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Spatial genomics: mapping human steatotic liver disease

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Abstract

Metabolic dysfunction associated steatotic liver disease (MASLD) is a leading cause of chronic liver disease worldwide. MASLD can progress to metabolic dysfunction associated steatohepatitis (MASH) with subsequent liver cirrhosis and hepatocellular carcinoma formation. The advent of recent technologies such as single cell and single nuclei RNA sequencing have transformed our understanding of the liver in homeostasis and disease. The next frontier is to contextualise this single cell information in its native, spatial orientation. This will markedly accelerate discovery science in hepatology, resulting in a further step-change in our knowledge of liver biology and pathobiology. Here we discuss up-to-date knowledge of MASLD development and progression, and how the burgeoning field of spatial genomics is driving exciting new developments in our understanding of human liver disease pathogenesis and therapeutic target identification.

Introduction

As the incidence of viral hepatitis decreases, steatotic liver disease is emerging as the leading cause of chronic liver disease worldwide. Metabolic dysfunction associated steatotic liver disease (MASLD) and metabolic dysfunction associated steatohepatitis (MASH) is the new nomenclature recently proposed for non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) respectively[1]. In both adolescents and adults, MASLD is a rapidly increasing contributor to liver-related mortality and morbidity[2,3]. MASLD is considered the hepatic manifestation of metabolic syndrome, and is commonly associated with obesity, dyslipidaemia, hypertension and diabetes[4], characterized by hepatic lipid deposition (steatosis) in the absence of excessive alcohol use or alternative causes. Although disease activity is dynamic, fluctuating over time, the natural history of MASLD is progression to MASH, cirrhosis and hepatocellular carcinoma (HCC), however the majority of individuals do not progress beyond MASLD.

Lifestyle changes have the potential to reverse steatosis, however steatohepatitis resolution takes substantial and sustained weight loss (>7%)[5], which the majority of patients find difficult to achieve. Furthermore, investigation into the antifibrotic impact of bariatric surgery on patients with advanced liver fibrosis found a persistence of fibrosis for many years[6]. Degree of fibrosis is considered the strongest predictive factor when correlating the progression of MASLD with life-threatening complications, however it must be noted that only a minority of patients will develop cirrhosis[7]. Nonetheless, due to a paucity of therapeutic options available MASH is the most rapidly increasing indication for liver transplantation[8], highlighting MASH as a huge unmet clinical need.

Single cell and single nuclei RNA sequencing (sc/snRNAseq) of healthy human liver [9-11] has deepened our understanding of this organ in homeostasis and provides a baseline from which to compare disease-related perturbations. However, while sc/snRNAseq approaches are transforming our understanding of liver disease pathogenesis, many of the current methods lose important spatial information. Spatial patterns of gene expression manifest at scales ranging from local (cell-cell interactions) to global (tissue zonation/organisation), and inclusion of these data is essential in facilitating a comprehensive understanding of the liver in health and disease. Although many studies in the liver have already harnessed sc/snRNAseq and spatial transcriptomics to delve into the mechanisms underpinning disease pathogenesis (Table 1), given the pace at which these technologies develop and the ever-increasing power of these approaches, there are undoubtedly many important discoveries still to be made in this area. Here we discuss how ongoing development of state-of-the-art spatial transcriptomics, applied in combination with other technologies, will provide valuable new insights into the cellular and molecular mechanisms driving MASLD progression.

Current knowledge of MASLD-MASH pathogenesis

A combination of metabolic, environmental, and genetic factors contribute to the development of MASLD pathogenesis[12,13]. The convergence in the liver of signals from external sources such as visceral adipose tissue and the gut microbiome, in addition to inflammatory cells, constitute a chronic metabolic challenge to hepatocytes[14]. This results in hepatocyte lipotoxicity and subsequent hepatocellular injury or death leading to liver inflammation and fibrogenesis (Fig 1). Despite an increase over the past two decades in our understanding of the mechanisms underlying MASLD progression to MASH, there remain significant gaps in our understanding of this transition, and which events along this pathway constitute critical points for therapeutic targeting.

Preclinical mouse models provide a very useful tool to study mechanisms of disease and novel treatment approaches and there are many transgenic, diet-induced and toxin-induced models available for studying MASH in mice. Nonetheless, none of these models completely recapitulate human MASH pathology, not least due to the differences in chronicity of disease. In humans MASH often develops over the course of 20+ years, whereas mouse and rat models typically run for between 6-24 weeks. For a detailed perspective on the use of mouse MASH models we refer the reader to this excellent review[15].

Changes in liver zonation in response to MASLD

The liver is comprised of repeating functional units called lobules, which are anchored around two key landmarks: the portal triad and the central vein. The portal triad comprises the hepatic artery supplying oxygen-rich blood, the portal vein supplying nutrient-rich blood from the gut, and the bile duct which drains bile from the liver. The central vein drains deoxygenated, nutrient-depleted blood to the systemic circulation (Fig. 2). Hepatocytes arranged across the lobule have distinct metabolic functional roles, and whilst understanding of liver zonation in humans and mice has advanced significantly in recent years[16], our knowledge of how zonation changes in the context of MASLD progression remains limited. In a murine diet-induced MASH model, alteration of liver sinusoidal endothelial cell (LSEC) zonation has been demonstrated[17,18], and in mice and humans age-related liver steatosis also results in disruption of LSEC zonation[19]. In mice, an age-related loss of pericentral LSEC C-kit expression and concomitant increase in the chemokine receptor CXCR4 expression was found to enhance inflammation and fibrosis in a MASH model. Splenic injection of C-kit^{+} LSECs in aged or diet-induced MASH mouse models decreased macrophage numbers and inhibited chemokine and inflammatory signalling pathways, improving overall health[19].

Mesenchymal cells in MASLD

Hepatic stellate cells (HSCs) have been identified as a major source of myofibroblasts in murine liver fibrosis[20] however other liver mesenchymal cells have also been found to contribute. ScRNASeq of murine portal mesenchymal cells has identified a small subpopulation with mesenchymal stem cell features which generate highly proliferative myofibroblasts when activated *in vitro*[21]. This population was further shown to exist in multiple human liver diseases including alcoholic liver disease, chronic hepatitis C, primary biliary cholangitis and MASH, and these cells localized with fibrotic septa. A key marker gene of this population, *Slit2*, is a known profibrotic factor in the liver, implicating this population in driving fibrogenesis. To characterize the molecular mechanisms underlying activation of human HSCs a recent study used scRNAseq from healthy livers, combining this data with published scRNAseq from cirrhotic human livers[22]. Integrative analysis identified potential intercellular signaling axes and master regulators responsible for HSC activation during fibrogenesis. In particular one master regulator, CREB3L1 was shown to be involved in the upregulation of 61 fibrosis-associated genes in HSCs. Ligand-receptor analysis of snRNAseq data from humans with advanced MASH identified multiple HSC autocrine signaling circuits, with over half of these requiring short-range, physical cell-cell proximity[23]. Interrogation of HSC projections in a mouse MASH model using the high-resolution, 3D tissue clearing approach iDISCO, demonstrated a marked and progressive increase in HSC-HSC interactions. One such HSC-specific autocrine signaling circuit conserved between mice and humans was found to be mediated by NTRK3, and knockdown of this *in vitro* inhibited HSC fibrogenicity and migration[23].

