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Profiling Cancer Biology

A novel method to minimize HIER-induced alterations on H&E staining in an integrated mIF-H&E workflow

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Background

Hematoxylin and eosin staining (H&E) is widely used as an anatomical assay for clinical diagnosis. Researchers and clinicians also rely on molecular *in situ* techniques, such as multiplexed immunofluorescence (mIF), to gain deeper insights on cellular phenotypes and tissue microenvironment. As a result, there has been significant interest in combining anatomical stains with molecular imaging techniques, most commonly by using a terminal H&E stain after mIF staining. This allows a combination of the two assays but has been observed to show alterations in the H&E staining pattern. In this work we identify heat-induced epitope retrieval (HIER) as the root cause of alterations of the terminal H&E stain after mIF. We further demonstrate a new workflow combining an initial H&E stain with subsequent InSituPlex assay to avoid these alterations and thus enable co-registration of highly sensitive multiplexed immunofluorescence with an unaltered H&E stain.

Methods

FFPE tissue slides were stained using a standard HIER protocol or a full mIF assay, followed by a standard H&E stain. Slides were imaged using a Zeiss Axioscan.Z1 scanner. Additional H&E-stained slides were prepared, and the H&E stain removed using a destaining protocol before carrying out InSituPlex® mIF staining for multiple markers. Image processing was carried out using STARVUE™ Image Data Science Platform: fluorescence and brightfield images of the same slide were overlaid using UltiStacker.AI™ to assess qualitative differences between pre- and post-mIF H&E, and UltiAnalyzer.AI™ was used to quantify cell densities and signal intensities between mIF image pre- and post-H&E.

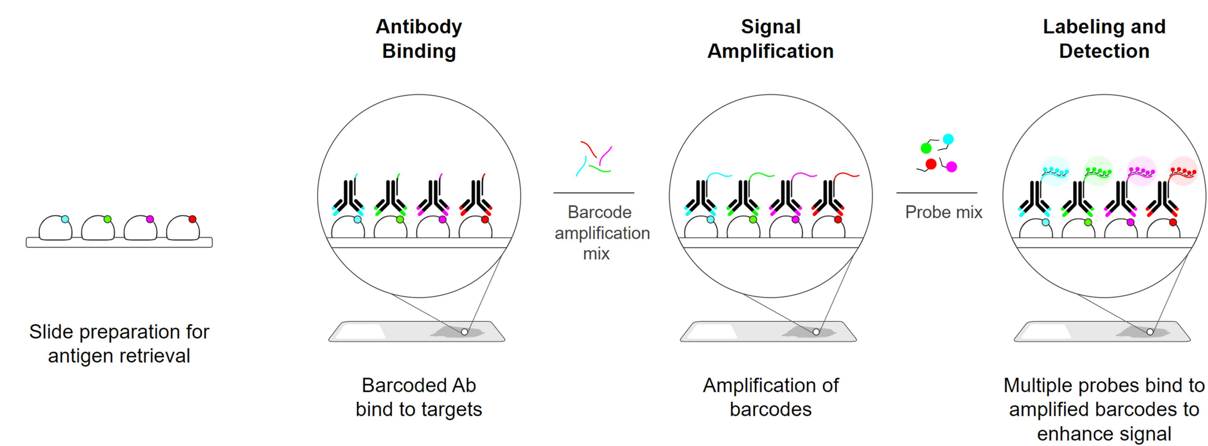


Figure 1. InSituPlex assay for multiplexed immunofluorescence staining.

Results

Alterations in H&E staining were observed between slides directly stained with H&E and slides stained with a terminal H&E after HIER alone or a full mIF assay. Calculated cell counts and tissue area were consistent throughout, but some tissue microstructures could be difficult to resolve after either HIER or full mIF. Our destaining protocol was successful in removing hematoxylin and eosin from directly-stained H&E slides and allowing subsequent mIF staining. For most biomarkers, qualitative and quantitative differences in InSituPlex® mIF staining were minimal. Brightfield and fluorescent images were co-registered with sub-micron accuracy using UltiStacker.AI™, enabling molecularly defined cellular phenotyping within a traditional H&E image.

