Histopathology

Histopathology 2024, **84**, 577–586. DOI: 10.1111/his.15093

REVIEW

Spatial transcriptomics and the anatomical pathologist: Molecular meets morphology

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Pang J-M B, Byrne D J, Bergin A R T, Caramia F, Loi S, Gorringe K L & Fox S B (2024) *Histopathology* **84**, 577–586. https://doi.org/10.1111/his.15093

Spatial transcriptomics and the anatomical pathologist: Molecular meets morphology

In recent years anatomical pathology has been revolutionised by the incorporation of molecular findings into routine diagnostic practice, and in some diseases the presence of specific molecular alterations are now essential for diagnosis. Spatial transcriptomics describes a group of technologies that provide up to transcriptome-wide expression profiling while preserving the spatial origin of the data, with many of these technologies able to provide these data using a single tissue section. Spatial transcriptomics allows expression profiling of highly specific areas within a tissue section potentially to subcellular resolution, and allows correlation of expression data with morphology, tissue type and location relative to other structures. While largely still research laboratory-based, several spatial transcriptomics methods have now achieved compatibility with formalin-fixed paraffin-embedded tissue (FFPE), allowing their use in diagnostic tissue samples, and with further development potentially leading to their incorporation in routine anatomical pathology practice. This mini review provides an overview of spatial transcriptomics methods, with an emphasis on platforms compatible with FFPE tissue, approaches to assess the data and potential applications in anatomical pathology practice.

Keywords: anatomical pathology, spatial transcriptomics, surgical pathology

Introduction

Pathologists have always been progressive in the introduction of ancillary tests to aid a patient's diagnosis and/or as predictive or prognostic markers. What starts as innovative cutting-edge research technologies such as immunohistochemistry, fluorescent and brightfield *in-situ* hybridisation and mutation testing, over time, are incorporated into standard-of-care diagnostic pathology. Thus, all these techniques are currently used for diagnosis and to identify aberrations that are biomarkers for targeted agents in many cancers.[1–5] They have also supported new ideas of tumour classification, as reflected in the WHO Classification of Tumours ('Blue Books') series. Furthermore, it is probable that with the development of techniques to study biological molecules in totality ('-omics') beyond the genome, such as the epigenome, transcriptome, proteome and metabolome, identification of additional biomarkers for disease are likely to emerge.

More recently a novel series of technologies which fuses some 'omics' with spatial location, so called 'spatial-omics', has become a powerful tool in unravelling biological processes. Indeed, spatial transcriptomics, Nature's method of the year in 2020[6] which, unlike bulk RNA sequencing where gene expression is an average across the tissue, allows up to whole-transcriptome expression to be linked to a specific

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spatial location with up to single-cell resolution. From a histopathologist's perspective it can easily be imagined that these techniques could become the ultimate modality to extract multiparameter diagnostic and prognostic/predictive information that is increasingly required from smaller and smaller biopsies. The potential of spatial transcriptomics can be seen in cancer where the assessment of numerous genotypical and phenotypical biomarkers located in neoplastic and non-neoplastic tumour components, including inflammatory cells and mesenchymal cells, are all important determinants of disease outcome.[7–15]

This review is targeted at diagnostic pathologists rather than biomedical researchers to introduce spatial transcriptomics, the most mature of the spatial 'omic methodologies', and therefore most likely to have immediate impact in clinical pathology.

Overview of Spatial Transcriptomics Methods

There are many published methods for capturing mRNA expression in a spatial context with their differing bioinformatic approaches for data analysis.[16–23] In general, spatial transcriptomic technologies can be arbitrarily divided into sequencing-based methods and imaging-based methods (Figure 1). Some of these methods can be combined with limited protein expression analysis of up to a few hundred protein targets.[22]

While a multitude of assays are available for fresh frozen tissue in the research setting, incorporation of spatial gene expression technology into anatomical pathology workflow requires reproducible, standardised assays compatible with formalin-fixed, paraffin-embedded (FFPE) tissue, which are more restricted in number (Table 1). FFPE-derived RNA is of generally poorer quality than fresh tissue-derived RNA, resulting in a lower proportion of reads mapping to coding regions and significant differences in the detected expression of certain transcripts.[24]

SEQUENCING-BASED METHODS

These methodologies use sequencing to quantify transcripts with spatial location provided by area of interest selection, *in-situ* sequencing or by barcoded array.

