Chapter 1

Single-Cell Transcriptomics of Immune Cells: Cell Isolation and cDNA Library Generation for scRNA-Seq

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Abstract

Single-cell RNA-sequencing (scRNA-seq) enables a comprehensive analysis of the transcriptome of individual cells by next-generation sequencing. ScRNA-seq offers an unbiased approach to investigate the cellular heterogeneity and dynamics of diverse biological systems, including the immune system. Optimization of the technical procedures performed prior to RNA-seq analysis is imperative to the success of a scRNA-seq experiment. Here, three major experimental procedures are described: (1) the isolation of immune CD8a$^+$ T cells from primary murine tissue, (2) the generation of single-cell cDNA libraries using the 10× Genomics Chromium Controller and the Chromium Single Cell 3′ Solution, and (3) cDNA library quality control. In this protocol, CD8a$^+$ T cells are isolated from murine spleen tissue, but any cell type of interest can be enriched and used for single-cell cDNA library generation and subsequent RNA-seq experiments.

Key words Single-cell suspension, CD8a$^+$ T cells, Single-cell cDNA libraries, 10× Genomics Chromium™, Single-cell RNA-seq

1 Introduction

The application of single-cell gene expression profiling technologies to immunological studies has revolutionized our molecular understanding of cell development, differentiation, and role of the immune system in health and disease. Single-cell RNA-sequencing (scRNA-seq) has provided a high-resolution investigation of the whole transcriptome of single cells, permitting the identification of novel regulators of immune cell differentiation and the interrogation of immune cellular heterogeneity [1–4]. In 2009, Tang et al. first published data analyses on whole-transcriptome sequencing of a single mouse cell, revealing novel insights into the complexity of the transcriptome at the single-cell level [5]. Over the past decade, numerous scRNA-seq platforms have been developed, including plate-based technologies such as STRT-seq [6], SMART-seq [7], SMART-seq2 [8], MARS-seq [9], CEL-seq [10],

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commercial microfluidics platform Fluidigm C1, and droplet-based and microwell technologies, including Drop-seq [11], InDrop [12], Seq-well [13], Microwell-seq [14], and commercial 10× Genomics Chromium Controller [15]. The technical details of these technologies have been extensively reviewed [16, 17]. Of importance, the success of a scRNA-seq experiment, irrespective of the type of platform used, is highly dependent on the integrity of the single-cell suspension obtained for analysis and quality of the single-cell cDNA libraries for sequencing, which are generated from these cells.

Here, the isolation of $CD8a^+$ T-cell suspensions from murine tissue, followed by single-cell cDNA library generation and library quality control procedures, are overviewed. In this protocol, cDNA library generation is performed using the 10× Genomics Chromium Controller and the Chromium Single Cell 3′ Solution Reagents Kit. The 10× Genomics Chromium Controller is a microfluidics platform, which enables gene expression profiling of 500–10,000 single cells per sample. GemCode Technology incorporates 10× barcodes to index the transcriptome of individual cells. Input cells are partitioned into gel bead-in-emulsions (GEMs), enabling the simultaneous generation of sequence-ready cDNA libraries of ≥500 cells. To produce GEMs, the 10× barcoded gel beads are combined with the 10× genomics master mix, sorted cells of interest ($CD8a^+$ T cells, as detailed in this chapter), and partitioning oil; then added onto the 10× Genomics Chromium Chip B, as designated in the Chromium Single Cell 3′ Solution Reagents Kits v3 user guide; and placed into the Chromium Controller. The incubation of the GEMs with 10× genomics reverse transcription reagents generates full-length cDNA from the polyadenylated mRNAs of one cell, all of which share the same 10× barcode and contain a unique molecular identifier (UMI). Following incubation, the GEMs are broken and first-strand cDNA is purified using Dynabeads MyOne SILANE magnetic beads. A PCR amplification of the barcoded, full-length cDNA is then performed to produce a sufficient cDNA yield for library generation. The construction of cDNA libraries involves fragmentation and size selection of the cDNA amplicons, end repair, A-tailing, adaptor ligation, and PCR to incorporate a sample index (the P5 and P7 primers, which are compatible with Illumina sequencing technology). cDNA quantification and cDNA library quality control are assessed using the Agilent 2100 Bioanalyzer and Qubit Fluorometer. The resulting single-cell cDNA libraries in this protocol can then be sequenced on Illumina Sequencers (MiSeq, NextSeq 500/550, HiSeq 2500, HiSeq 3000/4000, and NovaSeq).

2 Materials

Recommendations for working with associated materials in these experiments are as follows. Prepare all working solutions in a regulated biosafety cabinet to keep sterile. Store cell solutions and all other reagents at 4 °C, or as indicated. For cDNA library generation and quality control assessment, ensure that the laboratory workspace is clean and free of debris. Wipe down the workspace and all pipettes used with RNase cleaner prior to the start of the experiment. Use nuclease-free filtered pipette tips and gloves at all times.

