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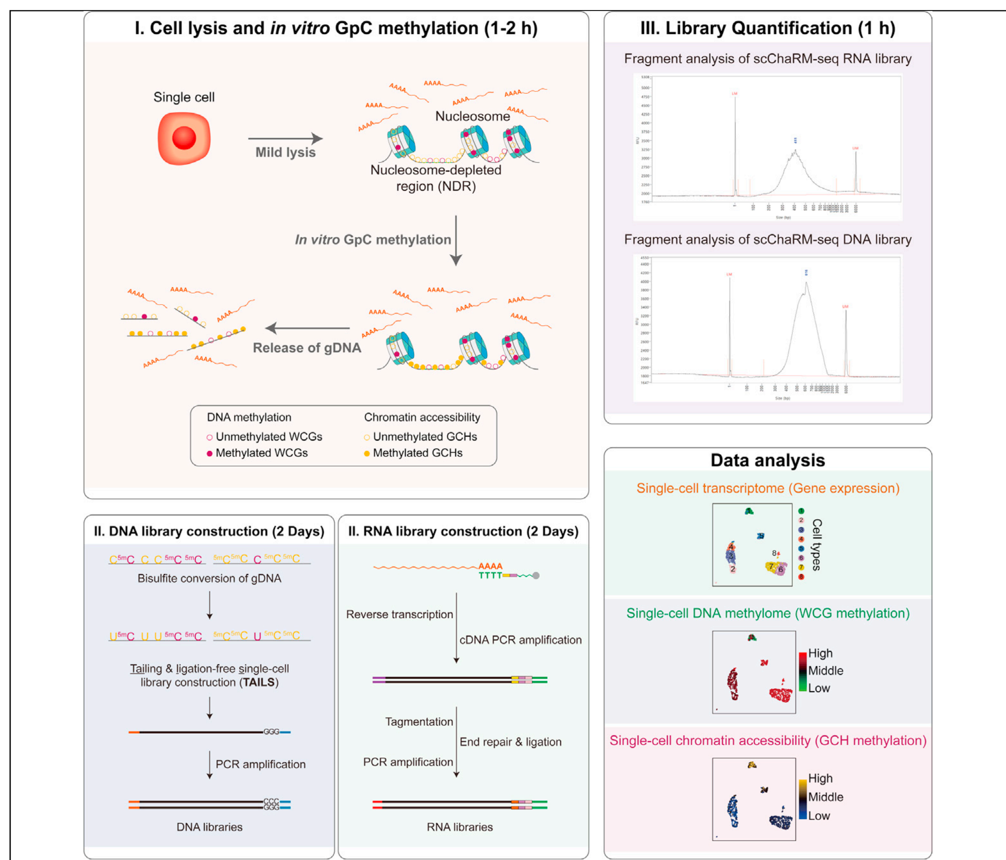
Protocol for scChaRM-seq: Simultaneous profiling of gene expression, DNA methylation, and chromatin accessibility in single cells



Single-cell multi-omics sequencing technology can infer cell heterogeneity and reveal relationships across molecular layers. Combining single-cell RNA sequencing, DNA methylation, and chromatin accessibility allows a multimodal understanding of cell function and epigenetic regulation within individual cells. Here, we offer a protocol to perform scChaRM-seq (single-cell chromatin accessibility, RNA barcoding, and DNA methylation sequencing), which has been applied to study *de novo* DNA methylation and its relationship with transcription and chromatin accessibility in single human oocytes.

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Highlights

Protocol for sequencing library construction of scChaRM-seq

Detailed description of single-cell RNA libraries procedures in scChaRM-seq

Detailed TAILS procedures to construct single-cell DNA libraries in scChaRM-seq

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Protocol for scChaRM-seq: Simultaneous profiling of gene expression, DNA methylation, and chromatin accessibility in single cells

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SUMMARY

Single-cell multi-omics sequencing technology can infer cell heterogeneity and reveal relationships across molecular layers. Combining single-cell RNA sequencing, DNA methylation, and chromatin accessibility allows a multimodal understanding of cell function and epigenetic regulation within individual cells. Here, we offer a protocol to perform scChaRM-seq (single-cell chromatin accessibility, RNA barcoding, and DNA methylation sequencing), which has been applied to study *de novo* DNA methylation and its relationship with transcription and chromatin accessibility in single human oocytes.
For complete details on the use and execution of this protocol, please refer to Yan et al. (2021).

BEFORE YOU BEGIN

Techniques for evaluation of single-cell DNA methylome with other molecular layers have been reported in recent years (Angermueller et al., 2016; Clark et al., 2018). We previously developed scCOOL-seq (single-cell Chromatin Overall Omic-scale Landscape sequencing) to perform multimodal sequencing of both DNA methylation and chromatin accessibility in single cells (Gu et al., 2019; Guo et al., 2017). Moreover, we have achieved high mapping efficiency for bisulfite-converted gDNA sequencing through a tailing- and ligation-free method for single cells (TAILS) (Gu et al., 2019). Recently, we combined single-cell barcoded RNA sequencing (Dong et al., 2018; Gu et al., 2019) with DNA methylation and chromatin accessibility profiling to generate a single-cell multi-omics sequencing method named scChaRM-seq (Yan et al., 2021). We have used scChaRM-seq to study the pattern of the DNA methylation establishment in human oocytes (Yan et al., 2021). In this STAR Protocol, we describe detailed steps on how to construct scChaRM-seq libraries (Figure 1) from mouse embryonic stem cells (ESCs). This protocol allows sequencing of up to 96 single-cell RNA (DNA) libraries in one batch, which is suitable for analyzing precious cell samples.

Note: Use RNaseZap and DNA-OFF to clean the workbench before starting the experiment.

Note: Multichannel pipettes are very useful to improve work efficiency and reduce the time costs.

