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Single-Cell, Spatial Biology in Formalin Fixed Paraffin-Embedded (FFPE) Tissues with the PhenoCycler Solution

HIGHLIGHTS:

- **Biomarker discovery in tissue samples requires unrestricted, non-ROI based imaging with single cell resolution.**

- **The PhenoCycler platform is part of a practical, end-to-end solution for ultra-high plex imaging of 40+ protein markers.**

- **Compatibility with FFPE tissues enables single cell discoveries in archived tissues.**

- **Whole tissue imaging of a breast cancer FFPE sample enabled the analysis of a rare cell population that could have been missed with an ROI-based approach.**

INTRODUCTION

Single cell biology is the simultaneous detection of dozens of biomarkers in the same cell and has become a dominant tool in biological discovery. Single cell biology techniques include scRNA sequencing, spatial transcriptomics and single-cell proteomics. These methods are ubiquitous in cancer research (Lawson et al, 2018), immunology (Papalexi et al, 2018), neurosciences (Tasic et al, 2018), infectious disease research (Avraham et al., 2016) and pharmaceutical drug development (Khan and Gerber, 2020). Due to the sheer amount of data that can be extracted from single cell biology experiments, we expect these techniques to dominate the life sciences for years to come.

Translational research and clinical pathology often depend on precious human tissue samples that are available as formalin-fixed paraffin embedded (FFPE) specimens. FFPE preservation is cheap and offers excellent tissue preservation and is thus the preferred preservation method for vast amounts of biobanked tissues. However, FFPE is incompatible with many single cell biology techniques and it is thus not possible to conduct single cell experiments in many of the clinically relevant tissues that are available to researchers.

In addition, single cell biology requires true single cell resolution. This is granted for techniques like scRNA-Seq that do not have spatial information. Other emerging spatial

biology platforms, on the other hand, have overcome the 'FFPE hurdle' but restrict their analyses to predefined regions of interest (ROIs) that exceed single cell diameters by several-fold. Such experiments are therefore not true single cell technologies.

CO-Detection by indEXing (PhenoCycler) pragmatically solves these problems through a modification to conventional multiplex immunofluorescence imaging (mIF). In doing so, the PhenoCycler technology provides a sensitive, reproducible, and highly multiplexed method for detecting 40+ proteins in FFPE tissues **(Figure 1)**.
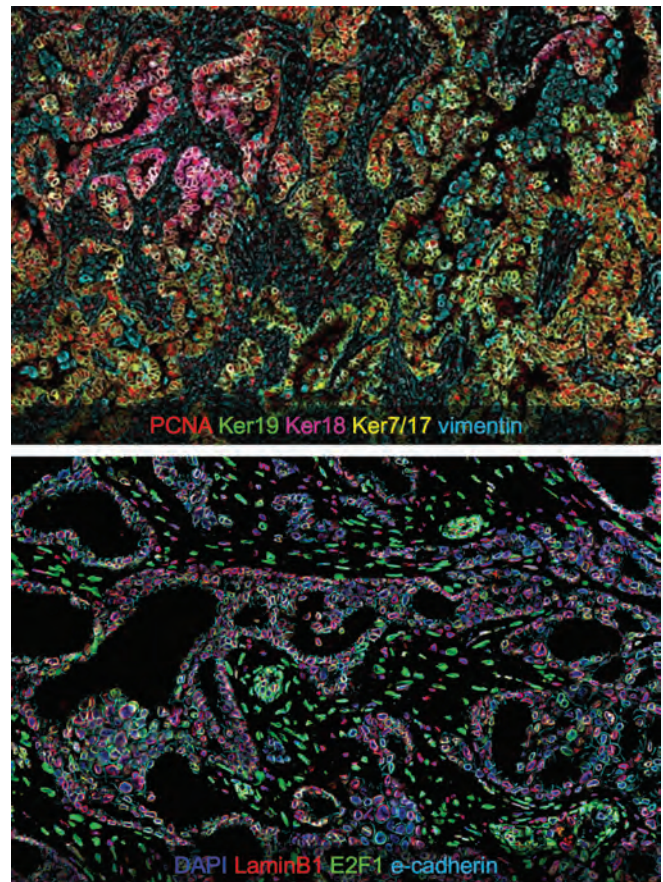

FIGURE 1. *Regional and single cell spatial biology data acquired with the PhenoCycler technology.* Top: Regional differences in Keratinocyte makeup are easily captured via unrestrained large-tissue multiplex imaging with PhenoCycler. Bottom: True single cell resolution enables visualization of subcellular components like the nuclear envelope (LaminB1) and the plasma membrane (e-cadherin).