2.1 The Isolation of Immune CD8a$^+$ T Cells from Murine Tissue

1. Cell media: Hanks balanced salt solution (HBSS) supplemented with 1% fetal bovine serum (FBS).
2. Red blood cell lysis buffer: Store at room temperature.
3. Cell isolation buffer: 1× Phosphate buffer saline, 0.5% FBS or 0.5% BSA, and 2 mM EDTA, pH 7.2–8.0.
4. Biotin-labeled antibodies against non-CD8a$^+$ T cells (MACS Miltenyi Biotec mouse CD8a$^+$ T cell isolation kit).
5. Anti-biotin microbeads.
6. MACS Miltenyi Biotec LS cell separation column: Store at room temperature in the dark.
7. MACS Miltenyi Biotec Manual Separator for magnetic bead isolation.
8. FACS Buffer: 1× Phosphate buffer saline, 5% FBS, 0.1% NaN_3 sodium azide, pH 7.2–8.0.
9. FITC anti-mouse CD3ε and APC anti-mouse CD8a$^+$ T-cell antibodies.
10. 1× PBS: Store at room temperature.
11. 15 and 50 mL conical tubes.
12. 1.5 mL Microcentrifuge tubes.
13. 70 μm Cell strainer.
14. Sterile 3 mL syringe.
15. 60 mm (diameter) × 15 mm (height) petri dish.
16. 5 and 10 mL serological pipettes.
17. P10, P200, P1000 pipettes and corresponding pipette tips.
18. Corning Falcon Test Tube with Cell Strainer Snap Cap.
19. Flow cytometer.

2.2 The Generation of Single-Cell cDNA Libraries

1. PCR tubes (0.2 mL 8-tube strips and caps).
2. 1.5 mL Nuclease-free microcentrifuge tubes.
3. Nuclease-free water.
4. Buffer EB: 10 mM Tris–Cl, pH 8.5, store at room temperature.

5. Pure ethanol (200 Proof, anhydrous): Store at room temperature in appropriate flammable-regulated storage area.
6. SPRIselect Reagent Kit (Beckman Coulter): Store at room temperature.
7. 50% Glycerol.
8. Vortex mixer.
9. Minicentrifuge with PCR 8-tube strip adapter.
10. Thermal cycler.
11. 10× Genomics Chromium Single Cell 3′ Reagents Kit: Store reagents at either room temperature, 4 °C, −20 °C, or −80 °C as indicated in the 10× Genomics Chromium Single Cell 3′ Reagents v3 user guide.
12. 10× Genomics Chromium Chip B and gasket.
13. 10× Genomics Chromium Controller.
14. 10× Genomics magnet.
15. Dynabeads MyOne SILANE (Thermo Fisher Scientific): Store at 4 °C.
16. Agilent 2100 Bioanalyzer instrument.
17. Agilent Bioanalyzer High Sensitivity DNA kit: Store reagents at 4 °C in the dark and Agilent Bioanalyzer High Sensitivity Chips at room temperature.

2.3 cDNA Library Quality Control

1. Agilent Bioanalyzer High Sensitivity DNA chip and kit reagents.
2. Agilent 2100 Bioanalyzer instrument.
3. Qubit dsDNA HS (High Sensitivity) Assay Kit (Invitrogen): Store kit reagents at 4 °C.
4. Qubit Fluorometer (Invitrogen).

3 Methods

3.1 The Isolation of Immune CD8a$^+$ T Cells from Murine Tissue

1. In a biosafety cabinet, mash spleen tissue in a 60 mm (diameter) × 15 mm (height) petri dish in 5 mL of cell media with the flat end of a sterile 3 mL syringe. Using a 5 mL serological pipet, pipet up the tissue suspension and pass through a 70 μm cell strainer placed atop a 50 mL conical tube. Add 2 mL of cell media to rinse the petri dish to attain as much of the tissue suspension and pass the excess tissue suspension through the same 70 μm strainer. Centrifuge the 50 mL conical tube at 300 × *g* for 5 min at 4 °C. Discard supernatant, leaving behind a red cell pellet.

2. To the red cell pellet, add 1 mL of red blood cell lysis buffer, vortex quickly to mix, and incubate at room temperature for 4 min. Following the 4-min incubation, add 3 mL of cell media to the conical tube and centrifuge it at 300 × *g* for 1 min at 4 °C. Remove and discard supernatant. Resuspend the cell pellet in 1 mL of cell isolation buffer. Count cells.
3. Perform enrichment of $CD8a^+$ T cells by incubating the spleen-derived cell suspension with a combination of biotin-conjugated antibodies against CD4, CD11b, CD11c, CD19, CD45R (B220), CD49b, anti-MHC-class II, Ter-119, and TCR γ/δ (*see* **Note 1**). This antibody cocktail is available from MACS Miltenyi Biotec as the mouse $CD8a^+$ T-cell isolation kit. Isolate $CD8a^+$ T cells by the depletion of magnetically labeled non-$CD8a^+$ T cells. Resuspend the cell pellet at 10^7 cells per 40 μL of cell isolation buffer (*see* **Note 2**) and transfer the cell suspension to a 1.5 mL microcentrifuge tube. Add 10 μL of the antibody cocktail to this cell suspension. Gently pipette mix the cell suspension mixture five times. Incubate the cells and the antibody mixture on ice for 10 min. Centrifuge the cell suspension at 400 × *g* for 1 min and discard the supernatant. Wash the cell pellet with 1 mL of cell isolation buffer, gently pipetting the cells to mix. Centrifuge the cells at 300 × *g* for 1 min and discard the supernatant.
4. Resuspend the cells in 30 μL of cell isolation buffer per 10^7 cells. Add 20 μl of an anti-biotin microbeads per 10^7 cells. Gently pipette the cell mixture five times to mix. Incubate on ice for 10 min. Centrifuge the cell suspension at 400 × *g* for 1 min and discard the supernatant. Wash the cell pellet with 1 mL of cell isolation buffer, gently pipetting the cells to mix. Centrifuge the cells at 300 × *g* for 1 min and discard the supernatant. Resuspend the cell pellet in 500 μL of cell isolation buffer and keep on ice.
5. Place an LS cell separation column in the magnetic field of the appropriate MACS Separator (MACS Miltenyi Biotec) and an empty 15 mL conical tube below the column for sample collection. Wash the column with 3 mL cell isolation buffer (*see* **Note 3**). Add 500 μL cell suspension to the column and collect the flow-through in the 15 mL conical tube. This is the negative fraction, containing the unlabeled $CD8a^+$ T cells (*see* **Note 4**). Wash the column three times, each with 3 mL of cell isolation buffer.
6. Centrifuge the 15 mL conical tube containing the unlabeled $CD8a^+$ T cells at 300 *g* for 5 min at 4 °C. Aspirate the supernatant carefully, leaving behind approximately 0.5 mL of supernatant. Gently resuspend the cell pellet in the remaining 0.5 mL and transfer single-cell suspension to a new 1.5 mL

