AKOYA
BIOSCIENCES®
THE SPATIAL BIOLOGY COMPANY®

We've rebranded some of our products. **CODEX®** *is now* **PhenoCycler™.**

Validation of Commercial PhenoCycler Antibodies

HIGHLIGHTS:

- The **PhenoCycler (formerly CODEX)** technology allows tissues to be stained with an entire antibody panel in a single step, thus decreasing experiment time and preserving sample integrity.

- The **PhenoCycler** antibodies perform similarly to other immunofluorescence (IF) and immuno-histochemistry (IHC) grade dye-conjugated antibodies.

- The **PhenoCycler** antibody validation process is thorough and quantitative, ensuring that specificity is achieved while optimizing sensitivity.

- There is minimal steric hindrance between multiple antibodies on a **PhenoCycler** panel.

MEET THE PHENOCYCLER SYSTEM:

The PhenoCycler System is the only benchtop platform that inte-grates with existing fluorescence microscopes to enable highly multiplexed immunofluorescence.
- Capacity to image 40+ biomarkers
- Spatial context for entire tissue sample
- FFPE-compatible
- End-to-end solution that includes reagents, instrument, and software
- Benchtop footprint
- Sample preserved for Region of Interest (ROI) analysis or H&E staining

INTRODUCTION TO THE PHENOCYCLER TECHNOLOGY

Understanding the biology and progression of cancer or complex immune disorders requires a comprehensive understanding of the spatial architecture within the tissue microenvironment.

Getting an accurate picture of cell neighborhoods and interactions in the tissue microenvironment requires a high level of multiplexed marker detection at the single-cell level with the spatial context. We have developed the PhenoCycler technology to provide a comprehensive solution for spatially-resolved, highly multiplexed biomarker analysis.

Figure 1. Key components of PhenoCycler technology: PhenoCycler Antibodies are pre-conjugated to PhenoCycler Barcodes. PhenoCycler Reporters are fluorophores conjugated to oligonucleotides for visualization of PhenoCycler antibodies. PhenoCycler Barcodes are activated oligonucleotides for custom-conjugation to third party antibody clones.

The PhenoCycler (CO-Detection by indEXing) technology, originally developed in the lab of Dr. Garry Nolan at Stanford University, uses antibodies conjugated to a proprietary library of oligonucleotides called Barcodes. This enables customizable panels of up to 40+ PhenoCycler 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.
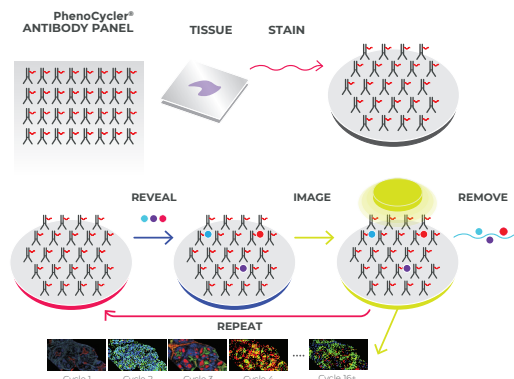

Figure 2. PhenoCycler chemistry: Single-step staining followed by multicycle imaging of 40+ biomarkers

TYPES OF PHENOCYCLER ANTIBODIES

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:

- Human FFPE tissues
- Human Fresh frozen tissues
- Mouse Fresh frozen tissues

VALIDATION OF PHENOCYCLER ANTIBODIES

Comparison of dye-conjugated and PhenoCycler antibodies

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.
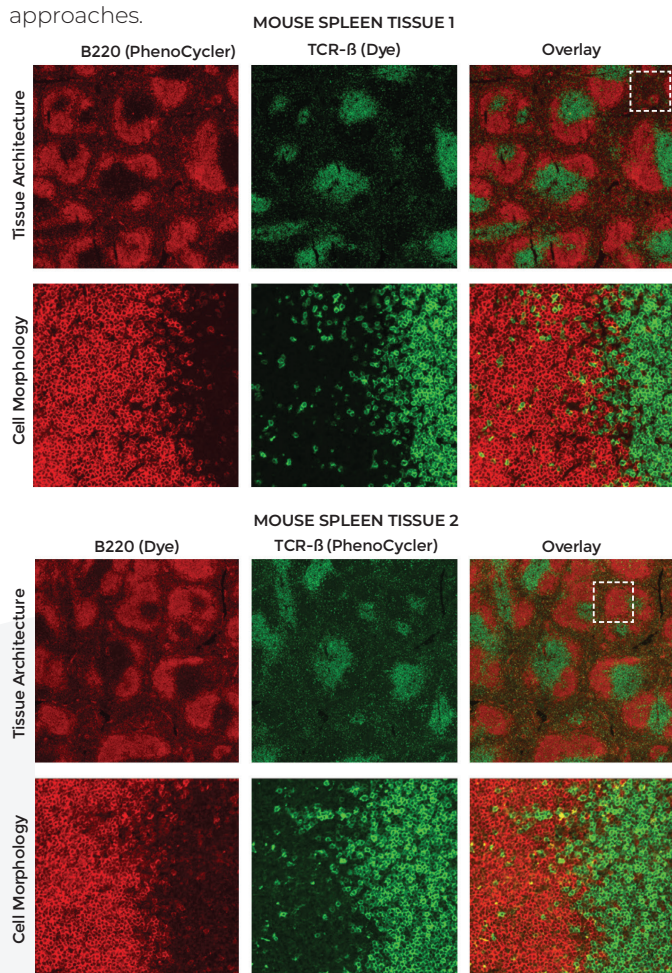

