Development and Validation of an Imaging Mass Cytometry and Multiplex Immunofluorescence Biologically-guided Cellular Segmentation Strategy

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Multiplex IF/IMC Analysis

Imaging Mass Cytometry and Multiplex Fluorescence staining approaches, require more stringent approaches for immune cell image segmentation & classification

Large panels of antibodies have been common in flow cytometry staining of cells in suspension, allowing for deep cell surface marker interrogation of the immune system.

Novel techniques (stain / strip immuno-fluorescence, or heavy metal-based antibody tagging, followed by imaging mass cytometry) allow highly multiplexed staining to be applied to the analysis of tissue sections.

Segmentation of these high multiplexed image sets to produce single cell counts for downstream analysis is more challenging than single marker studies, due to a strong desire to avoid mixing of signals from neighboring cells, and obtain “pure” readouts of biomarker abundance on a single cell level.

We have adopted a hybrid segmentation approach that utilizes biological domain knowledge to assist in the segmentation and classification of immune cell types in IMC and MxIF images

Two example methodologies are described here, first to adjust segmentation to account for biological differences, and the second to adjust the classification strategy used to identify cell types, overcoming bleedthrough of neighboring cells using a supervised feature-based random forest machine learning approach.

Selection of manually annotated cells to help guide supervised training allows for the reporting of quality metrics with respect to both segmentation (overall number of manually-scored cells versus segmented cells), and classification (per-class proportion of machine-scored immune cell subsets relative to gold standard of manual annotation)

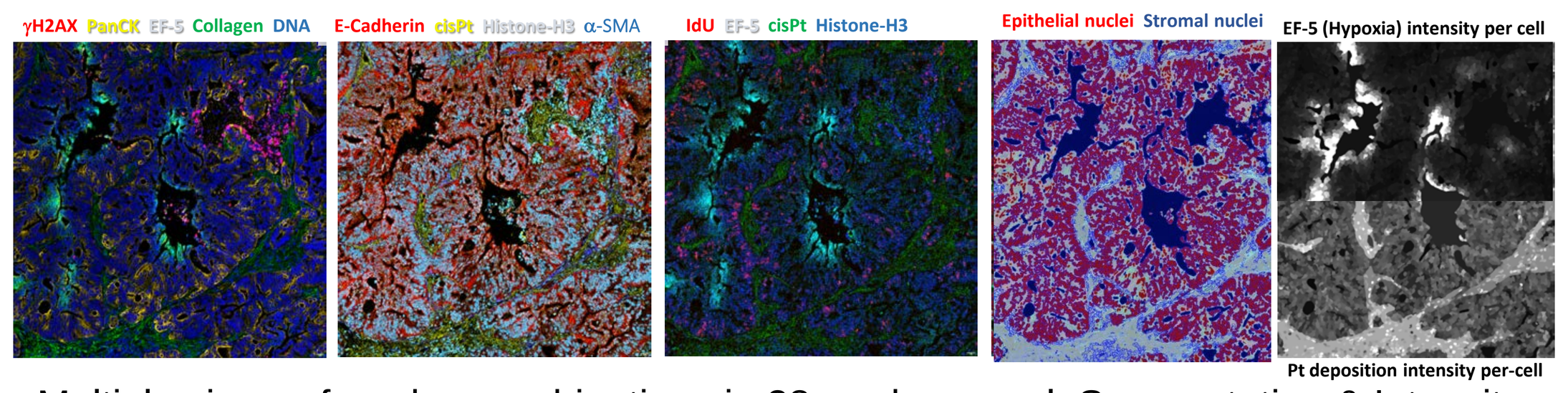
Future work could utilize this approach in a generalized segmentation and classification schema to improve analysis of immune subsets in highly multiplexed immunostained biopsies or tissue samples.

Image Segmentation

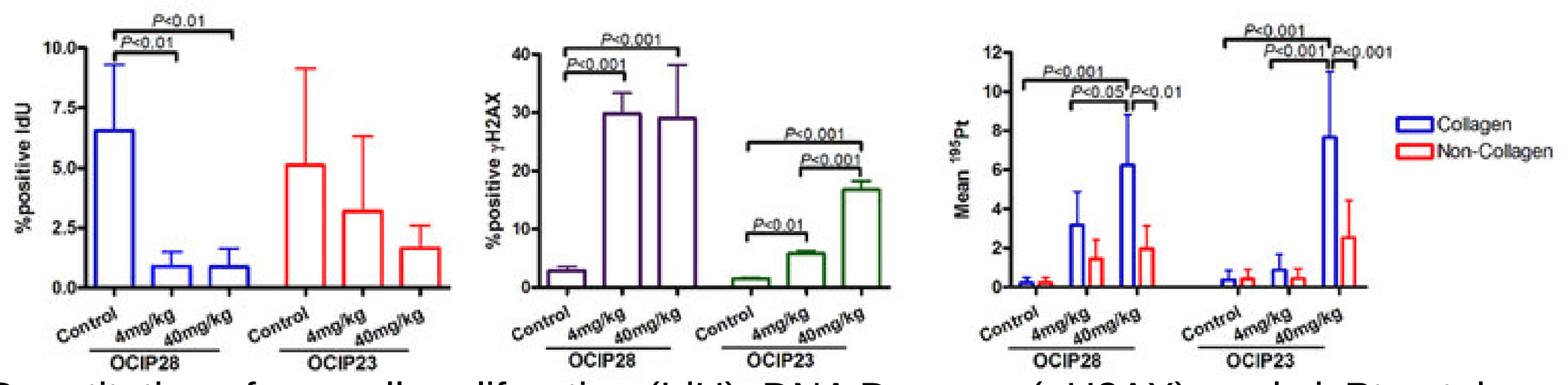
Cell Segmentation in IMC assists direct evaluation of platinum deposition in cisplatin treated pancreatic tumor xenografts

