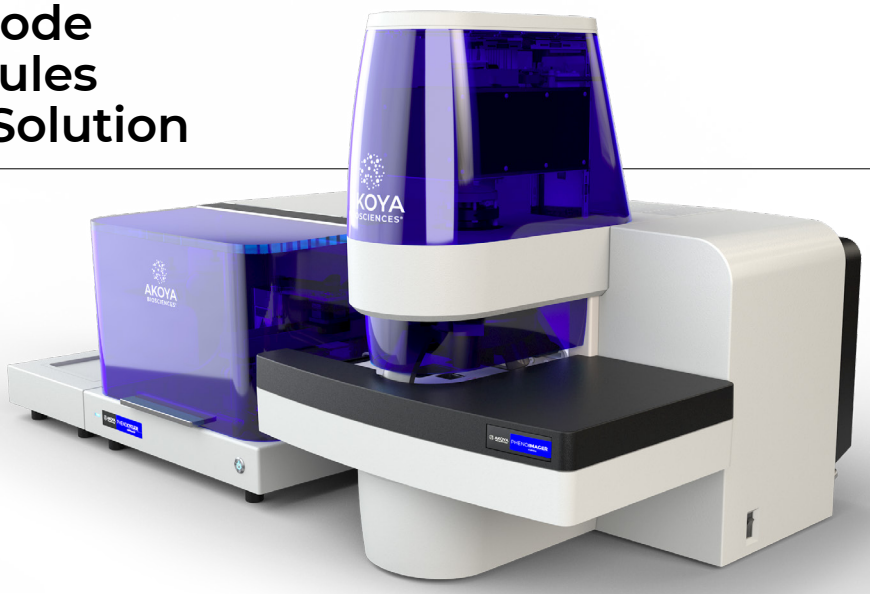
Validation of PhenoCode Discovery Panel Modules for the PhenoCycler Solution



HIGHLIGHTS

- The PhenoCycler® system allows tissues to be stained with an entire antibody panel in a single step, decreasing experiment time and preserving sample integrity
- PhenoCyler Antibodies are validated for specificity and sensitivity compared to gold standard techniques immuno-histochemistry (IHC) and immunofluorescence (IF)
- PhenoCode Discovery™ Panel Modules are pre-optimized with control and cancer tissues on PhenoCycler®-Fusion, enabling time and cost savings
- Minimal steric hindrance demonstrated between multiple antibodies across PhenoCode Discovery Panel Modules

THE PHENOCYCLER-FUSION SYSTEM

The PhenoCycler-Fusion is an integrated platform combining the strengths of Akoya's automated, ultrahigh-plex cycling platform, PhenoCycler, and its high-speed imaging platform, PhenoImager® (formerly Phenoptics), into an end-to-end, integrated workflow. The PhenoCycler uses antibodies conjugated to a proprietary library of oligonucleotides called Barcodes. This enables customizable panels of over 100 antibodies to be combined for a single tissue staining reaction. The PhenoCycler fluidics instrument automates iterative imaging cycles. For each cycle, up to three PhenoCycler Reporters, each with a spectrally distinct dye, are applied to the stained tissue to assay the corresponding Antibody Barcode. This process is repeated until all antibodies have been imaged.

