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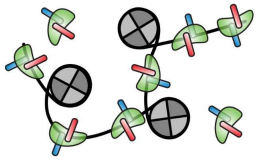
Isolation of nuclei from frozen tissue for snMultiome, snATAC-seq, snRNA-seq, and other epigenomic/transcriptomic assays V.2



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We use this protocol and it's working

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Abstract

This protocol enables the isolation of nuclei from frozen tissues for use in downstream single-nucleus Multiome (paired ATAC-seq and RNA-seq) experiments. These nuclei are suitable for use in bulk ATAC-seq, single-nucleus ATAC-seq, single-nucleus RNA-seq, ChIP-seq, HiC/MicroC, among other assays.

For single-nucleus Multiome and RNA-seq experiments we recommend addition of RNase inhibitor to the solutions and addition of Triton X-100 and Kollidon VA-64 to the homogenization buffer. These additions were strongly based on nuclei extraction protocols from the McCarroll and Macosko laboratories (**dx.doi.org/10.17504/protocols.io.bi62khge**). Additions of RNase inhibitor and Kollidon VA-64 are only required for single-nucleus RNA-seq and single-nucleus Multiome workflows.

Guidelines

The quality of the tissue at the time of freezing is a major factor in the quality of data downstream. If freezing tissue for use later, you may want to consider cryopreserving 50 mg tissue chunks in BAM Banker cryopreservative. If using previously frozen tissue, the ischemic time or post mortem interval should be kept as short as possible.

Nuclei can be cryopreserved in BAM Banker. In our experience, each freeze-thaw cycle results in minor decreases in single nucleus Multiome data quality. Ensure that thawing is carried out on ice and freezing is performed using a slow-freeze freezing container. We have had success isolating nuclei from every tissue we have tried including difficult to work with tissues such as heart.

Materials

STOCK BUFFERS

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

1.115x Homogenization Buffer Stable Solution - 200 ml

	A	B	C	D	E
	Stock	*Name*	*Final Conc. (M)*	*Fold Dilution (x)*	*Total Vol. (ul)*
	1	M Sucrose	0.27875	3.59	55750
	2	M KCl	0.02788	71.75	2790
	1	M MgCl2	0.005575	179.4	1120
	0.75	M Tricine-KOH pH 7.8	0.0223	33.63	5950
	-	Water	-	-	134400

Diluent Buffer - 100 ml

	A	B	C	D	E
	Stock	*Name*	*Final Conc. (M)*	*Fold Dilution (x)*	*Total Vol. (ul)*
	2	M KCl	0.15	13.33	7500.00
	1	M MgCl2	0.03	33.33	3000.00
	0.75	M Tricine-KOH, pH 7.8	0.12	6.25	16000.00
	-	Water	-	-	73500.00

50% Iodixanol Solution - 50 ml (Remake monthly for stability)

	A	B	C	D	E
	Stock	*Name*	*Final Conc.*	*Fold Dilution (x)*	*Total Vol. (ul)*
	-	Diluent Buffer	-	-	8333.33
	60	% Iodixanol	50 %	1.20	41666.67

Multiome Wash Buffer - 200 ml

	A	B	C	D	E
	Stock	*Name*	*Final Conc. (M)*	*Fold Dilution (x)*	*Total Vol. (ul)*
	1	M Tris-HCl pH 7.5	0.01	100.00	2000.00
	5	M NaCl	0.01	500.00	400.00

	A	B	C	D	E
	1	M MgCl2	0.003	333.33	600.00
	30	% BSA	1 %	30.00	6666.67
	-	Water	-	-	193000.00

SAME DAY BUFFERS

1x Homogenization Buffer Unstable Solution (HBUS) - 25 mL

**Note – cOmplete Protease Inhibitors come as tablets. It is difficult to use less than 1/2 tablet so we prepare the 1x Homogenization Buffer Unstable Solution in batches of 12 as outlined below.

