Exploration of renal space: navigating injury and repair through spatial omics

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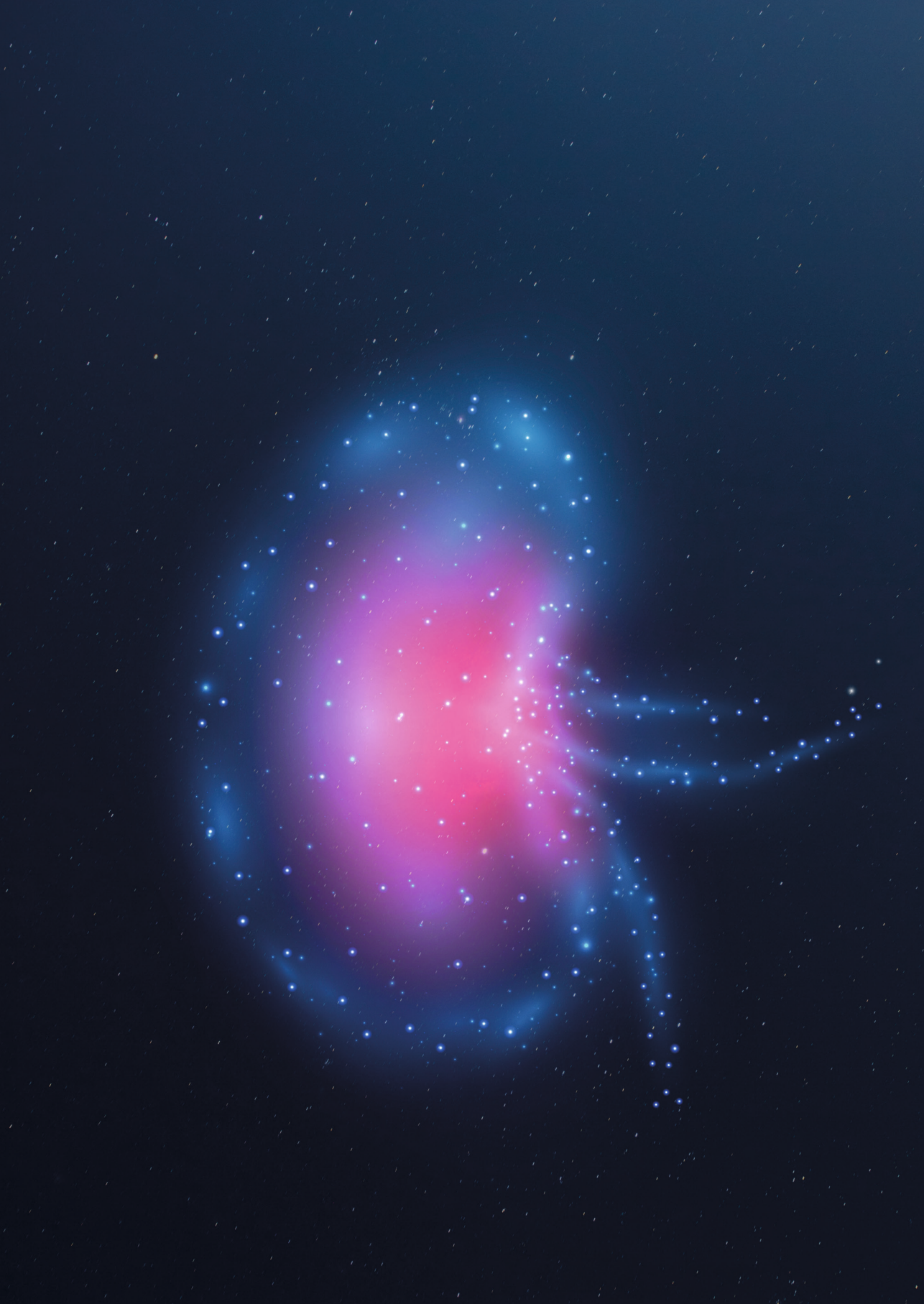

CHAPTER 1

Investigating kidney biology with spatial technologies – general introduction and thesis outline

