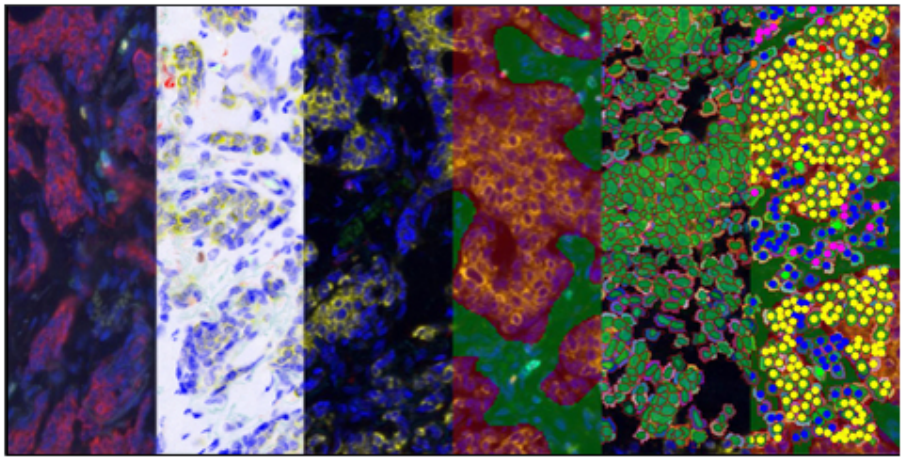
inForm® with MOTiF™

Advanced Image
Analysis Software

User's Manual



inForm® 2.5 Tissue Finder with MOTiF™

Advanced Image Analysis Software

US Patents 7,555,155; 7,953,264; 8,280,140; 8,639,043;
10,126,242 and patents pending.
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AKOYA BIOSCIENCES®

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This manual describes how to use inForm software.

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This software covered by US Patent 7,555,155; 7,953,264; 8,280,140; 8,639,043; 10,126,242 and patents pending.

Table of Contents

Chapter 1 Welcome to inForm 6

Chapter 2 Introduction 7

1 About inForm 7
2 Software Configurations 9
3 Key Terms 11
4 Using Online Documentation 12
5 Installing and Starting inForm 12
6 Activating the License 13
7 Contacting Akoya Biosciences 18

Chapter 3 Understanding the inForm Work Area 19

1 View Editor 25
2 Available Images Window 27
3 New Project Window 28
4 Spectral Library Window 29
5 Stain Store Management 31
    1 Managing Groups 32
    2 Choosing Stains and Fluors 33
    3 Managing Your Measured Spectra 36
    4 Managing Synthetic Spectra 37

Chapter 4 Common Image Analysis Tasks 40

Chapter 5 Working with Projects and Algorithms 41

1 Creating Projects 41
2 Opening a Project 44
3 Opening an Algorithm 46
4 Saving an Algorithm 46

Chapter 6 Opening Images 47

1 Opening Images in a Project 47
2 Viewing Images 49

Chapter 7 Drawing Processing Regions 51

Chapter 8 Preparing Images 52

1 Selecting the Image Format 52

2 Choosing a Spectral Library ... 54
3 Viewing a Spectral Library ... 60
4 Component Units ... 61
5 Completing the Prepare Images Step ... 64

Chapter 9 Manual Tissue Segmentation ... 65

1 Creating Tissue Categories ... 65
2 Drawing Tissue Category Regions ... 66
3 Editing Tissue Category Regions ... 66

Chapter 10 Trainable Tissue Segmentation ... 68

1 Adding Tissue Categories ... 68
2 Drawing Training Regions ... 69
3 Training the Tissue Segmenter ... 70
4 Adding Images to the Training Set ... 73
5 Editing Masks and Re-Training ... 75
6 Completing the Automated Tissue Segmentation Step ... 76

Chapter 11 Threshold ... 77

Chapter 12 Colocalization ... 80

Chapter 13 Segmenting Cells ... 82

1 Adaptive Cell Segmentation ... 82
1 Selecting Segmentation Components and Detecting Nuclear Pixels ... 83
2 Splitting Nuclear Clusters into Individual Nuclei ... 85
3 Refining Nuclear Shapes ... 87
4 Defining Cytoplasm ... 87
5 Defining Membrane ... 87
2 Cell Segmentation ... 89
1 Segmenting Nuclei ... 90
2 Segmenting Cytoplasm ... 94
3 Segmenting Membrane ... 96

Chapter 14 Phenotyping Cells ... 97

Chapter 15 Scoring IHC or IF ... 101

Chapter 16 Counting Objects ... 106

Chapter 17 Displaying the Extracted Data ... 108

1 Viewing the Color Image ... 109
2 Viewing the Component Image ... 110
3 Viewing the Composite Image ... 113

4 Viewing Data Tables ... 114
5 Viewing the Tissue Segmentation Data Table ... 115
6 Viewing the Cell Segmentation Data Table ... 116
7 Viewing the Score Data Table ... 118
8 Viewing the Colocalization Data Table ... 120
9 Viewing the Count Data Table ... 121
10 Viewing the Quant Data Table ... 122
11 Image Options ... 122
12 Component Stats ... 126
13 TMA Core Info ... 126
14 Slide Info ... 126
15 Position Stats ... 126
16 Shape Stats ... 128

Chapter 18 Exporting the Data ... 129

Chapter 19 Batch Processing ... 131

Chapter 20 Merging the Data ... 133

Chapter 21 Building Libraries ... 134

Chapter 22 Frequently Asked Questions ... 146

Chapter 23 Appendix A: Calculating Fractional Tissue Area ... 147

Chapter 24 Appendix B: Auto-Processing Data Received from Mantra Snap ... 150

Chapter 25 Appendix C: Phenotype Classification by Multinomial Logistic Regression ... 151

Chapter 26 Appendix D: Akoya Biosciences TIFF File Specification ... 155

Chapter 27 Appendix E: Segmentation Map TIFF Specification ... 162

Chapter 28 Appendix F: Adaptive Cell Segmentation Tips ... 164

Chapter 29 inForm® Software End User License Agreement ... 175

Index ... 185

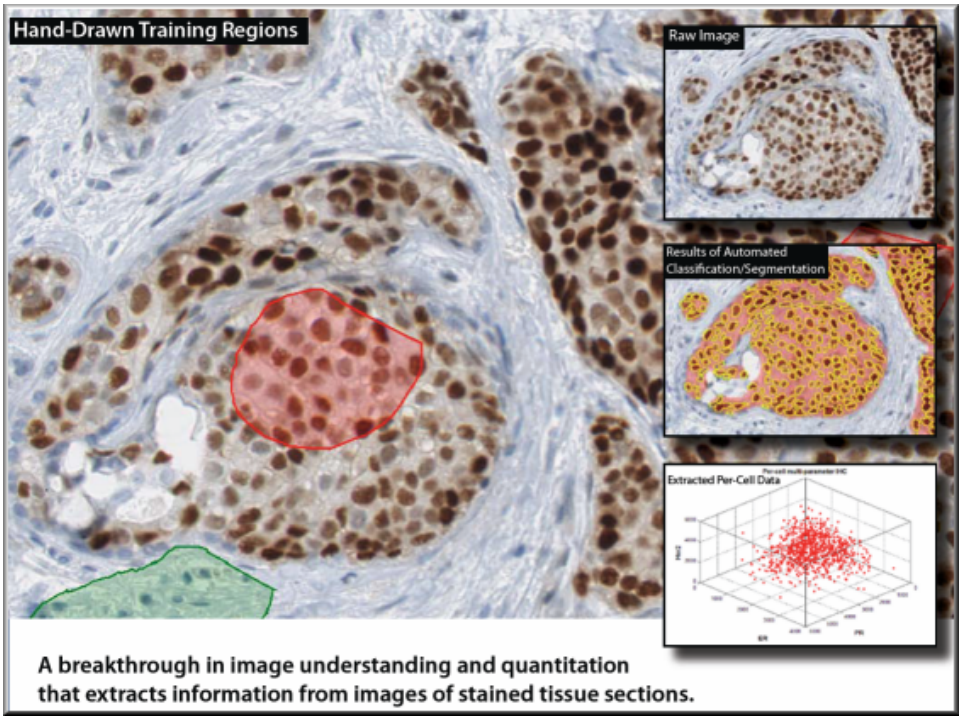
1 Welcome to inForm

Akoya's inForm® advanced image analysis software has been developed to address the biggest challenge facing microscopists once quantitative imagery has been acquired: extracting data. The challenge increases when dealing with multiplexed assays, since signals can be weak and highly mixed. inForm provides a powerful and fast solution with an intuitive, easy-to-use software interface. What might have been impossible or have taken days can now be done in minutes.

inForm solves challenging image analysis problems by combining fast and easy learn-by-example automated image processing with object recognition and data analysis tools. It is based on machine learning, which means that you can train the program to create an effective solution by simply selecting examples of what you want to find within the images. inForm then creates an algorithm that you can subsequently apply across the entire image or across as many images as you want.

Akoya's multispectral images and industry-standard RGB images, including TIFF files generated using the Akoya Biosciences Quantitative Pathology Imaging and Slide Scanning systems, can be read and analyzed by inForm.

inForm is for research use only. Not for use in diagnostic procedures.



Getting started – new users

- Be sure to review the topics in the Introduction 7 to familiarize yourself with the features and specifications of the software.

2 Introduction

The topics in this section provide basic information about inForm, what it is for, and what you can do with it.

How to get started

- See About inForm 7 for a general overview of the software features and specifications.
- See Software Configurations 9 to review the available software configurations and the capabilities of each configuration.
- Review Understanding the inForm Work Area 19 to familiarize yourself with the program.
- Read through Common Image Analysis Tasks 40 to familiarize yourself with the basic capabilities of inForm.
- Refer to Key Terms 11 to view definitions of terms used with inForm.

Other Information

- Find out how to Contact Akoya Bioscience 18 to place an order or receive technical assistance.

2.1 About inForm

inForm operates on monochrome, color, or multispectral images of tissue sections. Sections can be stained with standard stains, immunohistochemical stains (IHC), and immunofluorescence stains (IF, including Qdot®). Stains can be single or multiplexed up to 40 markers for multi-analyte analyses to support applications such as signaling pathway research in oncology.

inForm is available in different configurations, each providing additional capabilities for different applications.

- **inForm Tissue Finder** can be trained to find virtually any tissue type or structure, such as tumor, fibrosis, inflammation, stroma, granuloma or vessels.
- **inForm Cell Analysis** can be used to assess IHC or IF staining levels, on a cell-by-cell basis, for cell prototyping. It can also generate object counts.
- **inForm Viewer** can visualize component data, and reconstruct the unmixed signals into a composite image.

Features

- Compatibility with Akoya's digital slides (.qptiff) up to 40 channels, Akoya's multispectral images (.im3) and with conventional color images (.tif, .jpg, etc.).
- Full range of software configurations which can include capabilities from simple tissue area measurements to cell-as-a-unit multiplexed molecular phenotyping. To view the levels of inForm and the capabilities of each level, see Software Configurations 9.
- Computationally efficient; runs on a standard laptop.

Specifications

- Operates on Akoya Biosciences multispectral images (.im3), digital slides (.qptiff) up to 40 channels, and monochrome or color images (TIFF, BMP, JPEG, PNG)
- Computer Specifications: Microsoft® Windows® 10, 64-bit with 32GB RAM recommended. (Minimum: Microsoft® Windows® 10, 64-bit with 8GB RAM)

2.2 Software Configurations

inForm is available in the following software configurations. Each configuration enables specific tasks for viewing, selecting, segmenting, and counting.

The following inForm software configurations are available:

Viewer

inForm Viewer includes the following features:

- View images
- Unmix images
- View components and their signals
- View a composite of the component images
- Export images

Cell Analysis

inForm Cell Analysis includes all of the features available in inForm Basic Analysis, plus the following features:

- Manually segment tissue
- Create threshold maps
- Create colocalization maps
- Generate quantitative data for threshold and colocalization maps
- Save projects and algorithms
- Batch process images
- Export data tables
- Use cell segmentation to identify cells and cellular compartments
- Assign phenotypes to segmented cells
- Score segmented cells
- Use object segmentation to identify objects

Tissue Finder

inForm Tissue Finder includes all of the features available in inForm Cell Analysis, plus the following features:

- Train the Tissue Segmenter to automatically identify tissue categories
- Automatically segment the image into tissue categories
- Merge data files from a set of images or slides into summary data files

Steps Available in Each Software Configuration

The table below specifies which algorithm steps and software features are available in each software configuration.

	Viewer	Cell Analysis	Tissue Finder
Prepare Images	X	X	X
Manual Tissue Segmentation		X	X
Trainable Tissue Segmentation			X
Threshold		X	X
Colocalization		X	X
Cell Segmentation		X	X
Object Segmentation		X	X
Phenotyping		X	X
Score (only used with Cell Segmentation)		X	X
Export	X	X	X
Batch Processing		X	X
Review/Merge			X

2.3 Key Terms

The followingtab;e lists key tersm that are core to using the inForm software. Refer to the terms below when using the software.

Algorithm	Algorithms include all steps in processing an image, from image preparation, tissue segmentation and/or cell segmentation, object counting or scoring, to export. Algorithms may also include threshold or colocalization settings, depending on the project created to analyze the images.
Processing Region	Processing regions are user-drawn regions of interest. Use processing regions to only process specific regions on an image. All areas outside of the marked processing regions are ignored.
Processing Set	A set of images that will be processed using the settings in the Manual Analysis tab. Images in the processing set display a green square on the image thumbnail when viewing images in Single mode. All images added to a project are included in the processing set by default. To add or remove images from the processing set, right-click the large image in the image display area and click Processing Set. You can also click the Image List Editor button to open the Available Images window and then click the Load Images button.
Project	inForm projects include the images that were added to the project and the settings for each step in the algorithm. A new project that contains only the Prepare Images and Export Step is automatically created when opening inForm. You can work in the new default project, create a new project, or open a saved project.
Spectral Region	Spectral regions are user-drawn regions that identify autofluorescence on an image. Spectral regions can be used to create inForm spectral libraries that are exported for use with Lamina systems.
Tissue Category	Tissue Categories identify structures such as tumor cells vs. healthy cells that are present in the tissue samples. Regions in the image can be marked manually using inForm Basic Analysis or higher. Use inForm Tissue Finder to use the Tissue Segmenter to automatically classify tissue.
Tissue Segmenter	The Tissue Segmenter automatically identifies regions of tissue or regions that correspond to specific types of tissue. When you “train” a tissue segmenter, it learns how to identify structures in the images based on the training regions within each tissue category. The tissue regions or areas detected with the tissue segmenter can then be segmented to detect cells and perform IHC or IF scoring, or can be used for object counting. Only available with inForm Tissue Finder.
Training Set	The set of images used to train the Tissue Segmenter to identify tissue categories automatically. Images are automatically added to the training set if a training region is drawn on the image. To remove an image from the training set, right-click the large image in the image display area and click Training Set. When an image is removed from the training set, the training regions on the image are not used when training the Tissue Segmenter. (The training regions are not deleted from the image, so the image can be added back to the training set if desired.) You can

	also use the Available Images window to add or remove images from the training set.

2.4 Using Online Documentation

This manual contains detailed information about the inForm application. It is designed to be used as a reference tool in your everyday work with inForm. The manual explains how to use inForm through detailed explanations of features and step-by-step procedures for common tasks using realistic scenarios. The PDF file can be read online or can be printed for reference.

inForm also includes complete documentation in an HTML-based help system, which includes all of the information in this User's Manual. Online help provides three ways of locating information: Use the Contents tab to navigate through the document, the Index tab to find topics by keyword, or the Search tab to look up topics that contain specific words or phrases.

2.5 Installing and Starting inForm

Close all applications that are running before beginning the inForm installation. Installation requires an administrator account.

Installing the Software:

inForm requires R 3.1.0 and several R packages to be present on the machine prior to installation. If you have not done so already, please download and install R from the inForm section of the Akoya Biosciences software downloads site at https://www.akoyabio.com/support/software/#inform_tissue_analysis_software.

Once R is installed, launch the inForm installer. Follow the wizard prompts to install the software. inForm requires the Microsoft .NET 4.5 framework. The installer will install Microsoft .NET 4.5 if it is not already installed.

The installer asks which fluorophores are commonly in use at your lab. Selecting these choices sets the defaults for available Measured and Synthetic Spectra. You can change this default in Stain Store Management 31.

Starting inForm:

Select **Start > Akoya Biosciences > inForm**
OR
double-click the **inForm** shortcut on the Windows® desktop.

To use any software configuration other than inForm Viewer, see Activating the License 13.

2.6 Activating the License

After installing inForm, only the options for inForm Viewer are available. The customer license must be activated to enable any purchased software options. License activation requires an administrator account.

To activate a customer license:

1. Log into the computer using an account with Windows® Administrator permissions.
2. Start the inForm Licensing Application. **Start > Akoya Biosciences > inForm > inForm License App**

Figure 1. inForm License App

3. Click the **Activate** button. The Activate the Software window opens.
4. Select the **Activate** option and click the **OK** button.
5. Enter your 20 character license number.
6. If available, use the online activation method to complete your activation.
7. If online activation is not available, click the **Copy to Clipboard** button to copy the text onto the Windows® clipboard.
8. Email the copied text to the email address shown in the Activation window.
9. Click the **Cancel** button to close the Activation window.
10. Wait to receive the email that contains the text for the response key.
11. Type or paste the response key from the email into the Activation window and click the **OK** button.
12. Close the license application and start inForm.

Transferring a Software License

You can transfer the inForm license from one computer to another computer. The same version of inForm must be installed on both computers. When the license is transferred to the new computer, the license is removed from the current computer, leaving only the inForm Viewer options.

Transferring a license requires an administrator account on both computers, a USB thumb drive, and an available USB port on each computer.

Please reach out to support@akoyabio.com for assistance transferring an inForm license from one computer to another computer.

To transfer the license:

1. Log into the unlicensed computer with an administrator user name and password.
2. Plug the USB drive into an available USB port on the unlicensed computer.
3. Start inForm.
4. Click the **Activate** button or select **License** → **Activate**. The Activate the Software License window opens.

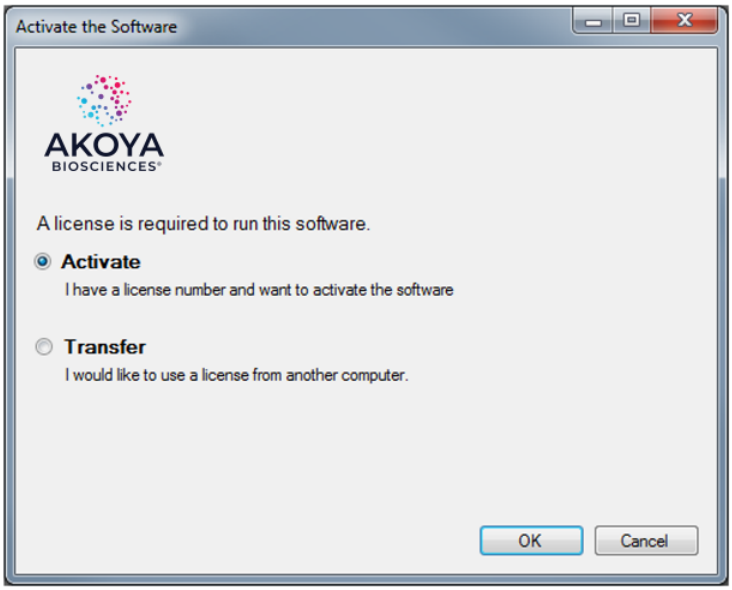


Figure 2. Activate the Software Window

5. Select the **Transfer** option and click the **OK** button. The Check Transfer Directory window asks if you have created a transfer directory.

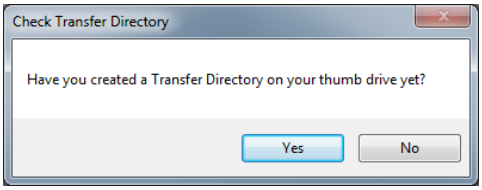


Figure 3. Check Transfer Directory Window

6. Click the **No** button. The Create Transfer Directory window instructs you to create a transfer directory on the USB flash drive.

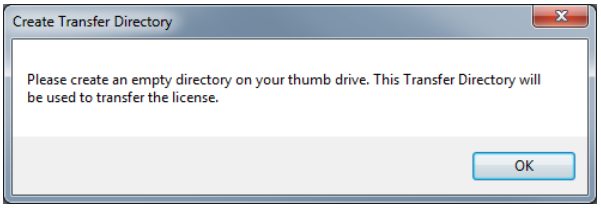


Figure 4. Create Transfer Directory Window

7. Click the **OK** button. The Browse for Folder window opens.

8. Browse to the USB drive and create a new directory. There are no restrictions on the name of the directory, but choose a name that is easy to identify.

9. Select the name of the new directory and click the **OK** button. The Transfer License Out window opens. Leave this window open, DO NOT click the OK button yet.

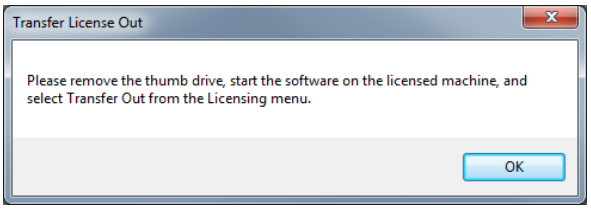


Figure 5. Transfer License Out Window

10. Remove the USB drive. (Leaving the Transfer License Out window open.)

11. Plug the USB drive into the licensed computer.

12. Start inForm on the licensed computer.

13. Select **License** → **Transfer** → **Transfer Out**. The Check Transfer Directory window asks if you have created a transfer directory.

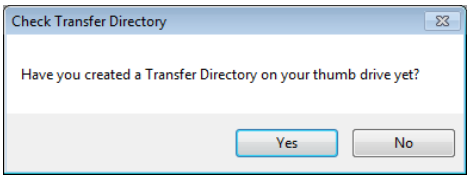


Figure 6. Check Transfer Directory Window

14. Click the **Yes** button. The Transfer License window asks if you want to transfer the license.

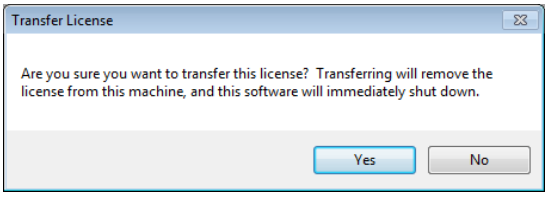


Figure 7. Transfer License Window

15. Click the **Yes** button. The Browse for Folder window opens.

16. Browse to the transfer directory on the USB drive and click the **OK** button. The License Error window displays a message that the software was unable to obtain a license.

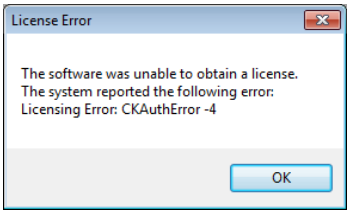


Figure 8. License Error Window

17. Click the **OK** button. The inForm software closes.

18. Remove the USB drive from the licensed computer.

19. Plug the USB drive into the unlicensed computer. The Transfer License Out window is still open on this computer.

20. Click the **OK** button. The Transfer License In window opens.

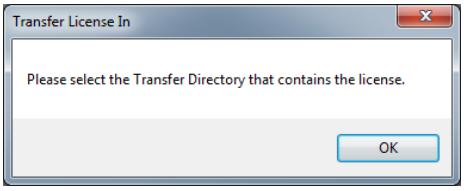


Figure 9. Transfer License In Window

21. Click the **OK** button. The Browse for Folder window opens.

22. Browse to the transfer directory, select the directory name, and click the **OK** button.

23. The license is copied to the unlicensed computer and the Restart Required window opens.

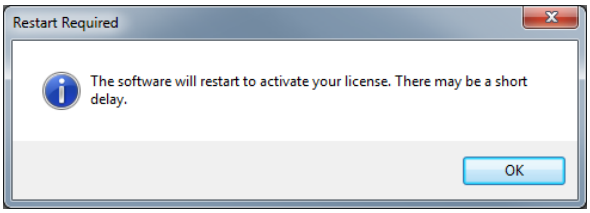


Figure 10. Restart Required Window

24. Click the **OK** button. The inForm software restarts with the licensed options available.

2.7 Contacting Akoya Biosciences

For more information, contact Akoya Biosciences or your local authorized distributor:

Akoya Biosciences, Inc.
100 Campus Drive, 6th Floor
Marlborough, MA 01752, USA

Technical Support:
Phone: +1 855-896-8401
Fax: +1 855-404-0061

Akoya Biosciences Web Site: http://www.akoyabio.com

Email: CustomerCare@akoyabio.com

Email (Outside the US): CustomerCareEMEA@akoyabio.com

3 Understanding the inForm Work Area

The inForm work area contains the step bar, process editor panel, image display toolbar, and image display area. The example below shows the Manual Analysis tab with a brightfield image that has been segmented into two tissue categories.

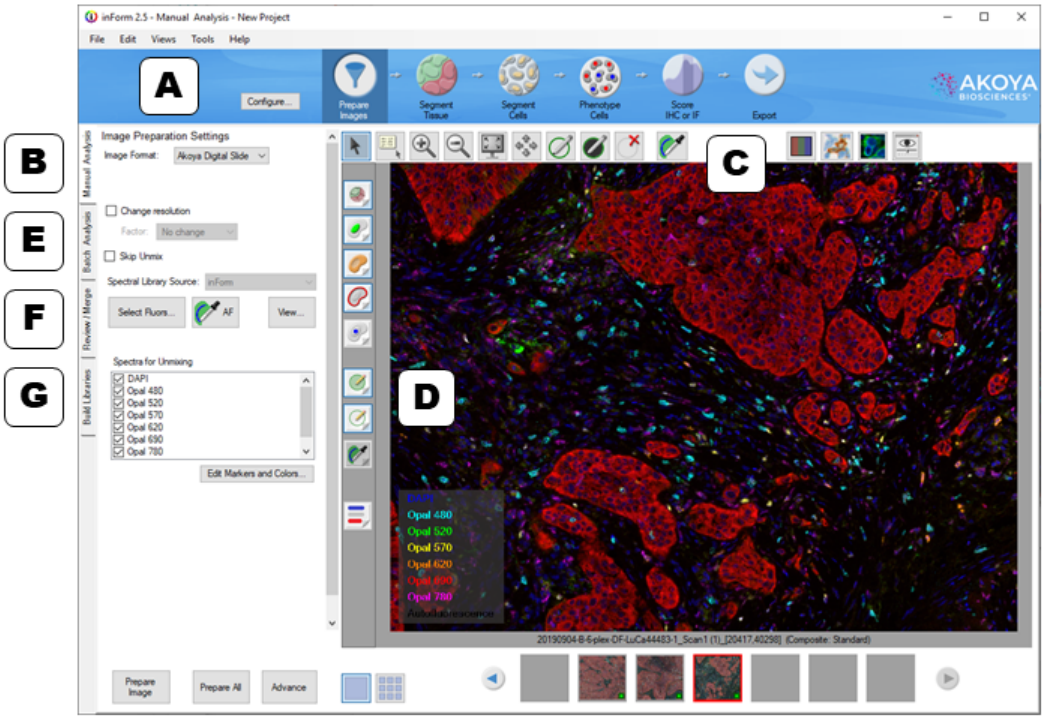
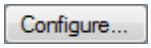


Figure 11. inForm Work Area with an unmixed composite image from a multiplexed fluorescence slide.

A. Step Bar

The Step bar displays an icon for each processing step included in the project that is open in the Manual Analysis tab. The Step bar only displays when the Manual Analysis tab is selected. A new project is automatically created when inForm opens. By default, the new project only includes the Prepare Images and Export steps. To create a new project with additional steps, see Creating Projects 41. To add, remove, or change the steps in the open project, see Configure a Project 41.

Click the icons in the step bar at the top of the work area to display the settings for each step in the Manual Analysis tab. For example, click **Prepare Images** to display the Image Preparation Settings panel in the Manual Analysis tab; click **Export** to display the Export Settings panel.



Configure button - Opens the Configure Project window to change the steps in the current project. See Creating Projects 41 for instructions. Vectra® Tissue Finder and Vectra HPF Finder projects cannot be configured.

Prepare Images - Use to specify the image format and sample format, change the image resolution, load a spectral library, convert images to optical density, and unmix images. See Preparing Images 52 for instructions.

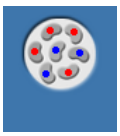
Manual Classification - Use to create tissue categories and manually draw tissue regions on the images in the project. Exported data includes position, shape, and component signal statistics for each segmented tissue region. See Manual Tissue Segmentation 65 for instructions. This feature is available with inForm Basic Analysis or higher.

Segment Tissue - Use to train the automated tissue segmenter and automatically segment the images. Exported data includes position, shape, and component signal statistics for each segmented tissue region. See Trainable Tissue Segmentation 68 for instructions. This feature is only available with inForm Tissue Finder.

Threshold - Use to create and analyze threshold maps. Exported data includes position, shape, and component signal statistics for each thresholded region. See Threshold 77 for instructions. This feature is available with inForm Basic Analysis or higher.

Colocalize - Use to create and analyze colocalization maps. Exported data includes position, shape, and component signal statistics for the colocalization region, as well as position, shape, and component signal statistics for the underlying component regions. See Colocalization 80 for instructions. This feature is available with inForm Basic Analysis or higher.

Segment Cells - Use to segment cell nuclei and optionally segment cytoplasm and membrane. Exported data includes position, shape, and component signal statistics for each segmented cell. See Segmenting Cells 82 for instructions. This feature is available with inForm Cell Analysis or higher.



Phenotype Cells - Use to create a phenotyping algorithm that can automatically phenotype cells after segmentation. See Phenotyping Cells for instructions. This feature is available with inForm Cell Analysis or higher.

Score IHC or IF - Use to quantify IHC or IF staining levels in segmented cells. The tools provided are intended to automate visual assessment (Positivity, 0-3+, 10-bin, 50-bin, and Double Positivity) and to provide histogram data of staining levels. Exported data includes the score for each selected cell compartment. See Scoring IHC or IF 101 for instructions. This feature is available with inForm Cell Analysis or higher.

Count Objects - Use to segment objects other than cells. Exported data includes the position, shape, and component signal statistics for each segmented object. See Counting Objects 106 for instructions. This feature is available with inForm Cell Analysis or higher.

Export - Use to select the export directory, the images to export, the data to export, and the file name options. See Exporting the Data 129 for instructions.

B. Manual Analysis Tab

The **Manual Analysis** tab on the left displays the settings for each step. Use these panels to select the settings for the algorithm or project. These settings can only be edited when viewing a single image, and not while viewing images in Gallery mode. See also Image Display Area 19.

C. Toolbar

The toolbar enables you to adjust the image zoom level, draw processing regions, draw training regions, and adjust the view settings. The tools available on the toolbar change based on the current processing step. The toolbar displays in the Manual Analysis tab.

Select: Return the cursor to the default pointer mode. Click and drag to pan around the image. Use the mouse wheel to zoom in and out at the cursor location.

View Data: Hover over the image to display the signal intensity of each component at any location, as well as any object information at that location.

Zoom In: Click on the image to re-center the image and to zoom in. Click and drag to zoom in on a specific region of the image.

Zoom Out: Click on the image to re-center the image and to zoom out. Double-click the on image with this tool to return magnification to 100%.

Zoom to Full: Click to view the image at 100%.

Pan: Click and drag the image to view the desired region in the window.

Draw Processing Regions: Click and drag to outline the desired processing region. Only the areas of the image within the processing regions are analyzed.

Draw Region of Disinterest: Click and drag to outline an area to ignore within the image. This area will not be processed.

Delete a Region: Click inside a processing region, region of disinterest, or training region to remove the region.

Draw Training Regions: Click and drag to identify regions of the image that represent the selected tissue category when training the tissue segmenter. This tool selects automatically when you select a tissue category from the category list. When in draw mode, you can continue drawing training regions. Only available during the **Segment Tissue** step.

Draw Classification Regions: Click and drag to identify regions of the image that contain the selected tissue category. Only available during the **Manual Classification** step.

Edit the Tissue Segmentation: Click the button to select the tissue category and then click and drag to manually draw the selected tissue category over an area. After segmenting the images, inspect the images to make sure the tissue regions are segmented as you intended. If areas are under the wrong masks (the tumor mask over non-tumor cells, for example), use this tool to draw the desired mask over an area. Only

available during the **Segment Tissue** step. Select Clear Edits to clear all manual edits to the tissue masks.

Select Regions to Unclassify: Click and drag to outline the area on the image to unclassify. Only available during the **Manual Classification** step.

Select Pixels to Unclassify: Click and drag over the specific pixels to remove from the classification region. Only available during the **Manual Classification** step.

White Picker: Click a white area on a Brightfield image to select the white background spectra prior to converting the image to OD (optical density). Available only during the **Prepare Images** step.

Autofluorescence Picker: Click and drag on an area of autofluorescence in the image to add autofluorescence to the spectral library. Available only during the **Prepare Images** step when creating a spectral library.

Spectral Curves: Hover over an area to see the spectral curve of that particular pixel. Available only during the **Prepare Images** step when the **Spectral Library Window** is open, and in the **Build Libraries** tab.

Add to Thresholded Regions: Click the button to select the desired unmixed component and then click and drag to outline the area on the image to be added to the selected threshold map. Only available during the **Threshold** step.

Erase Thresholded Regions: Click the button to select the desired unmixed component and then click and drag to outline the region to remove from the threshold map. Only available during the **Threshold** step.

Edit Phenotypes: Click on a segmented cell to assign a phenotype. Available only during the **Phenotype Cells** step.

Color Button: Click to select the conventional color view of your image. It performs the same function as selecting Color Image from the Data Displayed drop-down menu in the View Editor.

Component Button: Click to select the which unmixed component to view from a drop-down list. If inForm can identify a DAPI or hematoxylin counterstain, it will display the component as a conventional brightfield IHC stain on a hematoxylin counterstain. As a convenience, the up and down arrow keys on your keyboard let you switch rapidly amongst each component in the display window.

Composite Button: Click to select the composite image of the unmixed fluorescence image in the Image Display Area. See Displaying the Extracted Data 108 for more detailed information on viewing your color and unmixed component images.

View Editor: Click to open the View Editor Window 25 to choose the type of image or table to view and to change the display settings. You can also view or hide cells, masks,

and regions. Buttons to quickly show or hide image maps are available on the left side of the image display area.

Show/Hide Map Options: Click to show or hide the image maps. You can show or hide segmentation maps, training regions, and processing regions. (Most Show/Hide options are also available on the View Editor 25.) The buttons include:

Tissue Segmentation Map - Click to show/hide the Tissue Segmentation Map.

Nuclear Segmentation Map - Click to show/hide the Nuclear Segmentation Map.

Cytoplasm Segmentation Map - Click to show/hide the Cytoplasm Segmentation Map.

Membrane Segmentation Map - Click to show/hide the Membrane Segmentation Map.

Phenotype Map - Click to display a menu which allows you to show/hide individual phenotypes.

Training Regions - Click to show/hide the Training Regions.

Processing Regions - Click to show/hide the Processing Regions.

Spectral Regions - Click to show/hide the Autofluorescence Regions.

Legend - Click to show/hide the Legend of Components in view.

D. Image Display Area

The image display area in the Manual Analysis tab has two modes -- *Single* and *Gallery*.

- **Single mode** displays one image in the large Image area, and displays a row of thumbnails of all other open images below the large image. The image name and current display are listed below. Click any thumbnail to view the image in the display area. A blue square on a thumbnail indicates that the image is included in the training set; a green square indicates the image is in the processing set. The Algorithm settings in the left-hand panel can only be edited in *Single* mode.
- **Gallery mode** displays all the project images in the large Image area. Use the Image Size slider at the top of the workspace to adjust the number of open images shown in the image display area. In *Gallery* mode, all Algorithm settings are read-only. *Gallery* mode is for image and table review only; you cannot edit the Algorithm settings in this mode.

Right-Click Shortcut Menu

The following actions are accessible by right-clicking on an image in the Image Display area:

- **Save:** Saves a copy of the image (as shown in the Image Display Area) to a file in the selected format (.tif, .jpg, .png, .gif, or .bmp). (Default is the last selected image format.)
- **Delete Region:** Deletes the region that you right-clicked on. (Only available if you right-clicked on a region.)
- **Delete Regions:** Provides options to delete all training regions, all processing regions, or both, from an image. (Only available if training regions and/or processing regions have been created on the image.)
- **Show Regions:** Provides options to show/hide all training regions, processing regions, or both, on an image.
- **Processing Set:** Adds or removes the image from the processing set. If checked, the image is included in the processing set. If cleared, the image is not included in the processing set. Click the option to toggle the check mark.
- **Training Set:** Adds or removes the image from the training set. If checked, the image is included in the training set. If cleared, the image is not included in the training set. Click the option to toggle the check mark. Only displays if training images have been drawn on the image. (Draw training regions on the image or use the Available Images Window 27 to add images without training regions to the training set.)
- **View Editor:** Opens the View Editor 25 to select the display options.
- **Remove from Project:** Removes the image from the project.

E. Batch Analysis Tab

After a suitable project or algorithm for processing images has been created, validated, and saved, use the Batch Analysis tab to process large numbers of images. Use the Batch Analysis Editor 131 to select a project or an algorithm for batch processing, select export directory/folder preferences, and select the image/table options. (This feature is available with inForm Basic Analysis or higher.)

F. Review/Merge Tab

After completing a batch run, which often generates large numbers of output files, use the Review/Merge Editor 133 to combine the data from multiple output files into a single file for detailed and quantitative analysis. The Review/Merge Editor 133 has tools to filter out data from samples that are inadequately prepared, stained, and/or segmented, so that merged data sets are reliable. (This feature is only available with inForm Tissue Finder.)

G. Build Libraries

The Build Libraries 134 tab allows you to characterize stains and fluorophores by extracting the pure spectral information from them and adding them to the Stain Store. This allows you to build up your libraries of spectral data and easily separate multiplexed stains and fluorophores in single tissue sections. Stains and fluorophores in the Stain Store may be re-used with new images, so long as the images are from specimens that were prepared and imaged in the same manner.

3.1 View Editor

Use the View Editor to select the display options for images or data tables after processing. The images and data tables available are determined by the steps in the project. The steps available are determined by the inForm license. This section lists the views that are available for each software configuration. For definitions of the options, see Displaying the Extracted Data 108. To open the View Editor, click the **View Editor button** on the image toolbar.

Views Available In Viewer

Color Image - Shows the original color image plus the regions selected in the Image Options.

Component Image - Shows an image of a single unmixed component.

Composite Image - Shows a reconstructed image of components selected for viewing.

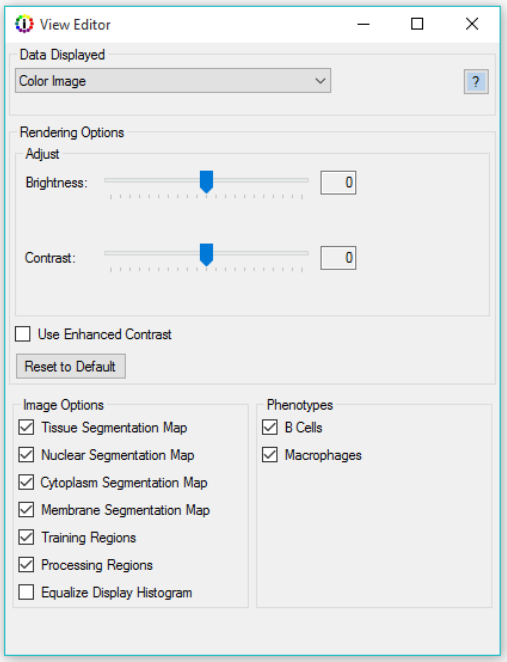


Figure 12. Views Editor

Views Available In Cell Analysis

Includes all views in Viewer plus:

Tissue Segmentation Data - This table is available when an algorithm contains a manual segmentation step. The table provides information on the manually segmented tissue regions. (See Viewing the Tissue Segmentation Data Table 115 for detailed information.)

Quant Data - This table is available when an algorithm contains a threshold or colocalization step. The table provides information on the thresholded regions. (See Viewing the Quant Data 122 for detailed information.)

Colocalization Data - These tables are available when an algorithm contains a colocalization step. (See Viewing the Colocalization Data 120 for detailed information.) There are three tables:

- **Colocalization Table** - Shows colocalization data of the selected components and positivity data for each selected component.
- **In Channel Table** - Shows the positivity of one component within another for all selected colocalization components.
- **Quant Table** - Shows stats for the whole image and colocalization region.

Cell Segmentation Data - This table is available when an algorithm contains a cell segmentation step. The table provides information on the segmented cells. (See Viewing the Cell Segmentation Data 116 for detailed information.) If the algorithm contains a phenotyping step, this table will contain the cell's phenotype information.

Object Segmentation Data - This table is available when an algorithm contains an object segmentation step. The table provides information on the segmented objects. (See Viewing the Count Data 121 for detailed information.)

Score Data - This table is available when an algorithm contains a score step. The available data changes depending on the type of scoring (positivity, 0-3+, 10-bin, 50-bin, and Double Positivity). (See Viewing the Score Data 118 for detailed information.)

Views Available In Tissue Finder

Includes all views in Cell Analysis. Tissue Segmentation data displays in the Tissue Segmentation Table. (See Viewing the Tissue Segmentation Data 115 for detailed information.)

3.2 Available Images Window

Use the Available Images Window to view a list of all images in the project and to add images to or remove images from the processing set or the training set. (The training set is only supported in inForm Tissue Finder when training the Tissue Segmenter.) Access this via **Tools > Image List Editor**.

To load additional images into the project, click the **Load Image** button. The new images must be compatible with the Prepare Images settings in the current project. New images must have the same spectral wavelength characteristics as the existing images in the project.

To remove an image, right-click the image name and click the **Remove** button.

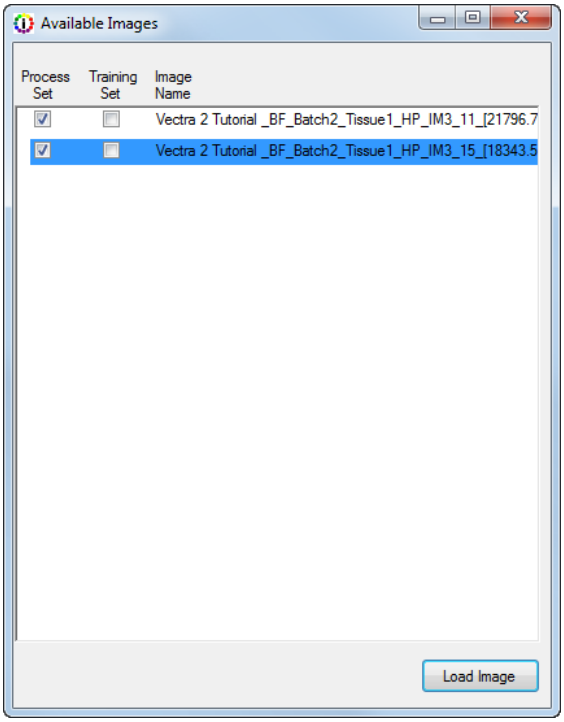


Figure 13. Available Images Window

3.3 New Project Window

Use the New Project Window to select the steps to include in the project. Certain steps are only available if a software option has been purchased and installed. (For available software configurations, see Software Configurations 9).

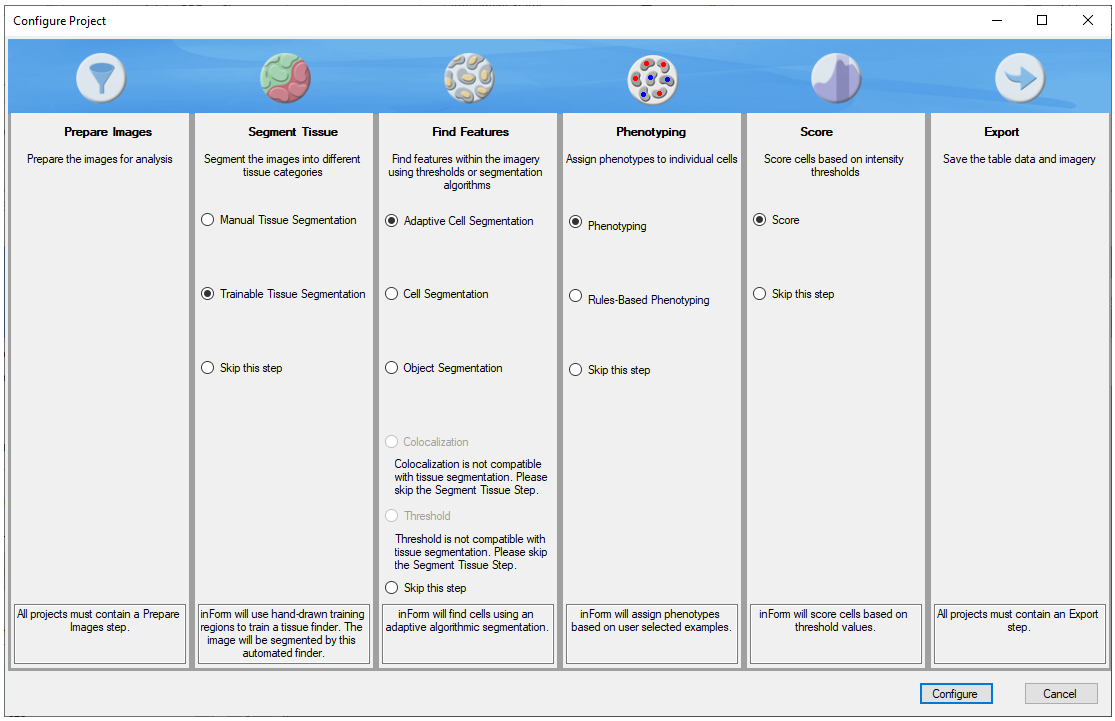


Figure 14. New Project Window

The New Project window contains the following options and buttons:

Prepare Images - Required for all projects. Specifies the Image Format and Sample format, changes the image resolution, loads a spectral library, unmixes the spectral components, and removes haze from the images. See Preparing Images 52 for details.

Segment Tissue - Segment the image into specific tissue categories.

- **Manual Tissue Segmentation** - Tissue categories are manually drawn on each image. See Manual Tissue Segmentation 65 for details.
- **Trainable Tissue Segmentation** - The tissue segmenter is trained to identify different tissue types in the images. Processing the images automatically assigns the appropriate tissue categories. See Trainable Tissue Segmentation 68 for details.

Find Features - Find features in the images using thresholds or segmentation algorithms.

- **Adaptive Cell Segmentation** - Identifies individual cells or subcellular objects in the image using an adaptive thresholding method. This algorithm is best suited for fluorescence imagery with membrane staining. See Segmenting Cells 82 for details.
- **Cell Segmentation** - Identifies individual cells or subcellular objects in the image using either thresholded or algorithmic segmentation. See Segmenting Cells 82 for details.
- **Object Segmentation** - Identifies objects on an image using either thresholded or algorithmic segmentation. See Counting Objects 106 for details.

- **Colocalization** - Identifies regions on an image where multiple unmixed component thresholds overlap. Threshold values for each component are specified individually. See Colocalization 80 for details.
- **Threshold** - Identifies regions on an image where a component is above a specific threshold. See Threshold 77 for details.

Phenotyping - Assign phenotypes to cells.

- **Phenotyping** - Train a phenotyping algorithm by assigning a few cells to each phenotype. Processing the images will automatically assign phenotypes to the remaining cells. See Phenotyping Cells for details.
- **Rules-Based Phenotyping** - Use a predefined algorithm that assigns phenotypes based on signals and exclusion rules. Available only when phenotyping the MOTiF™ Opal PD-a/PD-L1 Lung and Melanoma Panel kits.

Score - Score cells based on intensity thresholds.

- **Scoring** - Scores the segmented nuclei, cytoplasm, and/or membrane to count the percent positive of a specific component above a specified threshold. See Scoring IHC or IF 101 for details.

