Spatial embryo profiling (STEP) of primate implantation stages

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Method Article

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Abstract

Spatial embryo profiling (STEP) combines laser capture microdissection (LCM)-assisted sample collection for Smart-Seq2 profiling with virtual reconstruction of stereological immunofluorescence images of implanted primate embryos. Frozen, unfixed embryos O.C.T.-embedded *in utero* are cryosectioned, with every second to third embryo-containing section subjected to LCM for collection of tissue samples of one-to-three cells and processed individually for full-length transcriptome profiling. Remaining sections are fixed and processed for immunofluorescence staining, followed by stereological confocal microscopy, image registration, tissue segmentation and surface generation to create virtual 3D embryo models. Gaussian process regression following the integration of discrete, spatially defined transcriptome samples allows modelling of 3D gene expression patterns across the entire embryo model.

Introduction

We established SpaTial Embryo Profiling (STEP) to delineate early marmoset postimplantation development. STEP combines laser capture microdissection (LCM)-assisted sample collection for Smart-Seq2 profiling with virtual reconstruction of implanted marmoset embryos from stereological immunofluorescence stainings. At the implantation site, tissue samples of one to three cells were captured by LCM and processed individually by Smart-seq2 (Picelli et al., 2014) for full-length transcriptome profiling. STEP-transcriptomes were sequenced to high saturation, detecting on average >8,000 genes per sample and showed homogenous read-depths throughout lineages. Tracking the position of each LCM-sample allowed us to assign lineages according to transcriptome location within the embryo. To generate virtual models of Carnegie stage (CS) 5, 6 and 7 embryos, we performed stereological confocal microscopy, image registration and lineage segmentation of the implanted marmoset embryos. We integrated spatial transcriptomes into the virtual embryos and redeployed Gaussian process regression (GPR) to model gene expression between discrete samples and to determine genome-wide expression gradients.

Samples collected by LCM were collected for Smart-Seq2 library preparation by physical separation of genomic DNA (gDNA) from messenger RNA (mRNA) using an adapted version of previously established single-cell protocols for genome and transcriptome sequencing (G&T sequencing) adapted from (Clark et al., 2018), based on (Macaulay et al., 2017). Importantly, samples were not fixed prior to LCM-processing. GPR was applied onto spatially defined transcriptomes integrated into the established virtual embryo models. GPR is a nonparametric Bayesian approach widely used in machine learning (Rasmussen and

Williams, 2006) and most recently 2D spatial transcriptomics (Svensson et al., 2018), to model gene expression between discrete samples.

Alternative protocols

3D-transcriptomes have been generated of mouse embryo egg-cylinders of Embryonic day (E) 7.0 (Peng et al., 2016) and for E 5.5 to E 7.5 (Peng et al., 2019) previously. This approach has been recently adapted to non-human primates (Cui et al., preprint). In this study, cynomolgus E16, E18 and E20 were embedded in O.C.T. compound and populations of around 20 cells were collected by LCM and processed for low-input RNA sequencing.

We have used the mouse datasets to establish GPR-based 3D-transcriptome models to validate that our GPR-based approach accurately renders the well-characterised gene expression marker patterns from individual data points.

Reagents

Cryosectioning

Leica cryostat microtome (CM3050)

Naphthalate (PEN) membrane slides (Zeiss, 1.0PEN)

Histological slides (Superfrost Plus, Thermo Scientific)

Laser capture microdissection

Zeiss PALM microbeam laser capture station coupled to a Zeiss Axiovert 200 microscope

0.5 mL Eppendorf tube

RLTplus lysis buffer (1053393, Qiagen)

Micro-centrifuge (Fisher Scientific)

RNaseZAP (Sigma, R2020)

Transcriptome library preparation

96-well plates, LoBind semiskirted (Eppendorf, 30129504)

Microseal PCR Plate Sealing film (#MSB1001, Bio-rad)

Biotinylated oligo-dT30VN-tailed oligonucleotides (IDT)

Dynabeads Streptavidin C1 (65001, Invitrogen)

RNase-inhibitor (RNAsin; N2615, Promega)

Superscript II (Invitrogen, 200 U/µL)

template-switching oligos (TSO; Exiqon, 100 µM)

5x Superscript II first strand buffer (Invitrogen)

RNase-inhibitor (Promega, 1U/ µL)

MgCl2 (Invitrogen, 1M)

Betaine (Sigma, 5M)

DTT (Invitrogen, 100 mM)

dNTPs (Roche, 10mM)

KAPA HiFi HotStart Readymix (KK2601, Kapa)

IS PCR primers (IDT, 10µM)

AMPure XP beads (A63881, Beckman Coulter)

80% ethanol

Elution buffer (Qiagen)

MagnaBot® II Magnetic Separation Device (V8351, Promega)

DynaMag-2 Magnet (ThermoFisher, 12321D)

Eppendorf Thermomixer C, with 96W thermoblock (5306000006, Eppendorf)

