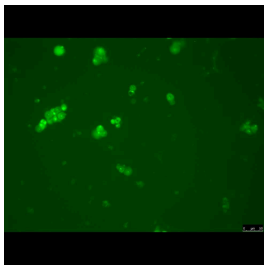
Jan 22, 2025

Dissociation of Anopheles sp midgut into single-cell suspension for scRNAseq



DOI
dx.doi.org/10.17504/protocols.io.j8nlke246l5r/v1

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OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.j8nlke246l5r/v1

Protocol Citation: Silvain Pinaud, Virginia.Howick, Mara Lawniczak 2025. Dissociation of Anopheles sp midgut into single-cell suspension for scRNAseq. **protocols.io https://dx.doi.org/10.17504/protocols.io.j8nlke246l5r/v1**

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

Created: April 16, 2020

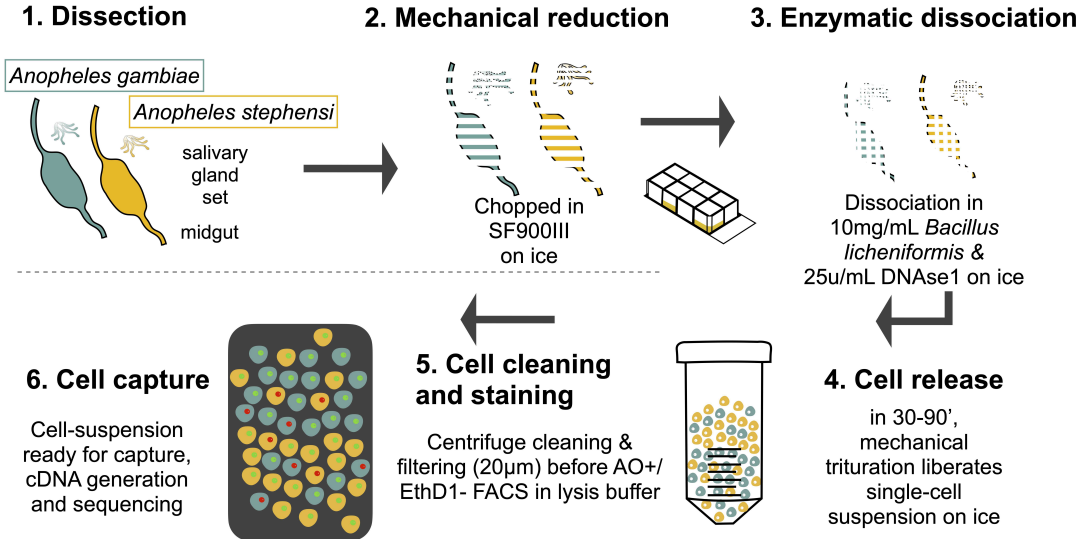
Last Modified: January 22, 2025

Protocol Integer ID: 35797

Keywords: Anopheles, scRNAseq, dissociation, cold-activated-protease, Bacillus licheniformis,

Abstract

Dissociation of Anopheles sp midgut into a single-cell suspension for scRNAseq



Here, we develop a method of cold dissociation for the mosquito midgut and salivary gland tissues that results in cell viability and recovery sufficient for scRNA-seq using a cold-activated protease from Bacillus licheniformis isolated from Himalayan glaciers **(Adam et al. 2017)**. Dissociation of fragile tissues at 4-6ºC has the potential to decrease stress-related artefacts compared to 37ºC proteolytic dissociation **(Adam et al. 2017; O'Flanagan et al. 2019)** . This method enabled us to recover sufficient cells from mosquito salivary gland and midgut tissues for scRNAseq.

Materials

MATERIALS

- Fuchs-Rosenthal disposable hemocytometer **INCYTO Catalog #**DHC-F015
- Protease from Bacillus Licheniformis **Sigma Catalog #**P5380
- Sf-900™ III SFM **Thermo Fisher Catalog #**12658019
- Nunc™ Lab-Tek™ Chambered Coverglass, 8 well, Borosilicate coverglass; 8-well **Thermo Fisher Catalog #**155411
- Ethidium Homodimer-1 (EthD-1) **Thermo Fisher Catalog #**E1169
- Leica TCS SP8 Confocal Microscope
- DNAse1 **Sigma Aldrich Catalog #**AMPD1-1KT
- Acridine Orange **Merck Millipore Sigma Catalog #**318337
- influx **BD Biosciences**

DNAse1 by Sigma AMPD1-1KT

Safety warnings

NB, prior trituration, tips and needle are primed with the digestion buffer to avoid cell to stick

Prepare dissociation buffer

1

Dissociation will be carry on in chambered slides that allow to handle low volume (<400µL) for the dissociation while being monitoring under a microscope.

