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# Generation of stem cell based mouse embryo-like structures

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## Abstract

In this chapter, we detail the experimental protocol leading to the generation of stem cell-based mouse embryo-like structures termed “ETiX-embryoids”. ETiX-embryoids are formed from combined embryonic stem cells, trophoblast stem cells and embryonic stem cells transiently induced to express Gata4. Cells are seeded into AggreWell dishes where they form aggegates that develop to resemble post-implantation mouse embryos following 4 days of culture. ETiX-embryoids establish an anterior signaling center and undergo gastrulation over the following 2 days. By day 7, ETiX-embryoids undergo neurulation and form an anterior-posterior axis with head folds at one end and a tail bud on the other. On day 8, they develop a brain and form a heart-like structure and a gut tube.

### Keywords

Embryo-like structures; ETiX-embryoids; Neurulation; Organogenesis; Stem cells

## 1. Introduction

In addition to the study of the natural embryo, the use of stem cell-based culture models has become increasingly prevalent in recent years[1-6]. Critically, they allow for the characterization of developmental processes without the requirement for large numbers of natural embryos. Moreover, they are amenable to genetic manipulations as well as live imaging techniques and therefore offer opportunities for molecular studies to be performed without the need for generation of knockout animals. In this chapter, we describe a procedure to generate ETiX-embryoids that over 8 days of culture are capable of recapitulating the development of the post-implantation mouse embryo up to early organogenesis.

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**Conflict of interest:** A patent application has been submitted by MZG, GA and CH based on these results.

## 2. Materials

### 2.1 Reagents

1. 2-β-mercaptoethanol (Gibco 31350010).
2. Advanced DMEM/F12 (Gibco, 21331-020).
3. AggreWell rinsing solution (StemCell Technology 07010).
4. B27 (Gibco 10889038).
5. β-estradiol (Tocris 50-28-2, stock: 10uM in DMSO).
6. CHIR99021 (StemCell Technology 72052).
7. DMEM (Gibco 41966052).
8. DMEM, bicarbonate-buffered, without glutamine (Gibco 11054).
9. DMEM low-glucose, with no bicarbonate buffer (Gibco 11880).
10. DMEM/F-12 (Gibco 21331020).
11. Doxycycline (Sigma-Aldrich D9891-5G, stock: 1mg/mL in water).
12. Foetal bovine serum (Gibco 10270106).
13. FGF4 (R&D Systems 7486-F4-025, stock: 100 ug/mL).
14. Gelatine type A (Sigma-Aldrich G1890).
15. D-(+)-Glucose (G8644-100ML).
16. GlutaMAX (Gibco 35050038).
17. Heparin (Sigma-Aldrich H3149-25KU, stock: 1 mg/mL in PBS).
18. HEPES (Thermo Fisher Scientific 15630056).
19. Human cord serum (Cambridge Blood and Stem cell Biobank, UK).
20. Human adult blood serum (BIOIVT,USA #HP1022).
21. ITS-X (Thermo Fisher Scientific 51500-056).
22. Leukaemia inhibitory factor LIF (StemCell Technologies 78056).
23. MEM non-essential amino acids (Gibco 11140035).
24. N-acetyl-L-cysteine (Sigma-Aldrich A7250, stock: 50 mM in water).
25. N2 Supplement (Life Technologies 17502048).
26. Neurobasal-A (Gibco 10888022).
27. PBS (Life Technologies 10010056).
28. PD0325901 (Cambridge Stem Cell Institute).
29. Penicillin/streptomycin (Gibco 15140122).

30. Progesterone (Sigma-Aldrich P0130-25G, stock: 1 mg/mL in DMSO).
31. Rat serum (adult rats, Charles River/Janvier Labs/BIOIVT).
32. RPMI 1640, no Glutamine (Thermo Fisher Scientific 21870076).
33. Sodium pyruvate (Gibco 11360039).
34. Trypsin-EDTA 0.05% (Life Technologies 25300054).

### 2.2. Experimental plates:

1. Tissue culture treated 6-well plates (e.g. Thermo Fisher Scientific 140675).
2. Suspension culture 6-well plate (CELLSTAR, Greiner Bio-One 657185).
3. Suspension culture 48-well plate (CELLSTAR, Greiner Bio-One 677102).
4. AggreWells (24-well format, StemCell Technology 34415).

