HALO 3.0

Spatial Analysis

Step-by-Step guide

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indica labs
informed pathology

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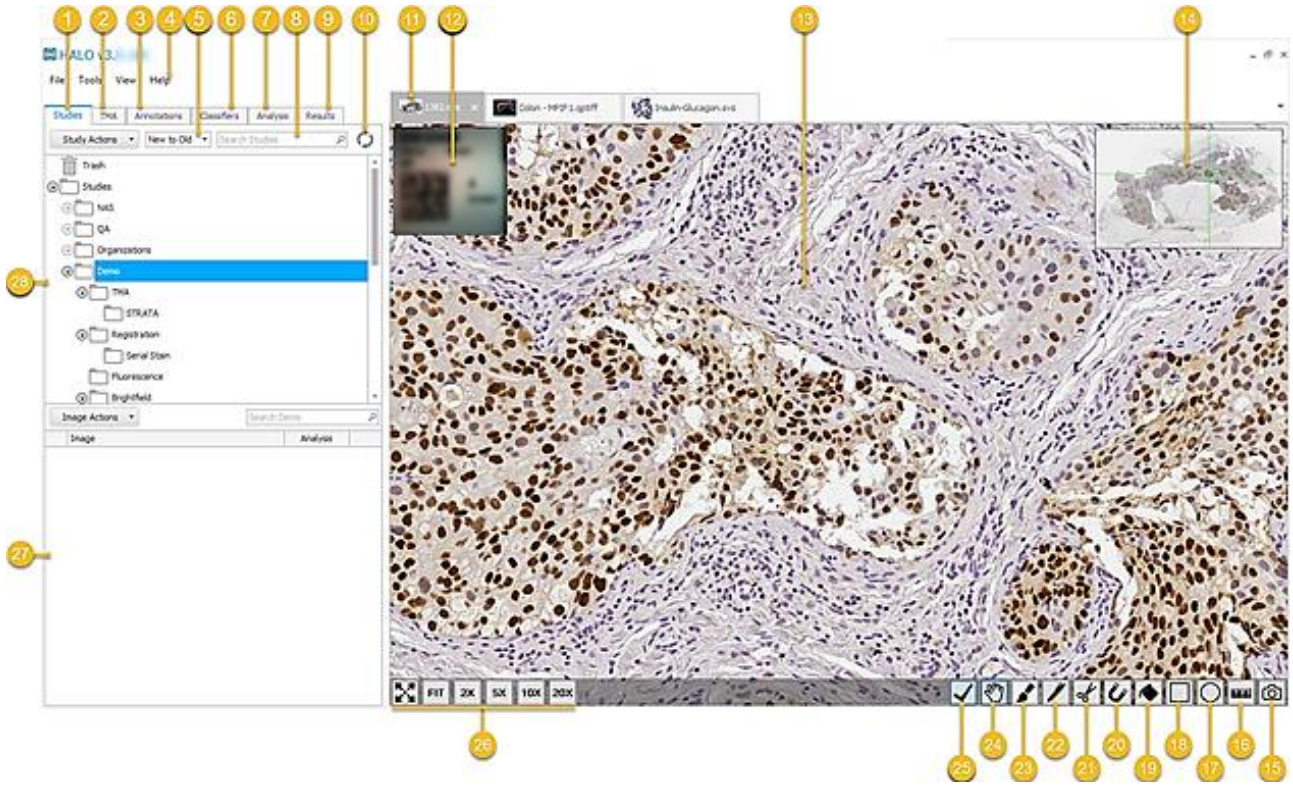
This guide will show how to use the Spatial Analysis module

After clicking the HALO icon to open you will be presented with the following. Please study the key features of the interface.



1	**Studies tab**: This tab is for organization of images and batch analysis mode is access from here.	2	**TMA tab**: Tissue Micro Array caters for analysis of slides containing multiple tissue or cellular cores. Only available if purchased.
3	**Annotations tab**: Annotations allow you to identify specific regions to include or exclude from analysis.	4	**Help Menu**: Information about software version, help guides, and reference popups.
5	**Study Sort:** Sort studies in ascending or descending order by creation date or alphabetical order.	6	**Classifiers tab**: Classifiers instruct the algorithm to delineate tissue based on its type [tumor, stroma etc.]. Only available if purchased.
7	**Analysis tab**: This tab is used to fine tune the analysis settings and load algorithms.	8	**Study Search**: Free text search for studies.
9	**Results tab**: Results summary and object results are located here.	10	**Refresh**: Refresh HALO (if part of a group license or Halo Link installation).
11	**Image Tab**: Open image tab (file name if unchanged)	12	**Slide Label**: Contains information about the sample
13	**Main image viewer**: The main working area, displays images after opening.	14	**Thumbnail**: Used to rapidly navigate around the image, may be reduced in size or removed.
15	**Snapshot**: Take a snapshot of the current viewing window.	16	**Ruler**: Measure the distance between two points on the image screen.
17	**Ellipse**: Draw an elliptical annotation.	18	**Rectangle**: Draw a rectangular annotation.
19	**Flood-Fill:** Automated tool to identify tissue boundaries using RBG pixel intensity and R-value.	20	**Magnetic Pen**: Automatically outline tissues or regions which have a well-defined edge.
21	**Exclusion Pen**: construct annotation regions to be excluded from analysis.	22	**Pen Tool**: Draw new polygon annotations or modify existing ones by drawing lines.
23	**Brush Tool**: Draw new polygon annotations or modify existing ones using brushstrokes.	24	**Pan tool**: This allows you to pan around the image.
25	**Toggle Mark-up:** Toggles between the analysis markup overlay and classifier overlay. Can hide both overlays.	26	**Zoom buttons**: Jump to a specified zoom.
27	**Image Actions pane**: Images are organized and analyzed here.	28	**Studies pane**: Study folders are organized here

The Spatial Analysis module offers a suite of subsequent analysis tools which can be used to identify proximity and relative spatial distribution of objects, cells, and/or features across single tissues or serial sections. This module is embedded within the HALO platform and can be used in conjunction with any of our cell-based brightfield or fluorescence analysis modules.

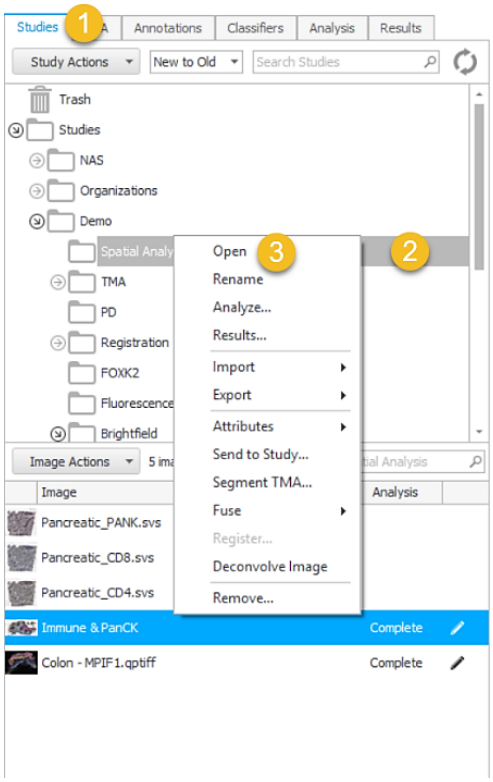
Nearest Neighbor: Determine the average distance and number of unique neighbors between any two cell or object populations.
Proximity: Calculate the number of objects or cells within a certain distance of another object or cell.
Infiltration: Determine the number of objects or cells within a set range of an annotated region of interest.
Density Heatmaps: Calculate and visualize the density of objects across an area of analysis.

The workflow for this module is broken down into sections in this user guide:

Contents

Generating Individual Spatial Plots

Step 1. Open image in the main viewing window



〉 Navigate to the **Studies** tab.