Agilent Bioanalyser high sensitivity chip system (5067-4626, Agilent Technologies)

Nextera XT DNA kit (FC-131-2001, Illumina)

Nextera XT 96-index kit (Illumina)

Nextera PCR master mix

Sequencing

Illumina HiSeq4000 platform

Haematoxylin and Eosin staining

4% paraformaldehyde (PFA; 15714S, Electron microscopy sciences)/ phosphate-buffered saline (PBS; Thermo Fisher)

Filtered haematoxylin (10052574, Fisher Scientific)

Millipore MQ water

Eosin (Fisher Scientific)

Distyrene/plasticiser/xylene mounting medium (DPX; 06522, Sigma-Aldrich)

Coverslips (12343138, Fisher Scientific)

Immunofluorescence staining

4% paraformaldehyde (PFA; 15714S, Electron microscopy sciences)/ phosphate-buffered saline (PBS; Thermo Fisher)

0.25% Triton X100 (13444259, Thermo Scientific)

0.3% polyvinyl pyrrolidone/PBS (Fisher Scientific)

Blocking buffer (2% donkey serum (116-4101Fisher Scientific)), 0.1% bovine serum albumin (BSA; A9418, Sigma), 0.01% Tween20 (BP337-100Fisher Scientific) in PBS)

DAPI (4',6-diamidino-2-phenylindole, Sigma)

Vectashield mounting medium (H-1200, Vector laboratories)

Pipettes (P2,10,20,200,1000)

Pipette tips (P2,10,20,200,1000)

Image acquisition

Inverted Leica SP8 confocal microscope (x0.75 air objective at 20x magnification) using acquisition software LASX (Leica microsystems)

ImageJ/Fiji software

Zeiss Apotome 2 widefield microscope with motorised stage (0.8 M27 air plan apochromat objective)

Image registration/alignment

Fiji package MultiStackReg

Lineage segmentation

CellProfiler 2.0 (open-source and available from the Broad Institute at www.cellprofiler.org)

MATLAB (Delauney triangulation built-in function), custom scripts (https://github.com/Boroviak-Lab/SpatialModelling)

3D surface generation and modelling

MATLAB (custom scripts: https://github.com/Boroviak-Lab/SpatialModelling)

Blender 2.81 (open-source 3D modelling and animation software, https://www.blender.org/)

Transcriptome sample projection and Gaussian Process regression

R package Seurat

MATLAB custom scripts (https://github.com/Boroviak-Lab/SpatialModelling))

Equipment

Laser capture microdissection (Zeiss Palm station)

*Confocal microscope (*e.g. Leica SP8 confocal microscope)

Procedure

Cryosectioning and Haematoxylin and Eosin staining of embryos

Section each O.C.T. block containing uteri with implanted embryos fully at a thickness of 12 µm using a Leica cryostat microtome (CM3050) to obtain consecutive slices of the whole organ

All embryo-tissue containing sections are collected, either on Naphthalate (PEN) membrane slides (Zeiss, 1.0PEN) or histological slides (Superfrost Plus, Thermo Scientific) for laser capture microdissection and immunostaining, respectively, and immediately transferred to dry ice and then stored at -80 until further processing.

During the cutting process, check slide for embryo tissue within the uterine cavity under a brightfield microscope. Confirm embryo tissue within the uterine cavity using haematoxylin and eosin staining.

Importantly, all following washing and staining steps are carried out using a P200 pipette due to the fragility of the tissue.

Haematoxylin and eosin staining: Thaw slides briefly at RT, then fix for 7 min in fixation solution (4% PFA/PBS). Remove fixation solution by two gentle washing steps using PBS. For nuclei staining, filtered haematoxylin is used. Apply for precisely 18 sec, and then thoroughly wash with tap water several times. Incubate samples in a drop of tap water on the slide for 1 min, followed by three wash steps using Millipore MQ water. For counterstaining of extracellular matrices and cytoplasm with eosin incubate for 23 sec. Wash slides with MQ water and dehydrate them sequentially in 25%, 50%, 70%, 90% and 100% ethanol (three washes each). Dry slides at RT and mount using distyrene/plasticiser/xylene mounting medium, and coverslips. After hardening of the mounting medium, store slides at RT until imaging.

Laser capture microdissection

Clean the whole set-up (Zeiss Palm station, microfuge, pipettes) thoroughly with RNaseZap to prevent RNA degradation. Label all Eppendorf tubes prior to LCM-processing, to minimise duration of processing.

Membrane slides are processed using a Zeiss PALM microbeam laser capture station coupled to a Zeiss Axiovert 200 microscope. Take unfixed sections from dry ice storage and thaw briefly (around 5 seconds) at room temperature. Identify tissue types based on morphology and topology using the PALM brightfield set-up in 5x and 10x magnification, and take pictures at all magnifications of the implantation site.

Take a brightfield microscope image at 20x magnification before sample collection.