Area of interest

Microdissection, either by laser microdissection or by automated mechanical microdissection, such as with the AVENIO Millisect system,[25] enables dissection of small areas of tissue or single cells from a known location followed by single-cell or single-nucleus RNA sequencing. This allows whole-transcriptome analysis at potentially single-cell resolution, but is tedious for analysis of large tissue areas. Nonetheless, several groups have used this approach in FFPE tissue to identify differential gene expression associated with progression from pre-invasive precursors to invasive carcinoma, including of the

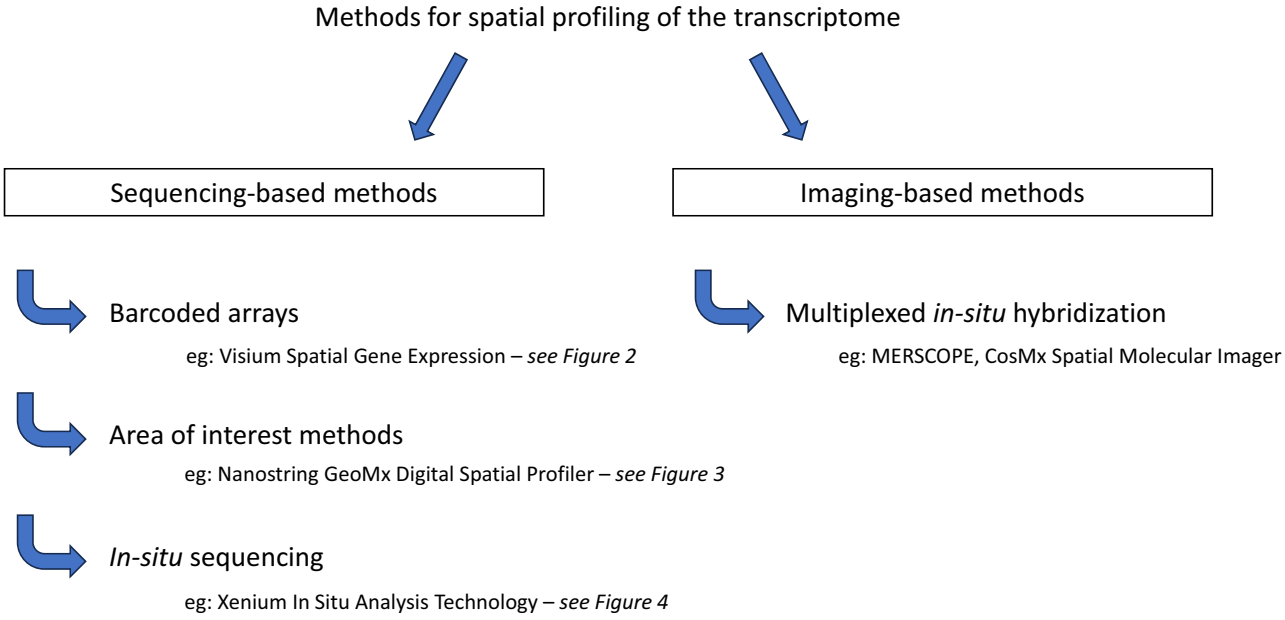

Figure 1. Tree showing an arbitrary classification of spatial transcriptomics methods with examples of FFPE-compatible assays.

