

Polygon AI Instruction Guide

By Rewire AI

1. **[Getting Started](#)**
 - a. **[Introduction](#)**
 - b. **[Installation](#)**
 - c. **[Registration and Login](#)**

2. **[Analysis Workflow](#)**
 - a. **[Starting a New Project](#)**
 - b. **[Importing Images](#)**
 - i. **[Importing Files or Folders](#)**
 - ii. **[Organizing Folders](#)**
 - iii. **[Supported File Types](#)**
 - iv. **[Stack Import](#)**
 - v. **[Splitting Large Images](#)**
 - c. **[Using Detection Models](#)**
 - i. **[Pre-trained Detection Models](#)**
 - ii. **[Recommend Model Tool](#)**
 - iii. **[Object Detection vs Segmentation Models](#)**
 - d. **[Annotating Images](#)**
 - i. **[Image Zoom, Scroll, & Pan](#)**
 - ii. **[Placing ROIs](#)**
 - iii. **[Changing ROI Shape & Color](#)**
 - iv. **[Manipulating ROIs](#)**
 - e. **[Detection Settings](#)**
 - i. **[Detection Sensitivity](#)**
 - ii. **[Overlap Removal](#)**
 - iii. **[Image Split](#)**
 - iv. **[Background Subtraction](#)**
 - v. **[Approve ROIs](#)**
 - vi. **[Export Results](#)**
 - vii. **[Import ROIs](#)**
 - f. **[Analysis Results Output Parameters](#)**
 - i. **[Setting Analysis Results Output](#)**
 - ii. **[Output Parameter Descriptions](#)**
 - g. **[Single-Channel Analysis Workflow](#)**

3. **[Advanced Analysis settings](#)**
 - a. **[Image View Settings](#)**
 - i. **[Brightness & Contrast Adjustments](#)**
 - ii. **[MinMax Pixel Adjustment](#)**
 - iii. **[LUT Operation](#)**
 - b. **[Image Analysis Settings](#)**
 - i. **[Area Selection](#)**

- ii. [Area Exclusion](#)
- iii. [Unit Scale](#)
- iv. [Invert Image](#)

4. Multi-Channel & Colocalization Analysis

a. Types of Colocalization

- i. [Object-based](#)
- ii. [Adjacent Objects](#)
- iii. [Multiple Objects Within](#)

b. Colocalization Analysis Workflow

5. Custom Model Training

a. When and Why to Train a Custom Model

b. Training a Custom Model

c. Testing and Improving Custom Models

d. Custom Model Training Workflow

6. Other Features

a. ROI Table

b. ROI Filtering

c. Z-Stack Analysis

d. Batch Detection

e. Touch-screen Mode

7. Account & License Options

a. Licenses

b. Set-up 2FA

8. Hotkeys

9. Support & Troubleshooting

10. eLab plugin

Getting Started

Introduction

Welcome to Polygon AI! From our humble origins as Pipsqueak, which started out as a Fiji/ImageJ plugin ([Slaker et al, 2016](#)), we are now proud to offer our own intuitive user interface with expanded capabilities and custom AI model training. Polygon AI offers unlimited potential to expand functionality to suit our users' diverse image analysis needs. Our software exists to remove tedious roadblocks when analyzing large amounts of microscopy data. Polygon is an elevated form of Pipsqueak, with new features, streamlined login and registration, and a free license option to align with our founding mission to improve image analysis for all.

While we transition from Pipsqueak to Polygon, your license will work the same in any version, and your experiments and custom models will be transferred from Pipsqueak to Polygon. Most importantly, the core analysis methods and algorithms remain the same so you don't need to worry about transferring mid-experiment.

Thanks for trying the new Polygon AI! We encourage you to report bugs or provide suggestions within the app via the bug report form. We'd also love to hear suggestions for future features that matter to you and your lab!

Installation

To use Polygon AI, *new users* will first need to download the software from our website. *Existing Pipsqueak users* can upgrade within the Pipsqueak app from the prompt that will appear whenever you open Pipsqueak. Unlike our original "PIPSQUEAK" software, Polygon AI does NOT require installation of Fiji/ImageJ.

Windows

For Windows users, run the downloaded .exe file and Polygon AI will automatically install and open. It will also appear as a start menu app in a Rewire folder. Polygon AI is compatible with Windows 10 or newer OS. If you receive a "Windows Protected your PC" message when installing Polygon AI, you'll need to select "More Info" and "Run Anyway".