ROSALIE G.J. RIETJENS

THE ULTIMATE RECYCLING SYSTEM

The average adult human body consists of more than half its weight in water. The majority of this water – two-thirds of the total body water - is contained within cells, also known as the intracellular fluid which helps with transport of ions, nutrients, and other molecules throughout the cell.[1] The remaining one-third of the water is found extracellularly, where it serves as a circulating reservoir and can be divided into the interstitial fluid, which bathes the outside of cells, and the intravascular fluid such as plasma and lymph. All this water plays a crucial role in a wide range of processes, from entire organ systems to the molecular level. By merely living our lives, toxic waste products such as urea and creatinine automatically build up in the fluid compartment, and the fluid balance gets challenged due to changes in solute composition.[2] In order to keep all processes functional and maintain homeostasis, it is important for waste products to be filtered out and for the fluid balance to be controlled. This is where the ultimate biological recycling system comes in: the kidney.

The kidney is a complex vital organ, and with currently available technologies over 20 different renal cell types have been characterized to exert its function.[3] These cell types are organized in functional units called nephrons, of which a single human kidney contains on average approximately one million (Figure 1).[4] These nephrons ensure that around 200 liters of blood gets filtered on a daily basis, thereby removing both waste products and excess fluid from the body. Additionally, kidneys regulate blood pressure, control electrolyte levels, regulate blood pH and produce important hormones.[5] To perform these functions effectively, different renal cell types have to collaborate properly and in a tightly regulated manner. In general, the nephron can be classified into distinct functional subunits: *i)* the renal corpuscle connected to *ii)* the tubular system which ends in *iii)* the collecting duct (Figure 1). The renal corpuscle consists of the glomerulus and Bowman's capsule. This first part of the nephron is where ultrafiltration occurs. Blood enters the glomerulus through the afferent arteriole, after which it moves to the glomerular capillaries. These capillaries consist of fenestrated endothelium, allowing plasma and small molecules to be pushed into Bowman's space due to hydrostatic pressure. This specialized endothelium, together with the glomerular basement membrane and filtration slits from podocytes, form the filtration barrier which orchestrates the composition of the filtrate.[6] This filtrate, also called pre-urine, flows into the renal tubular system where active and passive tubular reabsorption occur to move filtered water, electrolytes, glucose, amino acids, lipids and small proteins back into the bloodstream. The proximal convoluted tubule (PT-S1/S2) is located directly after the Bowman's capsule, located in the kidney cortex and contains a high number of ATP-driven transporters, as it is responsible for the bulk reabsorption of filtered solutes.[7] To be able to provide for the high energy demand, these proximal tubular epithelial cells contain a high number of mitochondria.

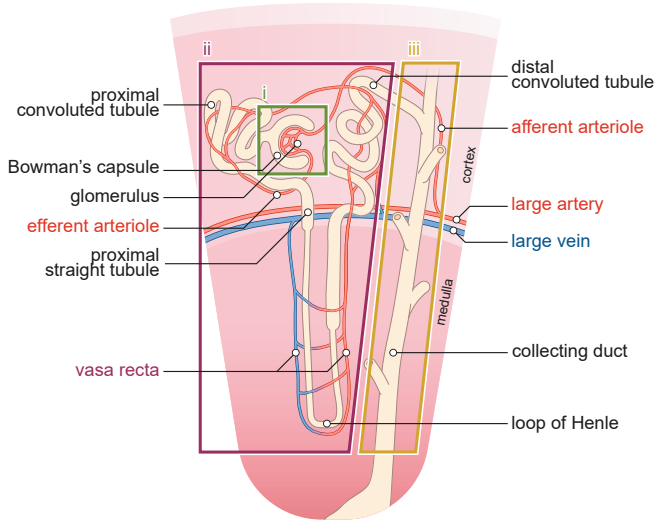

Figure 1. Anatomy of the nephron. The nephron consists of three subunits: *i)* the renal corpuscle, consisting of the glomerulus where blood flows in through the afferent arteriole, enters the glomerular capillaries and flows out through the efferent arteriole. *ii)* the tubular system with segments arranged in sequential order: the proximal convoluted tubule (PT-S1/S2), the proximal straight tubule (PT-S3), the loop of Henle (surrounded by the vasa recta) and the distal convoluted tubule. *iii)* the collecting duct.