Export - Save the table data and imagery

- **Export** - Required for all projects. Specifies the export settings for images and data tables after processing is complete. See Exporting the Data 129 for details.

3.4 Spectral Library Window

Use the Spectral Library window to view graphs of the emission spectral curve for each stain selected for unmixing. Viewing the spectral library can help when troubleshooting unexpected unmixing results. Ideally, the spectra should be easily distinguishable in each of the imaging bands. Typically, one filter is used for each stain. If the Spectral Library uses multiple filters, one graph displays for each filter. The figure below shows a spectral library with DAPI, CY3, and CY5 with good separation in each graph. The Spectral Library window updates dynamically if the Image Preparation Settings are changed.

To open this window, click the **View** button under the Spectral Library list box in the Prepare Images step after selecting a spectral library.

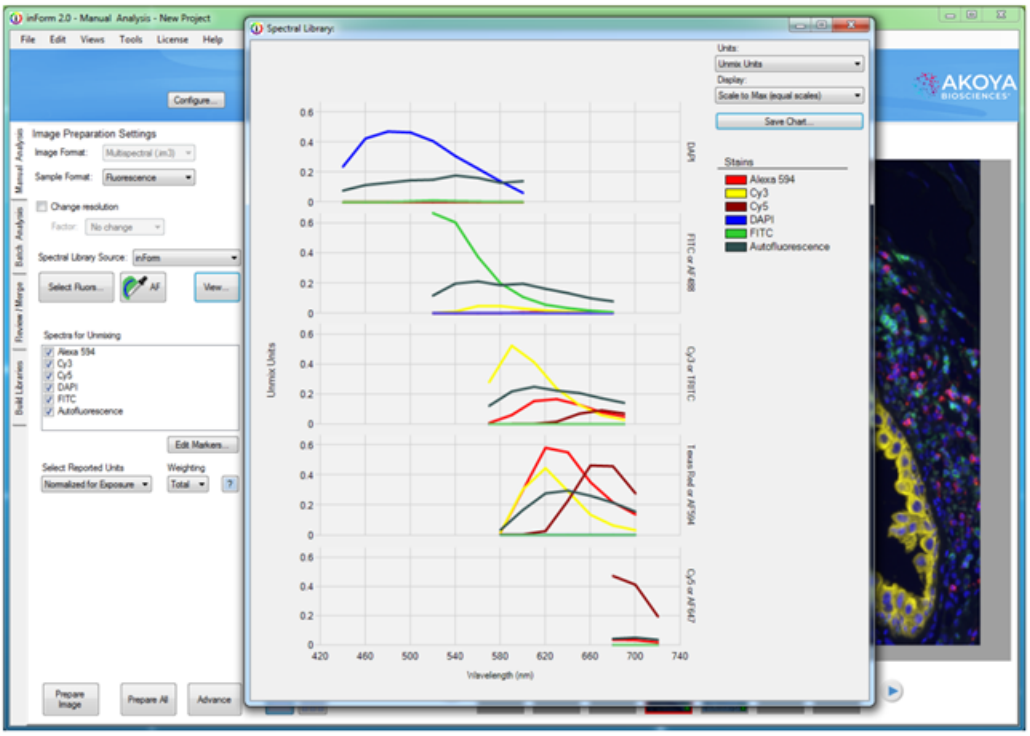


Figure 15. Spectral Library with multiple filters

Spectral Library Name: If a Nuance spectral library is loaded, displays the name of the current spectral library above the chart. The spectral library name is not displayed when an inForm spectral library is loaded.

Chart: The spectral library display can contain multiple graphs; one graph for each imaging band. Each graph displays the spectral segments within an imaging band. If there is only one data point in each band, all of the spectral segments display in one graph. Brightfield images only have one imaging band, so only one graph displays. After loading a library, select the check boxes for the spectra you want to use for unmixing in the Prepare Images step. The Spectral Library window automatically updates to show only the selected spectra. Hovering over the line in a spectral curve displays the name, wavelength (x value), and signal strength (y value) of the nearest data point. By default, the chart opens with Unmix Units and Scale to Max (equal scales) selected.

- **X Axis** - Spectral wavelength of light in nanometers.
- **Y Axis** - The units selected in the Units drop-down list.

Units drop-down list - Specifies the units on the Y Axis on the graphs. Only the units valid for the spectral library are available. For example, if a spectral library does not contain Raw units, the Raw option is not available.

- **Raw Counts -** Pixel intensity in unit counts. Typical Fluorescence values are from 0 to 4095. Typical Brightfield values are from 0 to 255.
- **Unmix Units** - Displays the signal used for unmixing. Can show artifacts that are not visible in other views. Usually the best unit for evaluating a spectral library.
- **Normalized for Exposure** - Signal is displayed in counts/second. Used to compare signal strength across bands. (Fluorescence libraries only)
- **OD** - Optical Density units (Brightfield libraries only)

Display drop-down list - Specifies the Y Axis scaling option for the graphs.

- **Scale to Max (equal scales)** - All graphs are scaled to the same value, which is the maximum signal value in all of the bands.
- **Scale to Max (varying scales)** - Each graph is scaled individually to the maximum signal unit for that graph. (This option is not available when there is only one imaging band, when the library is for a brightfield image, or when the units are OD.)
- **Normalized** - Each graph is scaled individually on the Y Axis to 1. Additionally, the signals are scaled so each spectrum has a maximum of 1. (This option is not available when units are Unmix Units.)

Save Chart Button - Opens the Save Chart window to specify the location, name, and file format for saving a graphic of the graphs displayed in the Spectral Library window. The chart can be saved as a .bmp, .gif, .png, .tif or .jpg and saved to a computer or network location. The graphic file includes the legend.

Legend - Displays the color for each stain in the chart. The colors are selected when the Spectral Library is created and cannot be changed in this view.

3.5 Stain Store Management

inForm has a set of factory-supplied synthetic fluorophores to choose from, as well as the capability to store new measured stains and fluorophores from your slides. All stains and fluorophores, synthetic or measured, can be grouped together under names of your own choosing.

The Stain Store Manager allows you to:

- Create and organize **Groups of Stains and Fluorophores**
- Designate which types of **Stains and Fluorophores Your Lab Uses**
- **Manage Your Own Measured Spectra**
- **Manage Your Synthetic Spectra**

Go to the top menu bar of inForm and choose: **Tools > Stain Store Manager**.

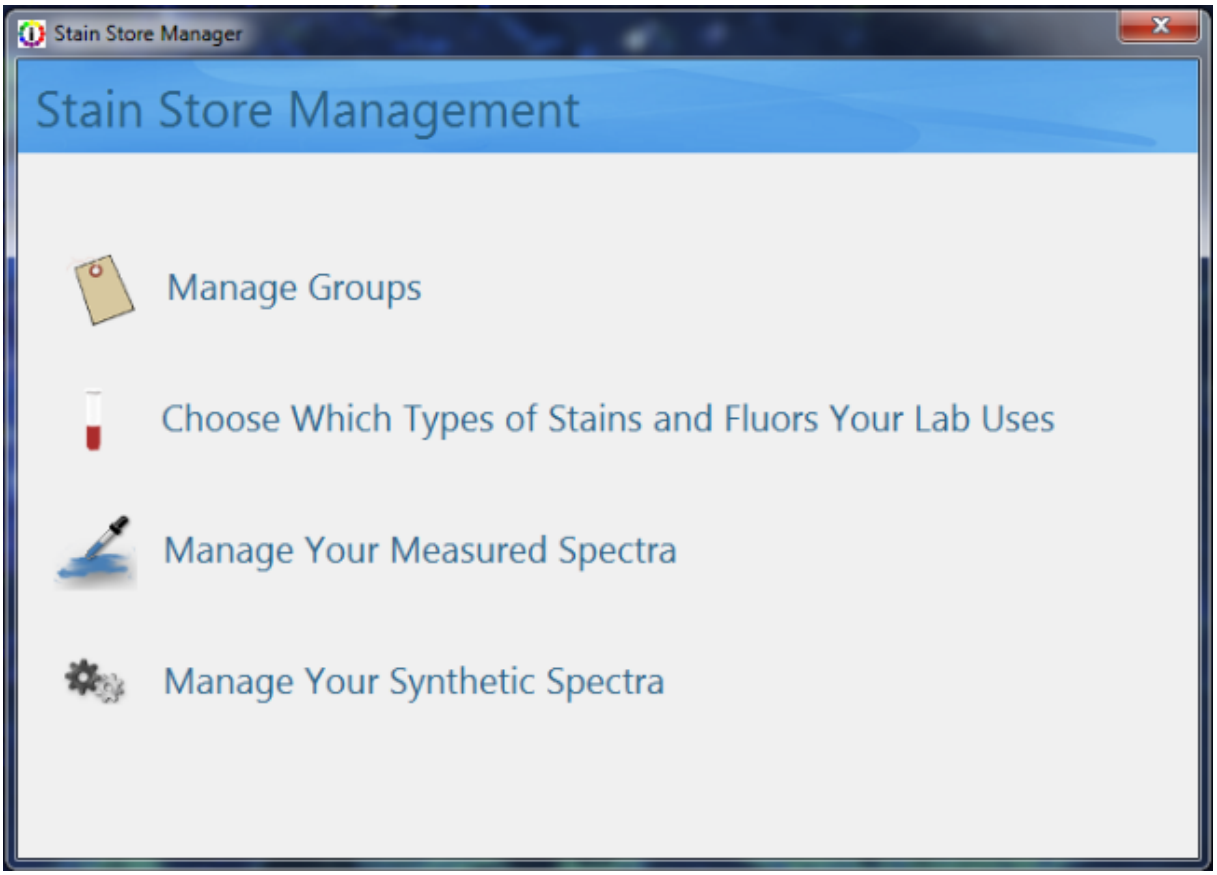


Figure 16. The home screen for the Stain Store Manager allows you to manage your stains and fluorophores from an intuitive interface, selecting only the stains and fluorophores that you use and allowing you to group them yourself, eliminating clutter and saving valuable time.

3.5.1 Managing Groups

To make finding the right stain or fluorophore easier, you can arrange them into Groups by clicking on **Manage Groups**. By default there are Groups named Favorites, Synthetic, and Measured present. These default Groups cannot be deleted, but they can be hidden by making them inactive. You can create new Groups using the **New** button, as well as **Rename** and **Delete** Groups. Only checked Groups will appear in the Select Fluors list.

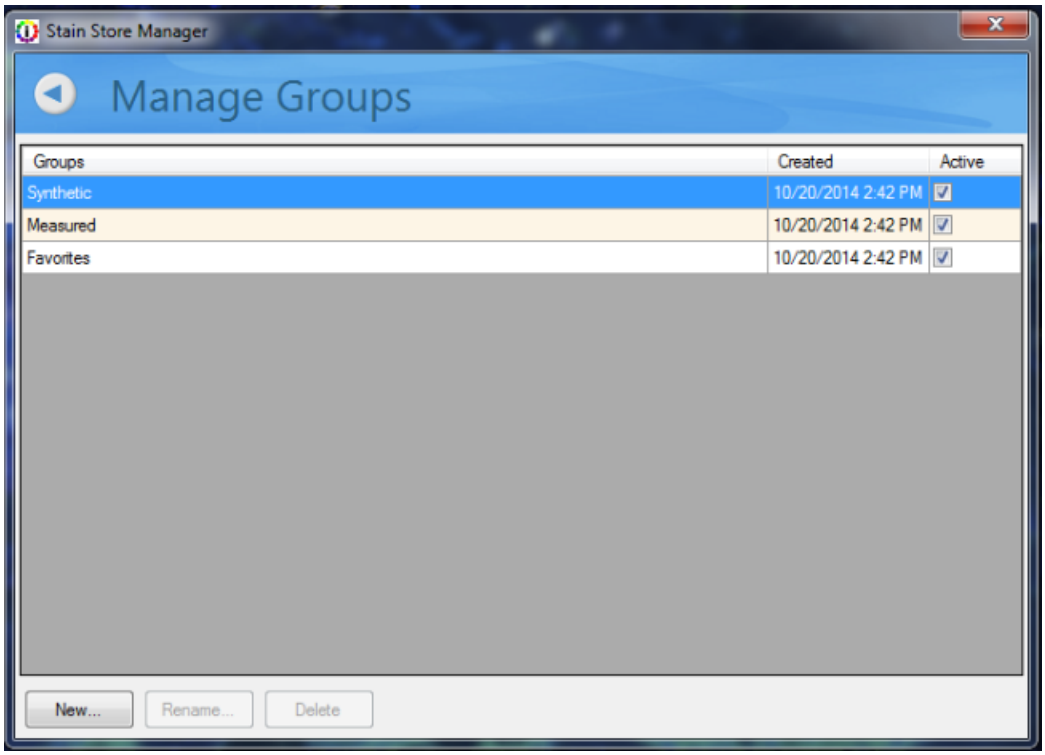


Figure 17. Manage Groups lets you arrange your stains and fluorophores into convenient Groups.

3.5.2 Choosing Stains and Fluors

Click **Choose Which Types of Stains and Fluors Your Lab Uses** in order to select from among a list of common stains and fluorophores grouped into families. For example, if you only use CY3 and CY5, you can uncheck CY3.5 and CY5.5 from the list in the Cyanine family. Only checked stains and fluorophores will appear in the Select Fluors list. This eliminates clutter and can save you time identifying the choosing the right stains and fluors for your particular experiment.

When the **'Other (User Specified)'** family name is selected, clicking on the **New** button lets you add a new custom stain or fluorophore. Only custom stains and fluorophores can be deleted.

> ***NOTE:*** *You cannot add new Synthetic Spectra. But you can, of course, add new measured spectra using the **New** button in the Choose Stains and Fluors window.*

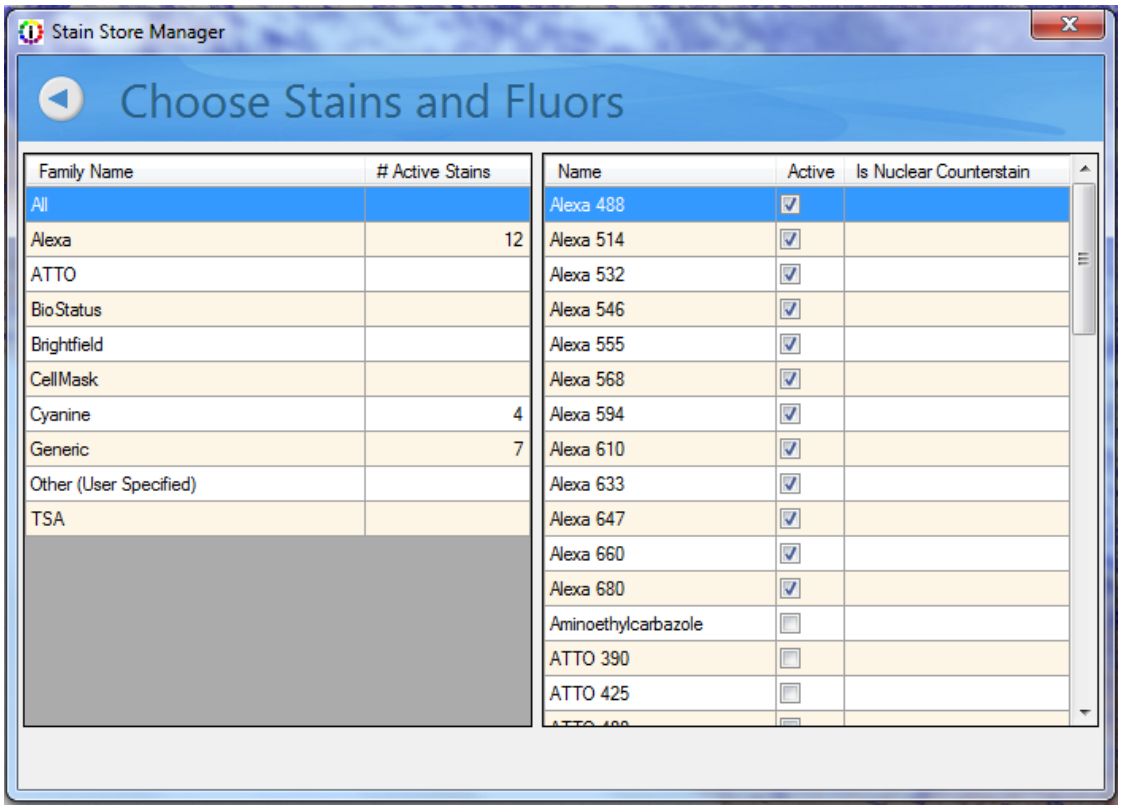


Figure 18. Stains and fluorophores are arranged by Family or can be seen all at once.

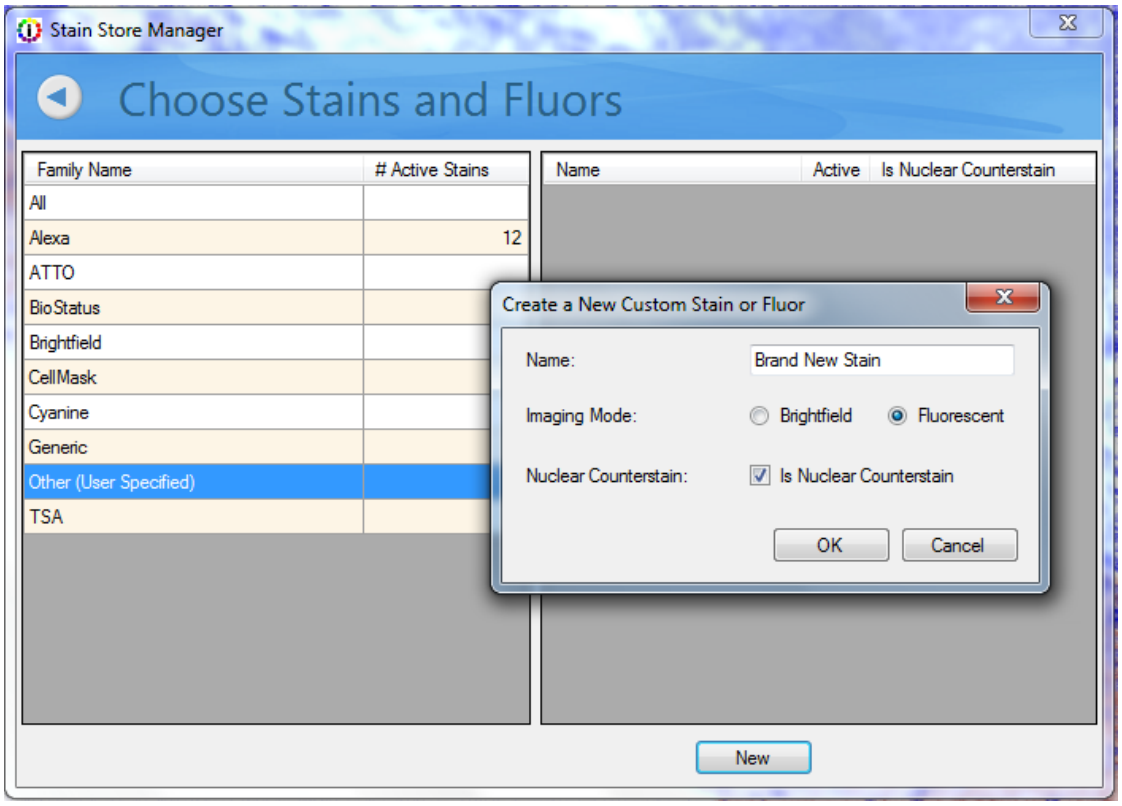


Figure 19. Adding a new stain or fluorophore can be done from within the Choose Stains and Fluors window.

See Building Libraries 134 for details on how you can measure and extract the signal and then save it to the Stain Store.

3.5.3 Managing Your Measured Spectra

Clicking on **Manage Your Measured Spectra** lets you see a list of all of the stains and fluorophores that you have acquired and measured. Only checked stains and fluorophores will appear in the Stains & Fluors list. Highlighting a particular stain or fluorophore will display the following characteristics on the right side of the window:

- **Name** of the stain or fluorophore
- **Groups** you have assigned to the spectrum
- **Creation Date** of the spectrum
- **Location** of the Source file (.im3)
- **Notes** that you can include for your own reference

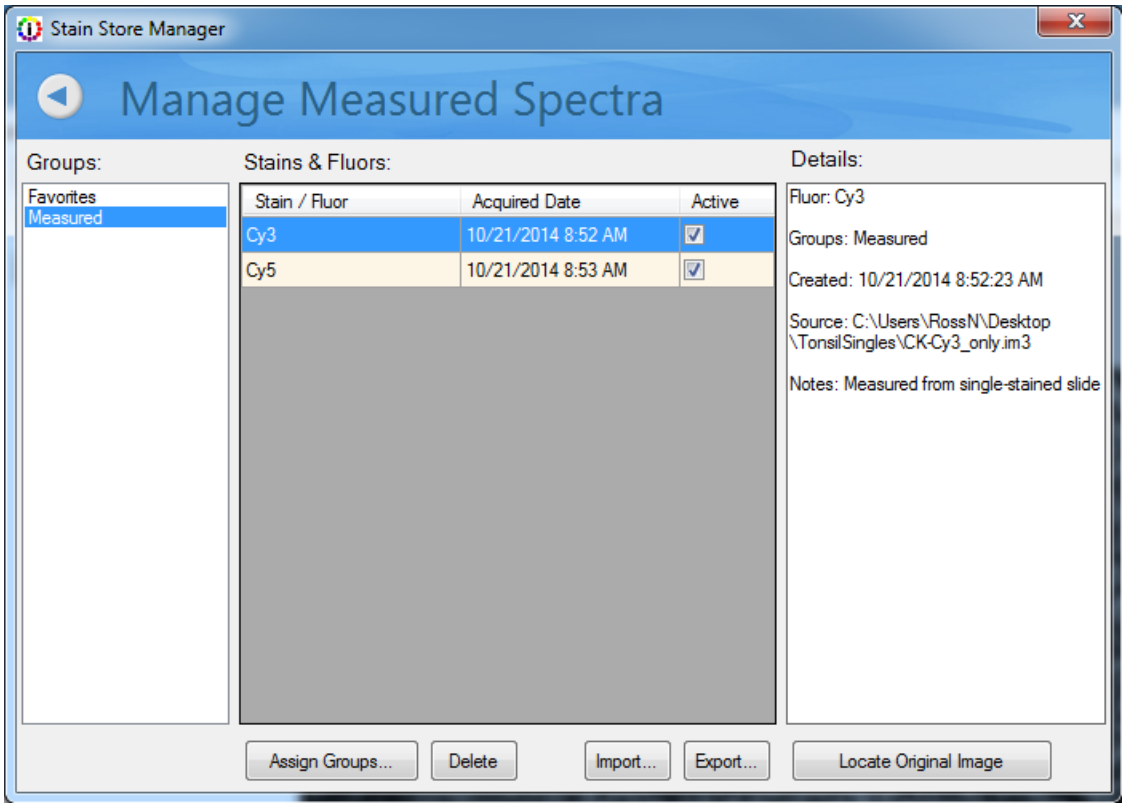


Figure 20. Manage Measured Spectra lets you select the spectra that you have measured.

You can:

- **Assign** one or more measured spectra to a group or groups

You can assign spectra to existing groups, or you can create a new group if needed. Each spectrum can belong to multiple groups.

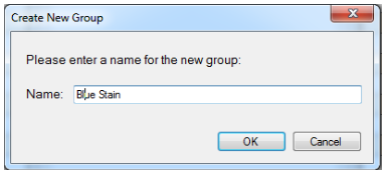


Figure 21. Assigning a new group.

- **Delete** a measured spectrum

- **Locate** the original source file from which you took your measurement, if it is present on your local system
- **Import** spectra from other inForm algorithms, projects, and Stain Store export files

To import measured spectra, click the **Import** button. Browse to the file containing the desired spectra. You can import from algorithms, projects, and .SSE files previously exported. After opening the file, you will be able to preview and select the spectra for import. Selected spectra will be added to the Stain Store and will be available to your projects.

- **Export** spectral data files

To export, click the **Export** button and select one or more measured spectra. You can save an .SSE file containing the spectra to a location of your choice. This can be very useful if you have done work on a single inForm workstation to develop ideal spectra for your experiment and then want to deploy the same spectra to multiple inForm workstations.

NOTE: *You cannot export synthetic spectra*

3.5.4 Managing Synthetic Spectra

Click **Manage Your Synthetic Spectra** and see a list of all the synthetic spectra that are available for your imaging system.

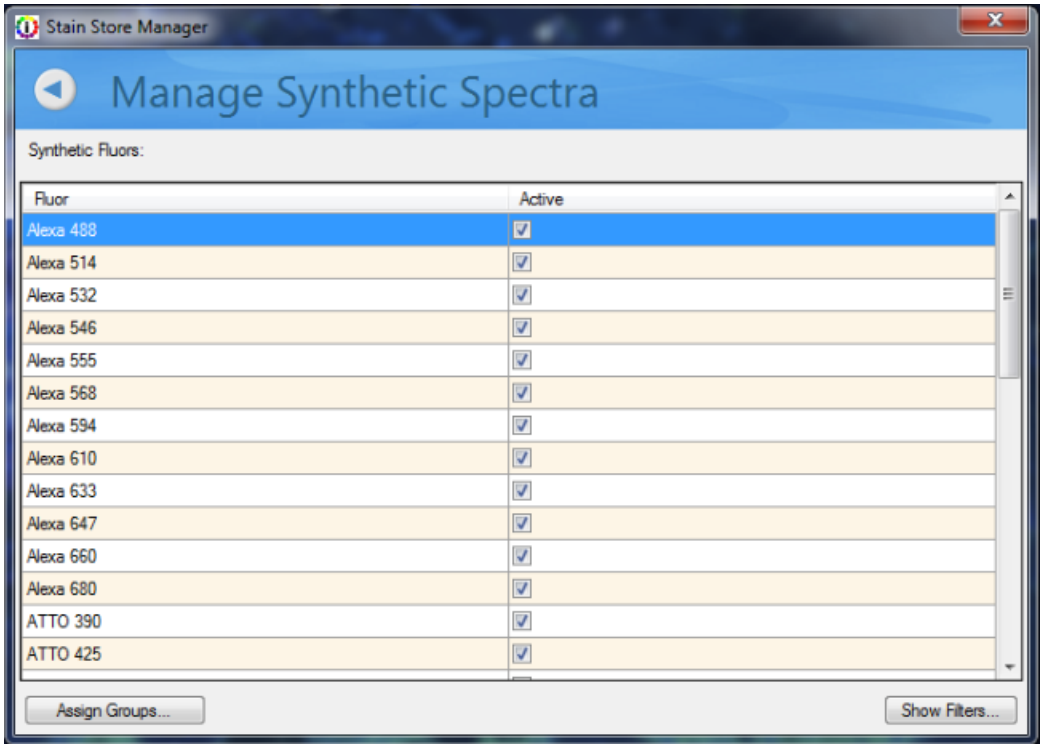


Figure 22. Choose which Synthetic Spectra will be displayed to choose for a spectral library in the Prepare Images step.

Check boxes in the Active column let you select which Synthetic Spectra in the Fluor column will be displayed for use in the Prepare Images step. You can click the **Assign Groups...** button to assign a particular Fluor (or a group of Fluors by highlighting more than one) to a new or existing Group. Each Fluor can be a member of more than one Group if you desire. If you would like to see the filters available to create the Synthetic Spectra, click the **Show Filters...** button.

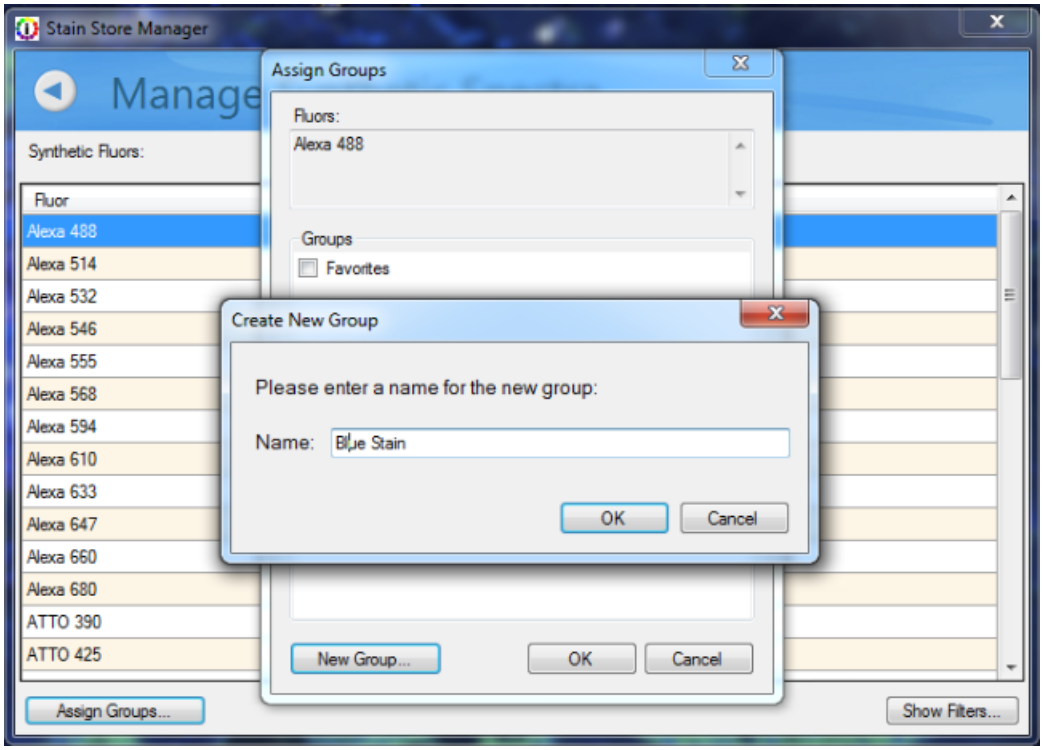


Figure 23. Assigning a Fluor to a new or existing Group is easy. You can specify the name of a Group and assign stains and fluorophores to multiple groups.

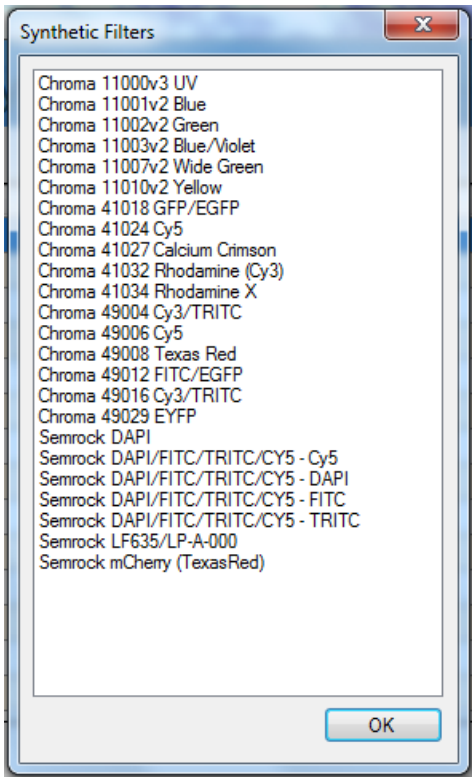


Figure 24. The list of filters available to generate Synthetic Spectra.

NOTE: *Under normal circumstances, you would capture images from single-stained slides in order to build up a 'library' of spectra (precise colors that are really unique multispectral profiles of your stains). To help get you started, Akoya Biosciences® includes some commonly used and characterized fluorescence spectra, referred to as 'synthetic' stains because they were not captured and characterized on your particular Mantra™ or Vectra™ system. However, because of the variability of stains and the staining process in brightfield IHC, there are no synthetic spectra provided for brightfield microscopy.*

4 Common Image Analysis Tasks

Use inForm to create automated image analysis routines to accurately extract data from Akoya images (QPTIFF, im3), or conventional RGB images (TIF, BMP, or PNG). (While it is also possible to process JPG images, this is not recommended. JPGs can contain compression artifacts that could adversely affect segmentation performance.)

Some common inForm image analysis tasks:

- inForm uses an AI-powered feature-based recognition algorithm to automatically identify and segment tissue types using training regions drawn by the user.
- inForm performs pixel-based analysis to quantitatively obtain component data. inForm can set thresholds (pixel-based) for all component signals and quantitate the thresholded component signals.
- inForm performs colocalization, which locates areas where components are simultaneously expressing on a pixel level.
- inForm identifies cells and finds nuclei, membrane, and cytoplasm, quantitates the component signal within those cell compartments and calculates consistent scoring judgments based on that quantitation.
- inForm identifies non-cellular objects, and can provide quantitative statistics.

Features can be combined to perform more advanced analysis, such as computing the double positivity of cells within a cancer region, or computing the colocalization of a multi-stained tissue sample.

The basic workflow to manually process images in inForm can be similar to the steps shown below:

1. Open a new project (see Working with Projects and Algorithms) 41. Follow the steps at the top of the window to process the images.
2. Open the desired images (see Opening Images) 47.
3. To use processing regions, draw the desired regions (see Drawing Processing Regions) 51.
4. Prepare the images for unmixing (see Preparing Images) 52.
5. If segmenting tissue, see either Manual Tissue Segmentation 65 or Trainable Tissue Segmentation 68.
6. Refer to the sections for the steps in the project (Threshold 77, Colocalization 80, Segmenting Cells 82, Scoring IHC or IF 101, and Counting Objects 106.
7. After images are processed, see Displaying the Extracted Data 108.

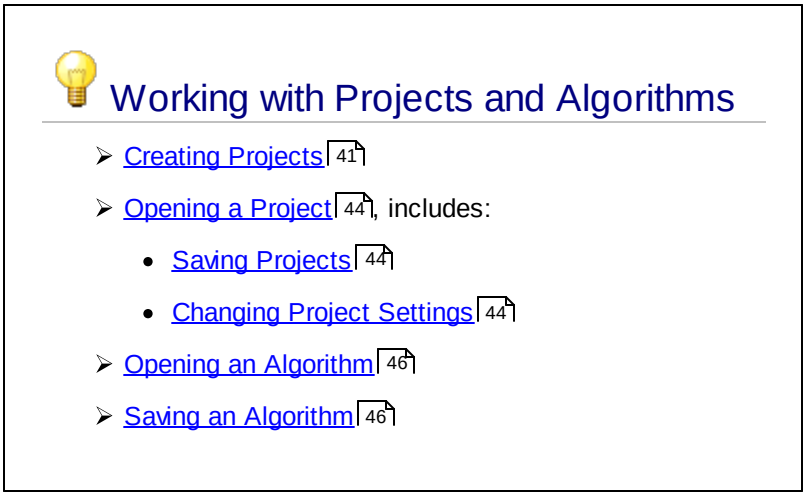
5 Working with Projects and Algorithms

An inForm project includes the images in the project, the algorithm to process the images, and any processing regions, training regions, manual tissue regions, and spectral regions.

The topics in this section describe how to create, open, and save projects, how to change the project settings, and how to open and save an algorithm.

An algorithm is used in batch mode to process images the same way each time.

- A project includes all the images so that you can go back and modify algorithm settings, including the trainable tissue classifier.
- An algorithm can be saved in a protected format so it cannot be edited.



5.1 Creating Projects

Each time inForm starts, a new project is created automatically. The new project contains only the **Prepare Images** step and the **Export** step. To create a project with additional steps, either Create a New Project 41 or Configure the Open Project 41. The name of the current project displays in the inForm title bar. Specify the desired project name when creating a new project or when configuring the project.

Create a New Project

The steps available when creating a new project are determined by the software configuration used to create the project. See Software Configurations 9 for information on the features available for each software configuration.

To create a new project:

1. Select **File > New > Project**. If an unsaved project is open already, inForm prompts you to save the current project. If you choose No, all unsaved settings are lost. The New Project window opens as shown in the figure below. Prepare Images and Export are automatically included in all projects.

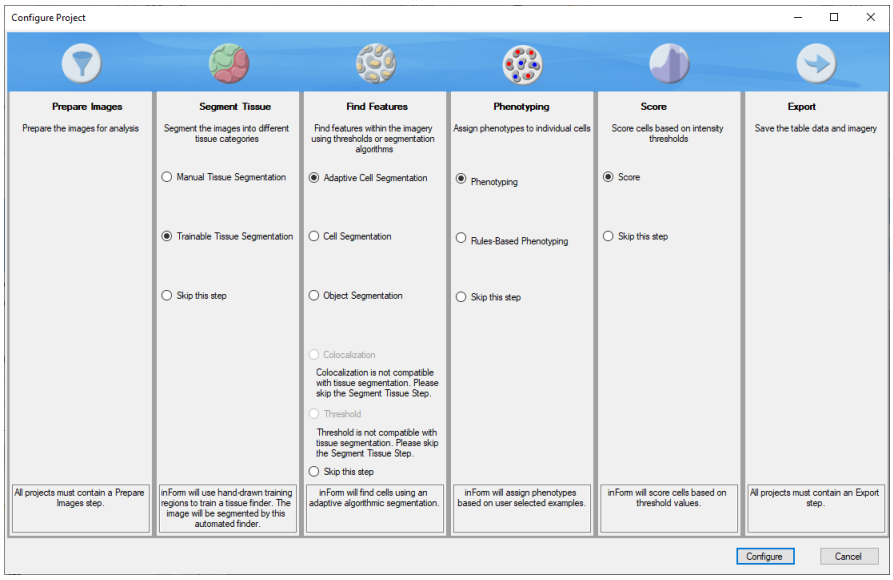


Figure 25. New Project Window

2. Type a name for the new project in the **New Project Name** text box.
3. To segment tissue, select the desired option under **Segment Tissue:**
   a. Select **Manual Tissue Segmentation** to manually mark tissue categories on the images.
   b. Select **Trainable Tissue Segmentation** to train the tissue segmenter to automatically identify areas as specific tissue categories.
   c. Select **Skip This Step** to skip tissue segmentation.
4. To find specific features, select the desired option under **Find Features**:
   a. Select **Adaptive Cell Segmentation** to identify cellular features, such as finding the nucleus, cytoplasm, and membrane for each cell using an adaptive thresholding algorithm.
   b. Select **Cell Segmentation** to identify cellular features, such as finding the nucleus, cytoplasm, and membrane for each cell.
   c. Select **Object Segmentation** to identify objects of interest.
   d. Select **Colocalization** to identify areas on the images where selected components are colocated.
   e. Select **Threshold** to identify the areas on the images where the strength of a component at each pixel exceeds a specific threshold.
   f. Select **Skip This Step** to skip finding features.
5. If **Cell Segmentation** was selected, select **Phenotyping** to assign phenotypes to each cell.
6. If **Cell Segmentation** was selected, select **Scoring** to calculate score statistics for the cells.
7. Click the **Create** button to create a new project with the selected steps.

8. See Opening Images 47 to add the desired images to the project.

Configure a Project

You can configure a project to add or remove steps from the project. inForm retains as many settings as possible, depending on which steps are added or removed from the project. For example, if you created a project without tissue segmentation and later decide that you want the project to include manual tissue segmentation, you can modify the project to add Manual Tissue Segmentation. Settings that are not affected by the changes are retained, but some settings may need to be adjusted due to the changes to the project.

The steps available when configuring a project are determined by the software configuration used to configure the project. See Software Configurations 9 for information on the features available for each software configuration.

1. Select **Tools > Configure Project** or click the **Configure** button.
2. Use the **Configure Project** window to select the desired steps. The **Configure Project** window contains the same options as the New Project Window 28.
3. Click the **Configure** button and inForm reconfigures the project.
4. Verify that all of the desired settings are selected in each step.

5.2 Opening a Project

To open a saved inForm project:

1. Select **File > Open > Project**, select the name of the project, and click the **Open** button. The images in the project open in Gallery mode as shown in the figure below. The View Editor and the Available Images window close if either one was open in the previous project.

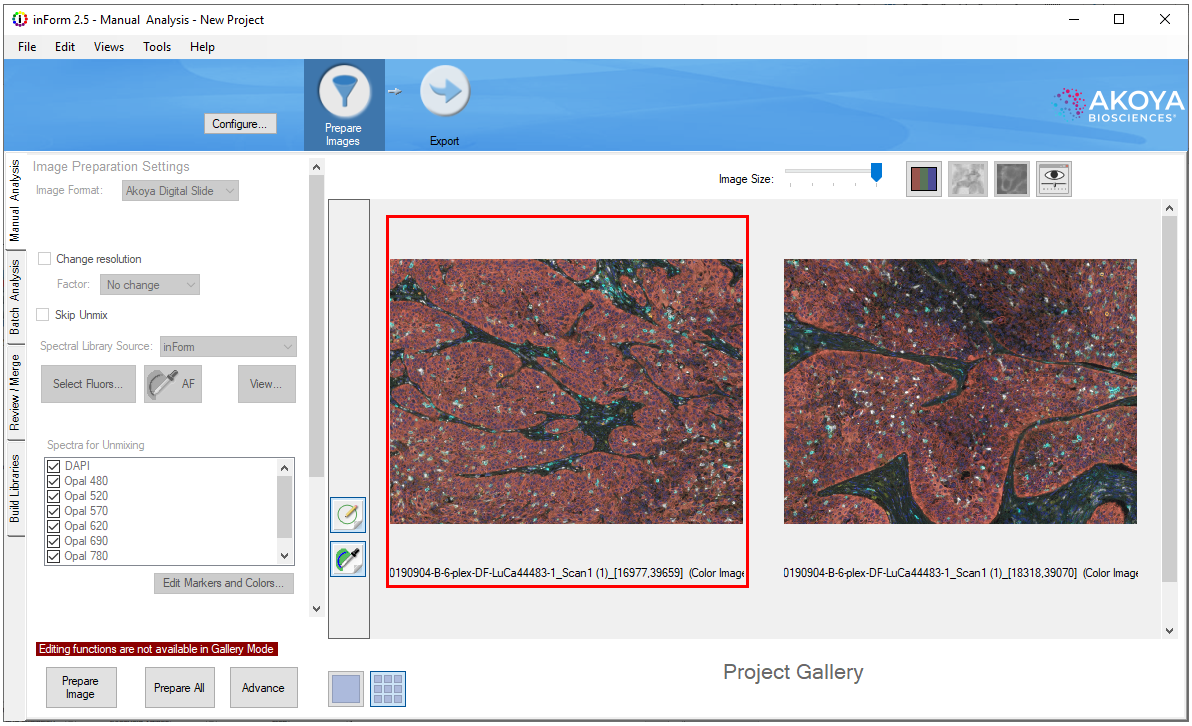


Figure 26. Gallery View

2. To edit algorithm settings, click the **Single** button to change to Single mode.

Saving Projects

Saved projects include the images, created composite views, the algorithm to process the images, and any processing regions, training regions, or manual tissue regions.

1. Select **File > Save > Project**.
2. Type a new file name, if desired, select the desired location, and click **Save**. A project file (.ifr) is saved in the specified location. A folder named <ProjectName>_TrainingImages is created in the same folder as the project file. Local copies of all the images in the project are saved in the training images folder. (To save only the algorithm and not the images, see Saving an Algorithm 46.)

NOTE: *Digital Slide projects do not store the images locally. Should the location of the data change, inForm will prompt you to find the slide data. When transferring data, please make sure that the study folder and its contents are moved as a unit.*

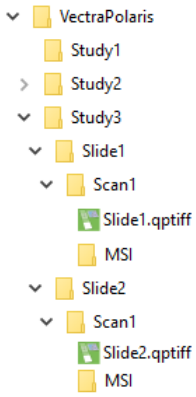


Figure 27. Data File Structure

Changing Settings

After a project has been created, the settings in the steps can be changed. Changing settings may require you to reprocess the images for later steps in the project.

5.3 Opening an Algorithm

Algorithms include all steps in processing an image: image preparation, image analysis, and export. You can load an algorithm into inForm and then open the images you want to process, or load the images first, and then apply the algorithm.

Algorithms can be used in Batch Processing 131 to automatically process multiple images at the same time using the same analysis settings. If a project is opened for use in Batch Processing, the project's algorithm is used to process the images.

To open an algorithm, select **File > Open > Algorithm**. If a project or image is already loaded, inForm warns you that all images will be reset to their initial state. Click **Yes** to discard all training regions, tissue regions, cell regions, object regions, thresholds, and component images. Select the desired algorithm, and click **Open**.

Open the desired images if they are not already open. To prepare the image(s), click the **Prepare Image** or **Prepare All** button. To review or change the settings in each algorithm step, click the **Advance** button to advance through each step or click the step buttons at the top of the window.

When algorithm development is complete, see Saving an Algorithm 46.

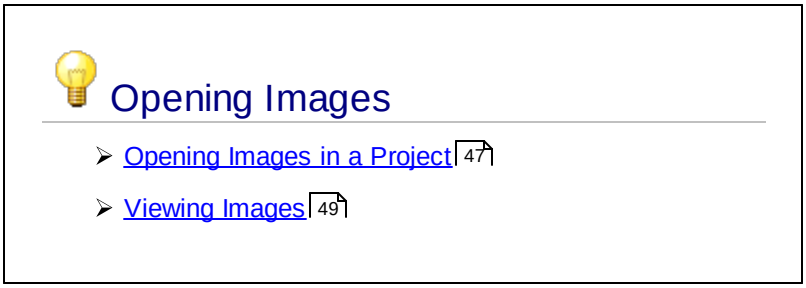
5.4 Saving an Algorithm

inForm Algorithms allow you to reuse the same settings to process compatible images or sets of images without re-selecting the settings for each new project. (Saving the project saves the images with the steps, see Saving a Project 44.)

To save the algorithm, select **File > Save > Algorithm**. Or to save the algorithm in a protected format that cannot be edited, select **Locked Algorithm**. Type the desired algorithm name, select the desired location, and then click **Save**.

6 Opening Images

The topics in this section describe how to open images in a project and describes the viewing options available.



6.1 Opening Images in a Project

Add the images to the project as described below. The images in a project can be included in the Processing set (the images to be processed by the algorithm) and/or can be included in the Training set if training a Tissue Segmenter.

The number of images that can be opened in a project depends on the size of the images (width and height in pixels) and the operating system.

Opening Images

To open the desired images:

1. Select **File > Open > Image**. The Select Image window opens. The **Files of Type** selector at the bottom of this window specifies the types of images to display: Digital Slides (.qptiff), Digital Slide Extract (.qpexport), Multispectral Fields (.im3), or other common image files such as TIFFs, BMPs, JPGs, etc.
2. Navigate to the location of the image files.
   - When loading Digital Slides, inForm will load all stamp annotations designated for inForm projects.
3. Select the desired image files. The image files must all be of the same image type (.qptiff, .im3, RGB, or Monochrome). Ctrl + click to select multiple image files. Shift + click to select a range of files.
4. Click the **Open** button to add the images to the project. The images are automatically included in the Processing set 11.

NOTE: *If a single image exceeds 16 megapixels (32-bit systems) or 110 megapixels (64-bit systems), the Change Resolution window opens and prompts you to select an image reduction factor to use to reduce the image resolution. The window also displays the number of images of a similar size that can be open at the same time. Select the desired factor and click the **OK** button to change the image resolution and open the image.*

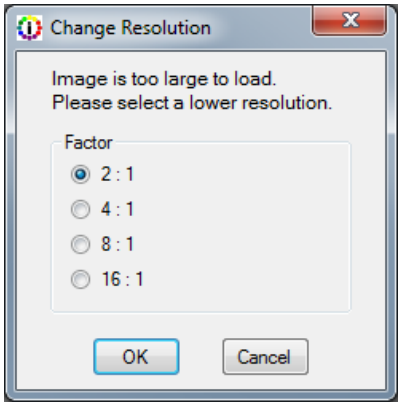


Figure 28. Change Resolution Window

5. To change the view of the images, see Viewing Images 49.
6. To process only part of an image, see Drawing Processing Regions 51.
7. To change the image resolution of all open images, convert to optical density, or unmix the images, see Preparing Images 52.

Opening Images Using the Available Images Window

You can add images to the project using the Available Images window.

1. Click the menu option **Tools > Image List Editor**. The Available Images Window 27 opens.
2. Click the **Load Image** button. The Select Image window opens,
3. Select the desired images and click the **Open** button.