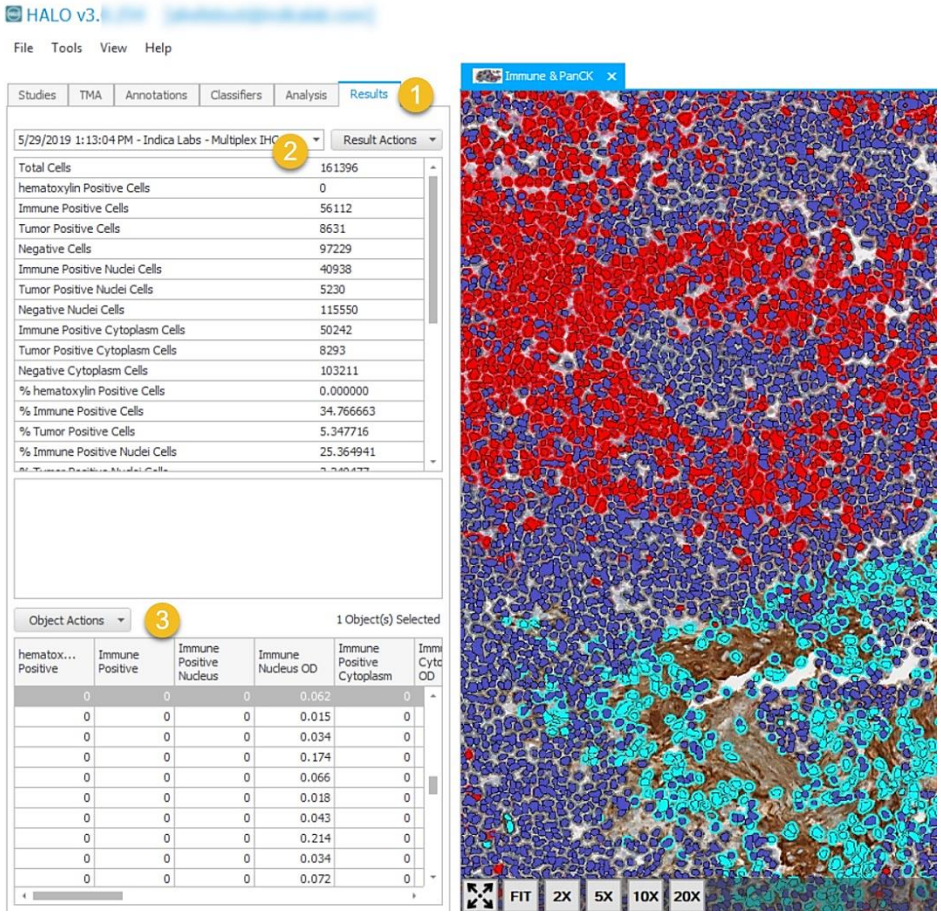
〉 Select the study folder containing your chosen images.

〉 Use the control key or the shift key with left mouse button to select all the images destined for analysis from the **Image Actions** pane.

〉 Click on the **Image Actions** drop down menu and click on **Open.** Alternatively, right click on the selected images and select **Open**.

Analysis tip: *To conduct spatial analysis HALO requires analysis results with object data. Check that images have been analyzed by looking for the label 'Complete' adjacent to the image in the Image Actions Pane.*

Step 2. Check that analysis results are suitable for spatial analysis



〉 Navigate to the **Results** tab.

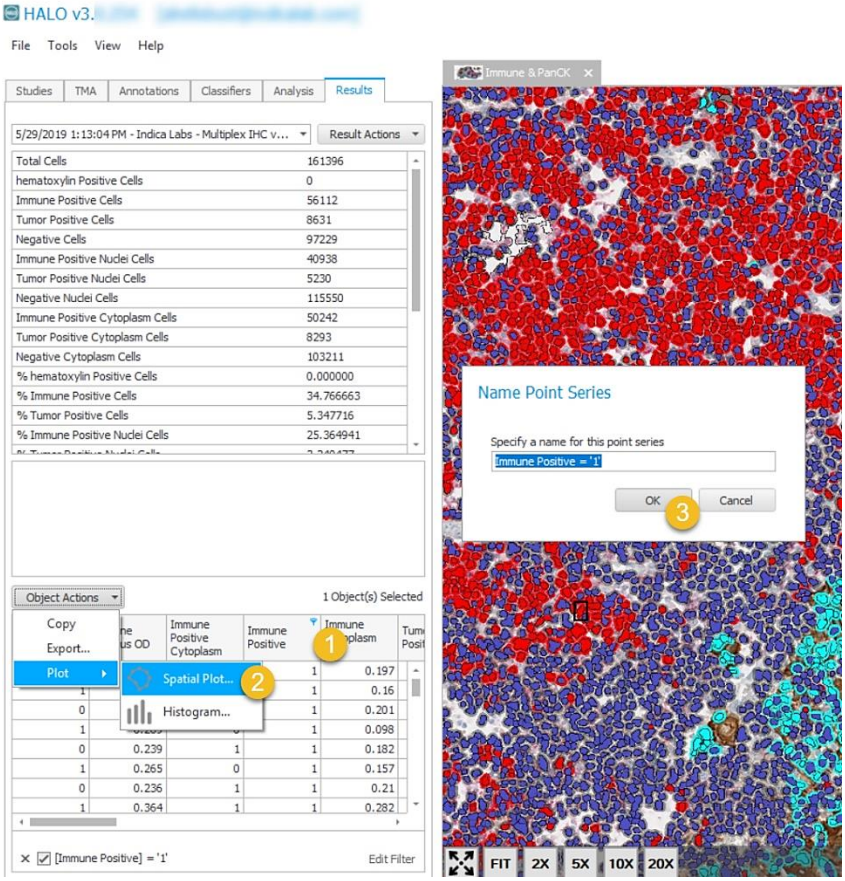
〉 Ensure that the correct result is selected using the drop down list. It is common to have more than one analysis result, especially following analysis optimization.

〉 Ensure there is object data present in the **Object** pane.

Analysis tip: *Spatial Analysis requires object data to be enabled. Please ensure when images are analyzed that 1) the module supports object data generation and 2) the Store Object Data parameter is set to 'True'.*

Area Quantification and Area Quantification FL modules do not generate object data!

Step 3. Create the spatial plot



› Click on the **filter funnel** in the upper right corner of the header of the object column to filter. "0" corresponds to a negative cell for a specific stain while "1" corresponds to a positive cell for that stain.

› Once the desired cell population is filtered in the object data table, click on the **Object Actions** button to open the drop-down menu and select **Plot** > **Spatial Plot**.

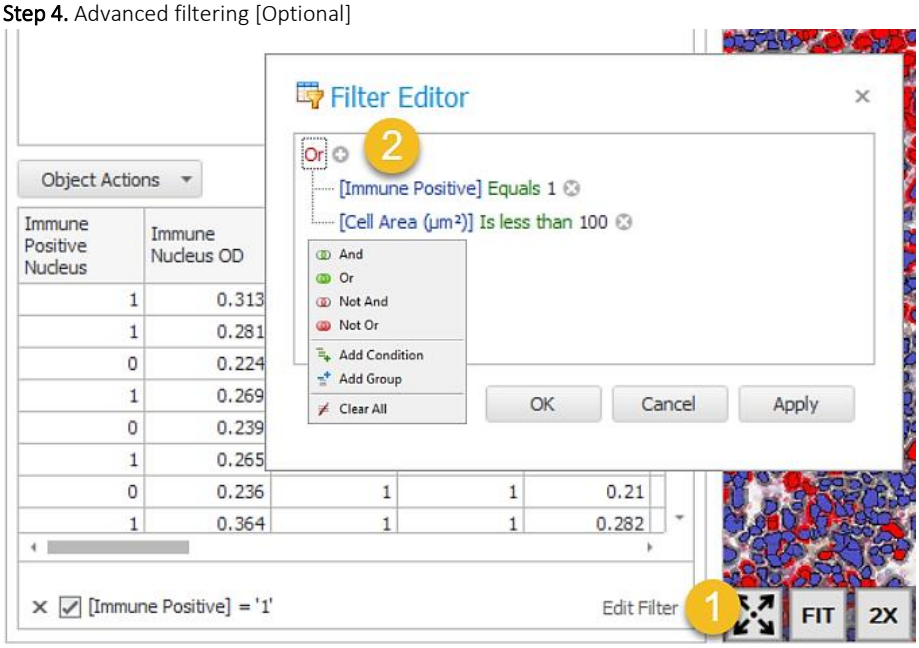
› In the **Name Point Series** window, input a name for the point series in the text field. This is the first layer of the spatial plot. It is recommended to use a descriptive name such as 'Immune Positive' to clearly identify the cell subset.

› Click on the **OK** button to progress to the **Plot** window.

› **Repeat** this filtering step as necessary until all cell populations and elements for spatial analysis are added to the spatial plot.

Analysis tip: Object data can be imported into the spatial plot from the same analysis result, different result from the same image, or from registered images.

Step 4. Advanced filtering [Optional]

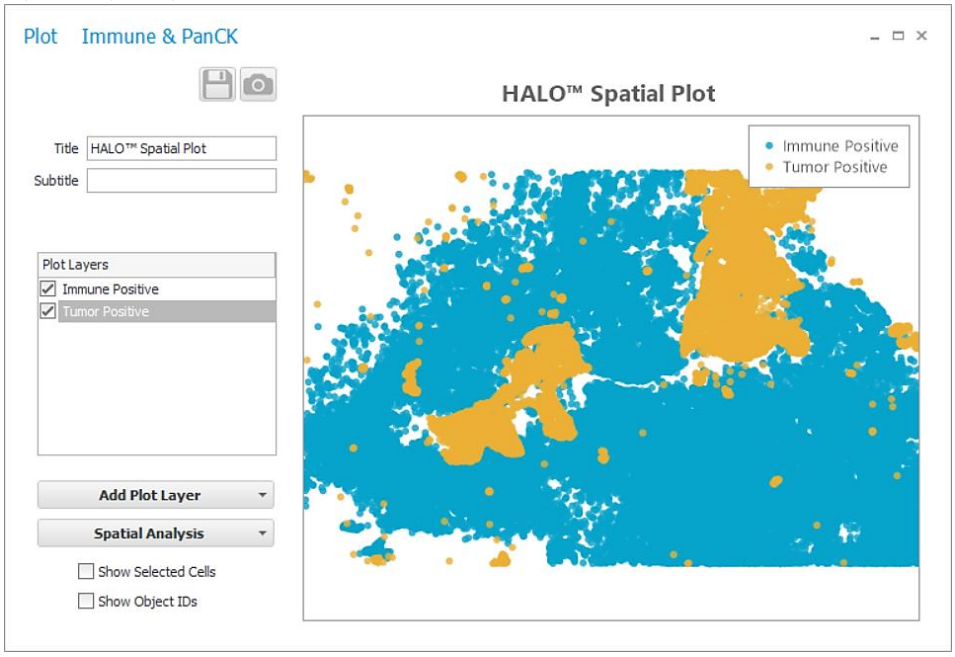


› Hover over and click the **Edit Filter** button at the bottom of the object data table. This option appears once at least one column is filtered.