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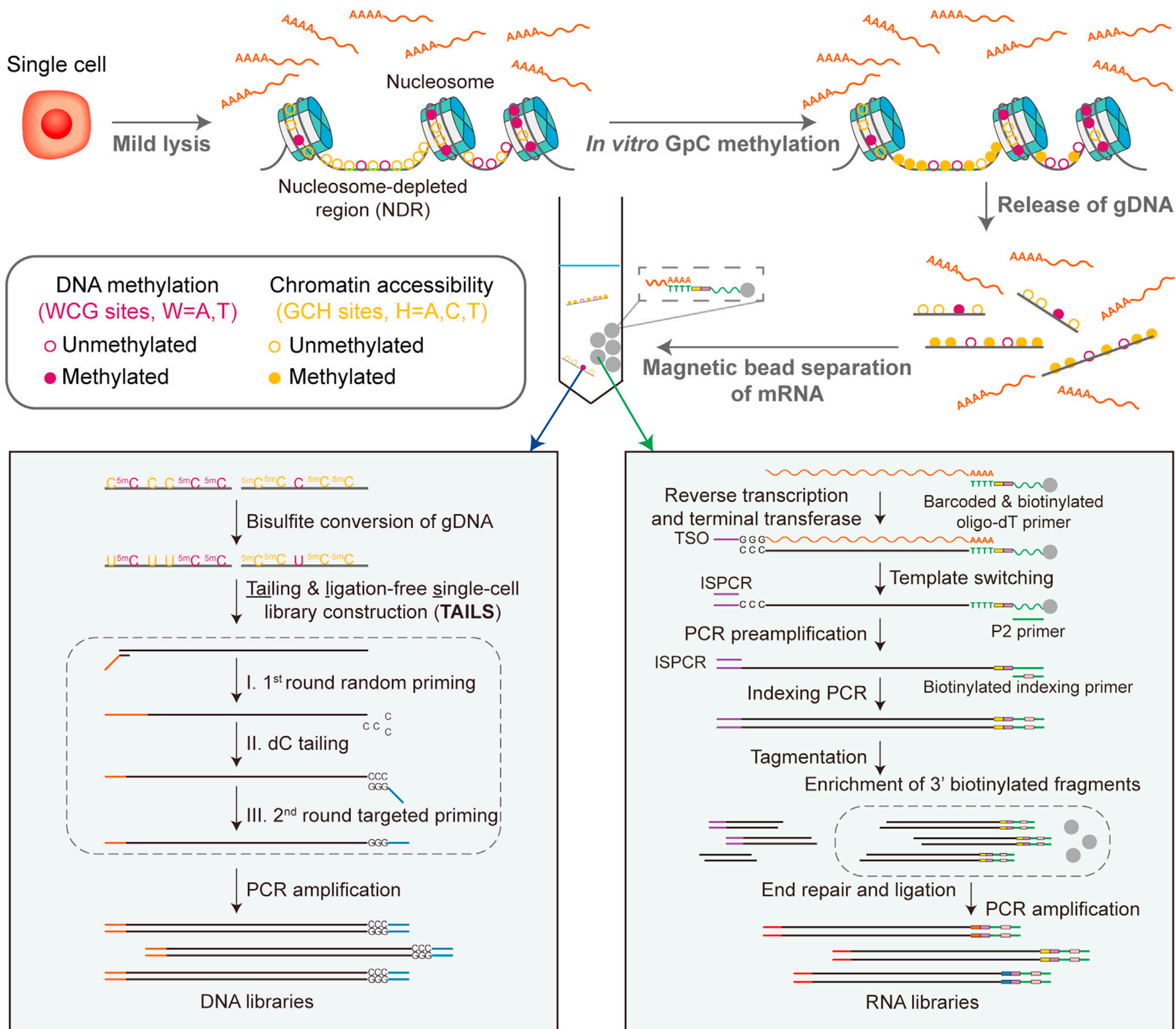

Figure 1. Scheme of scChaRM-seq

Individual cell was picked, lysed and underwent *in vitro* GpC methylation. mRNA was magnetically separated from released gDNA. Single-cell DNA and RNA were subjected to TAILS or RNA barcoding procedures respectively.

Note: Make sure that the pipette tips (filter tips) and centrifuge (or PCR) tubes used are both RNase-free and DNase-free.

Preparation of stock solutions

Timing: 1–2 h

Prepare the stock solutions according to "stock solution" in materials and equipment before the start of this experiment. Store them at a suitable temperature until use.

Conjugation of biotinylated oligo-dT primer to magnetic beads

Timing: 2–3 h

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Table 1. Twenty-four barcoded & biotinylated oligo-dT primers

Primer	Sequence
Biotin-oligodT-#1	/Biotin/TCAGACGTGTGCTCTTCCGATCTAACGTGATNNNNNNNNNT TTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#2	/Biotin/TCAGACGTGTGCTCTTCCGATCTAAACATCGNNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#3	/Biotin/TCAGACGTGTGCTCTTCCGATCTATGCCTAANNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#4	/Biotin/TCAGACGTGTGCTCTTCCGATCTAGTGGTCANNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#5	/Biotin/TCAGACGTGTGCTCTTCCGATCTACCACTGTNNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#6	/Biotin/TCAGACGTGTGCTCTTCCGATCTACATTGGCNNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#7	/Biotin/TCAGACGTGTGCTCTTCCGATCTCAGATCTGNNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#8	/Biotin/TCAGACGTGTGCTCTTCCGATCTCATCAAGTNNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#9	/Biotin/TCAGACGTGTGCTCTTCCGATCTCGCTGATCNNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#10	/Biotin/TCAGACGTGTGCTCTTCCGATCTACAAGCTANNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#11	/Biotin/TCAGACGTGTGCTCTTCCGATCTCTGTAGCCNNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#12	/Biotin/TCAGACGTGTGCTCTTCCGATCTAGTACAAGNNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#13	/Biotin/TCAGACGTGTGCTCTTCCGATCTAACAACCANNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#14	/Biotin/TCAGACGTGTGCTCTTCCGATCTAACCGAGANNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#15	/Biotin/TCAGACGTGTGCTCTTCCGATCTAACGCTTANNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#16	/Biotin/TCAGACGTGTGCTCTTCCGATCTAAGACGGANNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#17	/Biotin/TCAGACGTGTGCTCTTCCGATCTAAGGTACANNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#18	/Biotin/TCAGACGTGTGCTCTTCCGATCTACACAGAANNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#19	/Biotin/TCAGACGTGTGCTCTTCCGATCTACAGCAGANNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#20	/Biotin/TCAGACGTGTGCTCTTCCGATCTACCTCCAANNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#21	/Biotin/TCAGACGTGTGCTCTTCCGATCTACGCTCGANNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#22	/Biotin/TCAGACGTGTGCTCTTCCGATCTACGTATCANNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#23	/Biotin/TCAGACGTGTGCTCTTCCGATCTACTATGCANNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT
Biotin-oligodT-#24	/Biotin/TCAGACGTGTGCTCTTCCGATCTAGAGTCAANNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTT

