Micromass Chick Limb Cells Culturing Method

Procedures

Collect limb tips (5 dozen eggs)

1. Incubate eggs for 5 days. Spray 70% ethanol on eggs.
2. Open the eggs and get the embryos out into dish[1] containing EBSS w/ $Ca^{2+}$and Mg^{2+}.
3. Remove the outer membrane around the embryo and transfer them to a new dish containing EBSS w/ Ca^{2+}/ Mg^{2+}.
4. Use fine-tip forceps to cut off the limb tips, 0.3 mm from the very distal end and put them into a new dish containing EBSS with Ca^{2+}/ Mg^{2+}.
5. Collect tips and put them into a 15 ml centrifuge tube[2].

Removal of ectoderm w/ EDTA treatment

1. Pellet using speed 5 for about 2 min.
2. Remove EBSS with Ca^{2+}/ Mg^{2+}.
3. Add 5 ml of EBSS w/o Ca^{2+}/ Mg^{2+}[3] and 100 μl EDTA[4] (0.5M , pH 8).
4. Incubate for 20 minutes at 37°C(CO_2 5%). Do not close the lid too tight.
5. Spin at speed 5 to pellet.
6. Remove the media.
7. Add about 5 ml of fresh EBSS w/o Ca^{2+}/ $Mg^{2+}$and 100 μl of Fatal Bovine Serum (FBS[5]) to stop EDTA reaction.
8. Gently pipette up/down for 6-10 times to remove ectoderm.
9. Transfer tips to a clean dish (use the microscope to determine the cells which ectoderm is removed).
10. Pick out large pieces of mesenchyme.

Preparation of cells for incubation

1. Pellet tips at speed 5 for 5 mins.
2. Add 4.5 ml EBSS w/o Ca^{2+}/ $Mg^{2+}$and 500 μl Trypsine[6].
3. Incubate about 15-20 minutes at 37°C(CO_2 5%). Loose the lid a little bit.
4. Warm about 5 ml DMS.
5. Obtain a filter unit and place in a nylon filter[7] on the gasket[8].

[1] Fisherbrand Extra-Deep Stackable 100 x 20 mm Dishes, Fisher Cat No. 08-757-11Z
[2] Fisher Cat No. 14-959-49B. Falcon 352097 was used at UKMC.
[3] Gibco BRL Cat No. 14155-063
[4] Fisher Cat No. BP120-500
[5] HyClone Cat No. SH30071.01
[6] Gibco BRL Cat No. 15400-054
[7] Nytex 20-μm mono-filament nylon mesh, 13mm in diameter. TETKO, 333 South Highland Ave, Briarcliff Manor, Tel: 914-941-7767, or 1-800-995-0531
[8] Millipore swinnex 13. Millipore Cat No. SX0001300.

6. Obtain a 3 ml syringe[9] and 18 G needle[10].
7. After trypsinizing tips, close tube tightly. Spin at 5 for 5 mins to pellet.
8. Remove media.
9. Add 2.5 ml fresh pre-warmed DMS.
10. Place needle and syringe in the tube and pull up/down for 20 times to dissociate cells.
11. Suck up cells into syringe.
12. Remove needle, replace with the filter unit and gently push through into a clean 15 ml tube.
13. Take 10 μl cell suspension for cell counting.
14. Place 15 ml tube in centrifuge and pellet at setting 5 for 15 minutes.
15. Remove as much DMS from the 15 ml tube as possible. For the remaining, we consider it is 20 μl of medium left. Add proper amount of DMS for the desired cell density. The cell density could be about 1.6-2.0$\times 10^5$ cell/10μl. The amount of DMS(in μl) to be added can be calculated as below:

$$V = \frac{x * y}{z} - 20 \tag{1}$$

where x is # of cells in one cell counting chamber (0.1mm^3), y is volume of cell suspension (ml) and z is the target cell concentration ($\times 10^5$).

16. Use micropipette to pull up and down for 5-10 times, do not introduce air bubbles.
17. Add 10 μl of cell suspension into each well of the 24-well cell culture cluster[11] and keep them in CO_2 (5%) incubator (37°C).
18. Warm enough DMS to flood the cell culture wells.
19. After 30-40 minutes, flood each well with 0.5 ml or 1 ml of fresh and pre-warmed DMS.
20. Change medium (DMS) in each well every day (if flooded with 0.5 ml DMS) or every other day (if flooded with 1 ml DMS). Normally the cells will be alive for up to 7 days.