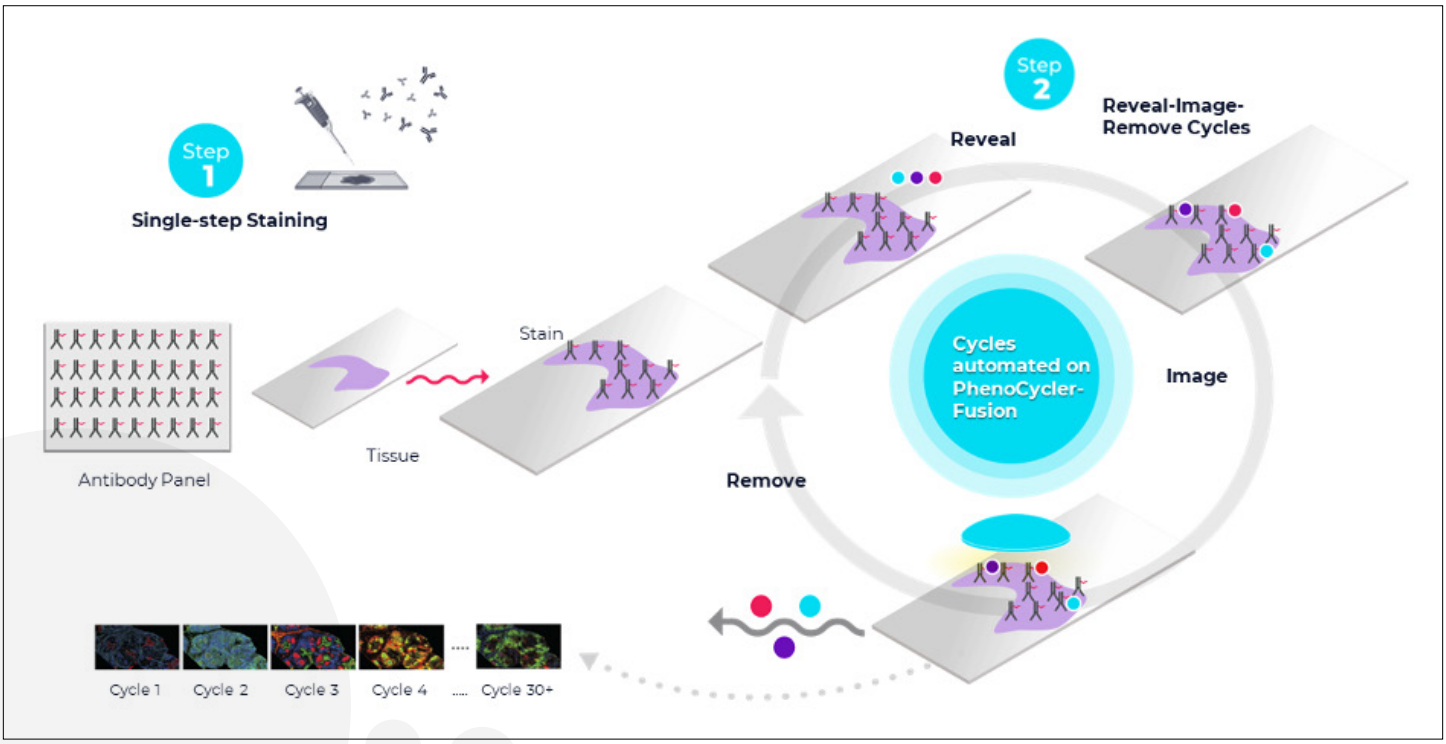


FIGURE 1. PhenoCycler Chemistry: Single-step staining followed by multicycle imaging of 100 + biomarkers.

PhenoCode Discovery Panel Module Development

The PhenoCycler Solution provides the flexibility to create panels comprised of commercially available, Akoya-validated antibodies and/or clones labeled with PhenoCycler Barcodes using Akoya's custom conjugation kit. Validated and inventoried PhenoCycler Antibodies are available for the following tissue types:

- FFPE Tissue
- Fresh Frozen Tissue

Validation of PhenoCycler Antibodies: FFPE Tissue

Each PhenoCycler antibody was conjugated to an oligo barcode and stained on human FFPE tissue. Staining specificity of each conjugated antibody was confirmed by comparing immunofluorescent results from PhenoCycler with a DAB chromogenic immunohistochemistry assay using the same antigen retrieval conditions. In each case, tissue morphology was equivalent between the DAB chromogenic immunohistochemistry assay and multiplex immunofluorescence using the PhenoCycler.

