Streamlining single-cell sequencing workflows using Cellometer and Cellaca high-throughput cell counters

Introduction

Single-cell analysis has evolved over the past three hundred years.[1] With the invention of the first microscopes in the 1660s, humans have been captivated by single cells existing within an organism. Humans are composed of approximately 3.72 x 10^{13} single cells within the different tissues and fluids in the body.[1] Unfortunately, genetic diseases, cancer, or neurodegeneration have caused devasting issues for humans and other organisms. To continue the betterment of human health, over the past three centuries, scientists have been trying to understand and characterize different aspects of single cells. Some of the early work in the 1850s correlated human diseases and abnormalities in single cells. By the late 1900s, the development of various novel staining and cytology techniques enabled further understanding of genetic differences of chromosomes in single cells. In the 1990s, the development of quantitative microarray methods was employed for genome-wide DNA and RNA analysis with some limitations regarding the large amount of input material required. By 2005, the first next-generation sequencing (NGS) technology was developed that enabled the genome-wide sequencing of DNA and RNA without input material limitation.[1] The most recent breakthrough technologies combined the use of microfluidics and nucleotide barcoding to encapsulate single cells in droplet microreactors (inDrop- and Drop-seq) for single-cell DNA and RNA sequencing (scDNA-seq, scRNA-seq), in 2015 (Figure 1).[1-4] These technologies were further optimized by companies such as 10x Genomics (Pleasanton, CA) and Singular Genomics (San Diego, CA).

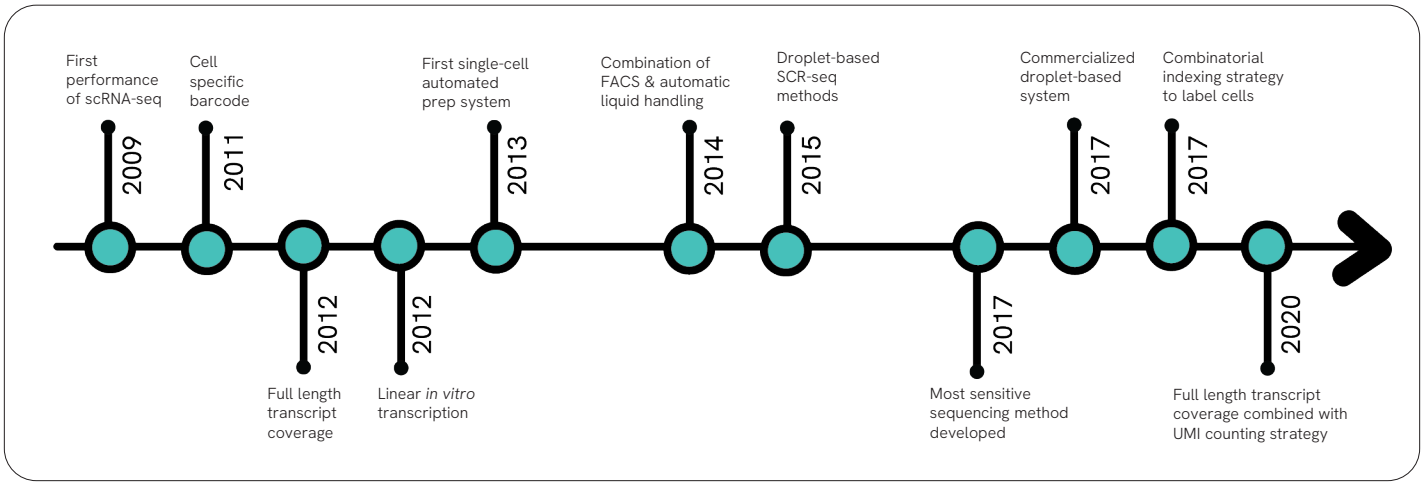

Figure 1: Timeline of single-cell sequencing milestones. Adapted from Development of Single-cell RNA Sequencing, https://encyclopedia.pub/entry/24618, accessed August, 2023.