You can also use the Available Images window to select which images are in the training and processing sets by selecting or clearing the check boxes next to each image name.

Adding Additional Images to a Project

After images have been added to a project, only files of the same type (Digital Slide, Digital Slide Extract, Multispectral Field, RGB, or Monochrome) can be added to the project. Add images to an open project either by selecting **File > Open > Image** or by clicking the **Load Image** button in the Available Images Window 27.

Removing Images from a Project

To remove images from the open project:

In **Single mode**, click the thumbnail of the image to be removed to show the image in the Image Display Area. Right-click the image in the image display area and select **Remove from Project**.

In **Gallery Mode**, right-click the image in the gallery and choose **Remove from Project**.

Images can also be removed from the project by right-clicking the image name in the Available Images Window 27 and selecting **Remove Image**.

6.2 Viewing Images

Images are viewed in the Image Display area in the inForm Window. View images in Single Mode to zoom in on an image, draw regions on an image, or edit the algorithm settings. View images in Gallery Mode to compare all the images in the project or to quickly locate specific images.

Gallery Mode

To view a gallery of images, click the **Gallery** button at the lower left of the display area. Note that when in Gallery mode, you cannot edit the Step settings.

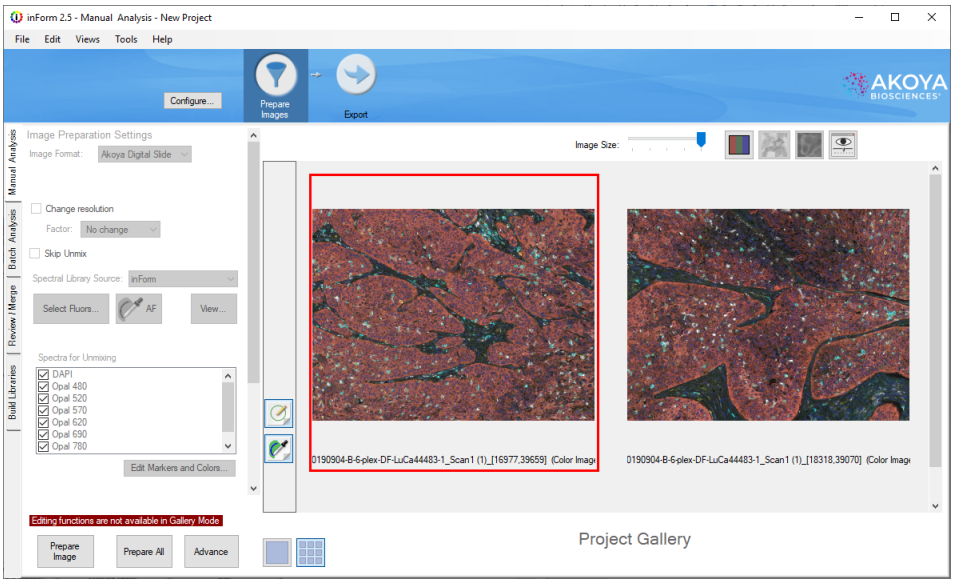


Figure 29. Gallery View

Single Mode

To view an image in Single Mode, click the **Single** button at the lower left of the display area.

Click the thumbnail of the image to view the image in the display area.

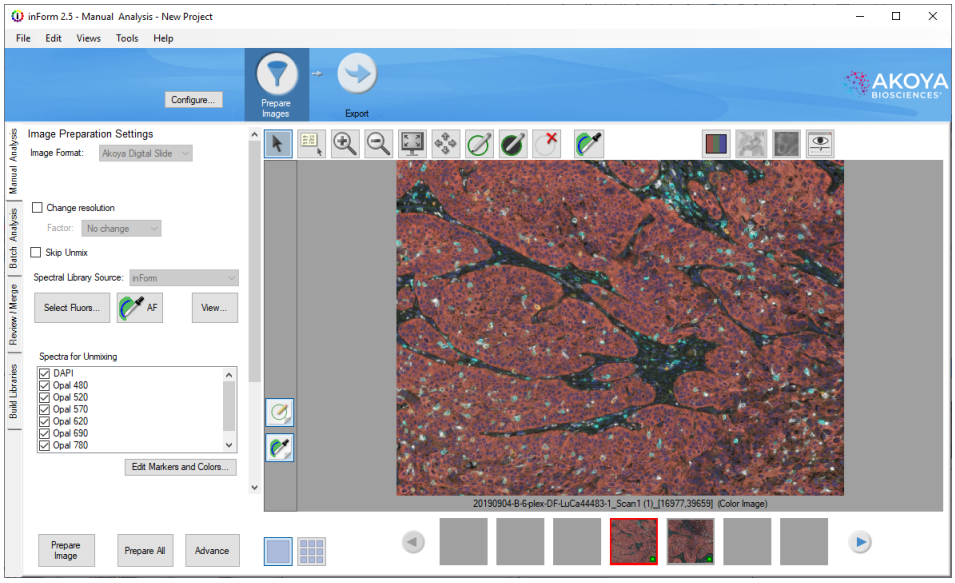


Figure 30. Single View

Cube Image

Select **Tools** > **Show Cube Image** to see more information about the selected image. Depending on how the image was taken, this may include image size, wavelengths acquired, exposure times, and instrument response data.

7 Drawing Processing Regions

Processing regions enclose areas of the image that will be processed using the steps in the project or algorithm. Areas of the image outside of the processing regions are ignored and are not included in any calculations or statistics. Processing regions are manually drawn on the images and cannot be used for Batch Processing 131.

To draw processing regions on an image:

1. In the open project, click the **Single Mode** button, and then click the desired image in the image thumbnails.
2. If desired, use the **Zoom In** button to zoom in on the image or the **Pan** button to move the image in the image display area.
3. Click the **Draw Processing Region** button on the toolbar.

4. Click and drag to outline the desired area of the image. Release the mouse button to connect the endpoint to the start point of the processing region. An ID number is assigned to the processing region and displays in the middle of the region. The ID number identifies the region in data tables.
5. Repeat step 4 to draw multiple processing regions on the same image.
6. Click each image and repeat steps 2 through 5 to define all desired processing regions on each image in the project.

Deleting Processing Regions

If a processing region doesn't enclose the desired region, delete the processing region and draw a new processing region.

To delete a processing region:

1. Click the **Delete Regions** button on the toolbar.

2. Click the processing region that you want to delete. The processing region is removed and the remaining processing regions with ID numbers higher than the deleted region are re-numbered.

You can also delete a processing region by right-clicking on the processing region and selecting **Delete Region** on the shortcut menu.

To delete all processing regions on an image:

1. Right-click anywhere on the image.
2. Select **Delete Regions > Delete Processing Regions** on the shortcut menu.

8 Preparing Images

The first processing step in an algorithm is the **Prepare Images** step. This section describes how to prepare the images for segmentation or analysis by changing image resolution, converting brightfield images to optical density, and unmixing the images.

Preparing Images

- Selecting the Image Format 52
- Choosing a Spectral Library 54 includes:
  - Converting to Optical Density 54
  - Choosing Spectra for Unmixing 54
  - Removing "Haze" Spectra 54
- Component Units 61
- Completing the Prepare Images Step 64

8.1 Selecting the Image Format

The **Image Preparation Settings** panel displays the Image Format, Sample Format, and Change Resolution options.

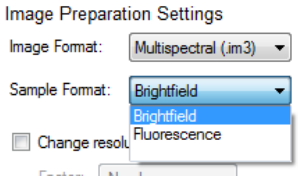


Figure 31. Image Format and Sample Format

Image Format

Before image analysis can begin, inForm needs to know the type of images in the project. Possible image types are Digital Slide (.qptiff), Digital Slide Extract (.qpexport), Multispectral Field (.im3), RGB, or Monochrome.

If you open a new project and then immediately add new images to the project, inForm automatically selects the correct Image Format. If you open TIFF, BMP, JPG, or PNG images, inForm recognizes the images as either RGB or Monochrome images.

Additionally, all images added to the project must be acquired at the same resolution. When using Vectra Polaris, imagery acquired at a resolution of 0.5um/pixel (20x) is fundamentally different than imagery acquired at 0.5um/pixel (20x binned from 40x); these images cannot be processed within the same project.

Sample Format

All images in the project must be the same sample format. For any RGB images and for Multispectral Field (.im3) images created with early versions of Nuance, you must select the correct **Sample Format** (Brightfield or Fluorescence). Monochrome images do not use a Sample Format. For all other image formats, inForm automatically detects the Sample Format.

For Vectra version 2.0.6 and earlier, and all Nuance versions, the imagery must have been acquired using the same filters in the same order. Later versions of Vectra™ and Mantra™ can vary in their filter order.

If desired, you can select the Image Format and Sample Format before adding images. In this case, only images that match the selected Image Format and Sample Format can be opened.

Change Resolution

Select the **Change Resolution** check box to change the resolution of all images in the project. You can lower the image resolution to increase the speed of training and tissue segmentation. If the set of images is so large that all of the images cannot be held in memory, lower the image resolution prior to unmixing.

8.2 Choosing a Spectral Library

inForm uses a process called spectral unmixing to decompose the image into its various components. For a simple example, a color image can be unmixed into its red, green, and blue components. But more powerfully, an image can also be unmixed into its stain components such as Hematoxylin and DAB for Brightfield images, which describe the contribution of each stain to the overall signal. The contributions of each stain are based on a spectral library that shows the characteristic color distribution of light associated with each stain. A fluorescence example could consist of stain components such as DAPI and FITC; the spectral library can additionally include the color distribution associated with tissue autofluorescence.

- For **Multispectral Fluorescent Digital Slides**, the spectral library can be synthetic, or extracted from singly stained samples.
- For **other Fluorescent Digital Slides**, a spectral library is not used.
- For **Brightfield Digital Slides**, a spectral library can be extracted from singly stained samples.
- For **Multispectral Field .im3 images acquired with Mantra, Vectra 3, or Vectra Polaris**, inForm can extract the spectrum from singly stained samples. In addition, a synthetic spectral library can be used for fluorescence imagery.
- For **all other Multispectral Field .im3 images**, the spectral library is created in the Nuance software.
- For **RGB images**, the spectral library can be created in the Nuance software or you can use the default RGB spectral library that is included with inForm.
- For **Monochrome Images**, spectral libraries are not used.
- For **Brightfield PKI TIFF Extracts**, the default RGB spectral library included with inForm can be used if desired. Spectral libraries are not used for fluorescent PKI TIFF Extracts.

Selecting an inForm Library

If you have previously created spectra by Building Libraries 134, you can select those spectra for use with your images. You must have an image loaded to select inForm libraries.

Select inForm as the Spectral Library Source.

Choose the spectra that represent the stains or fluorophores in your sample.

If this is a fluorescence sample, you may wish to select autofluorescence by drawing on an unstained portion of tissue with the Autofluorescence Picker.

If you need to reselect your stains or fluorophores, click the Select Stains/**Fluors**... button.

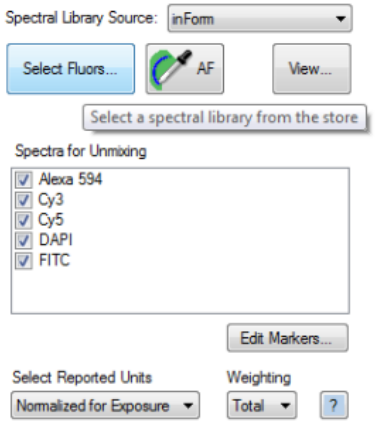


Figure 32. Select inForm as the Spectral Library Source to choose spectra created in inForm.

While choosing your stains or fluorophores, you can narrow your choices by selecting one or more Groups. Selecting All will show all spectra compatible with your imagery.

You can sort your spectra by name, creation date, and selection by clicking the column headers.

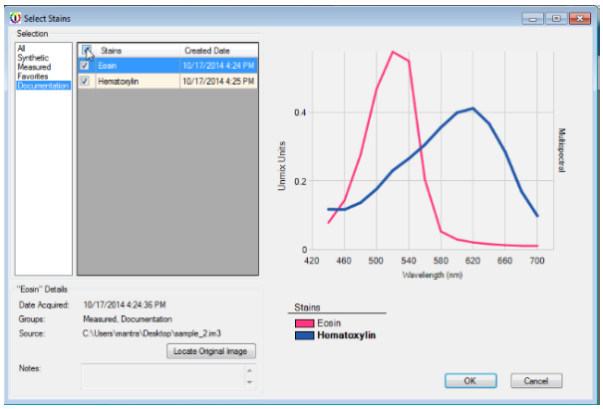


Figure 33. Available stains can be selected for preparing images.

You can preview the spectral shape for the highlighted stain or fluor, as well as your selected spectra, in the spectral chart. The details of the highlighted stain or fluor, including the acquisition date, groups, and notes, are shown below the listing. Clicking on a stain or fluor in the legend will highlight that stain and show its details.

Clicking the **Locate Original Image** button will bring up the folder containing the stain or fluorophore's source image, if available.

Selecting a Synthetic Spectral Library

If you have a fluorescence image taken with Vectra Polaris, Mantra™ or Vectra 2.0.7 and higher, you can use synthetic libraries within inForm.

Akoya Biosciences® includes some commonly used and characterized fluorescence spectra, referred to as 'synthetic' stains because they were not captured and characterized on your particular system.

Select inForm® as the **Spectral Library Source**. Select the **Synthetic Group** to limit your spectra to synthetic only.

Choose the spectra that represent the fluorophores in your sample and click the **OK** button.

You may wish to select autofluorescence by drawing on an unstained portion of tissue with the **Autofluorescence Picker**.

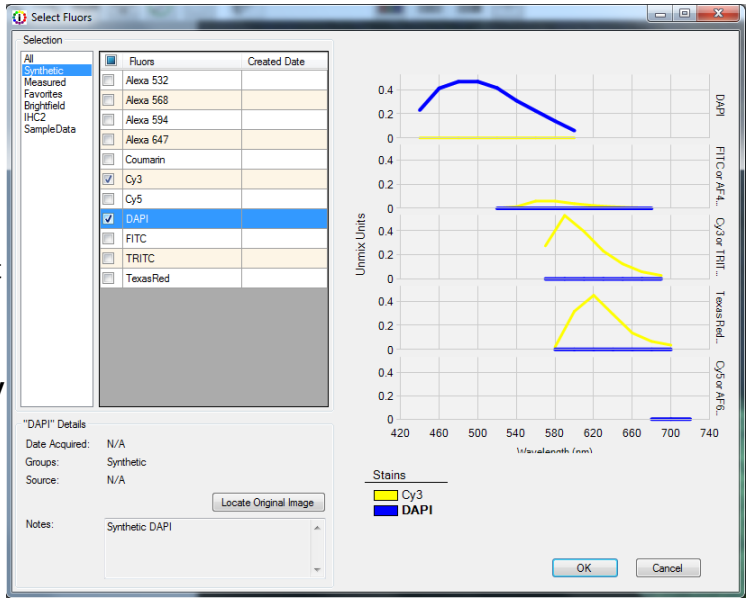


Figure 34. Selecting the Synthetic Group will only display synthetic fluors.

Selecting a Nuance Library

To select the spectral library:

1. For Brightfield RGB or for multispectral field .im3 images acquired with early versions of Vectra, convert to Optical Density as described in Converting to Optical Density 54 (below). Images acquired with recent versions of Vectra are already converted to Optical Density.
2. In the **Spectral Library Source** drop-down list, select **Nuance**. The Open Spectral Library window opens.
3. Select the desired Spectral Library (.csl) and click the **Open** button. The name of the selected spectral library displays. The components in the spectral library display in the **Spectra for Unmixing** list box. (Click the **Browse** button to change the selected spectral library.)
4. If this is a fluorescence sample, you may wish to select autofluorescence by drawing on an unstained portion of tissue with the **Autofluorescence Picker**.

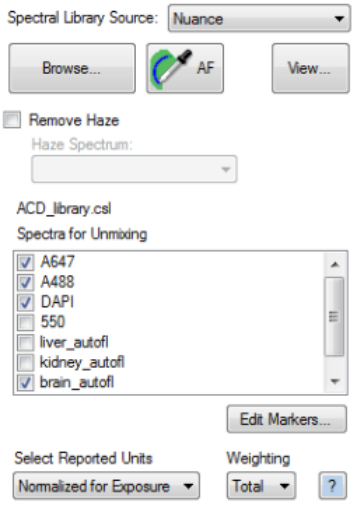


Figure 35. Load Spectral Library

5. For fluorescence images, see Remove Haze 54 for details on removing the background haze before unmixing.
6. In the **Spectra for Unmixing** list box, select the check boxes for the components that you want to unmix. (See Choosing Spectra for Unmixing 54 for more information.)
7. To view the selected Spectral Library, click the **View** button under the Spectra for Unmixing list box and see Viewing a Spectral Library 60.

Editing Markers

You can add the names of your markers to the spectra listed in the Spectra for Unmixing box in the Manual Analysis tab. Click the **Edit Markers and Colors...** button below the Spectra for Unmixing box. The Edit Markers and Colors button brings up the Edit Markers and Colors window, which allows you to enter the marker names and change the colors associated with your spectra.

You can type your marker name in each text box to the right of the name for each spectrum. You can also select the default color used for unmixing using the color drop-down list.

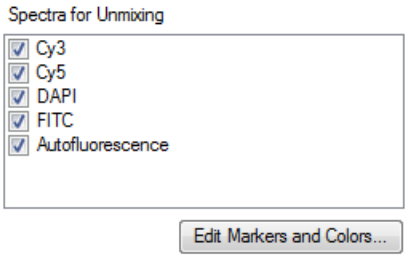


Figure 36. Edit Markers and Colors button

If you know that CD4 is your marker for FITC, type **CD4** in the text box to the right of FITC in the Edit Markers and Colors window. This puts the fluorophore name into parentheses next to the marker name in the list of spectra for unmixing.

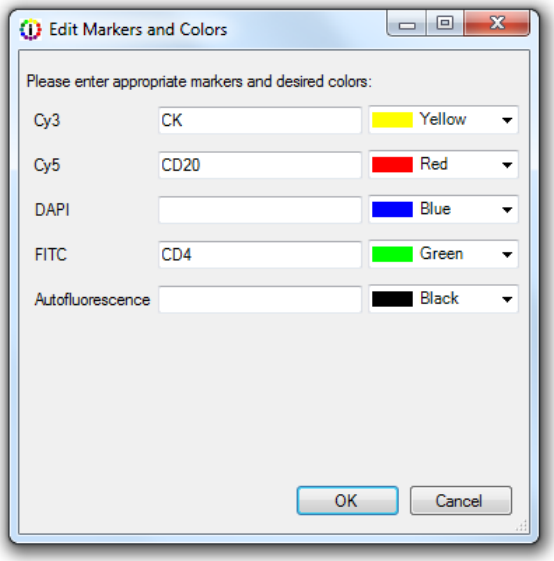


Figure 37. Enter the names of your markers next to the names of your spectra.

Converting to Optical Density

Brightfield multispectral and RGB images of IHC-stained samples need to be converted to optical density if the goal is to quantify stain "intensity" (optical density). Typically, brightfield multispectral images acquired with Nuance and recent versions of Vectra are already converted to optical density and do not need conversion as part of Prepare Images. Brightfield multispectral images acquired with early versions of Vectra are not converted to optical density when acquired, and require conversion as part of Prepare Images.

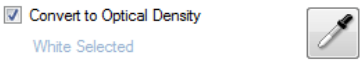


Figure 38. Convert to Optical Density

Selecting a "Reference White" for Optical Density conversion:

1. Select the **Convert to Optical Density** check box.
2. If the white areas on the image are small, zoom in on a white area.
3. Click the **White Picker** button.

4. Move the cursor (a white box) over a white region on the image and click. *"White Selected"* displays under the Convert to Optical Density check box. If the image appears very light, you may have clicked on an area that was not completely white. Clear the **Convert to Optical Density** check box to return to the original image, and then repeat the steps above.

Note: *The optical density conversion is required if you are working with brightfield images and plan to segment cells and measure stain intensity.*

Choosing Spectra for Unmixing

The **Spectra for Unmixing** list box is populated from the selected spectra or library. Check the stains you wish to use to unmix. If you have more than one spectral example from the same stain (e.g. multiple DAB spectra) in your list, you will only be able to use one for unmixing.

For Brightfield RGB images, if you do not load a library, the spectra for unmixing default to Blue, Green, and Red.

NOTE: *If Scale Views Based on Selected Images is selected in the View Editor 25 and then changes are made to the selected Spectra for Unmixing, the image scaling is reset to Scale Views Equally for All Images in the Project (default).*

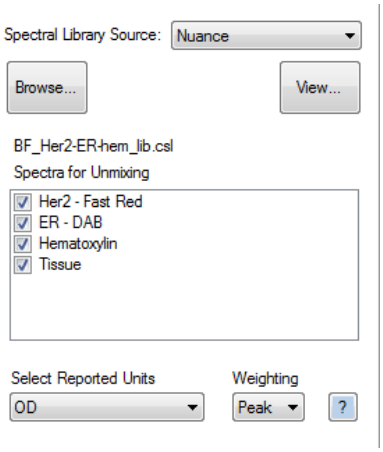


Figure 39. Spectra for Unmixing

Removing the "Haze" Signal

In fluorescence images, image contrast and unmixing accuracy can sometimes be improved if the general haze in the image, which has a characteristic spectra, is subtracted from the images first. When creating the spectral library for the images, create the background or "haze" spectrum and add it to the library. When the library is loaded, you can select the haze spectrum and remove it from the images.

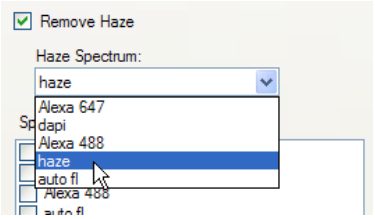


Figure 40. Remove Haze Spectrum

To remove haze:

1. Check the **Remove Haze** check box.
2. Select the **Haze Spectrum** to remove.

Haze is removed numerically (without scaling or other changes in value), wavelength-by-wavelength, from every pixel of the image. This alters the spectral shape of the remaining signals and their overall intensity. The magnitude of these changes depends on the intensity of the spectral signal being subtracted. Typically, when background spectra are small compared to the signals, spectral library entries derived from unsubtracted data sets can still be

used, but this should be validated when quantitative results are required. Ideally, the remaining spectra in the library should also have the haze spectrum subtracted (in Nuance).

Clicking the **Prepare Images** button will apply your settings and display the composite view.

8.3 Viewing a Spectral Library

The Spectral Library window displays graphs of the spectral segments in each imaging band in a spectral library. Only one graph displays for brightfield images or for fluorescent images with only one data point in each imaging band.

To view the spectral library:

1. Open the desired spectral library.
2. Select the spectra that will be used to unmix the images in the **Spectra for Unmixing** list box.
3. Click the **View** button below the Spectra for Unmixing list box to open the Spectral Library Window 29.
4. Select the desired **Units** to display the graphs in the chart. See Spectral Library Window 29 for complete descriptions.
5. Select the desired **Display** options for the chart.
6. To save an image of the chart with the currently selected display options, click the **Save Chart** button, type the desired name for the image file, select the desired image format, and click the **Save** button.
7. Click the Close (X) button in the upper right corner to close the Spectral Library window.

If a chart cannot be displayed because no spectra are selected or there are no compatible units, a message displays in place of the graphs.

8.4 Component Units

The components produced by unmixing are calculated using the settings selected in the **Select Reported Units** and **Weighting** drop-down lists. The Reported Units can be: Raw counts, counts Normalized for Exposure, or OD (optical density) units. The Weighting can be Total, Mean, or Peak.

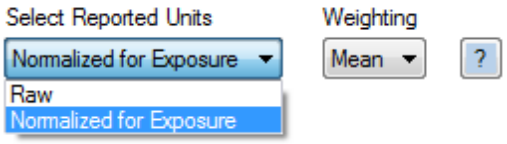


Figure 41. Component Units

Brightfield versus Fluorescence

Brightfield and fluorescence images use different measuring schemes because of the inherent differences of the images.

- For brightfield images, darker pixels indicate a higher expression of the biomarker.
- For fluorescence images, brighter pixels indicate a higher expression of a biomarker or reporter protein.

Therefore we use optical density for brightfield images and fluorescence counts (either raw or normalized) for fluorescence images.

Select Reported Units

Monochrome (black and white) images and **RGB** color images produce signals in counts.

For **multispectral images that are not converted to optical density**, component signal counts are either raw or *normalized for exposure*.

- **Raw Counts** reports the measured signal associated with a given component (such as DAPI, FITC, etc.).
- **Normalized for Exposure** reports the number of counts in the image, after accounting for all the camera settings in use (gain, binning, bit-depth, well depth, and exposure time). Using Counts Normalized for Exposure allows you to compare images taken using different camera settings or exposures, with the assurance that signal levels indicate the actual brightness at the sample, regardless of the instrument settings.

  Counts Normalized for Exposure = (counts x relative well depth)/ {$2^{\text{bit depth}}$ x exposure time x gain x binning area}, where relative well depth is the well depth of the camera sensor relative to a nominal Akoya sensor at the chosen gain setting, bit depth is the bit depth of the imagery, typically 8-bit (256 levels of gray) or 12-bit, exposure time is in seconds, gain is the gain setting of the camera, and binning area is 1 for 1x1, 4 for 2x2, etc.

  The values are small when using Mean weighting, and it may be hard to develop an intuition about what is normal. Selecting Total weighting (default) produces larger values.

For **brightfield images that are converted to Optical Density**, inForm produces unmixed component signals in **OD (Optical Density) units**. Optical Density indicates the amount of absorbing material present at a location. Optical Density = -log10 (pixel value / white reference pixel value).

Note: *If **Scale Views Based on Selected Images** is selected in the View Editor 25 and then changes are made to the Reported Units, the image scaling is reset to **Scale Views Equally for All Images in the Project (default)**.*

Weighting

Specifies whether to use the Mean, Peak, or Total signal to calculate the displayed units.

Total - The sum of all signals of the selected component across all wavelengths. Available for all images except images converted to Optical Density. (Default for fluorescence images.)

Mean - The average of all signals of the selected component across all wavelengths. Available for all units except Counts for Monochrome images. This is the equivalent of the settings in versions of inForm prior to V2.0.2. Examples: For RGB color images, inForm reports the average signal across the three color planes (red, green, and blue). A pure blue pixel with 240 counts would have a component strength of 80 (= 240 counts / 3 planes). For multispectral images, the signal is averaged across all wavelengths measured. So a pixel having a DAB signal of OD = 0.60 in the blue, OD = 0.20 in the green, and OD of 0.10 in the red would report a signal of 0.30 OD units (= [0.60 + 0.20 + 0.10] summed OD / 3 planes).

Peak - The peak signal of the selected component across all wavelengths. Available for images converted to Optical Density and for fluorescent multispectral images. (Default for brightfield images.)

Weighting Example

The simplified example below explains the difference between the three weighting options. (The example uses only two fluors and ignores noise and other sources of error.)

This example uses a spectral library with just two elements, Fluor A and Fluor B, and eight wavelengths (420, 460, 500, 540, 580, 620, 660, 700). The spectral library is shown in the figure below.

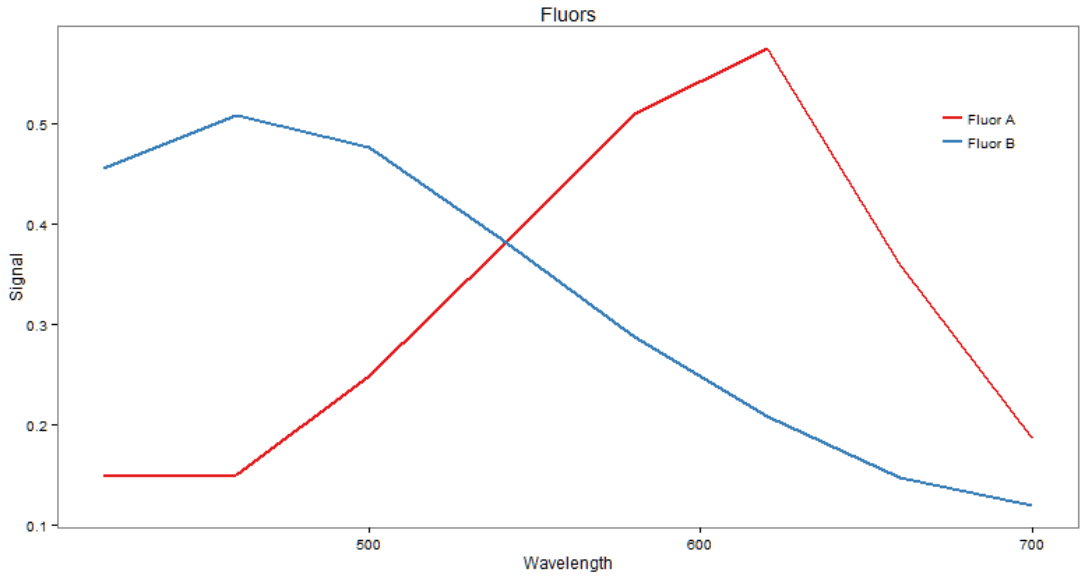


Figure 42. Spectral Library Example

If an image pixel expresses one part Fluor A and three parts Fluor B, the signal at the pixel is shown in the figure below.

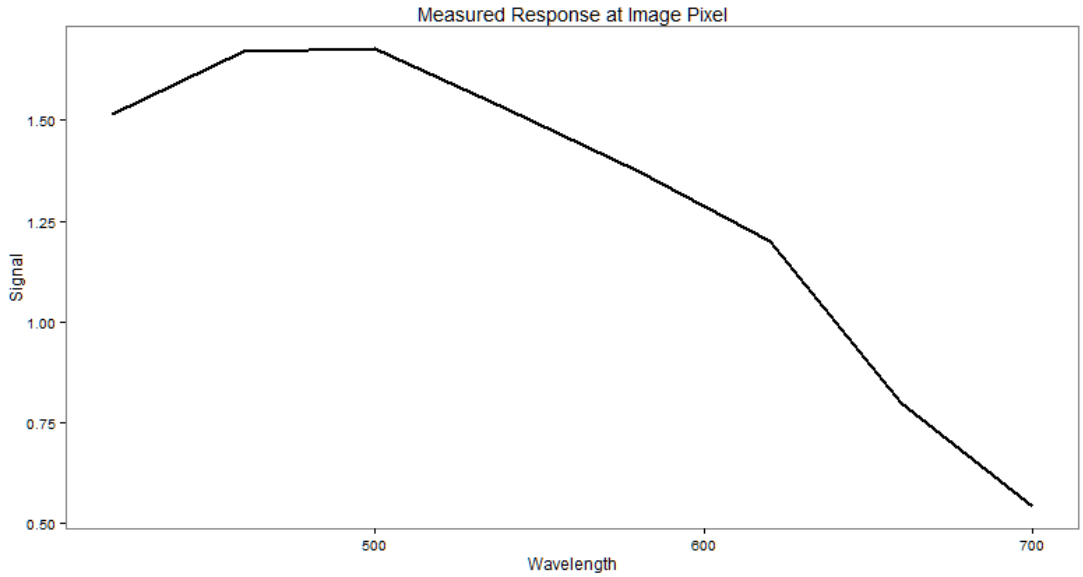


Figure 43. Signal at a Pixel

Unmixing recovers the original spectra, scaled according to the measured expression at the pixel as shown in the figure below.

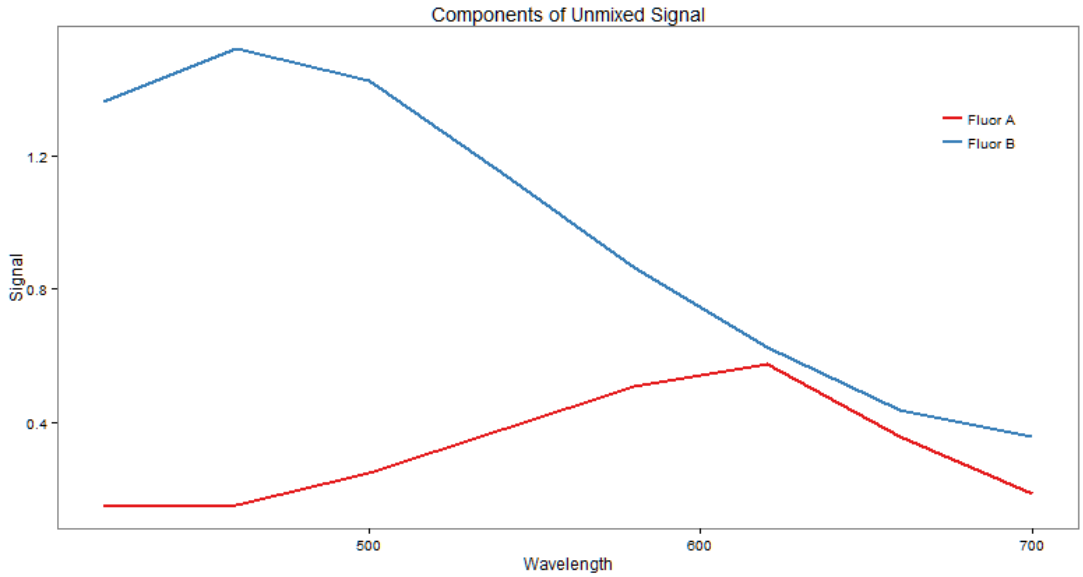


Figure 44. Original Spectra, Scaled

The challenge for the unmixed component image is to represent the unmixed spectrum using a single value. The three weighting options correspond to three possibilities for how to do this.

- Peak weighting represents the spectrum as the signal level at the brightest wavelength of the spectrum. In the example, this gives component values of
  Fluor A = 0.5739
  Fluor B = 1.5216

- Total weighting represents the spectrum as the sum of the signals at all wavelengths. In the example, this gives component values of

  Fluor A = 2.551
  Fluor B = 7.7514

- Mean weighting represents the spectrum as the average signal across wavelengths. In the example, this gives component values of

  Fluor A = 0.3189
  Fluor B = 0.9689

Notice that Total weighting is the Mean weighting multiplied by the number of wavelengths (eight in this example).

The figure below shows scaled spectra annotated with the weighting values:

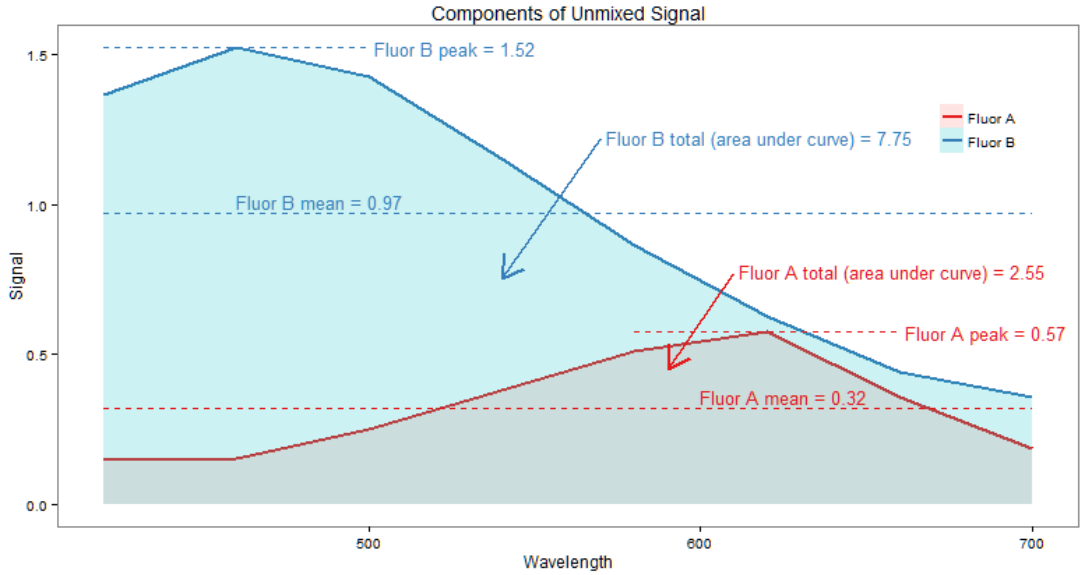


Figure 45. Weighting Values

8.5 Completing the Prepare Images Step

After all of the desired settings have been selected in the Image Preparation Settings panel, process the images using any of the methods below and then advance to the next step in the project.

- Click the **Prepare Image** button to apply the selected settings to the currently selected image.
- Click the **Prepare All** button to apply the selected settings to all images open in the project.
- Click the **Advance** button to apply the selected settings to all images open in the project and then display the next step in the project.
- Click the next step in the project in the Step Bar[19] at the top of the inForm window. All images are processed using the current settings, and then the settings panel for the next step displays.

9 Manual Tissue Segmentation

The topics in this section describe how to manually draw tissue categories on an image. The tissue categories can then be used to generate data or can be used during the Cell Segmentation, Scoring, or Object Counting steps. Algorithms that include a Manual Classification step cannot be used in Batch processing.

Manual Tissue Segmentation

- Creating Tissue Categories 65
- Drawing Tissue Category Regions 66
- Editing Tissue Category Regions 66

9.1 Creating Tissue Categories

Tissue categories divide the image into specific areas that correspond to specific types of tissue (for example, cancer, necrosis, stroma, etc.) or occasionally other structures of interest. The Manual Classification step (available in inForm Basic Analysis or higher) enables you to draw the appropriate tissue categories over areas of each tissue type on the images. Cell segmentation, scoring, and object counting can all be restricted to a specific tissue category.

1. Click the **Manual Classification** step to display the *Tissue Segmentation* panel.
2. Click the **New** button under the Tissue Categories section to create a new tissue category.

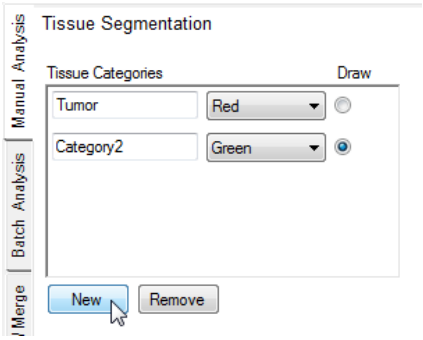


Figure 46. Tissue Categories - New Button

3. To change the tissue category name, highlight the name and then type the desired name for the tissue category.
4. To change the display color for the tissue category, click the **Color** pull-down and select the desired color.
5. Repeat steps 2 through 4 to create all of the desired tissue categories.
6. See Drawing Tissue Category Regions 66.

9.2 Drawing Tissue Category Regions

Draw tissue categories around the areas on the image that contain each defined tissue type. A single area on the image cannot belong to more than one tissue category.

To draw tissue category regions on an image:

1. Select the **Draw** radio button next to the desired tissue category.
2. Click and drag to outline the desired area on the image.
3. If the region intersects another region in the same tissue category, the two regions are merged into one region.
4. If the new region intersects a region in a different tissue category, the area becomes part of the new tissue category.
5. The unselected parts of the image file are covered with a gray mask. This may make it difficult to see the image to select additional tissue categories. To temporarily hide the tissue categories and the gray mask, click the **Show/Hide Tissue Segmentation Map** button.

   After the next tissue category is drawn on the image, the tissue segmentation map displays again.
6. See Editing Tissue Category Regions 66 if necessary.
7. When all of the desired tissue categories have been drawn, continue with the next processing step in the project. Either click the **Advance** button or click the next step button.

9.3 Editing Tissue Category Regions

After tissue categories have been drawn on an image, you can edit the regions to add areas to a region, remove areas from a region, or remove individual pixels from a region.

To add an area to manually selected tissue categories:

1. Zoom in or out on the image to the desired magnification.
2. Click the option button next to the tissue category that you want to add an area to.
3. If desired, use the **Tissue Segmentation Map** button to show or hide the tissue masks. Hiding the tissue masks makes it easier to see the colors on the original image.

4. If Processing Regions have been defined, use the **Process Regions** button to show or hide the process region masks.

5. Click and drag to outline the area to be included, intersecting the defined tissue classification areas as desired.
6. Release the mouse button to connect the end point to the start point. The outlined area is assigned to the specified tissue category.

To remove an area from the tissue category:

1. Click the **Select Regions to Unclassify** button.

2. Click and drag to enclose the area from which you want to remove the tissue classification.
3. Release the mouse button to connect the end point to the start point. The outlined area is removed from the tissue category.

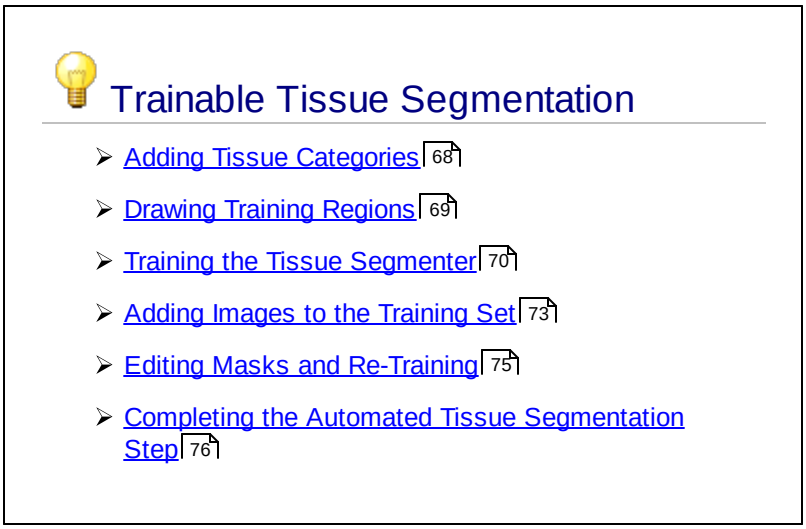
To remove single pixels from the tissue category:

1. Zoom in on the image.
2. Click the **Select Pixels to Unclassify** button.

3. Click a single pixel to remove it from the tissue classification region.
4. Click and drag to remove a line of pixels from the tissue classification region.

10 Trainable Tissue Segmentation

The topics in this section describe how to automatically classify tissue using the tissue segmenter. To set up the tissue segmenter, add tissue categories and draw training regions on images in the training set, train the tissue segmenter, segment images, and then fine tune the segmenter based on initial results.



10.1 Adding Tissue Categories

Tissue categories divide the image into specific areas that correspond to specific types of tissue (for example, cancer, necrosis, stroma, etc.) or occasionally other structures of interest. The Segment Tissue step (only available in inForm Tissue Finder) enables you to draw training regions around representative areas of tissue on images and then train the tissue segmenter to automatically locate similar regions of tissue in each image.

The **Tissue Segmentation Training** panel is used to define tissue categories, train segmenters, and segment images in the image processing set.

Creating Categories

1. Click the **Segment Tissue** step to display the *Tissue Segmentation Training* panel.
2. Decide how many tissue categories you want to segment the images into.
3. Click the **New** button and type the desired name of the tissue category in the text box.
4. Create the desired number of tissue categories for the images in the project.
5. To change the display color for a tissue category, click the **Color** pull-down and select the desired color.

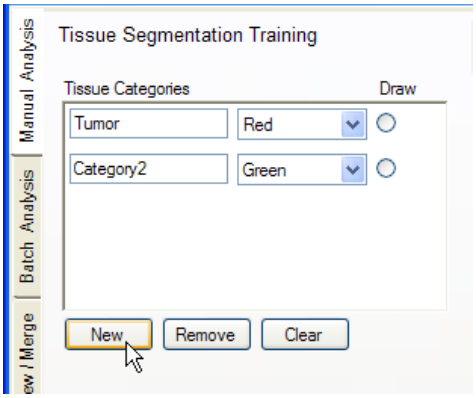


Figure 47. Creating Tissue Categories

Deleting Categories

To remove a category, select the category's radio button and click the **Remove** button.

To clear all tissue categories and start over, click the **Clear** button.

10.2 Drawing Training Regions

Training regions teach the tissue segmenter which cells should be included in each tissue category. It is important that you already know what to look for and are able to visually recognize structures characteristic of the tissue category. For example, if there are tumor cells present in any image that are negative (i.e., not stained), be sure to include those also. It is important to train on the full range of appearances of the tissue category, as in the example, where negatively-stained as well as the positively-stained cells are included.

To draw the training regions:

1. In the Tissue Segmentation Training panel, select the **Draw** radio button for a tissue category.
2. Click and drag to draw regions around groups of cells. (For example, if the tissue category is named *Tumor*, draw regions around groups of tumor cells).

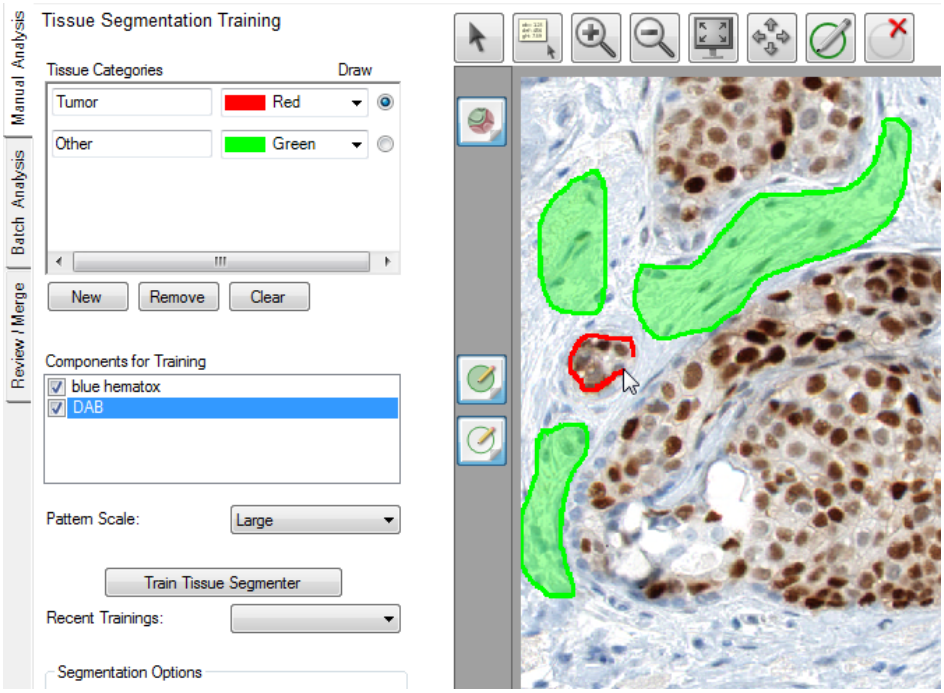


Figure 48. Drawing Training Regions

3. To delete a region, either click the **Delete Region** button and click inside the region to be deleted, or right-click the region and select **Delete Region** from the pop-up menu. (This menu also lets you delete all training regions or all within a selected category.)
4. When you are finished drawing regions for the first category, switch to the next category (e.g., non-tumor regions) and draw regions around cells characteristic of the next tissue category. Be sure to capture visual anomalies such as areas slightly out of focus, folds, bubbles, etc. Also capture stroma regions that have differing qualities of shape or orientation.
5. Repeat this process for all of the images in the training set.
6. When all training regions have been drawn, see Training the Tissue Segmenter 70.

10.3 Training the Tissue Segmenter

Only images that are included in the Training set are used to train the tissue segmenter. Images that have been removed from the training set are not used to train the tissue segmenter, even if the images include training regions.

Selecting Components for Training

Select the components that you want to use for training the tissue segmenter.

Components that provide distinguishing pattern information that is independent of signals to be quantified are preferred. For example, the counterstain component plane (e.g., DAPI or Hematoxylin) should be tried first for training because the counterstain reveals the structure of the tissue, and is less likely to produce a segmenter biased by the presence of the molecular marker you are trying to quantify. For example, if measuring the positivity of cells stained with Alexa Fluor® in a sample stained with both DAB and Alexa Fluor®, you would not want the segmenter to be dependent on the Alexa stain because that would bias the outcome.

