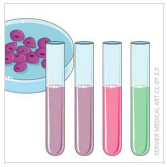

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Protocol to process Gastruloids for FACS (reporters only)

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ABSTRACT

Protocol to process reporter Gastruloids for FACS analysis of their endogenous reporters.

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KEYWORDS

Gastruloids, FACS

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GUIDELINES

This protocol assumes standard aseptic technique, and work in a laminar flow hood.

MATERIALS TEXT

Recipes:

Digestion solution (2mL per sample)
final concentrations: Collagenase IV [3mg/mL], Dispase [4mg/mL], DNaseI [100ug/mL] in PBS

- [3mg/mL] **Collagenase IV** [CAT#17104-019; Gibco™/Thermo Fisher Scientific] 1:4 from 12mg/mL stock
- [4mg/mL] **Dispase in H2O** [CAT#17105-041; Gibco™/Thermo Fisher Scientific] 1:2.5 from 10mg/mL stock
- [100ug/mL] **DNase I** [CAT#11284932001; Roche] 1:500 from 50mg/mL stock
- top up with **PBS-/-**

Staining buffer
Final concentrations: 10%ES-FBS, [100U/mL] Pen-Strep, [1mM] EDTA in PBS

- 1:10 final volume: **ES-FBS** [CAT#16141079; Gibco™/Thermo Fisher Scientific]
- 1:100 final volume: **Pen-Strep** [CAT#15140122; Gibco™/Thermo Fisher Scientific]
- 1:500 final volume: 500mM **EDTA** [CAT#15575020; Gibco™/Thermo Fisher Scientific]
- top up with **PBS-/-** [CAT#10010056; Gibco™/Thermo Fisher Scientific]

2% PFA in PBS-/-
(e.g. diluted 1:2 in PBS-/- from 4%PFA solution, CAT#15434389, Alfa-Aesar/Fisher Scientific)

BEFORE STARTING

Prepare enough FACS tubes/samples/controls to have a complete setup with both positive, negative, FMO (Fluorescence Minus One) references.

For a simple FACS setup of Gastruloids with a double reporter, in addition to

- your experimental samples (i.e. dissociated Gastruloids for each timepoint, with DAPI as live-dead discriminant)

you will thus also need

- double negative cells (ideally, the parental cells of the reporter, with no fluorescent insert; but use alternatively 2D cultured reporter cells if they do not express the reporters in the pluripotent state)
- cells that are RFP+ and have no GFP reporter (or RFP+ beads)
- cells that are GFP+ and have no RFP reporter (or GFP+ beads)
- Make sure to have a sample that not only has no reporter expression at all, but also no DAPI staining (to help set the gate for the DAPI signal).

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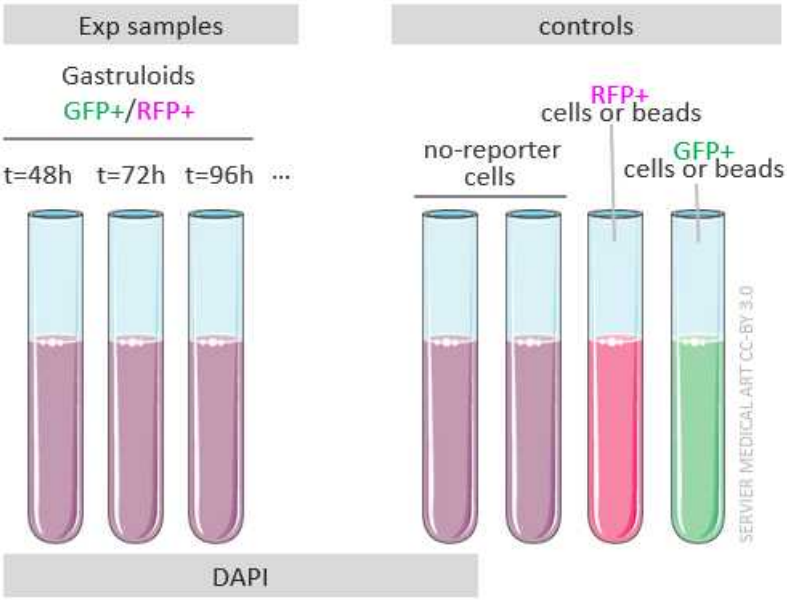

Gastruloid collection

1 Harvest Gastruloid from each well of a 96 well plate and collect them in a 15mL Falcon tube (pooled by timepoint or condition)

2 Once all Gastruloids have settled to the bottom of the tube, aspirate out the supernatant (N2B27 medium carried over with each Gastruloids)

3 Resuspend the Gastruloids in 5mL PBS-/-, to wash away traces of N2B27

4 Once all Gastruloids have re-settled to the bottom of the tube, aspirate out the PBS-/-.
Gastruloids are ready to be digested.

