysis of plasmalemma and cell fixation

The coverslips were put on a glass slide with cell-side up, and rinsed briefly with ice-cold phosphate-buffered saline (PBS; 20 mM sodium phosphate buffer, pH 7.4, 0.9% NaCl). Lysis buffer containing Surfact-Amps[®] NP-40 (10% aqueous solution of Nonidet P-40; Pierce, Rockford, IL, USA; various concentrations diluted in PBS) was added for various times at room temperature (RT). The coverslips were

rinsed with several changes of PBS. The cells were fixed by a sequential paraformaldehyde/methanol fixation procedure (incubation in 4% paraformaldehyde in PBS at RT for 10 min followed by incubation in methanol at -20°C for 20 min) (Moon et al. 2007).

Immunostaining

The fixed cells were immunostained with the indicated primary and secondary antibodies [Alexa Fluor 488-conjugated goat anti-mouse, Alexa Fluor 568-conjugated goat anti-rabbit, and Alexa Fluor 647-conjugated goat anti-chicken IgG (each diluted 1:1,000 in preblocking buffer; Invitrogen, Carlsbad, CA, USA)] as described previously (Moon et al. 2007).

Light and fluorescence microscopy

A Leica Research Microscope DM IRE2 equipped with I3 S, N2.1 S and Y5 filter systems (Leica Microsystems AG, Wetzlar, Germany) was used for phase-contrast and epifluorescence microscopy. Images ($1,388 \times 1,039$ pixels) were acquired with a high-resolution CoolSNAPTM CCD camera (Photometrics Inc., Germany) under the control of a computer using Leica FW4000 software.

Results and discussion

Lysis of plasmalemma and exposure of neuronal nuclei

We first evaluated this novel method with neuronal cells notorious for cytoplasm and nuclear cross-contamination. In order to lyse the plasmalemma and uncover the nuclei of rat hippocampal neurons in primary culture, we used NP-40, commonly used in nuclear fractionation (Borun et al. 1967). NP-40 is a non-ionic detergent that can break the plasmalemma, but is not powerful enough to disrupt the nuclear envelope. Ice-cold NP-40 (0.5%, v/v) was added to the coverslips of hippocampal neurons in culture (17 days in vitro; DIV 17). After 5 min incubation at RT, the coverslips were rinsed in PBS. Serial phase-contrast images were taken from the top to bottom (z -axis) of the cell. A typical microscopic field is shown in

Fig. 1B with three enlarged images in the insets (*b1*, *b2*, *b3* in Fig. 1B); the z -axis level corresponds to slightly above the top surface, middle of the nucleus, and bottom surface, respectively. Clear demarcation of the nuclei could be seen in both neuronal and glial cells (black and white arrows, respectively, in *b2* and *b3* of Fig. 1B). The findings were in contrast to the corresponding images from the NP-40-untreated cells (Fig. 1A). In the NP-40-untreated neurons, no images were obtained with clear demarcation of the nucleus at any level of the z -axis through the soma (*a1*, *a2*, *a3* in Fig. 1A). Identification of clear nuclear demarcation in cells treated with NP-40 indicates lysis of the plasmalemma, and thus exposure of the nuclei. The contour of the cells could still be identified in NP-40-treated neurons (Fig. 1 asterisks) indicating that some of the components of the cytoplasm adhere tightly to the substratum and could not be removed; this phenomenon was severer in older cultures (see below).

Optimization for plasmalemma lysis

We first varied the concentration of NP-40 to determine the optimal conditions for lysis of the plasmalemma. Since the most commonly employed concentration is 0.5%, in the literature (Spector et al. 1998), we tested 0.1, 0.5, and 1.0% of NP-40. Typical phase-contrast images of DIV 17 neurons before and after treating with NP-40 (for 5 min at each concentration) are shown in Fig. 2. Clear nuclear demarcation was evident at 0.1% of NP-40 (white arrow in the *inset* of Fig. 2B), indicating that the plasmalemma was lysed at this concentration. Similar clear nuclear demarcation was observed at 1.0% NP-40, indicating that the nuclear envelope was not disrupted at this concentration (white arrow in the *inset* of Fig. 2D). For these relatively mature neurons, somatic (white asterisks in *insets*) and large dendritic (black arrows) contours were easily identified, indicating that even 1.0% NP-40 cannot completely lyse and remove somatic and dendritic components that are tightly attached to the substratum. However, thin dendrites were completely lysed at a high NP-40 concentration. For example, in the images taken from 0.1% NP-40-treated neurons, thin dendrites could be seen frequently (arrowhead in Fig. 2B); whereas such thin dendrites mostly disappeared in the 0.5 and 1.0% NP-40 treated neurons. Therefore, the optimal concentration of NP-40 would be 0.5% for lysing the neuronal plasmalemma.

