

Co-immunofluorescence of MRPL12 and Nrf2 in HK2 Cells

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[Abstract] Immunofluorescence is a technique to visualize the localization of specific molecule targets within cells using the specificity of antibodies. Here, we describe a protocol to detect two different protein components in a cell simultaneously. Antibody concentrations to be used vary from cell to cell and should be optimized for different cell types. In this protocol, we perform co-immunofluorescence of mitochondrial ribosomal protein L7/L12 (MRPL12) and nuclear factor erythroid 2-related factor 2 (Nrf2), a potential transcription factor of MRPL12, in HK-2 cells, as an example. Taking advantage of the diverse set of antibodies raised in different species, we are able to analyze the colocalization and expression of these proteins.

Keywords: MRPL12, Nrf2, HK-2 cells, Co-immunofluorescence staining, Colocation, Semi-quantitative analysis

[Background] The mitochondrial ribosomal protein L7/L12 (MRPL12), a member of the mitochondrial ribosomal protein (MRP) family, plays pivotal roles in mitochondrial function, including mitochondrial ribosome assembling (Surovtsevaa *et al.*, 2011; Kühl *et al.*, 2016), mtDNA transcription (Surovtsevaa *et al.*, 2011; Nouws *et al.*, 2016), mitochondrial ATP production (Marty and Fort, 1996), and mitochondrial oxidative phosphorylation (OXPHOS) (Ma *et al.*, 2020). As a canonical molecule coordinating the expression of antioxidant genes (Hayes and Dinkova-Kostova, 2014; Dinkova-Kostova and Abramov, 2015), nuclear factor erythroid 2-related factor 2 (Nrf2) is predicted to be a potential transcription factor for MRPL12 (Gu *et al.*, 2021). To examine the regulation of Nrf2 on the expression of MRPL12, co-immunofluorescence was applied to identify the abundance and colocalization of the two proteins. Here, we performed an indirect immunofluorescence technique where the sample cells were incubated with primary antibodies raised in different species, which were detected by fluorophore-conjugated secondary antibodies. Using this co-immunofluorescence technique, we can simultaneously analyze the distribution of the proteins and perform a semi-quantitative analysis to elucidate the interaction between the two molecules indirectly.

Materials and Reagents

1. 24-well cell culture plate (Corning, Costar[®], catalog number:3524)
2. Cell culture flask, T25 (Corning, Costar[®], catalog number:430639)
3. Round coverslip, 9 mm (Biosharp, catalog number: BS-09-RC), place the coverslips in a 75% ethanol-containing centrifuge cube
4. Adhesion microscope slides (CITOTEST, catalog number: 80312-3161)
5. Parafilm (Bemis, catalog number:4157)
6. For cell preparation:
 - a. Dulbecco's Modified Eagle Medium (DMEM), 1g/L D-Glucose, with L-Glutamine (Gibco, catalog number: C11885500BT). Store at 2-8°C for 1 year
 - b. Fetal Bovine Serum (FBS) (Gibco, catalog number:10099141). Aliquot and store at -20°C
 - c. Penicillin-Streptomycin (10,000U/ml) (Gibco, catalog number:15140122). Store at -20~-5°C for 1 year
 - d. Phosphate Buffered Saline (PBS), sterile-filtered (Servicebio, catalog number: G4202). Store at 2-30°C for 18 months
 - e. 0.25% Trypsin-EDTA, (Gibco, catalog number: 25200-072). Store at -20~-5°C for 24 months
7. Phosphate buffered saline (PBS) powder (Absin, catalog number: abs9266). Store at room temperature (RT) for 2 years
8. 4% paraformaldehyde (PFA) (Solarbio, catalog number: P1110). Aliquot and store at -20°C for 1 year, and avoid repeated freezing and thawing. Store at 4°C for up to a month
9. Triton X-100 (Solarbio, catalog number: T8022). Store at RT for 2 years
10. Normal goat serum (Solarbio, SL038). Store at -20°C for 5 years
11. Primary antibodies:
 - a. MRPL12 antibody (Novas, catalog number: 3B12-1A3). Aliquot and store at -20°C. Avoid freeze-thaw cycles
 - b. Nrf2 antibody (Abcam, catalog number: ab62352). Aliquot and store at -20°C. Avoid freeze-thaw cycles
12. Secondary antibodies:
 - a. Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 488 (Thermo Fisher Scientific, Invitrogen, catalog number: A32723). Store at 4°C in the dark
 - b. Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 594 (Thermo Fisher Scientific, Invitrogen, catalog number: A32754). Store at 4°C in the dark
13. Hoechst 33342 (Life technologies, catalog number: HH-H3570). Aliquot and store at -20°C in the dark
14. Mounting medium, antifading (Solarbio, catalog number: S2100). Store at 4°C for 1 year
15. Cell culture medium (see Recipes)
16. PBS (see Recipes)
17. Permeabilization solution (see Recipes)

18. Blocking solution (see Recipes)

Equipment

1. Tweezers

Note: The tweezers should be sterile when conducting cell preparations. The subsequent steps do not require sterile conditions.

