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Nuclear Isolation of Post-Mortem Brain Tissue for snRNAseq

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Jonathan W Brenton^{1,2}, Carlo Sala Frigerio, Mina Ryten^{1,2}, Regina H Reynolds^{1,2}, e.gustavsson^{1,2}, Kylie Montgomery¹

¹Genetics & Genomic Medicine Dept, UCL GOS Institute of Child Health, 30 Guilford St, London WC1N 1EH;

²Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD, 20 815, USA

Jonathan W Brenton: jonathan.brenton.14@ucl.ac.uk;

Carlo Sala Frigerio: csalafrigerio@gmail.com

Mina Ryten: mina.ryten@ucl.ac.uk

Regina H Reynolds: regina.reynolds.16@ucl.ac.uk

e.gustavsson: e.gustavsson@ucl.ac.uk

Kylie Montgomery: kylie.montgomery@ucl.ac.uk

Ryten Laboratory



Jonathan W Brenton

UCL

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We use this protocol and it's working

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Abstract

This protocol is used to isolate nuclei from frozen brain tissue for single nuclear RNA sequencing using 10x Genomics GEM isolation using the Chromium accessory and Single Cell 3' Reagent Kits.

It is adapted from a protocol designed by Carlo Sala Frigerio.

The nuclei are homogenised by hand, run through a density gradient, resuspended, counted and diluted to an appropriate concentration to run in the Chromium.

This protocol is designed to be continued with the Chromium Single Cell 3' Reagent Kits User Guide (v3.1 Chemistry Dual Index) to isolate nuclei in GEMs.

Materials

Equipment

	A	B	C	D	E
	Manufacturer	Instruments	code	notes	website
	Beckman Coulter	Optima XPN-90			
	Beckman Coulter	SW41Ti rotor		swing-out, 6 slots	
	10X Genomics	Chromium		Firmware 4.00 at least	
	Logos bio	Luna-FL dual fluorescence cell counter			https://logosbio.com/automated-cell-counters/fluorescence/luna-fl
	Biorad	C1000 Touch with 96-Deep Well Reaction Module	1851197		https://www.bio-rad.com/en-uk/product/c1000-touch-thermal-cycler?ID=LGTW9415
	Any	Scale measuring to 0.01g or 0.001g			

List of Items

	A	B	C
	Item	Code	Manufacturer
	Tissue grinders, Potter-Elvehjem type, glass vessel, 2 mL	432-0200	VWR
	Tissue grinders, Potter-Elvehjem type, plain plunger, 2 mL	432-0206	VWR
	Tube, Thinwall, Ultra-Clear™, 13.2 mL, 14 × 89 mm (qty. 50)	344059	Beckman Coulter
	Eppendorf PCR Tubes, 0.2 mL, PCR clean, 8-tube strips, 120 pcs. (960 tubes)	0030124359	Eppendorf



	A	B	C
	DNA LoBind Tube Microcentrifuge Tube, 0.5 mL	0030108 035	Eppendorf
	DNA LoBind Tube Microcentrifuge Tube, 1.5 mL	0030108 051	Eppendorf
	DNA LoBind Tube Microcentrifuge Tube, 2.0 mL	0030108 078	Eppendorf
	Reagent reservoir, white PS, 50 mL, sterile	613-1184	VWR
	Falcon® 5 mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap	352235	Corning
	Protein LoBind Tubes 2mL	0030108 132	Eppendorf
	Protein LoBind Tubes 1.5mL	0030108 116	Eppendorf
	Nuclease-Free Water (Ambion)	AM9937	Thermo Fisher
	10% Tween-20	166- 2404	Bio-Rad
	Low TE Buffer (10 mM Tris- HCl pH 8.0, 0.1 mM EDTA)	12090- 015	Thermo Fisher
	Glycerol 50%	G5516- 500ML	Sigma-Aldrich
	Reagent, SPRIselect, 60 mL	B23318	Beckman Coulter
	Ethanol, Pure (200 Proof, anhydrous)	459836- 1L	Sigma-Aldrich
	Buffer EB	19086	Qiagen
	Sucrose, BioUltra, for Molecular Biology	84097- 1KG	Sigma-Aldrich
	Calcium Chloride	C5670- 100G	Sigma-Aldrich
	Magnesium acetate tetrahydrate	M5661- 50G	Sigma-Aldrich



