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Assembly of complete mouse embryo models from embryonic and induced stem cell types *in vitro*

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Abstract

The interaction between embryonic and extraembryonic tissues is critical in natural mouse embryogenesis. To enable such interaction *in vitro*, here we describe a protocol to assemble a complete mouse embryo model using mouse embryonic stem cells (ESCs) and induced ESCs to express *Cdx2* (or trophoblast stem cells) and *Gata4* to reconstitute the epiblast, extraembryonic ectoderm and visceral endoderm lineages respectively. The resulting complete embryo models recapitulate development from embryonic day 5.0 to 8.5, generating advanced embryonic and extraembryonic tissues that develop through gastrulation to initiate organogenesis to form a head and a beating heart structure as well as a yolk sac and chorion. Once the required stem cell lines are stably maintained in culture, the protocol requires 1 day to assemble complete embryo models and 8 further days to culture them until headfold stages, although structures can be collected at earlier developmental stages as required. This protocol can be easily performed by researchers with experience in mouse stem cell culture, although they will benefit from knowledge of natural mouse embryos at early post-implantation stages.

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Author contributions

K.Y.C.L. performed the experiments. K.Y.C.L. and G.A. developed the protocol with the guidance from M.Z-G. M.Z.-G. conceived and supervised the project. K.Y.C.L., G.A. and M.Z.-G. wrote the manuscript.

Editorial summary: Protocol to assemble complete mouse embryo models from embryonic and induced stem cells to generate advanced embryonic and extraembryonic organs

Proposed tweet: Assembly of complete mouse embryo models from embryonic and induced stem cell types *in vitro* until organogenesis

Proposed teaser: Complete mouse embryo models from stem cells

Competing interests

A patent ‘Generation of synthetic embryos from multiple stem cell types’ was filed by California Institute of Technology and the University of Cambridge under CIT File Number: CIT-8826-P and Serial Number: 63/344,251

Introduction

As the mouse embryo implants, it undergoes drastic morphological changes to form the egg cylinder consisting of epiblast (EPI), extraembryonic ectoderm (ExE) and visceral endoderm (VE), which give rise to embryo proper, placenta and yolk sac respectively. The complex spatiotemporal interaction between embryonic and extra-embryonic tissues is essential for body axis patterning and cell lineage allocation in the post-implantation mouse embryo[1]. While many mouse embryonic stem cell (ESC)-based *in vitro* models have been developed to capture discrete aspects of development[2–6], a complete embryo model should consist of both embryonic and extraembryonic tissues to fully recapitulate mammalian embryo development. We have reported the generation of two induced stem cell embryo models, that allowed us to reconstitute the three fundamental tissue lineages of the post-implantation embryo, EPI, ExE and VE, using ESCs, Cdx2-overexpressing ESCs or trophoblast stem cells (TSCs), and Gata4-overepxressing ESCs respectively. We showed that they are able to recapitulate development from embryonic day (E) 5.0 to 8.5, generating advanced embryonic tissues (including headfolds and a beating heart structure) and extraembryonic tissues (including a yolk sac and chorion)[7,8]. Here we describe the full protocol for their generation (see Box 1 for list of abbreviations used in this protocol).

Development of the protocol

Our efforts to fully model the mouse embryo began with the co-culture of ESCs and trophoblast stem cells (TSCs) suspended in Matrigel to form the ‘ETS embryo’ resembling the post-implantation egg cylinder[9] (with supporting protocol[10]). Our next step was to also incorporate extraembryonic endoderm (XEN) stem cells into our embryo model and in this way to create a complete embryo model with all its lineages, that we termed ‘ETX embryo’. We showed that the ETX embryo was able to undergo anterior-posterior patterning and gastrulation-related events, including the epithelial-to-mesenchymal transition (EMT), followed by cell migration between the epiblast and VE lineages, to form the mesoderm and definitive endoderm[11]. The ETX also formed the primordial germ cells[11]. To further extend our complete embryo model, we substituted XEN cells with ESCs that we induced to differentiate into the primitive endoderm lineage by transiently overexpressing the primitive endoderm master regulator, *Gata4*[12], upon doxycycline (Dox) induction (i*Gata4* ESCs). The resulting ‘ETiX embryo’ (also known as ‘iETX embryo’) better captured morphogenetic events, including migration of the anterior signalling centre, the anterior visceral endoderm (AVE) that specifies the anterior-posterior patterning, and completed gastrulation movements on the posterior epiblast to induce specification of the node[13]. By utilising the roller culture system that supports *in vitro* culture of post-implantation mouse embryos to organogenesis stages[14,15], we showed that this complete mouse ETiX-embryo model could undertake embryogenesis up to E8.5[7]. Finally, we replaced TSCs by inducing ESCs to differentiate into the trophectoderm by transiently expressing the TE master regulator, *Cdx2*[16,17] (i*Cdx2* ESCs) and created the double-induced embryo model, so-called ‘EiTiX embryo.’ We chose to express *Cdx2* based on reports showing that overexpression of Cdx2 is sufficient to drive embryonic cells to the trophectoderm lineage within the embryo[18], and that elimination of Cdx2 in the embryo prevents trophectoderm specification[17] as well as *Cdx2*-overexpressing ESCs transdifferentiating into TSC-like

cells[19]. To generate EiTiX embryos, we have made modifications to the ETiX embryo protocol to better support i*Cdx2* ESCs (see Table 1 for a summary of these *in vitro* embryo models).

Comparing the double-induced and single-induced embryo models (EiTiX embryo and ETiX embryo respectively), both models generated major post-implantation tissues, including embryonic tissues such as headfolds and heart, and extraembryonic tissues such as allantois, chorion and yolk sac blood islands. However, we found that ETiX embryos might be able to capture more cell types than EiTiX embryos[8], such as a larger number of primordial germ cells (PGCs) and cells of the ectoplacental cone, reflecting potential differences between TSCs and i*Cdx2* ESCs in their ability to form the extrembryonic tissues. ETiX embryos also have a higher initial formation efficiency on Day 4 than EiTiX embryos (21.7 and 15.5% respectively); however, they share similar efficiency to progress to late headfold stages (Table 2). An advantage of the EiTiX embryo over the ETiX embryo is that the EiTiX embryo is solely derived from ESCs, eliminating the use of TSCs that are typically cultured in undefined conditions[20,21]. Furthermore, TSCs are reported to contain heterogeneous subpopulations resembling different stages of trophoblast development[22], potentially increasing the variability of embryo formation. For completeness, we have provide detailed instructions to make both induced models we have generated – single and induced - ETiX and EiTiX embryos in this protocol. Steps specific for ETiX embryos (which involve the use of TSCs) are indicated in the method.

Applications of the method

Our complete embryo model is an *in vitro* system that allows the study of morphogenesis during embryonic development from very early post-implantation stages to stages of early organogenesis with headfold and heart formation. Since ETiX and EiTiX embryos are generated from three types of mouse stem cells to reconstitute the three fundamental cell lineages in the embryo, tissue-specific gene functions can be studied by genetic manipulation of the corresponding stem cell type (wildtype ESC for EPI lineage, i*Cdx2* ESCs or TSCs for ExE lineage and i*Gata4* ESCs for VE lineage). For example, CRISPR constructs can be used to knockout a gene-of-interest and reporter constructs can be transfected into ESCs to monitor spatiotemporal gene expression. Importantly, complete embryo models contain both embryonic and extraembryonic lineages and recapitulate their spatiotemporal chemical and mechanical interactions. With the ease of manipulating the culture condition of an *in vitro* system, for example by adding signalling molecules or pharmacological inhibitors, complete embryo models could be used to delineate the interplay and role of signalling pathways in post-implantation development, in processes such as symmetry breaking, anterior-posterior and left-right axes specifications and early organogenesis.

Comparison with other methods

While there are many existing *in vitro* models of mouse embryogenesis, to this date only our complete induced embryo models (EiTiX embryo[8] and ETiX embryo[7]) and the recent similar sEmbryo[23] have been reported to recapitulate development from gastrulation to headfold stages. sEmbryos use a similar induction strategy as we do in our EiTiX embryos

and have also been shown to develop up to E8.5. However, a longer induction period is used to drive *Cdx2* and *Gata4* overexpression to reconstitute the ExE and VE lineages for sEmbryo generation[23]. Prior to cell aggregation, i*Gata4* ESCs and i*Cdx2* ESCs are induced for 1 day and 1–14 days respectively to generate sEmbryos. In contrast, both inducible ESCs are induced for 6 hours only to generate EiTiX embryo. Moreover, sEmbryos are cultured on a roller culture incubator connected to an electronic device to regulate gas and pressure from Day 5 (early gastrulation stage). A similar apparatus is used to culture EiTiX embryos only from Day 7 and therefore EiTiX embryos can be cultured to early headfold stage without such apparatus and in static culture conditions.

Experimental design

Overview of the procedure—In this protocol we provide a detailed Procedure for our induced complete embryo generation from ESCs - EiTiX embryo - with modifications for ETiX embryo generation detailed in Box 1 The protocol of EiTiX embryo generation is summarised in Figure 1. Briefly, when the three types of ESCs are stably cultured after thawing (Steps 1–22), they are assembled and cultured for 4 days (Steps 23–42). EiTiX embryos displaying the correct morphology (resembling the E5.5 egg cylinder stage embryos) are selected on Day 4 (Steps 43–53) and are further cultured up to Day 8 (Steps 54–66), with selection of EiTiX embryos that successfully progress to the next day on each day from Day 4 onwards. From Day 0 to Day 4, a 24-well AggreWell plate is used, where each AggreWell contains 1,200 microwells that have an inverted pyramidal shape (Figure 2). Cell aggregates are formed individually in each microwell. This protocol is written based on setting up one well of a 24-well AggreWell plate, although 4–6 wells are typically set up in each experiment to generate a sufficient number of embryo models for culture until Day 8. The number of wells to be set up and the corresponding volumes of media required can be scaled up according to experimental need. On Day 4, EiTiX embryos are transferred and cultured in static conditions in a non-adherent 6-well plate. On Day 5 and Day 6, they are cultured individually in a 48-well plate also statically. On Day 7, EiTiX embryos are transferred to a rotating bottle culture chamber. An orbital shaker in a tissue culture incubator may also be used but EiTiX embryo development in the two apparatuses is yet to be compared. The generation of ETiX embryos follow the same overall experimental set-up, except the use of TSCs instead of iCdx2 ESCs and that only iGata4 ESCs require Dox induction. Procedures for the maintenance of MEFs, TSCs and the use of TSCs in ETiX embryo generation are detailed in Box 1. We also a detailed Procedure for fixation and immunofluorescence staining of our complete mouse embryo models (Steps 67–83).

Optimization of the iGata4 ESC line—We have previously described methods for generating new i*Gata4* ESC lines[13]. When establishing a new i*Gata4* ESC line for complete embryo model formation, it is important to isolate and expand several clonal lines for further quality control and testing. In the first instance, one should treat candidate clones with Dox for 6 hours, isolate mRNA from them and assess Gata4 mRNA expression level in Dox-treated and untreated cells to identify a clone with substantial, in our case 150–200-fold, increase in *Gata4* mRNA level. Following this initial test, selected clones should be treated with Dox for 6 hours, fixed, and analysed by immunofluorescence to assess for the upregulation of endodermal factors such as Gata4, Gata6 and Sox17. Clones that

successfully upregulate these transcription factors in response to *Gata4* overexpression are good candidates for the formation of complete embryo models.

Optimization of the iCdx2 ESC line—We have previously described methods for generating new i*Cdx2* ESC lines[8]. When establishing a new i*Cdx2* ESC line for complete embryo model formation, it should also be tested similarly as the i*Gata4* ESC line. First, several clonal lines should be isolated and expanded for further quality control and testing. In the first instance, one should treat the candidate clones with Dox for 6 hours, isolate mRNA from them and assess *Cdx2* mRNA expression level in Dox-treated and untreated cells to identify a clone in which the *Cdx2* fold change is substantial, in our case on average 180 folds. Following this initial test, selected clones should be treated with Dox for 6 hours, fixed, and analysed by immunofluorescence to assess for the upregulation of trophectoderm/trophoblast marker genes such as Eomes and Cdx2. Clones that successfully upregulate Eomes expression in response to *Cdx2* overexpression are good candidates for the formation of complete embryo models.

Testing of serum batches—From Day 5 and onwards the medium used for complete embryo model culture requires rat serum and human serum, and their quality is critical to support the development of the complete embryo model. Regardless of the source of these sera (purchased commercially or made in-house), one common issue affecting serum preparation is hemolysis. It usually occurs when blood is not handled properly during serum preparation and lysed blood cells release their contents into the serum. Properly prepared rat serum should be clear with a light brown/beige colour. Human serum should also be clear with a very bright yellow/straw colour. Hemolysis is reflected by a pink/orange/red hue of the serum. The darker the red hue, the more lysed blood is present. Hemolysis will affect serum performance and such serum samples should not be used. We test each serum batch, by culturing natural mouse embryos from E6.5 to E8.5. Suboptimal batches of serum will generally support natural embryo development from E6.5 to E7.5 but not beyond, and embryos will either die or show stunted growth or malformations. Only serum batches that successfully support mouse embryo development from E6.5 to E8.5 should be utilised to culture complete embryo models.