1. For each of the 24 barcoded & biotinylated oligo-dT primers (Table 1): add 5 μL of Dynabeads™ MyOne™ Streptavidin C1 beads to a 1.5-mL DNA LoBind Tube.
2. Place the tube on a magnetic rack for 30 s, then remove and discard the supernatant.
3. Wash the beads with 200 μL of 2× B&W buffer on the magnetic rack and remove the supernatant.
4. Carefully remove the tube from the magnetic rack, add 5 μL of 2× B&W buffer and 5 μL of 100 μM barcoded & biotinylated Oligo-dT primer to cover the beads.
5. Re-suspend the beads with a vortexer, then incubate for 20 min at 25°C on a rotator.
6. Place the tube on a magnetic rack for 30 s, and then remove and discard the supernatant.

Table 2. Four biotinylated indexing primers

Primer	Sequence
Index-primer-#1	/Biotin/CAAGCAGAAGACGGCATACGAGATCTCTACGTGACTGGAG TTCAGACGTGTGCTCTTCCGATC
Index-primer-#2	/Biotin/CAAGCAGAAGACGGCATACGAGATGCTACCGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC
Index-primer-#3	/Biotin/CAAGCAGAAGACGGCATACGAGATGCTCATGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC
Index-primer-#4	/Biotin/CAAGCAGAAGACGGCATACGAGATTGCCATGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC

7. Wash the beads 4 times with 200 μL of 1× B&W buffer on the magnetic rack (Dilute the 2× B&W buffer using nuclease-free water).
8. Wash the beads once with 200 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, PH 7.5) on the magnetic rack.
9. Carefully remove the tube from the magnetic rack, add 40 μL of TE buffer and keep at 4°C for less than 1 week.

Note: A total number of twenty-four different barcoded & biotinylated oligo-dT primers were synthesized (Integrated DNA Technologies, Inc.). This step allows preparation of barcoded oligo-dT-C1 beads sufficient for 96 single cells.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Dynabeads™ MyOne™ Streptavidin C1	Thermo Fisher Scientific	Cat# 65002
UltraPure 1 M Tris-HCl, pH 7.5	Thermo Fisher Scientific	Cat# 15567-027
0.5 M EDTA, pH 8.0, RNase free	Thermo Fisher Scientific	Cat# AM9260G
dNTP mix	Thermo Fisher Scientific	Cat# R0192
Nuclease-free water	Thermo Fisher Scientific	Cat# AM9932
Tween-20 (50% solution)	Thermo Fisher Scientific	Cat# 003005
λDNA	Thermo Fisher Scientific	Cat# SD0021
Phenylmethanesulfonyl fluoride (PMSF)	Thermo Fisher Scientific	Cat# 36978
dCTP	Thermo Fisher Scientific	Cat# R0151
Terminal Deoxynucleotidyl Transferase (TDT)	Thermo Fisher Scientific	Cat# EP0162
Superscript II reverse transcriptase	Thermo Fisher Scientific	Cat# 18064071
5M Sodium chloride solution (NaCl)	Sigma-Aldrich	Cat# S5150
Betaine	Sigma-Aldrich	Cat# 61962-50G
Nonidet P-40 substitute	Sigma-Aldrich	Cat# 11332473001
Recombinant RNase Inhibitor (40 U/μL)	Takara Bio	Cat# 2313B
GpC Methyltransferase (M.CviPI)	New England Biolabs	Cat# M0227L
Buffer RLT Plus	Qiagen	Cat# 1053393
Carrier RNA	Qiagen	Cat# 1068337
Klenow (3′→ 5′ exo-)	Qiagen	Cat# P7010-HC-L
Magnesium chloride ($MgCl_2$)	VMR	Cat# J364-100G
2× KAPA HiFi HS ReadyMix	Roche	Cat# 7958935001
Exo-SAP IT Express	Applied Biosystems	Cat# 75001
AMPure XP beads	Beckman coulter	Cat# A63882
Ethanol, absolute	In house	N/A
Critical commercial assays		
DNA Clean & Concentrator-5 Kit	ZYMO RESEARCH	Cat# D4014
EZ-96 DNA Methylation-Direct MagPrep Kit	ZYMO RESEARCH	Cat# D5045

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Zymoclean Gel DNA Recovery Kit	ZYMO RESEARCH	Cat# D4008
NEBNext Ultra II DNA Library Prep Kit	New England Biolabs	Cat# E7645L
NEBNext Multiplex Oligos for Illumina (Index Primers Set 1)	New England Biolabs	Cat# E7335
NEBNext Multiplex Oligos for Illumina (Index Primers Set 2)	New England Biolabs	Cat# E7500
Qubit dsDNA high-sensitivity kit	Invitrogen	Cat# Q32851
Oligonucleotides		
TSO primer: AAGCAGTGGTATCAACGCAGAGTACATrGrG+G	Integrated DNA Technologies	N/A
ISPCR primer: AAGCAGTGGTATCAACGCAGAGT	Integrated DNA Technologies	N/A
P2 primer: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC	Integrated DNA Technologies	N/A
QP2 primer: CAAGCAGAAGACGGCATACGA	Integrated DNA Technologies	N/A
Short Universal primer: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC	Integrated DNA Technologies	N/A
P5-N6-oligo: CTACACGACGCTCTTCCGATCTN_6	Integrated DNA Technologies	N/A
P7-G6-oligo: AGACGTGTGCTCTTCCGATCTG_6HN	Integrated DNA Technologies	N/A
Other		
DNA LoBind Tubes, 1.5 mL	Eppendorf	Cat# 0030108051
DNA LoBind Tubes, 2.0 mL	Eppendorf	Cat# 0030108078
50-mL High Clarity PP Centrifuge Tube	Corning	Cat# 352070
0.2-mL Thin Wall PCR Tubes with Flat Cap	Axygen	Cat# PCR-02-C
Magnetic Rack for 0.2-mL PCR tubes	Thermo Fisher Scientific	Cat# 492025
Magnetic Rack for 2.0-mL centrifuge tubes	Thermo Fisher Scientific	Cat# 12321D
Blue Light Gel Imager	N/A	N/A
microTUBE Snap-Cap	Covaris	Cat# 520045
Qubit Fluorometer	Thermo Fisher Scientific	Cat# Q33216
Focused-ultrasonicator	Covaris	Cat# M220
Fragment analyzer	AATI	N/A
Stereo microscope	Nikon	SMZ1270
Centrifuge, refrigerated	Eppendorf	5425R
Thermal Cycler	Thermo Fisher Scientific	ProFlex

Note: We suppose that the listed reagents, kits and consumable items from alternatives of commercial suppliers could also be used instead.

