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TITLE:

Single-cell RNA Sequencing and Analysis of Human Pancreatic Islets

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SUMMARY:

Here, we present a protocol to generate high-quality, large-scale transcriptome data of single cells from isolated human pancreatic islets using a droplet-based microfluidic single-cell RNA sequencing technology.

ABSTRACT:

Pancreatic islets comprise of endocrine cells with distinctive hormone expression patterns. The endocrine cells show functional differences in response to normal and pathological conditions. The goal of this protocol is to generate high-quality, large-scale transcriptome data of each endocrine cell type with the use of a droplet-based microfluidic single-cell RNA sequencing technology. Such data can be utilized to build the gene expression profile of each endocrine cell type in normal or specific conditions. The process requires careful handling, accurate measurement, and rigorous quality control. In this protocol, we describe detailed steps for human pancreatic islets dissociation, sequencing, and data analysis. The representative results of about 20,000 human single islet cells demonstrate the successful application of the protocol.

INTRODUCTION:

Pancreatic islets release endocrine hormones to regulate blood glucose levels. Five endocrine cell types, which differ functionally and morphologically, are involved in this essential role: α-cells

produce glucagon, β-cells insulin, δ-cells somatostatin, PP cells pancreatic polypeptide, and ε-cells ghrelin[1]. Gene expression profiling is a useful approach to characterize the endocrine cells in normal or specific conditions. Historically, the whole islet gene expression profiling was generated using microarray and next-generation RNA sequencing[2-8]. Although the whole islet transcriptome is informative to identify the organ-specific transcripts and disease candidate genes, it fails to uncover the molecular heterogeneity of each islet cell type. Laser capture microdissection (LCM) technique has been applied to directly obtain specific cell types from islets[9-12] but falls short of purity of the targeted cell population. To overcome these limitations, fluorescence-activated cell sorting (FACS) has been used to select specific endocrine cell populations, such as α- and β-cells[13-18]. Moreover, Dorrell et al. used an antibody-based FACS sorting approach to classify β-cells into four subpopulations[19]. FACS-sorted islet cells can also be plated for RNA sequencing of single cells; however, the plate-based methods face challenges in scalability[20-22].

To generate high quality, large-scale transcriptome data of each endocrine cell type, we applied microfluidic technology to human islet cells. The microfluidic platform generates transcriptome data from a large number of single cells in a high-throughput, high-quality, and scalable manner[23-27]. In addition to revealing molecular characteristics of a cell type captured in a large quantity, highly-scalable microfluidic platform enables identification of rare cell types when enough cells are provided. Hence, application of the platform to human pancreatic islets allowed profiling of ghrelin-secreting ε-cells, a rare endocrine cell type with little known function due to its scarcity[28]. In recent years, several studies have been published by us and others reporting large-scale transcriptome data of human islets using the technology[29-33]. The data are publicly available and useful resources for the islet community to study endocrine cell heterogeneity and its implication in diseases.

Here, we describe a droplet-based microfluidic single-cell RNA sequencing protocol, which has been used to produce transcriptome data of approximately 20,000 human islet cells including α-, β-, δ-, PP, ε-cells, and a smaller proportion of non-endocrine cells[32]. The workflow starts with isolated human islets and depicts steps of islet cell dissociation, single-cell capture, and data analysis. The protocol requires the use of freshly isolated islets and can be applied to islets from humans and other species, such as rodents. Using this workflow, unbiased and comprehensive islet cell atlas under baseline and other conditions can be built.

PROTOCOL:

1. Human islet dissociation

1.1. Obtain human islets isolated from cadaver organ donors of either sex, ages between 15-80 years, without pre-existing diseases unless islets from donors with specific demographics are required for the study purpose.

1.1.1. After isolation, have the isolated islets kept in the tissue culture facility for 2-3 days at the supplier. It often takes more than 1 day for islet damages to become visible.

1.1.2. Place the islets in a bottle and immerse it completely in the islet medium. Get it to the laboratory by overnight shipment.

1.1.3. Obtain the islet equivalent quantity (IEQ) of the shipped islets from the islet supplier.

1.2. Recover islets from the shipment on the day of islet arrival. Perform this step using a hood to minimize the chance of contamination.

1.2.1. Cool the complete islet media (CMRL-1066, 10% (v/v) FBS, 1x Pen-Strep, 2 mM glutamine) in a refrigerator.

1.2.2. Transfer the islets from the bottle to a 50 mL conical tube.

1.2.3. Add 10 mL pre-cooled complete islet media to the emptied bottle to wash out the remaining islets. Transfer the media to the conical tube.

1.2.4. Centrifuge the tube at 200 *x g* for 2 min to recover islets. Aspirate the supernatant leaving about 1-2 mL media with the pellet.

1.2.5. Resuspend the islets with pre-cooled complete islet media. Based on the IEQ provided by the supplier, add 12 mL media per 5000 IEQ.

1.2.6. Pour the islets in the media on a 10 cm non-treated tissue culture dish. Incubate overnight in a tissue culture incubator at 37 °C with 5% CO_2 in atmospheric air.

1.3. Dissociate islets as shown below. Perform this step following overnight incubation.

1.3.1. Pre-warm complete islet media and cell dissociation solution.

1.3.2. Prepare 1x PBS containing 0.04% BSA at room temperature.

1.3.3. Count and hand-pick 200-300 islets using a P200 pipette and transfer the islets to a 15 mL conical tube containing 5 mL pre-warmed complete islet media.

1.3.4. Collect the islets by centrifugation at 200 *x g* for 2 min. Gently aspirate the supernatant without disturbing the pellet on the bottom.

1.3.5. Add 1.0 mL pre-warmed cell dissociation solution and disrupt the pellet by pipetting gently up and down. Incubate the islets at 37 °C for 9-11 min. Every 3 min pipette up and down slowly for 10 s to dissociate the cells into single cells.

1.3.6. Once islet cells are well dissociated and the solution becomes cloudy, add 9 mL complete islet media and filter through a 30 µm cell strainer into a new 15 mL conical tube.

1.3.7. Wash the tube and cell strainer with 2 mL complete islet media to collect the remaining islets and add to the same tube.

1.3.8. Collect cells by centrifugation at 400 *x g* for 5 min.

1.3.9. Gently aspirate media and resuspend the cell pellet in 5 mL 1x PBS containing 0.04% BSA (PBS-BSA).

1.3.10. Filter through a new 30 µm cell strainer into a 15 mL conical tube and centrifuge at 400 *x g* for 5 min to collect cells.