Descending into the outer medulla of the kidney, the proximal straight tubule (PT-S3) continues the reabsorption process although to a lesser extent. Consequently, these PT-S3 epithelial cells have reduced mitochondrial density, which increases vulnerability of these cells to injury (discussed in the next section).[8] From the proximal tubules, the pre-urine flows into the loop of Henle which is a U-shaped tube extending into the renal medulla. Together with the vasa recta, the specialized capillary network surrounding the loop, the main function of the loop of Henle is to create a concentration gradient to concentrate the urine. The concentrated urine then reaches the distal convoluted tubule (DCT), which together with the collecting duct finetunes reabsorption, thereby ensuring the body's hydration and electrolyte balance. Taken together, the nephron is a remarkably intricate yet precisely orchestrated unit allowing the kidney to continuously adapt to the body's needs to sustain homeostasis.

KIDNEY INJURY AND REPAIR

Acute versus chronic kidney injury

There are many ways in which kidneys can undergo damage, but in general the distinction can be made between two categories: acute and chronic injury. Acute kidney injury (AKI) refers to an abrupt and often reversible impairment of kidney function.[9] Without timely and adequate treatment, kidney dysfunction can persist and gradually progress into chronic injury due to irreversible loss of nephron function. In recent years, the incidence of AKI has been increasing driven by various causes such as drug-induced kidney damage, volume depletion, sepsis, and severe trauma, in which ischemia-reperfusion injury (IRI) is the most common contributor.[10] Chronic kidney disease (CKD), the second damaging category, is defined by a progressive decrease of kidney function which is reflected by a decreased glomerular filtration rate and increased markers of kidney damage such as proteinuria, structural abnormalities or electrolyte imbalance.[11] The most common primary diseases leading to CKD are diabetes and hypertension, both causing damage to the renal microvasculature which eventually leads to irreversible loss of glomerular function. Currently, the combination of *i)* limited regenerative capacity of the kidney, *ii)* CKD therapies being merely able to slow down progression rather than curing the disease and *iii)* increased incidence of diabetes and cardiovascular disease, result in a rapidly increasing incidence of CKD. The only available treatment options for more advanced CKD and eventually end stage renal disease (ESRD) are either dialysis or renal replacement therapy, which place a significant burden on both the healthcare system as well as the patient's quality of life.

AKI and (mal)adaptive repair

Upon an acute kidney injury insult, hypoperfusion of the kidney and systemic inflammation cause damage to various renal cell populations (Figure 2).[12-14] Local oxygen delivery gets compromised due to early endothelial injury. In parallel a pro-inflammatory tissue environment is established, characterized by complement deposition, platelet aggregation and recruitment of inflammatory immunocytes due to expression of specific ligands and secreted factors such as kidney injury molecule-1 (KIM-1).[15] Given the high energy demand of the proximal tubule cells, altered oxygen availability poses a metabolic challenge to this nephron segment. Tubular injury and dysfunction are therefore a clear hallmark of AKI, especially within the PT-S3 segment considering its lower mitochondrial density. Secondary to tubular injury, glomerular filtration is reduced via tubuloglomerular feedback.

In the days following the acute phase of the injury, repair processes are instigated aimed at repair of the injured tubular cells (Figure 2). The surviving mature proximal tubule cells undergo a process of dedifferentiation, proliferation and redifferentiation to repopulate the tubular segment.[12,16,17] At the same time, immunocytes switch to a pro-repair phenotype to support regenerative processes. Upon successful completion of these adaptive repair processes, kidney homeostasis and function are restored. Although tightly regulated, the repair processes can also fail to succeed in establishing homeostasis and lead to so-called

maladaptive or failed repair.[18] This situation is characterized by a sustained inflammatory phenotype of infiltrated immunocytes, vascular rarefaction and tubular cell failure to reinstate their healthy, mature phenotype (Figure 2). Persistence of this phenotype is thought to be particularly relevant for processes driving kidney scarring such as epithelial and endothelial to mesenchymal transition as well as myofibroblast transformation leading ultimately to kidney fibrosis.