### 2.3 Cell culture media

#### 2.3.1. N2B27 medium (for 50 mL):

1. 25 mL of Neurobasal-A.
2. 25 mL of DMEM/F12.
3. 500 uL of P/S.
4. 500 uL of GlutaMAX.
5. 100 uL of 2-β-mercaptoethanol.
6. 500 uL of B27.
7. 250 uL of N2.
8. Supplement with LIF, Chiron and PD03 to make N2B27/2iLIF.

#### 2.3.2. Feeder Cell medium (FC, for 1 full bottle):

1. 1 bottle of DMEM (Gibco 41966052).
2. 90 mL of heat-inactivated FBS.
3. 5 mL of P/S.
4. 5 mL of GlutaMAX.
5. 5 mL of MEM non-essential amino acids.
6. 5 mL of Sodium pyruvate.
7. 1.2 mL of 2-β-mercaptoethanol.

#### 2.3.3. Trophoblast Stem Cell medium (TS, for 50 mL)

1. 50 mL of RPMI 1640.
2. 10.4 mL of FBS.

3. 520 uL of P/S.
4. 520 uL of GlutaMAX.
5. 520 uL of Sodium pyruvate.
6. 104 uL of 2-β-mercaptoethanol.
7. For 10 mL of TSF4H medium, add 10 uL of Heparin stock and 2.5 uL of FGF4 stock.

## 2.4 In vitro culture medium (IVC)[7]

### 2.4.1. Base for IVC (ADF+++)

1. 50 mL of Advanced DMEM/F12.
2. 500 uL of GlutaMAX.
3. 500 uL of ITS-X.
4. 125 uL of P/S.

### 2.4.2. IVC-20% FBS (10 mL)

1. 8 mL of ADF+++.
2. 2 mL of FBS.
3. 8 uL of β-estradiol.
4. 2 uL of Progesterone.
5. 5 uL of N-acetyl-L-cysteine.

### 2.4.3. IVC-30% FBS (10 mL)

1. 7 mL of ADF+++.
2. 3 mL of FBS.
3. 8 uL of β-estradiol.
4. 2 uL of Progesterone.
5. 5 uL of N-acetyl-L-cysteine.

### 2.4.4. DMEM/Rat serum medium/Human cord serum (DRH, for 1 mL)[8]

1. 250 uL of DMEM, bicarbonate-buffered, without glutamine (Gibco 11054).
2. 500 uL of rat serum.
3. 250 uL of human cord serum.
4. 10 uL of GlutaMAX.
5. 10 uL of P/S.

**2.4.5. Human and rat serum preparation—**Store at −80°C. Thaw slowly and heat-inactivate for 30 minutes at 56°C and filter-sterilized prior to use.

### 2.5. Roller system components

1. Rotary bottle system.
2. Gas mixer.
3. Gas cylinders ($O_2$, $N_2$ and $CO_2$).

The gas mixer has input from all three gas cylinders ($O_2$, $N_2$ and $CO_2$). The input pressure for each gases is maintained at 10 psi. The gases are mixed in the desired ratio (in this case 21% $O_2$ and 5% $CO_2$) and fed into the rotary bottle system. The final gas mixture is humidified before feeding into the rotatory bottles. The pressure of the gas (mixed + humidified) before at the entrance of the rotary bottle is 0.5 psi. The flow rate at the exit of the rotatory bottle system is 8-10 bubbles/sec. The bottles are rolled at 3 rpm.

## 3. Methods

### 3.1 Culture of mouse embryonic stem cells (ESCs):

Embryonic stem cells may be cultured in N2B27 /2iLIF medium on gelatinized, tissue culture-grade plates in an incubator set to 37°C with 5% $CO_2$ and 21% $O_2$. Cells should be routinely passaged every 3-4 days or when 70/80% confluent, with regular media change every day or every second day, in between passages (see Notes 1 and 2).

1. To passage, begin by preparing a tissue culture dish of the appropriate size for replating. To this end, add the appropriate volume of 0.1% gelatine solution to a well (i.e. add 1ml gelatine to 1 well of a 6-well plate) and set plate aside until needed. Pre-warm required media and solutions (N2B27 /2iLIF, FC, Trypsin) in 37°C water bath for no more than 10 minutes.

2. Aspirate the media from the well of cells and add appropriate volume of Trypsin (i.e. for 1 well of a 6-well plate add 1ml Trypsin).

3. Incubate the plate in the incubator for 3-4 minutes.

4. Gently pipette cells 2-3 times to produce a single-cell suspension and add 1-2 volumes of FC media to quench the Trypsin activity (see Note 3).

