Video Article

A Simple Cell-based Immunofluorescence Assay to Detect Autoantibody Against the N-Methyl-D-Aspartate (NMDA) Receptor in Blood

Chia-Hsiang Chen[1,2], Yu-Syuan Chang[1]

[1]Department of Psychiatry, Chang Gung Memorial Hospital-Linkou

[2]Department and Graduate Institute of Biomedical Sciences, Chang Gung University

Correspondence to: Chia-Hsiang Chen at cchen3801@gmail.com

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Abstract

The presence of anti-NMDA receptor autoantibody can cause various neuropsychiatric symptoms in the affected patients, termed anti-NMDA receptor autoimmune encephalitis. Detection of the specific autoantibody against the NMDA receptor in the blood or cerebrospinal fluid (CSF) is essential for the accurate diagnosis of this condition. The NMDA receptor is an ion channel protein complex that contains four subunits, including two mandatory NMDA receptor subunit 1 (NR1) and one or two NMDA receptor subunit 2A (NR2A), NMDA receptor subunit 2B (NR2B), NMDA receptor subunit 2C (NR2C), or NMDA receptor subunit 2D (NR2D). The epitope of anti-NMDA receptor autoantibody was reported to be present at the extracellular N-terminal domain of the NR1 subunit of the NMDA receptor. The goal of this study is to develop a simple cell-based immunofluorescence assay that can be used as a screening test to detect the presence of autoantibodies against NR1 subunit of the NMDA receptor in the blood to facilitate the clinical and basic research of anti-NMDA receptor autoimmune encephalitis.

Video Link

The video component of this article can be found at https://www.jove.com/video/56676/

Introduction

Anti-NMDA receptor autoimmune encephalitis is a newly recognized disease entity that can occur in patients of all ages, and affects predominantly female patients[1,2]. It is one of the most frequently diagnosed encephalitis among patients with initial unknown etiology of encephalitis[3]. Patients affected with anti-NMDA receptor encephalitis usually have prodromal symptoms of a headache or fever, followed by the quick development of consciousness level change and a variety of acute neuropsychiatric symptoms, including agitation, irritability, anxiety, insomnia, hallucinations, delusions, aggression, bizarre behaviors, movement abnormalities, autonomic dysregulation, and seizure attacks[4,5]. Early recognition of this condition and timely treatment with immunotherapy are important for a better outcome and even full recovery in affected patients[6]. Hence, it is suggested that anti-NMDA receptor autoimmune encephalitis should be considered as an important differential diagnosis of patients presenting with acute or new-onset psychotic features[7,8].

Besides clinical features, detection of autoantibody against the NMDA receptor in the blood or CSF is essential for the accurate diagnosis of anti-NMDA receptor autoimmune encephalitis[9]. Most of the immunological tests to detect the anti-NMDA receptor autoantibody are available in a few research laboratories[10,11], and there is only one commercially available cell-based immunofluorescence assay for screening the anti-NMDA receptor autoantibodies[12]. The goal of this study is to develop a simple in-house cell-based immunofluorescence assay that can be conveniently used in the laboratory to screen the presence of anti-NMDA receptor autoantibodies to facilitate the clinical research of anti-NMDA receptor autoimmune encephalitis. The NMDA receptor is a heterotetramer ion channel protein complex specifically expressed in the brain. It is made of two compulsory NR1 subunits, and the combination of one or two subunits of NR2A, NR2B, NR2C, or NR2D[13]. A previous study reported that the main epitope targeted by the antibodies was at the extracellular N-terminal domain of the NR1 subunit[5]. Hence, in this protocol, we express the human recombinant NR1-subunit protein of NMDA receptor tagged with green fluorescent protein (GFP) in the human embryonic kidney epithelial cell line (HEK293), and develop a cell-based immunofluorescence assay to detect the IgG class of anti-NMDA receptor autoantibodies in the blood.

Protocol

The study was approved by the Institutional Review Board of Chang Gung Memorial Hospital at Linkuo, Taoyuan, Taiwan (102-2577A3).

