Title

Opportunities for high-plex spatial transcriptomics in solid organ transplantation

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Abbreviations

FFPE	*Formalin-fixed paraffin embedded*
FISH	*Fluorescent in situ hybridization*
ISH	*In situ hybridization*
LCM	*Laser capture microdissection*
mRNA	*Messenger RNA*
RT-qPCR	*Real-time quantitative polymerase chain reaction*
scRNAseq	*Single cell RNA sequencing*
snRNAseq	*Single nucleus RNA sequencing*

Abstract

The last five years have seen the development and widespread adoption of high-plex spatial transcriptomic technology. This technique detects and quantifies mRNA transcripts *in situ*, meaning that transcriptomic signatures can be sampled from specific cells, structures, lesions, or anatomical regions whilst conserving the physical relationships that exist within complex tissues. These methods now frequently implement next generation sequencing, enabling the simultaneous measurement of many targets, up to and including the whole mRNA transcriptome. To date, spatial transcriptomics has been foremost used in the fields of neuroscience and oncology, but there is potential for its use in transplantation sciences. Transplantation has a clear dependence on biopsies for diagnosis, monitoring and research. Transplant patients represent a unique cohort with multiple organs of interest, clinical courses, demographics and immunosuppressive regimens. Obtaining high complexity data on the disease processes underlying rejection, tolerance, infection, malignancy, and injury could identify new opportunities for therapeutic intervention and biomarker identification. In this review, we discuss currently available spatial transcriptomic technologies and how they can be applied to transplantation.

Main text

Transplanted organs exist in a precarious state; their health and function can be impacted by surgical complications, ischemia reperfusion injury, senescence, recurrence of the primary pathology, drug toxicity, opportunistic infections related to immunosuppression and lifelong anti-donor immunological activity. All these factors demand careful patient monitoring, which is achieved through clinical assessments, indicators of graft function, and immunological activity. However, these are often proxy peripheral measurements for what is happening inside the allograft. The analysis of transplanted tissues themselves, through biopsy or resection, provides valuable information on the severity, type, and cause of transplant dysfunction. Understanding the nature of transplant pathology at the site of injury is essential to diagnosis, prognosis, and ultimately the identification of therapeutically targetable processes.

Range and utility of methods for tissue analysis

Pathological activity is dictated by the dynamic regulation and expression of the epigenome, transcriptome, and proteome. Current technologies can explore these molecular changes at different resolutions (from whole tissues to individual cells) and at different complexities (from a single target to the whole mRNA transcriptome).

The human transcriptome encodes ~20,000 protein-coding genes, which only accounts for 1-5% of cellular RNA at any given time[1]. In fact, the majority of cellular RNAs are transfer and ribosomal RNA that are part of >15,000 non-coding genes[1]. Most transcriptomic studies have implemented tissue dissociation to identify transcript changes in whole samples by quantifying a small number of genes at a time using real-time quantitative polymerase chain reaction (RT-qPCR), or hundreds of genes using microarray technologies. High throughput RNA sequencing was first implemented on mammalian cells between 2004 and 2008, developing a technique capable of quantifying the whole transcriptome[2,3]. These 'bulk' analyses provide an overview of transcriptomic changes, but their interpretation can be confounded by complex cell compositions[4]. The capacity to analyze RNA on a

single cell level was then developed between 2009 and 2012, although current high cell throughput (>100,000 cells) was only achieved around 2015, helping to determine mRNA expression in heterogenous cell populations 2015[5]. Fresh or frozen tissues are carefully dissociated into single cell suspensions for single-cell RNA sequencing (scRNAseq), and frozen or formalin-fixed paraffin embedded (FFPE) tissues are dissociated for single-nucleus RNA sequencing (snRNAseq). These high-throughput techniques can characterize hundreds of thousands of cells, but their interactions with each other, their anatomical distribution, and their co-localization with injury or infection are lost. Moreover, not all cell types survive tissue dissociation and processing equally, such as renal podocytes or endothelial cells. This creates inherent biases in single cell technologies that rely on viability and quality after tissue dissociation[6].