CKD

Chronic kidney disease is characterized by injury to the renal vasculature, glomerulus, or the tubulointerstitium, or a combination of these.[14] This injury leads to loss of nephrons, which initially activates compensatory mechanisms within the remaining nephrons such as hyperfiltration and tubular hypertrophy. Although these mechanisms help maintain the functional output of the kidney at first, over time they will increase the strain on these remaining nephrons and eventually cause destruction of kidney architecture and progressive loss of kidney function.[19] CKD is a complex, multifactorial disease of which the following events contribute to disease progression: *i)* pro-inflammatory immunocytes entering the damaged kidneys, *ii)* (myo)fibroblasts becoming activated and *iii)* extracellular matrix replacing the normal tissue architecture.[20] As stated, there currently is no effective treatment for CKD, and although the disease progression can be slowed down by lifestyle changes and medication, ultimately kidney function will regress to an extent where dialysis and renal replacement therapy become necessary.

Connection between AKI and CKD

The connection between AKI and CKD goes two ways; multiple sequential AKI events can eventually result into the transition towards CKD, however CKD patients can also undergo an AKI hit. When CKD patients suffer from an acute-on-chronic event, tubular epithelial cells often fail to properly de- and redifferentiate which accelerates deterioration of the kidney architecture and need for renal replacement therapy. Various theories are posed to explain the connection the other way around – AKI-to-CKD transition – of which three are briefly discussed.[13] Firstly, epigenetic changes following AKI can cause aberrant transcriptomic regulation, leading to altered gene expression of genes implicated in (recovery of) renal injury. Secondly, persistent expression of KIM-1 might play a role in transition. In the acute phase, KIM-1 expression promotes clearance of apoptotic cells, however prolonged expression could promote a pro-inflammatory and fibrotic phenotype. Thirdly, maladaptive repair can lead to tubular epithelial cells being arrested in a dedifferentiated state with ongoing production of profibrotic factors – a state called failed repair.[21] Lasting presence of these failed repair epithelial cells can lead to interstitial scarring as well as loss of glomeruli and capillaries.[22]

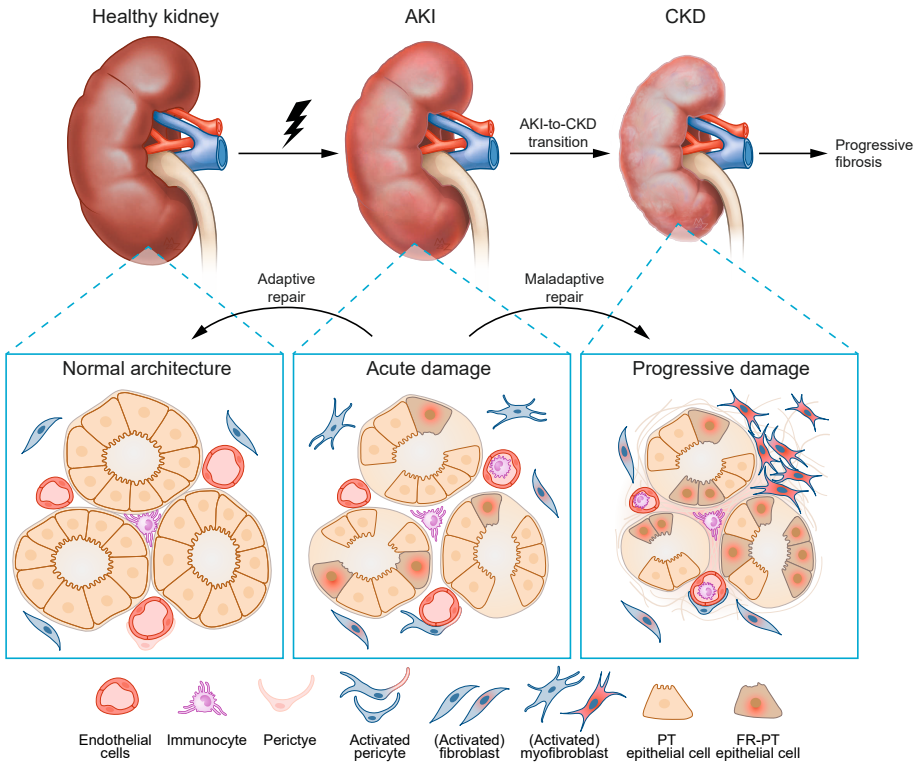

Figure 2. Histopathology of kidney injury and repair. Upon acute kidney injury, adaptive repair processes are aimed at epithelial de- and redifferentiation to repair the tubuli, supported by pro-regeneration immunocytes. A persistent inflammatory phenotype and failure of tubular cells to repopulate the tubuli – maladaptive repair – leads to progressive damage and AKI-to-CKD transition, ultimately resulting in progressive fibrosis and loss of kidney function.

