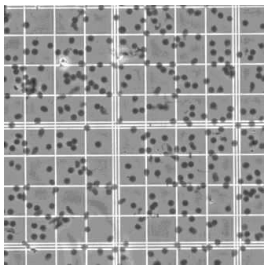
Aug 29, 2024

Nuclear Isolation and Purification Protocol for Single-Cell Methods



DOI
dx.doi.org/10.17504/protocols.io.5qpvo3kpxv4o/v1

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OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.5qpvo3kpxv4o/v1

Protocol Citation: Guillermo Barreto Corona, Amelia Hall 2024. Nuclear Isolation and Purification Protocol for Single-Cell Methods. **protocols.io** https://dx.doi.org/10.17504/protocols.io.5qpvo3kpxv4o/v1

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

Created: August 31, 2023

Last Modified: August 29, 2024

Protocol Integer ID: 87216

Disclaimer

As always, when starting to plan a protocol which implicates usage of animal or human tissues, please ensure that you are working within an IACUC (animal work) or IRB (human work) approved protocol. We highly recommend engaging with your institutional IACUC or IRB as part of the planning stages for experimentation. Post-mortem tissue is a gift, it is our collective responsibility as scientists to ensure we use these tissues safely and ethically.

Abstract

This protocol describes how to isolate and purify nuclei from solid tissue for use in single-cell library construction methods. The resulting nuclei are suitable for a variety of single-cell assays such as droplet-based methods like 10x Genomics Chromium and hybridization-based methods like SHARE-seq. Through sequencing we have verified that the resulting nuclei created through this protocol show a low percentage of mitochondrial RNA.

Guidelines

Use this protocol in accordance to your institution's human subject data policies if using any type of human tissue. As always, when starting to plan a protocol which implicates usage of animal or human tissues, please ensure that you are working within an IACUC (animal work) or IRB (human work) approved protocol. We highly recommend engaging with your institutional IACUC or IRB as part of the planning stages for experimentation. Post-mortem tissue is a gift, it is our collective responsibility as scientists to ensure we use these tissues safely and ethically.

Materials

Equipment needed:

- Eppendorf 5430R Centrifuge with S-24-11-AT Swinging Bucket Rotor (or equivalent swinging bucket centrifuge with soft ramp)
- Beckman Coulter Allegra X-30R Benchtop Centrifuge with SX4400 Swinging Bucket Rotor (or equivalent)
- Confocal Microscope system for visualization and cell counting
- Cryostat for sectioning

Plasticware:

- Eppendorf DNA LoBind 1.5 mL tubes
- Eppendorf DNA LoBind 2 mL tubes
- Eppendorf Conical Tubes 50 mL
- Pluriselect 40 µm pluriStrainers
- Pluriselect 10 µm pluriStrainers

RNase Inhibitors:

- Enzymatics RNAse Inhibitor (40 U/µL)
- SUPERase-In RNase Inhibitor (20 U/µL)

Optiprep:

- Optiprep Density Gradient Medium (D1556 - 60% Iodixanol)

Glassware:

- Wheaton Dounce Tissue Grinder, 7mL

Safety warnings

This protocol may be used on primate tissue (human and non-human) however it should be noted that even with proper screening of samples, this protocol produces aerosols that could be potentially damaging to your health. It is recommended to use effective personal protective equipment (PPE) and the nuclear isolation part of this protocol be done in a biosafety cabinet (BSC) to protect yourself, the sample, and the public.

Ethics statement

As always, when starting to plan a protocol which implicates usage of animal or human tissues, please ensure that you are working within an IACUC (animal work) or IRB (human work) approved protocol. We highly recommend engaging with your institutional IACUC or IRB as part of the planning stages for experimentation. Mouse brain tissue used to prototype this protocol was purchased from TransNetyx (**https://tissue.transnetyx.com/**).

1. Buffer Preparation

30m

1 The buffers that are noted with a '*' such as **HDT-2RI**, **HB**, and **NSB** are calculated for 2.5 reactions and should be adjusted for more reactions accordingly. The buffers that are able to be stored can be considered as stock buffers and are good for several iterations of the protocol. Make more PBS wash buffer as you see fit for the number of washes you would like to do after filtering (the recommendation is at least two).

