Jun 17, 2024

Zebrafish larvae dissociation for FACs sorting cells expressing fluorescent proteins

Forked from Zebrafish larvae dissociation for FACs sorting cells expressing fluorescent proteins

This protocol is a draft, published without a DOI.

Abigail Elliot[1], Yi Feng[1]

[1]Centre for Inflammatin Research, Institute for tissue repair and regeneration, Edinburgh Bioquarter, University of Edinburgh

Feng Lab (UoE)

RAUL BONNE HERNANDEZ
Federal University of São Paulo

Protocol Citation: Abigail Elliot, Yi Feng 2024. Zebrafish larvae dissociation for FACs sorting cells expressing fluorescent proteins. **protocols.io https://protocols.io/view/zebrafish-larvae-dissociation-for-facs-sorting-cel-dfp23mqe**

Protocol status: Working
We use this protocol and it's working

Created: October 26, 2021

Last Modified: June 17, 2024

Protocol Integer ID: 101850

Funders Acknowledgement:
Wellcome Trust
Grant ID: 100104/Z/12/Z
Cancer Research UK
Grant ID: C38363/A26931
Medical Research Council
Grant ID: MR/N013166/1

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Abstract

Large number of transgenic lines with cell/tissue specific expressing of fluorescent proteins are available in the zebrafish field. These tools provide an opportunity for isolating cell tpe of interest for gene expression analysis. Key to success for a FAC sorting experiment is to generate single cells suspension with maximum number of viable target cells preserved. Here we describe the protocol that we optimized in the lab for generate single cell suspension for FAC sorting cells expressing fluorescent proteins from zebrafish larvae. We used a combination of enzyme digestion (collagenase IV) and mechanical dissociation (rapid pipetting) to generate single cell suspension, which achieved the highest number of cell recovery and viable cells. We have used this protocol for larvae at 3, 4, 5 dpf and for downstream single cell RNA sequencing and RNA sequencing analysis.

Materials

Media A:

HBSS (no mag, no cal, plus phenol red)

HEPES 15mM [M] 15 millimolar (mM)

D-Glucose 25mM [M] 25 millimolar (mM)

2% Sterile Goat Serum [M] 2 % volume

Dissociation Solution:

Media A

100ul Collagenase (from 250 mg/ml stock) 100 µL from 250mg/ml stock

40um filter

Note

NB: Especially for the study of immune cells, use culture grade reagents and prepare solutions fresh in laminar flow hood.

Embryo Dissociation

27m

1 Collect anaesthetised larvae in 2ml Eppendorf 00:05:00 — 5m

> **Note**
>
> max 50 larvae per tube

2 Remove water and replace with 2ml Dissociation Solution with plastic pipette 00:05:00 — 5m

3 Transfer larvae and solution to well of 12-well plate with plastic pipette. 00:02:00 — 2m

4 Incubate on a shaker. 200-250 rpm, 28.5°C, 00:05:00 — 5m

5 Vigorously pipette up and down with P1000, 30s each well. 00:00:30 x Number wells — 5m

> **Note**
>
> Repeat incubation and pipetting 3 times or until fully dissociated.
> 3x is sufficient for larvae up to 4dpf but an extra round might be needed for 5dfp+

6 Transfer digest to pre-cooled 2ml Eppendorf on ice – if pooling multiple tubes, do so at this step into a 25ml falcon tube. — 5m

FACs preparation

20m

7 Centrifuge collected tubes. 300-500 x g, 4°C, 00:05:00 — 5m

> **Note**
>
> Do not exceed 500g since this may impact cell viability. Some cell types are more sensitive to centrifugation than others

8 Resuspend in Media A, 500ul / 50 larvae 5m

9 Filter through 40um filter, into FACS tube 10m

10 FACs collect cells in collect live cells in Media A + 10% Goat Serum

Note

Notes for 10X

After FACS sorting and prior to loading cells onto the 10X Chromium machine, one or preferably 2 wash steps (centrifuge @500g, 5 mins) are required to reduce contaminants and ambient RNA (released from dead/dying cells). The cell density should then be quantified using a cell counter such that an appropriate number of cells can be loaded.

The “optimal” situation according to the 10X user guide would be to FACS sort 100,000 cells, wash twice, and load 7,000 cells. However, this is often not possible due to limited cell numbers. In this case, FACS sorting 50,000 cells and washing once should allow the loading of 7,000 cells. The disadvantage of washing the cells only once is that this might lead to a higher amount of ambient RNA captured in the 10X droplets, but if this is the case there are computational tools that can detect and subtract ambient RNA (e.g. SoupX) from your samples during analysis and I have had success with this approach.

In my own experiments, using 120-150 whole larvae (3dpf-5dpf) I sorted 60,000 cells (50,000 macrophages +10,000 keratinocytes) and was able to comfortably load 7,000 cells. I think you can get more macrophages out than that (possibly 70,000), the keratinocytes were the most limiting factor for me

Note

On cell viability:
I use DAPI staining to measure cell viability during flow cytometry of live cells. In my hands, this protocol always gives >90% viability for macrophages. Remember that cells may die on their way through the sorter or whilst hanging about in the tube. When FACS sorting the first time or attempting to sort a new cell type I would recommend testing the post-sorting viability. There are a few ways to test post-sorting viability but I’ve found the best way is to run a small amount of sample back through the flow cytometer (in my case the % viable macrophages only fell by a couple of percent, demonstrating that they remain viable after sorting). I’d highly recommend doing a dry run of the sample preparation before attempting the actual sequencing experiment, and that is a good opportunity at which to check post-sort viability.