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1. Preparation of the NR1-GFP Expression Plasmid

1. Mix 10 ng of NR1-GFP plasmid with 100 µL of *Escherichia coli* competent cells strain DH5α in a sterile 1.5 mL centrifuge tube, pipet the mixture gently up and down 4-6 times, and incubate the tube on ice for 20 min.
2. Incubate the tube at 42 °C for 1 min in a water bath, remove the tube from the water bath, add 1 mL lysogeny broth (LB) medium into the tube, and incubate the tube at 37 °C in an incubator with shaking for 1 h.
3. Spread 50 µL of the mixture onto an LB agar plate containing ampicillin (0.1 mg/mL), and incubate the LB agar plate at 37 °C in an incubator overnight.
4. Pick a single colony from the overnight LB agar plate using a sterile tip, and swirl the tip in a in a bacterial culture tube containing 5 mL of LB medium and ampicillin (0.1 mg/mL). Incubate the tube at 37 °C in an incubator with shaking overnight.
5. Aliquot 3 mL of the overnight bacterial culture into a 500 mL flask that contains 250 mL sterile LB medium and ampicillin (0.1 mg/mL). Incubate the flask at 37 °C with shaking. Check regularly the optic density (OD) of the bacterial culture at wavelength of 600 nm using a spectrometer until the OD600 reaches 0.6-1.0.
   NOTE: Mix well 800 µL of the overnight bacterial culture with 200 µL glycerol to prepare the glycerol stock, and store the glycerol stock under -80 °C for future use.
6. Prepare NR1-GFP expression plasmid from the 250 mL bacterial culture
   1. Collect the cells by centrifugation at 6,000 x g for 15 min at 4 °C.
   2. Resuspend the bacteria pellet in 10 mL resuspension buffer containing RNase A; vortex thoroughly until no clump is seen.
   3. Add 10 mL lysis buffer to the bacterial suspension, gently invert the mixture 4-6 times, and incubate the lysate at room temperature (RT) for 5 min.
   4. Add 10 mL pre-chilled precipitation buffer to the lysate, gently invert the mixture 4-6 times.
   5. Pour the lysate into the barrel of a filter cartridge with the cap on; wait for 10 min at RT.
   6. Remove the cap from the cartridge outlet nozzle, insert a plunger into the cartridge, and gently press the plunger. Collect the filtered lysate into a 50 mL tube.
   7. Add 2.5 mL proprietary buffer from the manufacturer to the filtered lysate, mix the mixture by inverting the tube approximately 10 times, and incubate the mixture on ice for 30 min.
   8. Apply the filtered lysate mixture to a filter column that was pre-equilibrated with 10 mL equilibration buffer. Allow the column to drain by gravity.
   9. Wash the column with 30 mL wash buffer twice, then elute DNA from the column using 15 mL elution buffer.
   10. Add 10.5 mL (0.7 volumes) isopropanol to the eluted DNA buffer, mix well, and centrifuge the mixture at 20,000 x g for 30 min at 4 °C.
   11. Decant the supernatant carefully, wash the DNA pellet with 5 mL endotoxin-free 70% ethanol once, and centrifuge at 20,000 x g for 10 min.
   12. Decant the supernatant, dry the pellet for 5-10 min in a chemical hood, and dissolve the pellet in 300-500 µL of endotoxin-free buffer.
   13. Determine the plasmid concentration by measuring the absorption of the solution at UV 260/280 nm using a spectrophotometer.
       NOTE: Prepare the expression plasmid GFP using the same protocol.

2. Transfection of HEK293 Cells with NR1-GFP Expression Plasmid

1. Aliquot 200 µL of 2% gelatin solution to each well of a 48-well culture plate and incubate the plate at 37 °C for at least 30 min.
2. Aspirate the gelatin solution from the well, and seed 5 x 10^4 HEK293 cells in 200 µL of cell culture medium containing 10% fetal bovine serum (FBS) in each well. Incubate the plate at 37 °C in a humidified incubator supplied with 5% CO_2 overnight.
   NOTE: Cells were counted using a hemocytometer.
3. On the next day, replace the spent culture medium with 200 µL of fresh culture medium containing 10% FBS per well.
4. For each single well, prepare solution A by mixing 100 ng of NR1-tGFP plasmid with 20 µL culture medium, and solution B by mixing 0.8 µL transfection reagent with 20 µL culture medium. Multiply the number of wells needed to prepare the total volume for each experiment.
5. Prepare solution C by mixing solution A and solution B, and incubate the mixture at RT for 20-30 min.
6. Aliquot 40 µL of solution C to each well containing the HEK293 cells, swirl the plate gently, and incubate the plate at 37 °C in a humidified incubator supplied with 5% CO_2 overnight.
7. Check the expression of NR1-GFP recombinant protein in the host cells under fluorescent microscope with 40X-200X magnification (excitation/emission: 482/502 nm), and proceed to the cell-based immunofluorescence assay.
   NOTE: Transfect the expression plasmid GFP into the HEK293 cells using the same protocol.