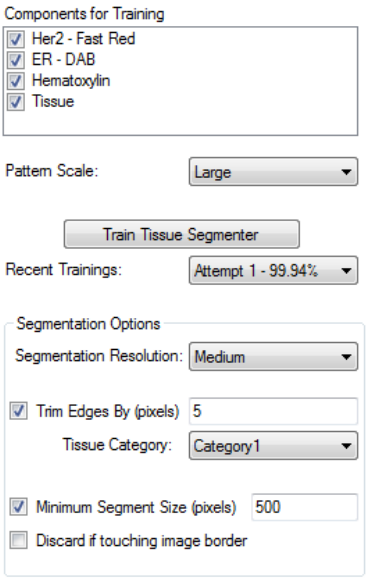


Figure 49. Components for Training

On the other hand, sometimes in brightfield applications where the presence of an IHC stain such as DAB blocks the staining of the counterstain, using both the counterstain and the IHC stain component planes for training yields a more robust tissue segmenter, even for the negative cases. In these cases where the target label is included in the training, it is important to include abundant negative cases in the training set.

Also, you should generally avoid using components that provide information unrelated to the desired segmentation.

Selecting a Pattern Scale

The recommended approach is to start with the default *Large* pattern scale. Once you have drawn training regions and trained a tissue segmenter, if segmentation accuracy for the training regions is low (<85%), increasing **Pattern Scale** may improve accuracy. However, the pattern scale needs to be compatible with the size of the training regions. Pattern scale refers to the spatial extent of patterns that are measured to support machine learning and image analysis.

If the selected pattern scale (e.g., *Maximum*) is too large for the drawn training regions, a grey mask displays on training regions that are too small after training and those training regions are not used to train the tissue segmenter.

You should use as large a pattern scale as is compatible with the training regions that you draw. If most of the training regions have gray masks over them after training, reduce the pattern scale (.e.g., from *Large* to *Medium*), and then retrain.

For example, if analyzing tumors in tissue sections in a 20x magnification image, the architecture is fundamentally on a large scale, and you probably want to select *Large*. For items with inherently fine

scale structures, select *Small* or *Medium*. An understanding of the different scales can be learned by seeing what size training regions are masked with grey after training.

Train the Segmenter

Click the **Train Tissue Segmenter** button to begin training the segmenter. The training process may take some time, so be patient.

The percent accuracy of the new tissue segmenter displays. If, for example, the segmenter was 99% accurate, this would mean that if the new segmenter was to be applied to all of the images used in training the segmenter, it would classify the pixels in the training regions with 99% accuracy. (Fewer than 1% of the pixels in all the drawn regions would be misclassified.)

The software continues to try to improve the accuracy. Click the **Stop** button to stop training when an acceptable level of accuracy is reached or the accuracy has stabilized. In general, segmenters should be at least 80% accurate. If the accuracy reaches 100%, a **Done** button displays to close the Algorithm Training dialog.

Recent Trainings

The **Recent Trainings** box records a history of segmenters trained using the current segmentation settings. For example, the first time you train is named “Attempt 1,” the second training is named “Attempt 2,” and so on. To re-classify the images using a previous segmenter, select the desired training attempt (i.e., segmenter) from this drop-down list. If you change any settings, the history of segmenters is cleared.

Segmentation Resolution

The **Segmentation Resolution** determines the resolution of the tissue category segmentation. There is a trade-off between resolution and segmentation speed, with an approximately 4x increase in segmentation speed for every step in resolution reduction. Select **Coarse** if you want to segment the images more quickly. Although the edges of the segmented regions appear pixelated, the segmenter still accurately differentiates structures well (e.g., tumor from stroma). Select **Fine** if the segmented regions need to have smoother edges, such as images for use in publications. Segmenting with **Fine** resolution takes longer than with **Coarse** resolution. Also, if you selected a small Pattern Scale, the Resolution must be set to **Medium** or finer.

Segmenting the Tissue in the Training Images

After you have trained the segmenter, you can segment the tissue in the current image or all of the images in the training set. You can segment just the current image first, to test the tissue segmenter. Then make any desired adjustments to the training parameters and retrain the segmenter. To see the Tissue Category mask on all images, click the **Segment All** button. When you are ready, advance to the next step in the project.

Fine Tuning the Segmenter

Observe the training regions following the segmenter training. As mentioned above, if the software grays out any region, this indicates that the region is too small for the selected **Pattern Scale** and was not used to train the tissue segmenter. You can ignore this if segmentation performance is adequate, delete the region and redraw a larger one, or try selecting a smaller pattern scale.

The **Trim Edges** option can be used to "erode" a tissue segmentation by removing the specified number of pixels from the periphery of the selected tissue category, making it smaller. This is useful if the tissue segmenter has a tendency to include pixels extending beyond the edges of objects or tissue categories of interest (e.g., tumor or vessels). For example, if you notice cancer regions extending into non-cancerous areas, select the **Trim Edges** option, choose the cancer tissue category, and enter the number of pixels you want to trim from the borders of the cancer regions.

The **Minimum Segment Size** option is an adjustable parameter (in pixels) that removes segments that have fewer pixels than this value. The removed segment is replaced by the tissue segment that is most abundant around the segment that was removed.

If the **Discard if Touching Image Border** check box is selected, tissue regions touching the edge of the image or process region are discarded. If not selected, all tissue regions are included.

10.4 Adding Images to the Training Set

When selecting images for the training set, it is recommended that you start with one image per distinct appearance. The training set should represent the range of staining levels and tissue architectures that you want to train the tissue segmenter to identify.

Whenever you draw a training region on an image (see Drawing Training Regions 69), inForm automatically adds the image to the training set. You can also add or remove images from the training set manually as described below.

1. Click the **Image List Editor** button to open the Available Images Window 27.

2. Select the **Training Set** check box next to the images to add the image to the Training set.
3. Clear the **Training Set** check box next to an image to remove the image from the Training set.
4. Close the Available Images window.
5. When you switch to Single mode, a small blue square on each thumbnail image at the bottom of the screen (see figure) indicates images are included in the training set. A green square indicates that the image is in the processing set.

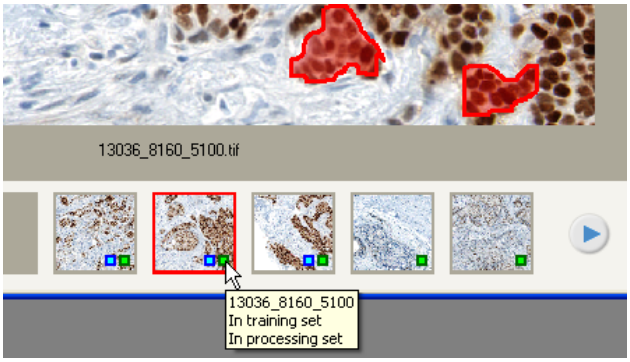


Figure 50. Training Set and Processing Set Indicators

6. See Drawing Training Regions 69 to add training regions to the desired images in the training set.

10.5 Editing Masks and Re-Training

After reviewing the segmented images, you might discover that some structures were not segmented correctly, or the segmenter may have selected some unwanted regions. The Tissue Segmentation masks should accurately cover their respective tissue categories.

To correct the tissue masks, you can either edit the mask manually, or you can re-train the tissue segmenter to detect the desired regions.

Manually Editing the Tissue Segmentation Mask

If the experiment or study involves few enough images, you can manually edit the Tissue Segmentation mask. Manual editing does not change the tissue segmenter and if the images are re-segmented, the changes to the Tissue Segmentation masks are lost.

To manually add areas to a Tissue Segmentation mask:

1. Click the **Edit Tissue Segmentation** button on the toolbar.
2. From the drop-down list, select the tissue segmentation mask you want to edit.
3. Draw around the areas on the image that you want to include in the selected Tissue Segmentation mask.
4. To continue processing the images using the edited Tissue Segmentation masks, advance to the next step without re-segmenting the images.
5. If the project contains a cell segmentation step and cell segmentation was performed before the mask was edited (see Segmenting Cells 82), you should click the **Segment Images** button in the **Cell Segmentation** editor to re-segment the cells in the edited tissue segmentations.

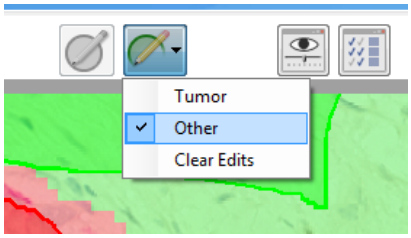


Figure 51. Editing Tissue Segmentation Masks

Re-Training the Tissue Segmenter

If some areas of the image were not classified as the desired tissue type, you can re-draw the training regions around the areas that were incorrectly classified and re-train the Tissue Segmenter.

To re-train the tissue segmenter:

1. In the Tissue Segmentation Training panel, select the **Draw** radio button for the tissue category that you want to draw additional training regions for.
2. Draw training regions around areas that were incorrectly classified.
3. Re-train the tissue segmenter and then re-segment the image. Verify that the desired regions are now correctly classified in the desired Tissue Segmentation mask.
4. To see the tissue segmentation on all images, segment all images. (Note that re-segmenting an image after retraining the tissue segmenter deletes any manual edits to the Tissue Segmentation mask.)

10.6 Completing the Automated Tissue Segmentation Step

After all of the desired settings have been selected in the Tissue Segmentation Training panel and the Tissue Segmenter has been trained, process the images using any of the methods below and then advance to the next step in the project.

- Click the **Segment Image** button to segment the currently selected image.
- Click the **Segment All** button to segment all images open in the project.
- Click the **Advance** button to display the next step in the project. (The tissue is segmented the next time images are processed.)
- Click the next step in the project in the Step Bar[19] at the top of the inForm window. The settings panel for the next step displays. (The tissue is segmented the next time images are processed.)

11 Threshold

Use the Threshold step to set threshold values for any of the unmixed components in an image and then to view the regions on the image that are above the threshold. Statistics are calculated for the thresholded regions and are displayed in the Quant Data table.

To set the thresholds and calculate the threshold statistics for the images:

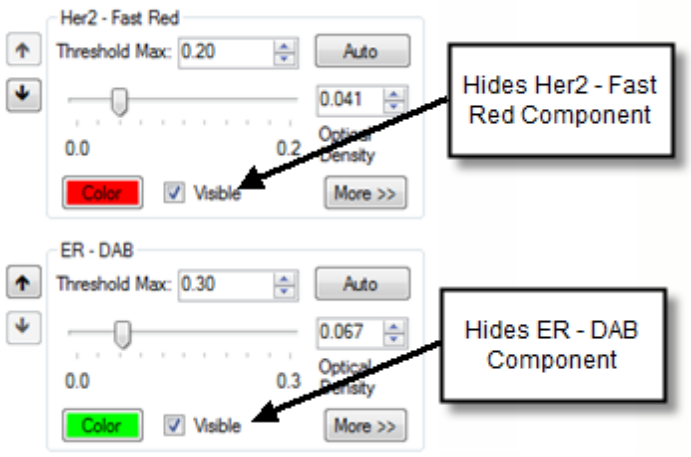
1. Click the **Threshold** button in the step bar.

2. In the **Components to Threshold** list, select each of the image components you want to create a threshold map for.
3. Click the **Auto** button to calculate a preliminary value for the Threshold Max and to choose a preliminary Threshold value. The **Threshold Max** is set to the value of the brightest pixel in the component. The slider range is set to (0 - Threshold Max). The threshold is set to an optimal value, based on the component values. (The threshold max and threshold can be set manually without clicking the Auto button, if desired.)
4. Set the desired Threshold for each unmixed component. Move the slider, type the desired value or click the up and down arrow buttons to select the threshold. The image display shows the areas that are above the threshold value as the slider is adjusted.
5. To change the color displayed for the regions, click the **Color** button and select the desired color.
6. To change the minimum size of the thresholded regions:
   - Click the **More** button under the name of the Threshold map that you want to change.
   - Set the desired value for **Minimum Connected Pixels** by either clicking the Up or Down arrow buttons or typing the value in the text box. The Minimum Connected Pixels specifies a minimum region size based on the number of pixels. Only regions that are larger than the Minimum Connected Pixels value are considered. Regions with fewer than this number of pixels are ignored.
7. If there are multiple images in the project, either select each image and apply the current settings to the image by clicking the **Accept for Image** button or click the **Accept for All** button to apply the Threshold settings to all of the images in the project. Verify that the threshold settings are appropriate for all images in the project and adjust if necessary.
8. If desired, change the threshold map view 78 or edit the threshold maps 79.
9. To view the statistics for the generated regions in the Quant data table, see Viewing the Quant Data Table 122, or to export the data, see Exporting the Data 129.

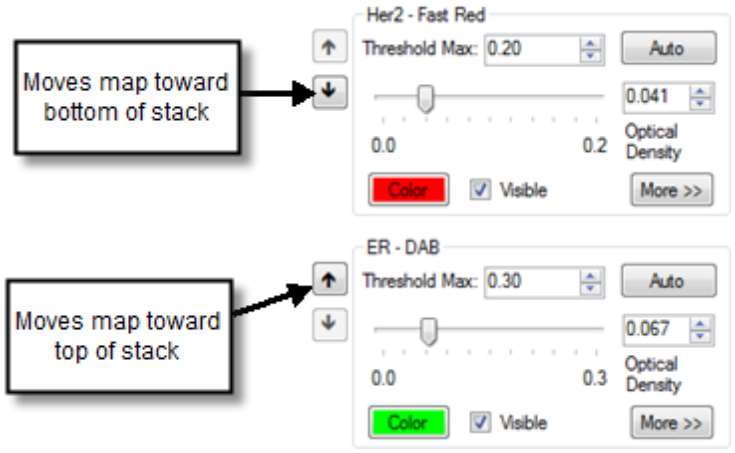
Changing the Threshold Map View

The threshold maps for each component are stacked in layers on top of the original graphic, with the top component in the Simple Threshold Settings panel as the uppermost layer in the image display. You can change the order of the threshold maps or hide threshold maps.

1. To hide a threshold map, clear the **Visible** check box under the component that you want to hide.



2. To move a threshold map up or down in the stack of maps, click the **Up or Down arrows** on the left of the **Color** button.



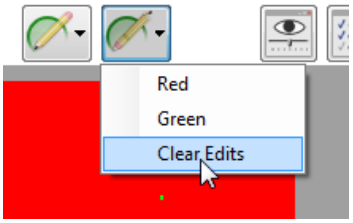
Editing Threshold Maps

Threshold maps can be edited manually. Additional areas can be drawn onto the threshold map or undesired areas can be erased from the threshold map. This procedure describes how to use the editing tools on the Simple Threshold Settings panel. The Add Thresholded Regions and Erase Thresholded Regions buttons on the toolbar can also be used.

1. To view editing options, click the **More** button under the name of the Threshold map that you want to edit.
2. To draw additional threshold regions, click the **Add** button under the component name and draw the desired regions on the threshold map.
3. To remove threshold regions, click the **Erase** button and draw around the area to be removed. Only the threshold areas for the selected component are erased.
4. To clear all manual edits from a threshold mask, click the **Clear** button under the name of the component. Only the manual edits on the threshold map for that component are cleared.

To clear all manual edits from all threshold maps:

1. Click the **down arrow** next to the **Erase Threshold Regions** button and click **Clear Edits**.



2. Click **Yes** in the Clear Mask Edits window.

12 Colocalization

Colocalization is a pixel-based analysis used to find overlapping components in images based on thresholds set for each component. Colocalization of multiple markers can be determined and displayed both visually and statistically. It is designed for analyzing and quantitating molecular markers and can be used to determine the amounts of colocalization of multiple markers.

1. Click the **Colocalization** button in the step bar.

2. Under **Markers for Colocalization**, select the markers for which you want to see overlap (colocalization) and positivity percentages.
3. Under **Denominator (Counterstain)**, select the marker(s) to use as the counterstains. To see colocalization of markers throughout the entire image and not just within a particular counterstain, select All Image Pixels.
4. For each component selected in the Markers or Denominator, a **Threshold** settings area displays below the Denominator check boxes.
5. To adjust the Threshold for each component:
   a. Click the **Auto** button to calculate a preliminary value for the Threshold Max and to choose a preliminary Threshold value. The **Threshold Max** is set to the value of the brightest pixel in the component. The slider range is set to (0 - Threshold Max). The threshold is set to an optimal value, based on the component values. (The threshold max and threshold can be set manually without clicking the **Auto** button, if desired.)
   b. Set the desired Threshold for each unmixed component. Move the slider, type the desired value, or click the **up** and **down** arrow buttons to set the threshold. The image display shows the areas that are above the threshold value as the slider is adjusted.
   c. To change the color of a threshold mask, click the **Color** button and select the desired color.

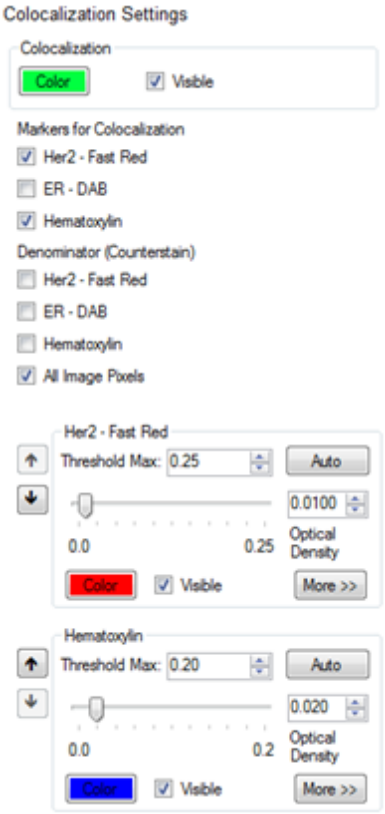


Figure 52. Colocalization Settings

6. To view the threshold for each component, clear the **Visible** check box for all components except the component that you are adjusting. Clear the **Visible** check box for Colocalization. Adjust the Threshold for the component until all of the desired areas are marked as positive.

7. If there are small regions that are marked as positive that should not be included, or if smaller regions should be marked as positive and are not, click the **More** button and adjust the **Minimum Connected Pixels** value as desired.

8. Under **Colocalization**, click the **Visible** check box to show the colocalization map. If desired, click the **Color** button and select the color to use to indicate areas of colocalization.

9. To view the Colocalization statistics, see Viewing the Quant Data Table 122 and Viewing the Colocalization Data Table 120.

10. To export the data, see Exporting the Data 129.

13 Segmenting Cells

The Segment Cells step is only available in inForm Cell Analysis and inForm Tissue Finder.

NOTE: Cells are considered to be an assembly of associated cellular components (nucleus, cytoplasm, and membrane). If you are segmenting tissue, some cells may straddle two segmented tissue regions. If a cell is not entirely contained within a single region, the "containing region" is undefined, and the Region ID in the Data tables is #N/A (see Displaying the Extracted Data 108).

inForm contains two approaches for segmenting cells:

1. Adaptive Cell Segmentation uses an adaptive thresholding algorithm to identify nuclear pixels, and is able to use both nuclear and membrane signals to guide nuclear splitting. It performs best on fluorescence imagery, especially imagery that contains membrane staining (see Adaptive Cell Segmentation 82).
2. Cell Segmentation uses classical segmentation methods to locate the nuclear, cytoplasmic and membrane compartments. It may outperform the Adaptive Cell Segmentation on brightfield imagery (see Cell Segmentation 89).

13.1 Adaptive Cell Segmentation

Adaptive Cell Segmentation allows you to use algorithms that account for variations in staining and background levels within and across images to identify cellular compartments from multiple image planes. These features provide improved segmentation performance when images contain mixtures of cell types with very different nuclear morphologies.

Adaptive cell segmentation consists of following steps. We recommend that you optimize the settings at each step before moving on to the next one.

1. Select cellular compartments and image planes to aid segmentation. See Selecting Segmentation Components 83.
2. Detect nuclear pixels using image planes associated with one or more nuclear stains. See Detecting Nuclear Pixels 83.
3. Split clusters of detected nuclear pixels into individual nuclei using:
   a. Image planes associated with nuclear stains (splits occur at valleys between nuclear signals). See Splitting Nuclear Clusters Using Nuclear Component Images 85.
   b. (optional) Image planes associated with assisting cytoplasmic or membrane stains (splits occur at ridges of positive membrane or cytoplasmic signal). See Splitting Nuclear Clusters Using Membrane or Cytoplasm Component Images 86.
4. Refine nuclear shapes. See Refining Nuclear Shapes 87.
5. (optional) Define cytoplasm and membrane around all nuclei. See Defining Cytoplasm 87 and Defining Membrane 87.

Use the **Cell Segmentation Settings** panel to set parameters for segmenting cells into their respective compartments. This allows you to extract and analyze data on a per-cell or per-compartment basis.

13.1.1 Selecting Segmentation Components and Detecting Nuclear Pixels

Use the following steps to choose which cellular compartments to segment, configure image planes, and detect nuclear pixels:

1. Choose which cellular compartments you want to segment: **Nuclei**, **Cytoplasm**, and/or **Membrane**. (**Note**: Adaptive Cell Segmentation can only detect cells with nuclear signal, so 'Nuclei' cannot be unselected). You can also choose to segment Cytoplasm and/or Membrane.

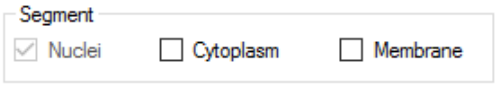


Figure 53. Adaptive Cell Segmentation Settings

NOTE: *We recommend that you wait to add Cytoplasm and Membrane options until you have optimized Nuclei detection and splitting settings. This will reduce visual clutter in the image. Adding those two options later will not alter any parameter adjustments to Nuclei settings.*

2. Click the **Add…** button to add image components to be used for segmentation. Then select the image component name from the drop-down list. Choose your strongest, most abundant nuclear component first. You can repeat this step to include additional nuclear, cytoplasmic, and/or membrane components if they will aid segmentation. **Only add components that show strong, specific staining. Adding components with weak or nonspecific signal can lead to undesired segmentation results**. All image components can still be analyzed within the segmented cell compartments even if those components are not configured to aid in segmentation here.

Figure 54. Adaptive Cell Segmentation Components

NOTE: *Priority will be given to nuclear splitting lines that are attributed to image components higher up in the list of components. So we recommend you add your strongest and most specifically stained components first in this list.*

3. Configure each component by pressing the '**...**' button. Select the compartment where this component is located.

4. For nuclear components, use the **Typical Intensity** slider to adjust the threshold used to detect nuclear pixels. This threshold is adaptive and is measured relative to nearby background. Increase this value if too much background is being detected as nuclear. Lower this value if faint nuclei are being missed. At this point, do not focus on whether nuclei are correctly split from each other (splitting will be addressed in subsequent steps). Only focus on whether the correct pixels are being detected as nuclear.

   NOTE: *When adjusting the Typical Intensity slider, you can monitor whether all the intended pixels are being detected by viewing only the image plane associated with the nuclear component you are configuring. Use the View Editor 25 to change the image view. Use the* ***show/hide regions*** *button to blink the segmentation masks on and off as needed.*

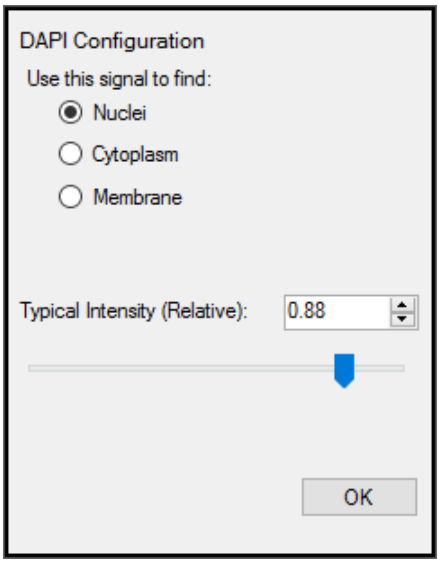


Figure 55. Adaptive Cell Segmentation Configuration

5. Once at least one image component has been configured, a live preview region will appear in the current image. This preview provides live feedback as you adjust settings such as the Typical Intensity. Click and drag the region to preview other areas in the image. Click and drag the image outside the preview region to drag the entire image.

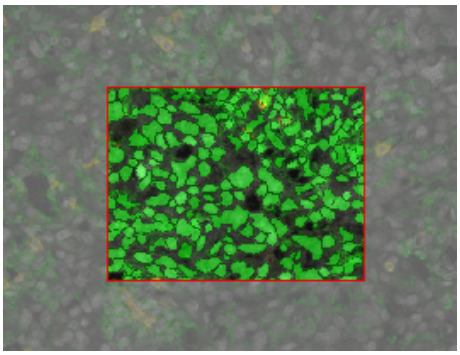


Figure 56. Live Preview Image

6. Click **Hide Preview** to hide the preview. Click the same button to show the preview again. The preview is only available when the selected segmentation settings are valid.

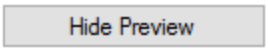


Figure 57. Hide Preview

7. If a component belongs to the cytoplasm or membrane, you can also select the **Use this signal to assist in nuclear splitting** check box. If that box is checked, signals in the associated image plane will be used to generate potential split lines within clusters of nuclei. We recommend only checking this box for image components that have strong, specific signals.

   NOTE: *All nuclear component planes are used to assist nuclear splitting regardless of which other cytoplasmic or membrane components have been selected. We recommend you wait to check the **Use this signal to assist in nuclear splitting** box until after you optimize nuclear splitting for nuclear component planes first. You may also want to assess the changes to the segmentation results after each box is checked individually so the added value of each component is more obvious.*

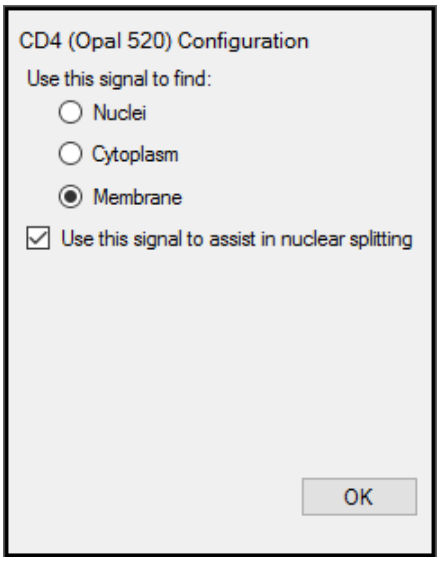


Figure 58. Use this Signal to Assist in Nuclear Splitting

13.1.2 Splitting Nuclear Clusters into Individual Nuclei

Clusters of nuclear pixels will be split into individual nuclei using image planes from nuclear components and from image planes from assisting membrane and cytoplasm components (if selected).

Splitting Nuclear Clusters Using Nuclear Component Images

Use the **Nuclear Component Splitting** panel to adjust splitting parameters used for image planes associated with nuclear components:

NOTE: *These settings will be applied to all nuclear components.*

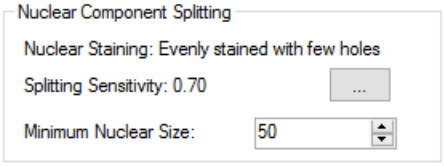


Figure 59. Nuclear Component Splitting

1. Configure the splitting sensitivity by clicking the '**...**' button. Choose the radio button that best describes the staining quality of all the selected nuclear planes (you may need to experiment with this.) Then use the slider bar to adjust the splitting threshold. Lower values will result in more splitting.
2. Set the Minimum Nuclear Size (in number of pixels). Splitting lines that generate nuclei smaller than this size will be ignored. Any isolated nuclei that are smaller than this value will also be discarded.

   NOTE: *You can position your cursor over segmented compartments in the image to display characteristics of the compartment, including nuclear size. This may help you choose an appropriate Minimum Nuclear Size.*

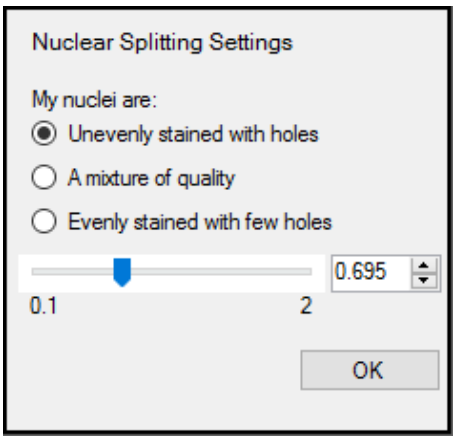


Figure 60. Nuclear Splitting Settings

Splitting Nuclear Clusters Using Membrane or Cytoplasm Component Images

Use the **Assisting Component Splitting** panel to adjust parameters for cytoplasmic and/or membrane image planes:

NOTE: *These settings will be applied to all cytoplasmic and membrane components that were selected with the **Use this signal to assist in nuclear splitting** check boxes. This panel will only be visible if at least one assisting component was selected.*

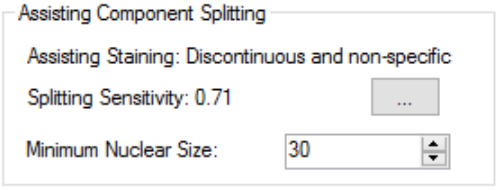


Figure 61. Assisting Component Splitting

1. Click the '**...**' button to configure the splitting sensitivity. Choose the radio button that best describes the staining quality of all the assisting membrane and cytoplasmic planes (you may need to experiment with this). Then use the slider bar to adjust the splitting threshold. Lower values will result in more splitting.
2. Set the Minimum Nuclear Size (in number of pixels). The system will ignore splitting lines that generate nuclei smaller than this size. If you are more confident in the splitting lines generated by the assisting components than those generated by the nuclear components, (or if the cells that have membrane signals tend to be smaller than those that do not), you may want to set this value to be smaller than the Minimum Nuclear Size selected in the **Nuclear Component Splitting** panel.

   NOTE: *For help choosing an appropriate Minimum Nuclear Size, you can position your cursor over segmented compartments in the image*

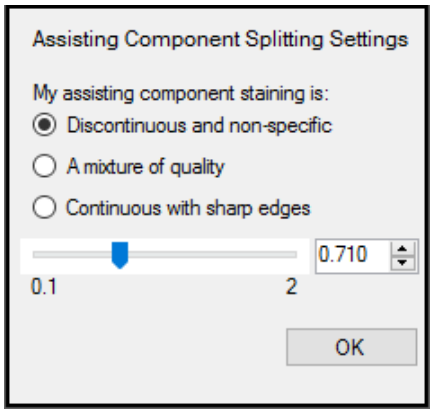


Figure 62. Assisting Component Splitting Settings

to display characteristics of the compartment, including nuclear size.

13.1.3 Refining Nuclear Shapes

After optimizing detection and splitting of nuclei, use the **Other Settings** panel to further refine the results:

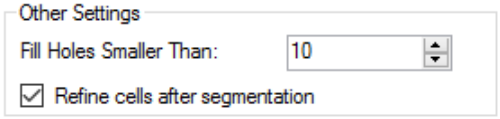


Figure 63. Other Settings

1. If you find that some nuclei contain empty 'holes', increase the **Fill Holes Smaller Than** value until those holes are filled.
2. If you find that the detected membrane shapes are more jagged or angular than desired, check the Refine cells after segmentation box to compact the cells into rounder shapes.

13.1.4 Defining Cytoplasm

Use the **Cytoplasm Settings** panel to segment Cytoplasm:

Figure 64. Cytoplasm Settings

1. Adjust the Cytoplasm Thickness to define the distance (in pixels) from the boundary of each cell's nucleus to the outer boundary of its cytoplasm. For each cell, all pixels between the nuclear boundary and the cytoplasm outer boundary will be defined as cytoplasm. This distance should be adjusted based on visual inspection of the results, so that cytoplasm segmentation approximately matches the average size of cells.

   NOTE: *Cytoplasm segmentation does not take into account any signals from cytoplasm component images.*

13.1.5 Defining Membrane

Use the **Membrane Settings** panel to segment Membrane:

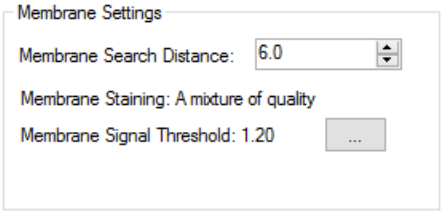


Figure 65. Membrane Settings

1. Adjust the Membrane Search Distance to define the distance (in pixels) from the boundary of each cell's nucleus to search for membrane signals.
   - If positive signals are detected between the nucleus boundary and the Membrane Search Distance, the membrane will be drawn where the positive signal is detected.
   - If no positive signal is found between the nucleus boundary and the Membrane Search

Distance, the membrane will be drawn at the nearest of three stop points:

- Membrane Search Distance
- A neighboring cell membrane
- Cytoplasm Thickness (if Cytoplasm segmentation was selected)

NOTE: *By setting the Membrane Search Distance to be larger than Cytoplasm Thickness, you allow the algorithm to search a large distance for true positive membrane signals, but to collapse back to a smaller cell size if no positive signals are found. For this added flexibility, you may want to include Cytoplasm segmentation even if you are not directly interested in the Cytoplasm compartment.*

NOTE: *If the membrane is drawn at positive membrane signals found at a distance between the Cytoplasm Thickness and the Membrane Search Distance, the resulting cytoplasm will span the entire area between the nucleus and the membrane, even if that results in a cytoplasm thicker than the chosen Cytoplasm Thickness value.*

2. Click the '**…**' button to configure the sensitivity of membrane signal detection. Select the best description of the staining quality of the configured membrane components (you may need to experiment with this). Then use the slider bar to adjust the membrane detection threshold. Lower values will result in detecting weaker signals.

NOTE: *These settings will be applied to all components configured as 'Membrane'.*

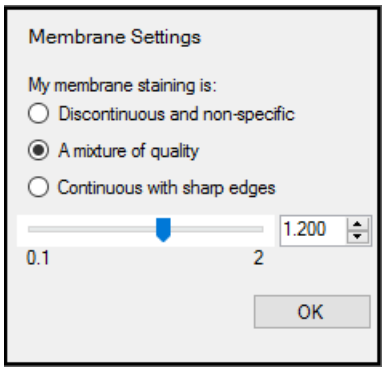


Figure 66. Membrane Signal Detection

NOTE: *If you selected both Cytoplasm and Membrane, you can set both via the Membrane and Cytoplasm Settings panel.*

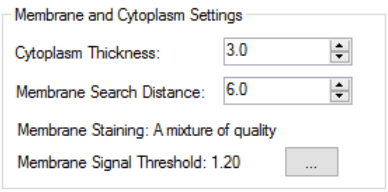


Figure 67. Membrane and Cytoplasm Settings

13.2 Cell Segmentation

Use the **Segment Cells** panel to set parameters for segmenting cells into their respective categories so that data can be extracted and analyzed on a per-cell basis.

To segment cells:

1. Select the desired cell components to segment: Nuclei, Cytoplasm, and Membrane. Cytoplasm and Membrane can only be selected after Nuclei is selected.
2. Select **Discard if Touching an Edge** to discard any cell where any cell component touches the edge of the image, process region, or tissue region, if applicable. Only the cells that are entirely within the image, a process region, if applicable, and the selected tissue category, if applicable, are included in the segmentation map. If not selected, cells are clipped at the edge of the image or region. If a cell is clipped at the edge of a process region or tissue category region, the size of the nucleus or cytoplasm inside the region may be smaller that the specified Minimum Size.
3. If Nuclei is selected, click the **Nuclei** tab and select the desired parameters. Use the preview window to determine the optimal parameters for segmentation. See Segmenting Nuclei 90 for descriptions of the parameters.
4. If Cytoplasm is selected, click the **Cytoplasm** tab and select the desired parameters. Use the preview window to determine the optimal parameters for segmentation. See Segmenting Cytoplasm 94 for descriptions of the parameters.
5. If Membrane is selected, click the **Membrane** tab and select the desired parameters. Use the preview window to determine the optimal parameters for segmentation. See Segmenting Membrane 96 for descriptions of the parameters.
6. Click the **Segment Image** button to segment the current image using the selected parameters.
7. If desired, change the parameters to obtain the desired results.
8. Click the **Segment All** button to segment all of the images.

13.2.1 Segmenting Nuclei

At the top of the **Cell Segmentation Settings** panel, select **Nuclei** in the **Compartments to Segment** box. (You can also select **Cytoplasm** and **Membrane** if you intend to find those as well.) Click the **Nuclei** tab.

Selecting a Tissue Category

Select the **Tissue Category** in which to find the cell nuclei or select All Categories to find cell nuclei in all tissue categories. Cells that are outside the chosen category are ignored. This option only displays if tissue has been manually or automatically segmented.

Tissue categories of interest might be *tumor* regions in oncology samples, and *islet* cells in pancreas samples.

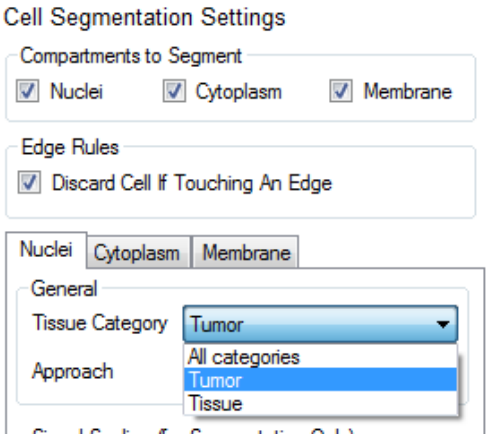


Figure 68. Cell Segmentation Settings for Nuclei

Selecting an Approach

- Select the **Pixel-Based (Threshold)** approach when there is a reliable nuclear counterstain, and nuclear pixels can be found by applying a simple threshold. This approach is purely pixel-based. This approach can also be used for other image analysis needs that can be satisfied with a simple threshold, such as detecting all pixels within a tissue category that stain positive for an IHC stain.
- Select the **Object-Based** approach if the nuclear counterstain does not provide consistent and specific staining of nuclear objects, and more advanced morphometry-based approaches are needed to detect nuclei.

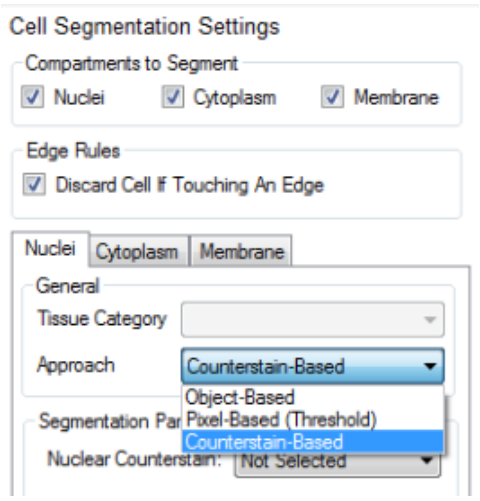


Figure 69. Cell Segmentation Approach

Pixel and Object-Based Settings

- **Auto Scale:** Select **Auto Scale** if you want the software to automatically scale each nuclear counterstain component plane individually before performing nuclei segmentation. This approach is a good place to start, and is useful if nuclear counterstain signals vary widely. Auto Scale is often sufficient for fluorescence applications.
- **Fixed Scale:** If you need better nuclear segmentation performance, and counterstain signals are consistent and reliable, you might get better results by selecting the **Fixed Scale** option. (The units are in parentheses.) This requires entering a scaling value, which can be determined by choosing the **View Component Data** tool from the toolbar and "mousing around" on the brightest nuclei to see the signal levels of the component being used for nuclear segmentation. Everything in a nuclear segmentation (indicated by a green region) should be nuclei.
    - Reduce the maximum **OD** or **Counts** value if nuclear segmentations are smaller than the nuclei, or if no nuclear segmentations occur when the segmenter is finished.
    - Increase the scale if segmentation is too sensitive and is finding many objects that are weakly stained and not nuclei. Adjust the scale until the unwanted regions are not detected.

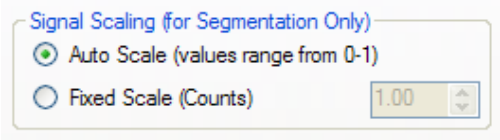


Figure 70. Signal Scaling

You can further add to what you consider nuclear pixels by including other components in the underlying signal. Pick an individual component signal as the primary, and then select secondary and tertiary components if desired. Enter a minimum signal value for each component. In **Auto Scale** mode, the signal range for each component is 0 to 1. If you selected the **Fixed Scale** option, the range for each component is from 0 to 2 for OD images, 0 to 255 for 8-bit images, and 0 to the Fixed Scale value for all other images. Also, whenever you switch between auto scale and fixed scale, the values are reset to zero.

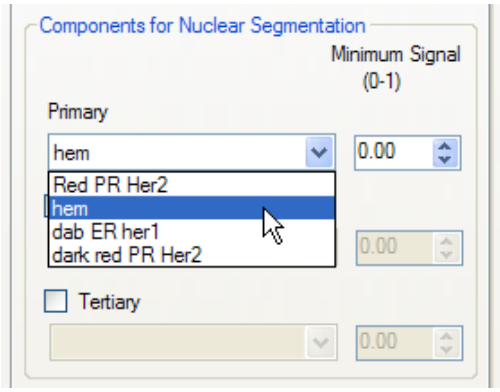


Figure 71. Components for Nuclear Segmentation

Only pixels above the minimum signal value are counted as nuclei. Using secondary and tertiary components is primarily useful in brightfield IHC applications where nuclear IHC stains are dark or "intense" and substantially block the Hematoxylin counterstain. In these cases, performing nuclear

segmentation on the logical intersection of the Hematoxylin and nuclear IHC signals is more effective. This approach does not work if the dark IHC stains reside outside the nucleus.

Once at least one component has been selected, a live preview region will appear in the current image. This preview provides live feedback as you adjust settings.

Click and drag the region to preview other areas in the image. Click and drag the image outside the preview region to drag the entire image.

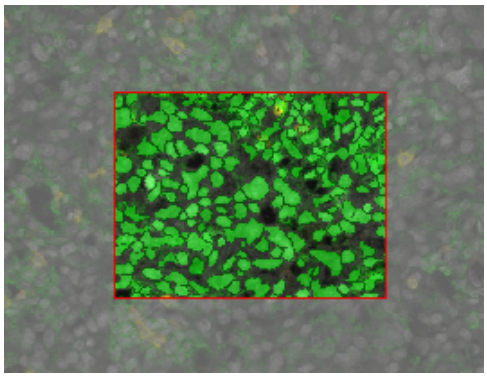


Figure 72. Live Preview Image

Click **Hide Preview** to hide the preview. Click the same button to show the preview again. The preview is only available when the selected segmentation settings are valid.

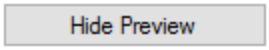


Figure 73. Hide Preview

Set the **Minimum Size**. If too many small, unwanted structures (e.g., not nuclei) are found during segmentation, try increasing the minimum size value. Any nuclei that have fewer pixels than this number are not segmented, and no associated data is collected. If a nucleus is clipped at the edge of a Process region or Tissue Category region, the original size of the nucleus is used to determine if it meets the minimum size threshold; the clipped size is reported in the tables. For images taken at 20x magnification, 100 is a good starting value.

Figure 74. Size Range

If appropriate, set the **Maximum Size**. This is required if you intend to select the **Refine Splitting** clean-up option discussed below. This sets an upper limit on nuclear segmentations (in number of pixels). Nuclear segmentations larger than this number are ignored. This can be useful if you want to ignore large clusters of nuclei, or if there are other objects in the classified region of interest that are not nuclei.

Select **Fill Holes** when some nuclei end up segmented with “holes” in them. This results from a common effect in tissue sections where nuclei appear hollow. Checking this check box automatically fills holes in nuclei.

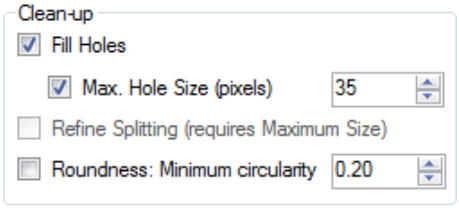


Figure 75. Clean-up options

If you want only those nuclei holes that are smaller than a certain size to be filled in, select **Max. Hole Size (pixels)** and enter the maximum number of

pixels. Nuclei with holes that are larger than this value are not filled.

If you select **Refine Splitting**, nuclei that are touching each other are split into separate nuclei instead of being detected as one large nucleus. This option is available only if **Maximum Size** is selected. Cell segmentation may take considerably longer when using this function. If you do not select **Refine Splitting**, nuclei that touch are detected as a single nucleus.

Select **Roundness: Minimum circularity** to find only nuclei that are more round. Zero returns any shape. Higher decimal values (up to 1) restrict selections to more round objects only.

13.2.2 Segmenting Cytoplasm

At the top of the **Cell Segmentation Settings** panel, select the **Cytoplasm** check box in the **Compartments to Segment** box. (Note that you must segment the nuclei in addition to segmenting cytoplasm. inForm only searches for cytoplasm where nuclei have been found.) Click the **Cytoplasm** tab.

Selecting Cytoplasm Shape Parameters

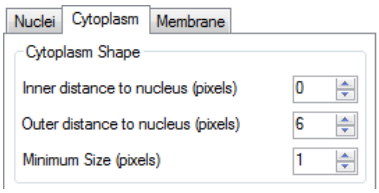


Figure 76. Cell Segmentation Settings for Cytoplasm

- **Inner distance to nucleus:** The distance (in pixels) from the edge of nuclear segmentations to the inner edge of the cytoplasm annular regions drawn around nuclei. This buffer distance should be large enough to ensure that imperfections in the nuclear segmentation do not lead to signal from nuclei inadvertently becoming part of cytoplasm signals.
- **Outer distance to nucleus:** The distance (in pixels) from the edge of the nuclear segmentation to the outer boundary of cytoplasm segmentation. This distance should be adjusted based on visual inspection of the results, so that cytoplasm segmentation approximately matches the average size of cells. This distance can be set large enough to include membrane signals if desired. In this case, it is important to note that the signal extracted from the segmenter for cytoplasm is a mixture of membrane and cytoplasm signal.
- **Minimum Size:** The minimum cytoplasm sample size (in pixels). If cytoplasm segmentations for detected nuclei have fewer pixels than this number, those cells are excluded from the analysis. This is common in cases where cells are tightly packed together without much space between nuclei for cytoplasm areas to be segmented. If the cytoplasm is clipped at the edge of a Process region or Tissue Category region, the original size of the cytoplasm is used to determine if it meets the minimum size threshold; the clipped size is reported in the tables.

Selecting Components to Include

You can further restrict what you consider “valid” cytoplasm based on the underlying signal. You can pick an individual component signal as the First, and then select a Second, a Third, and even a Fourth components if desired, and enter a signal range for each one. Or select *"Every Component"* or *“Any component”* and specify a signal range. Only pixels that fall within that signal range are counted as cytoplasm. (The signal range varies based on image type.)

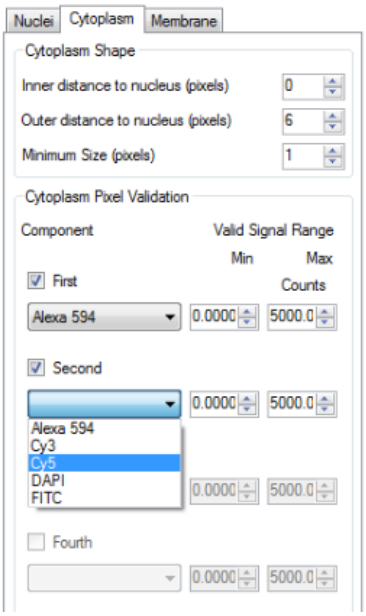


Figure 77. Cytoplasm Pixel Validation

13.2.3 Segmenting Membrane

At the top of the **Cell Segmentation Settings** panel, select the **Membrane** check box in the **Compartments to Segment** box. (Note that you must segment the nuclei in addition to segmenting membrane. inForm only searches for cytoplasm where nuclei have been found.) Click the **Membrane** tab.

Selecting Components for Membrane Segmentation

1. Select the **First** component signal for membrane segmentation.
2. Select the **Second, Third, and Fourth** component if desired.
3. For each component, set the **Full Scale:**
   - Select an image that is typical of all of the images to be analyzed.
   - Click the **Auto** button to find the brightest pixels in the image for each of the selected components. The brightest value for each component displays in the Full Scale box for each component. (The units can be counts or OD.)