1.1 **Buffers for Nuclear Isolation:**

Make **NIM2**:

	A	B
	NIM2	Volume
	H20	35 mL
	1M Sucrose	12.5 mL
	1M KCl	1.25 mL
	10% NP-40	0.5 mL
	1M Tris (pH 8.0)	0.5 mL
	1M MgCl2*6H20	0.25 mL
	1M DTT	50 µL
	Total	50 mL

Store at 4°C, good for 6-12 months

Make **PBS Wash Buffer**:

	A	B
	PBS Wash Buffer	Volume
	PBS	49.745 mL
	1m MgCl2	0.25 mL
	10% BSA (made in PBS)	5 µL
	Total	50 mL

Make Fresh

Make **Nuclear Suspension Buffer (NSB)***:

	A	B
	NSB	Volume
	PBS Wash Buffer	990 µL
	Superase RNase Inhibitor	5 µL
	Enzymatics RNAse Inhibitor	5 µL
	Total	1000 µL

Make Fresh

Make **HEPES-Tween Resuspension Buffer (HT-RSB)**:

	A	B
	HT-RSB	Volume
	H2O	48.75 mL
	1 M HEPES pH 7.3	500 µL
	10% Tween-20	500 µL
	5 M NaCl	150 µL
	1 M MgCl2	100 µL
	Total	50 mL

Store at 4°C, good for 6-12 months

Make **6X Homogenization Buffer (6X HB)**:

	A	B
	6X HB	Volume
	H2O	44.6 mL
	1 M Tris pH 7.5	3 mL
	1 M CaCl2	1.5 mL
	1 M MgAc2	900 µL
	Total	50 mL

Store at 4°C, good for 6-12 months

Make **HDT-2RI***:

	A	B
	HDT-2RI	Volume

	A	B
	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

Make Fresh

Make **Working 6X Homogenization Buffer (HB)***:

	A	B
	HB	Volume
	6X HB	1042.8 µL
	DTT	6.3 µL
	PMSF	0.875 µL
	Total	1050 µL
	+/- Yeast tRNA	63 µL

Make Fresh

1.2 The quantities below are calculated for 2.5 reactions - adjust the volumes to accommodate the number of gradients that you desire to make. It is suggested to plan to use 1 gradient per 1 million nuclei of a debris-dense sample but samples with less debris could be loaded with up to 10 million nuclei (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 or similar methods.

Buffers for Nuclear Purification:

Make **50% Iodixanol**:

	A	B
	50% Iodixanol	Volume
	OptiPrep (60% iodixanol in H2O)	916.65 µL
	Working 6X HB	183.5 µL

	A	B
	Enzymatics RNAse Inhibitor	2.75 µL
	Enzymatics RNase Inhibitor	2.75 µL
	Total	1105.7 µL

Make Fresh

Make **30% Iodixanol**:

	A	B
	30% Iodixanol	Volume
	OptiPrep (60% iodixanol in H2O)	829 µL
	H2O	282 µL
	Working 6X HB	275 µL
	1 M sucrose	264 µL
	Enzymatics RNAse Inhibitor	4.125 µL
	SUPERase RNase Inhibitor	4.125 µL
	Total	1658.25 µL

Make Fresh

Make **40% Iodixanol**:

	A	B
	40% Iodixanol	Volume
	OptiPrep (60% iodixanol in H2O)	1100 µL
	H2O	11 µL
	Working 6X HB	275 µL
	1 M sucrose	264 µL
	Enzymatics RNase Inhibitor	4.125 µL
	SUPERase RNase Inhibitor	4.125 µL
	Total	1658.25 µL

Make Fresh

2. Nuclear Isolation

1h 30m

2 The purpose of this part of this protocol is to isolate the nuclei from the tissue and other cellular debris. It is possible to proceed to fixation and skip the nuclear purification portion of this protocol if debris is not a concern for your project.

2.1 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 3-4cm of liquid nitrogen and once cold place your tissue sample inside the bottom component of your BioPulverizer. Re-combine the other components of the BioPulverizer and smash the top of the BioPulverizer using the accompanying about 5-10 times. Aliquot the resulting powdered tissue into either 1.5 mL or 2 mL tubes.

2.2 Weigh your samples and record the weights for your own records in mg. Proceed to freeze the sample at -80 degrees C (no flash freezing) until ready to use. Use dry ice to transport your tissue. *Note: It may be helpful to place any labels onto the corresponding tube prior to freezing.*

2.3 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.

2.4 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.*

2.5 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.

2.6 Dounce the sample using the tight pestle (or B) for 20-30 full motions.

2.7 Repeat step 5 with the tight pestle for a new resulting total of 6 mL of sample.

2.8 Transfer the entire sample from the dounce into a 50 mL conical tube and spin the sample 50xg (should be fine at any speed between 40-100xg) at 4ºC for 2 minutes.