Immune response in MASLD

A recent study has provided a comprehensive spatial and proteogenomic atlas of the immune cell landscape in healthy mouse liver[10]. Three distinct populations of macrophages have been shown to reside in the homeostatic liver, Kupffer cells (KCs) being the most prevalent. Also present are capsule/central vein associated macrophages, and a recently described small population of bile duct-associated macrophages, found specifically in close proximity to bile ducts within the portal triad[10,24,25].

Marked changes in immune cell subsets have been shown repeatedly in the context of MASLD and MASH and recent studies have focused on understanding how macrophage heterogeneity alters across MASLD development. KCs are found within liver sinusoids and largely act as filters of the bacterial rich portal blood flow[26]. Profound changes in vascular architecture occur in response to fibrotic liver disease in humans[27], and researchers have recently modelled and investigated these changes in a mouse model of chronic CCl_4-induced liver fibrosis. Using high-resolution intravital

imaging, a massive increase in collagen deposition and collateral vessel growth around sinusoids was shown to lead to displacement and impairment of KC function[28]. In response to this, bone marrow-derived monocytes formed multinucleated syncytia within these collateral vessels which performed bacterial clearance. Macrophage syncytia were also observed in multiple etiologies of human liver cirrhosis[28].

ScRNAseq of non-parenchymal cells from murine MASH liver has identified a population of 'MASH-associated macrophages' marked by high TREM2 expression and which correlate with disease severity in both mice and humans[18]. A similar TREM2$^+$ population has also been demonstrated in human and murine obesity[29], human liver cirrhosis[11] and human cholestatic liver disease[25], and localized to areas of fibrosis in humans and mice[11,25,30]. Systemic soluble TREM2 levels in mice correlate with MASH severity and TREM2 expression is required for effective lipid handling and ECM remodeling[30].

ScRNAseq of MASH mouse liver has also identified a significant increase in XCR1$^+$ conventional dendritic cells (cDCs) arising from MASH-induced proliferation of bone marrow progenitors[31]. Sequencing of interacting cDC-T cell pairs from liver draining lymph nodes demonstrated that cDCs promote T cell reprogramming to an inflammatory phenotype. Targeting XCR1$^+$ cDCs via an anti-XCL1 antibody, or genetic depletion using XCR1-diptheria toxin mice, reduced MASH activity score and markers of liver damage, implicating modulation of this cDC population as a possible future immunotherapeutic target[31].

CD8$^+$ T cells accumulate in the liver in human MASH[32,33], as well as animal models[34] and experimental depletion of these cells in animal models ameliorates MASH development[35], suggesting that CD8 T cells have a direct role in disease progression. ScRNAseq approaches have provided insights into their activation and functional role. Recent work has demonstrated in human MASH and mouse MASH models that gastrointestinal B cells are elevated in number and have enhanced expression of genes related to antigen presentation and B cell-receptor signalling[36]. These activated B cells were demonstrated to promote auto-aggressive T cell activation and exacerbate liver disease in mice. Functionally distinct subsets of CD8$^+$ T cells have been identified using scRNAseq approaches, and one such subset, CXCR6$^+$ T cells, have been demonstrated to accumulate in the liver in established MASH in mice and humans[32]. In early disease these CXCR6$^+$ T cells were shown to be activated by IL-15 and metabolic signals, including short-chain fatty acids and ATP, and subsequently found to initiate MHC1-independent hepatocyte death and subsequent MASH development[32]. Conversely CD69$^+$ CD103$^-$CD8$^+$ tissue resident cells have an important role in

promoting fibrosis resolution[37]. Adoptive transfer of these cells protected against fibrosis progression in a mouse model of MASH, possibly via attraction of HSCs through CCR5 signaling and induction of FasL-Fas mediated HSC apoptosis[37].

Hepatocytes in MASLD

Tight junctions between hepatocytes are essential for epithelial barrier function, and inflammation can lead to their disruption[38]. Claudin-1 (CLDN1) is a member of the tight junction protein family, and although the role of CLDN1 incorporated in tight junctions is well known, the function of nonjunctional CLDN1 (njCLDN1) is much less studied. A recent study demonstrated CLDN1 expression by hepatocytes, cholangiocytes and HSCs, and its expression is localised to the epithelial-stromal interface[39]. Targeting CLDN1 using highly specific monoclonal antibodies (targeting a conformation-dependent epitope of exposed njCLDN1) and *N*-acetylgalactosamine small interfering RNA (which allows specific targeting of hepatocytes) demonstrated that CLDN1 is a mediator and target for liver fibrosis[39]. Targeting CLDN1 reverted inflammation-induced hepatocyte profibrogenic signaling and suppressed the myofibroblast differentiation of hepatic stellate cells.

During MASLD hepatocytes can become 'activated' and directly signal to other cells including HSCs. Hepatocyte Notch signaling is activated in response to chronic liver injury in humans and mice, and is necessary and sufficient for MASH progression in mice[40]. Where understanding is currently lacking is which Notch ligand could be activating signalling, which cell type expresses this ligand, and how this is regulated. Analysis of Notch receptors and ligands in end-of-treatment liver biopsies from human MASH patients participating in a clinical trial demonstrated the Notch ligand, JAG1 to be lower in patients responding to treatment[41]. Further analysis demonstrated liver JAG1 gene expression to track with liver Notch activity and MASH severity in patients. In a mouse MASH model, increased JAG1 expression was mediated by hepatocyte Toll-like receptor 4 (TLR4)–nuclear factor κB (NF-κB) signalling in hepatocytes, and hepatocyte-specific JAG1 overexpression reversed the protection afforded by hepatocyte-specific TLR4 deletion. Conversely, hepatocyte-specific Jag1 knockout mice were protected from MASH-induced liver fibrosis despite steatosis and serum ALT (a marker of hepatocyte injury) levels remaining unchanged[41]. Together these data suggest JAG1 may be the Notch ligand driving MASH progression.