Mac

Double-click the downloaded Polygon AI .dmg file and a Finder window will open. In that window, drag and drop the Polygon AI icon into the Applications folder. From there you can open Polygon AI from Launchpad or from spotlight search. Polygon AI is compatible with MacOS High Sierra (10.13) or newer.

First Time Startup (Windows & Mac)

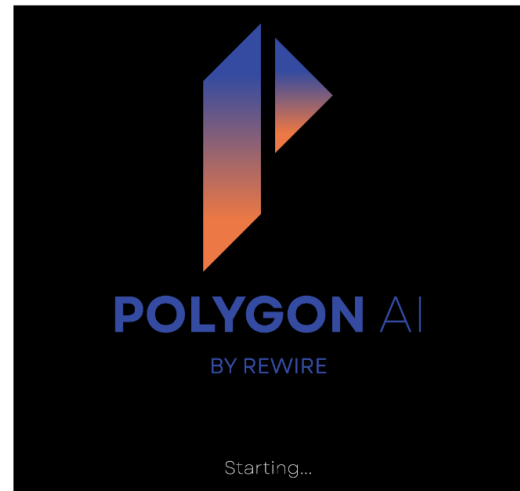
When Polygon AI is starting for the first time it can take a few minutes to download extra resources and updates in the background, so don't close the window until this process is finished. You will know it's finished when the splash screen disappears, and the application appears with the Sign-in window.

Registration and Login

New Users

You will need to register a new account within Polygon AI the first time you use the software. Your licence is tied to your registration email. A given account can be used on multiple computers but can only be used by one person at a time.

Existing Pipsqueak Users



If you've already used Pipsqueak in the past, then the same login information will work in Polygon AI and you do not need to register again. New users will automatically start with the Free license and existing Pipsqueak users will roll over their existing license type. See Account and License section for more information.

Analysis Workflow

Starting a New Project

When starting a new project, set up a unique project name and a description of the project to remind you of the details of your experiment. You'll also be prompted to select the data fields for the final .csv file export. To modify these later, right-click the *project name* in the *projects list* and select *Set Export Options*. See the [Analysis Results Output](#) section for more information on export options.

Importing Images

Importing Files or Folders

You can import selected image files or a whole experiment folder (max 500 MB file size per image). In a folder with mixed file types, only images will be imported. Import may take several minutes if you are uploading large images or a folder with many images, and the progress bar will indicate the number of files remaining to be imported. Note: if a full-sized image file exceeds our 500 MB import limit, you'll need to manually split it in another editing software like ImageJ before importing into Polygon.

Organizing Folders

When importing folders with subfolders, the organization within the folder will be preserved in Polygon. Folders can also be manually added below the project to group an experiment's images. For example, images from test subjects and control subjects can be placed in separate folders below the project. Subfolders can be added to further separate images. For instance, the test and control folders could have subfolders for images from male and female subjects. The appropriate folder setup will allow data to be conveniently grouped when exported for further analysis.

Supported File Types

Polygon can import most standard image types such as .tiff, .png, and .jpg. We recommend using the highest quality images possible (such as .tiff instead of .jpg) and keep image size and format consistent within an experiment. A mix of file types or compression levels may negatively impact detection model performance.

Stack Import

It is possible to upload several image files at once as a stack for 3D analysis. You can also import as a summed or max projection image for 2D analysis and colocalization. Within the Import Stack window, you can choose between *Step Selection* or *Pattern Selection*. In *Step Selection*, set the starting file and file increment to import only a subset of images in the folder. This is helpful when importing multi-channel images where several channels are present in the same experiment folder and alternate in a repeating pattern within the folder. Leaving the default settings of 0 for start index and 1 for step index will import every image file in the folder as one stack, whereas a start index and step size of 3 would start with the 3rd file in the folder and import every 3rd file after. *Pattern Selection* offers more flexible sorting, including using keywords to designate which files to import. This is helpful when files are disorganized in the folder.

Splitting Large Images

It is possible to split large images into smaller subsections within Polygon for easier analysis and/or improved cell/target detection. This can be done by right-clicking an imported image in the file-tree and selecting *Split Images*. From here you can designate the number of columns and rows to divide the image into. Subsections will be placed into a new folder within the project.

Image splitting can also be performed during cell/target detection **without** creating new image subsection files (see [Image Split](#) below). This option can also improve cell detection in large images and can be found in the *Analysis panel* (top tab on the far right).

Using Detection Models

Pre-trained Detection Models

We provide several pre-trained AI detection models for a wide variety of biomarker types. See our website [Help/FAQ](#) page for more information about each. Try different models to see which works best for your images, use the *Recommend Model* function (see more below), or create your own custom detection model using our intuitive in-app training process.