Cut a cluster of 1-3 cells (around 20-30 μm^2) at 20x magnification using the freehand tool. Using the RoboLCM programme, you can automatically pulse each sample individually. Pulse sample into the lid of an 0.5 mL Eppendorf tube containing 15 µL RLTplus lysis buffer.

Laser cutting speed, pulse intensity and focus has to be adjusted for each sample individually according to tissue type. Rough estimates are: Cut energy 50 to 60, LPC energy 80.

Take another picture after microdissection of this sample, to preserve its spatial identity, which allows lineage allocation after RNA-sequencing.

Incubate samples in lysis buffer for 2 minutes, spin down using a microfuge and immediately transfer to dry ice to prevent RNA degradation.

Transcriptome library preparation

a) Separation of genomic DNA and RNA

Prepare following solutions in 500 µL frozen aliquots (all plasticware is “Eppendorf low-bind”). Clean lab space using RNaseZap and 70 % EtOH before starting.

- Dynabead solution A

Reagent

Amount

NaOH (10 M)

500 µl (40 g NaOH pellets + 100 ml H2O)

NaCl (5 M)

500 µl

H2O UltraPure

49 ml

Total

50 ml

- Dynabead solution B

Reagent

Amount

NaCl (5 M)

1 ml

H2O UltraPure

49 ml

Total

50 ml

- Dynabead 2x Binding & wash buffer (B&W)

Reagent

Amount

Tris-HCl (pH 7.5, 1 M)

500 ul

EDTA (0.5 M)

100 ul

NaCl (5 M)

20 ml

H2O UltraPure

29.4 ml

Total

50 ml

- Dynabead 1x Binding & wash buffer (B&W)

Reagent

Amount

Dynabead 2x B&W

25 ml

H2O UltraPure

25 ml

Total

50 ml

- G&T-seq wash buffer

Reagent

Amount

Tris-HCl (pH 8.3, 0.1 M)

25 ml

KCl (2 M)

1.875 ml (2x 937.5 ul)

MgCl2 (0.5 M)

300 µL (diluted from 1 M stock)

DTT (1 M)

500 ul

Tween (50% vol/vol)

500 µL (diluted from 100% stock)

H2O UltraPure

21.8 ml

Total

50 l

Note: Beads can be stored for 1 month at 4C.

Preparation of oligo-dT beads

Add 50 µL of Dynabeads (Invitrogen, 65001, keep on 4C until use) to a 2.0 ml DNA LoBind Eppendorf tube (“Eppi”). Place it on a magnet rack (DynaMag-PCR until it gets clear (about 30 sec, use timer)

Leave it on the magnet, remove and discard supernatant (using P200 pipette to 100 ul)

Dynabead A

Remove Eppi from magnet, and add 200 µL of Dynabead A solution. Resuspend it by pipetting it up/down. It is normal for this stage to look messy, because beads stick to wall.

Place Eppi on magnet for 30sec, remove and discard supernatant, while keeping on magnet

Repeat wash step using 200 µL Dynabead A solution.

Dynabead B

Remove Eppi from magnet, add 200 µL Dynabead B solution. Resuspend it.

Place on magnet for 30 sec, remove and discard supernatant, while keeping on magnet.

Repeat wash step using 200 µL Dynabead B solution.

B& W buffer

Remove Eppi from magnet, add 50 µL of 2x B&W buffer and resuspend beads.

Place on magnet for 30 sec, remove and discard supernatant, while keeping on magnet.

Repeat B&W wash step using 50 µL of 2x B&W buffer.

Remove Eppi from magnet, add 50 µL of 2x B&W buffer.

Oligos

Add 50 µL of biotinylated oligo-dTs (100 µM) to the beads. Mix it with the pipette.

Incubate at least 15 minutes on Eppendorf Thermomixer C (programme: Sc Poly A Bead), while shaking gently at 600 rpm.

During incubation time:

Prepare a box with ice, place a cooling 96 well plate and 5x First strand buffer, 0,1 M DTT, dNTPs, betaine solution.

Thaw samples shortly, then keep them on ice.

Add 5.1 µL sample to a 96 well plate on ice. Double check with a pipette tip box, seal sample plate and keep it on the cooling rack.

Note: If 15 µL RLT buffer has been used for the sample, increase the bead resusupension volume by three times. The bead amount remains the same.

Place Eppi on magnet for 30 sec, remove and discard supernatant, while keeping on magnet.

Add 200 µL 1x B&W buffer (frozen aliquots). *Alternatively, dilute 2x B&W buffer to 1x B&W buffer by using 100µL buffer + 100 µL UltraPure H2O (Invitrogen).* Resuspend while taking off the magnet.

Place Eppi back to the magnet, until a pellet is formed (about 30 sec) Remove buffer by pipetting out of the middle, where a clear liquid is visible.