Consideration for stem cell culture in feeder cell (FC) medium—Routinely, our ESC lines are maintained in N2B27 2iLIF medium but during complete embryo model formation cells are aggregated in FC medium (see Reagent Setup). This culture medium change does not constitute an issue for the cell lines reported here because they are able to grow and develop in both media conditions. However, for cell lines that have not been tested before in this assay, it is be important to test whether they can tolerate, proliferate and differentiate in FC medium. This can be accomplished by culturing the cell lines in 2D with FC medium and assess whether they can differentiate. Successful differentiation can be identified by detecting upregulation of Otx2, Brachyury, FoxA2 and Sox17. Instead, if cell death occurs or these markers are not upregulated, it is likely that a specific cell line is not compatible with FC medium and is therefore not suitable for complete embryo model formation.

For ETiX embryo formation, one must also ensure that TSCs, which are routinely maintained in TSF4H medium, can be switched to FC medium during experiments. Similar to ESCs, if TSCs can differentiate without cell death in FC medium, they are likely to be compatible with the protocol to generate ETiX embryos. Successful differentiation can be indicated by the downregulation of Eomes and Cdx2 while the expression of Ap2γ is maintained.

Limitations

We have previously reported that the efficiencies of EiTiX embryo and ETiX embryo formation on Day 4 were 15.5% and 21.7% respectively (see also Table 2) and selection of successfully generated complete embryo models was performed on Day 4 for further culture[8,13]. This can be mitigated by the fact that the number of complete embryo structures generated can be easily scaled up by setting up more AggreWells. We include here the predicted number of structures that can be expected to develop successfully on each day of culture, starting from one AggreWell set up on Day 0 (Table 2). It is important to note that with the current protocol we were unable to predict which structures will successfully progress, therefore analyses at any given time are performed on both structures that will develop and structures that will not. It is also currently not known in which way well-developed induced or double induced embryos differ from the ones that fail to develop.

We have also reported that there were missing cell types in EiTiX and ETiX embryos, particularly in the ExE lineage, although there was still advanced development in the embryonic lineage as well as yolk sac and chorion development. The success of the protocol can also be affected by the starting condition of ESCs and TSCs (for ETiX embryos) and their genetic backgrounds. It is recommended to use CD1 wildtype ESCs, CD1 i*Gata4* ESCs and CAG-GFP i*Cdx2* ESCs (for EiTiX embryos) or CD1 wildtype TSCs (for ETiX embryos) to generate complete embryo models to headfold stages. Alternative ESC and TSC lines might be used but the resulting complete embryo models may have different developmental potential. Novel i*Gata4* and i*Cdx2* ESCs should be optimised following the methods described in ‘Experimental design.’

Materials

Biological materials

- **Cell lines**
  - Mouse embryonic stem cells (ESCs)
    - CD1 wildtype ESCs (a gift from Dr Takuya Azami and Prof Jennifer Nichols)
    - CAG-GFP i*Cdx2* ESCs (generated in-house)[8] (specific to EiTiX embryo formation)
    - CD1 i*Gata4* ESCs (generated in-house)[7]
  - Trophoblast stem cells (TSCs) (specific to ETiX embryo formation)
    - CD1 wildtype TSCs (generated in-house)[12]

- Mouse embryonic fibroblasts (MEFs) (Thermo Fisher Scientific, cat. no. A24903) (specific to ETiX embryo formation)

Cell lines are available from the corresponding author upon request.

CRITICAL It is critical that ESCs in culture should not be differentiating (as indicated by flattening colonies) as it can prevent the formation of complete embryo models. It is also critical to avoid maintaining ESCs in culture for more than 10 passages and to avoid using ESCs over passage number 30, which can impair embryo formation efficiency and development. Cell lines with other genetic backgrounds might be used but the resulting embryo models can have different developmental potential (see 'Limitations').

CRITICAL (specific to ETiX embryo model) It is critical that TSCs in culture should not be differentiating as it can prevent the formation of ETiX embryos. TSCs should also not be kept in culture for more than 10 passages and to avoid using TSCs over passage number 30, which can impair ETiX embryo formation efficiency and development. TSC lines with other genetic backgrounds might be used but the resulting ETiX embryos can have different developmental potential (see 'Limitations'). Unhealthy MEF cultures can also impair stem cell potential and growth of TSCs.

CAUTION The cell lines used in your research should be checked every 2 weeks to ensure that they are authentic and are not infected with mycoplasma.

- CD female rat serum

  We obtain CD female rat serum from Charles River Laboratories but it can also be produced in-house. It is recommended to use plain tubes rather than monovettes for the collection of blood during preparation of rat serum. See 'Reagent setup' for heat inactivation and storage of rat serum.

  CRITICAL The quality of rat serum should be validated according to the method described in 'Experimental design.'

  CAUTION Biological risk assessment might be required by the institute for the use of rat serum. It should be handled and disposed of according to institutional guidelines.

- Human cord serum

  We obtain human cord serum from the Cambridge Blood and Stem Cell Biobank. Adult human blood serum (Sigma-Aldrich, cat. no. H3667 or H5667) might be used instead of human cord serum but can result in lower efficiency of embryo development. See 'Reagent setup' for heat inactivation and storage of human serum.

  CRITICAL The quality of human cord serum should be validated according to the method described in 'Experimental design.'

  CAUTION Human cord serum should be screened to ensure it is free from any bloodborne viruses. Biological risk assessment might be required by the institute

for the use of human cord serum. It should be handled and disposed of according to institutional guidelines.

Reagents

Cell culture media and supplements

- DMEM/F12 (Gibco, cat. no. 21331–020)
- Neurobasal A (Gibco, cat. no. 10888–022)
- B27 (Gibco, cat. no. A1895601)
- N2 (made in-house, see ‘Reagent setup’)
  - DMEM/F12 (Gibco, cat. no. 21331–020)
  - Apo-transferrin (R&D Systems, cat. no. 3188-AT-100MG)
  - Bovine albumin fraction V (Thermo Fisher Scientific, cat. no. 15260037)
  - Human insulin solution (Sigma-Aldrich, cat. no. I9278–5ML)
  - Putrescine dihydrochloride (Sigma-Aldrich, cat. no. P5780–5G)
  - Sodium selenite (Sigma-Aldrich, cat. no. S5261–10G)
  - Progesterone (Sigma-Aldrich, cat. no. P8783–1G)
- Beta-mercaptoethanol (Gibco, cat. no. 31350–010)
- Penicillin/streptomycin (Gibco, cat. no. 15140–122)
- GlutaMAX (Thermo Fisher Scientific, cat. no. 35050–038)
- CHIR99021 (STEMCELL Technologies, cat. no. 72052)
- PD0325901 (STEMCELL Technologies, cat. no. 72182)
- Mouse leukaemia inhibitory factor (LIF) (STEMCELL Technologies, cat. no. 78056.1)
- DMEM (Gibco, cat. no. 41966–029)
- Foetal bovine serum (FBS) (Gibco, cat. no. 10270–098)
- NEAA (Gibco, cat. no. 11140–035)
- Sodium pyruvate (Gibco, cat. no. 11360–039)
- Heparin (Sigma-Aldrich, cat. no. H3149–25KU)
- Recombinant mouse Fgf4 (aa 67–202) protein (R&D Systems, cat. no. 7486-F4-025)
- RPMI-1640 (Thermo Fisher Scientific, cat no. 21870076)

Cell culture reagents

- Gelatin from porcine skin (Sigma-Aldrich, cat. no. G1890–100G)

- Trypsin-EDTA (Gibco, cat. no. 25300054)
- PBS (Gibco, cat. no. 10010–015)
- Nuclease-free water (Life Technologies, cat. no. AM9937)
- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, cat. no. D2650–5X10ML)

For setting up EiTiX embryos only

- Doxycycline (Dox) (Sigma-Aldrich, cat. no. D9891–5G)
- Anti-adherence rinsing solution (STEMCELL Technologies, cat. no. 07010)
- Y-27632 (STEMCELL Technologies, cat. no. 72304)

EiTiX embryo culture media and supplements

- Advanced DMEM/F12 (Gibco, cat. no. 12634–010)
- Insulin-transferrin-selenium-ethanolamine (ITS-X) (ThermoFisher Scientific, cat. no. 51500–056)
- β-estradiol (Sigma-Aldrich, cat. no. E8875–1G)
- Progesterone (Sigma-Aldrich, cat. no. P8783–1G)
- N-acetyl-L-cysteine (NAC) (Sigma-Aldrich, cat. no. A7250)
- DMEM, low glucose, pyruvate, no glutamine, no phenol red (Gibco, cat. no. 11880–028)
- HEPES (Gibco, cat. no. 15630–056)
- D-Glucose (Sigma, cat. no. G8644)

For fixation and immunofluorescence

- 16% formaldehyde solution (wt/vol) (ThermoFisher Scientific, cat. no. 28908) CAUTION Formaldehyde is a potential carcinogen and should be handled and disposed according to institutional safety guidelines
- Tween 20 (Sigma-Aldrich, cat. no. P9416–100ML)
- Triton X-100 (Sigma-Aldrich, cat. no. T9284–100ML)
- Glycine (Fisher Scientific, cat. no. BP381–1)
- Mineral oil (Fujifilm Irvine Scientific, cat. no. 9305)
- DAPI (Thermo Fisher Scientific, cat. no. D3571)
- Primary antibodies (see Table 3)
- Secondary antibodies (see Table 4)

Equipment

- Cell-culture treated plate (6-well: Thermo Fisher Scientific, cat. no. 140675; 12-well: Thermo Fisher Scientific, cat. no. 150628)

- Graduated filter tips (1ml: Starlab, cat. no. S1126-7710-C; 200µl: Starlab, cat. no. S1120-8810-C; 20µl: Starlab, cat. no. S1120-1710-C; 10µl: Starlab, cat. no. S1121-3810-C)
- Serological pipettes (50ml: VWR, cat. no. 734–1743; 25ml: VWR, cat. no. 734–1695; 10ml: VWR, cat. no. 734–1693; 5ml: VWR, cat. no. 734–1691)
- Centrifuge tubes (50ml: Fisher Scientific, cat. no. 10788561; 15ml: Fisher Scientific, cat. no. 10136120)
- 24-well AggreWell400 (STEMCELL Technologies, cat. no. 34415)
- Non-adherent 6 well multiwell plate (Greiner Bio-One, cat. no. 657185)
- Non-adherent 48-well multi-well plate (Greiner Bio-One, cat. no. 677102)
- 4-well cell culture dish (Thermo Scientific, cat. no. 176740)
- 96 well round (U) bottom plate (Thermo Scientific, cat. no. 163320)
- 0.45µm syringe filter (Sartorius, cat. no. 16555-K)
- 10ml syringe (BD, cat. no. SYR004)
- 7ml transfer pipette (VWR, cat. no. 612–1681)
- 35mm dish (Corning, cat. no. 351008)
- CO_2 incubator (Thermo Scientific, cat. no. Heracell 150i)
- Water bath (Grant, cat. no. SUB Aqua 12 Plus)
- Slide warmer (Ted Pella, cat. no. XH-2002)
- Stereomicroscope (Nikon, cat. no. SMZ1000)
- Centrifuge (Eppendorf, cat. no. 5702)
- Plate centrifuge (Eppendorf, cat. no. 5810 R)
- Haemocytometer (Sigma-Aldrich, cat. no. Z359629–1EA)
- Dumont #5 - Fine Forceps (Fine Science Tools, cat. no. 11254–20)
- Rotating bottle culture chamber (see ‘Equipment setup’)
  - BTC rotating bottle culture unit (Cullum Starr Precision Engineering Limited)
  - BTC Precision Incubator (Cullum Starr Precision Engineering Limited)
  - Electronic gas regulator (Arad Technologies)
  - OxyStreamer O_2 and CO_2 controller (BioSpherix)

Reagent setup

Reconstitution of media supplement

For the following reagents stored at −20°C, we recommend storing them as small aliquots to avoid repeated cycles of freezing and thawing, which can deteriorate the reagent and affect cell maintenance and experimental outcomes.