THE MULTIOMICS ERA

From blueprint to phenotype

Better understanding of the cellular mechanisms underlying CKD development as well as AKI-to-CKD transition are crucial to tackle the global pandemic of kidney disease. The cell and its processes are built up and regulated by various molecular layers, and proper interaction between these layers plays a significant role in determining cellular outcome. The complete set of molecules within one layer is referred to as the -ome (e.g. metabolome: the total set of metabolites) with the corresponding omics technology allowing collective characterization and investigation of this set (e.g. metabolomics: the study of the set of metabolites) (Figure 3). The blueprint of cellular potential is drawn in the genome and transcriptome. The genome (DNA) encodes instructions for which proteins could potentially be produced, whereas the transcriptome (RNA) reflects which parts of these instructions are being actively read. The representation of what is happening within the cell is reflected in

the proteome and fluxome. These two functional cellular readouts determine how biological processes are carried out, with the proteome providing a snapshot of the protein content of the cell and the fluxome providing an overview of the rates at which metabolic reactions occur. The molecular layer which most closely represents the actual state of the cell, is the metabolome. It reflects both genetic as well as environmental influences and provides a direct snapshot of the metabolites resulting from cellular activity.

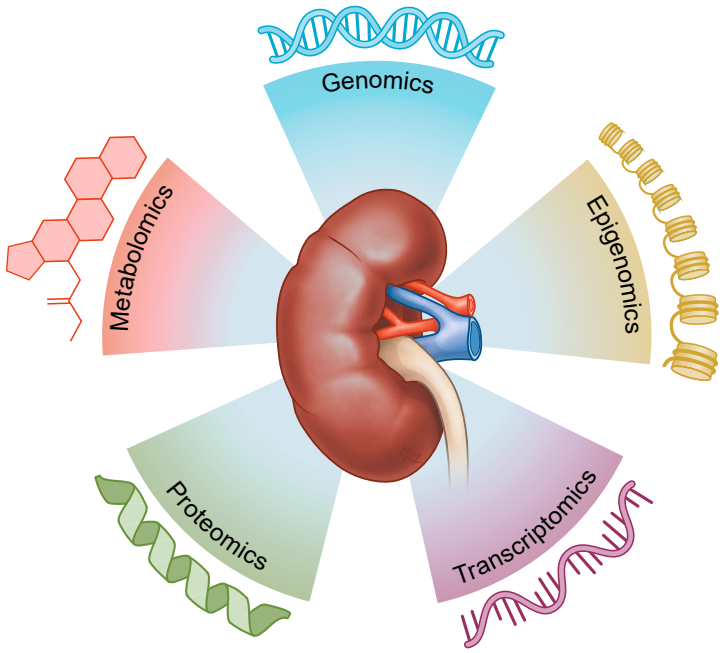

Figure 3. The multiomics landscape of the kidney.

Thus, each individual layer of -ome data identifies a set of features which can be linked to cellular state and phenotype. Although characterizing these individual features can be valuable and informative, in the end the multiome – where all the -ome layers are integrated – can reveal a comprehensive and interconnected view of how each molecular layer interacts and regulates processes like development, homeostasis, disease and regeneration. Although promising to provide new biological insights, multiome data integration still proofs to be a challenge given its complex and multidimensional nature. With computational approaches rapidly developing, there is no doubt that in the foreseeable future common pipelines will be put in place to analyze multiomic datasets.

The rise of spatial omics

With the growing interest in studying different -omes, omics technologies have rapidly advanced since the early 2000s. In 2003, the first complete human DNA sequence was mapped in the Human Genome Project.[23] In the same period, the advent of next-generation sequencing technology introduced the possibility to not only map DNA but also RNA.[24] The Human Protein Atlas, aiming to map all human proteins, was launched in 2003, and not long after the application of proteomics in the clinical setting also grew, leading to discovery of many new potential biomarkers.[25] The metabolomics field made great progress in this period as well, since online metabolomics databases allowed analysis of thousands of human metabolites – e.g. the Human Metabolome Database – combined with improved software to allow for high-throughput analysis of these extensive databases.[26] One challenge for the field of metabolomics is the high variety between molecules of the metabolome, which thereby necessitates a variety of instrument to cover analysis of the entire metabolome.