For many years, *in situ* hybridization (ISH) and fluorescent in situ hybridization (FISH) techniques were the principal methods of visualizing RNA in intact tissues at cellular and subcellular resolution. ISH and FISH reach high sensitivity in detecting target RNA, but the number of simultaneous targets can be limited by the capacity of fluorescent microscopes (2-4 channels). *In situ* sequencing was developed in 2010 with the precision to distinguish RNA polymorphisms in tissues to a subcellular resolution, despite a limited capacity for multi-plexing[7]. Analysis of gene expression in isolated histological structures and regions has been facilitated by laser capture microdissection (LCM). Although initially restricted to RT-qPCR analysis, studies implemented whole transcriptome RNA sequencing by 2014[8,9]. However, these early LCM approaches were limited by the depth and quality of the resulting transcriptomes. As an alternative, the informatic analysis of receptors and ligands attempts to reconstruct potential cell-cell interactions from single cell transcriptomics, such as CellPhoneDB or NicheNet[10,11]. These are useful tools to decipher cell-cell communication, but they can misdetect interactions and create false positive findings[12]. It has not been possible to directly explore the cell-cell interactions populations defined by sc/snRNAseq in the original tissue until the development of high-plex spatial methodologies over the last six years[13].

Outside of transcriptomic analysis, insight into tissue organization has traditionally been gained from histological dyes (e.g., Hematoxylin & Eosin) that use 1-3 contrasting colors to discriminate nuclei, cytoplasm, and connective tissue. Quantifying specific proteins and their localization within tissues can be achieved through imaging tissues after chromogen immunohistochemistry or immunofluorescence. Basic immunofluorescent microscopy measures ~4 targets, but recently immunofluorescent platforms like CODEX or imaging mass cytometry like Hyperion have enabled *in situ* simultaneous visualization of up to 60 proteins[14,15]. Methods that explore and quantify proteins have the same breadth of resolution and complexity as their transcriptomic counterparts. Total tissue dissociation can enable protein expression, interaction, and post-translational modification studies through mass spectrometry, blotting and immunoprecipitation. To assess the relationship between proteins and the cells who produce and interact with them, flow cytometry of single cell suspensions can directly quantify up to ~40 different proteins or isolate specific cell populations for further dissociation and analysis[16]. These methods are complementary to transcriptomics, visualizing the final outputs of transcriptional and proteomic regulation that manage cell function.

Methods for high-plex spatial transcriptomics

Spatial transcriptomics combines the high complexity of sequencing experiments with the topographic analysis of tissues. The aim is to understand cellular heterogeneity within and between tissues, to align cell phenotypes by location, and to study cell-cell interactions. In 2016, Ståhl et al. developed the first array based method for high-plex spatial transcriptomics[17]. Tissue sections (10-25µm thick) of mouse brain and human breast cancer were placed onto an array of positionally tagged oligonucleotides (barcodes) and permeabilised, resulting in the capture of tissue mRNA through hybridization in a spatially resolved manner. Barcoded cDNA is produced from the captured mRNA by reverse transcription reaction, which is then collected and sequenced to indicate how much of the target transcripts were present (~5000 unique genes captured per spot) and also where

on the slide they were found[17] (**Figure 1**). The methodology visualized an unprecedented heterogeneity of gene expression in invasive and in situ ductal cancers, potentially reflecting different subclone phenotypes within a single breast cancer biopsy. 10X Genomics acquired and developed this approach, improving the resolution by increasing the number of barcoded spots from 1007 to 4992 per capture area and decreasing the diameter of barcoded spots to 55 µm, resulting in a grid with an increased density of mRNA capture spots whose centre-to-centre distance is 100 µm. It is now commercially available as the **10X Visium platform**[18] and excels at creating unbiased atlases of gene expression based on the standardized grid of mRNA capture spots, each covering 1-50 cells, across the tissues and whole organs.