- *CHIR99021 stock solution (10mM):* Prepare a stock solution of CHIR99021 (10mM) by resuspending 1mg CHIR99021 powder in 214.9μl DMSO. Divide into 30μl aliquots. Store at −20°C for up to 6 months.
- *PD0325901 stock solution (10mM):* Prepare a stock solution of PD0325901 (10mM) by resuspending 1mg PD0325901 powder in 207μl DMSO. Divide into 20μl aliquots. Store at −20°C for up to 6 months.
- *Mouse LIF stock solution (10μg/ml):* Prepare a stock solution of mouse LIF (10μg/ml) by resuspending 10μg mouse LIF powder in 1ml H_2O. Divide into 200μl aliquots. Store at −20°C for up to 6 months.
- *Fgf4 stock solution (100μg/ml):* Prepare a stock solution of Fgf4 (100μg/ml) by resuspending 25μg Fgf4 powder in 250μl nuclease-free water. Divide into 6μl aliquots. Store at −20°C for up to 3 months.
- *Heparin stock solution (1mg/ml):* Prepare a stock solution of heparin (1mg/ml) by resuspending 2mg heparin powder in 2ml nuclease-free water. Filter and divide into 100μl aliquots. Store stock aliquots at −20°C for up to 6 months and thawed aliquots at 4°C for up to 1 month.
- *Dox stock solution (1mg/ml):* Prepare a stock solution of Dox (1mg/ml) by dissolving 0.005g Dox powder in 5ml nuclease-free water. Filter and divide into 50μl aliquots. Store stock solution at −20°C for up to 1 month and use fresh aliquots each time. CRITICAL Dox is light-sensitive and should be protected from light.
- *Y-27632 stock solution (20mM):* Prepare a stock solution of Y-27632 (20mM) by dissolving 5mg Y-27632 powder in 780μl DMSO. Divide into 30μl aliquots. Store at −20°C for up to 6 months.
- *β-estradiol stock solution (10μM):* Prepare a 10mM solution of β-estradiol by dissolving 0.0027g β-estradiol powder in 1ml DMSO. Dilute to final stock concentration of 10μM by adding 2μl 10mM solution in 2ml DMSO. Filter and divide into 50μl aliquots. Store at −20°C for up to 1 month.
- *Progesterone stock solution (1mg/ml):* Prepare a stock solution of progesterone (1mg/ml) by dissolving 0.005g progesterone powder in 5ml DMSO. Filter and divide into 30μl aliquots. Store at −20°C for up to 1 month.
- *NAC stock solution (50mM):* Prepare a stock solution of NAC (50mM) by dissolving 0.04g progesterone powder in 5ml nuclease-free water. Filter and divide into 50μl aliquots. Store at −20°C for up to 1 month.

Heat inactivation and storage of serum

- *FBS*: Thaw FBS at 4°C overnight and heat inactivate at 56°C for 30 minutes. Divide into 50ml aliquots. Store stock aliquots at −20°C for up to 6 months and store thawed aliquots at 4°C for up to 1 month.
- *Rat serum*: Thaw rat serum at room temperature (RT) (20–25°C) and heat inactivate at 56°C for 30 minutes. Centrifuge at 600g for 40 minutes at 4°C and remove any white precipitate on top. Divide into 1ml aliquots and store heat-inactivated aliquots at −80°C for up to 1 month. Store thawed heat-inactivated aliquots at 4°C and use within 2 days. CRITICAL It is crucial to ensure precipitates are removed, as residual blood clotting proteins in rat serum can impair EiTiX and ETiX embryo development.
- CRITICAL Do not refreeze thawed heat-inactivated aliquots.
- *Human cord serum:* Thaw human cord at RT and heat inactivate at 56°C for 30 minutes. Divide into 0.5ml aliquots and store heat-inactivated aliquots at −80°C for up to 1 month. Store thawed heat-inactivated aliquots at 4°C and use them within 2 days. CRITICAL Do not refreeze thawed heat-inactivated aliquots.

Preparation of culture media

- *N2:* Prepare N2 according to the following table. Divide into 400µl aliquots and store at −20°C for up to 1 year.

Composition	Volume (10ml)	Final concentration
DMEM/F12	5.357ml	
Apo-transferrin (100mg/ml)	1ml	10mg/ml
Bovine albumin fraction V (7.5% wt/vol)	1ml	0.75% (wt/vol)
Human insulin solution (10mg/ml)	2.5ml	2.5mg/ml
Putrescine dihydrochloride (160mg/ml)	100µl	1.6mg/ml
Sodium selenite (6mg/ml)	10µl	6µg/ml
Progesterone (0.6mg/ml)	33µl	2µg/ml

- *N2B27:* Prepare N2B27 medium according to the following table. Store at 4°C for up to 1 month.

Composition	Volume (40ml)	Final concentration
DMEM/F12	20ml	50% (vol/vol)
Neurobasal A	20ml	50% (vol/vol)
B-27	400µl	1% (vol/vol)
N2	200µl	0.5% (vol/vol)
Beta-mercaptoethanol	80µl	0.1mM
Pen/strep	400µl	1% (vol/vol)
GlutaMAX	400µl	2mM

- *N2B27 2iLIF:* Prepare N2B27 2iLIF medium according to the following table. Store at 4°C for up to 2 weeks. Warm medium in 37°C water bath for 5 minutes before use.

Composition	Volume (20ml)	Final concentration
N2B27	20ml	
CHIR99021 (10mM stock)	6µl	3µM
PD0325901 (10mM stock)	2µl	1µM
Mouse LIF (10µg/ml stock)	20µl	10ng/ml

- *Feeder cell (FC) medium:* Prepare FC medium according to the following table. Store at 4°C for up to 3 months. Prepare a 10 ml aliquot for thawing each vial of cells and prepare a 50 ml aliquot as a working stock for cell maintenance and culture of complete embryo models. Warm the aliquots in 37°C water bath for 5 minutes before use.

Composition	Volume (600ml)	Final concentration
DMEM	500ml	
Inactivated FBS	90ml	15% (vol/vol)
GlutaMAX	6ml	2mM
Pen/strep	6ml	1% (vol/vol)
NEAA	6ml	1% (vol/vol)
Sodium pyruvate	6ml	1mM
Beta-mercaptoethanol	1.2ml	0.1mM

- *FCF4H medium (specific to EiTiX embryo):* Prepare FCF4H medium according to the following table. Store at 4°C for up to 2 weeks. Warm medium in 37°C water bath for 5 minutes before use.

Composition	Volume (20ml)	Final concentration
FC medium	20ml	
Fgf4 (100µg/ml stock)	5µl	25ng/ml
Heparin (1mg/ml stock)	20µl	1µg/ml

- *FCF4H medium with Y-27632 (for use on Day 0) (specific to EiTiX embryo):* Prepare FCF4H medium with Y-27632 according to the following table. Warm FCF4H medium in 37°C water bath for 5 minutes before adding Y-27632. Prepare this medium fresh every time.

Composition	Volume (10ml)	Final concentration
FCF4H medium	10ml	
Y-27632 (20mM stock)	1µl	2µM

- *TS base medium (specific to ETiX embryo):* Prepare TS base medium according to the following table. Store at 4°C for up to 1 month. Prepare a 10 ml aliquot for thawing each vial of TSCs and warm in 37°C water bath for 5 minutes before use.

Composition	Volume (52 ml)	Final concentration
RPMI-1640	40ml	
Inactivated FBS	10.4ml	20% (vol/vol)
GlutaMAX	520μl	2mM
Pen/strep	520μl	1% (vol/vol)
Sodium pyruvate	520μl	1mM
Beta-mercaptoethanol	104μl	0.1mM

- *TSF4H medium (specific to ETiX embryo):* Prepare TSF4H medium according to the following table. Store at 4°C for up to 7 days. Warm TSF4H medium in 37°C water bath for 5 minutes before use.

Composition	Volume (20 ml)	Final concentration
TS medium	20ml	
Heparin (1mg/ml stock)	20μl	1μg/ml
Fgf4 (100μg/ml stock)	5μl	25ng/ml

- *Peri-implantation culture medium base (for use on Day 3 and Day 4):* Prepare peri-implantation culture medium base according to the following table. Store at 4°C for up to 1 month.

Composition	Volume (50ml)	Final concentration
Advanced DMEM/F12	50ml	
GlutaMAX	500μl	2mM
Pen/strep	125μl	0.25% (vol/vol)
ITS-X	500μl	1% (vol/vol)

- *Peri-implantation culture medium (20% FBS) (for use on Day 3):* Prepare fresh peri-implantation culture medium (20% FBS) according to the following table on Day 3. Equilibrate the medium in an incubator at 37°C with cap slightly loosened for 20 minutes before use.

Composition	Volume (10ml)	Final concentration
Peri-implantation culture medium base	8ml	80% (vol/vol)
Inactivated FBS	2ml	20% (vol/vol)
β-estradiol (10μM stock)	8μl	8nM
Progesterone (1mg/ml stock)	2μl	200ng/ml

Composition	Volume (10ml)	Final concentration
NAC (50mM stock)	5μl	25μM

- *Peri-implantation culture medium (30% FBS) (for use on Day 4):* Prepare fresh peri-implantation culture medium (30% FBS) according to the following table on Day 4. Equilibrate the medium in an incubator at 37°C with cap slightly loosened for 20 minutes before use.

Composition	Volume (10ml)	Final concentration
Peri-implantation culture medium base	7ml	70% (vol/vol)
Inactivated FBS	3ml	30% (vol/vol)
β-estradiol (10μM stock)	8μl	8nM
Progesterone (1mg/ml stock)	2μl	200ng/ml
NAC (50mM stock)	5μl	25μM

- *Post-implantation culture medium base (for use on Day 5 and Day 6):* Prepare fresh post-implantation culture medium base according to the following table on Day 5 and Day 6.

Composition	Volume (1ml)	Final concentration
DMEM (low glucose, pyruvate, no glutamine, no phenol red)	969μl	
GlutaMAX	10μl	1% (vol/vol)
Pen/strep	10μl	1% (vol/vol)
HEPES	11μl	11mM

- *Post-implantation culture medium base with glucose (for use on Day 7):* Prepare fresh post-implantation culture medium base with glucose according to the following table on Day 7.

Composition	Volume (1ml)	Final concentration
DMEM (low glucose, pyruvate, no glutamine, no phenol red)	849μl	
D-glucose	120μl	3mg/ml
GlutaMAX	10μl	1% (vol/vol)
Pen/strep	10μl	1% (vol/vol)
HEPES	11μl	11mM

- *Post-implantation culture medium (for use on Day 5 and onwards):* Prepare fresh post-implantation culture medium according to the following table on each day from Day 5. Filter and equilibrate the medium in an incubator at 37°C with cap slightly loosened for 1 hour before use.

Composition	Volume (1ml)	Final concentration	Comments
Post-implantation culture medium base	250µl	25% (vol/vol)	Use medium base with added glucose on Day 7
Inactivated CD female rat serum	500µl	50% (vol/vol)	
Inactivated human cord serum	250µl	25% (vol/vol)	

- *Gelatin solution (1mg/ml):* Prepare gelatin solution (1mg/ml) by dissolving 0.5g gelatin powder into 500ml deionised water. Autoclave solution within 4 hours of preparation. Divide into 50ml aliquots and store at RT for up to 1 year.
- *4% formaldehyde solution (wt/vol)*: Prepare 4% formaldehyde solution (wt/vol) by adding 250µl 16% formaldehyde solution (wt/vol) to 750µl PBS. Prepare it fresh prior to fixation.
- *Permeabilization buffer*: Prepare permeabilization buffer by mixing 30µl of 10% Triton X-100 in PBS (vol/vol), 100µl 1M glycine solution in PBS, and 870µl PBS. Prepare it fresh prior to permeabilization.
- *Blocking buffer:* Prepare blocking buffer by mixing 10µl of 10% Tween 20 in PBS (vol/vol), 100µl filtered FBS, and 890µl PBS. Prepare it fresh for immunofluorescence.

Equipment setup

Rotating bottle culture chamber

Place a rotating drum inside an embryo culture incubator (Cullum Starr Precision Engineering Limited) that maintains 37°C[13]. Connect glass culture bottles to the rotating drum by hollow rubber stoppers and rotate at 30 rpm. Culture bottles are continuously gassed with 5% CO_2 controlled by an electronic regulator (Arad Technologies). Flow rate is indicated by the rate of bubbles formation in the water bottle at the outlet and is set such that a continuous line of bubbles is first observed.

Alternative set up a rotating bottle culture system using OxyStreamer O_2 and CO_2 controller (BioSpherix) connected to O_2, CO_2 and N_2 gas cylinders to supply a mix of 21% O_2 and 5% CO_2 to glass culture bottles connected to the rotating drum (Cullum Starr Precision Engineering Limited). An orbital shaker in tissue culture incubator may also be used instead of a rotating bottle culture chamber but EiTiX embryo development in the two apparatuses is yet to be compared.

Procedure

Thawing and culture of mouse ESCs TIMING minimum of 1 week

CRITICAL All ESCs should be cultured in N2B27 2iLIF medium on gelatinised wells at 37°C, 5% CO_2. i*Cdx2* ESCs should be cultured in a 6-well plate while i*Gata4* ESCs and wildtype ESCs should be cultured in 12-well plates. ESCs should be passaged before they

reach 80% confluency, typically every 3–4 days. ESCs should be passaged at least one time after thawing before using them to generate EiTiX embryos. ESC colonies should be round and have defined boundary. Mycoplasma screening should be performed every 2 weeks.