Table 1. FFPE-compatible spatial gene expression assays

Assay	Type of assay	Area assessed	Target range	Resolution	Concurrent protein detection
Visium spatial gene expression[41]	Barcoded array	6.5 and 11 mm^2 options	Transcriptome-wide	55 μm spots, centre to centre interspot distance 100 μm	31 proteins related to immune cell and tissue compartments (Visium spatial protein expression human immune cell profiling panel for FFPE)
Nanostring GeoMx digital spatial profiler[32]	Digital spatial profiling	Potentially whole section, minimum UV illumination area 5 μm^2	Transcriptome-wide	Minimum UV illumination area 5 μm^2. UV illumination area recommended to encompass a minimum of 50–200 small-sized cells for RNA detection and 10–20 small-sized cells for protein detection	Panel of up to 96 proteins on the nCounter system
Laser capture microdissection	Laser microdissection followed by single cell RNA sequencing	Potentially whole section but in practice limited to small areas	Transcriptome-wide	Single cell	No
smFISH (single molecule RNA fluorescence *in-situ* hybridisation)[62]	FISH	Potentially whole section, but requires analysis as individual high magnification fields	Targeted, limited to a few genes per assay	Subcellular	No
RNAscope[63,64]	ISH	Potentially whole section, but requires analysis as individual high magnification fields	Targeted, up to 2 genes for brightfield ISH, up to 4 genes for FISH	Subcellular	No
MERSCOPE[44]	FISH	1 cm^2	Panels of up to 500 genes	Subcellular	Potentially yes
CosMx SPATIAL MOLECULAR Imager[43]	FISH	1 cm^2	Up to 1000 RNA targets	Subcellular	Co-detection of 100 proteins
Xenium *in-situ* analysis technology[37]	*In-situ* sequencing	12 × 24 mm	Panel-based, up to 500 targets	Subcellular	No

uterine cervix,[26] lung[27] and breast,[28] and to identify gene expression profiles associated with disease outcomes including in pancreatic adenocarcinoma[29] and advanced prostate carcinoma.[30]

The Nanostring GeoMx Digital Spatial Profiler assay (Figure 2) also uses region of interest selection to confer positional information. In this method, a tissue section is probed by up to four fluorescent markers, e.g. a pan-cytokeratin and immune cell markers, to delineate tissue compartments and identify cell types of interest. Probes to the transcriptome are added to the slide, which are themselves coupled to indexing sequences unique to the target by a UV photocleavable linker. Using the image generated by the fluorescent markers, specific regions of interest are selected ranging from 5 μm^2 to 660 × 785 μm.[31] Regions of interest can be manually delineated or selected based on tissue type using marker expression. The tissue can also be analysed spatially in a grid format or based on distance from an index structure. UV-light is applied to the regions of interest to release the cleaved indexing oligonucleotides, which are then aspirated off the slide and quantified on the NanoString nCounter system or on an Illumina sequencer, giving expression data for the specific area.[32] This method is FFPE tissue-compatible and allows protein co-detection for up to 96 protein targets using panels designed for the nCounter system and potentially hundreds of targets using panels designed for sequencing platforms.[32]

In-situ sequencing

In *in-situ* sequencing-based methods, mRNA is reverse-transcribed to cDNA within an intact tissue section to preserve spatial location. The cDNA is targeted by probes which are then amplified. Sequential ligation and imaging of fluorescent labelled probes to the target sequence allows detection of a unique fluorescent signal for the transcript (Figure 3).[33–36]

In-situ sequencing methods are suitable for FFPE tissue, and offer subcellular resolution.[19] Random primers allow potential whole-transcriptome coverage and detection of novel sequences; although with lower detection efficiency per transcript compared with transcript specific primers due to the high number of background sequences generated.[19]

Evolution of the technology has allowed direct targeting of RNA sequences, circumventing cDNA