Figure 3. Mouse fresh frozen spleen tissues showing tissue architecture (top rows) and cell morphology (bottom rows)

ANTIBODY TITRATION

Each inventoried PhenoCycler antibody is titrated to determine the optimal concentration to quantitatively optimize antibody sensitivity while maintaining specificity

1. Each commercial PhenoCycler antibody is titrated using a positive test tissue across four test concentrations.
2. An optimal titer is selected based on quantitatively maximizing the signal-to-noise ratio (SNR), and average signal while minimizing non-specific binding.
   In short, regions of interest are selected based on tissue quality, avoiding areas of tissue with rips or autofluorescence. Within these regions, a threshold is set for both signal and noise. A binary map is used to confirm that pixels associated with signal and noise, respectively match the raw antibody staining pattern. The SNR is extracted based on these values, and the signal within each region of interest is averaged. The objective is to maximize both signal and SNR while minimizing any nonspecific staining.
3. Each PhenoCycler antibody is titrated with at least one negative counterstain to assess non-specific staining or signal saturation.

In the first antibody titration example below **(Figure 4)**, the highest signal and SNR values occur at the highest titer. In this case, both the SNR and signal are high, there is no signal saturation, and qualitatively there is no detectable non-specific binding. Hence, the 1:250 titer is selected.
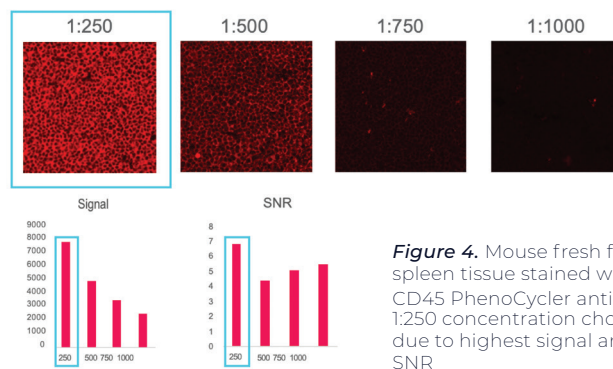

Figure 4. Mouse fresh frozen spleen tissue stained with CD45 PhenoCycler antibody. 1:250 concentration chosen due to highest signal and SNR

In the next example **(Figure 5)**, the signal is highest at the first and second titrations (1:250, 1:500). But because the signal is saturated, and there is evidence of non-specific binding, the SNR value is maximized at the third titration. All four parameters are considered to determine the best option for each antibody.
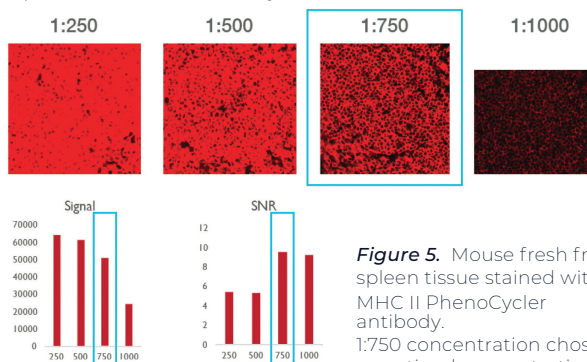

Figure 5. Mouse fresh frozen spleen tissue stained with MHC II PhenoCycler antibody. 1:750 concentration chosen as optimal concentration.

ANTIBODY SPECIFICITY

Once the optimal concentration of each PhenoCycler antibody has been determined, specificity is assessed.

A tissue stain is performed with each PhenoCycler antibody in combination with positive and negative counterstain(s) when possible. Counterstains are selected based on known expression patterns with either colocalization or exclusive staining patterns for the positive and negative controls, respectively.

