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Applicability of spatial transcriptional profiling to cancer research

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SUMMARY

Spatial transcriptional profiling provides gene expression information within the important anatomical context of tissue architecture. This approach is well suited to characterizing solid tumors, which develop within a complex landscape of malignant cells, immune cells, and stroma. In a single assay, spatial transcriptional profiling can interrogate the role of spatial relationships among these cell populations as well as reveal spatial patterns of relevant oncogenic genetic events. The broad utility of this approach is reflected in the array of strategies that have been developed for its implementation as well as in the recent commercial development of several profiling platforms. The flexibility to apply these technologies to both hypothesis-driven and discovery-driven studies allows widespread applicability in research settings. This review discusses available technologies for spatial transcriptional profiling and several applications for their use in cancer research.

INTRODUCTION

The study of tissue structures is fundamental to understanding living systems. From its rudimentary beginnings in the 17th century, the visual evaluation of tissue specimens has evolved into a distinct biomedical discipline unto itself. Today, histological assessment in clinical practice is ubiquitous and indispensable in accurate diagnosis of solid tumors. Conventional histochemical staining methods can reveal remarkably detailed tissue elements, particularly in formalin-fixed paraffin-embedded (FFPE) tissue sections. However, the advent of novel multiplexed immunohistochemical methods has significantly expanded the number of cellular and molecular targets that can be directly visualized on a single section with subcellular resolution (Tsujikawa et al., 2017). Moreover, the recent development of technologies such as imaging mass cytometry (Giesen et al., 2014), multiplexed ion beam imaging (MIBI) (Angelo et al., 2014), and the digital spatial profiling (DSP) platform (NanoString Technologies) (Merritt et al., 2020) expands the number of multiplexed protein targets available for analysis in frozen or FFPE tissue sections. Although the development of these powerful methods is impressive and reflects great technological advancements, they are nonetheless subject to the capacity limitations associated with antibody multiplexing.

Transcriptional profiling offers a different measurement approach for comprehensive molecular assessment of tissues. In particular, whole-transcriptome profiling circumvents some of the capacity constraints of these antibody-related methods by providing expression measurements across thousands of RNA species. Despite the immense amount of data generated by this approach, both bulk RNA sequencing (RNA-seq) and single-cell RNA sequencing (scRNA-seq) fail to retain anatomical or spatial information, because the tissues must be dissociated before sequencing. Traditionally, investigations seeking to obtain detailed gene expression profiling data and data pertaining to tissue architecture and organization were mutually exclusive, requiring that tissues are partitioned before downstream use. To remedy this, the early iterations of spatially resolved transcriptomics used microdissection to selectively remove portions of mounted tissue for subsequent gene expression profiling (Alevizos et al., 2001; Sriuranpong et al., 2004). These early labor-intensive procedures have been augmented by various strategies for concurrent visualization and identification of RNA targets while maintaining unbiased spatial context. Seemingly a daunting task a decade ago, *in situ* spatial transcriptional profiling is now a reality that holds great promise for the advancement of oncology research. This review summarizes the current state of spatial transcriptional profiling technology and discusses demonstrated and potential applications for cancer research.

COMPARISON OF SPATIAL TRANSCRIPTIONAL PROFILING PLATFORMS

Mapping the spatial organization of an intact tissue section using spatially resolved high-resolution transcriptomics is the next major step for deciphering tumor heterogeneity (Burgess, 2019). Several novel high-resolution spatial transcriptional profiling technologies, including sequential Fluorescence In Situ Hybridization (seqFISH) (Shah et al., 2017), Slide-seq (Rodriques et al., 2019), STARmap (Wang et al., 2018), High Definition Spatial Transcriptomics (HDST) (Vickovic et al., 2019), Multiplexed Error-Robust Fluorescence In Situ Hybridization (MERFISH)

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(Moffitt et al., 2016), Visium (Ståhl et al., 2016), fluorescent *in situ* sequencing (FISSEQ) (Lee et al., 2015), and GeoMx (Zollinger et al., 2020), exist and have been comprehensively reviewed by Asp et al. (2020). This review focuses on a subset of these platforms that has demonstrated potential to become widely accessible because of present or planned commercial development.

