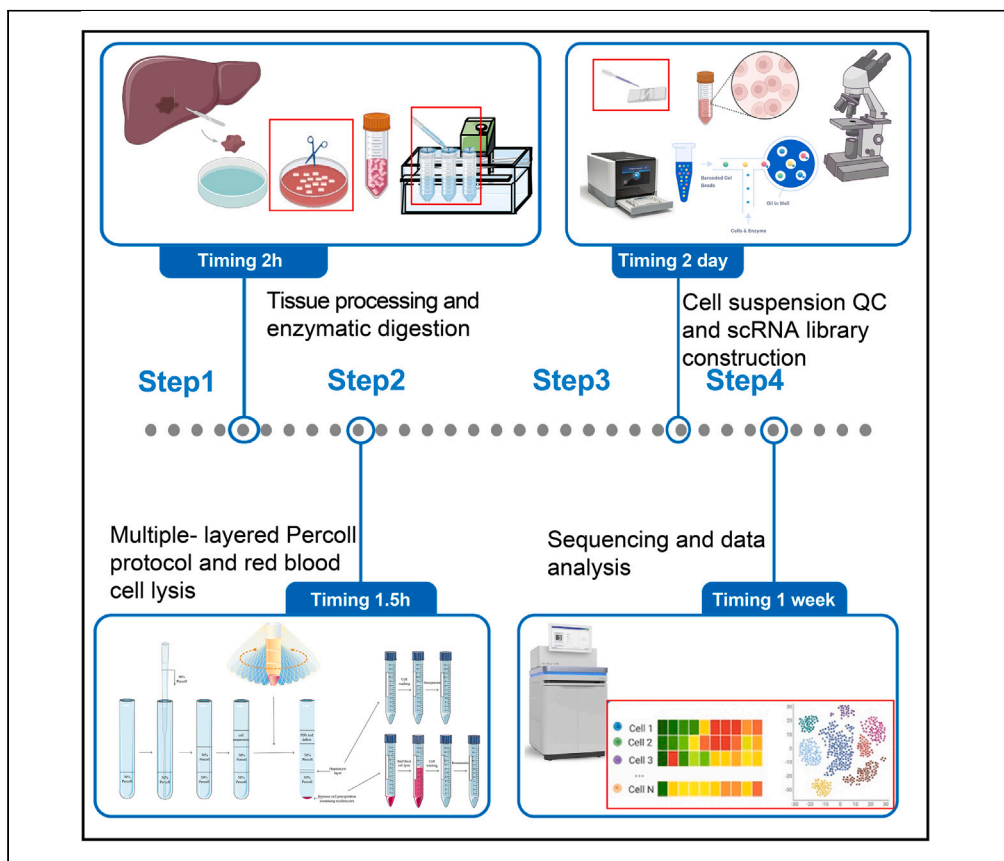**Protocol**

Protocol to dissociate and isolate wide-diversity single cells by density gradient centrifugation from human hepatoblastoma tissue

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Highlights

ScRNA-Seq reveals high heterogeneity of TME of hepatoblastomas

This protocol elevates cell viability and improves data quality

Multi-step dissociation retains wide-diversity cell types, notably hepatocytes

Single-cell transcriptome sequencing can characterize various cell types in human liver tissue and facilitate understanding of hepatoblastoma heterogeneity. Here, we present a protocol for isolating hepatocytes and immune cells from human hepatoblastoma samples with high viability. We describe steps for tissue processing, enzymatic digestion, Percoll density gradient separation, cell lysis, cell suspension quality control, and scRNA library construction. We then detail sequencing and data analysis. This protocol is applicable to preparing single-cell suspensions from other human liver tissue samples.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Protocol

Protocol to dissociate and isolate wide-diversity single cells by density gradient centrifugation from human hepatoblastoma tissue

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SUMMARY

Single-cell transcriptome sequencing can characterize various cell types in human liver tissue and facilitate understanding of hepatoblastoma heterogeneity. Here, we present a protocol for isolating hepatocytes and immune cells from human hepatoblastoma samples with high viability. We describe steps for tissue processing, enzymatic digestion, Percoll density gradient separation, cell lysis, cell suspension quality control, and scRNA library construction. We then detail sequencing and data analysis. This protocol is applicable to preparing single-cell suspensions from other human liver tissue samples.

BEFORE YOU BEGIN

The liver harbor a variety of structural and immune cells, also hepatocytes, which collectively facilitate crucial physiological processes and oncogenesis.[1,2] Preparing single-cell suspension from dissociated tissues has always been a prerequisite for obtaining high-quality data.[3,4] The protocol below describes how to prepare a single-cell suspension from hepatoblastoma tissue that were challenged with low cell yield, low cell viability, and cell type bias and how to collect enough hepatocytes for further study conditions. Moreover, this approach with minor modifications might be applied to isolate hepatocytes from liver cancer patients with tumor or adjacent normal tissue. The protocol was likewise suitable for corresponding mouse models.

Institutional permissions

This project was permitted and approved by the IRB of Shanghai Children's Medical Center Affiliated Shanghai Jiao Tong University School of Medicine (SCMCIRB-K2023006-1).

Preparation the environment and materials

© Timing: flexible before experiment

Use RNaseZap and DNA-Off to prepare the experiment bench before the experiment.

1. AMPureXP beads need to be balanced at room temperature for at least 30 min before use.

2. Calculate and prepare the required amounts of stock solutions and buffers ahead of the experiment.
    a. Mix 9 mL of Percoll stock solution with 1 mL of PBS (10×) to produce 100% Percoll gradient medium (abbreviated as 100% Percoll in following), and place on ice.
    b. Weigh an appropriate amount of collagenase II to prepare a 0.2% collagenase II lysis solution prior to conducting the experiment
3. Pre-cool the centrifuge to 4°C and pre-warm the water bath shaker to 37°C.

Note: We recommend preparing all reagents just before use to keep the solutions fresh and perform all steps on ice unless otherwise noted.

