Simple protocol for differential staining of inner cell mass and trophectoderm of bovine embryos

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This protocol is a slightly modified version of that described by Thouas et al. (Reprod Biomed Online 2001;3:25-29) and another one obtained from D.J. Walker and G.E. Seidel, Jr. at Colorado State University. An RNase step has been added to reduce cytoplasmic staining of propidium iodide.

Materials

1) 100 mM $NaPO_4$, pH7.4 (Stock solution)

To prepare 100 mM PO_4 pH 7.4, add sodium phosphate monobasic (1a) to 300 ml sodium phosphate dibasic until the pH reaches 7.4. Approximately, 100 ml of monobasic solution will be added.

1a. 100 mM sodium phosphate monobasic ($NaH_2PO_4.2H_2O$)
    13.8 g NaH_2PO_4
    1 L double–distilled (dd) ddH2O

1b. 100 mM sodium phosphate dibasic (Na_2HPO_4)
    14.2g $Na_2HPO_4.2H_2O$
    1 L ddH_2O

2) 10 mM PBS - Dilute 100 ml of 100 mM PO_4 pH 7.4 with 800 ml ddH_2O, add 9 g of NaCl and bring volume to 1000 ml with water. Check pH.

3) 10 mM PBS/PVP – Add 0.1 g polyvinylpyrrolidone (PVP) to 100 ml PBS.

3) 4% paraformaldehyde – Obtain 8% paraformaldehyde from Electron Microscopy Sciences (#15710-SP) and dilute 1:1 with PBS/PVP. Make fresh on the day of use and discard any leftover after using that day.

4) Triton X-100 – Add Triton X-100 to PBS/PVP (0.2 ml/100 ml) to obtain a final concentration of 0.2% (v/v).

5) Propidium Iodide (PI solution) – Prepare a 2.5 mg/ml stock 1 solution by dissolving 25 mg PI (Sigma P4170) in 10 ml of PBS/PVP and store at 4°C. Next, dilute stock 1 solution 1:25 (100 μl in 2.4 ml in Triton X-100) to give a working concentration of 100 μg/mL. Store stock 2 in a foil covered tube at 4°C for 3 weeks.

5) Hoescht 33258 (H Solution) – Prepare Stock 1 by dissolving 25 mg Hoechst 33258 (Sigma B2883) in 2.5 ml of distilled water (10 mg/ml). Store at 4°C. On the day of use, prepare Stock 2 by diluting the Stock 1 solution 1:1000 in 4% paraformaldehyde to give a working concentration of 1 μg/mL Discard whatever is not used on the day of use.

6) Microscope slides – Fisherbrand Superfrost slides (#12-550-14).

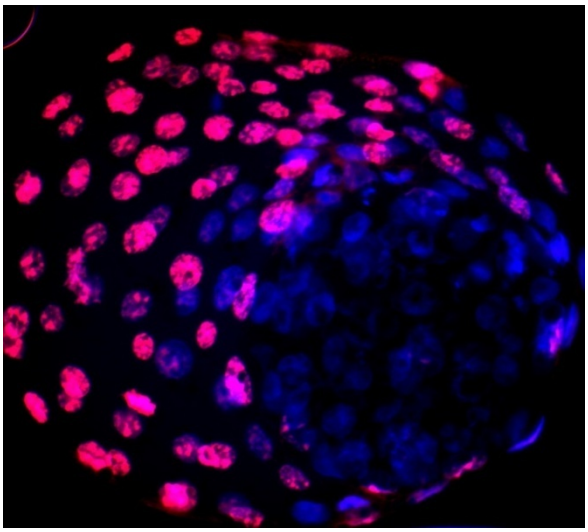
7) RNase **(RNase Solution)** – Stock 1 of RNase is supplied by Qiagen (#S23-24-26-36/37) at 100 mg/mL in DMSO. Store at room temperature. On day of use prepare Stock 2 by diluting stock 1 solution 1:100 in PBS/PVP. Discard whatever is not used on the day of use.

Procedure

1) Add RNase directly to microdrops of embryos. Drops should be 45 µL so remove medium as necessary and then add 5 µL of RNase Solution. Incubate for 1 hour at 38.5 ^{o}C.

2) For every set of embryos to be processed (up to at least 30), draw 4 circles with a PAP PEN on a large petri dish or other flat dish. We used grid plates from Fisher (Integrid 100 mm dish; cat. No. 08-757-149). Place a 50 µL drop of the PI solution within 1 circle. Add 50 µL of PBS/PVP in each of the other three circles.

3) On a second plate (or the lid of the grid plates), draw 4 circles with a PAP PEN for every set of embryos to be stained. Place a 50 µL drop of H Solution in 1 circle. Add 50 µL of PBS/PVP to each of the remaining three circles.



Representative example of a bovine blastocyst stained using the procedure described here. Red=PI and blue=Hoescht.

4) Place the grid plate on a slide warmer set to 39°C and allow the PI solution to warm up for 5-10 min prior to beginning the staining procedure.

TIP - *Conduct all following steps in a dark room to prevent bleaching. It may also help to cover the plate with foil.*

5) Remove blastocysts from culture in as little medium as possible and place into the drop of PI solution for 30 seconds. ***TIP*** *- load blastocysts into Drummond prior to end of 30 seconds in order to be able to put in wash solution immediately.*

6) After 30 seconds in PI solution, wash the embryos through the 3 drops containing PBS/PVP. ***TIP - TIMING IS CRITICAL*** *– too long and all the cells will be penetrated by PI; not long enough and the penetrance of TE cells will be incomplete.*

8) Place embryos into a 50 µL drop of the H solution and incubate at room temperature for 15 minutes.

9) Wash embryos 3 times in PBS/PVP by moving them through the 50 µL drops.

10) Place a small drop of glycerol onto microscope slide. Place embryos in a small volume of solution onto glycerol drop and mount coverslip. View embryos as soon as possible using a epifluorescent microscope with blue and red filters.