Applications of Spatial-Omics in Clinical Translational Cancer Research

By Dr Alison Finall, BSc MBBCh MRCS(Eng) MSc (Genomic Medicine) FRCPath FHEA
Consultant Cellular and Molecular Pathologist at Swansea Bay University Health Board, Swansea, UK

Don't let patients with TARGETABLE MUTATIONS get lost in the crowd

There are ~4,000 to 5,000 patients with ***METex14*** in mNSCLC per year in the United States.[1-2]

Nearly 1 in 2 patients with mNSCLC may have a targetable oncogenic mutation,[3-10] but many patients are not tested for all potential targets (prevalence of *MET*ex14 ~3%).[4,9,11-15]

The National Comprehensive Cancer Network® (NCCN®) recommends testing for *ALK, KRAS, BRAF, EGFR,* ***METex14****, NTRK1/2/3, RET, ROS1* and PD-L1 in eligible newly diagnosed mNSCLC patients.[16*]

Up-front broad molecular profiling may help optimize first-line treatment for mNSCLC.

MET, mesenchymal-epithelial transition; *MET*ex14, *MET* exon skipping; mNSCLC, metastatic non-small cell lung cancer.

*The NCCN Guidelines® for NSCLC provide recommendations for certain individual biomarkers that should be tested and recommend testing techniques but do not endorse any specific commercially available biomarker assays or commercial laboratories.

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Introduction

Spatial resolution of molecular pathological data has clear potential for use in future diagnostic pathology services for direct benefit of patients (e.g., mapping the heterogeneity of tumours). Spatial transcriptomics – the combining of digital cellular pathology with gene and protein expression data – has become a powerful tool for to address this potential (in fact, it was voted research technology of the year 2020 by Nature Methods).[1,2] This article explores potential applications of digital spatial profiling to aid advances in clinical diagnostic histopathology reporting and attempts to inspire future translational research projects for improvements in patient care.

What is Digital Spatial Profiling?

Spatial transcriptomic profiling of human tissues was borne out of developments in RNA fluorescence in situ hybridisation (FISH) for visualisation of subcellular location and semi-quantification in gene expression studies.[3] This is a simple technique which can enable specific detection of any gene expression target including genes that do not code for proteins. Rolling circle amplification methods allowed for FISH technology to be combined with sequencing technology for multiplex detection of up to 1000 gene expression products.[4,5]

With the development of digital histology slide imaging, researchers learned to combine the histological image with spatially resolved gene expression data by using capture probes attached to a glass slide.[6] Manual annotation of samples and critical features in frozen and formalin-fixed paraffin embedded (FFPE) tissue sections for spatial mapping of proteins and/or gene expression transcript requires experience in interpretation of microscopic morphology – particularly in pathological specimens. In the Visium™ platform designed by 10X Genomics, specially designed glass slides for spatial transcriptomics contain a fiducial frame for RNA capture that contains 5000 'spots' in an area 6.5mm^2. Each spot contains barcoded RNA poly-A capture probes covering the entire human protein coding genome; as useful as this technique is, however, it does not allow for assessment of non-coding RNA elements.

Commercial bioinformatic software for analysis matches the resulting sequencing data to each of the spots with the histological image overlay to produce a gene expression map of the tissue (see **Figure 1**). Spot resolution is 55µm across with the centre of adjacent spots being 100µm apart for standardized technologies, such as in the 10X Genomics Visium™ platform. This allows visualisation of groups of up to 10 cells, depending on cell size.

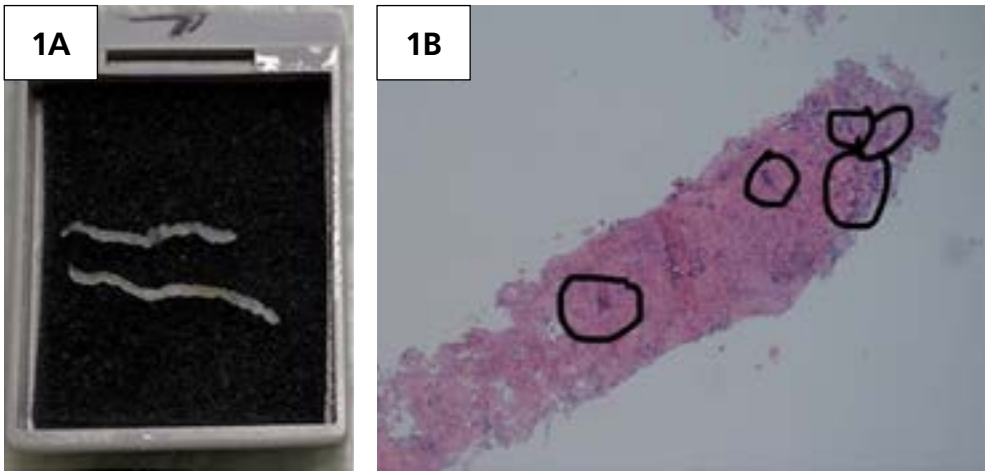


Figure 1: Illustration of (A, left) the small size of core needle biopsy samples which are ~ 1mm thick) and (B, right) a microscopic examination using medium power light microscopy to highlights a paucity of malignant cell content (black circles) in some cases.