Single-cell isolation must be performed in preparation for single-cell sequencing. Methods for the isolation of single cells include serial or limiting dilution, fluorescence- or magnetically activated cell sorting (FACS or MACS), manual or robotic cell picking, microfluidic platforms such as the HIVE scRNA-seq (Honeycomb, Waltham, MA), and laser capture microdissection (LCM) (Table 1).[1, 4, 5] After the cells are isolated using one of the techniques listed above, scDNA-seq and scRNA-seq can be performed to analyze the whole genome and transcriptome, respectively. Techniques for scDNA-seq such as PCR, multiple displacement amplification (MDA), multiple annealing- and looping-based amplification (MALBAC), or in vitro transcription and Phi29 DNA polymerase for scRNA-seq are all different methods for single cell analysis.[1, 4] scDNA-seq has three core capabilities termed fidelity, co-presence, and phenotypic association, used in combination to answer biological questions. scRNA-seq for transcriptomic analysis can be done in high resolution when compared to bulk average gene expression analysis.[3]

Single-cell sequencing is important for answering biological questions of many different applications such as neurobiology, germline transmission, organogenesis, cancer biology, clinical diagnostics, immunology, microbiology, tissue mosaicism, embryology, and prenatal-genetic diagnosis (Chart 1). Specifically, cancer research is of great importance in the current therapeutic landscape, where the characterization of individual cancer cells within an abundant population or rare population such as circulating tumor cells (CTCs) can shed some light on understanding how cancer cells metastasize in the blood.

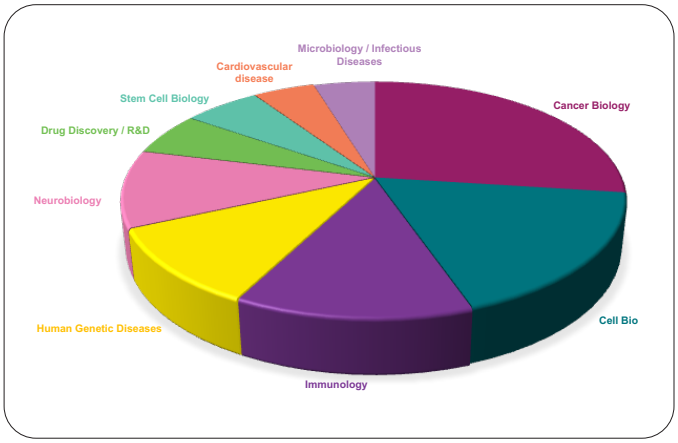

Chart 1: The percentages of single-cell sequencing in use by numerous fields show the versatility and importance of this analysis.

Cell counting is a critical step prior to single-cell isolation and sequencing

Common single-cell isolation techniques such as limiting dilution, FACS or MACS, and various microfluidic systems require accurate and precise cell counting before the isolation process. Low-quality cell counting may lead to inaccurate interpretation of the single-cell sequencing results which can waste time, resources, and precious sample materials.[2] Cell counting results can indicate sample quality and determine if the target cells can proceed to downstream single-cell sequencing processes. Depending on the single-cell isolation and sequencing method, the cell concentration, viability, and size are measured to determine if the cell samples are above a gated threshold to ensure a successful single-cell sequence experiment. For example, cell counting can identify the clumpiness of the cell sample and confirm whether the doublets or multiples have been properly removed.

It is critical to control the quantity and quality of the sample prior to single-cell sequencing specifically for samples such as PBMCs, which are used extensively in cell therapy research. Performing single-cell sequencing with 10x Chromium requires recommended sample preparation steps because low-quality cell samples, such as low viability, can cause unreliable cell recovery in 10x Chromium and generate suboptimal results due to background noise from ambient RNA of dead and apoptotic cells (Figure 2). Ultimately, this can yield low-quality results and impact time and cost.

