**Single-cell transcriptomics: a new research technique for precision medicine in nephrology**

Jisoo Kim[1], Jihwan Park[1,*]

[1]School of Life Sciences, Gwangju Institute of Science and Technology, Gwangju, Korea

[*]Correspondence to Jihwan Park

School of Life Sciences, Gwangju Institute of Science and Technology, 123 Cheomdan-gwagiro, Buk-gu 61005, Gwangju, Korea

Tel: +82-62-715-2503, E-mail: jihwan.park@gist.ac.kr



## Abstract

Due to the complex structure and function of the kidneys, the mechanism of kidney disease remains unclear. In particular, the present transcriptomics approaches at the bulk level are unable to differentiate the primary autonomous responses, which lead to disease development, from the secondary cell non-autonomous responses. Single-cell analysis techniques can overcome the fundamental limitations inherent in the measurement of heterogeneous cell populations and clarify the central issues regarding kidney biology and disease pathogenesis. Therefore, the application of single-cell sequencing helps in identifying the biomarkers and pathways related to the disease, stratifying patients, and setting appropriate treatment methods for each individual. This article covers various single-cell analysis techniques and single-cell transcriptomics studies performed in the field of nephrology. Moreover, we discuss the future of precision medicine in nephrology using single-cell analysis.

## Introduction

Defining gene regulatory mechanisms that determine cell function and behavior is fundamental for understanding the disease; however, due to the complex structure and function of the kidneys, the mechanisms governing development and progression of kidney disease remain unclear. Although, RNA sequencing has been used to understand the cause and progress of kidney disease, it is difficult to understand the biological differences between cell types as the bulk transcriptome presents average values for various cell types. As an alternative, single-cell RNA sequencing (scRNA-seq) was developed. scRNA-seq involves single-cell isolation, transcript capturing, library construction, sequencing and computational analysis, and enables the evaluation of basic biological properties of individual cells and cell populations at high resolution. It is a powerful approach not just to analyze the complex processes involved in kidney diseases but also to understand the causal mechanisms underlying disease development, which eventually facilitates precise identification of the therapeutic targets.

Precision medicine characterizes patients based on the information regarding all factors that can affect treatment, from dysregulated genes and cells to the patient's lifestyle, diet, and environment. First, precision medicine aims to identify the biomarkers that can predict the success or failure of the treatment for kidney diseases in less time than that required in standard determination. Second, it aims to identify pathways for therapeutic intervention using existing pharmacological or novel target-specific agents [1]. Identification of the specific target genes, crucial signaling pathways, and primary target cell types is the first step to enable this transition in nephrology. Although remarkable efforts have been made to identify the target genes, the cellular targets involved in the development of kidney diseases are less explored due to technical limitations.

Application of scRNA-seq can be a perfect solution to address the issues in precision medicine as it defines the cell types and states of complex diseases such as chronic kidney disease (CKD) and acute kidney injury (AKI. Moreover, scRNA-seq helps to identify potential biomarkers and signaling pathways for better diagnostics, prognostics, targeted therapy, early detection, and noninvasive monitoring (**Fig. 1**). Given the advantages, scRNA-seq would provide the foundation for realizing precision medicine in nephrology. In this review, we describe several techniques of single-cell analysis and scRNA-seq studies performed in the kidneys. Moreover, the application of single-cell analysis to precision medicine in nephrology has also been discussed.

**Figure 1. Application of single-cell transcriptomics in precision medicine for kidney diseases**

Single-cell analysis can be performed at various levels with kidney biopsy, blood, and urine samples from patients with kidney diseases. This approach provides a high resolution to observe

disease transitions in each kidney sample at a single-cell level. Single-cell transcriptomics will help identify disease-related biomarkers and pathways. Based on these findings, precision medicine can be achieved by classifying patients more accurately and administering treatments tailored for each patient group.

## Methodological overview

1. Sample preparation for single cell analysis

   scRNA-seq commences by separating tissues or organs into single cells. It involves the following processes: single-cell isolation, cell lysis, complementary DNA (cDNA) synthesis, cDNA amplification, library preparation, and sequencing.

   Obtaining high quality single cell suspensions is a key determining factor for successful single-cell studies. The single-cell preparation process is the biggest cause of unwanted technical variation and batch effects in all single-cell studies. To obtain a single cell suspension from kidney samples, the fresh tissue was dissociated by mechanical cutting and enzymatic digestion. An automatic tissue dissociator was utilized to minimize the batch effects. Because different tissues have different characteristics, the protocol must be optimized for each purpose and interest. Excessive tissue dissociation causes cell damage and low viability, which can result in unwanted transcriptional changes, ambient mRNAs and higher amounts of mitochondrial mRNAs [2, 3]. In contrast, insufficient tissue dissociation causes excessive multiplets in the data rather than single cells. In addition, the single cell suspension obtained through tissue dissociation should be filtered through cell strainers with an appropriate size or debris removal solution to remove the cell debris. Cell counting is another critical step because overloading the cell suspension to the equipment may cause problems in which multiple cells are captured together. In contrast, underloading can cause loss of information due to empty droplets. Multiplets can be removed using computational tools such as Scrublet and DoubletFinder [4, 5]. It is also important to determine the appropriate number of cells to be analyzed, which requires consideration of sample heterogeneity and abundance of the target cell type. A large number of cells have to be sequenced to analyze a smaller cell population in the kidneys (a total of 20,000 cells are required to obtain 100 target cells if the target cell type constitutes only 0.5% of the whole kidney), as approximately 60% of the whole kidney is composed of proximal tubule cells [6].

   scRNA-seq analysis is hindered when the cells are difficult to isolate during sample preparation or if they are damaged. Single nucleus RNA sequencing (snRNA-seq) overcomes these limitations and analyzes the transcripts by isolating single nuclei rather than individual cells [7, 8]. Although scRNA-seq requires fresh tissue samples, snRNA-seq can be performed

on frozen samples, and can also analyze transcripts of tissues that are difficult to separate because of intertwined cells [8]. Furthermore, snRNA-seq reduces cell stress and composition bias generated during the separation step. Nevertheless, information on cytoplasmic RNA cannot be obtained, as only RNA in the nuclei can be analyzed, and various intron sequences are observed. Therefore, it is necessary to select a suitable method based on the purpose of the experiment.