⚠ CRITICAL: All time-consuming steps (including the preparation of Percoll solution with different concentrations, cell dissociation solution, and the precooling of centrifuge, etc.) that can be performed prior to surgical sampling should be prepared and/or executed in advance since the quality of results critically depends on fast processing of liver cells after explanation.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Hepatoblastoma tissue	Shanghai Children's Medical Center	N/A
Chemicals, peptides, and recombinant proteins		
RNAseZAP	Thermo Fisher	Cat# AM9780
DNA-OFF	Takara	Cat# 9036
AMPureXP beads	Beckmann Coulter	Cat# A63881
PBS	Thermo Fisher	Cat# 10010-049
PBS (10×)	Thermo Fisher	Cat# 70011-044
DPBS, no calcium, no magnesium	Thermo Fisher	Cat# 14190250
FBS	Thermo Fisher	Cat# 26140079
DMEM	Thermo Fisher	Cat# 11965118
Ethanol 200 proof	Millipore Sigma	Cat# 7023-500ML
Collagenase II	Sigma-Aldrich	Cat# C2-28-100MG
Nuclease-free water	Qiagen	Cat# 129114
ACK Lysing buffer 100 mL	Thermo Fisher	Cat# 599451
Trypan blue stain (0.4%)	Thermo Fisher	Cat# T10282
BSA	Sangon Biotech	Cat#B600036-0001
Percoll	Solarbio	Cat# P8370-100mL
Cellometer ViaStain AOPI Staining Solution	Nexcelom Bioscience	Cat# CS2-0106-5
Miltenyi MACS Tissue Storage Solution	Miltenyi	Cat# 130-100-008
Critical commercial assays		
Chromium Next GEM Single Cell 3'Kit v3.1	10× Genomics, Inc.	Cat# 1000268
Chromium i7 Multiplex Kit	10× Genomics, Inc.	Cat# 120262
Chromium Next GEM Chip G Single Cell Kit	10× Genomics, Inc.	Cat# 1000120
Qubit™ dsDNA HS Assay Kit	Thermo Fisher	Cat# Q32854
KAPA Library Quantification Kits - Complete Kit (Universal)	Roche Sequencing	Cat# 07960140001
24 DNALabChipX-Mark	PerkinElmer	Cat# CLS145331
HT DNA NGS 3K Reagent Kit	PerkinElmer	Cat# CLS960013
NovaSeq 6000 S4 Reagent Kit v1.5 (300 cycles)	Illumina	20028312
Software and algorithms		
R Studio	R Studio Inc.	https://www.rstudio.com/
R Package: Seurat	Satija Lab	https://satijalab.org/seurat/

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
R Package: ggplot	Tidyverse	https://ggplot2.tidyverse.org/
Cell Ranger Software	10× Genomics, Inc.	https://support.10xgenomics.com/single-cellgene-expression/software/pipelines/latest/installation

Other		
Nikon ECLIPSE Ti2 Inverted Microscope Systems	Nikon	ECLIPSE Ti2-U
Countstar Altair Smart Cell Analyzer	Countstar	Cat# alt-002
Tween 20	Sigma-Aldrich	Cat# P9416-50ML
0.2 mL Maxymum recovery flat cap	Axygen	Cat# PCR-02-L-C
Axygen 0.2 mL polypropylene PCR tube strips, 8 tubes/strip, Clear, nonsterile	Axygen	Cat# PCR-0208-C
15 mL tubes	LabSelect	Cat# CT-002-15
1.5 mL DNA LoBind Tubes	Eppendorf	Cat# 22431021
2 mL DNA LoBind Tubes	Eppendorf	Cat# 22431048
20 µL filter tips	Axygen	Cat# TF-20-R-S
1000 µL filter tips	Biosharp	Cat# BS-1000-TRS
200 µL filter tips	Biosharp	Cat# BS-200-TRS
10 µL filter tips	Biosharp	Cat# BS-10-TRS
SmartStrainer 70 µm	Miltenyi Biotec	Cat# 130-098-462
SmartStrainer 30 µm	Miltenyi Biotec	Cat# 130-098-458
Invitrogen Countess Cell Counting Chamber Slides	Thermo Fisher	Cat# C10228
Qubit Assay Tubes	Thermo Fisher	Cat# Q32856

MATERIALS AND EQUIPMENT

Cell dissociation solution		
Reagent	Final concentration	Amount
Collagenase II	2 mg/mL	0.1 g
DMEM	-	50 mL
Total	N/A	50 mL

Cell suspension solution		
Reagent	Final concentration	Amount
FBS	10%	10 mL
DMEM	90%	90 mL
Total	N/A	100 mL

0.04% BSA solution		
Reagent	Final concentration	Amount
PBS	N/A	50 mL
BSA	0.4 mg/mL	20 mg
Total	N/A	50 mL

50% Percoll solution		
Reagent	Final concentration	Amount
100% Percoll	50%	5 mL
PBS	50%	5 mL
Total	N/A	10 mL

30% Percoll solution		
Reagent	Final concentration	Amount
100% Percoll	30%	3 mL
PBS	70%	7 mL
Total	N/A	10 mL

STEP-BY-STEP METHOD DETAILS

Sample collection

⏱ Timing: 10–30 min

This step describes how to take and transport samples.

1. Select tissue from the area of interest, trim as much of the discarded tissue as possible with a sterile scalpel and cut the tissue to a size of about 6 mm (Figure 1, avoid locations containing blood clots or erosive areas).
2. Wash the tissue twice with pre-cooled PBS or cell suspension solution, then place the tissue in tissue storage solution and transport on ice for further experiments within 30 min.

Processing of tissue

⏱ Timing: < 30 min

This step describes how to pre-treat the sample before enzymatic digestion.

3. Remove tissue from tissue preservation solution and place on ice in a 35 mm Petri dish, then rinse tissue 2–3 times using ice-cold PBS or cell suspension solution to remove any remaining blood.
4. Trim tissue blocks in PBS with sterilized scissors, selecting structurally intact tissue with a weight of about 0.2 g and avoiding blood clots.
5. Aspirate the PBS and unwanted tissue and gently cut the tissue with scissors into 1 mm^3 dimension (Figure 2).

   ⚠ CRITICAL: All instruments and consumables should be sterile and instruments such as scissors should be moistened with PBS or culture medium before use to avoid contact between tissue and dry items which may reduce viability.

   Note: If necessary, the clipped tissue can be gently rinsed with PBS and the supernatant carefully aspirated and discarded. This can further remove excess blood from the tissue.

Enzymatic digestion and mechanical dissociation of tissue

⏱ Timing: 60 min

This step details how enzymatic digestion in combination with gentle mechanical force allows for dissociation of solid liver tissue into single cell suspension.