Higher spatial resolution technologies are being developed that will allow for single cell resolution in the future.[7,8] Spatial proteomic profiling is also possible using a similar digital tissue morphology image overlay with digitally captured protein expression data which can be performed iteratively using a limited number of fluorescent markers, such as in the Lunaphore™ system.[9,10]

An alternative approach is to capture oligonucleotide sequences that recognise specific antibodies to protein epitopes. In the nanoString GeoMX™ platform these oligonucleotides are released for capture following exposure to UV light. This has the advantage of preserving the underlying tissue section, allows for focused analysis of protein expression. The number of proteins analysed in an experiment using the nanoString depends on the instrument used for differential expression; the nanoString nCounter can multiplex up to 96 proteins but can be far exceeded by using next generation sequencing (NGS) methods in the downstream workflow.[11-13]

What Clinical Pathology Research Questions Could be explored using Spatial profiling methods?

Biomarker evaluation in small volume disease

A common issue in clinical pathology practise is not having enough tissue to complete all the testing that may be required for a patient.[14] This issue is often encountered in thoracic pathology where DNA and RNA-based sequencing may be required to determine the best treatment for that individual patient where the diagnosis has been made on a core biopsy.

In a recent real-world study verifying rapid PCR testing for EGFR, 10% of our patients did not have

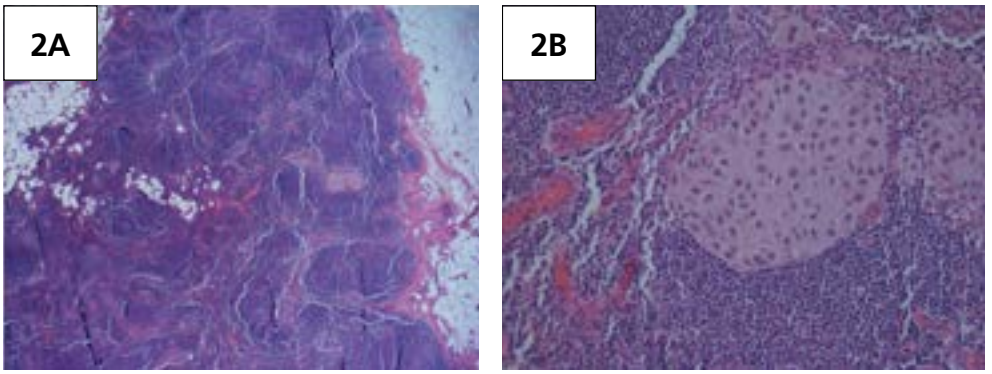
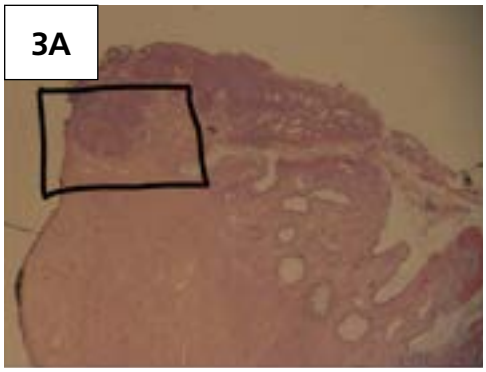
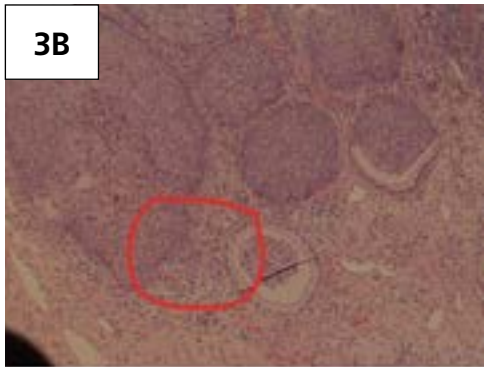


Figure 2: Illustration of the relatively low number of malignant melanoma cell nuclei in relation to surrounding lymphocytes which have a very high nuclear:cytoplasmic ratio. A) left, shows a micro-metastatic deposit of malignant melanoma in a lymph node (yellow arrow). B) right, shows a high-power view of the micro-metastatic deposit the illustrate difference in overall size and nuclear content.