2. Single RNA sequencing technologies

Various scRNA-seq methods have common procedures including RNA molecule capture, reverse transcription, cDNA amplification, sequencing library construction, and high throughput sequencing

Based on the library construction methods, scRNA-seq is largely divided into full-length scRNA-seq and tag-based scRNA-seq. Full-length scRNA-seq can be utilized not only to measure gene expression levels, but also to identify transcript isoforms, alternative splicing, and single nucleotide polymorphisms within the transcripts [9-12]. It presents high sequencing coverage and mapping efficiency; however, it requires limited cell throughput (hundreds of cells), relatively large batch effect, high sample preparation time, and high cost per cell because the samples must be prepared independently [9, 13-15]. Smart-seq2 and Quartz-seq are representative methods of full-length scRNA-seq [16, 17]. The tag-based scRNA-seq technique is mainly used to estimate the transcript abundance by sequencing the 3′-end of the transcripts in a large number of cells (tens and thousands to even millions of cells) [9, 13, 18, 19]. To distinguish different cells and to accurately measure the transcript copies, cDNA molecules are labeled with barcode sequences such as cell barcodes and unique molecular identifiers (UMIs). The tag-based scRNA-seq is again divided into droplet-based [19-21], microwell-based [22, 23], and split-pool barcoding-based technologies [24, 25], according to the labeling method. Droplet-based technology uses oil droplets and barcoded beads made of resin, to generate single droplets through water and oil in the chamber. Next, one cell and one bead pair are encapsulated in the droplets, and each single-cell mRNA is captured by oligo-dT of the barcoded bead. During cDNA synthesis, cell barcodes and UMIs are added to the cDNAs [19-21]. Although droplet-based barcoding obtains limited information on the 3′-end of the mRNA, it improves the throughput of single-cell analysis and remarkably reduces the experiment time, labor, cost per cell, and batch effect by simplifying the experimental procedure. Microwell-based technology captures mRNA by loading the cells on a plate with microwells, washing doublets with capillaries, and adding barcoded beads [22, 23]. It is simple and economical. Split-pool barcode methods identify a single cell by combinatorial indexing without the need for separation process to obtain a

single cell. Single-cell combinatorial indexing RNA sequencing (sci-RNA-seq) and split pool ligation based transcriptome sequencing (SPLiT-seq) are typical methods of split-pool barcoding[24, 25]. These techniques distribute numerous cells that are permeabilized to 96- or 384-well plates; the first molecular index is introduced to the mRNA of cells in each well, with *in situ* reverse transcription. After the first barcoding, the cells are pooled and numerous cells are distributed in another well plate again. Thereafter, a second barcode is sequentially added that provides a unique combination of barcode for each cell [24, 25].

Another challenge of scRNA-seq is the loss of cell location and orientation information during the tissue dissociation process. Remarkable efforts have been made to understand the cells by recreating a near-realistic cell environment. Because cells exist in a three-dimensional (3D) space and interact within that space, techniques have been developed to understand cell types and their locations through the spatial transcriptome analysis. Single-molecule fluorescence *in situ* hybridization (smFISH) was used to identify the RNA location and copy number; however, it could not measure multiple cells simultaneously [26, 27]. To overcome these limitations, multiplexed error robust FISH (MERFISH), a method that labels multiple RNAs in a single cell, and sequential-FISH (seq-FISH), which carries out multiple imaging FISH, have been developed [28, 29]. In addition, spatially resolved transcript amplicon readout mapping , which labels RNA with a DNA probe, and a fluorescent *in situ* sequencing method, which improves existing padlock probe shortcomings, have been developed [30, 31]. Visium, a spatial transcriptomics technology from 10× genomics, is a gene expression technique for morphological context. This technique provides a multidimensional view of tissue biology through high-throughput mRNA analysis of intact tissue sections [32]. Computing technologies such as Seurat, DistMap, and novoSpaRc are also simultaneously evolving [33-35], and efforts are made to understand the progression and function of cells in the spatial configuration.

There are several limitations to be overcome in scRNA-seq. In scRNA-seq experiments, dissociation may cause stress or change the cell proportion [2, 36]. The batch effect may be induced due to differences in protocols, sample handling, and platforms [37, 38]. In addition, the transcript efficiency per cell is relatively lower compared to bulk RNA-seq [39, 40]. The dropout event occurs in the droplet-based scRNA-seq method [20, 21, 41].

3. Computational analysis of scRNA-seq data

The raw data generated via sequencing is processed to obtain a gene by cell data matrix through analysis pipelines such as Cell Ranger [21], SEQC [42], and zUMIs [43].

Quality control filters include only high-quality cells, making it easier to identify distinct cell type populations when clustering cells, and identifying failed samples so that data can be recovered or removed from analysis. This requires generation of quality metrics such as the number of UMIs per cell, number of genes detected per cell, and percent of mitochondrial genes [44-46].

Normalization is essential for accurate comparison of gene expression between samples. Because the gene expression count depth of the same cells can vary due to the diversity inherent in each step, such as single cell capture, reverse transcription, and sequencing, the gene expression counts are scaled by the total number of sequencing reads or counts per cell [45, 47, 48].

After normalization, clustering analysis was performed to separate the cells based on gene expression patterns and identify the cell types. Seurat package allocates cells to clusters based on the principal component scores obtained from the expression of the most variable genes[49, 50]. For visualization of the cell clusters, t-distributed stochastic neighbor embedding and uniform manifold approximation and projection are mainly used [51, 52]. The identified clusters are then assigned into known cell types based on well-known cell type-specific markers or automatic cell assignment programs. Several cell marker databases for cell type classification also exist, such as Cell Finder [53], CellMarker [54], and PanglaoDB [55]. Despite these databases, marker-based cell-type classification allows markers to be represented by other cell types or complicate cell type classification by the heterogeneity of cell states. Various automated cluster annotation methods have been developed that combine annotation and clustering, and this paper presents a comparison of the various automatic cell identification methods [56]. Downstream analysis such as trajectory analysis, differential expression analysis, gene set analysis, and gene regulatory networks are well-summarized in the other reviews [45, 47, 57].