2. Paper towel

3. (Optional) Wet chamber

4. (Optional) Refrigerator (Haier, model: BCD-328WDPT)

5. Constant temperature incubator (Panasonic, model: MIR-262-PC)

6. Nikon inverted fluorescence microscope (Nikon, model: Ti-S)

7. Clean bench (Airtech, model: SW-CJ-1FD)

8. Directly heated CO₂ incubator (Thermo Fisher Scientific, model: 240i)

Software

1. ImageJ

2. Prism GraphPad

Procedure

A. Cell preparation

1. Place coverslips in 24-well plate

a. Take out coverslips from the 75% ethanol-containing centrifuge tube using sterile tweezers and place them on a sterile plate. Expose them to ultraviolet (UV) light for approximately 30 min with the other required material, including PBS, trypsin, culture medium (DMEM medium with 10% FBS and 1% penicillin-streptomycin, see Recipe 1), and 24-well cell culture plate.

b. Ensure that both sides of the coverslips are dry to avoid cells being killed by the remaining ethanol.

c. Drop approximately 5 μ l cell culture medium on each well. Place dry coverslips on the drop with sterile tweezers. Press the coverslips gently and remove the excess fluid to ensure they are firmly stuck to the bottom of the well.

2. Cell seeding and culture

a. Remove the medium from the cell culture flask (T25) containing the cells and wash with 2 ml PBS. Remove the PBS and add 500 μ l 0.05% trypsin for 2 min to detach cells from the cell culture flask wall.

b. Seed a number of cells according to the experimental requirements, e.g., transfecting with

siRNAs or overexpression plasmids (see Note 1). Shake the plate crosswise and lengthwise to prevent the local growth of cells from being too dense. Then allow cells to grow at 37°C in an incubator with 5% CO₂. Remember to add control groups when performing the colocalization (see Note 8).

- c. Treat cells in accordance with the experimental requirements. Aim at cell densities resulting in 70-80% confluency to avoid cells overlapping.

B. Immunofluorescence staining

1. When cells grow to 70-80% confluency, remove the medium and rinse once with 500 µl PBS for 5 min. The following steps do not need to be performed under sterile conditions.
2. Unfreeze the 4% PFA in advance. Add 300 µl of 4% PFA to each well for 20 min at RT.
3. Remove the fixative and rinse cells with PBS three times for 5 min (see Note 4).
4. Remove the PBS and add 300 µl of permeabilization solution to each well for 5 min at RT.
5. Remove the permeabilization solution and rinse with PBS three times for 5 min.
6. Block with 300 µl of blocking solution for 1 h at 37°C.
7. Remove the solution and rinse with PBS three times for 5 min.
8. Primary antibody incubation:
 - a. Dilute antibody A (MRPL12, 1:200) and antibody B (Nrf2, 1:300) with PBS (see Notes 5, 6, 7).
 - b. Add several drops of PBS on adhesion microscope slides, and then place a piece of parafilm on each slide. Expel bubbles to ensure the film tightly attaches to the slide.
 - c. Clamp out coverslips from the plate with tweezers and touch the edge of the paper towel to remove the remaining solution.
 - d. Put coverslips on the parafilm with cell-facing upward and add approximately 50 µl diluted antibody to cover it fully.
 - e. To avoid loss of the antibody by volatilization, incubate the slides in a wet chamber overnight at 4°C.
9. Optional step: After overnight incubation, keep slides at room temperature for approximately 30 min.
10. Lift up coverslips and put them back into wells with the cell-facing surface facing up. Rinse with PBS three times for 5 min.
11. Secondary antibody incubation:
 - a. Dilute the secondary antibody Alexa Fluor[®]-488 (1:1,000) and Alexa Fluor[®]-594 (1:1,000) with PBS (see Notes 5, 6).
 - b. Add 300 µl secondary antibody solution to each well.
 - c. Incubate the samples at 37°C for 1 h in the dark.
12. After secondary antibody incubation, protect the cells from light exposure. Remove the secondary antibody and rinse with PBS three times for 5 min.

13. To visualize nuclei, add the PBS-diluted Hoechst 33342 (1:1,000) to the coverslips for 10 min at RT.
14. Remove the solution. Rinse with PBS three times for 5 min.

