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Bead Beating Zebrafish Dissociation and Hashing with BS3/Methanol V.3

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We use this protocol and it's working

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Disclaimer

This protocol reflects how we currently process zebrafish embryos. However, optimizations are continually underway and will be included here as we adopt them.

Abstract

This protocol is for the mechanical dissociation of zebrafish embryos to isolate and barcode nuclei for sciPlex-RNA-seq3 using bead homogenization in a 96-well plate format. While this protocol was optimized for this specific assay, buffers may be substituted in order to make the bead homogenization compatible with other sequencing assays. This version is updated to reflect additional detergents added to the lysis buffer and different bead beating conditions to improve dissociation of the embryo tissues as well as a sucrose gradient step to remove debris from the nuclei.

Materials

Reagents

- Sodium phosphate dibasic (Na2HPO4) (Millipore Sigma, cat. no. S3264-250G)
- Sodium phosphate monobasic monohydrate (NaH2PO4-H2O) (Millipore Sigma, cat. no. 71507-250G)
- Potassium phosphate monobasic (KH2PO4) (Millipore Sigma, cat. no. 60218-100G)
- Sodium chloride (NaCl) (Millipore Sigma, cat. no. S3014-500G)
- Potassium chloride (KCl) (Millipore Sigma, cat. no. P9541-500G)
- Magnesium chloride solution (MgCl2), 2 M (Millipore Sigma, cat. no. 68475-100ML-F)
- Sucrose (Fisher Scientific, cat. no. S5-3)
- IGEPAL CA-630 (Millipore Sigma, cat. no. I8896-50ML)
- Diethyl pyrocarbonate (DEPC) (Millipore Sigma, cat. no. D5758-5ML)
- 10X Dulbecco's PBS (Thermo Fisher, cat. no. 14200075)
- Triton X-100 (Thermo Fisher, cat. no. A16046.AP)
- Tween20 (Fisher Scientific, cat. no. BP337-500)
- Digitonin (Millipore Sigma, cat. no. D141-100MG)
- DMSO (Millipore Sigma, cat. no. D2438-5X10ML)
- Methanol (MeOH) (Millipore Sigma, cat. no. 34860-2L-R)
- Bis(sulfosuccinimidyl)suberate (BS3) (Thermo Fisher, cat. no. 21580)
- Yoyo dye (Thermo Fisher, cat. no. Y3601)
- 100uM Hash oligos with 5' Amine Modifier C12 in the form 5'-/5AmMC12/GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG- [10bp-barcode]-BAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA-3' where B is G, C or T (IDT)

Equipment

- Bead Ruptor 96 (Omni International, SKU 27-0001, SKU 27-0002)
- Nunc 96-Well Polypropylene DeepWell Sample Processing & Storage Plates with Shared-Wall Technology (Thermo Fisher, cat. no. 278752)
- Nunc 96-Well Filter Plates (Thermo Fisher, cat. no. 278011)
- Breezliy 6mm Precision Steel Bearing Balls G25 304 Stainless Steel Ball (Amazon)
- Omni Sealing Mats for 2ml 96 Deep Well Plates (Millipore Sigma, cat. no. 27-530)
- Mesh strainers (Amazon)
- Refrigerated swinging bucket centrifuge with adapters to hold 96 well plates, 15ml and 50ml conical tubes, and 1.7ml microfuge tubes
- DNA/RNA LoBind tubes
- Chemical fume hood
- Multichannel pipettes and tips
- Wide bore 200ul pipette tips (Thermo Fisher, cat. no. 2069G)
- Cell counter with GFP channel

Reagent Preparation

1 **10x PBS hypotonic stock solution**

In a 500ml beaker, dissolve the following in approx. 250ml nuclease-free water:

- 5.45g Na2HPO4 (dibasic)
- 3.1g NaH2PO4-H2O
- 1.2g KH2PO4
- 1g KCl
- 3g NaCl

Once dissolved, bring final volume up to 500ml with nuclease-free water
Filter sterilize the solution
Store at 4°C for up to 6 months

Lysis buffer base

In a 500ml beaker, dissolve the following in approx. 250ml nuclease-free water:

- 50ml 10X PBS hypotonic stock solution
- 57g sucrose
- 750ul 2M MgCl2

Once dissolved, bring the final volume up to 50ml with nuclease-free water
Filter sterilize the solution
Store at 4°C for up to 6 months

