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Spatial-omics: Novel Approaches to Probe Cell Heterogeneity and Extracellular Matrix Biology

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Abstract

Complex intercellular interactions as well as biomolecular and biomechanical cues from the extracellular matrix (ECM) profoundly affect cellular functions. Traditional transcriptomic and proteomic approaches have provided insight into disease progression by identifying discrete cellular subpopulations or microenvironmental signatures characteristic of normal or pathological tissues, however these techniques do not examine how a given cellular state relates to its interactions with neighboring cells or its surrounding ECM with multiparametric characterization (i.e. ECM alignment, mechanical forces, crosslinking, etc.). Emerging spatial-omic techniques can provide high-resolution mapping of expression profiles similar to scRNA-seq and mass spectroscopy directly within tissues. The ability to preserve the spatial context of cells within samples, their cellular geometry, as well as their surrounding ECM gives spatial-omics the opportunity to interrogate previously unexplored signaling modalities, which has the potential to revolutionize ECM research and our understanding of fibrotic diseases. In this review, we present current spatial transcriptomic and proteomic techniques and discuss how they may be applied to investigate cell-ECM interactions.

Keywords

Spatial transcriptomics; spatial proteomics; matrisome; fibrosis; multiplex imaging

Introduction:

High-throughput or –omic approaches developed over the past decades have expanded studies of individual genes or proteins into the unbiased realm of transcriptomics or proteomics. This has dramatically transformed biomedical research [1] and considerably advanced our understanding of ECM biology and pathophysiology [2–4]. To list a few examples, single-cell RNA sequencing (sc-RNAseq) alone has permitted the classification

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of fibroblast subpopulations in human skin [5]; the identification of an alveolar macrophage population expressing pro-fibrotic genes in idiopathic pulmonary fibrosis (IPF) patients [6]; the distinction of central vein-associated hepatic stellate cells as the dominant contributor to pathogenic collagen production in a mouse model of centrilobular fibrosis [7]; and the finding that after injury in the murine lung the number of activated fibroblasts, determined by multiple genes, increases despite the total fibroblast population size remaining constant [8]. Furthermore, microarray analyses have demonstrated the alteration and enrichment of developmental pathways in IPF lungs [9]; mass-spectrometry-based proteomics can now profile the ECM composition of normal and fibrotic samples in an unbiased manner and identify signatures distinguishing normal vs diseased states [2, 10–13]; and global gene expression studies have provided insight into the active role of matrix metalloproteases (MMPs) in pulmonary fibrosis [14, 15]. Additionally, and of interest to researchers primarily focused on fibroblasts, sc-RNAseq can be harnessed to identify cell populations with no reliable surface marker [16, 17]. However, techniques capable of achieving truly -omic level information such as bulk RNA sequencing (RNAseq), single-cell RNASeq (sc-RNAseq), or mass spectrometry (MS) require the dissociation of cells from their native context, which results in the loss of any spatial information and other significant cellular information, including but not limited to cell shape, cell-cell, and cell-ECM interactions. Additionally, bulk –omic techniques mask tissue, cellular and sub-cellular heterogeneity by averaging gene expression or protein abundance across whole samples [18]. And yet, these pieces of information are imperative to fully understand biological mechanisms such as signal transduction cascades, differentiation states, and interactions of multiple cell types (e.g. immune-stromal).

Preserving tissue integrity using classic in-situ hybridization (ISH) has demonstrated the importance of transcript localization by facilitating discoveries such as the contribution of RNA gradients on cell polarization during embryogenesis, which ultimately dictates cell differentiation at different developmental stages [19]. Classic microscopy techniques provide spatial resolution for investigating known pathways such as epithelial-to-mesenchymal transition in fibrosis [20], but spectral overlap limits depth for the number of protein/RNA targets that can be simultaneously detected. Previous methods to circumvent this limitation involve overlapping serial sections comprising different markers. However, these methods assume that tissues are homogeneous in the z-direction across the stained sections, which is known to be untrue.

Spatial-omic techniques are now offering a new perspective. By quantify dozens to hundreds of genes, transcripts, or proteins, spatial-omics enable the collection of valuable molecular, cellular and microenvironmental information in the context of native tissue or cellular structures. In this review, we provide an overview of currently available methods for spatial transcriptomics and proteomics and further describe recent studies that have applied such methods to advance our understanding of ECM and fibroblast biology. Given our expertise in pulmonary fibrosis, many of the examples provided within this review will be in the context of the lung, but we will expand to other organ systems where the literature has expanded to demonstrate the relevance of these powerful techniques to ECM research. We propose that these methods can be used to investigate phenotypic traits in the context of cellular heterogeneity, microenvironmental, cellular and sub-cellular dynamics, and

eventually help us decipher the fundamental bases of pathologies. We invite our readers to refer to the following reviews for in-depth technical descriptions of spatial transcriptomic and proteomic technics [21–26].

Spatial transcriptomics or RNA imaging:

Recent advances in techniques and technology have enabled the *in-situ* visualization of RNA transcripts in tissue sections. For example, multiplex transcript technology such as RNAscope has enabled the visualization of up to 12 mRNA molecules commercially helping both clinicians and researchers characterize cells, similarly to the way immunohistochemistry identifies the relative abundance and spatial distribution for protein, even when the mRNA is lowly expressed [27]. Spatial transcriptomic approaches expand multiplex imaging to 100–1000s of transcripts and can be distinguished by whether they measure pre-determined targets or gather global expression data. In this section, we will review current techniques with respect to these categories while providing insight on each technique's advantages and limitations (Table 1).

