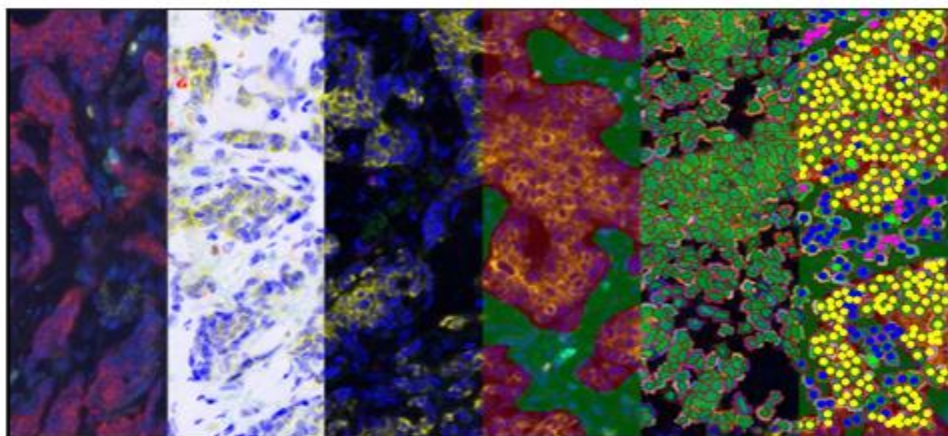


inForm User Guide

Advanced Image Analysis Software

3.0.0



inForm® 3.0.0 Tissue Finder Advanced Image Analysis Software

US Patents 7,555,155; 7,953,264; 8,280,140; 8,639,043;
10,126,242; 11,307,142 and patents pending.
© Copyright 2023 Akoya Biosciences, Inc. All Rights Reserved.



CONTENTS

Chapter 1. Welcome to inForm.....	5
Chapter 2. Introduction.....	7
2.1. About inForm.....	7
2.2. Software Configuration.....	8
2.3. Key Terms.....	10
2.4. Using Online Documentation.....	11
2.5. Installing and Starting inForm.....	12
2.6. Activating the License.....	13
2.7. Contacting Akoya Biosciences.....	14
Chapter 3. Understanding the inForm Work Area.....	15
3.1. View Editor.....	24
3.2. Available Images Window.....	26
3.3. New Project Window.....	27
3.4. Spectral Library Window.....	29
3.5. Stain Store Management.....	31
Chapter 4. Common Image Analysis Tasks.....	39
Chapter 5. Working with Projects and Algorithms.....	41
5.1. Creating an inForm Project.....	41
5.2. Saving an inForm Project.....	46
5.3. Editing the Step Settings for a Saved inForm Project.....	47
5.4. Creating and Saving an Algorithm.....	48
Chapter 6. Opening Images.....	51
6.1. Adding Images to an inForm Project.....	51
6.2. Adding or Removing Images from Project Processing Set or Training Set.....	55
6.3. Viewing Images and their Information in a Project.....	57
6.4. Removing Images from a Project.....	59
Chapter 7. Drawing Processing Regions.....	61
Chapter 8. Preparing Images.....	63
8.1. Selecting the Image Format.....	63
8.2. Choosing a Spectral Library.....	64
8.3. Viewing and Saving a Spectral Library.....	71
8.4. Exporting an Unmixing Library.....	72
8.5. Component Units.....	73
8.6. Completing the Prepare Images Step.....	78
Chapter 9. Manual Tissue Segmentation.....	79
9.1. Adding Tissue Categories.....	79
9.2. Drawing and Deleting Tissue Category Regions.....	81
9.3. Editing Tissue Category Regions.....	82
Chapter 10. Trainable Tissue Segmentation.....	85
10.1. Adding Tissue Categories.....	85