After mIF, alterations in H&E quality can be observed

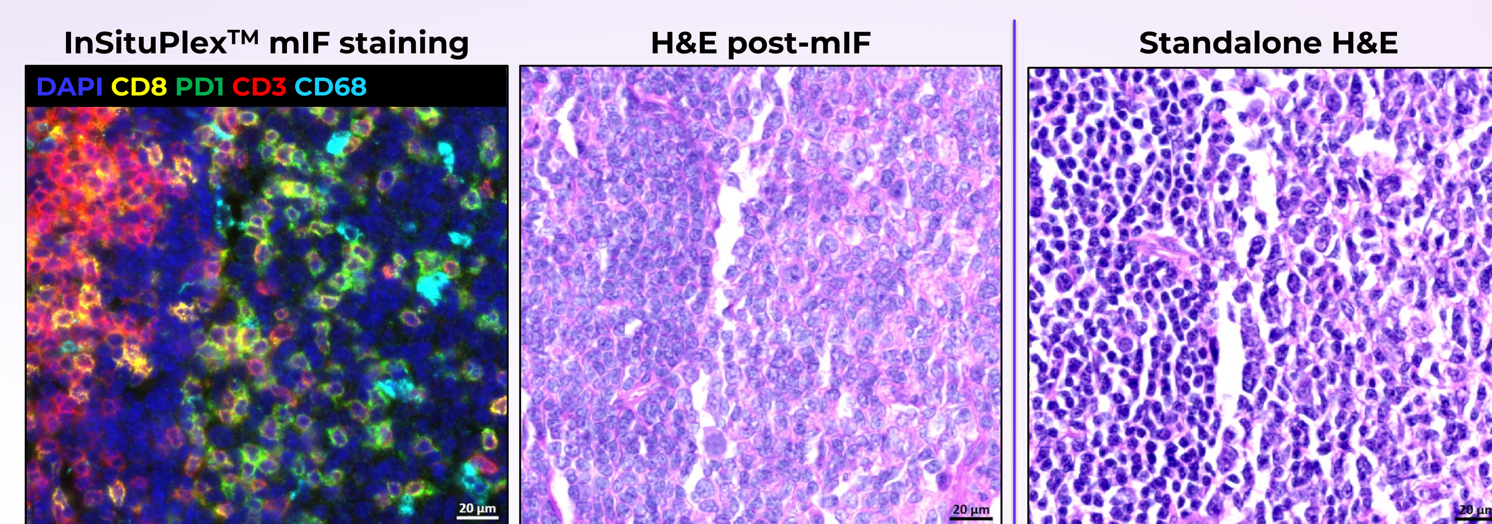


Figure 2: Quality of H&E after mIF staining shows differences in microscale tissue morphology when compared to a standalone H&E without prior mIF.