Imaging of pre-determined mRNA targets:

The first instance of imaging individual RNA species can be ascribed to single molecule fluorescent *in-situ* hybridization (smFISH), which provides absolute quantification of the copy number and localization of RNA molecules within cells. In smFISH, a series of fluorescently-conjugated oligonucleotides bind multiple complimentary sites of the same mRNA target to amplify the signal for visualization [28]. Not only can this approach detect nearly all transcripts present, effectively having close to 100% sensitivity, but smFISH also enhances specificity by increasing signal to noise ratio through the requirement of multiple successful binding events (~24 probes/target) to be detected by diffraction-limited fluorescent microscopy [21, 26]. smFISH is beneficial for the imaging of lowly abundant RNAs which require highly sensitive molecular tools to properly quantify their expression and determine their intracellular organization. In a recent study, Dobie *et al.* used smFISH on fibrotic liver samples and identified TREM2+CD9+ scar-associated macrophages in collagen-positive scar regions [29].

Originally smFISH was limited by the number of available fluorescent channels, but resourceful manipulations, such as combinatorial binding where pre-annotated color patterns are assigned to single targets [30], or temporal barcoding (also known as sequential FISH or seqFISH) where typically three targets are imaged per cycle allowing for the detection of F^n targets (F= fluorophores, n=hybridization cycles) [31], can be used to multiplex the system. Here, we are defining barcodes as a unique sequence (usually in the form of a synthetic DNA oligonucleotide) that is assigned to a specific cell, protein, or transcript of interest when performing multiplex imaging to identify that entity. Barcodes are essential for multiplexing the system in some spatial-omic techniques and therefore are widely deployed in the field of spatial-omics. Different forms of barcodes will be discussed throughout this review as well as visual examples for cells (Fig. 1) and proteins (Fig. 2B). For more depictions of the variations of spatial transcriptomic barcodes we recommend the following reviews [25, 26].

For example, a recent study conducted by Eng *et al.* expanded the capabilities of seqFISH (seqFISH+) to visualize 10,000+ genes within a single cell by altering the design of the barcodes. Their method vastly reduces the number of necessary multiplexing cycles by replacing standard 3–5 colored barcodes with 60 pseudocolored barcodes per image cycle, resulting in a total of 24,000 imaged genes after four cycles [32]. Combinatorial binding, however, increases the chance for misinterpreting signals due to hybridization error and temporal barcodes decrease detection sensitivity exponentially with each cycle [33]. By incorporating modified hamming codes (linear error-correcting codes previously used in telecommunication to automatically correct one bit errors) into the same workflow as temporal barcoded smFISH probes, multiplexed error robust FISH (MERFISH) can detect and correct errors while maintaining multiplexing to reduce RNA misidentification [23]. Xia *et al.* recently increased the gene throughput of MERFISH from the original ~1,000 genes [33] to ~10,000 transcripts [34]. This was achieved by applying expansion microscopy (ExM), a technique that expands a polymer to physically enlarge the biological structures and space out molecules within a cell, in combination with several other modifications to bring the field closer to genome-wide imaging (MERFISH and ExM were originally combined in this article [35]). A detailed description of the methods behind expansion microscopy can be found here [36]. Normal MERFISH requires transcripts longer than 3kb to accommodate the error-robust barcodes [33], but a study published in 2019 integrated branched DNA (bDNA) amplification into the MERFISH protocol to increase signal without altering fluorescent spot size, which will enhance shorter RNA imaging and increase imaging throughput [37]. Implementing additional amplification steps is an effective tool that has been previously incorporated into a number of other multiplexed FISH techniques [38–42] to increase signal and are described elsewhere [21, 26]. Amplification techniques must be applied carefully, however, as to avoid biasing the system with too many modifications, but the various derivatives of smFISH are advantageous for quantifying low abundant transcripts that would normally be undetected in methods such as scRNA-seq [43].

Rolling circle amplification (RCA) is used in another spatial transcriptomic technique known as *in-situ* sequencing (ISS). In ISS, single-stranded DNA oligonucleotides with 5’ and 3’ ends complimentary to the target sequence, also known as padlock probes, first bind cDNA libraries generated *in situ* from target RNAs [44]. The padlock probe hybridized to the cDNA forms a circle complex which undergoes RCA and sequencing by ligation to visualize over 100 transcripts per sample with single cell resolution [44]. ISS leverages padlock probes to heighten target binding specificity, allowing for the detection of non-coding and microRNA, but are expensive to produce and result in a laborious protocol. For this reason, Nagendran *et al.* developed a unique method known as proximity ligation *in-situ* hybridization (PLISH) to lower costs as well as increase scalability by replacing padlock probes with RNA-templated proximity ligation that requires two probes to adjacently bind onto the RNA sequence to form the circle complex [38]. In other instances the advantages of padlock probes can be built upon as demonstrated by spatially-resolved transcript amplicon readout mapping (STARmap), which requires the binding of both a padlock probe and RNA primer to induce RCA, creating a multi-cDNA containing amplicon [45]. After cDNA synthesis, the tissue is converted into an optically transparent 3D hydrogel to anchor DNA amplicons. A modified ISS technique is then applied to visualize and

decode pre-encoded five-base barcodes. STARmap can impressively read 1,000 genes in only six imaging cycles [45]. Although the previously described target methods are limited by library size, they are essential for acquiring a high detection efficiency and can be valuable tools for analyzing alterations in specific transcripts of interest within multiple fibroblast subpopulations simultaneously [46].

Genome-scale transcriptome sequencing:

For experiments that require an unbiased gene selection, spatial transcriptomic approaches that evaluate the whole genome were created. ISS is a primary example of a targeted method that has been modified to create an unbiased next generation fluorescent *in-situ* RNA sequencing (FISSEQ) technique [47]. Instead of using random primers and targeted padlock probes, FISSEQ uses specialized tagged-random primers that allow for RNA to be synthesized into cDNA amplicons *in situ*. All cDNA amplicons are then cross-linked together within cells for stabilization, followed by RCA and profiled by Sequencing by Oligonucleotide Ligation and Detection (SOLiD), effectively sequencing the whole transcriptome with single-nucleotide resolution [22, 47]. Implementing this method allowed for the localized examination of 8,102 human primary fibroblasts genes in a wound healing model to assess gene alteration in response to injury and further showed that contact-inhibited and migratory fibroblasts differentially express ECM genes [47].