- Imaging mass cytometry with heavy metal tagged antibodies allows for:
  - highly multiplexed tissue immunostaining
  - Integrated tissue & cell segmentation utilizing epithelial, stromal markers
  - Highly quantitative single cell analytics: intensity, spatial relationships
- Finding: Majority of injected platinum actually binds collagen, not tumor cells

Published: ***Chang et al, Scientific Reports 2016; 6:36641***



Multiple views of marker combinations in 28 marker panel. Segmentation & Intensity

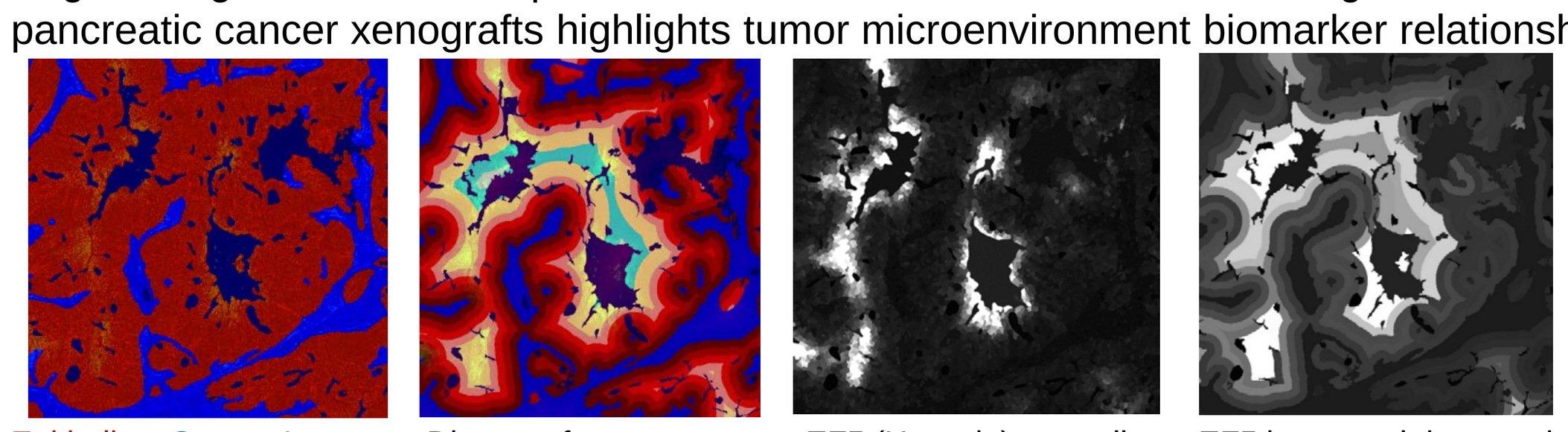


Quantitation of per-cell proliferation (IdU), DNA Damage (gH2AX), and cisPt uptake

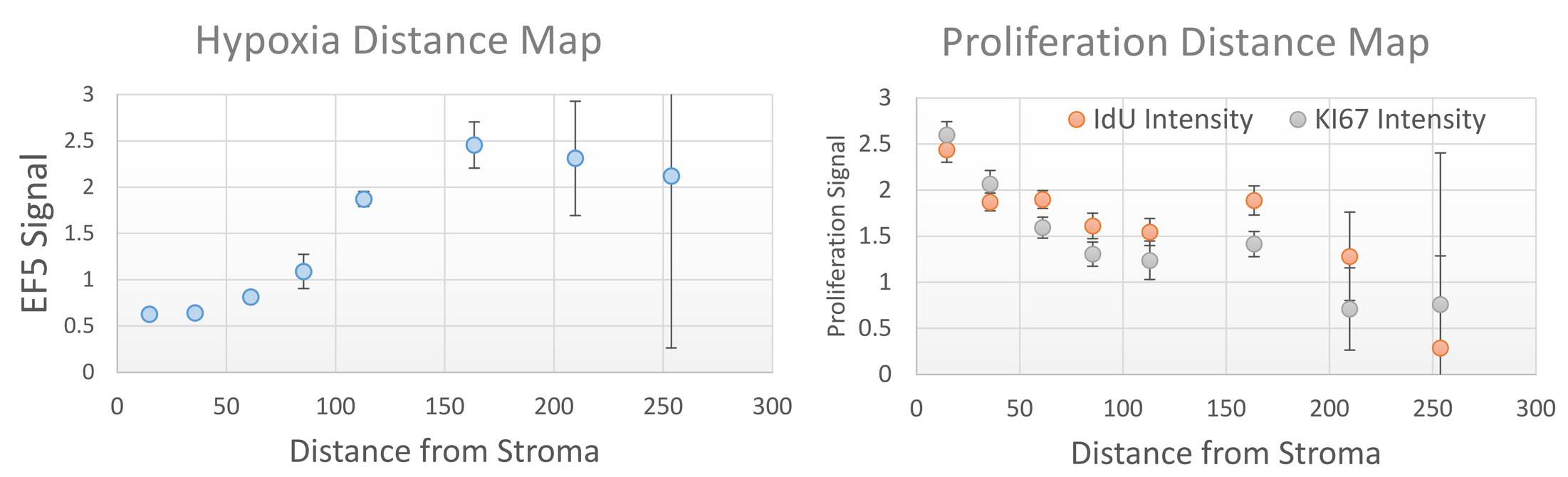
Laboratory of Dr. David Hedley, Princess Margaret Cancer Centre. Performed in collaboration with Fluidigm Canada Inc., manufacturers of Hyperion IMC system.

