# Ultra High-plex Spatial proteomic and transcriptomic profiling of head and neck cancers: Insights into immunotherapy response and resistance

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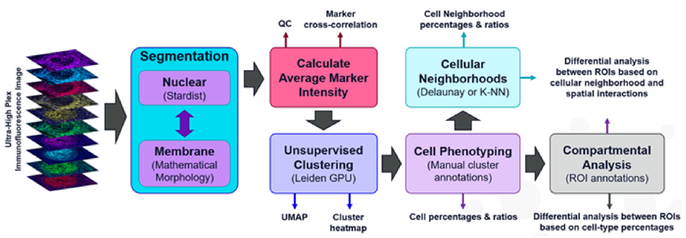
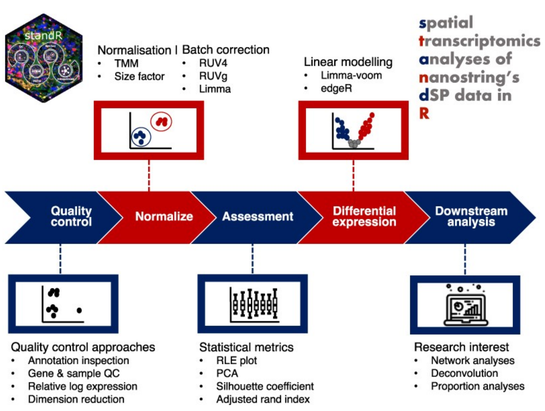
## Introduction

Head and neck squamous cell carcinomas (HNSCCs) are the seventh most common cancer and represent a global health burden. Immune checkpoint inhibitors (ICIs) have shown promise in treating recurrent/metastatic disease with durable benefit in ~30% of patients. Current biomarkers for HNSCC are limited in their dynamic ability to capture tumor microenvironment (TME) features with an increasing need for deeper tissue characterization. Therefore, new biomarkers are needed to accurately stratify patients and predict responses to therapy. Here, we have optimized and applied an ultra-high plex, single-cell spatial protein and whole transcriptome analysis in HNSCC. Tissues were analyzed with a panel of 101 antibodies that targeted biomarkers related to tumor immune, metabolic and stress microenvironments, and region of interest whole-transcriptome for tertiary lymphoid structures. Our data uncovered a high degree of intra-tumoral heterogeneity intrinsic to HNSCC and provided unique insights into the biology of the disease. In particular, a cellular neighborhood analysis revealed the presence of six unique spatial neighborhoods enriched in functionally specialized immune subsets. In addition, functional phenotyping based on key metabolic and stress markers identified four distinct tumor regions with differential protein signatures.

## Method

Retrospective HNSCC tissues were collected before treatment and stored as FFPE blocks from the Royal Brisbane and Women's Hospital (RBWH) with Human Research Ethics HREC Approval No. LNR/2020/QRBW/66744 and The University of Queensland ratification. Patients were treated with pembrolizumab or nivolumab and categorized based on response to therapy according to RECIST 1.1, including CR, PR, SD, and PD. Serial sections of hematoxylin and eosin (H&E) staining were prepared by Pathology Queensland and reviewed by a pathologist for tumor/stroma demarcation. Serial tissue sections were profiling using the Phenocycler Fusion (Akoya Biosciences) and Nanostring GeoMx Digital Spatial Profiler for targeted proteome (101-plex) and unbiased whole-transcriptome, respectively. Data was analysed using in-house computational workflows and previously published approaches (Liu et al., NAR 2023; Jhaveri et al., GEN 2023).