microcentrifuge tube. Centrifuge the microcentrifuge tube at 300 × *g* for 1 min at 4 °C. Remove supernatant carefully so as not to disrupt the cell pellet. Resuspend the cell pellet in 1 mL cell media and count the cells.

7. Use flow cytometry to confirm the purity of the enriched $CD8a^+$ T cells and to sort the purified $CD8a^+$ T-cell suspension for downstream library preparation and RNA sequencing. To stain the enriched $CD8a^+$ T cells for FACS sorting, first resuspend the cells at a concentration of 1–5 million cells/mL in 100 μL ice-cold staining buffer in a 1.5 mL microcentrifuge tube (*see* **Note 5**). Add 1 μL of FITC anti-mouse CD3ε and 1 μL APC anti-mouse $CD8a^+$ T-cell antibody to the cell suspension at a 1:100 ratio (*see* **Note 6**). Gently pipette up and down the cell suspension and antibody mixture to mix well. Incubate the cells and antibody mixture for at least 15 min on ice in the dark.
8. Following the 15-min incubation on ice, centrifuge the tube containing the cell suspension and antibody mixture at 300 × *g* for 1 min at 4 °C in a microcentrifuge. Discard the supernatant and add 0.5 mL ice-cold 1× PBS to the cells to wash. Centrifuge the cells at 400 × *g* for 1 min at 4 °C, and repeat with a second wash. Resuspend the cells in ~300 μL cell media. Filter the single-cell suspension using a Corning Falcon Test Tube with Cell Strainer Snap Cap (*see* **Note 7**).
9. Place the sample on ice and sort $CD8a^+$ T-cell-positive, CD3-ε-positive cells into cell media. Exclude dead cells and doublets during cell sorting. Record cell viability percentage and cell count of sorted purified $CD8a^+$ T cells. Keep sorted cells on ice.

3.2 Generation of Single-Cell cDNA Libraries

The generation of single-cell cDNA libraries for subsequent RNA-sequencing is performed according to the 10× Genomics Chromium Single Cell 3′ Reagents Kits v3 user guide, using the Chromium Controller and Chromium Single Cell Gene Expression Solution. It is recommended that the user follow the extensively detailed protocol in the Chromium Single Cell 3′ Reagents Kits v3 user guide, particularly for 10× Genomics master mix compositions as indicated in the procedures below. All steps are to be performed on the laboratory bench.

1. Centrifuge the sorted $CD8a^+$ T cells at 400 × *g* for 1 min at 4 °C and remove cell media. Wash the cell pellet in 200 μL of 1× PBS + 0.04% BSA (*see* **Note 8**). Spin down the cells at 400 × *g* for 1 min at 4 °C in the microcentrifuge and repeat with a second wash with 200 μL of 1× PBS + 0.04% BSA.
2. Prepare cells for cell capture in the 10× Genomics Chromium system according to the Chromium Single Cell 3′ Reagents Kits v3 user guide. Based on the cell suspension volume

calculator table of the Chromium Single Cell 3′ Reagents Kits v3 protocol and cell count of the sorted CD8a$^+$ T cells, calculate the volume of 1× PBS + 0.04% BSA required to suspend the sorted CD8a$^+$ T cells to form a cell stock concentration of 1200 cells/μL. This cell stock concentration is used for a targeted cell recovery of 6000 cells (*see* **Note 9**). Keep cells on ice.

