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JAX-Sen: Single Cell Dissociation of Mouse Placenta

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Cellular Senescence Net...

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We use this protocol and it's working

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Abstract

This protocol outlines the process of acquiring a single cell suspension prior to submission for single cell RNA sequencing. Whole mouse placentas were collected from male and female fetuses across 8 mouse strains (WSB, A/J, NOD, NZO, 129S1, PWK, CAST, C57Bl/6). Placentas were collected at 4 different timepoints for the C57Bl/6 strain (E9.5, E11.5, E13.5, and E17.5) and 1 timepoint (E13.5) for the other 7 strains. The whole mouse placenta is minced manually, undergoes two enzymatic digestions and ACK Lysis, and gets carefully filtered. Cells are then sorted to enrich for live cells and eliminate cellular debris.

Reagents and Materials

1 **Day before:**

- Scissors and tweezers (cleaned with ethanol)
- Autoclave bags and tape
- Autoclave tweezers and scissors

2 **Day-of:**

- Ice bucket and ice
- TrypLE
- Dispase
- RNase inhibitor
- DNase I
- Collagenase IV
- 1X PBS
- DMEM
- FBS
- EDTA
- BSA
- 15mL conical tubes
- 50mL conical tubes

Equipment:

- Centrifuge
- Benchtop centrifuge
- Rotating Incubator
- LUNA cell counter

2.1 Cool down centrifuge and benchtop centrifuge to 4^{o}C

2.2 Heat up rotator to 37^{o}C for Digest 2 (warm digest)

2.3 Fill ice bucket

2.4 Prepare reagents, listed below

2.5 Digest 1 (enough for 4 samples)

1. 1937.5uL TrypLE

2. 50uL Dispase
3. 12.5uL RNase inhibitor
4. 25uL DNase I

Digest 2 (enough for 5 samples)
1. 1.25mg collagenase IV
2. stock is 2mg/mL; need 0.625mL for one Digest 2
3. 12.5uL RNase inhibitor
4. Bring to 2.5mL with PBS

Ice-cold PBS
1. 50mL PBS

Stop buffer (DMEM with 10% FBS)
1. 45mL DMEM
2. 5mL FBS

CS buffer (need 6mL per sample, make according to orange chart)
1. EDTA
2. PBS
3. 10% BSA
4. FBS

	A	B	C	D	E	F	G	H	I
		Stock conc.	Working conc.	50 ml	100 ml	250 ml	500 ml	20 ml	
	EDTA	0.5 M	2 mM	0.2	0.4	1	2	0.08	ml
	BSA	10%	2%	10	20	50	100	4	ml
	FBS	100%	15%	7.5	15	37.5	75	3	ml
	DPBS	1X	make up	32.2	64.4	161	322	12.88	ml

*Keep samples/cells cold on ice throughout entire protocol

2.6 **Placenta Dissociation:**

- Ice bucket and ice
- Autoclaved tweezers and scissors
- Placenta samples & buffer (keep on ice)
- 5mL eppendorfs
- Ice-cold PBS (keep on ice)
- STOP Buffer (keep on ice)

- Digest 1 (keep on ice)
- 70um cell strainers
- 15mL conical tubes
- 2mL eppendorfs
- Digest 2 (keep on ice until otherwise indicated)
- ACK Lysing Buffer
- CS Buffer (keep on ice)
- Flowmi 40um cell strainers
- New 2mL tubes

2.7 **Cell Counting with LUNA**

- AOPI
- LUNA chip

2.8 **Cell Staining/FACS:**

- Ice bucket and ice
- Cell staining buffer
- Calcein
- PI
- FACS tubes
- 5mL eppendorfs
- 0.04% BSA
- Aluminum foil

Dissociation Protocol:

