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🌐 Overloading and Unpacking (OAK) V1

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Hayley Bennett¹, Bing Wu¹

¹Genentech

Single cell genomics



Hayley Bennett

Genentech

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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 3' Kit v3.1 reagents.

Materials

Plasticware:

- 2ml nuclease-free microfuge tubes.

Equipment:

- 10X Genomics Chromium Controller
- Swinging bucket centrifuge

Enzymes and reagents:

- Chromium Next GEM Single Cell 3' Kit v3.1 (4 rxns PN-1000269)
- 1 mM DTT
- Protector RNase inhibitor
- KAPA HiFi 2X mix

Buffers and solutions:

- Methanol
- PBS (Ca²⁺, Mg²⁺ free)
- 20X SSC (will be diluted to 3X)
- 7.5% BSA solution
- Nuclease-free water

General amplification primers:

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

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	AGTGTTAC CT	100 µM



	Primer name	Sequence	C	Stock concentration
		GTTACCTA CACTCTTT CCCTACAC GACGCTCT TCCGATCT		
	P5_plateTT_ B1_TruseqR1	AATGATAC GGCGACCA CCGAGATC TACACACA GTTTCGTTA CACTCTTT CCCTACAC GACGCTCT TCCGATCT	ACAGTTCG TT	100 µM
	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	CTTGATCG TA	100 µM



	Primer name	Sequence	C	Stock concentration
		GATCGTAA CACTCTTT CCCTACAC GACGCTCT TCCGATCT		
	P5_plateTT_H1_TruseqR1	AATGATAC GGCGACCA CCGAGATC TACACCGT ACCGTTAA CACTCTTT CCCTACAC GACGCTCT TCCGATCT	CGTACCGT TA	100 μ M

Primers for addition of P7 sequencing index (P7-i7index-TruseqR2) during 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 μ M
	P7_SI-TT-A2	CAAGCAGA AGACGGCA TACGAGAT TTTGATCC ACGTGACT GGAGTTCA GACGTGT	GTGGATCAAA	5 μ M
	P7_SI-TT-A3	CAAGCAGA AGACGGCA TACGAGAT TTTCGTAG TGGTGAAT GGAGTTCA GACGTGT	CACTACGAAA	5 μ M
	P7_SI-TT-A4	CAAGCAGA AGACGGCA TACGAGAT CTCGCTAG AGGTGACT GGAGTTCA GACGTGT	CTCTAGCGAG	5 μ M
	P7_SI-TT-A5	CAAGCAGA AGACGGCA TACGAGATA CAGGGCTA	GTAGCCCTGT	5 μ M



	Primer name	Sequence	Index sequence	Dilution of stock to use
		CGTGA CTG GAGTTCAG ACGTGT		
	P7_SI-TT-A6	CAAGCAGA AGACGGCA TACGAGAT TCACGCGT TAGTGA CT GGAGTTCA GACGTGT	TAACGCGTGA	5 µM
	P7_SI-TT-A7	CAAGCAGA AGACGGCA TACGAGATA CCCTTGGG AGTGA CTG GAGTTCAG ACGTGT	TCCCAAGGGT	5 µM
	P7_SI-TT-A8	CAAGCAGA AGACGGCA TACGAGAT GTATACTTC GGTGA CTG GAGTTCAG ACGTGT	CGAAGTATAC	5 µM
	P7_SI-TT-A9	CAAGCAGA AGACGGCA TACGAGAT CTCTCCAC TTGTGA CT GGAGTTCA GACGTGT	AAGTGGAGAG	5 µM
	P7_SI-TT-A10	CAAGCAGA AGACGGCA TACGAGAT GCATGTCA CGGTGA CT GGAGTTCA GACGTGT	CGTGACATGC	5 µM

Before starting

- 1 Ensure you have adequate aliquots of methanol at  -20 °C . We recommend storing in a cool block that can be pulled out and taken to a fume hood for use.

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

Pre-cool a centrifuge with a swinging bucket to  4 °C . A swinging bucket is crucial for cell recovery.

Prepare a stock of 3X SSC for washing cells after unpacking.

Prepare twenty labeled 200 µL tubes for aliquoting cells.

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

Fixation

- 2 Transfer between 100,000 and 2 million cells to a round-bottom 2 mL microfuge tube in 200 µL PBS.

Slowly add 800 µL ice-cold methanol dropwise to the cells with a P1000, swirling the tube gently.

Add another 800 µL ice-cold methanol in the same manner.

Place cells at  -20 °C for 15-30min.

Note: longer than 1 hr has been linked to poorer transcript recovery.

Post-fix wash

- 3 Prepare at least 200 µL resuspension buffer and place  On ice .

	Reagent	Final concentration	Stock conc.	Volume for 200 µL	Volume for 400 µL
	SSC	3X	20X	30	60
	BSA	2%	7.5%	26.7	53.4



	Reagent	Final concentration	Stock conc.	Volume for 200 μ L	Volume for 400 μ L
	Protect or RNase inhibitor	0.2 U/ μ L	40 U/ μ L	1	2
	DTT		1 mM	0.2	0.4
	Nuclease-free water	-	-	142.1	284.2

Remove cells from -20°C and place on On ice for 5 min.

Pellet in a swinging-bucket centrifuge at 1000G for 5 min at 4 °C

Remove as much supernatant as possible without disturbing the pellet and resuspend cells gently in 200 μ L resuspension buffer. If cell count is less than 8 million/mL then cells can be pelleted and resuspended again in a smaller volume - this limits the impact of any residual methanol in the cell suspension.

Overload 10X Chromium

- Count the fixed cells and, using the recommendations in the standard 10X Genomics Chromium (CG000204_ChromiumNestGEMSingleCell3_V3.1_Rev_D.pdf) as a guideline, prepare and load the chip with typically 10 times as many cells.

For example, with a solution of 10 million cells/mL, to target 100,000 cells, use 16.5 μ L of cell solution and 22.6 μ L of nuclease-free water in the reverse transcription mix.

Modified reverse transcription

- After GEM generation transfer the emulsion to a microfuge tube as described in the standard 10X protocol.

Place the reaction in a thermocycler at 53 °C for 45 min then down to 4 °C .

Note: Do not include 85°C step, as this will lyse the cells.

Proceed immediately to the next step.

Unpacking the GEMS



- 6 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 cells gently, without pipette mixing.

Proceed immediately to the next step.

Washing the cells

- 7 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 cells gently, without pipette mixing.
Pellet cells in a swinging-bucket centrifuge at 650G for 5 min at 4°C .

Distribute the cells into aliquots

- 8 **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 cell pellet, pipette up and down twice gently to resuspend, then with the same tip, aliquot 10 μL cell solution into twenty different 200 μL microfuge tubes.

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

Immediately store the cells at -80°C .

QC of cell yield

- 9 Using any spare volume of cells that were not aliquoted, at trypan blue 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 cells in the experiment and could be detrimental to data quality.

Clean up of cell aliquots



- 10 Heat aliquots to  -85 °C for 5 min.

Clean up using the Dynabeads Silane Viral NA kit (ThermoFisher, 37011D), following the protocol.

Elute cDNA in 35 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.

Amplification of cDNA adding 2nd index

- 11 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
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



Step	Temperature	Time	Cycle
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

- 12 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.

Library preparation

- 13 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 cells estimated from the aliquot cell count in Step 9.