10x Genomics Visium

First available in late 2019, the 10x Genomics Visium platform is the successor to the technology first known as Spatial Transcriptomics (Ståhl et al., 2016). The foundation of the Visium technology is the spatial gene expression slide, a glass microscope slide containing four regions on which tissue can be mounted for processing, imaging, and analysis. Each region measures 6.5 × 6.5 mm, limiting the size of the tissue and potentially requiring specimens to be trimmed or split over multiple capture areas. Preprinted within each tissue region are oligonucleotide probes arranged into an array of 5,000 features. Each probe is composed of a spatial barcode that is unique to an individual feature on the slide, a unique molecular identifier, and a poly(dT) region to facilitate the capture of poly(A)-tailed mRNA diffusing toward the slide surface from the overlaid tissue upon permeabilization (Ståhl et al., 2016). *In situ* synthesis of poly(A)-primed cDNA occurs directly on the slide and is followed by library generation. Once the libraries are made, short-read Illumina sequencing generates coding transcriptome data in which each read can be mapped back to the coordinates specified by its spatial barcode.

By decreasing the diameter of each feature and increasing the number of features fivefold, thus reducing the center-to-center distance between adjacent features, the commercially available product offers improved resolution over previous versions of the technology. Although this increases the coverage density, the current feature diameter of 55 μm does not yet allow single-cell resolution. An additional layer of information can be obtained by staining the tissue with hematoxylin and eosin (H&E) or fluorescently labeled antibodies before sequencing, although immunofluorescent staining may decrease the number of transcripts detected downstream (10x Genomics, 2020). Until recently, the Visium technology had only been compatible with fresh-frozen tissue due to a requirement for high-quality RNA; thus, image quality and histomorphological assessment of the tissue sections suffered. However, Villacampa et al. (2020) successfully applied this technology to FFPE tissue specimens. Although the study showed a significant correlation in the gene expression levels between fresh-frozen and FFPE specimens, fewer unique genes and unique molecules were detected from the FFPE specimens (Villacampa et al., 2020).

NanoString GeoMx DSP

The NanoString GeoMx DSP technology was commercially launched in the spring of 2019. The DSP workflow is designed to analyze spatial expression of a preselected panel of proteins or RNA species within user-defined regions of interest (ROIs) in a tissue section (Zollinger et al., 2020). The tissue is first permeabilized to expose the RNA, which is hybridized *in situ* to gene-specific probes containing a photocleavable molecular barcode. Following probe hybridization, the tissue is stained with fluorescently conjugated antibodies to reveal morphology and/or molecular markers of interest. DSP relies on fluorescent markers to visually guide ROI selection. ROIs may take the form of geometric shapes, contoured bands, or irregular masks derived from a fluorescent signal. ROIs can also be tiled to cover the entirety of a tissue specimen. Each ROI is individually exposed to UV light to liberate the photocleavable barcodes from the gene-specific probes. The cleaved barcodes are subsequently aspirated and identified with the NanoString nCounter system or by next-generation sequencing.

Conceptually, DSP is reminiscent of laser-capture microdissection (LCM), allowing a selection of specific regions to be analyzed. However, unlike LCM, DSP does not result in destruction of the tissue. Because this technology directly quantifies individual RNA molecules, up to 200 cells may be required per ROI to clear the limit of detection, although highly expressed transcripts have been detected in as few as 21 cells (Merritt et al., 2020). Curated panels of probes available from NanoString enable detection of up to 18,000 genes simultaneously. The probe-based chemistry also allows the detection of small RNA fragments, enabling high-confidence analysis of tissue sections from archival FFPE samples (Reis et al., 2011). Uniquely, DSP does not require specialized slides or tissue-mounting procedures. Furthermore, the usable area of 14.6 × 36.2 mm allows retrospective use of most tissue specimens mounted on standard microscope slides. It is also notable that gene expression is mapped back to a specific ROI rather than to a precise coordinate within the tissue. This limitation can functionally decrease the spatial resolution as the size of the ROI increases or if complex ROI shapes are used. For applications requiring greater resolution and precision, NanoString recently announced a new spatial molecular imaging platform that will allow spatial detection of up to 1,000 transcripts within an individual cell (NanoString Technologies, 2020). The platform, which will also be compatible with FFPE samples, is expected to be available in 2022.

