

In Situ Spatial RNA Detection of Type II Endometrial Carcinoma Tumor Microenvironment within FFPE Uterine Tissue

2022-12-23

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Abstract

This paper outlines the process of qualifying and optimizing Formalin-Fixed Paraffin-Embedded (FFPE) uterus tissue for in situ spatial RNA detection in Type II, Stage 1 endometrial carcinoma.

Keywords spatial transcriptomics, immunoncology, endometrial cancer, FFPE, RNA amplification, lock-and-roll, rolling circle

Institute (????)

Special thanks to Miguel Flores, Sarah McGuire, Tiffany Jones, and David Baumeister for their guidance and never-ending support.

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1 Introduction

In the never-ceasing fight against cancer, the immune microenvironment has become front and center. As B-cells and T-cells have became phenotyped, and immune population clusters divided into macrophage high and natural killer cell low groups, the spatial distribution of these immune soldiers become more and more relevant.(Makker, MacKay, Ray-Coquard, Levine, Westin, Aoki, and Oaknin (2021)) A prime example being tertiary lymphoid structures - distinct immune structures of varying immune cell composition, frequently found in tumor microenvironments. In the quest for higher resolution data, spatial transcriptomics has emerged as a worthwhile contender.

2 The Growing ECMA Caseload Problem

2.1 Setup

Endometrial cancer is increasing in incident rate, due to a number of factors. Some of these factors include obesity (sedentary lifestyle instead?) and delaying or never having children.(Shih, Wang, and Wang (2021)) While Type I EC has an relatively high recovery and survival rate, the same is not true for Type II EC. Early detection and early resection are key to a high chance of survival. It is anticipated that Type II case with continue to climb as the time marches on.(McAlpine, Temkin, and Mackay (2016)) Developing better tools for early, non-invasive diagnosis, as well as deeper investigative tools for disease pathology, therapy development, screening, and therapy qualifying.(Willvonseder, Stögbauer, Steiger, Jesinghaus, Kuhn, Brambs, Engel, Bronger, Schmidt, Haller, Weichert, Keller, Noske, Pfarr, and Boxberg (2021))

2.2 Comparison to Existing iTME Knowledge

In the never-ceasing fight against cancer, the immune microenvironment has become front and center.(Meyer and Zenclussen (2020)) As B-cells and T-cells have became phenotyped, and immune population clusters divided into macrophage high and natural killer cell low groups, the spatial distribution of these immune soldiers become more and more relevant.(van der Woude, Hally, Currie, Gasser, and Henry (2022)) A prime example being tertiary lymphoid structures - distinct immune structures of varying immune cell composition, frequently found in tumor microenvironments. In the quest for higher resolution data, spatial transcriptomics has emerged as a worthwhile contender.

Within the immune landscape of EC, there are many important players. The lineage of these cells is an important consideration as mutations within the surrounding tumor microenvironment are common. Myeloid-derived suppressor cells (MDSCs) originate from bone marrow stem cells, and are often upregulated in chronically inflamed regions, a result of altered hematopoiesis. Regions with high MDSC populations also exhibit T-cell suppression. The investigation of this mechanism throughout early stage endometrioid development would provide further pathological insight into EC.

2.3 Recent ECMA iTME Discoveries

2.3 Recent ECMA iTME Discoveries

Powerful therapy tools exist to combat cancer; among the many options, immune checkpoint inhibitors, CAR-T cell therapy, among many other treatments. When these therapies work, tumor rapidly shrink, metastasis is stopped in its tracks, and the risk of recurrence is low. However, often these expensive and taxing treatments results in no improvement. A growing consensus is the importance of the immune microenvironment in the treatment of malignancies in the body. With increased focus on the immune players of the tumor milieu, research has focused on categorizing immune populations, phenotyping T-cells, B-Cells, NKs, and macrophages, and single cell sequencing.(Yamamoto, Mabuchi, Yamasaki, Yoshimura, and Murata (2013)) Alongside immunohistology and the identification of tertiary immune structures within chronically inflammation tissue, high resolution, spatial RNA detection and distribution of immune cells and biomarkers are the newest frontiers.