10% IGEPAL

Mix 1ml IGEPAL CA-630 in 9ml nuclease-free water
Store at 4°C for up to 6 months

10% Triton X-100

Mix 1ml Triton X-100 in 9ml nuclease-free water
Store at 4°C for up to 6 months

10% Tween 20

Mix 1ml Tween 20 in 9ml nuclease-free water
Store at 4°C for up to 6 months

2% Digitonin

***Digitonin powder is hazardous - make this reagent in the chemical hood

1. Mix 5ml of DMSO with one 100g vial of Digitonin
2. Add 1ml or 2ml of DMSO to the vial first to resuspend the Digitonin powder by vortexing and pipetting. This makes it easier to transfer to a 15ml tube containing the remaining volume of DMSO
3. Make 200uL aliquots and store at -20°C for up to one year

0.3M SPBSTM

In a beaker, dissolve the following in approx. 250 nuclease-free water:

- 50ml 10X PBS
- 57g sucrose

Once dissolved, mix in the following:

- 5ml 10% Triton X-100
- 750ul 2M MgCl2

Bring the final volume up to 500ml
Filter sterilize the solution
Store at 4°C for up to 3 months

1.4M SPBSTM

In a beaker, dissolve the following in approx. 250 nuclease-free water (may require some mild heat to dissolve fully, but be careful not to burn the sugar):

- 50ml 10X PBS
- 239.5g sucrose

Once dissolved, mix in the following:

- 5ml 10% Triton X-100
- 750ul 2M MgCl2

Bring the final volume up to 500ml
Filter sterilize the solution
Store at 4°C for up to 3 months

100mM BS3

Mix one 50mg vial of BS3 with 873uL nuclease-free water
Aliquot and store at -80°C for up to one month. Do not freeze-thaw aliquot

Before Starting

2 **Prepare Equipment**

- Cool swinging bucket centrifuge to 4°C
- Prepare ice buckets - after addition of lysis buffer, keep everything on ice
- Clean the silicone sealing mat if necessary (see below)
- Clean beads if necessary (see below)

3 **Cleaning beads**

1. Empty the beads from the bead plate into a catch tray
2. Transfer the beads to a 50ml conical tube
3. Add in enough 10% bleach to completely submerge the beads, invert the tube a few times and let sit for ~3-5 minutes, then pour out into the strainer
4. Rinse the beads in the strainer under running deionized water for ~1 minute.

5. Empty the beads into a clean catch tray for easier transfer into a conical tube, then transfer into a 50ml conical tube.
6. Add in enough ethanol to completely submerge the beads, invert the tube a few times, then pour out into a dry strainer.
7. Transfer the beads into a clean container and let air dry (can air dry overnight).
8. Once dry, the beads can be stored in a closed container.

4 **Cleaning sealing mat**

1. Rinse thoroughly with 10% bleach, then DI water, then spray with 70% ethanol
2. Pat dry with a Kimwipe or let air dry, the mat can be used once it is dry

5 **Make BS3/MeOH fixative**

- For every 5ml of fixative needed, mix 75ul 100mM BS3 with 5ml MeOH
- Place on ice

6 **Make lysis buffer/IGEPAL aliquots**

- For every 1ml of lysis buffer needed, mix 1ml lysis buffer base with 10ul 10% IGEPAL, 10ul 10% Tween 20, 5ul 2% Digitonin, and 10uL DEPC
- Only mix the lysis buffer base and 10% IGEPAL, 10% Tween 20, and 2% Digitonin at this time
- Do not add in DEPC yet, DEPC has a very short half life and must be added right before use
- Calculate the volumes needed for each aliquot based on the number of embryos plus extra to account for pipetting error
- The first aliquot requires at least 200ul of buffer per embryo
- The second aliquot requires at least 50ul of buffer per embryo
- Place on ice

Nuclei Isolation

7 Place a single clean 6mm steel bead into each well of a deep well bead beating plate

- Bead beating plates with beads can be prepped ahead of time and kept covered
- Unused wells from filters and deep well plates can still be used. Be sure to mark used wells but still load a bead into the used wells for balancing

8 Transfer dechorionated embryos to a small petri dish containing clean zebrafish embryo media with 10% tricaine

- Manually dechorionate embryos before transfer to the clean zebrafish embryo media plate. Bead beating will not work if the embryos are still in their chorion.

