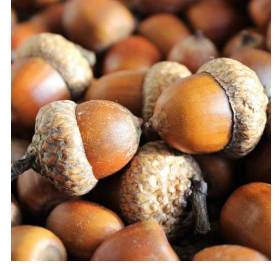


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Overloading and Unpacking (OAK) - Multiome V1

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We use this protocol and it's working

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Abstract

High-throughput single-cell sequencing is a powerful technique for investigating the cellular diversity of complex biological systems. Throughput, cost effectiveness, and experimental simplicity are crucial forefronts of technological advancement in profiling single cells. We developed "overloading and unpacking" (OAK), a method that enables robust profiling of hundreds of thousands of cells in a cost effective manner.

This protocol describes using OAK in conjunction with Chromium Next GEM Single Cell Multiome ATAC + Gene Expression Reagent Kits.

Materials

Plasticware:

- 2ml nuclease-free microfuge tubes.

Equipment:

- 10X Genomics Chromium Controller
- Swinging bucket centrifuge

Enzymes and reagents:

- Chromium Next GEM Single Cell Multiome ATAC Kit A, 16 rxns (PN-1000280, 10x Genomics)
- Chromium Next GEM Single Cell Multiome GEM Kit A, 16 rxns (PN-1000232, 10x Genomics)
- Chromium Next GEM Single Cell Multiome Gel Bead Kit A, 16 rxns (PN-1000231, 10x Genomics)
- 1 M DTT (P2325, Invitrogen) (dilute to 1 mM stock DTT just before use).
- Protector RNase inhibitor, 40 U/μL (03335402001, Roche Diagnostics)
- KAPA HiFi 2X Hotstart Ready mix
- NEBNext 2X master mix (M0541S, NEB)
- Single Index Kit N Set A (PN-1000212, 10x Genomics)

Buffers and solutions:

- 16% Formaldehyde (w/v), Methanol-free (28908, Pierce)
- PBS (Ca²⁺, Mg²⁺ free) (10010-023, Gibco)
- 20X SSC (will be diluted to 3X) (AM9770, Invitrogen)
- Nuclease-free water (AM9937, Invitrogen)
- 1 M Tris-HCl (T1275, Teko)
- 5 M NaCl (AM9759, Invitrogen)
- 1 M MgCl₂ (AM9261, Invitrogen)
- 10 % BSA in DPBS (A1595-50mL, Sigma)
- 10% Tween-20 (161-0781 Biorad)

General amplification primers:

	Primer name	Sequence	Stock concentration
	TSO enrichment primer	AAGCAGTG GTATCAAC GCAGAGT	100 μM
	Partial P5 Primer	AATGATAC GGCGACCA CCGAGA	5 μM

**Pre-amplification primer mix:**

Either use Pre-Amp Primers (2000271) from Chromium Next GEM Single Cell Multiome Amp Kit A, 16 rxns PN-1000233, or prepare an equimolar mix of the following primers:

	Primer name	Sequence	Stock concentration
	ATAC-fwd	AATGATAC GGCGACCA CCGAGA	100 μ M
	ATAC-rev	GTCTCGTG GGCTCGG	100 μ M
	RNA-fwd	CTACACGA CGCTCTTC CGATCT	100 μ M
	RNA-rev	AAGCAGTG GTATCAAC GCAGAG	100 μ M

Primers for addition of 2nd index (P5-i5index-TruseqR1) during cDNA amplification:

	Primer name	Sequence	C	Stock concentration
	P5_plateTT_ A1_TruseqR1	AATGATAC GGCGACCA CCGAGATC TACACAGT GTTACCTA CACTCTTT CCCTACAC GACGCTCT TCCGATCT	AGTGTTAC CT	100 μ M
	P5_plateTT_ B1_TruseqR1	AATGATAC GGCGACCA CCGAGATC TACACACA GTTTCGTTA CACTCTTT CCCTACAC GACGCTCT TCCGATCT	ACAGTTTCG TT	100 μ M