6. Aspirate 5 mL of cell lysis solution using a serological pipette.
   a. Add to the previous Petri dish containing the tissue.
   b. Transfer the tissue with the cell lysis solution into a 50 mL centrifuge tube using a serological pipette.
7. Put the 50 mL centrifuge tube to a 37°C water bath shaker, digest the tissue for 8 min, with the shaker speed set at 80–100 rpm.
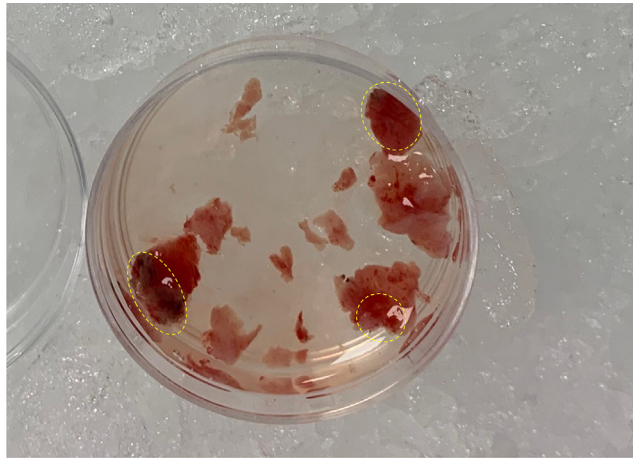
Figure 1. Tissue areas containing blood clots or eroded areas that need to be discarded

8. When the first enzymatic digestion is complete, pipette the tissue and liquid up and down for about 10 times using a 5 mL Pasteur pipette, so that the tissue and the enzyme solution can be fully mixed to further disrupt the tissue.

9. After the settlement of the remaining tissue, collect the suspension and discharge onto a 70 μm cell strainer placed on top of a 50 mL centrifuge tube on ice to terminate the enzymatic reaction and collect the cells.

10. Rinse the cell strainer with about 200 μL cell suspension solution and pass it through the cell strainer, to retrieve the maximal number of cells, absorb the cell suspension as much as possible for filtration.

11. Add 5 mL of fresh cell lysis solution into the remaining tissue fragments in the 50 mL centrifuge tube for a second round of tissue dissociation. Incubate in a 37°C water bath for 10 min.

   Note: Shake the centrifuge tube every 2 min for 5–10 s

12. When the second enzymatic digestion is complete, transfer the suspension to the 50 mL centrifuge tube from which the supernatant was previously collected and stored.

   Optional: Repeat the enzymatic digestion of the tissue precipitate in step 2 and collect the supernatant. Depending on the digestion of the tissue mass, we recommend that the total number of digestions is not more than 4. Please also note that a reduction of the tissue mass is usually observed after digestion.

   Note: Cell counts and viability counts can be performed on the supernatant obtained from each enzymatic digestion to determine the dissociations times. For example, obtaining less than one million cells per enzymatic digestion, or a cell viability of less than 70%, can be the end point of enzymatic digestion.

13. All the collected supernatant was filtered through a 70 μm filter and the filtrate was collected using a 50 mL centrifuge tube.

14. Collect the cells by centrifugation at 300 g for 6 min at 4°C

15. Resuspend the cell precipitate using cell suspension solution and transfer it to a 15 mL centrifuge tube. Repeat the previous centrifugation step and resuspend the precipitate with an appropriate amount of ice-cold cell suspension solution or PBS (~2–3 mL).

Density gradient centrifugation via multiple-layered Percoll
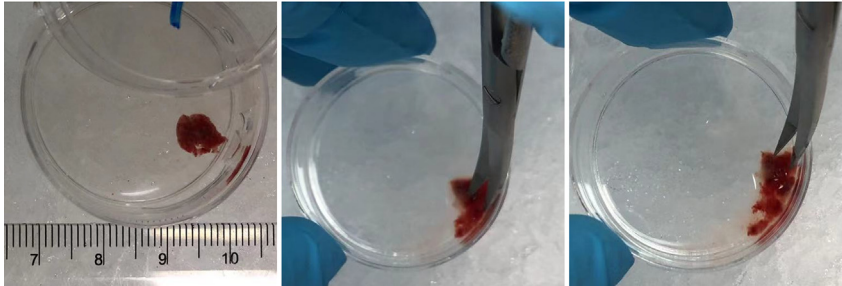
   ⏱ Timing: < 60 min

Figure 2. Mincing of hepatoblastoma tissue in digestion medium

This step details how the different components of the single cell suspension were separated by centrifugation using the Percoll density gradient protocol, which yielded a hepatocyte layer with the main component of hepatocytes and the immunocyte precipitation layer with the main component of immune cells and red blood cells. This method is used to remove the fragments from the single cell suspension and isolate the hepatocytes, so as to prevent the influence of the subsequent red cell lysis step on the activity of hepatocytes.

16. Add 3 mL of the 30% Percoll solution to the bottom of a 15 mL centrifuge tube.
    a. Using a Pasteur pipette, reach to the bottom of the tube and slowly add 3 mL of the 50% Percoll solution to the lower layer of the 30% Percoll solution.
    b. Slowly add 3 mL of the cell suspension onto the surface of the 30% Percoll layer, taking care not to disturb the gradient.

    Optional: It is also appropriate to add the 50% Percoll solution to the centrifuge tube first and then add the 30% Percoll solution along the liquid surface using a Pasteur pipette. Finally, add the cell suspension to the surface of the gradient.

    Note: Each layer needs to be added very gently to avoid disturbing the gradient.

17. Centrifugation at 400 g for 20 min at 4°C, set acceleration 4 and no brake (Figure 3).
18. Aspirate and discard the top layer of PBS, the layer of cellular debris between the PBS and 30% Percoll layer (there may be no cellular debris layer or it may not be visible) and the 30% Percoll layer in turn.
19. Carefully transfer the hepatocyte layer between the 30% Percoll layer and 50% Percoll layer to a 15 mL centrifuge tube, keeping the total volume to a maximum of 2 mL.

    Note: Transfer as little liquid as possible from the Percoll layer when transferring the hepatocyte layer.

Red blood cell lysis and cell washing

⏱ Timing: 30 min

This step washes the different layers of cells to remove participating Percoll medium and impurities and performs erythrocyte lysis on the immune cell layer.

20. Dilution of the cell suspension of the hepatocyte layer with 3 times volume of cell suspension solution and centrifugation at 300 g for 10 min at 4°C.