3. For GEM Generation and Barcoding, prepare the 10× Genomics master mix (RT Reagent, Template Switch Oligo, Reducing Reagent B, RT Enzyme C) on ice, using nuclease-free 1.5 microcentrifuge tubes and nuclease-free, filtered pipette tips. Per sample, add 33.4 μL of master mix into one tube of a PCR 8-tube strip on ice.
4. Prepare the cell suspension. For a targeted cell recovery of 6000 cells, add 8.0 μL of cells from the cell stock concentration of 1200 cells/μL with 38.6 μL of nuclease-free water, according to the volume calculator table of the Chromium Single Cell 3′ Reagents Kits v3 protocol. Keep cells on ice.
5. Carefully place the Chromium Chip B in the 10× Genomics Chip Holder (*see* **Note 10**). Add the appropriate volume of 50% glycerol solution into the chip wells that will not be used. For instance, if only one well of the 8-well chip will be used for one cell sample, fill the remaining 7 wells with 50% glycerol solution.
6. Gently pipette mix the cell suspension and add it to the tube of the PCR 8-tube strip containing the master mix. Gently pipette mix the cell suspension and master mix and carefully load 75 μL of this mixture into the bottom center of the first well in the row labeled 1 on the Chromium Chip B (*see* **Note 11**).
7. Place the 10× gel bead strip into the 10× vortex adapter and vortex for 30 s. To recover the gel beads after vortexing, flick the gel bead strip in a quick, downward motion, and ensure that the liquid levels in each tube of the gel bead strip look equal. Pierce open the foil seal of the gel bead strip and slowly pipette up 40 μL of gel beads. Gently dispense the gel beads into the first well in row labeled 2 on the Chromium Chip B (*see* **Note 12**). Add 140 μL of 10× genomics partitioning oil into the first well of row labeled 3 on the Chromium Chip B. Repeat with a second aliquot of 140 μL of 10× genomics partitioning oil for a total volume of 280 μL per well (*see* **Note 13**).
8. To attach the 10× gasket on top of the Chromium Chip B, align the top-left notch of the gasket to the top-left corner of the 10× chip holder. Ensure that the gasket is fastened onto the 10× chip holder and confirm that the gasket holes align with the wells (*see* **Note 14**).

9. On the Chromium Controller, press the eject button on its screen to eject the tray. Carefully place the 10× chip holder containing the Chromium Chip B and gasket on the tray, aligning the top-left notch of the chip holder with the controller tray. Confirm that the controller lists Chromium Single Cell B program on its screen and then press the play button on the controller to start the program.
10. Following the run, press the eject button on the controller and carefully remove the 10× chip holder and place it on the lab bench workspace. Discard the gasket. To open the chip holder, fold the lid backwards slowly until it clicks and the chip holder is positioned at 45°. The wells of the Chromium Chip B should now be exposed. Take caution to avoid any spillover of the 10× partitioning oil into other wells while opening the 10× chip holder (*see* **Note 15**).
11. To transfer the GEMs from the Chromium Chip B into a PCR tube, slowly pipette up 100 μL GEMs by placing the pipette tip to the lowest point of the recovery well in the top row of the Chromium Chip B. Remove the pipette tip from the well and visually analyze the GEMs in the pipette tip. The GEMs should appear opaque and uniform among all channels (*see* **Note 16**). Slowly dispense the GEMs into a clean tube of a PCR 8-tube strip on ice. Incubate the GEMs in a thermal cycler for reverse transcription (RT) with the program as follows:
    (a) Lid temperature: 53 °C; reaction volume of 125 μL; step 1: 53 °C for 45 min; step 2: 85 °C for 5 min; step 3: 4 °C hold.
    (b) Samples can be stored at 4 °C for 72 h or at −20 °C for 1 week.
12. To clean up the GEM-RT reaction, add 125 μL 10× genomics recovery agent to the sample at room temperature and let sit for 1 min without mixing. A biphasic mixture will appear, containing the 10× genomics recovery agent/partitioning oil at the bottom of the PCR tube (pink in color), and a clear aqueous phase on top.
13. Slowly remove 125 μL 10× genomics recovery agent from the bottom of the tube without removing any of the aqueous phase of the sample. Prepare the Dynabeads MyOne SILANE cleanup mix (10× Genomics Cleanup Buffer and Reducing Agent B, Dynabeads MyOne SILANE, and nuclease-free water) according to the Chromium Single Cell 3′ Reagents Kits v3 user guide on page 30.
14. Add 200 μL Dynabeads MyOne SILANE cleanup mix to each sample and pipette mix ten times. Incubate at room temperature for 10 min (*see* **Note 17**). Halfway through the incubation time, pipette mix the GEM-RT and Dynabeads mixture to resuspend the SILANE beads.