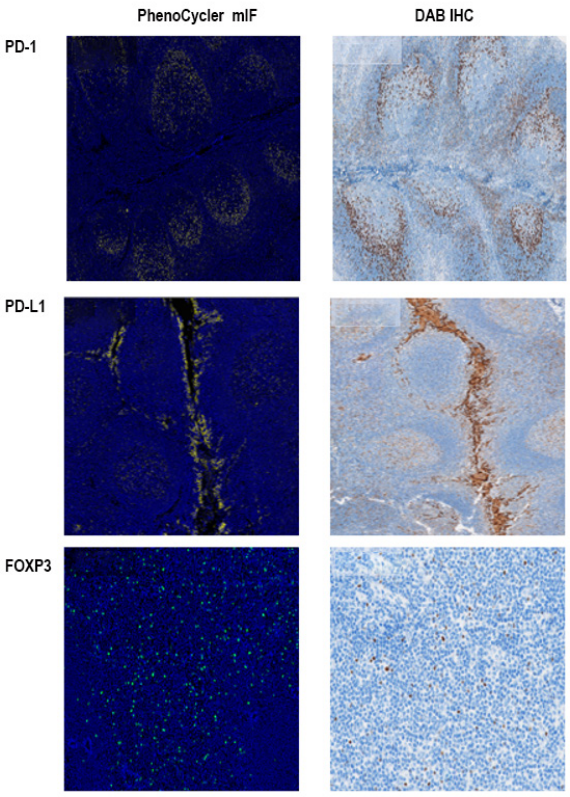


FIGURE 2. Comparison of PhenoCycler Antibody performance (left panel) with orthogonal DAB IHC (right panel) staining for PD-1, PD-L1 and FOXP3 shows comparable staining and expression patterns in human tonsil tissue.

Validation of PhenoCycler Antibodies: Fresh Frozen Tissue

Comparison of dye-conjugated and PhenoCycler Antibodies demonstrate equivalent staining patterns compared to dye-conjugated antibodies. Two fresh-frozen mouse spleen tissues were stained with anti-B220 and anti-TCR-ß antibodies as either dye-conjugated or PhenoCycler formats. In each case, the tissue morphology was equivalent between both antibody formats and matched the expected cell distribution based on the biology of the targets and test samples (Figure 3). This data demonstrates the viability of using oligonucleotide conjugated antibody moieties for tissue staining-based approaches.

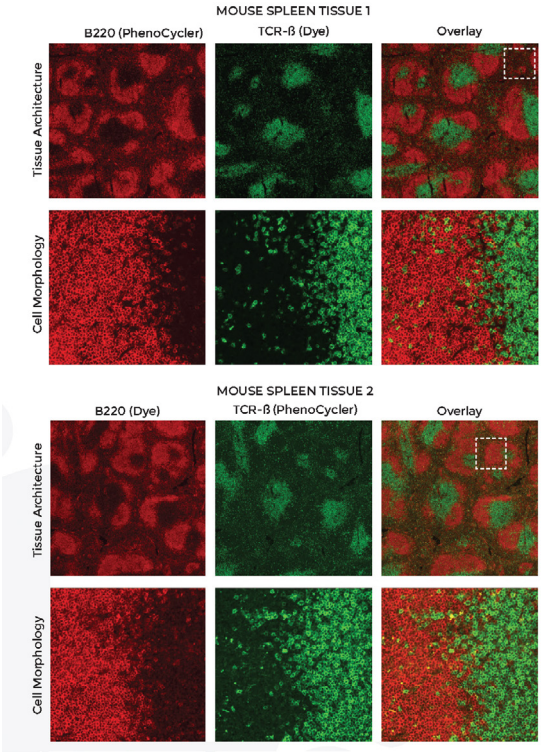


FIGURE 3. Mouse fresh frozen spleen tissues showing tissue architecture (top row) and cell morphology (bottom rows).