10.2. Drawing Tissue Segmentation Training Regions.....	88
10.3. Selecting Training Components.....	88
10.4. Adding Images to Training Set.....	92
10.5. Editing Masks and Re-Training.....	93
10.6. Completing Automated Tissue Segmentation Step.....	95
Chapter 11. Threshold.....	96
Chapter 12. Colocalization.....	97
Chapter 13. Segmenting Cells.....	99
13.1. Adaptive Cell Segmentation.....	99
13.2. Cell Segmentation.....	109
Chapter 14. Phenotyping Cells.....	120
14.1. Adding, Deleting, or Renaming a Phenotyping Schema.....	120
14.2. Adding and Deleting Phenotype Classifiers.....	122
14.3. Training a Phenotyping Classifier.....	124
Chapter 15. Scoring IHC or IF.....	130
15.1. Scoring Types.....	132
Chapter 16. Counting Objects.....	137
Chapter 17. Displaying Extracted Data.....	140
17.1. Viewing the Color Image.....	141
17.2. Viewing the Component Image.....	142
17.3. Viewing the Composite Image.....	146
17.4. Viewing Data Tables.....	149
17.5. Viewing the Tissue Segmentation Data Table.....	149
17.6. Viewing the Cell Segmentation Data Table.....	151
17.7. Viewing the Score Data Table.....	155
17.8. Viewing the Colocalization Data Table.....	157
17.9. Viewing the Count Data Table.....	159
17.10. Viewing the Quant Data Table.....	161
17.11. Image Options.....	163
17.12. Components Stats.....	167
17.13. TMA Core Info.....	167
17.14. Slide Info.....	168
17.15. Position Stats.....	168
17.16. Shape Stats.....	169
Chapter 18. Exporting Data.....	170
Chapter 19. Batch Processing.....	174
Chapter 20. Merging Data.....	176
Chapter 21. Building Libraries.....	178
21.1. Singly Stained Tissue Sections.....	179
21.2. Extracting a Stain or Fluorophore.....	180
21.3. Extracting a Custom Stain or Fluorophore.....	181
21.4. Extracting a Fluorophore with a Custom Filter.....	185
21.5. Extracting a Stain from a Color Image.....	185

21.6. Using Processing Regions	187
Appendix A. Calculating Fractional Tissue Area	191
Initial Setup	191
Create a PivotTable to Complete Fractional Tissue Area	191
Compute a Ratio	193
Saving the Table	193
Save as Tab-Separated Values	194
Appendix B. Auto-Processing Data Received from Mantra Snap	195
Appendix C. Phenotype Classification by Multinomial Logistic Regression	197
Introduction	197
Create a Training Set	197
Create Features	198
Train a Classifier	200
Apply the Classifier	201
Appendix D. Akoya Biosciences TIFF File Specification	202
Background	202
Data Format	202
Detection	203
Metadata	203
Appendix E. Segmentation Map TIFF Specification	213
Appendix F. Composite Image and Phenotype Map TIFF Specification	215
Appendix G. Adaptive Cell Segmentation Tips	217
What is Adaptive Cell Segmentation?	217
Considerations Before Use	217
Steps for Adaptive Cell Segmentation	218
Additional Tips and Tricks	230
Appendix H. Akoya Software Version Compatibility	232
Appendix I. inForm EULA	234

CHAPTER 1. WELCOME TO INFORM

Akoya's inForm® Tissue Finder™ advanced image analysis software has been developed to address the biggest challenge facing microscopists after quantitative imagery has been acquired: extracting data. Because signals can be weak and highly mixed, the challenge increases when dealing with multiplexed assays.

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.

inForm can read and analyze Akoya's multispectral images and industry-standard RGB images, including TIFF files, which are generated using the Akoya Biosciences Quantitative Pathology Imaging and Slide Scanning systems. (See Figure 1.1)

inForm is for research use only. It is not for use in diagnostic procedures.

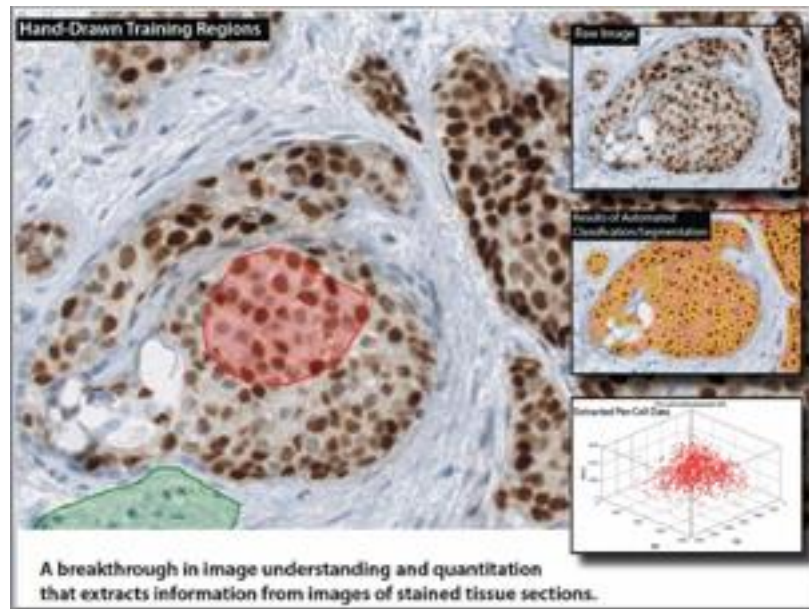


FIGURE 1.1. Sample inForm screen.