4. Integration of single cell transcriptomics with another omics data

Recently, a single-cell sequencing study has been developed to simultaneously analyze omics such as genome, epigenome, transcriptome, and proteome in one cell. By integrating genome and transcriptome information, it is possible to confirm the effect of DNA copy number variation on gene expression, transcript changes according to genomic changes, and the effect of mutations in the coding or non-coding region during transcript expression [58]. Genome and transcriptome sequencing (G&T-seq) is a technology that combines whole genome amplification (WGA) and Smart-seq2 [59]. As the relationship between DNA methylation and the transcriptome is crucial, a single-cell methylome and transcriptome (scM and T-seq) was developed to analyze this relationship in a single cell [60, 61].

Moreover, technologies that integrate chromatin information and the transcriptome have been considered. Single-cell combinatorial indexing-chromatin accessibility and mRNA (sci-CAR) integrates single-cell transcriptome analysis technology (sci-RNA-seq) and epigenetic analysis technology (sci-ATAC-seq) into one protocol [62]. Herein, it is possible to assess the relationship between the differentially expressed genes and chromatin regions that regulate these genes.

RNA and protein determine the properties of a biological system; however, because of the distinct half-life of mRNA and protein, and the effects of post-transcriptional modification, it is difficult to evaluate the correlation between mRNA and protein levels [58]. Simultaneous identification of the transcriptome and proteome at the single-cell level has helped in addressing this challenge by exploring RNA and protein abundance. Cellular indexing of transcriptomes and epitopes sequencing (CITE-seq) and RNA expression and protein sequencing (REAP-seq) assay can simultaneously analyze cell proteins and transcripts with oligonucleotide-labeled antibodies at a single-cell level, and can detect proteins barcoded into multiple antibodies and more than 20,000 genes [63, 64].

By analyzing the genome, epigenome, transcriptome, and proteome in a single cell, various techniques unaffected by cellular heterogeneity have been developed to overcome the limitations of single transcriptome studies. Through the continuous development of bioinformatics algorithms and experimental technology advances, more complex and important analyses will be possible. These single-cell multiomics can help researchers to precisely distinguish subtypes of cells from heterogeneous cell populations [65-67]. In addition, a more accurate lineage trajectory analysis is possible by integrating gene expression and epigenetic changes obtained during cell division and delivery to daughter cells [68]. Present single-cell multiomics data can reveal the correlation between different omics information, and presumably, may effectively reveal the causal relationship between omics and technology development.



## Approaches and utility of single cell transcriptomics in nephrology

- Kidney single-cell atlas

  Until now, various scRNA-seq studies have been conducted in the diseased kidneys and normal kidneys across humans and mice. Based on the results of these studies, various databases have been constructed. Table 1 summarizes the databases of scRNA-seq studies performed in the kidneys.

Table 1. List of single-cell analysis databases

| Definition | Resource | Target disease | Species | Cell number | Platform |
|---|---|---|---|---|---|
| Single-cell atlas from healthy mouse kidneys [6] | http://susztaklab.com/sc | Normal | Mouse | 57,979 | 10x Genomics |
| Single-cell atlas of the mouse glomeruli [69] | https://shiny.mdc-berlin.de/mgsca/ | Normal | Mouse | ~13,000 | Drop-seq |
| Single-cell RNA sequencing for each collecting duct type [70] | https://hpcwebapps.cit.nih.gov/ESBL/Database/scRNA-Seq/alpha-fraction.html | Normal | Mouse | 235 | Fluidigm C1 |
| Profiling kidney cell by lineage, gender, zonation [71] | https://cello.shinyapps.io/kidneycellexplorer/ | Normal | Mouse | 31,265 | 10x Genomics |
| Single-cell RNA sequencing of human kidney [72] | | Normal | Human | 23,366 | 10x Genomics |
| Profiling of human kidney development by single-cell RNA sequencing [73] | | Kidney development | Human | ~3,000 | STRT-seq |
| Inflammatory response in transplantation kidney [74] | http://humphreyslab.com/SingleCell/ | Transplantation | Human | 4,487 | InDrops |
| Single-nucleus RNA sequencing of human kidney [7] | | Normal | Human | 17,659 (nuclei) | Drop-seq |
| Single-cell atlas of the human kidney's immune system from mature and fetal kidneys [75] | https://www.kidneycellatlas.org/ | Normal | Human | 67,471 | 10x Genomics |
| Human kidney cell atlas by Mux-seq [76] | | Normal | Human | 45,000 | 10x Genomics |
| Single-cell RNA sequencing from patients with lupus nephritis [77] | | Lupus nephritis | Human | 4,019 | Fluidigm C1 |

| | | | | | |
|---|---|---|---|---|---|
| Kidney immune cell profiling by single-cell RNA sequencing in patients with lupus nephritis [78] | https://immunogenomics.io/ampsle/ | Lupus nephritis | Human | 8,455 | CEL-seq2 |
| Single-cell atlas of the human diabetes kidney [79] | http://humphreyslab.com/SingleCell/ | Diabetes | Human | 23,980 (nuclei) | 10× Genomics |
| Single-cell RNA sequencing of mouse glomeruli with diabetes [80] | | Diabetes | Mouse | 644 | Fluidigm C1 |
| Single-cell RNA sequencing from UUO mouse model [81] | https://argonaut.is.ed.ac.uk/shiny/katie.connor/mac_shiny/ | UUO | Mouse | 25,381 | 10x Genomics, SMART-seq2 |



The production of a single-cell atlas offers several advantages. First, by identifying the cell types of the differentiated cells, we can fathom the stage of development and differentiation. Second, it helps to better understand the mechanism of disease development and progression. By analyzing the diseased tissue of various patients at a single-cell level and comparing it with the cell atlas of a healthy person, it is possible to understand the heterogeneity of cells in the diseased tissue and precisely determine the cause of the disease. By analyzing the single cells in diseased tissues of various patients, the factors that contribute to differences between individuals can be revealed more accurately, making it possible to suggest suitable and personalized treatments for each patient. Eventually, it also facilitates the development of new drugs and biomarkers. Efforts to the build kidney's single-cell atlas are in process.

Park et al. constructed a cell atlas using scRNA-seq for 57,979 cells from the healthy mouse kidneys [6]. They identified previously defined 18 kidney epithelial and immune cell types as well as novel transitional cell types located between intercalated cells (ICs) and principal cells (PCs), which are distinct cells of the collecting ducts. Moreover, they revealed that Notch signaling is responsible for the transition of ICs to PCs. Karaiskos et al. performed single-cell profiling on mouse glomerulus cells [69]. They identified previously defined glomerular cell types such as podocytes, mesangial cells, and endothelial cells. They also revealed novel marker genes for all glomerular cell types and transcriptional heterogeneity of each cell type via sub-clustering the endothelial cells and the podocytes.