	A	B	C
	Ethylenediaminetetraacetic acid solution	03690-100ML	Sigma-Aldrich
	Phenylmethanesulfonyl fluoride	78830-5G	Sigma-Aldrich
	2-Mercaptoethanol	63689-25ML-F	Sigma-Aldrich
	Magnesium chloride hexahydrate	M2670-500G	Sigma-Aldrich
	Potassium chloride	P9333-500G	Sigma-Aldrich
	Digitonin 5%	BN2006	Thermo Fisher/Invi
	Nonidet P40 substitute Bioextra	74385-1L	Sigma-Aldrich
	Trizma HCl solution	T2194-1L	Sigma-Aldrich
	OptiPrep™ Density Gradient Medium	D1556-250ML	Sigma-Aldrich
	phosphate-buffered saline 1X w/o Ca and Mg	21-040-CV	Corning Cellgro
	RNasin Plus RNase Inhibitor, 10,000 units	N2615	Promega
	MACS BSA stock solution	130-091-376	Miltenyi Biotec GmbH
	High Sensitivity D5000 Ladder	5067-5594	Agilent
	High Sensitivity D5000 ScreenTape	5067-5592	Agilent
	High Sensitivity D5000 Reagents	5067-5593	Agilent
	High Sensitivity D1000 Ladder	5067-5587	Agilent
	High Sensitivity D1000 Sample Buffer	5067-5603	Agilent
	High Sensitivity D1000 ScreenTape	5067-5584	Agilent
	High Sensitivity D1000 Reagents	5067-5585	Agilent



	A	B	C
	LightCycler® 480 Multiwell Plate 384, white	4729749 001	Roche Lifescience
	KAPA library quantification kit for Roche Lightcycler LC480 (KK4854)	7960298 001	Roche Lifescience
	Falcon tubes, 50 mL		
	Falcon tubes, 15 mL		
	Foreceps		
	70% ethanol		

Stock Tables

	A	B	C	D	E	F
		g	stock (M)	FW (g/mol)	Volume (L)	
	sucrose	85.575	0.5	342.3	0.5	
	CaCl ₂	8.400	0.5	110.98	0.151378627	
	Mg Acetate*4H ₂ O	26.806	0.5	214.45	0.25	
	PMSF	0.120	0.1	174.19	0.006889029	in 2-propanol, store at 4C

Everything filter using 0.2 um filters

Beta-Mercaptoethanol

	A	B	C
		Dilution	Final Concentration (M)
	2-Mercaptoethanol	175 uL in 50 mL	0.05


Glycerol

mix 1:1 99% Glycerol and water, filter with 0.2um filter. Can be aliquoted and stored at -20C (equilibrate to room temperature before use). Or store in a 50mL falcon at room temperature.

Troubleshooting



Buffer Making for 6 samples (7x made to allow for waste)

1 Prepare buffers from stocks, keep  On ice .

1.1 Homogenisation Buffer (HB)

	A	B	C	D	E	F
	HB (Homogenisation Medium)	V (μl)	Stock conc		Final conc	
	Sucrose	17920.0	500	mM	320	mM
	CaCl ₂	140.0	1000	mM	5	mM
	Mg Acetate	168.0	500	mM	3	mM
	Tris pH 7,4 (Sigma)	280.0	1000	mM	10	mM
	EDTA	5.6	500	mM	0.1	mM
	NP40	280.0	10	%	0.1	%
	Digitonin	56.0	5	%	0.01	%
	PMSF	56.0	50	mM	0.1	mM
	β-mercaptoethanol	560.0	50	mM	1	mM
	Nuclease free water	8534.4				
	Total Volume	28000.0	4000	per sample		

Resuspend Digitonin first.