After choosing a model from the list, click the *Detect Cells* button. This tells the selected model to scan the active image to identify regions of interest (ROIs) based on what it is trained to detect. If none of the results look as expected, try modifying the [detection settings](#).

Recommend Model

If you're unsure which model to start with, select an image(s) in your file tree and try our *Recommend Model* feature in the model drop-down list. This feature will run your selected image(s) through automated detection by our 6 core Rewire models and produce ROIs for you to view which works best on your images.

One or more images of the same type can be selected for model recommendation. Selecting more than one image will improve the recommendation quality. To select multiple images, press *Shift* or *Control* (*Command* on MacOS) and select images in the *Project* panel. Then, right-click on the highlighted image names and select *Recommend Model*.

Alternatively, check out the Custom Model Training tutorial in Polygon to get started training your own model.

Object Detection versus Segmentation Models

Many of our pre-trained detection models are “object detection” models, which are especially good for detecting and counting objects with high-background or poorly defined edges. New in Polygon AI are “segmentation models” that can provide more detailed morphological information. We currently provide a General Segmentation model but will soon add segmentation versions of many of our core models. You can also train a custom segmentation model using our new *mask tool* under the polygon ROI drawing tool in the image window.

Annotating Images

Image Zoom, Scroll, and Pan

You can use the “*four-arrows*” button in the top left above the image to expand the image to the full center window size or set a specific zoom level using the number box. To zoom in and out within an image, use *Ctrl* or *Command* click with the mouse scroll wheel. You can also use *Ctrl+click* and move the mouse to pan around the image.

Placing ROIs

A Region of Interest (ROI) is a boundary around an object of interest (e.g. an individual cell, microorganism, subcellular puncta, or whatever discrete objects you are interested in identifying and quantifying in your images). Marking these objects with one of our ROI tools (boxes, circles/ovals, or polygons) is the basis for what regions of the image will be included in the *Analysis Results* output as well as important for telling the computer what it is looking for when training a custom detection model.

Changing ROI Shape and Color

You can change the default ROI color and shape under *File > Preferences (Polygon > Settings on MacOS)*.

Manipulating ROIs

You can move, delete, resize, and copy and paste individual ROIs. See the [Hotkeys](#) section or *Help > Hotkeys* in the Polygon AI app for further details. The *Select* tool allows you to draw around a region to select multiple ROIs at a time to delete them as a group. This is also useful to designate subsets of ROIs to modify with the [ROI Filtering](#) options. You can also hide or show your ROIs for ease of seeing your objects of interest in the image.

Hiding ROIs does not exclude them from the *Analysis Results* output (delete an ROI to exclude it from *Analysis Results* output).

Detection Settings

Detection Sensitivity

Adjust this to change the detection sensitivity of the model. This will change the number of ROIs detected in the image based on the model's confidence in its decision. The slider's number represents the minimum "certainty" accepted, with a lower value representing a higher confidence level, meaning fewer ROIs will be detected but the probability of false negatives (i.e. missed cells that you'll need to manually add) will be greater. A higher *Detection Sensitivity* setting, on the other hand, will return less certain results, meaning more ROI boxes will appear with a higher probability of false positives that will need to be manually deleted. When first identifying a model that works best for your images, you'll need to play around with the *Detection Sensitivity* until you find a balance where you have the most accurate detection. High *Sensitivity* settings are often used well in conjunction with higher *Overlap Removal* settings (see below).

Overlap Removal

This setting will change the amount of overlap allowed between two ROIs. A lower value will allow more overlap between ROIs to occur, which can help when cells are closely nested together. A higher value will merge ROIs that are overlapping, which helps with false positives detected near an isolated cell. In general, if there are many ROIs overlapping, increasing the removal setting can help.

Image Split

Splitting larger images into smaller pieces during detection can often improve results. This may help if your cells are a dramatically smaller size than those that were used to train the original model, due to different zoom levels and resolutions. To cut images into smaller files for annotation, use the *Split Images* option in the right-click menu in the file tree (see *Splitting Large Images* above).

Background Subtraction

The multi-faceted background subtraction in Polygon AI is designed to be highly customizable since many different methods are used to capture images from lab to lab. Our automatic background subtraction algorithm combines the *Rolling Ball algorithm*, native to ImageJ, with custom thresholding. Removing background may improve detection overall but risks the loss of faint ROIs. We recommend adjusting this consistently across images within an experiment.