	A	B	C	D	E
	Stock	*Name*	*Final Conc. (M)*	*Fold Dilution (x)*	*Vol per 12 samp. (ul)*
	1.115	x HB Stable Solution	1	1.115	22425.00
	1	M DTT	0.001	1000.00	25.00
	500	mM Spermidine	0.5	1000.00	25.00
	150	mM Spermine	0.15	1000.00	25.00
	10	% Triton X-100	1 %	10.00	2500.00
	-	mg/mL Kollidon VA-64	10	-	250 mg
	-	cOmplete Protease Inhibitor	-	-	0.50 Tablets

1x Homogenization Buffer Unstable Aliquot (with RNase Inhibitor)

	A	B	C
	Name	*Vol per sample (ul)*	*Volume to add (ul)*
	1X HBUS	1000	13000
	RNasin Plus RNase Inhibitor	5	65

30% Iodixanol Solution

	A	B	C	D	E	F
	Stock	*Name*	*Final Conc.*	*Fold Dilution (x)*	*Vol per sample (ul)*	*Total (ul)*
	-	1x Homog. Buffer Unstable	-	-	240.00	3200.00

	A	B	C	D	E	F
	50	% Iodixanol Solution	30 %	1.67	360.00	4800.00
	40	U/ul RNasin Plus RNase Inhibitor	0.2 U/ul	200	3	40

40% Iodixanol Solution

	A	B	C	D	E	F
	Stock	*Name*	*Final Conc.*	*Fold Dilution (x)*	*Vol per sample (ul)*	*Total (ul)*
	-	1x Homog. Buffer Unstable	-	-	120.00	1600.00
	50	% Iodixanol Solution	40 %	1.25	480.00	6400.00
	40	U/ul RNAsin Plus RNase Inhibitor	0.2 U/ul	200	3	40

Multiome Wash Buffer with Tween

	A	B	C	D	E	F
	Stock	*Name*	*Final Conc. (M)*	*Fold Dilution (x)*	*Vol per sample (ul)*	*Total (ul)*
	-	Multiome Wash Buffer	-	-	2500.00	30000.00
	10	% Tween-20	0.1 %	100.00	25.00	300.00
	1	M DTT	0.001		2.5	30.0
	40	U/ul RNasin Plus RNase Inhibitor	0.2 U/ul	200	12.5	150.0

Diluted Nuclei Buffer (1X)

	A	B	C
	Stock	*Name*	*Total Volume (ul)*
	20X	Nuclei Buffer (PN-2000207)	50
	1	M DTT	1
	40	U/ul RNasin Plus RNase Inhibitor	5
	30	% BSA	33.33
	-	Water	910.67

Reagents used in this protocol

	A	B	C
	Item	**Supplier**	**Cat Number**
	Eppendorf 2 ml Lo-Bind tubes	Sigma	Z666556-250EA
	Eppendorf 1.5 ml Lo-Bind tubes	Sigma	Z666548-250EA
	Nunc cryovials	Thermo	375418PK
	Iodixanol (comes at 60%)	Sigma	D1556-250ML
	Sucrose	Sigma	S7903-250G
	Triton X-100 (comes at 10%)	bioWorld	42030008
	Tricine	Sigma	T0377-25G
	Potassium Hydroxide (KOH)	Sigma	P5958-250G
	cOmplete Protease Inhibitors	Roche	11697498001
	MgCl2	Ambion (Therm o)	AM9530G
	KCl	Ambion (Therm o)	AM9640G
	DTT	Thermo	R0861
	Spermidine	Sigma	S2501
	Spermine	Sigma	S3256-1G
	70 um Flowmi cell strainers	Fisher	03-421-228
	70 um bucket-style cell strainers	BD Falcon	352350
	Tris-HCl pH 7.5	Invitrogen	15567-027
	NaCl	Ambion (Therm o)	AM9759
	Tween 20	Roche (Sigma)	11332465001
	H2O	Invitrogen	10977-015
	Dounce Tissue Grinder Set	Sigma	D8938-1SET
	INCYTO Disposable hemocytometers	Fisher	22-600-100
	BAM Banker	Wako Chemicals	302-14681
	RNasin Plus RNase Inhibitor	Promega	N2611/N2615
	Kollidon VA-64	BASF	Not commerically sold
	30% BSA Solution	Millipore-Sigma	A9576
	Optional: Countess 3 FL Automated Cell Counter	Thermo Fisher Scientific	AMQAF2000
	Optional: Countess Cell Coun	Thermo Fisher Sc	C10228

