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Jul 06, 2020

Archival preservation of cell suspensions for scRNA-Seq

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1 *Works for me* This protocol is published without a DOI.

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ABSTRACT

The inability to archive cells for subsequent high-quality single-cell analysis has hampered the interrogation of complex sample collections, such as those derived from clinical cohorts. Here, we describe a procedure for freezing FACS-sorted live cells in preservation medium, followed by thawing for single-cell RNA-seq (scRNA-seq) applications. By employing FBS-free preservation medium (Bambanker), this protocol achieves greater than 80% cell viability after one freeze/thaw cycle. Traditional preservation methods freeze cells in growth medium supplemented with 10% DMSO and 20-40% FBS. Although FBS improves cells viability during freezing and thawing, it needs to be removed for scRNA-seq due its inhibition of reverse transcription. FBS removal is typically achieved by washing in 1X PBS buffer, leading to inadvertent cell loss and transcriptional changes. Absence of FBS in the preservation medium makes it possible to apply thawed cell suspension directly to the 10X Chromium platform, by adding cells directly into reverse transcriptase reaction mix without the need for washing or buffer exchange. This approach minimizes sample handling to generate rapid (2-3 min for thawing), robust outcomes that avoid cell death or noticeable changes in the transcriptome.

PROTOCOL CITATION

Linas Mazutis, Ignas Masilionis 2020. Archival preservation of cell suspensions for scRNA-Seq . **protocols.io** https://protocols.io/view/archival-preservation-of-cell-suspensions-for-scrn-ba6qihdw

KEYWORDS

Cell preservation, cell freezing, thawing, scRNA-Seq

CREATED

Jan 09, 2020

LAST MODIFIED

Jul 06, 2020

PROTOCOL INTEGER ID

31664

MATERIALS

NAME	CATALOG #	VENDOR
DAPI (46-Diamidino-2-Phenylindole Dihydrochloride)	D1306	Invitrogen - Thermo Fisher
Fetal Bovine Serum	26140079	Gibco - Thermo Fisher
RPMI 1640 Medium	61870010	Gibco - Thermo Fisher
Bambanker	CS-02-001	
Protein LoBind tubes	022431081	Eppendorf
Falcon 15mL Conical Centrifuge Tubes	352097	Corning
Trypan Blue Solution 0.4%	15250061	Thermo Fisher Scientific

NAME	CATALOG #	VENDOR
Chromium Chip B Single Cell Kit 48 rxns	1000073	10x Genomics
Single Cell 3 GEM Library & Gel Bead Kit v3 16 rxns	1000075	10x Genomics

Preparation of single-cell suspension 1h

1 1. Using the protocol of your choice, dissociate the solid tissue sample into a single-cell suspension.

DAPI staining 5m

2 1. Resuspend the dissociated cells in 400 µl cell culture media (e.g RPMI, DMEM) supplemented with 2.5% FBS and 0.1 mg/ml DAPI dye. Aim for dilution of 500-2000 cells/µl.
2. Incubate on ice for 5 min.

FACS sorting 30m

3 1. Add 400 µl of Bambanker preservation media in a 15 ml FACS collection tube.
2. FACS-sort DAPI negative (live-cells) directly into the preservation media.
3. Collect >200,000 cells ideally, or 50,000 cells at a minimum.

NOTE: The preservation medium will get diluted by the FACS buffer during sorting. Avoid diluting more than 50%.

Concentrating cell suspension 10m

4 1. Transfer the FACS-sorted cell suspension into a 1.5 ml LoProtein Bind tube, but do not exceed 500 µl volume. If the sorted suspension constitutes more than 0.5 ml, then divide the solution into two tubes (e.g. each tube 400 µl).
2. Transfer the tubes to a swinging bucket centrifuge and spin down at **500 x g, 4°C 00:05:00**
3. **NOTE**: For centrifugation in a swinging bucket centrifuge place 1.5 ml tubes inside a 50 mL Falcon tube.
4. After centrifugation is complete, remove the medium without disrupting the pellet leaving ~20 µl of supernatant in the tube.
5. Dilute the cells with the preservation medium down to 1000-2000 cell/µl. Use the FACS quantification as a guide, but keep in mind that these numbers are often inflated.
6. Gently resuspend the cells using a 1000 µl or 200 µl pipette. Do not leave cell clumps behind.
7. At this step, one would typically expect to have 50-100 µl of preservation medium containing sorted live cells (at a concentration of 1000-2000 cells/µl).

NOTE: When resuspending the cells in preservataion medium, make sure to avoid bubbles and do not leave any cell clumps.

Freezing and storage 1d

5 1. Use slow freezing method (−1°C/minute) to freeze the cells at −80 °C.
2. Place the tube containing cells into a isopropanol-filled container and transfer it in a −80 °C freezer. Isopropanol-filled containers have a stated freeze rate of −1°C/minute, but performance may vary.
3. The next day, transfer the tube to liquid nitrogen tank for a long term storage.