3 Remove placenta from original tube with tweezers and place into labeled 5mL tube on ice

4 Add 1mL of the MACS buffer from each sample's original tube

5 Mince with scissors on ice

6 Add 2mL cold PBS; Coat wide P1000 with STOP buffer and pipette gently up and down to mix

7 Centrifuge at 800 x g, 4^{O}C for 5 minutes

8 Aspirate supernatant and resuspend pellet in 2mL ice-cold PBS, pipette with coated wide P1000 to mix

9 Centrifuge at 800 x g, 4^{O}C for 5 minutes

10 Aspirate supernatant and resuspend pellet in 2mL ice-cold PBS, pipette with coated wide P1000 to mix

11 Centrifuge at 800 x g, 4^{O}C for 5 minutes

12 Aspirate supernatant

13 Add 500uL of DIGEST 1, pipette up and down with coated wide P1000 after adding

14 Warm up DIGEST 2. Take samples in DIGEST 1 to cold room to rock; coat wide P1000 with STOP buffer and mix samples for 25 minutes
a. While DIGEST 1 runs, put DIGEST 2 in 37^{O}C incubator to warm up & prep tubes + 70um filters

15 Stop first digestion by adding 500uL STOP buffer

16 Filter cells through 70um cell strainers
a. First, retrieve 15mL tubes and 70um stainers for every sample. Label the 15mL tubes and strainer. Coat the strainers with 1000uL STOP buffer.
b. Also, label a 2mL tube for each sample

17 Wash 1mL of STOP buffer through the strainer after filtering cells

18 Collect the retentate on the strainer with wide P1000 and transfer to the new labeled 2mL tube

19 Add 500uL DIGEST 2 to the 2mL tube, pipette up and down with coated wide P1000 after adding

20 Put tubes in 37^{O}C rotator for 10 minutes
a. Halfway through, pipette up and down with coated wide P1000

21 Stop second digestion by adding 500uL STOP buffer, mix up and down

22 Filter contents of the 2mL tube through the same strainer as before

23 Wash 1mL STOP buffer through the filter

24 Centrifuge the 15mL tubes at 800 x g, 4^{O}C for 5 minutes to pellet cells

25 Aspirate supernatant and be careful not to disturb the pellet

26 Resuspend pellet in 2mL ACK Lysing buffer, mix up and down

27 Incubate on ice for 5 minutes

28 Add 5mL CS buffer to stop ACK lysis

29 Collect cells by centrifugation at 800 x g, 4^{O}C for 5 minutes

30 Resuspend pellet in 1000uL CS buffer, making sure the pellet is disrupted appropriately

31 Use P1000 to collect the cell suspension, then slowly pipette out through a Flowmi 40um cell strainer into a new 2mL tube

Cell Counting with LUNA:

31.1 Mix a 1:1 ratio of cell suspension and AOPI
a. 10uL cells and 10uL AOPI

31.2 Load 10uL of mixture into LUNA chip

31.3 Count with LUNA
a. Write down: Number of cells (cells/mL), viability (%), cell size (um)

31.4 After counting with AOPI, spin 2mL tubes down in centrifuge on bench to collect cell pellet
a. 800 x g, 5 minutes, 4^{o}C

31.5 Aspirate supernatant carefully with P1000 set to 1000uL and resuspend according to Cell Staining/FACS protocol below

Cell Staining/FACS:

31.6 Resuspend pellet in 300uL cell staining buffer
a. Resuspend pellet with highest cell count/viability combination in 350uL cell staining buffer

31.7 Transfer 50uL from the 350uL resuspension into separate FACS tube (to serve as unstained control)
a. Add 200uL cell stain buffer for total volume of 250uL

31.8 Stain all other samples with Calcein and PI
a. Live cells, Calcein: 10uL for every 300uL sample
b. Dead cells, PI: 1uL for every 100uL sample
    i. 300uL sample totals: 10uL calcein and 3uL PI
c. Add on ice and cover with tinfoil (stains are light sensitive), don't mix!
d. Write down time of staining on foil covering ice bucket

31.9 After staining with both Calcein and PI, pipette up and down to mix, then add to FACS tube
a. Pipette straight down and directly onto the filter

31.10 Create collection tubes for FACS
a. Add 100uL 0.04% BSA in PBS into a labeled 5mL microcentrifuge tube

31.11 Sort for live cells and submit for scRNAseq