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Podocoryna ACME cell dissociations - draft v1.0, May 13 2024

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Protocol status: Working

We use this protocol and it's working to produce scRNAseq datasets with ~500 - 3,500 cells per sample. Further optimization is expected.

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Abstract

This protocol is intended to dissociate and fix cells of the hydrozoan *Podocoryna carnea* for single-cell RNA sequencing on the 10X Chromium platform following a FACS sorting step. ACME-fixed *Podocoryna* cells maintain their morphology for use in microscopy and can also be frozen at -20C for storage and/or shipping.



Materials

Gloves

70% ethanol spray bottle

Glass dishes and petri dishes

0.2 um filtered seawater

Menthol crystals

Glass Pasteur pipettes

Razor blades

Microdissection razor and forceps

Dissecting microscope

15 mL and 50 mL Falcon tubes

N-acetyl cysteine powder

4 M MgCl_2

Glacial acetic acid

Methanol

DEPC H_2O

Glycerol

1X PBS

BSA powder

Microcentrifuge

Ice bucket

p1000 and p200 pipettes

Standard and wide-bore p1000 tips, p200 tips

Lo-Bind 1.5 mL/2.0 mL Eppendorf tubes

100 um, 40 um pluriSelect cell strainers

Hoechst/DRAQ-5 nuclear stain

Wheat germ agglutinin/Concanavalin-5 cytoplasm stain

Concave well microscope slides and coverslips

Compound fluorescent microscope with camera

5 mL FACS tubes

FACS instrument (ie. BD FACSymphony S6)



Prepare materials and reagents

- 1 Wear gloves for each step of protocol, clean bench spaces with RNase away and 70% ethanol prior to start.
- 2 Prepare 15 mL of 4% MgCl_2 in filtered seawater.
 - 13.5 mL of 0.2 μm filtered seawater
 - 1.5 mL of 4M MgCl_2
- 3 Prepare 15 mL of 4% MgCl_2 and N-acetyl cysteine (N-Ac) wash buffer.
 - 13.5 mL of 0.2 μm filtered seawater
 - 1.125 g of N-Ac powder
 - 1.5 mL of 4M MgCl_2
- 4 Prepare FACS tubes for cell sorting by coating with 5% BSA in 1X PBS. Only empty tubes of coating solution immediately prior to sorting, do not allow them to dry out. For 50 mL 5% BSA in PBS, add 2.5 g BSA to 50 mL tube with 40 mL PBS, dissolve for 10 mins at 4C, then fill to 50 mL total.
- 5 Prepare 20 mL of fresh ACME buffer.
 - 13 mL DEPC H_2O
 - 2 mL glacial acetic acid
 - 3 mL methanol
 - 2 mL glycerolStore in 1 mL aliquots at -20C if necessary
- 6 Prepare an ice cooler for the ACME dissociation steps.

Polyp ACME dissociation

- 7 Prepare for polyp dissection by placing a microscope slide with a *Podocoryna* colony in a glass dish with ~200 mL 0.2 μm filtered seawater. Add menthol crystals at a concentration of ~1 g/L to relax the polyps for ~10 minutes.
- 8 Use a clean razor blade to separate the *Podocoryna* colony from the microscope slide, removing the stolon and polyps together. Dissect relaxed polyps from the stolon using a clean microdissection blade and forceps, then collect the polyps into a petri dish use a glass Pasteur pipette. Transfer as little seawater and debris as possible. Approximately 300 polyps are recommended for good cell yields.
- 9 Transfer 50 polyps to a 1.5 mL tube for each dissociation reaction. Centrifuge tubes at ~10,000 rcf for 30 sec to collect polyps at the bottom of the tube, then remove as much seawater as possible with a p1000 or p200.



- 10 Add 500 μ L of the 4% MgCl_2 seawater and place tube on ice for 5-15 minutes. Centrifuge tubes at $\sim 10,000$ rcf for 30 sec to collect polyps, then remove as much liquid as possible (make a note if this relaxation step is omitted).
- 11 Add 100 μ L of the N-Ac wash buffer. Immediately, centrifuge tubes at $\sim 10,000$ rcf for 30 sec to collect polyps, then remove as much liquid as possible.

- 12 Add 1 mL of ACME buffer to start the ACME dissociation. Allow ACME to permeate the tissues for 1 min.

Next, begin pipetting using a wide-bore p1000 tip set to 500 μ L volume. Pipette at the top of the ACME liquid, aim to create small air bubbles and a "foam" that will disrupt the tissue. Avoid pipetting polyps into the tip, as they can become lodged above where the liquid will reach. Pipette for 1 min, then rest for 30 sec on ice, repeat 10 times for a total of 15 minutes of dissociation.

Alternatively, use a vortex at max speed for 5 sec at 30 sec intervals for 15 mins.

- 13 Verify that polyp tissues have dissociated and a single-cell suspension has been achieved. Small tissue chunks are acceptable but whole polyps should not be visible.