Pre-assay steps affect H&E stain quality

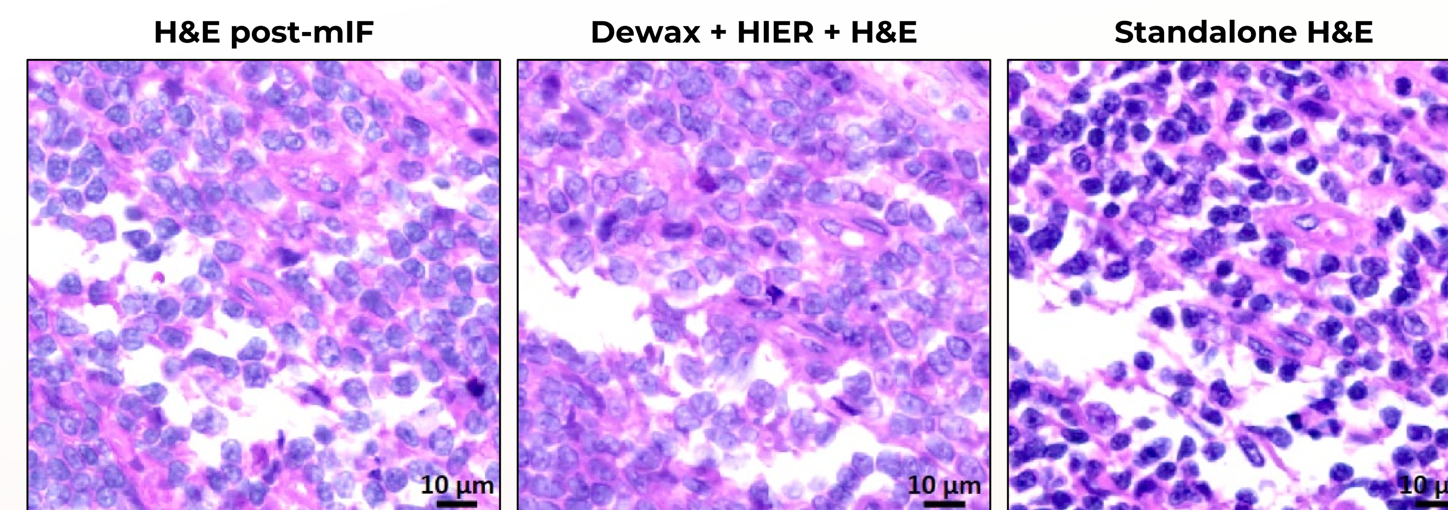


Figure 3. H&E post-mIF assay shows alterations compared to a standalone H&E assay. The same type of alterations are observed if the mIF assay is truncated solely to a Dewax and HIER step.