While FISSEQ is capable of directly sequencing a multitude of transcripts within the tissue, other unbiased spatial transcriptomic techniques rely on methods that allow them to employ bulk or single-cell RNA-seq and map the results back to prior images. Notably, the techniques mentioned till this point have z-scanning capabilities that are dependent upon the specifications of the microscope incorporated into the individual lab's setup, whereas the remainder of the spatial transcriptomic approaches lack z-scanning capabilities. These methods follow a similar workflow, where the tissue is imaged, cells are labeled based on spatial location, but are then disassociated, processed for sequencing, and then the transcripts are mapped back to the original image using computational methods. For example, advanced tools such as laser capture microdissection (LCM) are used to select single cells or entire populations of cells of interest to be sequenced post-imaging [48, 49]. Another technique called Visium by 10x Genomics uses specialized slides containing spatially-distinguishable capture probes within a 100μm diameter chip [50]. Cells are permeabilized within the tissue, releasing the mRNA contents of each cell to locally bind the positional molecular barcodes residing directly under the individual cells on the glass coverslip. RNA-seq data generated from the reverse transcripts of the captured mRNA are aligned to pre-permeabilization images of the tissue via spatial barcodes for 2D positional transcriptome analysis [50]. This method captures twice the amount of transcripts as LCM-seq with few additions to the normal RNA-seq pre-processing workflow [23]. As this method relies on RNA-seq, it is reasonable that the reported sensitivity is comparable to the ability of scRNA-seq to detect single RNA molecules, 5–40% [51], at 6.9±1.5% [23] and below the near 100% sensitivity of smFISH. A method recently developed by the Macosko lab, Slide-seq, reached an even finer resolution by employing coverslips coated in a monolayer of 10 μm microparticles ("beads") with distinct DNA-barcodes which can be mapped to their spatial address via sequencing of the barcode by oligonucleotide ligation

and detection (Fig. 1) [52]. Frozen tissue sections are placed on these slides, imaged, and then processed to release mRNA to be captured by the microparticles for 3' end RNA sequencing on commercial instruments. Although this method may be less sensitive to very low abundant transcripts, Slide-seq is highly scalable and achieves 10um spatial resolution, yielding the probing of localized gene expression within an individual cell [52].

Spatially-resolved proteomics:

RNAs and their protein counterparts are not expressed in a 1:1 ratio due to RNA degradation, post transcriptional, translational, and post-translational modifications [12, 53, 54], making it imperative to evaluate proteins, their concentration, interactions, and location. This was, for example, captured in the data collected for the atlas of the aging lung, where basement membrane collagen IV mRNA was downregulated, but protein was upregulated [10]. To achieve this objective many techniques have been developed that we have divided into 1) methods that require tissue dissociation and rely on computational mapping techniques or 2) methods that preserve tissue samples, often at the cost of decreased sequencing depth (Table 2).

Dissociative techniques:

Originally presented in 2014, imaging mass cytometry (IMC) is a method capable of dictating protein distributions with 1 micron resolution [55, 56]. Here rare earth metals are conjugated to antibodies rather than fluorochromes to allow for simultaneous imaging of ~40 proteins. Sections undergo a single staining step to reduce technical variability, and then the sample is nebulized one pixel at a time via a laser ablation system. Each ablated pixel is enriched for heavy metal reporter ions and quantified by time of flight (TOF) MS on a per cell basis for single-cell analysis of cell type and state [56]. Noise and channel spillover is reduced in this method as the metal isotopes have distinct detection peaks, unlike fluorophores which may "blead" due to spectral overlap, and these heavy metals are not commonly observed in vivo, ergo eliminating background noise and autofluorescence [24, 56]. Multiplexed Ion Beam Imaging (MIBI) also utilizes mass tagged antibodies to analyze highly multiplexed protein expression patterns with morphological context. Instead of using a laser as in IMC, an ion beam is used to generate secondary ions from the antibodies, which can image up to 100 targets simultaneously after performing secondary ion MS [57]. Recent adaptations of MIBI have shown that the ion beam can precisely image at various depths, unlike the laser used in IMC, to acquire multiple scans in the z direction and create 3D images with ~250 nm resolution in the axial dimension [58]. However, both these techniques require long imaging times in addition to being costly. The area sampled is also confined by the time it takes to process MS data obtained from each pixel and convert it to an image. For more details on these techniques please refer to the following reviews [24, 59–61].

Thousands of molecules, including metabolites, lipids, peptides, proteins, and glycans, can be unbiasedly analyzed through the use of MS. Spatially targeted optical micro-proteomics (STOMP) couples MS and affinity photolabeling to fully harnesses a global spatial proteomic approach [62]. In this method, regions of interest (ROI) are selected *in situ*, a hexa-histidine photo-tag is then cross-linked to ROI-labeled proteins using two-photon

excitation, which enables specific isolation of ROI proteins for mass spectrometry via anti-His bead pull-down. STOMP can identify proteins in any region of the cell with single micron resolution (.67um in xy axis and 1.48um axially), an entire order of magnitude greater than LCM [62]. Hadley *et al.*, using STOMP, identified proteins present in amyloid plaques, fully demonstrating the power of 1μm resolution [62]. Imaging MS can also be paired with matrix-assisted laser desorption/ionization (MALDI) to obtain unbiased, de novo peptide sequencing of 1,000s of analytes from specified, spatial areas [63]. Details on the various ways to implement MALDI-IMS have been reviewed elsewhere [64]. Briefly, a matrix is overlaid onto a tissue section and laser excitation causes absorption of the sample's ions into the matrix. Mass spectra generated at each xy coordinate are mapped back to the image to visualize xy spatial distribution [63]. MS methods are a valuable tool for spatial proteomics as they allow for the unbiased analysis of multiple analytes as well as post translational protein modifications like the citrullination of fibronectin which was shown to alter focal adhesion stability in fibroblasts [65], bringing us closer to an analysis that encompasses the whole proteome and distinguishes the ECM from cells. However, resolution often suffers with these approaches as compared to optical imaging in order to accommodate MS instrument sensitivity of lowly abundant species from small areas.