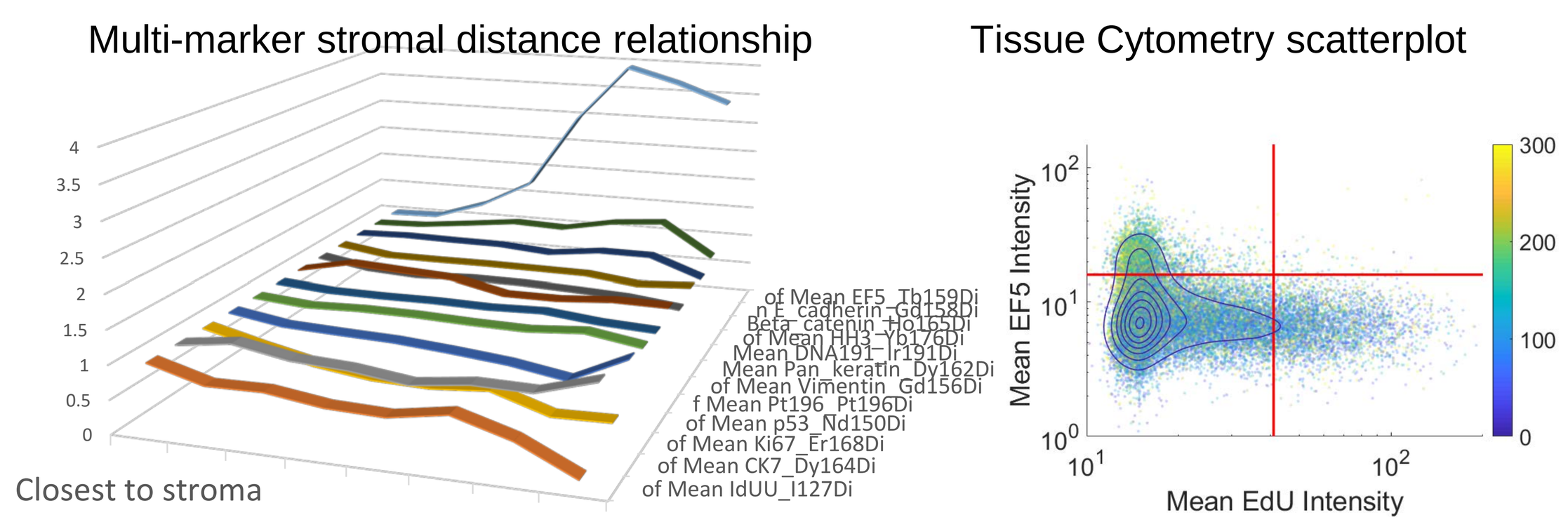
Stromal Distance Analysis of Hypoxia

Segmenting into bands of equal distance from blood vessels / stromal regions in pancreatic cancer xenografts highlights tumor microenvironment biomarker relationships



Epithelium Stroma Lumen — Distance from stroma — EF5 (Hypoxia) per-cell — EF5 in stromal dtstance bands



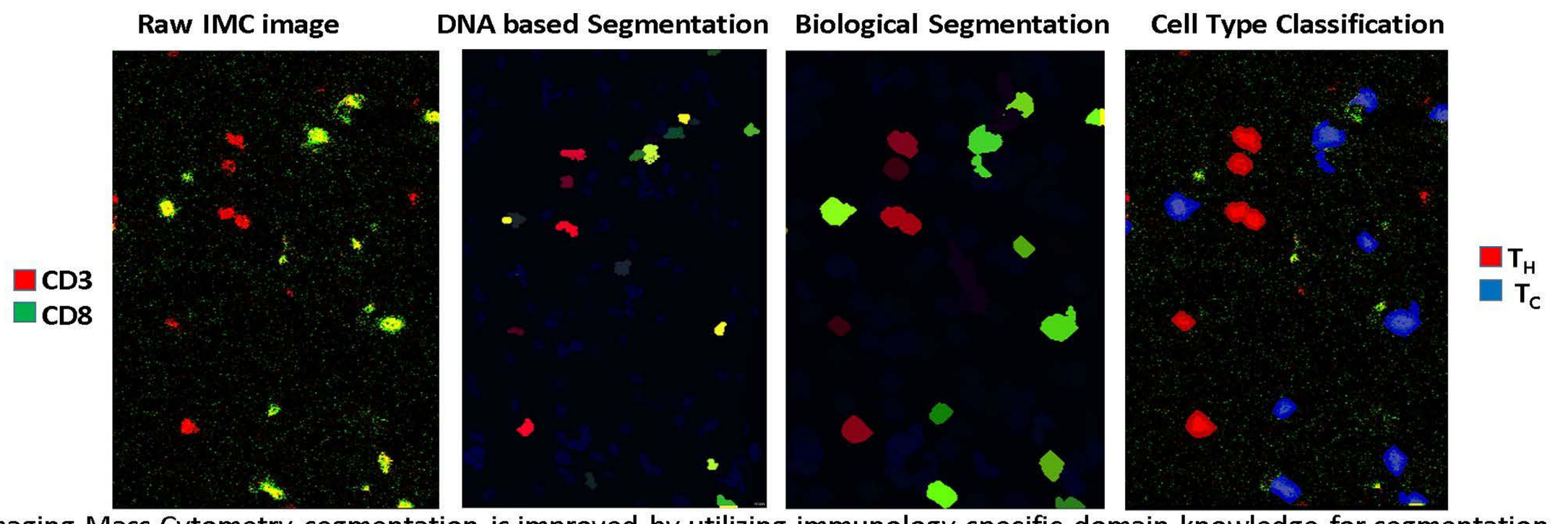


Zaidi et al 2019

This segmentation method allows for comparison amongst multiple biomarkers to highlight any putative microenvironmental relationships. Combining distance with cellular segmentation can also allow for multidimensional interrogation of biomarker relationships with distance, such as the “tissue cytometry” plot showing individual cell hypoxia and proliferative markers, with color scale denoting distance from each cell to nearest vessel.