Single Antibody/Multi-Antibody Stain Comparison

Even when large panels of PhenoCycler antibodies are used for staining, there is minimal steric hindrance. To demonstrate this, PhenoCycler antibody staining morphology and signal intensity were assessed in the context of antibody panels of differing sizes. Fifteen different mouse antibodies were stained in three different contexts:

1. Individually
2. In the presence of two other antibodies (positive and negative counterstains)
3. In the presence of 14 other antibodies

Qualitatively, it can be observed that the staining patterns didn't significantly change under the three different conditions (Figure 4).

Quantitatively, the SNR, signal, and noise were extracted from each stained tissue. Ten of the 15 PhenoCycler antibodies yielded a higher SNR in the multicycle relative to the single antibody stains (Figure 5). Three of the PhenoCycler antibodies displayed SNR values that were more than 15% lower in the multicycle relative to the single antibody stain, with two of these examples also displaying lower SNR values in the positive and negative counterstain images as compared to the single antibody stain. This result demonstrates that most PhenoCycler antibodies are unaffected in the presence of a larger PhenoCycler panel; however, there are some cases where the signal might be lower. In these cases, users should be aware of this

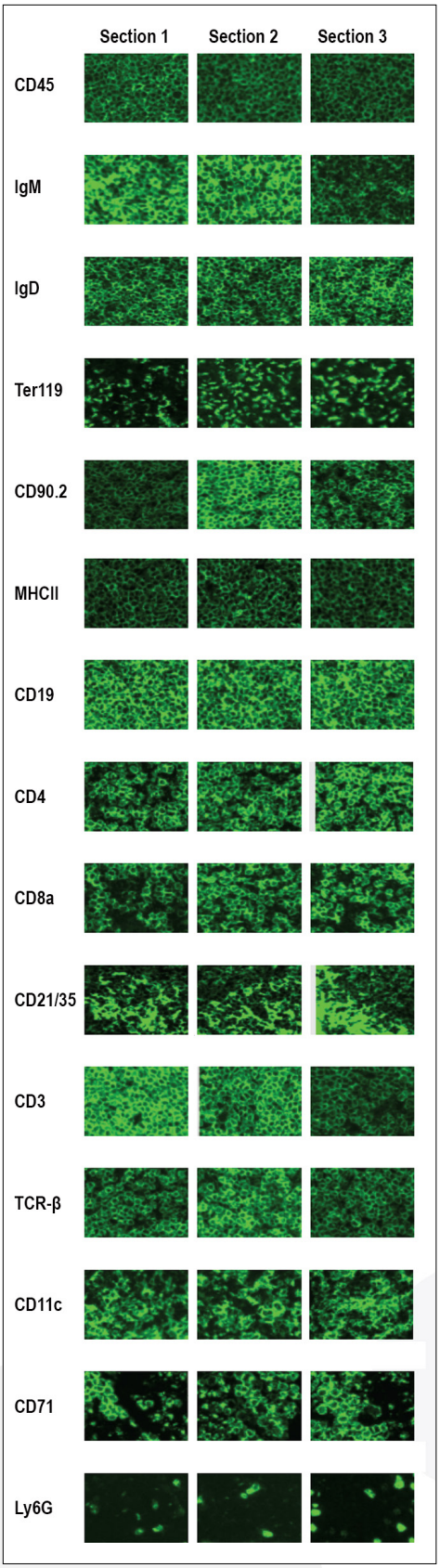


FIGURE 4. Representative images of PhenoCycler antibodies stained in the presence of other antibodies. Left column: Single antibody, Middle column: 3 antibody panel (positive and negative counterstains), Right column: 15 antibody panel.