Furthermore, Chen et al. performed scRNA-seq on mouse collecting duct cells [70]. Ransick et al. anatomically dissected the male and female kidneys to perform anatomy-guided scRNA-seq [71]. They confirmed sexual diversity along with spatial and temporal diversity in nephrons and the collecting system.

Liao et al. identified 10 normal human cell clusters via scRNA-seq with 23,366 kidney cells from 3 human donors [72]. Proximal tubule cells and collecting duct cells were classified into 3 and 2 subtypes, respectively. For gene expression profiling of human fetal kidney development, Wang et al. applied scRNA-seq to 3,543 renal cells spanning several embryonic stages and classified the major cell types of the human fetal kidney [73]. Moreover, they identified two subpopulations in the cap mesenchyme and reported heterogeneity of cap mesenchyme through differences in their molecular characteristics. Furthermore, they identified the transcription factor and signaling pathway involved in nephron tubule segmentation during fetal kidney development. Wu et al. performed scRNA-seq of human kidney allograft biopsy samples and confirmed the proinflammatory response of allograft rejection by comparing biopsy samples with healthy kidney epithelial transcriptomes [74]. Understanding the similarities and differences of organ-specific tumors and the origin of the tumors is crucial for suitable treatment. Young et al. reported single-cell profiling of human renal tumors and normal tissues from pediatric and adult kidneys [82]. They confirmed that Wilms tumor, a pediatric kidney cancer, was derived from abnormal fetal cells. The origin of the tumor was predicted by matching the transcriptome of adult kidney cancer to a specific subtype of the proximal convoluted tubular cells. Lake et al. optimized the snRNA-seq pipeline for clinical specimens to define the molecular transition states of more than 10 nephron segments across two major kidney regions, proximal tubules and collecting ducts [7]. Thus, this pipeline describes the anatomical nephron organization and provides a starting point for building a molecular and physiological atlas that can be used as an important reference for identifying variations in several kidney diseases. Stewart et al. attempted to solve the spatiotemporal immune topology in human kidneys via scRNA-seq [75]. The scRNA-seq of mature and fetal kidneys revealed the asymmetric distribution of immune cells via spatial distribution analysis of cells throughout the kidney, and the contribution of each cell type based on the nephrogenesis stage was analyzed. This kidney immune cell atlas can help understand the pathogenic mechanisms and identify the therapeutic targets in immune and infectious kidney diseases.

Recently, single-cell studies of human and mouse kidneys characterized the complexity of kidney tissues; however, some scRNA-seq studies missed the known cells and subtypes.

This occurs because sample preparation, dissociation method, and batch effects have an impact on the detection of rare or sensitive cells. A multiplex approach for droplet snRNA-seq (Mux-Seq) was applied to minimize batch variation [76]. This allows feasible application of several human biopsies to scRNA-seq at once, thereby enabling efficient and successful identification of different kidney cell communities in healthy and diseased models.

- Single-cell analysis of kidney diseases

Previous studies have reported the application of scRNA-seq to the kidneys of patients with lupus nephritis (LN). Der et al. reported that the type 1 interferon response in the patient's tubular epithelial cells was higher than that in the healthy controls, and also confirmed clinically relevant signatures related to the disease in kidney biopsy as well as a skin biopsy [77]. Arazi et al. performed scRNA-seq for the kidney tissues of patients with LN and healthy control, and confirmed that leukocytes in the kidney were active in the disease conditions and presented differences in activation status before and after the inflammatory reaction [78]. Subgroups of innate and adaptive immune cells expressing various transcription factors related to systemic lupus erythematous (SLE) were identified by analyzing cluster-specific expression of genes related to disease risk through genome-wide association studies. The scRNA-seq of LN tissues clinically confirmed the molecular signature associated with prognosis, which can improve the standard of current patient care and stratification.

Transcriptome profiling of the kidney tissue or isolated glomeruli provides insight into the pathogenesis of kidney fibrosis. Wilson et al. performed snRNA-seq using 23,980 nuclei from control and diabetic kidney cortex samples [79], which revealed gene expression changes in diabetic glomeruli, mesangial cells, endothelial cells, and diabetic proximal convoluted tubules and ascending limbs. In addition, intercellular signal changes were observed in the glomerular cell types through differentially expressed ligand-receptors. Differential expression analysis of the leukocytes revealed that infiltrating immune cells contribute to the generation of kidney risk inflammatory signature (KRIS) markers. These gene expression changes may be useful in identifying biomarkers and signaling pathways early in diabetic nephropathy. To better explain the mechanism of an early diabetic kidney disease development, Fu et al. performed scRNA-seq for gene expression analysis of kidney glomerular cells in a diabetic mouse model. They identified 5 distinct cell clusters and novel glomerular cell specific markers. Comparison of the scRNA-seq data between the diabetic and normal mouse kidneys revealed that the identified cell clusters were the same, but the immune cell population increased in diabetic mice. Moreover, they confirmed remarkable gene expression changes in endothelial and mesangial cells of

diabetic mouse kidneys. These analyses identify key factors involved in diabetic kidney disease progression and thereby help in implementing new treatment approaches [80]. Using the reversible unilateral ureteric obstruction model (R-UUO), changes observed in renal injury and repair were noted at the single-cell level [81]. The scRNA-seq analysis performed and revealed detailed characteristics of myeloid cell heterogeneity in damaged and recovering kidneys by identifying new monocyte and macrophage subsets that have not been observed in the kidney [81]. These data could identify potential therapeutic targets that can inhibit the progression of kidney disease and aid in the development of therapeutics for kidney diseases.