Utilizing Immune Markers to guide Segmentation

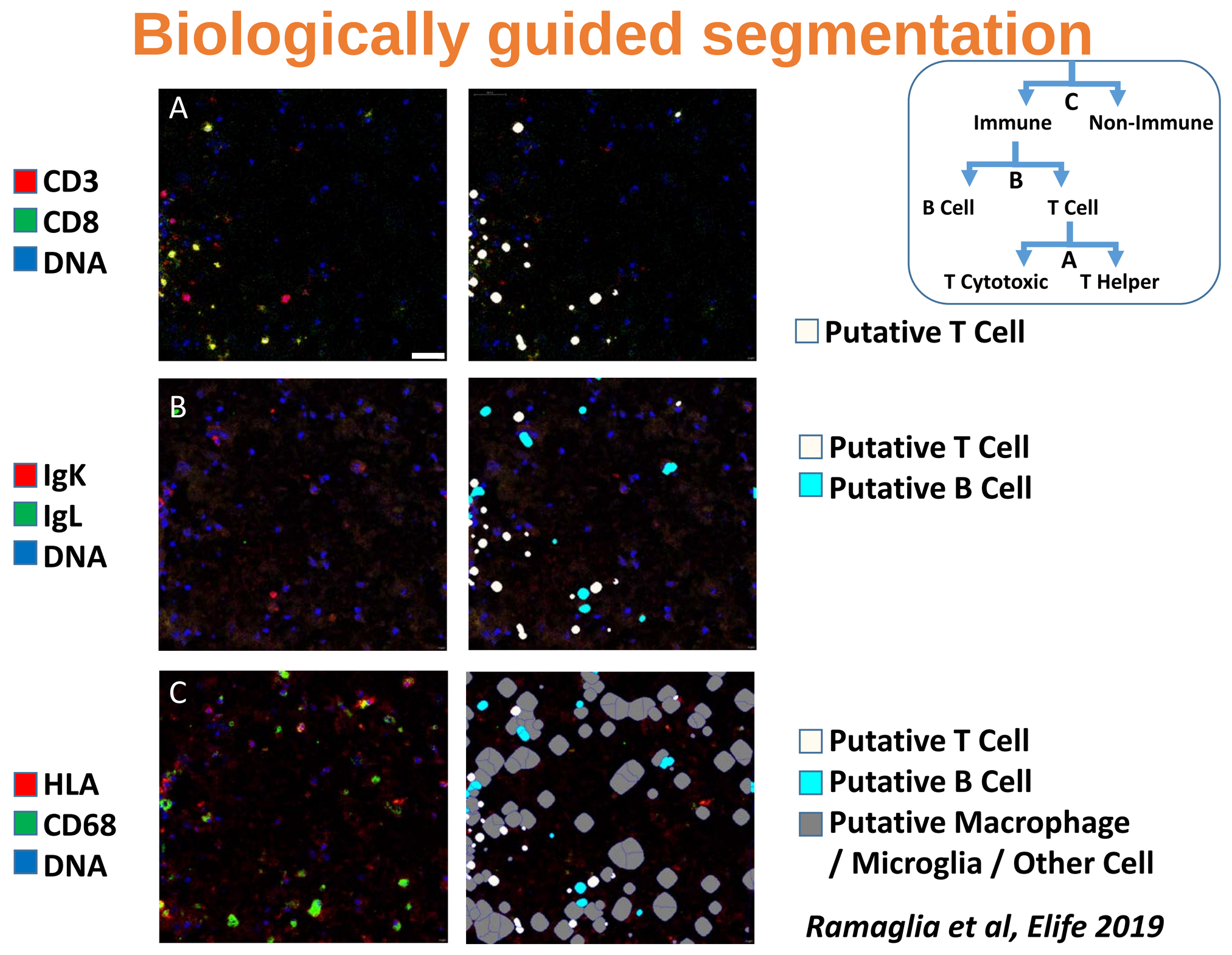
Pure DNA based segmentation can result in combination of biomarkers from neighboring cell types, resulting in implausible immune cell types (e.g. mixed T and B cell markers). By adopting an approach to combine DNA segmentation with biological domain knowledge (i.e. immune surface marker subtypes), we can improve segmentation.



Imaging Mass Cytometry segmentation is improved by utilizing immunology-specific domain knowledge for segmentation.