9 Transfer one embryo from the small petri dish directly onto a bead using 20ul embryo media with a wide bore tip, repeat for all embryos

10 In a fume hood, add the appropriate amount of DEPC to the first aliquot of the lysis buffer/IGEPAL/Tween 20/Digitonin mix. Be sure to vortex well and work fast because

DEPC has a short half-life of ~7 minutes.
- Add 10ul of DEPC for every 1ml of lysis buffer/IGEPAL/Tween 20/Digitonin mix
- After vortexing with DEPC, the solution should look cloudy
- ***Note: if performing assays other than RNAseq, this lysis buffer can be substituted with a suitable alternative (e.g. OMNI-ATAC for ATACseq).
- All subsequent steps with samples containing DEPC should be performed in a fume hood

11 Add 200ul of the completed lysis buffer to each well

12 Cover the plate with silicone sealing mat

13 Place in bead homogenizing machine and bead homogenize:
- Make sure to use another plate containing beads as a balance in the machine
- Recommendations for time and frequency using the Omni International Bead Ruptor 96 at different timepoints:
  6 hpf - 14 hpf: 3 minutes at 5.5Hz
  18 hpf - 24 hpf: 5 minutes at 5.5Hz
  24 hpf - 96 hpf: 7 minutes at 5.5Hz
- ***Note: Be sure to test these conditions before attempting to dissociate precious samples. Different machines or different buffers may require different settings

14 Centrifuge at 100 x g for 20 seconds at 4°C to get most of the buffer to the bottom of the well

15 Pipette the buffer up and down gently a few times while avoiding creating bubbles to resuspend nuclei, then transfer to a 20 um filter placed over a deep well catch plate

16 Add the appropriate amount of DEPC to the second aliquot of lysis buffer with IGEPAL, then add 50ul to the beads
- Add 10ul of DEPC for every 1ml of lysis buffer/IGEPAL mix

17 Transfer to same 20 um filter while avoiding creating bubbles

18 Centrifuge filter at 100 x g for 20 seconds at 4°C to get all buffer through the filter

sciPlex-RNA-seq Hashing, Fixing, Pooling, and Freezing

19 ***Note: The following steps are an adaptation from the sciPlex-RNA-seq3 protocol described in Martin et al, Nature Protocols 2023 to include single embryo hashing as was done in Saunders et al., Nature 2023. For other assays, these steps should be changed to follow the protocol of the assay being performed.

20 Add 2.5ul of 100uM hash to each well, pipette up and down gently a few times with a multichannel pipette set to 200ul using wide bore tips, then sit on ice for 5 minutes

21 Add 1000ul BS3/MeOH fixative into each well, pipette up and down gently a few times with multichannel pipette set to 200ul, then sit on ice for 15 minutes

22 Pool wells together into 50ml conical tubes and add in a volume of SPBSTM equal to 1000uL per well into the 50ml conical tubes
- A maximum of 3 columns (24 wells) can fit into one 50ml tube

23 Centrifuge 780 x g for 15 minutes at 4°C

24 Take off supernatant, being sure not to disrupt the pellet

25 Combine the pellets using 1ml SPBSTM across all tubes
- If you want to count the nuclei before putting them through the sucrose gradient or to look at the quality of the nuclei, count here using yoyo dye and a cell counter

26 Centrifuge 780 x g for 15 minutes at 4°C

27 Take off supernatant, being sure not to disrupt the pellet. Resuspend nuclei using 0.3M SPBSTM to a concentration of up to 5 million nuclei/ml
- The exact concentration of nuclei does not matter. 1ml of nuclei will be added to each sucrose gradient, with a maximum of 5 million nuclei per gradient, so add in 0.3M SPBSTM in multiples of 1ml to achieve 5 million nuclei or fewer per ml
- For example: if there are 7 million nuclei, it will get resuspended in 2mL 0.3M SPBSTM and placed into two sucrose gradients

28 Do a sucrose gradient to get rid of debris in the sample
- sonicate nuclei on low power for 6 seconds
- add 10ml 1.4M SPBSTM to a 15ml conical tube
- slowly layer 1ml of nuclei on top of the 1.4M SPBSTM
- centrifuge at 3,000 x g for 30 minutes at 4°C, with the acceleration set to 5 and brake set to 5
- take off supernatant and resuspend in 1ml 0.3M SPBSTM
- count nuclei using yoyo dye and a cell counter

29 Centrifuge 780 x g for 15 minutes at 4°C

30 Take off supernatant, being sure not to disrupt the pellet

31 Store nuclei at -80°C for up to 3 months