	A	B	C
	ting Chamber Slides	ientific	
	40 um Flowmi cell strainers	Fisher	14-100-150
	Trypan Blue solution, 0.4%	Thermo Fisher Scientific	15250061
	Optional: Ethidium homodimer I	Thermo Fisher Scientific	E1169

Before you start the protocol:

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 in a fridge.

3 Pre-chill all tubes. For each sample you are processing, you will need: (i) One 2 ml round-bottom LoBind tube for gradient separation (ii) One 1.5 ml LoBind tube for washing nuclei (iii) One 2 ml Nunc Cryotube for banking extra nuclei (iv) One 50 ml conical for filtration step (often optional)

4 Prepare all buffers. For faster dissolution, crush protease inhibitor tablets prior to addition to 1x Homogenization Buffer Unstable Solution. DTT, Spermidine, and Spermine are stored at -20 °C. All other detergents, Multiome wash buffer, and other buffers are stored at 4 °C. Keep all buffers on ice. Do not prepare transposition mix ahead of time. 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 Gradient Centrifugation

6 Remove samples from liquid nitrogen storage and keep on dry ice until use.

6.1 If the tissue you are using is fibrous or difficult to homogenize (for example heart, muscle, etc) then you may want to consider pre-crushing the tissue prior to Dounce homogenization. To do this, pre-chill a mortar and pestle on dry ice and use the pestle to break the tissue chunk into smaller pieces by repeatedly hitting the tissue. We don't recommend pulverizing the tissue into a powder - it should remain as small chunks. These small chunks may make it so that the Dounce gets stuck more frequently, but in our experience this pre-crushing can help liberate more nuclei from difficult tissues.

7 Place 20 mg frozen tissue into a pre-chilled 2 ml Dounce containing 1 ml cold 1x HBUS and let thaw for 00:10:00 on ice. For >30 mg tissue, use 2 ml 1x HBUS. For 10-20 mg tissue, use 1 ml 1x HBUS. For 50 um tissue sections, use 0.5 ml 1x HBUS. 10m

8 Since the Multiome RNA-based assay will be performed on these nuclei, add 5 µL RNasin Plus RNase Inhibitor per mL of 1x HBUS as instructed in Same Day Buffers and mix well.

9 Dounce with “A” loose pestle until resistance goes away (~10 strokes).

10 Place “A” pestle into beaker with sterile water to soak for cleaning later.

10.1 Optional – If residual un-homogenized tissue makes it difficult to Dounce, filter homogenate through a 70 um bucket-style cell strainer filter into a pre-chilled 50 ml conical prior to using tight pestle “B”.

11 Dounce with “B” tight pestle for 20 strokes.

11.1 The exact number of strokes necessary is tissue-specific and should be optimized for each specific application, before performing it on all experimental conditions. The first time handling a new tissue, vary the number of strokes of pestle “B” between 10-25. The release of nuclei can be verified using a hemocytometer and Trypan Blue stain. Nuclei should appear as trypan positive spheres, amid the tissue debris.

12 Gently place the “B” pestle into a beaker with sterile water to soak for cleaning later.

13 Load the homogenate into a 1 mL pipette tip, affix a 70 um Flowmi strainer to the end of the tip, and transfer homogenate to a pre-chilled 2 ml LoBind tube. Repeat as necessary until all volume has been
filtered and transferred.

14 Place Dounce into beaker with sterile water to soak for cleaning later.

15 Pellet nuclei by spinning 00:05:00 at 4 °C at 350 rcf in a fixed angle centrifuge.

16 Remove all but 50 ul of supernatant, which contains cytoplasmic components.

16.1 If the pellet is not clearly visible or the remaining 50 uL are difficult to remove, more supernatant may be left in the tube. Up to 400 uL of the supernatant may be left behind, however, proportionally less
of the 1x HBUS should be added in the next step.