Fig. 1 Serial z-stack phase-contrast images of rat hippocampal neurons cultured on coverslips (DIV 17). Typical phase-contrast images were taken from the top to bottom (z-axis) of the control (A) and cells treated with ice-cold NP-40 (0.5%, v/v) for 5 min (B). The boxed area, where the z-axis level corresponds to slightly above the top surface, middle of nucleus, and bottom surface, respectively, are shown enlarged

in the insets (a1-3, b1-3). Nuclear demarcation and cytoplasm components are marked by arrows and an asterisk, respectively. White arrows in B indicate nuclear demarcation of the glia. Note that the nuclear demarcation is clear in the NP-40 treated cells, indicating lysis of plasmalemma and exposure of 'naked' nuclei. Scale bar, 20 μ m

Optimal time for NP-40 treatment

Next, we sought to determine the optimum duration for NP-40 treatment. The experiments showed that it becomes more difficult to lyse dendritic and perikaryonic components as the neurons age in vitro (see below). Therefore, we used DIV 7 neurons, because at this DIV neurons usually entered the maturation stage (stage 5) (Dotti et al. 1988) but still exhibited sensitivity to NP-40. The neurons were treated with 0.5% NP-40, and serial images were taken up to 20 min. As shown in Fig. 3A, even 20 min of treatment did not completely lyse the dendrites (arrows), indicating that the dendritic components are intractable to lysis even at this stage of development, in vitro. However, they were significantly removed in the process of fixation and only traces of them could be seen (Fig. 3B, white arrows). In the images of fixed neurons, the nuclei were uncovered as noted by the observations of clear nuclear demarcation (black arrows in insets) in both 5 and 20 min-

treated samples. Therefore, treatment for 5 min appears to be optimal for the lysis of plasmalemma.

The nuclear envelope is not disrupted with NP-40 treatment

One would raise a possibility for the disruption of nuclear envelope and thereby leakage of nucleoplasmic components. To address this possibility, we have chosen two nucleoplasmic proteins, nucleolin and H2AX, and compared their distributions before and after NP-40 treatment. Nucleolin is one of the most abundant non-ribosomal proteins in the nucleolus (Bugler et al., 1982), and is also found in nucleoplasm and on the cell surface (reviewed in Mongelard and Bouvet, 2007). H2AX, which constitutes 2–25% of mammalian histone H2A, becomes rapidly phosphorylated on omega-4 serine upon induction of a DNA double-strand break (Paull et al. 2000; Redon et al. 2002; Rogakou et al. 1998). Both nucleolin and H2AX subserve as good markers for the nucleus. The

Fig. 2 Effects of NP-40 concentration on the lysis of plasmalemma. Rat hippocampal neurons (DIV 17) were treated with 0.0, 0.1, 0.5, and 1.0% NP-40 (A–D, respectively) for 5 min and washed. Typical phase-contrast images at the equatorial plane of nuclei on the z -axis were taken to show

the nuclear boundary (*arrows*). Somatic and large dendritic contours (*asterisks*) and thin dendrites (*arrowhead*) are marked. The *insets* represent the enlarged images of corresponding boxed areas. Scale bar, 20 μ m

rationale was such that their subcellular distributions would not be confined within the nuclei if the nuclear envelopes are to disrupt by NP-40 treatment. In NP-40-untreated images, arborisation of dendrites was clearly seen [Fig. 4A, NP-40 (–), phase], and nucleolin clusters were well confined within the nucleoplasm [arrowheads in Fig. 4A, NP-40 (–), nucleolin and merge]. After NP-40 treatment (0.5%, 5 min), most of the dendritic arborisation disappeared, and nuclei were uncovered [Fig. 4A, NP-40 (+), phase], indicating lysis of plasmalemma. However, the nucleolin clusters were still confined within the nucleoplasm [Fig. 4A, NP-40 (+), nucleolin and merge]. In fact, no single nucleolin cluster was found outside the demarcation of nucleoplasm in NP-40-treated samples in this study (the number of neurons observed = c.a. 500), and the average number of nucleolin clusters per nucleus was statistically not different from NP-40-untreated samples. Nuclear confinement and no difference in the average number of immunoreactive puncta per nucleus were also true for H2AX (Fig. 4B). Interestingly, a circle that