Techniques preserving tissue integrity:

In order to preserve morphological context and enhance resolution, a myriad of approaches that modulate standard antibody markers to present high-dimensional data have been developed to create non-destructive methods. Most of these techniques rely on iterative imaging of successive antibody staining cycles, where the sample is stained, followed by fluorophore bleaching, inactivation, or cleaving in preparation for a new batch of markers to be stained. These methods (MELC, SIMPLE, and MultiOmyx) are reviewed in detail elsewhere [24]. Instead, we will focus on improvements to the aforementioned and newer methods. For example, tissue-based cyclic immunofluorescence (t-CyCIF) is an advancement of a previous method developed initially by Gerdes *et al.* [66] and later adapted for cell culture by Lin *et al.* [67, 68], where multiplexed images are obtained by oxidizing fluorophores in a high pH hydrogen peroxide solution to erase the signal and image new proteins. In the span of three years the multiplexing capabilities of this technique expanded from imaging 16 channels after fluorescent-protein-based live cell imaging [68], to identifying 60 markers in formalin-fixed, paraffin-embedded (FFPE) samples [67]. Previous versions were limited by computational processing capabilities, but as analytical methods have improved the new constraint has become maintaining tissue integrity at higher cycles [67, 68]. The advantages of t-CyCIF are that it uses commonly available reagents, conventional microscopes that allow z-stacking, and commercial antibodies that can be selected to suite specific researcher interests. Our readers can refer to a recent study from the Hynes lab that reports the validation of ~100 antibodies recognizing ECM proteins [69]. However, the t-CyCIF protocol is time consuming as it requires an additional antibody incubation period for each imaging cycle. Alternatively, the CO-Detection by indEXing (CODEX) workflow facilitates imaging of up to 40 antigens after a single antibody incubation period comprising the entire antibody panel [70]. This method developed in the Nolan lab and now commercialized by Akoya Biosciences, combines in situ iterative indexing polymerization of unique DNA barcodes with automated assay performance via a

cyclic fluidic device and automated imaging acquisition. In each cycle, one non-fluorescent nucleotide binds to the 5' overhang of all oligo barcodes to index the system (Fig. 2). An additional fluorescent nucleotide, particular to that cycle, is incorporated only into the barcode of the antibody to be imaged that cycle [70]. The fluorophore is then cleaved by TCEP and washed away via the fluidics device in preparation for the next imaging cycle. The oligo on the previously imaged antibody is no longer polymerized as it is shorter than the following DNA barcodes. CODEX can also be integrated into any colored epifluorescence microscope, making it a versatile multiplexed imaging platform capable of spatial resolution in the lateral (xy) and axial (z) dimensions to create 3D images [70]. Although some of these techniques are capable of xyz resolution, most notably MIBI, t-CyCIF, and CODEX, they were designed with the intention of evaluating single cells and to our knowledge have not been applied to tissue sections thicker than 10 μm. These tools will be useful for further probing known location dependent protein-protein interactions, such as the decreased nuclear interaction of LEM domain-containing protein 3 and SMAD2/3 correlated to increased ECM stiffness in IPF tissue [71].

Multiplexed spatial-omics:

Few techniques have been developed to multiplex image RNA and protein in parallel to achieve -omics data on multiple molecular levels. Xia *et al.* demonstrated standard antibody staining in combination with MERFISH to identify localized RNA expression [34]. Techniques such as CITE-seq and REAP-seq have combined proteomic information with RNA-seq, but lack spatial context [72, 73]. Interestingly, recent studies such as those conducted in the Bodenmiller lab have been able to achieve imaging of 3 mRNA and 16 protein simultaneously with single-cell resolution [74]. The GeoMx™ Digital Spatial Profiling (DSP) (Nanostring Technologies, Inc.) system extends its method to accommodate the multiplexing of 40 proteins and up to 900 mRNA probes to be simultaneously analyzed with respect to spatial distribution, which can theoretically be extended to a maximum of 96 antibodies and an unlimited number of RNA readout through next generation sequencing (NGS) [75, 76]. This approach leverages oligo-tags attached to antibodies or RNA probes via a photocleavable linker. In this approach, pre-determined ROIs (identified by standard immunofluorescence) are exposed to photocleaving light directed by an automated digital-micromirror device, releasing the oligonucleotide tags to be aspirated into an automated microfluidics device and subsequently quantified through the nCounter System or NGS [75, 76]. Although DSP can overlay spatial transcriptome and proteome analyses through spatially aligned adjacent tissue sections, its protocol is optimized for interrogating tissue niche's or ROI, not single cell profiling on whole tissue sections [76]. DSP's pipeline, however, is compatible with the workflow of other spatial-omic techniques that do quantify expression with single cell resolution. RNA isoforms can also be identified through the integration of NGS in this system [75, 76]. Expanding these techniques will allow for -omic level profiling in various tissue regions, as well as allow for full paneling as transcripts can still provide information on proteins that do not have validated antibodies. In addition, continued progress must be made towards adding epigenetic and post-translational modifications, as well as profiling other biomolecules (lipids, glycans), if we want to

truly grasp the complexity, heterogeneity, and dynamics of cellular and pathophysiological processes.

-Omics and Spatial-omics Approaches to Study the Matrisome:

ECM proteins have different biochemical properties than those of intracellular compartments. The highly modified and cross-linked nature of ECM proteins not only confers resilience to tensile or compressive stresses at the tissue and cell level, but also poses challenges in studying these proteins with conventional biochemical methods. In this section, we will first present approaches recently developed to specifically study ECM proteins with high throughput. We will then discuss new emerging ideas that can be implemented to improve the resolution with which we can study the extent of ECM roles on pathophysiology.