MS: Manual Classifier

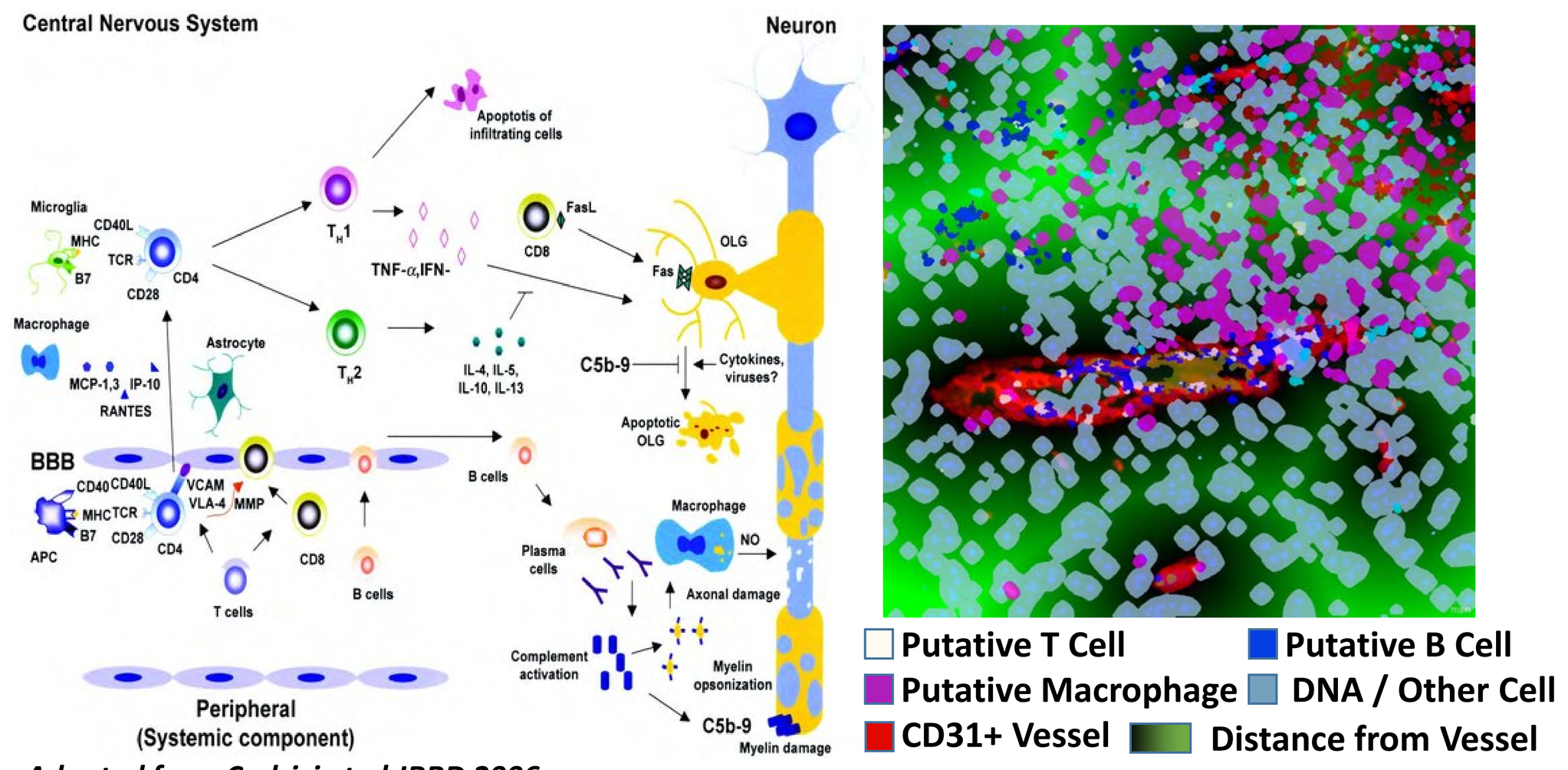
Biologically guided segmentation



Ramaglia et al, Elife 2019

MS: Immune cell blood vessel distance

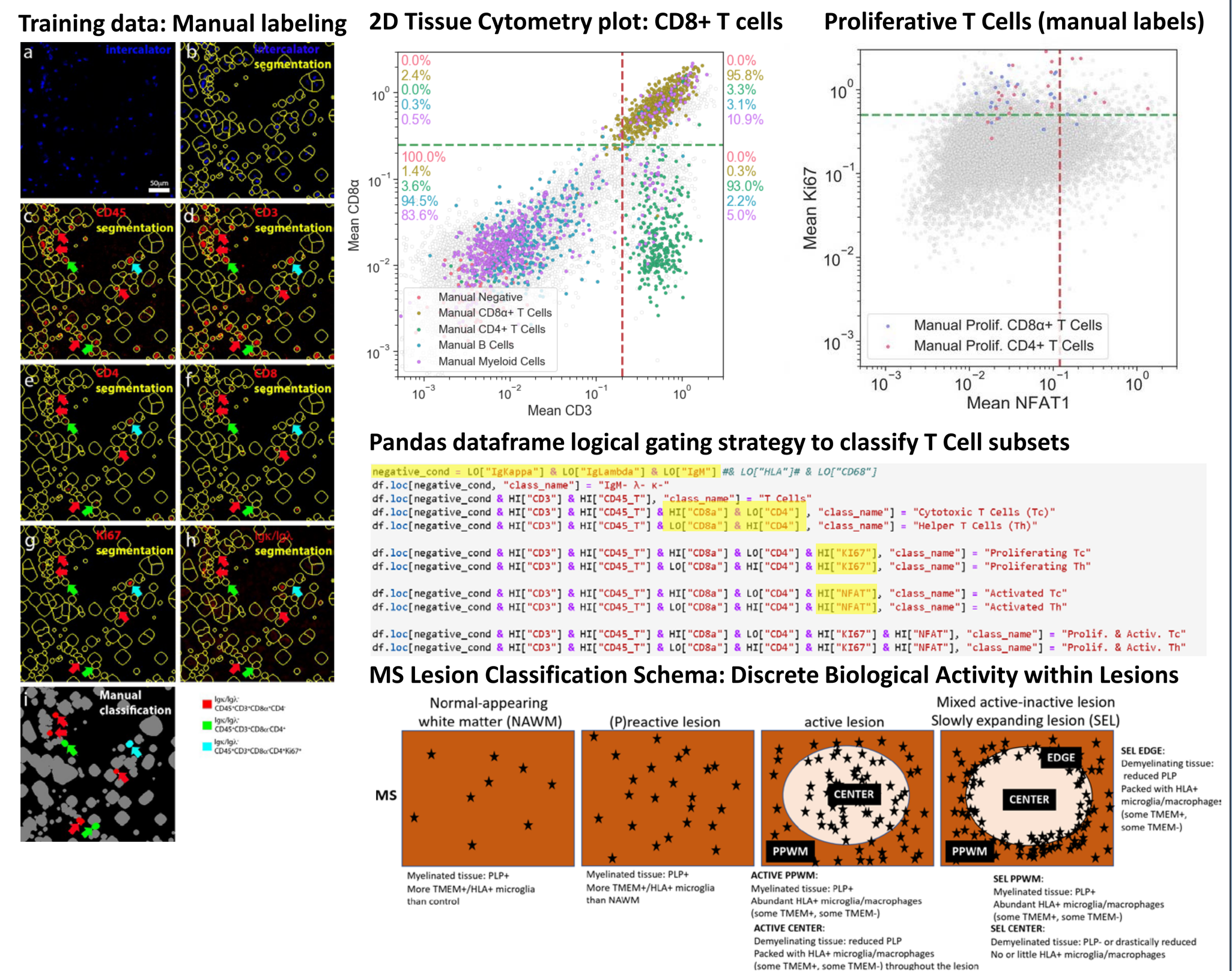
Immune influx from blood vessels plays an important role in progression of multiple sclerosis. Creation of a distance map utilizing CD31+ vessels permits direct measurement of distance to nearest (in-plane) vessel on a per-cell basis.