For Digitonin: If a precipitate forms in the 5% Digitonin solution, we performed the recommend steps in the manual. This requires heating the solution at 95 degrees C for 5 mins and vortexing slowly to dissolve the precipitate. Cool to room temperature prior to use. The 5% Digitonin solution stays in solution at room temperature for up to 1 week. Although in practice this tends to precipitate after a day so most likely this will need reheating after a day or two.

1.2 Gradient Medium



	A	B	C	D	E	F
	GM (Gradient Medium)	V (μl)	Stock conc		Final conc	
	CaCl ₂	105	1000	mM	5	mM
	Optiprep	17500	60	%	50	%
	MgCl ₂	63	1000	mM	3	mM
	Tris pH 7,4	210	1000	mM	10	mM
	PMSF	42	50	mM	0.1	mM
	β-mercaptoethanol	420	50	mM	1	mM
	Nuclease free water	2660.0				
	Total Volume	21000	3000	per sample		










1.3 ODN

	A	B	C	D	E	F
	<i>ODN</i>	<i>V (μl)</i>	<i>Stock conc</i>		<i>Final conc</i>	
	KCl	1342.6875	2000	mM	150	mM
	MgCl ₂	537.075	1000	mM	30	mM
	Tris pH 7,4 (Sigma)	1074.15	1000	mM	60	mM
	Sucrose	8951.25	500	mM	250	mM
	Nuclease free water	5997.3375				
	Total Volume	17902.5				





1.4 29% Cushion

	A	B	C	D	E	F
	29% Cushion	V (μl)	Stock conc		Final conc	
	Optiprep	1522 5	60	%	29	%
	ODN	1627 5				
	Total Volume	3150 0	4500	per sample		










Homogenisation

- 2 Rinse douncers with some HB buffer – leave these  On ice too.
- 3 Add  1 mL HB to douncer.
- 4 Put douncer on scale in any support (falcon tube holder was used here), zero the scale and add sample to douncer. Aim for between 80-150mg tissue. ~  100 mg of tissue works well.
- 5 Homogenise:~20 strokes with pestle A and then~80 strokes with pestle B; try to keep  On ice as much as possible. If meninges present following 10 strokes with pestle B remove with forceps as this can add a lot of resistance. Clean forceps in dH2O water, wipe clean and then spray with 70% ethanol and wipe and air dry.
- 6 Keep douncer with homogenised sample  On ice for and repeat steps 3-6 for other samples.
- 7 For each sample transfer ~  1 mL homogenate (everything in douncer) to 15mL falcon and add  1650 μL HB (total V =  2.65 mL). Mix by inverting as well as pipetting. All  On ice .





- 8 Add and mix  2.65 mL ( 0.883 mL x 3) Gradient medium to sample (total V =  5.30 mL). Mix by inverting gently as well as pipetting. All  On ice .

Gradient Preparation and Isolation by Centrifugation

- 9 Add  4 mL of optiprep cushion to an ultracentrifuge tube  On ice for each sample.
- 10 Layer  5.25 mL sample on top of cushion. To do this slowly add homogenate/gradient medium mixture just above the top of the optiprep cushion and keeping the pipette tip against the wall of the ultracentrifuge tube. One way to do this is to use a P200 first to layer  200 µL -  400 µL of sample on top of the cushion and the proceed with a P1000 to layer additional mLs of sample. Keep thumb under plunger of pipette to make sure addition of sample is gradual and is not pushed to the bottom of the gradient. Try and keep the bottom of the tube touching the ice as much as possible to keep the tube cool.
- 11 Check that sample's weight in ultracentrifuge tube is balanced (do in pairs – ie samples facing each other in ultracentrifuge should be equal in weight), in case adjust weight with HB.
- 12 Add samples to ultracentrifuge buckets.
- 13 Spin samples in SW41Ti  7700 rpm  00:30:00  4 °C - keep all tube holders in the machine even if empty and keep positions matched, ie holder 1 is in position 1. The ultracentrifuge should be pre-cooled to  4 °C prior to adding the samples. 30m