Intratumoral heterogeneity interferes with marker-based anticancer treatment because targeted therapy only removes a specific population of tumor cells while failing to detect others. To distinguish molecular and cellular heterogeneity in renal cell carcinoma, Kim et al. performed transcriptome profiling of primary and metastatic renal cell carcinoma at single-cell resolution [83]. The study demonstrated that metastatic cancer cells exhibit distinct gene expression patterns with increased metastatic and aggressive signatures compared to primary cancer cells. Based on transcriptome profiling and drug screening, drug sensitivity and activation status of signaling pathways were predicted, and the correlation between the predicted signature and measured data was verified. Based on single-cell transcriptome analysis, identifying a subgroup of cells that have an active state for signaling pathways and drug screening can lead to the most effective combination of drugs to eliminate the potentially targeted cancer cells. Such approaches will help overcome the intratumoral heterogeneity that interferes with the success of precision medicine. The immune cells of the tumor microenvironment (TME) are crucial in determining the response to cancer immunotherapy. Nevertheless, the role of immune cells in clear cell renal cell carcinoma (ccRCC) remains unclear, and most patients do not respond to these treatments. Vishwakarma et al. applied scRNA-seq to the tumors and immune cells in the blood from patients with ccRCC to characterize the TME of ccRCC [84]. They identified several intratumoral CD8 T cell states that characterize the effector, memory, and exhausted subpopulations along with the multiple cell states of tumor associated macrophages and dendritic cell types. Moreover, they demonstrated intratumoral cytotoxic and regulatory CD4 T cell clusters, establishing tumor-infiltrating effectors and memory programs. These results provide a basis for facilitating research on TME to identify new therapeutic targets and biomarkers.



## Application of single-cell analysis in precision medicine

Research on most diseases related to genetic or epigenetic alterations can make remarkable progress via single-cell analysis. Various biomedical fields such as microbiology, immunology, neurology, and oncology have already been studied by applying single-cell analysis in precision medicine, which has the potential to improve disease diagnostics, prognostics, targeted therapy, early detection, and noninvasive monitoring [85].

Existing drug screening methods provide only rough readouts such as cell survival, proliferation, altered cell morphology, or specific molecular findings revealing whether a specific enzyme is blocked. Therefore, most assays miss the cell state changes or subtle changes in gene expressions that can reveal the mechanisms appearing inside the treated cells. In addition, it may not be possible to detect unexpected side effects of the drug tested, or various responses between cells that are genetically identical to the same drug. During drug development, the application of single-cell sequencing provides more detailed information based on the genetic, epigenetic, and transcriptomic profiles of responders compared to those of nonresponders, and further helps in improving the efficiency and accuracy of drug development [86]. Due to limitations in studying the mechanism, heterogeneous response, and off-target effects of drugs, Srivatsan et al. introduced "sci-Plex," which combines nuclear hashing and conventional sci-RNA-seq to quantify the global transcriptional responses to numerous independent perturbations at single-cell resolution [87]. Sci-Plex can distinguish the distinct effect of a drug on a cellular subset, and can reveal the heterogeneity of a cellular response to perturbation. Furthermore, it is possible to measure changes in the relative proportions of a subset of distinct cells across the drugs. Shin et al. developed a multiplexed scRNA-seq method that can profile multiple experimental conditions, and evaluated whether the approach can simultaneously perform single-cell transcriptome profiling for multiple drugs [88]. After drug treatment, a 48-plex single-cell experiment was performed, and each drug revealed a unique transcriptome response and target specific gene expression signature at the single-cell level. They demonstrated that this method can be applied to screen drugs and their transcriptional responses in a high-throughput manner. Kim et al. performed drug screening for renal cell carcinoma based on single-cell transcriptome analysis. They identified cellular subpopulations with activation status for the signaling pathway based on single-cell transcriptome analysis, and derived effective combinations of drugs for cancer cell removal through drug screening [83]. Using single-cell transcriptome analysis, it is possible to understand the heterogeneous cellular pattern of diseases, and based on this understanding, drug screening will enable more precise and accurate treatments.

In recent years, immunotherapy has been used as a promising approach to treat advanced diseases. Remarkable progress has been made in the development of effective immunotherapy for specific cancers. However, it is still a relatively early step in understanding the complexity of the

immune system against human diseases. Single-cell sequencing will help in understanding tumor heterogeneity and highlight the need for this technique to develop safe and effective treatments for each patient. For instance, Krieg et al. analyzed immune cell subsets in the peripheral blood of patients before and after treatment at the single-cell level to investigate the immune signature related to the reaction of anti-programmed cell death protein-1 (anti-PD-1) immunotherapy [89]. The frequency of the immune cell subset of healthy controls, responders, and nonresponders was confirmed in the peripheral blood mononuclear cells of patients before and after immunotherapy. Using scRNA-seq, Kim et al. reported that the concentration of exhausting induction factors that cause T cells to lose their attacking power against abnormal cells can predict patient-specific reactions to immune cancer treatment [90]. Chimeric antigen receptors (CAR) are receptor proteins that are genetically modified to give T cells the ability to target specific proteins. CAR T cell therapy uses modified T cells to effectively target and destroy cancer cells. Suarez et al. designed a new CAR therapy targeting carbonic anhydrase (CAIX) to prevent T cell exhaustion [91]. It was confirmed that the anti-CAIX-CAR T cell secreting anti-PD-L1 reduces T cell exhaustion and improves CAR T cell treatment of ccRCC *in vivo*. Sheih et al. performed scRNA-seq to profile CD8+ CAR T cells isolated from patients treated with CAR T cell immunotherapy and from the infusion product (IP) [92]. These findings reveal that scRNA-seq can provide unique insights into the *in vivo* behavior of CAR T cells after adoptive transfer, and can improve CAR T cell immunotherapy through future studies.

As mentioned above, oncology is already implementing precision medicine via single-cell analysis; however, precision medicine in kidney diseases lags behind other fields, including cancer. As the immune mechanism is centrally involved in the progression of kidney disease, an insight into this mechanism will be an important step towards development of precision medicine [93]. To determine the key elements related to this mechanism, the heterogeneity of the tissue sample must be resolved. Single-cell analysis is the optimal method for solving the immune cell heterogeneity in the kidney diseases. A previous study reported the human kidney spatiotemporal immune topology at the single-cell level [75]. This profiling provides a description of the kidney's immune system and helps in the diagnosis and treatment of kidney-related diseases. Furthermore, as single-cell multiomics analysis can reveal correlations between different omics data, it is possible to analyze the disease-causing factors and their interaction. This overcomes the limitations of single omics data. Therefore, the application of single cell analysis in nephrology will contribute to the development of precision medicine (Fig. 1).