The matrisome: a comprehensive classification of ECM proteins:

High-throughput methods require careful annotation of the data generated in order to identify biologically relevant interactions among genes and proteins. Until recently, annotation tools only provided a partial view of the complex compartment that is the ECM [77]. To fill this gap in knowledge, we devised a computational pipeline to predict the “matrisome”, which is the collection of proteins that form ECM scaffolding (termed the core matrisome) but also those that interact with them (termed matrisome-associated components) [78]. This comprehensive collection accompanied by detailed nomenclature has allowed ECM research to enter the –omics era [2].

Indeed, the lists defined above can be used to annotate -omic datasets and map the matrisome of a given tissue or produced by a given type of cells. For example, Etich and collaborators discovered a unique matrisome-gene signature regulated in interferon-γ (IFNγ)- and dexamethasone-primed macrophages in vitro by revisiting previously published transcriptomic data [79]. They further validated the contribution of macrophage-secreted ECM proteins in skin wound repair in vivo [80]. A study conducted by Hamburg-Shields and collaborators highlights another example of how *the* list of matrisome genes are useful for the study of ECM biology. Here, they induced fibrosis in a mouse model by expressing a non-degradable form of β-catenin, then extracted and sequenced RNA from the dermis [81]. While all the changes observed in mutant skin were increases in gene expression, 36 of 175 (20.6 %) genes were matrisome genes, suggesting that β-catenin is one of the drivers of fibrosis [81]. With increased access to scRNA-seq technology, gene expression profiles of cells from fibrotic tissues have also been investigated. For instance, the analysis of the gene expression profile for different cell populations isolated from lung samples from either healthy control or IPF patients identified a new subtype of macrophages specific to IPF [6]. scRNA-seq of lung mesenchymal cells obtained from healthy donors vs. patients presenting with systemic sclerosis-associated interstitial lung disease identified different subpopulations of fibroblasts (one of them being characterized by a high level of the core matrisome gene MFAP5) and myofibroblasts [82]. However, these studies have left the matrisome largely unexplored, and annotating these datasets may reveal novel or unsuspected correlations that may lead to novel findings on the role of the ECM in pathophysiology.

Mass-spectrometry-based approaches to profile the ECM of tissues

Transcriptomic datasets convey a representative picture of the level of gene expression in a given condition, but do not fully reflect the state of protein abundance, since there is on average a 50% correlation between the two metrics [83]. Proteomic approaches such as those based on MS can be applied to take a snapshot of protein abundance in cells or tissue at a given time point or over time. However, as mentioned previously, ECM proteins present challenges for these methods mostly because of their insolubility [84]. This is further complicated by the quantity of peptides needed to perform an experiment, due to a lower sensitivity in proteomic tools compared to transcriptomic tools. To circumvent these challenges, we and others have developed methods to enrich and solubilize ECM proteins. While nuances between methods exist, they all follow a similar pipeline consisting of: ECM enrichment, protein solubilization and digestion into peptides, followed by MS analysis. For more details, we invite our readers to refer to a review we recently wrote on "matrisomics" [84]. The resulting proteomic data then can be annotated using the matrisome list, which can uncover ECM characteristics of the sample. Although much can be gained from the qualitative comparisons between matrisome profiles, labeled-based quantitative proteomic using isobaric tandem mass tag (TMT), or isobaric tag for relative and absolute quantification (iTRAQ) can provide additional information. In a 2017 study, we employed quantitative proteomics to characterize the changes in ECM composition in bleomycin-induced lung fibrosis and lung adenocarcinoma and found that the two diseases, both characterized by desmoplasia, shared a common ECM signature not observed in healthy lung tissues, but could also be distinguished from one another by the presence of specific ECM components [11]. This has similarly been observed in the context of liver fibrosis and hepatocellular carcinomas [85]. Novel proteomic approaches and bioinformatic tools are now focusing on probing post translational modifications of the ECM. One such study by Merl-Pham *et al.* investigates the effect of TGF-β on the ECM organization, and found novel sites of prolyl-3-hydroxylation site occupancy and lysine-O-glycosylation along with novel crosslinking enzymes that may help shed light on the process of fibril assembly [86]. The analysis of ECM proteins can be further extended with a technique called Quantitative Detergent Solubility Profiling (QDSP) [12]. As previously mentioned, most of the studies conducted on the ECM involve enrichment of the insoluble ECM by removing more soluble cellular components, yielding multiple fractions containing more soluble proteins in the process. Analysis of all the fractions (and not of only the ECM-enriched fraction) can shed light into mechanisms affecting ECM protein assembly and cross-linking during disease progression [12, 13, 87] or aging [10]. For example, high throughput degradomic and proteomic approaches were able to expand our knowledge of MMP cleavage specificity to establish regulatory roles of MMPs and design more effective therapeutics [88, 89].

Transcriptomic and proteomic characterizations of tissues or cells can be combined in multi-omic studies. This integration can reveal the different levels of regulations (transcriptional, translational, and post-translational) at play in healthy and diseased tissues. The Mann group has pioneered the use of multi-omics to study murine lung tissues in the context of bleomycin-induced injuries [12] and aging [10]. These approaches are particularly useful when genes expression level and protein abundance do not correlate. Together with changes in solubility and abundance seen in proteomic analyses, the transcriptomic analyses of

upstream regulators can provide a systems-biology view of the matrisome. Last, recent attempts have been made to integrate miRNA data into transcriptomic and proteomic data. One such study examined the mRNA and miRNAs profiles of pulmonary fibroblasts from IPF and scleroderma patients, and revealed specific fibrotic signatures at both the miRNA, ECM gene, and ECM protein expression levels [90].

