# Protocol for a developmental landscape of 3D-cultured human pre-gastrulation embryos

**Lifeng Xiang**
Yunnan Key Laboratory of Primate Biomedical Research; Institute of Primate Translational Medicine, Kunming University of Science and Technology

**Yu Yin**
Yunnan Key Laboratory of Primate Biomedical Research; Institute of Primate Translational Medicine, Kunming University of Science and Technology

**Tianqing Li** ( litq@lpbr.cn )
Yunnan Key Laboratory of Primate Biomedical Research; Institute of Primate Translational Medicine, Kunming University of Science and Technology

---

**Method Article**

**Keywords:** Human pre-gastrulation embryo, three-dimensional (3D) culture, primitive streak anlage, immunofluorescence imaging, single cell RNA-seq

**Posted Date:** December 12th, 2019

**DOI:** https://doi.org/10.21203/rs.2.16169/v1



---

# Abstract

Human embryogenesis is not well understood. Knowledge detailing human pre-gastrulation embryonic development including spatial self-organization and cell type ontogeny remains limited by available two-dimensional technological platforms. Here, we present a three-dimensional (3D) blastocyst-culture system, which enables human blastocyst development through primitive streak anlage (PSA). By the 3D-platform combined with immunofluorescence imaging and single-cell RNA-Seq, we reveal a developmental landscape of human pre-gastrulation embryos. Our protocol allows recording and analysis of embryo developmental landmarks and mechanisms from human blastocysts to pre-gastrulation stage (day 14 post- fertilization).


# Introduction

Early human embryogenesis, such as architecture formation and cell type specification, is obscure owing to technical challenge and unavailable materials. Recent *in vitro* implantation platforms using a two-dimensional (2D) culture approach have revealed some developmental landmarks of *in vivo* early human embryos[1,2]. However, these 2D-culture embryos are largely flattened, which creates an imperfect model of normal three-dimensional (3D) embryonic development *in vivo* and limits classification using equivalent Carnegie stages[3-5].

# Reagents

**Culture maintenance medium:**

Advanced DMEM/F12 (Thermo Fischer Scientific,12634-010)

DFBS (defined fetal bovine serum) (Biosera, bs-0003)

L-glutamine (Thermo Fisher Scientific, 25030)

ITS-X (Thermo Fisher Scientific, 51500-056)

β-Estradiol (Sigma-Aldrich, E8875)

Progesterone (Sigma-Aldrich, P0130)

N-acetyl-L-cysteine (Sigma-Aldrich, A7250)

Sodium Lactate (Sigma-Aldrich, L7900)

Sodium Pyruvate (Sigma-Aldrich, P4562)

Y27632 (Selleck, S1049)

Matrigel (Corning, 354234)

**Embryo thawing and zona pellucida removing medium:**

Kitazato Thawing Media Kit (Kitazato Corporation, VT102)

G-2 (Vitrolife, 10029)

Mineral oil (Vitrolife, 10029)

Tyrode's solution (Sigma-Aldrich, T1788)

**Fixation and Immunostaining:**

Paraformaldehyde (Sigma-Aldrich, 158127)

Triton X-100 (MP Biomedicals, 0219485483)

Tween-20 (Life Science Products & Services, TB0560)

BSA (Sigma-Aldrich, A8022)

PBS (Solarbio, P1010)

**Single Cell Isolation:**

0.5% Trypsin (Thermo Fisher Scientific, 15400054)

G-MOPS (Vitrolife, 10130)

PBS (Thermo Fisher Scientific, 14190114)

DFBS (defined fetal bovine serum) (Biosera, bs-0003)

SuperScript II (Invitrogen, 18064-014)

KAPA HiFi HotStart ReadyMix (KAPA Biosystems, KK2601)

# Equipment

$CO_2$ cell culture incubator (Thermo Fisher Scientific, 3111)

Confocal laser scanning microscopy (Leica, SP8)

Multi-photon microscope (Leica, TCS SP8 DIVE)

Inverted microscope (Leica, IV/2010)

Biosafety cabinet (Thermo Fisher Scientific, 1389)

Stereo microscope (Nikon, SMZ645)

# Procedure

All donated embryos in this study were surplus frozen embryos from couples who already had at least a healthy baby after IVF clinic treatment. The informed consent process for embryo donation compiled with International Society for Stem Cell Research (ISSCR) Guidelines for Stem Cell Research and Clinical Translation (2016) and Ethical Guidelines for Human Embryonic Stem Cell research (2003) jointly issued by the Ministry of Science and Technology and the Ministry of Health of People's Republic of China.

### Embryo thawing and zona pellucida removal

1. Before embryo thawing, human embryo culture medium G-2 was equilibrated in a

4-well plate overnight. 0.5 ml of G-2 medium was added to the well, and then 0.25 ml of mineral oil was used to cover the G2 medium.