## Discussion

Precision medicine is a new approach for disease treatment and prevention that takes into account an individual's gene variability, environment, and lifestyle. Based on specific genetic biomarkers and omics approaches, precision medicine has made remarkable impact on several areas of medicine, ranging from kidney cancer to acute and chronic kidney diseases. This approach will allow researchers to more accurately predict the groups that will receive treatments and prevention strategies for certain diseases, while also providing insights into the treatment approach. Precision medicine distinctly differs from the "one-size-fits-all" approach, which develops one-sided disease treatments and prevention strategies for patients with little consideration for individual differences. Identifying specific target genes, critical signaling pathways, and cell types is the first step to enable this transition from conventional to precision medicine in nephrology. In contrast to the remarkable efforts in identifying the target genes, fewer attempts have been made to identify the cellular targets in kidney disease development due to the technical limitations. Moreover, the present bulk measurements cannot differentiate primary cell autonomous responses, which lead to disease development, from secondary cell non-autonomous responses. scRNA-seq is an innovative approach that understands various cell types and cell states, and plays a pivotal role in development of precision medicine by characterizing the cells, pathways, and genes related to complex or heterogeneous diseases.

Single-cell transcriptome analysis can reveal new biological processes by combining multiple datasets, and correlation analysis between such multiomics datasets, thus more comprehensively describing the state of a single cell. Multiomics approaches characterize the disease states and identify target genes to identify therapeutic targets or biomarkers for kidney diseases. Various single-cell sequencing technologies have been developed, which can distinguish and analyze the cell types and states of the genome, epigenome, proteome, and transcriptome. Recently, single-cell sequencing technologies that can simultaneously analyze multiomics such as genome, epigenome, transcriptome, and proteome in a same single cell have been developed.

Integrative single-cell transcriptome data will enable us to untangle the complex pathological mechanisms of kidney diseases in different aspects and eventually, to assess disease risk and monitor disease states precisely for personalized medicine.



## Acknowledgement

This work was supported by "Global University Project (GUP)", "GIST Research Institute (GRI)" and "GIST Research Institute (GRI) IIBR" grants funded by the GIST in 2020.

## References


1. Wyatt, C.M. and D. Schlondorff, *Precision medicine comes of age in nephrology: identification of novel biomarkers and therapeutic targets for chronic kidney disease.* Kidney international, 2016. **89**(4): p. 734-737.
2. Denisenko, E., et al., *Systematic assessment of tissue dissociation and storage biases in single-cell and single-nucleus RNA-seq workflows.* Genome biology, 2020. **21**: p. 1-25.
3. Nguyen, Q.H., et al., *Experimental considerations for single-cell RNA sequencing approaches.* Frontiers in cell and developmental biology, 2018. **6**: p. 108.
4. Wolock, S.L., R. Lopez, and A.M. Klein, *Scrublet: computational identification of cell doublets in single-cell transcriptomic data.* Cell systems, 2019. **8**(4): p. 281-291. e9.
5. McGinnis, C.S., L.M. Murrow, and Z.J.. Gartner, *DoubletFinder: doublet detection in single-cell RNA sequencing data using artificial nearest neighbors.* Cell systems, 2019. **8**(4): p. 329-337. e4.
6. Park, J., et al., *Single-cell transcriptomics of the mouse kidney reveals potential cellular targets of kidney disease.* Science, 2018. **360**(6390): p. 758-763.
7. Lake, B.B., et al., *A single-nucleus RNA-sequencing pipeline to decipher the molecular anatomy and pathophysiology of human kidneys.* Nature communications, 2019. **10**(1): p. 1-15.
8. Wu, H., et al., *Advantages of single-nucleus over single-cell RNA sequencing of adult kidney: rare cell types and novel cell states revealed in fibrosis.* Journal of the American Society of Nephrology, 2019. **30**(1): p. 23-32.
9. Ziegenhain, C., et al., *Comparative analysis of single-cell RNA sequencing methods.* Molecular cell, 2017. **65**(4): p. 631-643. e4.
10. Deng, Q., et al., *Single-cell RNA-seq reveals dynamic, random monoallelic gene expression in mammalian cells.* Science, 2014. **343**(6167): p. 193-196.
11. Reinius, B., et al., *Analysis of allelic expression patterns in clonal somatic cells by single-cell RNA–seq.* Nature genetics, 2016. **48**(11): p. 1430.
12. Tseng, E. and J.G. Underwood, *Single-Cell Full-Length Isoform Characterization Using SMRT Sequencing: Pacific Biosciences' Iso-Seq method produces highly accurate long reads that can be used in combination with short-read RNA-seq approaches.* Genetic Engineering & Biotechnology News, 2020. **40**(3): p. 58-60.
13. Song, Y., et al., *Single cell transcriptomics: moving towards multi-omics.* Analyst, 2019. **144**(10): p. 3172-3189.
14. Ramsköld, D., et al., *Full-length mRNA-Seq from single-cell levels of RNA and individual circulating tumor cells.* Nature biotechnology, 2012. **30**(8): p. 777.
15. Hayashi, T., et al., *Single-cell full-length total RNA sequencing uncovers dynamics of recursive splicing and enhancer RNAs.* Nature communications, 2018. **9**(1): p. 1-16.
16. Picelli, S., et al., *Smart-seq2 for sensitive full-length transcriptome profiling in single cells.*