FISSEQ

First demonstrated in 2014 and currently being commercialized by ReadCoor, FISSEQ involves sequencing by ligation of cDNA amplicons, which have been crosslinked to the cellular matrix (Lee et al., 2014). The procedure begins with *in situ* reverse transcription of RNA molecules using random hexamer primers. The resulting cDNA is first crosslinked to the cellular environment and then circularized and amplified by rolling-circle amplification. Sequencing by ligation is then used to decode up to 30 bases of the amplicon sequence over several rounds of fluorescent probe hybridization. This methodology has been demonstrated to distinguish up to 8,100 distinct RNA entities (Lee et al., 2014). The approach can also be adapted for detection of coding variants and splice variants through the use of targeted primers. Although most extensively validated in cultured cells, the technique has been shown to be compatible with both fresh-frozen and FFPE tissue sections (Lee et al., 2015). In late 2020, ReadCoor was acquired by 10x Genomics, following the announcement of the RC2 product line for the simultaneous detection of DNA, RNA, and protein in a tissue using FISSEQ (ReadCoor,

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Table 1. Comparison of commercially available spatial transcriptional profiling platforms

	10x Genomics Visium	NanoString GeoMx DSP
Tissue compatibility	best compatibility with fresh-frozen	fresh-frozen and FFPE
RNA quality	RNA integrity number (RIN) $\geq$ 7 required	no requirement
Tissue preparation	mounting on a specialized gene expression slide	mounting on a standard microscope slide
Tissue size	maximum of 6.5 × 6.5 mm per capture area	maximum of 14.6 × 36.2 mm
Geographic bounds of detection	feature-based detection within the full capture area	user-defined regions of interest (ROIs)
Cellular resolution	approx. 10 cells/feature	approx. 20–200 cells/ROI
Direct RNA detection	no—cDNA amplification required	yes
Concurrent protein detection	yes—up to 3 multiplexed antibodies	yes—up to 3 multiplexed antibodies
Concurrent H&E staining	yes	no
Amount of data generated	whole coding transcriptome	panel-based detection
Type of RNA captured	poly-adenylated RNA only	any RNA to which a probe can be designed
Instruments required	fluorescent and bright-field imaging system; Illumina Next Generation Sequencing (NGS) platform	NanoString GeoMx Digital Spatial Profiler; NanoString nCounter or Illumina NGS platform
Sequencing depth required	min. of 50,000 read pairs per spot covered by tissue	no NGS required if using the nCounter instrument

2020). At the time of this writing, the commercial product had not yet been fully demonstrated and no release date had been set.

In situ sequencing using padlock probes

In situ sequencing (ISS) was first described in 2013 (Ke et al., 2013) in one of the earliest demonstrations of *in situ* spatial transcriptional profiling. The technique uses padlock probes, single-stranded DNA probes in which the regions of target complementarity are interrupted by an intervening non-specific sequence. Thus, following *in situ* reverse transcription, hybridization of the padlock probe to a cDNA target creates a loop of DNA, which can be ligated and amplified by rolling-circle amplification. Sequencing by ligation is then employed to determine the sequence of the DNA amplicons. The influence of this strategy is apparent in many subsequently developed techniques, including FISSEQ, described earlier. However, unlike the untargeted approach of FISSEQ, ISS requires deliberate target selection and probe design. In addition, ISS can resolve relatively few targets; improvement on the technology called HybISS (hybridization-based ISS) was able to resolve 119 targets in fresh-frozen brain tissue (Gyllborg et al., 2020). Nevertheless, the technology allows high resolution with a high signal-to-noise ratio and lends itself to the possibility of automation (Maïno et al., 2019). ISS was commercialized by Cartana, which offered reagent kits and probe panels before being acquired by 10x Genomics in late 2020.