Endometrial cancer is a sneaky beast - she can lie in wait and slowly grow, enduring a steady assault of T-cell and NK cells at her perimeter.(Souto, Vila, and Brú (2011)) But EC is clever and plays the long game, recruiting a sympathetic agent within the immune camp. MDSCs hold significant influence over T-cells, and, drunk on power from EC, MDSCs convince the T-cells that EC is no threat and that they really needn't do anything much at all about her. This can be observed by staining for CD11+ (1) in conjunction with stand H and E staining (?). Our main character is MDSC - and understanding her motivations, we must also know where she goes and to whom she talks.(Draghiciu, Lubbers, Nijman, and Daemen (2015))

3 An Overview of Spatial Transcriptomics

High spatial resolution within FFPE is a challenge, but a challenge worth undertaking. Endless bio-banks of data could be used if only the biomaterial were accessible. Both immunostaining and fluorescent in situ hybridization (FISH) are techniques currently used and commercially available for single-cell (is this true?) resolution,; however, these techniques perform considerably better on freshly frozen tissue, with no exposure to paraffin. Paraffin was not only creates difficulties in permeabilizing the tissue, but also with autofluorescence and background during fluorescence imaging.

3.1 In Situ RNA Detection

Lock'n'Roll, an in situ nucleic acid amplification technique (patent; how is this referenced?) paired with nucleic acid tags, has had early success on FFPE tissue. Lock'n'Roll is a form of rolling circle amplification, occurring in situ, “locked” onto the target RNA. This results in a “ ball” of amplified targets with a nucleic acid tag sequence embedded repeatedly within the Lock'N'Roll ball. Using a 4 sequence barcode, multiple fluorescent tags can be imaged and analyzed to call out specific targets whenever the correct colocalization is detected. The size of the Lock'n'Roll ball is important, allowing colocalization during imaging analysis. An example can be seen in ??.

3.1 *In Situ* RNA Detection



Figure 1 CD11+ (marking myeloid-derived suppressor cells, MDSCs) staining on uterine endometrioid carcinoma, stage I. Slide stained and provided by the Shih Laboratory, Johns Hopkins School of Medicine.