› Use **Filter Editor** to create advanced filtering conditions.

Analysis tip: Advanced filtering can be used to deal with biologically impossible double positive populations or to specify a specific *phenotype cell population.*

Step 5. Review spatial plot and plot layers



〉 Save the plot within HALO. The saved plot is associated with the analysis results.

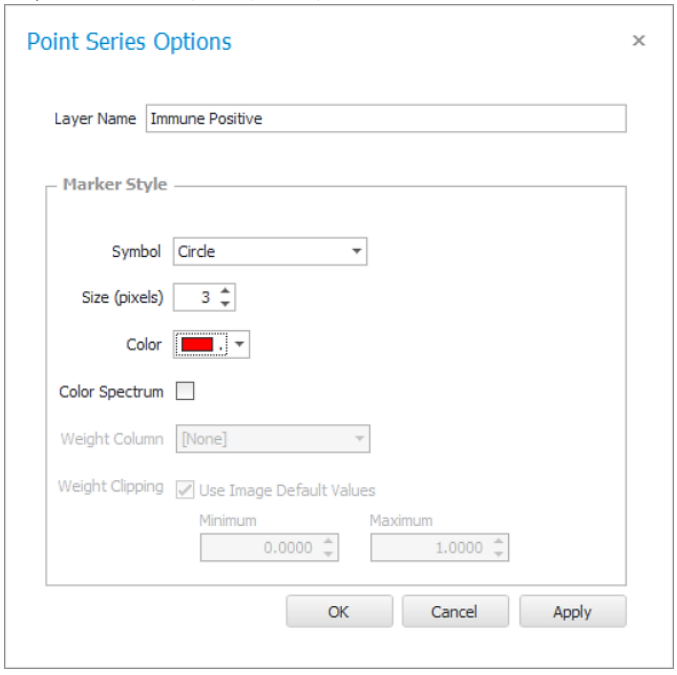
〉 Export the plot window as a .PNG image outside HALO.

〉 **Plot Layers** lists point series or annotation layers associated with the plot. Unchecking any **Plot Layer** will hide the layer in the plot. Double clicking on any **Plot Layer** will bring up the **Point Series Options** properties window. [Expanded upon in next step]

〉 From the **Add Plot Layer** button, select additional annotation layers or point series with previously used object filters.

〉 The **Plot Viewer** acts like the **Main Image Viewer**. The mouse can be used to **Pan** [**Left mouse** button] across the image. Scroll in or out to adjust the **Zoom** into the plot. Holding down the **right mouse** button over a data point will reveal the point **XY coordinates.**

Step 6. Customize spatial plot [Optional]



〉 Edit the **Layer Name** if necessary.

〉 Change the data point **Symbol** from **Circle** to **Square**, **Diamond** or **Triangle.**

〉 Change the **Size (pixels)** of each data point.

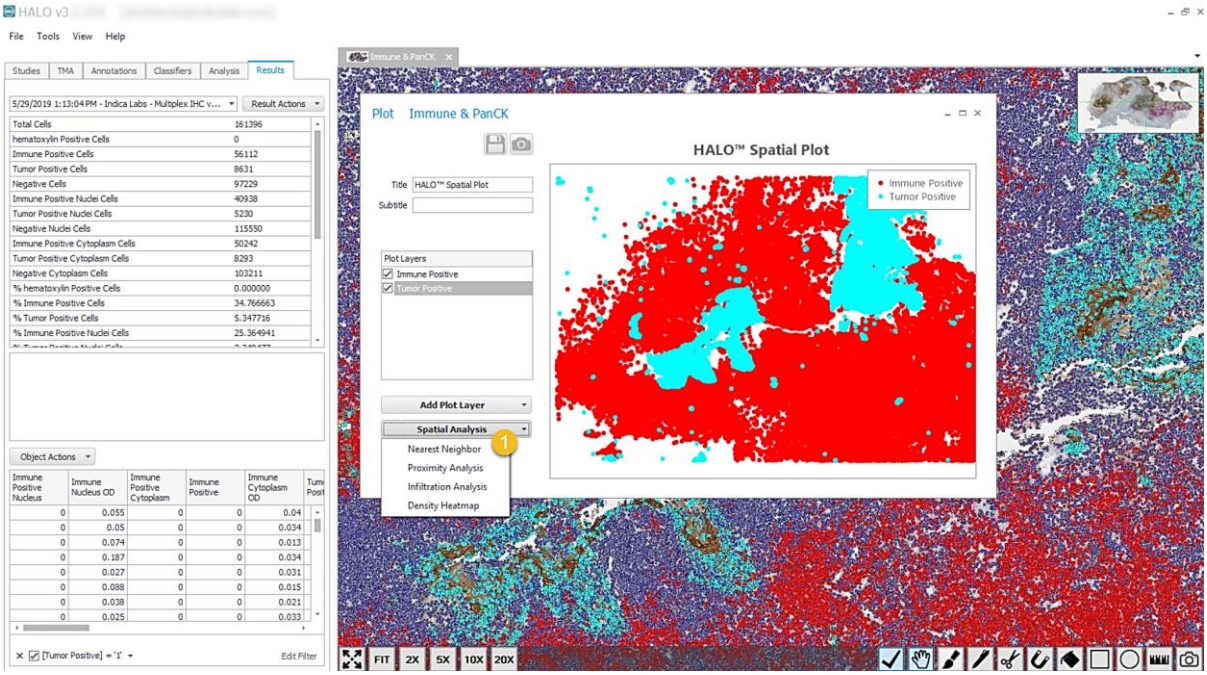
〉 Change the **Color** of each layer.

〉 Plot a data series on a **Color Spectrum** to view data points colored with a heatmap denoting a result value associated with that data point i.e. Nuclear Optical Density of Tumor by checking the **Color Spectrum** box.

〉 Select a parameter from the **Weight Column** for this feature to function.

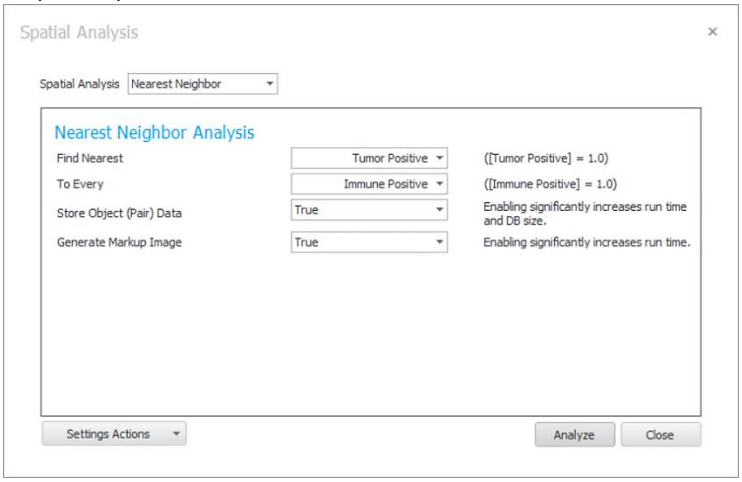
Run Spatial Analysis

Select type of spatial analysis



〉 From the **Spatial Analysis** button in the Plot window, select one type of spatial analysis from the drop down list.

a) Nearest neighbor analysis setup



〉 In the Spatial Analysis window, **Nearest Neighbor** should be selected in the drop-down.

