Enriching pathological analysis of FFPE tumor samples with spatial transcriptomics

Abstract
Formalin-fixed paraffin-embedded (FFPE) samples are a valuable resource for diagnosing prostate cancers. Properly stratifying disease severity and identifying the best therapeutic strategies could be aided by complementing traditional pathological assessment with spatial gene expression insights. Here, we used tissue-wide whole transcriptome analysis with Visium Spatial Gene Expression for FFPE to resolve the tumor microenvironment of two prostate cancer samples. We identify well-known prostate cancer markers and reveal the spatial organization of key cell types, including epithelial and immune cells.

Highlights
- Verification and refinement of traditional pathologist-led analysis
- Characterization of the spatial gene expression patterns of known prostate cancer markers
- Identification of disrupted spatial organization between basal and luminal cells in invasive carcinoma
- Visualization of immune cell composition and localization within the tumor microenvironment

Graphical abstract
**Introduction**

Diagnosis of prostate cancer relies on assessing a tumor’s histologic patterns according to the Gleason grading system (1). While this grading system is a powerful prognostic predictor, there is a wealth of additional information contained within these biopsies that could strengthen diagnosis and guide therapeutic development for patients on a personalized basis.

Clinical workflows commonly preserve the tissue architecture of biopsies by fixing in formalin and then embedding in paraffin (2). Cellular structures or protein markers can then be examined using histological dyes, such as hematoxylin and eosin (H&E), or immunohistochemical stains, respectively.

Hidden within these formalin-fixed paraffin-embedded (FFPE) samples is cellular gene expression information that has been challenging to access because FFPE processing damages RNA. Visium Spatial Gene Expression for FFPE (Visium for FFPE) is a next-generation molecular profiling solution that blends traditional histological analysis with whole transcriptome RNA sequencing for tissue-wide characterization of entire FFPE sections. Here, we demonstrate the tissue-wide tumor profiling of two human prostate cancer samples with Visium for FFPE. We illustrate that the spatial gene expression data complements pathologist annotations while additionally enabling the identification of cancer biomarkers and localization of specific cell types.

---

**Figure 1. Visium Spatial Gene Expression for FFPE takes you from block to data with a ready-to-use, robust workflow.**

A. A FFPE tissue section is placed onto a Visium Gene Expression Slide, and histological images are captured. Each Capture Area on a Visium slide has spots containing oligos with spatial barcodes. After sequencing libraries are prepared, the sequencing data is visualized to determine gene expression levels and locations.

B. The Visium for FFPE assay utilizes RNA-templated ligation, in which pairs of probes specific to genes in the protein-coding transcriptome are hybridized to their gene targets and then ligated to one another. The tissue is permeabilized to release ligated probe pairs to bind to capture probes on the slide, allowing for the capture of gene expression information. The probe pairs are extended to incorporate complements of the spatial barcodes and sequencing libraries are prepared.
Methods

Human normal prostate, stage III adenocarcinoma (Gleason score = 3 + 4 = 7), and stage IV acinar cell carcinoma (Gleason score = 4 + 3 = 7) biopsies which were FFPE preserved, were processed using the Visium Gene Expression for FFPE workflow (Figure 1).

FFPE tissues were prepared according to the FFPE – Tissue Preparation Guide (Document CG000408). Next, the sections were deparaffinized, stained with H&E, and decrosslinked following the appropriate Demonstrated Protocol (Document CG000409). Stained sections were imaged following the Visium for FFPE Imaging Guidelines (Document CG000436) and annotated by Agoko NV. Probe hybridization and barcoding were performed and then libraries were prepared following the User Guide (Document CG000407). The resulting libraries were sequenced on a NovaSeq 6000, analyzed using Space Ranger pipeline v1.3, and visualized on top of the tissue images in Loupe Browser v5.1. Pathologist annotations were imported into Loupe Browser as an additional classification category.

For immunohistochemistry (IHC), adjacent tissue sections were stained with anti-AMACR/p504s (clone:SP116) or anti-ERG (clone:EPR3864) Rabbit Monoclonal Primary Antibodies (Ventana, Roche Diagnostics). The Novolink Polymer IHC kit (Leica) was used according to the manufacturer’s instructions.

Figure 2. Whole transcriptome analysis with Visium for FFPE complements traditional histopathological methods. An H&E-stained image of normal (A, left) and stage III adenocarcinoma (B, left) prostate tissue was overlaid with pathologist annotations, graph-based spot clustering analysis, and pathologist annotations (middle), or total gene counts (right). C. Unbiased k = 2 spot clustering. D. Differential gene expression within the k = 2 clusters.
Enriching pathological analysis of FFPE tumor samples with spatial transcriptomics

Results

Tissue microenvironment profiling
Graph-based clustering of each spot, which contains mRNA from 1–10 cells, was performed by the Space Ranger pipeline and visualized using Loupe Browser. For both the normal and stage III adenocarcinoma samples, a comparison was drawn between the pathologist’s annotation of the tissue (Figure 2A and B, left) and the spatial gene expression clustering with reference to the H&E-stained tissue section (Figure 2A and B, middle). In the normal samples, the pathologist annotation confirmed the presence of normal prostate gland tissue, and Visium for FFPE identified seven clusters. Total gene counts for each spot were also overlaid on the H&E-stained tissue image (Figure 2A, right). A maximum of ~16,000 genes were detected in the tissue section, with a median of ~3,000 genes per spot.