Application of both snRNAseq and snATACseq in mouse and human MASH livers has identified a subset of hepatocytes demarcated by cell-autonomous inflammation and activated downstream of Notch signalling[42]. Ephrin type B receptor 2 (EphB2) was found to be the differential marker of this population. Hepatocyte-specific reduction of EphB2 reduced levels of fibrosis and inflammation in a

224 mouse MASH model but had no effect on liver steatosis or serum ALT, similar to JAG1 knockdown.
225 Given the long-standing link between MASH and Notch signalling[40] and the identification of these
226 Notch-linked, JAG1 and EphB2 hepatocyte subpopulations, further exploration of their origin and
227 pro-fibrogenic functions may provide valuable insights and novel therapeutic approaches for MASH.
228
229 Cellular senescence, the state of sustained cell cycle arrest, has been proposed as a potential driver of
230 MASLD progression to MASH[43]. Although many studies have shown correlations between markers
231 of senescence and MASLD, detailed mechanistic studies are required to fully understand the role of
232 this cell state in MASH pathogenesis. One study which demonstrated a strong association between
233 hepatocyte senescence and MASH severity in humans, subsequently used machine learning to
234 identify senescence markers[44]. Hepatocyte Gremlin-1 and BMP4 gene expression were found to be
235 most strongly associated with MASLD and MASH, and further investigation demonstrated that
236 BMP4 decreases cellular senescence and is anti-steatotic, anti-fibrotic and anti-inflammatory,
237 whereas Gremlin-1 acts as a brake on these beneficial effects. However senescence may be beneficial
238 in some liver cell types, such as HSCs, by reducing ECM deposition, as shown in a CCl_4-induced
239 murine liver fibrosis model[45]. A recent study also investigated senescence in the aging rodent liver,
240 identifying genes (*Glipr1, Clec12a and Phlda3)* enriched in the liver of aged mice. These genes were
241 also shown to increase in a stress-responsive manner in young mice treated with doxorubicin, an
242 anticancer drug commonly used to induce premature senescence[46]. Further studies are required to
243 fully understand the complex role of senescence across different cell lineages during MASH
244 progression.
245
246 Hepatocyte lipid loading due to an oversupply of saturated fatty acids and enhanced *de novo*
247 lipogenesis results in the generation of lipotoxic species which induce a cascade of events leading to
248 hepatocyte death. Molecules released from dying cells, called damage-associated molecular patterns
249 (DAMPs), are known to act as cell messengers and can modulate a wide variety of cellular
250 responses[47]. Using both bulk RNA sequencing and scRNAseq approaches Mederacke *et al*. found
251 mouse HSCs to highly express multiple DAMP receptors. Then, using a combination of proteomics
252 and ligand-receptor analysis of scRNAseq data, UDP-glucose, UDP-galactose and their ligand P2Y14
253 were uncovered as the ligand-receptor pair most strongly linking dying hepatocytes with HSC
254 activation[48]. In mice, HSC-targeted deletion of P2Y14 led to a substantial reduction in liver fibrosis
255 in five different liver injury models.
256
257 Lipotoxic hepatocytes may also have a direct role in mediating the immune response in MASLD
258 caused by replication stress subsequent to nucleotide pool imbalance[49]. Recent work by Donne *et al.*

in two mouse MASH models demonstrated replicating hepatocytes to have an imbalanced nucleotide pool. This hepatocyte nucleotide imbalance led to DNA damage which was subsequently found to activate the cGAS/STING pathway, an important mediator of the intrinsic immune response. Nucleotide pathway deregulation and cGAS/STING gene activation was additionally demonstrated in liver tissue from MASLD patients[49]. The cGAS/STING pathway has been presumed to have a tumour suppressor functional role[50], however growing evidence suggests it can also drive inflammation-mediated tumourigenesis[51]. To dissect transcriptional mechanisms regulated by lipotoxicity in hepatocytes, Perez-Schindler *et al.* examined combined transcriptomic, proteomic and chromatin accessibility data from human MASH biopsies and lipotoxic mouse hepatocytes, revealing several networks that were similarly dysregulated[52]. Independent deletion *in vitro* in lipotoxic mouse hepatocytes of two of the identified transcription factors, MAFK or TCF4, prevented the induction of genes linked with cell death and stress.

Hepatocyte death via apoptosis and necroptosis is a strong driver of MASH pathogenesis[53]. Necroptotic hepatocytes in mouse and human MASH liver have been shown to accumulate expression of CD47, an anti-phagocytic ligand[54]. This was also accompanied by an increase in macrophage SIRPα (CD47 receptor) expression, consistent with impaired clearance of necroptotic hepatocytes. Blockade of the CD47-SIRPα interaction in two mouse MASH models using either anti-CD47, anti-SIRPα, or AAV8-H1-shCD47 increased necroptotic hepatocyte uptake by macrophages, and decreased markers of HSC activation and fibrosis[54]. The mechanisms regulating cell death are difficult to study using sc/snRNAseq due to the RNA disruption associated with cell death[55]. Given the link between MASH pathogenesis and hepatocyte death[53], utilization of spatial transcriptomics to study these mechanisms in more detail, *in situ*, should yield valuable insights into the cellular niches and signaling pathways surrounding dying hepatocytes.

Using spatial transcriptomics to decode MASLD

High-resolution approaches such as sc/snRNAseq have provided paradigm shifts in our understanding of the cellular and molecular mechanisms regulating MASH. These gene-level insights into novel cellular subpopulations are often validated downstream using immunostaining (with sc/snRNAseq-derived targets) or *in situ* hybridisation of healthy and diseased tissue. These approaches are time consuming, often requiring optimisation of antibodies, and are limited in their scope. This multi-step methodological approach is now being short-circuited by the rapid development of spatial transcriptomics (ST) technologies which enable visualisation of the *in situ* cellular transcriptome within tissue sections. ST provides a singular platform from which to answer

multiple research questions for example: 1) what is the complex spatial relationship of multiple different cell types and subpopulations with each other and within the tissue environment? 2) what ligand-receptor interactions are present between these cell types? 3) how do these cellular and molecular elements spatially combine to drive organ fibrogenesis?

Understanding the spatial arrangement of the cellular niche and the key cell-cell interactions is paramount to understanding how fibrosis progresses. The optimal ST approach would potentially generate data regarding every gene and gene isoform, at subcellular resolution, in a whole tissue sample in three dimensions, however as yet no single ST technology has this ability. Compromises are required for each current approach in terms of resolution, sensitivity, ease of use or throughput, and no technology is able to provide 3D resolution (Table 2). Detailed evaluation of the various spatial technologies currently in development is outwith the scope of this review, however readers are referred to these excellent reviews for further information[56-58].