1.3.11. Aspirate the supernatant and resuspend the cell pellet in 200-300 µL 1x PBS-BSA solution.

1.3.12. Measure the cell concentration and adjust volume to a final concentration of 400-500 cells/µL.

2. Single cell suspension quality control

2.1. Determine the cell concentration using a fluorescence-based automated cell counter[34].

2.1.1. Mix 10 µL cells with 0.5 µL AO/DAPI. Pipette mix thoroughly. Load 10.5 µL onto the slide and run the cell count assay to determine count and viability.

2.1.2. Dilute and/or filter the cell suspension as necessary based on the cell count.

3. Single cell partitioning using a microfluidic chip. Follow protocol from microfluidic chip manufacturer[35].

3.1. Bring 3′ gel beads and reverse transcription (RT) reagents to room temperature (>30 min). Reconstitute the RT primer in TE buffer if needed.

3.2. Prepare RT master mix in a low bind tube as outlined in **Table 1**.

3.3. Determine the number of cells to be input for each sample. Calculate the cell suspension volume (X) necessary to deliver the desired target cell number. The calculated volume of nuclease-free water to add to each sample will be 33.8-X µL.

3.4. For each sample to be partitioned, add 33.8-X µL nuclease-free water into 0.2 mL PCR strip tube. Then, add 66.2 µL master mix to each strip tube. Do not add the cells to the strip tube at this point. Pipette gently to mix. Place the prepared strip tubes on ice.

3.5. Place a microfluidic chip into a chip case. Orient the chip case ensuring oil wells (row labeled 3) are closest to the person performing the experiment.

3.6. If running less than 8 samples, use 50% glycerol to fill unused channels in the following order:

3.6.1. Add 90 µL of 50% glycerol into the wells in row 1 for all unused channels.

3.6.2. Add 40 µL of 50% glycerol into the wells in row 2 for all unused channels.

3.6.3. Add 270 µL of 50% glycerol into the wells in row 3 for all unused channels.

3.7. Snap the gel beads into a vortex. Vortex at full speed for 30 s. Tap the strip on the bench top several times to collect beads. Confirm that there are no bubbles present.

3.8. Add X µL of cells into the prepared strip tubes. Pipette to mix 5 times. Without discarding the pipette tips, transfer 90 µL of cell mixture to row 1 of the chip.

3.9. Wait for 30 s, then load 40 µL of gel beads to row 2. Pipette very slowly for this step. Dispense 270 µL of partitioning oil to the wells of row 3.

3.10. Hook the chip gasket onto the tabs of the chip holder. Place the assembled chip holder into the single cell partitioning device and press the run button.

3.11. Immediately remove the assembled chip holder upon run completion.

3.12. Remove the chip gasket from the holder, open the chip case at a 45° angle, and remove 100 µL of the emulsion from the chip into a blue plastic 96-well plate.

4. Single cell cDNA amplification. Follow protocol from microfluidic chip manufacturer[35].

4.1. Reverse transcription.

NOTE: Perform this step under a clean PCR-only hood to prevent microbial and other contamination of the unamplified cDNA.

4.1.1. Seal the 96-well blue plate with a foil seal on a heated plate sealer.

4.1.2. Run the reverse transcription reaction in a thermal cycler as follows: 53 °C for 45 min → 85 °C for 5 min → 4 °C hold.

NOTE: This is a safe stopping point. Samples can hold at 4 °C for up to 72 h.

4.2. Post-RT purification

4.2.1. Bring the nucleic acid binding magnetic beads and nucleic acid size selection magnetic beads to room temperature and vortex to re-suspend. At this point, thaw the sample cleanup buffer for 10 min at 65 °C. Bring all other reagents to room temperature and vortex.

4.2.2. Prepare the buffers as shown in **Tables 2** and **Tables 3**.

4.3. Chemically break the emulsion and purify.

4.3.1. To do so, gently remove the foil seal from the plate.

4.3.2. Dispense 125 µL of pink emulsion-breaking reagent into each emulsion. Wait for 1 min, then transfer the entire volume to a clean 0.2 mL strip tube. Ensure that there is a layer of clear and a layer of pink in the strip tube.

4.3.3. Remove 125 µL of the pink layer from the bottom of the strip tube without disturbing the clear layer. It is normal for a small volume (~15 µL) of the pink layer to remain in the tube.

4.3.4. Add 200 µL of cleanup mix from **Table 2** to the strip tube and incubate at room temperature for 10 min.

4.3.5. Transfer the strip tube to a magnetic stand and allow the solution to clear. Remove the supernatant and discard, then wash the beads with 80% ethanol twice. Allow the beads to dry for 1 min.

4.3.6. Remove the strip tube from the magnet and add 35.5 µL of elution solution from **Table 3** to the beads. Pipette to resuspend the beads in the solution. Incubate for 2 min at room temperature.

4.3.7. Transfer the strip tube to a magnetic stand and allow the solution to clear. Remove the purified cDNA from the strip tube and dispense it to clean 0.2 mL strip tubes.

4.4. Amplify the cDNA.

4.4.1. Prepare amplification master mix in **Table 4**, below.

4.4.2. Add 65 µL of cDNA Amplification Master Mix to each sample. Place the strip tube in a thermal cycler and run the following program: 98 °C 3 min → 15 cycles of [98 °C 15 s → 67 °C 20 s → 72 °C 1 min] → 72 °C 5 min → 4 °C hold

NOTE: This is a safe stopping point. Samples can hold at 4°C for up to 72 h.

4.4.3. Purify the amplified cDNA with 0.6x nucleic acid size selection magnetic beads. Wash twice with 80% ethanol and elute with 40.5 µL.

4.4.4. Run the quality control cDNA using automated gel electrophoresis and fluorescence-based DNA quantitation assay[36,37].

NOTE: This is a safe stopping point. Samples can hold at 4 °C for up to 72 h or at -20°C indefinitely.

5. Sequencing library construction

5.1. Tagmentation and clean-up of cDNA[38].

5.1.1. Normalize cDNA to 50 ng in 20 µL of the total volume. Exact quantitation is critical in this step.

5.1.2. Make the tagmentation mix in **Table 5** and aliquot 30 µL to each 20 µL cDNA sample on ice. Put the samples in the thermal cycler and run the tagmentation protocol: 55 °C 5 min → 10 °C hold.

5.1.3. Perform the clean-up of tagmented cDNA using columns[38]. Add 180 µL DNA binding buffer to each sample. Transfer 230 µL to a spin column.