Repeat the wash step once again. Then remove all (!) supernatant. Resuspend in:

Mix for

1x

55x

110x

H2O UltraPure (freezer)

7.73 ul

425 ul

850 ul

ERCC (ready to use, in freezer)

0.18 ul

10 ul

20 ul

5x First strand buffer (SSII, Invitrogen, Y02321)

1.8 ul

100 ul

200 ul

RNase inhibitor (Promega, N251B)

0.27 ul

15 ul

30 ul

Total

9.98 ul

550 ul

1100 ul

Mix the reagents with a P1000.

Physical separation of m RNA and g DNA

1. Prepare 12 tubes and add 92 µL each.

2. Add 10 µL oligo-dt beads to each well of the 96-well sample plate, using a multipipette. Seal the plate.

3. Seal the plate and incubate for 20 min at RT on the Eppendorf Thermomixer C (2000 rpm, programme: SC Bead Bind). During incubation time:

4. Thaw Tween on Thermomixer at 99, then store at RT. Prepare in a 5 ml tube:

Mix for

1x

48 samples

96 samples

G&T seq wash buffer

41.25 ul

1980 ul

3960 ul

RNase inhibitor (Promega, N251B)

0.42 ul

20 ul

40 ul

Total

41.7 ul

2000 ul

4000 ul

RT-mastermix

Mix for

1x

55x

110 x

H2O UltraPure

3.59 ul

197.45 ul

394.9 ul

dNTP (10 mM, ready to use)

1 ul

55 ul

110 ul

TSO (100 uM, ready to use)

0.1 ul

5.5 ul

11 ul

MgCl2 (1M, Ambion)

0.06 ul

3.3 ul

6.6 ul

Betaine (5 M, Sigma, 130300-IVL)

2 ul

110 ul

220 ul

5x First strand buffer (SSII, Invitrogen, Y02321)

2 ul

110 ul

220 ul

DTT (0.1 M, Invitrogen, Y00147)

0.5 ul

27.5 ul

55

Superscript II (2000 U/ul, Invitrogen)

0.5 ul

27.5 ul

55 (add whole tube)

RNase-inhibitor (Promega, N251B)

0.25 ul

13.5 ul

27 ul

Total

9.99 ul

549.75 ul

1100 ul

1. Place the 96-well sample plate on the magnet for 1 min.

2. Prepare a semi-skirted 96-well plate for gDNA. Transfer 15 µL of the supernatant from the sample plate to the new plate, using a 20 µL multipipette.

1. Transfer remaining supernatant by pipetting a second time. Then place tips back into the box and continue with the next row.

2. Dispense 150 µL of G&T wash buffer with RNase inhibitor into a 12-tube lane.

3. Add 15 µL of G&T wash buffer to the beads, using a new box of tips, but only one row of tips for the whole plate.

4. Resuspend the beads in the G&T buffer by placing the plate off the magnet.

5. Seal the plate and vortex it for 5 min at RT. The colour should be homogenous brown, without particles, otherwise vortex longer. If some beads are still visible, centrifuge plate and vortex again.

When homogenous, centrifuge plate.

6. Place the 96-well sample plate on the magnet for 1 min. Transfer the supernatant into the gDNA plate, but this time only once.

7. Dispense 150 µL of G&T wash buffer with RNase inhibitor into a 12-tube lane.

8. Add again 15 µL of wash buffer, seal the plate, vortex it properly (5 min) until it is homogenous. Again if it is not homogenous, centrifuge/vortex it. Afterwards, centrifuge plate.

9. Place it on the magnet and transfer 14 µL supernatant to the gDNA plate. Repeat this step with another 14 µL to get all the supernatant out of the tubes.

10. Seal the gDNA plate and centrifuge it.

a. The total volume of sample in the gDNA plate should be 45 ul. Store on dry ice or at -20, for long-term storage at -80.

b) cDNA preamplification, tagmentation and cDNA library generation

1. Prepare a 12- tube lane and add 96 µL of RT-Mastermix into each tube.

2. Dispense 10 µL of RT-MM into each well of the bead- containing mRNA 96-well plate.

Reverse Transcription

1. Seal the plate and vortex it. Spin down the plate and place it into an Eppendorf Thermomixer C with Thermo Top using following conditions:

Note: This can also be done without an Eppendorf Thermomixer. Therefore, seal the plate, vortex it and spin it down. Incubate it for 1 h at 42C in a conventional PCR machine. Take it out before the 50C incubation step, and vortex again. Then continue using the PCR machine.

Cycle

Temperature

Time

Mixing (rpm)

1

42

2 min

2000

2

42

60 min

750

3

50

30 min

1500

4

60

10 min

1500

5

4

∞

1. Centrifuge the mRNA plate to collect the liquid to the bottom. If there are dark spots on the sealing film, this is from the machine and therefore no problem.

PCR pre-amplification

PCR mastermix is prepared at RT, using a 2ml LoBind Eppi.