1. Warm a 10 ml aliquot of FC medium (one aliquot for each vial of ESCs to be thawed) and N2B27 2iLIF medium in a 37°C water bath for 5 minutes.
2. Gelatin-coat tissue culture plates by adding 2ml (for a 6-well) or 1ml (for a 12-well) of gelatin solution. Shake the plate gently to make sure the entire surface of the well is covered with gelatin.
3. Thaw a frozen vial of ESCs (wildtype ESCs, i*Cdx2* ESCs or i*Gata4* ESCs) by shaking vigorously in a 37°C water bath until a small piece of ice remains.
4. Immediately add 1ml of FC medium from the 10ml FC medium aliquot to the vial and transfer the content from the vial back to the FC medium aliquot.
5. Rinse the vial with 1ml of FC medium from the 10ml FC medium aliquot and transfer the content from the vial back to the FC medium aliquot. Repeat for a total of 2 times.
6. Centrifuge at 200g for 4 min at RT.
7. Aspirate gelatin from the well prepared in Step 2 and add N2B27 2iLIF medium to tissue culture plate (1.5ml for a 6-well and 0.5ml for a 12-well).
8. After centrifugation, aspirate supernatant and resuspend cell pellet by add 0.5ml of N2B27 2iLIF medium and pipetting up and down for 4 times gently.
9. Add cell suspension to the well with N2B27 2iLIF. Shake the plate gently to distribute ESCs. Place the plate in a 37°C incubator.
10. Change medium (2ml for a 6-well, 1ml for a 12-well) the next day after thawing and then every 2 days. Add medium along the side of the well since ESCs colonies can easily be detached. *TROUBLESHOOTING*
11. Monitor ESCs and when they reach 80% confluency, passage them into a new well by following Steps 12–20.
12. Gelatin-coat tissue culture plates by adding 2ml (for a 6-well) or 1ml (for a 12-well) of gelatin solution. Shake the plate gently to make sure the entire surface of the well is covered with gelatin.
13. Aspirate culture medium and wash the well by adding 1ml of PBS along the side of the well.
14. Aspirate PBS and add trypsin-EDTA (500μl for a 6-well, 400μl for a 12-well). Incubate the plate for 4 min at 37°C.
15. Stop trypsinisation by adding FC media (2ml for a 6-well, 1ml for a 12-well). Gently pipette up and down across the area of the well for 8 times to facilitate cell detachment. Collect cell suspension in a 15ml Falcon tube.
16. Centrifuge ESC suspension to form a cell pellet at 200g for 4 min at RT.

17. Aspirate supernatant and add 1ml of PBS to cell pellet. Pipette up and down for 6 times to resuspend cell pellet.

18. Centrifuge ESC suspension to form a cell pellet at 200g for 4 min at RT.

19. Aspirate supernatant and resuspend ESC pellet by adding N2B27 2iLIF medium (1ml to a cell pellet originated from a 6-well (i*Cdx2* ESCs) and 500μl to a cell pellet originated from a 12-well (i*Gata4* ESCs and wildtype ESCs)) and pipetting up and down for 6 times.

20. Aspirate gelatin from the well prepared in Step 12 and add 2ml (for a 6-well) or 1ml (for a 12-well) of N2B27 2iLIF medium. Replate ESCs at 1:20 dilution for maintenance. (ESCs might be plated at 1:10 or 1:5 dilution if required for generating EiTiX embryo in 2 days or 1 day respectively. However, frequent passaging is not recommended for long term ESC maintenance.)

21. Change medium every 2 days.

22. 2–3 days before generating EiTiX embryo, passage wildtype, i*Gata4* and i*Cdx2* ESCs at 70–80% confluency by following Steps 12–20. i*Gata4* and i*Cdx2* ESCs need to be passaged into two wells each such that one well will be used for Dox induction and one well will be used for maintenance. If the experiment is to be set up in 2 days, passage and replate the cells by performing a 1:10 dilution. If the experiment is to be set up in 3 days, passage and replate the cells by performing a 1:20 dilution. *CRITICAL STEP* i*Gata4* ESCs and i*Cdx2* ESCs cannot be replated for ESC maintenance after Dox induction.

Assembly of mouse stem cells to form EiTiX embryos (Day 0) TIMING 7–8 hours

23. Induce i*Gata4* ESCs and i*Cdx2* ESCs by replacing medium (2ml for a 6-well, 1ml for a 12-well) with N2B27 2iLIF supplemented with 1μg/ml Dox (stock concentration: 1mg/ml, 1000X). Shake cell culture plate gently to distribute the medium. Induce cells with Dox for 6 hours.

24. After 6 hours, warm FC medium, FCF4H medium, trypsin-EDTA and N2B27 2iLIF medium in a 37°C water bath for 5 minutes.

25. Prepare the AggreWell plate by adding 500μl of anti-adherence rinsing solution to each well of the 24-well AggreWell plate. The volumes of media given for the following steps are based on setting up one well of a 24-well AggreWell plate. However, typically 6 wells are set up in each experiment to generate a sufficient number of embryos for culture till Day 8. The number of wells to be set up and the corresponding volumes of media required can be scaled up linearly as needed.

26. Prepare an appropriate balance plate and centrifuge the AggreWell plate at 2,000g for 5 minutes at RT. *CRITICAL STEP* Check if there are bubbles remaining in microwells as bubbles can prevent the deposition of cells into microwells. If needed, repeat centrifugation until all bubbles are removed. See

Figure 2A for image of microwells after centrifugation with anti-adherence rinsing solution.

27. Leave AggreWell plate with anti-adherence rinsing solution for at least 30 min inside the biosafety cabinet.

28. Trypsinise and collect cell suspensions of wildtype ESCs, i*Gata4* ESCs and i*Cdx2* ESCs by following Steps 13–19. If also replating wildtype ESCs for maintenance, gelatin-coat an unused 12-well (see Step 12), and replate wildtype ESC at 1:20 dilution (as detailed in Step 20).

29. Determine the cell density of cell suspensions of wildtype ESCs, i*Gata4* ESCs and i*Cdx2* ESCs by loading 10μl of cell suspension to a haemocytometer. During counting, place tubes with cell suspension in an 37°C incubator with cap slightly loosened. *TROUBLESHOOTING*

30. Determine the volumes of cell suspensions required for seeding into one well of an AggreWell plate. For each AggreWell, 6,000 wildtype ESCs, 6,000 i*Gata4* ESCs and 38,400 i*Cdx2* ESCs are required. Scale up the volume according to the number of AggreWells to be set up but prepare extra volume to account for loss from pipetting when distributing the cell mixtures across several wells (i.e. if setting up 6 wells, prepare a cell mixture for 7 wells).

31. Pipette cell suspensions up and down for 3 times to ensure cell suspensions are homogenous. Add the required volumes of the 3 cell suspensions to a 15ml Falcon tube.

32. Centrifuge the cell mixture at 200g for 4 min at RT.

33. During centrifugation of the cell mixture, aspirate the anti-adherence rinsing solution from the AggreWell and wash each well twice with 1ml of PBS each time. Aspirate PBS and add 500μl of FCF4H medium to each well.

34. Prepare FCF4H medium with Y-27632 by adding 1μl of Y-27632 to 10ml of FCF4H medium.

35. After centrifugation of the cell mixture from Step 32, remove supernatant by pipetting. *CRITICAL STEP* Remove supernatant by pipetting instead of using a vacuum aspirator since the cell pellet can be very small.

36. Resuspend the cell pellet with 1ml of FCF4H medium with Y-27632 by pipetting up and down for 4 times. If seeding more than one well, scale up the volume according to the number of wells to be seeded (1ml is required per well): first resuspend the cell pellet with 1ml of FCF4H medium with Y-27632 and the add the remaining volume using a serological pipette. Gently rotate the Falcon tube 3 times and pipette up and down for 4 times to ensure the cell suspension is homogenous.

37. Add the resuspended cell mixture drop by drop across the area of the well (1ml of cell mixture per well).

38. Prepare an appropriate balance plate and centrifuge the AggreWell plate at 100g for 3 min at RT.

39. Check the AggreWell under a microscope to confirm cell seeding and put it in a 37°C incubator. *CRITICAL STEP* Handle each AggreWell plate very carefully after cell seeding to avoid dislodging cells from microwells. *TROUBLESHOOTING*

Generation of EiTiX embryos (Day 1 to 3) TIMING 3 days

CRITICAL Medium changes on Day 1, Day 2 and Day 3 (Step 40 to 42) should be performed as close to 24-hour intervals from Step 39 as possible.

40. *Day 1*. Remove 1ml of medium slowly from the top of each AggreWell and add 1ml of FC medium slowly along the side of well. Repeat once so that 2 medium changes are performed for each AggreWell. *CRITICAL STEP* Remove and add medium slowly and avoid touching the bottom of the well such that cells deposited in microwells are not disturbed. Lift the lid of the AggreWell plate gently with one hand holding the bottom of the plate securely since the lid can be very tight and cells can be dislodged from microwells if the lid is not lifted carefully. *TROUBLESHOOTING*

41. *Day 2*. Remove 1ml of medium slowly from each AggreWell and add 1ml of FC medium slowly along the side of AggreWell. *TROUBLESHOOTING*

42. *Day 3*. Prepare peri-implantation culture medium (20% FBS) (see ‘Preparation of culture media’). 1.5ml of medium is required for each AggreWell and the volume can be scaled up linearly according to the number of wells set up (with an extra 1ml to account for loss from pipetting). Equilibrate the medium in a 37°C incubator for at least 20 min with the cap slightly loosened. Remove 1ml and then a further 200µl of medium slowly from each AggreWell. (Volume loss is expected due to evaporation. Therefore do not completely remove this last 200µl of medium if the AggreWell will become empty.) Add 1ml and then a further 500µl of peri-implantation culture medium (20% FBS) slowly along the side of the AggreWell. *TROUBLESHOOTING*

Selection of EiTiX embryos (Day 4) TIMING 1 hour

43. *Day 4*. Prepare peri-implantation culture medium (30% FBS) (see ‘Preparation of culture media’). For one AggreWell, prepare 10ml of medium and divide it into two wells of a non-adherent 6 well multiwell plate with 5ml of medium per well. Equilibrate the medium in a 37°C incubator for at least 20 min.

44. To collect all structures from one AggreWell, use a P1000 pipette with the tip cut off to create a bigger bore to first take up 1ml of medium from the AggreWell without touching the bottom of the well. Then, gently pipette up and down to flush the edge of the well 4 times. Transfer all the medium to a 15ml Falcon tube. Structures in suspension should be visible. *CRITICAL STEP* The P1000 pipette tip must be cut off, otherwise structures can be damaged when they pass

through the original bore opening. Do not flush the centre of AggreWell as structures can be damaged.

45. Add 1ml of PBS to each AggreWell along the side. Pipette up and down along the upper edge and lower edge of the well slightly more forcefully, four times each, to dislodge any remaining structures. Transfer to the 15ml Falcon tube from the previous step. Repeat washing with PBS if there are still structures left in the AggreWell. Keep the 15ml Falcon tube(s) in 37°C incubator with cap slightly loosened. *CRITICAL STEP* Minimise the time that the tube with collected structures is left outside of an incubator at RT since structures can start adhering to each other if left too long in a 15ml Falcon tube. If more than one AggreWell is set up, collect structures from up to 2 wells at a time (into 2 separate 15ml Falcon tubes) for selection of structures.

46. After all structures have settled to the bottom of the tube, transfer 100μl of supernatant to a 35mm dish for holding selected structures. Keep the dish on a 37°C slide warmer.

47. Using a P1000 pipette with tip cut off, remove excess media in the tube without disturbing the settled structures, leaving approximately 1ml in the tube. Gently pipette up and down for 4 times to resuspend the structures and divide the suspension to two 35mm dishes. Add the suspension to the centre of dish without spreading it to the side. Structures can be difficult to observe when they are on the side of the dish.

48. Transfer one dish with structures to a stereomicroscope for selection and transfer the other one to a 37°C incubator. *CRITICAL STEP* Minimise the time that the structures are left outside of the incubator at RT.

49. Swirl the dish gently to gather the structures in the centre and remove any bubbles on top. Look for EiTiX embryos with 1) a clear and thick epithelialised EPI-like layer, 2) an ExE-like compartment on top, 3) a complete outer VE-like layer, and 4) a central lumen (see Figure 3 for examples of selected structures). Use a P10 pipette to collect and transfer selected EiTiX embryos to the dish prepared in Step 46 under the stereomicroscope to ensure that they are transferred. It is expected that around 35–55 well-formed EiTiX embryos can be selected from each AggreWell. Remove any structures that are inadvertently transferred. Several EiTiX embryos can be taken up and transferred at one time to speed up the process. *CRITICAL STEP* The dish containing selected EiTiX embryos should be protected from light since they can be light-sensitive. Selection of EiTiX embryos should be performed within 10 minutes. This selection step is critical to the subsequent correct development. Structures that are 1) uncavitated, 2) with an inflated cavity or with only one inner compartment, or 3) with a thin EPI-like layer or with extra cavities should be discarded (see Figure 3 for examples of discarded structures). *TROUBLESHOOTING*

50. Transfer selected EiTiX embryos to the non-adherent 6 well multiwell plate with peri-implantation culture medium (30% FBS) as prepared in Step 43. Up to

50 EiTiX embryos can be transferred to each well of the non-adherent 6 well multiwell plate that has 5ml peri-implantation culture medium (30% FBS).