At this point, it is optional to centrifuge tubes at 1,200 rcf for 5 min at 4C to collect dissociated cells, then aspirate 1 mL ACME buffer and resuspend pellet in 200 μ L 1X PBS. Place 50 μ L in a well slide and view cells under a microscope to confirm dissociation quality (stain with 0.5 μ L Hoechst for 30 mins if preferred).

- 14 Prepare pluriSelect cell strainers above a new 1.5 mL tube, with a 100 μ m strainer above a 40 μ m strainer. Pass 1 mL of ACME buffer containing dissociated cells through the paired strainers.
- 15 Centrifuge tubes at 1,200 rcf for 5 min at 4C to collect dissociated cells, then aspirate 1 mL ACME buffer and resuspend pellet in 200 μ L 1X PBS.
- 16 Concatenate all resuspended cells into 1-2 tubes, with 600-800 μ L of cells per tube. Centrifuge tube(s) at 1,200 rcf for 5 min at 4C, aspirate supernatant and resuspend pellet in 900 μ L 1X PBS. Proceed directly to cell staining and FACS steps.

Medusa ACME dissociation

- 17 Collect as many medusae as possible using glass Pasteur pipettes. Place medusae in a petri dish with ~ 5 mL 0.2 μ m filtered seawater, and collect medusae to the center by gently swirling the dish in a circular motion. At least 300-500 medusae are recommended for good cell yields.
- 18 Transfer ~ 100 medusae to a 1.5 mL tube for each dissociation reaction. Centrifuge tubes at $\sim 10,000$ rcf for 30 sec to collect medusae at the bottom of the tube, then remove as much seawater as possible with a p1000 or p200.



19 Add 500 uL of the 4% MgCl_2 seawater and place tube on ice for 5-15 minutes. Centrifuge tubes at ~10,000 rcf for 30 sec to collect medusae, then remove as much liquid as possible (make a note if this relaxation step is omitted).

20 Add 100 uL of the N-Ac wash buffer. Immediately, centrifuge tubes at ~10,000 rcf for 30 sec to collect medusae, then remove as much liquid as possible.

21 Add 1 mL of ACME buffer to start the ACME dissociation. Allow ACME to permeate the tissues for 1 min.

Next, begin pipetting using a wide-bore p1000 tip set to 500 uL volume. Pipette at the top of the ACME liquid, aim to create small air bubbles and a "foam" that will disrupt the tissue. Avoid pipetting medusae into the tip, as they can become lodged above where the liquid will reach. Pipette for 1 min, then rest for 30 sec on ice, repeat 10 times for a total of 15 minutes of dissociation. STOP after 4 or more rounds if tissues appear completely dissociated!

Alternatively, use a vortex at max speed for 5 sec at 30 sec intervals for 15 mins.

22 Verify that medusae have dissociated and a single-cell suspension has been achieved. Small tissue chunks are acceptable but whole polyps should not be visible.

At this point, it is optional to centrifuge tubes at 1,200 rcf for 5 min at 4C to collect dissociated cells, then aspirate 1 mL ACME buffer and resuspend pellet in 200 uL 1X PBS. Place 50 uL in a well slide and view cells under a microscope to confirm dissociation quality (stain with 0.5 uL Hoechst for 30 mins if preferred).

23 Prepare pluriSelect cell strainers above a new 1.5 mL tube, with a 100 um strainer above a 40 um strainer. Pass 1 mL of ACME buffer containing dissociated cells through the paired strainers.

24 Centrifuge tubes at 1,200 rcf for 5 min at 4C to collect dissociated cells, then aspirate 1 mL ACME buffer and resuspend pellet in 200 uL 1X PBS.

25 Concatenate all resuspended cells into 1-2 tubes, with 600-800 uL of cells per tube. Centrifuge tube(s) at 1,200 rcf for 5 min at 4C, aspirate supernatant and resuspend pellet in 900 uL 1X PBS. Proceed directly to cell staining and FACS steps.

Cell staining and FACS

26 Stain cells in 900 uL PBS with 5 uL of wheat germ agglutinin and 1 uL of Hoechst. Vortex tube(s) and leave covered in the dark on ice for 30-60 mins.

27 Centrifuge tube(s) at 1,200 rcf for 5 min at 4C, aspirate supernatant and resuspend pellet in 450 uL 1X PBS.



- 28 Place 50 uL from each tube in a well slide and view cells under a fluorescent microscope to confirm dissociation and staining quality. Take photos to document cell morphology and viability, if possible.
- 29 If satisfied with dissociated cells, combine into a single 5 mL coated FACS tube (~800 uL if using 2 tubes) and proceed to FACS sorting. Sort cells into a 5 mL coated FACS tube with 100 uL 1X PBS.
- 30 To collect sorted cells, centrifuge FACS tube at 1,200 rcf for 10 min at 4C, aspirate supernatant and resuspend pellet in 900 uL 1X PBS. Add 100 uL DMSO (10%, adjust volume as necessary) and freeze at -80C.