C. Mounting, storage, and imaging

1. Label microscope slides with the sample information. Drop approximately 5 μ l antifade mounting medium for each coverslip on the glass slide. Then, place the coverslips on the mounting medium with the surface containing the cells facing down and ensure that there are no bubbles. Remove the excess fluid if needed.
2. Samples can be stored for approximately 1 week at 4°C or 2 months at -20°C in the dark. Longer storage is not recommended.
3. Use a Nikon inverted fluorescence microscope to capture images from five different fields on each sample; select regions randomly (Figure 1). The excitation wavelengths of Hoechst 33258, Alexa Fluor 488, and Alexa Fluor 594 are 345 nm, 499 nm, and 591 nm, respectively. Record fluorescence from individual channels separately.

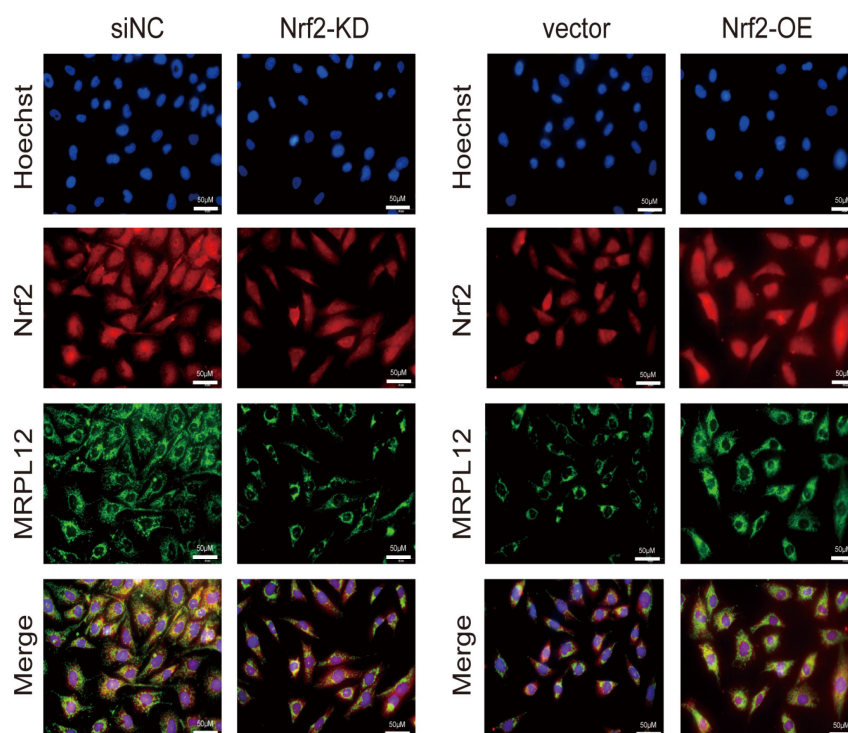


Figure 1. Immunofluorescent staining in Nrf2 overexpressed or knocked down HK-2 cells. HK-2 cells were fixed with methanol. Nrf2 (red), MRPL12 (green), and nuclei (blue) were stained as described in the protocol. Images were captured and merged using a Nikon inverted fluorescence microscope. Scale bar is 50 μ m. Figure 1 reprinted with permission from Gu *et al.* (2021).

Data analysis

In our published studies, we quantitatively analyzed the fluorescence intensity of HK2 cells among compared groups.

1. Fluorescence intensity determination

The fluorescence in stained HK2 cells is estimated using ImageJ. To quantify the fluorescence, first open the image in ImageJ, then choose Image > type > 16-bit to convert the image to grayscale. Afterward, go to Analyze > Set Measurements and choose “Area,” “Mean gray value (Mean),” and “Integrated intensity (IntDen)” as measurement parameters. Next, choose Analyze > Tools > ROI Manager. Randomly select a stained cell followed by Add[t] > Rename. Then mark another two cells and three spots of fluorescence background through the above method. Select all items and click “Measure” (Figure 2).