    Note: Add at least 3 times the volume of hepatocyte layer liquid and mix gently and well to prevent loss of cells after centrifugation due to unmixed Percoll.
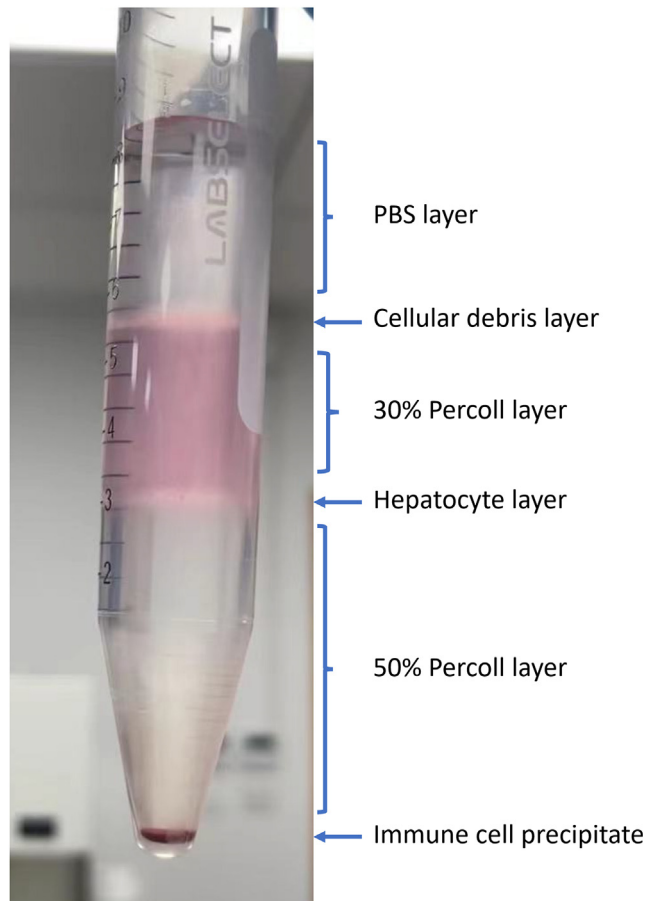
Figure 3. Density gradient centrifugation via multiple-layered Percoll

21. The precipitate below 50% Percoll layer contains immune cells and erythrocytes. Aspirate and discard all supernatants (remaining 50% Percoll layer), then resuspend the immune cell precipitate using 1 mL of ACK Lysing buffer and let it react for 3 min at room temperature to lyse the erythrocytes.
22. To terminate the erythrocyte lysis reaction, the erythrocyte lysed immune cell fluid was filled with 10 mL cell suspension solution in 15 mL centrifuge tubes and the immune cells were collected by centrifugation at 500 g for 10 min at 4°C (Figure 4).
23. Resuspend the cell precipitates from steps 20 and 22 respectively with 2 mL cell suspension solution or 0.04% BSA solution and perform quality control on the cell suspensions (this can be adjusted after an initial count if necessary to get optimal cell concentrations for counting).

Cell viability, concentration and singularity evaluation and counting

⊙ Timing: < 20 min

In this step, the activity of the immune cell resuspension and hepatocyte layer suspension was examined and recorded under the microscope. Subsequently, the two cell suspensions were combined and quantified.

24. Aspirate 10 μL of Percoll density gradient centrifuged hepatocyte layer suspension and immune cell resuspension after erythrocyte lysis respectively from step 23 and stain with 10 μL of Trypan blue stain (0.4%), then examine the cell status using ECLIPSE Ti2 inverted microscope. The results are shown in Figure 5.
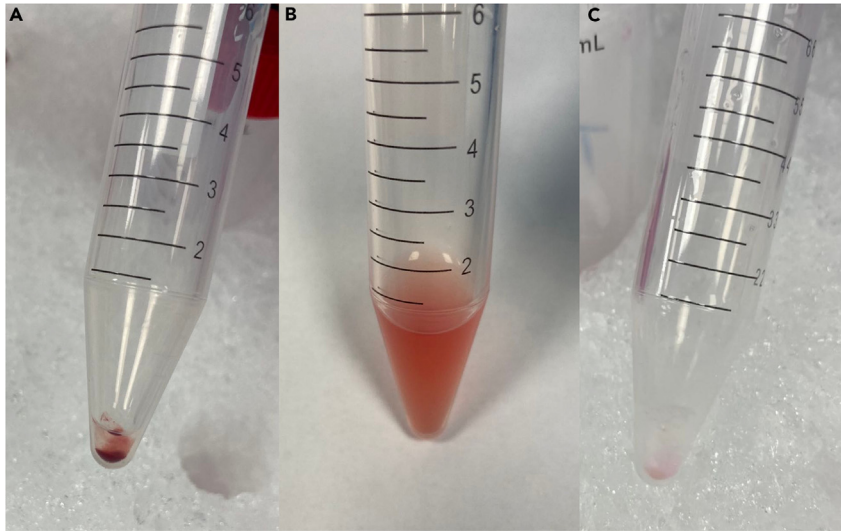
Figure 4. Cell pellets before, during and after red blood cell lysis

25. Mix the above two single cell suspensions (hepatocyte suspension and immune cell suspension), centrifuge them at 400 g for 10 min at 4°C, and then resuspend in 5 mL cell suspension solution or 0.04% BSA solution. Afterward, proceed to the next step of detecting the viability and concentration of the single cell suspensions.

    Note: The volume of cell suspension solution required for resuspension is determined by the requirements of subsequent experiments. We believe that a final concentration of 1000–3000 cells/µL is favorable for counting, while for 10× Genomics Single-cell transcriptome library construction, a final concentration of 800–1300 cells/µL is favorable for obtaining stable results.

26. To determine cell viability and concentration, 10 µL of the final single-cell suspension from step 25 was stained with 10 µL of Cellometer ViaStain™ AOPI Staining Solution (AOPI) and analyzed using the Countstar Altair Smart Cell Analyzer. The results of the automated counts are shown in Figure 6.

    Note: Survival rate should be at least 80%. A high percentage of dead cells indicates a problem with the processing of the tissue, e.g., due to problems with enzyme concentration, incubation time and other processing steps.

    a. If cell viability lower than 80%, refer to troubleshooting.
    b. If the cell count is below 500 cells/mL, please refer to troubleshooting.
    c. If cell viability is ≥ 80% and cell count is ≥ 500 cells/mL, proceed to the next step.

    Note: When using an automated cell counter, it is important to ensure that the correct focus is set to count all surviving cells of different sizes and shapes. Examining cell morphology under a bright-field microscope is a good way to make a quick visual inspection of the results of the automated cell counter.