	Primer name	Sequence	C	Stock concentration
	P5_plateTT_C1_TruseqR1	AATGATAC GGCGACCA CCGAGATC TACACCAA GGATAAAA CACTCTTT CCCTACAC GACGCTCT TCCGATCT	CAAGGATA AA	100 µM
	P5_plateTT_D1_TruseqR1	AATGATAC GGCGACCA CCGAGATC TACACGCT TGTCGAAA CACTCTTT CCCTACAC GACGCTCT TCCGATCT	GCTTGTCG AA	100 µM
	P5_plateTT_E1_TruseqR1	AATGATAC GGCGACCA CCGAGATC TACACCTG TCCTGCTA CACTCTTT CCCTACAC GACGCTCT TCCGATCT	CTGTCCTG CT	100 µM
	P5_plateTT_F1_TruseqR1	AATGATAC GGCGACCA CCGAGATC TACACAGC GGGATTTA CACTCTTT CCCTACAC GACGCTCT TCCGATCT	AGCGGGAT TT	100 µM
	P5_plateTT_G1_TruseqR1	AATGATAC GGCGACCA CCGAGATC TACACCTT GATCGTAA CACTCTTT CCCTACAC GACGCTCT TCCGATCT	CTTGATCG TA	100 µM
	P5_plateTT_H1_TruseqR1	AATGATAC GGCGACCA CCGAGATC TACACCGT ACCGTTAA CACTCTTT CCCTACAC GACGCTCT TCCGATCT	CGTACCGT TA	100 µM

Primers for addition of P7 sequencing index (P7-i7index-TruseqR2) during GEX sequencing library amplification:

	Primer name	Sequence	Index sequence	Dilution of stock to use
	P7_SI-TT-A1	CAAGCAGA AGACGGCA TACGAGAT CGCATGTT ACGTGACT GGAGTTCA GACGTGT	GTAACATGCG	5 µM
	P7_SI-TT-A2	CAAGCAGA AGACGGCA TACGAGAT TTTGATCC ACGTGACT GGAGTTCA GACGTGT	GTGGATCAAA	5 µM
	P7_SI-TT-A3	CAAGCAGA AGACGGCA TACGAGAT TTTCGTAG TGGTGA GGAGTTCA GACGTGT	CACTACGAAA	5 µM
	P7_SI-TT-A4	CAAGCAGA AGACGGCA TACGAGAT CTCGCTAG AGGTGACT GGAGTTCA GACGTGT	CTCTAGCGAG	5 µM
	P7_SI-TT-A5	CAAGCAGA AGACGGCA TACGAGATA CAGGGCTA CGTGA GAGTTCAG ACGTGT	GTAGCCCTGT	5 µM
	P7_SI-TT-A6	CAAGCAGA AGACGGCA TACGAGAT TCACGCGT TAGTGA GGAGTTCA GACGTGT	TAACGCGTGA	5 µM
	P7_SI-TT-A7	CAAGCAGA AGACGGCA TACGAGATA CCCTTGGG AGTGA	TCCCAAGGGT	5 µM



	Primer name	Sequence	Index sequence	Dilution of stock to use
		GAGTTCAG ACGTGT		
	P7_SI-TT-A8	CAAGCAGA AGACGGCA TACGAGAT GTATACTTC GGTGACTG GAGTTCAG ACGTGT	CGAAGTATAC	5 μ M
	P7_SI-TT-A9	CAAGCAGA AGACGGCA TACGAGAT CTCTCCAC TTGTGACT GGAGTTCA GACGTGT	AAGTGGAGAG	5 μ M
	P7_SI-TT-A10	CAAGCAGA AGACGGCA TACGAGAT GCATGTCA CGGTGACT GGAGTTCA GACGTGT	CGTGACATGC	5 μ M



Before starting

- 1 Prepare your single-cell suspension. We recommend starting with over 500K cells if possible, to ensure a visible pellet after the fixation step.

Prepare 4 mL nuclei wash buffer per sample, and place  On ice .