Gastruloid digestion 8m

5 Digest the Gastruloids by adding 1mL **Digestion Solution** (3mg/mL Collagenase IV, 4mg/mL Dispase, 100ug/mL DNAseI, in PBS-/-), **37 °C (waterbath)** , **00:04:00** min 4m

6 After the 4min incubation has elapsed, use a P1000 coated in Staining Buffer to try to mechanically disrupt the Gastruloids

7 Place the tube back again at **37 °C (waterbath)** , **00:04:00** min; to complete the digestion 4m

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8 After the last 4min of incubation have elapsed, use a P1000 coated in Staining Buffer (see recipe in "Materials" section) to mechanically disrupt the Gastruloids. Gastruloids should break easily and give rise to a single cell suspension.

Cell filtering 4m

9 Prewet the blue cap of a filter-FACS tube (e.g. CAT#352235, Falcon/Corning), by pipetting 1mL of Staining Buffer through it.

This step is very imprtant. Not prewetting the filter can lead to loss of a high number of cells at the filtering step.

10 *Working* **On ice**

Filter your cell suspension (digested Gastruloids) through the cap of the FACS tube

11 **On ice**

Wash the filter by passing 1mL more of DIgestion Solution through it.

12 **On ice**

Transfer the contents of the FACS tube to a clean 15mL Falcon tube.

13 **On ice**

Collect any leftover cells by flushing the old FACS tube with 2mL Staining Buffer, and transferring in the 15mL Falcon tube with the rest of the cells

14 **On ice**

Add 5mL more Staining buffer into the tube Falcon tube with your cells suspension, to completely stop the digestion reaction. This tube now contains a filtered, single-cell suspension of your sample for FACS.

4m

15 Spin down the cell suspension, **200 x g, 4°C** , **00:04:00** min

Cell collection 4m

9m

16 For samples that come from 2D culture (e.g. 2D-grown reporter cells as negative controls, or 2D-grown single-color cells as positive controls):

- aspirate out the medium from the culture dish
- add 3mL PBS-/- to wash away any trace of medium, and aspirate it out again
- Add 500uL Accutase, to detach the cells, **00:05:00** min, **Room temperature**
- Transfer the detached cells +Accutase to a clean Falcon tube
- Add ~5mL 10% Serum to stop the reaction
- Spin down the cell suspension, **200 x g, 4°C** , **00:04:00** min

The cells are ready to be processed just as any Gastruloid sample (proceed to the next step)

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DAPI staining and fixation 23m

17 For each sample, prepare 500uL of 0.2ug/mL DAPI in Staining Buffer

> From a stock solution of DAPI 1mg/mL, use 1:5000.
> E.g. add 0.1uL DAPI in 500uL Staining Buffer

15m

18 **On ice**

Resuspend the cell pellet in the DAPI solution, and incubate **00:15:00** min, **On ice**

4m

19 Spin down the cell suspension, **200 x g, 4°C** , **00:04:00** min

20 **FIxation:**

On ice

Resuspend the DAPI-stained cell pellet in 2mL 1%PFA,

4m

21 Spin down the cell suspension, **200 x g, 4°C** , **00:04:00** min

22 Resuspend in 300uL Staining Buffer and transfer to a clean FACS tube pre-coated with Staining Buffer

Processing/Storage 23m

23 Cells can be kept in Staining Buffer, **4 °C** , in the dark (wrapped in alluminium foil) for at least a week (e.g. while waiting to collect samples from later on timepoints)

24 When ready for FACS, **On ice** ,distribute your cells in 300uL per FACS tube and proceed for sorting/analysis.

Beads samples 23m

10s

25 If using beads as positive references, prepare bead tubes just before FACS processing.

For GFP BrightComp eBeads™ Compensation Bead Kit (Invitrogen/Thermo Fisher Scientific, CAT#A10514):

- vortex vial for **00:00:10** s
- Dispense 1 drop into a clean FACS tube
- Resuspend in 1mL Staining Buffer

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