Single-cell transcriptome library construction
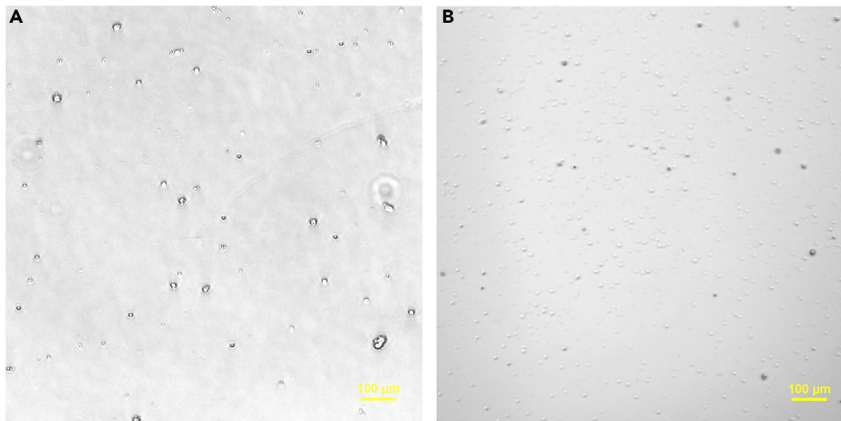
⏱ Timing: 1 day

Figure 5. Results of Taipan Blue staining of different cell layers after multiple-layered Percoll density gradient separation
(A and B) (A) shows the hepatocyte layer with a larger overall cell diameter, and (B) shows the immune cell layer with a smaller overall cell diameter.

A brief procedure for the construction of single cell transcriptome library from the cell suspensions obtained by step 25 via Chromium Next GEM Single Cell 3′ Kit v3.1 (10× Genomics). Chromium Next GEM Single Cell 3′ Kit v3.1 was used for mRNA barcoding, reverse transcription, and single-cell transcriptome library preparation, detailed operating manual refer to: https://www.10xgenomics.com/support/single-cell-gene-expression/documentation/steps/library-prep/chromium-single-cell-3-reagent-kits-user-guide-v-3-1-chemistry-dual-index.

27. Following the manual for Chromium Controller to load the cells onto Chromium Chip G to generate GEM and barcode cells and our aimed target cell recovery is 10000.
    a. At the end of this step, transfer GEMs to a new 0.2 mL centrifuge tube.
    b. Execute the GEM-RT incubation procedure: 53°C, 45 min; 85°C, 5 min; 4°C, hold; with the lid temperature at 53°C.

    ⏸ Pause point: The products of GEM-RT Incubation can store at 4°C for up to 72 h or at −20°C for up to a week, or proceed to the next step.

    Note: We recommend a pause at this step to end the first day of the experiment, other pause points in the library construction step can refers to the detailed operating manual.

28. Following the steps in the manual for recovery, amplification, and purification of reverse transcription products. The PCR cycles of amplification in this process is determined by "Targeted Cell Recovery". See the table below for details:

Targeted cell recovery	Total cycles
<500	13
500–6,000	12
>6,000	11

As our aimed target cell recovery is 10000, >6000, so the PCR cycles of cDNA amplification is 11, and the purification step is completed by using AMPure XP beads and the product concentration was determined by the Qubit™ dsDNA HS Assay Kit.
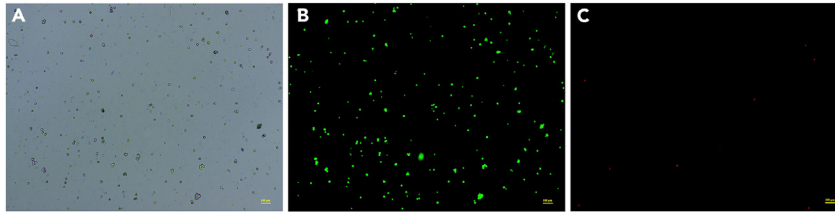
Figure 6. Results of AOPI staining of single cell suspensions
(A) illustrates the composition of a single cell suspension.
(B) presents the live cell count within the same field of view.
(C) depicts the dead cell count in the identical area. The total cell concentration reached 1.13×10^6/mL, with a live cell concentration of 1.09×10^6/mL (96.5%) and a dead cell concentration of 3.97×10^4/mL (3.5%).

29. Perform cDNA quality control and quantification.
30. Follow the steps in the manual for cDNA fragmentation, end-repair, size selection, adapter ligation, amplification and purification, and other library construction steps. The amount of cDNA input determines the number of PCR cycles of amplification in this process. Refer to the table below for more details:

cDNA input	Total cycles
0.25–25 ng	14–16
25–150 ng	12–14
150–500 ng	10–12
500-1,000 ng	8–10
1,000–1,500 ng	6–8
>1500 ng	5

Since the amount of cDNA we input is between 25–150 ng, the number of PCR cycles is generally 12–14, and the purification step is completed by using AMPure XP beads and the product concentration was determined by the Qubit™ dsDNA HS Assay Kit.

31. Determine the distribution of the final DNA library by diluting 1 μL sample with 9 μL water and then loading 1 mL of diluted DNA library on an Agilent Bioanalyzer High Sensitivity chip. The expected DNA traces are shown in Figure 7. Select the region encompassing 35–10,000 bp and record the DNA concentration.

    Note: The expected cDNA amount should be at $\geq$ 5.0 ng/mL. Refer to troubleshooting table if DNA amount is lower.

    ⏸ **Pause point:** At this step, libraries can be frozen at −20°C or −80°C, and stored for several months.

Next-generation DNA sequencing

🕐 **Timing: 1 day**

This step briefly describes the steps for sequencing a single cell transcriptome library.

32. Using 10 mM Tris-HCl [pH 8.5] dilute each DNA library down to 2 nM concentration.
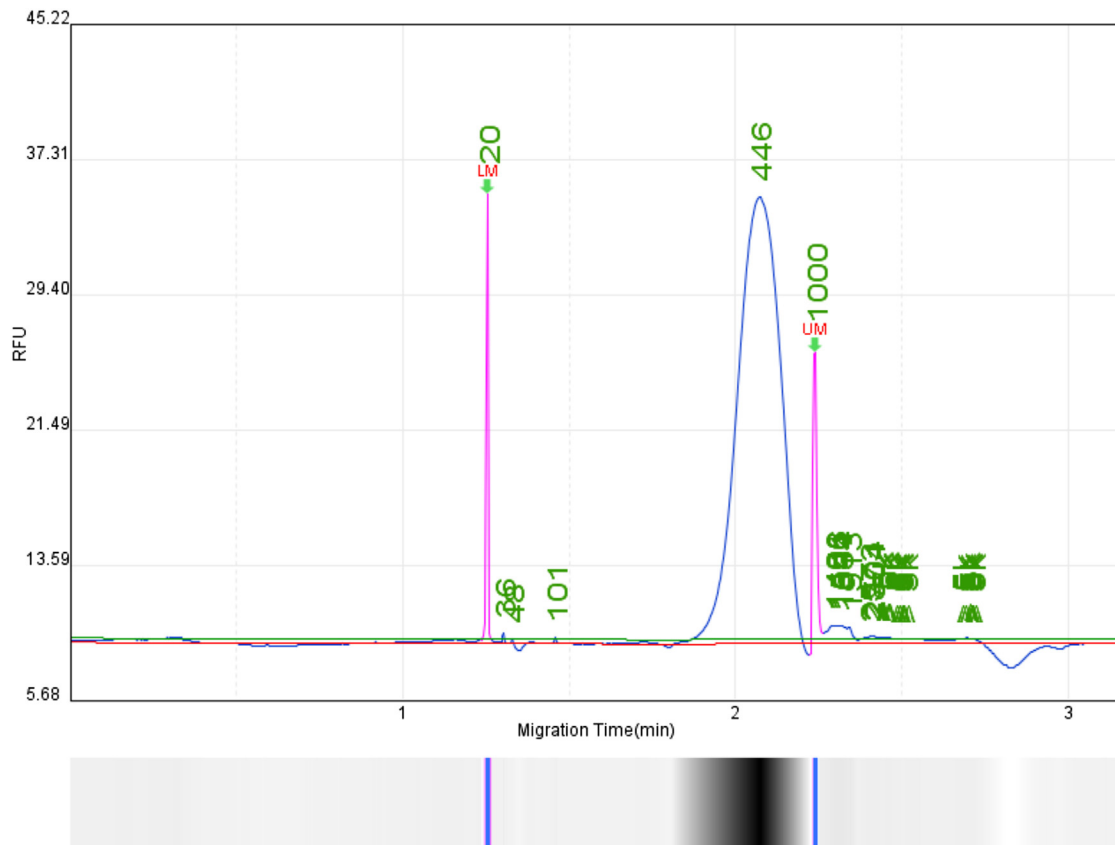33. Pool individual DNA libraries into one tube.