5. Transfer the cell suspension to a 15 ml Falcon tube and centrifuge at 0.2 x g (RCF) for 4 min.

6. Aspirate supernatant leaving a tiny amount above the cell pellet so as not to disturb it. Wash the cells with PBS (1ml for 1 well of a 6-well plate) and repeat centrifugation step as above (step 5).

7. Aspirate supernatant as above and resuspend cells in 1ml N2B27 /2iLIF medium.

8. Aspirate the gelatin solution from the prepared plate (step 1) and add appropriate volume of N2B27 /2iLIF (2 ml for 1 well of a 6-well plate).

9. Plate the cells at desired dilution (i.e. 1:5, 1:10, 1:20).

10. Distribute the cells evenly by gently moving the plate in a figure of 8 motion and place in the incubator (see Note 4).

### 3.2 Culture of mouse trophoblast stem cells (TSCs):

Trophoblast stem cells may be cultured in TSF4H on inactivated MEFs in an incubator kept at 37°C with 5% $CO_2$ and 21% $O_2$. As with ESCs, cells should be routinely passaged every 3-4 days or when 70/80% confluent, with regular media top-up in between passages (see Note 5).

Passaging of TSCs is similar to that of ESCs with the following distinctions:

1. Medium conditioning for ~5-6hrs is advisable and helps prevent differentiation of TSCs. To condition media aspirate the FC medium from a well coated with iMEFs and add the appropriate volume of TSF4H.

2. After 6 hours, begin passage by aspirating the media from the well of TSCs, and washing with PBS.

3. Add the appropriate volume of Trypsin and incubate the plate for 4 minutes.

4. Quench the Trypsin activity with 2ml of TS base medium.

5. Gently dissociate cells by pipetting and centrifuge in 15ml Falcon tube at 0.2 x g (RCF) for 4 minutes.

6. Aspirate supernatant and resuspend cells with 1ml of TSF4H.

7. Dilute at 1:10 or 1:20 and plate in the well of iMEFs pre-conditioned with TSF4H (step 1).

### 3.3 Culture and inactivation of mouse embryonic fibroblasts (MEFs):

Culture and expand active MEFs only up until P5. Cell harvests decrease with the passage number. Cells should not be passaged more often that at 3-day intervals. Should the cell number at confluency be lower or equal to the number of cells platted the cells need to be inactivated immediately, regardless of passage number. Overcrowding promotes the outgrowth of neural cell types.

To passage active MEFs:

1. Aspirate the media and add PBS to wash once.

2. Aspirate the PBS and add TryplE for 2-7minutes or until cells are visibly detached.

3. Add at least 2 volumes of FC media and wash the surface to ensure all the cells are removed. Add cells to a 50ml Falcon tube.

4. If passaging multiple plates, pool all the cells and quantify cell number using a haemocytometer.

5. Seed appropriate number of cells to dishes:

| Size | Seeding number |
|---|---|
| 15 cm dish/T150 | $1.3x10^6$ |
| T75 | $6.5x10^5$ |
| T25 | $2.2x10^5$ |

MEFs may be inactivated through irradiation or with mitomycin-C. For mitomycin-C inactivation proceed as follows:

1. Aspirate FC media from plates and add appropriate volume of fresh FC (enough volume to cover the surface, i.e for 15cm plate use 10ml).

2. Add the appropriate volume of mitomycin-C for a final concentration of 10 ug/ml dropwise to the cells taking care to distribute the drops across the entire dish. Mix by swirling the dish very gently.

3. Incubate treated cells for 1,5 hours at 37°C with 5% $CO_2$ and 21% $O_2$.

4. Remove media and dispose of all mitomycin-C containing media appropriately.

5. Wash cells with PBS 5 times prior to the addition of TryplE.

6. Incubate for 2-7 minutes or until the cells begin to detach.

7. Add at least 2 volumes of FC media and wash the surface to ensure all the cells are removed. Add cells to a 50 ml Falcon tube.

8. Quantify cell number and plate/freeze down in appropriate numbers as needed (i.e. for TSC culture 0.5 x $10^6$ iMEFs frozen down can be subsequently thawed and used for 3 wells of a 6-well dish).

### 3.4 Preparation and seeding of cells for ETiX-embryoid formation

1. One or two days prior to the experiment, passage tetO-Gata4 ESCs at a dilution of roughly 1:5. A well with many small colonies is preferable to fewer larger colonies as it ensures a homogenous response to doxycycline.