Probing the Regional Characteristics of ECM Composition and Architecture:

The studies described above have greatly advanced our understanding of the complexity of the ECM and have shed light on the multiple cell types and subtypes that can contribute to the production and remodeling of the ECM in healthy and diseased tissues. However, because of their destructive natures, the methods mentioned above do not allow researchers to probe sub-tissular heterogeneity of the ECM. To overcome this, approaches have focused on isolating a ROI in order to capture and evaluate smaller groups of cells or a defined microenvironment that are otherwise lost in the bulk processing of the samples. LCM is one of those methods, as it allows for the manual selection of a specific region to be excised from a tissue slide for analysis. LCM allows isolation of a particular compartment, as demonstrated by the isolation of glomeruli for the characterization of the glomerular basement membrane [91].

Another approach to characterize the localization and pattern of ECM protein distribution within tissues, is ECM Image Mass Spectrometry (ECM-IMS) [92]. This technique builds upon MALDI (see above), which records the mass-to-charge (m/z) ratio resulting from each pixel that is ionized. The resulting data can be represented by the distribution of m/z ratios that can be turned into a characteristic signature, allowing for the differentiation of, for example, a pathological tissue from its normal counterpart. One of the earlier studies utilizing the ECM-IMS aimed to find ECM signatures distinguishing young vs. old skin samples. Once age-dependnet m/z signal identified, the authors later identified proteins that were more abundant in young skin samples; these included collagen IV, collagen VII, collagen XVII, and nidogen I [93]. Another study using ECM-IMS compared low grade lung adenocarcinoma (LUAD) samples to normal lung from tissue microarrays by identifying and imaging peptides obtained from collagenase type III digestion. This resulted in a regional distribution tissue map of peptides of specific m/z ratios plotted with respect to their intensity. The authors were thus able to identify a characteristic m/z signature composed of 25 peptides that distinguished LUAD samples from normal tissues. Furthermore, because these spectra were assigned to spatial coordinates, the authors could also identify peptides preferentially found in the tumor core, around blood vessels, or in normal tissues adjacent to tumors [94]. To the best of our knowledge, other spatial proteomic approaches described above have not yet been applied to specifically study the ECM composition of tissues. However, in a recent study, Alföldi and collaborators used cytometry by time-of-flight (CyTOF) to profile protein expression changes in lung cancer cells cultured in 2D, 3D, or grown in vivo and among the 12 markers used in the study, one of them, Galectin-3 is a matrisome-associated proteins [95].

Importantly, to fully capture the complexity of the ECM, one needs, in addition to profiling its biochemical composition, to determine, in a time- and regionally-resolved manner, its

architectural organization and biophysical and mechanical properties. These methods do not per se fall under the term of –omic approaches, and will thus not be discussed here, but we are listing for our readers recent and comprehensive reviews illustrating methods (second harmonic generation, atomic force microscopy) that permit these kinds of analysis [96–98].

Future Directions and Challenges:

Multiplex spatial-omic techniques will soon become prevalent, due to their ability to provide quantitative data on dozens of protein or RNA species within the same sample. Multiplex spatial-omics will help resolve the cellular complexities that contribute to overall cell phenotype/state, cell-cell interactions, as well as how these molecular identities link to their respective tissue architecture, and therefore tissue function by obtaining insight from multiple spatial scales [99, 100]. These methods although beneficial, still require advances to increase the resolution and throughput of the system and decrease acquisition time, as well as cost. There is also a need to increase the depth of these systems. At the RNA level, splicing variants are traditionally difficult to detect, and yet determining which splicing variant of a given ECM protein is being actively translated is of paramount importance [101, 102]. In the case of spatial image-based proteomics, construct libraries for each image cycle are limited by the number of variant (either barcodes, fluorescent dyes, or rare metals) available. Furthermore, antibodies must be validated and only rarely detect isoforms.

Interestingly, none of the current spatial-omic approaches presented in this review are capable of performing multiplexed spatial-omics on live cells *ex vivo* or *in vivo*. Researchers must be cognizant that these molecular profiles are representative of a single snapshot within the tissue. The observed phenomenon that cells operate through discontinuous transcription dictated by stochastic bursts, or “transcription bursts”, indicates that genetic profiles we observe in fixed samples may simply be an artifact of expression heterogeneity. Bulk sequencing techniques mask heterogeneity, as they are analyzing cells at a single time point; confining cell identification to the cell’s active transcriptional state at that moment, while other cells with similar functional roles are in a transcriptionally dormant state and are not accounted for [103].

What is possibly the most considerable challenge spatial-omics users will encounter is finding proper statistical tools for analyzing the vast amount of data produced by these systems. Furthermore, standardizing the way researchers present and analyze this data will require a community effort where computational algorithms and techniques are made publicly shared. Some of the commercialized spatial-omic techniques described in this review provide helpful software for cell segmentation and identification, but these methods still could benefit from enhancements to accommodate more complex samples with unusual cell shapes and sizes, such as those observed in IPF. Machine learning algorithms, such as CellDissect [104], can be implemented and have the potential to address the aforementioned issue. Visualizing structures that are typically difficult to evaluate through standard procedures due to microscope and affinity reagent limitations, such as lipid rafts, will need creative solutions in order to benefit from spatial-omic techniques as well. To facilitate the movement of spatial-omic platforms into clinical settings, approaches will have to continue to be developed in a cost and time effective manner.