Figure 7. Distribution of the scRNA-seq library

34. Follow the NovaSeq 6000 Denature and Dilute Libraries Guide to denature and dilute libraries (Standard Loading).
35. When using NovaSeq 6000 S4 Reagent Kit v1.5 (300 cycles) sequence the DNA library using following recommended number of cycles: Read1, 28 cycles; i7 index, 10 cycles; i5 index, 10 cycles; Read 2,90 cycles.
36. Aim to achieve sequencing depth of 30,000–50,000 reads per cell, or 120–200 million reads per DNA library (e.g., a pool of 8 libraries may require up to 1,600 million reads).

   Note: If the sequencing depth does not meet this standard, refer to troubleshooting.

Data processing

⏱ Timing: 10 h

This step aligns the FASTQ file to the reference genome via the Cellranger count pipeline and generates a results file for further analysis.[5]

37. Run Cellranger counts, process raw FASTQ data by alignment, filtering, barcode counts and UMI counts and generate single cell feature counts for a single library.

```
> /Your/Path/To/cellranger count --id=output_file_name --transcriptome=/Your/Path /To/re-
fdata --fastqs=/ Your/Path/To/Fastq/ --sample=sampleID
```

Note: For other types of raw data, other functions of the pipeline, see https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/what-is-cell-ranger for details.

Data visualization

⏱ Timing: 1 week

This step mainly uses the Seurat pipeline to filters and downscales the expression matrix obtained by Cellranger, and performs analysis and visualization.

Note: The Seurat package offers a wealth of data handling methods, and we have provided only the basic module descriptions. For multiple sample analysis and other individual parameters see[6]: https://satijalab.org/seurat/

38. Load the required packages and data

```
library(dplyr)

library(Seurat)

library(patchwork)

setwd('/Your/Path/.../output_file')

data <- Read10X(data.dir = "/Your/Path/.../filtered_feature_bc_matrix")

HBdata <- CreateSeuratObject(counts = data, project = "sampleID", min.cells = 3, min.features = 200)
```

Code Box 1. R code for loading data.

39. Perform quality control by visualizing data using the VlnPlot() function and further filter gene expression, reads, and mitochondrial expression data using the subset() function (See Figure 8).

```
HBdata[["percent.mt"]] <- PercentageFeatureSet(HBdata, pattern = "^MT-")

VlnPlot(HBdata, features = c("nFeature_RNA", "nCount_RNA", "percent.mt"), ncol = 3)

HBdata <- subset(HBdata, subset = nFeature_RNA > 400 & nCount_RNA > 3000 & percent.mt < 20)
```

Code Box 2. R code for quality control

Note: The thresholds for filtering are chosen appropriately based on the results of the violin plot and the characteristics of the sample.

40. Normalize and find variable features. Highly variable features of the dataset are identified by the FindVariableFeatures() function and the first 2000 (or other appropriate number of features) are used for downstream analysis.

```
HBdata <- NormalizeData(HBdata, normalization.method = "LogNormalize", scale.factor = 10000)
```

```
HBdata <- NormalizeData(HBdata)

HBdata <- FindVariableFeatures(HBdata, selection.method = "vst", nfeatures = 2000)
```

Code Box 3. R code for normalizing and finding variable Features.

41. Downscale and cluster the data. Use the ScaleData() function to linearly transform the dataset for further Principal Component Analysis and to determine the data dimensions for further analysis. Cluster cells using the FindNeighbors() and FindClusters() functions and visualize the dataset using the RunUMAP() method.

```
all.genes <- rownames(HBdata)

HBdata <- ScaleData(HBdata, features = all.genes)

HBdata <- RunPCA(HBdata, features = VariableFeatures(object = HBdata))

HBdata <- FindNeighbors(HBdata, dims = 1:15)

HBdata <- FindClusters(HBdata, resolution = 0.8)

HBdata <- RunUMAP(HBdata, dims = 1:15)

DimPlot(HBdata, reduction = "umap")
```

Code Box 4. R code for data downscaling and clustering.

