**FixNCut v1.0**

Preparation of DSP (Oz Soup) stock and working solutions

DSP (dithiobis(succinimidyl propionate)) also known as Lomant's Reagent and can be purchased from Thermo: <https://www.thermofisher.com/order/catalog/product/22585>.

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

-Dispense the stock into 100 uL aliquots and store at −80 °C.

-Immediately before use prepare 500 uL of 1 mg/mL DSP working solution (DSP 1x) in molecular biology grade 1x PBS as follows: aliquot 10 uL of stock DSP in a 1.5 mL eppi tube and while vortexing (VERY IMPORTANT) add 490 uL of PBS (room temp) dropwise using a P200.

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

Fixation protocol

1) Submerge a ~3x3 mm (the smaller the better) piece of tissue (or organoids) in 500 uL of the Oz soup and incubate for 45’ at RT. [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 uL of the Oz soup and incubate for 30’ at RT.]

20 At the 45’ mark, add 10 uL of 1 M Tris-HCl pH 7.5, mix well by vortexing for 2-3” and sit at room temp for at least 15’.

3) Pellet the pieces of tissue 20” at 500xRCF or a 5-10” in minispinner, and remove supernatant. [For cells, mix by vortexing 2-3” and pellet cells 5’ at 500xRCF at RT]

4) Add 1000 mL of PBS, mix by vortexing 2-3”, and pellet pieces (or cells) as above, remove supernatant. [For cells, mix by vortexing 2-3” and pellet cells 5’ at 500xRCF at RT]

For cells only:

Repeat 4) once more for a total of 2 washes. Continue on step 13-16) below. If sorting or shipping samples follow step 12).

For tissues only:

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

6) Incubate at 37 °C for 30’ with agitation 800 rpm. At 15’ mark pipette up and down 5 times.

7) After digestion, filter the digestion reaction through a 70 um mesh.

8) Add 10 mL ice-cold PBS and pellet cells for 5’ at 500xRCF at 4 °C (swinging bucket rotor). Pre-Wash.

9) Remove supernatant and add 10 mL of cold PBS+1% BSA and resuspend the pellet before pelleting again (5’ at 500xRCF at 4 °C). Wash 1

10) Remove supernatant and add 10 mL of cold PBS+1%BSA  and resuspend the pellet before pelleting again (5’ at 500xRCF at 4 °C). Wash 2

11) Remove supernatant and add 10 mL of cold PBS+1%BSA  and resuspend the pellet before pelleting again (5’ at 500xRCF at °C). Wash 3

12) (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 -80C until use. After storage, thaw in water bath at 37 °C and wash cells twice with PBS+0.5-1%BSA).

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

14) Filter cells through Flowmi 40 um

15) Count cells and bring concentration to 1000-1500 cells/uL

16) Load Chromium as per manual.

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.

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 -80C in a bag (with silica if possible).

-bring tubes at room temp and prepare the fixation a few minutes (no longer than 10') 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 uL.

-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.