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GRO nuclear prep for SHARE-seq

This protocol was initially created by a combination of the CVDi cardiovascular tissue sectioning protocol and Surya's brain nuclei prep using an iodixanol gradient to remove debris. The resulting nuclei are usable for input to both 10x and SHARE-seq. Note: We no longer freeze nuclei in storage buffers and rather opt for a dry pellet. The most recent version of this protocol has been edited by Guillermo Barreto Corona with the assistance of Amelia Weber Hall, Nancy Osei and Nick Poirier to be optimized for SHARE-seq.

Buffers:

NIM2 (store at 4°C, good for 6-12 months)

250mM sucrose, 10mM Tris pH 8.0, 25mM KCl, 5mM $MgCl_2$, 1uM DTT, 0.01% NP-40

1 M sucrose (1 M = 17.12 g in 50 mL)	12.5 mL
H_2O	35 mL
1 M KCl (1 M = 3.72 g in 50 mL)	1.25 mL
10% NP-40	0.5 mL
1 M Tris pH 8.0	0.5 mL
1 M $MgCl_2$*6H_20 (1M = 10.16 g)	0.25 mL
1 M DTT	50 µL
Total	**50 mL**

PBS Wash buffer (make fresh, enough for at **least** 2 samples)

0.1% BSA, 5mM $MgCl_2$, 1x PBS

PBS	49.745 mL
1 M $MgCl_2$	250 µL
7.5% BSA	6.67 µL
Total	**50 mL**

NSB (make fresh)

0.01% BSA, 5 mM MgCl2, 0.4 U/uL RNase inhibitor

PBS wash buffer	990 µL
Superase RNase Inhibitor	5 µL
Enzymatics RNase Inhibitor	5 µL
Total	**1000 µL**

HEPES-Tween Resuspension Buffer

(HT-RSB; stored at 4°C, good for 6-12 months)

H_2O	48.75 mL
1 M HEPES pH 7.3 (10 mM HEPES)	500 µL
10% Tween-20 (0.1% Tween-20)	500 µL
5 M NaCl (10 mM NaCl)	100 µL
1 M $MgCl_2$ (3mM $MgCl_2$)	150 µL
Total	**50 mL**

6x Homogenization Buffer

(6X HB; stored at 4°C, good for 6-12 months)

H_2O	44.6 mL
1 M Tris pH 7.5 (60 mM Tris pH 7.5)	3 mL
1 M $CaCl_2$ (30 mM $CaCl_2$)	1.5 mL
1 M $MgAc_2$ (18 mM $Mg(Ac)_2$)	900 µL
Total	**50 mL**

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HDT-2RI
(Make Fresh)

HT-RSB	7.5 mL
7.5% BSA	40.13 µL
5% digitonin	15 µL
Enzymatics RNase Inhibitor	18.75 µL
SUPERase RNase Inhibitor	9.38 µL
Total	**7583.3 µL**
+/- Yeast tRNA	75 µL

Working 6X Homogenization Buffer (HB)
(Make Fresh)

6X HB	1042.8 µL
DTT (1M)	6.3 µL
PMSF (200mM)	0.875 µL
Total	**1050 µL**
+/- Yeast tRNA	63 µL

Iodixanol Gradient:

The quantities below are calculated for 2.5 reactions - adjust the volumes to accommodate the number of gradients you desire to make. It is suggested to make 1 gradient per 1 million nuclei of a debris-dense sample but samples with less debris could be loaded with more (such as mouse brain and pancreas). The iodixanol gradient purification should be done the same day as the nuclear isolation part of the protocol if the nuclei are intended to be used in SHARE-seq.

50% iodixanol
(Make Fresh)

OptiPrep (60% iodixanol in H_2O)	916.65 µL
Working 6X HB	183.5 µL
Enzymatics RNase Inhibitor	2.75 µL
SUPERase RNase Inhibitor	2.75 µL
Total	**1105.7 µL**

30% iodixanol
(Make Fresh)

OptiPrep (60% iodixanol in H_2O)	**829** µL
H_2O	282 µL
Working 6X HB	275 µL
1 M sucrose	264 µL
Enzymatics RNase Inhibitor	4.125 µL
SUPERase RNase Inhibitor	4.125 µL

40% iodixanol
(Make Fresh)

OptiPrep (60% iodixanol in H_2O)	1100 µL
H_2O	11 µL
Working 6X HB	275 µL
1 M sucrose	264 µL
Enzymatics RNase Inhibitor	4.125 µL
SUPERase RNase Inhibitor	4.125 µL