In the **ABL Slide-seq** method, fresh frozen tissue (10µm) sections are placed onto a monolayer of 10µm beads each identifiable by unique DNA sequences. Tissues are permeabilised, releasing the mRNA that is then captured by the adjacent oligonucleotide-tagged beads (**Figure 1**). The subsequent libraries represent mRNA expression data at each 10µm interval (bead diameter), enabling high spatial resolution for the transcriptomic output[19]. High definition spatial transcriptomics (**HDST**) similarly uses 2µm beads to improve this resolution and is compatible with histological imaging[20].

Instead of capturing mRNA with immobilized probes, **in situ sequencing** converts the mRNA to cDNA in the tissue and then detects it by padlock probes, which become circular molecules after binding to their target sequence enabling rolling probe amplification (**Figure 1**). This amplification generates *in situ* barcodes that can be read through epifluorescence and recent developments have facilitated the detection of up to 99 genes at a single cell resolution[21,22]. Intratissue sequencing is also the basis of **STARmap** (spatially-resolved transcript amplicon readout mapping), which is a method for measuring 160 to 1020 genes simultaneously in three dimensions at a resolution less than 2 µm[23].

Direct mRNA quantification conducted by immunofluorescent assays such as the **RNAscope HiPlex** v2 overcome this limitation with iteratively staining, imaging, removing the fluorescence and re-

staining to measure up to 48 targets[24]. This complexity can be drastically upscaled through using the **MERFISH** (Multiplexed Error Robust Fluorescence In-Situ Hybridization) method, which has been developed into the Vizgen MERSCOPE platform[25]. MERFISH can map between 100 and 1000 transcripts at a time[25,26]. Initially only available for fixed or fresh frozen tissues, a protocol for preparing FFPE tissues for MERFISH has now been released[27,28]. To run on the MERSCOPE platform, tissues are gel-embedded and cleared before probe hybridization. The RNA probes are tagged with 'readout sequences' that bind fluorescent reporters in sequential rounds of hybridization and imaging to produce a recognizable barcode for each transcript detected (**Figure 1**).

The **Nanostring GeoMx digital spatial profiler** (DSP) offers an image guided approach for sampling the transcriptome in manually selected areas. FFPE or fresh frozen tissues exposed to probe-nucleotide complexes during *in situ* hybridization protocols. The RNA probes are attached to oligonucleotides, indicating which transcripts are represented, by a photocleavable linker. Tissues are subsequently stained for markers of interest using standard immunofluorescence or FISH to inform the manual selection of ROI. Once a region is selected, focused UV light cleaves the linker, releasing the oligonucleotide that can then be collected and sequenced (**Figure 1**). This technique permits manual sampling of specific cell populations or histological features, contouring around features or random sampling. The sensitivity for gene expression is dependent on the size of the selected areas of interest with limited sensitivity at 15 cells and robust analyses over 100 cells[29].

For 'geographical position sequencing' (**Geo-Seq**), the areas were dissected by LCM and lysed before the construction and sequencing of libraries. Recent advances in this LCM-based technique have improved sensitivity for low RNA input, enabling analysis of ~20 cells at a time[30,31]. Users benefit from high-plex transcriptomic results of manually selected areas and features, but obtain relatively low cellular resolution as the LCM can compromise RNA stability and cell integrity[8,30].

In a different approach, the **ProximID**, **ClumpSeq** or **paired-cell sequencing** platforms perform mild dissociation protocol to stochastically break up a complex tissue into small tissue fragments, which

are then sequenced[32–34] (**Figure 1**). Cell interactions are preserved and can be then used to reconstruct spatial expression, but there is poor control over which interactions stay intact or the size of the obtained fragments.