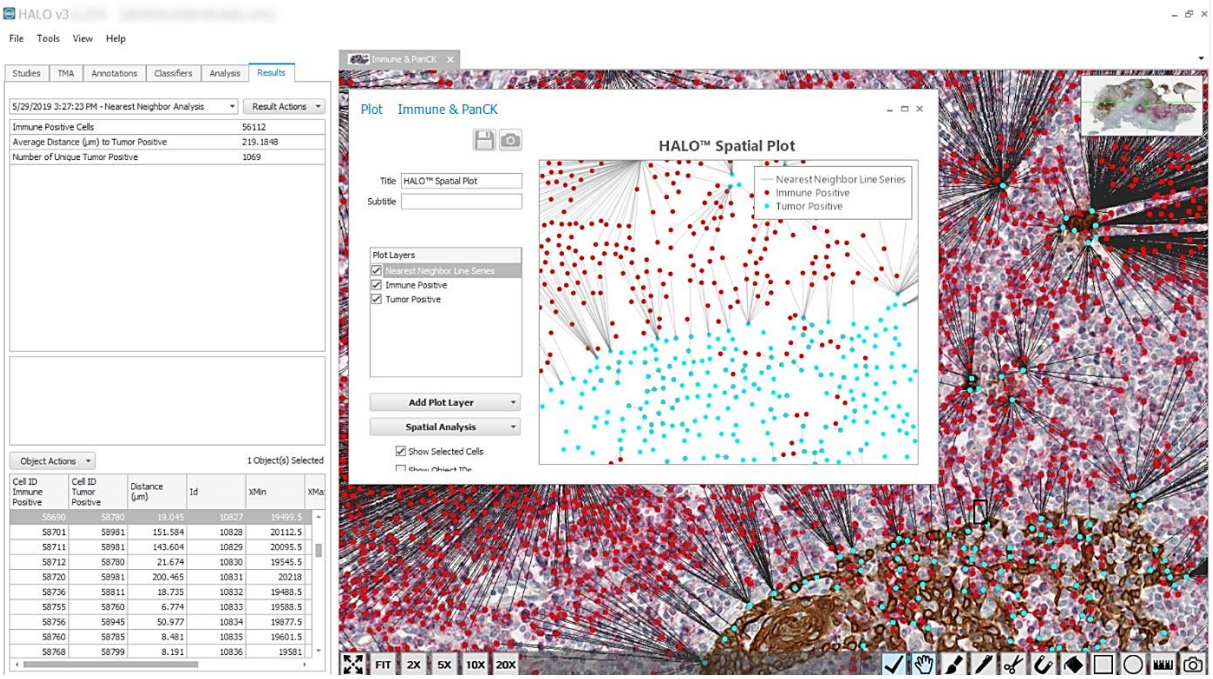
〉 Using the drop-down menus, select which data series to measure as nearest neighbors. The **order** is important. Changing the order of the analyzed populations will change the results. The filter used to generate these series is displayed to the right.

〉 Change the **Store Object (Pair) Data** and **Generate Markup Image** parameters to true if that additional data is desired. Enabling these features can significantly increase analysis run time.

〉 Click the **Analyze** button.

〉 After Analysis is complete HALO will 1) update the spatial plot to contain a new layer, 2) add a Nearest Neighbor line series to the plot, 3) generate new summary result under the Results tab, 4) generate object data (if **Store Object (Pair) Data** was set to true), 5) generate a markup image (if **Generate Markup Image** was set to true).

Viewing the nearest neighbor analysis results



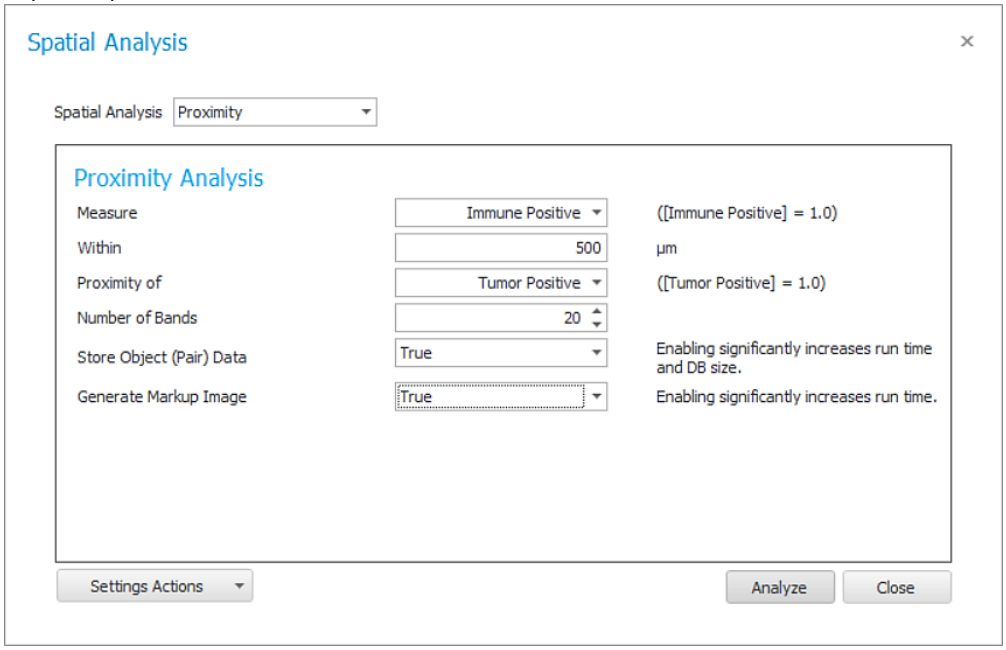
〉 The summary results are displayed in the summary results pane in the **Results** tab. To switch between spatial analysis results and previous results use the drop-down menu.

〉 A grey Nearest Neighbor Line Series is now displayed as a new plot layer. This may be hidden by unchecking the box to the immediate left of the layer. To remove the layer completely, right click on the layer to access more option and select **Remove**.

〉 If **Store Object (Pair) Data** was set to true, the object results pane will now display object data (shown above).

〉 If **Generate Markup Image** was set to true, a markup image will be displayed in the main image viewer (shown above). Use the ☑ button to toggle the markup on and off.

b) Proximity analysis setup



〉 In the Spatial Analysis window, **Proximity** should be selected in the drop-down.

〉 Using the drop-down menus **Measure** and **Proximity of,** select which cell type should be measured within the pre-defined proximity radius of the other. The filter used to generate these series is displayed to the right.

〉 Set the proximity radius (µm) using the **Within** text field. Cells outside the boundary will not be lost from the analysis.

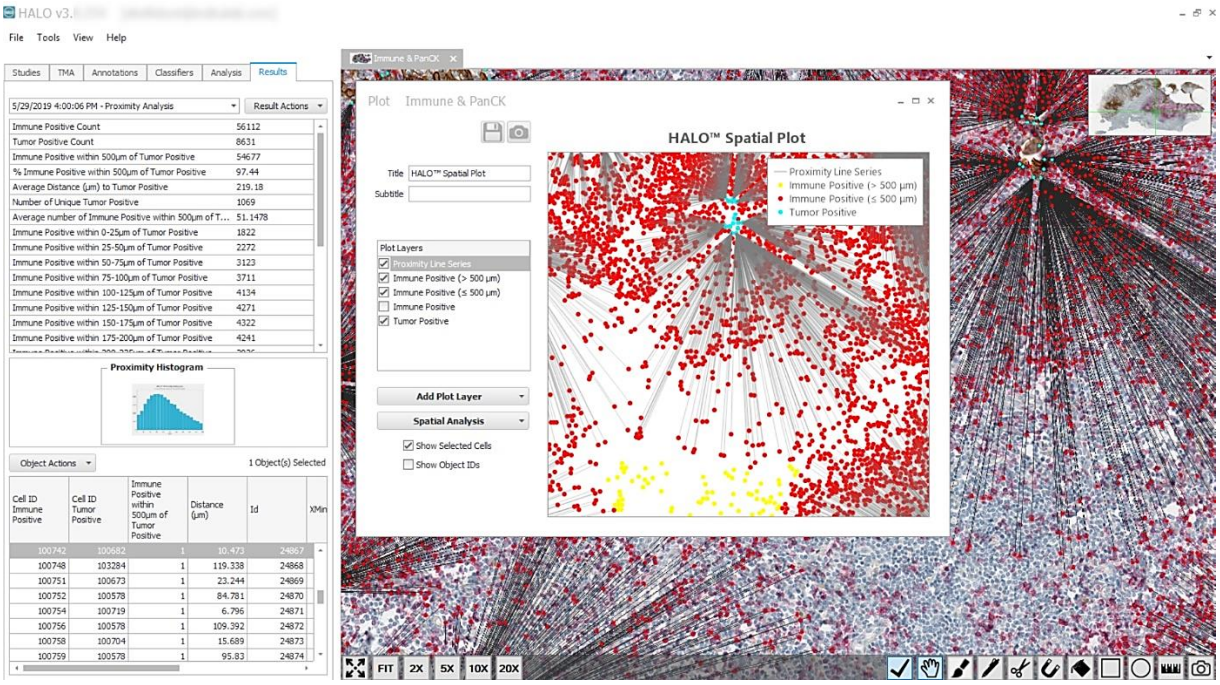
〉 Set **Number of Bands.** to define how many bins to divide the distance analyzed to get a better understanding of cell distribution. If "Within" is set to 500µm and "Number of Bands" set to 20. You will output 20 bands of 25 µm in width.

〉 Change the **Store Object (Pair) Data** and **Generate Markup Image** parameters to true if that additional data is desired. Enabling these features can significantly increase analysis run time.