17 Gently resuspend nuclei in the required volume to give a **total volume of 400 ul 1x HB**. For example, if 50 ul was in the tube in the previous step, add 350 ul 1x HB. Make sure nuclei are fully resuspended without clumps.

18 Add 1 volume (400 ul) of 50% Iodixanol Solution (with RNasin Plus RNase inhibitor) and mix well by pipetting.

19 Slowly layer 600 ul of 30% Iodixanol solution (with RNasin Plus RNase inhibitor) 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. To avoid bubbles, only push pipette to the first stop.

20 Slowly layer 600 ul of 40% Iodixanol solution (with RNasin Plus RNase inhibitor) 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. To avoid bubbles, only push pipette to the first stop.

20.1 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.

21 In a pre-chilled swinging bucket centrifuge, spin for 00:20:00 at 4 °C at 3000 rcf with the brake off. Handle tubes gently so as to not disturb the gradient.

21.1 Iodixanol is meant to be used at higher speeds 10000 rcf but high-speed swinging bucket centrifuges are not always readily available so we perform this step at 3000 rcf and have not had any issues.

21.2 It is critical to use a swinging-bucket centrifuge for this step and to have the brake turned off.

22 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.

23 Using a 200 ul volume, collect the nuclei band and transfer to a fresh tube. To do this, place the pipette tip just above the 30%-40% interface and carefully circle the tip back and forth while slowly releasing the plunger to “vacuum” up the nuclei band.

23.1 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.

24 Transfer the 200 uL of nuclei and add to 800 uL of Multiome Wash Buffer in pre-chilled 1.5 mL Lo-Bind Eppendorf tubes.

25 Filter the nuclei (1 mL) using a 1000 µL pipette tip and 40 µm Flowmi filter into the same 1.5 mL Lo-Bind Eppendorf tubes.

25.1 This is intended to remove some of the debris from the nuclei samples. If working with samples with low nuclei counts, this step can be skipped if nuclei loss from the Flowmi filtration is a concern.

26 Count the nuclei in each of the individual samples. Transfer 10 ul of the nuclei solution to a clean 1.5 mL tube and add 10 ul of Trypan Blue stain. Mix well by pipetting and load 10 uL into a disposable hemocytometer slide. An automatic cell counter compatible with Trypan Blue (such as the Countess) can also be used.

26.1 For samples potentially containing debris, using Trypan Blue may be difficult to distinguish nuclei from debris. In these instances, more accurate counts can be obtained using a fluorescent dye (ethidium homodimer-I) and a fluorescence compatible cell counter. Dilute the ethidium homodimer-I stock (1 mM) 1:100 and mix 10 µL of this with 10 µL of nuclei solution.

27 If using a hemocytometer, count the number of intact nuclei in the 4 large corner squares. Take the total number in all 4 squares and multiply by 5000 to obtain the concentration of nuclei per mL. This
is taken from the equation: (total cells counted)/(4 squares counted)*10^4*(dilution factor of 2).

27.1 If using a standard, non-fluorescence automatic cell counter nuclei should stain and be labelled as "dead".

27.2 If using a fluorescence compatible cell counter (Countess 2 FL, Countess 3 FL, Cellaca MX FL5, Luna-FL, etc) and ethidium homodimer-I to count nuclei, take the RFP positive nuclei count.

Note: we have commonly used the Countess 3 FL for nuclei in Multiome assays. If using the Countess 3 FL for 10x Genomics Fixed RNA Profiling Assays (eg. RNA Flex Kits) we have experienced difficulties counting nuclei with fluorescent dyes post-probe hybridization.

28 We recommend pooling nuclei samples prior to transposition (using human samples) to mitigate technical variability between reactions and running the pool across multiple channels of the 10x Genomics Multiome chip.

28.1 This pooling strategy requires some genotype information for the individuals that are part of the pool to facilitate linking reads in the sequencing data back to the original samples.

28.2 In certain instances such as mouse samples, pooling different samples together will not be feasible. In these cases, process each sample individually through the single-nucleus Multiome protocol. If not pooling samples, perform an individual high-volume wash for each sample.