The choice between these techniques depends on the cost, availability of platforms, but primarily the experimental question (**Table 1**). An unbiased atlas of transcriptomics across a tissue or organ would benefit from the Visium or Slide-Seq approaches, whilst the evaluation of a well-defined histological feature would benefit from the selectivity of GeoMx or LCM/Geo-Seq. Moreover, investigation of high-resolution cell-cell interactions in a tissue may benefit from semi-dissociative techniques such as ProximID, ClumpSeq or paired-cell sequencing. The correct use of spatial transcriptomic technologies can be extremely efficient at extracting large amounts of information from small tissue fragments. Whilst the quality and method of tissue processing and preservation must be taken into account to ensure the efficacy of these techniques, there is potential to utilize them for the in-depth transcriptomic analysis of rare and archived tissues.

Applications of spatial transcriptomics

Since the first uses of high-plex spatial transcriptomics in 2016, the availability of commercial platforms and the development of whole transcriptome testing, the number of publications referring to spatial transcriptomics has doubled each year. The majority describe technological advances and the development of bioinformatic tools for data analysis and interpretation (**Figure 2A**). In the fields of medical research, 40% of spatial transcriptomic studies are being conducted in oncology, neuroscience, and developmental biology (**Figure 2A**).

In neuroscience, spatial transcriptomic analysis using the 10X Visium platform of human dorsolateral prefrontal cortices identified the six layers, confirming known and revealing new laminar markers[35]. By screening for genes associated with Alzheimer's disease and autism spectrum disorders, they established that distinctive patterns of laminar expression were associated with different clinical

symptoms[35]. Mapping snRNAseq onto the spatial transcriptomic signatures showed distributions of disease-associated neuronal cells in the cortex that had not been previously appreciated[35].

The boom of spatial transcriptomics coincided with the COVID-19 pandemic in 2020 and 2021, sparking in-depth studies of post-mortem lung tissue following SARS-CoV-2 infection. The GeoMx platform was used to profile intra-tissue and inter-tissue heterogeneity comparing alveolar vs bronchiolar regions, pan-cytokeratin positive (epithelial) cells vs pan-cytokeratin negative cells, SARS-CoV-2 infected vs uninfected areas, and acute vs late COVID-19 lung phenotypes[36–39]. These studies identified key chemokines (CXCL10, CXCL11, CXCL2 and CXCL3) associated with areas of high SARS-CoV-2 load[36,38]. Inflamed areas were defined by innate activation along neutrophil degranulation and interferon pathways, whilst transcripts for epithelial barrier maintenance were downregulated[38,39]. IFNγ and IFNα responses were strongly associated with alveolar and airway areas in early COVID-19 but resolved into only alveolar areas by late COVID-19[37]. Airway, alveolar and vascular areas had different relationships with myogenesis, apoptosis and IL-6/JAK/STAT3 pathways over the course of the illness[37]. This implies that COVID-19 deaths are related to two temporal stages of lung injury driven by different pathological processes, from early pathogen and innate inflammatory responses to failing tissue repair and may require different strategies for clinical management.

The combination of spatial transcriptomics and open access data repositories has also promoted the generation of accessible whole organ or tissue gene expression atlases[38,40–44]. Stahl's original technology[17] was applied to generate an atlas of the entire adult mouse brain by hybridizing 75 sequential tissue sections on barcoded arrays and then reconstructing the transcriptomic profile of the brain in three dimensions[41]. Spatial transcriptomics, single cell transcriptomics and in situ sequencing were combined to create a spatially resolved cellular map of the human embryonic heart across three developmental phases[43]. The 10X Visium and scRNAseq have also led to the description of 9 novel cell populations in the developing human intestine, adding to the understanding of cell

differentiation and organ formation in the human foetus[45]. Data availability opens the opportunity for using related datasets for hypothesis generation as there are many papers now addressing cancer and physiology across a range of organs (**Figure 2B**).

Current applications in transplantation research

Very few papers have yet implemented spatial transcriptomics in transplantation. Of 532 Pubmed publications citing spatial transcriptomics, two publications mention spatial profiling of ischemic tissues[46,47], whilst only one publication examined T cell-mediated rejection in a renal transplant biopsy[48] (**Figure 2A**). No relevant studies were found on preprint servers (medRxiv or bioRxiv), but given the novelty of this technique, studies are likely underway.