51. Repeat Steps 44 to 50 until all structures have been selected.

52. If needed, prepare more peri-implantation culture medium (30% FBS) such that each well of the non-adherent 6 well multiwell plate does not contain more than 50 EiTiX embryos (each well requires 5ml of medium).

53. Shake the suspension plate gently to distribute the EiTiX embryos since they can fuse to each other. Return the plate to the 37°C incubator.

Culture of EiTiX embryos to headfold stages (Day 5 to 8) TIMING 4 days

54. *Day 5*. Transfer 100μl of medium from the non-adherent 6 well multiwell plate to a 35mm dish for holding selected EiTiX embryos. Keep the dish on a 37°C slide warmer.

55. Swirl the non-adherent 6 well multiwell plate gently to gather the EiTiX embryos in the centre. Using a P1000 pipette with the tip cut off, transfer all EiTiX embryos from up to 2 wells to a 35mm dish. Minimise the volume of medium transferred and add the suspension to the centre of the dish without spreading it to the side.

56. Swirl the dish gently to gather all EiTiX embryos to the centre. Select structures with 1) a clear and thick epithelialised EPI-like layer (ideally one side is thicker, resembling the gastrulating posterior EPI) and 2) an asymmetric anterior visceral endoderm (AVE)-like region (appears as a protruding region in the outer VE-like layer away from the distal tip of the EiTiX embryo) (see Figure 4 for example). Use a P10 pipette to collect and transfer selected EiTiX embryos to the dish prepared in Step 54 under the stereomicroscope to ensure that they are transferred. Several EiTiX embryos can be taken up and transferred at one time to speed up the process. The dish containing selected EiTiX embryos should be protected from light since they can be light-sensitive. Selection of EiTiX embryos should be performed within 10 minutes. *CRITICAL STEP* Do not select EiTiX embryos with a prominent symmetric AVE-like region at the distal tip since they are unlikely to establish an anterior-posterior axis for subsequent development. *TROUBLESHOOTING*

57. Transfer all selected EiTiX embryos back to one well of the non-adherent 6 well multiwell plate and count the number of EiTiX embryos selected. Transfer unselected EiTiX embryos to another well.

58. Repeat Steps 54 to 57 until all EiTiX embryos have been selected. Among these selected EiTiX embryos, we typically select the best 10–15 structures for further culture due to the costs of rat serum and human cord serum.

59. Based on the number of EiTiX embryos selected, prepare post-implantation culture medium (see ‘Preparation of culture media’). Prepare 250μl of medium per EiTiX embryo, with an extra 400μl to account for media loss due to filtering.

After filtering, divide medium into a non-adherent 48 well multiwell plate, 250μl per well. Equilibrate the medium in 37°C incubator for at least 1 hour. CAUTION Handle and dispose of rat serum and human cord serum according to institutional guidelines.

60. After the medium has been equilibrated, observe selected EiTiX embryos to confirm the selection and look if there are any well-developed EiTiX embryos left among the unselected ones. Transfer 1 selected EiTiX embryo to each well of the non-adherent 48 well multiwell plate with post-implantation culture medium.

61. *Day 6*. Check if any EiTiX embryos have failed to progress. Successful Day 6 EiTiX embryos should resemble an elongated egg cylinder with a thick EPI-like layer. A node-like indentation may be observed at the distal tip and sub-compartments resembling the exocoelomic cavity and ectoplacental cavity might be observed in the ExE-like compartment. Examples of successful Day 6 EiTiX embryos can be found in Figure 4. Prepare post-implantation culture medium (250μl per successful EiTiX embryo, with an extra 400μl to account for media loss due to filtering) and equilibrate in a 15ml Falcon tube with cap slightly loosened in 37°C incubator for at least one hour. Withdraw 100μl of medium slowly from each well without touching the EiTiX embryo and add 250μl of fresh media slowly along the side of the well. *TROUBLESHOOTING*

62. *Day 7*. Check if any EiTiX embryos have failed to progress. Successful Day 7 EiTiX embryos should develop headfold-like structures with neural tube-like structure along the body axis, enclosed completely by a yolk sac-like membrane. A region resembling the chorion should be visible on one side of the outer membrane, opposite to the presumptive headfolds. Examples of successful Day 7 EiTiX embryos can be found in Figure 4. *TROUBLESHOOTING*

63. Turn on the gas supply and temperature control of the rotating bottle culture chamber. Equilibrate the chamber until it is at 37°C, 5% CO_2.

64. Prepare post-implantation culture medium supplemented with glucose (see ‘Preparation of culture media’). 2ml of medium are required for each rotating bottle which can hold up to 3 EiTiX embryos. An extra 400μl medium should be added to the required volume to account for media loss. Transfer the medium to a bottle and equilibrate it in the rotating bottle culture chamber for at least 1 hour. The flow rate should be set such that the bubbles just start forming a continuous stream.

65. Using a 7ml transfer pipette (tip may be cut off for bigger bore opening), carefully transfer up to 3 EiTiX embryos to one rotating bottle within minimal carry-over of medium. Place the rotating bottle inside the culture chamber and check for the flow rate. Protect the whole culture chamber from light. *CRITICAL STEP* EiTiX embryos can be light-sensitive and the yolk sac may be ruptured by rough handling.

66. Check the flow rate after 15 min and adjust it if necessary. *CRITICAL STEP* Flow rate can fluctuate over time and should be checked regularly and adjusted if needed.

67. *Day 8*. Successful Day 8 EiTiX embryos should have distinctive regions resembling more developed headfolds, beating heart, tail, allantois and chorion. Red-pigmented areas resembling blood islands might be observed on the yolk sac-like membrane. *TROUBLESHOOTING*

68. To fix EiTiX embryos, transfer EiTiX embryos to individual 35mm dishes and dissect away the yolk sac-like and amnion-like membranes with fine forceps, as the membranes can prevent the penetration of fixative. Using a 7ml transfer pipette (tip may be cut off for bigger bore opening), first transfer EiTiX embryos to a 35mm dish with PBS to wash off media, before transferring to 1ml of 4% formaldehyde solution in a 4-well plate. Fix EiTiX embryos for 20 min at RT on a rocker. *CAUTION* Formaldehyde is a potential carcinogen and should be handled and disposed according to institutional safety guidelines

69. Wash EiTiX embryos with 1ml of PBST (0.1% Tween 20 in PBS, a 50 ml working stock can be prepared and stored for up to 1 month) 3 times, 5 min each. EiTiX embryos can be kept in 1ml of PBST in a parafilm-sealed 4-well plate at 4°C. *PAUSE POINT* EiTiX embryos can be kept for up to 1 week at 4°C although it is recommended to proceed with immunofluorescence on the same day or the next day for best results.

Immunofluorescence of EiTiX embryos TIMING 3 days

CRITICAL The immunofluorescence procedure detailed here can be applied to structures from Day 4 to Day 8. The fixation procedure for all stages is as detailed in Steps 68–69. While Day 7 and Day 8 EiTiX embryos should be dissected to remove extraembryonic membranes before fixation, this is not required for Day 4 to Day 6 EiTiX embryos. The maximum numbers of EiTiX embryos that can be fixed together in one well of a 4-well plate are: 20 (Day 4), 10 (Day 5), 5 (Day 6), 3 (Day 7 and Day 8).

70. Transfer EiTiX embryos with PBST to a round bottom 96-well plate. The maximum numbers of EiTiX embryos that can be transferred per well are: 10 (Day 4 and Day 5), 4 (Day 6), 3 (Day 7) and 2 (Day 8).

71. Remove as much PBST as possible without disturbing EiTiX embryos and add 200μl of permeabilisation buffer per well.

72. Permeabilise for 30 minutes (Day 4 to 5 EiTiX embryos) or 35 minutes (Day 6 to 8 EiTiX embryos) on a rocker at RT.

73. Remove permeabilisation buffer and wash with 200μl of PBST for 3 times, 5 minutes each on a rocker at RT.

74. Dilute primary antibodies in blocking buffer at the appropriate dilution ratios (Table 3). 100–200μl of primary antibody mix is required per well according to the number of samples in each well.

75. Remove PBST and add the primary antibody mix to EiTiX embryos.

76. Incubate overnight on a rocker at 4°C.

77. Remove the primary antibody mix and wash with 200μl of PBST for 3 times, 5 minutes each on a rocker at RT.

78. Dilute secondary antibodies (1:1000, Table 4) and DAPI (1:1000, 5 μg/ml final concentration) in blocking buffer. 150–250μl of antibody mix is required per well according to the number of samples in each well.

79. Centrifuge the secondary antibody mix at 16,900g for 5 minutes.

80. Remove PBST and add 100–200μl of secondary antibody mix to EiTiX embryos, leaving the bottom 50μl which might contain unwanted crystals or precipitates.

81. Incubate on a rocker for 3 hours at RT or overnight at 4°C.

82. Remove the secondary antibody mix and wash with 200μl of PBST for 3 times, 5 minutes each on a rocker at RT. *PAUSE POINT* Immunostained EiTiX embryos can be stored at 4°C for up to 1 week although it is recommended to proceed with imaging on the same day or the next day for best results.

83. Prepare the imaging dish by adding drops of PBS to a glass-bottomed dish and cover with mineral oil. Transfer EiTiX embryos to individual drops for imaging using confocal microscopes.

Timing

- Thawing and maintenance of ESCs (Steps 1–22), minimum of 1 week
- Day 0: assembly of mouse ESCs to form EiTiX embryos (Steps 23–39), 7–8 hours
- Day 1–3: generation of EiTiX embryos (Steps 40–42), 30 minutes per day
- Day 4: selection of EiTiX embryos (Steps 43–53), 1 hour (longer if more than one AggreWell is set up)
- Day 5–8: culture of EiTiX embryos to headfold stages (Steps 54–69), 2 hours per day
- Immunofluorescence of EiTiX embryos (Steps 70–83), 3 days

Protocol alternative: ETiX embryo

- Thawing and maintenance of MEFs (Steps 1–10, Box 1), 1 day
- Thawing and culture of mouse TSCs (Steps 11–30, Box 1), minimum of 1 week
- Assembly of mouse stem cells to form ETiX embryos (Box 1), 7–8 hours

Troubleshooting

Troubleshooting guide can be found in Table 5.

Anticipated results

After seeding the three types of ESCs into AggreWell, ESCs aggregate with each other to form round cell aggregates in 2 days. On Day 3, an outer cell layer is visible and a central lumen can be observed. On Day 4, structures that resemble the post-implantation egg cylinder (with a central lumen, two cellular compartments surrounded by an outer cell layer) can be observed. Representative images of cell aggregates in AggreWell can be found in Figure 2B.

The representative morphology of Day 4 to Day 8 EiTiX embryos can be found in Figure 3–4, which is also largely similar to ETiX embryos. On Day 4, a spectrum of structures should be expected. Apart from correctly organised EiTiX embryos that resemble the post-implantation egg cylinder (Figure 3a), one will also obtain structures that are 1) uncavitated, 2) with an inflated cavity or with only one inner compartment, or 3) with a thin EPI-like layer or with extra cavities (Figure 3b). However, these incorrect structures should not be used for further culture. On Day 5, EiTiX embryos should have 1) a clear and thick epithelialised EPI-like layer where one side is thicker and 2) an asymmetric AVE-like region as indicated by a protruding region in the outer VE-like layer away from the distal tip of the EiTiX embryo. On Day 6, EiTiX embryos should resemble elongated egg cylinders with a thick EPI-like layer. A node-like indentation may be observed at the distal tip and sub-compartments resembling the exocoelomic cavity and ectoplacental cavity might be observed in the ExE-like compartment. Day 7 EiTiX embryos should develop headfold-like structures with neural tube-like structure along the body axis, enclosed completely by a yolk sac-like membrane. A region resembling the chorion should be visible on one side of the outer membrane, opposite to the presumptive headfolds. On Day 8, EiTiX embryos should have distinctive regions resembling more developed headfolds, beating heart, tail, allantois and chorion. Red-pigmented areas resembling blood islands might be observed on the yolk sac-like membrane. These developmental milestones and the corresponding immunofluorescence images of EiTiX embryos from Day 4 to Day 8 can be found in Figure 5.

For each AggreWell, typically 35–55 well-formed EiTiX or ETiX embryos can be selected on Day 4 under stereomicroscope. Since 6 AggreWells are usually set up, this gives 210–330 Day 4 EiTiX or ETiX embryos per experiment. Table 2 describes the efficiencies of EiTiX or ETiX embryos progressing to the next day, where successful structures are selected on each day. We have also included in Table 2 the predicted number of EiTiX or ETiX embryos that can be expected to develop successfully on each day of culture, starting from one AggreWell set up on Day 0.

Acknowledgments

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Data availability

Supporting data of this study can be found in our previous publications[7,8,13] and are available from the corresponding author upon request.