CONSIDERATIONS FOR EXPERIMENTAL DESIGN

The commercial availability of two end-to-end spatial transcriptional profiling technologies, 10x Genomics Visium and NanoString DSP, provides greater opportunity for the use of these specific platforms in most research settings. Fundamental differences between the two (Table 1) make each platform better suited for specific experimental applications. The ability to select ROIs for analysis makes DSP ideal for interrogating rare cell populations or comparing transcriptional profiles based on known tissue markers. Thus, the DSP platform has a greater focus on hypothesis testing, providing precise information pertinent to the investigator's interest. In contrast, no user-defined constraints are provided for data collection on the Visium platform, with greater focus on untargeted, discovery-driven approaches. Because the Visium platform generates sequencing data for all transcribed genes in a sample, the platform is species agnostic and is suitable for use with any organism provided that a reference genome is available for read mapping. In contrast, the current commercially available panels for the DSP platform are designed against human and mouse genes. Despite these differences, the two platforms can be viewed as complementary.

Before experimentation, consultation and/or collaboration with an anatomic pathologist is highly encouraged to ensure that the tissue is of sufficient quality for downstream processing. For instance, tissues with substantial regions of hemorrhage are problematic for the DSP platform, because the intrinsic autofluorescence of red blood cells can obscure antibody-specific fluorescence and confound ROI identification. Furthermore, relying on the experience of an anatomic pathologist allows more comprehensive interpretation of the molecular data within the necessary histopathological context. For example, expert pathological assessment can discern cell aggregates from multinucleated giant cells or identify focal regions of necrosis that may produce high background or decreased barcode or Unique Molecular Identifier (UMI) counts.

Platform flexibility

The 10x Genomics Visium platform offers a post hoc targeted enrichment protocol in which cDNA molecules of interest are captured from the Visium sequencing library, using biotinylated gene-specific oligonucleotide probes, and collected using streptavidin beads. After washing, the enriched molecules of interest are amplified and purified, resulting in a targeted library that is fully barcoded and indexed. The molecules are then ready for sequencing using Illumina sequencing technology.

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NanoString DSP allows the addition of custom, gene-specific barcoded probes to the assay, enabling the spatially resolved quantification of genes not represented on the commercially available panels. The platform also affords great flexibility in the shapes and quantities of ROIs selected for analysis, allowing a multitude of investigator-specific experimental strategies. In addition to antibody-based ROI selection, RNAscope probes can be used to identify ROIs within the tissue based on gene expression. Finally, as described earlier, the use of UV illumination to release photocleavable barcodes is a unique feature of the assay that leaves the tissue intact for subsequent histological or molecular analyses if desired.

Cellular resolution

An important limitation of both the 10x Genomics Visium and the NanoString DSP technologies is that the limit of detection ranges from tens to hundreds of cells within a given region. This can prove confounding in a tumor environment in which cancer cells are often directly adjacent to intermixed immune and stromal cells. Although these companies have made substantial effort to improve resolution, alternative strategies with higher resolution have been developed. These include imaging-based strategies such as seqFISH (Shah et al., 2017) and MERFISH (Moffitt et al., 2016), which use high-resolution microscopy to resolve fluorescently labeled probes hybridized directly to RNA targets. For profiling of large tissue sections, Slide-seq, a recently developed capture-based strategy, may be more suitable. Slide-seq uses a slide coated with uniquely barcoded, 10 μm beads onto which a tissue sample is overlayed (Rodriques et al., 2019). Slide-seq therefore provides a workflow similar to that of 10x Genomics Visium but with a smaller feature size that approaches single-cell resolution. Thus, experimental designs requiring high-resolution spatial transcriptional profiling may employ various established, albeit not commercially available, techniques.