## Results

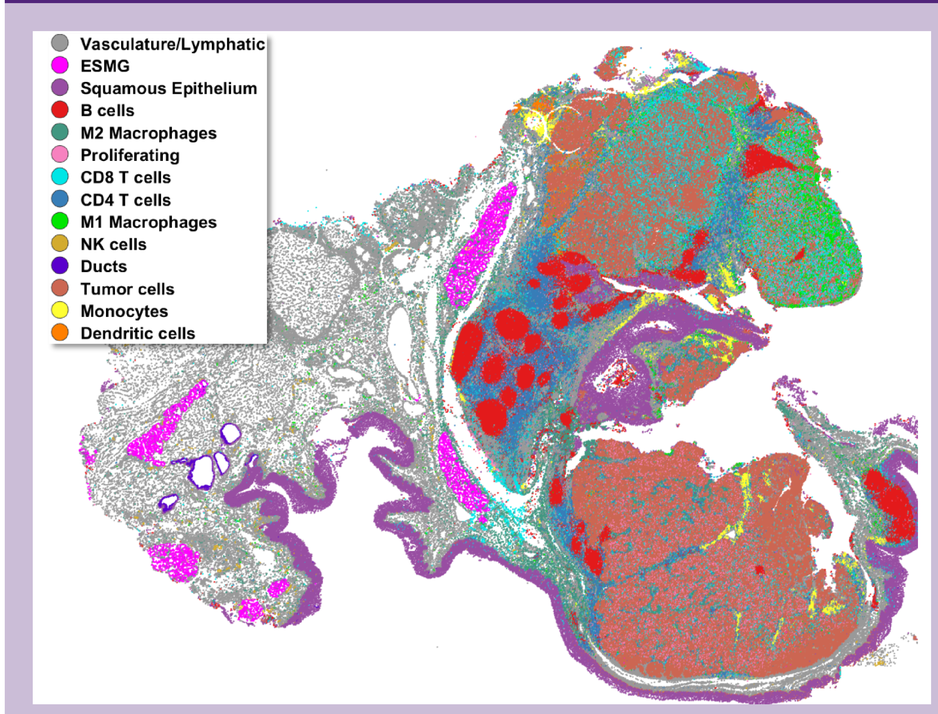



Figure 1. Spatial proteomic profiling of HNSCC (101-plex) to demarcate major cell types through the TME.

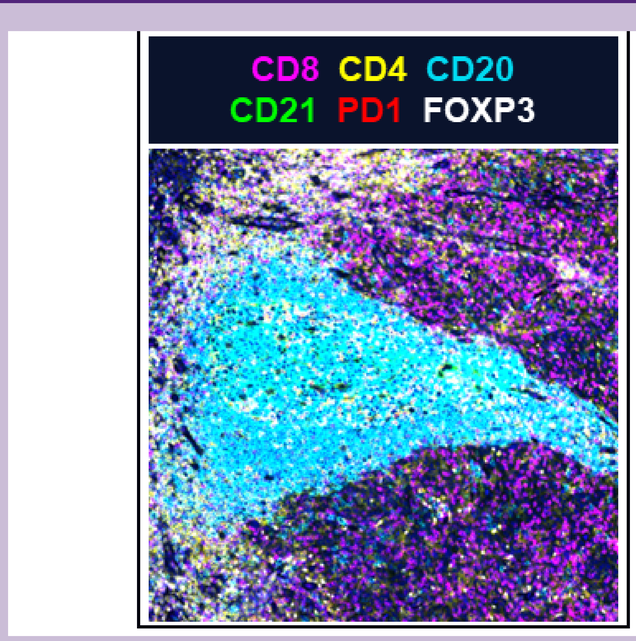



Figure 2. Multi-protein definition of Tertiary lymphoid Structure (TLS) - top right of TME.

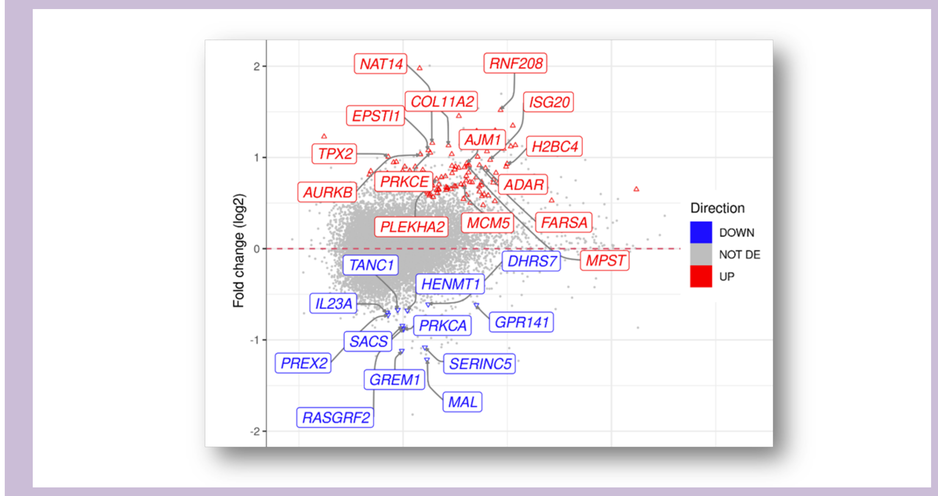



Figure 3. Differential expression between the TLS and normal germinal centres. Genes upregulated in TLS (Red), upregulated in normal tonsil (blue)