MATERIALS AND EQUIPMENT

Stock solutions

Name	Reagents amount
PMSF (100 mM)	17.4 mg Phenylmethanesulfonyl fluoride (PMSF) in 1 mL isopropanol. Heat to 37°C to dissolve and store at −20°C (stable at least for 6 months).
1 M $MgCl_2$	952.1 mg $MgCl_2$, fill up to 10 mL with nuclease-free water and store at 4°C (stable at least for 6 months).
5 M Betaine	14.65 g Betaine, fill up to 25 mL with nuclease-free water and store at −20°C (stable at least for 6 months).
Elution buffer	500 μL Tris-HCl (1 M, pH 7.5), fill up to 50 mL with nuclease-free water and store at 4°C (stable at least for 6 months).

2× B&W buffer		
Reagent	Final concentration	Amount
Tris-HCl (1 M, pH 7.5)	10 mM	0.5 mL
EDTA (0.5 M)	1 mM	0.1 mL
NaCl (5 M)	2 M	20 mL
Nuclease-free water	n/a	29.4 mL
Total	**n/a**	**50 mL**
Store at 4°C (stable at least for 6 months).		

DNA wash buffer		
Reagent	Final concentration	Amount
5× Superscript II first-strand buffer	1×	2 mL
DTT (100 mM)	10 mM	1 mL
Tween-20 (50% solution)	0.05%	10 μL
Nuclease-free water	n/a	6.99 mL
Total	**n/a**	**10 mL**
Store at 4°C for up to 1 week. Right before use, add 10 μL RNase inhibitor (40 U/μL) per 1 mL of buffer.		

⚠ CRITICAL: Handle DTT in a draft chamber and wear protective gloves as it is toxic upon inhalation.

Work solutions

Re-suspend buffer		
Reagent	Final concentration	Amount
5× Superscript II first-strand buffer	1×	2 μL
RNase inhibitor (40 U/μL)	1 U/μL	0.25 μL
Nuclease-free water	n/a	7.75 μL
Total	**n/a**	**10 μL**
Prepare right before use.		

LM buffer		
Reagent	Final concentration	Amount
Tris-HCl (1 M, PH 7.5)	50 mM	0.125 μL
NaCl (5 M)	50 mM	0.025 μL
DTT (100 mM)	10 mM	0.25 μL
EDTA (5 mM)	0.25 mM	0.125 μL
10% NP-40	0.5%	0.125 μL
λDNA (1 pg/μL)	n/a	1.0 μL
PMSF (10 mM)	0.25 mM	0.063 μL
RNase inhibitor (40 U/μL)	1 U/μL	0.063 μL
M.CviPI (4 U/μL)	1 U/μL	0.625 μL
SAM (8 mM)	160 μM	0.05 μL
Nuclease-free water	n/a	0.049 μL
Total	**n/a**	**2.5 μL**
Prepare right before use.		

RT reaction buffer		
Reagent	Final concentration	Amount
Nuclease-free water	n/a	1.795 μL
dNTP mix (10 mM each)	1 mM each	0.5 μL
TSO primer (100 μM)	1 μM	0.05 μL
$MgCl_2$ (1 M)	6 mM	0.03 μL
Betaine (5 M)	1 M	1 μL
5× Superscript II first-strand buffer	1×	1 μL
DTT (100 mM)	5 mM	0.25 μL
Superscript II reverse transcriptase (200 U/μL)	10 U/μL	0.25 μL
RNase inhibitor (40 U/μL)	1 U/μL	0.125 μL
Total	**n/a**	**5 μL**

Prepare right before use.

PCR preamplification mixture		
Reagent	Final concentration	Amount
2× KAPA HiFi HS ReadyMix	1×	6.25 μL
ISPCR primer (10 μM)	0.2 μM	0.25 μL
P2 primer (10 μM)	0.6 μM	0.75 μL
Nuclease-free water	n/a	0.25 μL
Total	**n/a**	**7.5 μL**

Prepare right before use.

Indexing PCR mixture		
Reagent	Final concentration	Amount
2× KAPA HiFi HS ReadyMix	1×	25 μL
ISPCR primer (10 μM)	0.4 μM	2 μL
Indexing primer (10 μM)	0.4 μM	2 μL
Total	**n/a**	**29 μL**

Prepare right before use.

NEB final PCR mixture		
Reagent	Final concentration	Amount
2× NEBNext Ultra II Q5 Master Mix	1×	12.5 μL
QP2 primer (10 μM)	0.3 μM	0.75 μL
Short Universal primer (10 μM)	0.3 μM	0.75 μL
Total	**n/a**	**14 μL**

Prepare right before use.

1st round priming mixture		
Reagent	Final concentration	Amount
10× Blue buffer	1×	1.25 μL
Klenow (3′ → 5′ exo-)	4 U/μL	1 μL
P5-N6-oligo (10 μM)	0.4 μM	0.5 μL
dNTP (10 mM each)	0.4 mM	0.5 μL
Total	**n/a**	**3.25 μL**

Prepare right before use.

dC tailing mixture		
Reagent	Final concentration	Amount
Nuclease-free water	n/a	3.3 μL
10× Blue buffer	1×	0.5 μL
dCTP (100 mM)	1 mM	0.2 μL
TDT enzyme	1 U/μL	1 μL
Total	n/a	**5 μL**
Prepare right before use.		

2nd round priming mixture		
Reagent	Final concentration	Amount
Nuclease-free water	n/a	0.5 μL
Klenow (3′ → 5′ exo-)	4 U/μL	2 μL
10× Blue buffer	1×	0.5 μL
P7-G6-oligo (10 μM)	0.4 μM	1 μL
dNTP (10 mM each)	0.4 mM	1 μL
Total	n/a	**5 μL**
Prepare right before use.		