Mix for

1x

55x

110x

H2O MQ

0.75 ul

41.25 ul

82.5 ul

HiFi HotStart ReadyMix (2x, Kapa biosystems, KM2602)

11 ul

605 ul

1210 ul

IS PCR primers (10 uM, IDT)

0.25 ul

13.75 ul

27.5 ul

Total

12 ul

660 ul

1320 ul

1. Distribute mastermix into a 12-tube lane, 105 µL per tube.

2. Add 12 µL of PCR mastermix to the 96-well sample plate. Use each tip just once: Dip tip into the sample, mix it up/down a few times.

3. Seal the plate and vortex it. The total single reaction volume is 22 µLl.

4. Run the PCR program under following conditions (duration 2.5-3h):

Cycle

Temperature

Time

Repeats

1

98C

3 min

-

2

98

20 sec

20x

3

67

15 sec

-

4

72

6 min

-

5

72

5 min

-

6

4

∞

hold

5. Store the plate on dry ice, then freeze it at -80C.

PCR purification

1. Place Ampure XP beads to RT to equilibrate them to RT. Thaw cDNA plate at RT.

2. Vortex the bead solution and pour it into a basin. Add 20 µL to each sample well, and mix it up/down after pipetting once.

3. Pour the bead solution back into the bottle afterwards. Seal the plate and vortex it. Incubate the plate for 10 min at RT.

4. Prepare a 80% EtOH solution in the meantime, using EtOH (VWR) and MilliQ . Pour EtOH solution in a basin. Take gel for Bioanalyzer out of the fridge.

5. Place the plate on the magnetic stand (MagnaBot II) for 3-5 min, until liquid is clear.

6. Remove the supernatant without disturbing the beads, using 60 µL multi-pipette.

7. Wash beads with 100 µL 80% EtOH on the magnet, by taking it up with a multipipette from the basin. Pipette EtOH down into the sample wells, take it up slowly again using the same tip and discard the tips.

8. Repeat the washing step, but empty the EtOH in the tip into the bin, go back into the well and remove everything.

9. Dry the bead pellets by incubation at RT for 3-5 min, to a max of 10 min, or until a crack appears on the surface of the beads. Keep it for this on the magnetic stand.

10. Transfer 2250 µL elution buffer (EB) from bottle into a 5 ml tube. Dispense 200 µL EB in a 12-tube lane. Remove the 96-well plate from the magnetic stand when dried, and add 20 µL EB per sample well.

11. Seal the plate and vortex it, then incubate the plate for 10 min at RT.

12. Place the plate on the magnetic stand for 3 min. You can store the plate at -20C without the magnet.

Bioanalyzer

1. Follow the manufacturer's protocol for the Agilent High Sensitivity DNA Kit to determine concentration of 10 randomly selected individual samples (1 µL of sample). Check that electrodes are cleaned before and after each run, and that Gel-Dye mix is <6 weeks old from preparation date.

2. The peak should be between 700 and 7000 bp. Peaks may also be very sharp.

Tagmentation reaction (Illumina Nextera XT DNA sample preparation kit)

1. Take samples out of the -20C freezer, and thaw them on the magnet.
2. Prepare a 1.5 ml LoBind tube on ice with following reagents:

Mix for

1x

55x

110x

Tagmentation DNA buffer (TD, 2x, freezer)

2 ul

110 ul

220 ul

Amplicon tagment mix (ATM, freezer)

1 ul

55 ul

110 ul

Sample DNA

1 ul

1 ul

1 ul

Total reaction volume

4 ul

166 ul

331 ul

1. Place a cooling rack on the ice. Prepare a 12-tube lane, and distribute 27.5 µL per tube.
2. Distribute 3 µL of the reaction mix in a semi-skirted 96-well plate
3. Spin down the sample plate using the prepared balance. Add 1 µL of sample to the semi-skirted plate.
4. The total single reaction volume is 4 µL per well.
5. Seal the plate with the beads, and store it on the -80C.
6. Seal the tagmentation reaction plate, and perform following PCR program :

Cycle

Temperature

Time

Repeats

1

55C

5 min

1

2

4

∞

hold

1. Put the samples back on ice.
2. Prepare a 12-tube lane with NT buffer, add 10 µL per tube.
3. Add 1 µL NT buffer to each sample, using a multipipette.
4. Seal the plate and vortex it. Then spin down using the prepared balance. From now on, keep at RT.

Tagmentation reaction (Illumina Nextera XT DNA sample preparation kit)

1. Add 2 µL of indexing primers using a multi-channel pipette from the already prepared 96-well plate. Avoid bubbles by avoiding pipetting completely down.

Amplification of adapter-ligated fragments

1. Add 26.7 µL Nextera PCR mastermix to each tube of a 12-tube lane (in total 320 ul).
2. Add 3 µL of Nextera mastermix to each well of the sample plate. The total reaction volume is now 10 ul.
3. Perform the indexing PCR under following conditions :

Cycle

Temperature

Time

Repeats

1

72C

3 min

-

2

95

30 sec

-

3

95

10 sec

12

4

55

30 sec

-

5

72

30 sec

-

6

72

5 min

-

7

4

hold

Library pooling

1. Pool the samples by pipetting 2 µL into a 8-tube lane, then pool this together into a 1.5 ml Eppi. You should have 192 µL sample solution.

