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

Investigation of Autofluorescence Best Practices

Background fluorescence

Background fluorescence, otherwise known as autofluorescence, can be a serious problem in fluorescence imaging of tissue sections, especially in the fluorescence channels used for AF488 and ATTO550. This problem can be more relevant for FFPE than for fresh-frozen tissue sections but is likely to be found in both sample types. Representative unstained tissue sections should be imaged in each fluorescent channel prior to staining with PhenoCycler™ Antibodies to exclude highly autofluorescent samples/regions and channels. In general, for FFPE tissues we recommend using the AF750 channel instead of AF488 for this reason.

Check for Autofluorescence

We recommend to pre-screen tissues for autofluorescence before performing validation of conjugated antibodies or running a PhenoCycler multicycle run. Sections from the same tissue block and sectioning session that will be used for PhenoCycler studies can be screened as representative of the expected autofluorescence level.

Fluorescence Imaging

Using the fluorescence microscope dedicated to the PhenoCycler run, select 20x magnification and image a 5-7 mm2 area of the representative tissue with the coverslip side facing the microscope objective. Use the DAPI channel to focus, then image all channels using the recommended standard exposure times for the microscope. Evaluate the level of autofluorescence to make sure that samples meet quality standards for multichannel fluorescence imaging. Examples on how to evaluate the background level in fluorescence images can be found in the vendor's microscope manual.

Instructions for "No Dye" Control

Perform the tissue staining protocol without any antibodies added (as described in Chapter 5 of the user manual) followed by tissue mounting without reporters (but with DAPI) (Appendix C) and finish with fluorescence imaging.

Autofluorescence Detection and Conjugation Considerations

If a tissue sample displays high autofluorescence, we recommend revealing rare biomarkers or less-abundant antigens using reporters that emit at higher wavelength, such as Cy5 for fresh-frozen and AF750 for FFPE tissues. This is because antibodies corresponding with lessabundant antigens may fluoresce with a lower signal intensity than their high-abundance counterparts and may not be able to be parsed from the autofluorescence signal. These suggested fluorescence channels present intrinsically lower autofluorescence levels, resulting in images with lower background noise.

Sample	**Tissue sections adherent to poly-lysine coated coverslips**	
	Fresh-frozen	**FFPE**
Lab Equipment	Fluorescence microscope used for PhenoCycler runs equipped with DAPI, AF488/FITC/GFP, Atto550/TRITC, Cy5 filters	Fluorescence microscope used for PhenoCycler runs equipped with DAPI, Atto550/TRITC, Cy5, AF750/Cy7 filters

DN-00133 Rev A