Nature methods, 2013. **10**(11): p. 1096-1098.
17. Sasagawa, Y., et al., *Quartz-Seq: a highly reproducible and sensitive single-cell RNA sequencing method, reveals non-genetic gene-expression heterogeneity.* Genome biology, 2013. **14**(4): p. 1-17.
18. Hashimshony, T., et al., *CEL-Seq2: sensitive highly-multiplexed single-cell RNA-Seq.* Genome biology, 2016. **17**(1): p. 77.
19. Macosko, E.Z., et al., *Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets.* Cell, 2015. **161**(5): p. 1202-1214.
20. Klein, A.M., et al., *Droplet barcoding for single-cell transcriptomics applied to embryonic stem cells.* Cell, 2015. **161**(5): p. 1187-1201.
21. Zheng, G.X., et al., *Massively parallel digital transcriptional profiling of single cells.* Nature communications, 2017. **8**(1): p. 1-12.
22. Han, X., et al., *Mapping the mouse cell atlas by microwell-seq.* Cell, 2018. **172**(5): p. 1091-1107. e17.
23. Gierahn, T.M., et al., *Seq-Well: portable, low-cost RNA sequencing of single cells at high throughput.* Nature methods, 2017. **14**(4): p. 395-398.
24. Cao, J., et al., *Comprehensive single-cell transcriptional profiling of a multicellular organism.* Science, 2017. **357**(6352): p. 661-667.
25. Rosenberg, A.B., et al., *Single-cell profiling of the developing mouse brain and spinal cord with split-pool barcoding.* Science, 2018. **360**(6385): p. 176-182.
26. Femino, A.M., et al., *Visualization of single RNA transcripts in situ.* Science, 1998. **280**(5363): p. 585-590.
27. Raj, A., et al., *Imaging individual mRNA molecules using multiple singly labeled probes.* Nature methods, 2008. **5**(10): p. 877-879.
28. Eng, C.-H.L., et al., *Transcriptome-scale super-resolved imaging in tissues by RNA seqFISH+.* Nature, 2019. **568**(7751): p. 235-239.
29. Wang, G., J.R. Moffitt, and X. Zhuang, *Multiplexed imaging of high-density libraries of RNAs with MERFISH and expansion microscopy.* Scientific reports, 2018. **8**(1): p. 1-13.
30. Wang, X., et al., *Three-dimensional intact-tissue sequencing of single-cell transcriptional states.* Science, 2018. **361**(6400): p. eaat5691.
31. Lee, J.H., et al., *Fluorescent in situ sequencing (FISSEQ) of RNA for gene expression profiling in intact cells and tissues.* Nature protocols, 2015. **10**(3): p. 442.
32. Ståhl, P.L., et al., *Visualization and analysis of gene expression in tissue sections by spatial transcriptomics.* Science, 2016. **353**(6294): p. 78-82.
33. Nitzan, M., et al., *Gene expression cartography.* Nature, 2019. **576**(7785): p. 132-137.
34. Karaiskos, N., et al., *The Drosophila embryo at single-cell transcriptome resolution.* Science, 2017. **358**(6360): p. 194-199.
35. Satija, R., et al., *Spatial reconstruction of single-cell gene expression data.* Nature biotechnology, 2015. **33**(5): p. 495-502.

36. van den Brink, S.C., et al., *Single-cell sequencing reveals dissociation-induced gene expression in tissue subpopulations.* Nature methods, 2017. **14**(10): p. 935-936.
37. Chen, W., et al., *A comparison of methods accounting for batch effects in differential expression analysis of UMI count based single cell RNA sequencing.* Computational and structural biotechnology journal, 2020.
38. Tran, H.T.N., et al., *A benchmark of batch-effect correction methods for single-cell RNA sequencing data.* Genome biology, 2020. **21**(1): p. 1-32.
39. Stoeckius, M., et al., *Cell Hashing with barcoded antibodies enables multiplexing and doublet detection for single cell genomics.* Genome biology, 2018. **19**(1): p. 1-12.
40. Shin, D., et al., *Multiplexed single-cell RNA-seq via transient barcoding for simultaneous expression profiling of various drug perturbations.* Science advances, 2019. **5**(5): p. eaav2249.
41. Zilionis, R., et al., *Single-cell barcoding and sequencing using droplet microfluidics.* Nature protocols, 2017. **12**(1): p. 44.
42. Azizi, E., et al., *Single-cell map of diverse immune phenotypes in the breast tumor microenvironment.* Cell, 2018. **174**(5): p. 1293-1308. e36.
43. Parekh, S., et al., *zUMIs-a fast and flexible pipeline to process RNA sequencing data with UMIs.* Gigascience, 2018.
44. AlJanahi, A.A., et al., *An introduction to the analysis of single-cell RNA-sequencing data.* Molecular Therapy-Methods & Clinical Development, 2018. **10**: p. 189-196.
45. Luecken, M.D. and F.J. Theis, *Current best practices in single-cell RNA-seq analysis: a tutorial.* Molecular systems biology, 2019. **15**(6): p. e8746.
46. McCarthy, D.J., et al., *Scater: pre-processing, quality control, normalization and visualization of single-cell RNA-seq data in R.* Bioinformatics, 2017. **33**(8): p. 1179-1186.
47. Hwang, B., et al., *Single-cell RNA sequencing technologies and bioinformatics pipelines.* Experimental & molecular medicine, 2018. **50**(8): p. 1-14.
48. Lun, A.T., K. Bach, and J.C. Marioni, *Pooling across cells to normalize single-cell RNA sequencing data with many zero counts.* Genome biology, 2016. **17**(1): p. 75.
49. Peyvandipour, A., et al., *Identification of cell types from single cell data using stable clustering.* Scientific reports, 2020. **10**(1): p. 1-12.
50. Townes, F.W., et al., *Feature selection and dimension reduction for single-cell RNA-Seq based on a multinomial model.* Genome biology, 2019. **20**(1): p. 1-16.
51. Maaten, L.v.d. and G.J. Hinton, *Visualizing data using t-SNE.* Journal of machine learning research, 2008. **9**(Nov): p. 2579-2605.
52. Becht, E., et al., *Dimensionality reduction for visualizing single-cell data using UMAP.* Nature biotechnology, 2019. **37**(1): p. 38-44.
53. Stachelscheid, H., et al., *CellFinder: a cell data repository.* Nucleic acids research, 2014. **42**(D1): p. D950-D958.
54. Zhang, X., et al., *CellMarker: a manually curated resource of cell markers in human and*