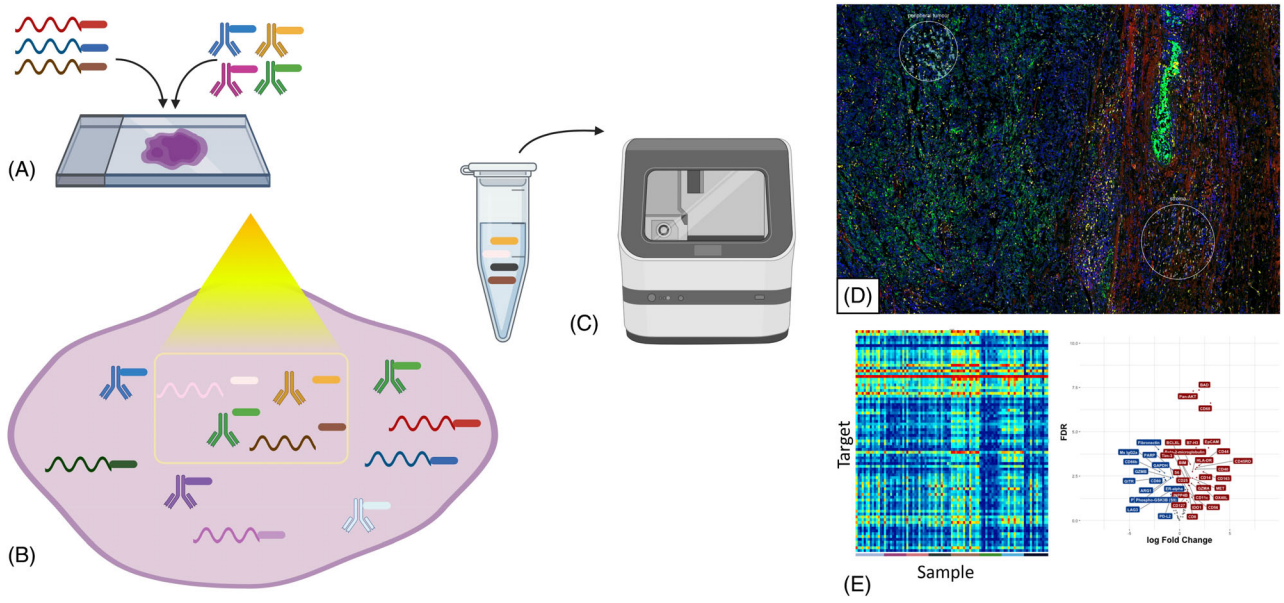

Figure 2. Working principle of the digital spatial profiler (Nanostring GeoMx Digital Spatial Profiler). **A,** Oligonucleotide-tagged antibodies and RNA probes are added to the tissue section. **B,** Antibodies and RNA probes bind to targets within the tissue. UV light is illuminated in the area of interest (represented by the yellow line in the figure), causing the oligonucleotide tags to be released. **C,** The released oligonucleotide tags are quantified using the nCounter platform or next-generation sequencing platforms. **D,E,** Example of an experiment using Nanostring GeoMx Digital Spatial Profiler. **D,** In this case, four fluorescent markers (pan-cytokeratin, CD3, CD68 and SYTO13 nucleic acid stain) delineate tissue compartments (shown as red, blue, green and yellow in the figure). In this experiment, two areas of interest (peripheral tumour and stroma) are manually selected (circles). The tissue can also be evaluated by tissue compartment or profiled across the tissue in a grid pattern or according to distance from a certain structure. UV light is illuminated over the selected areas of interest releasing tagged oligonucleotides which are quantified to generate expression data shown in the heat-map and volcano plot (**E**). FDR, false discovery rate.

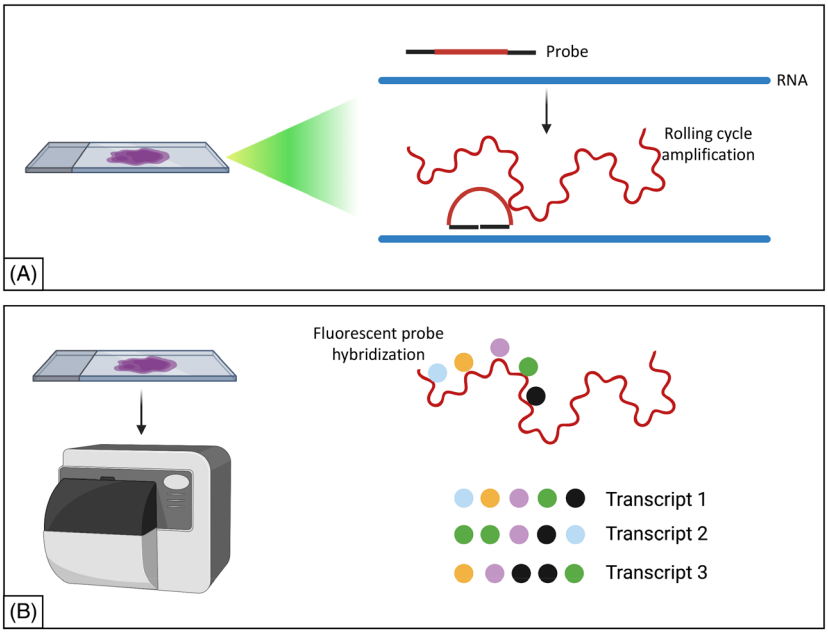

Figure 3. Working principle of *in-situ* sequencing, such as Xenium *in-situ* analysis technology. **A,** The tissue section is placed on the special analysis slide, and following pre-treatment probes bind to the target RNA sequence forming a padlock-like shape. The probes are amplified (rolling cycle amplification). **B,** Multiple rounds of fluorescent probe hybridisation, imaging and removal of fluorescent probes occur to generate a signal unique to the transcript.