Getting Started – New Users

Be sure to review the topics in the [Introduction](#) to familiarize yourself with the features and specifications of the software.

CHAPTER 2. INTRODUCTION

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

Section	Page
2.1. About inForm	7
2.2. Software Configuration	8
2.3. Key Terms	10
2.4. Using Online Documentation	11
2.5. Installing and Starting inForm	12
2.6. Activating the License	13
2.7. Contacting Akoya Biosciences	14

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), or immunofluorescence stains (IF), including Qdot[®]. Stains can be single or multiplexed up to 50 markers for multi-analyte analyses to support applications such as signaling pathway research in oncology.

inForm is available in different configurations, with each configuration 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 then reconstruct the unmixed signals into a composite image.

Features

- Compatibility with Akoya's digital slides (.qptiff) up to 50 channels, Akoya's multispectral images (.im3) and 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 [Chapter 2.2. Software Configuration](#).
- Computationally efficient; runs on a standard laptop.

Specifications

- Operates on Akoya Biosciences multispectral images (.im3), digital slides (.qptiff) up to 50 channels, and on 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 CONFIGURATION

inForm is available in the following software configurations, where each configuration provides for specific tasks for viewing, selecting, segmenting, and counting.

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 the features that are 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 the features that are 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 algorithm steps and software features available in each software configuration are summarized below. (See Table 2.1.)

TABLE 2.1. Algorithm steps and software features available

	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 following table lists the key terms that are core to using the inForm software. Refer to the terms below when using the software. (See Table 2.2.)

TABLE 2.2. inForm software terminology

Term	Definition
Algorithm	Algorithms include all the steps in processing an image, from image preparation, tissue segmentation and/or cell segmentation, object counting or scoring, to export. Depending on the project created to analyze the image, algorithms can also include threshold or colocalization settings.
Processing Region	Processing regions are user-drawn regions of interest. Use processing regions to process only specific regions on an image. All areas that fall outside of the marked processing regions are ignored.

Term	Definition
Project	inForm projects include the images that you 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 that the tissue segmenter detects can then be segmented to detect cells and perform IHC or IF scoring, or can be used for object counting. Available only with inForm Tissue Finder.
Training Set	The set of images used to train the Tissue Segmenter to identify tissue categories automatically. If you draw a training region on an image, then image is automatically added to the training set. To remove an image from the training set, right-click the large image in the image display area, and then click Training Set. If you remove an image from the training set, then the training regions that are on the image are not used when training the Tissue Segmenter. (The training regions are not deleted from the image, so you can always add the image to the training set again.) You can also use the Available Images window to add or remove images from the training set.

2.4. USING ONLINE DOCUMENTATION

This guide contains detailed information about the inForm application and is designed to be used as a reference tool in your everyday work with inForm. The guide 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 the information in this User Guide. Online help provides three ways of locating information:

Use the Contents tab to navigate through the document; use the Index tab to find topics by keyword; or use the Search tab to look up topics that contain specific words or phrases.

2.5. INSTALLING AND STARTING INFORM

Before you begin the inForm installation, close all open applications. 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.

After R is installed, launch the inForm installer and follow the wizard prompts to install the software. inForm requires the Microsoft .NET 4.5 framework. The installer installs 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 default fluorophore values for available Measured and Synthetic Spectra. You can change this default in [Chapter 3.5. Stain Store Management](#).

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 [Chapter 2.6. Activating the License](#).

2.6. ACTIVATING THE LICENSE

After inForm is installed, the only options that are available are for inForm Viewer. You must activate the customer license 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. From the Windows start menu, start the inForm Licensing Application: Start > Akoya Biosciences > inForm License App. (See Figure 2.1.)



FIGURE 2.1. inForm License Application window to activate your license.

3. Click Activate License.
The Activate the Software dialog box opens.
4. Select Activate, and then click the OK.
The Enter Your License Number dialog box opens.
5. Enter your 20-character license number.
The Activation Options are enabled: Internet and Offline.
6. Activate your license.
 - a. If the Internet (online) activation is available, then select this option.
After you successfully activate inForm online, the Enter Your License Number

dialog box closes. You can then close the inForm License Application dialog box and start inForm.