NB: The dissociation needs to be visually monitored using a microscope to check the proportion of single cells vs surrounding debris. The use of chambered slides or low-volume culture plates is perfectly on purpose.

1.1

Prepare 750 µL of dissociation buffer (DB) per well. Each well must contain 1-20 midguts.
Mix [M] 10 mg/mL of Bacillus licheniformis protease and 25 u/ml of DNAse1 in Sf900III medium and store on ice.

NB: 200µL will be add first in the well, then 3x 100µL. Remaining volume is used to prime tips.

1.2 Prepare 1 canonical 50 mL tube per well by adding 20 mL of Sf900III on ice and in the dark. This tube with this large volume is required to dilute the dissociation buffer when collecting/rescuing the single-cells.

Midgut dissection

2 Dissect 10 mosquito guts in a droplet containing Sf900III and transfer on ice. Check under a dissection scope that tissue is not too affected by dissection or had dried. Remove any contaminant tissues surrounding midguts.

Dissociation setting

3 Rinse guts and cut into pieces before transferring them gently with forceps in a well that contains 200 µL DB. Disrupt pieces of tissue by using a P1000 pipette to pipette up and down 30 times slowly (two seconds up, two seconds down, etc).

The whole dissociation process must not be longer than 90'.

Start the clock when step 3 done. 01:30:00

3.1 Incubate on ice on an aluminum foil (± 4-6°) in the dark.

Trituration/Collection

4 ***NB, prior trituration, tips and needle are primed with the digestion buffer to avoid cell to stick***

After 00:20:00 , the tissue suspension is mixed 15 times again using P1000 primed pipette tip with 2s per up or down.

4.1 The larger pieces of tissue are allowed to settle for 00:05:00

4.2 Single-cells are collected from the supernatant by gently removing 100 µL of supernatant using a primed P200 pipette.

Single-cells are transferred into a 50mL conical tube containing 20 mL of Sf900III on ice and in the dark (this large volume is required to dilute the dissociation buffer).

4.3 Add 100 µL per well of fresh DB to the remaining tissue.

5 **Repeat step 4. two times.**
The dissociation cycle of 30’ (mixing, 20’ of incubation, mixing, 5’ of settling, followed by collection of single cells) is carried out two other times.

5.1 Step 4. -> 4.1 -> 4.2 -> 4.3 for 00:30:00

5.2 Repeat on dissociation cycle: Step 4. -> 4.1 -> 4.2 -> 4.3 for 00:30:00

Final trituration

6 At the end of the 90', the remaining tissue from the chambered slides is mixed in 100-200µL of fresh DB with a primed P200 pipette (20 up/down, 3s each) to increase shear force, the remaining cells are transferred to the same collection tube.

Well rinsing

6.1 Rinse chamber-slide well with 200µL of cold Sf900III and transferred into the collection tube

6.2 Repeat 6.1

Single-cell suspension cleaning

7 Cells are then gently pelleted in the canonical 50mL tube 700g 5-10' at 4 °C and the supernatant is removed (but can be saved to check for single-cells) with a serological pipette without disturbing the pellet.

7.1 Cell pellets is resuspended in 200µL-1.5mL of fresh Sf900III, filtered accordingly of the FACS use (40µm) and transferred in a fresh 1.5mL tube, kept at 4 °C .

Single-cell staining

8 Cells are washed once with cold SF900III at 500g/4°C.

8.1 Cells are resuspended in 1mL of Sf900III and stained with a combination of Acridine Orange (AO, 318337 from Merck) at [M] 0.5 µg/ml and Ethidium Homodimer-1 (EthD1, E1169 from ThermoFisher) at [M] 1 µg/ml for 5-15' at 4 °C in the dark.