5.1.4. Centrifuge at 1300 *x g* for 2 min and discard the flow-through.

5.1.5. Wash twice with 300 µL DNA wash buffer. Centrifuge an additional 2 min at 1300 *x g* to ensure ethanol removal.

5.1.6. Elute purified tagmented cDNA by adding 31 µL of elution buffer to the column and incubate at room temperature for 2 min.

5.1.7. Centrifuge for 2 min at 1300 *x g* to recover the purified product.

5.2. Sample index PCR.

5.2.1. Choose barcodes that do not overlap during a multiplexed sequencing run.

5.2.2. Make a Sample Index PCR master mix as shown in **Table 6**.

5.2.3. Add 60 µL of Sample Index PCR master mix to 30 µL of the purified sample.

5.2.4. Add 10 µL of a 20 µM, 4-oligo sample index to each sample (record index used). The total reaction volume is now 100 µL.

5.2.5. Place in a thermal cycler with the lid set to 105 °C. Run the following program: 98 °C 45 s → 12-14 cycles of [98 °C 20 s → 54 °C 30 s → 72 °C 20 s] → 72 °C 1 min → 4 °C hold.

NOTE: This is a safe stopping point. Samples can hold at 4 °C for up to 72 h.

5.3. Purify libraries with double bead clean up.

5.3.1. Add 100 µL of nucleic acid size selection magnetic beads to the sample and mix thoroughly with a pipette. Incubate at room temperature for 5 min.

5.3.2. Transfer to a magnet and let it stand until the solution clears. Remove and discard the supernatant.

5.3.3. Wash twice with 200 µL of 80% ethanol.

5.3.4. Dry the beads on the magnet for 2 min. Remove from the magnet and add 50.5 µL of EB Buffer to the bead pellet. Pipette to re-suspend the beads in the buffer.

5.3.5. Incubate at room temperature for 2 min. Transfer to a magnet and let stand for 2 min. Transfer 50 µL of eluted sample to a clean strip tube.

5.3.6. Add 40 µL nucleic acid size selection magnetic beads to the sample and incubate at room temperature for 5 min. Transfer to a magnet and let the solution clear. Remove and discard supernatant.

5.3.7. Wash twice with 125 µL of 80% ethanol.

5.3.8. Dry the beads on the magnet for 2 min. Remove from the magnet and add 60.5 µL of EB Buffer to the bead pellet. Pipette to re-suspend the beads in the buffer.

5.3.9. Incubate at room temperature for 2 min. Transfer to a magnet and let it stand for 2 min. Transfer 50 µL of eluted sample to a clean strip tube. This is the final library.

5.3.10. Hold samples at 4 °C for up to 72 h or at -20 °C indefinitely. Note that this is a safe stopping point.

5.4. Quantify and run quality control of final libraries using automated gel electrophoresis and fluorescence-based DNA quantitation assay[36,37]. Dilute the samples 1:10 prior to running quality control.

6. Library sequencing

6.1. Normalize each sample to be sequenced to 2 ng/µL and pool 3 µL of each normalized sample together.

6.2. Measure the pool concentration with fluorescence-based DNA quantitation assay[37].

6.3. Dilute the pool to 0.25 ng/µL.

6.4. Denature the pool as follows: 12 µL of diluted pooled sample (0.25 ng/µL) + 1 µL DNA control (1 nM), 2 µL EB Buffer + 5 µL NaOH (0.4N). Let this incubate for 5 min, then add 10 µL of 200 mM Tris pH 8.0.

6.5. Load 4.05 µL into 1345.95 µL HT1. Load 1.3 mL into the sequencer's cartridge and run according to manufacturer's guidelines[39] using a sequencing recipe with 26 cycles (Read 1) + 8 cycles (i7 Index) + 0 cycles (i5 Index) + 55 cycles (Read 2).

7. Read alignment (Supplemental File 1)

7.1. Run Cell Ranger (v2.0.0) to demultiplex raw base call (BCL) files generated by sequencing into FASTQ files. Align FASTQ files to human B37.3 Genome assembly and UCSC gene model to obtain expression quantification.

7.2. Alignment quality control.

7.2.1. Generate alignment metrics and check Q30 bases, valid barcode fraction, cell-associated read fraction, mapped read fraction and reads detected in each cell.

7.2.2. Examine the barcode rank plot to make sure the separation of the cell-associated barcodes and the background.

8. Data analysis (Supplemental File 2)

8.1. Cell quality control and preprocess.

8.1.1. Exclude cells with < 500 detected genes, < 3000 total number of unique molecular identifier (UMI), > 0.2 viability score as previously described[32]. Adjust the cutoffs according to tissue and cell types.

8.1.2. Remove doublets.

8.1.2.1. Assess the five endocrine hormone genes (glucagon - *GCG*, insulin - *INS*, somatostatin - *SST*, pancreatic polypeptide - *PPY*, and ghrelin - *GHRL*) for bimodal expression pattern (high- and low-expression mode) using R package *mclust*[40].

8.1.2.2. Remove cells that express more than one hormone gene, i.e., with two or more hormone genes in the high-expression mode.

8.1.3. Normalize gene expression by the total UMI and multiply by the scale factor of 10,000 at cell level using R package *Seurat*[41].

8.1.4. Remove genes detected in less than 3 cells.

8.1.5. Detect variable genes using average expression and dispersion of all cells. Adjust the cutoffs according to the tissue and cell types.

8.2. Perform the principal component analysis with the variable genes. Cluster cells with the selected number of principal components. Derive cell-cluster enriched genes by comparing one cell cluster with the rest of the cells.

REPRESENTATIVE RESULTS:

The single-cell RNA sequencing workflow consists of three steps: dissociating intact human islets into single cell suspension, capturing single cells using a droplet-based technology, and analyzing RNA-seq data (**Figure 1**). Firstly, the acquired human islets were incubated overnight. The intact islets were examined under the microscope (**Figure 2A**). The integrity of dissociated islet cells has been validated using RNA fluorescence in situ hybridization (RNA-FISH). As shown in **Figure 2B**, dissociated α- and β -cells were visualized using *GCG* and *INS* mRNA probes, respectively.

Cell count and viability need to be determined before the single-cell capture step. Cells with low viability or high debris are not suitable for further processing. A good cell concentration usually ranges from 400 to 500 cells/μL. Approximately 6000 cells were loaded to the microfluidic chip in the single-cell partitioning step, and 100 μL of gel beads in emulsion were removed from the chip. **Figure 3A** exemplifies a successful example of emulsion following the partitioning step. The liquid in each pipette tips is uniform pale cloudy with minimal partitioning oil separated from the gel beads. In contrast, **Figure 3B** shows a poor-quality emulsion with clear phase separation between the gel beads and oil. This could be due to a clog during the chip run.