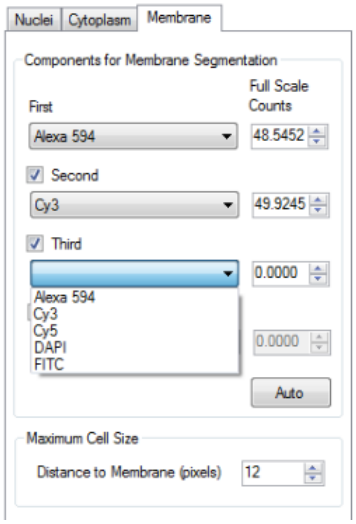


Figure 78. Components for Membrane Segmentation

Specifying a Maximum Cell Size

The **Distance to Membrane** value specifies the maximum size of the cells, defined here as the distance (in pixels) from the edge of the nucleus to the outer edge of the membrane. A value of 12 for images taken at 20x magnification is usually a good starting value. If the algorithm is finding a membrane that is either too large or too small based on your knowledge of the nucleus/cell size, then try reducing (or increasing) the **Distance to Membrane** value. This value limits how far from the nucleus the algorithm is permitted to go when segmenting the cell membrane.

Figure 79. Maximum Cell Size

Earlier versions of inForm provided a "Membrane Priority" option. This has been set to always prioritize membrane. New membrane splitting options are available in Segmenting Nuclei 90.

14 Phenotyping Cells

You must have first performed cell segmentation in order to proceed with phenotyping. Naturally, accurate cell segmentation is required in order to obtain accurate cell phenotyping. Like inForm's tissue segmentation features, inForm's phenotyping feature is trainable, saving you from the burden of directly writing algorithms and manually programming the software.

Load a project if one is not already open in inForm, and configure the project to include the Phenotyping step. Perform your segmentation operations if you have not already done so. When you advance to the Phenotyping step, you will see the Phenotyping Settings to the left of the image display area.

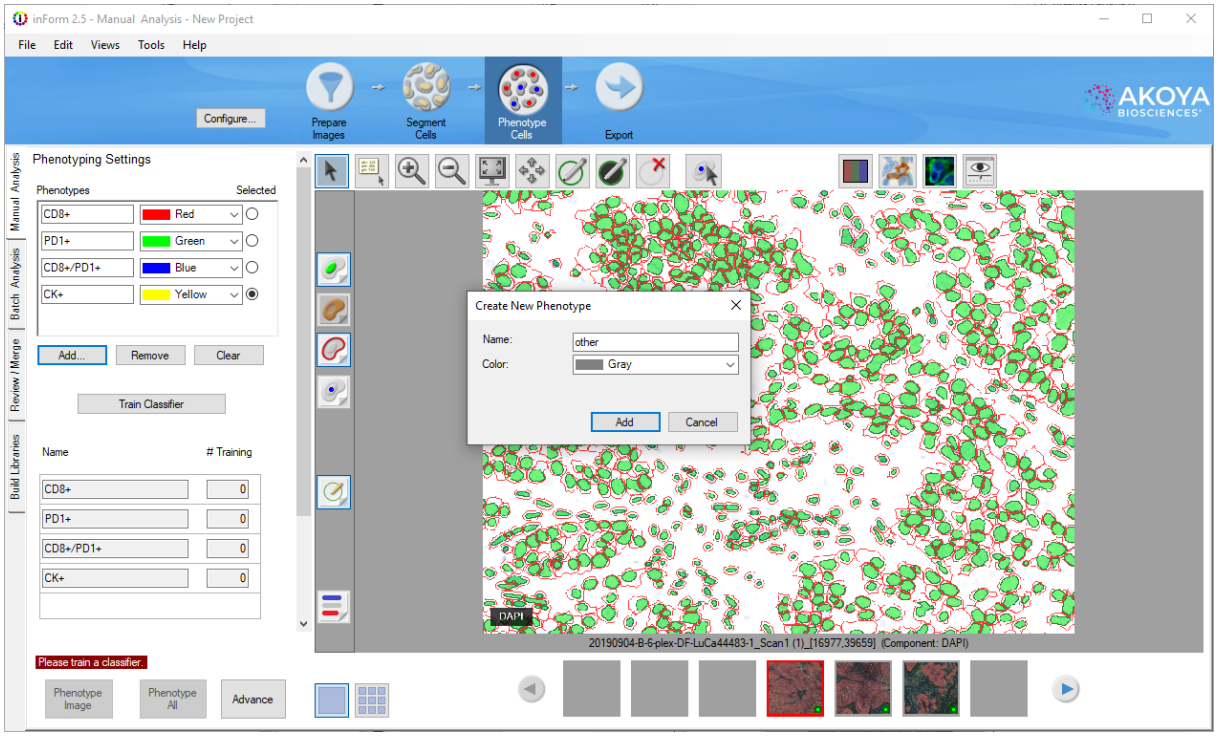


Figure 80. After you have performed your cell segmentation, you can advance to the Phenotyping step. Here a new phenotype is being created, named Other and assigned the color Gray.

Use the **Add…** button to add phenotypes to the Phenotypes list. You can select a color for the phenotype. Selecting a phenotype **Selected** radio button and clicking the **Remove** button will remove that phenotype. Clicking the **Clear** button will remove all phenotypes.

Below the phenotype selection area is the **Train Classifier** button and the Training table that lists each phenotype, the number of cells of each phenotype that you have selected for training purposes, and the total number of cells that you have selected.

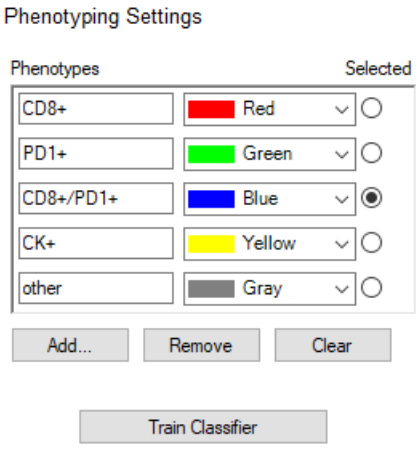


Figure 81. Phenotyping Settings

Click **Edit Phenotypes** to assign a phenotype to a cell. Click a particular cell to bring up a drop-down menu, allowing you to choose the phenotype and then move on.

> **NOTE:** *You need to identify at least five (5) cells in each phenotype in order to proceed. Akoya Biosciences recommends choosing 25 or more cells of each phenotype for optimal results. You may wish to start with a small number of cells, then add mis-classified cells to the training set by correcting their phenotype.*

You may choose cells across multiple images if, for example, you cannot find many cells of a particular phenotype in one image. Of course, you should choose images from the same tissue or from tissue sections that you are confident have been stained and then segmented identically or very similarly.

If you decide to **Add, Remove** or **Clear** phenotypes after you have already started training the classifier, you will need to begin training again.

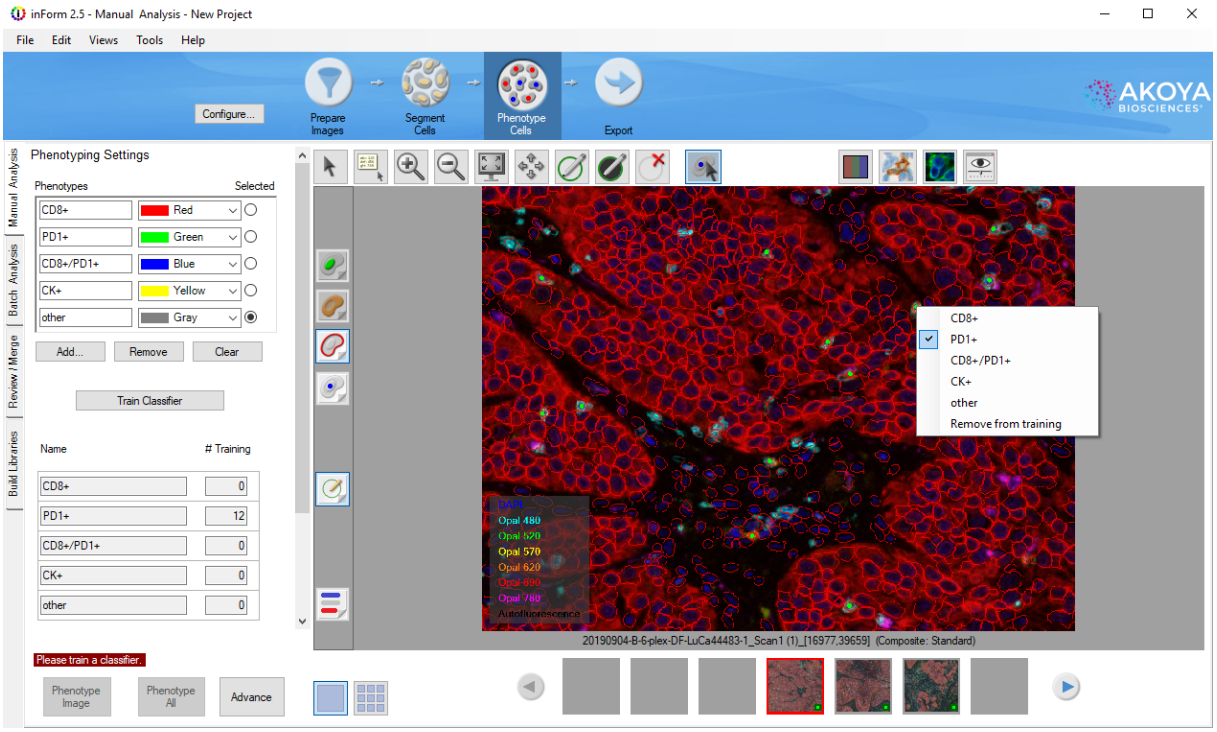


Figure 82. Click on a cell and choose a phenotype from the drop-down menu. It may help to turn off the visualization of the nuclei. You can also toggle back and forth amongst the viewing modes to help identify the cell type.

You may find that turning off the visualization of the nuclei allows you to better select the cell phenotypes that you recognize. When you are done selecting your cells for training, click the **Train Classifier** button.

NOTE: *The first time you train a classifier, the training step may take longer than subsequent steps because the features that inForm has been trained to recognize are cached in memory for use in training steps after the first time.*

Once the classification process is finished, you can move your cursor over any particular cell to reveal the phenotype that inForm has assigned, as well as the confidence level in percent of that classification.

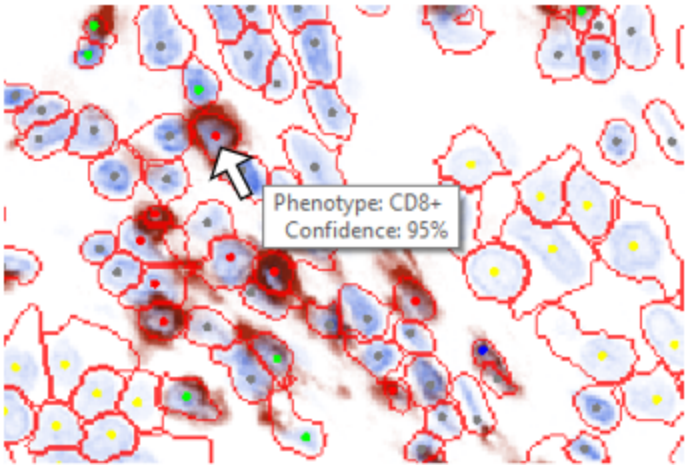


Figure 83. Cell phenotype and confidence.

Each cell's phenotype and confidence can also be viewed in the Cell Segmentation Data table.

If you wish to correct errors in classification, you can click **Edit Phenotypes**, click any particular cell, and then chose the phenotype that you believe to be the correct one. inForm will then re-classify that cell at 100% confidence in your choice. You can also remove cells from a training set. You may also add correctly classified cells to the training set by selecting their (correct) phenotype from the drop-down menu.

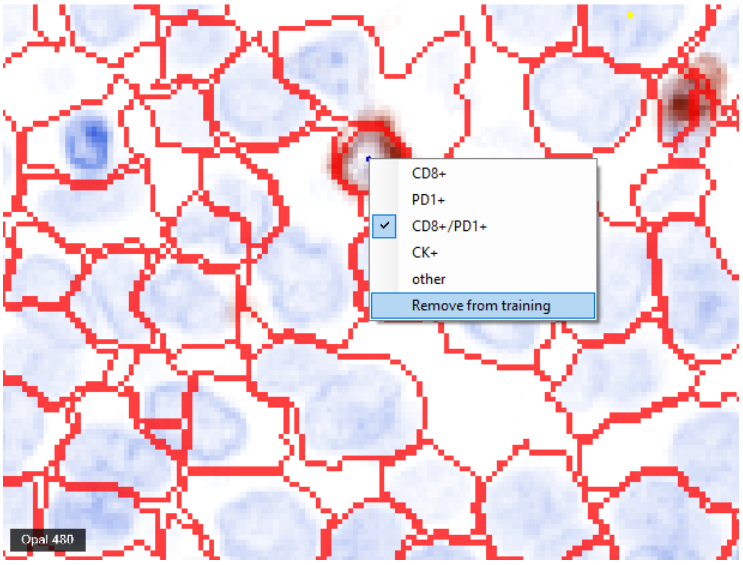


Figure 84 You can remove cells from training if you do not know which phenotype it should belong to.

The actual parameters for classification can be viewed in the inForm log file, accessed from **Help > View Log**... The parameters are logged after training and can be found in the log written on the date of the training. The log shows the normalized non-zero coefficients used in the linear classifier for each phenotype. Normalized coefficients are scaled by the standard deviation of the associated feature. This puts all the coefficients on the same scale and allows comparing the relative importance of features. A confidence level, in percent, is reported for each phenotype with 25 or more training examples. See Appendix C: Phenotype Classification by Multinomial Logistic Regression 151 for a detailed description of the phenotyping algorithm and the parameters in the log file.

15 Scoring IHC or IF

After segmenting nuclei, cytoplasm, and membrane, the detected cell compartments are quantified. The options in the **Score IHC or IF Settings** panel provide functionality similar to that which is done manually or visually when users assess chromogenic immunohistochemical stain levels. The Score IHC or IF settings specify the component thresholds and "score" the images.

1. **Tissue Category** - Select the tissue category to use for scoring. Cells that are outside the chosen tissue category are ignored. This option only displays if tissue has been segmented. Only tissue categories in which cells have been segmented are available.
2. **Scoring Type** - There are five Histogram score types, which are used to assess the intensity of each label. Select a score type from the **Scoring** box. Descriptions of each method are given below.
3. **Compartment** - Select whether you want to score the *Nuclei,* the *Cytoplasm*, or the *Membrane* cell compartment. If desired, you can score the nuclei, save the data, and then score the cytoplasm and/or membrane separately. The scoring data is a result of binning a histogram of all pixels within the selected compartment.
4. **Component** - Select the component to use for calculating scoring data.

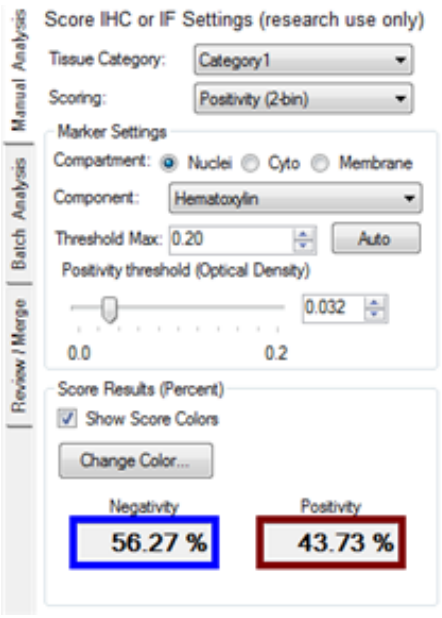


Figure 85. Score IHC or IF Settings

5. **Threshold Max** - Click the **Auto** button to calculate the **Threshold Max** for the selected component. The Threshold Max specifies the maximum value of the **Threshold** sliders. If necessary, adjust this value up or down as needed. For OD converted images, 3.0 is the maximum possible value.
6. **Positivity Threshold** - Specifies the threshold values for the selected Scoring Type. Drag the slider, type the desired value in the text box, or click the **up** and **down** arrow buttons to change the Positivity Threshold value. See Scoring Types 101 for descriptions of each Threshold.
7. **Show Score Colors** - If selected, the score colors display on the image and around the results percentage text boxes. If not selected, the colors do not display on the image or around the results percentage text boxes.
8. **Change Color** - Opens the Change Color window to select the colors for each bin or to restore the default colors to the bins.
9. When you score the image(s), the results display at the bottom of the Score IHC or IF settings panel. See Scoring Types 101 for descriptions of the results for each scoring type.

Scoring Types

Positivity

This bins the spectrally unmixed nuclear, cytoplasm, or membrane signals into two bins, negative or positive, with a single threshold. (See Figure 30 above.) It provides data in percent. Decreasing the **Positivity Threshold** slider increases the percent positivity.

The **Score Results (Percent)** box shows the negativity and positivity percentages of the cell nuclei, cytoplasm, or membrane.

Double Positivity (2x2-bin)

Select this scoring method to classify cells, according to co-expression, into four classes: double negative, single positive for one marker, single positive for another marker, or double positive. The Negativity and Positivity percentages for each of the two markers are also shown.

Double Positivity contains the same options as Positivity with the addition of the following:

Second Marker Settings: Select the Compartment, Component, Threshold Max, and Positivity Threshold for the second marker after selecting the first marker settings.

Show Score Colors: Select the score colors to display on the image: Double Positivity, First Marker, or Second Marker.

To select the Double Positivity settings:

1. If the tissue has been manually or automatically segmented, select the **Tissue Category** as described above.
2. Select **Double Positivity (2 x 2-bin)** in the **Scoring** list box.
3. Under **First Marker Settings**, select the **Compartment** and **Component**, and then click the **Auto** button.
4. Under **Second Marker Settings**, select the **Compartment** and **Component**, and then click the **Auto** button.
5. To adjust the First Marker Threshold, select **First Marker** in the **Histogram Results** box and then adjust the **First Marker Positivity** slider or type the desired value in the **Positivity** text box. Positive regions are indicated by the Single Positive 1 color. Negative regions are indicated by the Double Negative color.
6. To adjust the Second Marker Threshold, select **Second Marker** in the **Histogram Results** box and then adjust the **Second Marker Positivity** slider or type the desired

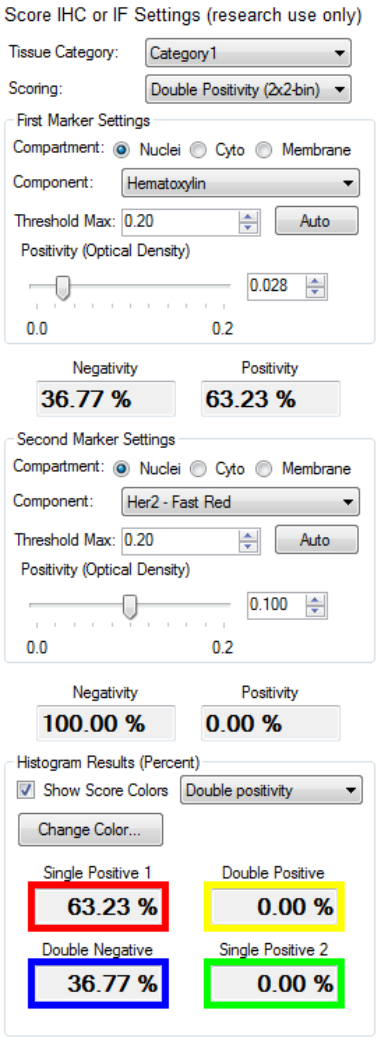


Figure 86. Double Positivity Scoring

value in the **Positivity** text box. Positive regions are indicated by the Single Positive 2 color. Negative regions are indicated by the Double Negative color.

7. To view the double positivity colors, select **Double Positivity** in the **Histogram Results** box.

0-3+ (4-bin)

This option bins spectrally unmixed signals in nuclei, membrane, or cytoplasm into four bins. This score type can be used to calculate H-scores with nuclear stains.

0/1+ Threshold: Specifies the value below which to include cellular objects in the 0 bin.

1+/2+ Threshold: Cellular objects above the 0/1+ threshold and below the 1+/2+ threshold are included in the 1+ bin.

2+/3+ Threshold: Cellular objects above the 1+/2+ threshold and below the 2+/3+ threshold are included in the 2+ bin. Cellular objects above this value are included in the 3+ bin.

Score Results (Percent): Shows the percentage positivity of the cell nuclei, membrane, or cytoplasm within each bin.

H-Score: The calculated H-Score of the image. The H-Score is calculated using the percentages in each bin and ranges from 0 to 300.

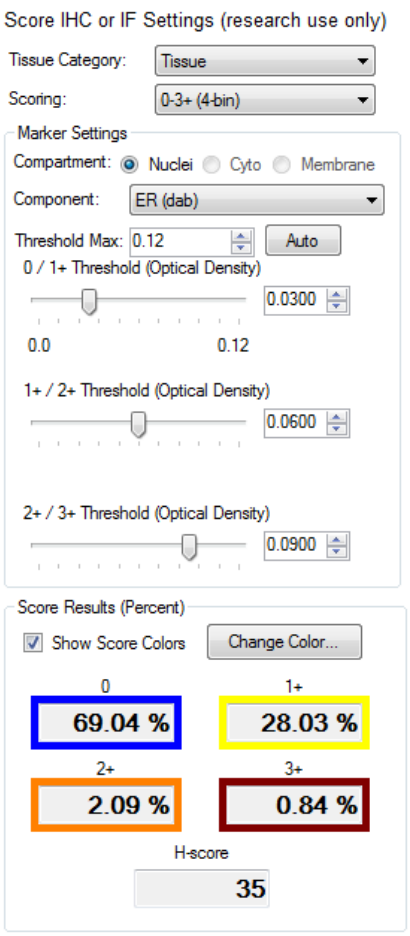


Figure 87. 0-3+ Scoring

Ten-bin

This histogram bins the data into ten bins for more quantitative work. The **10th Bin Nominal Level** slider specifies the ninth bin lower threshold. The other eight thresholds are equally spaced between zero and the ninth threshold.

The **Histogram Results** boxes display the percentages of pixels falling into the bins determined by the nominal level slider position.

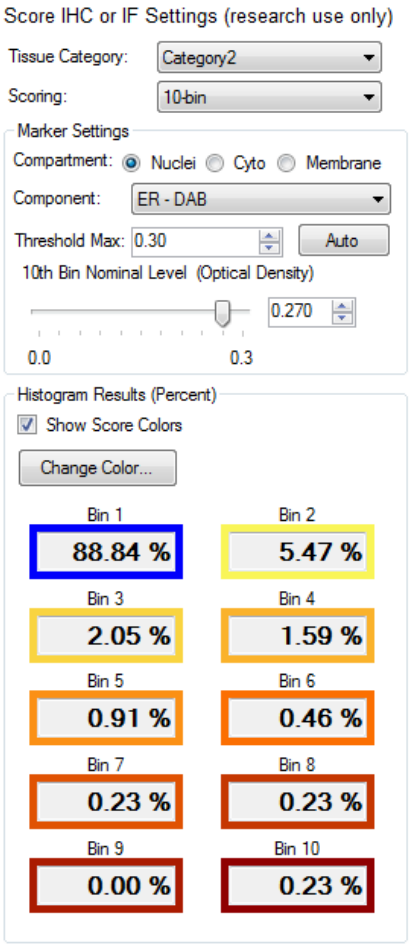


Figure 88. 10-bin Scoring

Fifty-bin

Divides signals into 50 equal bins.

The **50th Bin Nominal Level** slider specifies the lower threshold for the 50th bin. The remaining thresholds are equally spaced between zero and the 50th bin threshold.

For display, the 50 values are summed into 10 values, each value being the sum of 5 bins. The actual values for all 50 individual bins display in the Score table.

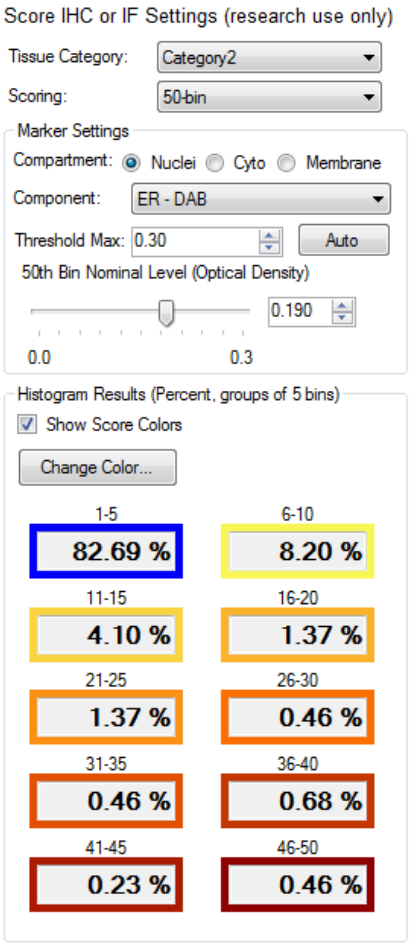


Figure 89. 50-bin Scoring

16 Counting Objects

Count Objects locates individual irregularly-shaped objects within an image field or within a selected tissue category if tissue has been segmented.

1. Click the **Count Objects** button in the step bar to display the **Object Counting Settings** panel.

2. To exclude any objects that are touching the edge of the image, process region, or tissue region, select the **Discard Object if Touching an Edge** check box.
3. If tissue has been segmented, select the **Tissue Category** in which you want to find the objects. Objects outside of the selected tissue category are not counted. (This option only displays if the project includes a Manual Classification or Segment Tissue step.)
4. Select the desired **Approach** to use to identify objects: **Object-Based** or **Pixel-Based (Threshold)**. Select the **Pixel-Based (Threshold)** approach when there is a reliable or consistent stain, and object pixels can be found by applying a simple threshold. This approach is purely pixel-based. Select the **Object-Based** approach if the stain does not provide consistent and specific staining of objects, and more advanced morphometry-based approaches are needed.
5. Select the desired **Signal Scaling**:

- **Auto Scale:** Automatically scales each component plane individually before performing object segmentation.
- **Fixed Scale:** If you need better segmentation performance, and stain signals are consistent and reliable, you might get better results by selecting the **Fixed Scale** option.

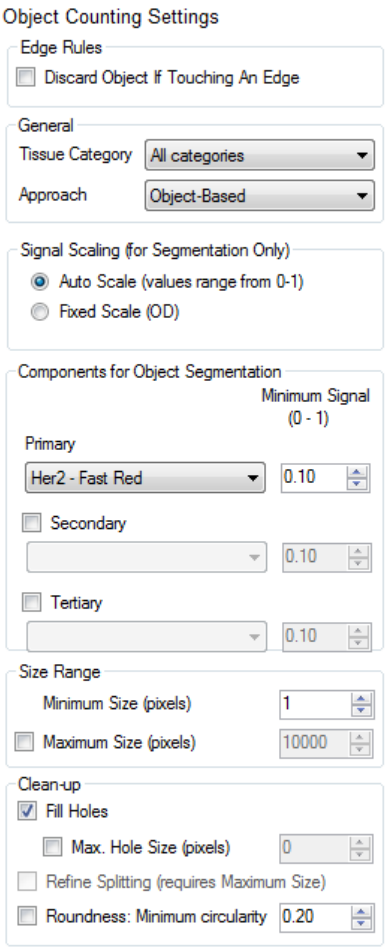


Figure 90. Object Counting Settings

6. Select the **Primary** component for object segmentation from the drop-down list.
7. Adjust the **Minimum Signal** value for the primary component to the desired threshold value.
8. If desired, select **Secondary** and **Tertiary** components and minimum signal values.
9. To exclude objects smaller than a specific number of pixels, increase the value in the **Minimum Size (pixels)** text box.
10. To exclude objects larger than a specific number of pixels, select the **Maximum Size (pixels)** check box and specify the largest object size in pixels.
11. To automatically fill holes in objects, select **Fill Holes**. To limit the size of the holes that are automatically filled, select the **Max Hole Size** check box and specify the size in pixels of the largest holes that should be filled.

12. To detect objects that touch other objects as individual objects, instead of as one object, select the **Refine Splitting** check box. The **Maximum Size (pixels)** check box must be selected to enable this option.
13. To exclude objects based on the roundness of the object, select the **Roundness** check box and specify the desired **Minimum Circularity**. Zero returns any shape. Higher decimal values (up to 1) restrict selections to more round objects only.
14. After processing, use the View Editor 108 to view the resulting data tables. You can also export the Count Data table by advancing to the Export step (Exporting the Data 129).

17 Displaying the Extracted Data

After analyzing the images, use the View Editor 25 to customize the view of the images and extracted data. These options do not change the underlying images, they only change how the images are displayed. The options below are available in the Data Displayed list (depending on which software configuration is installed and which steps are included in the project):

Displaying the Extracted Data

- Viewing the Color Image 109
- Viewing the Component Image 110
- Viewing the Composite Image 113
- Viewing Data Tables 114
- Viewing the Tissue Segmentation Data Table 115
- Viewing the Cell Segmentation Data Table 116
- Viewing the Score Data Table 118
- Viewing the Colocalization Data Table 120
- Viewing the Count Data Table 121
- Viewing the Quant Data Table 122
- Image Options 122
- Component Stats 126
- TMA Core Info 126
- Slide Info 126
- Position Stats 126
- Shape Stats 128

17.1 Viewing the Color Image

Select **Color Image** in the Data Displayed list, then choose the desired options.

The following options are available for the Color Image:

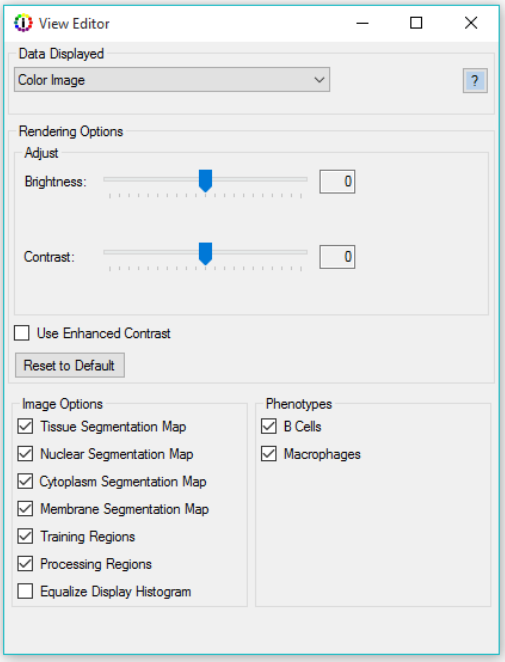


Figure 91. View Editor (Color Image)

Rendering Options

- **Brightness -** Change the value to change the brightness of the selected image. Lighten or darken the image using the Brightness percentage slider.
- **Contrast -** Change the value to change the contrast between light and dark portions of the selected image. Increase or decrease the contrast using the Contrast percentage slider.
- **Use Enhanced Contrast -** Select to artificially increase the vividness of the image.
- **Use Equal RGB Color Weighting -** (only for Fluorescence images) Select to distribute the wavelength range equally in the red, green, and blue components. If not selected, the image displays as it looks to the human eye.
- **Reset to Default button** - Click to reset the Rendering Options back to the default settings.

Image Options - See Image Options 122.

17.2 Viewing the Component Image

Select a **Component** in the Data Displayed list, then choose the desired options.

The following options are available for the Component Image:

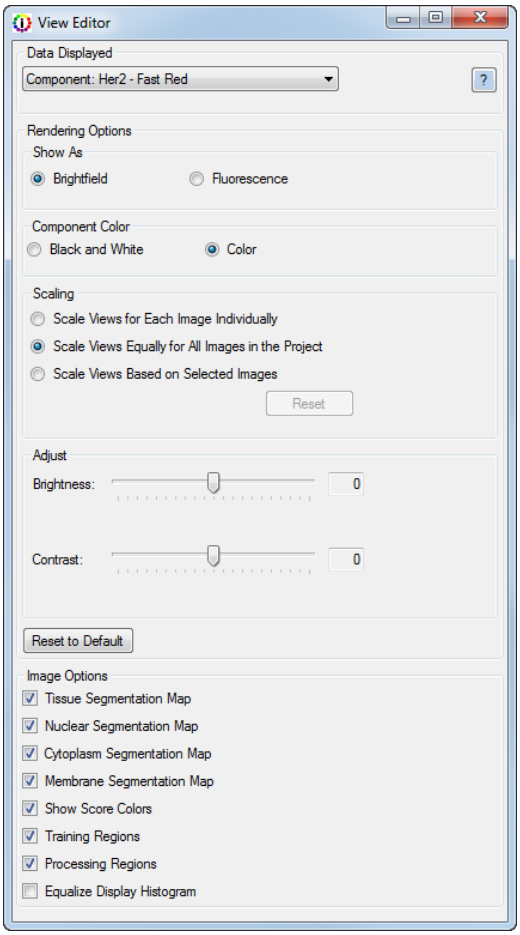


Figure 92. View Editor (Component Image)

Rendering Options

Show As:

- **Brightfield** - Select to display the component as if it were conventionally stained along with a counterstain. Stains will be displayed as DAB, autofluorescence will be displayed as eosin, and DAPI will be displayed as hematoxylin. This style is available when inForm can identify a hematoxylin or DAPI counterstain. If no counterstain is apparent, inForm will display the component signal only.
- **Fluorescence** - Select to display only the component signal on a black background

Component Color-

When a component is shown without its counterstain, you can select the display color for the component.

- **Black and White** - Select to show the component as a white signal against a black background.
- **Color** - Select to show the component in color. This color is calculated from the spectral shape of the component, i.e. the color you would see when viewed through a microscope.

Scaling -

- **Scale Views for each Image Individually** - Select to show each image scaled individually. Dim images are brightened to show the components. The relative brightness of components cannot be compared between images.
- **Scale Views Equally for All Images in the Project** - Select to show the brightness of components for each image all scaled relative to each other. Bright components appear bright and dim components appear dim. All images are rescaled relative to all images in the project whenever images are removed from or added to the project.
- **Scale Views Based on Selected Images** - Select to show the brightness of components for each image based on the brightness range of the selected images. Bright components appear bright and dim components appear dim. The first time the option is selected, all current images in the project are selected. The scaling limits remain the same even if images are added to or removed from the project. Click the **Reset** button to open the Scaling Image Selection Window 112 to change the images

selected to determine the scaling limits. See Scaling based on Selected Images 111 for the suggested workflow.

When this option is selected, the scaling limits are saved with the algorithm and project. If the algorithm or project is used in Batch Mode, then all images processed in the batch job use the saved scaling limits.

NOTE: *If Scale Views Based on Selected Images is selected and then changes are made to the selected Spectra for Unmixing or to the Reported Units in the Prepare Images step, the image scaling is reset to Scale Views Equally for All Images in the Project (default).*

Brightness - Change the value to change the brightness of the selected image. Lighten or darken the image using the Brightness percentage slider.

Contrast - Change the value to change the contrast between light and dark portions of the selected image. Increase or decrease the contrast using the Contrast percentage slider.

Reset to Default button - Click to reset the Rendering Options back to the default settings.

Image Options - See Image Options 122.

Scaling Based on Selected Images

Images with bright signals in each component, such as positive controls or positively expressing images, should be used to scale all images in the project.

When **Scale Views Equally for All Images** or **Scale Views Based On Selected Images** is selected, the scaling limits are saved with the algorithm and project. If the algorithm or project is used in Batch Mode, then all images processed in the batch job use the saved scaling limits.

1. Load only the images of the positive controls for the images.
2. Process the images as desired.
3. Open the View Editor and select any component in the **Data Displayed** drop-down list.
4. Select the **Scale Views Based on Selected Images** option. The scaling limits are set based on the positive control images that are open in the project.
5. Save the algorithm and/or the project.
6. Use the settings for batch processing by loading the algorithm or project in the **Batch** tab.
7. If the positive controls change, open the project and click the **Reset** button on the View Editor. Select the new positive controls in the Scaling Image Selection window 112 to set the new scaling limits.

Scaling Image Selection Window

Use the Scaling Image Selection Window to choose the images used to scale the brightness of all images in the project. The images that are brightest in at least one component are at the top of the list and are selected by default. Images must be unmixed to be used for the scaling calculations. If an image is not unmixed, it displays at the bottom of the list and the **Select For Scaling** check box is disabled.

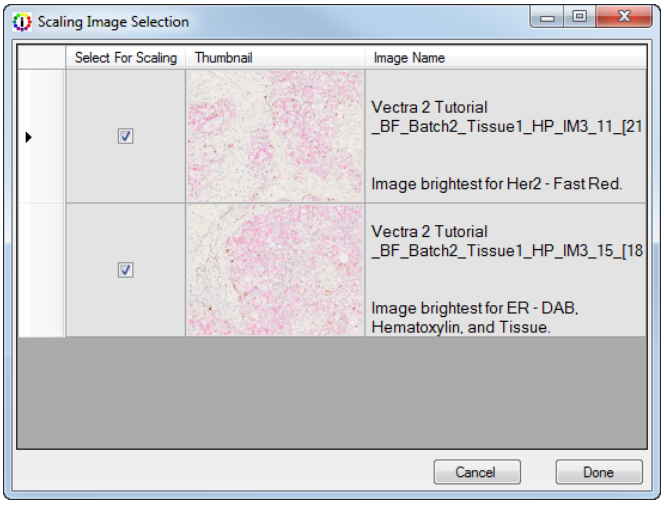


Figure 93. Scaling Image Selection Window

Select For Scaling Check boxes - The scaling limits are calculated using all selected images. These scaling limits are saved and used when processing images in Batch Mode.

Use the **Thumbnail** and **Image Names** to identify the images in the project. The Image Name column also identifies images that are the brightest for each component.

Cancel button - Closes the Scaling Image Selection Window without recalculating any image scaling limits. The limits do not change.

Done button B Recalculates the image scaling limits using the selected images and then closes the window.

17.3 Viewing the Composite Image

Select **Composite Image** in the Data Displayed list, then choose the desired options.

The following options are available for the Composite Image:

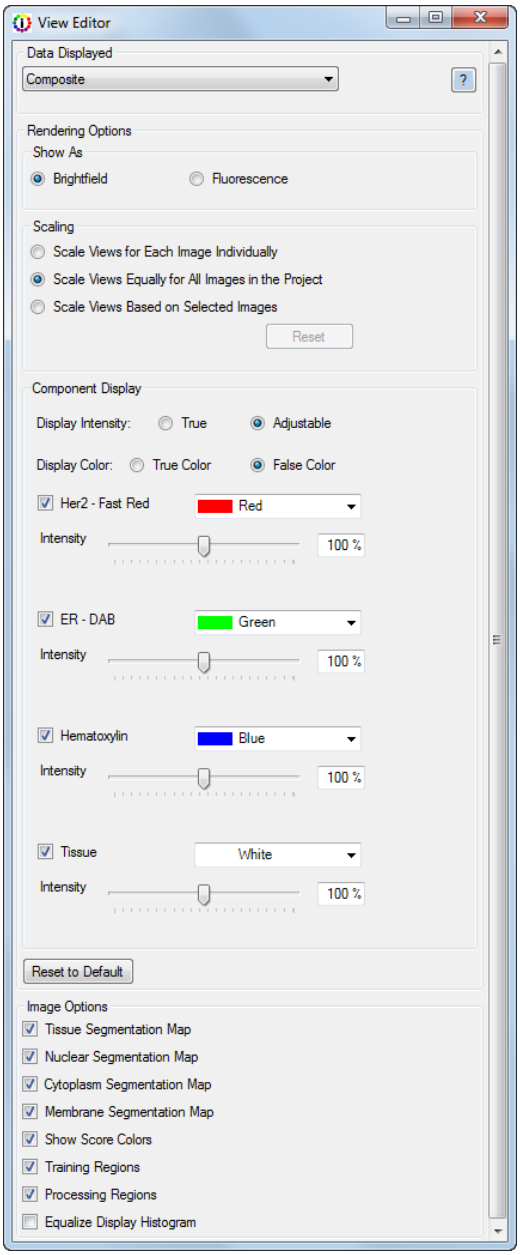


Figure 94. View Editor (Composite Image)

Rendering Options

- **Show As:**
  - **Brightfield -** Select to display the composite image on a white background.
  - **Fluorescence** - Select to display the composite image on a black background.
- **Scaling:**
  - **Scale Views for each Image Individually** - Select to show each image scaled individually. Dim images are brightened to show the components. The relative brightness of components cannot be compared between images.
  - **Scale Views Equally for All Images in the Project** - Select to show the brightness of components for each image all scaled relative to each other. Bright components appear bright and dim components appear dim. All images are rescaled relative to all images in the project whenever images are removed from or added to the project. (Default.)
  - **Scale Views Based on Selected Images** - Select to show the brightness of components for each image all scaled relative to the selected images. Bright components appear bright and dim components appear dim. The first time the option is selected, all current images in the project are selected. The scaling limits remain the same even if images are added to or removed from the project. Click the Reset button to open the Scaling Image Selection Window 112 to change the images selected to determine the scaling limits.

NOTE: *If Scale Views Based on Selected Images is selected and then changes are made to the selected Spectra for Unmixing or to the Reported Units in the Prepare Images step, the image scaling is reset to Scale Views Equally for All Images in the Project (default).*

- **Display Intensity:** Select **True** to display each component in the composite image at the original intensity. For example, if Component A is dimmer than Component B, they display that way in the composite. Select **Adjustable** to display each component in the composite image at the intensity selected on the slider.
- **Display Color:** Select **True Color** to display each component in the calculated color from the spectral shape of the component. Select **False Color** to display each component in the selected false color. Select the False Color using the color picker next to each component (only visible when False Color is selected).
- **Component check boxes:** Select to display each component. If not selected, the component does not display. (The Intensity Slider for each component only displays if the Display Intensity is Adjustable. The Color Selector for each component only displays if the Display Color is False Color.)
- **Reset to Default button** - Click to reset the Rendering Options back to the default settings.

Image Options - See Image Options 122.

17.4 Viewing Data Tables

The data tables that are available to view are determined by the steps selected in each project. To view a data table, select the desired table in the **Data Displayed** drop-down list in the View Editor 25.

Changing the selections in the View Editor immediately changes the columns and rows displayed in the table.

To sort the table, click anywhere in the desired column header. The up or down arrow in the column header indicates the sort order of the data. If summary rows are displayed, the summary rows remain at the top of the table.

17.5 Viewing the Tissue Segmentation Data Table

The Tissue Segmentation Data Table is available when an algorithm contains a manual tissue segmentation or trainable tissue segmentation step. The table provides information on the manually or automatically segmented tissue regions. Select **Tissue Segmentation Data** in the Data Displayed list, then choose the desired options under **Table Contents**. Each option you select adds one or more data columns or rows to the table.

During image analysis, segmented tissue categories are assigned ID numbers. These numbers are used in the data tables to identify the tissue category for stats such as area (pixels), area (percent), etc.

The following options are available for the Tissue Segmentation Data Table:

Components - Select all or individual components for which to display component statistics. Each selected component creates one column for each component stat selected.

Tissue Categories - Select all or individual tissue categories for which to display data. An entry row is created for each Region ID in the tissue category.

Component Stats - Select the values to display for each component signal. See Component Stats 126 for descriptions of each option.

Position Stats - Select which values to display for each segmented object. See Position Stats 126 for descriptions of each option.

TMA Core Info - Select the options to include in the data table. See TMA Core Info 126 for descriptions of each option.

Shape Stats - Select the options to include in the data table. See Shape Stats 128 for descriptions of each option.

Coordinate Units - Select the unit of measure for your table statistics, microns or pixels. This option is only available for for imagery taken with Mantra, Vectra 3, and Vectra Polaris.

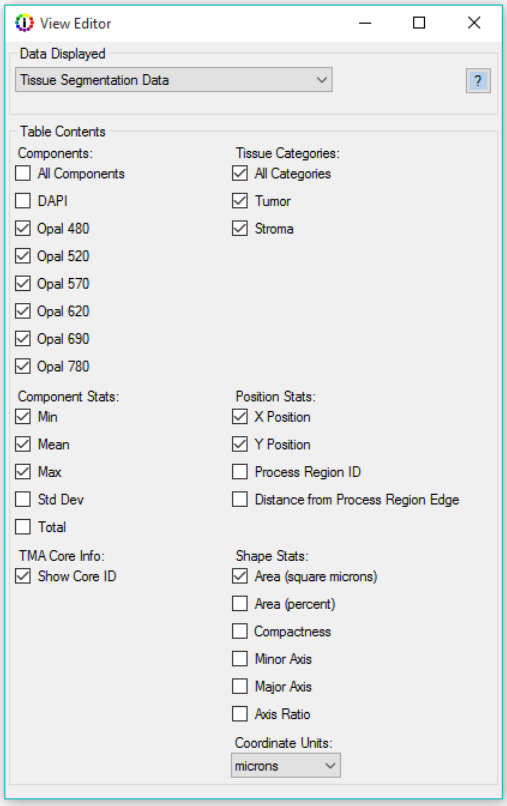


Figure 95. View Editor (Tissue Segmentation Data)

17.6 Viewing the Cell Segmentation Data Table

The Cell Segmentation Data Table is available when an algorithm contains a cell segmentation step. The table provides information on the segmented cells. Select **Cell Segmentation Data** in the Data Displayed list, then choose the desired options under **Table Contents**. Each option you select adds a data column or row to the table.

The table only shows statistics for cell compartments that have been segmented (nuclei, cytoplasm, membrane). For example, if cytoplasm is not segmented, the Cytoplasm column is not included in the table.

The following options are available for the Cell Segmentation Data Table:

Components - Select all or individual components for which to display component statistics. Each selected component creates one column for each component stat selected. If there is no data for a component, the component will not be displayed in the list.

Tissue Categories - Select all or individual tissue categories for which to display data. If selected, a Tissue Category column displays in the Cell Segmentation table to specify the Tissue Category for each cell. This selection does not display if there is only one Tissue Category defined or if there is no Segment Tissue step in the project.

> **Note:** *Rarely, small areas of tissue are not classified by the tissue segmentation step. Cells within these unclassified areas are not reported in the table.*

Component Stats - Select the values to display for each component signal. See Component Stats 126 for descriptions of each option. Each selection adds one column for each segmented cell component and one column for the entire cell.

For Entire Cell statistics:

- Statistics are calculated using the segmented compartments; for example, if cytoplasm is not segmented, the statistics do not include that compartment in the calculation.
- Statistics are calculated using over the entire pixel area of the cell; so the Min stat looks at all pixels across the available compartments in the cell.

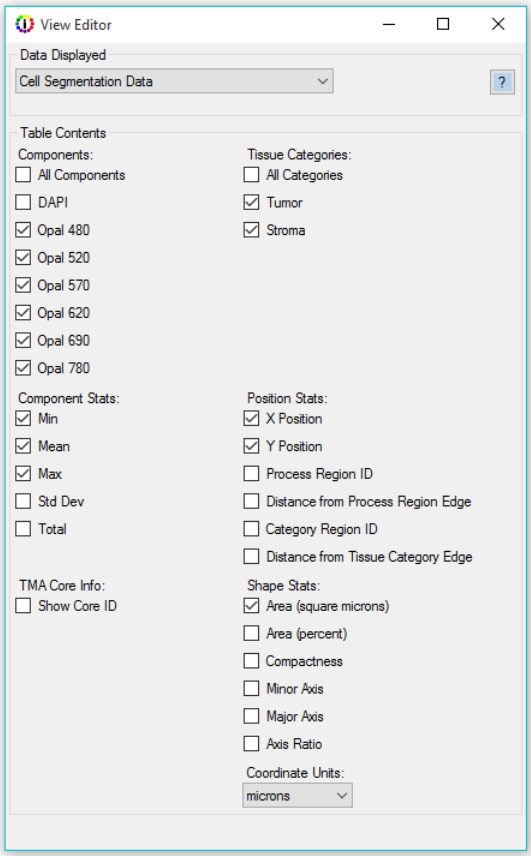


Figure 96. View Editor (Cell Segmentation Data)

When calculating the mean, averaging across all pixels is equivalent to taking the weighted average of the compartment means, where the weight is the pixel area of the compartment. This can be

verified in Excel®; computing:

Cell Mean = [(Nucleus Area * Nucleus Mean) + (Cytoplasm Area * Cytoplasm Mean) + (Membrane Area * Membrane Mean)]/Cell Area

results in the same number as the reported Cell Mean.