Heat-induced epitope retrieval and not dewaxing causes altered H&E

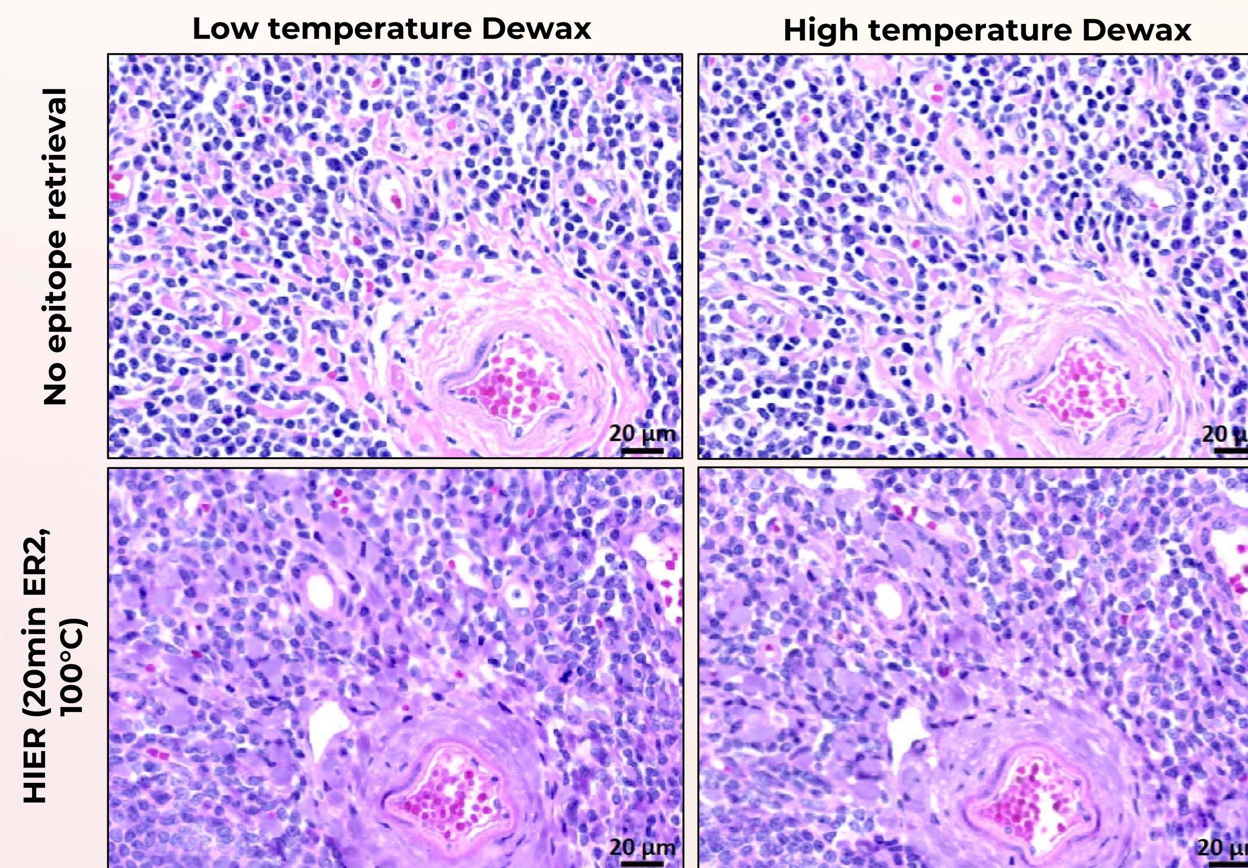


Figure 4. HIER and not Dewax is the single step causing H&E alterations in post-mIF H&E protocols. Dewax was carried out at high temperature (4x30sec, 72°C) or low temperature (3x10min, ambient temp), and optionally followed by HIER for 20 minutes (pH 9, 100°C).