Spatial transcriptomics approaches

Broadly there are two approaches to spatial transcriptomics (Table 2). Building on *in situ* hybridisation methods, high-plex RNA imaging approaches (such as single molecule fluorescence *in situ* hybridisation or direct *in situ* sequencing) localise hundreds to thousands of genes in intact tissue by specifically tagging individual mRNA transcripts with fluorescent probes using hybridisation directly in tissues (imaging-based ST) (Fig. 3a). The probes are then imaged by fluorescence microscopy, and using image analysis methods are associated with a cell nucleus and spatial gene expression is analysed. Fluorescence-based RNA detection methods have been used for many years, however the number of detectable fluorescent molecules has been restricted due to spectral bandwidth limitations. Recent advances in imaging-based ST has overcome this by using multiple sequential imaging rounds and combinatorial strategies for detection of an ever increasing number of transcripts, and the limits of this approach are constantly being pushed. Robust downstream analysis of imaging-based ST depends on accurate segmentation of cell membranes and structures. This has proven hard to automate largely due to factors such as cell density (immune infiltrate, solid tumours) or complex cell shapes (endothelia, neurons) however utilisation of deep learning frameworks has facilitated significant advances in the creation of generalisable tools for cell segmentation[59,60].

An alternative approach is sequencing-based ST (Fig. 3b). A slide surface is coated with barcoded DNA primers that are each uniquely marked, enabling downstream mapping *in silico* at the analysis stage. Tissue sections are placed over this barcoded area and mRNA is then captured either by diffusion of RNA from the tissue to slide, or diffusion of barcoded primers into the tissue. The

captured mRNA is then removed from the slide and sequenced. The gene expression information can then be mapped back onto the tissue using the barcoded primers for spatial visualisation of each transcript. Capture spot resolution continues to improve, reaching a resolution smaller than the diameter of a typical single cell, improving the likelihood that only a single cell is contributing to the gene readout. However, the number of unique RNAs captured continues to be a limiting factor for sequencing-based ST. This can be overcome by using imaging-based ST but this approach is currently limited by transcriptome coverage. However methods are improving and up to 1000 genes can now be visualised.

Integration methods have been developed and are broadly grouped into mapping and deconvolution. Mapping approaches seek to determine where sc/snRNAseq assigned cell populations reside within regions or niches in the ST data. Deconvolution estimates the proportion of different cell types contained within a mixed mRNA capture spot[61-63] and is mainly used for sequencing-based ST which are currently often not at single cell resolution. These utilise external information from gene enrichment strategies or sc/snRNAseq to deconvolve the sequenced spots, and from there downstream analyses can be performed.

Homing in on disease-associated cell types and interactions

Previous sc/snRNAseq studies have demonstrated the importance of cellular cross-talk in driving MASLD progression[11,23,64], however these data lose the spatial relationships of cells and other approaches (such as immunostaining) only allow for a limited analysis of pre-determined interactions at any one time. Analysis approaches such as CellPhoneDB[65], NicheNet[66] and CellChat[67] have been developed to interrogate ligand-receptor interactions within sc/snRNAseq data, generating a multitude of interactions and hypotheses. However these putative interactions must be carefully validated and interrogated via approaches such as immunostaining and *in vivo* and *in vitro* analyses[68]. Further functional validation approaches include imaging of live tissue slices or intravital microscopy. Recent work utilising IVM has demonstrated progressive remodelling of the liver vasculature during fibrogenesis, with KC maladaptation within an altered fibrotic niche rescued by monocytes forming KC-like syncytia to capture bacteria[28]. Another recent study used snRNAseq to identify a novel ANXA2$^+$ migratory hepatocyte subpopulation which emerges during human liver regeneration, and a corollary migratory hepatocyte subpopulation was also found in APAP-induced mouse liver injury[69]. 4-D intravital imaging identified motile hepatocytes at the edge of the necrotic area in APAP-induced mouse liver injury, enabling collective migration of the hepatocyte sheet to effect wound closure. Lastly, live imaging of murine precision-cut lung slices demonstrated the

requirement of EGR2, a molecule identified using scRNAseq, in the differentiation of alveolar macrophages and their function in tissue repair[70].

Applied to scRNAseq of human chronic liver disease, CellPhoneDB has been used to interrogate interactions between scar-associated cell populations, identifying TNFRSF12A, PDGFRA and Notch signalling as key regulators of mesenchymal cell function within the fibrotic niche[11]. This study also identified a population of TREM2^{+}/CD9^{+} scar-associated macrophages that have since been further resolved in other fibrotic tissues[71] and share some marker expression with 'lipid-associated' and 'MASH-associated' macrophages[10,18,29,72]. Guilliams *et al.* applied NicheNet to a mouse MASH model to understand the role of 'lipid-associated macrophages' and found limited ligand-receptor interactions for these cells, suggesting that metabolites rather than cell-cell interactions may be driving this cellular phenotype[10]. However, given the overlap of gene expression for these scar-associated macrophages across studies and the proposed combination of multiple markers to confirm their identity[71], unbiased and rich single-cell resolution ST data should provide further insights into the phenotype of these macrophage subpopulations in different tissues and biological contexts.

For example, a recent study in human high-grade serous ovarian cancer (HGSC) integrated ST with scRNAseq identifying spatially resolved cancer associated fibroblasts (CAF)-associated biomarkers with potential prognostic significance[51]. The authors developed a method to investigate region-specific ligand–receptor interactions between HGSC and neighboring CAF subregions, identifying the ligand–receptor pair, APOE-LRP5, in a subtype of CAFs and their neighboring HGSC cells with prognostic significance. Following myocardial infarction (MI), pathogenic remodelling of the left ventricle can occur, potentially resulting in high morbidity and mortality. To shed light on the cellular and molecular mechanisms driving this remodelling process researchers have combined scRNAseq, sequencing-based ST and epigenomic analyses of human cardiac tissue, generating an integrated spatial multi-omic map[73]. Changing cell states and gene-regulatory networks of cardiomyocytes, endothelia, myeloid cells and fibroblasts during disease progression were identified. By clustering spots based on gene expression, distinct inflammatory and fibrotic molecular niches were identified with a macrophage *SPP1*$^{+}$ subset being predictive of myofibroblast co-residence. Ligand-receptor analysis of these two populations revealed complex cellular crosstalk that was validated in human post-MI tissues and found to be enriched in the ischaemic zone, pointing towards a clear spatial association of this myeloid subset and myofibroblasts. Further investigation of the signalling pathways identified will potentially reveal therapeutic targets to modulate post-MI cardiac tissue remodelling. To understand the cell types enriched within the fibrotic regions of human end-stage cirrhotic livers sequencing-based ST has been combined with previously published scRNAseq data[74].

Fibrotic and cirrhotic tissue regions were identified, and through analysis of gene content, the frequency of cell types distributed between these two regions was investigated.

However the MASLD disease process is not uniform across the liver, with some areas more actively fibrogenic than others[11,71]. Further, given MASLD to MASH progression is not a linear process with disease activity waxing and waning, harnessing ST across the spectrum of disease will be key to help further elucidate the mechanisms regulating disease progression. Firstly identification and interrogation of disease-associated cellular niches, key regions where pro-regenerative or pro-fibrogenic activity is taking place, and subsequent cell and molecular level interrogation of these regions is essential. Sequencing-based ST has recently been deployed to investigate the changes in gene expression across tissue regions in human acetaminophen (APAP)-induced acute liver injury[69]. Drawing analysis trajectories between the remnant viable and necrotic regions of the tissue facilitated identification of an interface peri-necrotic region which displayed molecular signatures of proliferation, cellular differentiation and methionine salvage (involved in reactive oxygen species scavenging). This was distinct from the necrotic region where signatures for collagen organisation, integrin signalling and T-cell differentiation dominated.