List of abbreviations

A	Anterior
Al	Allantois
AVE	Anterior visceral endoderm
Ch	Chorion
DMSO	Dimethyl sulfoxide
Dox	Doxycycline
EPI	Epiblast
ESC	Embryonic stem cell
ExE	Extraembryonic ectoderm
FBS	Foetal bovine serum
FC	Feeder cell
H	Heart
HF	Headfolds
i*Cdx2* ESCs	Embryonic stem cells with doxycycline inducible *Cdx2* expression
i*Gata4* ESCs	Embryonic stem cells with doxycycline inducible *Gata4* expression
ITS-X	Insulin-transferrin-selenium-ethanolamine
LIF	Leukaemia inhibitory factor
NAC	N-acetyl-L-cysteine
NT	Neural tube
P	Posterior
PS	Primitive streak
RT	Room temperature
T	Tail

TSC	Trophoblast stem cell
VE	Visceral endoderm
WT	Wildtype
XEN	Extraembryonic endoderm

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Key points:

- This protocol is for the assembly of complete mouse embryo models from embryonic and induced stem cells. These embryo models develop to generate advanced embryonic and extraembryonic tissues.
- While there are many existing *in vitro* models of mouse embryogenesis, these complete induced embryo models are among the only models reported to recapitulate development from gastrulation to headfold stages.

Key papers

- Amadei, G. *et al.* Embryo model completes gastrulation to neurulation and organogenesis. *Nature* **610**, 143–153 (2022). https://doi.org/10.1038/s41586-022-05246-3
- Amadei, G. *et al.* Inducible Stem-Cell-Derived Embryos Capture Mouse Morphogenetic Events In Vitro. *Dev. Cell* 1–17 (2021) https://doi.org/10.1016/j.devcel.2020.12.004
- Lau, K. Y. C. *et al.* Mouse embryo model derived exclusively from embryonic stem cells undergoes neurulation and heart development. *Cell Stem Cell* **29**, 1–14 (2022). https://doi.org/10.1016/j.stem.2022.08.013

Box 1:

Protocol alternative: ETiX embryos

Complete embryo models can also be generated by using TSCs in lieu of i*Cdx2* ESCs. TSCs are grown on MEFs and maintained in TSF4H medium. The steps below describe how to establish and maintain TSC culture and use them for complete embryo model formation.

Thawing and maintenance of MEFs TIMING 1 day

MEFs are cultured in FC medium on gelatinised wells at 37°C, 5% CO2 in 6-well plates, at a density of 170,000 cells per well. Inactive MEFs do not proliferate and should not be kept for more than 2 weeks in culture.

1. Warm FC medium (a 15ml aliquot for each vial of MEFs and an additional 50 ml aliquot) in a 37°C water bath for 5 minutes.
2. Gelatin-coat tissue culture plates by adding 2ml of gelatin solution (for a 6-well). Shake the plate gently to make sure the entire surface of the well is covered with gelatin.
3. Thaw a frozen vial of MEFs by shaking vigorously in a 37°C water bath until a small piece of ice remains.
4. Immediately add 1ml of FC medium from the 10ml FC medium aliquot to the vial and transfer the content from the vial back to the FC medium aliquot.
5. Rinse the vial with 1ml of FC medium from the 10ml FC medium aliquot and transfer the content from the vial back to the FC medium aliquot. Repeat for a total of 2 times.
6. Centrifuge at 200g for 4 min at RT.
7. Aspirate gelatin from the well prepared in Step 2 and add 2ml of FC medium per a 6-well.
8. After centrifugation, aspirate supernatant and resuspend cell pellet by adding 0.5ml of FC medium and pipetting up and down for 4 times gently.
9. Add the required volume of MEF suspension to each well such that the cell density is 170,000 cells per well. Shake the plate gently to distribute MEFs. Place the plate in a 37°C incubator overnight.
10. Change medium the next day after thawing and then every 2 days. Add medium along the side of the well to avoid disturbing the MEFs. Discard MEF plates that are more than two weeks old. *TROUBLESHOOTING*

Thawing and culture of mouse TSCs TIMING minimum of 1 week

All TSCs should be cultured in TSF4H medium on MEF plates at 37°C, 5% CO2. TSCs should be passaged before they reach 80% confluency, typically every 3–4 days. TSCs should be passaged for at least one time after thawing before using them to generate

ETiX embryos. TSC colonies should be round and have defined boundaries. Screening for mycoplasma should be performed every 2 weeks.

1. Replace the FC medium in one well of MEFs from the plate set up in Step 9 with 2ml of TSF4H medium and place the plate back in the incubator. Wait for 6 hours to allow the MEFs to condition the medium.
2. Warm a 10ml aliquot of TS base medium (for each vial of TSCs to be thawed) and TSF4H medium in a 37°C water bath for 5 minutes.
3. Thaw a frozen vial of TSCs by shaking vigorously in a 37°C water bath until a small piece of ice remains.
4. Immediately add 1ml of TS base medium from the 10ml TS base medium aliquot to the vial and transfer the content from the vial back to the 10ml TS base medium aliquot.
5. Rinse the vial with 1ml of TS base medium from the 10ml TS base medium aliquot and transfer the content from the vial back to the TS base medium aliquot. Repeat for a total of 2 times.
6. Centrifuge at 200g for 4 min at RT.
7. After centrifugation, aspirate supernatant and resuspend the cell pellet by adding 1ml of conditioned TSF4H medium from the MEF well (from Step 94) and pipette up and down for 4 times gently.
8. Add cell suspension to the well of MEFs with the remaining TSF4H medium. Shake the plate gently to distribute TSCs. Place the plate in a 37°C incubator.
9. Change medium the next day after thawing. On each following day until passage, top up medium by adding 1ml of TSF4H medium. *TROUBLESHOOTING*
10. Monitor TSCs and when they reach 80% confluency, passage them into a new well by following Steps 21–29.
11. Remove FC medium from one well of MEFs from the plate set up in Step 9 and add 2ml of TSF4H medium. Place the plate in the incubator and wait for 6 hours to allow MEFs to condition the medium.
12. Add 2ml of gelatin to one well of a six-well dish and shake the plate gently to make sure the entire surface of the well is covered with gelatin.
13. Aspirate culture medium from the TSCs well and wash the well by adding 1ml of PBS along the side of the well. Repeat this step twice.
14. Aspirate PBS and add 500μl of trypsin-EDTA. Swirl the plate around and immediately aspirate the trypsin. Add another 500μl of typsin-EDTA and incubate the plate for 4 min at 37°C.
15. Stop trypsinization by adding 2ml of TS base media. Gently pipette up and down across the area of the well for 8 times to facilitate cell detachment. Collect cell suspension in a 15ml Falcon tube.

16. Centrifuge TSC suspension to form a cell pellet at 200g for 4 min at RT.

17. Aspirate supernatant and add 2ml of TSF4H medium to cell pellet. Pipette up and down for 6 times to resuspend cell pellet. *TROUBLESHOOTING*

18. Remove gelatin from the well (from Step 22) and plate the TSC suspension. Shake the plate gently to promote even cell distribution. Place the plate in the incubator for 30 minutes to allow MEFs and differentiated TSCs to attach to the plate (Gelatin depletion step)

19. Collect the supernatant from the well into a 15ml Falcon tube. Plate the cells in the MEF-coated well containing the conditioned TSF4H (from Step 104). A 1:10 dilution will reach confluency in 2–3 days while a 1:20 dilution will reach confluency in 3–4 days.

20. On the next day, wash the TSC well with 1ml of PBS and replace with 2ml of TSF4H medium. On each following day until the next passage, top up with 1ml of TSF4H medium.

Assembly of mouse stem cells to form ETiX embryos (Day 0) TIMING 7–8 hours

Prepare TSCs for the experiment as described in above. 1–2 days before TSCs reach confluency, passage i*Gata4* and wildtype ESCs at 70–80% confluency to prepare all three cell types for ETiX embryo generation. Passage i*Gata4* ESCs into two wells each such that one well will be used for Dox induction and one well will be used for maintenance. If the experiment is to be set up in 2 days, passage and replate the cells by performing a 1:10 dilution; if the experiment is to be set up in 1 day, passage and replate the cells by performing a 1:20 dilution.

Follow Steps 23 to 39 from the main Procedure to prepare the other two cell types (wildtype and i*Gata4* ESCs), disregarding instructions pertaining to i*Cdx2* cells (which are functionally replaced by TSCs). In Steps 30, 19,200 TSCs, 6,000 wildtype ESCs and 6,000 i*Gata4* ESCs are required for each AggreWell. In the steps that require FCF4H medium (Steps 33, 34 and 36), use FC medium instead of FCF4H medium. Remaining steps and procedures are the same for EiTiX and ETiX embryos.

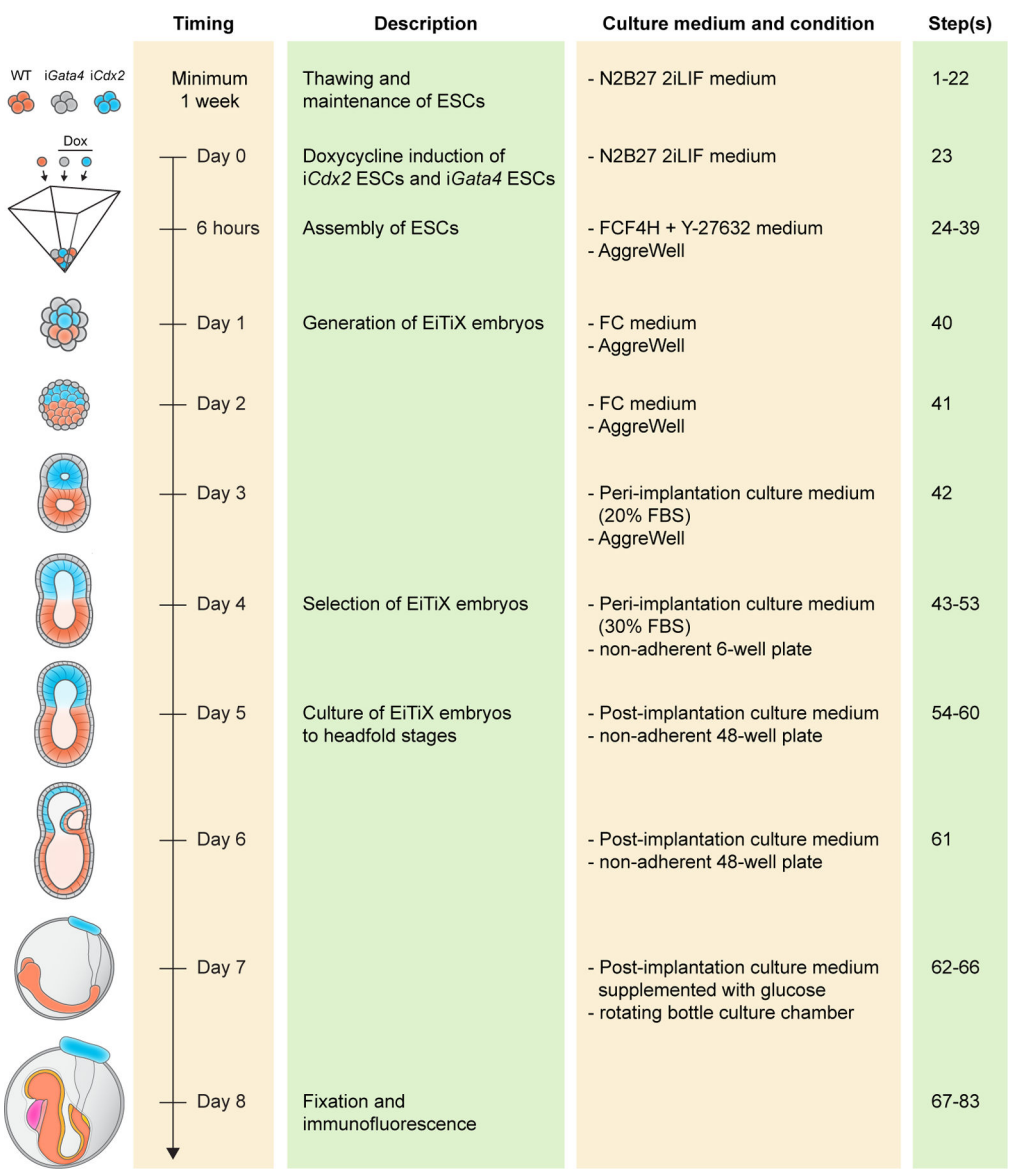

Figure 1. Overview of EiTiX embryos generation.

Schematic diagram summarising the key steps of generating EiTiX embryos. Once the three ESC lines (wildtype (WT) ESCs, i*Gata4* ESCs and i*Cdx2* ESCs) are stably maintained in culture, they are aggregated to assemble EiTiX embryos, which develop over 8 days to reach headfold stages. Drawings of EiTiX embryos are adapted from our previous publication[8].