*mouse.* Nucleic acids research, 2019. **47**(D1): p. D721-D728.
55. Franzén, O., L.-M. Gan, and J.L.J.D. Björkegren, *PanglaoDB: a web server for exploration of mouse and human single-cell RNA sequencing data.* Database, 2019. **2019**.
56. Abdelaal, T., et al., *A comparison of automatic cell identification methods for single-cell RNA sequencing data.* Genome biology, 2019. **20**(1): p. 194.
57. Lafzi, A., et al., *Tutorial: guidelines for the experimental design of single-cell RNA sequencing studies.* Nature protocols, 2018. **13**(12): p. 2742-2757.
58. Macaulay, I.C., C.P. Ponting, and T. Voet, *Single-cell multiomics: multiple measurements from single cells.* Trends in Genetics, 2017. **33**(2): p. 155-168.
59. Macaulay, I.C., et al., *G&T-seq: parallel sequencing of single-cell genomes and transcriptomes.* Nature protocols, 2015. **12**(6): p. 519-522.
60. Angermueller, C., et al., *Parallel single-cell sequencing links transcriptional and epigenetic heterogeneity.* Nature methods, 2016. **13**(3): p. 229-232.
61. Hu, Y., et al., *Simultaneous profiling of transcriptome and DNA methylome from a single cell.* Genome biology, 2016. **17**(1): p. 1-11.
62. Cao, J., et al., *Joint profiling of chromatin accessibility and gene expression in thousands of single cells.* Science, 2018. **361**(6409): p. 1380-1385.
63. Peterson, V.M., et al., *Multiplexed quantification of proteins and transcripts in single cells.* Nature biotechnology, 2017. **35**(10): p. 936.
64. Stoeckius, M., et al., *Simultaneous epitope and transcriptome measurement in single cells.* Nature methods, 2017. **14**(9): p. 865.
65. Luo, C., et al., *Single-cell methylomes identify neuronal subtypes and regulatory elements in mammalian cortex.* Science, 2017. **357**(6351): p. 600-604.
66. Tang, X., et al., *The single-cell sequencing: new developments and medical applications.* Cell & Bioscience, 2019. **9**(1): p. 53.
67. Ortega, M.A., et al., *Using single-cell multiple omics approaches to resolve tumor heterogeneity.* Clinical and translational medicine, 2017. **6**(1): p. 46.
68. Hu, Y., et al., *Single cell multi-omics technology: methodology and application.* Frontiers in cell and developmental biology, 2018. **6**: p. 28.
69. Karaiskos, N., et al., *A single-cell transcriptome atlas of the mouse glomerulus.* Journal of the American Society of Nephrology, 2018. **29**(8): p. 2060-2068.
70. Chen, L., et al., *Transcriptomes of major renal collecting duct cell types in mouse identified by single-cell RNA-seq.* Proceedings of the National Academy of Sciences, 2017. **114**(46): p. E9989-E9998.
71. Ransick, A., et al., *Single-Cell Profiling Reveals Sex, Lineage, and Regional Diversity in the Mouse Kidney.* Developmental cell, 2019. **51**(3): p. 399-413. e7.
72. Liao, J., et al., *Single-cell RNA sequencing of human kidney.* Scientific Data, 2020. **7**(1): p. 1-9.
73. Wang, P., et al., *Dissecting the global dynamic molecular profiles of human fetal kidney*

*development by single-cell RNA sequencing.* Cell reports, 2018. **24**(13): p. 3554-3567. e3.

74. Wu, H., et al., *Single-cell transcriptomics of a human kidney allograft biopsy specimen defines a diverse inflammatory response.* Journal of the American Society of Nephrology, 2018. **29**(8): p. 2069-2080.
75. Stewart, B.J., et al., *Spatiotemporal immune zonation of the human kidney.* Science, 2019. **365**(6460): p. 1461-1466.
76. Schroeder, A.W., et al., *Novel Human Kidney Cell Subsets Identified by Mux-Seq.* bioRxiv, 2020.
77. Der, E., et al., *Tubular cell and keratinocyte single-cell transcriptomics applied to lupus nephritis reveal type I IFN and fibrosis relevant pathways.* Nature immunology, 2019. **20**(7): p. 915-927.
78. Arazi, A., et al., *The immune cell landscape in kidneys of patients with lupus nephritis.* Nature immunology, 2019. **20**(7): p. 902-914.
79. Wilson, P.C., et al., *The single-cell transcriptomic landscape of early human diabetic nephropathy.* Proceedings of the National Academy of Sciences, 2019. **116**(39): p. 19619-19625.
80. Fu, J., et al., *Single-Cell RNA Profiling of Glomerular Cells Shows Dynamic Changes in Experimental Diabetic Kidney Disease.* Journal of the American Society of Nephrology, 2019. **30**(4): p. 533-545.
81. Conway, B.R., et al., *Kidney single-cell atlas reveals myeloid heterogeneity in progression and regression of kidney disease.* bioRxiv, 2020.
82. Young, M.D., et al., *Single-cell transcriptomes from human kidneys reveal the cellular identity of renal tumors.* Science, 2018. **361**(6402): p. 594-599.
83. Kim, K.-T., et al., *Application of single-cell RNA sequencing in optimizing a combinatorial therapeutic strategy in metastatic renal cell carcinoma.* Genome biology, 2016. **17**(1): p. 80.
84. Vishwakarma, A., et al., *Mapping the Immune Landscape of Clear Cell Renal Cell Carcinoma by Single-Cell RNA-seq*. bioRxiv, 2019: p. 824482.
85. Wiedmeier, J.E., et al., *Single-Cell Sequencing in Precision Medicine*, in *Precision Medicine in Cancer Therapy*. 2019, Springer. p. 237-252.
86. Xu, X., et al., *Single-cell exome sequencing reveals single-nucleotide mutation characteristics of a kidney tumor.* Cell, 2012. **148**(5): p. 886-895.
87. Srivatsan, S.R., et al., *Massively multiplex chemical transcriptomics at single-cell resolution.* Science, 2020. **367**(6473): p. 45-51.
88. Shin, D., et al., *Multiplexed single-cell RNA-seq via transient barcoding for simultaneous expression profiling of various drug perturbations.* Science advances, 2019. **5**(5): p. eaav2249.
89. Krieg, C., et al., *High-dimensional single-cell analysis predicts response to anti-PD-1 immunotherapy.* Nature medicine, 2018. **24**(2): p. 144.
90. Kim, K., et al., *Single-cell transcriptome analysis reveals TOX as a promoting factor for T*

*cell exhaustion and a predictor for anti-PD-1 responses in human cancer.* Genome medicine, 2020. **12**(1): p. 1-16.

91. Suarez, E.R., et al., *Chimeric antigen receptor T cells secreting anti-PD-L1 antibodies more effectively regress renal cell carcinoma in a humanized mouse model.* Oncotarget, 2016. **7**(23): p. 34341.
92. Sheih, A., et al., *Clonal kinetics and single-cell transcriptional profiling of CAR-T cells in patients undergoing CD19 CAR-T immunotherapy.* Nature Communications, 2020. **11**(1): p. 1-13.
93. Kurts, C., et al., *The immune system and kidney disease: basic concepts and clinical implications.* Nature Reviews Immunology, 2013. **13**(10): p. 738-753.