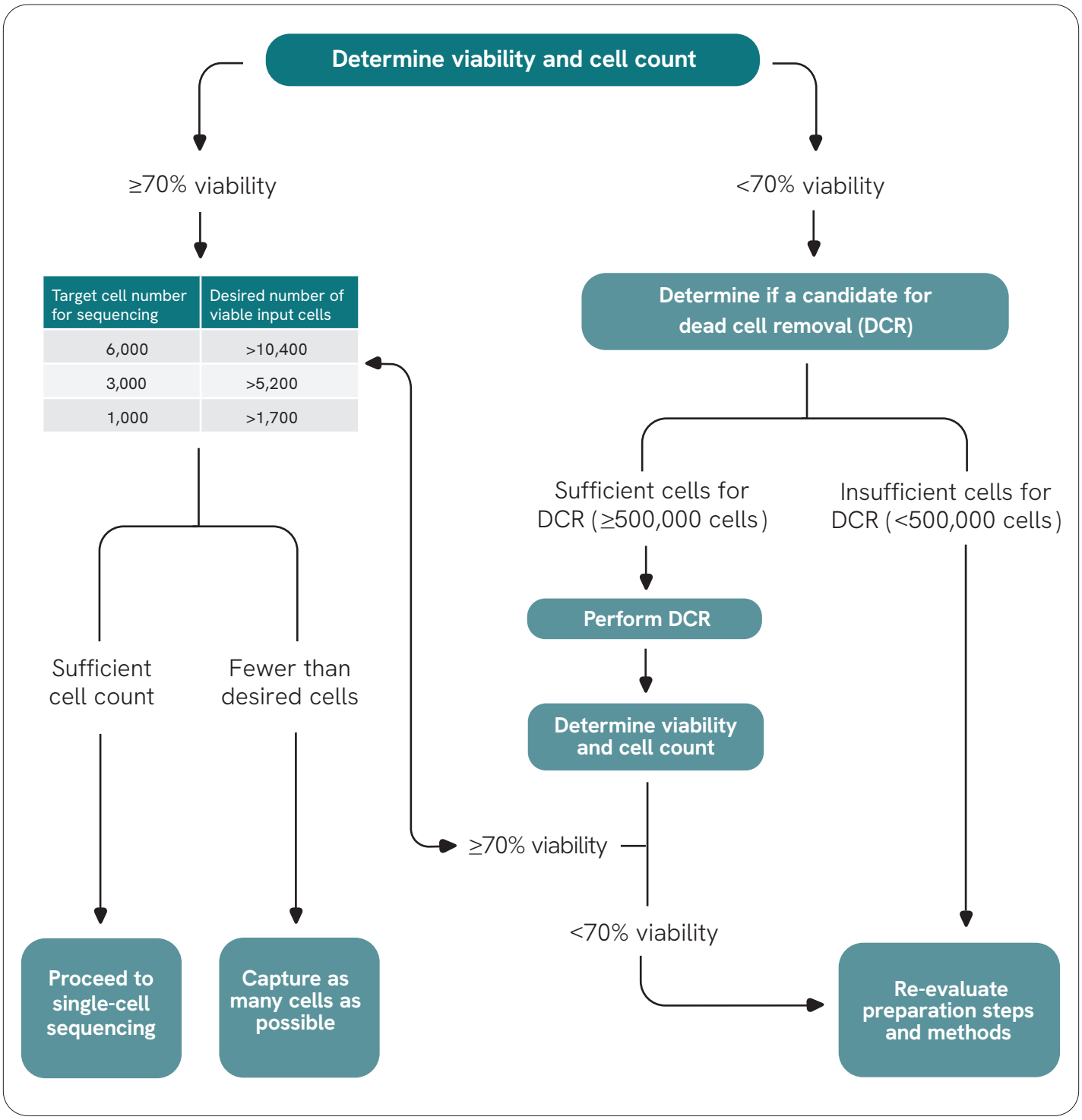

Figure 2: Sample processing workflow for single-cell projects showing best practices for sample preparation.

Table 1: Single-cell isolation methodologies.

Techniques	Definition
Fluorescence-activated cell sorting (FACS)	Microdroplets with single cells are isolated by electric charge at high pressure
Magnetic-activated cell sorting (MACS)	Microdroplets with single cells are isolated by magnetic separation
Laser capture microdissection (LCM)	Cells are cut with lasers from a tissue section on a slide under a microscope
Manual cell picking	Cells are isolated by a glass pipetteMicrodroplets with single cells are isolated by magnetic separation
Microfluidic platforms	Microfluidic chips isolate single cells in flow channels
Robotic micromanipulation	Single cells are isolated using a robotic-controlled micropipette
Nuclei isolation	Separation and identification of nuclei from frozen samples or tissues

Currently, the International Organization for Standardization (ISO) has issued three publications on cell counting and characterization that provide guidance for evaluating, comparing, and selecting a cell counting method.[6, 8] The first two publications, ISO 20391-1:2018 and 20391-2:2019 under Biotechnology – Cell Counting – Part 1 and Part 2, respectively, guide the measurement considerations and procedures to evaluate cell counting.[6, 7] The third publication, ISO 23033:2021 under Biotechnology – Analytical Methods, provides general considerations for testing and characterizing cellular therapeutic products, which includes some guidance on cell viability measurement.[8] The purpose of this guidance is to ensure scientists can establish a high-quality cell counting method for cellular therapy products that can translate to other biological assays such as single-cell sequencing.

