Feb 08, 2024

Single-cell RNA-seq

DOI
dx.doi.org/10.17504/protocols.io.eq2lyjy5mlx9/v1

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Protocol Citation: patricia.garcia 2024. Single-cell RNA-seq. **protocols.io** https://dx.doi.org/10.17504/protocols.io.eq2lyjy5mlx9/v1

Protocol status: Working
We use this protocol and it's working

Created: February 08, 2024

Last Modified: February 08, 2024

Protocol Integer ID: 94895

Keywords: single cell, cell rna, seq harvesting, rna, seq, cell

Abstract

Harvesting and performing single-cell RNA-seq.

Troubleshooting

Harvesting cells for single-cell RNA-sequencing

1 Samples were trypsinised or scraped from the culture surface and placed in a 15mL conical tube.

2 Tubes were centrifuged at 800g in a refrigerated centrifuge for 5 minutes, and the culture media was decanted.

3 The pellet was resuspended in 10mL chilled PBS per tube by pipetting, then centrifuged again using the above parameters before decanting the PBS.

4 Pellets were resuspended in 1mL PBS, and 100,000 cells were transferred to a 1.5mL falcon tube.

5 These were centrifuged at 1000rpm for 3 minutes at 4°C.

6 Cells were resuspended in 20µL chilled DPS.

7 180µL chilled 100% methanol was added dropwise to the cells while gently vortexing to prevent cell clumps, before fixing the cells on ice for 15 minutes.

Single-cell RNA-sequencing protocol

8 Between 2400 to 4000 cells were loaded for each sample into a separate channel of a Chromium Chip G for use in the 10X Chromium Controller (cat: PN-1000120).

9 The cells were partitioned into nanoliter scale Gel Beads in emulsions (GEMs) and lysed using the 10x Genomics Single Cell 3′ Chip V3.1 GEM, Library and Gel Bead Kit (cat: PN-1000121).

10 cDNA synthesis and library construction were performed as per the manufacturer's instructions.

11 The RNA was reversed transcribed and amplified using 12 cycles of PCR.

12 Libraries were prepared from 10µL of the cDNA and 13 cycles of amplification. Each library was prepared using Single Index Kit T Set A (cat: PN-1000213) and sequenced on the HiSeq4000 system (Illumina) using 100 bp paired-end run at a depth of 20-50 million reads. Libraries were generated in independent runs for the different samples.

Single-cell RNA-sequencing data processing

13 Reads were aligned to the human reference genome (Ensembl release 93, GRCh38) using Cell Ranger v3.0.2.

14 The analysis was carried out using Seurat v3.0 (REF- Butler, 2018 ,Stuart, 2019 #645) following Seurat's standard workflow.

15 Cells expressing fewer than 200 genes were excluded from the subsequent analysis. In addition, we excluded cells with more than 3000 detected genes to remove suspected cell doublets or multiplets.

16 Due to the nature of the cells used for this experiment, a 10% cut-off was applied for the percentage of mitochondrial genes expressed to filter out likely apoptotic cells.

17 Using default parameters of Seurat, data for each sample were log normalised across cells and the 2000 most highly variable genes identified.

18 Using the canonical correlation analysis ('CCA') to identify anchors, we integrated the samples using Seurat v 3 (Butler, 2018; Stuart, 2019), followed by regression of the effect of cell cycle and scaling of the data. Dimensional reduction was performed using 50 PCs.

19 Clustree v0.4.4 and Seurat's plots functions were used to visualise the expression of astrocytic and neuronal marker genes across different cluster resolutions.

20 A clustering resolution of 0.25 was selected.

21 The differentially expressed genes between the clusters of interest were identified using Seurat's FindMarkers() and the default 'Wilcox' test.