PCR purification

1. Place Ampure XP beads to RT to equilibrate them to RT.

2. Vortex the bead solution. Add 96 µL (0.5) beads to the tube, vortex it well and spin it down.

3. Incubate the tube for 10 min at RT.

4. Prepare a 80% EtOH solution in the meantime, using EtOH (VWR) and MQ H2O.

5. Place the tube on the magnetic stand (DynaMag-2, ThermoFisher) for 3-5 min, until it gets clear.

Short length clean-up step

1. Take up the supernatant, and transfer it to a 1.5 ml LoBind Eppi. Add 38.4 µL beads, vortex it well and spin it down. Incubate the tube for 10 min at RT. Place the tube on the magnetic stand for 3-5 min, until it gets clear.

2. Wash beads with 300 µL 80% EtOH on the magnet. Pipette EtOH down into the sample wells, take it up slowly again using the same tip and discard the tips.

3. Repeat the washing step, but empty the EtOH in the tip into the bin, go back into the well and remove everything (!).

4. Dry the bead pellets by incubation at RT for 3-5 min, to a max of 10 min, or until a crack appears on the surface of the beads. Keep it for this on the magnetic stand.

5. Remove the sample from the magnetic stand when dried, and add 19.2 µL EB .

6. Vortex the sample, then incubate the plate for 10 min at RT.

7. Place the plate on the magnetic stand for 5 min. Transfer the supernatant to a new, labelled (date-Plate no.- embryo code) 1.5 ml tube. Store the tube on ice.

Quality check of the final cDNA library

1. Measure the DNA concentration using a Bioanalyzer, following manufacturer protocols. DNA libraries of sufficient concentration can now be sequenced on the HiSeq400 Platform.

Immunofluorescence staining

All washing steps were carried out by manual pipetting using a P200 pipette due to the fragility of the tissue.

Thaw slides at room temperature (RT) and fix for 8 min in 4% paraformaldehyde (PFA)/phosphate-buffered saline (PBS) solution.

Wash three times using PBS.

Permeabilise in 0.25% Triton X100 in 0.3% polyvinyl pyrrolidone/PBS for 30 min at RT.

Wash three times using PBS.

Incubate for 30 min in blocking buffer (2% donkey serum, 0.1% bovine serum albumin, 0.01% Tween20 in PBS) at RT.

Incubate primary antibodies in blocking buffer at given concentration in a humidified chamber overnight at 4C.

Wash three times using PBS.

Supplement secondary antibodies with nuclear staining DAPI (4',6-diamidino-2-phenylindole, Sigma) in blocking buffer and incubated for 60 min at RT.

Wash three times using PBS.

Mount slides using Vectashield mounting medium (H-1200, Vector laboratories) and coverslips.

Image acquisition

Stained immunofluorescence slides of CS5 and CS6 embryo are imaged with an Inverted Leica SP8 confocal microscope with 2µm z-intervals with a x0.75 air objective at 20x magnification using acquisition software LASX (Leica microsystems).

Perform tile scanning to image the uterine cavity with embryo in its entirety, automatically merge tile-scanned images by the acquisition software.

Process obtained confocal images obtained using ImageJ/Fiji software. Adjust histogram of images appropriately (so that values of grey with low number of pixels excluded) and apply 'remove outliers' option where necessary to remove background speckles for presentation purposes. For z-projections, apply 'maximum intensity projection'.

Immunofluorescence-stained slides of CS7 embryos are imaged with a Zeiss Apotome 2 (widefield microscope with motorised stage) using an 0.8 M27 air plan apochromat objective to obtain whole uterine cavity images.

Acquire several images to cover the whole endometrial area and merge automatically using the Apotome software.

Process images similarly to CS5 and 6 using ImageJ software.

Image registration/alignment

All imaged serial transverse sections with visible, intact embryo structures are used for embryo reconstruction. Broken sections are removed.

Register images using the Fiji package MultiStackReg. Each image is registered to the DAPI channel of the previous image using an affine transformation and applied to all other channels. Crop registered

images to an identical region of interest.

For sections processed by LCM, manually annotate and track LCM-sample locations in Fiji.

Lineage segmentation

Lineage segmentation is performed by sequential nuclear segmentation and lineage annotation using CellProfiler 2.0 (open-source and available from the Broad Institute at www.cellprofiler.org)[59].

Segment nuclei with the “IdentifyPrimaryObjects” module on DAPI images using intensity to identify objects and propagation methods to declump.

Use the “MeasureObjectIntensity” module on all stained channels to store fluorescence intensity, and manually trace composite images with the “MaskObjects” module to segment lineages based on morphology and marker expression.