A big leap in all omics technologies was the ability to interrogate the molecular content of a single cell. In 2009, the first single-cell RNA sequencing study was published and following that technological improvement drastically reduced the costs of performing large scale single cell RNA sequencing experiments.[27] The field of proteomics and metabolomics took a fraction longer to reach the single cell stage, as proteins and metabolites respectively could not as easily be amplified like transcripts. However, with increased sensitivity of instrumentation and improved sample preparation methods, also these omics technologies now allow molecular interrogation of single cells.[28]

One common denominator of single cell omics technologies is the need for homogenization of the tissue of interest to obtain a single cell suspension. This leads to loss of information on the architecture and potential interplay of single cells in their native tissue environment. As the need arose for more in-depth understanding of cellular interactions and organization within a tissue of interest, spatial omics technologies began to enter the field of molecular technologies.[29] By now, spatial biology has been recognized twice by the renowned *Nature* publishing group as its "Method of the Year" – first for spatial transcriptomics in 2020 and later for spatial proteomics in 2024.[30,31] Often, researchers make the analogy with serving fruit. Drinking a fruit smoothie gives you insights into what kind of fruit has gone into the blender, similar to bulk omics where an averaged overview of the analyzed cells is obtained. Fruit salad allows for identification of each individual fruit piece, but it is still mixed, similar to single cell omics analysis. Finally, the fruit tart gives an exact overview of where each individual piece of fruit is located in relation to all the other fruit pieces: spatial omics.[32] A major advantage of spatial omics, is that beyond characterization of the cells itself it also allows characterization of the tissue microenvironment or cellular niches. These niches, defined by a group of cells and factors surrounding those cells that have an effect on cell behavior, play an important role in kidney injury and repair.[33]

The types of fruit tart analyzed in this thesis, are based primarily on metabolites and RNA transcripts. For spatial metabolome analysis, we employed matrix-assisted laser desorption-ionization (MALDI) mass spectrometry imaging (MSI). Chapter 2 provides an extensive review on spatial metabolomics and its application in tissue injury and regeneration. In short, a chemical matrix is applied to thinly sliced tissue sections, which allows interrogation of specific metabolite classes located in their original location in the tissue.[34] For spatial transcriptome analysis, the recently developed method spatial enhanced resolution omics sequencing (Stereo-seq) was used. This platform made a grand entrance with a multi-publication launch in 2022, showcasing its potential across multiple tissue types for different purposes.[35-38] The strength of this technique compared to others in the field, is the combination of high sensitivity, ability to go down to single-cell resolution, and a large field of view. In chapter 6, we concurrently apply MSI and Stereo-seq to samples of ischemia-reperfusion injury kidney samples, thereby combining the topics introduced in this first chapter.

THESIS OUTLINE

In this thesis, we aimed to investigate the cellular and molecular processes involved in kidney injury and regeneration using available spatial biology tools. Starting with **chapter 2**, we introduce the key spatial tool used throughout this thesis: spatial metabolomics. Expanding our view beyond the kidney, we describe in which ways this technology can be applied to study tissue injury and regeneration, as well as how insights from these studies could reveal new therapeutic targets. Next, in **chapter 3**, we employ this tool in a study aimed at investigating the early cellular renal changes upon diabetes. Using MSI, we found that phosphatidylinositol lipid metabolism was altered specifically in PT-S3 epithelial cells at a point of disease progression where histopathological changes had not yet occurred. In **chapter 4** and **chapter 5**, we continue to describe both the experimental (chapter 4) and data analysis (chapter 5) strategies which allow researchers to go beyond imaging a snapshot of metabolism and study the metabolic fluxome using stable isotope tracing. This strategy allowed us to visualize the metabolic anomalies introduced by the Warburg effect in renal cell carcinoma. In **chapter 6**, spatial multiomics is applied to study IRI at the multi-molecular level. Using spatial metabolomics and spatial transcriptomics concurrently, we were able to reveal a persistent injury niche-specific metabolic defect two weeks after the ischemic insult. Finally, **chapter 7** presents preliminary data of spatial epigenomics and guided spatial proteomics applied to relevant renal samples, which highlight the potential future perspectives for the line of research presented in this thesis.

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