〉 Click the **Analyze** button.

〉 After Analysis is complete HALO will 1) update the spatial plot to contain two new layers (e.g. Immune Positive ≤500µm) and (e.g. Immune Positive >500µm) , 2) add a Proximity line series to the plot, 3) generate new summary result under the Results tab, 4) add a Proximity Histogram to the Plot Carousel, 5) generate object data (if **Store Object (Pair) Data** was set to true), and 6) generate a markup image (if **Generate Markup Image** was set to true).

Viewing proximity analysis results



› The summary results are displayed in the summary results pane in the **Results** tab. To switch between spatial analysis results and previous results use the drop-down menu.

› A grey Proximity Line Series is now displayed as a new plot layer. This may be hidden by unchecking the box to the immediate left of the layer. To remove the layer completely, right click on the layer to access more option and select **Remove**.

› Two new plot layers (e.g. Immune Positive ≤500µm), (e.g. Immune Positive > 500µm) replaced the original Immune Positive layer.

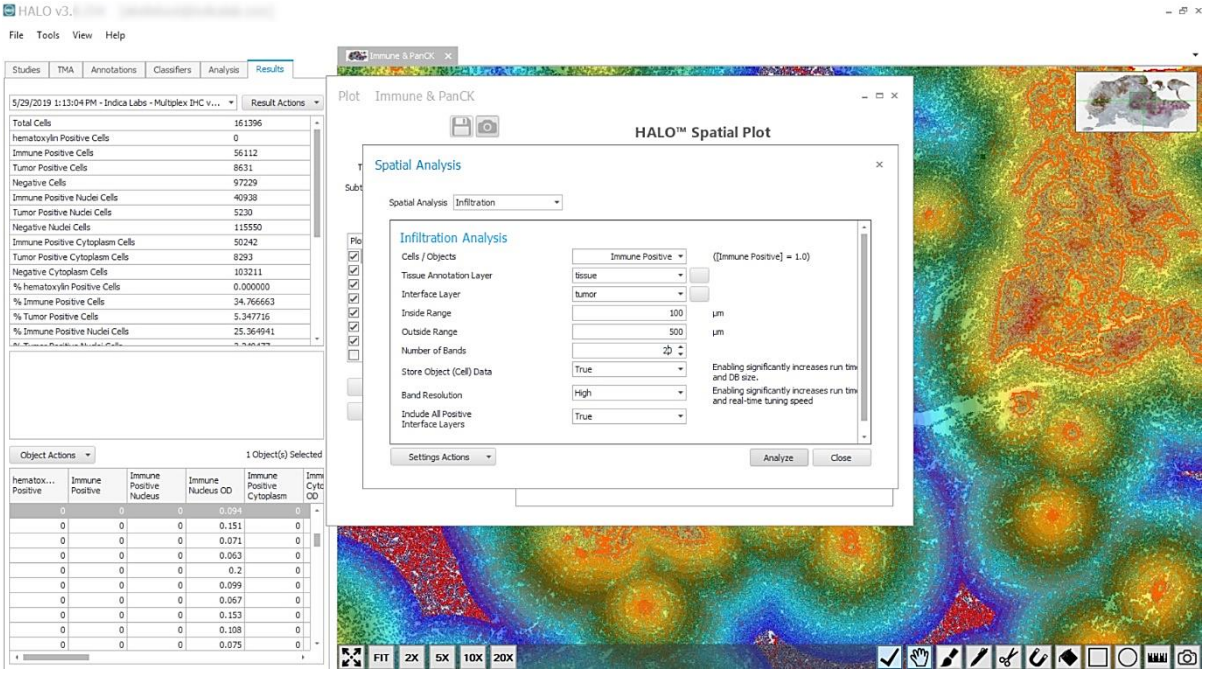
› A **Proximity Histogram** was added to the **Plot Carousel**. Double clicking on the histogram will open it within HALO and allow it to be modified or exported.

› If **Store Object (Pair) Data** was set to true, the object results pane will now display object data (shown above).

› If **Generate Markup Image** was set to true, a markup image will be displayed in the main image viewer (shown above). Use the ☑ button to toggle the markup on and off.

Analysis tip: New Object Layers created by Proximity Analysis (Immune Positive ≤500µm and Immune Positive > 500µm) can be used as objects in other spatial analyses.

c) Infiltration analysis setup



〉 In the Spatial Analysis window, **Nearest Neighbor** should be selected in the drop-down.

〉 Using the drop-down menus **Cells/Objects** select which cell population to measure. The filter used to generate this series is displayed to the right.

〉 Use the **Tissue Annotation Layer** to specify your region of interest to measure the cell density of a specific cell population of interest. Use button to freehand draw the tissue annotation layer. If left empty the whole data series imported from the results will be used.

〉 Set **Interface Layer** to define which annotation is to be used in the infiltration analysis. Use button to freehand draw the interface layer.

〉 Set the **Inside Range** and **Outside Range** as the distance either side of the boundary to measure the cell population of interest. This is set to 500µm as default.

〉 Set the **Number of bands** to define how many bins to divide the distance analyzed to get a better understanding of cell distribution. This is set to 10 as default. If Inside and Outside Range are left at default 500µm and Number of Bands left at default 10. You will output 10 bands of 100 µm in width.

〉 Change the **Store Object (Pair) Data** parameter to true if that additional data is desired. Enabling this feature can significantly increase analysis run time.

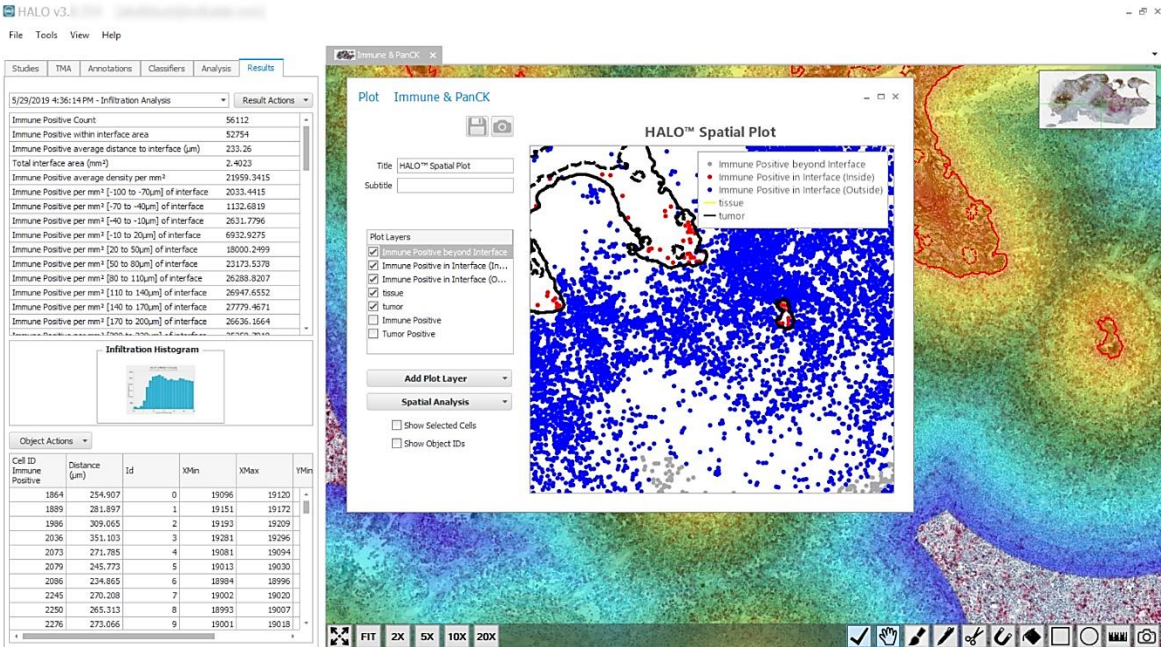
〉 Set the **Band Resolution** to increase the resolution of the bands. This may be necessary for large images. **Default** corresponds to a map 1000x1000 pixels, **high** corresponds to a map of 2500x2500, and **highest** corresponds to a map 5000x5000 pixels. Increasing the resolution can significantly increase analysis run time.

〉 Set the **Include All Positive Interface Layers**. **False** is the default behavior. This behavior is displayed in the markup image.

〉 Click the **Analyze** button to generate **Infiltration Analysis** spatial analysis plot and dataset.

〉 After Analysis is complete HALO will 1) update the spatial plot to contain new annotation layers (e.g. Tissue and Interface Layer), 2) update the spatial plot to contain three new layers (e.g. *Immune Positive beyond the Interface*, *Immune Positive beyond in Interface (Inside)*, and *Immune Positive beyond in Interface (Outside)*), 3) apply a markup image in the main viewer distinguishing size of bands around the interface layer as defined in the input settings, 4) generate new summary result under the Results tab, 5) add an Infiltration Histogram to the Plot Carousel, and 6) generate object data (if **Store Object (Pair) Data** was set to true).