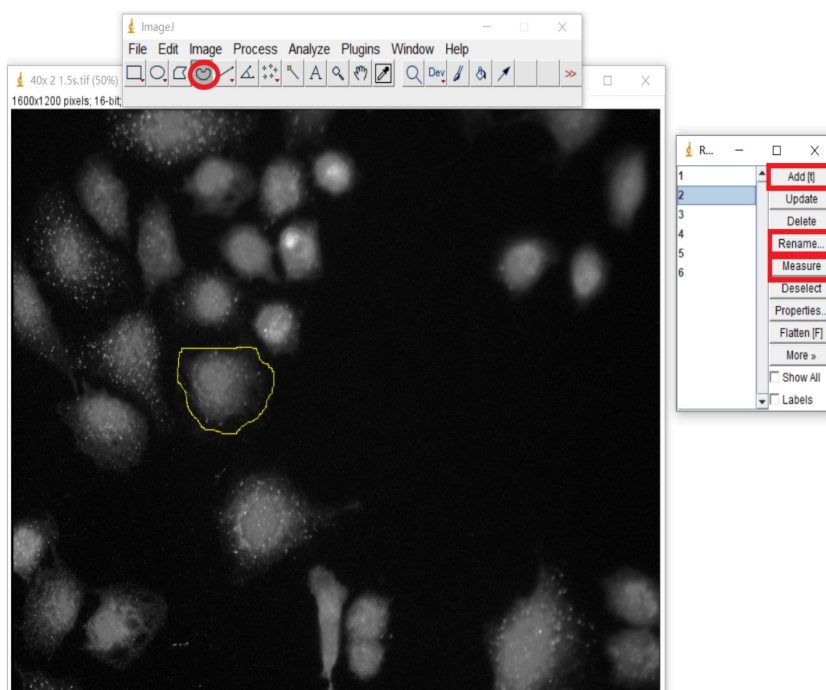


Figure 2. Fluorescence intensity determination using ImageJ. Select the measuring object and calculate with ROI Manager. Circle a cell randomly (oval highlighted) followed by Add[t] (rectangle highlighted) > Rename (rectangle highlighted). Three cells and three spots of fluorescence background are totally marked. Select all items, then choose “Measure” (rectangle highlighted).

2. Statistical analysis

Use the following formula to determine the corrected integrated intensity values for each cell:
Corrected IntDen (cell) = IntDen (cell) - Area (cell) × Mean (background).

Plot the corrected integrated intensity values of HK-2 cells into column graphs and assess statistical significance with the Student’s *t*-test using Prism GraphPad (Figure 3).

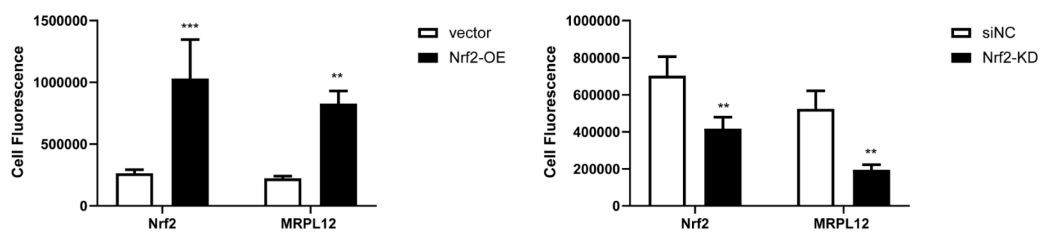


Figure 3. Statistical analysis for fluorescence intensity of Nrf2 and MRPL12 expression in Nrf2 overexpressed or knocked down HK-2 cells. Data are presented as the mean \pm S.D. (n = 3). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Figure 3 reprinted with permission from Gu *et al.* (2021).

Notes

1. It is also feasible to transfect siRNAs or overexpression plasmids in 6-well plates and passage the transfected cells to a 24-well plate.
2. Protect the cover glass from drying out throughout the entire procedure. For example, change solution quickly and ensure that solutions perfectly cover the cells.
3. To ensure cells will not be rinsed out during the whole protocol, place the pipette tip at the edge of the well to draw up the fluid and gently add the new solution to the sidewall of the well.
4. Pause point: There could be a pause after rinsing the fixative. The fixed samples can be stored at 4°C for approximately 1 week if required. Nevertheless, we suggest continuing with the next procedures immediately. Longer storage is not recommended.
5. The two antibodies must have different species origins, and the secondary antibodies must have different exciting light wavelengths.
6. The concentration of the antibody may be determined based on antibody instructions or on data from a concentration gradient test done as a preliminary experiment to find the optimum dosage.
7. To reduce antibody use, it is advisable to put coverslips on the parafilm to incubate the antibody. You can directly add the diluted antibody to the well without putting the cover glass on the parafilm.
8. To control for nonspecific staining, “no primary antibody” and “no secondary antibody” controls should be performed for each new antibody. In addition, at least a “no primary antibody control” should be performed for each colocalization experiment.

Recipes

1. Cell culture medium
Low glucose (1g/L) DMEM medium containing:
10% FBS
1% penicillin-streptomycin
2. PBS

Dissolve the PBS powder in double distilled water, adjusting the pH to 7.2-7.4.

3. Permeabilization solution

PBS containing:

0.5% Triton X-100

Note: It should be freshly prepared.

4. Blocking solution

PBS containing:

1% normal goat serum

Note: It should be freshly prepared.

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Competing interests

The authors declare no financial or non-financial conflicts of interest within this work.

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