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Abbreviations:

ISH	in-situ hybridization
sc-RNAseq	single-cell RNA sequencing
IPF	idiopathic pulmonary fibrosis
MMPs	matrix metalloproteases
ECM	extracellular matrix
RNAseq	RNA sequencing
MS	mass spectrometry
smFISH	single molecule fluorescent in situ hybridization
seqFISH	sequential FISH
MERFISH	multiplexed error robust FISH
ExM	expansion microscopy
ER	endoplasmic reticulum
bDNA	branched DNA
ISS	in situ sequencing
RCA	rolling circle amplification
PLISH	proximity ligation in situ hybridization
STARmap	spatially-resolved transcript amplicon readout mapping
FISSEQ	fluorescent in situ RNA sequencing
SOLiD	Sequencing by Oligonucleotide Ligation and Detection
LCM	laser capture microdissection
EMT	epithelial to mesenchymal transition
IMC	imaging mass cytometry

CyTOF	cytometry by time-of-flight
MIBI	multiplexed ion beam imaging
STOMP	spatially targeted optical micro-proteomics
ROI	regions of interest
MALDI	matrix-assisted laser desorption/ionization
MELC	multi-epitope-ligand cartography
SIMPLE	sequential immunoperoxidase labeling and erasing
t-CyCIF	tissue-based cyclic immunofluorescence
FP	fluorescent protein
CODEX	CO-Detection by indEXing
CITE-seq	Cellular Indexing of Transcriptomes and Epitopes by Sequencing
REAP-seq	RNA expression and protein sequencing assay
DSP	Digital Spatial Profiling
NGS	next generation sequencing
T1D	Type I Diabetes
IFNγ	interferon-γ
TMT	tandem mass tag
TGF-β	transforming growth factor beta
QDSP	Detergent Solubility Profiling
LUAD	low grade lung adenocarcinoma

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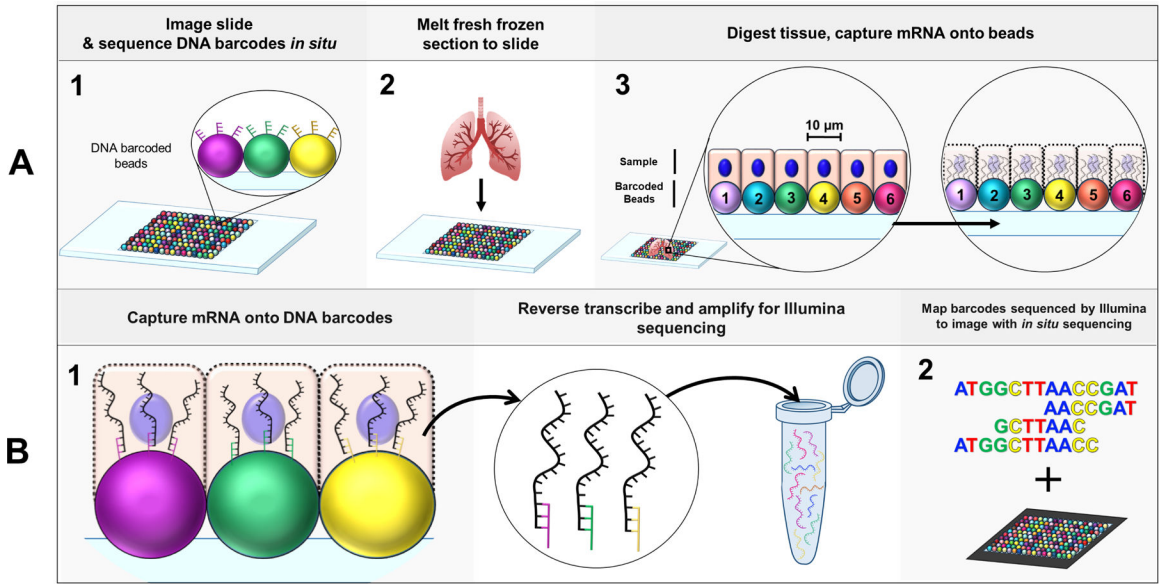

Figure 1: Slide-seq multiplex imaging workflow

(A) Schematic of initial Slide-seq steps (A1) A rubber coated coverslip is covered in a monolayer of 10µm micro-particles (“beads”) to create a “puck”. Each bead is conjugated to its own unique barcode whose sequence is determined *in situ* via SOLiD (Sequencing by Oligonucleotide Ligation and Detection) for high resolution localization of each bead on the puck. (A2) Fresh frozen tissue can then be melted onto the puck, (A3) where the cells (approximately one cell/bead) overlap with the DNA barcoded beads. The tissue is digested and the mRNA of each cell is captured onto the DNA barcodes residing directly beneath the respective cell. **(B)** Schematic of library preparation for Slide-seq. (B1) mRNA are reverse transcribed to incorporate in a 3’-end, barcoded RNA-seq library preparation. Products are then amplified and undergo Illumina sequencing. (B2) Transcript profiles associated with a barcode sequence obtained from Illumina sequencing are mapped to their specific tissue location by matching the Illumina data to the barcode sequence from the initial *in situ* sequencing step in A1 [52].

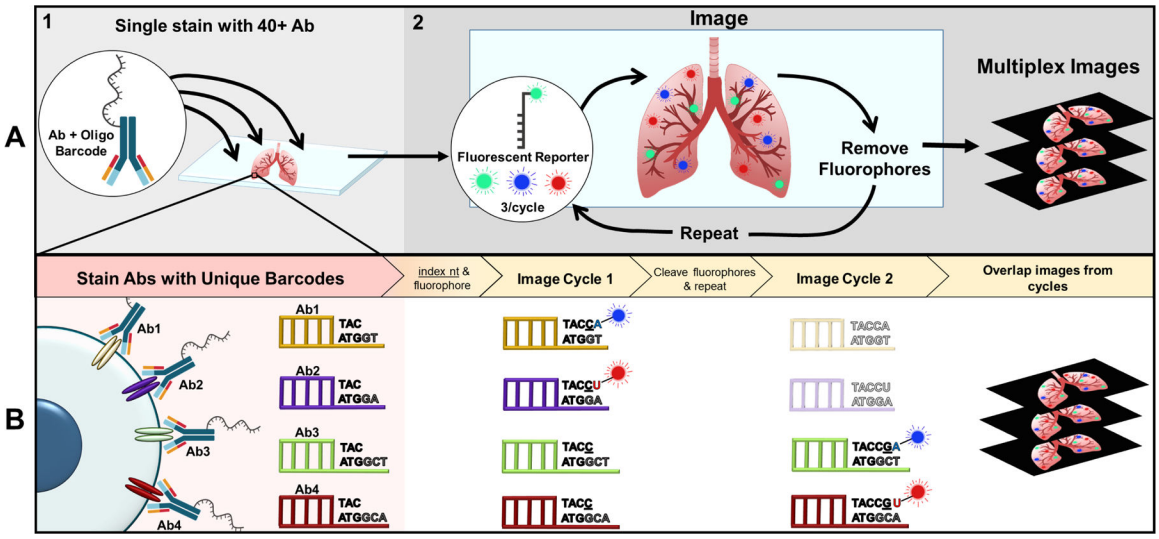

Figure 2: Co-Detection by indEXing (CODEX) multiplex imaging workflow.