Viewing infiltration analysis results



› The summary results are displayed in the summary results pane in the **Results** tab. To switch between spatial analysis results and previous results use the drop-down menu.

› Two new annotation plot layers (Tissue Boundary and Interface line) are added. Layers may be temporarily hidden or made visible by unchecking the box to the immediate left of the layer. To remove a layer completely, right click on the layer to access more option and select **Remove.**

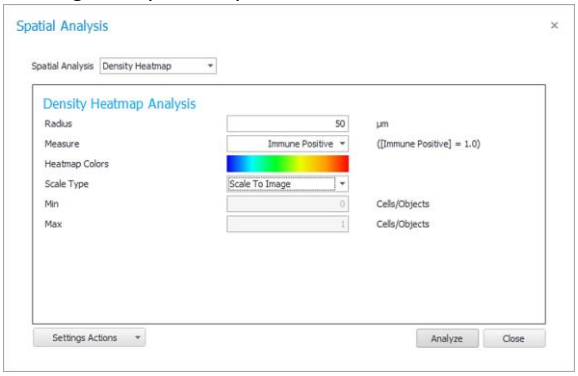
› Three new plot layers (e.g. *Immune Positive beyond the Interface*, *Immune Positive beyond in Interface (Inside)*, and *Immune Positive beyond in Interface (Outside)*) replaced the original Immune Positive layer.

› An **Infiltration Histogram** was added to the **Plot Carousel**. Double clicking on the histogram will open it within HALO and allow it to be exported. Negative values correspond to within the interface annotation and positive values corresponds to outside the interface annotation.

› A rainbow markup image is displayed in the main image viewer (shown above). Use the ☑ button to toggle the markup on and off. Color only distinguishes bands.

› If **Store Object (Pair) Data** was set to true, the object results pane will now display object data (shown above).

d) Creating density heatmaps



› In the Spatial Analysis window, **Density Heatmap** should be selected in the drop-down.

› Using the drop-down menus, select which data series to **Measure**. The filter used to generate this series is displayed to the right.

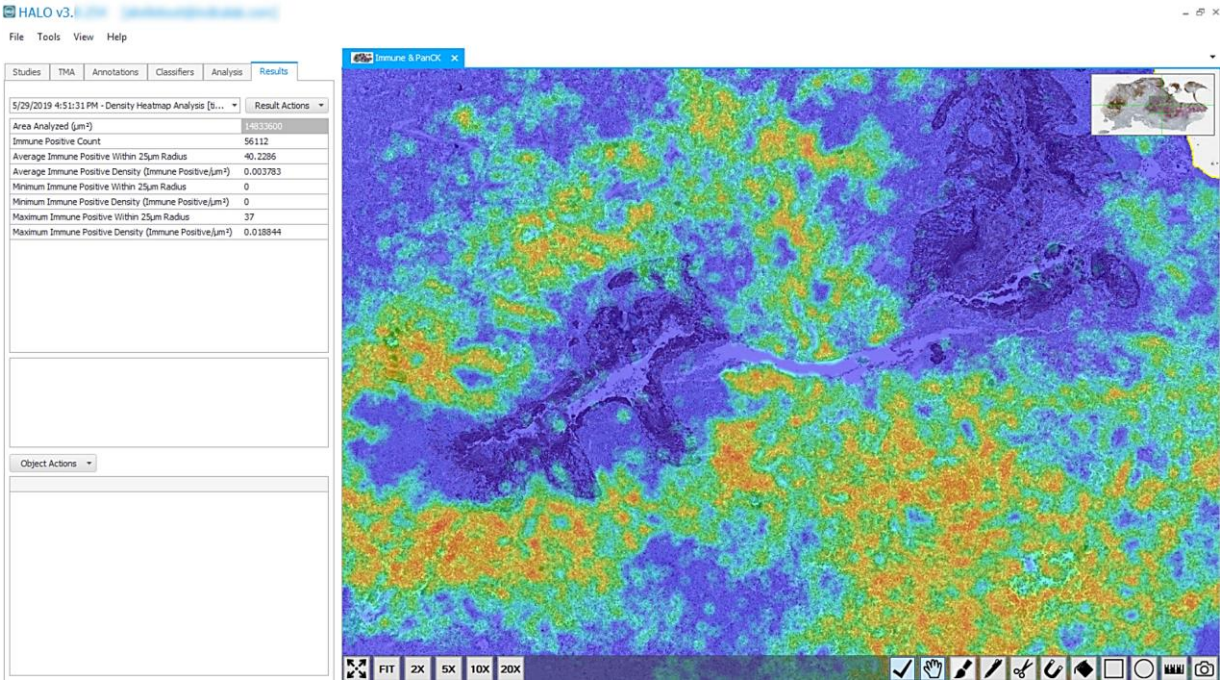
› Change the **Store Object (Pair) Data** and **Generate Markup Image** parameters to true if that additional data is desired. Enabling these features can significantly increase analysis run time.

› Click the **Analyze** button.

› After analysis is complete HALO will generate new summary result under the Results tab and a markup image.

Analysis tip: Open the Edit Color Bar window to select a different color scheme by double clicking on the Heatmap Colors color bar.

Viewing density heatmap results

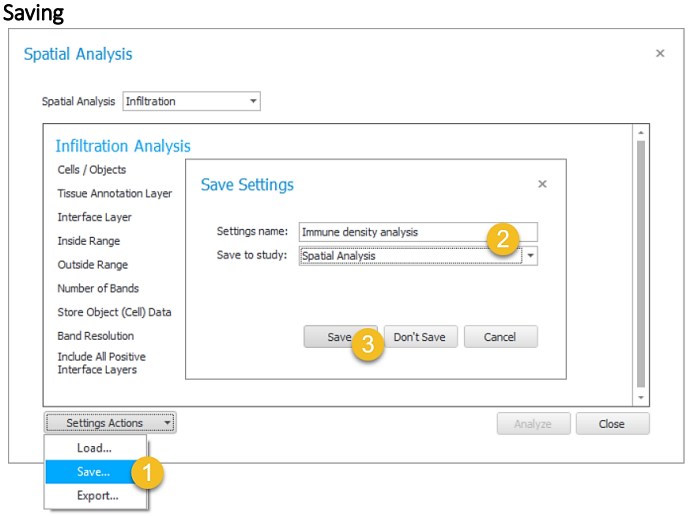


› The summary results are displayed in the summary results pane in the **Results** tab. To switch between spatial analysis results and previous results use the drop-down menu.

› A markup image will be displayed in the main image viewer (shown above). Use the ☑ button to toggle the markup on and off.

Saved spatial analysis settings

Saving



〉 Finalize settings in the **Spatial Analysis** window.

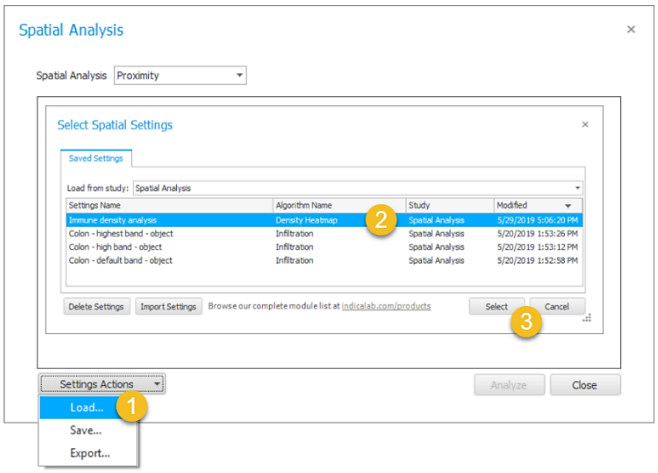
〉 From the **Settings Actions** drop-down select **Save...**.

〉 In the **Save Settings** window. give the settings a unique name and select the study to save the settings to.

〉 Click the **Save** button.

Analysis tip: From the **Settings Actions** dropdown select **Export...** and select the location and name for the exported file.

Loading



〉 Navigate to the **Spatial Analysis** window.

〉 From the **Settings Actions** drop-down select **Load...**.

〉 In the **Select Spatial Settings** window, select the study from which the settings come from and highlight the settings of interest.

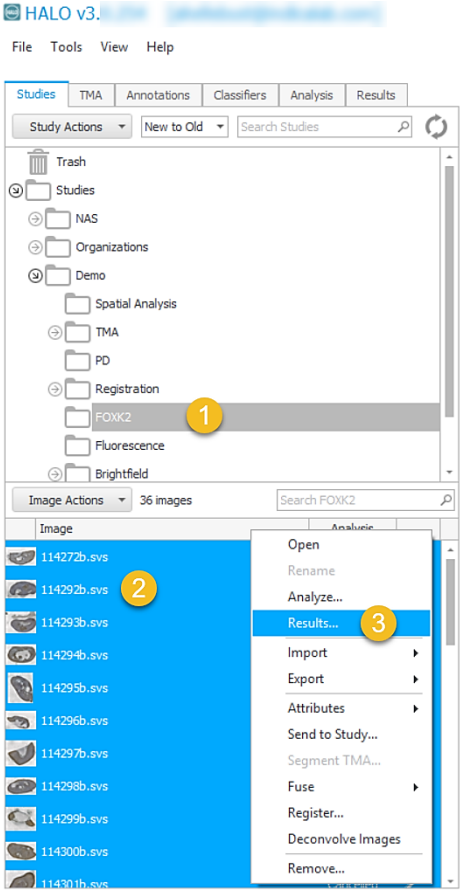
〉 Click the **Select** button.