SPATIAL DISSECTION OF THE TUMOR MICROENVIRONMENT

The solid tumor microenvironment is composed of various clonal populations of malignant cells, immune cells, non-immune stromal cells, extracellular vesicles, matrix proteins, metabolites, and chemical mediators that function in concert to promote tumor growth and disease progression (Quail and Joyce, 2013). Although bulk RNA-seq and, more recently, scRNA-seq have provided insight into the molecular underpinnings of the tumor microenvironment across an array of malignancies, these methods fail to elucidate the organizational and architectural details of the tumor tissue. On its own, spatial transcriptional profiling addresses this critically important knowledge gap. The importance of spatially resolved gene expression was illustrated in a study by Berglund et al. (2018), in which prostate cancer cells in the center of a tumor were found to be transcriptionally distinct from those at the periphery. Notably, the study was able to detect differentially enriched transcriptional pathways promoting altered metabolism, inflammation, cell motility, and cell proliferation within different regions of the tumor (Berglund et al., 2018). Because the cells were not histologically distinct, the observed differences would not have been appreciated during routine histopathological examination. In a separate study, spatial transcriptional profiling of the metastatic melanoma microenvironment revealed the coexistence of distinct region-specific melanoma gene expression profiles and demonstrated that histologically defined lymphoid areas adjacent to tumor cells had transcriptional profiles that differed from those of distal lymphoid regions within the same tissue (Thrane et al., 2018). Spatial transcriptional profiling of aggressive B cell lymphoma identified gene signatures that discerned dark-zone from light-zone germinal centers (Tripodo et al., 2020). Moreover, the authors demonstrated the prognostic value of these dark-zone and light-zone gene expression signatures.

Identifying and localizing transcriptionally distinct cellular populations

Various computational methods have been developed to deconvolute both bulk RNA-seq and scRNA-seq datasets to identify distinct cellular populations (Aran et al., 2017; Newman et al., 2015, 2019; Yoshihara et al., 2013; Abdelaal et al., 2019; Menden et al., 2020). Likewise, over the past several years, numerous metagene signatures and pathway analysis algorithms have been developed to extract biologically and clinically relevant information from complex gene expression datasets. The integration and application of these widely adopted tools to spatial transcriptional profiling datasets have provided a more detailed understanding of the complex heterogeneity of tumor tissues. Svedlund et al. (2019) used ISS to map regional variation in the expression of intrinsic subtype gene signatures, OncotypeDX recurrence scores, and immune infiltrates in breast cancer specimens. The creation of these spatially derived OncoMaps highlighted the clonal heterogeneity present within and across the tumor specimens analyzed. Deconvolution and pathway analysis of spatial transcriptional profiling data derived from multifocal prostate cancer revealed the distribution and organization of regions enriched in non-immune stromal cells, normal glandular epithelium, prostatic intraepithelial neoplastic glands, carcinoma cells, and immune cells (Berglund et al., 2018).

Although useful, the aforementioned cellular deconvolution tools were not designed to identify the multitude of tissue-specific cellular subsets present within a given specimen; thus, these tools provide broad, generalizable cell-type categories. The integration of scRNA-seq and spatial transcriptional profiling has circumvented this limitation by generating contextually relevant, tissue-specific cellular gene expression profiles that are directly applicable to the spatial profiling data. Several methods have been developed to integrate scRNA-seq and spatial transcriptional profiling datasets for the purposes of deconvoluting cellular mixtures. These include RCTD (robust cell type decomposition) (Cable et al., 2020), SPOTlight (Elosua et al., 2020), SpatialDecon (Danaher et al., 2020), JSTA (joint cell segmentation and cell-type annotation) (Littman et al., 2020), DSTG (deconvoluting spatial transcriptomics data through graph-based convolutional networks) (Su and Song, 2020), cell2location (Kleshchevnikov et al., 2020), SpaGE (spatial gene enhancement using scRNA-seq) (Abdelaal et al., 2020), and stereoscope (Andersson et al., 2020). This integrated approach has proven successful in identifying, characterizing, and localizing rare and/or unique cellular populations within tumor tissues. For example,

