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# Development of an AI-based Algorithm to Quantify Eosinophils in H&E Images from Colorectal Cancer (CRC) Tissue Sections Guided by Biomarker Staining using Multiplex Immunofluorescence Imaging

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## Background

Eosinophils are innate immune granulocytes that migrate to areas of inflammation to combat against infection and disease. There is growing interest in the involvement of eosinophils in cancer as they are routinely observed in the tumor microenvironment (TME) and, depending on the cancer type, have been shown to drive other immune cells to either suppress or promote tumor growth - In colorectal cancer (CRC), eosinophil infiltration into the TME has been linked to a favorable prognosis. However, the behavior of eosinophils and their effect on associated immune mediators in the TME remains poorly understood. Currently, eosinophils are primarily identified from H&E stained tissue sections based on morphological features by a pathologist, but it can be challenging to reliably and efficiently identify all eosinophils by visual inspection alone. Here, we present an image analysis workflow to establish an AI-based cell classifier which can accurately quantify eosinophils in H&E stained CRC tissue sections by leveraging biomarker staining of eosinophils using multiplex immunofluorescence (mIF) imaging to guide classifier development and validation.

| Granulocytes | | Histological Features in H&E Stained Tissue |
|---|---|---|
| neutrophil |  | Multi-lobed nucleus, cytoplasm stained by both acidic (eosin) and basic (hematoxylin) stains. Diameter ~ 12 µm |
| eosinophil |  | Bi-lobed nucleus, cytoplasm stained pink by eosin, typically contains dark red/pink granules. |
| basophil |  | Bi or tri-lobed nucleus, cytoplasm stained blue/purple by hematoxylin, typically contains dark granules. |

## Methods

### Specimen Preparation

- mIF images: CRC tissue sections were labeled with DAPI and a 4plex biomarker panel (CD15, CD16, CD68, and panCK), and imaged (40x) by multiplex immunofluorescence (mIF) using the PhenoImager HT platform (Akoya).
- Post mIF H&E images: Biomarker fluorescence was quenched, sections stained by H&E, and imaged (40x) using an Aperio slide scanner (Leica).
- Serial H&E images: A serial section of each CRC specimen was stained by H&E and imaged (40x) using an Aperio slide scanner.
- mIF and H&E images were imported into the HALO® v3.6 platform for development of a cell classifier to detect eosinophils in H&E images using the Halo AI object phenotyper module.

| mIF panel | |
|---|---|
| **Biomarker** | **Opal dye** |
| CD15 | 520 |
| CD16 | 570 |
| CD68 | 620 |
| CK | 690 |

| mIF phenotype | |
|---|---|
| **Cell type** | **Biomarker expression** |
| eosinophil | CD15+CD16-CD68- |
| neutrophil | CD15+CD16+ |
| macrophage | CD68+ |
| tumor cell | CK+ |

### AI Cell Classifier Development & Validation Workflow

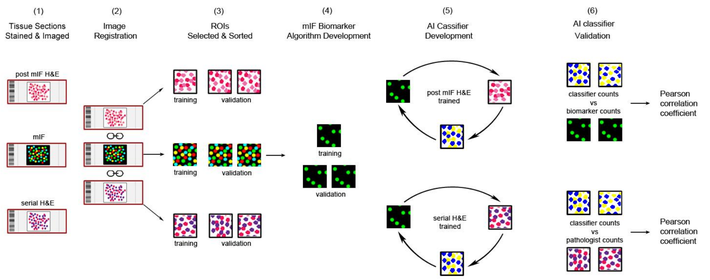


**Figure 1: AI classifier workflow.** 1) mIF, post mIF H&E, and serial H&E sections were stained and imaged as described above. 2) Images were imported into Halo v3.6 and registered for synchronized navigation. 3) Corresponding ROIs were selected from each image and separated into training and validation sets. 4) An algorithm was developed for the detection of eosinophils in mIF images based on the biomarker expression profile. 5) A Halo AI classifier was trained using the mIF eosinophil overlay image to guide the selection of eosinophil in the post mIF H&E and serial H&E images. 7) Classifier performance was evaluated on the validation image set by comparing eosinophils detected by the classifier in the post mIF H&E images to the number of eosinophils detected in the mIF images, and by comparing eosinophils detected by the classifier in the serial H&E images to the number of eosinophils detected by a pathologist.