Adapted from Cudrici et al JRRD 2006

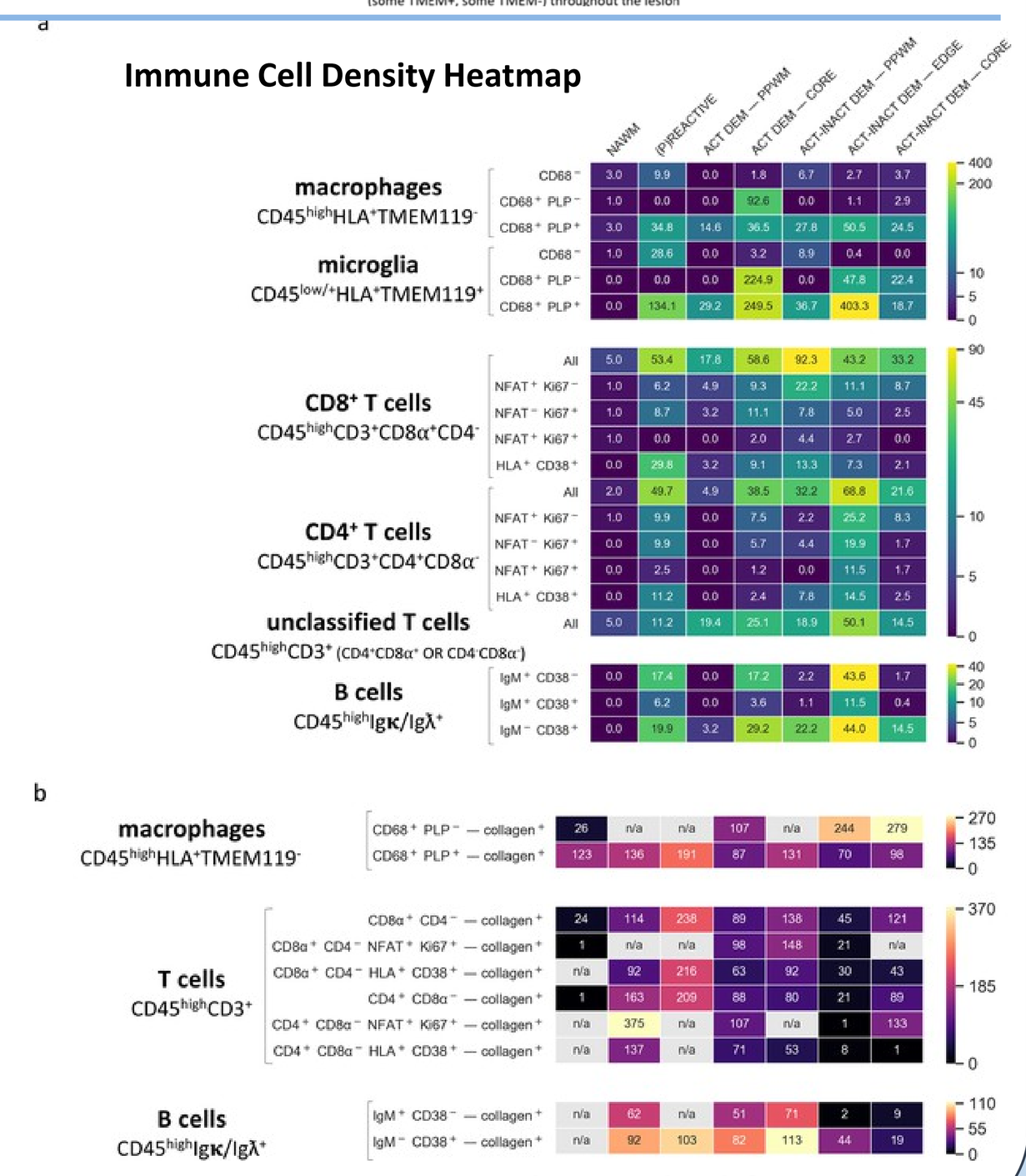
Gating strategy using manual annotations

Classification of distinct immune cell subsets was performed by first manually labeling these subsets, then determining manually identified “gates” / thresholds by inspection of 2D scatterplots of markers of interest, to identify positivity cutoff



The resulting heatmap was obtained by first segmenting every cell within specific lesions (corresponding to regions of interest with discrete biological activity), then applying the gating strategy determined by manual labeling of discrete immune cell types. The cell subtype count is reported in units of cells / mm2 across each region of interest.

In addition to the immune cell density, the average distance to nearest blood vessel for each immune cell subset is reported here.