Begin background subtraction by clicking the checkmark box to the left of *Background Subtraction* in the *Analysis panel* on the right side of the app. We offer both *automatic* and *manual* selection for background sampling. *Automatic* background sampling is used by default. This method places 22 square ROIs around the perimeter of the image, distributed uniformly.

If *manual* sampling is preferred, a user can edit or place their own ROIs for background sampling. Background ROI positions can be edited by clicking *Set* under the *Background ROIs* title. Adjust, delete, or draw your own ROIs as needed (boxes, circles, or polygons are acceptable), then press *Save*. It is recommended that at least 5 ROIs are placed, but the algorithm will still perform if fewer are placed. These ROIs are not counted in your data export and will not appear on your image after saving them. Background subtraction will remain enabled for this

image as long as the blue checkmark is visible.

After background ROIs are saved, the *Rolling Ball algorithm* smoothing is performed across the entire image. The ball's radius is set to 50 pixels by default, but it is **highly recommended that the user changes this to be around 1.15-1.25 times the average size of the object of interest, in pixels**, for best results. Learn more about the [Rolling Ball algorithm here](#).

We calculate background subtraction from the mean pixel intensity of the Background ROIs and then discard the brightest 33% and dimmest 33% from the sample. We then calculate the mean pixel intensity and standard deviation across the remaining Background ROIs. These values are then used to set all pixels dimmer than the calculated mean + two standard deviations to be "*Not a Number*" (NaN). This results in the suppressed background pixels being excluded from the measured cell intensity values within the ROIs. This background value can also be subtracted from the *Mean Intensity* value to calculate the *Mean-Background* value of pixels above background (i.e., the resulting Mean-Background pixel intensities represent *signal* rather than the mean of signal + background). [Our background method has been previously published here](#).

Approve ROIs

After final annotation of all objects of interest in an image, select the *Approve ROIs* button (looks like a checkmark in a box) below the *Detect Cells* button. This will show a checkmark next to that image file in the Project's file tree and allow you to keep track of which images you've finished. Clicking the *Approve button* followed by *Export Results* will include the current image's results in your *Analysis Results* output. Note: approval of ROIs is also necessary to make the image available for *Custom Model Training*.

Export Results

Save your results for further analysis outside of Polygon by clicking the *Export Results* button (looks like an "up" arrow) directly below the *Detect Cells* button in the *Analysis panel*. This process will create the following *Analysis Results* output files and save them in the selected destination folder:

- *[image_name]_analysis_export.csv* A .csv file with the detailed data for each image that can be opened in a wide variety of statistical programs.
- *[image_name]_user_rois.zip* A .zip file containing the placement of ROIs in a format that can be imported into ImageJ or back into Polygon via the Import button

Successful Export will briefly show a popup notification with a link to the file save location. See the *Analysis Results Output* section below for more information.

Multiple results from multiple images can be batch exported into a single *Analysis Results* output file by right-clicking on the *Project* name, folder name, or multiple highlighted image files, then selecting *Export Results*. This will include all approved image data in one .csv file in addition to individual image files.

Import ROIs

Users can import an externally generated ROI set saved in ImageJ format or re-import ROIs generated, approved, and exported during previous Polygon analyses (most likely from the same image file). To use this feature, select the *Import ROIs* button (looks like a "down" arrow) in the analysis panel, select the file with the ROIs ready to be imported, and click "Import."

Analysis Results Output

Setting Analysis Results Output

When first creating a new project you'll be prompted to set which types of data to export in the *Analysis Result* output file for that image set. You can modify this at any time by right-clicking on the project folder and

selecting *Set Export Options*.

“Legacy” versions of most export parameters are available in the *Export Options window* to enable data replication of cited results. “Legacy” designation in the output files references an older algorithm for that metric (as opposed to our “non-legacy” versions). Newer “non-legacy” versions use ImageJ’s method for calculating that variable. In all cases, the “Legacy” and “non-legacy” results are very similar, but not identical. **We recommend using the “non-legacy” versions.**

Output Parameter Descriptions

Below you can find definitions for all output that can be included in your analysis results export.

Image Index

Ascending sequential numbering of images included in the Analysis Results output file. Used to help sort rows during post-Polygon statistical analysis.

Cell Number

Ascending sequential numbering of ROIs included in the Analysis Results output file. Number is not unique to that *Image Index*. Used to help sort rows during post-Polygon statistical analysis.