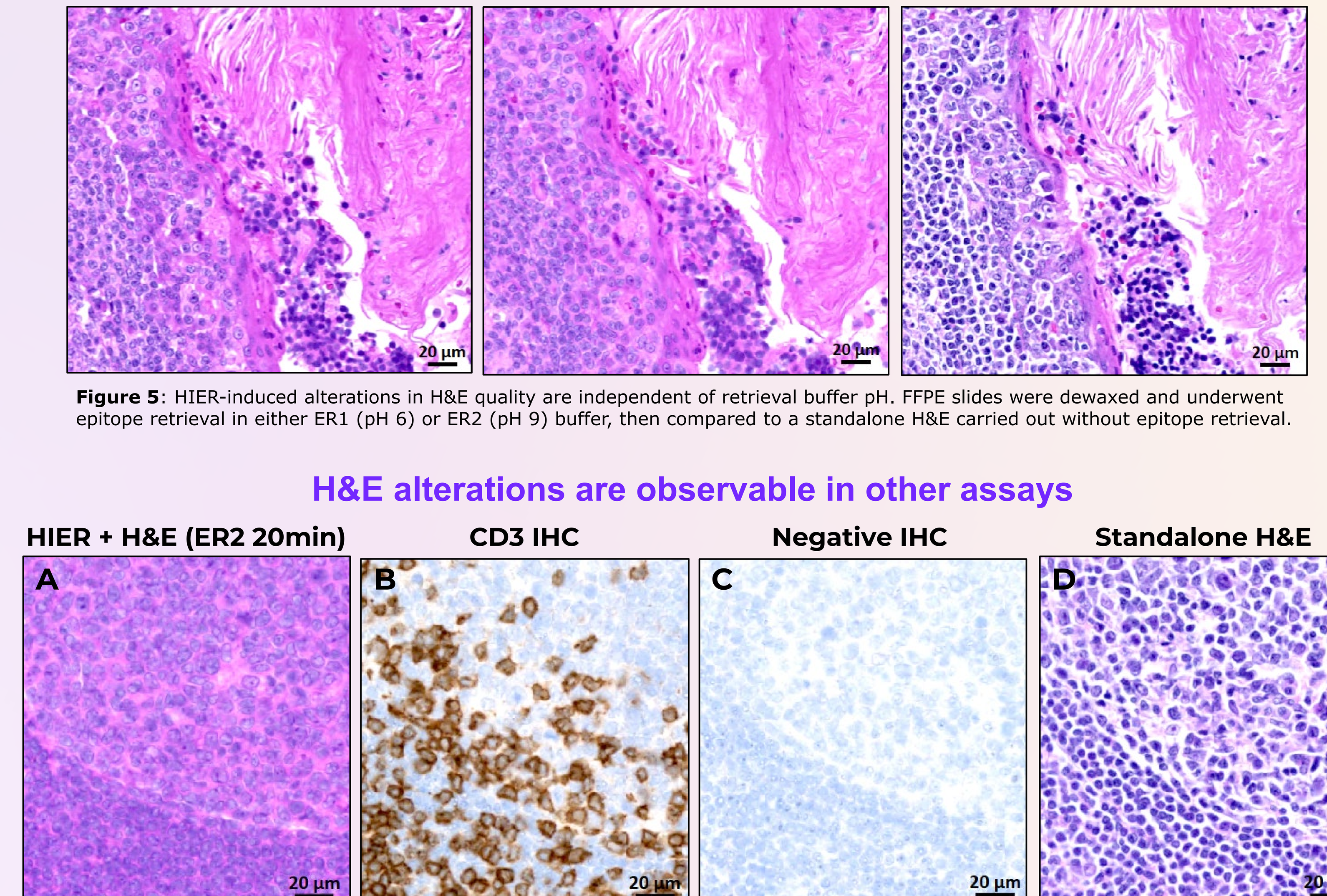
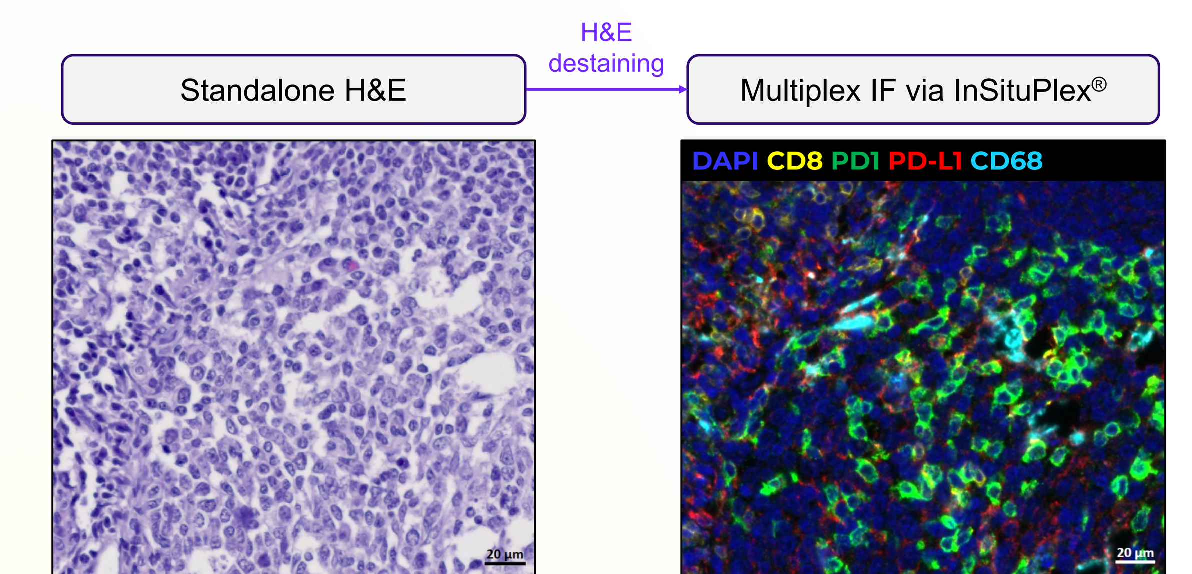


Figure 5: HIER-induced alterations in H&E quality are independent of retrieval buffer pH. FFPE slides were dewaxed and underwent epitope retrieval in either ER1 (pH 6) or ER2 (pH 9) buffer, then compared to a standalone H&E carried out without epitope retrieval.