THE PHENOCYCLER WORKFLOW

PhenoCycler is based on immunofluorescence imaging and is thus deployable without the need for complicated instrumentation or operational infrastructure. The PhenoCycler chemistry is an iterative workflow that relies on a DNA-based tagging approach, whereby antibodies are labeled with specific oligonucleotide tags (barcodes) and dye-oligonucleotides (reporters) are iteratively hybridized and dehybridized across multiple cycles. This process is completely automated through the PhenoCycler instrument and reporter readouts are acquired using standard epifluorescence optics. Sample preparation for PhenoCycler follows established guidelines and employs readily available commercial reagents **(Figure 2)**.

FIGURE 2 *The PhenoCycler workflow and chemistry.*
Top: The PhenoCycler Instrument is part of a complete workflow that also includes reagents and an analysis and visualization software suite. The PhenoCycler system integrates with the Keyence BZ-X 700/800 inverted epifluorescent microscope stage through a custom stage insert. Bottom: The single step staining preserves sample integrity for re-analysis of specific regions. The Reveal-Image-Remove cycles are completely automated on the PhenoCycler instrument.

The large number of cells and biomarkers that can be imaged in a single PhenoCycler experiment produce rich and complex datasets. To that end, we have developed the Multiplex Analysis Viewer (MAV), an end-to-end software package that accompanies the PhenoCycler system.

MAV is free to use and available for download at **help.PhenoCycler.bio/PhenoCycler/**. The MAV user does not require bioinformatic training and will be able to undertake cell phenotyping and cell sorting functions via intuitive and easy-to-use interfaces. Built-in MAV functions include signal gating, unbiased clustering, T-SNE plotting, cell sorting, nearest neighbor analyses and visualization of spatial interactions.

The following section gives an overview of a PhenoCycler dataset that was acquired from human FFPE sample tissue and analyzed entirely with the MAV software. The result is a demonstration of single cell discovery in a clinically relevant tissue.

PHENOCYCLER IS DESIGNED FOR UNRESTRICTED SINGLE CELL DISCOVERY IN FFPE TISSUES

Biological discovery requires unrestricted access to a research substrate so that even minute details can be resolved and measured empirically. Multiplex imaging technologies are ideally suited for discovery research, especially if a researcher can freely scan entire tissue sections. PhenoCycler enables researchers to scan tissues comprehensively and to record each cell on a tissue; the only limit is the optical resolution imposed by the objective lens. Because of this, even rare cell populations can be detected. This is illustrated in the case study below.

A 36-plex PhenoCycler antibody panel was used to study epithelia, oncogenes and immune cells in a stage II ductal adenocarcinoma breast tissue. The list of markers is shown in **Table 1**. Representative images are shown in **Figure 3**.