synthesis and improving transcript detection efficiency.[34] An example of commercial *in-situ* sequencing technology is CARTANA, acquired by 10× Genomics, who offer targeted panels combined with detection and analysis software as Xenium *in-situ* analysis technology.[37]

Barcoded Methods

Barcoded assays include the method first described by Stahl *et al.*[38] as 'spatial transcriptomics', which has since evolved into the commercial product 10× Genomics Visium spatial gene expression (Figure 4). These methods use special slides covered by uniquely sequence-barcoded spots or beads to confer positional information. In the original version of the assay, an intact tissue section is placed on the Visium slide and primers associated with the spots or beads allow reverse transcription of mRNA contained in the tissue section. This process has been modified in the currently available assay, and now probes are hybridised to tissue on normal glass slides and the transcriptomic probes are then transferred onto the Visium slides within a CytAssist machine.[39] The resultant cDNA transcripts are then removed from the Visium slide and undergo sequencing with the software using the barcodes incorporated into the sequence to provide spatial positioning. These methods potentially capture a large proportion of the transcriptome and are FFPE tissue-compatible. Importantly, resolution relies upon the size and spacing of the barcoded spots and the size of the barcoded beads. For fresh frozen tissue a resolution of 0.5–0.8 μm[40] is possible. For FFPE tissue, the Visium spatial gene expression assay currently has a resolution of up to 55 μm, with increased resolution anticipated in future versions. Combined protein expression is possible using the Visium spatial protein expression human immune cell profiling panel for FFPE, which includes tissue compartment-specific proteins and 31 protein targets focused upon immune cell phenotype, including PD-1 and PD-L1.[41]

IMAGING-BASED METHODS

Imaging-based methods use *in-situ* hybridisation (ISH), similar to brightfield ISH and fluorescent ISH (FISH) used in routine histopathology. ISH-based methods initially targeted a limited number of transcripts, but development of sequential hybridisation and fluorescent signal combinations has allowed visualisation of thousands of RNA transcripts in parallel.

