Chapter title:

Protocol for the study of Connexin and DNA interactions.

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Summary

Connexins (Cxs) are transmembrane proteins which form hemichannels and gap junction channels at the plasma membrane. These channels allow the exchange of ions and molecules between the intra and extracellular space and between cytoplasm of adjacent cells respectively. The channel function of Cx assemblies has been extensively studied, however "non-canonical" functions have emerged in the last few decades and have capture the attentions of many researchers, including the role of some Cxs as gene modulators or transcription factors. In this chapter, we describe a protocol to study the interaction of Cx46 with DNA in HeLa cells. These methods can facilitate understandanding the role of Cxs in physiological processes and pathological mechanisms, including, for example, the contribution of Cx46 in maintaining stemness of glioma cancer stem cells.

Keywords: Connexins, Chromatin Immunoprecipitation (Chip), DNA interactions, immunoprecipitation, Cx46

1. Introduction

Connexins are a family of proteins that usually are present at the plasma membrane forming hemichannels or gap junction channels [1]. Although hemichannels and gap junction channels are formed by the same types of proteins, they participate in different cellular functions. Thus, while hemichannels allow the exchange of molecules and ions between the cytoplasm and the extracellular milieu, gap junction channels allow the exchange of molecules and ions directly from a cytoplasm on one cell to the cytoplasm and neighboring cells [2, 3]. The channel-dependent way of action is known as "canonical" function, and it has been extensively studied over many years. However, also a "non-canonical" role of Cxs

has emerged in the last years [4]. A very interesting mechanisms of action is related to the capacity of Cx43 and Cx46 to be localized in the cell nucleus and modulate gene expression [5–7]. However, the molecular mechanisms underlying these phenomena, remain elusive.

Here, we present a protocol for cell transfection, Cx46-DNA interaction and analysis of Cx46-DNA interaction (see Figure 1). This protocol will be helpful for future experiments in the field of Cx-based gene regulation in both cellular physiology and pathology.

2- Materials

2.1 Cell culture

1. HeLa cells.
2. Transmitted light microscope.
3. Bench cooled-centrifuge.
4. 60 mm cell culture plates.
5. Incubator maintained at 37°C and 5% CO_2.
6. Borosilicate glass Pasteur pipettes.
7. Cell culture medium: Dulbecco's Modified Eagle Medium (DMEM) low glucose, supplemented with 10% Fetal bovine serum (FBS) and Penicillin (1,000 U/mL)/streptomycin (1,000 U/mL) (Complete DMEM). Stored at 4°C, warmed before use.
8. 100 ml autoclaved glass bottle.
9. Sterile biosafety cabinet.
10. Trypsin/EDTA stock solution (10x, 0.5% trypsin) is stored at -20°C. The working (1x) solution is stored at 4°C and warmed before use.

2.2 HeLa cells transfection

1. Transmitted light microscope.

2. 35 mm cell culture plates.
3. pCAG-Cx46 mammalian expression plasmid.
4. Cooled bench centrifuge.
5. Incubator maintained at 37°C and 5% CO_2.
6. Vacuum line.
7. Complete DMEM, stored at 4°C.
8. Lipofectamine™ 3000 Reagent, stored at 4°C.
9. Phosphate-Buffered saline (PBS) (Sodium chloride 0.137M, Potassium Chloride 0.027M, Sodium Phosphate Dibasic 0.01M, Potassium Phosphate Monobasic 0.018M, pH 7.4), stored at 4°C.
10. Opti-Minimal Essential Medium (OptiMEM), stored at 4°C.
11. Sterile biosafety cabinet.
12. Blasticidine-HCl selection antibiotic.

2.3 Nuclear extracts and DNA fragmentation

1. 100 mm cell culture plates.
2. 37% Formaldehyde stock.
3. Orbital shaker.
4. 1.25 mM Glycine stock solution.
5. Cell Scraper.
6. 15 ml Falcon tube.
7. Cooled bench centrifuge.
8. Lysis buffer (50mM HEPES pH: 7.8, 3mM MgCl2, 20mM KCl, 0.1% NP-40, 2mM phenylmethylsulfonyl fluoride (PMSF), 2 µg/mL Aprotinin, 2 µg/mL Leupeptin and 1 µM Pepstatin).
9. Tripan blue.
10. Basket with ice.
11. Tight Dounce homogenizer.
12. 1.5 mL Eppendorf tubes.