## Eosinophil Classifier Development & Validation

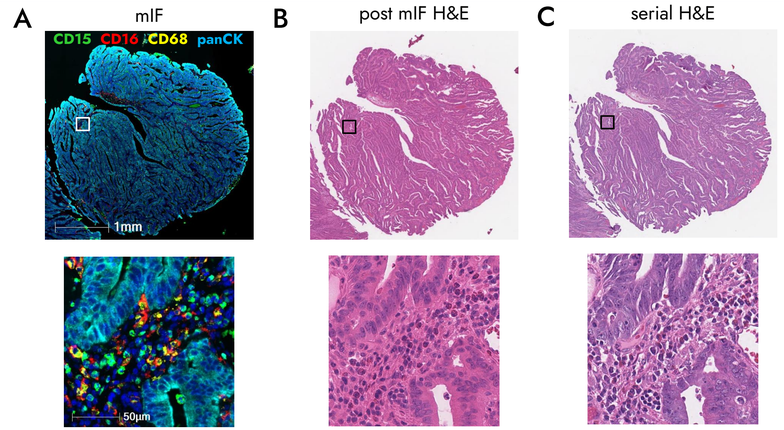


**Figure 2: mIF guided training of an AI classifier to detect eosinophils in H&E images.** Representative mIF (A), post mIF H&E (B), and serial H&E (C) images of a CRC tissue specimen. Top row shows macroscopic view and bottom row a 50x magnified view of the ROI in the top image. Images were registered for synchronized navigation, and the mIF staining profile for eosinophils (CD15+CD16-CD68-CK-) was used to guide the training of an AI classifier to detect eosinophils in the post mIF H&E and serial H&E images.

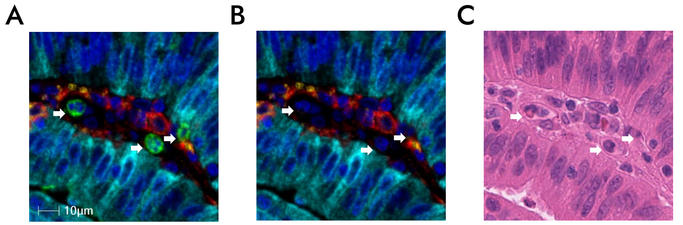


**Figure 3: Evidence of CD16- neutrophils in CRC specimens.** CD15+ cells observed in the mIF image (A) lacked expression of CD16 (B) despite staining appearance and morphological features in the post mIF H&E (C) characteristic of neutrophils. A-C) Arrows indicate neutrophils. Cells like these were not marked as eosinophils during training of the AI classifier.