Figure 6: HIER-induced alterations in hematoxylin staining are observable after IHC, due to HIER step. Tonsil slides were dewaxed and stained using either (A) H&E after HIER (ER2, 20min, 100C), (B) immunohistochemical staining for CD3 [BC33], including HIER (ER2, 20min, 100C), (C) negative control IHC staining for no primary antibody, and (D) H&E without prior HIER (Standalone H&E). In all cases where HIER has been performed, nuclei are more diffuse and have less sharp borders.

Integrating mIF and H&E staining



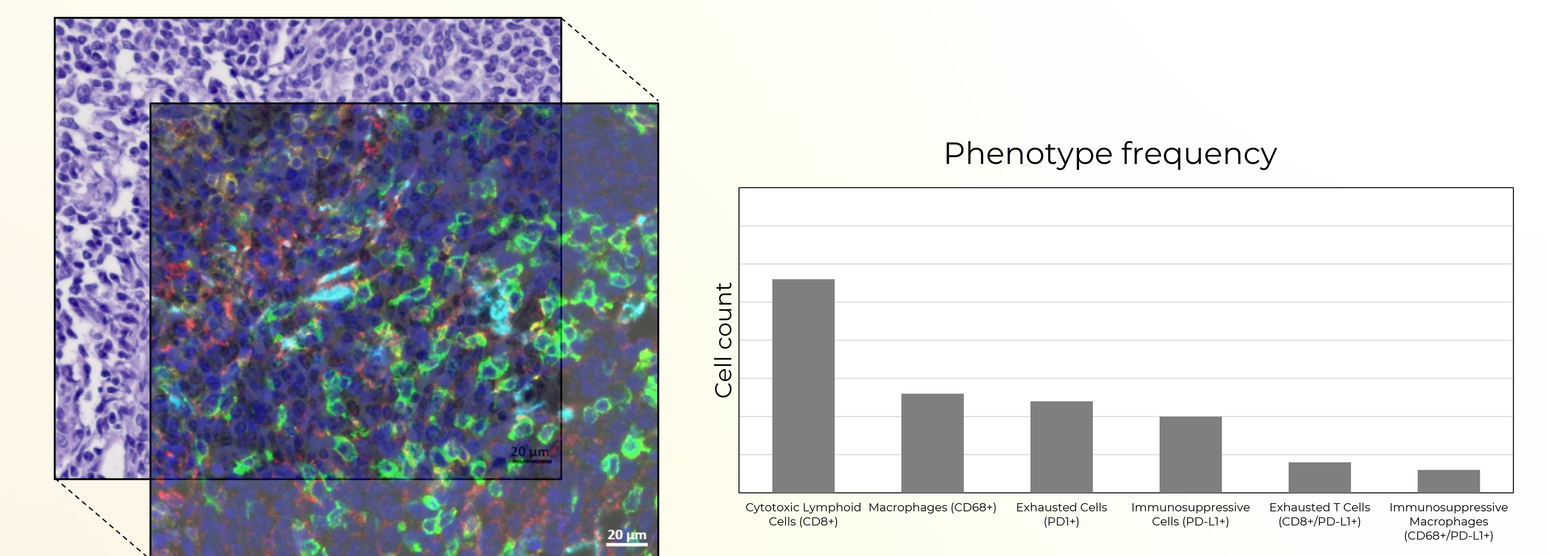


Figure 7: Same-slide H&E and mIF can be carried out using an H&E-first protocol.