15. After the 10-min incubation, place the PCR tube on the 10× magnet-high position until the solution becomes clear. Remove the supernatant and add 300 μL of 80% ethanol to the pellet while the PCR tube is still on the magnet (*see* **Note 18**). Let sit for 30 s, and then remove the ethanol. Repeat the ethanol wash with 200 μL of 80% ethanol. Let sit for 30 s, and then remove the ethanol. Centrifuge the PCR tube briefly and place it on the 10× magnet-low position. Remove the excess ethanol and air-dry for 1 min.
16. Remove the PCR tube from the magnet and add 35.5 μL 10× elution solution I (buffer EB, 10% Tween 20, 10× reducing reagent B) (*see* **Note 19**). Pipette mix and incubate at room temperature for 2 min. Place the PCR tube on the 10× magnet-low position until the solution clears. Transfer 35 μL sample to a new PCR tube.
17. For cDNA amplification, prepare the 10× genomics cDNA amplification reaction mix (Amp mix and cDNA primers) on ice. Add 65 μL of cDNA amplification reaction mix to 35 μL of the eluted sample. Pipette mix and centrifuge briefly. Incubate in a thermal cycler with the program as follows:
    (a) Lid temperature: 105 °C; reaction volume: 100 μL.
    (b) Step 1: 98 °C for 3 min; step 2: 98 °C for 15 s; step 3: 63 °C for 20 s; step 4: 72 °C for 1 min; step 5: go to step 2. The number of cycles depends on the cell load. Based on the 10× genomics cycle number optimization table on page 32 of the v3 user guide, a cell load of 500–6000 cells requires 12 cycles; step 6: 72 °C for 1 min; step 7: 4 °C hold.
18. For cDNA cleanup, the SPRIselect reagent is used. Vortex the SPRIselect reagent to resuspend any settled beads. Add 60 μL (0.6× the volume of cDNA amplification reaction volume) of SPRIselect reagent to the cDNA sample in the PCR tube and pipette mix 15 times. Incubate at room temperature for 5 min.
19. Place the PCR tube on the 10× magnet-high position until the solution clears. Discard the supernatant. Add 200 μL of 80% ethanol to the pellet and let sit for 30 s. Remove the ethanol while the PCR tube is still on the magnet. Repeat with a second wash of 200 μL of 80% ethanol added to the pellet. Incubate at room temperature for 30 s before removing the ethanol. Centrifuge the PCR tube quickly and place back onto the 10× magnet-low position. Remove the remaining ethanol and let air-dry for 2 min (*see* **Note 20**).
20. Remove the PCR tube from the 10× magnet, add 40.5 μL Buffer EB to the PCR tube, and gently pipette mix. Incubate at room temperature for 2 min. Place the PCR tube on the 10× magnet-high position until the solution clears. Transfer 40 μL of the sample to a new PCR tube. Samples can be stored at 4 °C for 72 h or at −20 °C for 4 weeks.

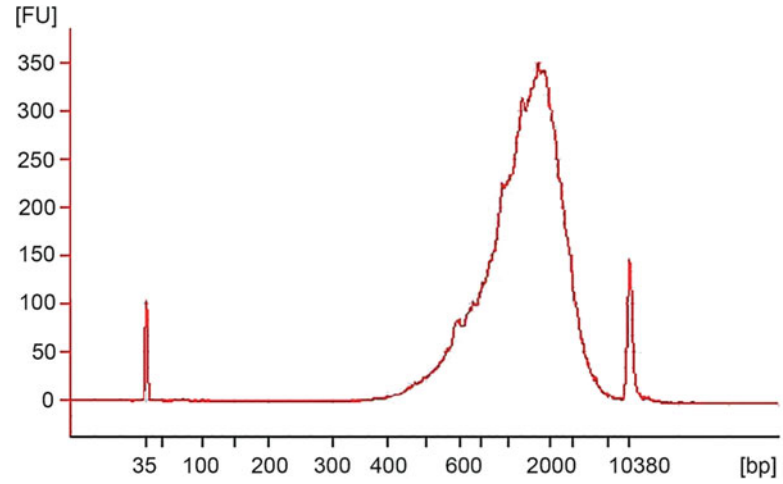

Fig. 1 Representative Agilent Bioanalyzer Trace of CD8a$^+$ T-cell cDNA. Shown is an electropherogram of 1 μL diluted (1:10) cDNA. The cDNA content range is between ~400 and ~9000 bp

21. To assess the cDNA quality, use the Agilent Bioanalyzer High Sensitivity DNA chip on an Agilent 2100 Bioanalyzer instrument (*see* **Note 21**). Dilute the cDNA sample 1:10 and run 1 μL of cDNA on an Agilent Bioanalyzer High Sensitivity chip (*see* **Note 22**). Evaluate the electropherogram of the sample (Fig. 1).
22. To obtain the concentration of the cDNA sample, in the Agilent 2100 Bioanalyzer software, manually select the region on the electropherogram between ~200 and ~9000 bp. The cDNA concentration in this region will be shown in pg/μL in the software. To calculate the cDNA total yield in ng, use the following formula:

$$\text{Total cDNA yield (ng)} = \text{concentration of cDNA (pg/L)} \times \text{Elution volume (e.g.40 L)} \times \text{Dilution Factor (e.g.10)}/1000\ \text{(pg/ng)}$$

23. According to the 10× Genomics Chromium Single Cell 3′ Reagents Kits v3 user guide, use 25% of total cDNA yield to generate the cDNA libraries (0.25 × total cDNA yield (ng)). Transfer 10 μL (25%) of cDNA sample to a PCR tube on ice. Store the remaining cDNA sample at −20 °C for up to 4 weeks.
24. Generation of single-cell cDNA libraries first involves fragmentation, end repair, and A-tailing steps. Prepare the 10× genomics fragmentation mix (fragmentation buffer, fragmentation enzyme) on ice, as on page 37 of the v3 user guide. To the 10 μL of cDNA, add 25 μL buffer EB and 15 μL 10× genomics fragmentation mix. Pipette mix the contents

15 times on ice, and then briefly centrifuge the PCR tube (*see* **Note 23**). Place the PCR tube in a pre-cooled thermal cycler and incubate the reaction with the program as follows:

(a) Lid temperature: 65 °C; reaction volume: 50 μL; fragmentation step: 32 °C for 5 min; end repair and A-tailing step: 65 °C for 30 min; step 3: 4 °C hold.