42. Identify the cell types of the main cell clusters and use the subset() function to separate the different cell clusters for further identification of fine cell types. The cell populations and their markers identified include: Macrophage (CD14, FCGR1A), cDC (CD1C, CSF2RA, CLEC10A), pDC (GZMB, IRF7), T (CD3D, CD3E), B (MS4A1, CD79A), Monocyte (FCN1), LVEC (GNG11), Endothelial progenitor cells (PECAM1, CD34), Endothelial cells (AQP1), Periportal LSEC (MGP, ALB, APO), Entral venous LSEC (FCGR2B), Dividing endothelial cells (TUBA1B), Epithelial (AFP), Malignat cells (AFP), Hepatocyte (ALB, APOA1), Hepatic stellate cells (TPM2, CPLEC11), Hepatic progenitor cell (KRT19, KRT7), Kupffer (C1QA, FCGR3A).

```
HBdata.markers <- FindAllMarkers(HBdata, only.pos = TRUE, min.pct = 0.25, logfc.threshold = 0.25)

VlnPlot(HBdata, features = "PTPRC")

new.cluster.ids <- c('Tissue_secific', 'Tissue_secific', 'Tissue_secific', 'Tissue_secific', 'Tissue_secific', 'Tissue_
secific', 'Immune', 'Endothelial', 'Stromal', 'Immune', 'Stromal', 'Immune', 'Immune', 'Tissue_secific', 'Immune', 'Im-
mune', 'Tissue_secific')

names(new.cluster.ids) <- levels(HBdata)

HBdata <- RenameIdents(HBdata, new.cluster.ids)

DimPlot(HBdata, reduction = "umap", label = TRUE,raster=FALSE)

Immune <- subset(data24T, cells= WhichCells(data24T, idents = c("Immune")))

Endothelial <- subset(data24T, cells= WhichCells(data24T, idents = "Endothelial"))

Stromal <- subset(data24T, cells= WhichCells(data24T, idents = "Stromal"))

Tissue_secific <- subset(data24T, cells= WhichCells(data24T, idents = c("Tissue_secific")))

## Repeat Data downscaling and clustering reference to step 41 and define clusters by marker gene expression
```
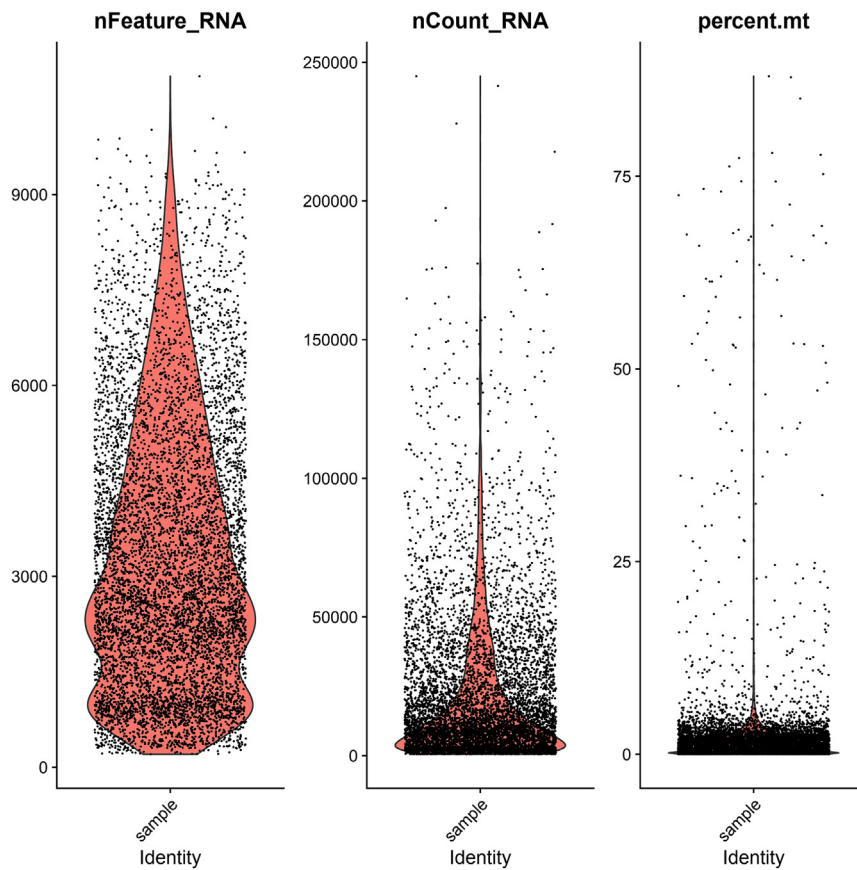
Figure 8. Gene expression, reads and mitochondrial expression of the example data

Code Box 5. R code for identifying cell types in different cell clusters

EXPECTED OUTCOMES

The aim of this protocol is to provide an easy-to-handle procedure to obtain large amount, high viability and more diversity single cells from human liver and processing workflow that has been performed under high containment biological laboratories and is thus suitable for infectious disease research. Processing hepatoblastoma tissue according to steps 1–26 could provide a single cell suspension containing approximately 2×10^6 cells, including T cells, B cells, dendritic cells, monocyte, endothelial cells, hepatocyte, and et al. from 0.2 g tissue. the singularity and viability rates of the obtained single-cell suspension is above 90%, thereby fulfilling the necessary technical quality criteria for subsequent single-cell RNA-sequencing.

> *Note:* Cell numbers can vary, depending on age, disease, infectious agent, and time point of analysis.