The AO is a cell-permeable fluorescent dye that will emit a green fluorescence at 525 nm when bound to DNA at neutral pH. It was used as a cell-specific marker in our suspension. Meanwhile, EthD1 is a cell-impermeant dye that binds to DNA and emits red fluorescence (617nm). It was used as a counterstain for dying and dead cells in both fluorescent microscopy and FACS in conjunction with the AO.

8.2 Cells are washed twice with 1.5ml of Sf900III by centrifugation at 500g, 4 °C for 5-10'

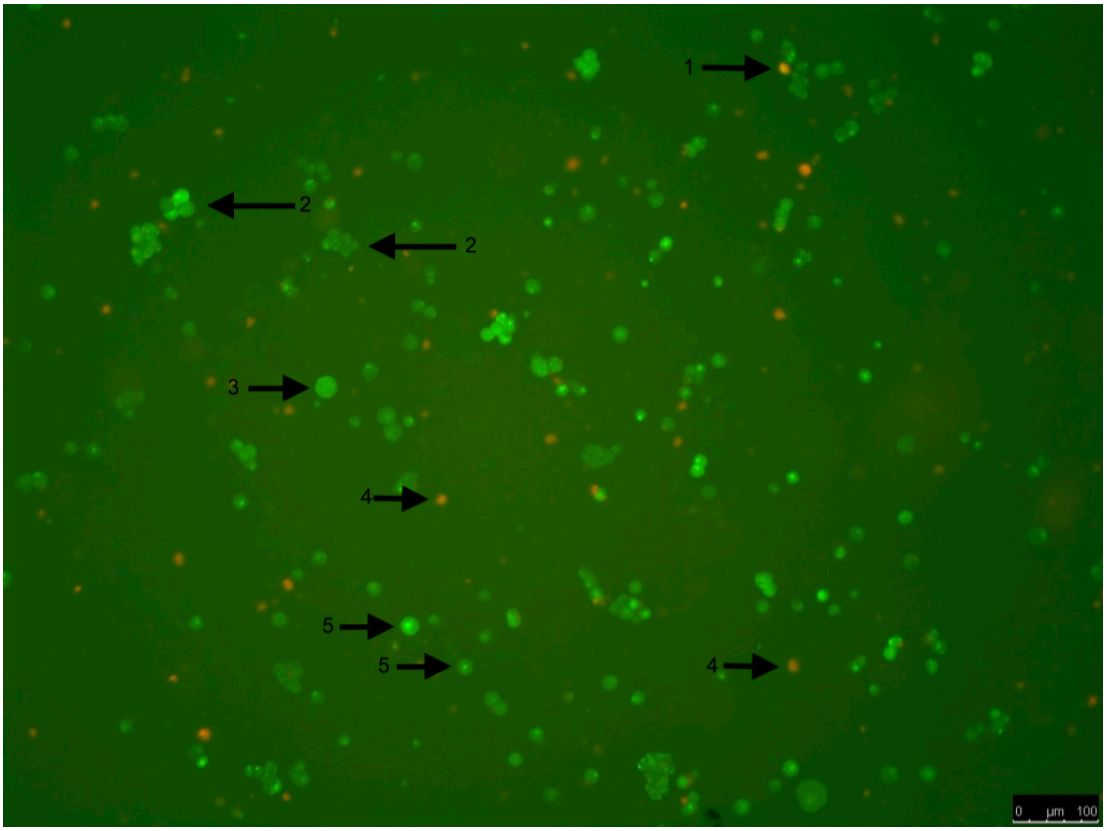
8.3 Cells are resuspended in FACS-compatible tubes in 0.4%BSA-SF900III cold media for sorting and store on ice in the dark until the sort or the load for scRNAseq.

Cell viability check

9 Cell concentration and viability is checked under a fluorescent microscope (or using a automated fluorescent cell counter).

525nm Green for the AO
617nm Red for the EthD

AO+/EthD1- -> All the healthy cells
AO+/EthD1+ -> Dying cells
AO-/EthD1+ -> Dead cells



1 dying cell
2 cluster of individualised single-cells, are clotted on slide but would be single in flow
3 nucleus is hardly defined, the whole cell is stained, cell is maybe dying, would be discard by the FSC/SCC gating
4 dead cell
5 high quality cell. Nucleus is well defined and stained compared to the cytoplasm.