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scRNA-seq identified various subpopulations of carcinoma cells, ductal epithelial cells, immune cells, and fibroblasts in pancreatic ductal adenocarcinoma (PDAC) tissue, and subsequent spatial transcriptional profiling localized these cell populations to distinct regions within the tissue and provided information regarding spatial coenrichment of immune cells and cancer cells (Moncada et al., 2020). In a zebrafish model of melanoma, an integrated scRNA-seq and spatial transcriptional profiling approach was used to identify and characterize a unique subpopulation of tumor and muscle cells that specifically localized to the tumor-normal interface, suggesting a spatiotemporal transcriptional regulation of genes involved in melanoma invasion (Hunter et al., 2020). Sharma et al. (2020) used a combination of scRNA-seq and spatial transcriptional profiling to reveal the similarities between the fetal liver and the hepatocellular carcinoma microenvironment and discovered fetal-like subsets of endothelial cells and tumor-associated macrophages.

Spatially resolved interrogation of tumor niches

Tumor tissues are also known to contain specialized cellular niches that promote tumor growth and disease progression (Butler et al., 2010; Peinado et al., 2017; Plaks et al., 2015). Spatial transcriptional profiling is suited to interrogate different niche compartments and can provide a great deal of information regarding the cellular composition, transcriptional programs, specific function, and location of these niches within the tissue. Employing a multiparametric approach consisting of scRNA-seq, spatial transcriptional profiling, and MIBI, Ji et al. (2020) identified a unique, tumor-specific keratinocyte population residing in an immunosuppressive fibrovascular niche at the leading edge of cutaneous squamous cell carcinomas. The authors further demonstrated that the tumor-specific keratinocytes may increase the malignant potential of adjacent normal cells and contribute to tumor progression.

Understanding the function of the bone marrow niche and leveraging that information to investigate metastatic disease is an active area of research with significant biological and clinical implications. scRNA-seq profiling of resident cells isolated by LCM of spatially isolated 1.45 mm^2 regions of murine bone marrow revealed that the bone marrow is a heterogeneous collection of unique niches whose organization is dictated by regionally restricted differential cytokine expression (Baccin et al., 2020). Although this study did not use the spatial transcriptional profiling platforms discussed in this review, the findings demonstrate the importance of such an approach in tumor niche biology. The increased spatial resolution afforded by technologies such as 10x Genomics Visium and NanoString DSP positions these, and other high-resolution spatial transcriptional profiling platforms, as the most obvious platforms to use for detailed investigations into tumor niches.

Relationship between microenvironment and treatment response

The ability to confidently identify tumor clones and interrogate tumor-specific cellular processes can be beneficial to determining therapeutic vulnerabilities. In an analysis of a prostate tumor, Wang et al. (2020) applied spatial transcriptional profiling to assess metabolic networks, which often demonstrate spatial variability because of gradients of nutrients and metabolites. *In silico* modeling of the data then identified metabolic vulnerabilities that could be targeted by known small-molecule compounds. In cases for which pre- and post-treatment samples are available, spatial transcriptional profiling can provide unique insight into treatment response. A recent study discovered that treating patients with prostate cancer using high-dose-rate brachytherapy resulted in region-specific responses in the tumors, hallmarked by the increased expression of genes associated with immune activation and inflammation (Keam et al., 2020). In an excellent demonstration of the potential of spatial transcriptional profiling, the authors showed that the observed response was driven specifically by tumor-adjacent stroma and made key observations about the densities of, and distances between, responding immune cell subsets (Keam et al., 2020). In comparing PDAC specimens from treatment-naive versus neoadjuvant-treated patients, Hwang et al. (2020) used an integrated approach to highlight the phenotypic plasticity of the tumor cells in response to therapy and showed that distinct immune niches were differentially associated with basal-like versus classical-like tumor cells.