2. On the day of the experiment, six hours before cell dissociation: add fresh media (N2B27 /2iLIF) to all ESCs, add Dox (1:1000 dilution) to the tetO-Gata4 ESCs, and top up the medium (TSF4H) on TSCs.

3. Six hours following Dox addition, plating of the experiment is started.

4. Begin by adding 0.5ml of anti-adherence rinsing solution to the desired number of AggreWells and centrifuge the plate at 2000 x g (RCF) for 5 min. Check to ensure no bubbles are present and set aside at room temperature until cells have been quantified. If bubbles are present, centrifuge once more for another 5 min.

5. Prepare a volume of FC that is 2x that of the number of AggreWells (for 1 well prepare 2ml) in a Falcon tube and equilibrate in the incubator with a loose cap. This media will be used for washes of the AggreWells and for cell resuspension following the combination of each of the three cell types.

6. Prepare 1-2 wells with 1 mL of gelatin and set aside at room temperature. This is required for MEF depletion from the TSC suspension.

7. Dissociate ESCs and TSCs as described above. Following dissociation resuspend TSCs in 2 ml of TSF4H and plate in the gelatinized dish prepared in the previous step.

8. Allow MEFs to stick down for 20-30 min in the incubator. During this time, quantify the ESC lines. Then collect the TSC suspension.

9. Quantify the remaining cells with a haemocytometer (use 10 ul of the cell suspension). Place the Falcon tube with remaining cells in the incubator until needed.

10. Calculate volumes yielding 6,000 ESCs, 6,000 tetO-Gata4 ESCs and 19,200 TSCs (per AggreWell) and transfer these to a 15 mL Falcon tube.

11. Centrifuge for 4 min at 0.2 x g (RCF).

12. In the meantime aspirate the rinsing solution from the AggreWell and rinse the wells once with 1 mL of PBS, and once with 0.5 ml of equilibrated FC (step 5). Aspirate again and add 0.5 ml equilibrated FC.

13. Place the AggreWell plate back in the incubator until the cells are ready to be plated.

14. Add Rock inhibitor at a final concentration of 0.5 nM to the remaining medium in the incubator (1 ml per AggreWell) and use this to resuspend the cells at the end of the centrifugation (step 11).

15. Add cells to the AggreWell dropwise on the centre of each well.

16. Centrifuge the plate for 3 min at 100 x g (RCF), and placed the plate in the incubator (day 0).

17. The next day (day 1), cells should have come together and single cells are no longer observed under the microscope. On this day, perform two media changes. Begin by using a pipette to carefully remove 1ml of media from each AggreWell. Gently replace this with 1 ml fresh FC equilibrated for ~20 min prior in the incubator (no Rock inhibitor added). The media changes need to be performed very gently exercising great care as not to disturb

the structures forming within the wells. Media is removed and added slowly by placing the pipette tip against the side of the wells, avoiding formation of bubbles.

18. Place plate in the incubator for a minimum of 5 min before performing another media change as above.

19. On day 2, change media once with 1 ml of fresh FC medium (as in step 17).

20. On day 3, removed 1 ml media from AggreWell and add 1.5 ml of IVC-20% FBS equilibrated for ~20 min prior in the incubator.

21. On day 4, ETiX-embryoids in the AggreWell are transferred to CELLSTAR 6 well multiwell plate for suspension culture (Greiner Bio-One 657185). Prepare 5 ml of IVC-30% FBS per well.

22. Equilibrate IVC-30% FBS in the incubator for a minimum of 20 min.

23. All ETiX-embryoids are collected from AggreWell for analysis at 4 days of development and analysed under a stereomicroscope. It is recommended to collect one individual AggreWell at a time. To collect cut the end of the tip off from a P1000 and gently pipette to dislodge structures from AggreWells. Collect in a Falcon tube. Wash each AggreWell with 1 ml PBS and combine this with the rest.

24. Add structures to a plastic dish and carefully examine the morphology. Select ETiX-embryoids with cylindrical morphology and two clearly defined cellular compartments (an ESC compartment and TSC compartment) surrounded by an outer cell layer, the VE-like layer. The ESC compartment should be epithelialized with a lumen. The TSC compartment is more variable in appearance and therefore, a wider range of appearances for the TSC compartment is acceptable (Fig. 1).

25. Transfer selected ETiX-embryoids with the ESC and TSC compartments surrounded by a VE-like layer to equilibrated IVC-30% FBS media to continue their culture.