The landscape of acute kidney injury in ischemia reperfusion injury and sepsis has been assessed in two papers using the 10x Visium platform. Ferreira *et al.* (2021) used multiple approaches to identify the cell types and states in human nephrectomy tissue, mapping out the distribution of 28 cell types *in situ*[46]. In mice, acute kidney injury (AKI) incited by ischemia reperfusion injury (IRI) and sepsis (cecal ligation puncture model) were compared. Even with minimum visible histopathological injury at 6 hours after the initial insult, different transcriptome pathways were activated including metabolic changes, IL-17 signaling and neutrophil migration in IRI and p53 activation, TNF signaling and macrophage differentiation in sepsis. The odds ratio of immune and epithelial cell colocalisation identified neutrophil engagement with injured proximal tubules in the outer stripe of the medulla, in IRI but not sepsis or sham surgeries. Analysis of genes expressed by the injured tubules interacting with neutrophils identified epithelial *Atf3* as relating to this interaction. *Atf3* was most highly expressed in this epithelial cell type in both spatial transcriptomic and single cell sequencing results. Immune-epithelial crosstalk during sepsis identified inflammatory macrophages associated with proximal tubules through the cortex[46]. *Mdk* (Midkine) expression was associated with macrophage presence and recruitment and is expressed broadly by cortex tubule epithelial cells. This approach

identified genes implicated in immune recruitment with specific anatomical regions promoting injury[46]. Dixon et al. (2022)[47] also explored ischemia reperfusion injury in in mice to decipher acute and long-term fibrotic changes in kidneys after 4 hours, 12 hours, 2 days, and 6 weeks. Cell states from single nuclei RNA sequencing were mapped onto the kidney tissue identifying the persistent co-location of T cells and macrophages with injured proximal tubules up to 6 weeks into recovery, but not with the fibroblasts despite the accumulating fibrosis[47].

NanoString GeoMx DSP platform has been used to explore regional transcriptomics in T cell-mediated rejection (TCMR) and to define the specific transcriptomic profile of histological features, such as collapsing glomeruli. Salem et al. (2022) performed a case study of gene expression across different pathohistological features in a biopsy with chronic active TCMR[48]. The 5 sampled tubular regions demonstrate variability in their pathological features (scarring, interstitial fibrosis and tubular atrophy (IF/TA), and tubulitis) and the most variable genes between them were associated with renal epithelium, stromal cell development and responses. There is potential for much larger studies to define the nature and development of heterogenous tissue damage, focusing on the immune-stromal cell interactions driving injury in rejection. Smith et al. (2022) capitalized the GeoMx platform's capacity to manually select and sample small histological structures, such as glomeruli[49]. They asked what the molecular drivers of virus-associated collapsing glomerulopathy are. Comparison of normal and collapsed glomeruli revealed dysfunction with a reduction in podocyte-lineage genes and an increase in proliferation markers. This approach implicated novel genes in the pathology, compared to previous bulk or laser capture microdissection methods. Notably collapsed glomeruli from SARS-CoV-2 and HIV infected patients were morphological similar, yet the glomeruli still had disease-specific gene and protein expression indicating diverse disease-injury interactions[49].