3.1 *In Situ* RNA Detection

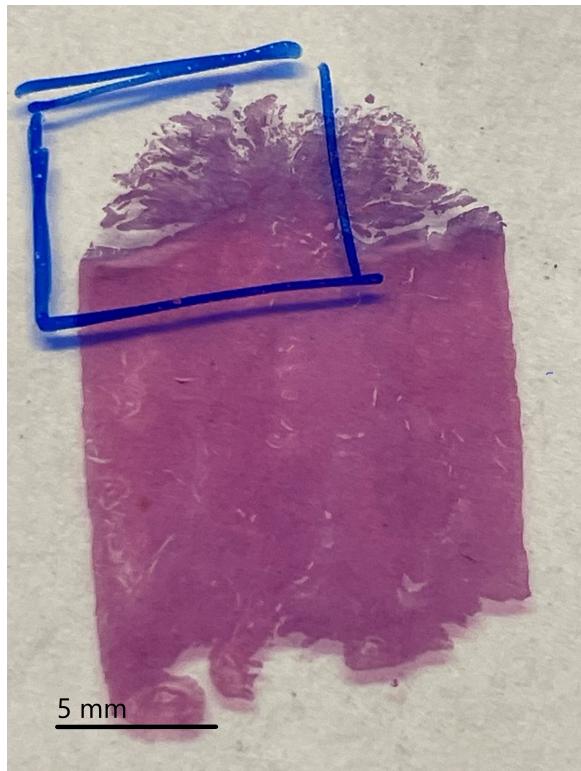
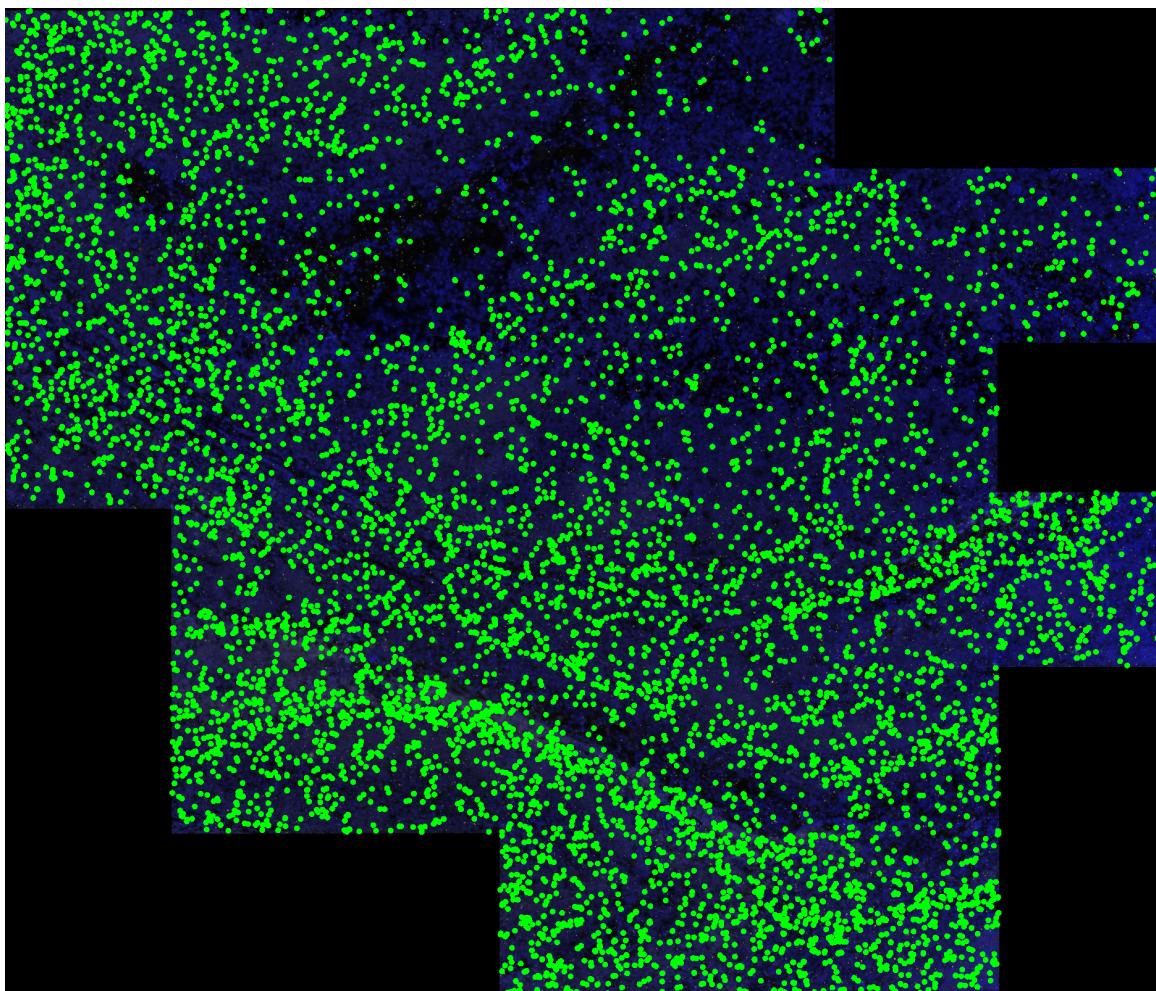


Figure 2 Hematoxylin and Eosin staining on uterine endometrioid carcinoma, stage I. Slide stained and provided by the Shih Laboratory, Johns Hopkins School of Medicine

3.1 *In Situ* RNA Detection

Figure 3 Compiled imaged of transcript callouts within FFPE human tonsil tissue.
DAPI is stain in blue, B2M RNA is called out in green.