Nuclei wash buffer:

	Reagent	B	Volume for 4 mL (μL)	Volume for 8 mL (μL)
	Tris-HCl	1 M	40	80
	NaCl	5 M	8	16
	MgCl ₂	1 M	12	24
	BSA	10 %	400	800
	Tween-20	10 %	40	80
	DTT	1 M	4	8
	RNase inhibitor	40 U/μL	100	200
	Nuclease-free water		3400	6800

Prepare 1 mL 1X nuclei buffer (part number 2000207, 10x` Genomics) and place

 On ice

1x nuclei buffer:

	Reagent stock	Volume (μL)
	Nuclei buffer (20X)	50
	1 M DTT	1
	RNase inhibitor (40 U/μL)	25
	Nuclease-free water	924



Pre-cool a centrifuge with a swinging bucket to  4 °C . A swinging bucket is crucial for cell recovery. The cooled centrifuge is required for sections 2, 3, 5, 7 and 8 of the protocol.

Prepare 200 µL tubes for transposition step.

Prepare twenty labeled 200 µL tubes for aliquoting cells.

Optional: Pre-coat 2 mL microfuge tubes with BSA.

Nuclei isolation and fixation

- 2 Prepare nuclei as suggested in the protocol provided by 10x Genomics (https://assets.ctfassets.net/an68im79xiti/G2F46chiuUKzVPL5tuiyy/1dc1908b5a77f5cd3abef59a750a2b54/CG000365_DemonstratedProtocol_NucleiIsolation_ATAC_GEX_Sequencing_RevA.pdf) or by your preferred method.

During the wash steps prepare the fresh 0.3% formaldehyde fixative buffer and store on wet ice. To do this add 18.75 µL 16% methanol-free formaldehyde solution to 981 µL PBS to make 1 mL total.

Wash the nuclei in the nuclei wash buffer in a round-bottom 2 mL microfuge tube. In the final wash, if necessary filter the nuclei with a 40 µm filter, then centrifuge between 500,000 and 2 million cells at 500 G for 5min.

Remove the supernatant and resuspend the nuclei pellet in the fixative buffer, pipette mix on addition to prevent the formation of clumps.

Place nuclei  On ice for 10 min.

Post-fix wash

- 3 Pellet nuclei in a swinging-bucket centrifuge at 500G for 5 min at  4 °C .
Remove supernatant and dispose responsibly (contains formaldehyde). Add 1.5 mL nuclei wash buffer and centrifuge at 500G for 5 min at  4 °C .

Remove as much supernatant as possible without disturbing the pellet and resuspend cells gently in 200 µL diluted 1X nuclei buffer (part number 2000207, 10x Genomics).

Count the nuclei and adjust to a concentration of approximately 4000 nuclei/µL.

Transposition



- 4 Dispense 5 μL of nuclei (20000 per transposition reaction) into 200 μL microfuge tubes.

Scale the number of reactions to the number of nuclei per channel. As some nuclei are inevitably lost prior to loading, we recommend transposing 200,000 nuclei in 10 reactions.

Prepare the mixture of TDE1 and TDE1 buffer

	Reagent	Volume (μL)	Mastermix, 10 reactions (μL)
	TDE1 buffer	7	77
	TDE1	3	33

Dispense the mastermix to each tube and place in the thermocycler at  37 $^{\circ}\text{C}$ for 60 min, as you would do for one tube in the standard 10x Genomics protocol (CG000338_ChromiumNextGEM_Multiome_ATAC_GEX_User_Guide_RevF).

Note: We have tried transposing the nuclei en masse in one reaction, instead of separate tubes, the results were similar to separate reactions run in parallel on the same sample, however with $n=1$ we are not quite confident to make this recommendation yet.

Combine transposed nuclei and overload 10X Chromium

- 5 Using a single low-retention P200 pipette tip, combine all the reactions into one 2 mL microfuge tube.

Using 150 μL 1x nuclei buffer (part number 2000207, 10x Genomics), wash the sides of the tubes from which the nuclei were combined, and add to 2 mL microfuge tube containing the nuclei.