Spatial interrogation of the epithelial response in MASLD

As outlined earlier, hepatocyte lipotoxicity is regarded as being the initiating factor in MASLD, initiating a cascade of events which shape disease progression (Fig 1). *In situ* spatial profiling of hepatocyte molecular changes as lipid loading occurs, and the key cell types and ligand-receptor interactions in the niche surrounding these hepatocytes, will deepen our understanding of the pathogenesis of MASLD. Sequencing-based ST has recently been used to determine spatial gene expression in healthy human liver[69,75,76]. Using sequencing-based ST one study compared zonation in healthy human liver versus APAP-injured human liver demonstrating plasticity of function in the remnant hepatocytes in APAP-injured human liver[69]. Further interrogation of this using both imaging-based ST and immunostaining demonstrated that some hepatocytes in the remnant viable region express both portal and central-associated genes. This suggests that the liver compensates for the loss of peri-central hepatocytes following APAP-induced liver injury by altering the functional phenotype of the surviving hepatocytes. Similar analyses of hepatocytes across the various stages of MASLD progression would provide much needed granular information regarding the key molecular processes occurring in hepatocytes during MASLD pathogenesis. These types of studies would also help to shed light on how the various hepatocyte subpopulations present in MASLD interact with non-parenchymal cell populations, such as the liver mesenchyme, to drive fibrogenesis.

Spatial profiling of microbiota in MASLD

Animal studies have demonstrated a potential causal role of the gut microbiota in MASLD pathogenesis and there is increasing evidence in human-based studies that the gut microbiome may represent a driver of human MASLD pathophysiology[77].

Although there is a wealth of data on dysbiosis in MASLD there is no current evidence demonstrating a direct causal link and it is not clear if the dysbiosis precedes MASLD development or results from it[78]. Proposed hypotheses as to how the gut microbiota might contribute to MASLD include increased intestinal permeability and the action of microbially produced metabolites[79]. Further, bacterial translocation from the gut to the liver has been found to occur in human autoimmune hepatitis, primary sclerosing cholangitis and liver cirrhosis[80-82]. Investigation of host-microbiota responses in MASLD using ST will help further our understanding of the microbial contribution to the disease pathogenesis by interrogating the spatial distribution of microbes within tissue and the keys cells they interact with.

For example, sequencing-based ST has been used to determine the identity and location of intratumoral microbial communities within tissues from patients with oral squamous cell carcinoma and colorectal cancer[83]. Higher resolution imaging-based ST was used to compare microbial-positive regions to microbial-negative regions. The microbial-positive niches were found to be less vascularised, highly immuno-suppressive and spatially associated with malignant cells. Development of a scRNAseq method incorporating a primer specific for bacterial 16S ribosomal RNA, allowed the researchers to interrogate cell-associated bacteria and the host cells they interact with, revealing that bacteria-infected cancer cells are more migratory and recruit more immune cells. In oral squamous cell carcinoma, bacterial cellular load positively correlated with the neutrophil chemoattractant CXCL8 and negatively with the T cell receptor CD3E. Application of these types of approaches will hopefully rapidly accelerate our understanding of the key tissue-microbial niches involved in MASLD pathogenesis.

Probing the liver cancer microenvironment

Previous studies have shown that genetic factors play an important role in MASLD development and progression to MASH[84] and genome-wide association studies (GWAS) have identified gene variants that are related to MASLD risk[85,86]. Genome-wide copy number variations (CNVs) have previously been inferred from gene expression in single cells, successfully identifying regions of chromosomal gain and loss[87-89], and this approach has now been applied spatially[90]. Sequencing-based ST was used to infer CNVs in human prostate cancer samples and enabled the identification of small clonal units

468 not evident morphologically, and provided further granularity on the clonal patterns present within
469 tumours[90]. Although not analysed in this study, the spatial identification and mapping of clonal niches
470 would further facilitate understanding of the environmental effects and cellular crosstalk leading to
471 the generation of these clones and their potential phenotypic switch from benign to malignant cells.
472 A deeper understanding of this in the context of MASLD and other forms of chronic liver disease
473 will be crucial in discovering novel therapeutic targets for HCC.

474

475 The MASLD liver is a dynamic inflammatory environment and how inflammation, aberrant
476 metabolism and proliferation interact to contribute to DNA instability and HCC development is still
477 poorly understood. Monotherapeutic approaches to HCC treatment such as immune checkpoint
478 inhibitors have modest clinical efficacy and may be more effective in combination with other
479 therapeutic agents, however a significant proportion of patients still fail to respond. Biomarkers and
480 ways to predict patient response in order to determine the most beneficial treatments for patients are
481 lacking. ST has been used to profile the tumour microenvironment of HCC patients undergoing a
482 clinical trial using the combination treatment cabozantinib and nivolumab, where 5 out of 15 patients
483 had a response to treatment[91]. Sequencing-based ST uncovered differences in the tumour
484 microenvironment between the treatment responders and non-responders, highlighting B cell-rich and
485 CAF-rich areas in the responders. One responder experienced early HCC recurrence and analysis
486 showed this tumour to have a distinct immune cell-poor region resembling the non-responder
487 microenvironment.

488

489 This study highlights the need for more detailed spatial analyses of the tumour microenvironment in
490 HCC patients, and combining these datasets with sc/snRNAseq data will provide further orthogonal
491 information from which to identify the key pathogenic processes involved. This approach was taken
492 in a recent study in pancreatic ductal adenocarcinoma (PDAC), a highly aggressive and treatment
493 refractory cancer[92]. The authors combined snRNAseq and imaging-based ST on matched specimens
494 from treated or treatment-naïve patients, mapping the snRNAseq-identified malignant programs,
495 CAF programs and immune cell composition onto tissue. Using ligand-receptor analysis, spatially-
496 defined interactions between multicellular communities and between specific cell types were
497 identified that were differentially correlated by treatment status[92]. In a study of human squamous cell
498 carcinoma, an immunosuppressive tumour-specific keratinocyte subpopulation was localised to a
499 fibrovascular niche at the tumour border[93]. This population expressed genes associated with
500 immunotherapy resistance and numerous ligands inferred to modulate cancer-associated fibroblasts,
501 suggesting potential ways in which tumour subpopulations may promote local immunosuppression.
502 Studies in other cancer types have also leveraged this combination of approaches[94,95].