**Note:** The G-2 medium must be pre-equilibrated in the incubator overnight or for at least 6 hours.

2. Human blastocysts (day 5 or 6 post-fertilization, d.p.f 5-6) were thawed using a Kitazato Thawing Media Kit by strictly following the manufacturer's instructions.

3. After culturing in drops of equilibrated G-2 medium for 4 hours, the embryos were transferred to acidic Tyrode's solution to remove the zona pellucida.

**Note:** The Pasteur's pipette diameter should be larger than the embryo diameter to avoid damaging the embryos. No more than two embryos were treated in the acidic Tyrode's solution each time. Once the zona pellucida vanished, the embryos were immediately transferred to G-2 medium to avoid damage to the embryos by the acidic Tyrode's solution.

4. Embryos were washed in G-2 medium for two times and then transferred to *in vitro* culture medium.

### Evaluation of embryo quality

According to the Gardner's scoring system[6], thawed blastocysts were given numerical scores from 1 to 6 based on their expansion degree and hatching status. The blastocyst with expansion and hatching status above 3 and with visible inner cell mass above grade B were included in the study. Based on morphologies, normal embryos had to meet the two following requirements: obvious expansion during culturing and absence of obviously dead or broken (fragmented) cell mass during development. Otherwise, they were excluded in this study.

### *In vitro* three-dimensional culture of human embryos

1. The culture conditions were as follows: 37.2℃, 6% $CO_2$ and saturated humidity

2. 150 μL/well mIVC1 was added to a low-attachment 96-well plate (3474, Corning) and the culture medium was pre-equilibrated for at least six hours

3. The day 5-6 embryos without zona pellucida were washed in pre- equilibrated mIVC1 three times and then transferred to a new well for each embryo

**Note:** Embryos should be washed at least 3 times in mIVC1 to remove the G-2 residue.

4. On day 6-8 post-fertilization (d.p.f 6-8), the culture medium was mIVC1

5. At d.p.f 8, 50% of mIVC1 medium was replaced by mIVC2

6. At d.p.f 9, the embryos were transferred to new wells in mIVC2 including 10% Matrigel

**Note:** Matrigel should be thawed at 4℃, and all operations should be performed on ice.

7. Afterwards, 50% of the culture medium was replaced with new mIVC2 including 10% Matrigel every day.

**Note:** The time that the embryos are outside of the incubator should be minimized when replacing medium or taking photos. mIVC1 and mIVC2 should be pre-equilibrated in the incubator for at least six hours before use.

### Embryo frozen section

1. Embryos were fixed with 4% PFA for 40 mins in room temperature

2. Embryos were washed three times in PBS in room temperature

3. Dehydrated with 15% sucrose for 1 min in room temperature

**Note:** The dehydration time can be extended appropriately if the sample does not sink

4. Embryos were embedded in a plastic mold with O.C.T.

**Note:** Embryos should be washed in O.C.T three times before transferred to the mold

5. The embedded embryos were sectioned by a Leica frozen slicer.

6. The slices were baked on a heating plate for 40 mins at 45 degree centigrade

7. The slices were stored in -20 refrigerator

## Embryo section staining and taking photos

1. Embryo sections were placed in dark box and washed with PBS three times
2. Samples were permeabilized by 0.2% Triton X-100 for 30 minutes at room temperature
3. Wash with PBS one time
4. Samples were blocked by 3% BSA for 4 hours at room temperature
5. Wash with PBS one time
6. Add primary antibodies with 1% BSA
7. Incubate primary antibodies overnight in a 4℃ refrigerator.
8. Samples were washed with 0.05% Tween-20 in PBS for three times, 3-5mins per time
9. Add secondary antibodies with PBS for 2 hours at room temperature
10. Samples were washed with 0.05% Tween-20 in PBS for three times, 3-5 mins per time
11. Cover slips were covered with 50% glycerine
12. Taking photos by Leica SP8

**Note:** Samples must be kept moist all the time and without drying out

## Whole embryo staining and taking photos

1. Embryos were fixed with 4% PFA in a normal 96-well plate, 200 μL/well, 40 mins in room temperature
2. Embryos were transferred to a new well with 200μL PBS
3. Embryos were transferred to a new well with 200μL 0.5% Triton X-100 for 2 hours

in room temperature

4. Embryos were transferred to a new well with 200μL 3% BSA for 4 hours in room temperature
5. Embryos were transferred to a new well with 100μL primary antibody diluted by

1% BSA

6. Incubate primary antibodies overnight in a 4℃ refrigerator

7. Embryos were washed with 0.05% Tween-20 in PBS for three times, 15 mins per time

8. Embryos were transferred to a new well with 100μL secondary antibody diluted by

PBS

9. Embryos were washed with 0.05% Tween-20 in PBS for three times, 15 mins per time

10. Embryos were washed with 50% glycerine and transferred to 8-well IbiTreat μ plates to take photos

11. To make the 3D videos, embryos were mounted in on aqueous solution of 80% glycerol, and a multi-photon microscope (Leica TCS SP8 DIVE) was used to take photos.