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OptiPrep (60% iodixanol in H_2O)	**829** µL
H_2O	282 µL
Total	**1658.25 µL**

OptiPrep (60% iodixanol in H_2O)	1100 µL
H_2O	11 µL
Total	**1658.25 µL**

Nuclear Isolation:

1. Before nuclear isolation either section or pulverize the tissue sample:
    a. To section the sample, use a cryostat to section the tissue into 30-40 sections that are 100 µm thick. To do this let the cryostat cool down, set section thickness to 100 µm, and attach the back of your tissue sample to a target using Optimal Cutting Temperature (OCT). Once the OCT hardens, place your target on the target holder platform and insert your blade. Proceed by positioning the cryostat and begin sectioning. Place sections into either a 1.5 mL or 2 mL tube.
    b. To pulverize the tissue use a BioPulverizer. Clean your BioPulverizer with a disinfectant followed by RNaseZap to ensure best quality and place the BioPulverizer into an ice bucket. Fill the space around the BioPulverizer with about 1 inch of liquid nitrogen and once cold place your tissue sample inside the bottom component of your BioPulverizer. Re-combine the other components of the Biopuliverizer and smash the top of the BioPulverizer using the hammer provided about 5-10 times. Aliquot the resulting powder into either 1.5 mL or 2 mL tubes.
2. Weigh your samples using a tare tube of the same brand and with the appropriate blank labels attached. Record the weights for each BioSAli in LIMS in mg. Proceed to freeze the sample at -80 degrees C (no flash freezing) until ready to use. Note: It may be helpful to place any labels onto the corresponding tube prior to freezing.
3. Begin by cooling a 7 mL dounce on wet ice and resuspend your sectioned or pulverized tissue sample with 2 mL of NIM2 buffer. Using a wide-bore tip and a 1 mL pipette, transfer the entire sample into the dounce on ice. Triturate the sample with the same wide-bore tip 10x or until the sample is easy to pipette.
4. Dounce the sample using the loose pestle (or A) for 20-30 full motions. Note: Cleaning the pestles with 70% ethanol and RNaseZap and placing the pestles momentarily in the freezer before douncing helps prevent potential early degradation of your sample.
5. Use an additional 2 mL of NIM2 to wash down any sample stuck to your pestle into the dounce (total volume = 4 mL). Set your loose pestle aside and let your sample sit on ice for 3-5 minutes.
6. Dounce the sample using the tight pestle (or B) for 20-30 full motions.
7. Repeat step 5 with the tight pestle for a new resulting total of 6 mL of sample.
8. Transfer the entire sample from the dounce into a labeled 50 mL conical tube and spin the sample 50 x g (should be fine at any speed between 40-100 x g) at 4 degrees C for 2 minutes.
9. While waiting to pellet the larger material in your sample, with a new 50 mL conical tube on ice attach a 10 µm filter followed by a 40 µm filter on top. Once your sample is done spinning, transfer the complete supernatant while avoiding the pellet through the top 40 µm filter. Note: Some tissue types are harder to get through the filters than others. In this case pipetting the sample within the base of the filter can help the sample pass through. Additionally, carefully disconnecting the two filters from each other can release tension and also help the sample pass through.

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10. Once the entirety of the supernatant passes through, remove the 40 µm filter and discard it. Wash the 10 µm filter with 6 mL of PBS Wash Buffer. Pipetting periodically like in step 9 can help clear clogs if your sample is not passing through.
11. Spin the conical tube with your sample in it at 550 x g for 5 minutes at 4 degrees C to pellet your nuclei.
12. Carefully remove the supernatant and add 6 mL of PBS wash buffer to the conical tube without disturbing the nuclei pellet. Note: Set aside your supernatant in a separate conical tube as a back-up (in the case you lose your nuclei you can restart the wash/pelleting steps of this protocol.
13. Spin again at 550 x g for 5 minutes at 4 degrees C.
14. Very carefully and without disturbing the pellet, remove the supernatant to isolate the pellet of nuclei.
15. Resuspend the pellet in 435 µL of freshly made NSB. Transfer the suspended nuclei into a new 2 mL tube that has been BSA coated. Note: To BSA coat a tube add 200 µl of 7.5% BSA to a tube, vortex it, and remove the remainder of the BSA with a pipette.
16. Count your nuclei using a hemocytometer and a microscope at 10x:
    a. If using trypan mix 10 µL of sample with 10 µL of trypan blue 0.4% solution. To calculate an estimate for the amount of nuclei in your sample, count an entire larger square grid (like 5x5 or 4x4) or multiply a subsection accordingly. Multiply your count by 20,000 and then multiply by the amount of your sample in mL (i.e. if you have 365 µL of sample, multiply by 0.365).
    b. If using propidium iodide (PI) mix 25 µL of sample with 1 µL of PI. To calculate an estimate of the amount of nuclei in your sample use the same calculation as above but multiply by 10,000 instead of 20,000 to account for the amount of sample used for this count. Note: Use PI to count samples that you expect to be particularly debris-dense.