13. Sonication Buffer (50 mM HEPES pH: 7.8, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium Deoxycholate, 1% Sodium Dodecyl Sulfate, 2mM PMSF, 2 µg/mL Aprotinin, 2 µg/mL Leupeptin and 1 µM Pepstatin).
14. Bioruptor sonication tubes (Diagenode).
15. Neubauer Chamber.
16. Bioruptor Pico sonication device.

2.4 Agarose gel

1. Agarose.
2. TAE Buffer (40 mM Tris base, 20 mM Acetic acid and 1 mM EDTA).
3. DNA samples.
4. UV transilluminator.
5. SYBR safe nucleic acid gel stain.
6. 1 kb DNA ladder.
7. Any commercial 6X DNA Loading Buffer.

2.5 Chromatin Immunoprecipitation.

1. Fractioned DNA samples.
2. Sonication buffer without Sodium Dodecyl Sulfate.
3. Mouse IgG linked to protein G.
4. Orbital shaker.
5. Cooled bench centrifuge.
6. M-280 Sheep Anti-Mouse IgG dynabeads.
7. Anti-Connexin 46 antibody (Santa Cruz Biotechnology, C-3).
8. Magjet rack 2x1,5 mL (Thermo Scientific)
9. Immunoprecipitation (IP) buffer (100 mM Tris HCl pH: 8.0, 500 mM LiCl, 1% NP-40 and 0.1% Sodium Deoxycholate).
10. TE buffer (10mM Tris-HCl and 1 mM EDTA), pH: 8.0.
11. Elution buffer (50 mM NaHCO3, 1% SDS).
12. Refrigerator at -20°C.

2.6 Decrosslinking and purification of DNA

1. 5 M NaCl.
2. , 10 mg/mL RNase stock solution.
3. Thermoregulated bath.
4. 50 mg/mL proteinase K solution.
5. TE buffer, pH 8.0.
6. Phenol:Chloroform:Isoamyl Alcohol (1:1:1).
7. Chloroform:Isoamyl Alcohol (24:1).
8. Cooled bench centrifuge.
9. 1.5 ml Eppendorf tubes.
10. Absolute ethanol.
11. 3 M sodium acetate, pH 7.0.
12. 20 mg/mL glycogen solution.
13. Refrigerator at -20°C.
14. Fluorimeter for DNA quantification.

3- Methods

3.1 Culturing HeLa cells

1. Warm complete DMEM in a 37°C water bath for 15 minutes prior to use.
2. Culture HeLa cells in sterile 60 mm tissue culture dishes that are housed in an incubator maintained at 37°C and 5% CO_2 (*see* **Note 1**).
3. Passage HeLa cells once they reach a ~90% confluence (*see* **Note 2**).
4. For subculturing, 60 mm tissue culture dishes are removed from the incubator. Check cell viability using a light microscope and tripan blue exclusion.
5. Attach an autoclaved borosilicate glass Pasteur pipette to a vacuum line and aspirate the growth medium from the dish.
6. Gently rinse the cell monolayer with 1–2 mL of sterile PBS. Use the Pasteur pipette to aspirate PBS between washes.

7. Add 1 ml of trypsin/EDTA 0.05% and place the dish in the incubator for 3 min. Remove the dish from the incubator and check whether cells have detached using a light transmitted microscope.
8. Once cells are detached, add 1 mL of complete DMEM to inhibit the trypsin.
9. Remove cells using a 1000 ml pipette and place the cell suspension in a 2 ml Eppendorf tube.
10. Centrifuge the tube at 2,000 rpm for 5 min at room temperature.
11. Remove trypsin and add 1 mL of complete DMEM. Gently resuspend the cell pellet by pipetting up and down to break apart any cell aggregates.
12. Count the cell number using a Neubauer chamber.
13. Calculate the volume required for seeding $1x10^5$ cells and add this volume to a new 60 mm tissue culture dishes.
14. Add 4 mL of thawed complete DMEM.