Document lineage segmentation by pre-drawing traces on confocal images and compare to output images of traced nuclei from “MaskObjects”.

Compile lineage-annotated nuclei coordinates into matrices in MATLAB and integrate with spatial sample information.

Optional: Evaluate segmentation by plotting antibody fluorescence intensity stored from “MeasureObjectIntensity” to ensure marker expression corresponds to the expected lineage (i.e. TFAP2C showed high expression in trophoblast nuclei and mid-level expression in amnion nuclei).

Integrate spatial LCM sample coordinates into the nuclear segmentation matrix. Align nuclei and spatial sample coordinates according to tissue center of mass on adjacent slides and scale to appropriate dimensions between sections.

3D surface generation and modelling

Generate surfaces from segmented nuclei coordinates using MATLAB built-in functions (Delauney triangulation) and custom scripts (https://github.com/Boroviak-Lab/SpatialModelling).

Extend tissues by scaling the last processed cross section using an inverse function with custom MATLAB scripts (https://github.com/Boroviak-Lab/SpatialModelling).

Import generated surfaces into Blender 2.81 (open-source 3D modelling and animation software, https://www.blender.org/) as object files (.obj) for smoothing into representative models.

Guide sculpting by imported confocal images placed at equivalent coordinates in the model.

Apply subdivision-surface (Catmull Clark) to reduce poly-count and produce smooth surfaces between sections. Apply digital sculpting tools under Blender 'sculpt mode' (inflate, smooth, crease, elastic deform) to ensure mesh cavities are consistent with imported confocal images and generate non-overlapping tissues. Apply Boolean modifiers to generate tight seams between lineages.

Create manifold final surfaces using Mesh Clean-up tools and vertex editing. Convert surfaces to quad-mesh with the "Remesh" tool to generate even topology with defined voxel size and export as Wavefront (.obj) files. Voxel size is maximised to minimise file size.

Re-project spatial LCM samples onto final surfaces using custom scripts in MATLAB (https://github.com/Boroviak-Lab/SpatialModelling).

Generation of 3D-transcriptomes

Time: A few seconds per stage per gene. Inference of individual genes or embryo stages can be done in parallel.

Project 3D gene expression patterns onto reconstructed Blender models using Gaussian process (GP) regression (Rasmussen and Williams, 2006). For each LCM sample, normalised gene expression patterns were first calculated using $\log_e$ Counts per 10000 in Seurat, although other normalised expression levels such as $\log_e$CPM would also be applicable.

Construct a Gaussian process model for an individual tissue e.g., EmDisc CS5, by regressing the gene expression for a specific gene against the sample 3D (x,y,z) co-ordinates, assuming a squared exponential covariance function and Gaussian additive noise. Hyperparameters are optimised using Type II MAP estimates, and constitutes a spatially varying model of gene expression.

(Optional) A second GP model can be fitted to the data using a squared-exponential covariance function with fixed arbitrarily large length-scale hyperparameter, constituting a model with no spatial variability in expression.

All other hyperparameters can be inferred using Type II MAP estimates, and a likelihood ratio can be used to determine if the expression pattern for that gene in that tissue is spatially varying or not.

Using the Gaussian process model (or), infer gene expression values at arbitrary points of the corresponding embryonic or extraembryonic tissue based on the posterior mean of the GP e.g., after training on the EmDisc_CS5 LCM samples, expression patterns over the entire EmDisc_CS5 Blender model could be generated, based on the posterior mean of the GP. To exploit the probabilistic nature of these models, the posterior variance or bootstrapped samples from the posterior mean could also be projected onto the embryo.

Construct a separate set of GP models for all tissue types, for all genes of interest, and at all stages.

As well as inferring 3D patterns, GP models could be used to infer expression patterns in the corresponding tissues for virtual sections or along arcs that bisected the tissue of interest. In the latter case, a GP model evaluated along an arc is used as a basis for identifying tissue specific spatial gradients.

For construction of virtual micropatterns, GP models can be used to infer gene expression patterns along an arc running from the anterior to posterior end of the embryo. Expression at a random distance, d, along this arc was projected onto a circle at radius d and a random angle.

Custom MATLAB code for generation of 1D/2D/3D transcriptomes is available at https://github.com/Boroviak-Lab/SpatialModelling

Troubleshooting

Step

Problem

Possible reason

Solution

LCM-processing

Estimate the location of desired tissue within embryo while LCM-processing: To choose desired LCM

sample location under brightfield microscope, expertise and experience with embryo morphology is required.

Prior staining for markers is not possible due to potential RNA degradation during processing.

Molecular characterization of embryos prior to LCM-processing to obtain an overview and get

expertise on morphology and topology.

LCM-processing

Estimate right size of microdissection area to obtain single-cell samples: It is challenging to estimate

the size of one cell during LCM-processing.

Due to tissue being unlabelled, cell boundaries are difficult to distinguish.