4.1 Automated Multi-round Fluorescence Microscope Imaging

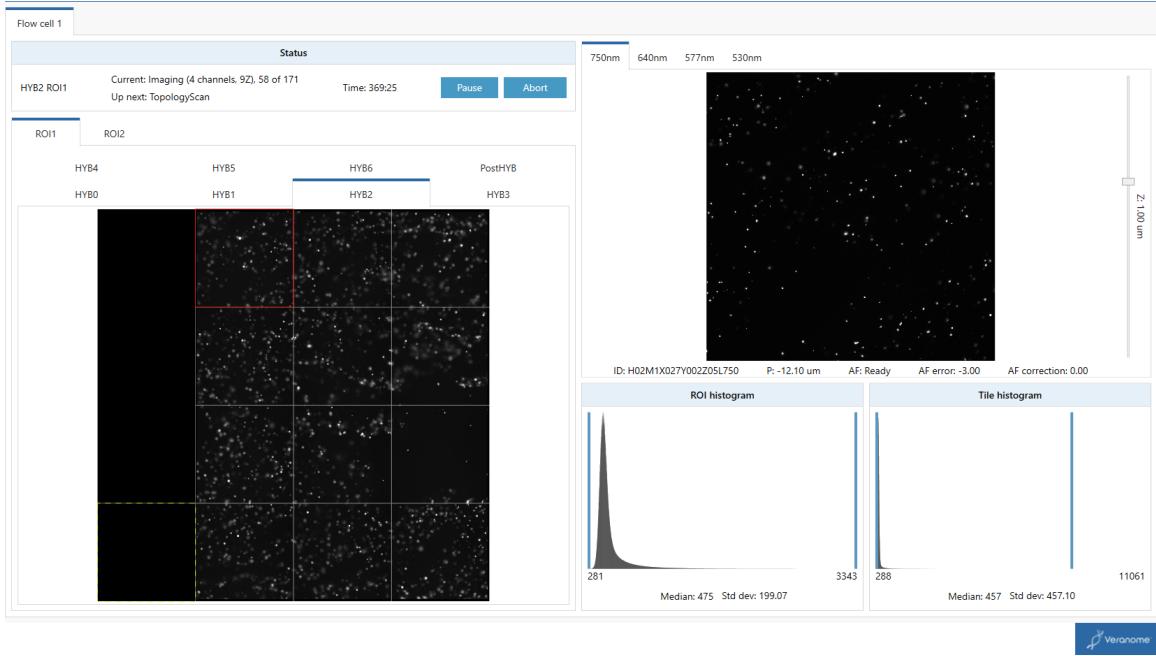


Figure 4 Automated, Multiround Imaging of In Situ Fluorescent Readout Probes

4 Methods

4.1 Automated Multi-round Fluorescence Microscope Imaging

4.2 Calculation of Codewords with Error Correction

As discussed in Chen, Boettiger, Moffitt, Wang, and Zhuang (2015), the probability of a mis-identified transcripts can be calculated and corrected.

Beginning with the probability of properly identified RNA transcripts with a 4 'ON' bits of 24 total bits, 1.

$$(1 - p_1)^m (1 - p_0)^{N-m} \quad (1)$$

The impact of a '0' that should be a '1', or a '1' that should '0' is equivalent; both result is a wrongly identified transcripts. However, the probability of a '0' (no spot) being misidentified as a '1' is lower than a '1' (true spot) being misidentified as a '0'. This is based on the logic that a fake spot is likely than a missed spot. These errors are weighted accordingly in 2

$$\frac{1}{2^N} \sum_{m=0}^N \binom{N}{m} (1 - p_1)^m (1 - p_0)^{N-m} \quad (2)$$

Finally, to measure the accuracy of the decoded analysis, a Mis-Identification Rate (MIR) can be calculated using ???. This is a ratio of the true RNA transcripts that

5.1 Highly Expressed Transcripts Across Tumor Border

have been misidentified as the *wrong* RNA transcripts compared to *true positive* RNA transcripts.

$$1 - \frac{1}{2^N} \sum_{m=0}^N \binom{N}{m} (1-p_1)^m (1-p_0)^{N-m} \quad (3)$$

5 Results

This experiment is in-processed with limited data available at this time.