surrounds nucleoplasm could be identified in NP-40-treated samples [Fig. 4B, NP-40 (+) H2AX, arrow]. This circle was not seen in NP-40-untreated samples [Fig. 4B, NP-40 (–), H2AX, arrow]. Since the immunostain signal was sharply demarcated by this circle, it strongly suggests that this circle represents nuclear envelope. Confinement of nucleolin and H2AX clusters were also true for murine hepatoma cell line (Hepalcl1c7), human colon adenocarcinoma cell line (HT-29), and human breast cancer cell line (MDA-MB-231), and a circle demarcating nucleoplasm was frequently observed in H2AX immunostains (data not shown). Taken together, these results indicate that nuclear envelopes remain intact after NP-40 treatment.

Neuronal age-dependent sensitivity to dendritic lysis

As mentioned above, we observed that the amount of somatodendritic remains, after NP-40 treatment, depends on the age of the neurons in culture.

Fig. 3 Effects of NP-40 treatment time on the lysis of plasmalemma. Rat hippocampal neurons (DIV 17) were treated with 0.5% NP-40. Phase-contrast images of live cells after 2, 5, 10, 20 min (**A**), and after washing and fixing (**B**). Dendrites are marked by *white arrows*. Inset **B**, nuclear demarcation (*black arrows*) and cytoplasm contours (*asterisks*) are marked. Scale bar, 20 μ m

Therefore, we investigated the sensitivity of dendritic lysis at different ages in culture. The neurons at various DIV (4 h to DIV 21) were treated with 0.5%

NP-40 (5 min, RT), and representative images are shown in Fig. 5. To monitor the lysis of dendrites, the neurons were immunostained with an antibody to the

Fig. 4 Nuclear envelopes are not disrupted after NP-40 treatment. Rat hippocampal neuronal cultures (DIV 17) were treated with NP-40 (5%, 5 min), fixed and immunostained with nucleolin (**A**) or H2AX (**B**) antibody. Phase-contrast (*phase*), immunostain, and merge images are shown. Dendritic arborisations are clearly seen in NP-40 untreated samples [NP-40 (–)] but not in NP-40-treated ones. Note that the nucleolin clusters and H2AX puncta (*arrowheads* in **A** and **B**, respectively) were confined within the nucleoplasm, and note the sharp nuclear demarcation line in the H2AX immunostain images of NP-40-treated cells [**B**, NP-40 (+), *inset*, *arrow*] but not in NP-40-untreated ones (*arrow* in the *inset* of **B**). Scale bar, 20 μ m

α -subunit of tubulin, a component of microtubules in somatodendritic compartments. The dendrites of neurons at early developmental stages (4 and 17 h in culture) were completely lysed (Fig. 5, Phase and Tubulin). However, residual dendrites were noted in DIV 3 neurons, and there were more and more dendritic remains observed as the number of DIVs increased (Fig. 5, arrows). The more extensive and tighter attachment to the substratum may have resulted in a more incomplete lysis of the dendrites. The integrity of the nuclei was well maintained, as evidenced by phase-contrast and immunostaining images of H2AX, a nuclear marker.

Application to non-neuronal cells

To test if this novel method works on other types of cells, we first applied the present protocol to study human embryonic kidney 293T (HEK293T) cells. Similar to neuronal cells, treatment with 0.1% NP-40 for 5 min was enough to lyse the plasmalemma and uncover the nuclei (Fig. 6A, arrow). However,

extensive remnants of the cytoplasm were present at this concentration (Fig. 6A, 0.1%, asterisk). Increasing the NP-40 concentration to 0.5% significantly reduced the presence of cytoplasmic remnants (Fig. 6A, 0.5%, asterisk). However, higher NP-40 concentration (1.0%) did not differ from 0.5%. Similar results were obtained with other cell types such as Hep1c1c7, HT-29, and MDA-MB-231 (Fig. 6B–D, respectively). Therefore, 0.5% of NP-40 for 5 min appears to be optimal for lysing both neuronal and non-neuronal plasmalemma.