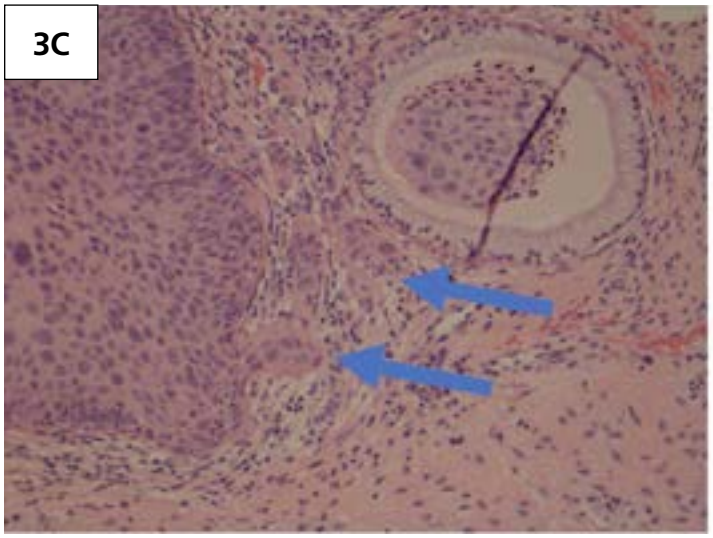


Figure 3: (A, upper left) shows a tiny focus of early invasive squamous carcinoma (SCC) of the cervix arising in a large area of high-grade, cervical intra-epithelial neoplasia (CIN3) which colonises underlying endocervical glands as demonstrated at medium power (B). Photograph B corresponds to the area in the black rectangle in photomicrograph A. (C) is a high-power photomicrograph of nests of invasive SCC comprising around 10 cells each.

sufficient remaining malignant tissue for DNA NGS after diagnosis.[15] Subsequent to this study, RNA NGS panel sequencing was commenced in our institution as a standard requirement for detection of a range of large structural rearrangements including NTRK 1,2,3, ALK-1, ROS-1 MET exon 14 skipping lesions and RET fusions. The failure rate using this technique can be as high as 50% in our experience and this is in part due to insufficient tissue for testing but also due to the fragile nature of RNA molecules in comparison with DNA.[16,17]

We know that rapid, automated PCR using platforms (e.g., the Idylla™ system) not only can save time in generating information for clinical action but also consume less tissue.[15,18,19] DNA panel NGS in our reference laboratory requires 60µm thickness of sectioned tissue and RNA panel NGS a further 58µm.15 Being able to use spatial transcriptomics to assay for novel fusion proteins and/or large structural DNA rearrangements using a set of predefined probes that target commonly known breakpoints would be of great benefit to patents with non-squamous non-small cell lung cancer. Currently, it is not possible to find and image gene expression or protein targets resulting from gene fusions and large structural rearrangements using existing spatial technologies so far. This gap in the clinical requirement from a pathologist's point of view is begging to be filled by commercial development and research.

"Spatial mapping of biomarkers is ideally suited for experimental interrogation of key molecular aspects of invasion and metastasis because we now have the ability to focus on those cells that have acquired genetic alterations that can withstand circulation in an intravascular space through lymphatic or blood vessels. Those malignant genotypes that exit via these vessels are able to 'seed' tissue distant to, and likely biochemically distinct from, its site of origin."

A similar problem exists in lymph node sampling in a setting of metastatic malignancies where the abnormal DNA/RNA/protein for analysis of therapeutic biomarkers is swamped in a sea of wild-type background lymphocytes.[17] Picking up abnormal gene expression profiles is much more likely to be successful by using spatial techniques to target small foci of micro-metastasis. Of course, this can apply to any malignancy where there are small metastatic deposits but has particular relevance in a clinical context of malignant melanoma where there is a large cell size relative to the surrounding lymphocytes (see **Figure 2**).[20] It is also important to note that some metastatic carcinomas of the breast have clinical prognostic significance when micro-metastatic disease is found.[21,22]

Adjunct to Diagnosis of Early invasion

A difficult diagnostic dilemma for anatomical pathologist is identifying early invasion in both squamous carcinomas and adenocarcinomas arising in a variety of body organs.[23,24] Gene expression signatures from tiny foci of early invasive carcinomas may be 'drowned-out' in signals from background normal structures and in-situ dysplastic epithelia in experimental studies that utilise bulk RNA sequencing methods.[25] Spatial transcriptomics has the potential to highlight unique transcriptomic and proteomic signatures associated with the earliest detection of invasion.[26]

Identification of such gene expression signatures could be used as an adjunct method for diagnosis of early invasive carcinomas in difficult cases where there is significant background inflammation. Such cases may occur in vulva or pancreas tissue where morphological features may be subtle and open to interobserver variation in diagnosis. Spatial proteomic data could also be used to inform translational development of novel immunohistochemical assays that could directly impact the practice of diagnostic histopathology.