TABLE 1 *36-plex PhenoCycler Custom Panel Design for Breast Cancer FFPE Analysis*
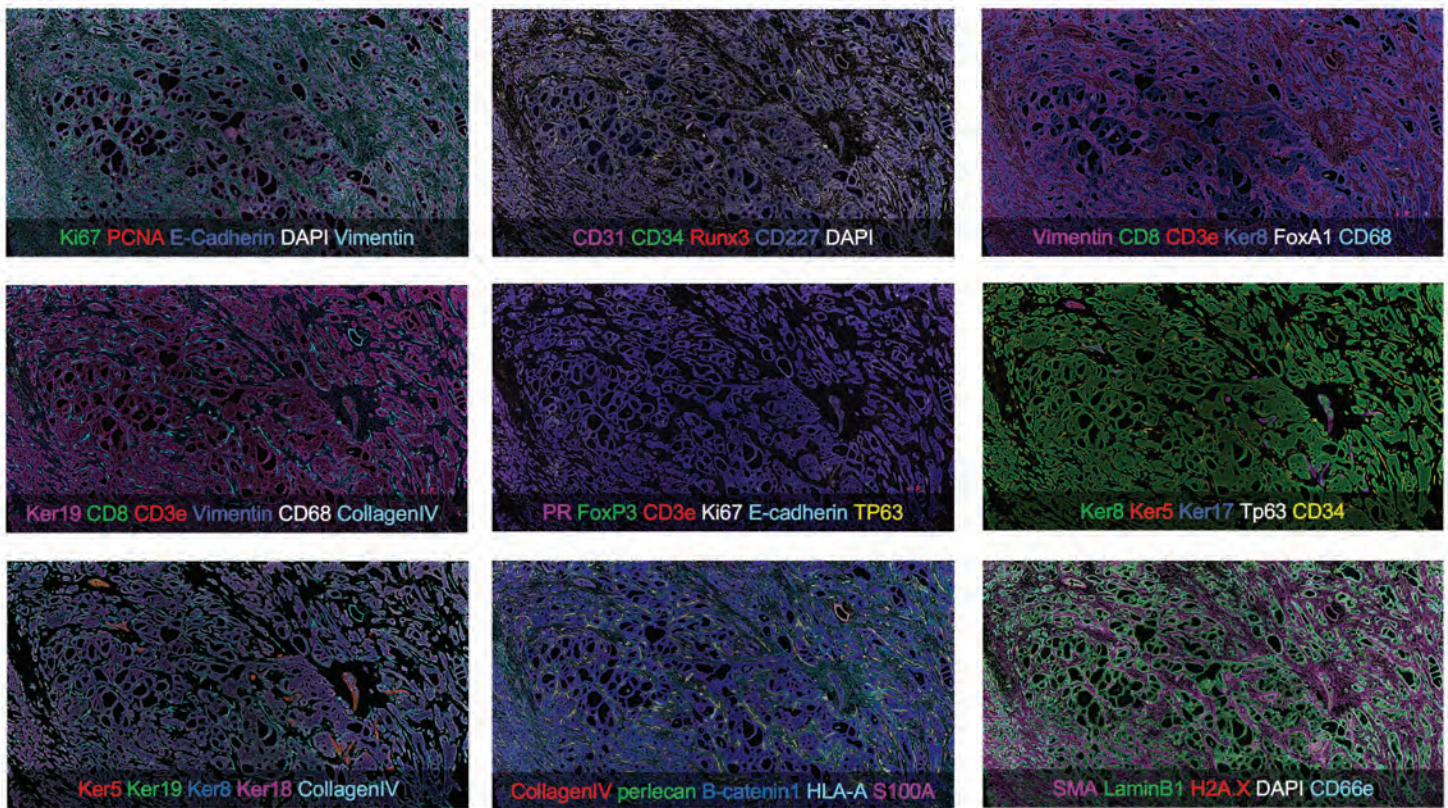
FIGURE 3 *Multiplexed image series of human breast FFPE tissue:* A Stage II Ductal Adenocarcinoma sample (size: 6.76 mm²) was stained with 36 antibodies and imaged with the PhenoCycler System.

Human breast tissue is composed of diverse epithelia, which comprise basal and luminal layers in secretory and non-secretory lobular and ductal collecting structures, respectively (Nguyen et al, 2018). Luminal and basal epithelia are abundant, easily detected and selectively identifiable with certain biomarkers. **Figure 4** shows an example in which basal (Ker5 and Ker 17) epithelia are purple and clearly distinguishable from Ker8 positive luminal epithelia (green).
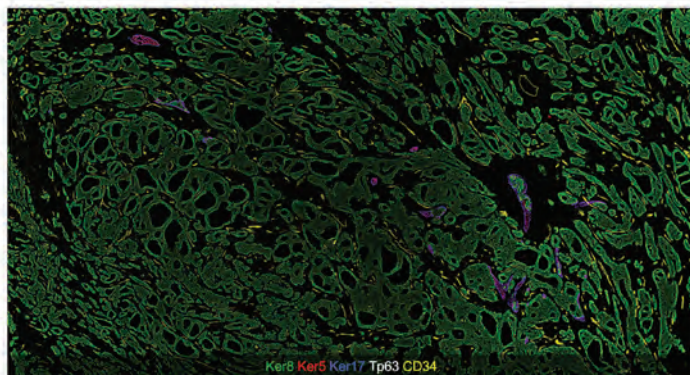

FIGURE 4 Markers to distinguish basal (purple, Ker 5 and Ker 17) and luminal epithelia (green, Ker 8)