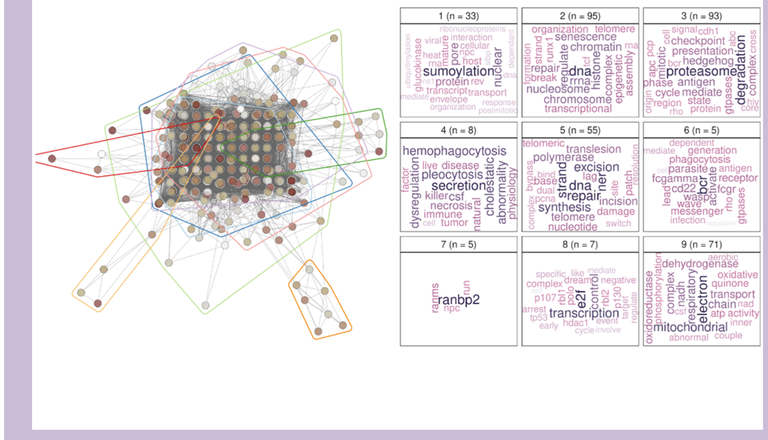



Figure 4. Visee plot of top DEG between TLS and germinal centres (upregulated pathways in TLS vs normal germinal centres)

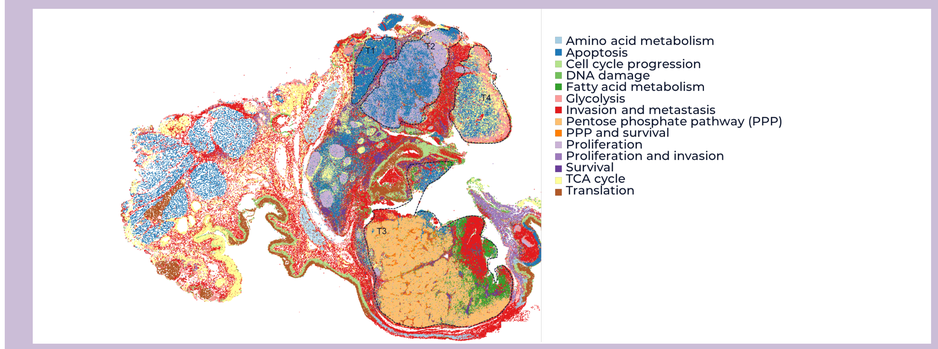



Figure 5. Spatial Metabolic map of the whole tumour highlighting areas of response (increased proapoptotic pathways - increased expression of BAX, BAK) and resistance (increased expression of GLUT1, G6PD)

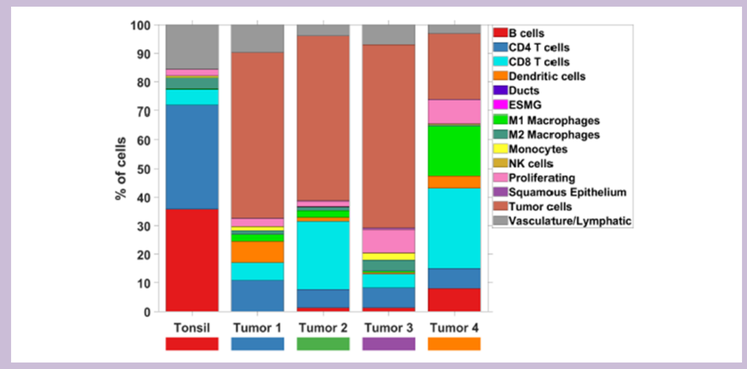



Figure 6. Multi-compartment analysis of the subtumours shows that M1 macrophages are co-localised in T4, wheras M2 macrophages are found in T3

## Conclusion

This dichotomy of immune activation-induced death and tumor progression in the same sample demonstrates the heterogenous niches and competing microenvironments that may underpin variable clinical responses. Our data integrate single-cell ultra-high plex spatial information with the functional state of the TME to provide insights into HNSCC biology and differential responses to ICI therapy. We believe that the approach outlined in this study will pave the way toward a new understanding of TME features associated with response and sensitivity to ICI therapies.