Currently, no immunohistochemical biomarkers are available to support a diagnosis of early invasion in squamous carcinomas, which, consequently, remains a subjective morphological assessment and therefore open to the possibility of interobserver variation and human error.[24] The advantage of immunohistochemistry (IHC) is that it is fast, cheap and reliable for morphological interpretation; infrastructure is well developed in diagnostic immunohistochemistry laboratories across the globe to adopt new antibody-based protein detection methods rapidly.[27] This is of particular importance in support of healthcare systems that are limited in financial resources and lack the ability to perform or out-source relatively expensive sequencing assays. Perhaps of greater importance, research and development activity needs to be directed towards developing IHC biomarkers of high clinical utility that allow for determination of primary site in squamous carcinomas.

Thoracic pathologists often encounter this

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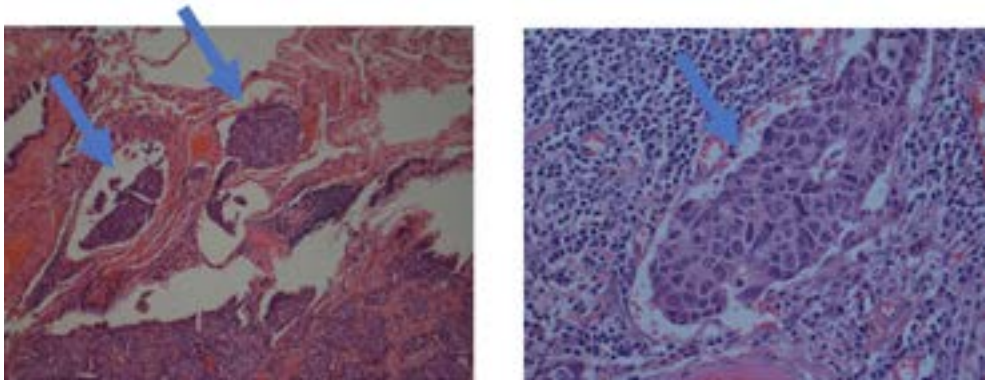

Figure 4: Showing foci of microvascular tumour emboli (blue arrows) now available for targeted molecular scrutiny with the advent of spatial transcriptomics and proteomics.

clinical diagnostic dilemma in a context of metastatic malignancy to the lung where the primary site is unclear. We can use a $p16^{INK4A}$ IHC assay as surrogate to probe for squamous carcinomas driven by human papilloma virus (HPV) infection; but this is only of clinical relevance where the suspected primary site is in oropharynx, cervix, or perineum.[28] Research into squamous carcinomas has often relied on bulk sequencing of tissue samples with the inevitable loss of granularity of detail that spatial analysis can bring.[29]

Spatial mapping of squamous carcinoma of the skin has highlighted unique signals within tumour specific keratinocytes involving interferon-based intercellular signalling between the host immune system.[26] Ji *et al* also showed that expression of *mixed metalloproteinase 10 (MMP10)* was focussed within cells at the leading invasive edge of the tumour with a stromal rich expression of endothelial and cancer-associated fibroblast transcripts.[26]

MMP10 protein is able to degrade fibronectin and a variety of collagen types through activation of pro-collagenase enzymes and is associated with the risk of developing invasive carcinoma in large scale genetic studies.30 Further research is needed to determine whether an IHC-based assay for *MMP10* protein expression rather than mRNA expression could be developed to support a pathological diagnosis of invasion to early carcinomas. An MMP10 assay may also stratify risk of impending invasion in *in-situ* precursor neoplasia of squamous epithelia in a variety of organs.

Seeing the small foci of invasive disease arising in a background of large areas of in-situ neoplasia (see **Figure 3**) helps one to understand how proteomic and transcriptomic signals would be lost in bulk sequencing and flow cytometry-like single-cell RNA sequencing that uses proteomic gating methods to enrich for cells of interest with an inherent loss of tissue architectural context.

"In the context of tumour mutational burden scoring for lung cancer, high levels of expression of CD56 protein and CD4 in areas of lymphocytes density associated with non-small cell lung cancer were found to predict clinical response more accurately to checkpoint inhibitors than NGS."

Understanding Factors Involved in Metastasis

Much cancer research of the past has been inspired to look at facets of carcinogenesis as described by Hanahan and Weinberg.[31] They detailed the molecular mechanisms involved in promotion of invasion and metastasis; resisting cell death; induction of angiogenesis; maintaining drivers signals for cell proliferation – all in the service of the seemingly infinite ability for cell division and avoidance of tumour growth suppression.[31] The reality of clinical diagnostic pathology recognises that all these factors are at play in the pathogenesis of benign mass lesions that may have no clinical impact on patient symptomatology nor affect lifespan (with the exception if invasion and metastasis).[32]

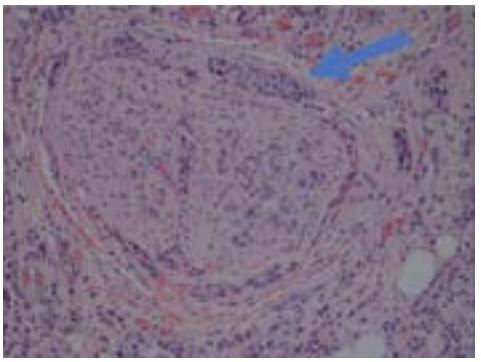

Figure 5: Perineural invasion in cutaneous squamous carcinoma. Arrow indicates groups of malignant cells under the perineural sheath.