25. Following the thermal cycler program, vortex to mix SPRIselect reagent (*see* **Note 24**). Add 30 μL (0.6×) of the SPRIselect reagent to the sample, pipette mix, and incubate at room temperature for 5 min. Place the PCR tube on the 10× magnet-high position until the solution becomes clear. Transfer 75 μL of the supernatant to a new PCR tube.

26. Add 10 μL of the SPRIselect reagent (0.8×) to the sample, pipette mix, and incubate at room temperature for 5 min. Place the tube on the 10× genomics magnet-high position until the solution becomes clear. Discard 80 μL of the supernatant without disrupting any of the beads. Add 125 μL of the 80% ethanol to the PCR tube while still on the 10× genomics magnet. Incubate for 30 s. Carefully remove the ethanol and repeat to perform a second 80% ethanol wash. Briefly centrifuge the PCR tube and place the tube on the 10× genomics magnet-low position until the solution becomes clear. Carefully remove any excess ethanol (*see* **Note 25**).

27. Remove the PCR tube from the magnet, add 50.5 μL of buffer EB, and pipette mix to fully resuspend the pellet. Incubate at room temperature for 2 min. Place the PCR tube on the 10× genomics magnet-high position until the solution becomes clear. Transfer 50 μL of the sample to a new PCR tube, being careful not to aspirate any SPRIselect reagent into the sample.

28. To complete the generation of single-cell cDNA libraries for subsequent RNA-seq involves adaptor ligation and sample index PCR steps. Prepare the 10× genomics adaptor ligation mix (ligation buffer, DNA ligase, adaptor oligos) as on page 39 of the v3 user guide, on ice. Add 50 μL of the 10× genomics adaptor ligation mix to 50 μL sample from the previous step. Pipette mix 15 times and briefly centrifuge the mixture. Incubate the sample in a thermal cycler with the program as follows:

    Lid temperature: 30 °C; reaction volume: 100 μL; step 1: 20 °C for 15 min, step 2: 4 °C hold.

29. Add 80 μL (0.8×) of the SPRIselect reagent to the sample, pipette mix, and incubate at room temperature for 5 min. Place the PCR tube on the 10× genomics magnet-high position until the solution becomes clear, and then carefully remove the supernatant. Add 200 μL of the 80% ethanol to

the pellet and incubate for 30 s. Remove the ethanol and repeat for an additional wash. Briefly centrifuge the PCR tube and place it on the 10× genomics magnet-low position. Carefully remove any excess ethanol and air-dry for precisely 2 min.

30. Remove the PCR tube from the magnet, add 30.5 μL of buffer EB, and pipette mix. Incubate at room temperature for 2 min. Place the PCR tube on the 10× magnet-low position until the solution becomes clear.
31. Transfer 30 μL of sample to a new tube, being careful not to aspirate any SPRIselect reagent.
32. For the sample index PCR, record the 10× genomics sample index well ID on the 10× Genomics Chromium i7 Sample Index Plate that is used for each sample (*see* **Note 26**). Prepare the 10× genomics sample index PCR mix (10× Amp mix, SI primer) as on page 41 of the v3 user guide on ice. Pipette mix 60 μL of sample index PCR mix with 30 μL of sample, and add 10 μL of one 10× Genomics Chromium i7 Sample Index to the sample + sample index PCR mixture. Pipette mix and briefly centrifuge the contents. Incubate the mixture in a thermal cycler with the program as follows:
    (a) Lid temperature: 105 °C; reaction volume: 100 μL; step 1: 98 °C for 45 s; step 2: 98 °C for 20 s; step 3: 54 °C for 30 s; step 4: 72 °C for 20 s; step 5: go to step #2 for # cycles (*see* **Note 27**); step 6: 72 °C for 1 min; step 7: 4 °C hold.
33. After the thermal cycler program is complete, add 60 μL (0.6×) of the SPRIselect reagent to the sample, pipette mix, and incubate at room temperature for 5 min. Place the PCR tube on the 10× genomics magnet-high position until the solution becomes clear. Carefully transfer 150 μL of the supernatant into a new PCR tube.
34. Add 20 μL of the SPRIselect reagent to the sample, pipette mix, and incubate at room temperature for 5 min. Place the PCR tube on the 10× magnet-high position until the solution becomes clear. Remove 165 μL of the supernatant. To the pellet, add 200 μL of the 80% ethanol and let sit for 30 s. Remove the ethanol and repeat the ethanol wash once more. Briefly centrifuge the PCR tube and place it on the 10× genomics magnet-low position. Remove any excess ethanol.
35. Remove the PCR tube from the magnet, add 35.5 μL of buffer EB, pipette mix, and incubate at room temperature for 2 min. Place the PCR tube on the 10× genomics magnet-low position until the solution becomes clear. Transfer 35 μL of supernatant into a new PCR tube, labeled cDNA library. Store the cDNA library at −20 °C for a long term.