Pathologist annotation segregated the stage III adenocarcinoma section into seven regions (Figure 2B, left) with a large portion annotated as invasive carcinoma (blue). Spatial gene clustering analysis identified nine clusters (Figure 2B, middle) with graph-based clusters 1 and 2 (light green and red, respectively) overlapping with the pathologist-annotated invasive carcinoma. Total gene counts for each spot were also overlaid on the stained tissue image (Figure 2B, right). In this sample, a maximum of ~16,000 genes were detected in the tissue section, with a median of ~5,000 genes per spot. Demarcation of invasive carcinoma from benign prostate parenchyma, determined by unbiased clustering (k = 2; Figure 2C), also correlated well with the pathologist’s annotations. Examination of differentially expressed genes in these k = 2 clusters revealed that several well-known prostate cancer markers (CRISP3, TMEFF2, NPY, AMACR, and ERG) are overexpressed in the identified tumor-cell population (Figure 2D, red boxes).

IHC validation of Visium for FFPE data
The use of biomarkers for stratifying tumor severity and guiding treatment decisions has become increasingly important in prostate cancer diagnosis (3). Alpha-methylacyl-CoA racemase (AMACR) is one such biomarker whose overexpression has a high correlation with prostate cancer risk and diagnosis (4). AMACR transcripts were observed to be overexpressed (assessed by Visium for FFPE) in the invasive carcinoma region of the stage III adenocarcinoma sample (Figure 2D). We examined AMACR protein expression with immunohistochemistry (IHC) staining and found it to be low in the normal prostate sample (Figure 3A, left). In line with the transcriptome data from Visium for FFPE, the protein-based IHC showed a granular, cytoplasmic AMACR staining pattern in malignant glands and cells in the stage III adenocarcinoma (Figure 3A, middle) and a stage IV acinar cell carcinoma (Figure 3A, right).

ETS-related gene (ERG) RNA was also overexpressed in the invasive carcinoma region of the stage III adenocarcinoma sample (Figure 2D). Several studies have shown that ERG overexpression is linked to high Gleason score, tumor stage and aggressiveness, and survival outcomes (5). Expression of ERG RNA (assessed by Visium for FFPE) and protein (assessed by IHC staining) were found to be minimal in normal prostate tissue (Figure 3B). There was a high correspondence between RNA expression levels observed in the Visium for FFPE spatial gene expression data and the IHC protein staining for ERG in the stage III adenocarcinoma (Figure 3B, middle) and the stage IV acinar cell carcinoma (Figure 3B, right) samples.
Figure 3. Validation of cancer biomarker expression by IHC. Protein (assessed by IHC) and RNA expression (assessed by Visium for FFPE) levels of key prostate cancer genes, AMACR (A) and ERG (B). The color-coded scale bars indicate log2 RNA expression levels.
Spatial organization of luminal and basal cells

Most prostate cancers originate from epithelial cells. The human prostate contains two major epithelial cell subtypes, basal cells and luminal cells (6; Figure 4A). Using canonical markers associated with basal and luminal cells, we sought to map the spatial organization of these two cell types (Figure 4B, C). Within the normal prostate sample, basal and luminal cells were observed to have similar spatial organization (Figure 4D, top). In the stage III adenocarcinoma sample, this spatial organization is lost, with the luminal cell population exhibiting a substantial expansion in the invasive carcinoma region that does not occur with basal cells (Figure 4D, bottom). Understanding this disorganization of luminal cells could provide insights into tumor dynamics.

Figure 4. Spatial organization of basal and luminal cells is disrupted in adenocarcinoma. A. Schematic of the cellular architecture of the normal prostate epithelium. B–C. Expression levels and spatial organization of basal and luminal cells in normal prostate and in the stage III adenocarcinoma sample. D. Overlay of the spatial organization of both cell types.
Characterization of immune cells

Immune cells are one of the key non-tumor cell populations present in tumor microenvironments influencing both tumor suppression and progression (7). Pathologist annotation of the stage III adenocarcinoma sample identified a small population of immune cells within the sample, outlined in yellow (Figure 2B, left). No immune cells were identified in the stage IV acinar cell carcinoma by pathologist annotation (data not shown). Since the Visium for FFPE assay measures over 18,000 genes in the human transcriptome, any number of genes can be viewed in combination and analyze together. However, by assessing unique cellular signatures based on canonical markers for immune cells, Visium revealed a large presence of immune cells in both tumor samples (Figure 5) and was able to distinguish between immune cell types, specifically B and T cells in this example. Of particular note is the distribution of plasma B cells along the invasive carcinoma border of the stage III adenocarcinoma (Figure 5A, left). No specific pattern was observed in the acinar cell carcinoma (Figure 5B, right). T cells were observed to be scattered across the whole section for both tumors. Further investigation and more detailed characterization of these immune cell populations may provide additional insights into disease progression or treatment options.

A. Plasma B-cell (IGHA1, IGHG1, JCHAIN, IGKC, IGLC1) localization

B. T-cell (CD3D, CD3E, CD4, CD8A, CD247) localization

Figure 5. Visium for FFPE reveals immune cell infiltration, distribution, and characterization in the TME. Spatial gene expression patterns (shown as UMI counts) for markers associated with plasma B cells (A) and T cells (B) in both tumors.
Conclusion
Prostate cancer can exist in patients while posing little to no threat to mortality. However, it can also manifest as an extremely aggressive tumor that requires immediate life-saving interventions. Spatial gene signatures can significantly evolve our understanding of the tumor microenvironment, enabling development of better diagnostic and prognostic approaches, including personalized cancer treatment.

Visium Spatial Gene Expression for FFPE enabled comprehensive profiling of two different prostate cancer samples. The tissue-wide, whole transcriptome gene expression data obtained from Visium for FFPE complements pathological annotations, while providing additional insights on tumor heterogeneity, biomarker expression patterns, and spatial organization of cell types in the microenvironment.

References

Resources
Explore the Visium for FFPE data from these samples further by downloading the following datasets:
- Normal human prostate (download)
- Human prostate adenocarcinoma (download)
- Human prostate acinar cell carcinoma (download)