Spatial transcriptomic and proteomic research should focus on those defining features of malignancy (viz., the ability to invade and metastasise) so that translational research projects should have the maximum impact for patients – for it is these two malignancies that are responsible for mortality in 90% of patients.[32,33] Spatial mapping of biomarkers is ideally suited for experimental interrogation of key molecular aspects of invasion and metastasis because we now have the ability to focus on those cells that have acquired genetic alterations that can withstand circulation in an intravascular space through lymphatic or blood vessels. Those malignant genotypes that exit via these vessels are able to 'seed' tissue distant to, and likely biochemically distinct from, its site of origin.[33,34]

The low-volume nature of tumour micro-emboli in tissue section is best appreciated by microscopic examination (see **Figure 4**). Our knowledge of many proteins and gene expression characteristic tumour emboli observed in different cancer types and organs of origin have yet to developed, let alone published. As we develop this knowledge in the coming years, the findings could be of direct relevance to patients by identifying signatures in invasive tumours for risk stratification, management strategies, or surgical intervention before they metastasise.

Perineural invasion is more common in some malignancies than vascular invasion – examples include prostate cancer,[35,36] basal cell carcinoma of skin[37-39] and adenoid cystic carcinoma of the lacrimal and salivary glands.[40-42] The same principal application of spatial mapping to investigate molecular biomarkers involved in the interaction of malignant cells with the perineural sheath will apply as in tumour interaction with endothelial cell lining lymphatic and vascular spaces (see **Figure 5**).

Immuno-oncology

Spatial transcriptomic profiling has been used to interrogate tumour microenvironments for discovery of immune interactions with the malignancies for greater understanding of cell-cell communication events. A good example would be the identification of interferon-associated transcripts in the basal component of squamous cell carcinomas of the skin.[26] Targeted evaluation of the interaction between malignancies and their adjacent stroma is also important for the development of novel immunotherapeutic drugs in an oncological setting.[26,43,44]

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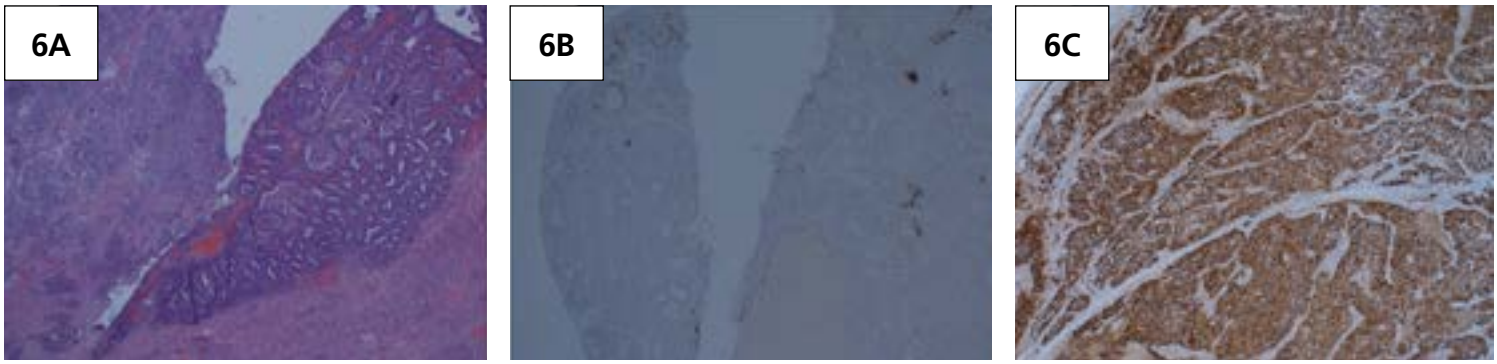


Figure 6: Photomicrograph illustrating heterogeneity of grade within an endometrioid adenocarcinoma of the ovary arising in a background of ovarian endometriosis. FIGO grade 3 disease is seen with a solid pattern (A) directly abutting an area of more well-differentiated FIGO grade 1 disease in the same section from the same tumour mass in the same patient. The heterogenous appearance of the tumour is also reflected in IHC protein expression with loss of oestrogen receptor expression in grade 3 areas (B) and retention in better differentiated areas (C).