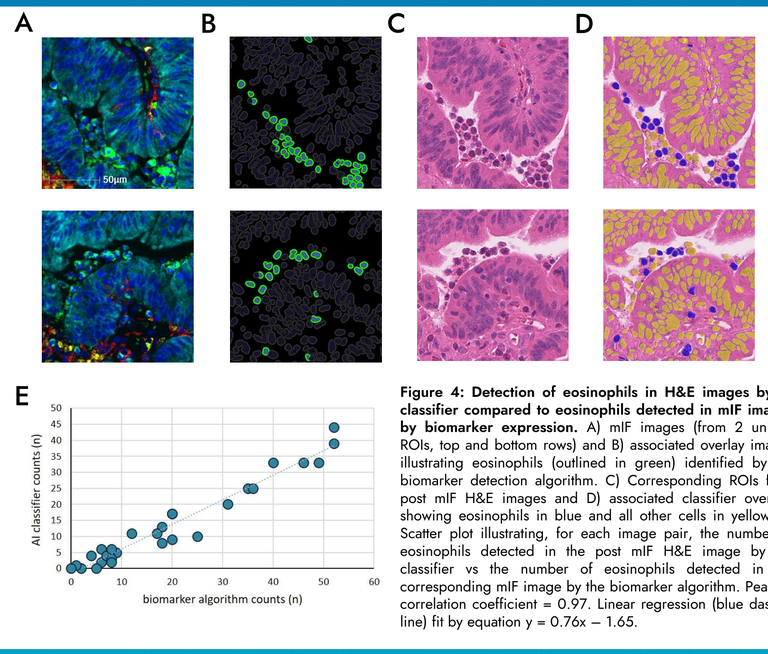


**Figure 4: Detection of eosinophils in H&E images by AI classifier compared to eosinophils detected in mIF images by biomarker expression.** A) mIF images (from 2 unique ROIs, top and bottom rows) and B) associated overlay images illustrating eosinophils (outlined in green) identified by the biomarker detection algorithm. C) Corresponding ROIs from post mIF H&E images and D) associated classifier overlays showing eosinophils in blue and all other cells in yellow. E) Scatter plot illustrating, for each image pair, the number of eosinophils detected in the post mIF H&E image by the classifier vs the number of eosinophils detected in the corresponding mIF image by the biomarker algorithm. Pearson correlation coefficient = 0.97. Linear regression (blue dashed line) fit by equation y = 0.76x – 1.65.

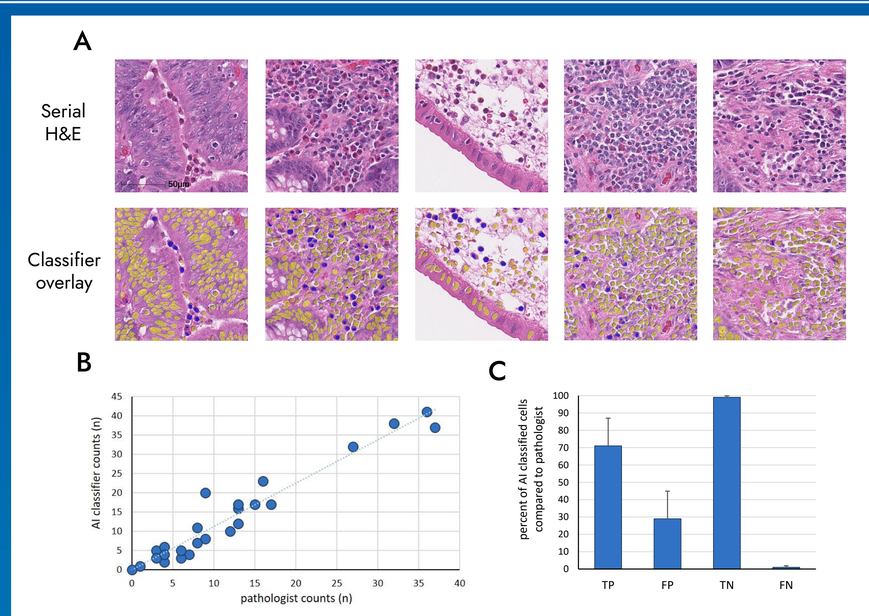


**Figure 5: Detection of eosinophils in serial H&E images by AI classifier compared to pathologist.** A) Top row shows representative ROIs from serial H&E images and bottom row shows classifier overlays for the same ROIs with eosinophils indicated in blue and all other cells in yellow. B) Scatter plot demonstrating, for each ROI analyzed, the number of eosinophils detected by the AI classifier vs. the number of eosinophils visually identified by a pathologist. Pearson correlation coefficient = 0.97. Linear regression (blue dashed line) fit by equation y = 1.12x – 0.09. C) Classifier accuracy was categorized, for each ROI, as the percent of true positives (TP), false positives (FP), true negatives (TN), and false negative (FN) detected by the classifier in comparison to the pathologist. Bars show mean ± SD from 24 ROIs.

## Summary

- Biomarker labeling of mIF images offers the potential to guide training of AI cell classifiers on H&E images specimens but requires careful consideration when designing the biomarker panel.
- Weak neutrophil expression of CD16 in CRC specimens resulted in an overestimation of the numbers of eosinophils in mIF images when analyzed with the eosinophil biomarker detection algorithm, limiting the utility of concordance analysis between eosinophil counts determined by the trained AI classifier on post mIF H&E images.
- The trained Halo AI eosinophil classifier performed well when compared to pathologist scoring of serial H&E images, demonstrated by a high Pearson correlation coefficient and an accuracy for correctly identify eosinophils of ~ 70%.
- Future development will focus on improving the performance of the AI classifier by expanding the sizes of the training and validations sets with the goal of establishing a classifier capable of high-throughput detection and quantification of eosinophils from H&E stained tissue sections with a greater than 90% accuracy.

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