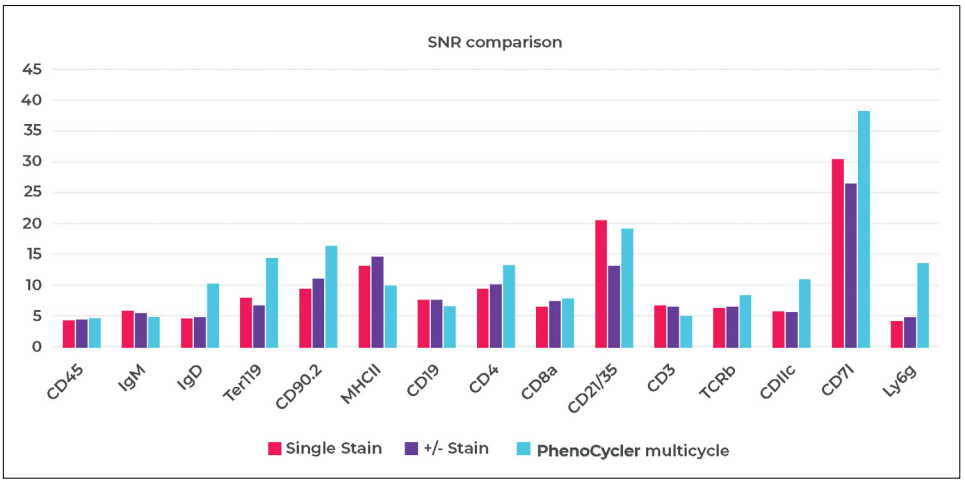


FIGURE 5. Signal-to-Noise Ratio value for each PhenoCycler antibody under the 3 different staining conditions (single antibody, 3-antibody panel with positive and negative counterstains, and 15-antibody panel).

possibility and perform proper controls for specific antibodies and combinations of interest. There were some observed examples where the signal was lower in the context of the larger PhenoCycler panel. However, in all but one of these instances, the noise was also lower, so the overall SNR was not affected. In some cases, it might be necessary to follow-up on these observations.

Antibody Titration and PhenoCode Discovery Panel Module Development

Once the conjugated antibodies have been assessed for specificity, they are configured in a panel module that contains 10 to 15 antibodies and titrated. Using tonsil tissue and at least one cancer tissue, each PhenoCycler Antibody in the panel module is tested across three different dye channels (Figure 6) and titrated as needed. Fluors are assigned to optimize signal to background ratios, as well as balance the antibodies across the three different fluors to maximize cycle efficiency. Exposure times are optimized to avoid saturation and maximize signal.

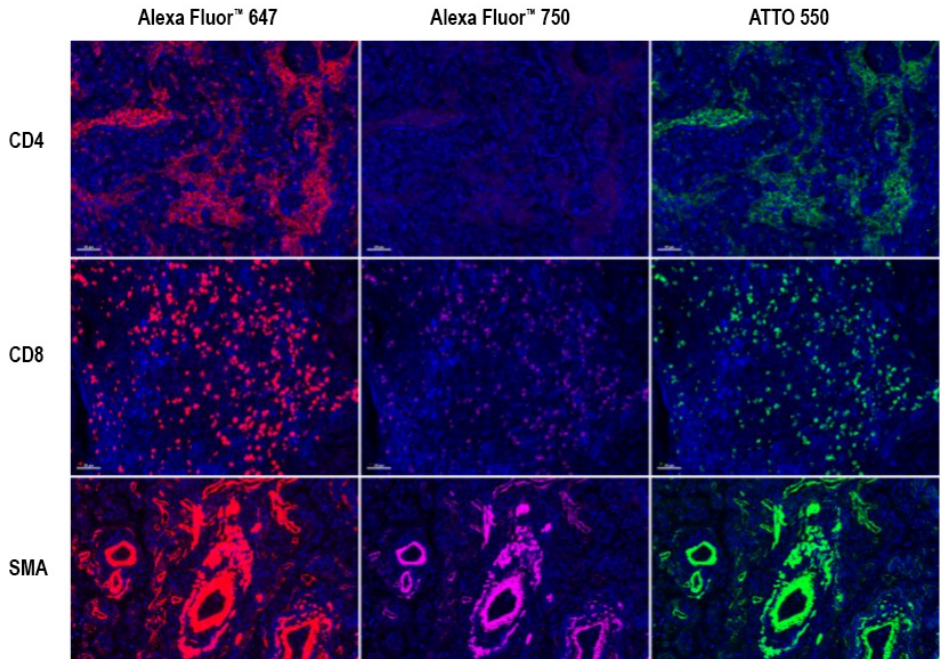


FIGURE 6. Antibodies were tested across multiple fluors on lung cancer tissue.