Following single cell partitioning, cDNA amplification was performed. **Figure 4A** illustrates a representative fragment size distribution after cDNA amplification. The typical peak for a good quality cDNA sample resided near 1000-2000 bp. Interestingly, a spike near 600 bp was specific to islet cDNA. The fragment size distribution for the RNA-seq libraries was between 300 and 500 bp (**Figure 4B**).

After sequencing, we employed a set of read alignment metrics to evaluate single-cell RNA-seq data quality (**Table 7**). The first three metrics well summarized single-cell sequencing library quality. On an average, 92% of reads were derived from intact cells and 72% of reads were mapped to exons. Out of all exon reads captured in droplets, 90% of them were produced by intact cells and the rest was likely ambient RNAs in cell-free droplets. These alignment metrics suggest good data quality. The ratio between exon reads and UMI was an empirical measurement to evaluate sequencing saturation and usually, 10:1 ratio was a good indicator. Additionally, the number of detected genes (UMI > 0) was a useful feature to characterize different cell types. For human islet cells, the number of detected genes is about 1,900 in each cell.

We sequenced a total of 20,811 islet cells from 12 non-diabetic donors. Expression of more than one hormone was detected in about 6% of the cells. These multi-hormonal cells are most likely

doublets because our previous work showed that less than < 0.1% of single islet cells co-expressed more than one endocrine hormone[33]. We removed all the identified multi-hormonal cells. It is also important to exclude low-quality cells based on total UMI, detected genes, and cell viability[33]. After these quality control steps, 19,174 remained for further analysis. The clustering analysis revealed 12 cell types: α-, β-, δ-, PP, ε-cells, acinar, ductal, quiescent stellate, activated stellate, endothelial, macrophage, and mast cells (**Figure 5**). As expected, endocrine cells were the majority (**Table 8**). The top enriched genes in α-cells (i.e., *GCG*, *TTR*, *CRYBA2*, *TM4SF4*, *TMEM176B*) and β-cells (i.e., *IAPP*, *INS*, *HADH*, *DLK1*, *RBP4*) are consistent with other studies[13,15-18,20-22,29-31,33]. Interestingly, both α- and β-cells consisted of several subpopulations. Three β-cell subpopulations, Beta sub1, 2, and 3, were similar with small numbers of subpopulation-enriched genes (18 in Beta sub1, 33 in Beta sub 2, and 18 in Beta sub 3). The fourth subpopulation had 488 enriched genes. The small α-cell subpopulation (Alpha sub3) comprised proliferating cells, characterized by enriched expression of *MKI67*, *CDK1*, and *TOP2A*.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic diagram of single-cell RNA sequencing workflow.

Figure 2: Representative images of intact and dissociated human islets. (**A**) An image of islets taken after overnight incubation. (**B**) Dissociated islet cells visualized by RNA-FISH staining for *INS* (white) and *GCG* (red).

Figure 3: Examination of the quality of single-cell emulsion prior to reverse transcription. (**A**) A single-cell emulsion of good quality. The liquid in each pipette tip was homogeneously cloudy. (**B**) A single-cell emulsion of poor quality. The liquid in the pipette tip was not homogeneous and showed separation between oil and the gel beads.

Figure 4: Examination of the quality of single-cell cDNA and library. (**A**) A representative cDNA traces. This cDNA was of good quality and yield, with the main peak for the sample occurring near 1000-2000 bp. The spike in the trace around 600 bp was typical and distinctive of islet cDNA. (**B**) A representative final sequencing library trace. This library was of good quality and yield, with the main peak occurring between 300-500 bp.

Figure 5: Cell types and subpopulations identified in single-cell RNA sequencing of human pancreatic islets. Cells were clustered by distinctive cell types in the space of t-distributed stochastic neighbor embedding (tSNE) dimensions. The analysis also revealed three subpopulations in α-cells and four in β-cells.

Table 1: Reverse transcript mix.

Table 2: Cleanup mix.

Table 3: Elution solution.

Table 4: cDNA amplification mix.

Table 5: Tagmentation mix.

Table 6: Sample index PCR master mix.

Table 7: Read alignment metrics.

Table 8: Cell type composition. Total number of cells for each cell type and average cells for each donor in each cell type.

Supplemental File 1: Commands used for sequence alignment.

Supplemental File 2: R scripts to perform cell quality control, cell clustering, and to identify cell-type enriched genes.

DISCUSSION:

Single-cell technologies developed in recent years provide a new platform to characterize cell types and study molecular heterogeneity in human pancreatic islets. We adopted a protocol of droplet-based microfluidic single-cell isolation and data analysis to study human islets. Our protocol successfully produced RNA sequencing data from over 20,000 single human islet cells with relatively small variations in sequence quality and batch effects.

In particular, two steps are critical in this protocol for high-quality outcomes. Caution needs to be taken when dissociating human islets. It is important to not over digest the islets. Single-cell partitioning is another key step for a successful single-cell experiment. We demonstrated examples of good- and poor-quality emulsions in **Figure 3**. A clear emulsion is usually an indicative of inadequate number of cells being collected in the partitioning step.

The access to isolated primary human islets is a rate-limiting step to generate large-scale human islet single-cell transcriptomes. Isolated islets from individual cadaver donors are usually processed at different times, thus potential sample-dependent batch effects should be carefully examined during data analysis. Integrative analysis can be used to identify common cell types and subpopulations across individual batches[41]. The batch effect can also be adjusted by batch-corrected expression quantification[42]. Another challenge to analyze single-cell RNA-seq data is to identify doublets. In the data pre-processing, we took measures to remove endocrine doublets by identifying cells expressing multiple hormone genes (*GCG*, *INS*, *SST*, *PPY*, and *GHRL*). Identification of doublets formed by two different cell types is a relatively easy task due to the extremely high expression of endocrine hormones. The real challenge is to identify within-cell-type doublets, e.g., doublets by two α-cells. Because higher UMI and higher number of detected genes are suggestive of potential doublets, one solution is to remove outliers with a high number of genes and UMI during the cell QC step. Additionally, tools to detect doublets are available[43-45].