LCM-processing at 20x to better see cell boundaries under brightfield microscope. Settings for LCM cut and pulse should be optimised for each objective used.

LCM-processing

Collection of mixed samples during microdissection: During microdissection two or more stacked

cells may be collected, resulting in mixed samples.

Relatively thick slices (more than single-cell width) need to be collected to avoid compromising integrity of the fragile embryo structures.

Optimised section thickness to 12µm to minimise mixed sampling and preserve tissue integrity.

LCM-processing

Obtain samples from all areas within a tissue: Spreading out sample acquiring throughout whole area of tissues caused several samples to drop out during quality control.

RNA amount or quality was not sufficient for samples obtained from extreme ends/the margins of tissues. Adjusted sample size and shape according to location. Especially crucial for samples obtained from the tissue margins.

LCM-processing

Parts of tissue breaking off during processing: Tissue break samples causing a high drop-out rate during quality control.

Tissue can become easily too dry during prolonged sampling resulting in tissue breaks. Tissue breaks

causing mixed samples and/or quality control dropout.

Fast and efficient processing, prior molecular characterization to obtain overview of embryo for better

aimed sampling.

Sequencing and analysis

Obtain clean, unique marker profiles of cell lineages: Limited number of samples can hinder the generation of unique marker profiles for each lineage.

Scarcity of material due to the ethical and legal constraints in primate research prevents higher sample numbers/embryos.

Processed every second-to-third section of embryo for LCM. High-quality and in depth analysis of given samples to increase resolution as best as possible.

Sequencing and analysis

Clustering of samples from different tissue types: Some tissues of different origins have similar expression patterns making it difficult to confidently unmix (e.g. amnion and trophoblast).

Similar expression profiles have biological reasons, as early embryo expresses several genes in different tissues. Thorough sequencing expression analysis allows to determine unique tissue marker profiles to distinguish tissues.

Time Taken

Estimated total time per embryo: **10 - 16 weeks**

Estimated time for different protocol parts:

Cryosectioning and H&E staining: 5-10 days per pregnant uterus

Laser capture microdissection: 60-90 minutes per section

Transcriptome library preparation: 3 days / 96-well plate

Sequencing: 2-3 weeks / sequencing lane

Immunofluorescence staining: 2 half-days / section (sections can be processed in batches or iteratively)

Image acquisition: 20-30 minutes per slide (Confocal or Apotome)

3D model reconstruction: Timing depends on file size and embryo complexity

Image registration/alignment: 1 hour / embryo

Lineage segmentation: 5-10 hours / embryo

3D surface generation: 2-3 hours / embryo model

Modelling: 3-5 days / embryo model

Generation of 3D-transcriptomes: 2-3 seconds / gene / stage

Anticipated Results

STEP generates 3D-transcriptome models of implanted embryos or any complex tissue. 3D-transcriptomes can be used to visualise gene expression from various lineages, genome-wide analysis of expression gradients or spatial identity mapping of single-cell transcriptomes of another specimen, e.g. in vitro cultured cells.

References

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

Clark, S.J., Argelaguet, R., Kapourani, CA. *et al.* scNMT-seq enables joint profiling of chromatin accessibility DNA methylation and transcription in single cells. *Nat Commun* **9,** 781 (2018). https://doi.org/10.1038/s41467-018-03149-4

Macaulay, I. C., Ponting, C. P. & Voet, T. Single-Cell Multiomics: Multiple Measurements from Single Cells. *Trends Genet **33**, 155-168* (2017). https://doi.org/10.1016/j.tig.2016.12.003

Rasmussen, C. E. & Williams, C. K. I. Chapter 2 - Regression. in *Gaussian Processes for Machine Learning* (2006). doi:10.1093/bioinformatics/btq657

Svensson, V., Teichmann, S. & Stegle, O. SpatialDE: identification of spatially variable genes. *Nat Methods* **15,** 343–346 (2018). https://doi.org/10.1038/nmeth.4636

Peng, G. *et al.* Spatial Transcriptome for the Molecular Annotation of Lineage Fates and Cell Identity in Mid-gastrula Mouse Embryo. *Dev Cell* **36**, 681–697 (2016). https://doi.org/10.1016/j.devcel.2016.02.020

Peng, G., Suo, S., Cui, G. *et al.* Molecular architecture of lineage allocation and tissue organization in early mouse embryo. *Nature* **572,** 528–532 (2019). https://doi.org/10.1038/s41586-019-1469-8

Niu, Y. *et al.* Dissecting primate early post-implantation development using long-term in vitro embryo culture. *Science* ***366****, 6467* (2019). https://doi.org/10.1126/science.aaw5754

Cui, G. *et. al.* Spatial and molecular anatomy of germ layers in the gastrulating Cynomolgus monkey embryo. *bioRxiv* (2022) 2022.01.26.474719. https://doi.org/10.1101/2022.01.26.474719

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