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Nuclei Isolation from Tissue for 10x Multiome by Iodixanol

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We use this protocol and it's working

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Abstract

We are using the iodixanol gradient method to extract nuclei for 10X single cell and Multiome assays. We have tested this protocols with a range of primary brain samples, developing, postnatal and adult. We obtain clean nuclei, with least amount of ambient RNA. Using this protocol, we are able to remove most of the myelin from adolescent and adult samples, which generally interfere the nuclei counts before loading the 10X Controller. We have generated high quality GEX and ATAC-sequencing libraries with our samples from a range of ages, across different projects.

Troubleshooting

Before you start the protocol:

1 1) All steps should be performed on ice or at 4°C. Pre-chill a swinging bucket centrifuge and a fixed angle centrifuge to 4°C.
2) Pre-chill all Dounces and pestles to 4°C on ice.
3) Pre-chill all tubes.
4) Prepare all buffers. For faster dissolution, crush protease inhibitor tablets prior to addition to 1x Homogenization Buffer Unstable Solution. DTT, Spermidine, Spermine, and digitonin are stored at -20°C. All other detergents and buffers are stored at 4°C.
a. Remember that the catalog number provided for iodixanol from Sigma comes as a 60% solution (not 100%).
5) Fill up a 2 L beaker with 500 ml sterile water to soak the used Dounces and pestles.

Isolation of Nuclei via Dounce Homogenization and density centrifugation for 10x single-cell multiome:

2 1) Remove samples from liquid nitrogen storage and keep on dry ice until use.
2) If you are working with a tissue type that is particularly hard to dissociate via Douncing, it can be helpful to pre-crush (do not pulverize) your tissue fragment using mortar and pestle. The tissue must remain frozen and cold during this entire process. Otherwise proceed to Step 3.
3) Place 20-50 mg frozen tissue or crushed tissue into a pre-chilled 7 ml Dounce containing 1 ml cold 1x HB.
4) Dounce with “A” loose pestle until resistance goes away (~10 strokes).
5) Place “A” pestle into beaker with sterile water to soak for cleaning later.
a. Optional – If residual un-homogenized tissue makes it difficult to Dounce, filter homogenate through a pre-chilled 50 ml conical using a 70 um bucket-style cell strainer filter prior to using tight pestle “B”.
6) Dounce with “B” tight pestle until resistance goes away (~15 strokes).
7) Place “B” pestle into beaker with sterile water to soak for cleaning later.
8) Filter during transfer using a 40-um cell strainer (PluriSelect Cat: 43-10040-60) to a pre-chilled 2 ml LoBind tube.
9) Place Dounce into beaker with sterile water to soak for cleaning later.
10) Pellet nuclei by spinning 5 min at 4°C at 350 xg in a fixed angle centrifuge.
11) Remove all supernatant, if the pellet is not clearly visible, you can leave 50 ul supernatant in the tube.
12) Gently resuspend nuclei in 350 ul 1x HB, but make sure the total volume of nuclei suspension is 400 ul. Make sure nuclei are fully resuspended without clumps.
13) Add 1 volume (400 ul) of 50% Iodixanol Solution and mix well by pipetting

14) Slowly layer 600 ul of 30% Iodixanol solution under the 25% mixture. To avoid mixing of layers, wipe the side of the pipette tip with a Kimwipe to remove excess Iodixanol solution from the external surfaces of the pipette tip.
15) Layer 600 ul of 40% Iodixanol solution under the 30% mixture. To avoid mixing of layers, wipe the side of the pipette tip with a Kimwipe to remove excess Iodixanol solution from the external surfaces of the pipette tip.
a. During this step, you will need to gradually draw your pipette tip up to avoid overflowing the tube. However, the tip of your pipette must stay below the 30%-40% interface at all times.
16) In a pre-chilled swinging bucket centrifuge, spin for 20 min at 4°C at 3,000 xg with the brake off. Handle tubes gently so as to not disturb the gradient. Set acceleration level at 1, deceleration level at 0. Set centrifuge time to 25 min and after it's done, it takes another 13 min to stop.
a. Iodixanol is meant to be used at higher speeds (10,000 xg) but high-speed swinging bucket centrifuges are not always readily available, so we perform this step at 3,000 xg and have not had any issues.
17) Using a vacuum, aspirate the top layers down to within 200-300 ul of the nuclei band at the 30%-40% interface. Be careful not to get too close as you will disrupt the nuclei band.
18) Using a 200 ul volume, collect the nuclei band and transfer to a fresh tube. Do not aspirate more than 200 ul at this step as this can cause you to take too much of the 40% layer which sometimes contains debris.
19) Dilute nuclei by adding 200 ul of wash buffer. Mix gently by pipetting. Filter nuclei suspension to 1.5 ml LoBind tube with 40 um Mini-Strainer (PluriSelect Cat: 43-10040-60).