Post-mIF H&E has consistent analytical results as standalone H&E

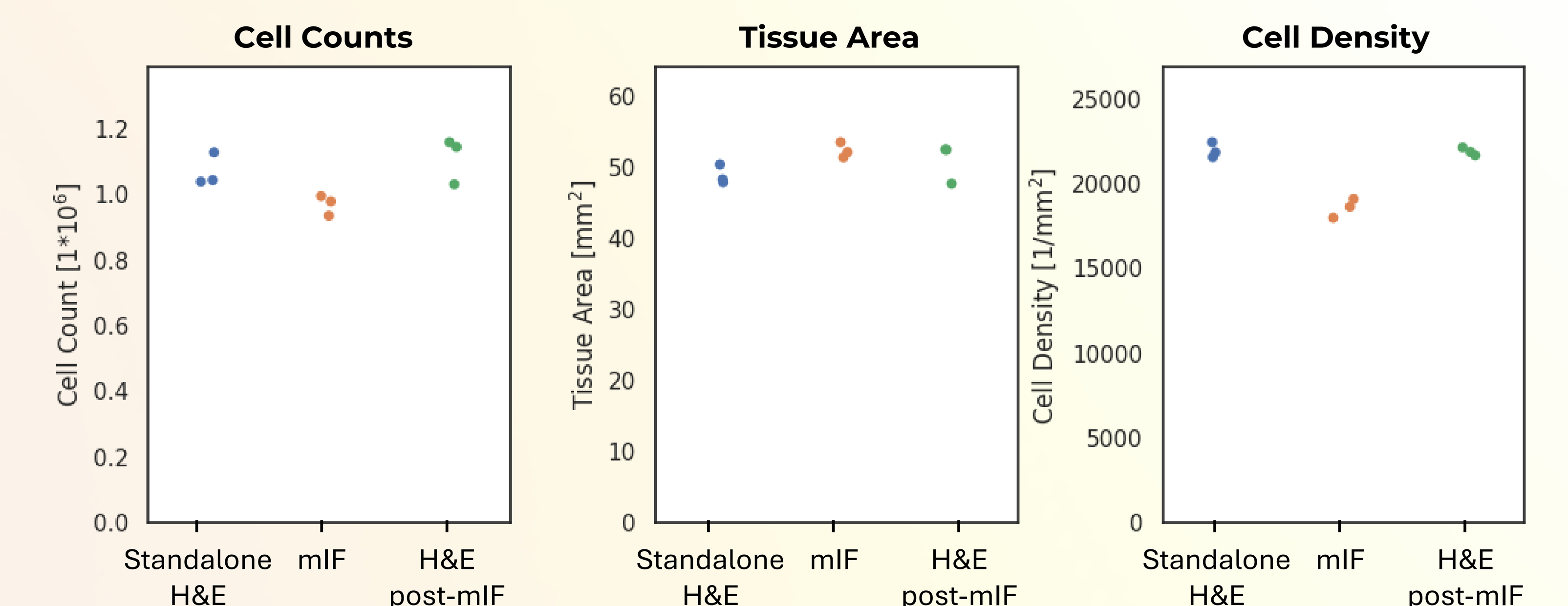


Figure 8: H&E staining after mIF shows minimal changes in cell count, tissue area, or measured cell density compared to standalone H&E. Serial tonsil slides were H&E stained directly (Standalone H&E), then H&E destained using a proprietary method. The slides were then stained using an InSituPlex™ multiplex immunofluorescence assay (mIF) followed by H&E (H&E post-mIF). Cell counts, tissue areas, and cell density were calculated using UltiAnalyzer.AI™ software using a Deep Learning instance segmentation method (stardist, Uwe et al. 2018) and tissues were segmented using texture descriptors and thresholding. The high degree of consistency between all three measurements demonstrates that the InSituPlex assay is gentle enough to permit successive H&E staining without tissue loss and that analysis of both standalone and terminal H&E images is possible using UltiAnalyzer.AI™.

Conclusions

While the mIF to H&E workflow is widely used and is capable of allowing combined anatomical and cellular phenotyping, alterations in the H&E stain exist due to the use of HIER in most mIF protocols. An alternative method is possible in which H&E-stained slides are destained and then restained for mIF or other spatial assays, providing the same valuable combination of assay information but without the HIER-induced alterations in H&E staining pattern.