Opportunities for spatial transcriptomics in transplantation research

Transplantation depends on the prevention or resolution of alloimmune responses that lead to **rejection**. After engraftment, alloantigens are presented to and recognized by allospecific T cells in the vascular or lymphatic systems causing lymphocyte activation, differentiation, proliferation, and migration into the intragraft[50]. This loss of control produces TCMR and immune-driven tissue damage. In antibody-mediated rejection (ABMR), T helper cells prime alloreactive naïve and memory B cells in draining lymph nodes for differentiation into plasma cells and production of donor-specific antibodies (DSA)[51]. T cell subsets can also enhance B cell activity and promote the formation of ectopic germinal centers within allografts, potentially promoting local DSA production pathways[52]. Alloantibody binding to the allograft endothelium results in the activation of the complement cascade and myeloid cells, coordinating cytotoxicity, chemotaxis, inflammation, chronic lesions, dysregulated tissue repair and fibrosis. The processes involved in rejection span multiple anatomical locations, diverse hematopoietic-stromal cell interactions and temporal progression of the pathology that results in distinct manifestations of inflammation and injury. For example in kidney transplantation, TCMR manifests as substantial interstitial inflammation, tubulitis and intimal arteritis, whilst ABMR presents with microvascular inflammation and glomerular dysfunction[53]. These spatially distinct features have significant clinical value providing the basis for the diagnosis of the current six subclassifications of renal transplant rejection[53]. Likewise pathology is found following other solid organ transplants, including lung allografts whose histological features range from cellular rejection, to antibody-mediated rejection and to chronic lung allograft dysregulation[54]. In-depth analysis of histological features surrounding rejection would define the underlying immune-endothelial-epithelial-stromal interactions that contribute to damage, for example identifying how B cell infiltrates or innate-like B cells may be retained, activated or expanded in the cardiac and renal allografts[55–57]. Understanding these cellular interactions could lend insight into the development and nature of subclinical and borderline rejection episodes, of specific acute and chronic histological features, and of the contributions of donor and recipient immune cells to injury[58–60].

In parallel to rejection, the high-resolution spatial analysis of **tolerance** and operational tolerance may reveal novel pathways to exploit therapeutically, for example of regulatory lymphoid structures that can promote kidney allograft tolerance[61] or bronchus-associated lymphoid tissues from tolerant lung grafts that can promote peripheral tolerance[62]. Operational tolerance is associated with specific gene profiles in 'bulk' analyses of liver biopsies, although this has not be deconvoluted into a cellular or compartmental mechanisms[63]. Furthermore, T cell repertoires inside liver allografts tend to be distinct from those in the blood until rejection takes place; analysis of the trajectory between quiescence and pathology might identify pivotal changes that disturb homeostasis. Interventional clinical trials can result in unanticipated changes to the graft, such as an increase in lobular inflammation in liver transplants within 4 weeks of low dose IL-2 treatment[64] or atypical focal inflammatory infiltrates in renal transplants after adoptive Treg transfer[65]. Plus, the true efficacy of immunotherapies may be only be characterized in the allograft itself as opposed to circulating leukocyte responses, which can be discordant such as after the treatment of intestinal transplants with infliximab[66]. High-plex characterization of biopsies could infer the significance of such histological features and their impact on trial outcomes[67].

The systemic immunosuppression of transplant patients leaves them vulnerable to **opportunistic infections** and **cancer** development. The combination of the allogenic transplant, strong immunosuppression and the initial injury creates a patient cohort with the potential of altered immune responses and pathology progression when compared to non-transplanted patients. Infections vary in their cell specificity and presentation, but exhibit overlap with alloimmune pathology in cases such as BK virus nephropathy, pulmonary bacterial infections[68], or **recurrence** of hepatitis C in liver transplants[69,70]. Study of tumors, their microenvironment, infected cells, and neighboring cell phenotypes could determine key pathways relevant to the uniquely immunosuppressed transplant cohort.

The first published studies to have explored renal **ischemia reperfusion injury** have shown organ-wide transcriptomic changes with a temporal evolution from acute inflammation and hypoxia to long-term fibrosis[42,46,71]. These studies demonstrated change even in the absence of histological changes and could be applied to different organs or provide insight into the efficacy of surgical refinements and organ preservation methods.

Challenges and future directions in spatial transcriptomics

The interpretation of spatial transcriptomic studies is intrinsically linked to the study design. The sampling strategy may require an impartial array or manually selected areas, it may profile histological structures (like glomeruli) or require a subcellular resolution, it may quantify the whole gene-coding transcriptome or a selected panel. Given the differences between technologies, the chosen method and sampling strategy must be appropriate for the hypothesis.