The expected unsorted (not enriched or depleted for any specific cell type) physiological frequencies of pulmonary cell types after barcoding, library preparation and sequencing are displayed in Figure 9. We divided the cells into four broad categories - immune, endothelial, stromal, and tissue-specific and further divided them in more detail, with their cell types and proportions shown in Figures 9A–9D respectively. Table 1 shows the quality of the library after sequencing and processing through the Cellranger pipeline.
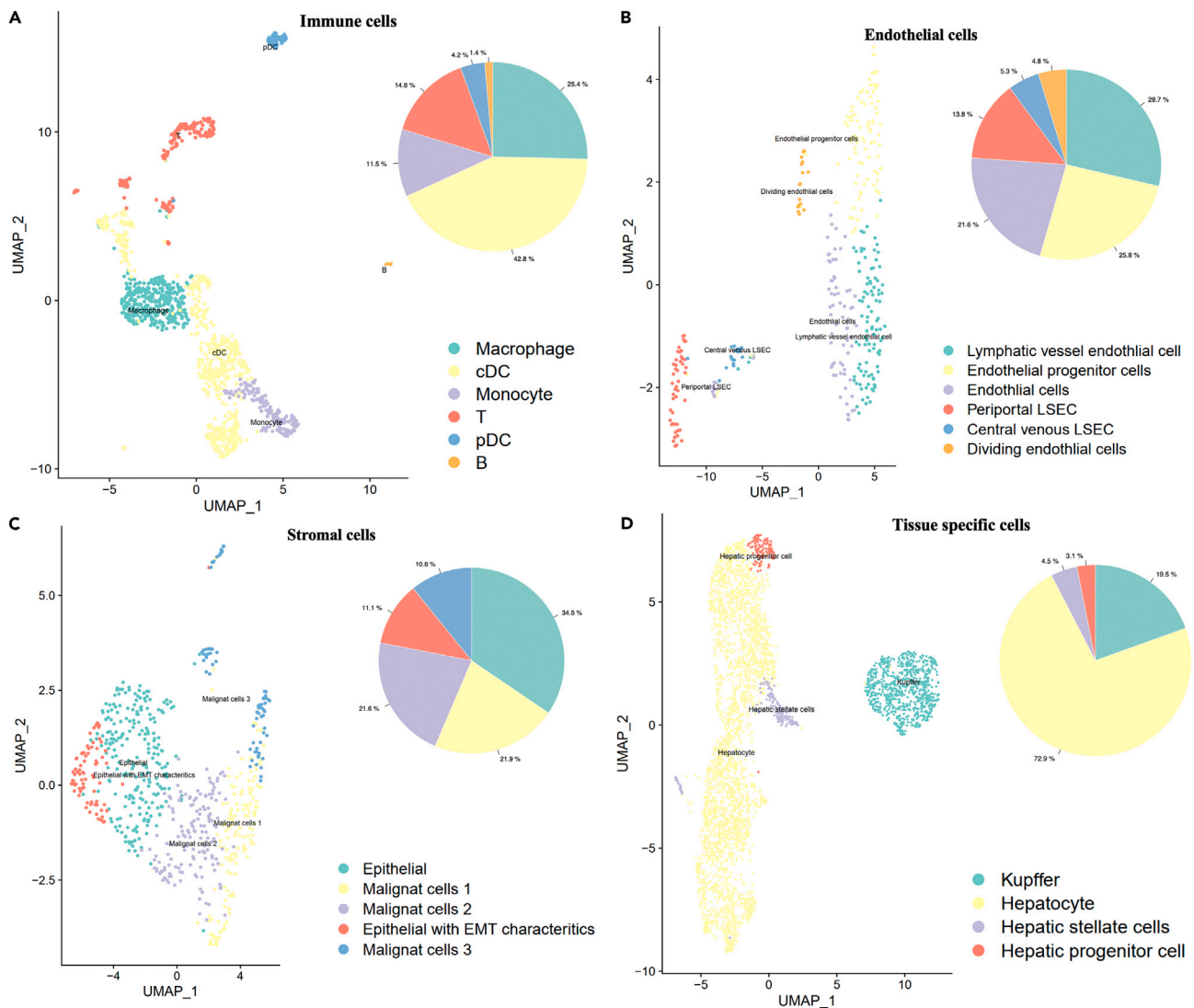
Figure 9. UMAP plot displaying clustering and annotation of different cell types and pie chart indicating proportions of sequenced and annotated cell types in hepatoblastoma patients

(A–D) Figures 7A–7D are UMAP plots and pie charts of cell subpopulation ratios of Immune cells (A), Endothelial cells (B), Stromal cells (C), and Tissue specific cell populations (D), respectively.

LIMITATIONS

This protocol is used to dissociate liver tissue to obtain single cell suspensions and perform single cell sequencing with a typical cell viability rate of approximately 95%. However, due to sample differences, the enzyme concentration and reaction time used will need to be adjusted accordingly, as will the downstream data analysis.

Table 1. Key library quality information after Cellranger pipeline processing

Number of reads	Valid barcodes	Q30 bases in barcode	Q30 bases in RNA read	Q30 bases in UMI
502,468,713	98.7%	96.5%	93.1%	96.1%
Total genes detected	Mean reads per cell	Median UMI counts per cell	Median genes per cell	Estimated number of cells
25,717	64,944	11,255	2,833	7,737

TROUBLESHOOTING
Problem 1
Low yield of cells after dissociation.

Potential solution

- Use more tissue as well as more enzymatic solution, ensuring that the tissue is cut small enough (Step 5) and that the temperature of the enzymatic reaction is stable and the speed of the shaker is appropriate (Step 7).
- Increase the enzyme concentration and the number of dissociations according to the cell viability obtained.

Problem 2
Low cell viability.

Potential solution

- The duration of each digestion or the concentration of the enzyme is reduced and the cellular activity of each digestion is observed to determine whether the conditions are suitable (Step 6–12).
- Shorten the processing time from obtaining the sample to obtaining the cell suspension and ensure that the corresponding storage and reaction temperatures are appropriate.
- If the above methods do not significantly improve cell viability, treat the cell suspension appropriately with a live cell enrichment kit, such as Dead Cell Removal Kit (Miltenyi Biotec, 130-090-101).

Problem 3
Failure of the multilayer Percoll density gradient separation method to isolate different cell types.

Potential solution

- If it is difficult to add multiple layers of Percoll, try using phenol red stained medium to differentiate the layers, for example, using DMEM medium with phenol red instead of PBS to prepare 30% Percoll (Figure 10), which facilitates the observation of different density junctions when adding different densities of Percoll medium in drops and facilitates the removal of unwanted layers (Step 16).
- Ensure proper acceleration and braking speed of the centrifuge, and prevent mixing between the different layers when adding Percoll medium and cell suspension.

Problem 4
Cell clumping in the final single cell suspensions before processing 10× chromium controller.

Potential solution

- Addition of appropriate amount of DNAase I (concentration below 0.1 mg/mL) to the cell final single cell suspensions (Step 25).
- Collect the cells by centrifugation the cell suspension at 400 g for 5 min at 4°C, and resuspend cells with 0.05% trypsin, mix and reacted at 37°C for 5–10 min, then terminate the digestion with a medium containing 10% FBS. Centrifugation and resuspension of the cell suspension with culture medium.

Problem 5
Single cell suspensions containing debris impurities.

Figure 10. Stratification pattern of Multiple- layered Percoll protocol after replacement of PBS in 30% Percoll with DMEM medium containing phenol red

Potential solution

- For larger piece of debris, the cell suspension can be centrifuged for a short time (less than 10 s) by setting the centrifuge to a minimum speed (usually less than 50 g) and taking the supernatant as the final cell suspension. The cell suspension can also be filtered through a 30 μm filter membrane (Step 25).
- For smaller debris, cells can be enriched by low-speed centrifugation at 200 g for 5–10 min at 4°C.

Problem 6
Abnormal distribution of single-cell transcriptome library.

Potential solution

- If the distribution of cDNA library fragments is abnormal, the experiment may not proceed. If the final DNA library fragment distribution is abnormal, the library can be re-constructed using the excess cDNA library (Step 30).

- Detailed library quality control criteria can be found in the user guide of Chromium Next GEM Single Cell 3′ Kit v3.1.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, [Jian He] (jih003@sjtu.edu.cn).

Materials availability

No new material was generated using this protocol.

Data and code availability

The datasets supporting the current study have not been deposited in a public repository because further research is ongoing but are available from the corresponding author on request.

AUTHOR CONTRIBUTIONS

J.H., M.M., X.Z., Y.W., R.G., Methodology, Reviewing and Editing; Y.S., Reviewing; J.H., S.G., Supervision; J.H., Conceptualization; J.H., X.Z., Writing - Original Draft.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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