TMA Core Info - Select the options to include in the data table. See TMA Core Info 126 for descriptions of each option.

Position Stats - Select which values to display for each segmented object. See Position Stats 126 for descriptions of each option.

Shape Stats - Select the options to include in the data table. See Shape Stats 128 for descriptions of each option.

The columns below are always visible in the Cell Segmentation Data table:

- **Cell ID** - Displays a unique ID number for each cell, based on the location of the nuclei within each tissue category, if tissue has been segmented.
- **Total Cells** - The total number of cells scored in the image.
- **Tissue Category Area (Pixels)** - If tissue has been segmented (either automated or manual), the area of the selected tissue category, measured in number of pixels. If there is no tissue segmenting step, this column is the total number of pixels in the whole image.
- **Cell Density (per megapixel)** - If tissue has been segmented (either automated or manual), displays the cell density for each tissue category: the number of cells in the tissue category area / (the number of pixels in the tissue category area / 1,000,000). If there is no tissue segmenting step, displays the cell density for the entire image: Total cells / (the number of pixels in the image / 1,000,000).
- **Phenotype and Confidence** - If the algorithm contains a Phenotype Cells step, cell phenotypes and confidence levels are automatically added to the table.
- **Coordinate Units** - Select the unit of measure for your table statistics, microns or pixels. This option is only available for for imagery taken with Mantra, Vectra 3, and Vectra Polaris.

17.7 Viewing the Score Data Table

The Score Data Table is available when an algorithm contains a Score step. The available data changes depending on the type of scoring. Select **Score Data** in the Data Displayed list, then choose the desired options under **Table Contents**. Each option you select adds a data column to the table.

Table Contents - Select the options to include in the data table. See TMA Core Info 126 for descriptions of each option.

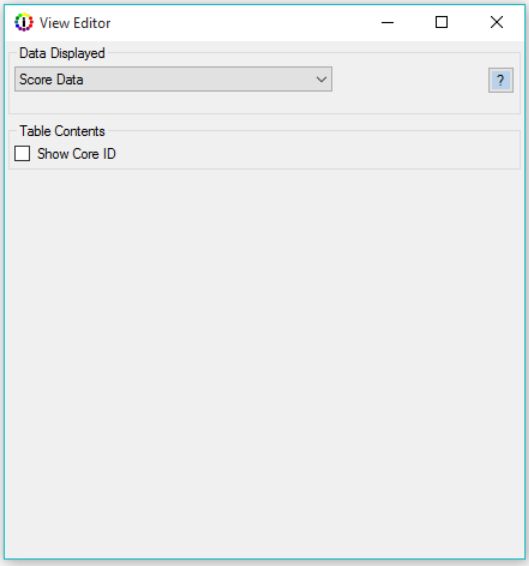


Figure 97. View Editor (Score Data)

The columns below are always visible in the Score Data table:

- **Tissue Category** - If tissue has been segmented (either automated or manual), the tissue category used for scoring displays in the table. If there is no tissue segmenting step, this column is blank.
- **Tissue Category Area (Percent)** - If tissue has been segmented (either automated or manual), the area of the selected tissue category displays in the table. If there is no tissue segmenting step, this column is 100% (the whole image).
- **Number of cells** - The number of cells scored in the image.
- **(First) Cell Compartment** - The cell compartment used for scoring.
- **(First) Stain Component** - The stain used to create the threshold for scoring.

The following additional columns are visible when **Positivity (2-bin) scoring** is selected:

- **Positivity** - The scored positivity.
- **Positivity Threshold** - The threshold value used to set the positivity.

The following additional columns are visible when **Double Positivity (2x2)** scoring is selected:

- **Second Cell Compartment** - The secondary cell compartment used for scoring
- **Second Stain Component** - The secondary stain used to create the threshold for scoring.
- **Double Negative** - The percent of cells that is negative (less than the threshold) for both components.
- **Single <Component1>** - The percent of cells that is positive for component 1 and negative for component 2.
- **Single <Component2>** - The percent of cells that is positive for component 2 and negative for component 1.
- **Double Positive** - The percent of cells that is positive for both markers.
- **<Component1> Threshold** - The threshold used for component 1.

- **<Component2> Threshold** - The threshold used for component 2.

The following additional columns are visible when **0-3+ (4-bin) scoring** is selected:

- **0+** - The percentage of cells whose component strength is less than the first threshold.
- **1+** - The percentage of cells whose component strength is greater than or equal to the 0/1+ threshold and less than the 1/2+ threshold.
- **2+** - The percentage of cells whose component strength is greater than or equal to the 1/2+ threshold and less than the 2/3+ threshold.
- **3+** - The percentage of cells whose component strength is greater than or equal to the 2/3+ threshold.
- **H-score** - The calculated H-Score of the image. The H-Score is calculated using the percentages in each bin and ranges from 0 to 300.
- **Threshold 0/1+** - Clicking the **Auto** button sets this value to 1/4 of the Threshold max. This value can be changed.
- **Threshold 1+/2+** - Clicking the **Auto** button sets this value to 1/2 of the Threshold max. This value can be changed.
- **Threshold 2+/3+** - Clicking the **Auto** button sets this value to 3/4 of the Threshold max. This value can be changed.

The following additional columns are visible when **10-bin or 50 bin scoring** is selected:

- **Bin X (where X ranges from 1-10 or 1-50 per scoring type)** - Columns showing the percent of scored values that fall in each scoring bin. The user sets the lower threshold for the last bin. The last bin is the percent of cells whose component strength is greater than the threshold the user set. The thresholds for the remaining bins are spaced evenly from 0. For example, the 49th bin has a lower limit of (48 * threshold) / 49.

17.8 Viewing the Colocalization Data Table

The Colocalization Data Tables are available when an algorithm contains a Colocalization step. Select **Colocalization Data** in the Data Displayed list, then choose the desired options for each table.

The following tables are included in the colocalization data:

Colocalization Table - Shows how much the numerator components are colocated with the denominator.

- **Percent Stats -** Shows the percent colocalization (where all colocalization pixels are positive within the selected denominator) and the percent positivity of each selected component in colocalization.
- **Pixel Stats -** Shows the actual pixel count for the colocalization, component positivities, and selected denominator.

In Channel Table - Shows the positivity of one component within another for all selected colocalization components.

- **Percent Stats -** Shows the percentage of inter-component positivity.
- **Pixel Stats -** Shows the pixel count of the component positivity.

Quant Table - Shows stats for the whole image and colocalization region.

Components - Select all or individual components for which to display component statistics. One column per numerator component is shown.

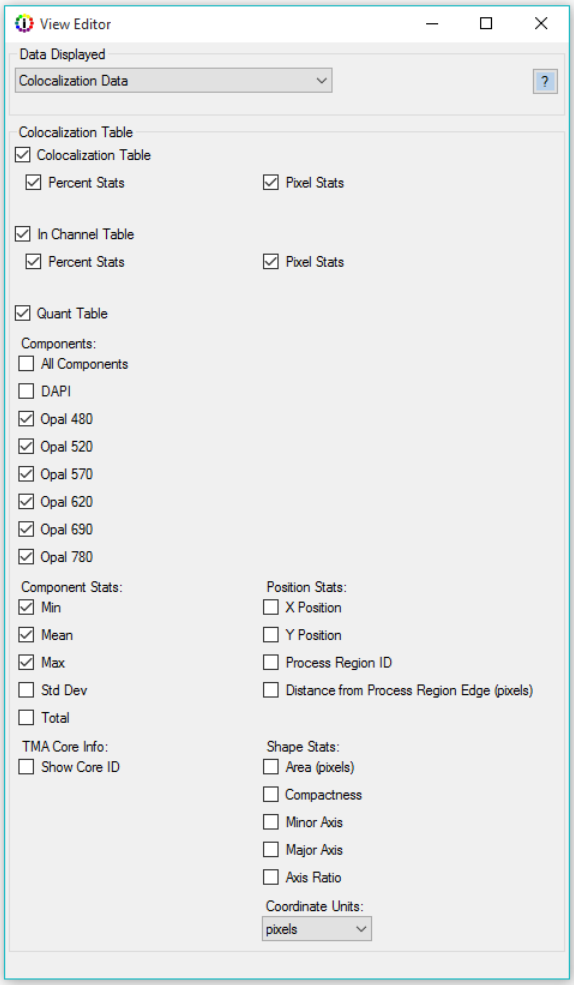


Figure 98. View Editor (Colocalization Data)

Component Stats - Select the values to display for each component signal. See Component Stats 126 for descriptions of each option.

TMA Core Info - Select the options to include in the data table. See TMA Core Info 126 for descriptions of each option.

Position Stats - Select which values to display for each segmented object. See Position Stats 126 for descriptions of each option.

Shape Stats - Select the options to include in the data table. See Shape Stats 128 for descriptions of each option.

Coordinate Units - Select the unit of measure for your table statistics, microns or pixels. This option is only available for for imagery taken with Mantra, Vectra 3, and Vectra Polaris.

17.9 Viewing the Count Data Table

The Count Data Table is available when an algorithm contains an Object Segmentation step. Select **Count Data** in the Data Displayed list, then choose the desired options under **Table Contents**. Each option you select adds one or more data columns or rows to the table.

The following options are available for the Count Data Table:

Components - Select all or individual components for which to display component statistics. Each selected component creates one column for each component stat selected.

Tissue Categories - Select all or individual tissue categories for which to display data. If selected, a Tissue Category column displays in the Count Data table to specify the Tissue Category for each cell. This selection does not display if there is only one Tissue Category defined or if there is no Segment Tissue step in the project.

Component Stats - Select the values to display for each component signal. See Component Stats 126 for descriptions of each option.

TMA Core Info - Select the options to include in the data table. See TMA Core Info 126 for descriptions of each option.

Position Stats - Select which values to display for each segmented object. See Position Stats 126 for descriptions of each option.

Shape Stats - Select the options to include in the data table. See Shape Stats 128 for descriptions of each option.

Coordinate Units - Select the unit of measure for your table statistics, microns or pixels. This option is only available for for imagery taken with Mantra, Vectra 3, and Vectra Polaris.

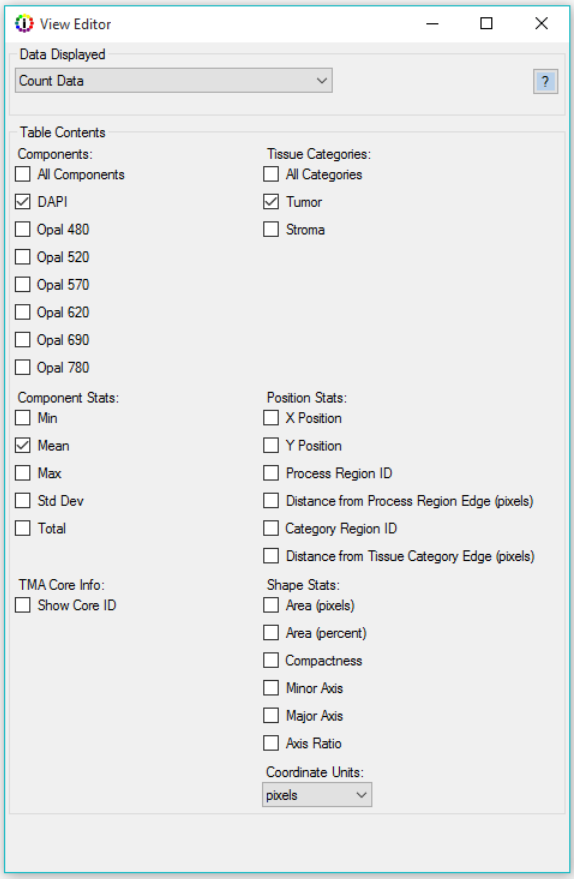


Figure 99. View Editor (Count Data)

17.10 Viewing the Quant Data Table

The Quant Data Table is available when an algorithm contains a Threshold or Colocalization step. Select **Quant Data** in the Data Displayed list, then choose the desired options under **Table Contents**. Each option you select adds one or more data columns or rows to the table.

The following options are available for the Quant Data Table:

Components - Select all or individual components for which to display component statistics. Each selected component creates one column for each component stat selected.

Component Stats - Select the values to display for each component signal. See Component Stats 126 for descriptions of each option.

TMA Core Info - Select the options to include in the data table. See TMA Core Info 126 for descriptions of each option.

Position Stats - Select which values to display for each segmented object. See Position Stats 126 for descriptions of each option.

Shape Stats - Select the options to include in the data table. See Shape Stats 128 for descriptions of each option.

Coordinate Units - Select the unit of measure for your table statistics, microns or pixels. This option is only available for for imagery taken with Mantra, Vectra 3, and Vectra Polaris.

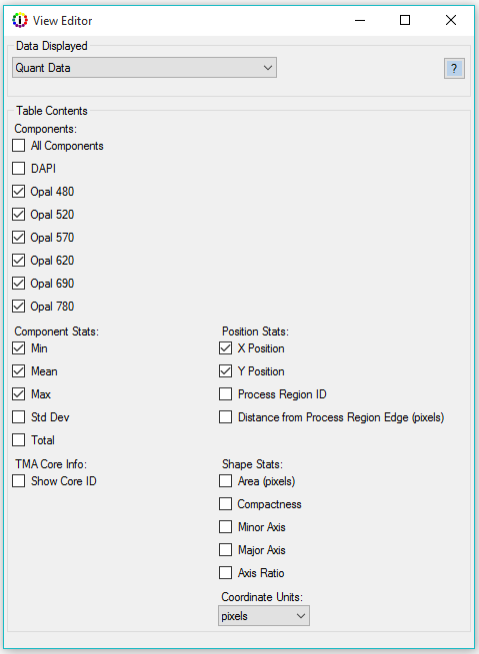


Figure 100. View Editor (Quant Data)

17.11 Image Options

The Image Options below are available on the View Editor 25 when an image is selected in the **Data Displayed** list. Select or clear the check boxes described below to show or hide maps or regions on all images in the project. The buttons on the left side of the image display area also show or hide the maps or regions. Show Score Colors is also accessible on the Score Settings panel. The Equalize Display Histogram can only be shown or hidden here.

- **Tissue Segmentation Map** - Select to display the Tissue Segmentation Map on the image. (Only for projects that include a Manual Classification or Segment Tissue step.)
- **Nuclear Segmentation Map** - Select to display the Nuclear Segmentation Map on the image. (Only for projects that include a Segment Cells step.)
- **Cytoplasm Segmentation Map** - Select to display the Cytoplasm Segmentation Map on the image. (Only for projects that include a Segment Cells step.)
- **Membrane Segmentation Map** - Select to display the Membrane Segmentation Map on the image. (Only for projects that include a Segment Cells step.)

- **Show Score Colors** - Select to display the Score Colors Map on the image. (Only for projects that include a Score IHC or IF step.)
- **Object Segmentation Map** - Select to display the Object Segmentation Map on the image. (Only for projects that include a Count Objects step.)
- **Training Regions** - Select to display the Training Regions on the image. (Only for projects that include a Segment Tissue step.)
- **Processing Regions** - Select to display the Processing Regions on the image.
- **Equalize Display Histogram** - Select to map the pixels so the image histogram has approximately the same number of pixels assigned to each tonal value of the histogram. This gives the best display of the whole dynamic range of dim and bright signals. If not selected, the lowest 0.01% of the pixels are mapped to 0, the highest 0.01% are mapped to 255, and the remaining pixels are linearly interpolated between those values. This prevents a few bright or saturated pixels from skewing the display.

See Displaying the Extracted Data 108 for descriptions of other options available for each image type or data table.

Shortcuts for the image views (Color, Component, and Composite) are available on the toolbar. To toggle across displays, use the color, component, and composite buttons.

Figure 101 Image View Shortcuts enable the quick toggle between views and selection between different component and composite views.

Simulated Brightfield Views (Pathology Views)

When viewing fluorescence imagery, the component button lets you display components in a simulated brightfield mode (Pathology Views™). Pathology Views renders fluorescence images in a manner that is familiar to anyone trained in histopathology. displaying them as simulated H&E images or as simulated DAB/Hematoxylin images. Components are displayed as if you were looking at serial sections that are single-stained. The difference is that this method displays multiplexed stains on a single tissue section with high contrast and perfect registration.

- Simulated H&E: Simulated Eosin (using the autofluorescence component) and simulated Hematoxylin (using the DAPI component) are displayed against a white background.
- Simulated DAB/Hematoxylin: Individual components are displayed as a simulated DAB stain with a simulated Hematoxylin counterstain (from the DAPI signal) against a white background.

To toggle between components, click the component button located in the upper right hand corner of your screen . A drop-down list of all available components will display. Select the component you would like to display; the legend will always display the currently shown component.

You can also toggle from one component to the next by using the up and down arrows on your keyboard.

For brightfield images, this button allows you to select different component views.

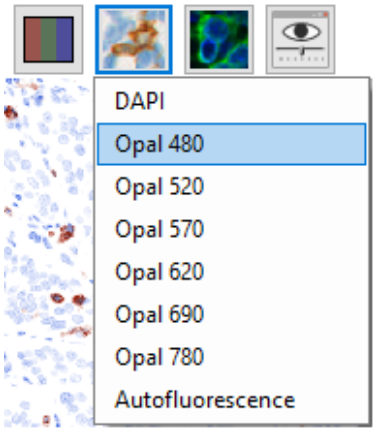


Figure 102 The component button allows you to select simulated brightfield views of fluorescence images.

Fluorescence Composite Views

While inForm can open imagery containing up to 40 components, only 10 channels can be displayed at time. When an image is first opened, inForm will default to displaying the Standard View, which is the first 10 components designated in the scan protocol.

The composite button allows you to select different composite views.

Please Note: inForm cannot open views that were created using the Phenochart software. You will need to create new views using inForm. Views can be saved as part of a project or algorithm.

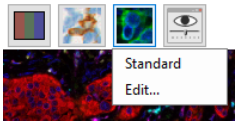


Figure 103. Default Composite View Selection

The composite views editor allows you to select which components you would like to display. You can edit existing views, create new views, and select which components to display per view. To access the composite views editor, click the composite button and click Edit.

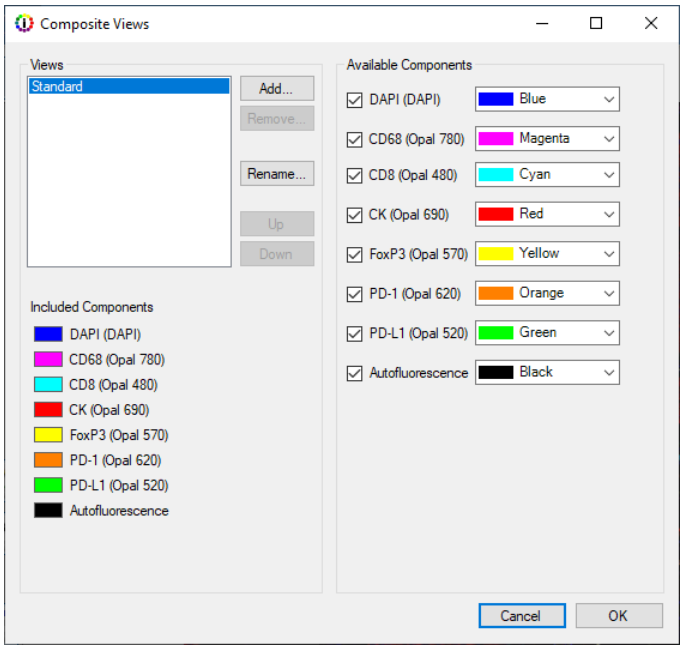


Figure 104. Composite Views Editor

Editing Composite Views

Click Rename to change the name of this view.

To change which components are displayed you can select individual components from the Available Components list displayed on the right half of the window. inForm can display up to 10 components. Change the color of each component by selecting any of the preset colors, or choose Custom to create a custom color.

Click Add to create additional Views for display. You will first be prompted to name your view. After adding a name, select **OK**. You can change the view name at a later time by selecting the view from the Views list and clicking **Rename**. To change components, first click the checked boxes next to the name of the components you wish to remove and then select the new components from the Available Components list. Assign each component a desired color. There is no limit to the number of views that can be created.

The display order of all Views can be changed. To change the location of a view, select the desired view and click the **Up** and **Down** buttons.

To permanently remove a view, select the desired view and click the **Remove** button. To prevent accidental deletion of a view, you will be prompted to click the **OK** button in the Removing Composite View confirmation before a view is permanently deleted.

Viewing Composite Views

To toggle between Views, click the composite button. A drop-down list of all **Views** created will display. Select the view name you want to display; the Legend shows which components are included within the selected Composite View.

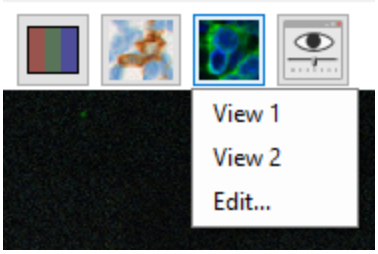


Figure 105. The composite button allows you to select which view to display

You can also toggle between views by using the up and down keyboard arrows.

17.12 Component Stats

The data tables contain the options below.

- **Min** - Displays one column containing the minimum value of the component in each region. For Cell Segmentation Data Table, displays one column for each cell compartment that was segmented, and one column for the entire cell.
- **Mean** - Displays the mean of the component in the region. The summary line displays the weighted mean value of the component across all regions, which is the average of the region means, weighted by region size.
- **Max** - Displays the maximum value of the component in the region.
- **Std Dev** - Displays the standard deviation of the values for the component in the region. The summary line displays the standard deviation of the individual means.
- **Total** - Displays the sum of the value of the component across all pixels in the region.

17.13 TMA Core Info

The data tables contain the options below.

- **Show Core ID** - If the image is of a core on a TMA slide scanned with a Vectra scanner, the Sector, Row, Column and Field of the core display in the table. If there is no core information, the columns contain zeros.

17.14 Slide Info

Exported Data tables will contain the Lab ID, Slide, ID, and Field ID for appropriate data formats. These fields are shown in the image name rather than the table display in inForm projects.

17.15 Position Stats

The data tables contain the options below. Units are in pixels.

- **X Position and Y Position** - The X and Y location of the center of the bounding box for the region.

- **Process Region ID** - If process regions are defined for the image, displays the ID number of the process region containing the tissue category. Displays #N/A if an object spans multiple process regions.
- **Distance from Process Region Edge (Pixels)** - The distance, in pixels, from the center of this region's bounding box to the nearest edge of the containing process region. Displays #N/A if the distance cannot be determined.
- **Category Region ID** - If tissue is segmented, displays the Region ID of the tissue category the segmented object is in. (Only available in the Cell Segmentation and Count Data Tables if tissue has been segmented.)
- **Distance from Tissue Category Edge (Pixels)** - The distance, in pixels, from the segmented object to the nearest edge of the tissue category. Note that if the distance is reported as #N/A, this indicates that the distance is unknown or indeterminate, for example if a cell is in a tissue region that abuts the edge of the image. The cell may be closer to the edge of the image than to any other region in the image, so it cannot be determined whether the distance to an in-image point would be nearer than a point that lies beyond the edge of the image. (Only available in the Cell Segmentation and Count Data Tables if tissue has been segmented.)

17.16 Shape Stats

The data tables contain the options below.

- **Area (pixels)** - The size, in pixels, of the region.
- **Area (percent)** - The region size as a percent of the total processed area. If there are no processing regions, it is the total image area. Otherwise it is the total area of the processing regions.
- **Compactness** - The compactness of the region. Compactness is the ratio of the area of the region to the area of a circle (the most compact shape) having the same perimeter. A circle has a compactness of 1. Objects that are elongated or irregular are less compact, as are objects with irregular or reticulated edges.
- **Minor Axis** - The smaller of the two axes of the minimum area bounding box enclosing the region.
- **Major Axis** - The larger of the two axes of the minimum area bounding box enclosing the region.
- **Axis Ratio** - The ratio of the major axis / minor axis of the minimum area bounding box enclosing the region.

18 Exporting the Data

After analyzing the images, use the Export step to export images, component images, maps, and data tables. Images are exported in the format selected in the Export Settings step. Data tables are exported to tab-separated text files, which can be opened in Microsoft® Excel® and many other data analysis programs.

1. Click the **Export** button to display the **Export Settings** panel.

2. Click the **Browse** button to select or create the desired **Export Directory**.
3. Select the **Image Output Format** you want to export images to, either JPEG or TIFF. (Generally, TIFFs retain more resolution and are usually larger. JPEGs are compressed slightly and are often better for emailing and publishing on the web.)
4. From the **Images to Export** list, select the images and maps to export. Images can be exported with or without the maps. Exporting the images with maps can be used with Review/Merge to view the segmentation for all images in a batch.

   Composite imagery can be exported with or without the Legend, and will be exported as a multi-image TIFF.
5. Select **Pathology Views** to export the components as a set of single stain + hematoxylin counterstain brightfield images.
6. Select the **Component Images (multi-image TIFF)** option to save a multi-image TIFF file of component data for analysis using third party analysis software. The values in the Component images are in the units selected in the Prepare Images step.

   See Appendix D: Akoya Biosciences TIFF File Specification 155.
7. The Tissue, Cell, and Object maps are exported in a single, multi-image TIFF file. If there were processing regions or regions of disinterest, a processing map is also included. Select **Segmentation Maps** to export one or more of these items. These images are designed to be used by image processing tools; see Appendix E: Segmentation Map TIFF Specification 162 for more information.

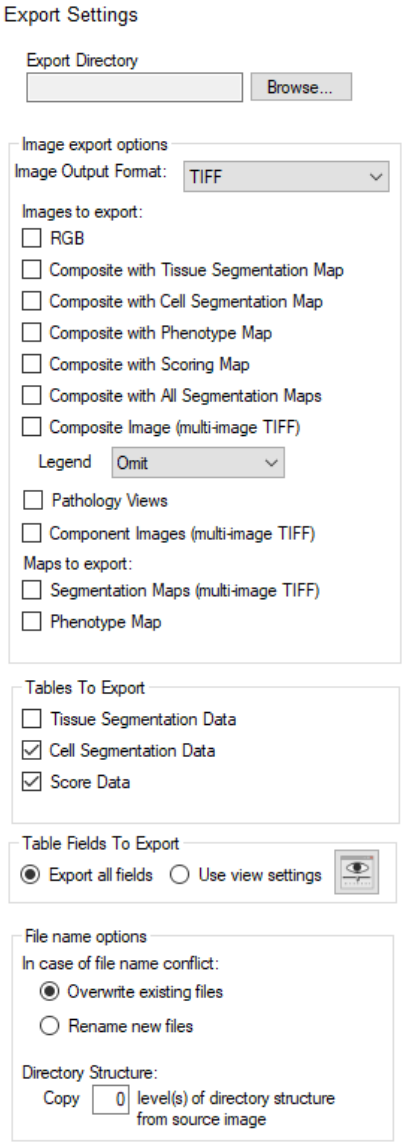


Figure 106. Export Settings

NOTE: *In Adaptive Cell Segmentation, each membrane object is assigned to a specific cell, and its label will match the corresponding nuclear label. In all other cell segmentation implementations, membrane is a shared boundary, and the image will only contain a binary map of membrane presence.*

The phenotype map is a color image with one dot representing each phenotyped cell.

8. Select the desired **Tables to Export.** The tables available for export depend on which steps are included in the algorithm or project. Data tables are exported to tab-separated text files, which can be opened in Microsoft® Excel® and many other data analysis programs
9. In **Table Fields to Export**, select whether to export all available table data fields, or only the fields that are visible in the table view. To select which fields are visible in the table, use the **View Editor** (see Displaying the Extracted Data) 108.
10. Select **File Name Options:** If files are already saved in the selected export directory, select how to name the new files. You can:
    - overwrite existing files if the new file names match, or
    - rename the new exported files to preserve any existing files in the export directory.
11. If you want to replicate the directory structure of the source images to the export directory, enter the number of directory levels to replicate in the **Copy __ level(s)** text box. For example, by entering "2" in this field, data in a *"CancerStudy\Sample001"* directory would be exported to the *\<ExportDirectory>\CancerStudy\Sample001* directory. This helps keep the exported images organized.
12. Click **Export For Selected** or **Export for All** to export the data.

The figure below shows a sample list of exported files.

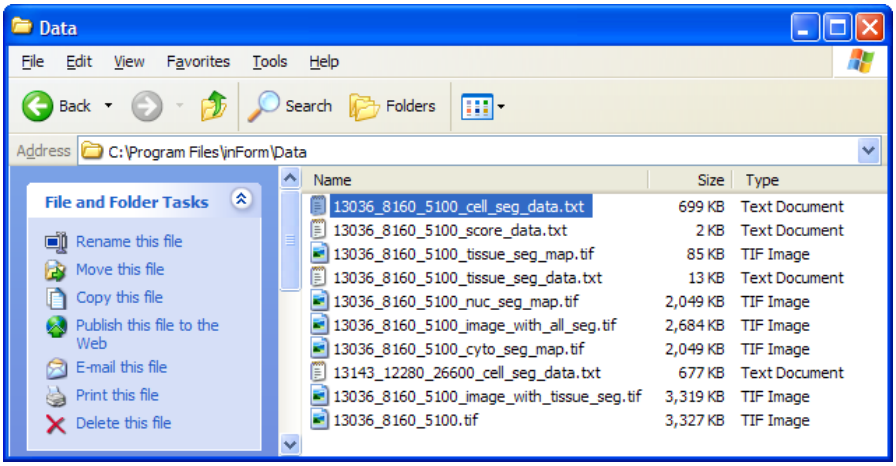


Figure 107. Exported Data Files

19 Batch Processing

Batch Analysis processes a large number of images quickly and effectively, with just one click of the batch **Run** button. Before beginning batch analysis, create and test an algorithm or project on a set of images that is representative of the entire data set. Adjust the algorithm or project as necessary until the images are processed as desired and then save the algorithm or project.

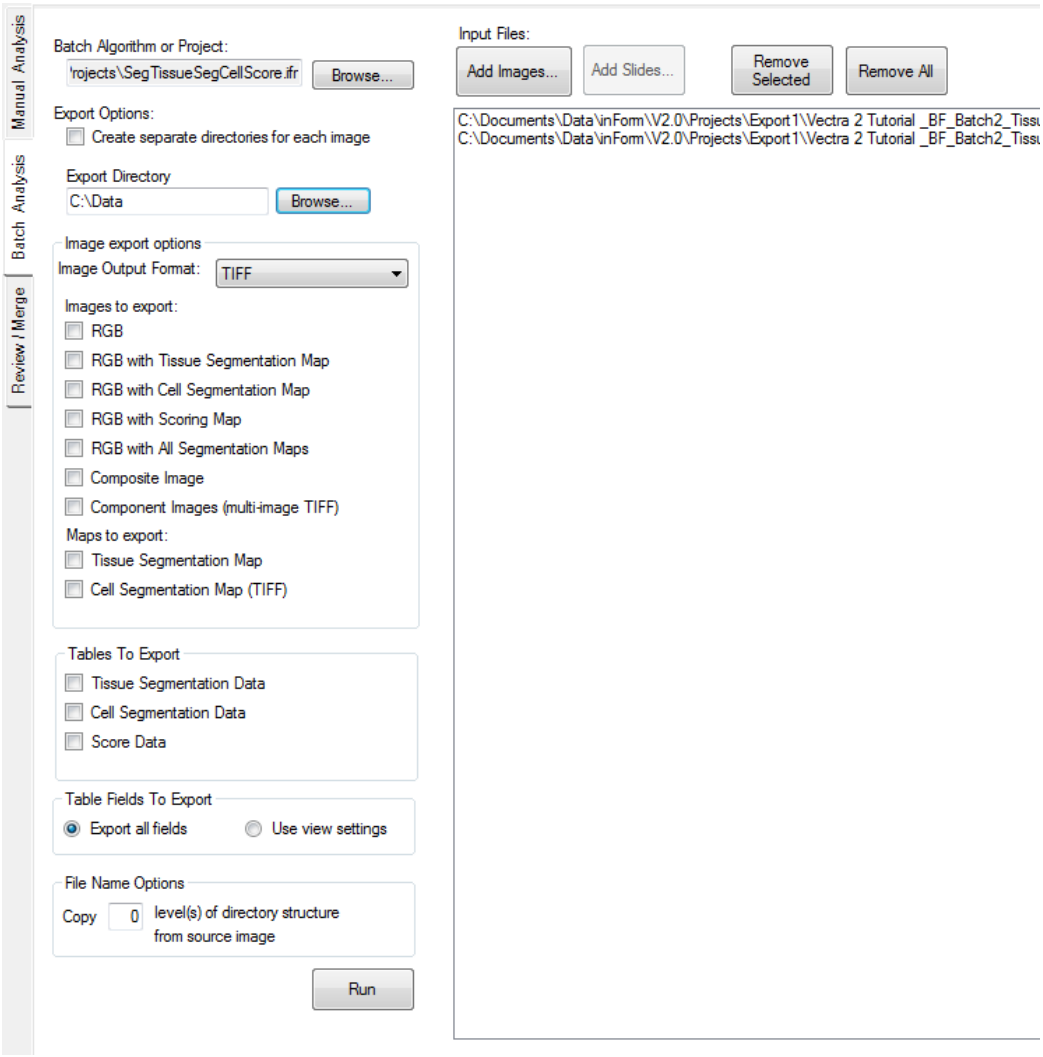


Figure 108. Batch Analysis Tab

1. Click the **Batch Analysis** tab to configure the batch settings and select images for processing. You are prompted to save the open project. Click **Yes** to save the open project or **No** to close without saving.
2. Algorithms that contain the Manual Classification step are not compatible with batch processing. If the open project contains this step, the software will post a warning. Open a project that does not contain steps that require user interaction, and then click the Batch tab.

3. Select a **Batch Algorithm or Project.** When you first switch to the **Batch** panel, the **Algorithm** settings are copied from the active project. You can run a batch using these settings, or click the **Browse** button to select an Algorithm (.ifp) or Project (.ifr) file that contains the desired settings. When loading a project, only the algorithm settings are used. The images from the project are not loaded or processed.

4. If desired, select the **Create separate directories for each item/image** option to save the exported files in a separate folder for each of the images or slides in the data set. This is recommended for large batch processes that result in many data files and images.

5. Select the desired export settings to export images, component images, maps, and data tables. Export settings are explained in detail in Exporting the Data 129.

6. Click the **Add Images** button to select images to process. Click the **Add Slides** button to select slides scanned by Vectra 3 or Vectra Polaris; inForm will process all acquired multispectral fields or inForm batch slide regions, depending on the algorithm selected. Annotations that have been ignored or deleted will not be processed.. To load older slide imagery from Vectra 2, hold the control button down before clicking **Add Slides**. If you need to change the Vectra Data Directory you are looking at, select **Change Browser Location…** from the slide browser's gear menu.

7. If necessary, use the **Remove Selected** button to remove selected images or slides from the batch or use the **Remove All** button to remove all of the images or slides from the batch.

8. Click the **Run** button. inForm processes the items one at a time and exports the specified data.

9. When the image processing is complete, click the **Done** button in the Batch Progress window.

20 Merging the Data

Use the Review/Merge tab to combine the data of a completed batch run. Data tables from each field can be merged into a single file. For example, all Cell Segmentation data from a set of batched images can be merged into one Cell Segmentation data file.

1. After running a batch, click the **Review/Merge** Tab.
2. The **Batch directory** defaults to the directory of the current batch process, or the last directory used for batch processing. Click the **Browse** button to choose a different batch results directory.
3. The data sets that can be merged display in the list. All data sets are selected by default.
4. Review the list of data sets and select the data sets to be reviewed:
   - Select or clear the check marks in the list to include or exclude specific data sets.
   - Click the **Include All** button to select all data sets in the list.
   - Click the **Include None** button to clear the check boxes for all data sets in the list.
5. When a data set is selected in the list, thumbnails of the available exported images display at the bottom of the window. Click a thumbnail to view the image in the large image display area.
6. Review the images and optionally reprocess the data:
   - Use the left or right arrow buttons next to the **Gallery** thumbnails (or Merge hot keys listed towards the bottom left) to scroll through the images for each data set.
   - If the imagery contains areas that you would like to exclude, you can draw regions of disinterest using the toolbar.
   - If you would like to limit the data to a specific area instead, you can draw processing regions.
   - Once your regions are drawn, click **Re-process** to re-process the imagery using the new regions.

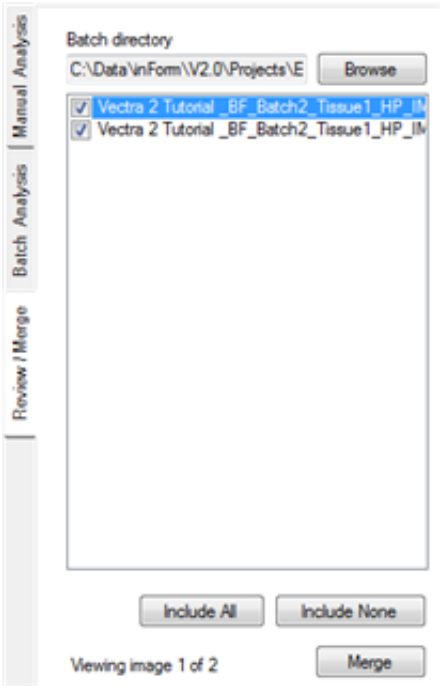


Figure 109. Review/Merge Tab

7. Accept or reject each data set by clicking the **Yes** or **No** button, or by using the Merge hot keys.
8. Review the images and optionally reprocess the data: When all data sets have been reviewed, click the **Merge** button. Type a file name prefix to identify the new files as the merged files. The merged data files are saved as tab separated text files in the batch directory with the specified file name prefix followed by the name of the data table. Data from any excluded data sets are included in the <prefix>_rejected_... files.

21 Building Libraries

IMPORTANT: *This feature works with images taken with Vectra software and with all Mantra™ images. It will not work with fluorescence Nuance images.*

There are three ways to extract the spectra from a stain or fluorophore using the Build Libraries tab:

1. Use a singly stained slide of a stain or fluorophore in the Stain Store.

   Open a Digital Slide or Multispectral Field of a singly stained slide, extract and then select an existing stain or fluorophore in the Stain Store.

2. Use a singly stained slide of a new or custom stain or fluorophore.

   Create a new stain or fluorophore in the Stain Store, open a Digital Slide or Multispectral Field of the singly stained slide, extract and then select the new stain or fluorophore in the Stain Store.

3. Use a color image of a stain in the Stain Store.

   Open an RGB (color) image containing the new stain, convert the image to optical density (OD), extract and then select an existing stain in the Stain Store.

Singly Stained Tissue Sections

The most precise and repeatable way of obtaining quantitative fluorescence data from tissue with multiple fluorescence labels present is to measure and extract the signals from the individual labels in singly stained tissue sections and store them for later use. Having an unstained tissue section is also useful for obtaining the spectra of the autofluorescence in order to reduce or eliminate it from images of your stained tissue.

This also applies to brightfield data obtained from slides with multiple brightfield stains applied to single tissue sections: ideally you will also want to have slides available that have only single brightfield stains applied to the tissue. Of course, the fluorescence technique itself allows for many more labels, especially using Akoya Bioscience's method of reducing or removing the confounding autofluorescence signal from your slides.

IMPORTANT: *Akoya Biosciences imaging systems and software programs will give you optimal results when the tissue sections have been stained well, using protocols that give you consistent slides suitable for multilabel brightfield or fluorescence imaging. The best results from any imaging system come from good samples. Consider using high-quality, well-tested staining kits such as Akoya's Opal™ multiplex reagent kits for fluorescence and a protocol developed for multilabel staining and imaging.*

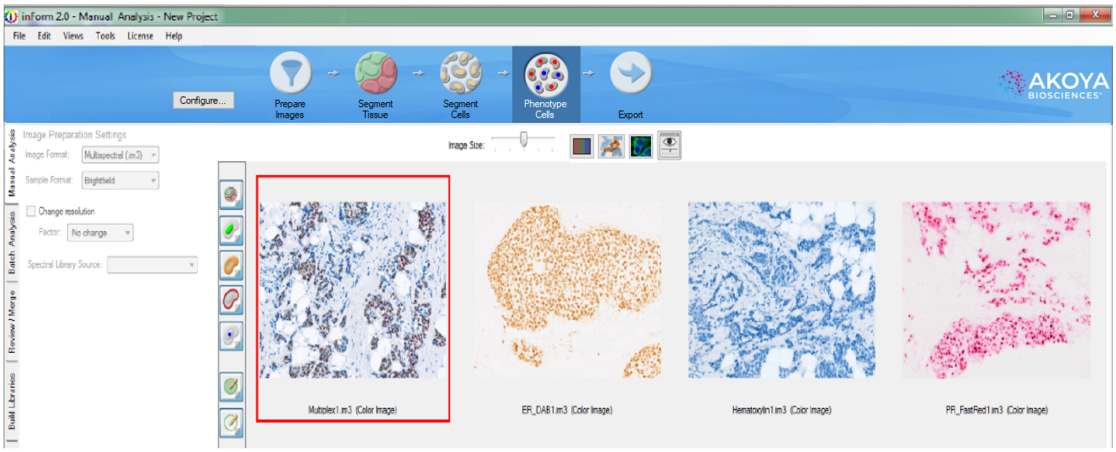


Figure 110. You should strive to utilize measured and extracted signals from singly stained slides that you can store for later use. Left to right: multiplexed IHC stained tissue section, DAB-only stained tissue section, hematoxylin-only stained tissue section, and Fast Red only stained tissue section.

Extracting a Stain or Fluorophore

Click the **Build Libraries** tab and then click the **Browse** button to load an image of a singly stained sample.

NOTE: *When loading Digital Slides with inForm Project annotations, only the first annotation will be loaded.*

Once the image opens, you can use the **Spectral Curves tool** and move your cursor over the image and see the spectral curves on the right side of the window in real time. You can set the units of the chart. Choose the stain or fluorophore from the drop-down list and click the **Extract** button. Confirm that the image displayed (you can view the original **Color** image or the individual **Component** image) is accurate, then click the **Save to Store** button.

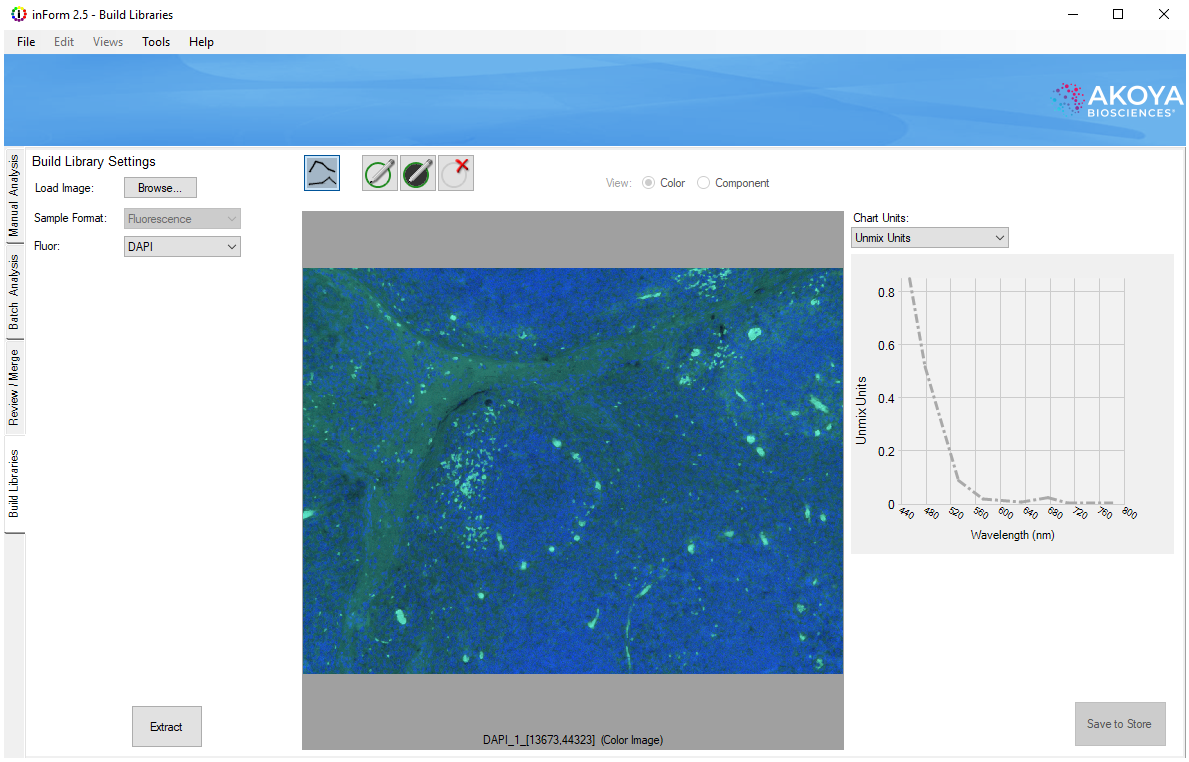


Figure 111. An opened DAPI image before the stain has been extracted.

Select the display color, add notes, and choose groups for your extracted stain or fluorophore before saving it.

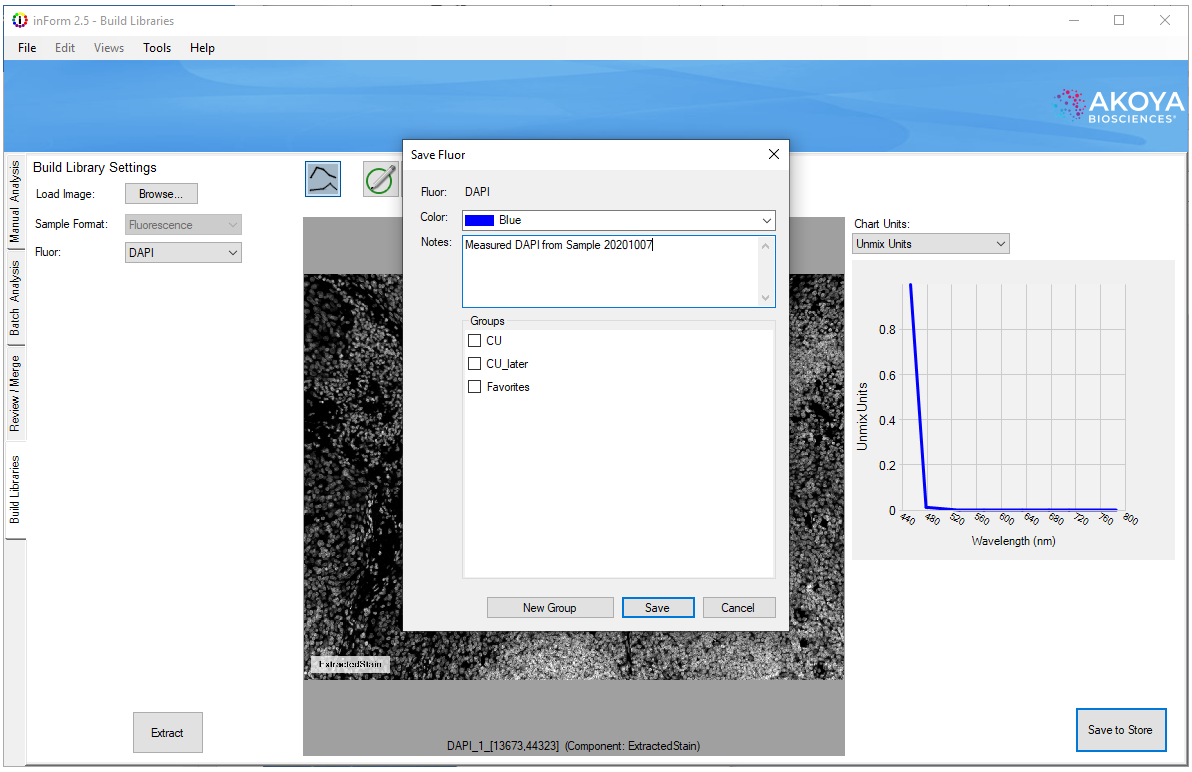


Figure 112. Clicking Extract allows you to extract and save a measured single stain to the Stain Store, which serves as your library of spectra for different stains and fluorophores.