A major limitation of single-cell RNA sequencing is low sensitivity. Using spike-in External RNA Controls Consortium (ERCC) RNAs, we estimated that only 10% of all expressed genes were detected using the current protocol and that detected ones were biased toward high abundance genes[46]. Pancreatic endocrine cells express extremely high-level of hormone genes (i.e., *GCG*, *INS*, *SST*, and *PPY*). As a result, the mRNAs of these genes have the risk to become ambient RNA. Such background noises cannot be entirely avoided. However, this step-by-step protocol will help researchers minimize undesired experimental noises. The current protocol is designed for freshly isolated tissues. Other technologies, such as single-nucleus RNA sequencing[47,48], are available for RNA-seq of fresh, frozen, or lightly fixed tissues. Additionally, a recently developed cell hashing technology[49] can be considered as an advanced microfluidic single-cell protocol allowing sample multiplexing.

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All authors are employees and shareholders of Regeneron Pharmaceuticals, Inc.

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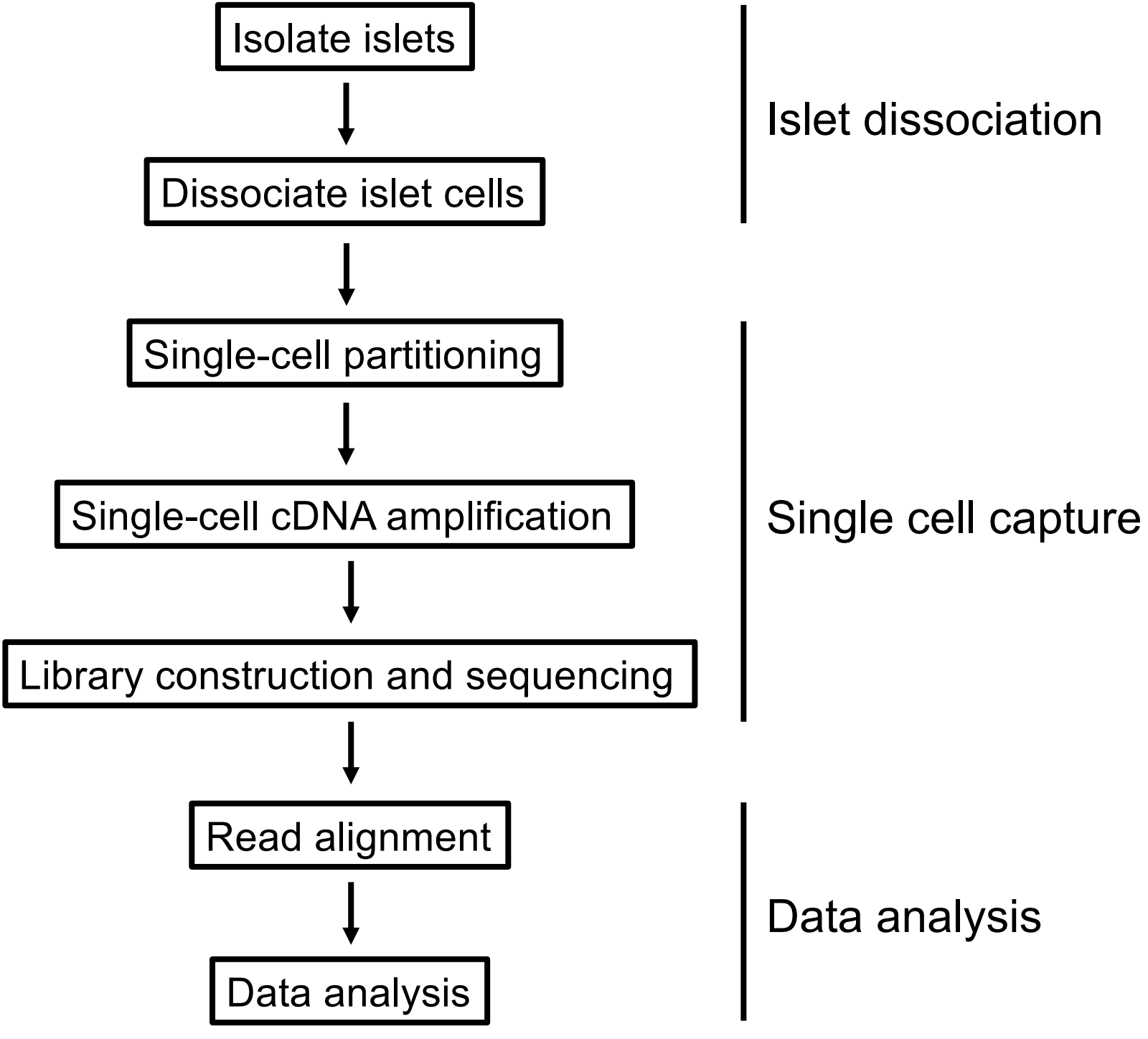
Figure 1