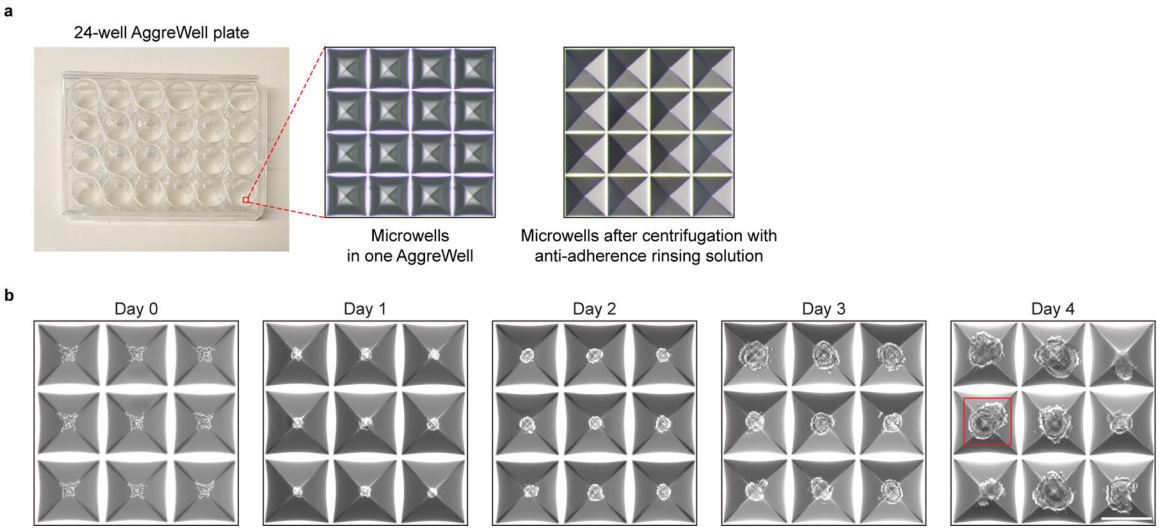

Figure 2. Assembly of three ESC types in AggreWell to generate EiTiX embryos.

(a) Photograph of a 24-well AggreWell plate and zoomed-in images showing a fraction of microwells (16 out of 1,200 microwells) from one well of the AggreWell plate (red square). Microwells should not contain any bubbles after centrifugation with anti-adherence rinsing solution. (b) After seeding of ESCs, they aggregate form round cell aggregates from Day 1 to Day 2. On Day 3, an outer cell layer is visible and a central lumen can be observed. On Day 4, EiTiX embryos that resemble the post-implantation egg cylinder (with a central lumen, two cellular compartments surrounded by an outer cell layer) can be observed. Red square shows an example of EiTiX embryos. Scale bar, 150μm. Panel b is adapted from our previous publication[8].

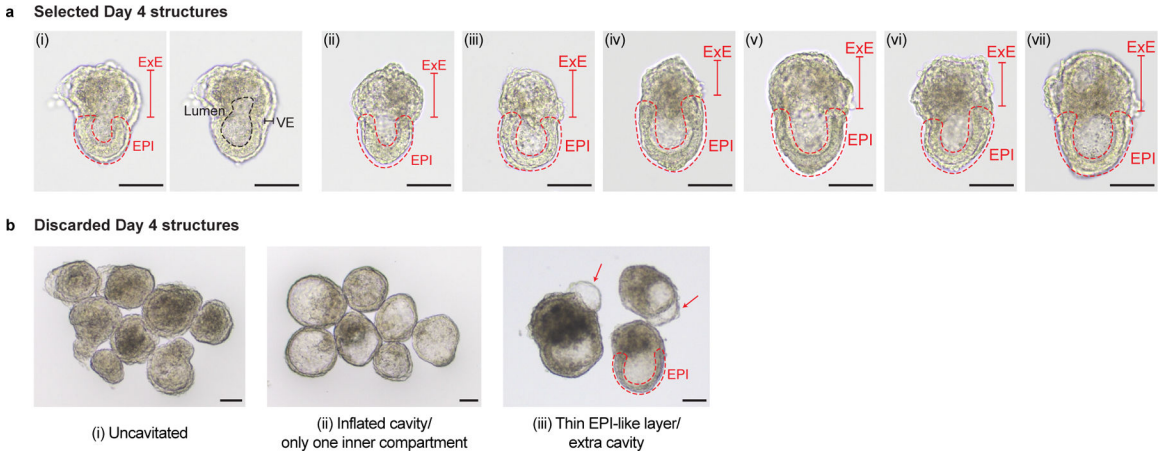

Figure 3. Selection of EiTiX embryos on Day 4.

Representative brightfield images of selected (a) and discarded (b) EiTiX embryos on Day 4. (a) Examples of EiTiX embryos (i-vii) that resemble the post-implantation egg cylinder, containing an extraembryonic ectoderm (ExE)-like compartment (red bracket), a thick epiblast (EPI)-like epithelial layer (red dotted line). They also have an outer visceral endoderm (VE)-like cell layer (black bracket) and a central lumen (black dotted line). (b) Examples of discarded structures that are (i) uncavitated, (ii) with an inflated cavity or with only one inner compartment, or (iii) with a thin EPI-like layer (dotted line) or with extra cavities (arrow). Scale bar, 50μm.

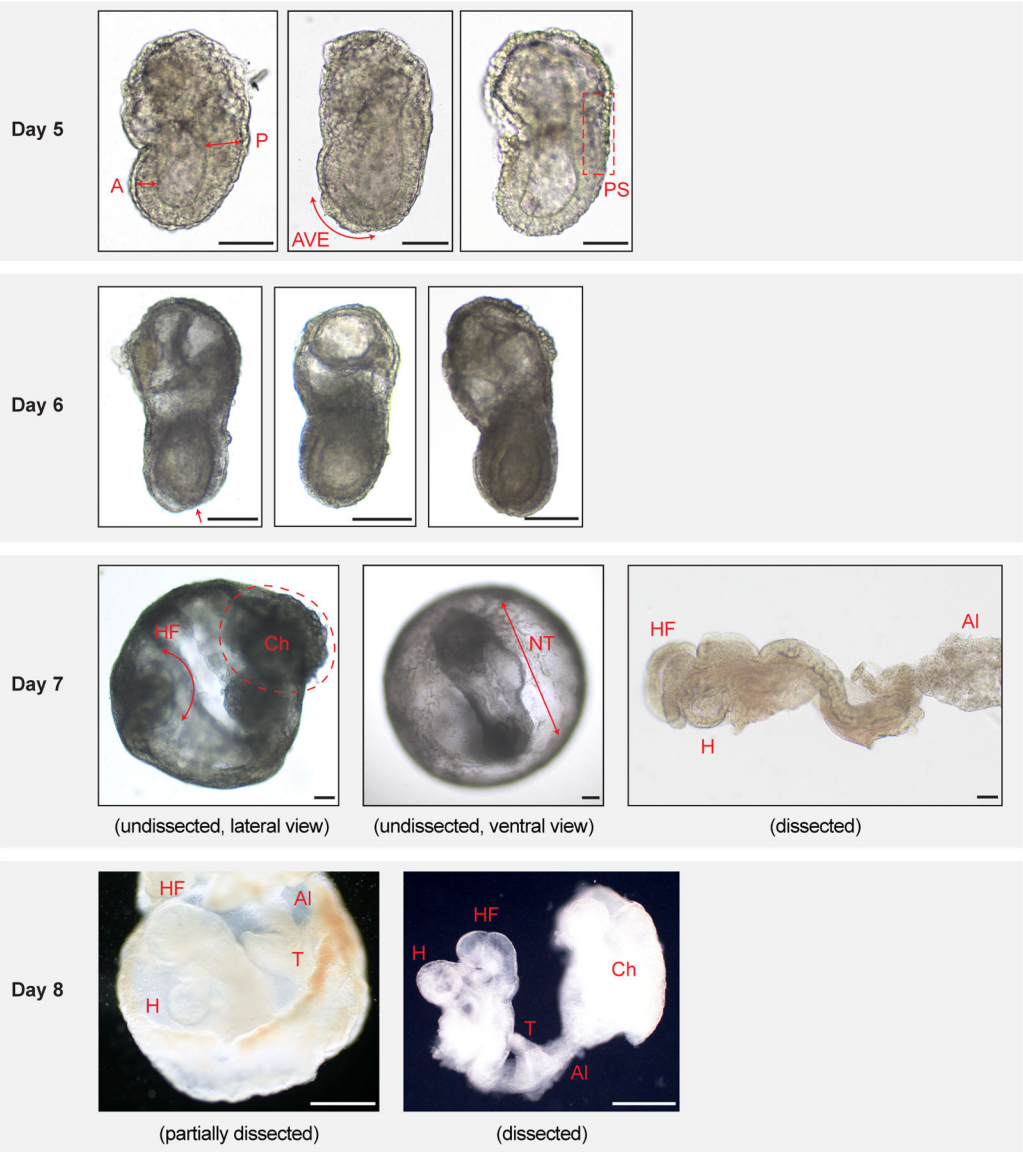

Figure 4. Development of EiTiX embryos from gastrulation to headfold stages.

Representative brightfield images of successful EiTiX embryos from Day 5 to Day 8. *Day 5*: EiTiX embryos have an elongated egg cylinder shape. An anterior(A)-posterior(P) axis is reflected by the thicker EPI layer on one side (arrows). An asymmetric anterior visceral endoderm (AVE)-like region and a primitive streak (PS)-like region appear as a protruding region in the outer VE-like layer away from the distal tip (arrow) and an emerging cell layer at the posterior EPI-like layer (box) respectively. Scale bar, 50μm. *Day 6*: Successful Day 6 EiTiX embryos resemble an elongated egg cylinder with a thick EPI-like layer. A node-like indentation may be observed at the distal tip (arrow) and sub-compartments resembling the exocoelomic cavity and ectoplacental cavity may be observed in the ExE-like compartment. Scale bar, 100μm. *Day 7*: EiTiX embryos develop an early heart (H)-like structure and headfold (HF)-like structures (arrow) with neural tube (NT)-like structure (arrow) along the body axis, enclosed completely by a yolk sac-like membrane. A region

resembling the chorion (Ch) is visible on one side of the outer membrane (circle), opposite to the presumptive headfolds, and is connected to the embryonic region by an allantois (Al)-like structure. Both undissected and dissected structures are shown. Scale bar, 100μm. *Day 8*: Successful Day 8 EiTiX embryos have distinctive headfolds (HF)-like, beating heart-like, tail (T)-like, allantois (Al)-like and chorion (Ch)-like regions. Red-pigmented areas resembling blood islands may be observed on the yolk sac-like membrane. Partially and fully dissected structures are shown. Scale bar, 500μm. Images of Day 7 and Day 8 EiTiX embryos are different structures, each dissected and positioned differently for imaging. Some images are adapted from our previous publication[8].

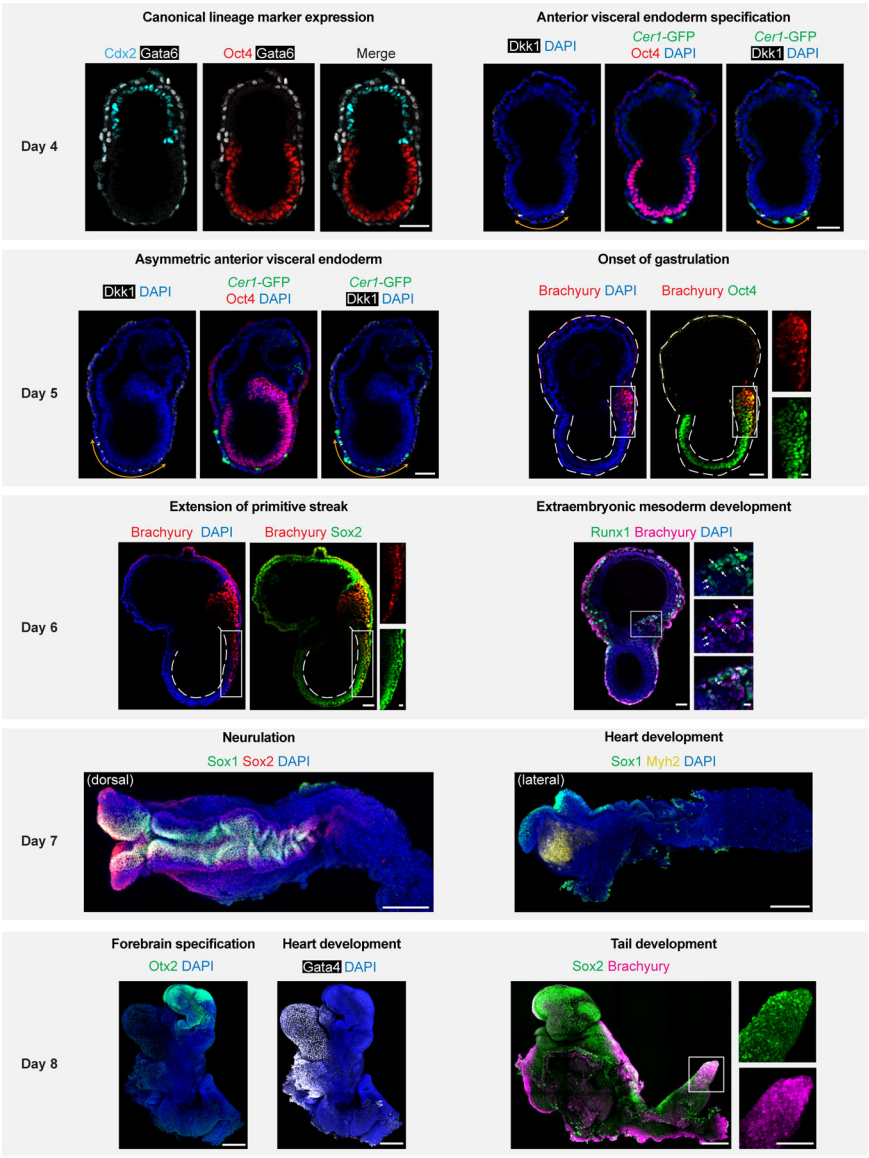

Figure 5. Developmental milestones of EiTiX embryos.