Analysis tip: Click on the **Import Settings** button and select the saved settings file to import.

Batch Spatial Analysis

Two types of batch spatial analysis are available in HALO. The first option requires batch spatial plots to be generated and runs the analysis on the local machine (see step 2a). This option must be used for spatial analysis between cell populations on serial sections. The second option does not require batch spatial plots but does require saved spatial analysis settings and runs server side (see step 2b).

Step 1. Identify images for batch spatial plotting



› A **Prerequisite** to batch spatial plotting is that cell filters for at least one image in the study of interest **must have** already been created. Steps 1-6 must have already been completed for at least one image in study.

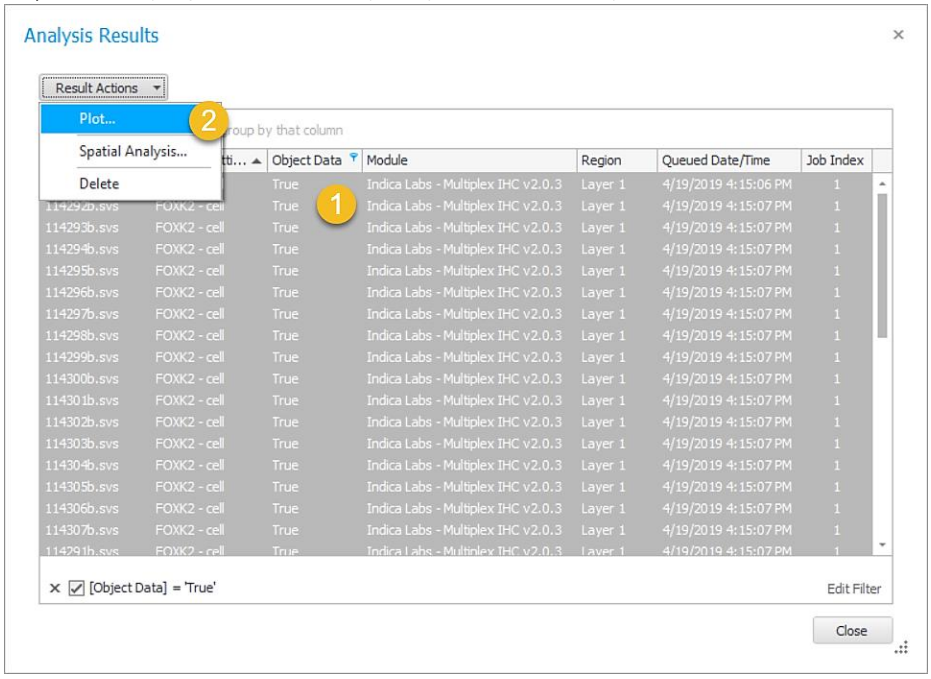
› Navigate to the **Studies** tab.

› Select the study folder containing your chosen images.

› Use the control key or the shift key with left mouse button to select all the images destined for batch spatial analysis from the **Image Actions** pane.

› **Right Click** and select **Results...** from the options available.

Step 2a. Identify object data to build spatial plots from the analysis results window

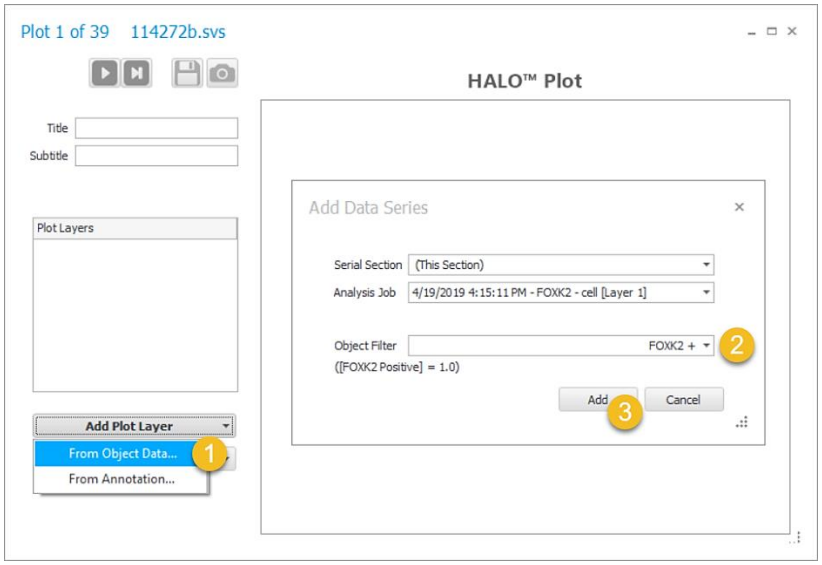


› Use **Analysis Results** table to create filters to identify appropriate analysis results to build spatial plots.

› Drag and drop column headers to create advanced filters for sorting through results.

› Ensure that the correct result file is selected. It is common to have more than one analysis result especially following analysis optimization.

› Once appropriate results are selected to generate spatial plot click **Results Actions** followed by **Plot...**



Analysis tip: *Analysis results and plots are associated with the individual images. To view results, open the individual images.*

› If object filters **have not** been previously created for this study, please see and perform steps 1-3 for one image and generate cell population filters.

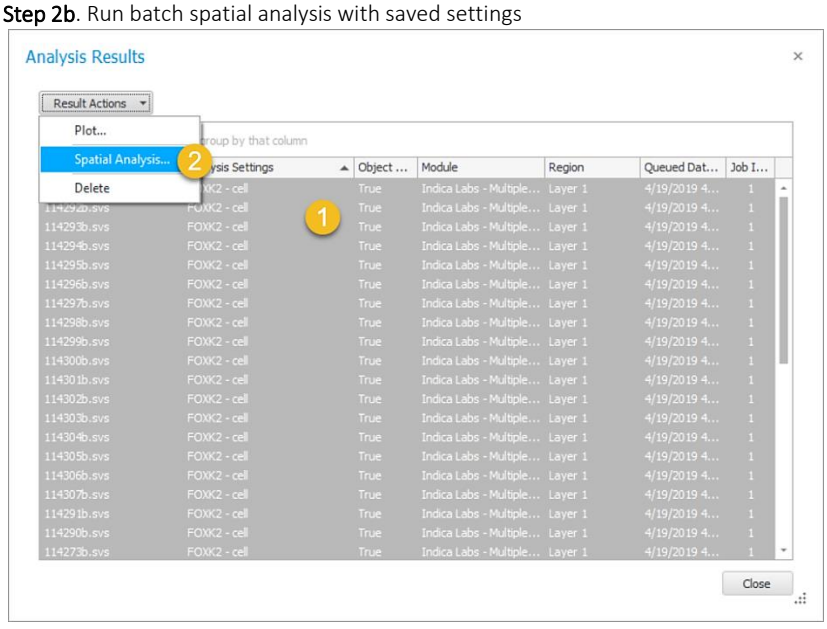
› If cell populations have been previously created, click on **Add Plot Layer** and select **From Object Data…** and select the cell population from the **Object Filter** drop down menu. Select **Add** to add cell population in batch to all applicable spatial plots. Loading object data may take a moment.

› Once objects have been loaded into the Spatial Plots proceed with adjusting object properties. These adjustments will be applied in batch to all associated spatial plots.

› Once objects have been loaded into the Spatial Plots proceed with performing Spatial Analysis following the same process as for individual analysis.

› Use  to navigate the various spatial plots being generated.

Step 2b. Run batch spatial analysis with saved settings

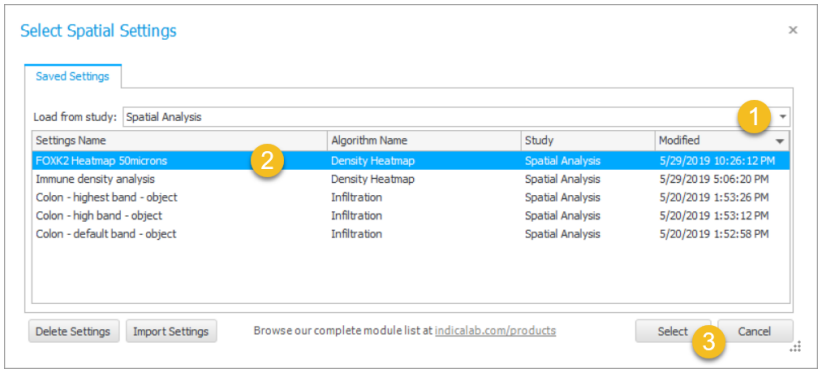


› Use **Analysis Results** table to create filters to identify appropriate analysis results to build spatial plots.