Centrifuge the combined nuclei at 500G for 10 min  4 $^{\circ}\text{C}$. Remove supernatant leaving 16 μL solution around the pellet or expected pellet position. (Note: because of the lower number of nuclei the pellet may be clearly visible, we recommend keeping the supernatant until loading).

Resuspend the nuclei in the 16 μL solution with gentle pipetting. At this point you may want to take 1 μL and dilute in 1x nuclei buffer for counting in a haemocytometer, and to confirm a high concentration of nuclei.



Once ready, load the 10X Chromium as described in the standard multiome 10x Genomics protocol (CG000338_ChromiumNextGEM_Multiome_ATAC_GEX_User_Guide_RevF).

Modified reverse transcription

- 6 After GEM generation transfer the emulsion to a microfuge tube as described in the standard multiome 10x Genomics protocol.

Proceed with the reaction in a thermocycler at  37 °C for 45 min then  25 °C for 30 min before cooling to  4 °C as described.

As soon as the thermocycler program is complete proceed immediately to the next step.

Unpacking the GEMS

- 7 Add 125 µL 10x Genomics recovery agent to the GEMS and allow separation to occur. Do not invert or remove the recovery agent.

Using a low-retention P200 pipette tip, carefully transfer approx 80 µL aqueous solution into a 2 mL microfuge tube.

Add 800 µL of 3X SSC to the nuclei gently, without pipette mixing.

Proceed immediately to the next step.

Washing the cells

- 8 Pellet cells in a swinging-bucket centrifuge at 650G for 5 min at  4 °C .
Remove supernatant, being careful not to disturb the pellet.
Add 1 mL of 3X SSC to the nuclei gently, without pipette mixing.
Pellet cells in a swinging-bucket centrifuge at 650G for 5 min at  4 °C .

Distribute the cells into aliquots

- 9 **Work quickly for the best cell recovery.**

Remove supernatant, being careful not to disturb the pellet.

Using a low-retention P200 pipette tip add 215 µL to the nuclei pellet, pipette up and down twice gently to resuspend, then with the same tip, aliquot 10 µL nuclei solution into twenty different 200 µL microfuge tubes.

Note: the number of nuclei aliquots is flexible, however we recommend for loads of 100,000-200,000 nuclei, twenty aliquots to reduced the collision rate of indexing.

Immediately store the dispensed nuclei at  -80 °C .

QC of cell yield

- 10 Using any spare volume of nuclei that were not aliquoted (recommend 1:5 dilution in 3x SSC), 1:1 with trypan blue solutin and count on a haemocytometer. This will give an expected number of cells per aliquot, this information is useful to allow you to sequence sub-libraries at the correct depth.

Note: some debris is expected, a large amount of debris may indicate some lysis of nuclei in the experiment and could be detrimental to data quality.

Clean up of cell aliquots

- 11 Heat aliquots to  -85 °C for 5 min.
Clean up using the Dynabeads Silane Viral NA kit (ThermoFisher, 37011D), following the protocol.
Elute each aliquot of cDNA in 23 µL by heating in a thermocycler to  50 °C for 10 min, tapping the tube at the midway point.
Place on a magnet and transfer the eluted cDNA to a new 200 µL microfuge tube.

Pre-amplification step

- 12 Perform the pre-amplification step on the aliquots by adding the following mix. Do not combine the aliquots.

	Reagent	Volume (µL)	Volume in mastermix for 10 reactions (µL)
	NEBNext 2X PCR master mix	25	275
	Pre-Amp Primer Mix (10x Genomics 2000271, or own mix)	2	22
	Template	23	Add individually



Reagent	Volume (μL)	Volume in mastermix for 10 reactions (μL)
Total volume	50	X

The thermal cycler program is as described in the standard 10x multiome protocol. CG000338_ChromiumNextGEM_Multiome_ATAC_GEX_User_Guide_RevF. However we recommend using 11 cycles of PCR.

Clean up the pre-amplification reactions

- On each aliquot, do a 1.6X SPRI bead clean up (use 80 μL beads) for the pre-amp products. Elute each aliquot in 40 μL EB.