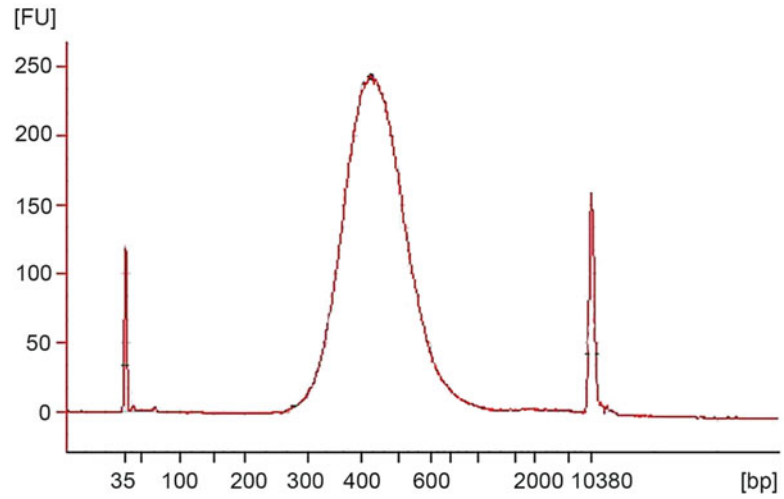

Fig. 2 Representative Agilent Bioanalyzer Trace of single-cell cDNA libraries. Shown is an electropherogram of 1 μL diluted (1:10) cDNA library with peak of the fragment distribution curve at ~450 bp

3.3 cDNA Library Quality Control (QC)

1. For cDNA library QC, use the Agilent Bioanalyzer High Sensitivity DNA Chip and Agilent 2100 Bioanalyzer instrument. Run 1 μL of 1:10 diluted cDNA library on an Agilent Bioanalyzer High Sensitivity Chip.
2. The average fragment size of the single-cell cDNA libraries can be determined from the Agilent Bioanalyzer trace (Fig. 2). Manually select the region between ~35 and ~10,000 bp for each cDNA library sample. It is expected that the majority of 10× Genomics Chromium library inserts will be between 400 and 600 bp in size (*see* **Notes 28** and **29**).
3. Determine the concentration of the cDNA library (ng/μL) using the Qubit dsDNA HS (High Sensitivity) Assay Kit and Qubit Fluorometer (Invitrogen). The concentration of the cDNA libraries for each sample is required to calculate the library input concentration (pooled or non-pooled samples) in downstream sequencing experiments on an Illumina sequencer. Calculate the cDNA library concentration as follows:

$$\begin{aligned}&\text{cDNA library concentration (nM)}\\&\quad = \text{cDNA library concentration (ng/L)}/660\ \text{g/mol}\\&\qquad \times \text{average fragment size of cDNA library (bp)} \times 10^6.\end{aligned}$$

4 Notes

1. The procedure outlined describes the enrichment of $CD8a^+$ T cells by negative selection. An alternative method of cell enrichment is positive selection, in which the cells of interest are

bound to biotin-labeled antibodies. In positive selection, the negative fraction containing the untargeted cells is eluted through the LS column into the collection tube, while the positive fraction containing the cells of interest is flushed out of the LS column by a plunger that is supplied with the column (MACS Miltenyi Biotec).

2. Keep the cell isolation buffer on ice in between steps of the cell enrichment procedure.
3. Do not let the LS column become dry while on the magnetic separator in between the first wash of the column to sample elution. If the cells are not ready to load onto the column immediately after the first wash of the column, remove the column from the magnetic separator until ready for use. This is to maintain the integrity of the column for efficient cell isolation by magnetic bead enrichment.
4. When adding the cell sample to the LS column, ensure that the cells have not settled and remain fully suspended in the buffer. Gently pipette the cell suspension up and down prior to loading onto the LS column.
5. For FACS sorting, cells can be incubated with fluorophores in either one of 1× PBS, cell media, or FACS buffer as the staining buffer. Cells can be stained in 1.5 mL centrifuge tubes or 96-well round-bottom plates on ice.
6. In general, 0.1–10 μg/mL of a fluorophore-conjugated antibody is used for flow cytometry staining, but it is important to predetermine the optimal concentration of each fluorophore-conjugated antibody prior to any experiment. This can be achieved by titrating the antibody in FACS buffer, followed by incubating the cells of interest with different concentrations of antibody. Differences in signal intensities as a result of the titrations can be analyzed on the flow cytometer at the time of sample acquisition. For multi-fluorophore flow cytometry experiments, it is essential to include controls. Negative (unstained cells) and single-stain controls (cells stained with only one of the fluorophore-conjugated antibodies) should be included in the flow cytometry panel design for compensation purposes on the cytometer, and to identify the positive expression of proteins of interest on the cells analyzed. Alternative controls include the use of isotype control antibodies and FMO (fluorescent-minus-one) controls.
7. Filtering the cell suspension prior to FACS sorting will remove any potential cell clumps, which is important to help minimize the chance of cell clogs in the cytometer during sample acquisition.
8. Store 1× PBS + 0.04% BSA at 4 °C. This solution is used for washing the cells prior to cell capture on the 10× genomics

chromium to minimize cellular aggregates and dead cells to maintain good integrity of the live cells.