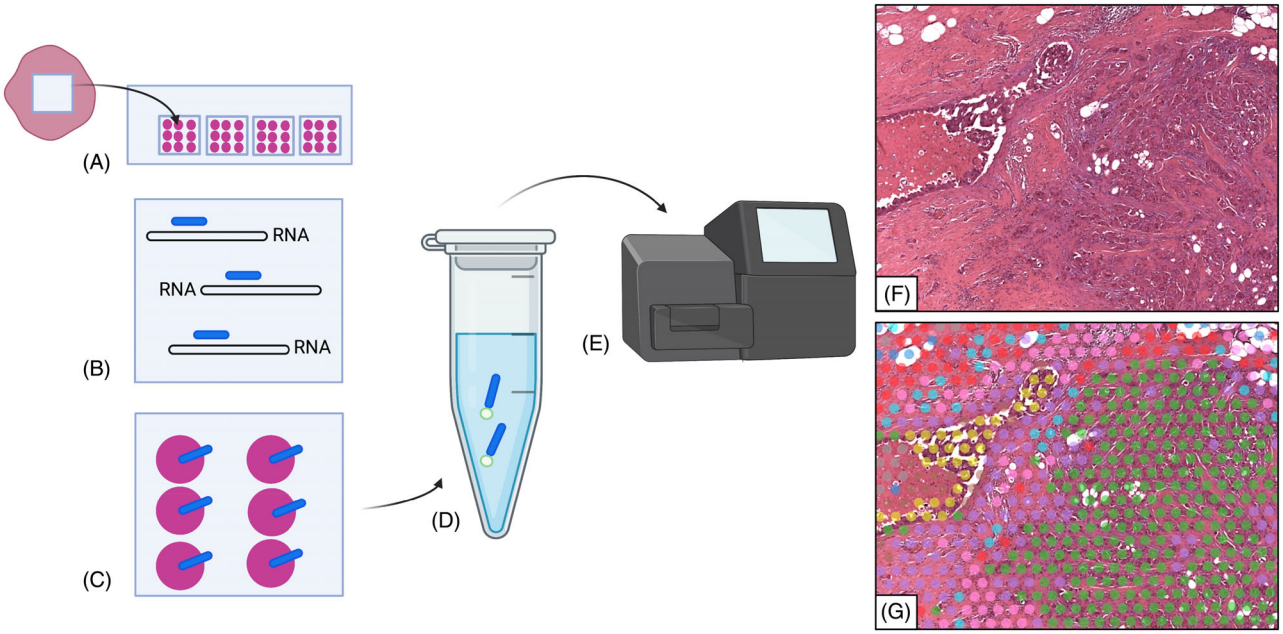

Figure 4. Working principle of barcoded array-based spatial transcriptomics, such as the Visium spatial gene expression assay. **A,** The tissue section containing the area of interest is placed on special slides bearing barcoded spots. **B,** RNA probes bind to target RNA and RNA is digested. **C,** Probes are captured by the spots and barcodes added. **D,** The barcoded sequences are eluted off the slide. **E,** Sequencing using next-generation sequencing platforms. **F–G,** Example of data output from the Visium spatial gene expression assay overlaid on the corresponding H&E-stained tissue section of breast carcinoma. The colour of the capture spots represent gene expression at the spot site.

ISH-based methods allow subcellular spatial resolution and are not subject to amplification bias, but can be limited by optical crowding by multiple transcripts, the ability to target only known transcripts and are low-throughput requiring multiple rounds of hybridisation and imaging. It is also subject to artefacts that also occur in other ISH applications, such as non-specific binding of probes and autofluorescence, in the case of FISH.[19,42] ISH is the basis of MERSCOPE (Vizgen) and CosMx spatial molecular imager (Nanostring), which streamline the process by performing automated rounds of hybridisation with potential protein co-detection, integrated with analysis and visualisation software.[43,44]

Analysis of spatial transcriptomic data

Given the recent development of different spatial transcriptomic technologies and varied contexts of its use, there is no set pipeline for analysis of spatial transcriptomics data. Some assays, such as 10× Genomics' Visium spatial gene expression and the Nanostring GeoMx digital spatial profiler provide software for very basic data analysis and visualisation. Results can be visualised as graphs, tables or as a spatial map, with some technologies allowing overlay of the data on a corresponding histological image.

However, in general, data analysis requires advanced bioinformatic skills and use of specific R and Python-language based tools for each step, recently reviewed by Liu *et al.*[18] The need for dedicated bioinformatics support is a major limitation in the implementation of such technologies in anatomical pathology, largely constraining their current use to exploratory analyses in the research realm.

Potential uses of spatial transcriptomics in anatomical pathology

Spatial transcriptomics data have the potential to improve understanding of normal and disease states and may have potential clinical use with improvements in cost, resolution, throughput, informatics and analysis (Figure 5). Potential clinical uses include, but are not limited to:

1. Establishing normal gene expression of specific tissue types in specific anatomical locations,[45] and when integrated with protein expression[46] providing an atlas of normal and abnormal expression, potentially contributing to the understanding of the