Recently, spatial technologies have been employed to show that increased expression *VISTA, LGALS9* and *TNFRSF14* genes, involved in T-lymphocyte activity suppression where present at the invasive front of cutaneous squamous cell carcinomas.[26] Even in established areas of clinical practice using PD-L1, there is room for exploration using digital spatial profiling techniques to improve the tools we have at our disposal. In the context of tumour mutational burden scoring for lung cancer, high levels of expression of CD56 protein and CD4 in areas of lymphocytes density associated with non-small cell lung cancer were found to predict clinical response more accurately to checkpoint inhibitors than NGS.[45] The advantage of this finding is that IHC for CD56 and CD4 is fast and cheap and can be multiplexed in situations of limited diagnostic tissue.[9] The variation upon this experimental theme is vast but should be explored to identify new and better targets of future monoclonal antibody therapies in addition to TMB, CTLA4, PD-1 and PD-L1.[11,13,46-48]

Molecular Sub-typing of Malignancy

Pathologists have long been aware that malignancies are heterogenous with multiple patterns of invasive malignancy present within a single lesion and that focal de-differentiation of a malignancy can be responsible for the development of metastatic disease.[49,50] As such identification of subclones within a malignant tumour and characterising their gene and protein expression requires a spatial context for accuracy and clinical relevance. In some tumour types, it is molecular heterogeneity itself which is associated with metastases as was shown in one of the first papers published that examined malignant melanoma using digital spatial profiling techniques.[51]

The production of gene and protein expression maps from tissue sections of malignancy allows for exploration of heterogeneity to advanced understanding of pathogenesis and pattern recognition for advances in the diagnostic specialty that is histopathology. Furthermore, linking data from spatial transcriptomic and proteomic studies to clinical outcomes can highlight gene expression signatures for recurrence risk and prognosis prediction. A good example is RNA sequencing for sub-typing of colorectal adenocarcinomas which has highlighted particular morphological patterns of adenocarcinomas that correlate with particular gene expression signatures and have an effect on clinical parameters progression-free and overall survival.[52] Another example of heterogeneity of grade within an endometrioid adenocarcinoma of critical interest is an endometrioid adenocarcinoma of the ovary arising in the background of ovarian endometriosis (see **Figure 6**). These cancer types place morphological diagnosis at the centre of precision medicine within tumour boards/ multidisciplinary team meetings and allow stratification of patients based on visual recognition at the H&E section level and can therefore be utilised with little expense across all continents and low- income countries.[53,54]

"Furthermore, linking data from spatial transcriptomic and proteomic studies to clinical outcomes can highlight gene expression signatures for recurrence risk and prognosis prediction. A good example is RNA sequencing for sub-typing of colorectal adenocarcinomas which has highlighted particular morphological patterns of adenocarcinomas that correlate with particular gene expression signatures and have an effect on clinical parameters progression-free and overall survival."

Research examining heterogeneity of prostate cancer using spatial methods has revealed greater accuracy in identifying well-differentiated patterns of adenocarcinoma (Gleason grades 1 and 2) which is known to be an area of difficulty in practice for histopathologists.[55]

In addition, spatially focussed techniques have generated further sub-classifications in haemato-oncology with more precise descriptions of diffuse large B-cell lymphoma.[56]

Prognostication

Understanding the molecular pathological basis of breast cancer has resulted in an updated classification giving four broad subgroups: luminal types (A and B); HER-2 overexpression; basal phenotype; and normal-like. Gene expression studies have shown that it is possible give a risk score of for developing metastases for these subgroups over a ten-year follow-up period in lymph-node negative early-stage disease.