› Drag and drop column headers to create advanced filters for sorting through results.

› Ensure that the correct result file is selected. It is common to have more than one analysis result especially following analysis optimization.

› Once appropriate results are selected to generate spatial plot click **Results Actions** followed by **Spatial Analysis…**

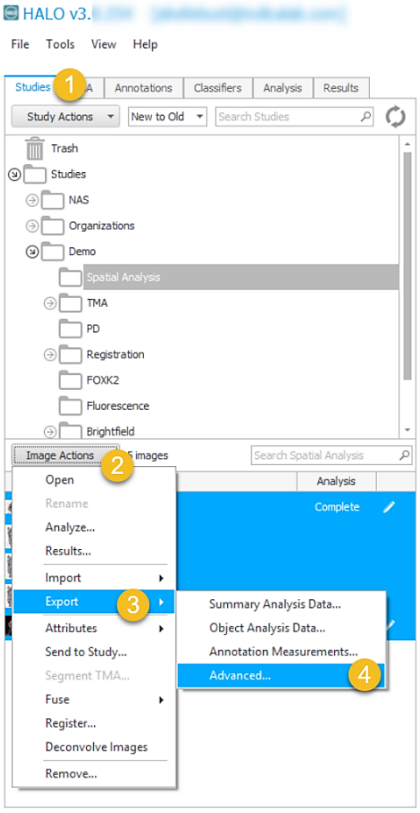


› In the **Select Spatial Settings** window, select the study from which the settings come from and highlight the settings of interest.

› Click the **Select** button to add the analyses to the analysis queue.

Export Analysis Data

Step 1. Select images



› Navigate to the **Studies** tab.

› Highlight the image(s) from any complete analysis for data export. Left mouse button + the Control/Shift keys can be used to select multiple images.

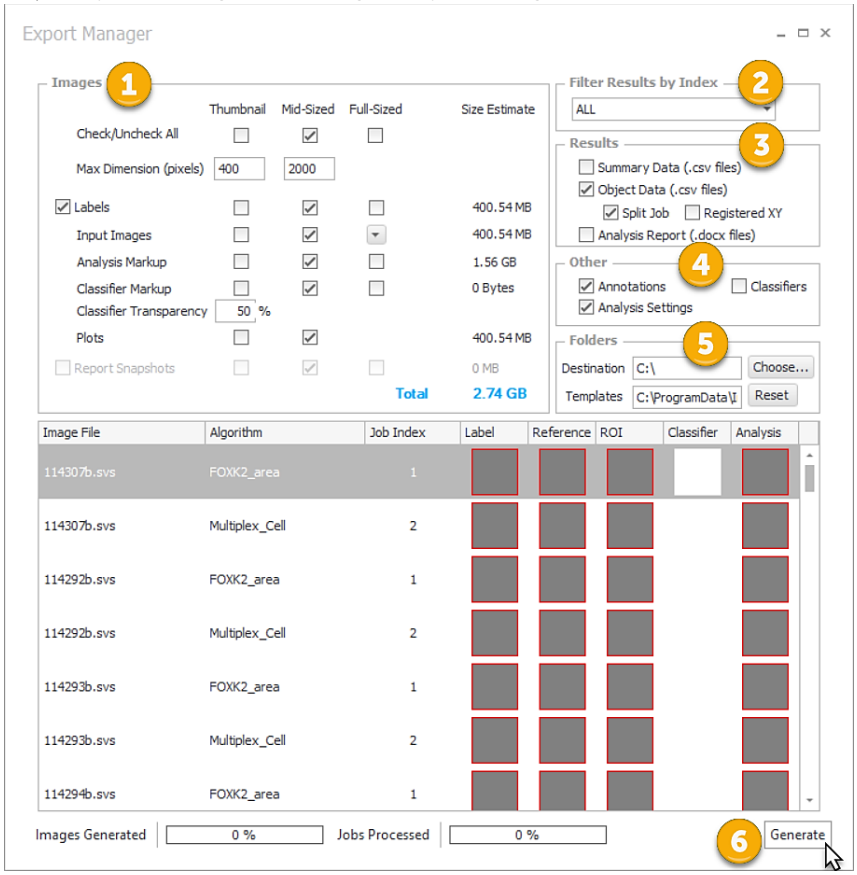
› Right click on the selected images or click on the **Image Actions** button to open the more options menu.

› Select **Export.**

› Select **Summary Analysis Data**, **Object Analysis Data**, or **Advanced** to proceed to the **Export Manager**. The former two options will export results to a .csv file.

› If **Advanced** is selected please proceed to the next step.

Step 2. Export the image results using the Export Manager and create an archive



› In the **Images** sub-section check boxes to export image **Labels**, **Analysis Markup** image, **Classifier Markup** image. Check boxes to export **Thumbnail** image, **Mid-sized** images or **Actual** sized images. Enter values to set the pixel dimension of the **Thumbnail** or **Mid-Sized** image if this option is selected. Enter values to set transparency of classifier mark-up image.

› In the **Filter Results by Index** sub-section use the drop-down menu to select which results to export if multiple analysis runs are present.

› In the **Results Data** sub-section check boxes to export **Summary Data (.csv files)**, **Object Data (.csv files)** or generate **Analysis Report (.docx files)**.

› In the **Other** sub-section check boxes to export **Annotations** (if present), **Analysis Settings** in the form of an Analysis protocol and **Classifiers** in the form of a non-editable classifier file.

› In the **Folders** sub-section input the file path in which HALO will create an archive.

› Click the **Generate** button.

Output Parameters

This image analysis module reports a set of results for each distinct analysis region and for the entire digital slide if analyzed.

The results from **Nearest Neighbor Analysis** include the following output parameters:

- ***Population A* Cells** – Total ***Population A*** objects detected.
- **Average Distance (µm) to *Population B*** – Average Distance (µm) of "Population A" objects to "Population B" objects.
- **Number of Unique *Population B*** – Total unique ***Population B*** objects detected.

The results from **Proximity Analysis** include the following output parameters:

- ***Population A* Count** – Total ***Population A*** objects detected.
- ***Population B* Count** – Total ***Population B*** objects detected.
- ***Population A* within *input range* µm of *Population B*** – Total ***Population A*** objects detected within input range to **Population B**.
- **% *Population A* within *input range* µm of *Population B*** – Percentage of ***Population A*** objects detected within input range to ***Population B***.
- **Average Distance (µm) to *Population B*** – Average Distance (µm) of *Population A* objects to *Population B* objects.
- **Number of Unique *Population B*** – Total unique ***Population B*** objects detected.
- **Average number of *Population A* within *input range* µm of *Population B*** – Average number of *Population A* objects within the *range* of a *Population B* object.
- Depending on the number of bands selected in the input parameter a breakdown of number of ***Population A* detected within a distance to *Population B*.**

The results from **Infiltration Analysis** include the following output parameters:

- ***Population A* Count** – Total ***Population A*** objects detected.
- ***Population A* within interface area** – Total ***Population A*** objects detected within interface area.
- ***Population A* average distance to interface** – Average Distance (µm) of ***Population A*** to interface area.
- **Total interface area (mm^2)** – Total interface area.
- ***Population A* average density per mm^2** – Average Density of ***Population A*** per mm^2.
- Depending on the number of bands selected in the input parameter a breakdown of ***Population A*** density per mm^2 detected within a distance to interface.
- Depending on the number of bands selected in the input parameter a breakdown of ***Population A*** count detected within a distance to the interface.
- Depending on the number of bands selected in the input parameter a breakdown of ***Band area in mm^2*** detected within a distance to the interface.

Output Parameters

The results from the **Density Heatmap Analysis** include the following output parameters:

- **Area Analyzed (µm²)** – Total area analyzed in µm².
- ***Cell Population* Count** – Total ***Cell Population*** cells detected.
- **Average *Cell Population* within *Distance* µm Radius** – Average number of objects counted within the user defined area around each object.
- **Average *Cell Population* Density (*Cell Population*/µm²)** – Average density of objects counted within the user defined area around each object.

$$= \frac{\text{Average } Cell\ Population \text{ within } Distance \text{ µm radius}}{\pi * radius^2}$$

- **Minimum *Cell Population* within *Distance* µm Radius** – Minimum number of objects counted within the user defined area around each object.
- **Minimum *Cell Population* Density (*Cell Population*/µm²)** – Minimum density of objects counted within the user defined area around each object.

$$= \frac{\text{Minimum } Cell\ Population \text{ within } Distance \text{ µm radius}}{\pi * radius^2}$$

- **Maximum *Cell Population* within *Distance* µm Radius** – Maximum number of objects counted within the user defined area around each object.
- **Maximum *Cell Population* Density (*Cell Population*/µm²)** – Maximum density of objects counted within the user defined area around each object.

$$= \frac{\text{Maximum } Cell\ Population \text{ within } Distance \text{ µm radius}}{\pi * radius^2}$$