During Ultracentrifugation



- 14 Take out 10X beads from  -80 °C and GEM kit from  -20 °C . Prepare Template Switch Oligo as per 10x instructions if needed.
- 15 Prepare the resuspension buffer:






15.1 RNAseq Resuspension Buffer (for 6 samples + 1 for waste). Prepare and keep on ice.

	A	B	C	D	E	F
	RNAseq Resuspension Buffer					
		V (uL)	stock		final	
	1x PBS (w/o Ca and Mg)	9464				
	BSA	1680	10	%	1.5	%
	RNase IN (Promega Purple cap)	56	40	u/uL	0.2	u/uL
	final volume	11200				

Resuspension and filtering



- 16 Gently take away supernatant using plastic Pasteur or P1000, then P200 Gilson. Nuclei will be a white smear at the bottom of the tube, the tube will gain condensation quickly so can wipe it to get a better look at the nuclei at the end and try and hold up the tube to a dark surface to get a look at the nuclei (although not all can be seen). Keep tube steady and try not to mix and shake, which may resuspend the nuclei. Remove as much supernatant as possible by pipetting from the surface of the liquid and following the layer down to the lip of the ultracentrifuge tube. At this point remove as much as possible before nuclei start to come along with supernatant. Usually  200 µL -  500 µL remains at the bottom of tube.




When in doubt leave supernatant as not to decrease yield or keep supernatant in a separate tube and filter as a separate sample that could be combined later in case of low yield. However, the vast majority of nuclei will be present at the bottom of the tube and this typically doesn't add many nuclei.


- 17 Add in  200 µL of suspension buffer, transfer to chilled 1.5 or 2mL LoBind tube (either protein or DNA)  On ice . All further steps should also be done  On ice




and nuclei/samples should be kept cool as much as possible to prevent nuclei clumping.

18 Wash out ultracentrifuge tube with further  200 μL of suspension buffer, pool in the LoBind tube  On ice .


19 Add a further  400 μL of suspension buffer to the LoBind tube  On ice ( 800 μL total suspension buffer).

20 Mix gently by pipetting and filter through a filter cap Falcon tube and add to a new 1.5 or 2ml LoBind tube (protein or DNA)  On ice .





21 Keep  On ice until the other samples have been resuspended and filtered.

22 Centrifuge  500 rcf for  00:05:00 and at  4 °C .



5m

23 Nuclei can now be seen, after wiping away condensation, as a white smear on the side of the tube in the DNA LoBind tubes or more of a pellet if using protein LoBind tubes. Remove supernatant and resuspend nuclei in  400 μL resuspension buffer by pipetting.

24 Filter through a filter-cap Falcon tube.

25 Transfer to LoBind Eppendorf  On ice and centrifuge  500 rcf for  00:05:00 and at  4 °C .





5m

26 Remove supernatant and resuspend nuclei in  400 μL ( 200 μL if nuclei pellet looks small in Lo Bind tube) resuspension buffer by pipetting.

27 Filter through a filter-cap Falcon tube. Keep  On ice .

Nuclei Counting



- 28 Count each aliquot with Luna FL ( 18 μL sample +  2 μL Acridine Orange/Propidium Iodide). Add  10 μL to slide. Save images and counts.
- 29 Create a new RNAseq aliquot of 1000 nuclei/uL in  80 μL by diluting original sample.
- 30 Count diluted sample and record number (save files if needed but adds time for each sample).
- 31 Round count to nearest 100 and use this when loading the chip. Ie if one sample has concentration of 1100 and another 900 use two different cell concentrations in 10x protocol to load rather than rediluting sample to achieve 1000 cells.