Cell sample composition increases the difficulty of generating high-quality cell counting results

Cell counting was treated as a trivial procedure for decades until the rise of cell and gene therapy. In the early years of cytology, scientists were dealing with blood samples that were stained with differential staining to identify different types of cells. For decades, cultured cells were utilized for experimentations, which were generally easier to measure. As we progress into the cell and gene therapy era, cell samples have become extremely challenging due to a wide range of biological sample types, which are complex, dynamic, and heterogeneous. These cell samples require various media formulations and lengthy bioprocessing steps. For instance, patient tumors or tissue digests can be incredibly messy, making cell counting extremely difficult in contrast to the typical cultured cell lines.

Accurate and precise cell counting and viability measurements are required for single-cell isolation and sequencing

In the past century, manual cell counting has been considered the gold standard for cell counting. The past two decades of improvements in imaging technology and analysis have enabled the development of automated cell counters. Cellometer® cell counters from Revvity are among the first image-based cell counters introduced to provide more consistent and accurate results compared to manual cell counting.[9-25]

The brightfield-only systems from Revvity such as the Cellometer Mini and Auto T4 have been used to count cultured cell lines or processed blood samples stained with Trypan Blue. As cell samples become more complex and heterogeneous, fluorescence-based cell counting is required, necessitating instruments such as the Cellometer K2, Auto 2000, and Spectrum. These instruments utilize a dual-stain combination of acridine orange (AO) and propidium iodide (PI) to fluorescently label the nucleus of live and dead cells with green and red fluorescence, respectively. This methodology enables accurate and consistent counting of messy samples like tumor digests or apheresis samples without the need for further purification steps, significantly reducing the time needed for the cell counting process. In addition to cell count and viability measurement, Cellometer systems can also measure cell size, which can be used to determine the percentage of clumpy cells.

Finally, with the rise of cell and gene therapy, multiple patient samples and conditions may be required per experiment, with the Cellaca® MX and Cellaca® PLX instruments typically used for high-throughput cell counting. The Cellaca platform can perform cell counts in a 24-chamber plate in as little as 3 min using Trypan Blue and 5 min using AO/PI.

Cellometer and Cellaca systems used for single-cell sequencing published in peer-reviewed journals

In recent years, the Cellometer and Cellaca systems have been employed prior to the single-cell sequencing process for applications such as cancer biology, immunology, neurobiology, and most recently infectious disease and microbiology. We have identified example publications that show the integration of Revvity cell counters into the standard 10x Genomics Chromium single-cell sequencing workflow, which are described in the next sections.

Immuno-oncology and immunotherapy

"Baseline Frequency of Inflammatory Cxcl9-Expressing Tumor-Associated Macrophages Predicts Response to Avelumab Treatment" was published by the Cancer Immunology Discovery Group at Pfizer (San Diego, CA) in Cell Reports (2020). It was reported that the sorted CD45+ cells obtained from dissociated tumors were counted using the Cellometer K2 before loading on a Chromium Single Cell Chip for scRNA-seq studies.[26]

"Single-cell Characterization of the Cellular Landscape of Acral Melanoma Identifies Novel Targets for Immunotherapy" was published by The Moffitt Cancer Center & Research Institute (Tampa, FL) in Clinical Cancer Research (2022). In this work, nine human acral melanoma samples were collected from eight patients, digested, and enriched for live cells using FACS. The single-cell suspension from the human melanoma tissue was counted to measure cell concentration and viability using the Cellometer K2 prior to performing scRNA-seq using 10X Genomics Chromium.[27]

"An Optimized Workflow for Single-Cell Transcriptomics and Repertoire Profiling of Purified Lymphocytes From Clinical Samples" was published by the Sanofi Immunology and Inflammation Research Therapeutic Area (Cambridge, MA). Cellaca MX was employed with AO/PI staining and the 24-well counting plate (CHM24-A100) for counting human peripheral blood mononuclear cells (PBMC). In this work, Cellaca MX with AO/PI was compared directly with manual and automated counting with Trypan Blue. The results showed that Cellaca MX with AO/PI was highly comparable with manual counting with Trypan Blue, while automated counting with Trypan Blue can overestimate viability. Therefore, Cellaca MX was recommended as a cell counting method for 10X Genomics.[28]