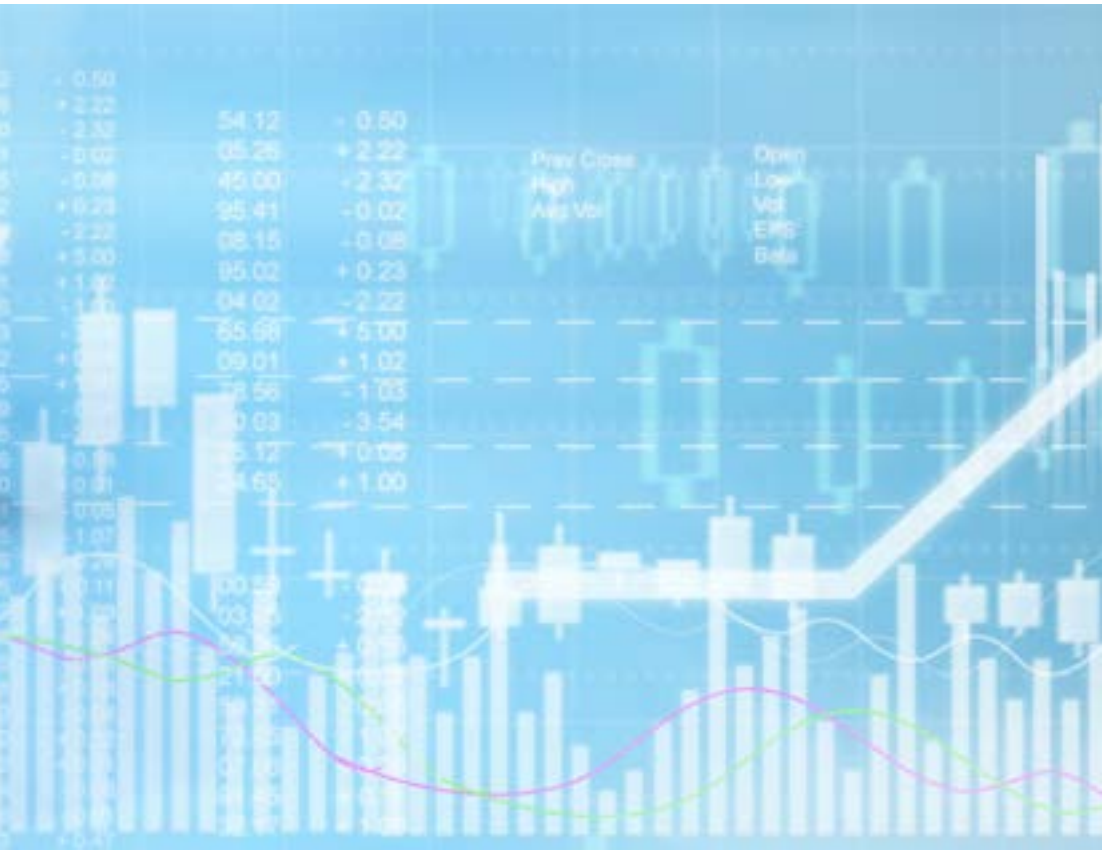
A number of commercially available assays have been developed and approved by regulatory agencies for predicting prognosis but not for treatment selection or predicting treatment responses.[57] Such assays include Mammaprint™, a 70 gene expression signature; OncotypeDx™, based on a profile of 21 genes (5 of which are control) and PAM50-risk of recurrence score which includes 50 cancer genes and 22 housekeeping/ control genes.[57] The OncotypeDx™ gene expression panel, for example, requires tissue sections totalling 45µm of tissue for use in breast cancer recurrence risk prediction.

For those patients with limited tissue available for analysis, this may not be possible to achieve. Multiplex gene expression based on use of a single 5µm FFPE tissue section could represent a significant improvement in patient care pathways by preventing the need for repeat invasive procedures to retrieve more malignant tissue and potentially save time.[19] Furthermore, these assay types should be used with caution in a context of rare histopathological subtypes beyond the 4-tier molecular classification described above,58 and they should always be interpreted in a context of the pathology assessment with an overlay of gene expression data on the digitised morphological image. Such an approach leads to refinement of meaning and interpretation of gene expression data for future patients.[58]

Figure 7: Examples of panels of immunohistochemical markers to assess subtypes and origin to malignancy of unknown origin. The photograph illustrates the tissue consumption required to accommodate the need for multiple protein biomarkers.

One of the first studies to use spatial transcriptomics to interrogate breast cancer was performed by Stahl, *et al* in 2015.[6] They revealed the underlying molecular heterogeneity present within both the invasive component of breast cancer and also in precursor ductal carcinoma in-situ (DCIS) which remains bound by basement membrane and therefore has no potential for metastases. They showed a distinct gene expression profile of genes involved in epithelial-mesenchymal transition such a *KRT17* and *GAS6* and hypothesised that spatial information combined with RNA-seq data could yield more focussed and meaningful insights for patients.[6]

The value of spatial transcriptomics is also emphasised by the observation that the clinically useful prognostic information obtained from gene expression data is limited beyond what is already gleaned from histological factors of tumour grade and lymph node status.[59] A prognostic risk score based on a combination of anatomical pathology with gene expression is expected to be more accurate than gene expression-based assay scores alone.[59,60] To date, the gene expression assays in breast cancer do not convey information from gene expression in stroma nor the interaction with inflammatory cells at the invasive interface. Incorporating information regarding DNA mutations along with gene expression data will also add a layer of nuance that could explain why



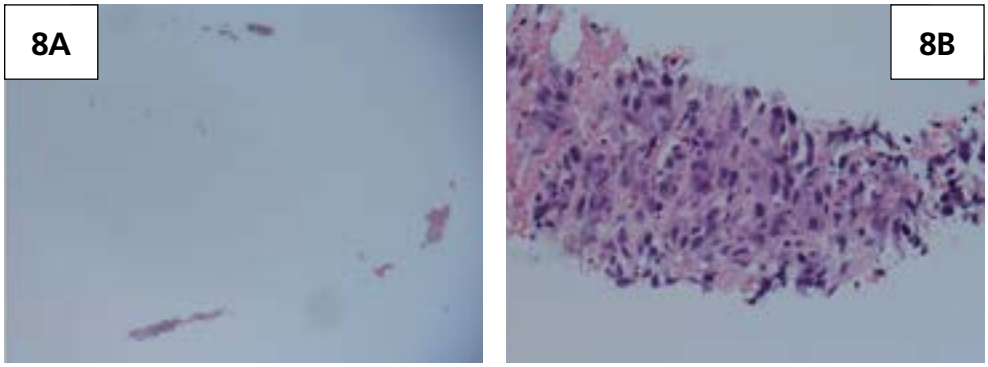


Figure 8: (A) Low power photomicrograph highlighting the paucity of needle biopsy material available for microscopic analysis and (B) an example of an undifferentiated malignancy which could be one of several possible types of carcinoma, sarcoma, germ cell tumour, lymphoma or melanoma in view of a lack of morphologically informative cytological and architectural features.