RECAPITULATION OF INDEPENDENTLY PUBLISHED FINDINGS

The above-mentioned basal and luminal cell types comprise most of the epithelial cells known in human breast tissue, but lesser known epithelial cells also exist. Ngyuen et al. (2018) identified a rare epithelial cell type that expressed both Ker 14 and Ker 8; this cell had previously only been described in mice, and since only once in humans. We designed an antibody panel to study breast tissue that included both Ker 14 and Ker 8 markers **(Table 1)**. We then asked if we could detect the rare Ker14/Ker8 cell population via PhenoCycler imaging of a large tissue sample **(Figure 5)**. Overall, we imaged 63'056 cells on the tissue sample shown in Figures 3 and 4. We then segmented the entire cell population and clustered individual cell phenotypes via unbiased K-means clustering built into our MAV software. The resulting T-SNE plot is shown in **Figure 5** and a single cell population of 44 cells (0.07% of total) is indicated in cyan. This small population of 44 cells labels with both, Ker14 and Ker8 antibodies, as is shown in the imaging data on the right hand side of **Figure 5**. Ker8 labeling in these cells is both sparse and weak and it would be easy to overlook this cell population if data were a) analyzed manually and b) restricted by setting of imaging ROIs *a priori*. Comprehensive multiplex imaging and unbiased cell phenotyping, however, captures these cells as a distinct population as is shown in **Figure 5**.
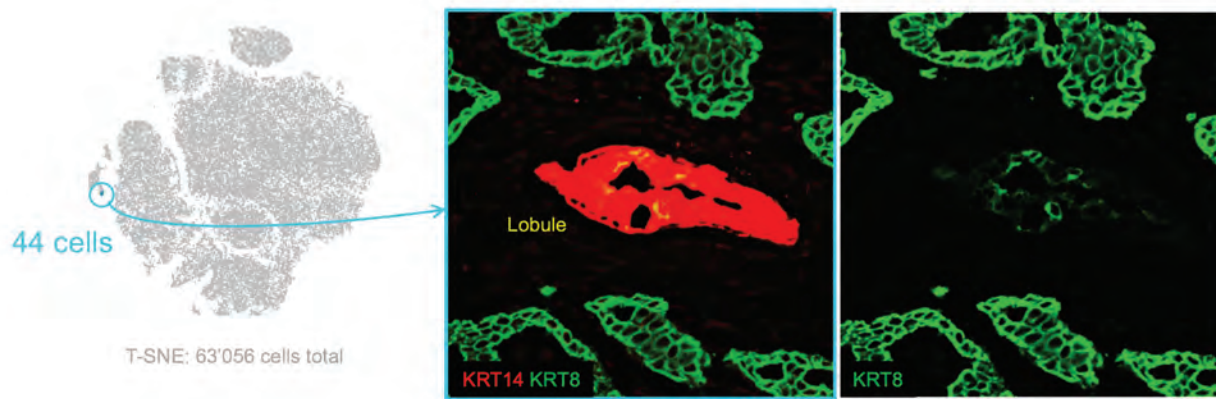
FIGURE 5 *Detection of rare cells with PhenoCycler.* Left: T-SNE population map of 63'056 cells clustered from image shown above. Right: anatomical data from the same experiment confirming the Ker14/Ker8 phenotype of rare cell population.

We confirmed the Ker 14 / Ker8 cells to be the same cell population that was previously described in Ngyuen et al. (2018). This case study thus demonstrates the ability of the PhenoCycler technology to resolve rare cell populations via unrestricted imaging of large tissue samples. This
is an important feature of our technology, given that we still know little about the etiology and origin of many diseases. There are reasons to believe that rare cell populations harbor answers to these questions, which underscores the usefulness of unrestricted highly multiplexed imaging.

SUMMARY AND CONCLUSIONS

Spatial biology is a rapidly evolving research discipline and many technologies have been designed to cater to this market. This report has summarized a key feature of PhenoCycler – namely FFPE compatibility and unrestricted access to tissues at single cell resolution - and demonstrated how these aid cancer research and discovery. In closing, we will now introduce a recent external study, which used the PhenoCycler technology for just this purpose – to discover new biological mechanisms that may underlie the etiology of colon cancer.

Schürch et al. (2020) recently exploited the PhenoCycler technology to produce an in-depth account of the composition and spatial organization of the tumor

microenvironment in colorectal cancer. The study was conducted entirely with the PhenoCycler technology and relied on precious tissue microarray samples (TMA) that were sourced from a clinical biobank. Owing to the unprecedented biomarker depth and spatial resolution of this study,
the authors were able to discover new compositional and organizational principles of the colon cancer tumor microenvironment. These principles describe how cells and 'cellular neighborhoods' organize and interact with one another and how tumor tissue modulates and controls the responses of endogenous immune cells.

Taken together, the data in these studies verify the ability of PhenoCycler to drive unbiased, single cell biological discovery in FFPE tissue samples.

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