"Single Cell T Cell Landscape and T Cell Receptor Repertoire Profiling of AML in Context of PD-1 Blockade Therapy" was published by the University of Texas MD Anderson Cancer Center (Houston, TX) in Nature Communications (2021). In this work, bone marrow (BM) from adult patients with acute myeloid leukemia (AML) was collected and stored in liquid nitrogen. The BM samples were thawed and counted with the Cellometer Mini using Trypan Blue before 10X Chromium analysis.[29]

Immunology

"Simultaneous Trimodal Single-Cell Measurement Of Transcripts, Epitopes, And Chromatin Accessibility Using Tea-Seq", *"Optimized Workflow For Human Pbmc Multi-Omic Immunosurveillance Studies"*, and *"Barware: Efficient Software Tools For Barcoded Single-Cell Genomics"* were all published the Allen Institute (Seattle, WA) in eLife (2021), STAR Protocols (2021), and BMC Bioinformatics (2022), respectively.[30, 31, 32] In all three manuscripts, the authors used both Cellometer Spectrum and Cellaca MX to perform single- or multi-sample-based cell counting and viability measurement of PBMCs prior to single-cell sequencing with 10X Chromium. Specifically, the second publication recommended the integration of Cellometer and Cellaca systems in the 10X Chromium and high dimensional flow analysis workflow under STAR Protocols.

Cancer biology

"Intra-Tumoral Activation of Endosomal TLR Pathways Reveals a Distinct Role for TLR3 Agonist Dependent Type-1 Interferons in Shaping the Tumor Immune Microenvironment" was published by the Cancer Immunology Discovery Group from Pfizer (San Diego, CA) in Frontiers in Oncology (2021). In this work, harvested tumors were dissociated into single-cell suspensions and sorted into $CD45^+$ cell populations. The cells were then counted using the Cellometer K2 prior to 10X Chromium scRNA-seq analysis.[33]

"HyPR-seq: Single-cell Quantification of Chosen RNAs via Hybridization and Sequencing of DNA Probes" was published by the Broad Institute of MIT and Harvard (Cambridge, MA) in PNAS (2020). In this work, a new sequencing method, Hybridization of Probes to RNA (HyPR-seq), was introduced and compared to 10X Chromium scRNA-seq. Kidney organs were collected from mice and digested into single-cell suspensions. The red blood cells were lysed, and the entire sample was filtered before counting on a Cellometer Auto T4 with Trypan Blue staining.[34]

"A Multi-center Cross-platform Single-cell RNA Sequencing Reference Dataset" was published by the Center for Genomics at Loma Linda University (Loma Linda, CA). In this work, the authors present a benchmark scRNA-seq dataset from two distinct cell lines across four sequencing centers. Human breast cancer (HCC1395) and B lymphocyte (HCC1395BL) samples were measured on the Cellometer Auto T4 with Trypan Blue prior to the Illumina HiSeq system for scRNA-seq.[35]

Virology

"*Circuits Between Infected Macrophages and T cells in SARS-CoV-2 Pneumonia"* was published by the Division of Pulmonary and Critical Care Medicine from the Feinberg School of Medicine at Northwestern University (Chicago, IL) in Nature (2021). In this work, the bronchoalveolar lavage (BAL) fluid samples were collected from 88 patients with SARS-CoV-2-induced respiratory failure and 211 patients with pneumonia for flow cytometry and bulk transcriptomic profiling. Some of the samples were subjected to scRNA-seq with 10X Chromium. The BAL samples were counted using Cellometer K2 with AO/PI for flow cytometry, bulk, and scRNA-seq.[36]

"Inhibition of SARS-CoV-2 Infections in Engineered Human Tissues Using Clinical-Grade Soluble Human ACE2" was published in Cell (2020) by various institutions in Sweden, Spain, Austria, and Canada. The authors engineered human kidney organoids to study the ACE2 markers within the kidney tubules. The organoids were homogenized, dissociated, and filtered. The single-cell suspension was analyzed by the Cellometer K2 prior to 10X Chromium scRNA-seq.[37]