Spatial information and sequencing are often interpreted by comparisons of cells or areas within and between tissues and patients. Careful attention should be given to powering technical and biological replicates for meaningful comparisons. The choice of comparator tissues also requires consideration as there are numerous technical factors that can influence transcriptomic readouts including RNA stability, temperature, age of samples, time before fixation, fixation method, sectioning thickness, then hybridization and sequencing batch effects[72]. In addition, biological factors have an impact on comparisons such as tissue orientation and composition, cell sizes, cell numbers, cell densities, confounding subclinical diseases, and consistency in the severity and stage of tested pathologies.

Spatial transcriptomics is applied to thin sections of fixed tissue (4-25 μm), which whilst informative removes the dimensions of depth and time from cellular interactions. Some studies have circumvented the former by the analysis of sequential slides and post-hoc 3D reconstruction the tissue, which is chiefly possible in small animal organs. Clinical research relies heavily on small

biopsies, where organ pathology is interpreted from a single fragment and may not be able to show the full extent and breadth of non-uniform diseases. Tissues must be fixed to conserve structural and transcriptomic integrity, but this creates a snapshot which does not necessarily represent the strength or duration of dynamic cell-cell interactions. To overcome this, experimental studies select samples across developmental stages or time post-injury, or infer developmental and cellular differentiation trajectories through 'pseudotime' analyses to retrieve latent temporal information from dynamic biological processes[73].

Data from spatial transcriptomic experiments can be obtained relatively quickly, yet analysis and interpretation can be significant challenges to newcomers. New bioinformatic tools and analytic approaches are an evolving discussion that makes up most academic publications (**Figure 1B**). This technique generates layers of data combining image analysis, sequencing processing and gene expression analysis that requires significant time and training to exploit properly and reliably. The use of spatial transcriptomic data from repositories comes with additional challenges if not linked to the histological map.

Analytic complexity is augmented by complementing spatial transcriptomics with spatial, single cell or bulk proteomic, lipidomic, genomic, epigenomic datasets from sequential, distal, or unrelated tissues or patients. Supporting spatial transcriptomics with these datasets can overcome the relatively low mRNA capture efficiency compared to single cell or bulk RNA sequencing methods, by promoting cell deconvolution, identifying disease-associated genes and validating the functional outcomes of transcript expression[74,75]. An important caveat to transcriptomic research is that transcript expression is not necessarily proportional to protein expression or function due to post-transcriptional and post-translational regulation[76]. Examples include significant HIF-1α mRNA whilst the protein is efficiently degraded in normoxia; T helper cells CD4 is a lineage marker as a protein yet there is poor correlation between transcript abundance and protein expression[77]; and neutrophils are defined by their stockpiles of cytotoxic granules but mRNA of granule transcripts are

downregulated with maturity[78]. Commercial platforms currently assess protein coding mRNA, but this does not preclude future studies from interrogation of non-coding RNA such as miRNA distribution.

There is an evolving capacity for higher spatial resolution and greater transcript detection sensitivity. The upcoming **NanoString CosMx** Spatial Molecular Imaging (SMI) platform will employ cycles of *in situ* hybridization for up to 1000 RNA targets at z-stacked subcellular resolution, enabling high throughput analysis of up to 2 million cells in a given fresh frozen or FFPE tissues[79]. In parallel, the 10X Genomics is expected to release the **Visium HD** that will improve their resolution to a single cell level, although no data has yet been published. 10X Genomics has also announced the launch of their **Xenium** analyzer for the *in situ* analysis of up to 400 transcripts to a subcellular resolution; this non-destructive method may allow for simultaneous protein detection. In the meantime, current methods are being adapted for specific uses. The Visium protocol has been modified to spatially resolve T cell receptor (TCR) sequences, enabling T cell clones to be associated with intra-tissue niches[80]. In fact, this method showed that specific CD8+ T cell clones had preferential transcriptomic phenotypes and locations in the tumor microenvironment of lung carcinoma and melanoma[81]. The resolution of *in situ* TCR sequencing was then rapidly advanced using an indexed bead array comparable to Slide-Seq (**Slide-TCR-seq**) to enable spatial and transcriptomic study of T cell clones with a 10µm resolution[82]. Visium protocols have also been modified for the preparation of long-read libraries, which allows the measurement of regions changes in transcript isoforms after splicing[83]. In parallel to transcriptomics, the first high-plex *in situ* epigenomic assays have now been established to assess spatial distributions of chromatin accessibility[84]. **Spatial-ATAC-seq** utilizes the barcoding systems, familiar to spatial transcriptomics, to spatially resolve accessible genome loci and provide novel insight into region epigenetic heterogeneity related to the tissue microenvironment[84]. **Epigenomic MERFISH** uses *in situ* tagmentation and transcription followed by MERFISH imaging to visualize the cellular and subcellular organization of chromatin changes of over 100 targets simultaneously[85].