When a brightfield image is displayed and extracted, you will only see one spectral curve to the right of the image in the Build Libraries tab. However, when a fluorescence image is displayed, you will see as many spectral curves as you had epi-fluorescence filter sets in use at the time of the image acquisition.

In brightfield, you may receive a message that more than one stain was detected in your particular specimen. This is common for DAB, and has been seen in other IHC stains. Confirm that your slide is singly stained (without a counterstain); if so, it's safe to proceed.

To extract your next stain or fluorophore, click the **Browse** button to load a new image. If you've left an extracted stain or fluorophore unsaved, the system will prompt you before loading your next signly stained sample.

Extracting a Custom Stain or Fluorophore

If you want to extract and save the spectra for a stain or fluorophore that does not appear in the Stain Store (perhaps you are developing new stains or fluorophores), you can create a new custom stain or fluorophore in the Stain Store.

Go to **Tools > Stain Store Manager** and click **Choose Which Types of Stains and Fluors Your Lab Uses** 31. Select the **Other (User Specified)** line under Family Name, then click the **New** button and enter the Name of your new or custom stain or fluorophore. Choose whether the stain or fluorophore will be imaged in brightfield or fluorescence and if it is a nuclear counterstain. Click **OK** and close the Stain Store window.

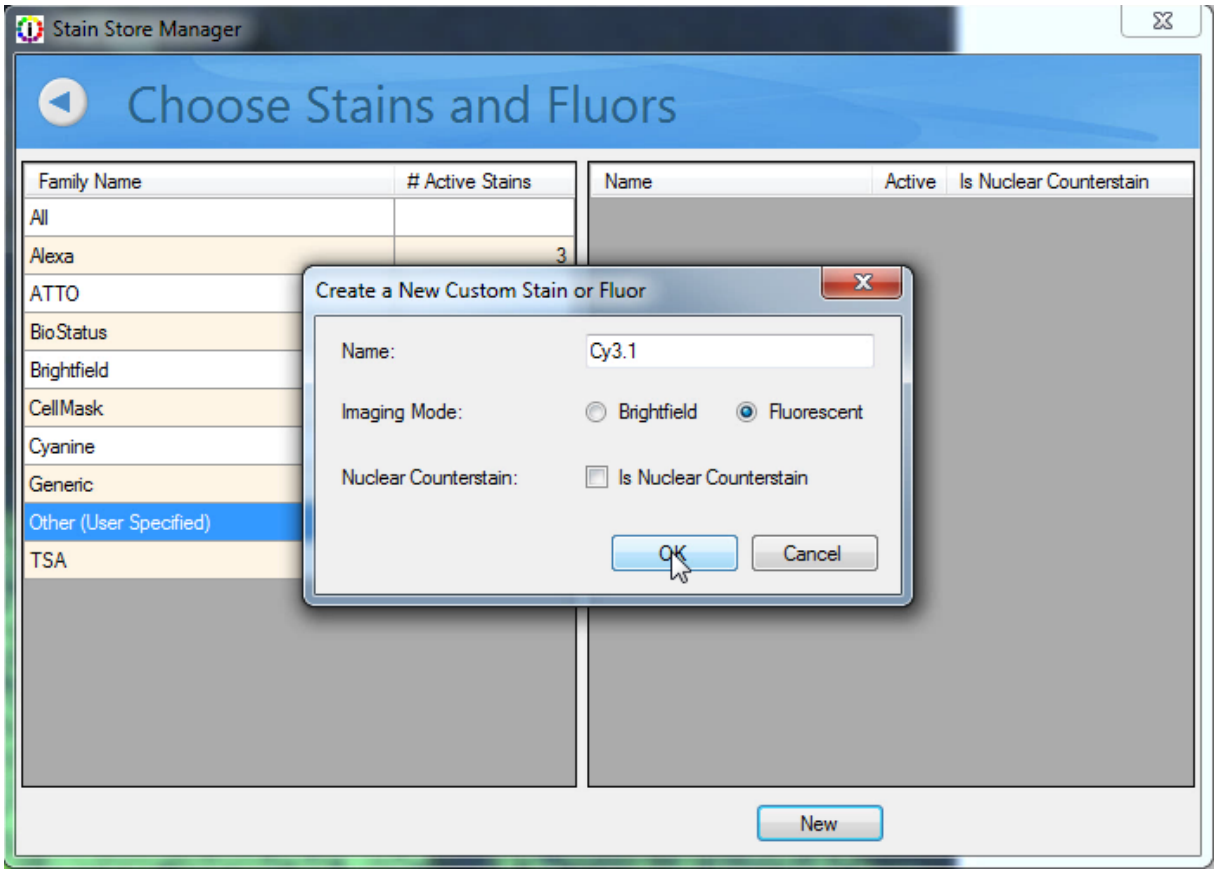


Figure 113. If you have a new stain or fluorophore that does not appear in the Stain Store, you can create a new listing for one.

Select the **Build Libraries** tab and click the Load Image **Browse** button. Select the image of your singly stained slide, then chose the name of your new/custom stain or fluorophore in the Stain/Fluor drop-down list.

For brightfield images, click the **Extract** button, confirm the results are accurate, then click the **Save to Store** button to save the extracted spectra to the Stain Store.

For fluorescence images, when the image opens, move your cursor over the pure signal and observe the curves that appear to the right of the image. These display how much of the signal appears within each fluorescence emission 'band',

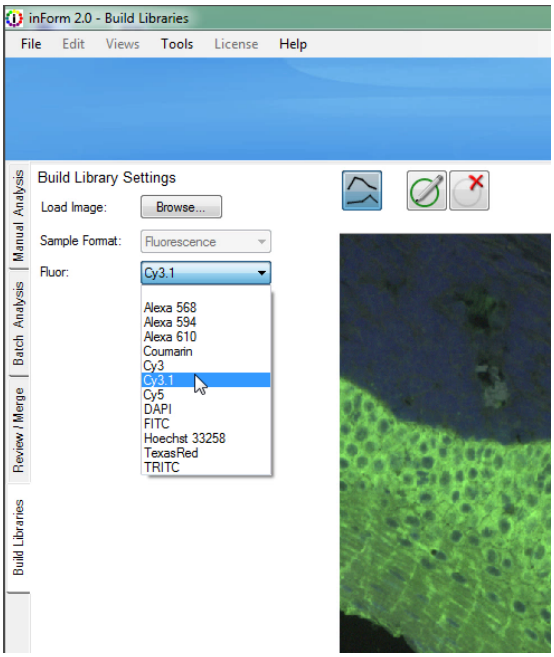


Figure 114. In the Build Libraries tab, you can select your new/custom stain or fluorophore in the Stain/Fluor drop-down menu.

corresponding to each of your epi-fluorescence filters.

You will have as many curves as you have epi-fluorescence filters in use. The Akoya Biosciences Mantra™ system that was used to acquire the image in Figure 76 had 5 filters, and thus 5 bands and 5 curves. From the drop-down menus, choose the **Signal** band where the fluor most strongly expresses and a **Dark** band where the fluor does not express at all. Then click the **Extract** button.

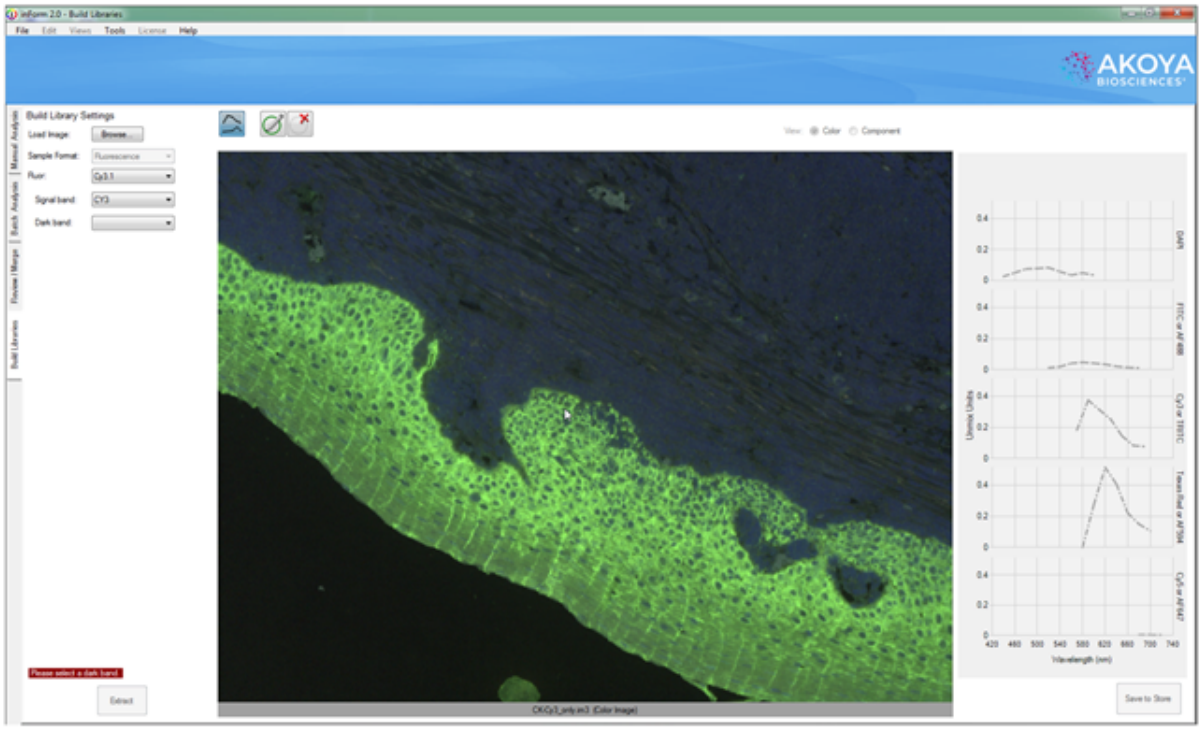


Figure 115. You can use the curves to the right of your image that display the spectra under your cursor to selec the 'Signal Band' where the majority of the signal of your new/custom fluorophore appears, and also the 'Dark Band' where there is little or no signal.

After the pure signal has been extracted, you will see a black-and-white image where the lighter pixels represent the signal of your new/custom stain or fluorophore. Confirm that the image displayed (you can view the original **Color** image or the black-and-white individual **Component** image) is accurate, then click the **Save to Store** button.

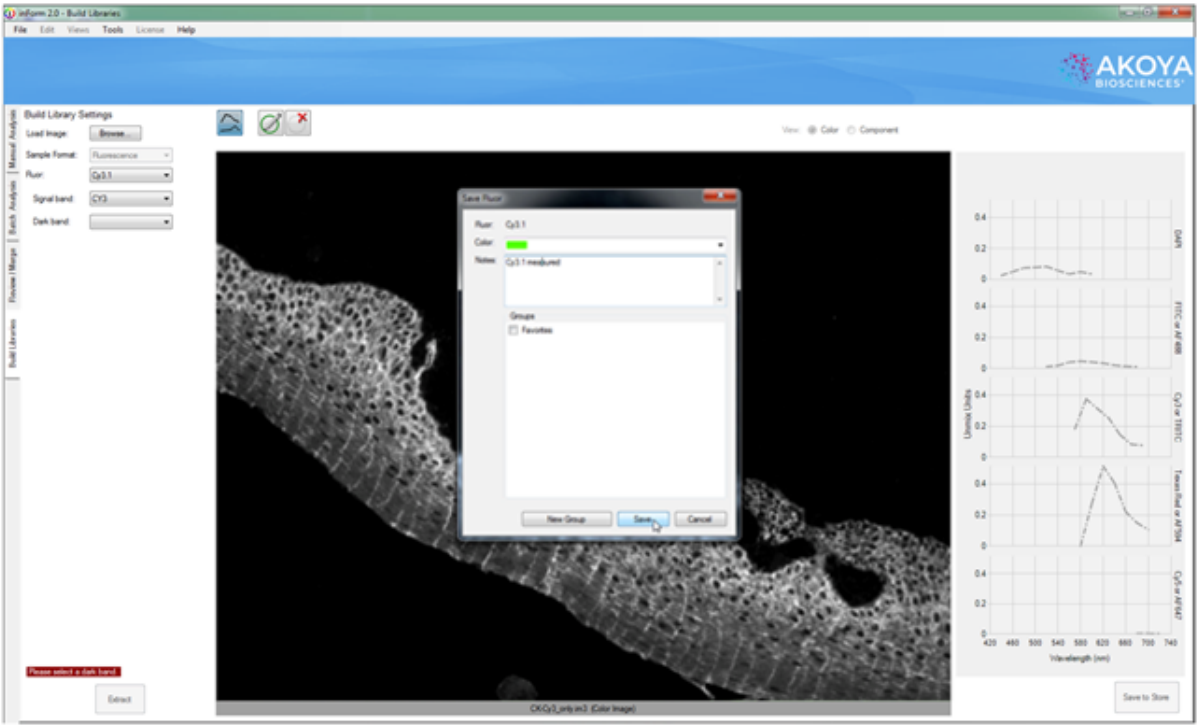


Figure 116. Once the spectra from your new/custom stain or fluorophore has been extracted, you can save it to the Stain Store.

Extracting a Fluorophore with a Custom Filter

inForm has built-in settings for many manufacturers' epi-filter cubes. If you are using a custom filter, inForm requires more information before extraction.

Click the **Build Libraries** tab and click the Load Image **Browse** button. Select the image of your singly stained sample. Then select the fluorophore you wish to extract.

inForm will present two drop-down menus which have been prefilled with the names of the bands used in the image. Choose the **Signal band** where the fluor most strongly expresses and a **Dark band** where the fluor does not express at all. Note that your custom filter may not be needed in these selections.

When you have made your selections, click the **Extract** button. Confirm that the image displayed is accurate, and then click the **Save to Store** button.

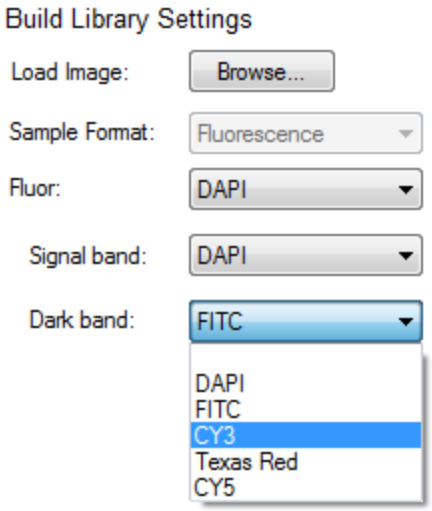


Figure 117. Dark Band Drop-Down List

Extracting a Stain from a Color Image

Occasionally you may have RGB images from another source that you wish to unmix using inForm. In this case you can extract the pure signal from a color (RGB) image of a singly-stained sample. The image must be of high quality and not contain multiple labels.

Select the **Build Libraries** tab and click the Load Image **Browse** button. If you are loading an extremely large image, you may be asked to bin it to reduce the size.

Select the color image (.BMP, .JPG, .TIF, or .PNG file) of your singly stained slide and then chose the name of the corresponding stain in the Stain drop-down menu.

Using the **Convert to OD** tool, select the brightest white area in the image. inForm will convert the image to optical density, which is required in order to properly quantify stain intensity.

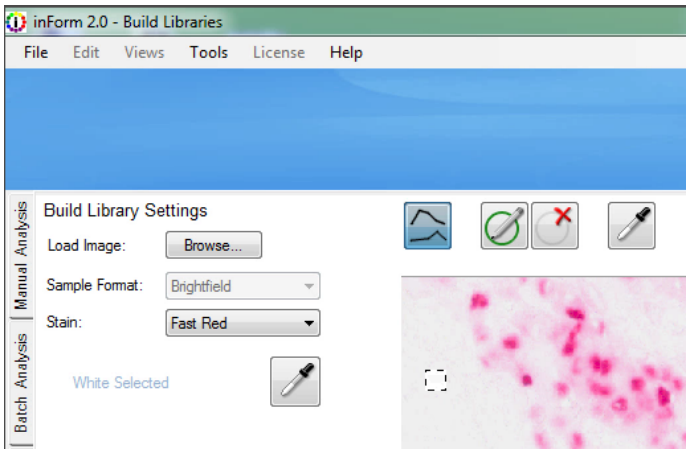


Figure 118. When you open a color (RGB) image of a singly stained slide, you need to select a white area in order to help the software convert the image to optical density, which is necessary in order to properly quantify stain intensity.

Click the **Extract** button and when the black-and-white image of the extracted pure signal appears, confirm that the image displayed (you can view the original **Color** image or the black-and-white individual **Component** image) is accurate and click the **Save to Store** button.

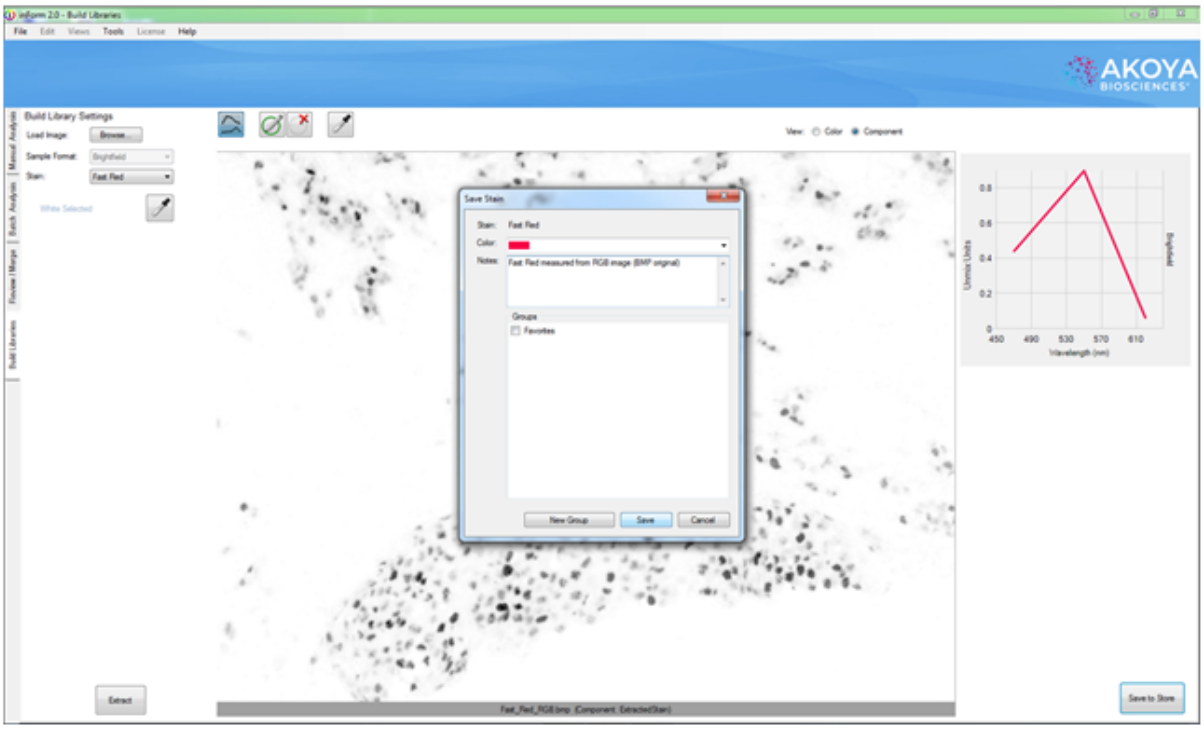


Figure 119. You can extract a pure signal from a color (RGB) image after it is converted to optical density, but the resulting spectra will not be as detailed as the pure signal extracted using a multispectral .IM3 file. Note the lack of a smooth spectral curve representing the Fast Red stain. Instead of information from many wavelengths, a color (RGB) image only contains information for three broadly overlapping regions in the visible wavelength range.

Using Processing Regions

Sometimes you will get an error message when clicking the **Extract** button. If this occurs, you can help the inForm algorithm extract the pure signal by using the **Process Regions** tool to select areas containing the pure signal for brightfield images and also to select areas with both the primary signal and autofluorescence for fluorescence images.

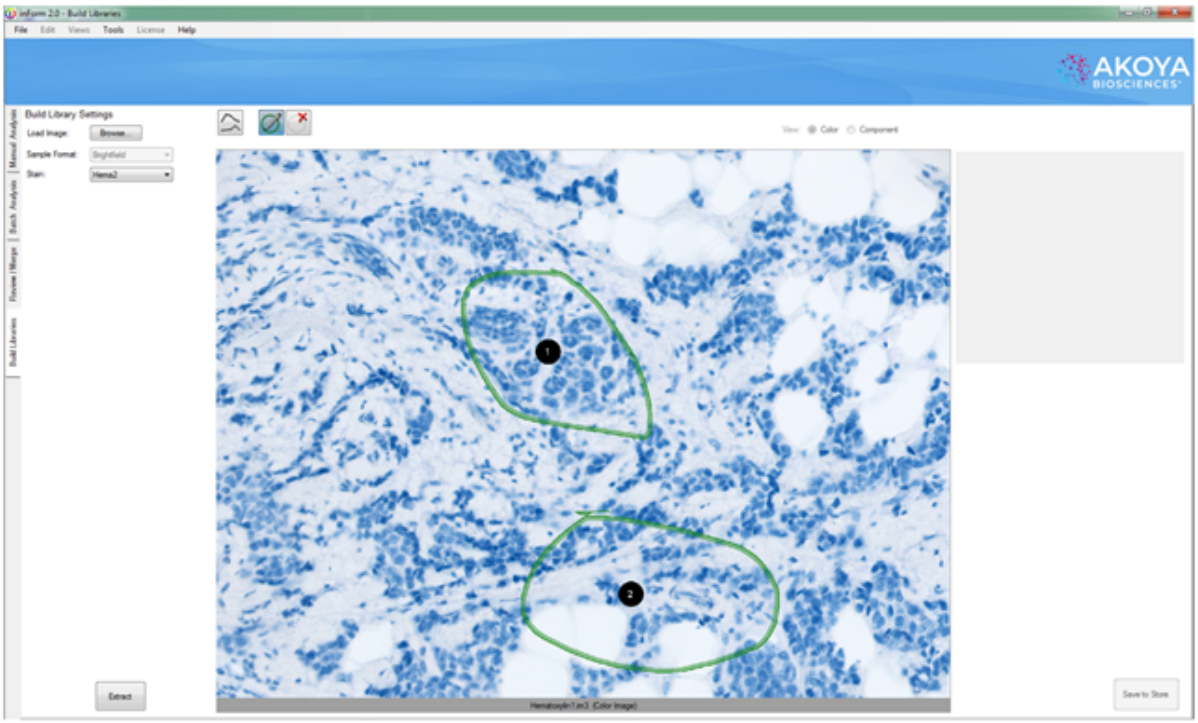


Figure 120. You may have a slide that contains small areas of your stain or fluorophore, and clicking the Extract button results in an error. Click the ROI button (diagonal pencil over a green circle) and select areas to assist the inForm extraction algorithm. You may need to select regions based on different criteria, depending on whether your image is a brightfield or a fluorescence one.

For brightfield images, you might need to select several areas that mostly or only contain the signal that you are interested in, avoiding large empty white areas. For fluorescence images, do not try to select areas that just contain the signal that you are interested in. Select areas that contain both the signal and autofluorescence, perhaps minimizing but not completely avoiding areas with large voids or no signal. Do not hesitate to try selecting different areas: you can delete areas using the **Delete** button.

You might also receive an error message if inForm detects the presence of more than one stain or fluorophore in the sample). Although Akoya Biosciences highly recommends using singly stained slides, perhaps you have a small amount of inadvertent contamination from other stains in your otherwise singly stained slides. Below is an image containing a large amount of eosin and a much smaller amount of hematoxylin. You can draw a region or regions around the tissue where just the eosin is present, ignoring the rest of the image.

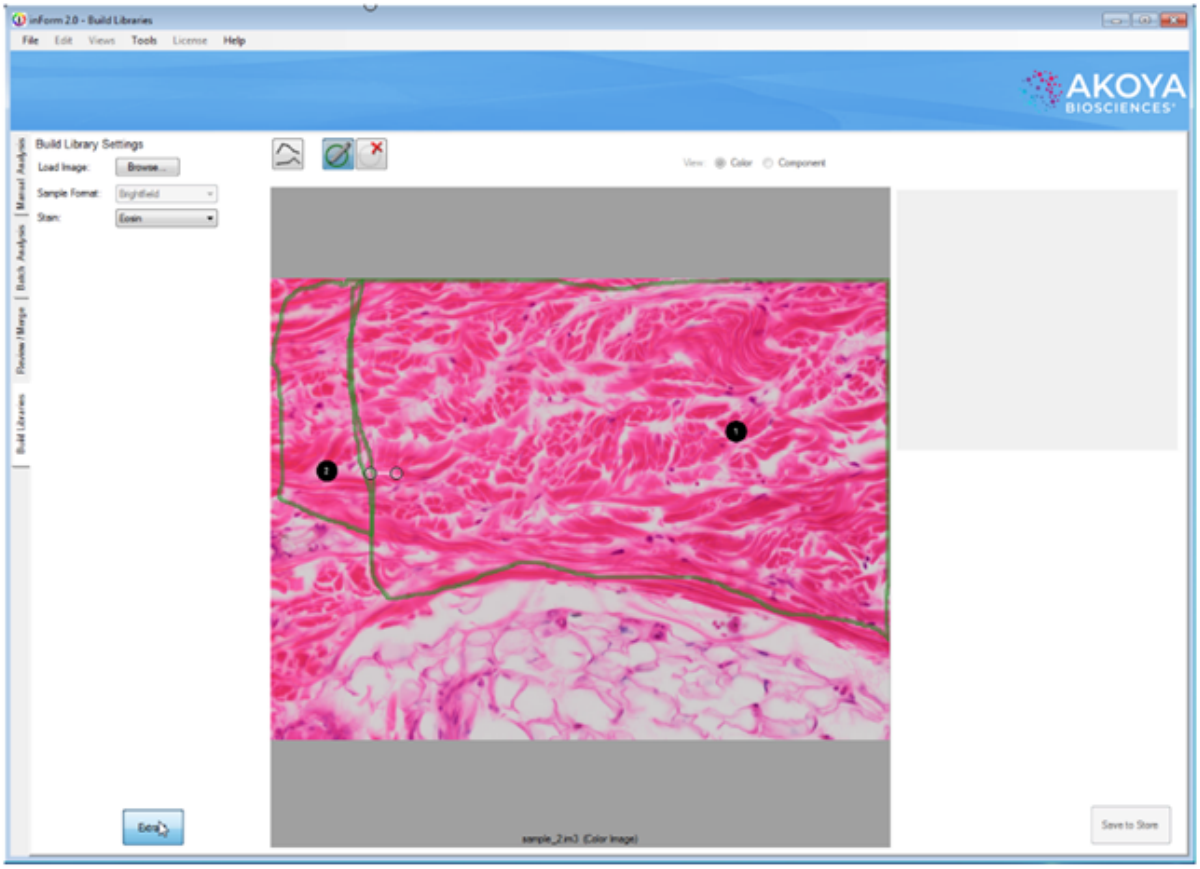


Figure: 121. In the Build Libraries tab, you can select regions within your specimen that contain reasonably pure spectra from a single stain. In this case pink eosin, avoiding areas where there are very small amounts of blue hematoxylin present.

inForm will extract what it believes to be the pure spectra and display the spectral curves to the right of a black-and-white image. The black-and-white image represents the presence of the pure stain or fluorophore in your sample. You can toggle back and forth amongst the **Color** and **Component** views in order to check and verify proper selection using the View selector above the large displayed image.

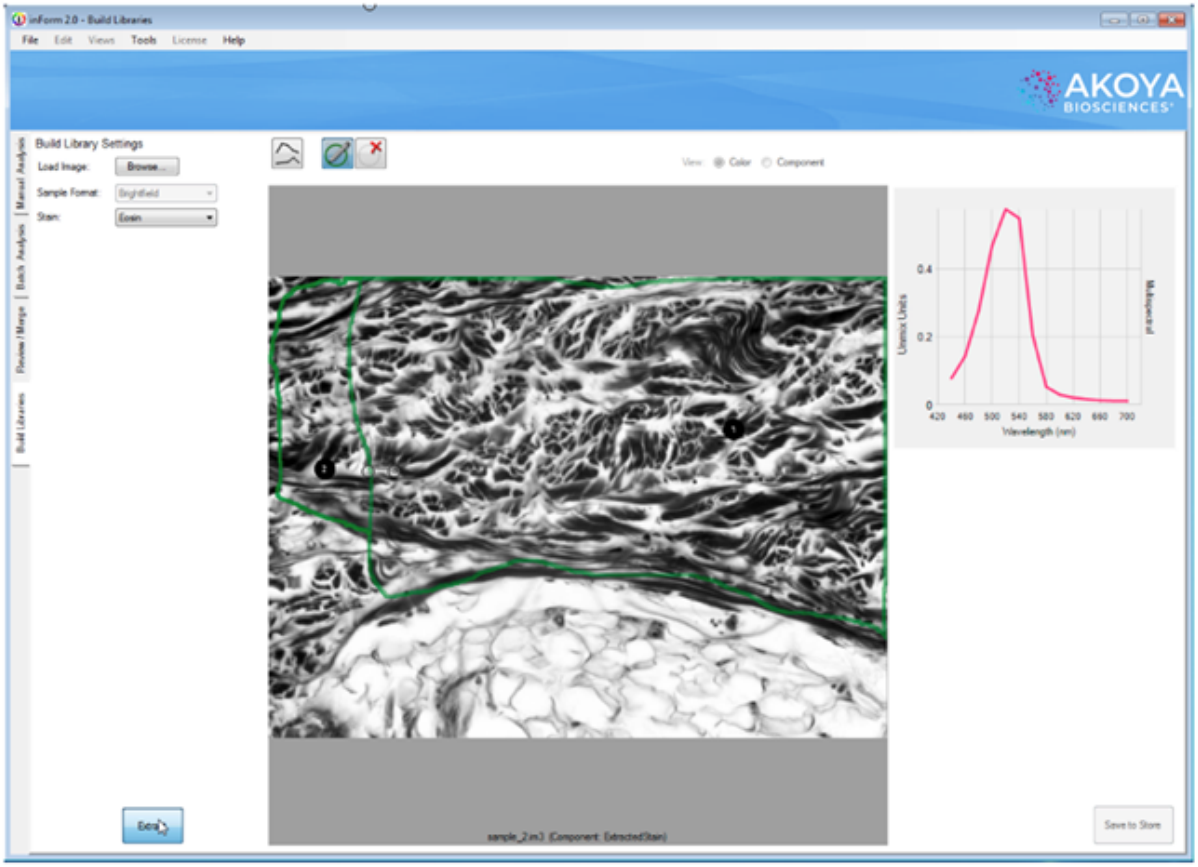


Figure 122. If there is enough of the pure spectra present for the stain or fluorophore that you chose from the drop-down menu, inForm will show you the spectral curve that it has extracted and a black-and-white image representing the presence of that stain or fluorophore.

Click the **Extract** button and when the black-and-white image of the extracted pure signal appears, confirm that the image displayed is accurate and click the **Save to Store** button

22 Frequently Asked Questions

I am trying to extract a pure hematoxylin signal from a singly stained slide, but after I open the image that I took in Mantra in the Build Libraries tab in inForm, I don't see hematoxylin in the Stain drop-down menu. How can I add hematoxylin to the choices?

The **Stain** drop-down list items in the Build Libraries tab are populated from the Stain Store, as described in Understanding the inForm Work Area 19 and Building Libraries 134.

You might not have selected hematoxylin as a stain that your lab uses. Go to **Tools > Stain Store Manager** and click **Choose Which Types of Stains and Fluors Your Lab Uses**.

Select **Brightfield** under Family Name and then select the **Hematoxylin** check box on the right.

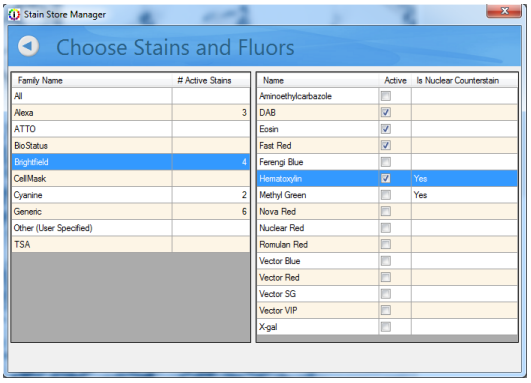


Figure 123. You can add stains and fluorophores to the Stain drop-down menu in the inForm Build Libraries tab.

Close the Stain Store Manager and return to the inForm Build Libraries tab. Hematoxylin will now appear in the drop-down menu.

Of course, you can also measure new stains and fluorophores that do not already exist in the inForm Stain Store. See Building Libraries 134.

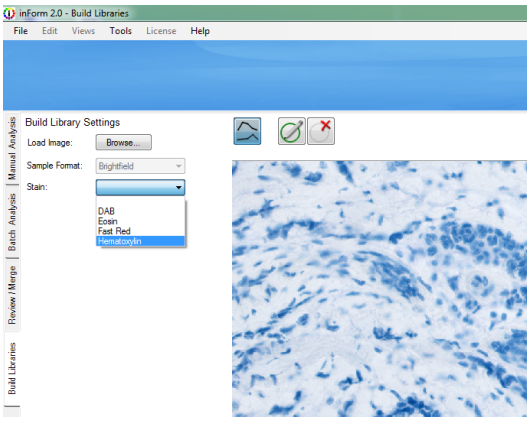


Figure 124. Once you have selected a stain or fluorophores in the Choose Which Types of Stains and Fluors You Lab Uses window of the Stain Store Manager, the stain or fluorophore will appear in the Stain drop-down menu in the inForm Build Libraries tab.

23 Appendix A: Calculating Fractional Tissue Area

Fractional tissue areas can be calculated from inForm data using an Excel® PivotTable. Examples of this type of calculation are:

- **Tumor load** - tumor area / (tumor + stroma)
- **Lung fibrosis** - fibrosis area / (normal lung + normal collagen + fibrosis)
- **Breast tissue lobular involution** - acini area / (acini + inflammation + lobular stroma)

It is also possible to calculate tissue fractions where the numerator and denominator are different tissue categories, such as:

- **Kappa/lambda ratio in lymphoid tissue** - kappa tissue area / lambda tissue area

This calculation can be performed per image or per slide with inForm (V2.0.1 or higher) and Vectra.

Initial Setup

These instructions assume that you already have an inForm algorithm capable of automatically segmenting the tissue categories of interest, and that you have processed several images using the algorithm and merged the tissue segmentation tables into a single table.

To compute tissue fraction by slide, you must have a column in the data table for slide ID. inForm includes this column for Vectra images.

The instructions in this section are for Excel® 2010 and may need modification for other versions of Excel®.

Create a PivotTable to Compute Fractional Tissue Area

To create the PivotTable:

1. Start Excel® and open the data file. In the Open dialog, select **Text Files** in the file type drop-down list, then select the inForm data file. The file name should end with "tissue_seg_data_summary.txt".
2. In the Text Import Wizard, if you are using a file from inForm V1.4, change the value for **Start import at row** to **3**. This skips the first two rows. For inForm V2.0 or higher, leave the value at **1**. Click the **Finish** to open the file.
3. In the Insert ribbon, click **PivotTable**. Leave the defaults: use the entire table and put the table on a new worksheet. Click **OK** to create the PivotTable. A new, empty PivotTable opens. On the right side, the PivotTable Field List shows all the fields in the source table.
4. Select the check box next to the name of the field to summarize by, usually Sample Name or Slide ID. It could also be TMA row and column. The selected field is shown in the Row Labels box and as a column in the pivot table.
5. In the PivotTable Field List, select the check box next to **Tissue Category** to add Tissue Category to the Row Labels list.
6. Drag **Tissue Category** from Row Labels to Column Labels.

7. Some tissue categories in the data may not be used in the tissue fraction calculation, for example a Background category. In this case, click the drop-down list next to **Column Labels** and de-select any categories that are not of interest.
8. In the PivotTable Field List, select the check box next to **Area (pixel)** to add Area (pixel) to the Values box.
9. You should now have a PivotTable that shows total pixel area for each tissue category of interest and a Grand Total. The table has one row for each Sample Name or Slide ID, depending on the selection for Row Labels.
10. To display pixel area as a percent, right-click **Grand Total** and select **Value Field Settings**. Select the tab **Show Values As**. In the drop-down list, select **% of Row Total** and click **OK**.

You should now have a pivot table that displays the percent of total area for each tissue category. The values of interest can be read directly from the table. For example, a tumor category displays the tumor load.

Compute a Ratio

The PivotTable does not easily display a ratio of two categories; it only shows each category as a fraction of the total. Additional ratios can be added as new computed columns outside the pivot table. Use explicit row/column identifiers in the formula, such as “D3”. Don’t use the GETPIVOTDATA () function that Excel® provides if you click target cells. For example:

1. Click the cell in the first empty column in the first data row.
2. Type the desired formula such as “=C5/B5” and press Enter.
3. Select the entire range in the target column, starting with the cell just entered.
4. Type CTRL+D to fill the column range with the desired formula.

Saving the Table

You can save the entire Excel® workbook and/or export the PivotTable data as a text file for further processing with other programs. If exporting the PivotTable, you may want to refine the table for cleaner output. Below are some suggestions.

Remove the Grand Total Row at the Bottom

1. Open PivotTable Options by clicking the **Options** button at the left of the **PivotTable Tools > Options** ribbon.
2. In the Totals & Filters tab, deselect **Show grand totals for columns**.

Display Fractional Area

To display the tissue fraction as a decimal fraction rather than as a percent, click the drop-down list next to the field name in the **Values** box. Select **Value Field Settings**. In the dialog box click **Number Format** and set the desired formatting.

Save as Tab-Separated Values

To save the file as tab-separated text:

1. In the **File** ribbon, choose **Save As**.
2. Select **Text (Tab delimited)** in the **Save as type** drop-down list. To save as a CSV file, select **CSV (Comma delimited)**.
3. Type the desired file name, select the desired location, and click **Save**.
4. Two warning messages display. First, The selected file type does not support workbooks that contain multiple sheets. Click the **OK** button. Then, "[FileName] may contain features that are not compatible with Text (Tab delimited)." Click the **Yes** button.

24 Appendix B: Auto-Processing Data Received from Mantra Snap

New for inForm version 2.1 is auto-processing of data from the Mantra™ Quantitative Pathology Imaging System. This allows you to capture ('snap') images using the Mantra workstation with the Mantra Snap software program and have those images automatically processed in the inForm program that comes with the workstation.

By default, each time you snap a picture using the Mantra instrument, the Mantra Snap program saves your image to the location that you selected for your Destination folder. But Mantra Snap can also be configured to automatically launch inForm and perform a pre-arranged operation, such as tissue and cell segmentation and phenotyping, on each of your images—even while you continue to snap pictures.

Chose the Launch inForm Analysis option in the Post Processing editing area and the inForm program will be launched, if it is not already running, after you snap a picture. If inForm is already running and an algorithm is loaded, the Mantra image will be processed by inForm using that algorithm.

For example, you may be trying to develop a robust phenotyping algorithm on a set of slides or a slide with a tissue microarray on it. You can take pictures using the Mantra instrument and Mantra Snap. Taking the first image launches inForm, which you then use to develop an algorithm that you test by taking more images using Mantra Snap. You can make changes to the algorithm based on the results that inForm shows you, and then take more images with Mantra Snap, an iterative process.

After you have a robust algorithm or if you simply wish to apply the algorithm to many images as you take them in Mantra Snap, you can select the **Batch Process Using inForm** option which will not bring up the actual inForm user interface, but will run a specified algorithm (selected using the **Browse…** button) on each picture you snap in the background.

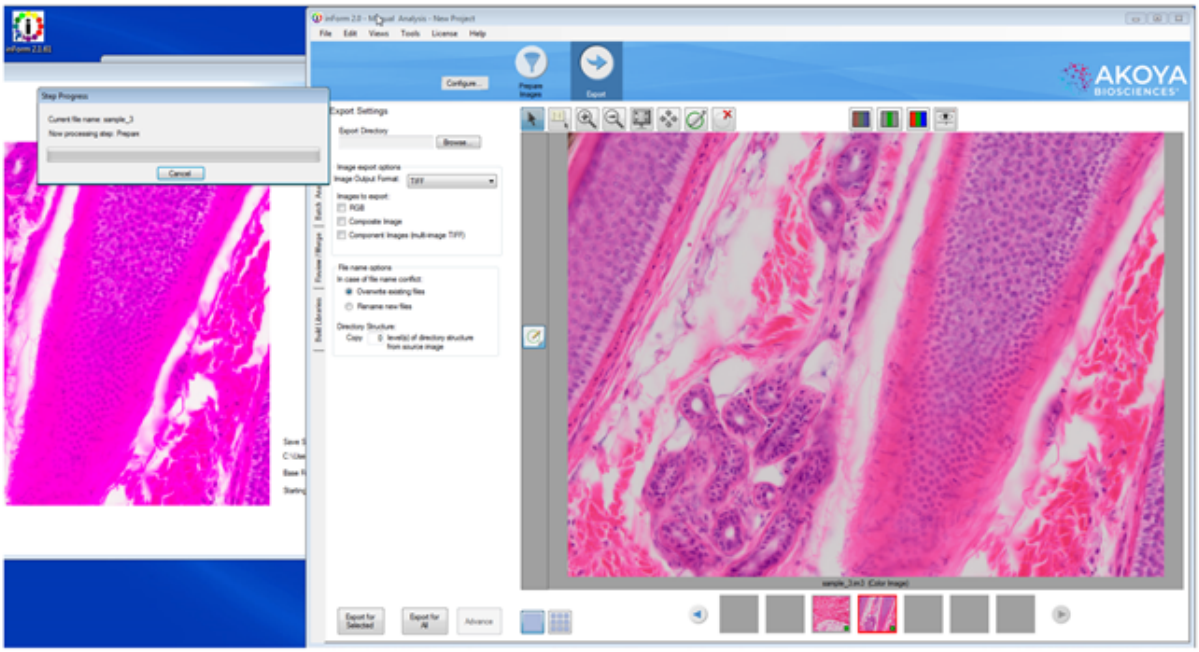


Figure 125. A Mantra image was used to develop an inForm algorithm and then a second image taken on the Mantra workstation. Immediately after clicking the Snap button, the second image has automatically been opened in inForm and the open algorithm is being applied to the image.

25 Appendix C: Phenotype Classification by Multinomial Logistic Regression

inForm uses a training set, image features and machine learning to classify cells into user-defined phenotypes. The user provides the training set. Image features are derived from texture analysis and cell segmentation. The `glmnet`[1] package from **R**[2] creates the machine learning classifier. While the creation of the classifier is complex, the result is a relatively simple multinomial logistic regression classifier[3] with softmax output. The classifier is applied to the features to assign phenotypes to cells.

This document presents the steps in this process.

Introduction

Phenotyping cells in an inForm image (or images) requires four steps:

1. Create a training set by marking up the image(s).
2. Create features from the image(s).
3. Train a classifier using features of the training set.
4. Apply the classifier to the full image(s).

The first step is controlled by the user. In the second step, inForm uses features from the cell segmentation table and adds texture features similar to the ones used for tissue classification. In the third step, the training set features are passed to `glmnet` which uses lasso regularization with cross-validation to select features and create a model. Finally, the model is applied to the full feature table to classify all cells.

Create a training set

The user interacts with inForm to create a training set containing representative samples of each phenotype. This process is documented in Phenotyping Cells 97. The training set must contain at least five examples from each phenotype.

Create features

inForm creates features for each cell in an image. The features come from two sources: cell segmentation and component textures.

Cell segmentation features

The cell segmentation features consist of most of the columns in the full cell segmentation table. The columns that are omitted, and the reasons for the omissions, are

- empty columns and columns containing only or predominantly a single value
- percents - these duplicate the non-percent data
- data specific to an image or the location of a cell within an image:
  - Path

- Sample Name
- Cell ID
- Cell X Position
- Cell Y Position
- Category Region ID
- Process Region ID
- Distance from Process Region Edge
- Lab ID
- Slide ID
- TMA Sector, Row, Column, Field

- Tissue Category - this might be informative but it is removed because the classifier requires quantitative features
- Features which are exact linear combinations of other features; usually just Entire Cell Area

The number of cell segmentation features varies depending on how many components are in the image and how many cell compartments were segmented. Each compartment contributes five morphological features (area, compactness, minor axis length, major axis length, axis ratio) plus five features per component (minimum, mean, maximum, standard deviation, total). If more than one compartment is segmented, features will be created for Entire Cell as well. Thus with $n_{compartment} > 1$ compartments and $n_{component}$ components, the number of cell segmentation features will be approximately

$$(n_{compartment} + 1) * (5 + 5 * n_{component}) - 1$$

With three compartments and five components, this gives **(3 + 1) * (5 + 5 * 5) - 1** or 119 cell segmentation features.

Refer to the inForm manual for detailed descriptions of the cell segmentation features.

Texture features

Texture features are created for each component of the image. The features are computed within a 16x16 pixel kernel placed at each possible location on a 4x4 grid across the image. The grid locations are assigned to cells by matching the center points of the kernels with the cell segmentation masks. The feature values for the kernels assigned to each cell are averaged to give a single value per cell for each feature. There are 10 texture features, so this process gives 10 features per component for each cell. With three compartments and five components, there are 50 texture features and 119 cell segmentation features per cell, a total of 169 features.

inForm uses three kinds of texture features: spatial frequency measurements, co-occurrence features and simple first- and second-order statistics.

Spatial frequency features

The spatial frequency features represent the relative intensity of four spatial frequency bands computed with a two-dimensional Fast Fourier Transform. The full range of frequencies is divided into four equal bands. The intensity within each band is summed and normalized by the total intensity of all frequencies.

These make the FFT1, FFT2, FFT3 and FFT4 features. FFT1 represents the lowest frequency and FFT4 the highest.

Co-occurence features

Grey-level co-occurence features[4] are commonly used for texture classification. They were originally defined by Haralick et. al. (1973)[5] . A detailed description of these features is beyond the scope of this document; please refer to the references. In the language of the references, the features used by inForm are Angular Second Moment (ASM), Entropy, Inverse Difference Moment (Homogeneity) and Contrast. The texture features are spatially-invariant (computed in four directions) and use an offset distance of one.

Simple statistics

The texture features include the mean and standard deviation of the component value in the kernel. To some extent these features duplicate the mean and standard deviation features in the cell segmentation table but they are computed differently and the values are not exactly the same.

Train a classifier

The preceding steps create a training set labeled with user-assigned phenotypes, and a feature vector for each cell in the training set. These are given to the `cv.glmnet` function in the `glmnet` package to create a multinomial logistic regression classifier.

`cv.glmnet` uses lasso regularization[6] to select from the available features. Lasso regularization sets some parameters of a regression model to zero, so it can be used for feature selection. The number of zero parameters is controlled by a meta-parameter **λ** whose value is chosen by ten-fold cross-validation. The value of **λ** is chosen which minimizes the cross-validation error.

The algorithm used to select features and create the classifier is complex (see Friedman et. al. (2010)) but the result is fairly simple. For each of the **K** phenotypes in the training set, `glmnet` constructs a linear predictor $f_i(X) = X * \beta_i$ from the available features ***X***. Each β_i is a vector whose length is the number of features; lasso regularization forces many of the coefficients β_{ij} to be zero. For the i^{th} phenotype, the probability that a cell with features ***X*** belongs to phenotype ***i*** is given by the softmax function:

$$P(i \mid X) = \frac{e^{f_i(X)}}{\sum_{k=1}^{K} e^{f_k(X)}}$$

The phenotype for a cell is then taken to be the one with the highest probability, i.e. $\mathbf{argmax}_i P(i \mid X)$ The confidence reported by inForm is the probability of the winning phenotype, $P(i \mid X)$, expressed as a percent.