Thawing 5m

6

CRITICAL: When thawing cells, it is of critical importance to proceed quickly, within 5-10 minutes. Handling thawed cells for longer than 15 min may result in poor quality data.

Therefore, make sure that the scRNA-seq platform is set up and reagents are ready before starting the thawing procedure.

1. Remove the tube from liquid nitrogen and transfer to water-bath set at 37 ºC
2. Incubate the tube for 1-2 minutes, during which the frozen cells should thaw completely.

Cell counting 2m

7

NOTE: Proceed quickly, keeping in mind that any extended incubations may result in transcriptome changes. Thawed cells can be processed directly and no buffer change is needed.

1. Count the thawed cells by mixing 5 µl of cell suspension with 5 µl of 0.1% (w/v) Trypan Blue.
2. Evaluate cells under bright field microscopy (or alternatively, using the Countess II instrument).
3. Proceed to Cell Encapsulation step.

Cell Encapsulation 20m

8

NOTE: Cells are typically washed in 1X PBS buffer + 0.04% (w/v) BSA to remove preservation medium. However, we have found that Bambanker preservation medium does not cause detectable transcriptional changes; thus, cells should be loaded directly onto the microfluidics chip without washing or buffer exchange.

1. When using 10X Genomics Chromium system to perform scRNA-Seq follow the "Cell Suspension Volume Calculator Table" in the CG000204 RevD manual here.
2. Load the cells directly onto a chip and place the chip into the Chromium instrument.
3. Proceed according to 10X Genomics protocol (here).

scRNA-Seq 2d

9 1. Follow the 10X Genomics manual CG000204 RevD here to construct the scRNA-Seq library.

2. We use a NovaSeq 6000 instrument to sequence the final DNA libraries using pair-end sequencing option, R1 read - 26 cycles, R2 read - 70 cycles, and index read - 8 cycles, aiming for ~150M reads per ~5,000 single-cells.

3. We process FASQ files using the SEQC pipeline and mapped to a reference genome with the default SEQC parameters to obtain the gene-cell count matrices.

Expected results

10 To evaluate how the freezing/thawing procedure affects cellular transcriptomes, we performed scRNA-seq on cells derived from human lung.

We dissociated fresh human lung tissue into a single-cell suspension (step 1), we FACS-sorted live cells (steps 2-6), and divided the sorted live cells we collected into three tubes with equal volumes:

Tube 1. Cells were processed for scRNA-Seq immediately following 10X Genomics manual CG000204 RevD here.
Tube 2. The preservation medium in which cells were suspended was replaced with RPMI 1640 by centrifugation at 400 g for 5 min, and processed for scRNA-seq in parallel with Tube 1.
Tube 3. FACS-sorted cells were frozen (step 5) and incubated in liquid nitrogen for 2 weeks. Next, cells were thawed, counted and processed for scRNA-Seq (steps 6-9).

The results are summarized in Figures 1-2 below:

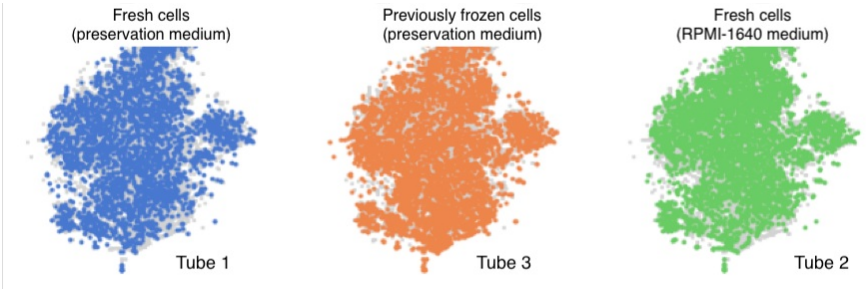

Figure 1. Comparison of gene expression profiles of fresh and frozen single cells derived from human lung. tSNE plot of all cells (grey) derived from a fresh human lung sample, colored by whether they were suspended in preservation medium before freezing (blue), previously frozen in preservation medium (orange), or never frozen cells suspended in RPMI 1640 medium (green).

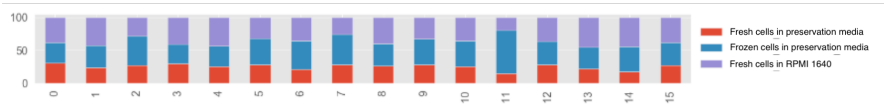

Figure 2. Comparison of cell types in fresh and previously frozen samples. Color bars indicate proportions of cell types. Y-axis, percentage of cells; X-axis, Phenograph cluster numbers corresponding to distinct cell types. Red, dissociated cells suspended in preservation medium and processed immediately; blue, dissociated cells frozen in the preservation medium and processed after 2 weeks in liquid nitrogen; purple, dissociated cells resuspended in RPMI medium and processed immediately.