ATAC-Seq library construction from the pre-amplification product

- Use 10 μL pre-amplification product from each aliquot to create sequencing libraries of the accessible chromatin component.

Reagent	Volume (μL)	Volume in mastermix for 10 reactions (μL)
NEBNext 2X PCR master mix	50	550
P5_partial (100 μM) (SI- PCR Primer B)	0.6	6.6
Nuclease-free water	36.9	405.9
Sample Index N (PN-1000212 Single Index Kit N Set A)	2.5	Add individually
Template	10	Add individually
Total volume	100	X

Use the following thermocycler program to amplify and add barcodes to the ATAC-Seq library. We recommend 15 cycles of PCR.



Step	Temperature	Time	Cycle
Initial denaturation	98 °C	30 s	1x
Denaturation	98 °C	10 s	
Anneal	67 °C	30 s	
Elongation	72 °C	20 s	Go to step 2. n times
Final elongation	72 °C	2 min	1x
Hold	4°C	Hold	

Note: the extension time is 20S as opposed to 30S to minimize larger fragments.

Perform double-sided clean-up as described in the standard 10X Genomics Chromium protocol. Take 3 µL for QC and store the library at  4 °C for up to 72hr or

 -20 °C until sequencing.

Use Qubit for quantification and Tapestation D1000 for size estimation.

Sequence to recommended depth based on the number of nuclei estimated from the aliquot cell count in Step 10.

Amplification of cDNA adding 2nd index

- 15 This amplification uses a primer against the TSO sequence, and a long P5 primer that adds an index to the barcoded cDNA.

Reagent	B	C
2X KAPA HiFi mix	50 µL	
100 µM TSO enrichment primer	0.4 µL	
100 µM P5-i5index-TruseqR1	0.4 µL	Note index used for each sample



Reagent	B	C
Nuclease-free water	14.2 µL	
cDNA	35 µL	

For the number of cycles, use that recommended by the standard 10X Genomics Chromium protocol plus two.

Step	Temperature	Time	Cycle
Initial denaturation	98 °C	3 min	1x
Denaturation	98 °C	30 s	
Anneal	63 °C	30 s	
Elongation	72 °C	1 min 15 s	Go to step 2. n times
Final elongation	72 °C	5 min	1x
Hold	4°C	Hold	

Store at 4 °C for up to 72hr or -20 °C for up to a week.

Clean up amplified cDNA

- 16 SPRIselect and QC is performed on the amplified cDNA as described in the standard 10X Genomics Chromium protocol.

Store at 4 °C for up to 72hr or -20 °C for up to four weeks.

Gene expression (GEX) library preparation from amplified cDNA

- 17 Fragmentation, End Repair & A-tailing and adaptor ligation steps with clean-up are all performed on the amplified cDNA as described in the standard 10X Genomics Chromium protocol.

At the PCR stage, since we have already added the P5 index earlier, we use a primer to retain this index and add an i7 index.



	A	B	C
	2X KAPA HiFi mix	50 µL	
	100 µM Partial P5 primer	0.4 µL	
	5 µM P7-i7index-TruseqR2	10 µL	Note index used for each sample
	Nuclease-free water	9.6 µL	
	Cleaned up sample after adaptor ligation	30 µL	

For the number of cycles, use that recommended by the standard 10X Genomics Chromium protocol based on cDNA input.

Step	B	C	D
Initial denaturation	98 °C	45 S	1x
Denaturation	98 °C	20 s	
Anneal	54 °C	30 s	
Elongation	72 °C	20 s	Go to step 2. n times
Final elongation	72 °C	1 min	1x
Hold	4°C	Hold	

Perform double-sided clean-up as described in the standard 10X Genomics Chromium protocol.

Take 3 µL for QC and store the library at  4 °C for up to 72hr or  -20 °C until sequencing.

Use Qubit for quantification and Tapestation D1000 for size estimation.

Sequence to recommended depth based on the number of nuclei estimated from the aliquot cell count in Step 10.