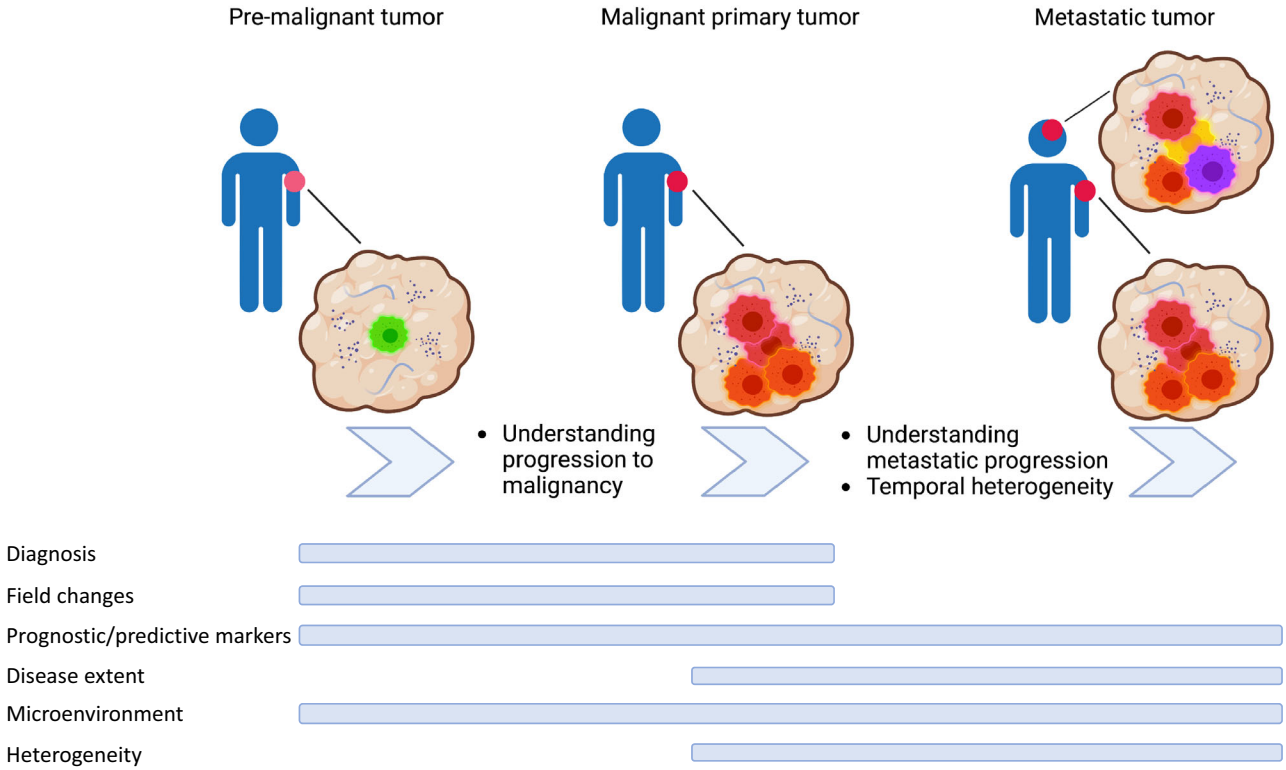

Figure 5. Potential uses of spatial gene expression assays in the course of malignancy.

pathogenesis of neoplastic and non-neoplastic conditions[47] and opening novel therapeutic areas.

2. Identification of changes in gene expression preceding morphological evidence of disease that may influence surveillance regimens and allow early detection of clinically relevant disease. For example, changes in gene expression in chronic active T cell-mediated renal transplant rejection have been identified by spatial transcriptomics not reflected by morphology-derived rejection scores, suggesting biological changes that are unable to be identified morphologically.[48]
3. Diagnosis and classification of disease, assessment of disease extent and assessment of prognostic and predictive biomarkers. Spatial transcriptomic profiles have been demonstrated to distinguish regions of pre-invasive carcinoma, invasive carcinoma and normal tissue in breast and prostate;[49,50] and between metastatic melanoma, lymphoid tissue and stroma in lymph nodes.[51] Stromal gene expression profiles have been reported to differ according to proximity to tumour epithelium,[50–52] and tumour microenvironment gene expression patterns in pancreatic ductal adenocarcinoma are reported to be associated with tumour differentiation and response to chemotherapy.[10] Spatial gene expression could replace quantitative immunohistochemistry assays, especially predictive biomarkers such as PD-L1 where interobserver concordance is particularly poor,[53] and may result in better correlation with treatment outcomes and relieve pathologists of the need to score challenging immunohistochemical assays that have different scoring methods and cut-points. The ability to assess multiple targets in a single assay is particularly useful for biopsies with limited tissue requiring a panel of markers and with further development, spatial transcriptomics/proteomics technology may eventually replace immunohistochemistry for expression profiling.
4. Identifying heterogeneity within a tumour. Gene expression may confirm morphological heterogeneity[54] or identify heterogeneity in the absence of morphological changes.[51,55] Spatial transcriptomics can identify subpopulations with gene expression patterns associated with prognosis or response to targeted treatments such as mRNA fusion transcripts,[56] gene expression patterns associated with chemotherapy response[57] and treatment-resistant subpopulations. The presence of tumour heterogeneity, including of the tumour microenvironment, is associated with poorer clinical outcomes in several cancer