Lung cancer cells commonly metastasise to the brain and recent work has spatially profiled matched primary and metastasized non-small cell lung carcinoma patient samples[96]. This study identified extensive remodelling occurring within the brain tumour microenvironment, creating an immunosuppressive and fibrogenic niche for the metastasising cells. Therefore, in addition to understanding the tumour microenvironment of HCC, ST will also allow for investigation of liver metastatic niches. Resection of colorectal cancer (CRC) metastases in the liver is one of the most common indications for liver surgery. Approximately 50% of CRC patients develop liver metastases during their disease course[97] and surgical resection is the only treatment that offers a chance of cure and long-term survival. The impact of MASLD on colorectal liver metastasis remains unclear with some studies suggesting that MASLD reduces the risk of colorectal liver metastases, while others suggest the opposite[98]. Given the 'seed-soil' hypothesis for cancer metastasis and the historical focus on the metastatic cell (being the seed), ST would greatly aid in the investigation of the 'soil' or liver microenvironment and the cues and signals arising from this which aid in the 'seeding' of CRC metastases in the liver.

As discussed above, application of ST in multiple disease settings has provided further granularity and rich information regarding disease pathogenesis, and importantly data derived from cells in their native, *in situ* state. In many cases these data, with further validation and functional interrogation, have the potential for translatable impact in the clinical setting[91]. As such, the use of ST approaches to decode the cellular and molecular processes driving steatosis to MASH to cirrhosis and HCC development will allow us to home in on the key cellular pathways and targets driving disease progression. ST in combination with other approaches will provide rich substrate from which to identify and develop novel efficacious therapies for patients with liver disease.

Multimodal spatial profiling in MASLD

Immunostaining has been a valuable tool for validating populations and cell states uncovered using sc/snRNAseq. With the possibility of combining high-plex immunostaining with spatial transcriptomics on the same tissue[10,99], comprehensive analyses can be undertaken on cell composition and spatial arrangement in tissues in health and disease.

Spatial transcriptomics technologies are advancing at spectacular pace. Gene profiling depth and spatial resolution are continually increasing and an ever-expanding suite of commercial instruments are becoming available thereby opening this technology up to the whole scientific community. Current ST approaches tend to use thin 2D tissue slices and while highly informative, there is loss of

information outside the sectioning plane which is critical for a detailed understanding of tissue niches. This issue has been somewhat circumvented via the production of serial thin sections and computational methods for data realignment and 3D reproduction[100], however it is expensive in terms of reagents and processing time. Increasingly powerful ST approaches combining comprehensive gene coverage with full 3D reconstructions will no doubt be developed in the coming years.

Emerging spatial technologies

In MASH, the collaborative and integrative analysis of ST data with data generated from other technologies will yield a wealth of rich information on disease mechanisms (Box 1). Current spatial profiling technologies are being extended to include simultaneous protein and mRNA quantification. The barcoding technologies developed for scRNAseq have been used to develop highly multiplexed protein imaging, where antibodies are conjugated to oligonucleotides and subsequently sequenced[101,102]. Although not yet widely applied, these approaches will allow visualisation and localisation of hundreds of proteins in tissue and enable co-mapping with transcriptomic information. The ability to spatially co-register protein, histological, and RNA data together into a single image has been used to identify new biomarkers of human breast tumour subtypes, enhancing differential gene expression analysis across tissue regions[99,103]. Further, high-plex protein and whole transcriptome co-mapping has been used to reveal early immune activation in response to COVID-19 vaccination in humans[104]. In the context of MASH pathogenesis, protein and transcriptome co-mapping should provide rich and highly detailed information on the various cell states and phenotypes regulating disease progression, and in time may also yield important information which could facilitate disease diagnosis, stratification and prognostication in the clinical setting.

Spatial profiling of genomic and epigenomic measurements is increasingly being used to provide high resolution data on genome organisation in healthy and diseased tissue. Spatial information of epigenetic modifications is critical to understanding how the epigenome controls cell states and shapes the development of cell types in the native context of complex tissues and the imaging-based ST approaches, MERFISH and seqFISH+, have been used to explore the relationships between nuclear organisation and cell states[105-107]. In cancer, spatial analysis of open chromatin and histone modifications in tissue have enabled the discovery of distinct tumour clones and their inherent copy number variations associated with clone-specific genetic aberrations and the local tumour microenvironment[108]. Extending this, two technologies for spatially resolved, genome-wide, joint profiling of the epigenome and transcriptome have been published[109]. Co-sequencing of chromatin accessibility and transcriptome, or histone modifications and transcriptome, was applied to the adult human brain on the same tissue section at near-single cell resolution, uncovering details as to the

573 epigenetic mechanisms controlling transcriptional phenotypes and cell dynamics. A current limitation
574 of most sc/snRNAseq and ST approaches is their ability to only retrieve single-ended polyadenylated
575 mRNA, rather than full-length mRNAs. This hinders alternative splicing analysis and prohibits
576 detection of many long non-coding, short non-coding and non-polyadenylated protein-coding
577 transcripts, features that have been linked with liver disease[110,111]. A recently developed method, vast
578 transcriptome analysis of single cells by dA-tailing (VASAseq) provides high-sensitivity, full-length
579 transcriptome coverage at the single cell level, and is further adapted for use on high-throughput
580 droplet-based platforms[112]. However, an important consideration regarding this method however is
581 the high cell input number required for this workflow.

582

583 Current sc/snRNAseq methods allow a snapshot in time of the transcriptional activity of cells. Given
584 the dynamic nature of disease progression, as demonstrated by intravital liver microscopy in mice[28,69],
585 exploration of the transcriptome at the single cell level *in vivo* would provide important functional
586 information. Recent work has built on earlier research which aimed to study transcriptome dynamics
587 in live cells *in vitro*. Pioneering work used a transcriptome *in vivo* analysis (TIVA) tag which, once
588 photoactivated, enabled mRNA capture from single cells in live tissue[113]. Similarly, ZipSeq uses
589 patterned illumination and photocaged oligonucleotides to serially print 'zipcodes' onto live cells in
590 intact tissues, in real time[114]. Using a different approach, the cytoplasm of live cells has been biopsied
591 (Live-seq) enabling further downstream molecular or functional analyses on the same cell, however
592 this method is limited to *in vitro* experiments[115].