(A) Top left moving right depicting a macro-view of CODEX workflow. (A1) Tissue section (either FFPE or fresh frozen) is simultaneously stained with the entire panel of antibodies tagged with their own unique oligonucleotide barcode by the user. Of note, the commercial system currently provides ~40 barcodes that can be conjugated to any antibody of interest. (A2) The stained section then undergoes several imaging cycles automated within the fluidics device where three fluorophores are imaged and subsequently removed via TCEP cleavage each cycle (14 cycles for 40 antigens). Images are then overlapped to create a ~40 protein multiplexed image from a single tissue section. **(B)** Bottom depicting cellular-level view of CODEX workflow with two fluorophores (for simplicity) and indexing technology. Each antibody is conjugated to a unique barcode with a 5' overhang. The indexing nucleotide (denoted by underlining) and two nucleotides bound to fluorophores are added to the section. As shown in cycle 1, the indexing nucleotide is incorporating into all of the barcodes, while the fluorescent nucleotides are only able to bind to their respective pre-designated barcodes to image the antibodies denoted for that cycle. Fluorophores are removed to repeat the process. Barcodes for antibodies in earlier cycles will be shorter than later cycle oligo-tags. After the antigen of interest for that cycle is imaged, the oligonucleotide will no longer be indexed, as denoted by the lighter shading in cycle 2 [70].

Abbreviations: Antibody (Ab); nucleotide (nt); FFPE (Formalin Fixed Paraffin Embedded); Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)

Table 1:

Overview of spatial transcriptomic techniques discussed

Method	Number of Targets	Advantages	Limitations
Single Molecule Fluorescent in situ Hybridization (smFISH) [28]	See derivatives of technique (MERFISH and seqFISH)	Absolute quantification (100% sensitivity); High signal:noise; High sensitivity to image RNA in low abundance	Target must be selected, biasing the results
Multiplexed Error Robust FISH (MERFISH) [33–35, 37]	~10,000	Detects and corrects errors; Same as smFISH	Requires transcripts longer than 3kb; Special equipment
Sequential FISH (seqFISH) [31, 32]	10,000+	Single cell resolution; Reduces the number of necessary multiplexing cycles; Same as smFISH	Decreased detection sensitivity with each cycle; Time consuming protocol
In-situ sequencing (ISS) [44]	100+	Heighten target binding specificity; Can detect Small Nuclear Polymorphisms (SNPs)	Padlock probes are costly; Time consuming protocol
Proximity Ligation in situ Hybridization (PLISH) [38]	Unlimited targeted detection 4 transcripts/cycle	High signal to noise; Cost effective; Detects low abundant RNAs	Laborious protocol; Time consuming protocol
Spatially-resolved Transcript Amplicon Readout Mapping (STARmap) [45]	1,000 transcripts/6 imaging cycles	1,000 genes in only six imaging cycles	Padlock probes are costly
Spatial Transcriptomics [50]	Entire transcriptome/non-targeted	Similar protocol as RNA-seq; High transcript count	Lower sensitivity for low abundant transcripts; Not single cell resolution (~100um); Special slide needed; Sequencing data analysis
Slide-Seq [52]	Entire transcriptome/non-targeted	Single cell resolution (10um)	Lower sensitivity for low abundant transcripts; Sequencing data analysis
Fluorescent in situ RNA Sequencing (FISSEQ) [47]	Entire transcriptome/non-targeted	Unbiased sequencing; Maintains sample integrity	Low target detection; Very time consuming protocol

Note: For other comprehensive tables from previous reviews on spatial transcriptomics please refer to [23, 25].

Table 2:

Overview of spatial proteomic techniques discussed

Method	Number of Targets	Advantages	Limitations
Imaging Mass Cytometry (IMC) [55, 56]	~40 antibodies	1 micron resolution; Single section and staining; Low signal spillover; No autofluorescence	Limited by number of rare metals; Biased by target selection; Special equipment; Costly; Long imaging time; Data analysis
Multiplexed Ion Beam Imaging (MIBI) [57]	Capable of ~100 targets, commercially 40+	200–300 nm resolution; Parts-per-billion sensitivity; Low signal spillover; No autofluroescence	Biased by target selection; Costly; Special equipment; Long imaging time; Data analysis
Spatially Targeted Optical Micro-Proteomics (STOMP) [62]	1,000s of analytes	1 micron resolution; Analyzes post translational protein modifications; Unbiased target selection	Special equipment needed; Trouble measuring low abundant proteins; Data analysis
Matrix-Assisted Laser Desorption/Ionization (MALDI) [49]	1,000s of analytes	Analyzes post translational protein modifications; Unbiased target selection	Low resolution; Trouble measuring low abundant proteins; Requires matrix; Data analysis
Tissue-based Cyclic ImmunoFluorescence (t-CyCIF) [67, 68]	60 antibodies	Cost effective; Uses common lab items; Enhanced signal to noise with each cycle	Time consuming
Co-Detection by indEXing (CODEX) [70]	~40 antibodies	Single section and staining; Cost effective; Uses common microscope	Requires special reagents and equipment

Note: For other comprehensive tables from previous reviews on spatial proteomics please refer to [24].