Immunolocalization of GATA6, NANOG and CDX2 in Bovine Embryos

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This technique allows for identification and counting of primitive endoderm ($GATA6^+$) AND epiblast ($NANOG^+$) cells in the ICM. The concurrent identification of TE cells ($CDX2^+$) improves the accuracy of ICM identification and also allows for determination of TE cell number.

Materials

Triton X-100

Tween 20

Polyvinylpyrrolidone (PVP)

Paraformaldehyde

BSA Fraction V

PBS (in our lab we use Dulbecco's PBS)

Slides

Cover slips

96 well plates

Slow fade® Gold antifade reagent (S36936, Life Technologies)

Hoescht 33342

Primary antibodies

- Rabbit polyclonal anti-human GATA6 (H-92) (sc 9055, Santa Cruz technology).
- Mouse polyclonal anti-human NANOG (14-5768-82, eBioscience)
- Mouse monoclonal anti-human CDX2 (CDX2-88, Biogenex)
- IgG from rabbit serum (I8140, Sigma)- aliquots in A9, -20°C freezer – 10 mg/ml.
- IgG from mouse serum (I8765, Sigma)- aliquots in A8, -20°C freezer – 100 µg/ml.

Secondary antibodies

- Alexa Fluor 555 conjugated goat polyclonal anti-rabbit IgG (A21428, Life Technologies)
- FITC conjugated goat polyclonal anti-mouse IgG (ab 6785, Abcam)
- Alexa Fluor 350 conjugated goat polyclonal anti-mouse IgG (A11045, Invitrogen)

Preparation of Solutions

1. DPBS/PVP: add 0.2 g PVP to 100 ml DPBS; store at 4°C.
2. Antibody buffer: add 0.10 ml Tween 20 and 1 g BSA to 100 ml DPBS; store at 4°C.
3. 4% paraformaldehyde: add 100 µl 8% (w/v) paraformaldehyde to 100 µl DPBS/PVP; prepare before use.
4. Wash buffer: add 0.10 ml Tween 20 and 0.1 g BSA to 100 ml DPBS; store at 4°C.

5. Permeabilization solution: add 0.5 ml Triton X-100 to 100 ml DPBS; store at 4°C.
6. Blocking buffer: add 5 g BSA to 100 ml DPBS; store at 4°C.
7. Primary antibodies:
    a. Rabbit anti-human GATA6 antibody: dilute in antibody buffer to 1 µg/ml.
    b. NANOG antibody: dilute in antibody buffer to 1 µg/ml.
    c. CDX2 antibody: sold ready to use; the concentration is 0.4 µg/ml.
    d. Anti-rabbit IgG and anti-mouse IgG are used as negative primary antibody controls. Dilute in antibody buffer to same concentration as primary antibodies.
8. Secondary antibodies:
    a. GATA6: Anti-rabbit IgG conjugated with Alexa Fluor 555: dilute in antibody buffer to 1 µg/ml.
    b. NANOG: Anti-mouse IgG conjugated with FITC: dilute in antibody buffer to 1 µg/ml.
    c. CDX2: Anti-mouse IgG conjugated with Alexa fluor 350: dilute in antibody buffer to 1 µg/ml.

Procedure

EMBRYO COLLECTION AND FIXING

1. Select embryos to be collected and wash them 3 times in 50 µl drops of cold DPBS/PVP.
2. Place embryos in a drop of 4% paraformaldehyde for 20 minutes.
3. Wash embryos 3 times in DPBS/PVP.
4. Proceed to permeabilization or store embryos in DPBS/PVP at 4°C for up to 1 week.

PERMEABILIZATION AND BLOCKING

1. Place embryos in 50 µl of permeabilization solution for 30 minutes at RT.
2. Place embryos in 50 µl of blocking buffer for 1 h at RT.

IMMUNOSTAINING

1. Separate a few embryos (around 5) for use as negative primary antibody controls.
2. Place embryos in 50 µl of GATA6 primary antibody overnight at 4°C.
3. Place negative control embryos in 50 µl of rabbit IgG.
4. Wash embryos 3 times in wash buffer (30 – 50 µl drops).
5. Place embryos in 50 µl of anti-rabbit IgG conjugated to Alexa Fluor 555 for 1h at RT in the dark.
6. Wash embryos 3 times in wash buffer (30 – 50 µl drops).
7. Place embryos in 50 µl of NANOG primary antibody for 1h at RT in the dark.
8. Place negative control embryos in 50 µl of mouse IgG for 1h at RT in the dark.
9. Wash embryos 6 times in wash buffer (30 µl drops).
10. Place embryos in 50 µl of anti-mouse IgG conjugated to FITC for 1h at RT in the dark.

11. Wash embryos 6 times in wash buffer (30 µl drops).
12. Place embryos in 50 µl of CDX2 primary antibody for 1h at RT in the dark.
13. Place negative control embryos in 50 µl of mouse IgG for 1h at RT in the dark.
14. Wash embryos 6 times in wash buffer (30 µl drops).
15. Place embryos in 50 µl of anti-mouse IgG conjugated to Alexa fluor 350 for 1h at RT in the dark.
16. Wash embryos 6 times in wash buffer (30 µl drops).

MOUNTING EMBRYOS IN SLIDES

17. Place embryos in groups of 5 – 10 in slides containing drops (5 – 10 µl) of anti-fade solution.
18. Carefully place a coverslip over each drop.
19. Keep slides at 4°C in the dark until examining them under the fluorescence microscope.

TIPS FOR IMPROVING RESULTS

1. Once you start working with antibodies, it is recommended that you keep the embryos away from direct light exposure at all times possible (except when working under the microscope). Reducing the lights in the laboratory is useful.
2. Examination of the embryos under the fluorescence microscope should preferably be performed on the same day. If needed, slides can be kept at 4°C IN THE DARK overnight. The fluorescence signal starts to diminish within hours, so evaluation and imaging should be done as soon as possible.

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