Qualitative assessment: Images are assessed at different levels of architectural details to confirm PhenoCycler antibody staining specificity and cellular morphology. Zoomed-out and zoomed-in views of the tissue are observed. The larger architectural regions are considered in different combinations, checking for overlap from the positive counterstain, and checking for no overlap from the negative counterstain. **Figure 6** shows an example of the positive and negative counterstaining patterns for fresh frozen mouse CD4 PhenoCycler antibody. As expected, colocalization is seen with TCR-ß and there's no overlap with B220.

Quantitative assessment: A co-localization value is calculated based on the number of shared pixels within a specified region of interest. The co-localization value is determined by defining high quality regions of interest (no tissue torn or missing, and limited auto-fluorescence). The number of co-localized pixels for the PhenoCycler antibody is quantified with both the positive and negative counterstains. For example, the colocalization values for fresh frozen mouse CD4 PhenoCycler antibody were 60% with TCR-ß (positive counterstain, **Figure 6. G and I**) and 7% with B220
(negative counterstain, **Figure 6. F and I**). The shared pixel localization with B220 was confirmed to be at the borders of follicle regions and is therefore attributed to an artifact in the calculation. Thus, the specificity for each commercial PhenoCycler antibody is determined through a combination of this qualitative and
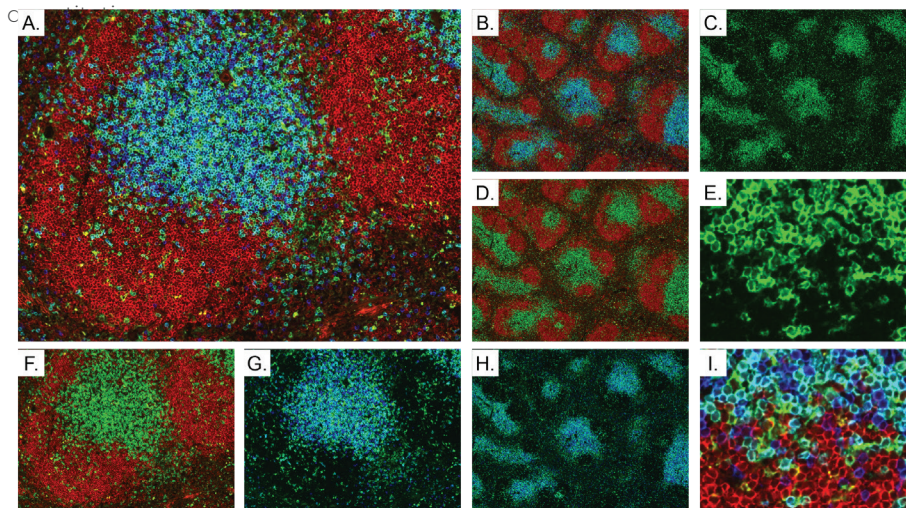

Figure 6. Mouse fresh-frozen spleen section stained with CD4 PhenoCycler antibody and relevant counterstains. A fresh-frozen mouse spleen tissue section was stained with PhenoCycler antibody CD4-BX026 (RM4-5), TCR-ß-BX003 (H57-597), and B220-BX010 (Ra3-6B2). Representative imaging regions are shown above with different combinations of markers depicted with the following color assignment: CD4 (green), TCR-ß (blue) and B220 (red) A.) CD4, TCR-ß and B220 medium zoom, B.) CD4, TCR-ß and B220 zoomed-out, C.) same region as B with just CD4, D.) same region as B with CD4 and B220, E.) zoomed-in region with just CD4, F.) same region as A with CD4 and B220, G.) same region as A with CD4 and TCR-ß, H.) same region as B with CD4 and TCR-ß and I.) Same region as E with all channels.
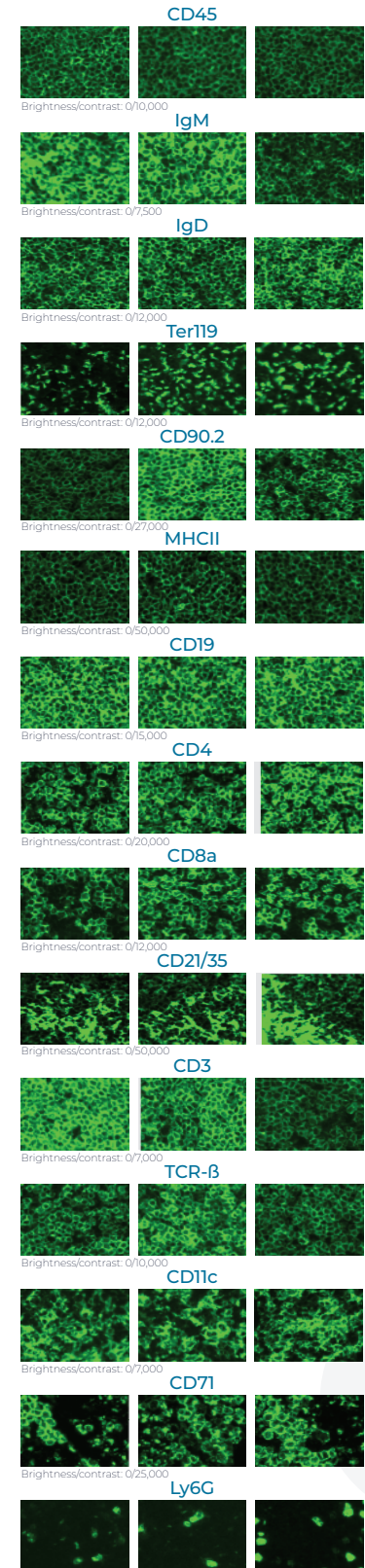

