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We use this protocol and it's working

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FixNCut v1.0

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ABSTRACT

This protocol details reversible fixation for cells and tissues for subsequent use in sc/snRNA, sc/snATAC or Multiome. Spatial-Omics compatibility is being validated. For more information check this preprint: https://www.biorxiv.org/content/10.1101/2023.06.16.545221v2

ATTACHMENTS

748-1909.docx

GUIDELINES

Notes:

All washes and centrifugations need to be done at 4 °C unless otherwise specified.

IMPORTANT: Washing volumes may change accordingly to your needs. If you want to change the protocol, let's discuss just in case. Time, temperatures and concentrations must be maintained.

DSP has been used before for fixing cells and prep RNA, it works fine. For single cell or tissue following dissociation is what we have been studying and works well. It's still work in progress, but the key is to keep the stock fixative away from water because it neutralizes the NHS-esters quickly.

Points to take into account:

- **CRITICAL**: Prepare working solution (1x) right before fixation (no more than 5 minutes). For larger pieces replace with fresh 1x fixative a couple of times.
- make single use aliquots (20-50 uL) for 2 to 5 fixations. What's not used do not re-freeze (it is fine to re freeze let's not give them the option)
- keep aliquots at -80 °C in a bag (with silica if possible).
- bring tubes at Room temperature and prepare the fixation a few minutes (no longer than 10 min) before fixing. This will ensure that the NHS-ester isn't in contact with aqueous solution for too long.
- evaluate small precipitation during fixation. Too much ppt: bad. You should see a small ppt on the walls of the tube, like in the attached photo. You will notice that the first 2 drops of PBS will be generate the precipitate but will precipitate as you add more.
- do not prepare aliquots larger than 500 µL.
- do not store 1x solutions.
- We have noticed some performance variability from vial to vial purchased from Sigma.
- Viability is not a good measure because PI or Trypan Blue don't enter after fixation and the cells look alive.
- So far, the best test has been the small shift in the LMO-FAM or LMO-Cy5 on cells.

MATERIALS

Liberase™ TM Research Grade **Merck MilliporeSigma (Sigma-Aldrich) Catalog #5401127001**,
Nuclei Isolation Kit: Nuclei EZ Prep **Merck MilliporeSigma (Sigma-Aldrich) Catalog #NUC101-1KT**

Preparation of DSP (Oz Soup) stock and working solutions

1 30m

Note

DSP (dithiobis(succinimidyl propionate)) also known as Lomant's Reagent and can be purchased from Thermo: https://www.thermofisher.com/order/catalog/product/22585.
DSP (dithiobis(succinimidyl propionate)) Lomants Reagent **Thermo Fisher Scientific Catalog #22585**

Equilibrate DSP vial at Room temperature for 00:30:00 and then prepare 50x stock solution of DSP (50 mg/mL) in molecular biology grade dimethyl sulfoxide (Sigma, cat. no. D8418-50ML).

2 Dispense the stock into 100 µL aliquots and store at -80 °C.

3 Immediately before use prepare 500 µL of 1 mg/mL DSP working solution (DSP 1x is also known as OzSoup) in molecular biology

grade 1x PBS as follows: aliquot 10 µL of stock DSP in a 1.5mL eppi tube and while vortexing (VERY IMPORTANT) add 490 µL of PBS (Room temperature) dropwise using a P200.

4 Filter DSP working solution using a 40-µm Flowmi strainer (Sigma, cat. no. BAH136800040-50EA).

Fixation

1h 30m

5 Submerge a ~3x3 mm (the smaller the better) piece of tissue (or organoids) in 500 µL of the OzSoup and incubate for 00:45:00 at Room temperature . 45m

5.1 For cells in suspensions, wash cells in cold PBS at least twice (no media + FBS should be present). Pellet cells and resuspend (up to 2 milliion) cells in 500 µL of the Oz soup and incubate for 00:30:00 at Room temperature . 30m

6 At the 45 min mark, add 10 µL of [M] 1 Molarity (M) Tris-HCl pH 7.5 , mix well by vortexing for 2-3" and sit at Room temperature for at least 00:15:00 . 15m

7 Pellet the pieces of tissue at 500 rcf, 00:20:00 or a 5-10" in minispinner, and remove supernatant. 20m

7.1 For cells, mix by vortexing 2-3min and pellet cells 500 rcf, Room temperature, 00:05:00 . 5m

8 Add 1 mL of PBS, mix by vortexing 2-3 min, and pellet pieces (or cells) as above, remove supernatant.

8.1 For cells, mix by vortexing 2-3 min and pellet cells 500 rcf, Room temperature, 00:05:00 . 5m

Fixation: For cells only

1h 30m

9 Repeat 8 once more for a total of 2 washes. Continue on step 18-21 below. If sorting or shipping samples follow step 17.

Fixation: For tissues only

50m

10 Add 1 mL of 200 µg/mL Liberase (Liberase™ Research Grade Sigma-Aldrich 5401127001, https://www.sigmaaldrich.com/US/en/product/roche/libtmro) in PBS (80 µL of 2.5 mg/mL Liberase + 920 µL PBS).

11 Incubate at 37 °C for 00:30:00 with agitation 800 rpm. At 15 min mark pipette up and down 5 times. 30m

12 After digestion, filter the digestion reaction through a 70 µm mesh.

13 Add 10 mL * ice-cold PBS and pellet cells for 500 rcf, 4°C, 00:05:00 (swinging bucket rotor). Pre-Wash. 5m

Note

For pre-wash and Washes 1-3 10 mL is a starting point. One can use less as per preference.
*This volume can be adjusted for small tissues sizes and/or pellets. Optimization may be required.

14 Remove supernatant and add 10 mL * of cold PBS+1% BSA and resuspend the pellet before pelleting again (500 rcf, 4°C, 00:05:00). 5m

Wash 1.

Note

*This volume can be adjusted for small tissues sizes and/or pellets. Optimization may be required.

15 Remove supernatant and add 10 mL * of cold PBS+1% BSA and resuspend the pellet before pelleting again (500 rcf, 4°C, 00:05:00). 5m

Wash 2.

Note

*This volume can be adjusted for small tissues sizes and/or pellets. Optimization may be required.

16 Remove supernatant and add 10 mL * of cold PBS+1% BSA and resuspend the pellet before pelleting again (500 rcf, 4°C, 00:05:00). 5m

Wash 3.

Note

*This volume can be adjusted for small tissues sizes and/or pellets. Optimization may be required.

17 **Optional**: If storing for later processing or shipping samples, freeze cells using a cryopreservation strategy as if the main goal was to keep the cells alive. For example, use CryoStor10 and Mr Frosty for slow freezing. Include 1-2 U/uL of RNAse inhibitor per sample for storage. Store -80 °C until use. After storage, thaw in water bath at 37 °C and wash cells twice with PBS+0.5-1%BSA.

18 Remove supernatant and resuspend cells in 0.5-1 mL of PBS+1% BSA (optionally add +0.5-1 U/uL RNAse Inhibitor).

19 Filter cells through Flowmi 40 um.

20 Count cells and bring concentration to 1000-1500 cells/uL.

21 Load Chromium as per manual.

Note

For ATAC or Multiome kits prpware nuclei using EzLysis Buffer (Sigma-Aldrich, Cat: NUC101-1KT), SaltyEz10/50 protocols (dx.doi.org/10.17504/protocols.io.bx64prgw) or alternatives you are familiar with.