9. Selection of the targeted cell recovery number can vary per experiment and will depend on the nature of the experimental design (e.g., how many single cells are desired to be analyzed and sequenced per experiment) and limitations of cell number availability.
10. Hold the 10× Genomics Chromium Chip B horizontal and avoid touching the barcode on the chip. It is important to fill all unused input wells on the chip (of all rows labeled 1, 2, and 3) with 50% glycerol solution prior to loading the wells that will be used. Do not add 50% glycerol to the recovery wells on the chip.
11. It is important to slowly load the cell suspension and the master mix into the center of the appropriate well on the Chromium Chip B to avoid introducing any air bubbles. The presence of air bubbles will interfere with the microfluidics in the chromium controller and may negatively affect the efficiency of cell capture and GEM generation. Visually assess each well containing the master mix and the cell suspension for any air bubbles. Hold a pipette tip by hand to gently pop and remove any air bubbles present.
12. To pierce open the foil of the 10× genomics gel bead strip, take a pipette tip in one hand and puncture the center of the foil seal of the gel bead strip to create a small hole. Using the same pipette tip, gently widen the size of the hole on the foil seal in order to fully open the seal. To aspirate the gel beads for loading onto the Chromium Chip B, place the pipette tip to the bottom of the well of the gel bead strip and slowly pipette up the beads without introducing any air bubbles during pipetting.
13. Do not forget to add the 10× partitioning oil, as failure to add this reagent at this step can cause chromium controller damage.
14. When attaching the 10× gasket onto the 10× chip holder, keep the gasket horizontal and do not touch the surface of the gasket that aligns with the Chromium Chip B wells. Do not press on the gasket when it is fastened to avoid any movement of the Chromium Chip B and spillover of the reagents in the wells.
15. Analyze the volumes in all rows labeled 1 through 3. An unequally high volume in any well can indicate a clog occurred during the controller run.
16. When assessing the quality of the GEMs, if there is partitioning oil in the pipette tip containing the GEMs, a clog may have occurred during the controller run.

17. After addition of the Dynabeads MyOne SILANE mix to each sample and during the incubation step, do not close the PCR tubes to avoid any overflow and loss of reagents.
18. Prepare a fresh solution of 80% ethanol every time for the post-GEM-RT cleanup. Prepare 80% ethanol using ethanol, pure (200 proof) and nuclease-free water. Keep the time duration for each 80% ethanol wash (30 s) and air-dry step (1 min) precise.
19. Prepare the 10× elution solution I before the Dynabeads MyOne SILANE cleanup mix to ensure that the elution solution is ready for its immediate addition to the PCR tube after the ethanol washes and air-drying step.
20. For this air-drying step, it is important to not exceed the 2 min of incubation at room temperature, as this will decrease the cDNA elution efficiency.
21. Equilibrate reagents of the Agilent Bioanalyzer High Sensitivity to room temperature in the dark approximately 30 min prior to using the Agilent 2100 Bioanalyzer instrument. Ensure that all sample wells contain marker reagent, including unused wells. Do not forget to load the ladder in the appropriate well on the chip. When loading the marker reagent and sample by pipetting, do not introduce air bubbles into the wells of the chip.
22. For cells with low-RNA material (less than 1 pg RNA/cell), 1 μL of undiluted cDNA can be run on the Agilent Bioanalyzer High Sensitivity Chip.
23. Prepare the 10× genomics fragmentation mix on ice and pre-cool the thermal cycler to 4 °C until ready for use.
24. Vortex the SPRIselect reagent to fully resuspend it before any sample addition as the SPRIselect reagent has a tendency to settle. Do not discard the supernatant at this step.
25. It is critical to perform these steps as quickly as possible to not let the sample overdry. Overdrying the sample will decrease the elution efficiency.
26. In multiplexed sequencing experiments, i.e., when the cDNA libraries from more than one sample or experimental condition will be sequenced together in the same run, it is critical to ensure that different (i.e., nonoverlapping) sample indices from the 10× Genomics Chromium i7 Sample Index Plate are used in order to identify each sample during the RNA-seq data analysis.
27. The number of cycles will depend on the concentration (ng) of the cDNA input (25% of total cDNA which was used in library construction) as determined in **steps 18** and **19**. The recommended number of index PCR cycles has been previously

determined as indicated on page 41 of the 10× Genomics Chromium Single Cell 3′ Reagents Kits v3 user guide.

28. Shown in Fig. 2 is a representative Agilent Bioanalyzer trace of one cDNA library sample with the majority of insert sizes (peak of the fragment distribution curve) at 500 bp. If the peak of the cDNA libraries fragment distribution curve on the Bioanalyzer trace ranges from 400 to 1000 bp, this indicates the presence of larger fragments in the libraries, which can still be submitted for sequencing.
29. Unexpected peaks in the electropherogram of cDNA libraries might appear at peak sizes <100 bp (indicates primer or primer dimer) or ~120 to ~125 bp (indicates adapter dimer). It is highly recommended to remove potential dimers in the cDNA libraries prior to RNA-seq. To remove the potential dimers, perform an additional SPRIselect cleanup step using 0.8× the total volume of library sample. Run 1 μL of undiluted cDNA library on an Agilent Bioanalyzer High Sensitivity DNA Chip and analyze the trace. The unexpected peaks at <125 bp should no longer be present.

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