DNA final PCR mixture		
Reagent	Final concentration	Amount
2× KAPA HiFi HS ReadyMix	1×	12.5 μL
NEBNext Universal Primer (10 μM)	0.3 μM	0.75 μL
NEBNext Index Primer (10 μM)	0.3 μM	0.75 μL
Total	n/a	**14 μL**
Prepare right before use.		

Alternatives: This protocol uses Fragment analyzer (AATI) to assess the fragment size distribution of the RNA and DNA libraries. There are also several commercial capillary electrophoresis systems that can implement the analysis of library size distribution. For example, the Bioanalyzer (Agilent), P/ACE MDQ (Beckman) or LabChip (PerkinElmer). Researchers should choose appropriate instrument to analyze fragment size distribution of sequencing libraries.

STEP-BY-STEP METHOD DETAILS

The scChaRM-seq could be applied to various types of mammalian cells (if a cell contains poly-A tailed mRNA, DNA methylation modification and chromosomes), including cell lines and tissue cells. In this protocol, we took use of mouse embryonic stem cells (ES cells or ESCs) as an example cell line to describe step-by-step method details.

Preparation single-cell suspension of mouse ESCs

Timing: 3 days

Routine culture of mouse ES cells follows the published protocol (Samuelson and Metzger, 2006) under either 2i- or serum- containing media (Ficz et al., 2013). Single-cell suspension is prepared during propagation after cell detachment by using trypsin. The viability of mouse ES cells should be above 95% (Figure 2).

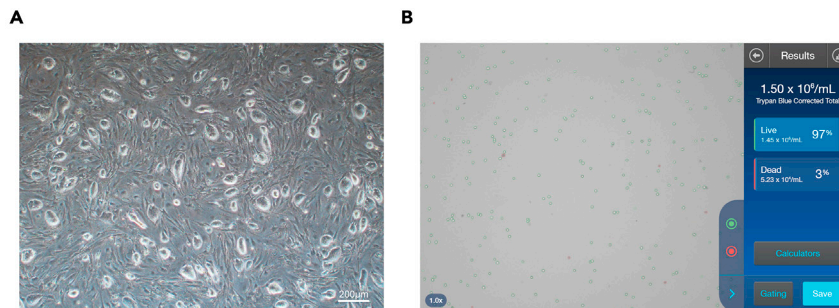

Figure 2. Typical morphology and viability of mouse ES cells
(A) Clone morphology of mouse ES cells.
(B) Examination of cell viability by trypan blue staining (CountessTM 3 FL Automated Cell Counter).

Single-cell lysis and *in vitro* GpC methylation

⏱ Timing: 1–2 h

Note: All the buffer and reaction mixtures should be prepared on ice.

1. Prepare lysis & *in vitro* methylation buffer (LM buffer) for individual cell.
2. Add 2.5 µL LM buffer to each 0.25-mL PCR tube and place the tube on ice.
3. Manually pick individual cell under the stereo-microscope with mouth-pipette, and transfer single cell into 0.25-mL PCR tube containing ice-cold LM buffer.

Note: One needs practice to perform step 3. For how to use mouth-pipette to manually selecting single cells, please refer to Lipovsek et al. (2020) as a learning example (Lipovsek et al., 2020).

4. Vortex briefly and centrifuge at 1,000× *g* for 1 min at 4°C, then immediately place the tube back on ice.
5. Incubate single cell sample in a thermal cycler at 37°C for 15 min.
6. After incubation, centrifuge at 7,500 × *g* for 1 min at 4°C and put the sample back on ice.
7. Add 5 µL of Buffer RLT Plus to each single cell sample.
8. Vortex briefly and incubate the mixture at 25°C for 5 min.
9. Quickly centrifugation (7,500 × *g* for 30 s) and proceed to the next step, or transfer samples to a −80°C freezer.

Pause point: The *in vitro* methylated cell samples can be stored at −80°C for several days.

⚠ CRITICAL: Because PMSF is toxic, wear protective gloves, eye/face protection and protective clothing to avoid accidentally inhalation while handling.

Separation of mRNA from lysate

⏱ Timing: 0.5–1 h

10. For each barcoded oligo-dT-C1 beads: transfer 10 µL of oligo-dT-C1 beads to a 0.25-mL PCR tube, and place it on a magnetic rack for 1 min, and then remove and discard the supernatant.
11. Remove tube from magnetic rack, re-suspend the beads with 10 µL of re-suspend buffer and keep it on ice.
12. Add 10 µL of oligo-dT-C1 beads to the 7.5 µL of single cell lysate.

13. Vortex briefly and incubate for 10 min at 25°C.
14. Briefly spin down the sample on a benchtop mini centrifuge, place the tube on a magnetic rack for 1 min and transfer the supernatant to a new 0.25-mL PCR tube, keep it on ice.
15. Wash the beads on the magnetic rack once with 30 μL of DNA wash buffer for 5 min and transfer the supernatant to the same tube containing gDNA from a single cell.
16. Collect the released gDNA from a single cell (47.5 μL in total) on ice and keep it at −80°C until further processing.

Pause point: The gDNA can be stored at −80°C for several weeks.

⚠ CRITICAL: The mRNA captured on the oligo-dT-C1 beads needs to be processed to the next step immediately.

Reverse transcription

Timing: 2 h

17. Prepare the reverse transcription reaction buffer (RT reaction buffer).
18. Add 5 μL of RT reaction buffer to the remaining beads containing mRNA from a single cell.
19. Vortex briefly and spin down quickly.
20. Incubate the reaction in a thermal cycler with a heated lid (105°C), as detailed below:

PCR cycling conditions

Steps	Temperature	Time	Cycles
1	25°C	5 min	1
2	42°C	60 min	1
3	50°C	30 min	1
4	70°C	10 min	1
5	4°C	Hold	

PCR preamplification

Timing: 3 h

21. Centrifuge the RT sample at 7,500 × *g* for 1 min.
22. Prepare the PCR preamplification mixture for a single cell.
23. Add 7.5 μL of PCR preamplification mixture to each single-cell sample. Vortex briefly and spin down quickly.
24. Perform the PCR in a thermal cycler (lid temp. 105°C) by using the following program:

PCR cycling conditions

Steps	Temperature	Time	Cycles
1	95°C	3 min	1
2	98°C	20 s	4 cycles
3	65°C	30 s	
4	72°C	5 min	
5	98°C	20 s	18 cycles
6	67°C	15 s	
7	72°C	5 min	
8	72°C	5 min	1
9	4°C	Hold	

25. Quantification the concentration of single-cell cDNA by the Qubit Fluorometer.

Note: The total number of PCR cycles depends on the RNA content in different type of cells. 18 cycles indicated in this protocol are suitable for mouse embryonic stem cells. The number of cycles can be increased for cells which have limited mRNA (e.g., cumulus cells, 20 cycles) or decreased for cells which have abundant mRNA (e.g., oocytes, 16 cycles).