current commercially available gene expression assays in breast cancer are not able to predict responses to treatments.[59] Spatial data, may however, address the potential for a minor subclone in the malignancy being responsible for lack of treatment response in some women.[61,62]

A multitude of other prognostic gene expression signatures could be translated to the glass slide by pathologists including those relating to colorectal, prostate and lung cancer.[63] Pathologists will need to refine the translation of signatures to slides in terms of histological subgroup and staging context for each patient. Hence, many clinical trials will need to be completed to achieve genuinely personalised oncology in the future.

Malignancy of Unknown Origin (MUO) / Carcinoma of Unknown Primary (CUP)

Spatial proteomics has become an integral part of cellular diagnostic pathology to determine malignancy type and origin. A common problem for clinical pathologists is to help patients with disseminated, late-stage malignancies reach better understanding of their diagnosis and disease. Pathologists employ immunohistochemical stains to help answer both questions (see **Figure 7**).

Clearly the ability to do extensive panels of immunohistochemistry is limited to large tissue, and often require the excision of specimens. The case illustrated in Figure 8 highlights the problem of limited diagnostic material as previously discussed above. This could be overcome by digital image capture for multiplex immunohistochemistry.[9,64]

Malignancy of unknown origin can account for up to 5% of cases of disseminated malignancy,[65,66] but this group of patients represents a diverse collection of neoplasms that make them difficult to study using morphology as a shared characteristic. Malignancies of unknown origin, as a group, includes a wide variety of tumour types including divergent forms of malignancy such as sarcomas, carcinomas, lymphomas and germ cell tumours, all of which have different clinical behaviours and prognosisUsing molecular biomarkers in an agnostic fashion (that is, without a specific histopathological diagnosis) may desirable for determination of eligibility for molecular-based oncological drugs.[67,68] A good example of this approach to patient care is use of Entrectinib therapy for treatment of neurotrophic receptor

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tyrosine kinase (NTRK)-fusion positive solid malignancies.[69,70] The only disappointing clinical feature of NTRK fusions in malignant solid tumours is its low incidence across many tumour types, whether of known or unknown origin.[71]

Beyond Oncology

Spatial transcriptomics and proteomics have applications beyond cancer research. To prepare for the next viral pandemic, the world would benefit from closer collaboration between, and colocation of, virology and cellular pathology departments. Research collaboration across specialities, such as cellular pathology and virology for example, could help to create maps of specific human cell types infected by viruses and describe the specific membrane protein epitopes exploited for viral entry. This kind of knowledge could drastically improve our ability to convert research effort to understanding, treating, and preventing novel viruses. COVID-19 tissue maps have been produced using tissue samples from autopsies for various organs to understand pathological impacts of SARS CoV2 infection and inform development of vaccines and monoclonal antibody mediated treatments.[72-74]

This kind of tissue map could be generated to help combat many other viruses as well. In a similar vein, applications of digital spatial profiling can be applied to any area of disease that can be detected and characterised by tissue pathological process. Examples include understanding epithelial-stromal cell interactions in endometriosis,[75] investigating glomerulonephropathy,[76,77] understanding autoimmune diseases,[78] analysing cirrhosis of liver,[79,80] and researching neurodegenerative conditions such as Alzheimer's disease.[81] As always, the aspirational goal is to convert this level of detail to yet more precise treatments. JPM

Take home lessons

1. Gene and protein expression with spatial cartography opens new doors for discovering pathological mechanisms in microscopic disease states such as early invasive carcinoma and factors involved in developing metastases.
2. There is an opportunity to translate gene expressions assays currently used in clinical practice for prognostication to the spatial sphere. Bringing functional genomics to the glass slide will allow for meaningful integration of morphological and molecular data and will transform practice of diagnostic cellular pathology.
3. Correlation of tumour gene expression heterogeneity with micro-morphological variation and clinical outcomes data is likely to yield new sub-classifications within established diagnostic categories of malignancy.
4. The list of possible clinically applicable new pathological insights is endless. Spatial cartography heralds an exciting time for translational pathological research. There are likely to be seismic shifts in the practice of stratified medicine in the coming years as a result.

Dr Alison Finall

Alison is a consultant histopathologist based in the UK with sub-specialist interests in gynaecological and thoracic pathology. She is an Honorary Associate Professor at Swansea University with current research projects using spatial technologies in ovarian malignancies and placenta. She is the 2020 holder of the BAGP Research Award in Gynaecological Pathology and recently completed an MSc in Genomic Medicine with distinction.

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