26. When selecting at D5: i) the ESC and TSC compartments should have a single merged lumen; ii) the beginning of gastrulation on one side of the ETiX-embryoids should be apparent (a thickening of the proximal region of the ESCs on one side); iii) the AVE should have migrated to the ESC-TSC boundary and be opposite to the forming streak. ETiX-embryoids with the AVE stuck at the tip of the structure or not at the boundary should be excluded. If the ESC line used contains a reporter line this can be used as an additional validation of asymmetry (Fig. 2).

27. On day 5 transfer each ETiX-embryoid to a single well of 48-well, non-adherent dish with 250 uL of DRH or EUCM medium.

28. On day 6, change media on each ETiX-embryoid using 250 uL of DRH/EUCM.

29. On day 7, ETiX-embryoids resemble E8.0 natural embryos with a clear anterior-posterior axis and bifurcating neural folds extending into a neural tube and culminating in a

tail bud. Move the ETiX-embryoids to a rotating bottle culture chamber apparatus in DRH media supplemented with of 3.0 mg/mL of D-(+)-Glucose (Sigma G8644). Each rotating bottle contains 2 mL of medium and 3 ETiX-embryoids.

30. On day 8, change media to DRH medium supplemented with 3.5 mg/mL of D-(+)-Glucose. In each rotating bottle, culture 2 in 3 mL of medium.

31. At the end of day 8, dissect and fix the ETiX-embryoids in 4% PFA for 20 min at room temperature in preparation for downstream analyses (Fig. 3).

## 4. Notes

1. Ensure cells are not displaying signs of stress (i.e. abnormal morphology, cell debris, excess differentiation/cell death) and are cycling normally prior to the beginning of an experiment. Passage at least once after thawing a fresh vial before using for experiment.

2. Where possible use cells with young passage numbers.

3. When passaging and prior to quenching the Trypsin activity ensure a single cell suspension has been achieved. If unsure, check cells briefly under an optical microscope.

4. When returning the passaged cells to the incubator close the door gently as vertical vibrational movements can transmit through the culture dish and cause unwanted cell crowding (particularly if incubator door contains magnetic strip). Avoid opening/closing the door of the incubator following passaging if possible.

5. For TSCs that are more than 50% confluent, perform media changes as follows: remove 50% of the media and top up with fresh TSF4H.

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**Figure 1:**
A selection of ETiX-embryoids on day 4. ESC compartments are lumenized with a cup-shaped morphology while TSC compartments show greater morphological variability. A layer of VE-like cells enveloping the two compartments is clearly visible.