Pause point: PCR products can be stored at −20°C for several weeks.

cDNA purification

Timing: 1–1.5 h

26. Pool 6 μL of the PCR products from each single-cell sample which are labeled by one of twenty-four different barcodes.
27. Purify once with Zymo DNA Clean & Concentrator-5 Kit according to the manufacturer's protocol, elute in 50 μL elution buffer.
28. Quantification the concentration of single-cell cDNA by the Qubit Fluorometer.
29. Add 40 μL of equilibrated AMPure XP beads to each single-cell sample and mix by vortex to make the solution homogeneous.
30. Incubate the mixture for 10 min at 25°C to let the DNA bind to the beads.
31. Place the tube on the magnetic rack for 5 min until the solution is clear and the beads have been collected at one corner of the well.
32. Carefully remove and discard the liquid without disturbing the beads. Wash the beads twice with 200 μL 80% (vol/vol) ethanol solution.
33. Remove any trace of ethanol and let the beads dry completely (usually 3–5 min).
34. Before removing tube from the magnetic rack, add 30 μL elution buffer. Mix by vortex, incubate the tube off the magnetic rack for 2 min.
35. Place the tube on the magnetic rack and leave it until the solution appears clear and beads have accumulated in a corner of the well.
36. Collect the supernatant without disturbing the beads and transfer it to a fresh 1.5-mL DNA LoBind Tube.
37. Measure the concentration of cDNA by the Qubit Fluorometer.

Pause point: Single-cell cDNA can be stored at −20°C for several weeks.

Quality check of the cDNA

Timing: 1 h

38. Check the size distribution of the cDNA on a Fragment analyzer. A good cDNA sample should show a peak approximately at 1–2 kb, and the yield of the cDNA sample is approximately more than 50 ng.

Indexing PCR amplification

Timing: 1 h

39. Prepare the indexing PCR (Table 2) mixture for pooled single-cell cDNA. Use ~40 ng cDNA as template, add 29 μL of indexing PCR mixture to each single-cell sample to a total volume of 50 μL. Vortex briefly and spin down quickly.
40. Perform the PCR in a thermal cycler (lid temp. 105°C) by using the following program:

PCR cycling conditions			
Steps	Temperature	Time	Cycles
1	95°C	3 min	1
2	98°C	20 s	3 cycles
3	67°C	15 s	
4	72°C	5 min	
5	72°C	5 min	1
6	4°C	Hold	

41. Add 40 µL of equilibrated AMPure XP beads to PCR products, purify once and elute with 30 µL elution buffer (For detailed steps of purifying DNA with AMPure XP beads, please refer to "cDNA purification" above).
42. Quantification the cDNA concentration by the Qubit Fluorometer.

Pause point: PCR products can be stored at −20°C for several weeks.

cDNA library construction

Timing: 3 h

43. Following the manufacturer's protocol, fragmentation of cDNA sample by the Covaris™ to achieve peak distribution around 300–350 bp. The default programs and settings are pre-installed by the manufacturer, and could be easily selected to use.
44. After sonication, purify once with Zymo DNA Clean & Concentrator-5 Kit according to the manufacturer's protocol, elute in 50 µL elution buffer.
45. Purify once with 50 µL of equilibrated AMPure XP beads, elute in 50 µL nuclease-free water (For detailed steps of purifying DNA with AMPure XP beads, please refer to "cDNA purification" above).
46. Take 10 µL of Dynabeads™ MyOne™ Streptavidin C1 beads into 1.5-mL tube, wash the beads once with 200 µL 2× B&W buffer on magnetic rack, re-suspend beads with 50 µL 2× B&W buffer by vortex.
47. Incubate 50 µL of eluted cDNA sample with re-suspended beads at 25°C for 30 min.
48. After incubation, put each tube on magnetic rack, then discard the supernatant.
49. Add 100 µL 1× B&W buffer to wash beads, then add 100 µL elution buffer to wash beads again. Re-suspend beads in 50 µL nuclease-free water and keep it on ice.
50. Following the manufacturer's protocol of the NEBNext Ultra II DNA Library Prep Kit, then perform "End repair" and "Adapter ligation" steps.
51. After incubation with the USER enzyme for 15 min, spin down and put each tube on magnetic rack, discard the supernatant.
52. Add 100 µL elution buffer to wash beads, then discard the supernatant.
53. Re-suspend beads in 11 µL nuclease-free water.

PCR amplification

Timing: 2 h

54. Add 11 µL of adaptor ligated cDNA fragments to 14 µL of NEB final PCR mixture.
55. Perform PCR amplification (lid temp. 105°C) as follows:

PCR cycling conditions			
Steps	Temperature	Time	Cycles
1	98°C	30 s	1
2	98°C	10 s	9 cycles
3	65°C	75 s	
4	65°C	5 min	1
5	4°C	Hold	

56. Purify the PCR mix twice by equilibrated AMPure XP beads and elute in 30 μL of elution buffer (For detailed steps of purifying DNA with AMPure XP beads, please refer to "cDNA purification" above).
57. Measure the concentration of RNA library by the Qubit Fluorometer.

Quality check of the final RNA library

Timing: 1 h

58. Check the size distribution of the final RNA library on a Fragment analyzer. A good RNA library is approximately more than 100 ng, and usually ranges from 200 bp to 800 bp.

Purification of genomic DNA

Timing: 1 h

59. Purify the gDNA with 47.5 μL of equilibrated AMPure XP beads (For detailed steps of purifying DNA with AMPure XP beads, please refer to "cDNA purification" above). Elute gDNA with 10 μL of nuclease-free water, and transfer the gDNA to a new 0.25-mL PCR tube.