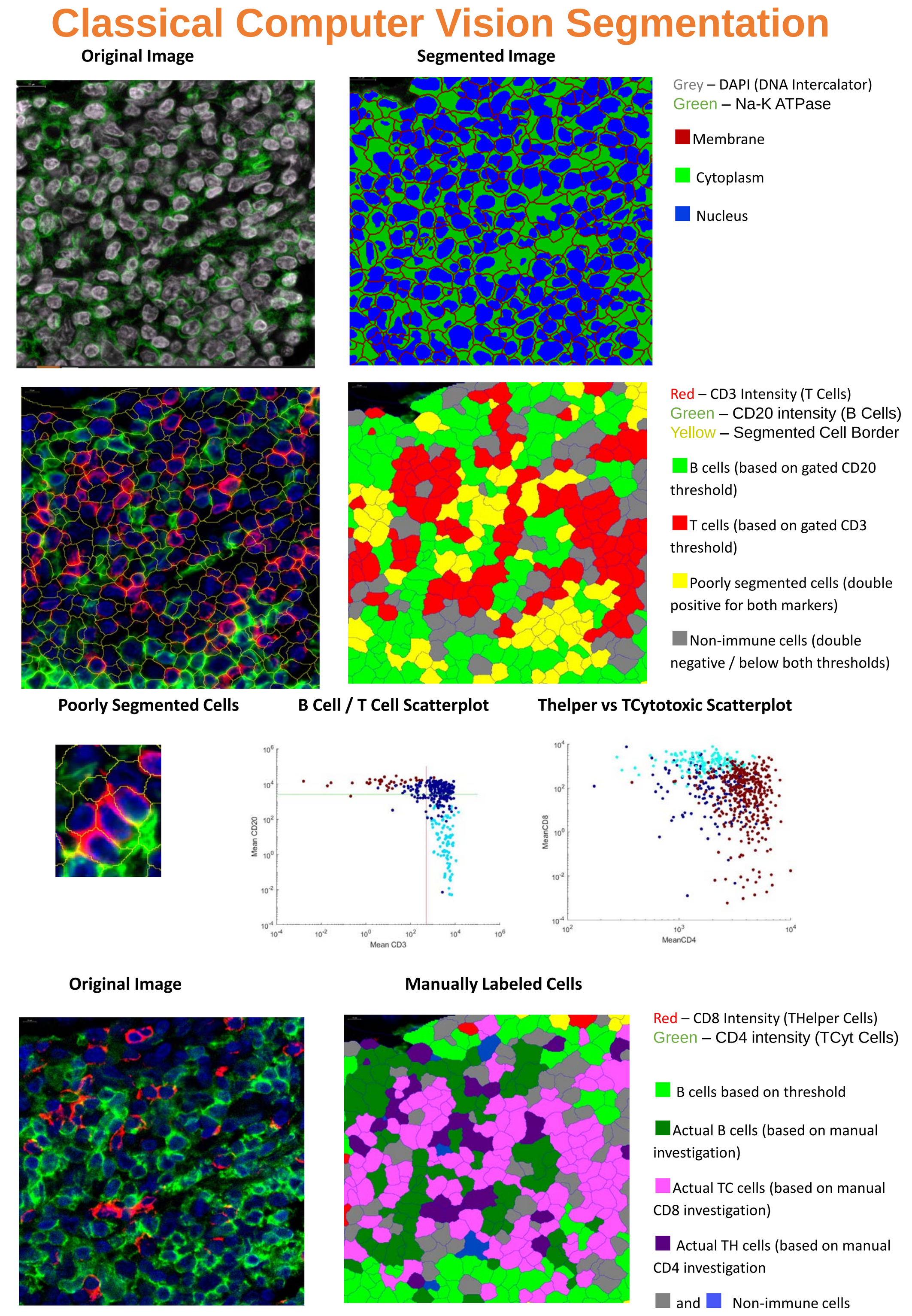
Ramaglia et al, Elife 2019

Random Forest Classifier

Breast cancer: Immune infiltrate classification

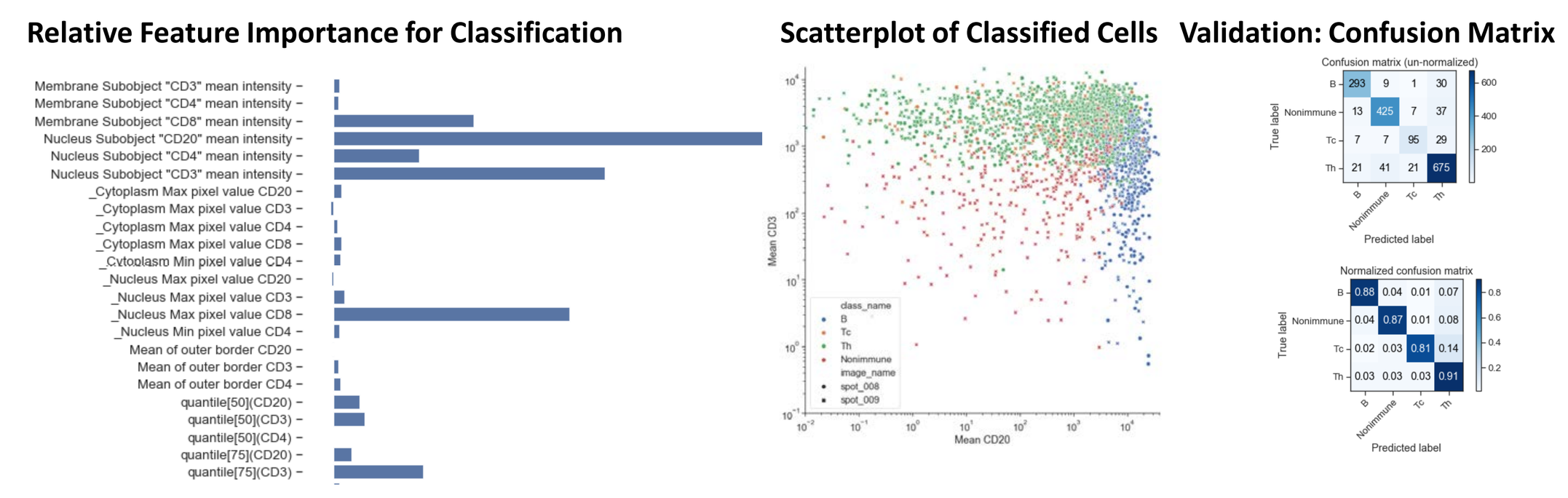
Knowledge of the immune content of the tumor microenvironment, and the proportions of distinct regulatory and cytotoxic immune cell components surrounding and within the tumor, is critical for the delivery of optimal therapy. Multiplexed immunofluorescence, using sequential staining, stripping and re-staining, can highlight immune cell subsets. However, challenges remain with segmentation and classification of these cell types. The higher resolution of these images, and greater density of immune cells, made segmentation adjustments more challenging; we optimized the segmentation to balance under- and over-segmented cells; and utilized a Random Forest machine learning strategy to “train” an immune classifier using supervised labeled training data.