Spatially resolved T cell clonality

Understanding the clonal dynamics of T cells in both the intratumoral and the peritumoral microenvironments has biological and clinical significance. Tu et al. (2019) described a capture-based enrichment protocol to recover T cell receptor (TCR) transcripts from barcoded 3′ scRNA-seq libraries and subsequently performed targeted TCR sequencing to investigate T cell clonality. The method described is conceptually similar to the 10x Genomics Visium post hoc targeted enrichment protocol. This suggests that with assay optimization and accessibility to high-quality fresh-frozen tissues from which cDNA molecules of sufficient length can be reverse transcribed, spatially resolved TCR repertoire analysis may indeed be achievable. This approach is not applicable to FFPE specimens due to the decreased nucleic acid integrity associated with fixation and paraffin embedding. Given the similarities between TCR and B cell receptor (BCR) sequencing, the method described by Tu et al. (2019) can presumably be extended to BCR sequencing to spatially assess both T cell and B cell clonality. The implications of this approach are profound, especially as it relates to immuno-oncology.

SPATIAL RESOLUTION OF FUSION GENES AND ALTERNATIVELY SPLICED TRANSCRIPTS

Oncogenic fusion genes are observed in upward of 25% of soft-tissue tumors (Bridge, 2014) and in various other tumor types, including prostate carcinoma (e.g., *TMPRSS2-ERG*) (Tomlins et al., 2005) and lung cancer (e.g., *EML4-ALK*) (Soda et al., 2007). Differences in the relative expression levels of fusion transcripts can impose various transcriptional programs and cellular functions that act both cell autonomously and non-cell autonomously. For instance, the abundance of the *EWSR1-FLI1* oncogenic fusion gene, the product of the diagnostic translocation in Ewing sarcoma, differentially regulates various processes in Ewing sarcoma cells, including stemness, differentiation, motility, proliferation, and cell-matrix interactions, and thus contributes

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to the cellular heterogeneity of this disease (Franzetti et al., 2017). Although molecular cytogenetics have been used for decades to identify translocations at single-cell resolution, these approaches are incapable of simultaneously generating spatially resolved expression profiles for thousands of genes.

Friedrich and Sonnhammer (2020) developed a computational method, STfusion, to infer the expression alternatively spliced fusion transcripts from data generated on the 10x Genomics Visium platform. Using the Berglund dataset (Berglund et al., 2018), the authors revealed the spatial distribution of *SLC45A3-ELK4* chimeric transcripts in prostate cancer tissues (Friedrich and Sonnhammer, 2020). Because the algorithm works by quantifying imbalances in the abundance of 3′ sequencing reads from the fusion partners in question, it is plausible that this tool can also be leveraged to spatially resolve the expression of oncogenic fusion genes.

The Cancer Transcriptome Atlas (CTA) assay for the NanoString DSP platform relies on counting barcoded probes that directly hybridize along the length of a given mRNA molecule, with an average of 5 probes per gene product. The counts of individual probes for a given gene product are then combined to generate an expression value. By quantifying the representation of individual barcoded probes corresponding to *PTEN*, Merritt et al. (2020) discovered probe dropout in CCRF-CEM cells and interpreted the data as evidence of alternative splicing. CCRF-CEM cells are a *PTEN* null line; thus, the observed probe dropout may reflect alternative splicing or a different deleterious aberration, illustrating that caution must be exercised when interpreting these indirect inferences. Furthermore, because this assay does not generate actual sequence data, inferred fusions would require confirmation via an independent method, such as targeted amplicon sequencing, digital droplet PCR, or fluorescent *in situ* hybridization. Moreover, inferring deleterious mutations in this manner is specific to the CTA assay because the more comprehensive Whole Transcriptome Atlas assay is designed to query approximately 18,000 genes using a single barcoded probe per gene.