The study of transplantation is informed by and rooted in tissue analysis. High-plex spatial transcriptomics offers new opportunities to analyze transplant pathology and tolerance, to identify biomarkers and therapeutic targets, and to assess the impact of therapeutic interventions in high resolution. This approach is ideal for descriptive and mechanistic research in ‘bench to bedside’ medicine, refining the findings for translation into clinical settings. The potential incorporation into personalized medicine would require a vast bank of reference tissue date and robust rapid analytic pipelines. For now, the scientific community is generating accessible organ atlases that the transplant community could explore, adopt, and supplement.

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Technology name	# Target genes	Imaging	Min. resolution	Tissue preparation	Tissue thickness	Max. tissue area	Pro
10X Visium	~18,000	Histological dyes / IF	55µm (1-50 cells per spot)	Fresh frozen/FFPE	10µm	6.5 x 6.5mm	Unbiased mapping of transcriptome across whole tissue
Slide-seq	~18,000	Reconstruction from bead array	10µm	Fresh frozen	10µm	3mm diameter spots	Unbiased mapping of transcriptome to whole tissue
Geo-seq	~18,000	Histological dyes	20 cells	Fresh frozen	15µm	< 50 x 24mm *	Manual selection of structures of interest
NanoString GeoMx	~18,000	IF (4 channels)	15 cells	FFPE/Fresh frozen	5µm	35 x 14mm	Manual selection of structures of interest
ProximID	~18,000	None	2 to 20 cells	Dissociated fresh tissues	-	-	Cell interactions preserved and relatively high sequencing depth
MERFISH	1000	IF	<100nm	Fresh frozen/FFPE	10µm	< 50 x 24mm *	Cellular and subcellular resolution

In situ sequencing	99	DAPI nuclear staining	<3µm	Fresh frozen	4-10µm	10 x 6mm	Cellular and subcellular resolution

Table 1: Comparison of capabilities of selected spatial transcriptomic methods. *Dependent on microscope or LCM platform.

Figure legends

Figure 1: Different methodological approaches to high-plex spatial transcriptomics. The interactions between cells can be reconstructed through tissue dissociation and single cell analysis (**A**), the transcriptomic signature of groups of cells or a histological structure could be defined by microdissection or the GeoMx in situ mRNA capture (B and E). The high-resolution location and quantification of mRNA transcripts can be detected through in situ sequencing and FISH (C-D), whilst unbiased transcriptome can be assessed by laying the tissue over an array of capture areas (E). Created with BioRender.com.

Figure 2: Spatial transcriptomic publications are becoming increasingly prevalent in the fields of oncology, neuroscience and developmental biology. (**A**) Publications from the Pubmed search results were manually annotated according to the overall research topic. (**B**) Publications from Pubmed search results were manually annotated for their primary organ of focus. The top 13 organs are shown in purple bars, and the proportion of cancer/malignancy/tumor-related studies are shown in orange bars. Pubmed search criteria included one of the following in the title or abstract: “spatial profiling”, “digital spatial profiling”, “spatial transcriptom*”, “spatial transcriptional profiling” or “spatially resolved transcriptom*”. Pubmed search results were restricted to publication between 2016 and 2022 and excluded publication types: “Review”, “Comment”, “Editorial” and “Letter”.