types.[10,51] Gene expression differences between disease sites and over the disease course may help to elucidate the biology of disease progression, mechanisms of treatment response or resistance and inform clinical management for each disease stage.[58,59]

Challenges of spatial transcriptomics in clinical practice

The significant barriers to integration of spatial transcriptomics into routine anatomical pathology practice include the cost of the assays, reagents and associated equipment amounting to thousands of dollars per sample, workflow time-lines of approximately 6–8 weeks and the need for scientists and pathologists with appropriate wet-laboratory and analytical skills. In particular, the skills required for data analysis are currently highly specialised, given the current lack of streamlined analytical solutions that would be required for clinical implementation. These issues currently limit the use of these technologies to research laboratories, institutions or via specialised service providers with significant molecular pathology and bioinformatics capabilities, as well as the required equipment.

In addition, few of the commercially available whole-transcriptome assays are compatible with FFPE tissue, and assays offering single-cell resolution for FFPE tissue have only been commercially available very recently. As analysis regions can span multiple cells, any analyses would be confounded for cells not present in clusters; for example, myoepithelial cells in the transition from *in-situ* to invasive carcinoma, or tumours where malignant cells are vastly outnumbered by tumour microenvironment cells such as Hodgkin lymphoma. Transcriptionally active cells may also mask expression of less transcriptionally active cells. At present, cellular composition of the tissue within the analysis region can only be computationally inferred from single-cell mRNA sequencing data sets, a process known as cellular deconvolution.

Normal gene expression for specific tissue types in specific anatomical locations has not been defined and the significance of gene expression at a single time-point is uncertain, given that gene transcription does not have a constant state but rather occurs in bursts.[60,61] The degree of heterogeneity within a given tissue type is unknown, and with the area of analysis in practical terms being limited to a fraction of a whole tissue section, whether the analysed area is representative of the entire tumour/disease process needs to be addressed before diagnostic use.

Correlation of transcriptional data with protein expression is required and, importantly, the clinical significance of expression data for a specific disease and site is currently unknown, and will only be established with time as evidence from studies accumulate. The robustness of the various platforms across institutes and under differing tissue preparation methods also remains to be determined.

Nevertheless, as with other techniques that have been successfully adopted into anatomical pathology, improvements in chemistry, workflows, small agile sequencing platforms, automation, informatics and possibly digital pathology and machine-learning solutions may allow its use in diagnostic laboratory settings. Thus, with further development, one might anticipate clinical use of these technologies into some laboratories in some form within the next decade, providing pathologists with further diagnostic and prognostication information to augment and integrate with histopathology findings.

Conclusion

Spatial transcriptomics is a new group of technologies which interrogate potentially transcriptome-wide gene expression within a spatial context. Spatial transcriptomics offers the potential to improve understanding of disease biology and provide clinically meaningful information. Its use in anatomical pathology is currently very significantly limited by cost, logistics and uncertainty regarding interpretation of the data in a clinical context, issues which are likely to be addressed in the near future given the large-scale investment by many companies and exponential development in the spatial transcriptomics field.

Acknowledgements

J.M.P. received departmental support funding from the Peter MacCallum Cancer Centre Research Division towards this work. S.L. is supported by the National Breast Cancer Foundation of Australia Endowed Chair and the Breast Cancer Research Foundation, New York. K.L.G. is supported by the Peter MacCallum Foundation. S.B.F. is supported by NHMRC Investigator grant GNT1193630. The figures were created with BioRender.com.

Conflicts of interest

The authors declare that they do not have competing financial, personal or professional interests in relation to the subject of this paper.

Data availability statement

Data sharing is not applicable to this article, as no data sets were generated or analysed during the current study.

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