Figure 7. Representative images of PhenoCycler antibodies stained in the presence of other antibodies. Left column: Single antibody, Middle column: 3 antibody panel (positive and negative counterstains), Right column: 15 antibody panel

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 7)**.

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 8)**. 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 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. In conclusion, PhenoCycler antibodies are shown to perform similarly to dye-conjugated antibodies, the PhenoCycler antibody validation process is demonstrated to be thorough and quantitative, thus minimizing the amount of validation required by users. Finally, the data here demonstrates an assay for measuring potential steric hinderance from PhenoCycler antibody panels and shows there are minimal effects for this example.
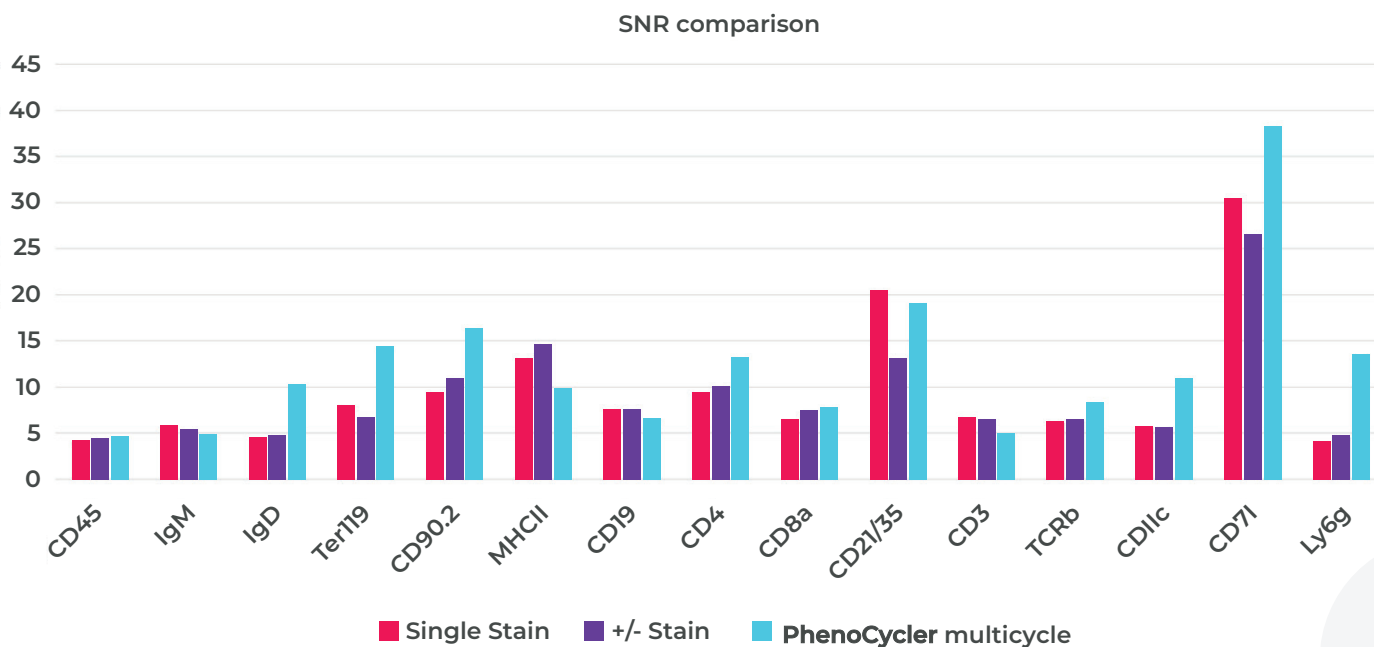

Figure 8. SNR values for each PhenoCycler antibody under the 3 different staining conditions (single antibody, 3-antibody panel with positive and negative counterstains, and 15-antibody panel).