Bisulfite conversion of gDNA

Timing: 3.5 h

60. Use EZ-96 DNA Methylation-Direct MagPrep Kit following the manufacturer's protocol to conduct CT conversion of gDNA from single cell. DNA was bisulfite converted as follows:

PCR cycling conditions			
Steps	Temperature	Time	Cycles
1	98°C	8 min	1
2	64°C	3.5 h	1
3	4°C	hold	

61. Add 10 ng of carrier RNA to each sample before purification and elute DNA with 10 μL of elution buffer.
62. Incubate at 55°C for 4 min and transfer 9.25 μL to a new 0.25-mL PCR tube.

DNA libraries construction by TAILS

Timing: 5 h

63. Incubate the 9.25 μL of bisulfite converted and purified gDNA at 65°C for 3 min; 4°C for pause to stretch the DNA strand.
64. Add 3.25 μL of 1st round priming mixture to each single-cell DNA sample.
65. Perform the first random priming as follows:

PCR cycling conditions			
Steps	Temperature	Time	Cycles
1	4°C	5 min	1
2	20°C	5 min	1
3	37°C	60 min	1
4	4°C	Hold	

66. Add 3 μL of Exo-SAP IT Express to each single-cell sample to remove the remaining primers as follows:

PCR cycling conditions			
Steps	Temperature	Time	Cycles
1	37°C	60 min	1
2	80°C	10 min	1
3	4°C	Hold	

67. After incubation, transfer immediately to ice. Add 5 μL of dC tailing mixture to single-cell sample.
68. Incubate sing-cell sample as follows:

PCR cycling conditions			
Steps	Temperature	Time	Cycles
1	37°C	15 min	1
2	70°C	15 min	1
3	95°C	90 s	1
4	4°C	Hold	

69. After incubation, transfer immediately to ice. Add 5 μL of 2nd round priming mixture to single-cell sample.
70. Synthesize the second DNA strands as follows:

PCR cycling conditions			
Steps	Temperature	Time	Cycles
1	4°C	5 min	1
2	20°C	5 min	1
3	37°C	90 min	1
4	4°C	hold	

71. After incubation, transfer immediately to ice.

Pause point: After the second-round priming, samples can be stored at −20°C for 1 day.

Purification and PCR amplification

Timing: 1.5–2 h

72. Purify the single-cell DNA samples once with equilibrated AMPure XP beads, elute with 12 μL of nuclease-free water and transfer 11 μL to a new 0.25-mL PCR tube (For detailed steps of purifying DNA with AMPure XP beads, please refer to "cDNA purification" above).
73. Add 14 μL DNA final PCR mixture to the 11 μL of purified DNA sample, vortex and spin down briefly.
74. Perform the PCR amplification (lid temp. 105°C) as follows:

PCR cycling conditions

Steps	Temperature	Time	Cycles
1	95°C	3 min	1
2	98°C	20 s	20 cycles
3	65°C	30 s	
4	72°C	1 min	
5	72°C	3 min	1
6	4°C	Hold	

Pooling and gel recovery

Timing: 2–2.5 h

75. Pooling the PCR products together: 12.5 μL of each single-cell's DNA for 24 samples.
76. Purify with Zymo DNA Clean & Concentrator-5 Kit according to the manufacturer's protocol, elute in 20 μL of nuclease-free water.
77. After 1.5% agarose electrophoresis, recover the DNA between 400-bp and 1,000-bp by using the Zymoclean Gel DNA Recovery Kit according to the manufacturer's protocol.
78. Purify the DNA with equilibrated AMPure XP beads, elute with 20 μL of elution buffer (For detailed steps of purifying DNA with AMPure XP beads, please refer to "cDNA purification" above).

Quality check of the final DNA library

Timing: 1 h

79. Quantify the libraries and check the size distribution with a Fragment Analyzer. The yield of the DNA library is approximately more than 30 ng, and the size distribution is between 400 bp and 1 kb.

EXPECTED OUTCOMES

This protocol describes the construction of scChaRM-seq libraries. The constructed libraries can be applied for next-generation sequencing (NGS) to obtain multi-omics datasets including transcriptome, DNA methylome and chromatin accessibility. For more details, please refer to our recent publications (Yan et al., 2021).

The yield of the RNA and DNA libraries can be firstly analyzed using Qubit Fluorometer. Generally, the yield of the RNA library is approximately more than 100 ng, and the yield of the DNA library is approximately more than 30 ng.

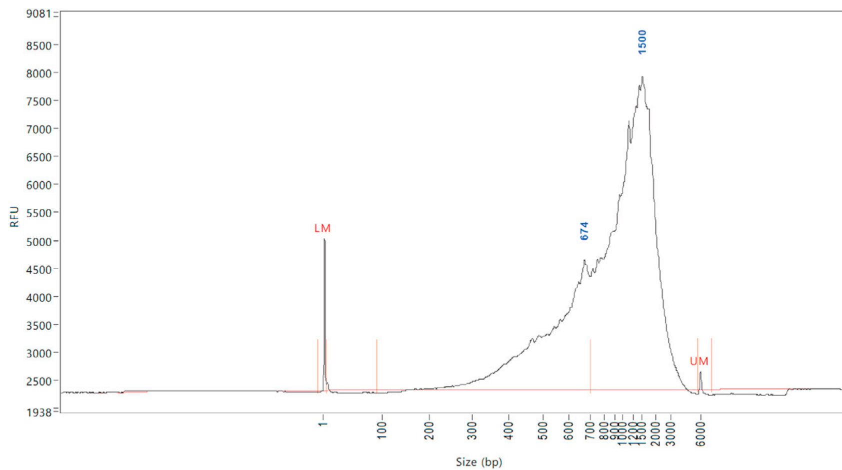

Figure 3. The size distribution of scChaRM-seq cDNA
A typical result of scChaRM-seq cDNA sample from the AATI Fragment Analyzer.

The fragment analyzer can be used to check the size distribution of both the DNA and RNA libraries. When construction of the RNA library, inspect the quality of the cDNA are also necessary. A good cDNA sample should show a peak approximately at 1–2 kb (Figure 3).

For the final RNA library, the distribution of the fragments usually ranges from 200 bp to 800 bp, the expected peak should be around 400 bp (Figure 4).