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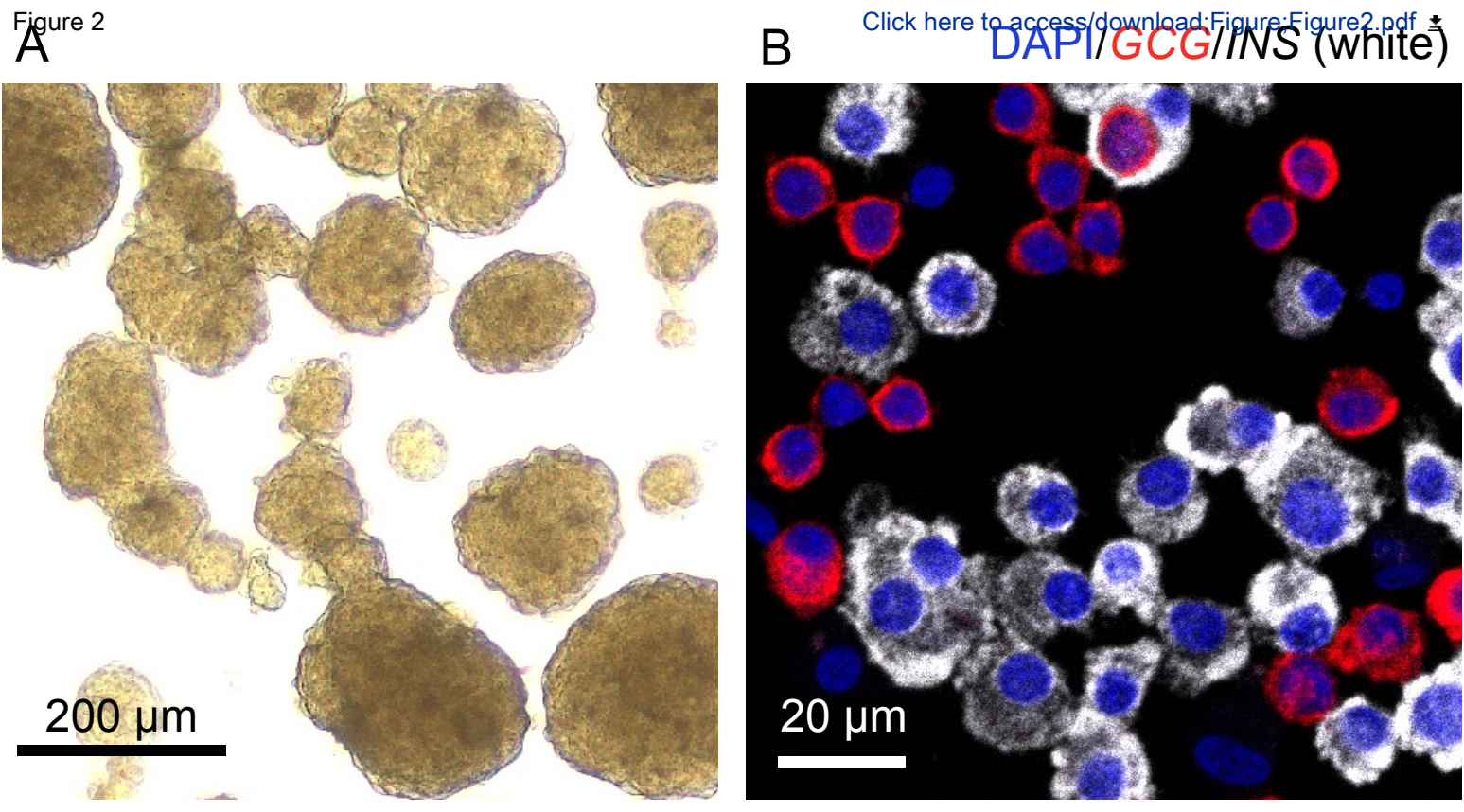

Figure 3

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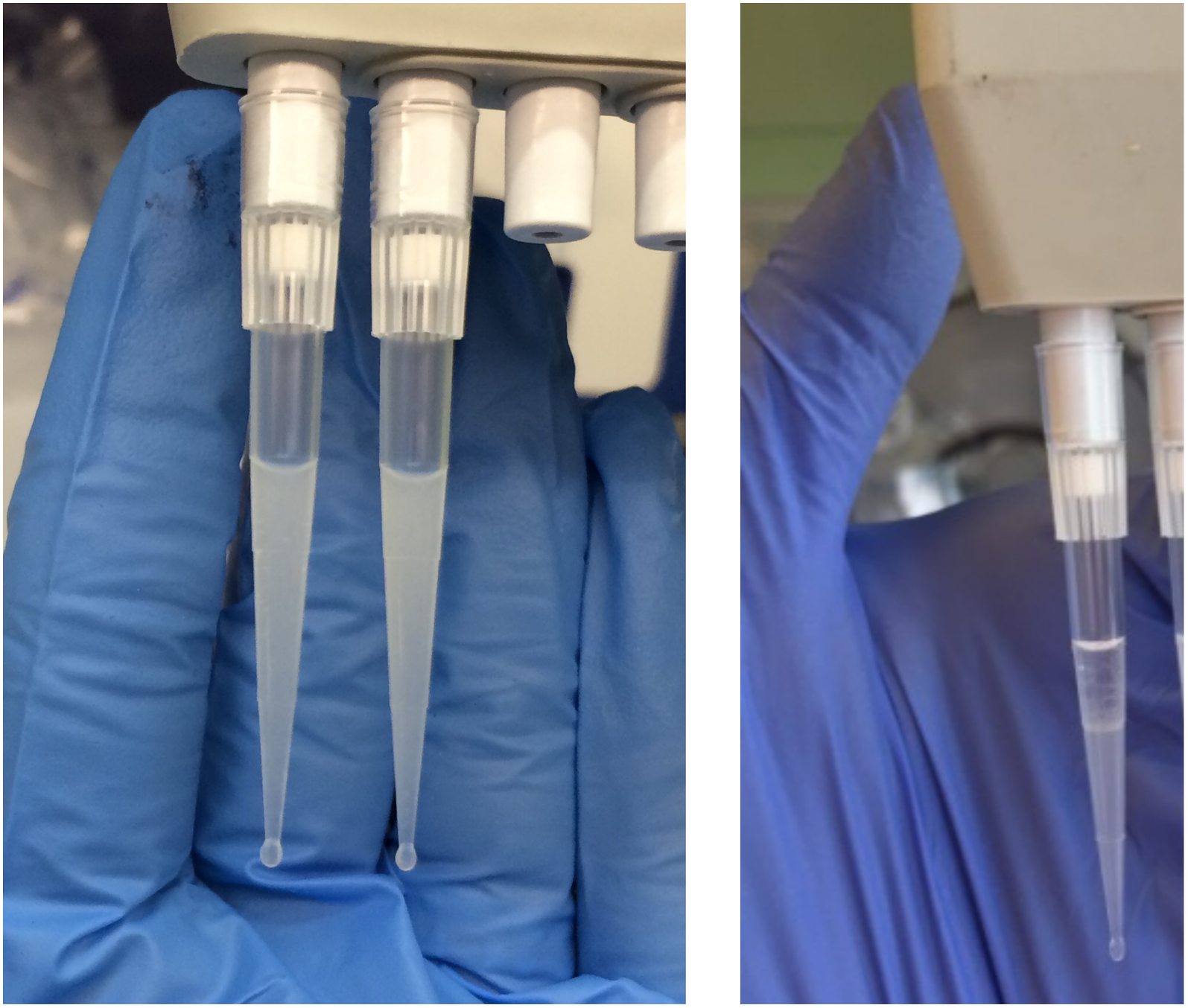
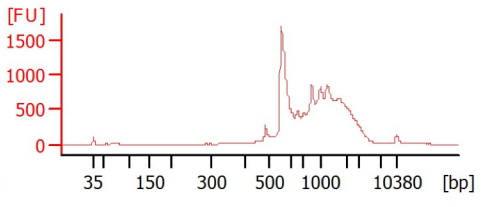