As mentioned earlier, the DSP platform allows flexibility in experimental design that can be exploited to support the spatially resolved quantification of fusion genes and alternatively spliced transcripts. Conceptually, the addition of exon-specific barcoded hybridization probes that both flank and physically span the junction or junctions in question should be sufficient to detect both fusions and alternatively spliced transcripts *in situ*. This approach has been used to successfully detect the expression of fusion genes in FFPE material using earlier generations of the NanoString gene expression technology (Guerreiro Stucklin et al., 2019; Reguart et al., 2017). However, at the time of this writing, there was a lack of available published or preprint data to objectively evaluate the efficacy and reproducibility of applying such an approach to spatial transcriptional profiling. Nonetheless, given the immense amount of interest in this technology, it is anticipated that such datasets will emerge in the near future.

INFERRING COPY NUMBER ALTERATIONS FROM SPATIAL TRANSCRIPTIONAL PROFILING DATA

Various informatic methods infer DNA copy number alterations (CNAs) from scRNA-seq datasets, such as inferCNV (Tirosh et al., 2016), hidden markov model integrated Bayesian approach for detecting copy number variants (CNVs) and LOHs from single-cell RNA-seq data (HoneyBADGER) (Fan et al., 2018), single cell inferred chromosomal copy number variation (sciCNV) (Mahdipour-Shirayeh et al., 2020), and clonal architecture with genomic clustering and transcriptome profiling of single tumor cells (CACTUS) (Darvish Shafighi et al., 2020). STARCH (spatial transcriptomics algorithm reconstructing copy-number heterogeneity) is a newly developed method designed to infer CNAs from spatial transcriptional profiling data (Elyanow et al., 2020). Both inferCNV and STARCH have demonstrated success in revealing CNAs from simulated and experimentally derived spatial profiling data (Elyanow et al., 2020). Similar to the implementation of cellular deconvolution tools for use with spatial data, this study demonstrated that the existing copy number analysis tools designed for scRNA-seq datasets could potentially be used to reveal CNAs within their spatial context.

LOOKING FORWARD

Although spatial transcriptional profiling is still in its infancy, the field is already looking past this approach as a standalone methodology. The era of integrated spatial multiomics is quickly being ushered in as the number of studies combining spatial transcriptional profiling with scRNA-seq and/or highly multiplexed spatial proteomic profiling continues to increase. Although not covered in this review, the NanoString DSP platform can also be used for highly multiplexed immunohistochemistry, enabling the quantitative spatially resolved assessment of up to ~100 molecularly barcoded antibodies on a single tissue. Using both protein and RNA assays on serial sections allows an integrated multiomic approach, albeit on separate sections. The development of a truly integrated multiomic platform in which different molecular species are assayed in a single specimen may soon become a reality with the announcement of ReadCoor's RC2 spatial profiling platform. The multiomic FISSEQ-based platform promises high-resolution spatial profiling of DNA, RNA, and protein within a single sample (ReadCoor, 2020). In addition, a recent publication debuted a spatially resolved microRNA profiling method, allowing researchers to look beyond the coding transcriptome and begin to investigate transcriptional regulation *in situ* (Nagarajan et al., 2020).

As this convergence of technologies and methodologies begins to take place, new computational tools will need to be developed to harmonize these data across the multiple platforms to allow integrated data analysis and visualization. Such integration pushes the informatic needs of the research community well beyond the standardized data processing pipelines of a commercial vendor. Rather than borrowing tools from single-cell analysis, true multiomic integration will require the development of informatic tools that include image analysis and machine learning to better understand complex tumor biology.

Nonetheless, systematic head-to-head comparisons will have to be conducted to objectively identify the relative strengths, weakness, and biases of any individual platform and computational method. It is crucial that a series of benchmark datasets and best practices are established to facilitate reproducible

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and transparent evaluation of each computational method. To this end, a searchable database complete with raw data, processed data, associated clinical and experimental annotations, and image overlays will need to be created and managed to increase the utility of these data and analyses to the broader cancer research community.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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