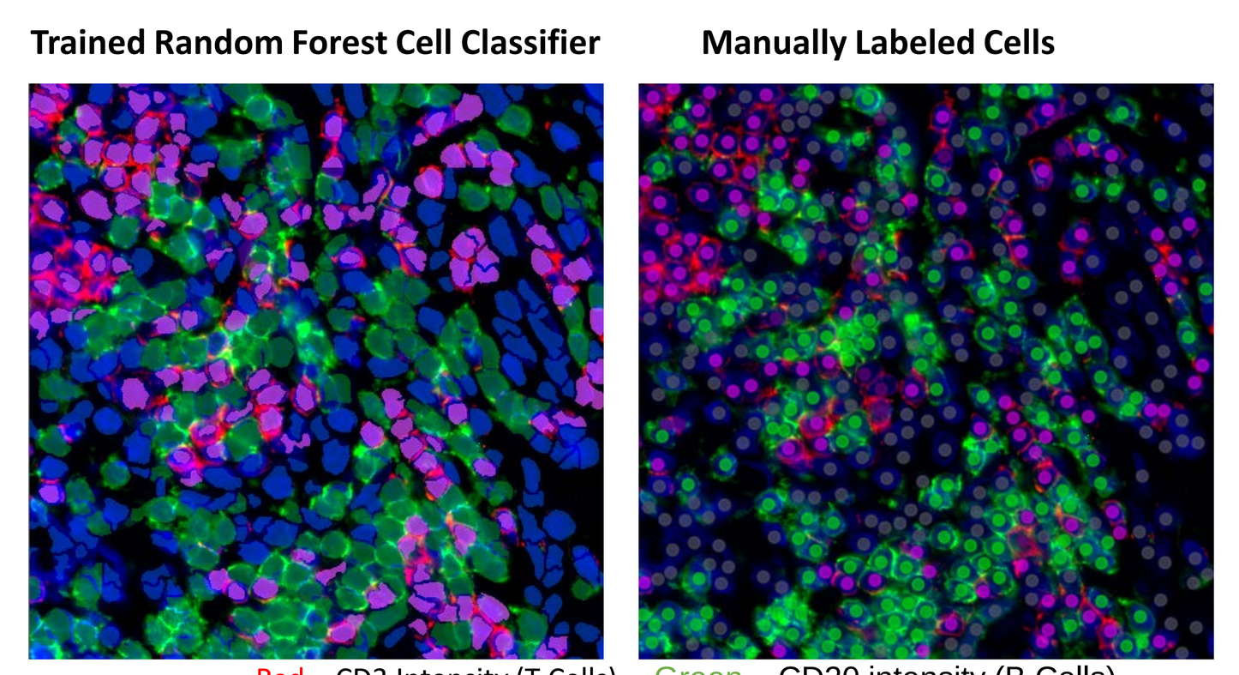
DNA (Nuclear) and Na-K ATPase (Membrane) Classical Computer Vision Segmentation



Random Forest-based Immune Cell Classifier

Computer vision-based segmentation was tuned to balance under- and over-segmented cells (utilizing thresholds for DAPI and membrane markers). For each detected cell, ~50 features were exported, corresponding to pixel intensities for sub-regions and histogram-based intensity subsets. These were used with manually labeled data to train a Random Forest classifier to properly identify immune cell subsets.



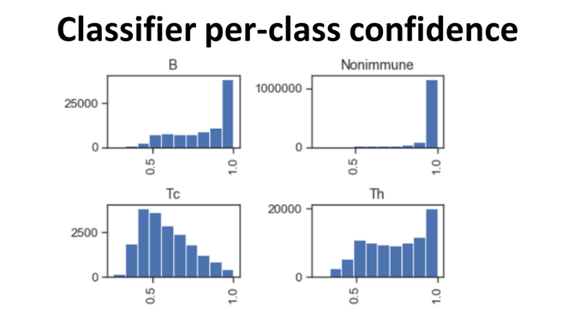


Red – CD3 Intensity (T Cells) Green – CD20 intensity (B Cells)

Total Counts in this ROI

	Manual	Classifier
B Cell	145	165
T Helper	102	126
T Cytotoxic	11	2
Non-Immune	175	141

Classifier per-class confidence



Trained classifier achieves an accuracy of 0.87, f1-score (macro) of 0.84; Precision of: [0.86, 0.88, 0.74, 0.89], and recall of [0.88 0.88 0.74 0.88] when trained on labeled cells from representative tumor microarray cores. This approach is useful when segmentation is challenging; allowing for extraction of immune cell density data for a number of classes, for better evaluation of the tumor microenvironment.