5.1 Highly Expressed Transcripts Across Tumor Border

Initial imaging during the optimization process reveals high expression among cell types typically crucial in hot immune environments. These transcripts are expressed to create a locally cytotoxic microenvironment due to inflammation caused by the carcinoma.(Vanderstraeten, Luyten, Verbist, Tuyaerts, and Amant (2014))

Key Transcripts in Endometrial Carcinoma with "Hot" Immune Prescence			
Transcript	Protein Type	Specific Function	Role
CD8B	T-cell Surface Glycoprotein	Activates CD4+ CTLs	Immune function
CX3CR1	Immature WBCs Chemokine Receptor	Recruits NK cells through inflammation	Immune function Chemotaxis
TGFB1	Growth Factor	Promotes Th17 & Tregs cells	Immune function Normal Development
CD69	T cell, B cell, NK cell Post-activation Antigen	Promotes lymphocyte proliferation	Immune function
IL-6	Cytokine	Stimulates B cells Differentiates CD4 T cells	Immune function Tissue Regeneration
PRF1	Perforin Protein	Creates pore in cell	Programmed Death
CD1C	T-cell Surface Glycoprotein	Presents antigen to TCR and NK cells	Immune function
CXCL11	Chemokine	Attracts & induces Ca+ release in activated T cells	Immune function Chemotaxis
TGFB3	Growth Factor	Stimulates Growth	Immune function Normal Development

Table 1

Key Transcripts in ECMA Local Tumer Immune Microenvironment

Broad Institute (2022) ICGC/TCGA Pan-Cancer Analysis of Whole Genomes Consortium (2020)

5.2 Computational Challenges

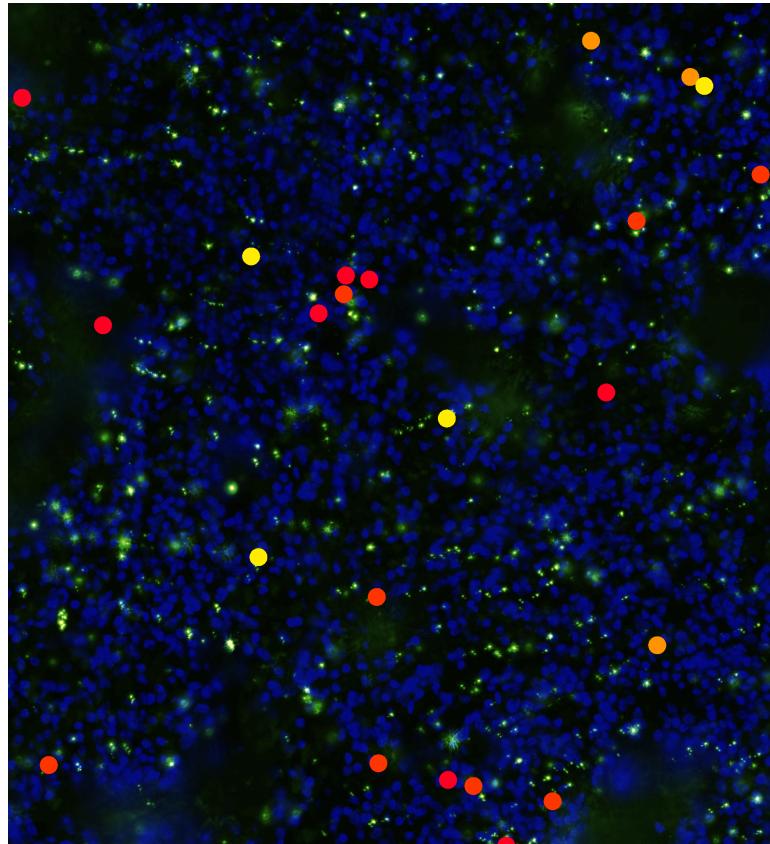


Figure 5 Compiled imaged of transcript callouts within FFPE human spleen tissue. DAPI is stain in blue, RNA is called out in green. Note the large frequency of RNA spots uncorresponding to a transcript.

5.2 Computational Challenges

A repeated challenge through analysis was computational overcorrection for errors. This can be observed in 5, where RNA spots are visible in yellow, but positive transcripts cannot be identified by the decode executable.

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5.3 Conclusion

As the project continues, more data will become available, allowing a conclusion section to be thoroughly written. Stay tuned!

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