2.9 While waiting to pellet the larger material in your sample, on a new 50 mL conical tube on set up 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 tissues are harder to get through the filters than others. In this case pipetting the sample at the base of the filter can help the sample pass through. Additionally, carefully disconnecting and then reconnecting the two filters from each other can release tension and also help the sample pass through.*

2.10 Once the entirety of the supernatant passes through the 40 µm filter, 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 2.9 can help clear clogs if your sample is not passing through.

2.11 Spin the conical tube with your sample in it at 550xg for 5 minutes at 4ºC to pellet your nuclei.

2.12 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.*

2.13 Spin again at 550xg for 5 minutes at 4ºC.

2.14 Without disturbing the pellet, remove the supernatant to isolate a crude nuclei pellet.

2.15 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.*

2.16 Count your nuclei using a hemocytometer and a microscope at 4-10x:

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 (e.g. 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 400 µL of sample, multiply by 0.4).

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: It is highly
recommended to count with both Trypan and PI but it is highly recommended to use PI to count samples that you expect to be particularly debris-dense.*

3. Nuclear Purification

1h

3 The previous section for nuclear isolation should have left you with 400 µL of nuclei. You can proceed with the entirety of your sample or split you sample to multiple tubes. Using the counts you recorded you should aim to use about 1 million nuclei per gradient however it is possible to over for tissue types with less debris without overloading the gradient.

3.1 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).*

3.2 Mix 400 µL (1 volume) of **50% iodixanol** to each gradient to obtain a final concentration of 25% iodixanol. Mix well.

3.3 Aspirate 600 µL of **30% iodixanol** with a 1 mL pipette and use a kimwipe 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 layer above.

3.4 Aspirate 600 µL of **40% iodixanol** with a 1 mL pipette and use a kimwipe 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.*

3.5 Using a swinging-bucket centrifuge, spin your gradients at 3000xg 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.

(!) It is critical to have the centrifuge on soft ramp/soft brake or acceleration at 0 in order to get a clear band of nuclei at the interphase of the 30% and 40% layers.

3.6 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 for best yield.

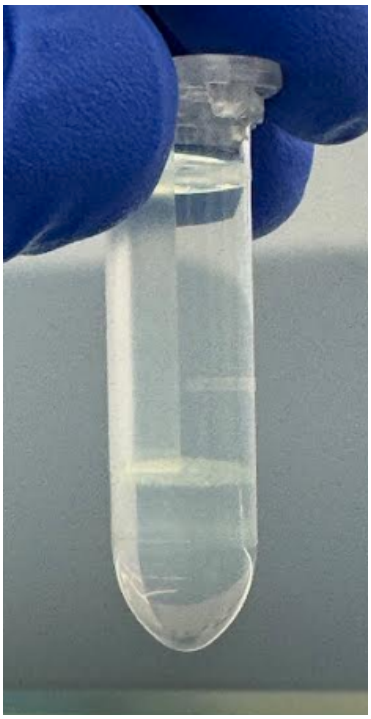

Photo of nuclei band

3.7 Count your nuclei (refer to step 2.16 of Nuclear Isolation).

3.8 Add ~1mL of **HDT-2RI** to dilute iodixanol and spin down at 750xg for 5 min at 4ºC to pellet nuclei. Remove supernatant and place the tube with your nuclei pellet on ice until you can proceed with fixation. For SHARE-seq go to fixation immediately.

4. Fixation

30m

4 Fixation of your nuclei is required for SHARE-seq if not done on the same day. If your project doesn't require fixation you can ignore this step and use your crude nuclei pellet.

4.1 Add 1 mL of room-temperature **HDT-2RI** to your pelleted nuclei and resuspend it. Transfer tube to a room-temperature rack.

4.2 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.

4.3 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, and 13.3 µL of 7.5% BSA. Mix using a pipette. Incubate on ice for 10 minutes.

4.4 Spin at 750xg for 5 min at 4ºC.

4.5 (Optional) Count your nuclei (step 2.16) again here if you want an accurate count of how many you have and to check if your nuclei are in good condition. It is also acceptable to use the counts (from step 3.7) as rough estimates for your downstream experiments.

4.6 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 for multiple experiments, split your sample into different BSA coated tubes during this step. Label tubes as desired.

4.7 Spin at 750xg for 5 min at 4ºC.

4.8 Remove supernatant and freeze dry pellets at -80 degrees C. *Note: We have found success at just placing the dry nuclei pellet into a freezer without flash freezing.*