Medium and Suppliments

Media

EBSS w/ Ca^{2+}/ Mg^{2+}(1 liter)

- 1 pack of Earles Balanced Salts, modified[12].
- 2.20 g Sodium Bicarbonate ($NaHCO_3$).

DMEM (1 liter)

- One pack of Dulbeccos Modified Eagles Medium[13].
- 3.70 g Sodium Bicarbonate ($NaHCO_3$).

[9] BD No.309585, Fisher Cat No. 14-823-40
[10] BD No.305196, Fisher Cat No. 14-826-5D
[11] Corning 3526
[12] Sigma Product No. E6132
[13] Gibco BRL Cat No. 12100-046, or 11971-025 (used at UKMC)

F-12 (Ham) w/o L-glutamate and sodium bicarbonate (1 liter)

- 1 pack of F-12[14].
- 1.18 g sodium bicarbonate ($NaHCO_3$).

DM (1 liter)

F-12	600 ml
DMEM	400 ml
10X transferrin	500 μl
10X Ascorbic Acid	500 μl
10X Insulin	500 μl
1X hydrocortisone	100 μl
Pen-Strep	10 ml

DMS (100 ml)

DM from above	90 ml
FBS[15]	10 ml

Supplements

Transferrin 10 mg/ml in DM (10X)

- 200.0 mg transferrin[16].
- 20 ml DM (60 ml F-12 + 40 ml DMEM).

Ascorbic Acid (Vitamin C) 100 mg/ml (10X)

- 2 g ascorbic acid[17].
- 20 ml DM, filter.

Insulin 10 mg/ml (10X)

- 10 mg/ml Insulin[18] in 0.01N HCl (5 μl 12N HCl into 6 ml distilled H_2O).

Hydrocortisone[19] (1X) 1mM in distilled H_2O

Penicillin-Streptomycin w/ Glutamine (Pen-Strep[20])

How to make F-12 and DMEM media

Add (F-12 or DMEM) powder and other necessary chemicals into a beaker with stirrer bar inside. Add 800 ml distilled water and stir for 5 minutes or until chemicals are fully dissolved. Add more distill water to 1000 ml and stir for 1 more minutes. Filter medium through bottle-top filter kit [21] in the hood into an autoclaved bottle.

[14] Gibco BRL Cat No. 21700-075
[16] Sigma C0880. C7786 was used as substitute at UKMC because Sigma did not have C0880 in smaller amount and C0880 was around $500.00 for 1g.
[17] Sigma A4403
[18] Sigma I1882
[19] Sigma H0135
[20] Invitrogen 1514-0148
[21] Millipore Steritop Sterile Vacuum Bottle-Top Filters with 500 ml or 1000 ml funnel. Note: the thread size may be different (33 or 45mm) depending on what kind of bottle used.

How to clean nylon filter and filter holder

The nylon filters and filter holders can be reused after each experiment. To clean nylon filter, rinse with DI water or distilled water, put in ethanol (70%) for 4-6 hours and expose to UV light overnight. To clean filter holder, rinse with DI water and expose to UV light overnight.

Comments and Extension of This Culture Method

Label Cells with DiI

Two ways were tried at UKMC (June, 2004).

Adding DiI into cell suspension

The first way I tried in E002 was to add dissolved DiI (10μl, 10mg/ml in ethanol) into cell suspension (about 1.5 or 2ml). Got a lot of undissolved DiI crystals in the culture. So, this is NOT a good way to label cells with DiI in micromass cell cultures.

Injecting DiI into limb first

The other way I tried in E004 was to inject DiI into chick limb (put the embryo on agar can help injection) before the limb tips were cut off. It turned out to be a much better way to have cells labeled because I did not see any DiI crystals in brightfield and cells were labeled well.

The ratio of labeled cells to unlabeled cells could not be controlled well. However, to get less cell labeled, just inject less spots of DiI into chick limbs. In E004, 3 DiI spots were injected on each limb and about 10-15% cells were labeled with DiI.