3.2 HeLa cells transfection

1. Warm complete DMEM and OptiMEM in a 37ºC water bath.
2. Check that cell confluence is below 60% using a light microscope.
3. Label two 1.5 mL microcentrifuge tubes for each transfection reaction.
4. Thaw aliquots of plasmid pCAG-Cx46 at room temperature, and place in biosafety cabinet along with Lipofectamine 3000.
5. For each transfection reaction, prepare a tube containing 117.5 µL OptiMEM and 7.5 µL Lipofectamine™ 3000 Reagent. Prepare another tube containing a volume equivalent to 1 µg plasmid DNA and 2 µL of P3000 reagent in a final volume of 125 µL of OptiMEM.
6. After 5 min, mix both tubes by gently pipetting.
7. Incubate the tube with the mixture for 5 minutes at room temperature.
8. Remove the 35 mm dishes containing HeLa cells and place them in the safety chamber.
9. Add the transfection solution drop-by-drop onto the cells throughout the 35 mm cell culture dish containing 2 mL of complete DMEM.
10. Return the cells to the incubator and incubate overnight.
11. Wash twice with PBS and replace the media-containing plasmid with complete DMEM.

12. After 2 weeks, it is possible to select stable transfected cells by adding 10 μg/mL of blasticidine to the complete DMEM,
13. If cells reach more than 90% confluence, repeat steps from 6 to 11 adding 10 μg/mL of.blasticidine

3.3 Nuclear extracts

1. Rinse twice the cell cultured in 100 mm plates (at ~80% confluence) with 5 mL of PBS at room temperature.

2. Add 5 mL of formaldehyde at 37% in PBS to bring it to a final concentration of 1% for each plate and perform crosslinking for 10 minutes in orbital agitation (*see* **Note 3**).

4. Add 0.5 mL of 1.25 mM glycine solution and incubate for 5 min at room temperature and constant orbital agitation.

3. Discharge fixing solution and rinse with 5 ml of PBS x 3 times (*see* **Note 4**).

6. Discharge the last PBS rinse and harvest cells using 5 mL of PBS containing protease inhibitors.

7. Place the cell suspension is in a 15 ml Falcon tube and centrifuge for 5 min at 2,000 g and 4°C.

8. Resuspend the pellet in 1.5 mL lysis buffer and count the cells with trypan blue to obtain the total number of cells.

9. Incubate the cell suspension on ice for 10 min.

10. Homogenize the cell suspension with a 2 mL Tight Dounce homogenizer by applying ~10 strokes. Check with trypan blue if the nuclei have been purified.

11. Transfer the homogenized to a 1.5 mL Eppendorf tube.

12. Centrifuge for 5 min at 500 g and 4°C, and discharge supernatant.

13. Resuspend the pellet in 2 mL of Sonication Buffer and transfer it to a Bioruptor sonication tubes (Diagenode). Add a maximum volume of 500 μL of resuspended nuclei.

16. Sonicate for 50 min at 30 s intervals using a Bioruptor Pico sonication device. Check for rupture of the nuclei using trypan blue in a Neubauer Chamber.

17. Centrifuge at 10,000 g for 5 minutes at 4°C.

18. Transfer the supernatant to a new 1.5 mL Eppendorf.

19. To assess the degree of chromatin fragmentation, perform rapid decrosslinking of an aliquot in a thermocycler at 95°C for 25 minutes, then add 2 µg/mL of RNase A and incubat at 37°C for 15 minutes. Finally, add 50 µg/mLof Proteinase K and incubate for 15 minutes at 58°C.

20. Qunatify uncrosslinked fragmentated chromatin using the Qubit dsDNA BR Assay Kit on a Qubit Fluorometer following the manufacturer's instructions.

3.4 Agarose gel

1. Prepare a 1% agarose gel in TAE buffer.

2.- Mix 100 ng of each DNA sample, 3 µl of 6X loading buffer and enough H_2O to a final volume of 18 µl.

3. Load this mixture in the agarose gel.

4. Run gel at 100 Volts until the sample buffer is located at approximately 50% of the gel.

5. Place the gel in a UV transilluminator and examine the level of chromatin fragmentation, ideally, it should be between 200 and 300 bp (*see* **Note 5**).

3.5 Chromatin immunoprecipitation protocol

1. Take 200 ng of DNA from non-immunoprecipitated samples and keep it as control (Input) at -20ºC.

2. To 400 ng of fragmented DNA (from point 3.3.16) add enough sonication buffer without Sodium Dodecyl Sulfate to complete a final volume of 500 µL.