3. Cell-based Immunofluorescence Assay

1. Aspirate the spent medium from the wells, then wash each well with 200 µL of phosphate buffered saline (PBS) three times.
2. Add 200 µL of 4% paraformaldehyde solution to each well; incubate the plate at RT for 15 min.
3. Aspirate the paraformaldehyde solution from the wells, and wash each well with 200 µL of PBS three times.
4. Add 200 µL of 10% skim milk in PBST (0.1% Tween-20 in PBS) solution to each well, and incubate the plate at RT for 1 h with gentle shaking.
5. Aspirate the 10% skim milk in PBST solution from the well, then wash the wells with 200 µL PBST once.
6. Incubate the wells with diluted plasma (1:100 in PBST) as the primary antibody with gentle shaking at RT for 1 h.
   NOTE: Plasma is obtained from the blood of patients suspected to have autoimmune encephalitis.
7. Aspirate the diluted plasma from the wells, and wash each well with 200 µL of PBST three times.
8. Add 200 µL of goat anti-human IgG conjugated with Alexa Fluor 594 (1:1,000 dilution in PBST) to each well, and incubate the culture plate at RT with gentle shaking for 1 h.
9. Aspirate the goat anti-human IgG conjugated with Alexa Fluor 594 solution, and wash each well with 200 µL of PBST three times.
10. Add 100 µL glycerol solution (50% glycerol in PBS and 1:100,000 of 4',6-diamidino-2-phenylindole (DAPI)) to each well.

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11. Observe and image the cells under a fluorescent microscope using a 10X eyepiece, and 4X, 10X, and 20X objectives.
NOTE: Use the correct filter for each dye: for NR1-GFP (excitation/emission = 482/502 nm), for Alexa Fluor 594 (excitation/emission = 561/594 nm), for DAPI (excitation/emission = 358/461 nm). Conduct the negative control using the HEK cells expressing GFP in the same way.

Representative Results

On average, we could obtain 200-300 µg endotoxin-free expression plasmid NR1-GFP and GFP from 250 mL bacterial culture following the procedures described in section 1 of the protocol. An amount of 100 ng/well of the expression plasmid was used for the transfection of the HEK293 cells cultured in the 48-well plate as described in the section 2 of the protocol. 24-30 h after transfection, the cells expressed the NR1-GFP, and GFP recombinant proteins could be detected under fluorescent microscope. **Figure 1A** shows the image of the host cells that express the NR1-GFP, while **Figure 1D** shows the cells expressing GFP. The green signal can be used as a quality controller of the assay: approximately 30% of the cells had the green signals under the fluorescent microscope. **Figure 1A** and **1D** show the image of the host cells that express the NR1-GFP. The green signal can be used as a quality controller of the assay: approximately 30% of the cells had the green signals under the fluorescent microscope. The cells expressing NR1-GFP were used for screening the presence of anti-NMDA receptor autoantibodies in the human plasma sample.

In the cell-based immunofluorescence assay as described in the section 3 of the protocol, the positive control sample (obtained from a commercial source, see **Table of Materials**) was added to the pooled plasma in 1:10 dilution according to the manufacturer's instructions. The pooled plasma was prepared from 5 adult males and 5 adult females. **Figure 1B** is the Alexa Fluor-594 image of positive control sample after incubation with the cells expression NR1-GFP, while **Figure 1E** is the Alexa Fluor-594 image of positive control sample after incubation with the cells expression GFP. **Figure 1C** is the merged image of **Figure 1A** and **Figure 1B**: there are significant overlaps of green signals and red signals in the same cell, indicating the co-localization of NR1-GFP and the antibodies against the NR1 subunit of NMDA receptor (arrows). A plasma sample that shows greater than 30% overlaps of green and red signals will be interpreted as positive in this case. **Figure 1F** is the merged image from **Figure 1D** and **Figure 1E** that serves as the negative control for **Figure 1C**. The weak red color is background fluorescence of the Alexa Fluor-594 image. There is little overlap of green and red signals, indicating no binding of the anti-NMDA receptor autoantibody against the recombinant protein.

During the establishment of the experimental procedures, we observed a low signal-to-noise ratio of the Alexa Fluor-594 image when the plasma samples were tested. We attempted to optimize the signal-to-noise ratio by conducting a serial dilution of the plasma form 1:10, 1:50, 1:100, and 1:200, and testing different concentrations of Alex Fluor-594 labeled secondary antibody from 1:500 to 1:2,000. We found that 1:100 dilution of the plasma and 1:1,000 or 1:2,000 dilution of the Alexa Fluor-594 were the optimal conditions for the interpretation of the data.