Figure 4

A



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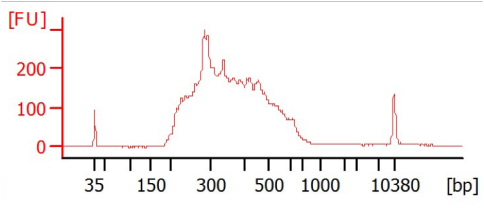

Figure 5

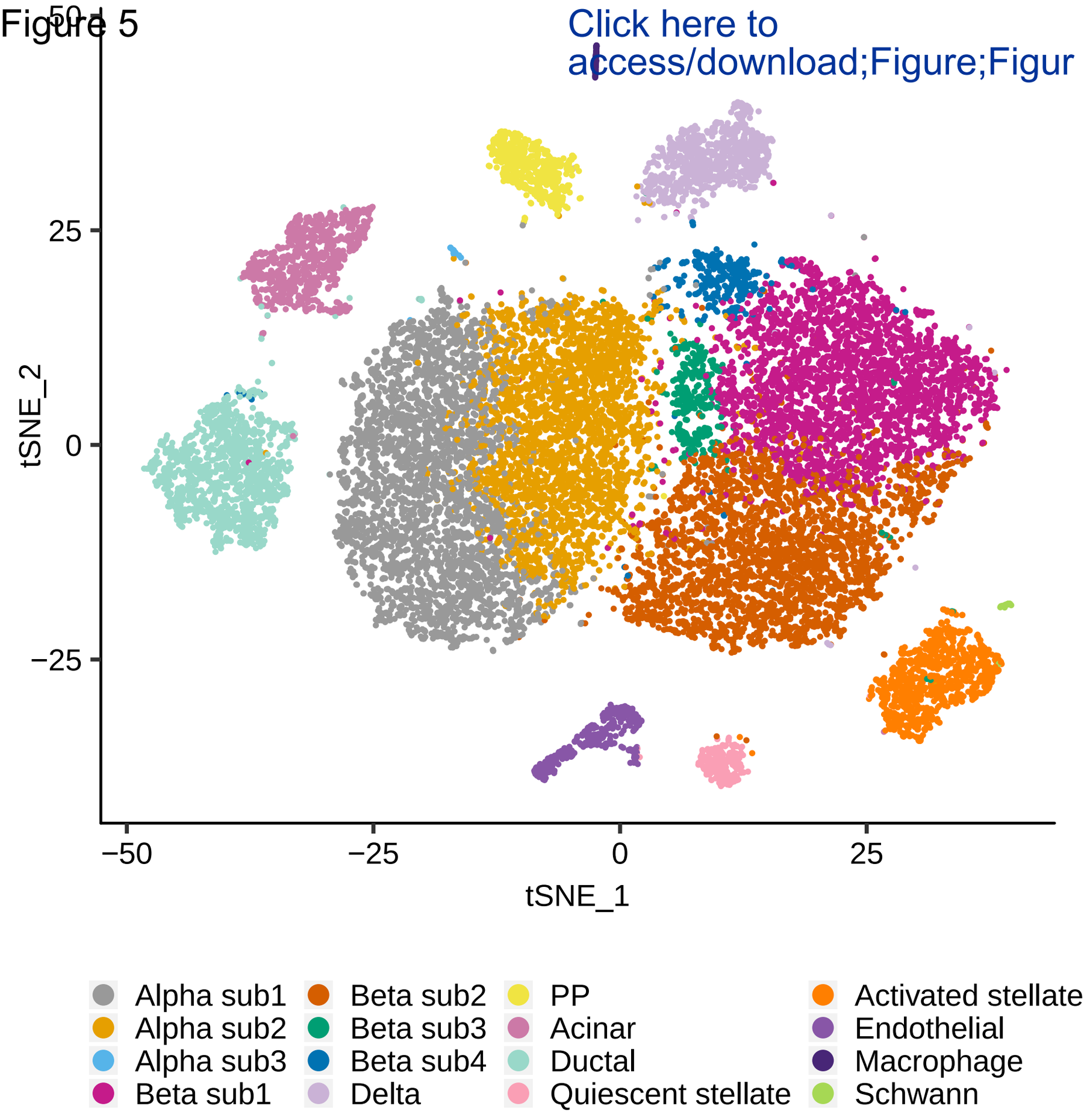

Table 1

Reagent Name	Vol. to Use (µL) per reaction
RT Reagent Mix	50
RT Primer	3.8
Additive A	2.4
RT Enzyme Mix	10
Total	**66.2**

Table 2

Reagent Name	Volume to Use (uL) per reaction
Nuclease-free water	9
Buffer Sample Clean Up 1	182
Dynabeads MyOne Silane	4
Additive A	5
Total	**200**

Table 3

Reagent Name	Volume to Use (uL) per reaction
Buffer EB	98
10% Tween 20	1
Additive A	1
Total	**100**

Table 4

Reagent Name	Volume to Use (uL) per reaction
Nuclease-free water	8
Amplification Master Mix	50
cDNA Additive	5
cDNA Primer Mix	2
Total	**65**

Table 5

Reagent Name	Volume to Use (µL) per reaction
Tagmentation Enzyme	5
Tagmentation Buffer	25
Total	**30**

Table 6

Reagent Name	Volume to Use (µL) per reaction
Nuclease-free water	8
Amplification Master Mix	50
SI-PCR Primer	2
Total	**60**

Table 7

Sample ID	% Reads with Valid Cell Barcodes	% Exon Reads in Captured Cells among Total Cells	% Exon Reads among Total Reads	Mean Exon Reads per Cell	Median UMI per Cell	Median Genes per Cell
Sample-1	92%	93%	76%	142,015	10,310	1,747
Sample-2	92%	91%	74%	151,395	11,350	1,754
Sample-3	94%	92%	75%	120,538	19,604	2,180
Sample-4	95%	93%	67%	160,657	11,870	2,111
Sample-5	94%	92%	62%	177,809	13,821	2,288
Sample-6	95%	89%	67%	138,208	8,235	1,296
Sample-7	94%	89%	72%	147,484	13,606	2,272
Sample-8	94%	91%	69%	159,793	9,505	1,865
Sample-9	95%	92%	72%	168,436	12,794	2,389
Sample-10	83%	83%	74%	88,067	13,323	1,805
Sample-11	82%	88%	77%	67,752	9,295	1,278
Sample-12	91%	85%	74%	194,781	14,877	1,746