Pooling Samples and High Volume Wash of Nuclei

29 If using human samples, we recommend pooling nuclei from different samples to avoid channel-to-channel technical variability that may confound downstream sample comparisons. Include equal proportions of nuclei in each sample to be included in the pool (if possible).

29.1 Important: Do not include more than one sample from a particular individual in a pool.

30 Transfer nuclei from each individual sample to a pre-chilled 15 mL Falcon tube. Fill the Falcon tube on ice up to 15 mL using Multiome wash buffer.

30.1 The number of samples to pool and number of nuclei per individual sample to add to the pool depends on the specific experiment. For large cohorts with human postmortem samples we typically pool nuclei from 10-12 individual samples and add 200,000 nuclei to the pool. We account for nuclei loss due to the low-speed centrifugation and removing the resultant supernatant, even if very careful.

31 Mix the pooled nuclei in the high-volume wash (15 mL total) by inverting up and down 5 times.

31.1 The aim of this high-volume wash is to dilute out ambient RNA signals that are known to contaminate true signals once Gel Beads in Emulsions (GEMs) are formed

32 Centrifuge 15 mL Falcon tube for 00:10:00 at 250 rcf at 4 °C in a pre-chilled large swinging bucket centrifuge. The nuclei pellet should be visible afterwards. 10m

32.1 The low-speed centrifugation reduces mechanical damage to the nuclei and nuclear membrane during the pelleting step. This helps prevent the nuclei from leaking valuable RNA signal. This also lowers ambient RNA levels from exogenous signals in the downstream single-nucleus RNA-seq data.

33 Prepare 1 mL of Diluted Nuclei Buffer (1X). Vortex briefly, spin down, and chill on ice.

34 If pooling samples in the 15 mL tube, carefully aspirate the supernatant down to 1 mL with a 10 mL serological pipette. Remove down to 200 uL with a p1000 pipette. Remove the last 200 uL with a p200 pipette very carefully without disturbing the nuclei pellet.

35 Resuspend pooled nuclei in 100 µL of Diluted Nuclei Buffer. This volume may differ depending on the targeted final nuclei concentration before transposition and the number of nuclei added to the high-volume wash.

Pipette up and down 10 times gently on ice. Transfer the resuspended nuclei to a new, pre-chilled, 1.5 mL Lo-Bind Eppendorf tube.

36 Transfer 10 µL of the nuclei solution to a clean 1.5 mL tube and add 10 µL of Trypan Blue stain. Mix well by pipetting and load into a disposable hemocytometer slide

36.1 If using Ethidium Homodimer, dilute the 1 mM stock solution 1:100. Mix 10 uL of the nuclei solution with 10 uL of Ethidium Homodimer and load into a disposable Countess Cell Counting slide. Let sit for at least one minute before loading into a fluorescent-capable Countess. Use the cell count displayed in the RFP channel.

37 Count the number of intact nuclei in the 4 large corner squares. Take the total number in all 4 squares and multiply by 5000 to obtain the concentration of nuclei per mL. Adjust this concentration to nuclei per ul before proceeding with the 10x Genomics Multiome protocol.

38 If necessary, add more Diluted Nuclei Buffer (1X) to the nuclei to get to the desired concentration to start with the 10x Genomics Multiome protocol. We tend to overload the chip and dilute the nuclei to 5000 nuclei/uL. The final nuclei pool concentration may be different depending on the original samples and the specific experiment.

38.1 We typically add 5 ul volume of nuclei to the transposition reaction. Using a concentration of 5000 nuclei/ul corresponds to loading 25,000 nuclei per transposition reaction and well of the 10x Genomics chip. This is more than 10x Genomics recommends and is not officially supported by 10x Genomics. In our experience this concentration does not lead to more clogs in the 10x Chromium GEM Generation step.

38.2 We have had much success and similar data quality when loading 35,000 and 40,000 nuclei per transposition reaction and channel of the 10x Genomics Next GEM Chip J. This overloading assumes there is little to no debris in the final nuclei sample at this stage.