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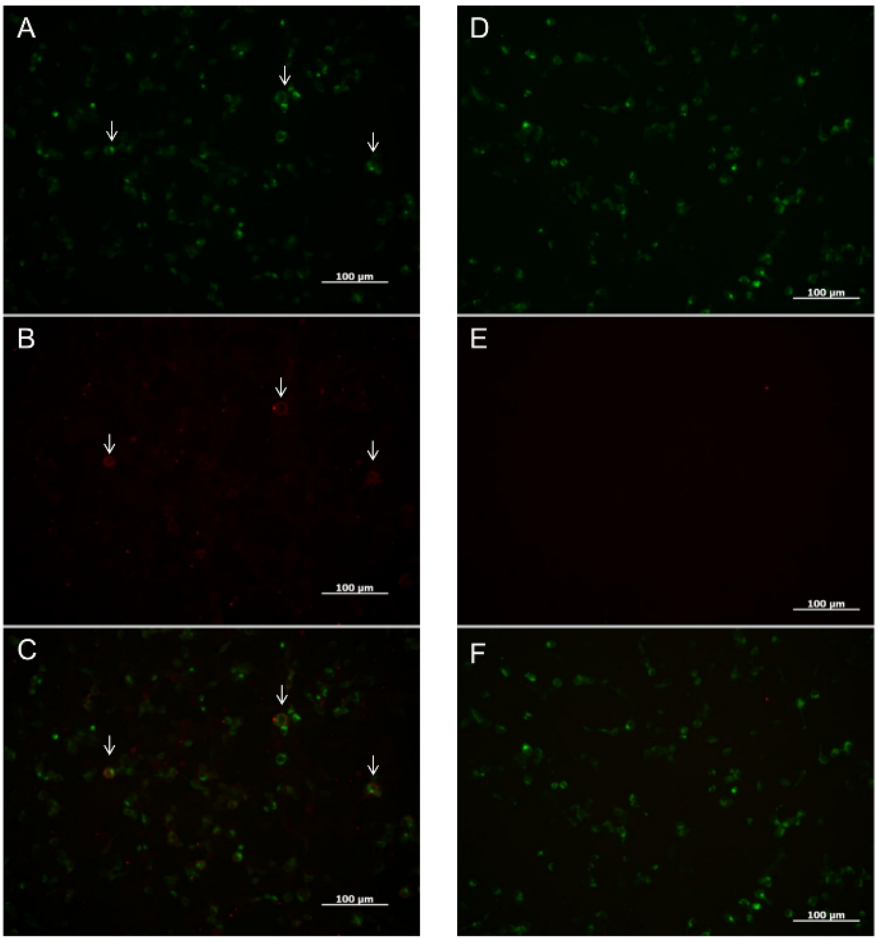

Figure 1: Representative images of the cell-based immunofluorescence assay to detect anti-NMDA receptor autoantibody. (**A**) Image of the HEK293 cells that express NR1-GFP. (**B**) The Alexa Fluor-594 image taken from the same field of **A** after incubation with the positive control sample. Red signal indicates the binding of the anti-NMDA receptor autoantibodies to the NR1 expressed in the HEK293 cells. (**C**) The merged image of **A** and **B**. Yellow color indicates the co-localization of the NR1 (green signal) and the anti-NMDA receptor autoantibodies (red signal). Arrows indicate examples of some prominent cells showing co-localization of NR1-GFP and anti-NMDA receptor autoantibodies. (**D**) Image of the HEK293 cells expressing NR1-GFP. (**E**) The Alexa Fluor-594 image taken from the same field of **D** after incubation with the positive control sample. Weak red signal indicates the background noise of Alexa Fluor-594 image. (**F**) The merged image of **D** and **E**. This image serves as a negative control, as there is little yellow signal observed. The negative control image shows the specific binding of the anti-NMDA receptor antibodies to the NR1-GFP in the assay. All the images were taken under 10X eye lens and 20X lens objective. Please click here to view a larger version of this figure.