Nuclear Purification (Iodixanol Gradient):

1. Each iodixanol gradient should be done in a 2 mL BSA-coated tube. Split the sample into the desired amount of gradients and bring up the total volume of the sample to 400 µL with any leftover NSB. Note: It is recommended that debris-dense/most samples be processed at 1 million nuclei per gradient, however cleaner samples can be potentially loaded with more (such as mouse brain or human pancreas).
2. Mix 400 µL (1 volume) of 50% iodixanol to each gradient to obtain a final concentration of 25% iodixanol. Mix well.
3. Aspirate 600 µL of 30% iodixanol with a 1 mL pipette and use a kim wipe to wipe off any iodixanol from the outside of the pipette tip. Bring the tip all the way to the bottom of the tube and slowly add the 30% iodixanol layer being careful not to mix with the top layer.
4. Aspirate 600 µL of 40% iodixanol with a 1 mL pipette and use a kim wipe to wipe off any iodixanol from the outside of the pipette tip. Bring the tip all the way to the bottom of the tube and slowly add the 40% iodixanol layer being careful not to mix with the top two layers. Note: With this layer slowly bring the top upwards when dispensing the layer to avoid overflowing the gradient.
5. Using a swinging-bucket centrifuge, spin your gradients at 3000 x g for 20 min at 4ºC with the soft brake feature set “on.” Note: If the centrifuge does not have a soft brake feature make sure acceleration/deceleration is set to 0 since this is a key parameter to ensure you do not disrupt your gradients.
6. While the gradient is on ice, use a 1 mL pipette to aspirate 500 µL from the top of the gradient (the purpose of this is to aspirate some of the topmost layer so the sample doesn’t overflow

when aspirating your nuclei band). With the same pipette use a new tip and aspirate 400-600 µL of the nuclei band and transfer to a new 1.5 µL BSA-coated tube on ice. Note: The nuclei band may be hard to see or cloudy. Bring the gradient to a light source and tilt slightly towards you to see where the interphase between the two layers is and aspirate what you can from that area. It is recommended to only attempt to aspirate the nuclei band all at once.

7. Count your nuclei (refer to step 16 of Nuclear Isolation).
8. Add 500-1000 µL of HDT-2RI to dilute iodixanol. Spin down at 750 x g for 5 min at 4ºC to pellet nuclei. You may notice the diluted iodixanol forms a layer after centrifugation. Remove supernatant and place the tube with your nuclei pellet on ice until you can proceed with fixation. For SHARE go to fixation immediately.

Nuclei Fixation (Required for SHARE-seq):

1. Add 1 mL of room-temperature HDT-2RI to your pelleted nuclei and resuspend it. Transfer tube to a room-temperature rack.
2. At room-temperature add 13.34 µL of methanol-free formaldehyde (16% stock solution) for a final concentration of 0.2% formaldehyde in your nuclei. Close the tube and nutate for 5 minutes at room-temperature.
3. To quench fixation, per reaction, add 56.1 µL of fresh 2.5M Glycine (0.94g per 5mL stock), 50 µL of 1M Tris pH 8.0, 13.3 µL of 7.5% BSA, mix using a pipette. Incubate on ice for 10 minutes.
4. Spin at 750 x g for 5 min at 4ºC.
5. Count Nuclei again here if you want accurate counts for LIMS since there is noticeable loss here and you can check if your nuclei are in good condition. If you don't mind exact counts you can skip this step.
6. Remove supernatant and add 500 µL of cold HDT-2RI to resuspend the nuclei pellet. If the goal is to aliquot nuclei into more tubes split your sample into different BSA coated tubes during this step. Label tubes accordingly.
7. Spin at 750 x g for 5 min at 4ºC.
8. Remove supernatant and freeze dry pellets at -80 degrees C. Record on LIMS.