"A Single-Cell Atlas of Lymphocyte Adaptive Immune Repertoires and Transcriptomes Reveals Age-Related Differences in Convalescent COVID-19 Patients" was published in Frontiers in Immunology by institutions in Switzerland. In this work, the scientists profiled the adaptive immune response by single sequencing the lymphocyte immune repertoire and transcriptome from COVID-19 patients. The T and B cells were collected from the patients and analyzed using Cellometer Spectrum with AO/PI before 10X Chromium scRNA-seq.[38]

Reproduction biology

"Preparation of Single-cell Suspensions From the Human Placenta" was published by the Perinatology Research Branch at NICHD/NIH/DHHS (Bethesda, MD) in Nature Protocols (2022). This manuscript provides detailed instructions on single-cell preparation of the human placenta for scRNA-seq using 10X Chromium, where Cellometer Auto 2000 is used with AO/PI for cell count and viability measurement.[39]

"A Single-cell Atlas of Murine Reproductive TIssues During Preterm Labor" was published by the Perinatology Research Branch at NICHD/NIH/DHHS (Bethesda, MD) in Cell Reports (2023). The scientists used a model of infection-induced preterm labor in mice to generate an atlas of single-cell analysis. Murine uterus, decidua, and cervix tissues were collected, dissociated, and filtered into a single-cell suspension. The cells were counted on a Cellometer Auto 2000 with AO/PI before scRNA-seq using 10X Chromium.[40]

"*Cell–Cell Communication at the Embryo Implantation Site of Mouse Uterus Revealed by Single-Cell Analysis"* was published in the International Journal of Molecular Sciences (2021) by the College of Veterinary Medicine at the Gansu Agricultural University in China. In this work, scientists are investigating the molecular mechanisms involved in embryo implantation. They were able to identify the global gene expression associated with the site of implantation using single-cell analysis. The whole uterus was dissociated and filtered into single-cell suspensions. The cells were counted on a Cellometer Auto 2000 with AO/PI prior to scRNA-seq using 10X Chromium.[41]

Neurobiology

"Single Cell RNA Sequencing Detects Persistent Cell Type- and Methylmercury Exposure Paradigm-Specific Effects in a Human Cortical Neurodevelopmental Model" was published by Vanderbilt University (Nashville, TN), Purdue University (West Lafayette, IN), and Albert Einstein College of Medicine (Bronx, NY) in Food Chemical Toxicology (2021). The researchers investigate the lasting effects of methyl mercury on human brain development by scRNA-seq using a differentiating cortical human-induced pluripotent stem cell (hiPSC) model. The differentiated neural lineage samples were collected and counted using the Cellometer Auto T4 before scRNA-seq with 10X Chromium.[42]

"An optimized protocol for retina single-cell RNA sequencing" published by the Department of Neurobiology at NIH (Bethesda, MD) in Molecular Vision (2020). The authors presented a protocol for optimal dissociation of retina sample preparation to generate high-quality single-cell suspension for scRNA-seq using 10X Chromium. The Cellometer Auto 2000 was utilized to count the cells and measure cell viability before scRNA-seq. The cell concentration should be diluted to ~ 900–1,400 cells/µl.[43]

Organogenesis

"*Single-cell Transcriptome Profiling of an Adult Human Cell Atlas of 15 Major Organs*" was published in Genome Biology (2020) by Sun Yat-Sen University in Guangzhou, China. The scientists performed single-cell transcriptomes of 84,363 cells derived from 15 tissue organs of one adult donor to generate an adult human cell atlas. The tissue organs were collected, dissociated, and filtered into single-cell suspensions, as well as counted on a Cellometer Auto 2000 prior to 10X Chromium scRNA-seq.[44]

Conclusion

In conclusion, single-cell analysis has evolved over the past few centuries and with the current technologies, scientists can better understand the biological landscape on a single-cell basis instead of bulk analysis. The information gathered through this process can paint an entire picture representing the genome atlas for many applications. To ensure a successful single-cell analysis experiment, accurate and consistent cell counting is required. Cellometer and Cellaca platforms have demonstrated cell counting with high accuracy and precision that is fit-for-purpose for a wide range of biological samples. The publications reviewed show that using brightfield with Trypan Blue or fluorescence with AO/PI enables proper analysis of cell concentration and viability, as well as cell size, to prepare single-cell sequencing. Revvity will continue to advance cell counting technologies and the science of cell counting to ensure scientists use the most accurate, consistent, and fit-for-purpose methods for their biological assays.

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