Discussion

There are several cell-based immunological assays to screen the presence of autoantibodies against NMDA receptor reported in the literature, including live cell-based immunofluorescence assay[11], fixed cell-based immunofluorescence assay[9], and flow cytometry-based assay[14]. The live cell-based immunofluorescence assay should be performed shortly after the preparation of cells ectopically expressing the NR1 protein, while the flow cytometry-based assay requires trained personnel and special equipment. A fixed cell-based immunofluorescence assay is a more convenient assay that can be conducted for clinical settings. Hence, many studies used the commercial indirect fixed cell-based immunofluorescence assay to screen anti-NMDA receptor autoantibody in serum and CSF specimens[12,15]. The kit used (see the table of materials) contains the fixed HEK293 cells that ectopically express the recombinant NR1 subunit of NMDA receptor. The assay has been evaluated to have excellent performance in detecting the anti-NMDA receptor IgG antibody in the serum and CSF specimens[12]. In our protocol, we used the same approach as that recommended by the kit manufacturer, except that we use NR1-GFP expression plasmid. The NR1-GFP protein can be used to monitor the transfection efficiency and distribution of NR1 protein in each experiment. Hence, it can be used as a quality assurance of the assay.

The transfection efficiency of NR1-GFP expression plasmid into the HEK293 cells is approximately 30% in the protocol presented here, which is sufficient for the immunofluorescence assay. However, we found that the intensity of the green fluorescence is not evenly distributed among the transfected cells, suggesting differences in efficiency of transfection or expression ability of individual cells. The varying expression levels of recombinant NR1-GFP in different cells cause heterogenous intensity when the green image was merged with the red image of Alexa Fluor 594. The goat anti-human IgG conjugated with Alexa Fluor 594 was used as the secondary antibody to detect the presence of human autoantibody IgG class. We found that the brightness of Alexa Fluor 594 is relatively weaker than that of the green fluorescent protein. We tried to optimize the signal intensity by increasing the concentration of the anti-human IgG conjugated with Alex Fluor 594, however, the background increased

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as well. Hence, the optimal concentration for the anti-human IgG conjugated with Alex Fluor 594 is 1:1,000 in this laboratory. Also, some plasma samples had a significantly high background signal in the Alexa Fluor 594 image. Different dilutions of plasma were tested, and it was found that a minimal dilution of 1:100 is necessary to obtain a clean background for most of the samples. Hence, we recommend 1:100 dilution of plasma as the standard step in this protocol. Compared with the other cell-based assays that can detect the serum sample in 1:10 and 1:20 dilution of blood sample[11,16], this current protocol does not have enough sensitivity to detect low titer anti-NMDA receptor autoantibodies, which is a limitation of the protocol.

The current protocol is a fixed cell-based immunofluorescence assay. The culture plate can be stored in 4 °C refrigerator after fixation for later use for up to 1 month, in comparison to the live cell-based assay that cannot be stored for a long time. However, the NR1 protein will be denatured and lose its natural conformation during the fixation procedure, which may affect the conformation of the epitope that binds to the anti-NMDA receptor autoantibodies. Hence, some studies adopted live cell-based immunofluorescene[11,17]. Our protocol can be alternatively modified to become a live cell-based immunofluorescence assay to preserve the natural conformation of the NR1 protein, if we incubate the plasma sample with the live cells first, and fix the cells later. Thus, there is flexibility in the protocol to meet the need of the experiment.

The NMDA receptor is a heterotetramer complex protein consisting of NR1, NR2A, NR2B, NR2C, and NR2D. The current protocol was designed to detect the IgG class of autoantibody against NR1 subunit of the NMDA receptor. The protocol can also be modified to detect different isotypes of autoantibodies, as long as the anti-human IgG secondary antibodies are replaced by the other specific class of secondary antibodies. Furthermore, the current protocol can be modified to detect the presence of autoantibodies against NR2A, NR2B, NR2C, and NR2D, if the NR1-GFP expression plasmid is replaced with the corresponding expression plasmid, respectively.

In this protocol, we used a plasma specimen instead of serum, because we routinely collect blood sample with anti-coagulant for various study purposes, but the protocol can apply to serum or CSF specimens. The titer of the autoantibodies in the plasma can be quantified by serial dilution of the sample. In the laboratory, we also tested the African green monkey kidney fibroblast-like cell line (COS1) as the host cells using the same protocol. The results are the same as those using the HEK293 cells, except that the COS1 cells have a slightly lower transfection efficiency than the HEK293 cells.

To ascertain the presence of the anti-NMDA receptor autoantibodies, it is suggested that additional experiments such as immunohistochemistry assay using rodent brain tissues and immunocytochemistry using primary cultured rodent neurons be performed[5,9]. Hence, the current protocol can only be considered as a screening assay. Although its experimental validity was verified using the positive control sample in this study, the sensitivity and specificity of this assay in clinical settings need to be established in future study.

Disclosures

The authors have nothing to disclose.

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