Note that the confidence will always be between **1/K** and 1. If all **K** phenotypes are nearly equally likely, the confidence will be slightly greater than **1/K**. If only one phenotype is likely, it will have confidence near 1.

Apply the classifier

Given the β_i parameters for each $f_i(X)$ and a set of features, it is straightforward to compute the $f_i(X)$ and $P(i \mid X)$ and select a phenotype. inForm uses the `predict.cv.glmnet` function of `glmnet` to perform this computation and return the selected phenotype and confidence.

Cells in the training set may be misclassified by the `glmnet` model. For training set cells, the model classification is always overridden by the training set classification, and the confidence is set to exactly 1.

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1. Jerome Friedman, Trevor Hastie, Robert Tibshirani (2010). Regularization Paths for Generalized Linear Models via Coordinate Descent. Journal of Statistical Software, 33(1), 1-22. http://www.jstatsoft.org/v33/i01/.

2. R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.

3. http://en.wikipedia.org/wiki/Multinomial_logistic_regression

4. http://www.fp.ucalgary.ca/mhallbey/tutorial.htm

5. R. M. Haralick, K. Shanmugam, Its'Hak Dinstein (November 1973). Textural Features for Image Classification. In Systems, Man and Cybernetics, IEEE Transactions on, Vol. SMC-3, No. 6. (November 1973), pp. 610-621, doi:10.1109/tsmc.1973.4309314

6. Tibshirani, R. (1996). Regression shrinkage and selection via the lasso. J. Royal. Statist. Soc B., Vol. 58, No. 1, pages 267-288). http://www-stat.stanford.edu/~tibs/lasso/lasso.pdf

26 Appendix D: Akoya Biosciences TIFF File Specification

Background

This describes a TIFF format that Akoya Biosciences uses for its tissue images. The imagery may be a simple RGB image, a set of components extracted from a field or region, or a whole-slide scan. In the latter case, it may be a brightfield (BF) color RGB image or a multiband fluorescence (FL) image.

The goal is to use the same syntax and metadata for all these kinds of images, and minimize the semantic distinctions where possible. Specifically, an extracted component represents signal attributed to a stain or fluorescent dye in a sample. The signal values incorporate image preprocessing such as normalization for exposure time and spectral unmixing when that technique is used.

Data format

The files are TIFF or BigTIFF images, depending on image size, with multiple images per file.

For images larger than about 2K x 2K pixels, tiled format is used, and the image is provided in several resolutions (pyramidal tiled images). Tile size is 512 x 512 pixels. Images smaller than 2K x 2K use stripped format.

The highest resolution (baseline) image(s) appear first in the file. For each resolution there are N baseline images where N depends on the contents. For BF images, N=1 and each image is an RGB image. For FL images or unmixed component images, N = number of bands, which is usually > 1, and each image is a grayscale image.

A thumbnail RGB image is provided, and this is a good image to use as an icon in graphical image lists. This comes after the baseline images, meaning it is the second image in BF (RGB) images, and the (N+1)st image for FL images or unmixed component images.

Next come the reduced-resolution images (if present). The pyramid contains enough levels that the image size is no larger than 2K x 2K at the coarsest resolution.

For whole-slide scans, there are two more non-tiled images after these: an optional RGB image of the label, and a macro (low-resolution) RGB image of the whole slide.

Overall, the arrangement is:

Table 1. Images

Description	RGB/mono	Title/Strip	Resolution	Notes
Baseline image	Varies	Varies	Full	Tiled if > 2K x 2K RGB for BF, else mono
More full-resolution images	Mono	Varies	Full	If N > 1
Thumbnail	RGB	Stripped	~500 x 500	
Half-resolution images	Varies	Varies	Half	Only if baseline is tiled
Quarter, eighth, etc.	Varies	Varies	Quarter, eighth, etc.	Continues until 2K x 2K or smaller

Macro (overview) image of whole slide	RGB	Stripped	~2000 x 4000	Required for whole-slide scans Optional for simple RGB images and extracted components
Label image	RGB	Stripped	~500 x 500	Optional, whole-slide scans

Detection

Readers can recognize Akoya tissue images via the contents of the “Software” TIFF tag (see below). The file suffix is .qptiff for whole slide scans. Some TIFF tags contain “PerkinElmer” for historical reasons; they remain to retain 3rd party reader compatibility.

Metadata

Metadata is contained in two locations: within standard TIFF tags as listed in the table below, and within the ImageDescription string, using a set of XML tags described below. These are provided for each image (IFD) in the file, and describe that image rather than the baseline image or the scan as a whole. The ScanProfile tag is only provided on the first, baseline image as it may be large.

Table 2. TIFF tags

TIFF Tag	Optional?	Description of contents
Software		Starts with “PerkinElmer-QPI”
ImageDescription		Further metadata in XML format (see next section)
ImageWidth		Width of the image in pixels
ImageLength		Height of the image in pixels
ResolutionUnit		Unit used for resolution and position (see below)
XResolution		Pixel X resolution (see below)
YResolution		Pixel Y resolution (see below)
XPosition	Y	Sample X location in ResolutionUnits. This is ULHC location except for Macro image which reports its image center.
YPosition	Y	Sample Y location in ResolutionUnits. This is ULHC location except for Macro image which reports its image center.
SampleFormat		Integer (1) for BF, FL; or float (3) for extracted components
SMinSampleValue		Minimum signal value in the image
SMaxSampleValue		Maximum signal value in the image
BitsPerSample		8 (FL); 8, 8, 8 (RGB); or 32 (unmixed component)
SamplesPerPixel		1 (FL or unmixed component) or 3 (RGB)
NewSubfileType		0 for full-resolution images, 1 for reduced res images
TileWidth	Y	Tile width (512) if tiled format is used
TileLength	Y	Tile height (512) if tiled format is used
TileOffsets	Y	List of tile offsets, if tiled format is used
TileByteCounts	Y	Size of each (compressed) tile, if tiled format is used

StripOffsets	Y	List of strip offsets, if tiled format is not used
RowsPerStrip	Y	Number of rows per strip, if tiled format is not used
StripByteCounts	Y	Size of each (compressed) strip, if tiled format is not used
PlanarConfiguration		1 (chunky) for RGB images, 2 (planar) otherwise
PhotometricInterpretation		2 (RGB) for RGB images, 1 (BlackIsZero) otherwise
DateTime		Acquisition time
Compression		May be None, CCITT Group 3, PackBits, LZW, or JPEG
JPEG fields	Y	JPEG fields are defined when JPEG compression is used

ResolutionUnit, XResolution and YResolution are required fields in a valid TIFF file. When the true resolution of the image is known, ResolutionUnit will be 3 (cm) and XResolution and YResolution will be pixels/cm. When the true resolution is not known, ResolutionUnit will be 2 (inch) and XResolution and YResolution will be 96 (pixels/inch). Pixels from Akoya Biosciences instruments are always square so XResolution and YResolution will always have the same value.

The TIFF spec is not explicit about the data type and value for SMinSampleValue and SMaxSampleValue; the writer uses the same data type as the image pixels (byte or float).

Image Description contents

The ImageDescription tag contains a string in XML format. The string contains a top-level < PerkinElmer-QPI-ImageDescription> element. Nested within this element are child elements with the tag names and values as listed in the table below. Elements appear in the order listed. Values are stored as text content of the element. Elements are required unless otherwise specified. See the example below.

Table 3. Image Description tags

Tag	Optional?	Contents
DescriptionVersion		Version of the image description field itself, a single number. This document describes version 2 of the field.
AcquisitionSoftware		Software used to acquire the image
Identifier		GUID in string format. This is an identifier for the image file itself.
SlideID	Y	ID of the slide that this image was taken from.
Barcode	Y	Barcode text of the slide this image was taken from.
ComputerName	Y	Name of the computer on which the slide was scanned.
ImageType		A string identifying the type of image within the file (Table 1), with the following values: • FullResolution • ReducedResolution • Thumbnail • Overview • Label
IsUnmixedComponent		“True” for unmixed multispectral images, otherwise “False”.
ExposureTime		Exposure time as an integer number of microseconds.

		For unmixed images, this is the exposure time for the dominant wavelength band for the component (FL); or the brightest wavelength in the cube (BF).
SignalUnits		A byte *wwww tttt* where the *tttt* nibble indicates the signal unit type from the following: • – raw counts • – normalized (counts/second/gain/full-scale/binning) • – OD (optical density) • – dark-corrected counts and the *wwww* nibble indicates how the signal is weighted across the spectral bands (or colors): • – average across all bands • – total summed signal across all bands • – peak signal in highest-valued band Thus, for example, a value of 68 (hex 44) encodes OD units with peak-signal weighting.
Name	Y	Band name for FL whole slide scans, component name for extracted components. Not present for RGB images
Color	Y	Color to use when rendering this band, as decimal r,g,b byte triplet. Present for FL whole slide scans or extracted components. Not present for RGB images.
Responsivity	Y	Instrument responsivity, if available, for FL whole-slide and unmixed images. See below for details.
Objective	Y	Objective name, if known, otherwise not present.
ScanProfile	Y	Element containing scan and/or and unmix parameters. It is valid XML whose contents are opaque to most readers. It is only provided on the first (baseline) image, and is omitted from all other IFDs.
ProtocolName	Y	Name of the acquisition protocol.
ValidationCode		Used for internal data integrity checks – readers can ignore this.
ImagingCycle	Y	Zero-based acquisition cycle for CODEX imagery
Biomarker	Y	String indicating target such as CD8, Ki67, etc.
ScaleFactor	Y	After allowing for bit-depth conversion, pixel values in this dataset have been scaled by this factor. For example, if a 12-bit image is stored in an 8-bit image, and counts were scaled from 4095 to 255, this would be 1; if counts were scaled from 3102 to 255, ScaleFactor would be 1.3201. Default is 1.
AutofluorescenceSubtracted	Y	String containing ‘true’ or ‘false’, indicating whether this dataset has been subtraction-corrected by a background (autofluorescence) image. Treated as ‘false’ by default.
LampType	Y	String with the FL lamp type • Unknown (default) • PriorLumen200 • PriorLumen220 • XCiteMultiBandLed • PriorMimicLed • SolaLed • Colibri7
InstrumentType	Y	String with the instrument type, one of the following:

		• Unknown (default) • Keyence700 • Keyence800 • AxioObserver • Dmi8 • Polaris • Vectra3
CameraType	Y	String with the camera sensor type, one of the following: • SonyICX285AL • HamamatsuC11440 • IMX265 • IMX421
ExcitationFilter	Y	Description of excitation filter with sub-elements listed below If present, all its sub-elements must be provided.
Name		String with the filter public name
Manufacturer		String with the manufacturer or OEM supplier
PartNo		String with the manufacturer part number
Bands		Collection of bands each as described by sub-elements below
Band		Description of a band with sub-elements noted below
Name		String with the band name
Cuton		Cuton wavelength of this band in nm
Cutoff		Cutoff wavelength of this band in nm
Active		True if this band is excited, False if not
EmissionFilter	Y	Description of emission filter with sub-elements listed below. If present, all its sub-elements must be provided.
Name		String with the filter public name
Manufacturer		String with the manufacturer or OEM supplier
PartNo		String with the manufacturer part number
Bands		Collection of bands each as described by sub-elements below
Band		Description of a band with sub-elements noted below
Name		String with the band name
Cuton		Cuton wavelength of this band in nm
Cutoff		Cutoff wavelength of this band in nm
CameraSettings	Y	Description of the camera settings used for this image with sub-elements listed below. If present, all its sub-elements must be provided
Gain		Gain factor, such as 1, 3, etc.
Binning		Binning extent, such as 1 for full-resolution, 2 for 2x2 binning
OffsetCounts		Offset count setting for the camera
BitDepth		Bit resolution used to read out the camera. This may differ from the resolution used to store this image (for example, it may be acquired with 12-bit resolution but saved in a QPTIFF file with 8-bit resolution)
Orientation		String containing one of the following values: • 'Normal' (default) • 'Rotate180' • 'MirrorV' • 'MirrorH'

ROI	Y	Description of the sensor ROI contributing to the image. Coordinates are sensor pixels before binning. Even if the CameraSettings are provided, this field is optional
X		X origin of ROI. Left-most pixel in sensor array is 0.
Y		Y origin of ROI. Top-most pixel in sensor array is 0.
Width		Width of ROI
Height		Height of ROI

For whole slide images (BF and FL), SignalUnits will be 64 (hex 40) (raw counts, peak signal). For unmixed images, SignalUnits will reflect the unmix settings.

Instrument Responsivity

The <Responsivity> tag is a container for a list of normalized instrument response values. This tag is present for whole-slide FL images from Vectra Polaris and unmixed FL multispectral images originating from Vectra 3 and Vectra Polaris.

For whole-slide images, the <Responsivity> tag will contain one <Filter> tag. The < Filter> tag contents will be different for each image within the TIFF file, reflecting the filter used to take the image.

For unmixed component images, the <Responsivity> tag will contain one <Band> tag for each band in the original image file. The <Band> tags are repeated for each unmixed component image.

The overview, thumbnail and label images do not have <Responsivity> tags.

Each <Filter> or <Band> tag describes the instrument responsivity for acquisitions using that filter or band. The contents of the <Filter> and <Band> tags are described below.

Table 4: Contents of Filter and Band tags

Tag	Contents
Name	The name of the filter (whole-slide image) or band (component image)
Response	The instrument response to the reference artifact, normalized for exposure. This is raw counts / ($2^{\text{bit depth}}$ × exposure time × gain × binning area), where bit depth is the bit depth of the imagery, exposure time is in seconds, gain is the gain setting of the camera, and binning area is 1 for 1×1, 4 for 2×2, etc.
Date	The date and time of the reference image in UTC, ISO 8601 format.
FilterID	Detailed description of the acquisition filter, as {ExcitationFilter band name}_ {ExcitationFilter manufacturer}:{ExcitationFilter part number} / {EmissionFilter manufacturer}:{EmissionFilter part number} The {ExcitationFilter band name}_ prefix may be omitted if there is only a single excitation band.

Sample ImageDescription

Sample ImageDescription for the DAPI band of a FL whole-slide image, containing a single <Filter> tag:

```
<?xml version="1.0" encoding="utf-8"?>
```

```
< PerkinElmer-QPI-ImageDescription >
    <DescriptionVersion>1</DescriptionVersion>
    <AcquisitionSoftware>VectraScan 1.0.0</AcquisitionSoftware>
    <ImageType>FullResolution</ImageType>
    <Identifier>AABED946-BB58-44FB-95B3-48E177E3BB83</Identifier>
    <IsUnmixedComponent>False</IsUnmixedComponent>
    <ExposureTime>50</ExposureTime>
    <SignalUnits>64</SignalUnits>
    <Name>DAPI</Name>
    <Color>0,0,255</Color>
    <Responsivity>
      <Filter>
        <Name>DAPI</Name>
        <Response>30.7</Response>
        <Date>2015-10-22T13:10:18.0618849Z</Date>
        <FilterID>Semrock:FF02-409/LP-25 Emission / Semrock:FF01-377/50-25
Excitation</FilterID>
      </Filter>
    </Responsivity>
    <Objective>4x</Objective>
    <ScanProfile><!-- this will be a serialized scan protocol. It is valid
XML but otherwise opaque -->
    </ScanProfile>
    <ValidationCode>4281ff86778db65892c05151d5de738d</ValidationCode>
</ PerkinElmer-QPI-ImageDescription >
```

27 Appendix E: Segmentation Map TIFF Specification

inForm now exports the tissue segmentation map, the object / cell compartment label images, and a process region label image in a single file. The file is a multi-image TIFF file where all images are 32-bit images. The tissue segmentation map, if present, is the first image; the object or cell label images follow, lastly the process region image, if present. Cell compartments come in order {nucleus, cytoplasm, membrane} ; only segmented compartments are included.

Metadata about each image is stored as XML within the image's ImageDescription tag.

Tissue Segmentation

The tissue segmentation image is a class map; the value at each pixel is that pixel's tissue category ID. In certain cases the class cannot be calculated, and for such pixels the value is 255. Category IDs begin at 0.

The XML structure for the Tissue Segmentation Image Description is:

```
<?xml version="1.0" encoding="utf-16"?>
 <TissueClassMap>
  <Version>1</Version>
  <Entry>
   <Name>{first tissue class name}</Name>
   <Color>{alpha},{red},{green},{blue}</Color>
   <ID>{guid string}</ID>
  </Entry>
...
  <Entry>
    <Name>{last tissue class name}</Name>
    <Color>{alpha},{red},{green},{blue}</Color>
    <ID>{guid string}</ID>
  </Entry>
 </TissueClassMap>
```

The XML contains one <Entry> element for each tissue category. The position of the <Entry> element corresponds to the category ID. Colors are specified as four decimal integers in the range 0-255.

Object and Cell Segmentation

The object and cell segmentation images are label images; the value of each pixel is the ID of the object or cell which contains that pixel. The map will report 0 if no object was present; which may mean that the area was background, or it may mean that a particular compartment was not found within that cell. The object ID is the same as the ID reported in inForm tables. A label image can contain over 2 billion objects.

The XML structure for both the Object and Cell Segmentation Image Descriptions is the same:

```
<?xml version="1.0" encoding="utf-16"?>
 <SegmentationImage>
  <Version>1</version>
<CompartmentType>{type}</CompartmentType>
 </SegmentationImage>
```

where {type} is Nucleus, Cytoplasm, or Membrane for cells, or "Object" for object segmentation.

Process Regions

The process region image is a label image; the value of each pixel is the ID of the process region which contains that pixel. The map will report 0 if no region was present, which means that portion of the image was not processed. The region ID is the same as the ID reported in inForm tables.

The XML structure for the Process Region Image Description is:

```
<?xml version="1.0" encoding="utf-16"?>
<ProcessRegionImage>
<Version>1</Version>
</ProcessRegionImage>
```

28 Appendix F: Adaptive Cell Segmentation Tips

This section contains tips for Adaptive Cell Segmentation.

What is Adaptive Cell Segmentation?

Adaptive Cell Segmentation allows you to use algorithms that account for variations in staining and background levels within and across images to identify cellular compartments from multiple image planes. These features provide improved segmentation performance compared to previous versions of inForm when images contain mixtures of cell types with very different nuclear morphologies. With inForm 2.4, you can segment individual immune and cancer cells in densely packed, complex morphologies, including areas with lots of inflammation, for more accurate multi-parameter cell identification and spatial analysis using the new cell segmentation capabilities of inForm®.

With inForm version 2.4, now you can:

- Segment highly varying tumor and lymphocyte nuclei, when nuclei differ in size, density, and homogeneity, and when there is background signal.
- More accurately segment individual nuclei in densely packed fields of lymphocytes and when cells are a complex mix of immune and cancer cells, compared to previous versions of inForm.
- Include markers in addition to DAPI to identify nuclei.
- Optimize segmentation with a move-able real-time preview window.

Considerations Before Use

- Adaptive cell segmentation should only be used when commencing new projects.
- Projects developed and saved using inForm 2.3 that include cell segmentation cannot be opened using inForm 2.4.
- If you want to apply new inForm 2.4 functionality to an existing project, save the project developed using v2.3 with post-segmentation image analysis steps removed. Then it can be opened in inForm 2.4.

Steps for Adaptive Cell Segmentation

Configure New Project

1. Select the **Adaptive Cell Segmentation** radio-button in the **Find Features** column.

   NOTE: *The classic **Object-based** cell segmentation algorithm is still available using inForm 2.4, but the **Counterstain-base** option is no longer available.*

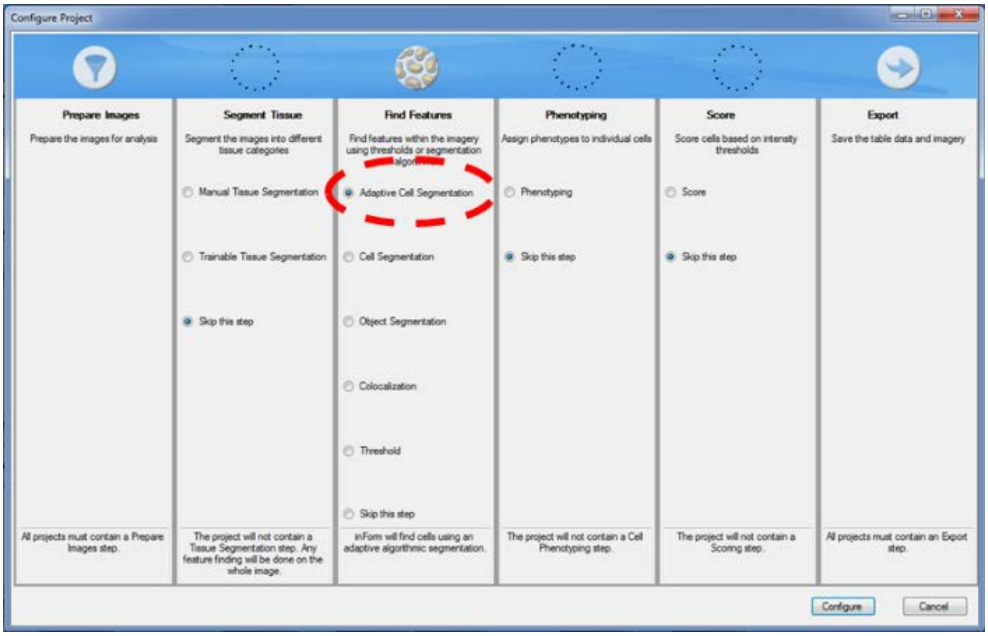

Figure 126. Configure Project - Adaptive Cell Segmentation

Choose Which Cellular Compartments to Segment

NOTE: *As with previous versions, the adaptive cell segmentation algorithm can only detect cells with nuclear signal.*

1. If you choose to segment the membrane compartment, we highly recommend also segmenting the cytoplasm.
2. To reduce visual clutter, turn off the membrane and cytoplasm segmentation masks until after optimizing the nuclear splitting.

Add Nuclear Stains

NOTE: *We recommend choosing the strongest, most abundant nuclear component first. In many cases, this will be the DAPI stain.*

1. Click the **Add…** button to add stains to be used for segmentation.
2. Configure the DAPI signal by clicking the '**…**' button.
   - o New with inForm 2.4, when you start a configuration, a live preview region displays in the current image. This preview provides immediate feedback as you adjust parameters throughout the segmentation steps.
   - o The window area is set and cannot be changed, but it can be dragged to any area within the tissue image. To zoom in on the window, use the scroll function of the mouse or select the Zoom button.
   - o The Live Preview Window provides immediate feedback as segmentation parameters are adjusted with each step. The dotted red circle indicates where to choose which cellular compartments to segment. Red arrows indicate required selections for DAPI configuration.

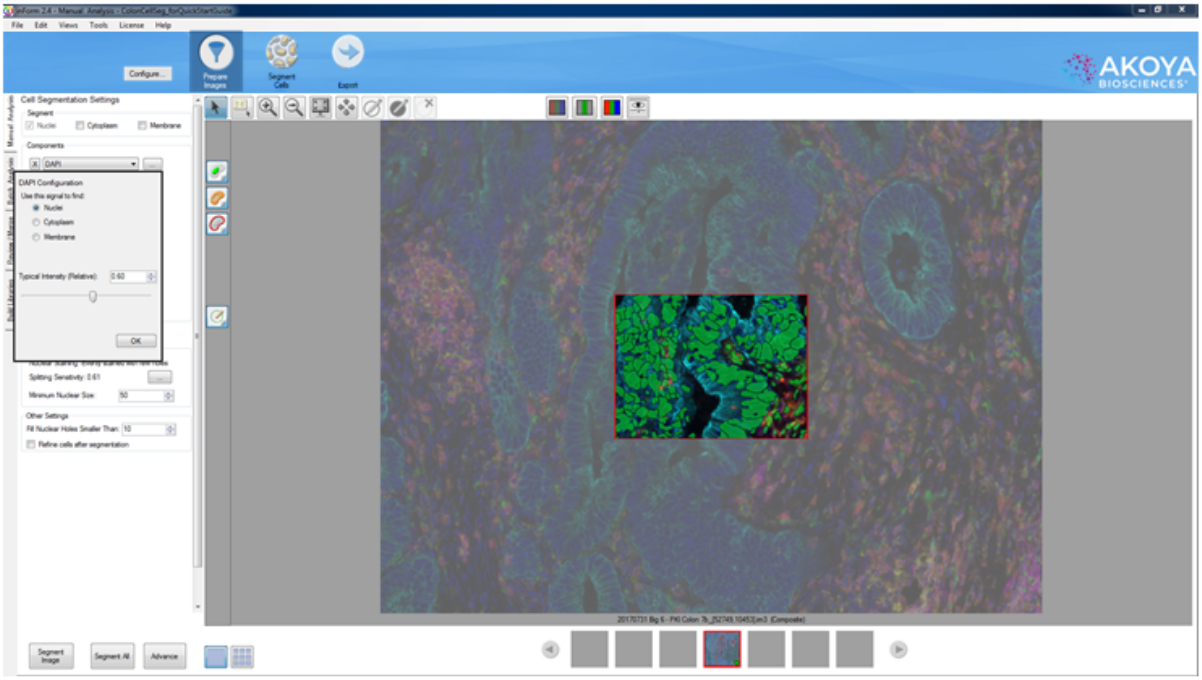

Figure 127. Live Preview - DAPI Configuration Window

a. Use the **Typical Intensity Slider** to adjust the threshold until you have detected all pixels expressing nuclear signals. This step is ONLY to collect signal you believe is real for the field-of-view at hand.

NOTE: *We recommend turning off all other stains during this step. It may be easier to switch to the simulated brightfield pathology view.*

NOTE: *Moving the Typical Intensity Slider left (lower value) collects more signal from faint nuclei (see Figure 74). Moving slider right (higher value) collects less signal (see Figure 75). Yellow boxes indicate regions of DAPI signal that may require fine tuning to determine validity for serial section analysis. Red arrows indicate settings requiring configuration for DAPI detection.*

- Moving the slider left (lower value) collects more signal from faint nuclei.

Figure 128. Slide Left - Lower Value - Settings Requiring Configuration

- Moving the slider right (higher value) collects less signal from faint nuclei.

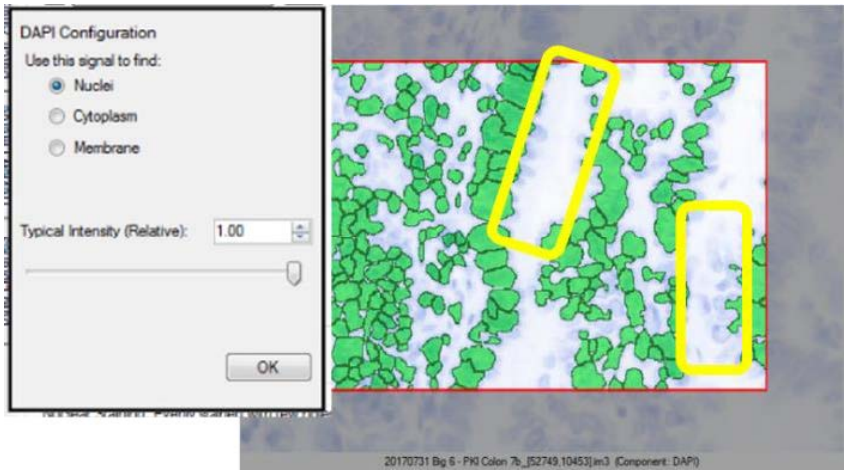

Figure 129. Slider Right - Higher Value

3. If smaller nuclei are being missed, an adjustment to the Minimum Nuclear Size setting is required - this can be adjusted in the **Nuclear Component Splitting** panel.

Multiple Nuclear Stains

With inForm 2.4, it is now possible to select more than one nuclear signal for segmentation. For example, FoxP3, with strong staining, can at times cover the DAPI signal. With previous versions of inForm, this could result in the cell not being detected. Using adaptive cell segmentation, you can have the confidence that all cells expressing a nuclear stain are identified.

1. Click the **Add** button to add additional nuclear stains to be used for segmentation.
2. Click the '**...**' button to configure the stain.

NOTE: *It is possible to select more than one nuclear signal for segmentation. For example, FoxP3, with strong staining, can at times block the DAPI signal. Yellow circles indicate*

regions of FoxP3 signal. Red arrows indicate required selections for FoxP3 configuration.

a. Use the Typical Intensity (Relative) Slider to adjust the threshold until you have detected all pixels expressing nuclear signals.

- Moving the slider right (higher values) reduces the amount of splitting.
- Moving the slider left (lower values) increases the amount of splitting.

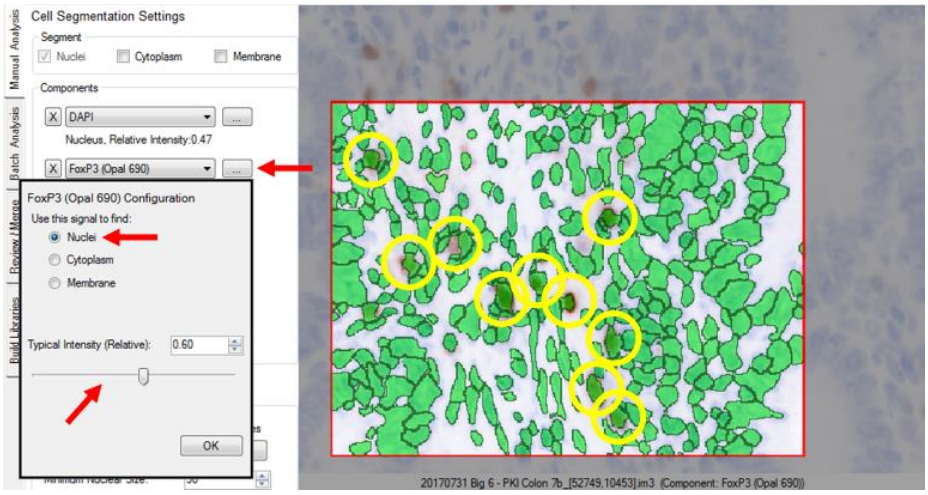

Figure 130. Multiple Nuclear Stains

Split Nuclear Clusters into Individual Nuclei

It is now possible to describe the quality of the nuclear stain. This will permit better splitting of cells, especially when the nuclear stain is non-homogeneous with holes.

1. After you have identified all the nuclear stains and captured all pixels expressing nuclear signal, use the Nuclear Component Splitting panel to adjust splitting parameters.
2. Configure the nuclear splitting sensitivity by clicking the '**...**' button.

NOTE: *Moving the Nuclear Splitting Settings Slider right (higher value) reduces the amount of splitting (see Figure 77). Moving the slider left (lower value) increases the amount of splitting (see Figure 78). Red arrows indicate the settings requiring configuration for nuclear splitting.*

a. Use the Nuclear Splitting Settings Slider to adjust the splitting threshold:

- Moving the slider right (higher values) reduces the amount of splitting.

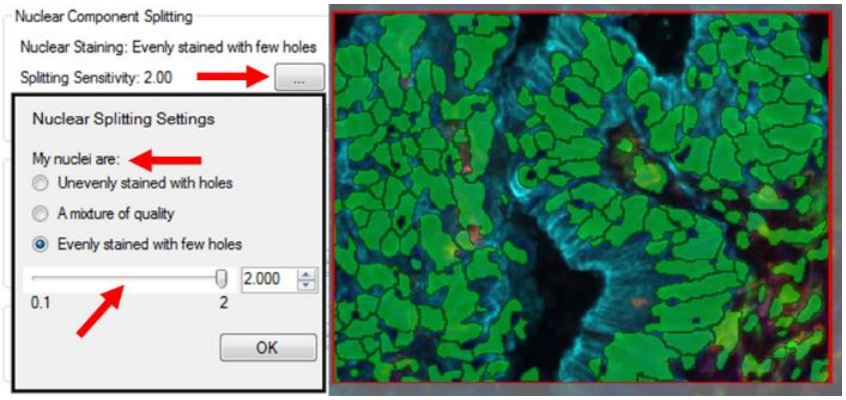

Figure 131. Slider Right - Higher Values

- Moving the slider left (lower values) increases the amount of splitting.

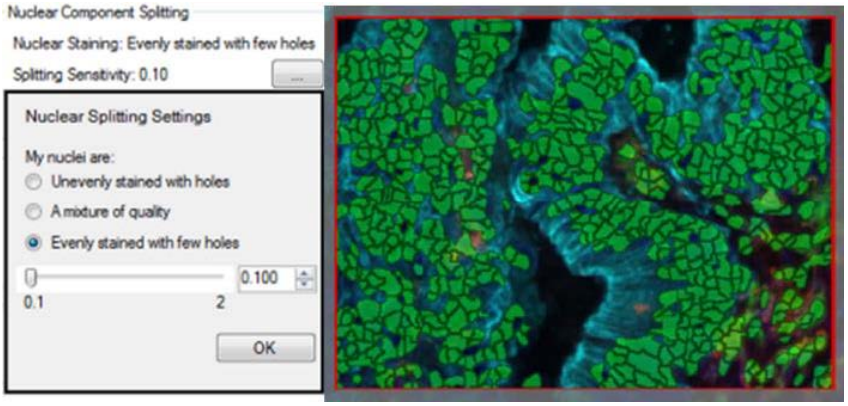

Figure 132. Slider Left - Lower Values

We recommend optimizing this step before identifying assist markers.

Select Membrane or Cytoplasm Stains to Aid Nuclear Segmentation

NOTE: *When cells are highly clustered, the use of membrane or cytoplasm stains may become necessary to better split individual nuclei.*

Add Membrane or Cytoplasm Stains

1. Select the **Cytoplasm** and **Membrane** check boxes. Choose stains that can best assist your nuclear staining.

   - We recommend adding the strongest and most specifically stained membrane components first. The assist segmentation algorithm gives priority to nuclear splitting lines that are attributed to image components higher up in the list of components to be segmented. Please note, for successful segmentation, not all components need to be selected to aid nuclear splitting.

   - We recommend optimizing splitting before adding the next component.

   NOTES: *With adaptive cell segmentation, having a 'pan immune marker' such as CD45 may result in better identification of all immune cells.*

Cytoplasm segmentation does not take into account any signals from identified cytoplasm components. Pixels to designate cytoplasm are identified based on proximity to nuclear edge, within the user-selected distance, and inside of membrane compartment.

Configure the Component

1. Click the '**...**' button next to the first added stain.
2. Select the cellular compartment that will find the stain's signal and then select the **Use this signal to assist in nuclear splitting** check box.

   NOTE: A membrane stain can be used to both assist nuclear splitting and to segment the membrane compartments. It places rings around segmented nuclei according to user-selected parameters, as per Segment Cytoplasm 172.
3. Repeat for other added stains.

Split Using Membrane or Cytoplasm Stains

1. Use the **Assisting Component Splitting** panel to adjust parameters. This panel is only available when the **Use this signal to assist in nuclear splitting** check box has been selected.

   NOTE: *Moving the **Assisting Component Splitting Settings Slider** right (higher value) reduces the amount of splitting. Moving the slider left (lower value) increases the amount of splitting. Yellow boxes indicate tumor regions where nuclear splitting is affected by assist marker; the stroma remains unaffected. Red arrows indicate settings requiring configuration for the assisting component.*

   a. Use the Assisting Component Splitting Settings Slider to adjust the splitting threshold:

   - Moving the slider right (higher values) reduces the amount of splitting.

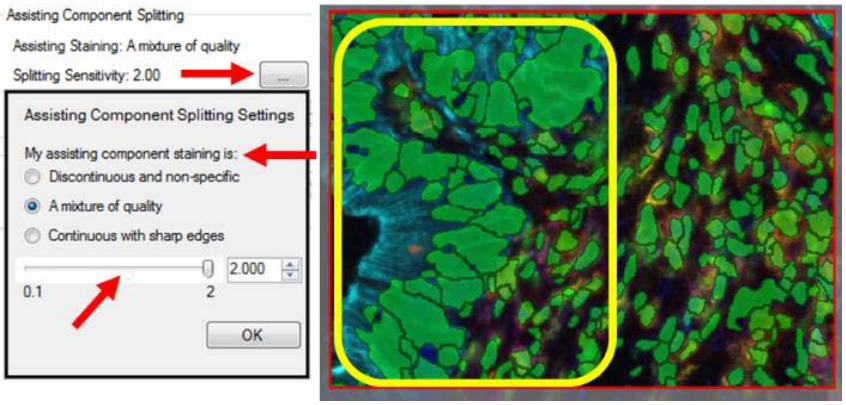

Figure 133. Slider Right - Higher Value

   - Moving the slider left (lower values) increases the amount of splitting.

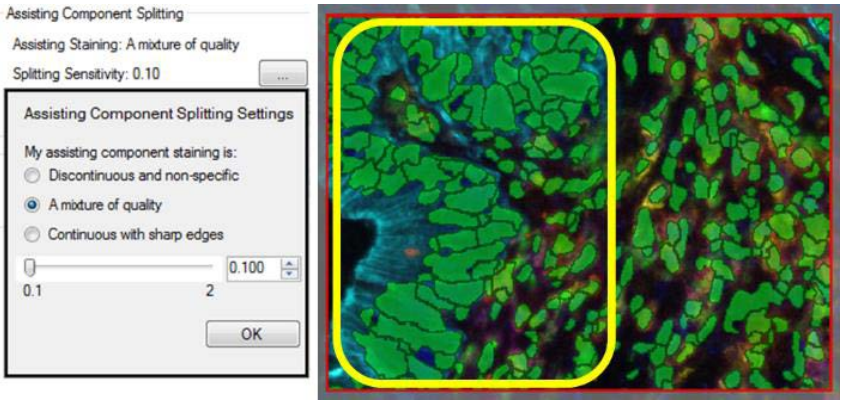

Figure 134. Slider Left - Lower Values

NOTE: *Adjustment of the splitting sensitivity will affect all markers you have selected to assist in nuclear splitting.*

Further Refinement of Nuclear Shapes

1. In the **Other Settings** panel, it is possible to further refine the nuclear segmentation results. This setting is "on" by default.
    - Any nuclei with an empty hole can be filled in by increasing the 'Fill Holes Smaller Than' value.
    - We generally recommend selecting the Refine cells after segmentation option, which rounds out sharp edges on the nuclear segmentation mask in addition to compacting the nuclear compartment slightly.

Segment Cytoplasm

1. Adjust the **Cytoplasm Thickness setting** to define the distance (in pixels) from the boundary of each cell's nucleus to the out boundary of its cytoplasm.

    NOTE: *The Cytoplasm Settings panel is only available when the Cytoplasm compartment has been selected for segmentation.*

    - With many samples, we find a Cytoplasm Thickness setting of 6 (pixels) works well, but adjust accordingly. This distance should be adjusted on visual inspection of the results, so that cytoplasm segmentation approximately matches the average size of cells.

Segment Membrane

NOTE: *The Membrane Settings panel is only available when the Membrane compartment has been selected for segmentation.*

1. Set the **Membrane Search Distance**.
    a. Adjust the **Membrane Search Distance** to define the distance (in pixels) from the boundary of each cell's nucleus to search for membrane signals.
        - The Membrane Segmentation algorithm will detect or define the membrane compartment based on detected peak signals between the edge of the nucleus and the Membrane Search Distance. If no significant peak signal is detected, the membrane is set to the nearest stop point:
            - (a) Membrane Search Distance
            - (b) Neighboring Cell Membrane
            - (c) Cytoplasm Thickness, if Cytoplasm segmentation was selected. If Cytoplasm Segmentation is not selected, (c) becomes half of the search distance.
        - When segmenting the membrane compartment, we strongly recommend selecting the cytoplasm for segmentation. The default membrane segment will collapse to the nucleus if no peak signals are found, with a distance determined by the Cytoplasm Thickness. This is particularly helpful with lymphocytes with no membrane signal, or have a small cytoplasm compartment.

- The interface requires that the **Membrane Search Distance** is set larger than the **Cytoplasm Thickness**, so the algorithm can search for signal over larger distances, but collapse back to a smaller nuclear size if no signals are found.

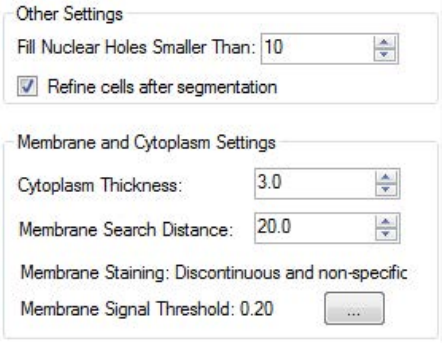

Figure 135.Membrane Search Distance and Cytoplasm Thickness

- With many samples, we find a Membrane Search Distance of 20 (pixels) works well, but adjust accordingly.

2. Configure the Membrane.

   a. Click the '**...**' button.

   NOTE: *It is now possible to describe the quality of the membrane stain. This will permit better splitting of cells, especially when the membrane stain is discontinuous or non-specific.*

   b. Use the slider to adjust the membrane detection threshold:

   - Moving the slider to the right (higher value) collects less signal.
   - Moving the slider to the left (lower value) collects more signal from weaker membrane signals.

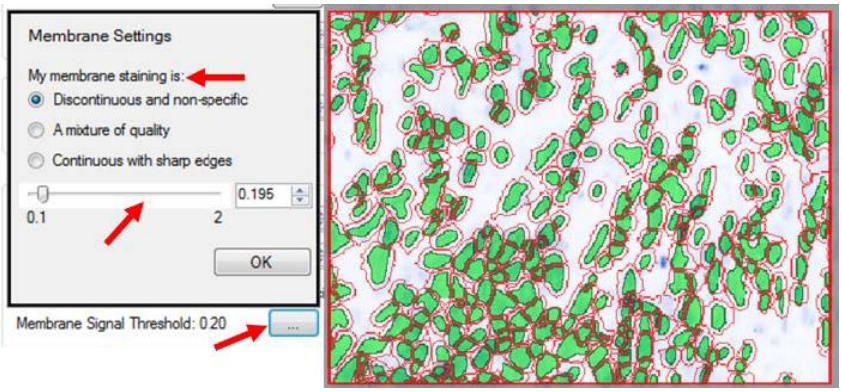

Figure 136. Membrane Detection Threshold

Please follow the youtube link below to watch a video demonstrating each step of the inForm 2.4 adaptive cell segmentation process:

https://www.youtube.com/watch?v=VAX92f2ar-M&feature=youtu.be

Additional Tips and Tricks

1. What signals are used when configuring the splitting threshold?
    - The Nuclear Component Splitting panel only uses stains configured for the nuclear compartment.
    - The Assisting Component Splitting panel only uses stains where the **Use this signal to assist in nuclear splitting** check box is selected.
2. Only when a user chooses to segment the cytoplasm and membrane will the membrane and cytoplasm settings show. We recommend having the cytoplasm component selected when segmenting the membrane as this helps to increase the search distance for the membrane stain ridge.
3. How does the Minimum Nuclear Size found in the Nuclear Component Splitting and Assisting Component Splitting panels differ?
    - The Minimum Nuclear Size found in Nuclear Component Splitting plays two roles:
        - Before any splitting occurs, any individual islands of nuclear pixels that are smaller than this value are discarded.
        - When splitting based on nuclear components (i.e., splitting due to a valley in DAPI intensity), no individual splits are made that create nuclei smaller than this value.
    - The **Minimum Nuclear Size** found in **Assisting Component Splitting** has one role:
        - When splitting based on membrane/cytoplasm components (i.e., splitting due to a peak in membrane intensity), no individual splits are made that create nuclear smaller than this value. If no membrane signal is found overlaying a particular island of nuclear pixels, this parameter does not matter, defaulting to settings in the **Nuclear Component Splitting**.
        - If the nuclear signal is patchy, you might want to keep the minimum size in **Nuclear Component Splitting** large to avoid getting a large tumor nucleus split into many tiny nuclei. At the same time, if you're confident in the membrane staining, you could make the minimum size in **Assisting Component Splitting** much smaller so you're allowed to split very dense immune cells into smaller objects using the membrane signal. That way you can keep your larger tumor cells from being over-split while the immune cells can be more aggressively split by membrane-assist.

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Index

- 0 -

0-3+ (4-bin), scoring 103

- A -

About inForm 7
Adaptive Cell Segmentation 82
  Defining Cytoplasm 87
  Defining Membrane 87
  Detecting Nuclear Pixels 83
  Refining Nuclear Shapes 87
  Selecting Segmentation Components 83
  Splitting Nuclear Clusters 85
Adding images to a project 47
Algorithm 11, 46
  Definition 11
  Opening 46
  Saving 46
Auto scale 91
Available Images Window 27

- B -

Batch processing 131

- C -

Calculating Fractional Tissue Area 147
Categories, adding 68
Cell segmentation (classic method) 89
Cell segmentation data, viewing 116
Cell segmentation, overview 82
Cell segmention, adaptive 82
Classifying Tissue
  automatically 68
  manually 65
Colocalization 80
Colocalization data, viewing 120
Color Image view 109
Compartment 101
Component data
  Viewing 108
Component for scoring 101
Component units 61
component view 110
Components for training 70
Composite Image, viewing 113
Contact Akoya Biosciences 18
Count data, viewing 121
Counting objects 106
Cytoplasm, segmenting
  adaptive cell segmentation method 87, 94
  classic cell segmentation method 94

- D -

Data
  Displaying extracted 108
  Exporting 129
  Merging 133
  Viewing tables 114
Down-sample 53
Drawing Processing Regions 51
Drawing training regions 69

- E -

Editing masks 75
Excel 147
Export settings 129
Exporting the data 129
Extracted Data, viewing 108

- F -

Features 8
Fifty-bin, scoring 105
Fixed scale 91
Fractional Tissue Area, calculating 147

- I -

ID number 51
IF scoring 101
IHC scoring 101
Image
  Displaying 108

Image
Format 52
Viewing 49
Image analysis tasks, summary of 40
Images
Adding to a project 47
Adding to a training set 73
Opening 47
Opening in a project 47
inForm window 19
Inner distance to nucleus 94
Installing inForm 12
Introduction to inForm 7

- K -

Key Terms 11

- L -

License, activating 13

- M -

Masks, editing/re-training 75
Membrane, segmenting
adaptive cell segmentation method 87
classic cell segmentation method 96
Merging the data 133
Minimum size 94

- N -

New project 41
New Project Window 28
Nuclear Clusters, splitting 85
Nuclear Pixels, detecting 83
Nuclei, segmenting 90

- O -

Object-based approach 90
Objects, counting 106
Online documentation 12
Open
Algorithm 46
Project 44
Opening images 47
Opening images in a project 47
Outer distance to nucleus 94

- P -

Percent total area 147
PivotTable 147
Pixel-based approach 90
Positivity, scoring 102
Printed documentation 12
Processing Regions 47
Deleting 51
Drawing 51
Project 11
Adding images 47
Creating New 41
Definition 11
Open 44
Opening images 47

- Q -

Quant data, viewing 122

- R -

Region ID number 51
Removing images from a project 47
Re-training 75

- S -

Sample format 53
Saving
algorithms 46
Scale Views Based on Selected Images 110
Scale Views Equally for All Images in the Project 110
Scale Views for each Image Individually 110
Scaling Image Selection window 110
Score data, viewing 118
Scoring IHC or IF 101
Scoring type 101

Segmenting adaptive cell segmentation components 83
Segmenting cells 82
Segmenting membrane 96
Segmenting nuclei 90
Segmenting tissue 68
Select Reported Units 61
Sorting tables 114
Specifications 8
Spectral library
viewing 60
Spectral Library window 29
Spectral Region 11
Splitting Nuclear Clusters into Individual Nuclei 85
Support, Technical 18
Supported image formats 7

- T -

Tables, viewing 114
Technical Support 18
Ten-bin, scoring 104
Threshold 77
Threshold maximum 101
Tissue
automatically classifying 68
manually classifying 65
Tissue Category
Definition 11
For scoring 101
Tissue segmentation 68
Tissue segmentation data, viewing 115
Tissue Segmenter, definition 11
Tissue Segmenter, training 70
Training regions, drawing 69
Training Set
Adding images 73
Training the tissue segmenter 70
Transferring the license 13

- U -

Units
library 30
User interface 19

- V -

View Editor 25

- W -

Weighting 61
Welcome 6