593

594 *Integrative analysis of ST with other technologies*

595 Analysis of the metabolic changes occurring during MASLD development have been largely limited
596 to the systemic level and little is known regarding the changes occurring at the hepatic cellular level,
597 largely due to technological limitations. Changes in liver metabolism have a role in compensating
598 for, or exacerbating, MASLD disease progression and could be a source of therapeutic targets. Mass
599 spectrometry imaging (MSI) and in particular, matrix-assisted laser desorption-ionization time of
600 flight mass spectrometry (MALDI-)MSI has been used to help understand tissues at the proteomic,
601 lipidomic and metabolomic levels[116]. MALDI-MSI is a label-free technique that produces 2D ion
602 density maps of a tissue section and allows the determination of the spatial distribution of hundreds
603 of particles within a single imaging run, with no *a priori* knowledge required. MALDI-MSI has been
604 used to study the zonation of lipids in mouse MASH models, which demonstrated complete loss of
605 zonation in MASH[117]. Further analysis revealed that increased expression of the LPCAT2 enzyme in
606 the pericentral region may be linked to increased oxidative damage in this area. The application of
607 MALDI-MSI to *in vitro* cultures of a hepatocyte cell line stimulated with fatty acids has shown a

divergence in their response, outlined by distinct metabolic states[118]. Although this technology has been applied to biological and clinical samples widespread adoption of this approach is currently limited by low-throughput, custom instrumentation requirements and lack of easily accessible computational methods for data analysis. However the integration of ST and MALDI-MSI could be a powerful approach for deciphering the metabolic changes regulating MASH progression.

The emerging technology of artificial intelligence (AI), including machine learning and artificial neural networks, has arisen in response to the generation of highly complex data and is increasingly at the core of biomedical research, paving the way for significant breakthroughs, enhancing drug discovery strategies and facilitating clinical practice. For example, combining label-free collagen microscopy with AI approaches has allowed the standardised evaluation of MASH features in patients enrolled in a clinical trial, and showed greater sensitivity than conventional scoring[119]. Furthermore, combining machine-learning based histopathology data with bulk RNAseq in humans, a 5-gene expression signature was shown to correlate with risk of MASH progression and highlighted the role of Notch signalling[120]. An AI-based platform, FibroNest, has been used to comprehensively annotate fibrosis in a mouse MASH model providing statistical analysis of collagen morphometry and architecture[23]. This technology has also been applied to human MASLD where collagen morphometric information was used to predict the development of cirrhosis and HCC[121]. A deep learning algorithm, ST-Net, combines ST with histology images and has been used to predict spatial variation in gene expression across tissue and was able to capture intra-tumour heterogeneity in human breast cancer[122]. Another approach, XFuse, combines spatial barcoding and histology sections to predict gene expression at single cell resolution[123]. Application of these approaches, i.e. combining AI-based interrogation of immunostained images with integrated spatial transcriptomic data, across the MASH spectrum should greatly increase our understanding of disease pathogenesis.

Furthermore, integration of powerful AI-based histology analysis with multimodal spatial profiling and rich clinical metadata will very likely drive a new era in liver disease diagnostics, stratification and prognostication. This should also in turn increase the precision of clinical trial design, which will hopefully accelerate the discovery of potent new therapies for patients.

Conclusions

In order to fully understand the complexity of disease a combination of approaches is essential, as no one methodology will capture all the relevant information. While sc/snRNAseq and ST provide information regarding the transcriptomic changes occurring in cells and tissue, their integration with other technologies such as genomics, epigenomics, proteomics, and metabolomics, will in time

643 provide a rich and highly comprehensive view of MASH progression. This will greatly facilitate the
644 discovery of relevant and precise therapeutic targets. However analysis of these multimodal datasets
645 requires sophisticated integration methods and integration of disparate data types (including patient
646 metadata), and this is currently a major area of focus in the field[124].

647

648 There are inherent challenges in studying human disease with multi-omics approaches. Variability in
649 access to and quality of tissue procurement and fixation, coupled with patient heterogeneity driven
650 by multiple variables such as age, sex, ethnicity and co-morbidities are just some of the reasons why
651 it is important to include a sufficiently high number of samples to allow generation of impactful data.
652 Furthermore, procurement of early-stage diseased tissue to allow investigation of changes occurring
653 during disease progression is challenging, with many diseased human liver samples only becoming
654 accessible at the time of explant, when the disease under study is end-stage. Very careful
655 consideration should always be given to the main research question, as this dictates selection of the
656 optimal single cell genomics or spatial profiling approach. From a logistical perspective, the ability
657 to perform snRNAseq on frozen tissue has now circumvented the requirement for fresh tissue,
658 although for some cell types (eg specific populations of leukocytes) scRNAseq is still preferred to
659 generate optimal data. Finally, initial ST technologies required fresh-frozen tissue which can be
660 challenging to source, however newer approaches utilise formalin-fixed paraffin embedded (FFPE)
661 samples (with comparable quality of data to frozen tissue), enabling the use of the vast amounts of
662 FFPE tissue stored in biobanks around the world.

663

664 MASLD is a complex, dynamic disease whose steatohepatitic and fibrotic activity waxes and wanes,
665 in response to a variety of genetic, epigenetic, and environmental modifiers. The use of sc/snRNAseq
666 has markedly accelerated our understanding of the cell types and states present in MASLD, and this
667 in combination with cutting-edge ST approaches will further deepen our understanding of the key
668 cellular and molecular mechanisms regulating MASLD pathogenesis. ST now adds a further step-
669 change in our ability to interrogate healthy and diseased human liver, importantly allowing the
670 investigation of the key pathogenic processes *in situ*, without the need to disrupt tissue into single
671 cells. The ability to interrogate these processes in their spatial context and the subsequent
672 development of integration approaches to combine this spatial resolution with other powerful
673 readouts available (sc/snRNAseq, epigenomics, proteomics, and metabolomics) will allow us to
674 understand, characterise and classify the various stages and subtypes of MASLD at levels that were
675 previously unimaginable.

676

Figure legends

Figure 1: Schematic broadly outlining the micro-architectural changes occurring in the liver during MASLD pathogenesis.

Figure 2: Schematic overview of the liver lobule (upper) and the hepatic sinusoid (lower). PV, portal vein; CV, central vein; HA, hepatic artery; HSC, hepatic stellate cell; LSEC, liver sinusoidal endothelia cell.

Figure 3: Schematic overview of the main spatial transcriptomic approaches currently used. A) Imaging-based ST localises a few to thousands of genes in intact tissue, by specifically tagging individual mRNA transcripts with fluorescent probes. Fluorescent probes are then imaged and deconvolved B) In sequencing-based ST, tissue is applied to a slide surface coated with barcoded DNA primers each uniquely marked, enabling downstream mapping *in silico* at the analysis stage.

Box 1: Emerging spatial technologies in MASLD. Enhancement of existing technologies to improve cell resolution, segmentation and gene profiling depth. Extension of existing technologies to include the separate or simultaneous analysis of DNA, epigenome and protein. Integrative analysis of ST with AI and MALDI-MSI approaches.

Table 1: Research utilising scRNAseq, snRNAseq, ST or a combination to study human liver biology. HCC-hepatocellular carcinoma; MASLD-metabolic dysfunction -associated steatotic liver disease; MASH-metabolic dysfunction associated steatohepatitis; ALD-alcoholic liver disease; PBC-primary biliary cholangitis; PSC-primary sclerosing cholangitis; NAS-; APAP-ALF-acetaminophen-induced acute liver failure; NAE-ALF-non-hepatitis A-E acute liver failure; HBV-Hepatitis B virus.