## Isolation of single cells

1. Three drops of 30 μL 0.25% trypsin were added on a 35mm petri dish (Corning, 351008), 3 mL mineral oil was covered on the trypsin for each dish

2. The dishes were incubated in a 37℃ incubator for 30 mins

3. Embryos were washed in PBS 3 times

**Note:** Wash the embryo at least 3 times to remove the residue of medium

4. Embryos were washed in 0.25% trypsin drops for 2 times and then transferred to the third drop of trypsin

5. Embryos were incubated in a 7℃ incubator for 15 mins

6. 2 μL DFBS was added to the third drop to terminate the digestion

7. Each embryo was transferred to a drop of G-MOPS in 35mm petri dish covered by mineral oil in room temperature

8. Use different diameters of Pasture pipette to blow the embryo into single cell

9. Each single cell was washed in DPBS

10. Each single cell was pipetted into a PCR tube with 2 μL lysis solution

# Troubleshooting

**Zona pellucida remove of embryos:** When the embryo develops *in vivo*, it hatches itself from the zona pellucida. We need to remove the zona pellucida to ensure the embryo's continued development. The digestion process of Tyrode's solution is very violent, excessive digestion will affect the quality of embryos. The thickness of zona pellucida was various of different embryos, so that embryos should be digested at a maximum number of two in the same time.

**Note:** The diameter of Pasteur pipette should be larger than the embryo, or the embryo would be damaged.

During digestion, the embryos must be observed all the times to ensure that the digestion time is just right.

Before embryos transferred to mIVC1, make sure the residue of Tyrode's solution was washed out.

**Optimizing the components of culture medium:** To optimize the culture medium, we add sodium lactate and sodium pyruvate to supplement the energy required for embryo metabolism, add Y27632 to delay apoptosis. We removed penicillin/streptomycin and performed strict aseptic operations.

**Note:** strictly aseptic operations should be performed.

**Establish of 3D culture system:** In the process of repeating 2D culture system, we found that at d.p.f 11 the trophoblast became apoptosis and the diameter of the embryos cannot be enlarged. In order to simulate the development process of embryos *in vivo*, we attempted to add a substance during the embryo implantation stage so that the embryo can grow in three dimensions. Therefore, we used low-attachment plate during pre-implantation stage then added Matrigel at the implantation stage until d.p.f 14.

**Confirm the concentration of Matrigel:** To preserve the three-dimensional structure of the embryo, we tried four experimental conditions of Matrigel and finally we confirmed that 10% Matrigel was the best condition.

**Note:** All the operations of Matrigle should be carried on the ice.

# Time Taken

3D culture of embryos from blastocyst: 9 days

IF staining of frozen sections: 2 days

IF staining of whole embryos: 2 days

# Anticipated Results

# References


1 Shahbazi, M. N. *et al.* Self-organization of the human embryo in the absence of maternal tissues. *Nature Cell Biology* **18**, 700-708 (2016).

2 Deglincerti, A. *et al.* Self-organization of the in vitro attached human embryo. *Nature* **533**, 251-254 (2016).

3 Rossant, J. Human embryology: Implantation barrier overcome. *Nature* **533**, 182-183 (2016).

4 Rossant, J. & Tam, P. P. L. New Insights into Early Human Development: Lessons for Stem Cell Derivation and Differentiation. *Cell Stem Cell* **20**, 18-28 (2017).

5 Wamaitha, S. E. & Niakan, K. K. in *Current Topics in Developmental Biology* Vol. 128 (eds Berenika Plusa & Anna-Katerina Hadjantonakis) 295-338 (Academic Press, 2018).

6 Gardner, D. K., Lane, M., Stevens, J., Schlenker, T. & Schoolcraft, W. B. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. Fertility and sterility 73, 1155-1158 (2000).

7 Picelli, S. *et al.* Full-length RNA-seq from single cells using Smart-seq2. *Nat Protoc* **9**, 171-181, doi:10.1038/nprot.2014.006 (2014).


# Acknowledgements


This work was supported by the National Key Research and Development Program (2018YFA0108500), the Yunnan National Key R&D Program (2018ZF007-02 and 2018FA008), the National Natural Science Foundation of China (31760268 and 81660266), Chinese Postdoctoral Science Foundation (2017M623317XB), Yunnan Provincial Innovation Team (2018HC003 and 2017HC009) and Yunnan Innovation Talents of Science and Technology (2013HA027).