The present protocol is useful for distinguishing nuclear from perikaryonic expression

Our laboratory has been studying the developmental expression of *N*-acetylglucosamine kinase (NAGK) in rat hippocampal neurons in culture. ICC experiments showed that the immunostaining signals increased in the soma until developmental stage 3 (axonal outgrowth) (manuscript in preparation). However, it was not clear whether the signals were associated with the

Fig. 5 Neuronal age-dependent sensitivity to dendritic lysis. Rat hippocampal neurons of the indicated DIV (4 h to DIV 21) were treated with 0.5% NP-40 for 5 min, and doubly immunostained with antibodies against H2AX (B) and α -tubulin (C), a nuclear and a somatodendritic marker, respectively. Phase-contrast (phase) (A) and fluorescence images are shown in parallel. Dendrites are marked by arrows. Scale bar, 20 μ m

nucleus or perikaryon (the cytoplasm around nucleus) or both (Fig. 7A). Such ambiguities could be resolved by the novel method reported here. Immunostaining after lysing the plasmalemma revealed several NAGK immunoreactive puncta in the nucleus (Fig. 7B, arrowheads). The rest of the signals observed by conventional ICC were not noted, indicating that most of the signals in the soma were actually from the perikaryon. Therefore, our new protocol, ‘immunonucleochemistry’ (‘INC’) clearly distinguished nuclear from perikaryon signals.

The present protocol can be applied to any nuclei isolated from different sources. The strategy for isolation of nuclei should depend on their source. When nuclei are isolated from tissues, a homogenation

step is incorporated, after which the resulting homogenate is passed over a sucrose gradient to purify the nuclei (Spector et al. 1998). With regard to isolation of nuclei from cells grown in suspension, the cells can be lysed either by osmotic swelling followed by homogenation in a Dounce-type homogenizer or by treatment with mild non-ionic detergent such as Triton X-100, under isotonic conditions, followed by passing through a needle (Belgrader et al. 1991; Spector et al. 1998). However, the hypotonic swelling step can lead to a certain degree of leakage of the nuclear components (Weinberg and Penman 1968; Zieve and Penman 1976). Although isotonic conditions reduce leakage, the long procedure increases the possibility of leakage occurring.

Fig. 6 Efficient lysis of non-neuronal plasmalemma by the present protocol. **A** Human embryonic kidney 293T (HEK293T) cells. Cells were treated with 0.0, 0.1, 0.5 and 1.0% NP-40. Phase-contrast images after 5, 10 min, and after washing are shown. Nuclear demarcation (*arrows*) and cytoplasmic remnants (*asterisk*) are marked. Note that a clear nuclear boundary is seen in the 0.1% NP-40 treated cells; however, there are significant cytoplasmic remnants at this concentration. (*B–D*) Other cell types. Murine hepatoma cell line (Hepa1c1c7) (**B**), human colon adenocarcinoma cell line (HT-29) (**C**), and human breast cancer cell line (MDA-MB-231) (**D**) were treated with 0.5 and 1.0% of NP-50 for 5 min, and typical phase-contrast images are shown. Sharp nuclear demarcations are not seen in NP-40-untreated samples (*before*), but were evident in NP-40-treated samples (*arrows*). Scale bar, 20 μ m

Conclusion

We have presented a novel method for immunostaining ‘naked’ nuclei after lysing of the plasmalemma. The suggested optimal conditions for lysing the

plasmalemma included treatment with 0.5% NP-40 for 5 min for both neuronal and non-neuronal cells in culture. However, it may be necessary to adjust the concentration of NP-40 and treatment time depending on the particular cell type. The procedure is

Fig. 7 Isolation of nuclear signals from perikaryonic expression by the present protocol. Developmental stage 3 (axonal outgrowth) neurons in culture were immunostained with an antibody against NAGK with (B) or without (A) NP-40 treatment according to the present protocol (0.5%, 5 min).

Lysis of the plasmalemma by the present protocol removed strong immunostaining signals from the soma (A), and isolated several NAGK immunoreactive puncta in the nucleus (B). Scale bar, 20 μ m

Fig. 8 Flow chart of the present protocol for ‘immunonucleochemistry’

schematically presented in a flow chart in Fig. 8. This new protocol referred to as ‘immunonucleochemistry’ (‘INC’), would be useful for isolating nuclear signals from those that originate in the cytoplasm.

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