ROI ID

Numeric identifier of the *Detection Model* or manual annotation that generated the ROI/contour. This number is a unique numeric identifier of an ROI that is generated from a Detection Model and ROI combination, thus useful for finding a unique cell across channels in a multiplexed image. The first three digits are specific to a *Detection Model* (e.g. “001”) or manual annotation (“FFF”). However, *ROI ID* numbers will be repeated between two images using the same *Detection Model* (i.e., the *ROI ID* of the third cell of two images analyzed using the “WFA” *Detection Model* will both be “001-003.”)

Layer

Z-Stack specific data export. Numerical identifier of the z-step on which the ROI is located. Note that ROI IDs are repeated between layers and do not correspond to ROIs on adjacent layers (i.e., the ROI ID number is random). See “In Sets” variable below for determining colocalization of ROIs and association with a common cell/biomarker in Z-Stacks.

In Set

Z-Stack specific data export. Numerical identifier of family of ROI colocalizing in the z-dimension around a common cell or biomarker. All ROIs given a particular *In Set* number (e.g., “5”), are associated with a single cell/biomarker across multiple *Layers* (or z-steps) of the z-stack. Note that ROI IDs between layers are random and do not indicate colocalization or association.

ROI Source

Name of the *Detection Model* (or “hand-drawn” manual annotation) that generated the ROI/contour.

CoM X and CoM Y

Brightness-weighted average of the x and y coordinates of all pixels in the image or selection. These coordinates are the first order spatial moments. *Center of Mass (CoM)* on the X and Y axis is an ImageJ-based calculation called “*Center of Mass*” and uses the XM and YM headings.

Pixel Area

Area of selection in square pixels. Area is in calibrated units, such as square millimeters, if *Image Analysis Settings > Unit Scale* was used to spatially calibrate the image. Pixel Area does not include pixels set to NaN.

Pixel Area is also an ImageJ-based calculation called "[Area](#)."

Background

We calculate background from the mean pixel intensity of the *Background ROIs* and then by discarding the brightest 33% and dimmest 33% *Background ROIs* from the sample. We then calculate the mean pixel intensity and standard deviation across the remaining Background ROIs. These values are then used to set all pixels that are dimmer than the calculated mean+ two standard deviations to be "*Not a Number*" (NaN). This results in the suppressed background pixels being excluded from the measured cell intensity values within the ROIs. This background value can also be subtracted from the *Mean Intensity* value to calculate the *Mean-Background* value of pixels above background (i.e., the resulting Mean-Background pixel intensities represent *signal* rather than the mean of signal+background). [Our background method has been previously published here](#).

Mean Intensity

Mean intensity is also an ImageJ-based calculation called "[Mean Gray Value](#)." It is the average intensity of pixel values within an ROI (sum/ number of pixels). These pixel intensities are influenced by the Rolling Ball subtraction step and Background thresholding to NaN. In other words, the mean value ignores background pixels. **These values are arbitrary units.**

Median Intensity and Mode Intensity

Median: The median value of the pixels in the image or selection. *Mode*: Most frequently occurring gray value within the selection. Corresponds to the highest peak in the histogram. *Median and Mode* are also [ImageJ-based calculations](#).

Stdev

Standard deviation of the gray values used to generate the mean gray value. *Stdev* is also an ImageJ-based calculation called "[Standard Deviation](#)."

Mean-Background

In addition to the *Mean Intensity*, we also offer a *Mean-Background Intensity*, which takes the *Mean Intensity* calculation value one step further and subtracts the mean background value of the image (as calculated by the method described above). This value is representative of the signal above the background within the ROI. This value was published and described in [Slaker et al 2016](#).

Integrated Density

Integrated Density is a calculation based on multiplying the Area of an ROI by the Mean intensity value of the ROI. Thus, Integrated Density = Area * Mean. [ImageJ's definition of that value can be found here](#).

Min and Max

Minimum and maximum gray values within the selection. *Min* and *Max* are also ImageJ-based calculations called "[Min & Max Gray Level](#)."

Feret's Diameter

The longest distance between any two points along the selection boundary, also known as maximum caliper. Uses the Feret heading. *Feret's* metrics are also [ImageJ-based calculations](#).

Ferets X and Y

The starting coordinates of the Feret's diameter (FeretX and FeretY) are also displayed.

Ferets Angle

FeretAngle (0-180 degrees) is the angle between the Feret's diameter and a line parallel to the x-axis of the image.

Ferets Min

MinFeret is the minimum caliper diameter.

Circularity

$4\pi \cdot \text{area} / \text{perimeter}^2$. A value of 1.0 indicates a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated shape. Values may not be valid for very small particles. Based on [ImageJ-based calculations](#).

