General Protocol for Double Staining Immunocytochemistry

Overview

The following procedure is a general protocol for immunocytochemistry (ICC) of fixed cells. The reagent amounts listed below are intended for use in a 24-well format. Amounts suggested can be scaled up or down, depending on plate format and well size.

Required Reagents and materials

- 24-well tissue culture plates
- PBS (without Mg^{2+} or Ca^{2+})
- 4% Paraformaldehyde
- Triton X-100
- Bovine Serum Albumin (BSA)
- DAPI or mounting medium
- Primary Antibodies
- fluorophore-conjugated secondary antibody

Required Equipment

- Fluorescence microscope with image capturing capabilities
- Pipettes and tips
- Class II Biosafety cabinet
- Aluminum foil

Stemgent® Primary Antibodies

OCT4 Antibody, Rabbit anti-Mouse/Human	Cat. No. 09-0023
NANOG Antibody, Rabbit anti-mouse/Human	Cat. No. 09-0020
Sox2 Antibody, Rabbit anti-Mouse/Human	Cat. No. 09-0024
TRA-1-60 Antibody, Mouse anti-Human	Cat. No. 09-0010
TRA-1-81 Antibody, Mouse anti-Human	Cat. No. 09-0011
SSEA-4 Antibody, Mouse anti-Human	Cat. No. 09-0006

Block Buffer Preparation

Prepare appropriate volume of blocking buffer to obtain 5% BSA-PBS solution. Filter sterilize using a 0.2µm filter and store at 4°C for up to one month. Use this blocking buffer to perform blocking steps. In a sterile centrifuge tube prepare 1% BSA-PBS. This solution can be used to dilute antibodies.

Preparing permeabilization solution

In a sterile centrifuge tube prepare 0.2% Triton-X-PBS. This solution can be stored at room temperature for up to three months.

ICC Procedure

1. Seed and culture cells in a 24-well plate until ready for ICC analysis. We recommend 30-40% confluency for iPSCs.
2. Wash each well 3 times with 0.5 ml of room temperature PBS.
3. Fix each well by adding 0.25 ml of 4% paraformaldehyde in PBS and incubating for 20 minutes at room temperature.
4. Aspirate the 4% paraformaldehyde and then wash each well 3 times with 0.5 ml of PBS for 5 minutes with gentle agitation. Fixed cells may be stored in 1mL of PBS at 4°C overnight.
   Note: If visualizing intracellular markers perform this step. Otherwise proceed to step 7.
5. Aspirate the PBS and add 150 µL 0.2% Triton-X-PBS to each well. Incubate for 10 minutes at room temperature.
6. Aspirate the 0.2% Triton-X-PBS and wash the wells with 0.25 mL PBS per well.
7. Add 0.5 ml blocking buffer per well and incubate at room temperature for 1 hour.
8. Dilute the primary antibodies in blocking buffer according to the manufacturer's instructions.
   e.g. for a 0.1% solution, add 10 µl of antibody to 10 ml blocking buffer
9. Aspirate the blocking buffer and add 250 µl of each diluted primary antibody to each well. Incubate at 4°C for at least one hour. We recommend incubation overnight.
10. After the incubation time, wash each well 3 times with 0.5 ml of PBS for 10 minutes with gentle agitation.

Note: If using a conjugated antibody, skip steps 11 through 14 and go directly to step 15. If using a purified primary antibody, continue to step 11.

11. Dilute the fluorophore-conjugated secondary antibodies in blocking buffer, according to the manufacturer's instructions, and add 250 µL of each antibody to each well.

12. Incubate at room temperature for one hour, protecting the plate from light.
Note: You will need to protect the plate from light throughout steps 12 – 15.

13. Following incubation, wash each well 3 times with 0.5 ml of PBS for 10 minutes with gentle agitation.

14. Prepare a 2 µg/ml working solution of DAPI by diluting in PBS. Add to each well and incubate for 10 minutes at room temperature.

15. Wash each well once with 0.5 ml of PBS for 5 minutes with gentle agitation.

16. Aspirate any reaming PBS and add 1 to 2 drops of mounting medium to each well to stain the nuclei and preserve the samples for fluorescence microscopy imaging. Alternatively prepare a 0.2 µg/mL DAPI solution to stain the nuclei and visualize the cells.

17. Analyze cells using a microscope with image capturing abilities and annotate the images as follows:

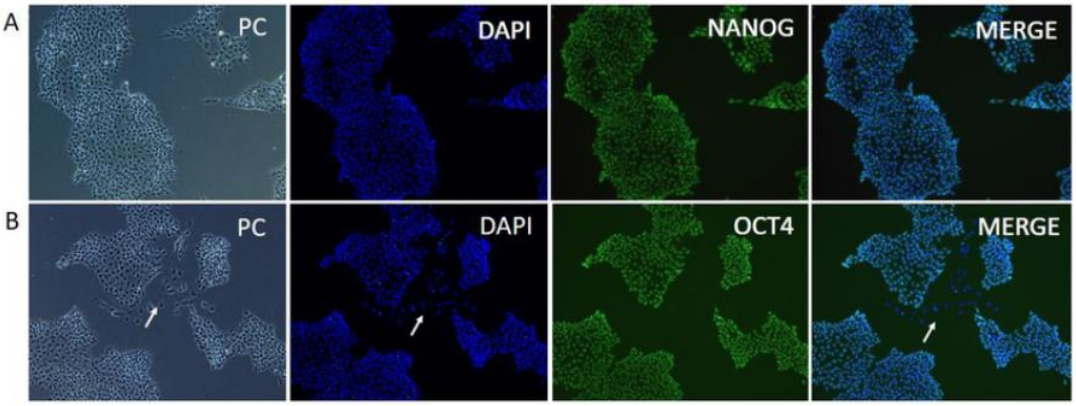

Fig 1. Results from characterisation of intracellular pluripotency markers by single staining ICC. Images were taken at 10X magnification. PC, Phase contrast microscopy. A, Fibroblast derived hiPS cells characterised by expression of NANOG. B, Blood derived hiPS cells characterised by expression of OCT4. White arrows indicate area of differentiated cells in the middle of the panel.

Useful controls to use for ICC

Positive control: Use a cell line which has been
tested positive for the expression of the desired markers by ICC.

Negative controls:
No primary control: Allocate one well for “Blocking only” to check for secondary antibody specificity.
Primary only control: Allocate a well for “primary antibody only” for each antibody to check for autofluorescence.

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