1. Take a sample and count nuclei using Trypan Blue.
2. Centrifuge at 500 xg for 5 min at 4°C.
3. Remove the supernatant without disrupting the nuclei pellet.
4. Resuspend in x uL chilled Diluted Nuclei Buffer. (The volume can be changed based on counting results from step 13, target for 8,000 nuclei/uL)
5. Take a sample and count nuclei using Trypan Blue.
6. Dilute to 3220 nuclei/uL and proceed immediately to 10x single cell Multiome user guide. Use 5 uL nuclei.

Stock Buffers

3 All stock solutions should be filtered using a 0.22 um PVDF/PES filter system. All solutions except for the 50% Iodixanol solution are stable at 4°C for at least 6 months.

1 M Sucrose (300 mL)

Substance	Stock Conc.	Amount	Final conc. in working solution
Sucrose	-	102.69 g	1 M
Water	-	235.5 mL	-

1.0616x Homogenization Buffer Stable Solution (200 mL)

Substance	Stock Conc.	Amount	Final conc. in working solution
Sucrose	1 M	53.1 mL	0.2653 M
KCl	2 M	2.66 mL	26.6 mM
$MgCl_2$	1 M	1.06 mL	5.31 mM
Tricine-KOH pH 7.8	0.75 M	5.67 mL	21.2 mM
Water	-	137.5 mL	-

Diluent Buffer (100 mL)

Substance	Stock Conc.	Amount	Final conc. in working solution
KCl	2 M	7.5 mL	150 mM
$MgCl_2$	1 M	3 mL	30 mM
Tricine-KOH pH 7.8	0.75 M	16 mL	120 mM
Water	-	73.5 mL	-

50% Iodixanol Solution (50 mL) **Remake monthly for stability

Substance	Stock Conc.	Amount	Final conc. in working solution
Diluent Buffer	-	8.3 mL	-
Iodixanol	60%	41.7 mL	50%

1x Homogenization Buffer Unstable Solution (8 mL) for 4 reactions prepare fresh

Substance	Stock Conc.	Amount	Final conc. in working solution
HB Stable Solution	1.0616X	7536 uL	1X
DTT	1 M	8 uL	1 mM
Spermidine	500 mM	8 uL	0.5 mM
Spermine	150 mM	8 uL	0.15 mM
NP40	10%	240 uL	0.3%
cOmplete PI (Diluted in HB stable)	100X	80 uL	1X
RiboLock	40U/uL	120 uL	0.6U/uL

30% Iodixanol Solution per reaction prepare fresh

Substance	Stock Conc.	Amount	Final conc. in working solution
HB unstable	-	240 uL	-
50% Iodixanol Solution	50%	360 uL	30%

40% Iodixanol Solution per reaction prepare fresh

Substance	Stock Conc.	Amount	Final conc. in working solution
HB unstable	-	120 uL	-
50% Iodixanol Solution	50%	480 uL	40%

Wash Buffer (1mL) for 4 reactions prepare fresh

Substance	Stock Conc.	Amount	Final conc. in working solution
Tris-HCl pH 7.4	1M	10 uL	10mM
NaCl	5M	2 uL	10mM
$MgCl_2$	1M	3 uL	3mM
BSA	30%	33.3 uL	1%
Tween-20	10%	10 uL	0.1%
DTT	1M	1 uL	1mM
RiboLock	40U/uL	15 uL	0.6U/uL
Ultrapure water	-	925.7 uL	-

Diluted Nuclei Buffer (2mL) for 4 reactions prepare fresh

Substance	Stock Conc.	Amount	Final conc. in working solution
Nuclei Buffer (from 10x Multiome kit)	20X	50uL	1X
DTT	1M	1 uL	1mM
RiboLock	40U/uL	15 uL	0.6U/uL
Ultrapure water	-	934 uL	-