Aspect Ratio

Major axis/minor axis. Enable "Fit Ellipse" in *Analyze>Set Measurements* to have the major and minor axis displayed. Based on [ImageJ-based calculations](#).

Roundness

$4 \cdot \text{area} / (\pi \cdot \text{major_axis}^2)$, or the inverse of the aspect ratio. Based on [ImageJ-based calculations](#).

Solidity

Area/convex area. The *Edit>Selection>Convex Hull* command makes an area selection convex. Based on [ImageJ-based calculations](#).

Skewness

The third order moment about the mean. The documentation for the ImageJ [Moment Calculator](#) plugin explains how to interpret spatial moments. Based on [ImageJ-based calculations](#).

Kurtosis

The fourth order moment about the mean. Based on [ImageJ-based calculations](#).

Filename

Name of the image file (unique if images are named uniquely).

Analysis date

Date the *Analysis Results* output file was exported.

Single-Channel Analysis Workflow

To analyze images with only one channel/biomarker, see the workflow outlined below. For multi-channel images and colocalization analysis see the [Colocalization Analysis Workflow](#).

1. Go to the projects tab and create a new project with a description of your experimental details.
2. Import image files into your new project. Delete and rename as needed by right-clicking the image or folder. You can drag images and whole folders into existing folders to create subfolders to further organize your images. Images are stored locally and tied to their original location on your computer.
3. Click on an image in the left side *Projects* panel to bring it up as the active image in the center window. This is the only image that will be affected by the settings in the right-side *Analysis* panel.
4. Select an appropriate detection model from the pull-down menu in the top right-side window. Don't

know what model to choose? If your cell or biomarker of interest isn't in the list, choose one that most closely resembles your marker from the General Models list. Try this with different models until you get one that works best or try our "Recommend Model" feature in the model drop-down list. Selecting more than one image will improve the recommendation quality. To select multiple images, press *Shift* or *Control* (*Command* on MacOS) and select images in the *Project* panel. Then, right-click on the highlighted image names and select *Recommend Model*. The model won't be perfect because it wasn't trained on your images, but you can manually fix errors in the next step.

5. Once you've selected a model, click *Detect Cells* and wait a moment to see the detected ROIs appear on your image. Try different image settings until you get the best detection. See the Analysis Settings section above for more information on what the settings do.
6. You can click to select an ROI on the image and delete or resize the ROI by clicking and dragging the edges. Click and drag with your mouse to add new ROIs, which will be automatically added to the ROI list with the "FFF" prefix on the *ROI ID*. You can even change the color of the ROI box under options.
7. After you're satisfied with your dataset, click "Approve ROIs" which will add a checkmark next to that image in the *Project* panel and add it to your data output file.
8. When you're done analyzing all of your images, click "Export Results" to export your data to an *Analysis Results* output ".csv" file.

Didn't find a good detection model or settings for your images? Add your annotated images to a custom detection model. Check out our *Custom Model Training* tutorial in Polygon and the Custom Model Training section below.

Advanced Analysis Settings

Image View Settings

These settings only adjust the way you view the image to assist in visualizing your biomarkers or cells. They do not change the way the detection model functions or affect image quantification in the Analysis Results output.

Brightness and Contrast Adjustments

You can adjust the brightness and contrast of the active image to bring out weaker signals.

MinMax Pixel Adjustment

These settings allow you to set the minimum and maximum intensity values in the active image. Raising the Min + lowering the Max can work better than brightness/contrast to accentuate the middle ground. Raising the Min level can also help remove some background noise.

LUT Operation

False color grayscale images to suit your preference. Useful for visualizing differences in pixel intensities with different colors.

Image Analysis Settings

These settings will affect the detection model results and your resulting data output!

Area Selection

This allows you to analyze only a selected subregion(s) of your image. Draw an area(s) to include using the square, circle, or polygon drawing tools.

Area Exclusion

This allows you to exclude a selected subregion(s) of your image. Draw an area(s) to exclude using the square, circle, or polygon drawing tools.

Unit Scale

The size of the image and the physical scale are properties that won't directly affect the cell detection or intensity measurement. However, some labs choose to report cell intensity as mean intensity/area, in which case you would need to double check the area calculation. If pixel scale information is not provided, metrics such as area will be returned in pixels rather than physical distance. Calculate your scale from your microscope software or using a scale bar watermark on images exported from your microscope.

Invert Image

Creates a reversed image, similar to a photographic negative, of the entire image or selection. Pixel intensities remain relative to non-inverted values and can be used for quantification. This tool is useful for detecting cells/targets stained with immunohistochemical methods (dark staining on a bright background) using *Detection Models* trained on fluorescent stains (bright staining on a dark background) and vice versa.