Developmental milestones and the corresponding marker characterisations by immunofluorescence of EiTiX embryos from Day 4 to Day 8 (see Table 3 for the spatial expression of the markers and the primary antibodies used). Arrow, Dkk1-positive domain on the VE-like layer; dotted line: outline of EiTiX embryos and the central lumen; box: region of the zoomed-in panels. Scale bar, Day 4: 50μm, Day 5 and 6: 50μm, 15μm (zoomed), Day 7: 200μm, Day 8: 200μm, 100μm (zoomed). Images are adapted from our previous publication[8].

Table 1:

Summary of stem cell-derived *in vitro* embryo models

Name	Cell types used	Aggregation conditions	References
ETS embryo	ESCs and TSCs	Matrigel	9, 10
ETX embryo	ESCs, TSCs and XEN cells	AggreWell	11
ETiX embryo (or iETX embryo)	ESCs, TSCs and i*Gata4* ESCs (induced XEN cells)	AggreWell	7, 13
EiTiX embryo	ESCs, i*Cdx2* ESCs (induced TSCs), i*Gata4* ESCs (induced XEN cells)	AggreWell	8

Table 2:

Predicted number of well-formed structures based on observed experimental efficiencies.

	EiTiX embryo			ETiX embryo		
Day of experiment	**Predicted number of structures**	**Average experimental efficiency of progression to the next day (n= 3–5 experiments)**	**Standard deviation of experimental efficiency**	**Predicted number of structures**	**Average experimental efficiency of progression to the next day (n= 17 experiments)**	**Standard deviation of experimental efficiency**
0	1200 (per AggreWell)	/	/	1200 (per AggreWell)	/	/
4	186	15.5%	0.68	261	21.7%	1.33
5	45	24.4%	0.85	54	21.0%	8.50
6	29	65.4%	14.8	40	74.7%	23.2
7	20	72.1%	13.2	30	74.6%	19.2
8	15	75.0%	16.7	22	72.1%	22.8

Day 4 efficiency was determined by immunofluorescence analysis of all structures collected from one AggreWell (typically around 700–1000 structures, with some loss of structures during the washing steps in immunofluorescence). Correct Day 4 structures display the expected spatial expression of canonical lineage markers as shown in Figure 5.

Day 5, 6, 7 and 8 efficiencies were calculated by the percentage of successful structures out of those selected on the previous day. Note that on Day 4, 35–55 structures were selected from each AggreWell set up on Day 0 (see Step 49), while on Day 5, 10–15 structures obtained from 6 starting AggreWells were selected for further culture and the subsequent experiment efficiencies (see Step 58).

Table 3:

Primary antibodies used to characterise EiTiX embryos

Marker	Spatial expression	Antibody	Dilution used
Ap2γ	ExE	R&D Systems, cat. no. AF5059 (RRID: AB_2255891, https://scicrunch.org/resolver/RRID:AB_2255891)	1:500
Brachyury	Primitive streak	R&D Systems, cat. no. AF2085 (RRID: AB_2200235, https://scicrunch.org/resolver/RRID:AB_2200235)	1:500
Cdx2	ExE	BioGenex, cat. no. MU392-UC (RRID: AB_2335627, https://scicrunch.org/resolver/RRID:AB_2335627)	1:500
		Abcam, ab76541 (RRID: AB_1523334, https://scicrunch.org/resolver/RRID:AB_1523334)	1:500
Cerberus1	AVE	R&D Systems, cat. no. MAB1986 (RRID: AB_2275974, https://scicrunch.org/resolver/RRID:AB_2275974)	1:500
Dkk1	AVE	R&D Systems, cat. no. AF1096 (RRID: AB_354597, https://scicrunch.org/resolver/RRID:AB_354597)	1:1000
Eomes	ExE	Abcam, cat. no. ab23345 (RRID: AB_778267, https://scicrunch.org/resolver/RRID:AB_778267)	1:500
FoxA2	Definitive endoderm	Cell Signaling Technology, cat. no. 8186 (RRID: AB_10891055, https://scicrunch.org/resolver/RRID:AB_10891055)	1:200
Gata4	VE, heart	Santa Cruz Biotechnology, cat. no. sc-9053 (RRID: AB_2247396, https://scicrunch.org/resolver/RRID:AB_2247396)	1:500
Gata6	VE	R&D Systems, cat. no. AF1700 (RRID: AB_2108901, https://scicrunch.org/resolver/RRID:AB_2108901)	1:500
Hand1	Chorion	Santa Cruz Biotechnology, cat. no. sc-390376 (RRID: AB_2935660, https://scicrunch.org/resolver/RRID:AB_2935660)	1:100
Lefty	AVE	R&D Systems, cat. no. AF746 (RRID: AB_355566, https://scicrunch.org/resolver/RRID:AB_355566)	1:1000
Myh2	Heart	R&D Systems, cat. no. MAB4470 (RRID: AB_1293549, https://scicrunch.org/resolver/RRID:AB_1293549)	1:500
Nkx2.5	Heart	R&D Systems, cat. no. AF2444 (RRID: AB_355269, https://scicrunch.org/resolver/RRID:AB_355269)	1:500
Oct3/4	EPI	Santa Cruz Biotechnology, cat. no. sc-5279 (RRID: AB_628051, https://scicrunch.org/resolver/RRID:AB_628051)	1:250
Otx2	Fore-/mid-brain	R&D Systems, cat. no. AF1979 (RRID: AB_2157172, https://scicrunch.org/resolver/RRID:AB_2157172)	1:500
Runx1	Haematopoietic progenitor	Abcam, cat. no. ab92336 (RRID: AB_2049267, https://scicrunch.org/resolver/RRID:AB_2049267)	1:500
Sox1	Neuroepithelium	Cell Signaling Technology, cat. no. 4194 (RRID: AB_1904140, https://scicrunch.org/resolver/RRID:AB_1904140)	1:500
Sox2	EPI, neuroepithelium	Santa Cruz Biotechnology, cat. no. sc-365823 (RRID: AB_10842165, https://scicrunch.org/resolver/RRID:AB_10842165)	1:200
		Thermo Fisher Scientific, cat. no. 14–9811-82 (RRID: AB_11219471, https://scicrunch.org/resolver/RRID:AB_11219471)	1:500
Sox17	Definitive endoderm	R&D Systems, cat. no. AF1924 (RRID: AB_355060 https://scicrunch.org/resolver/RRID:AB_355060)	1:500

ExE: extraembryonic ectoderm, AVE: anterior visceral endoderm, VE: visceral endoderm, EPI: epiblast

Table 4:

List of secondary antibodies used in immunofluorescence

Secondary antibody	Source	RRID
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific, cat. no. A-21202	AB_141607
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific, cat. no. A-21206	AB_2535792
Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific, cat. no. A-11055	AB_2534102
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568	Thermo Fisher Scientific, cat. no. A10037	AB_2534013
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568	Thermo Fisher Scientific, cat. no. A10042	AB_2534017
Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568	Thermo Fisher Scientific, cat. no. A-11057	AB_2534104
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	Thermo Fisher Scientific, cat. no. A-31571	AB_162542
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	Thermo Fisher Scientific, cat. no. A-31573	AB_2536183
Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	Thermo Fisher Scientific, cat. no. A-21447	AB_2535864
Donkey Anti-Rat IgG H&L (Alexa Fluor® 647) preadsorbed antibody	Abcam, cat. no. ab150155	AB_2813835

Table 5:

Troubleshooting guide

Step	Problem	Possible reason	Solution
10	Differentiating ESC colonies	Suboptimal culture medium and culture of ESC	Prepare fresh N2B27 2iLIF medium and store at 4°C for only up to 10 days.
			Passage ESCs before they reach 80% confluency. Individual colonies should not be touching.
29	There are clumps of ESCs, making it difficult to accurately count the cell density	Insufficient trypsinisation and pipetting to break up ESC colonies	Extend trypsinisation.
			Pipette up and down for more times after stopping trypsinisation.
39	Some microwells of AggreWell are empty after seeding of ESCs	There are bubbles remained in microwells before cell seeding	Check for any remaining bubbles in microwells. Repeat centrifugation of AggreWell plate with anti-adherence rinsing solution until there are no bubbles.
		Insufficient treatment of AggreWell with anti-adherence rinsing solution	Ensure anti-adherence rinsing solution is left in the AggreWell for at least 30 min.
	Unequal distribution of cells across microwells	Cell suspension is non-homogenous and not distributed across the AggreWell	Resuspend final cell pellet with sufficient pipetting and add the suspension of cell mixture drop by drop across the area of the well.
		AggreWell plate is not balanced during centrifugation	Ensure AggreWell plate is balanced precisely during centrifugation.
40–42	Cell aggregates are dislodged from microwells	Medium change is too vigorous	Remove medium very slowly and add medium slowly along the side of AggreWell.
		Improper handling of the AggreWell plate	Move the AggreWell plate carefully and avoid dragging of the plate. Lift the lid gently with one hand holding the bottom of the plate.
49	EiTiX embryos do not form or low yield of EiTiX embryos	Missing one or more cell type(s) in the cell seeding mixture or miscounting of cell density for seeding	Ensure all three cell types have been added. Clumps of ESC should not be observed when counting on a haemocytometer (see troubleshooting for Step 29).
		ESCs are differentiating in culture	Ensure ESC colonies are round and have a defined boundary (see troubleshooting for Step 10). Thaw a new vial of ESCs if necessary.
		ESCs have been cultured for too long	Thaw a new vial of ESCs with lower passage number and avoid culturing ESCs for more than 10 passages. If possible, avoid using ESCs with passage number over 30.
		Contamination with mycoplasma or other microorganisms	Perform mycoplasma testing every 2 weeks and look for signs of contamination.
	EiTiX embryos are damaged or disintegrated	EiTiX embryos are dislodged from microwells too vigorously or transferred too vigorously	Pipette up and down gently to dislodge EiTiX embryos from AggreWell and only pipette along the boundary of the well.
			Cut a wider bore opening of the P1000 pipette tip.
56	EiTiX embryos fuse together	Overcrowding of EiTiX embryos	Gently shake the plate containing selected Day 4 EiTiX embryos to distribute them.
			Avoid culturing more than 50 EiTiX embryos in a 6-well.
56, 61, 62, 67	Low efficiency of EiTiX embryos progressing to the next day	EiTiX embryos are left at RT for too long or exposed to excessive temperature fluctuation	Perform selection of EiTiX embryos and medium change as quickly as possible. If possible, designate an incubator only for EiTiX embryo culture and minimise opening of incubator.
		Suboptimal peri-implantation culture medium	Test the batch of FBS.
			Make new stock solutions of β-estradiol, progesterone and NAC. Store at −20°C for up to 1 month and avoid repeated freezing and thawing.

Step	Problem	Possible reason	Solution
		Suboptimal rat serum in post-implantation culture medium	Produce rat serum in-house if possible.
			Validate the quality of the batch of rat serum by using it to culture natural mouse embryos.
			Remove any blood clotting proteins appearing on top after centrifuging at 600g for 40 min at 4°C.
			Do not refreeze heat-inactivated rat serum and use thawed aliquots within 2 days.
	Contamination of culture medium	Exposure of culture medium to microorganisms	Filter post-implantation medium.
			Minimise opening of culture plate and use sterile filtered pipette tips.
93	MEFs do not attach to the plate	MEFs did not survive thawing	Ensure steps involved in the thawing of MEFs are performed quickly.
	MEFs do not extend processes to cover the well	Unhealthy MEFs	Purchase MEFs of good quality.
102	TSCs are differentiating	The medium is not well conditioned by MEFs	Ensure that MEFs are healthy and have not been in culture for more than 2 weeks.
			Ensure to condition the MEFs well for at least 6 hours before plating TSCs.
		Suboptimal heparin and/or Fgf4	Prepare fresh batch of heparin and/or Fgf4.
110	TSCs are not dissociating properly	Insufficient washing of well with PBS before the addition of trypsin-EDTA, insufficient trituration	Ensure to wash TSCs sufficiently with PBS before adding trypsin-EDTA.
			Triturate TSCs well to break down any cell clumps.