Table 2: Summary of the currently available spatial transcriptomic technologies.

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710 Table 1.

Publication (ordered by year)	Methodologies	Tissue	Reference
Camp et al. 2017	scRNA-Seq	Foetal (10.5 and 17.5 weeks gestation) and adult (n=3) healthy liver	[125]
Zheng et al. 2017	scRNA-Seq	HCC tissue (n=6)	[126]
MacParland et al. 2018	scRNA-Seq	Healthy livers (n=5)	[9]
Aizarani et al. 2019	scRNA-Seq	Healthy livers (n=9)	[127]
Ho et al. 2019	scRNA-Seq	HCC tissue (n=1)	[128]
Ramachandran et al. 2019	scRNA-Seq	Healthy (n=5) and cirrhotic (n=2 MASLD, n=2 ALD, n=1 PBC) livers	[11]
Cavalli et al. 2020	snRNA-Seq	Healthy liver (n=1)	[129]
Massalha et al. 2020	scRNA-Seq	Cholangiocarcinoma (n=2), colorectal liver metastases (n=3), benign liver cyst (n=1)	[130]
Zhao et al. 2020	scRNA-Seq	Healthy liver (n=3)	[131]
Diamanti et al. 2021	snRNA-Seq	Healthy liver (n=1)	[132]
Dudek et al. 2021	scRNA-Seq	Intrahepatic $CD3^+$ T cells from patients with steatosis (n=4) or MASH (NAS $\geq$ 5) (n=3)	[32]
Hou et al. 2021	scRNA-Seq ST	Embryonic livers (n=2: 8 and 17 post-conception weeks)	[133]
Payen et al. 2021	scRNA-Seq	Healthy livers (n=2)	[134]
Wang et al. 2021	scRNA-Seq	Healthy liver (n=6) compared with previous fibrotic datasets.	[22]
Wu et al. 2021	ST	21 tissue specimens from 7 patients (n=5 HCC, n=1 intrahepatic cholangiocarcinoma, n=1 combined HCC/ cholangiocarcinoma), tissues spanning normal/edge/tumour	[135]
Andrews et al. 2022	scRNA-Seq snRNA-Seq ST	Healthy livers (n=4)	[136]
Chung et al. 2022	scRNA-Seq ST	End-stage cirrhotic livers (n=4 PSC, n=2 PBC, n=2 ALD)	[74]
Filliol et al. 2022	snRNA-Seq	HCC (n=2) and adjacent cirrhotic (n=2), healthy (n=2), MASLD cirrhotic (n=2), and data for n=2 additional healthy[136]	[137]
Guilliams et al. 2022	scRNA-Seq snRNA-Seq CITE-Seq	Healthy (n=14), >10% steatosis with no fibrosis (n=5)	[10]
Meng et al. 2022	scRNA-Seq	Liver cancer tissues (n=15 patients)	[138]
Wen et al. 2022	scRNA-Seq snRNA-Seq	HCC tissue (n=1)	[139]
Ye et al. 2022	scRNA-Seq ST	n=8 Control (4 choledochal cyst resection, 4 post chemotherapy with hepatoblastoma), n=6 biliary atresia infant livers (scRNA-Seq on all, ST on one of each)	[140]
Yu et al. 2022	ST	Healthy livers (n=2)	[76]
Zhang et al. 2022	scRNA-Seq	Healthy (n=2), cirrhotic (n=3), acute-on-chronic liver failure (n=5) livers	[141]
Andrews et al. 2023	scRNA-Seq snRNA-Seq ST	Healthy (n=24) and PSC (n=5) livers	[75]
Kotsiliti et al. 2023	scRNA-Seq	$CD45^+$ cells healthy and cirrhotic MASH livers (n=1 healthy, n=1 cirrhotic, also previous datasets.	[36]
Li et al. 2023	scRNA-Seq ST	Healthy (n=1), MASLD (n=1) and MASH (n=2) livers	[142]
Matchett et al. 2023	snRNA-Seq ST	Healthy (n=9), APAP-ALF (n=10), and NAE-ALF (n=12)	[69]
Starlinger et al. 2023	ST	Liver resection tissue; n=1 patient with dysfunctional liver regeneration, n=1 patient with functional liver regeneration	[143]
Wang et al. 2023	snRNA-Seq	Control (non-tumour tissue from liver metastasis resections) (n=3) and MASH (n=9) liver	[23]
Xiao et al. 2023	snRNA-Seq	Healthy (n=3) and MASH (n=3)	[42]
Yu et al. 2023	ST	Liver of chronic HBV patients (n=18)	[144]
Zhang et al. 2023	ST	HCC liver (n=7 patients; n=4 responders; n=3 non responders to neoadjuvant cabozantinib and nivolumab)	[145]

711

712

Table 2.

Imaging-based ST	Brief outline	Considerations	T
seqFISH	DNAse I-based digestion and sequential staining/imaging rounds to decode transcripts in space.	Long imaging times Cell segmentation required Imaging interference from high-copy genes Needs pre-determined list of genes	F
MERFISH	Employing error correction in barcode assignment for robust barcode calling in noisy FISH-based images.		
Is situ sequencing	Sequencing by ligation (ISS, STARmap) or sequencing by synthesis (BaristaSeq).		
seqFISH+	Genome-scale transcriptome investigation separating individual transcripts into fluorescent spectra, employing 20 probes per each encoding round.		
Sequencing-based ST			
Spatial transcriptomics	Barcoded oligos are randomly arranged on a functionalised surface which captures mRNA released from the mounted tissues/cells.	Not single cell resolution	F F
Slide-seqV2	Employs random spatial bead spreading and *in situ* sequencing decoding	Beads can span more than one cell	F
HDST (High-definition Spatial Transcriptomics)	Deposits beads with combinatorial barcodes on patterned wafers which are then decoded with serial hybridisation.	Beads can span more than one cell	F
Seq-Scope/Stereo-seq	Utilises Illumina or MGI sequencing for oligo patterning on flow cells, and barcode calling is performed direct on the sequencer.	High sequencing cost due to resolution. Lateral mRNA diffusion	F
DBIT-seq	Delivers barcoded oligos directly to tissue through orthogonal microfluidics in a predetermined spatial distribution.	Not single cell resolution.	F
XYZseq	Tissue is placed on a spatially barcoded microwell array for an initial round of reverse transcription after which whole cells are removed and undergo single cell sequencing.	Limitations to number of cells captured.	F
Sci-Space	Tissue is placed on a glass slide bearing spatially gridded hashing oligos. Tissue is then permeabilised to allow for oligo transfer and then imaged. Nuclei are then extracted, fixed and sequenced.	Limitations to number of nuclei captured. Lower transcript capture.	F

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