Multi-channel and Colocalization Analysis

Colocalization mode allows users to analyze multiple biomarkers in an image via split channels and assess the number and degree of colocalization.

Types of Colocalization Analysis:

1. ***Object-Based***: This is best for objects that overlap a majority of their area, such as a nuclear marker within a cell body marker. This method uses the *Minimum Overlap Percentage* value to define area overlap sufficient for colocalization.
2. ***Adjacent Objects***: This is best for assessing the colocalization of distinct objects that are in proximity to each other.
3. ***Multiple Objects Within***: This option is for users that have multiple smaller objects within a larger object. You will need to specify the channel with the larger object as the "Marker Image". This method uses the *Minimum Overlap Percentage* value to define area overlap sufficient for colocalization.

Colocalization Analysis Workflow

1. To colocalize images, either select a folder or the relevant individual images with ctrl- or shift-click, then right-click and select "Colocalize."
2. ***If you do not have a merged image***: Set the Image Color Channels to the colors you want for each image and click the "Create Merged Image" button. A new merged image should appear in the middle window alongside the original images.
3. If you have your own merged image, you can select it alongside the single-channel images in step 1 and set the detection model in the drop-down menu for that image to "Merged/None."
4. ***If you haven't yet run detection and annotated your images***: Once you've finished adding and editing your annotations, click "Run Colocalization" and the overlapping ROIs will appear on the merged image.

5. You can then "Export Results". The export parameters will be the same as for Single-Channel analysis as described in the *Export Parameters* section, but unique to Colocalization are columns indicating which ROIs colocalized with others.

Custom Model Training

When and Why to Train a Custom Model

A custom detection model allows you to train your own unique model to detect your biomarker of interest. If none of our existing detection models worked well for your images, then this may be a good option for you. With relatively small upfront effort, you can create a model that will dramatically reduce your analysis time while improving the consistency of your results. See the Custom Model tutorial under the Help menu within Polygon AI or keep reading for more information.

Training a Custom Model

When setting up a new custom model, please provide a brief but descriptive name for your unique model. This will be displayed in the community models list if you decide to publicly share your model. We recommend including your lab or last name and an abbreviated biomarker or cell type name. A more detailed description about your experiment and image dataset will also be helpful for future users and allow you to differentiate between multiple custom models more easily. Private models can also be shared between users with Premium licenses: either add the account email address(es) when first creating a new model, or right-click an existing model and select Edit. You can then add emails in the *Shared User* window and press *Add User*.

To get started with the custom model training process, you'll need to annotate at least 5 images **but more is always better** (see the Single-Label Analysis Workflow above). It is important to annotate all the objects of interest on each image, and each ROI should have its corresponding object fully within it. See our associated [blog post](#) for annotation best practices. After annotating and approving the training images, you can add the image set to a custom model. From here you will be prompted to provide detailed contours for at least 20 random objects (**again, more is better**) in the 5 training images. Once you reach these minimums, use the "Submit" button to start the training process.

If you are training a segmentation model with masks, all your annotations will be automatically converted to contours during custom model training.

After submitting your custom model for training, we'll get back to you as soon as a few hours, but no more than a few business days with a new custom model to test and refine as needed.

Testing and Improving Custom Models

Once the model is done training it will appear in the model drop down menu. Test it on images of the same type of objects, but that you did NOT use for training. You can submit more annotated images and contours as needed to improve the detection quality.

Custom Model Training Workflow

1. After fully annotating an image, click "Approve ROIs", which will add a checkmark next to that image in the *Project* panel file tree. Only approved images will appear in the *Custom Model Training* submission window. Repeat for several images – we recommend a minimum of 5 images with at least 500 annotations. **Important:** each training image must all be the same single biomarker. Multi-channel images must be split into single channels prior to annotation and training.
2. Create a new model in the Custom Model tab by clicking the "Create Model" button.

3. Give your model a unique name. We recommend at least Lab/Company name + Biomarker, e.g. “My Awesome Rewire Parvalbumin model”. In the Model Description box, you can provide additional details about the experiment to distinguish between future models. This information will also be useful to other users if you choose to share your model publicly.
4. Private models can be shared between users with Premium licenses by adding the account email address(es) in the *Shared User* window and pressing *Add User*.
5. Choose the Project that contains your annotated training images.
6. Select your new model from the custom model list and click “Add Images”
7. All Approved images in the Project will be automatically selected. Deselect any images you don’t want to include in the training dataset and click “Add”.
8. Once the blue progress bar fills up, indicating you have enough images and annotations, click “Train Model” and click “Train” to submit. We’ll contact you within a few business days to let you know your model is ready. Once deployed, your custom model will appear in the model drop down list.