Table 8

Cell type	Number of cells	Ave. cells per donor (standard deviation)
Alpha	6546	546 (258)
Beta	7361	613 (252)
Delta	922	77 (37)
PP	545	45 (25)
Epsilon	11	1 (1)
Acinar	836	70 (71)
Ductal	1313	109 (95)
Quiescent stellate	225	19 (14)
Activated stellate	890	74 (58)
Endothelial	408	34 (21)
Macrophage	80	7 (6)
Schwann	37	3 (3)

Name of Material / Equipment	Company	Catalog Number	Description
30 µm Pre-Separation Filters	Miltenyi Biotec	130-041-407	Cell strainer
8-chamber slides	Chemometec	102673-680	Dell counting assay slides
Bioanalyzer High Sensitivity DNA Kit	Agilent	5067-4626	for QC
Bovine Serum Albumin	Sigma-Aldrich	A9647	Single cell media
Chromium Single Cell 3' Library & Gel Bead Kit v2, 16 rxns	10X Genomics	120237	Single cell reagents
Chromium Single Cell A Chip Kit v2, 48 rx (6 chips)	10X Genomics	120236	Microfluidic chips
CMRL-1066	ThermoFisher	11530-037	Complete islet media
EB Buffer	Qiagen	19086	Elution buffer
Eppendorf twin-tec PCR plate, 96-well, blue, semi-skirted	VWR	47744-112	Emulsion plate
Fetal Bovine Serum	ThermoFisher	16000-036	Complete islet media
Human islets	Prodo Labs	HIR	Isolated human islets
L-Glutamine (200 mM)	ThermoFisher	25030-081	Complete islet media
Nextera DNA Library Preparation Kit (96 samples)	Illumina	FC-121-1031	Library preparation reagents
NextSeq 500/550 High Output Kit v2.5 (75 cycles)	Illumina	FC-404-2005	Sequencing
Penicillin-Streptomycin (10,000 U/mL)	ThermoFisher Life	15140-122	Complete islet media
Qubit High Sensitivity dsDNA Kit	Technologies	Q32854	for QC
Solution 18	Chemometec	103011-420	Cell counting assay reagent
SPRISelect Reagent	Fisher Scientific	B23318	Purification beads
Tissue Culture Dishes (10 cm)	VWR	10861-594	for islet culture
TrypLE Express	Life Technologies	12604-013	Cell dissociation solution
Zymo DNA Clean & Concentrator-5, 50 reactions	VWR	77001-152	Library clean up columns

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We proofread the manuscript.

2. Please provide an email address for each author.

We provided an email address for each author.

3. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

We rephrased the Summary following the suggestion.

4. Please rephrase the Long Abstract to more clearly state the goal of the protocol.

We rephrased the Long Abstract to clearly state the goal of the protocol.

5. Please expand the Introduction to include all of the following:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application

We have expanded the Introduction to address above five points.

6. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: TrypLE Express, Chemometec NC250, Ao/DAPI, Eppendorf twin-tec 96-well plate, blue Eppendorf plate, Agilent Plate-Loc sealer, DynaBeads MyOne SILANE, SPRIselect Reagent, additive A, Recovery Agent, SPRIselect, Qubit, etc

We removed all commercial language.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences

wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We changed the Protocol to comply with the guideline. We mentioned to use hoods when applicable.

8. The Protocol should contain only action items that direct the reader to do something.

We changed the Protocol to comply with the guideline.

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.

We changed The Protocol to comply with the guideline.

10. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.

We adjusted the numbering.

11. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

We added more details throughout the Protocol.

12. 1.1: Is there any specifics for obtaining islet cells? Any age, sex, disease bias? Please include from whom were these islets collected.

The islet donor information has been added to step 1.1.

13. 1.1.6: How do you calculate islet equivalent in your experiment? Do you know how much islet is present? Do you count? If yes, how? This step needs more detail.

We do not count islet equivalent quantity (IEQ). The number is provided by the islet supplier. This is clarified in steps 1.1.3. and 1.2.6.

14. 1.2.2: Again, how do you know how much ml is 1 islet equivalent?

Based on the IEQ provided by the islet supplier as explained in step 1.2.6.

15. 4.3: how is this done?

It is done chemically. The information in step 4.3.

16. 7,8: how is this done. Please provide screenshots as supplemental files for the steps. If codes are used, please include it as supplemental file.

We have included codes used in steps 7 and 8 as Supplemental Files 1 and 2, respectively.

17. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have shortened the highlighted steps within the limit.

18. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

This is not applicable as we are not reusing any figures from a previous publication.

19. Please expand the journal titles in the reference section.

We expanded the journal titles.

20. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials. Please alphabetically sort the materials table.

We removed he trademark (™) and registered (®) symbols. We sorted the materials table alphabetically.

Response to Reviewer #1's comments:

1. The authors may want to include the following sentence or something similar to "Here in this section we describe single islet cell encapsulation using 10X genomics" in their single cell partitioning section for better clarity to readers.

We changed the title of step 3. to "Single cell partitioning using microfluidic chip". We avoided the use of "10X genomics" because the term is a commercial language, which is prohibited to use in the manuscript.

2. Potential 'pause' points may be included wherever possible. For eg., prior to "Post RT purification" step, the authors can include a note saying "The cDNA can be stored in -20 if post RT purification step cannot be started immediately or on the same day.

We included two potential pause points in step 4.

3. The authors may want to include some references that help detect 'doublets'.

We expanded the last paragraph of the Representative Results and the Discussion to describe identification and removal of doublets in detail.

Response to Reviewer #2's comments:

1. In Step 1.1.2, how long should the islets be immersed in the islet media?

For the duration of the overnight shipment. We revised step 1.1.2 to clarify this point.

2. In Step 1.2.5, how to ensure the islets are completely dissociated?

The cell solution becomes cloudy once cells are completely dissociated. We revised step 1.3.8 to clarify this point.

3. In Step 1.2.10, is the 1X PBS buffer pre-chilled?

The 1X PBS buffer should be at room temperature. We revised step 1.3.1 to clarify this point.

4. How to identify and exclude the doublets during data analysis?

We revised the Protocol to describe doublet removal, and expanded the last paragraph of the Representative Results and the Discussion to describe identification and removal of doublets in detail.

5. The information of 30 μm cell strainer, flat-bottom bottle and non-treated culture dish should be provided in the Material section.

We added 30 μm cell strainer and non-treated culture dish to the Material section and removed flat-bottom bottle from the manuscript as the bottle does not need to be flat-bottom.

Supplemental File 1

Click here to access/download

Supplemental Coding Files

supplemental_file_1.sh

Supplemental File 2

Click here to access/download

Supplemental Coding Files

supplemental_file_2.R