PhenoCode Discovery Panel Module Verification and Validation

Each panel module is run in triplicate on tonsil tissue and at least one cancer tissue, where each antibody is stained at the recommended concentration and the optimal Reporter. A negative control is run where the same tissue block is run without the PhenoCycler Antibodies to ensure that staining is specific to the antibodies in the panel module (Figure 7). A pathologist reviews the images to ensure that the antibodies are staining the intended targets.

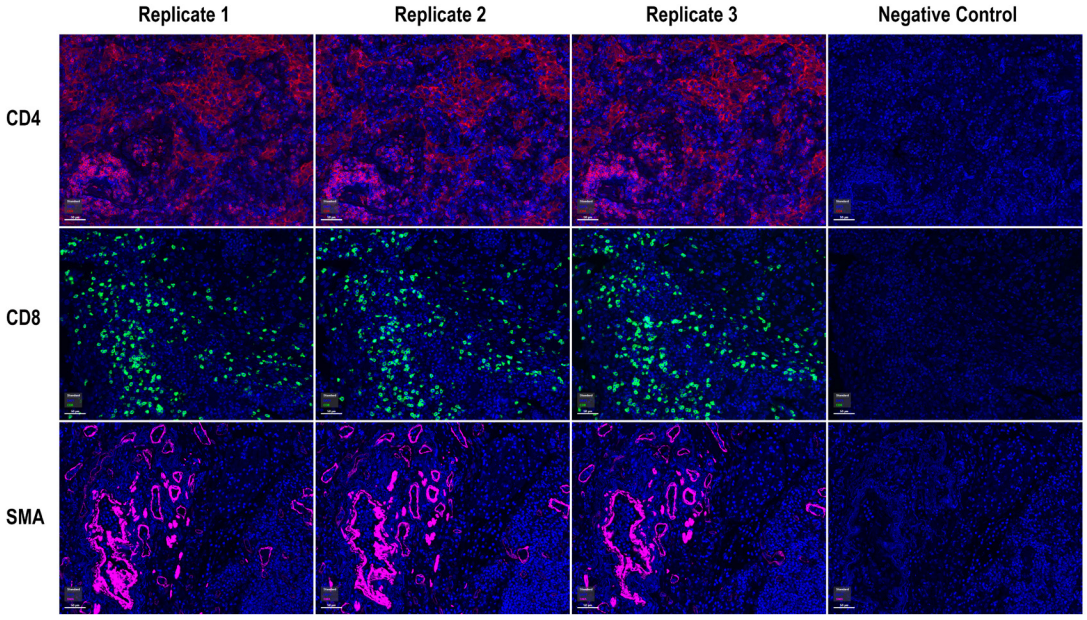


FIGURE 7. Antibodies stained in triplicate and with negative control on lung cancer tissue.

Conclusion

PhenoCode Discovery Panel Modules go through extensive screening, optimization, and validation to maximize performance and efficiency. Each of the panel modules is pre-optimized and has been designed to seamlessly work together to achieve ultrahigh-plex detection with minimal steric hindrance, enabling time and cost savings. Together, PhenoCode Discovery Panels and the PhenoCycler-Fusion System enable rapid and deep spatial phenotyping across whole tissue at scale.

PD-0000XX Rev A

AKOYA BIOSCIENCES®
THE SPATIAL BIOLOGY COMPANY®