**Workflow Summary: Replicating Analysis from ACAT1 Inhibition Study in Microglia**

### 📁 Section 1: Image Analysis of Stained Tissue Sections

**Tools Used:** - Nanozoomer 2.0HT (Hamamatsu) – Whole-slide scanner - NDP.view2 – Image viewer software - ImageJ Fiji – Open-source image quantification software - GraphPad Prism v9 – Graphing and statistical testing

**Steps & Purpose:** 1. Scan tissue slides at 20x magnification using Nanozoomer – for digitization. 2. Review scanned images using NDP.view2 – to select representative regions. 3. Choose 5 representative tumor-region images per sample – ensure consistency. 4. Use ImageJ Fiji: - Threshold and quantify % area stained (WNT5A, LRP5, CD44, SPP1) - Count phospho-histone 3-positive nuclei/mm² (cell proliferation) 5. Use GraphPad Prism to perform statistical comparisons and generate plots.

### 📊 Section 2: scRNA-seq Tissue Preparation & Processing

**Tools Used:** - 10X Genomics Chromium platform – single-cell capture - CellRanger v5.0.0 – RNA alignment and quantification - Seurat v4.3.0 (R) – normalization, clustering, scoring - ggplot2 v3.5.0 (R) – visualization

**Steps & Purpose:** 1. Dissect GBM tumors from mice, dissociate into single-cell suspensions. 2. Perform 10X Chromium scRNA-seq – to barcode and capture transcripts. 3. Align reads to mm10 + Rfp + RCAS custom genome using CellRanger. 4. Load results into Seurat for: - QC filtering, normalization, dimensionality reduction. - Use CellCycleScoring() to assign S/G2M (cycling) scores. - Use AddModuleScore() with Neftel GBM signatures to classify cell states.

### 🔢 Section 3: Differential Expression & Network Modules

**Tools Used:** - muscat v1.12.1 (R) – scRNA-seq DE analysis across groups - DESeq2 – statistical engine for DE genes - hdWGCNA v1.72 (R) – co-expression module detection - clusterProfiler v4.6.2 (R) – pathway enrichment analysis

**Steps & Purpose:** 1. Run muscat::pbDS() to identify DE genes across sample conditions (uses DESeq2 under the hood). 2. Use hdWGCNA to build gene co-expression networks. 3. Run clusterProfiler::enrichGO() or enrichKEGG() to discover overrepresented pathways.

### 🚀 Section 4: RNA Velocity

**Tools Used:** - scVelo v0.3.2 (Python 3.9.13) – inference of RNA dynamics

**Steps & Purpose:** 1. Input filtered, normalized data into scVelo. 2. Estimate spliced/unspliced transcript ratios. 3. Visualize projected cell state trajectories.

### 🛡️ Section 5: Ligand–Receptor Signaling Analysis

**Tools Used:** - CellPhoneDB v2.1.7 (Python) – ligand-receptor inference tool

**Steps & Purpose:** 1. Input expression matrix + cell type annotations. 2. Run CellPhoneDB to infer statistically significant ligand–receptor pairs. 3. Compare interaction strength between experimental groups.

### 📈 Section 6: Bulk RNA-seq (TCGA GBM Samples)

**Tools Used:** - cgdsr (R) – download TCGA GBM bulk RNA + mutation data from cBioPortal

**Steps & Purpose:** 1. Group samples by GBM subtype using mutations (EGFRvIII, NF1, PDGFRA). 2. Calculate mean log10 gene expression for marker genes per sample. 3. Compare expression programs identified from scRNA-seq with bulk data.

### ⚖️ Section 7: Statistical Testing

**Tools Used:** - GraphPad Prism v9 or R base functions

**Tests Used & When:** - Student’s t-test – for normally distributed data - Mann–Whitney U test – for non-normal distributions - Fisher’s exact test – for binary/categorical comparisons

**Note:** Always read the figure legends and supplementary methods of the paper to verify exact parameters and thresholds used in each analysis.

**This summary is designed for practical replication of methods from a published study on ACAT1 inhibition and autophagy in microglia.**