For the DNA library, a peak will be observed around 600bp and the size distribution is between 400 bp and 1 kb (Figure 5). It is important that the lower molecular weight fragments (below 150bp) should not be present in both RNA and DNA libraries, this will affect sequencing results (Figures 4 and 5).

LIMITATIONS

The scChaRM-seq method has achieved multi-omics profiling with moderate throughput, combining the multiplex sequencing of single-cell RNA with DNA methylation and chromatin accessibility. However, the scChaRM-seq is on the low throughput for scRNA-seq compared with other single-cell RNA-seq methods such as 10× Genomics platform (Zheng et al., 2017). Efforts on elevating the throughput for single-cell multi-omics sequencing are still needed.

TROUBLESHOOTING

Problem 1

The cell viability is low (step 3).

Potential solution

In general, cells should be at least 90% viable before use. When the cell viability is low, RNA quality from single cells may be poor. We usually use automated cell counter to measure cell viability; one could also use a blood counting chamber for cell counting after trypan blue staining.

Problem 2

The yield of the cDNA sample is very low (step 37).

Potential solution

Generally, the yield of the cDNA sample is approximately more than 50 ng when quantified using Qubit Fluorometer. If you got a very low cDNA yield, it might be because the total number of

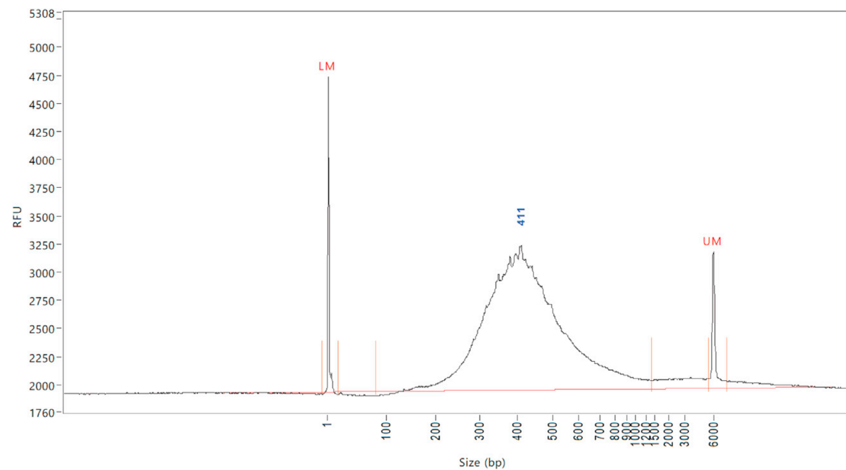

Figure 4. The size distribution of scChaRM-seq RNA library

A typical result of scChaRM-seq RNA library from the AATI Fragment Analyzer.

PCR cycles is not enough. The number of PCR cycles depends on the RNA content in different type of cells. 18 cycles indicated in this protocol are suitable for mouse embryonic stem cells. You can increase the number of cycles for cells which have limited mRNA.

Problem 3

The quality of the cDNA sample is poor (step 38).

Potential solution

As shown in Figure 3, a good cDNA sample should show a peak approximately at 1–2 kb. If your cDNA sample showed a huge peak lower than 500bp, the mRNA might be degraded. You can use RNase*Zap* to clean work bench and equipment before starting the experiment. Make sure that the pipette tips (filter tips) and centrifuge (or PCR) tubes are both RNase-free. Besides, it is also very important to examine the cell viability before picking up single cells and perform downstream experiments.

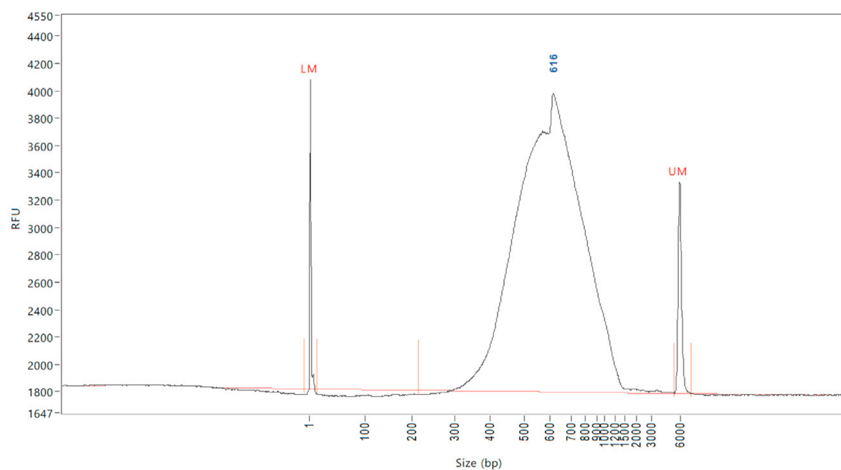

Figure 5. The size distribution of scChaRM-seq DNA library

A typical result of scChaRM-seq DNA library from the AATI Fragment Analyzer.

Problem 4

The DNA recovery efficiency is low (step 59–78).

Potential solution

During the construction of single-cell DNA libraries, the amount of input DNA is very limited. Therefore, one needs to ensure that DNA is efficiently recovered in the purification steps. It's better to use the DNA LoBind tubes to store DNA samples. Moreover, when introducing the DNA recovery kit or AMPure XP beads for purification procedures, ensure those reagents are still in usable date and follow the manufacturer's instructions.

Problem 5

The yield of the DNA library is very low (step 79).

Potential solution

Generally, the yield of the DNA library is approximately more than 30ng when quantified using Qubit Fluorometer. If you got a very low DNA yield, there might be two possible reasons. The first one is the loss of input gDNA during purification steps, you may try to use DNA low-bind tubes and increase the number of PCR cycles. The second one is gDNA might be degraded by DNase, you should use DNase-free tips and tubes when handling single-cell DNA samples. Additionally, you can use reagent to minimize DNase contamination from work bench before starting the experiment. The amount of the gDNA from a single cell is very limited, it is important to avoid loss of input gDNA when handling single-cell samples.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Fan Guo (guofan@ioz.ac.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

For complete details on data analysis or an example of datasets generated by using scChaRM-seq, please refer to Yan et al. (2021).

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AUTHOR CONTRIBUTIONS

F.G. developed the scChaRM-seq method. R.Y. and X.C. performed the experiments. F.G. and R.Y. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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