39 These nuclei are now ready to proceed with the ATAC transposition reaction as input to **Step 1.1e** of the 10x Genomics Multiome protocol. These nuclei can also be used for single-nucleus RNA-seq, single-nucleus ATAC-seq, and other epigenomic and transcriptomic assays. Nuclei can also be cryopreserved for later use as described below.

Transposition of Nuclei

40 From here, follow the steps in the 10x Genomics Chromium Next GEM Single Cell Multiome ATAC + Gene Expression User Guide (CG000338).

41 Prepare Transposition Mix containing the ATAC Buffer B (PN-2000193) and ATAC Enzyme B (PN-2000265/PN-2000272) kit on ice. Pipette mix 10x and centrifuge briefly.

42 Add 10 ul Transposition Mix to a tube of a PCR 8-tube strip on ice for each sample. Briefly pulse centrifuge and maintain on ice.

43 Confirm nuclei are at the correct concentration in Diluted Nuclei Buffer. This protocol uses 5000 nuclei/ul but other concentrations may be used depending on the experiment and starting sample amount.

44 Gently pipette the Nuclei Stock on ice immediately before adding to the transposition reaction. Add 5 ul of Nuclei Stock to the tube containing the Transposition Mix. Gently pipette mix 6x (pipette set to 10 ul). Do not centrifuge.

45 For the transposition reaction incubate in a thermal cycler for 01:00:00 at 37 °C . 1h

On the thermal cycler program the lid temperature to be 50 °C and the reaction volume to be 15 µL

46 Proceed immediately with the subsequent steps of the 10x Genomics Chromium Multiome ATAC + Gene Expression protocol

Cleanup and Cryopreservation of Nuclei and Homogenate

47 If you would like to save extra nuclei for other assays or to potentially use in additional experiments downstream, you may follow these steps. However, we note that in our hands there is a statistically significant reduction in data quality when comparing freshly isolated and cryopreserved nuclei:

47.1 Pellet remaining nuclei by centrifugation for 00:10:00 at 500 rcf at 4 °C .

47.2 Carefully aspirate supernatant using two pipetting steps (p1000 then p200) as above.

47.3 Gently resuspend nuclei pellet in 100 ul of cold BAM Banker media and transfer to a pre-chilled 2 ml Nunc cryovial.

47.4 Slow-freeze nuclei in a freezing container and move to -80 °C or liquid nitrogen storage the next day.

48 Cleaning Dounces and pestles: Rinse all Dounces and pestles thoroughly with sterile water (2x) followed by 70% ethanol (2x). Let Dounces and pestles dry on a Kimwipe or paper towel for a few hours to overnight.

Using Nuclei for Single-Nuclei Experiments with 10x Genomics after Freezing

49 Thaw nuclei on ice or use directly after isolation.

50 Aliquot desired number of nuclei into 1 mL of Multiome Wash Buffer in a 1.5 mL Lo-Bind microcentrifuge tube. The number of nuclei you use depends on the specific experiment. Gently invert the tube 5x.

51 Pellet at 500 rcf at 4 °C for 00:05:00 in a fixed-angle rotor. You should be able to see a small pellet if you used more than 25,000 nuclei.

52 Aspirate supernatant using 2 pipetting steps as above (p1000 then p200).

53 Resuspend nuclei in the desired volume of 1x Diluted Nuclei Buffer (use the same recipe as in the Materials section). Nuclei tend to clump more after freezing and thawing so addition of 1-2% BSA helps reduce clumping.

53.1 After this step, you will use 5 ul of nuclei in the transposition reaction.

54 Transfer 5 ul of nuclei suspension containing nuclei into 10 ul of transposition mix from the 10x protocol.

55 Mix by pipetting, do not centrifuge or vortex and avoid introducing bubbles. Proceed to transposition incubation as indicated in the part of the 10x protocol.

Protocol references

Chromatin accessibility profiling by ATAC-seq https://www.nature.com/articles/s41596-022-00692-9

An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues https://www.nature.com/articles/nmeth.4396

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