Other Features

ROI Table

This shows a list of the ROIs drawn on the active image, with the corresponding X,Y coordinates in the image as well as the size of the ROI box in pixels. IDs contain number prefixes for ROIs detected by the model and letter prefixes for ROIs added manually by the user. Hovering over an ROI in the table will highlight the corresponding ROI box in the active image. Conversely, hovering over an ROI in the image will move to the corresponding ROI in the table. ROIs with a blue checkmark will be included in the final data export. ROIs that are manually deleted from the image will automatically disappear from the ROI table.

ROI Filtering

We now offer several ROI filtering options in the ROI Filter tab on the right-hand analysis menu.

Z-stack Analysis

See the Importing Images section for detailed information about importing z-stacks. After import, you can view your stacks as summed or max projected images. 3D Trace allows detection of individual objects through layers. Premium users can right-click a stack and select Batch Detection to run analysis on all images in the stack. Exporting *Analysis Results* from different z-stack views (summed, max projection, or 3D trace) results in identical *Analysis Results* output files. See Export Parameters above for more information on *Analysis Results* output column variables and column variables specific to z-stacks.

Note: You cannot yet use colocalization in stacks

Batch Detection

This Premium License feature allows users to speed up their workflows by successively analyzing multiple images in sequence and outputting their data in aggregate for a given experimental folder. To perform *Batch Processing*, select multiple images in the *Project* panel, then right-click > *Batch Detection*. Images will open as a grid in the center window. Begin by selecting a single Detection Model and Detection Settings to apply to all images, then click Detect Cells. Images will process in parallel and display a checkmark in the Project panel when complete. ROIs can then be checked and edited for accuracy, and then marked Accepted for Export. See Export Results section for detail on batch export. It is also possible to use Batch Detection across layers in a Z-Stack.

Touch-screen Mode

Polygon AI is compatible with touch screen computers running Windows or MacOS. Unfortunately, at this time it

cannot run on Google Chrome tablets or smartphones. For best performance we recommend using a mouse, but it is possible to draw ROIs with a finger or stylus. You can also double-tap to add ROIs. Touch screen compatibility is automatically available on any touch device, with no need to turn on any mode or settings.

Account & License Options

Licenses

The basic free license limits a user to 2GB of image storage and 2 custom models at a time. Your count towards these limits will appear in the bottom right corner of the app. You can test out the Premium license features under the Polygon Demo Project that automatically loads with your installation. This gives you ample time to test it out before you decide if a full-featured 1 year license is right for you.

Set-up Multi-factor Authentication

For those who use an authenticator app, you can turn on 2FA from the Account window.

Hotkeys

We provide several useful Hotkeys to streamline your analysis, as shown below. Also see the list under *Help > Hotkeys* in the Polygon AI app.

- Hide/unhide all ROIs on an active image: **ctrl + t**
- Remove ROIs on an active image (as well as from the ROI list): **Delete** or **Backspace**
- Collapse the left window: **ctrl + <**
- Expand the left window: **shift + ctrl + <**
- Collapse the right window: **ctrl + >**
- Expand the right window: **shift + ctrl + >**
- Reset zoom to original size: **ctrl –**
- Zoom with mouse scroll wheel: **hold ctrl + scroll**
- Pan around image with mouse: **hold ctrl + click & drag image**

Suggestions for additional useful Hotkeys or features? Let us know with the feedback suggestion window, accessed from the help “?” icon in the lower right corner of the app.

Support & Troubleshooting

We offer a wide variety of support documents, including this comprehensive instruction document. See our [Help & FAQ](#) webpage for frequently asked questions, trouble-shooting tips, and links to video tutorials (coming soon). Free license users can refer to the above material for assistance. You can also report a bug or provide feedback with the “?” icon in the lower right corner of the app. Premium users will have access to email support (replies within 48 business hours) and up to 1 hour of Zoom lab training per paid license year. Additional live video training may be purchased as an add-on as needed. We also encourage users to attend our webinars for demonstrations of features and live Q&A sessions. Sign up for our email newsletter and social media accounts to stay informed of upcoming webinars.

eLabNext Plug-in

eLabNext digital lab notebook users can take advantage of our Polygon plug-in to easily add images and results from their analysis into eLab. You must first log in with your eLab credentials via the eLab tab.