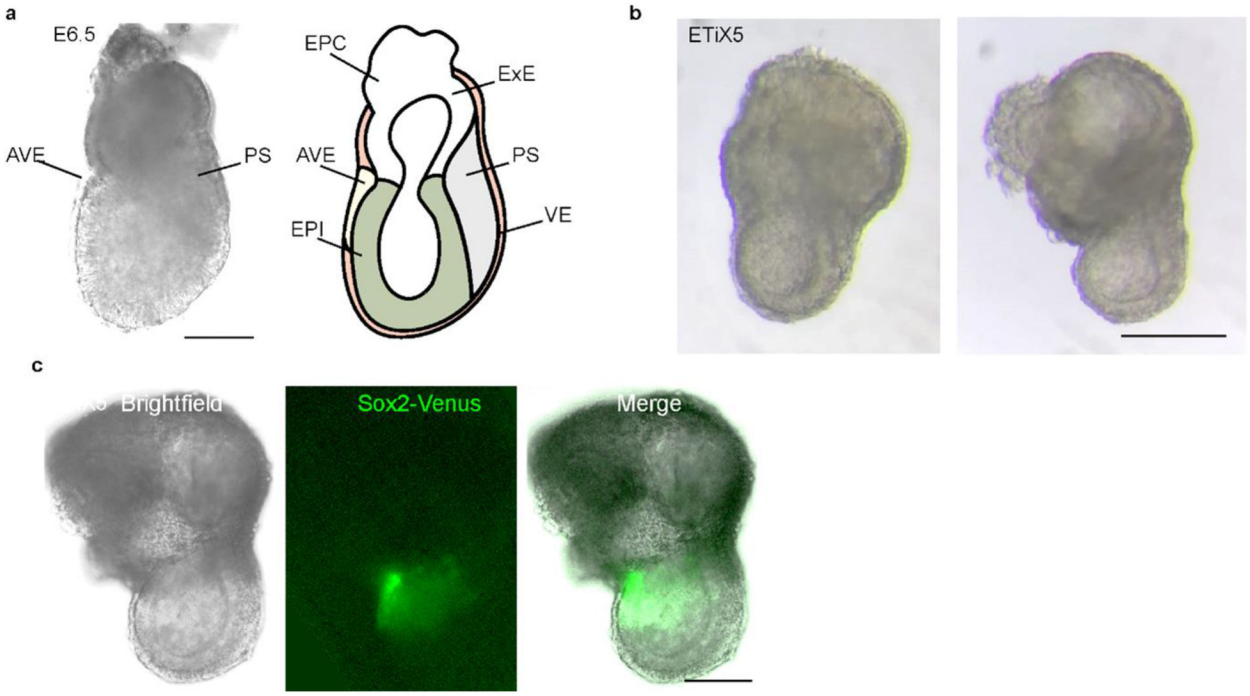


**Figure 2:**

(a) E6.5 natural embryo (left) and diagram highlighting key morphological features (right). (b) Representative examples of ETiX-embryoids selected at day 5. (c) ETiX-embryoid at day 5 made with ESCs containing a Sox2-Venus reporter. Sox2 signal displays a gradient of expression across the epiblast with the strongest signal apparent in the anterior.

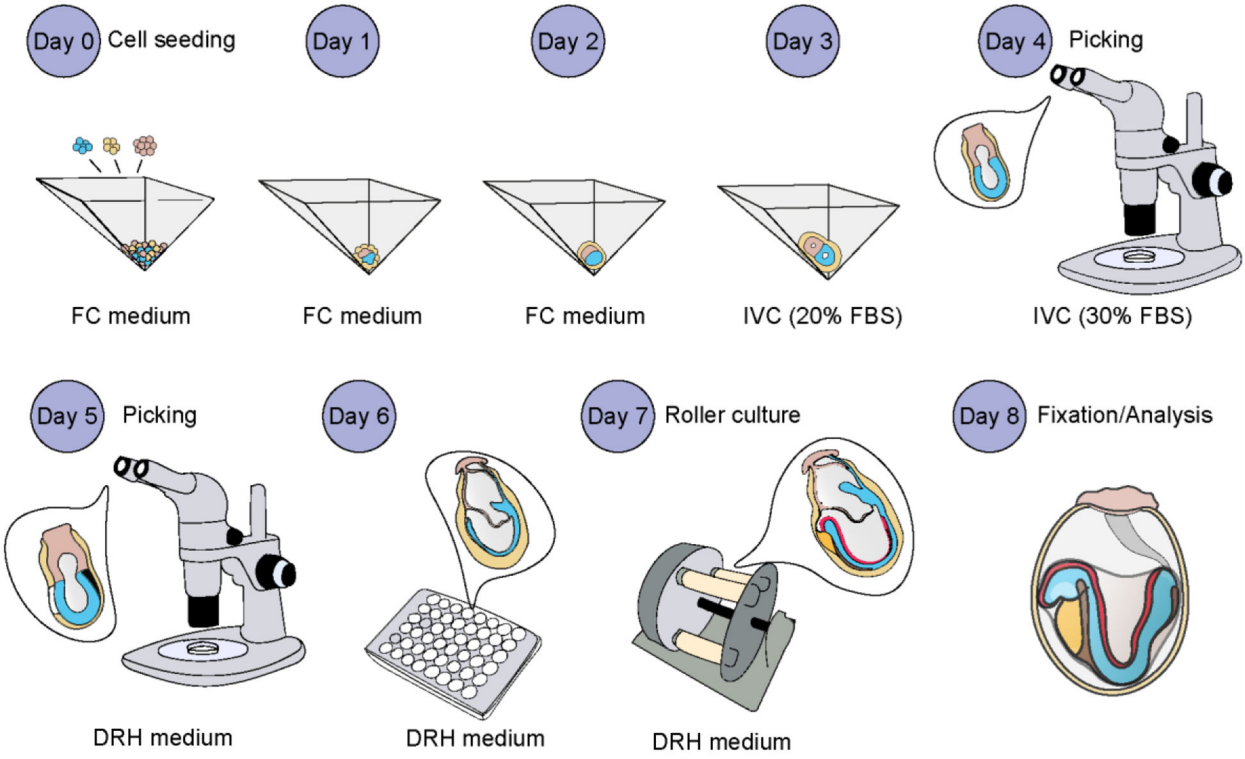


**Figure 3:**
Schematic depicting the preparation of ETiX-embryoids.