3. Add 2 µL of mouse IgG (12-371, Merck Millipore) linked to protein G in the tube containing fragmented chromatin.

4. Incubate this mixture for 2 h under orbital agitation at 4°C.

5. Centrifuge at 2,000 g for 5 minutes at 4°C and transfer the supernatant to a new 1.5 mL tube.

6. Add 50 µL of M-280 Sheep Anti-Mouse IgG dynabeads and 2 µg of anti-Connexin 46 antibody (Santa Cruz Biotechnology, C-3).

7. Incubate overnight under constant rotation at 4ºC.

8. Place the tube over a magnet until all the beads are concentrated at the bottom.

9. Discharge the supernatant and rinse the pellet with 500 µL of IP wash buffer for 5 minutes in orbital shaking.

10. Repeat steps 8 and 9 once more.

11. Discharge the supernatant and rinse the beads with 500 µL of TE buffer, pH: 8.0, for 5 minutes in orbital shaking.

12. Repeat steps 8 and 9 once more.

13. In the last rinse, remove the supernatant and resuspend the in 100 µL of elution buffer. Place in in a vortex mixer and run at 2,800 rpm.

14. Incubate samples 15 min at 65 °C in a thermoregulated bath, then store the supernatant at -20°C.

3.6- Decrosslinking and purification of DNA

1. Add the necessary volume of elution buffer to the control samples (Inputs) to reach a final volume 100 µL (*see* **Note 6**).

2. Add 4 µL of 5 M NaCl to the immunoprecipitated what and to the input tubes to obtain a final concentration of 200 mM.

3. Add 2 µg/mL of RNase A and incubate overnight in a thermoregulated bath at 65°C.

4. Add 50 µg/mL of Proteinase K and incubate in a thermoregulated bath for 2 hours at 50°C.

5. Add 200 µL of TE buffer (pH 8.0) and 300 µL of Phenol:Chloroform:Isoamyl Alcohol (1:1:1).

6. Invert the tubes upside down and then returns it to its normal position 4-6 times.

7. Centrifuge at 12,000 rpm for 10 minutes at 4°C

8. Recover the aqueous phase and place it in a 1.5 tube Eppendorf.

9. Add 300 mL of Chloroform:Isoamyl Alcohol (24:1) and invert the tube upside down and then return it to its normal position 4-6 times.

10. Centrifuge at 12,000 rpm for 10 minutes at 4°C.

11. Recover the aqueous phase and place it in a 1.5 tube Eppendorf. Add 2.5 volumes of absolute ethanol, 0.1 volumes of 3 M Sodium Acetate pH 7.0 and 20 µg of Glycogen.

12. Place the mixture at -20°C overnight.

13. Centrifuge at 7,500 g for 25 minutes at 4°C.

14. Rinse the pellet with 500 µL of 70% ethanol.

15. Centrifuge again for 10 min at 7,500 x g for 25 minutes at 4°C (*see* **Note 7**).

16. Discharge supernatant and resuspend the pellet in 30 µL of TE buffer or 100 µL in the case of inputs.

14. Measure DNA concentration using the Qubit HS dsDNA Assay Kit (see Note 8).

15. The DNA samples are now ready for qPCR or sequencing.

PLACE FIGURE 1 HERE

4- Notes

Note 1. This protocol was adjusted for HeLa cells. However, it can be applied also to other cell lines.

Note 2. It is important not let the cell grow beyond 90% confluence, to avoid cell stress, which can alter expected results.

Note 3: If the type of interactions between a given Cx and the DNA is unknown, different crosslinker can be used with different spacer sizes, like EGS (Ethylene glycol bis (sulfosuccinimidyl succinate).

Note 4. The fixing solution must be descarded in a proper vessel.

Note 5. The size of fragmentated DNA could be more than 300 bp but never more than 500 bp.

Note 6. Control samples are fragmented butnot immunoprecipitated DNA.

Nota 7. Let the pellet dry but not too much, because otherwise resuspension turns problematic.

Nota 8. Dilute the DNA 1:10 in water because the quantification is more reliable than in TE.

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Figure Legends:

Figure 1. Scheme of Chip protocol. Briefly, cells are grown in a plastic dish, then proteins that are interacting with the DNA are crosslinked, to preserve these interactions. Cell nuclei are purified and sonicated to disrupt their content and to break down the DNA in small fragments. Subsequently, an antibody against Cx46 is added to the fragmented DNA, incubated and precipitated to obtain only DNA fragments that interact with Cx46. Finally, Cx46 and DNA crosslink is reversed, and free DNA fragments can be analyzed by different techniques, as for example, sequencing.

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