- b. If the Internet (online) activation is not available, select Offline to open the Offline Activation dialog box, and then carry out the following steps to activate your software offline:
 - i. Click Copy to Clipboard to copy the text that is displayed in the Offline Activation dialog box to the Windows clipboard.
 - ii. Email the copied text to the email address that is shown in the Offline Activation dialog box, and then wait to receive the email that contains the text for the response key.
 - iii. Enter or paste the response key from the email where indicated in the Offline Activation dialog box, and then click OK.
 - iv. The Offline Activation dialog box closes. You can then close the inForm License Application dialog box and start inForm.

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

CHAPTER 3. UNDERSTANDING THE INFORM WORK AREA

Section	Page
3.1. View Editor	24
3.2. Available Images Window	26
3.3. New Project Window	27
3.4. Spectral Library Window	29
3.5. Stain Store Management	31

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. (See Figure 3.1.)

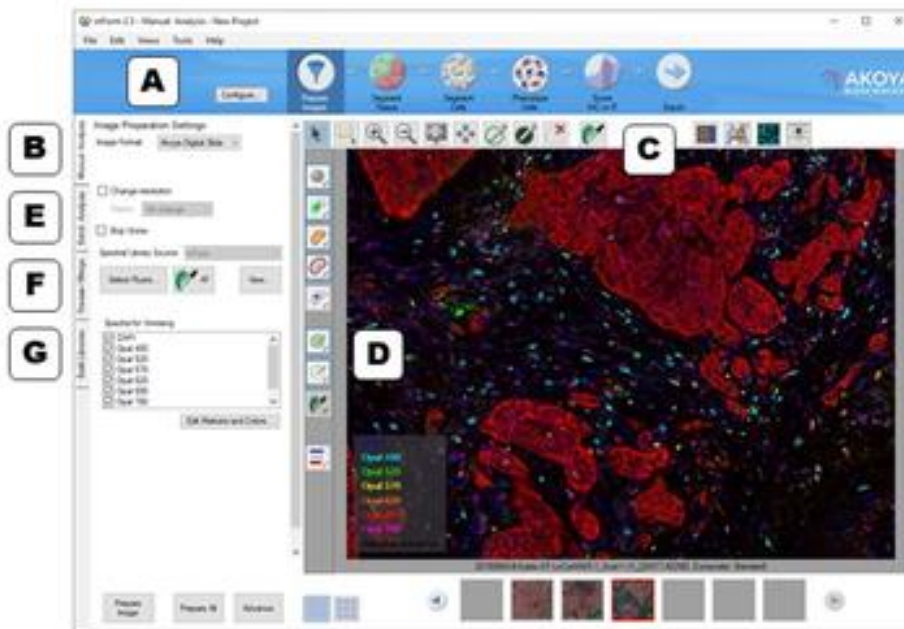






FIGURE 3.1. inForm work area with an unmixed composite image from a multiplexed fluorescence slide. (A) Step Bar. (B) Manual Analysis Tab. (C) Toolbar. (D) Image Display Area. (E) Batch Analysis Tab. (F) Review/Merge Tab. (G) Build Libraries.

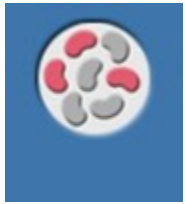

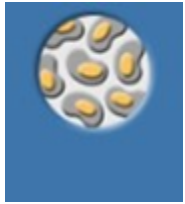
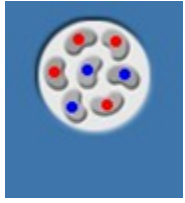
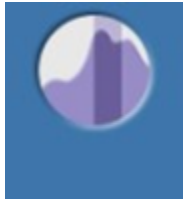
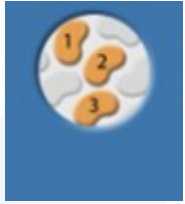
A. Step Bar


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 is displayed only 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 [Chapter 5.1. Creating an inForm Projects](#). To add, remove, or change the steps in the open project, see [Chapter 5.2. Reconfiguring a Saved inForm Project](#).

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. (See Table 3.1.) 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

TABLE 3.1. Step Bar icon names and descriptions

Button	Name	Description
	Configure	Opens the Configure Project window to change the steps in the current project. See Chapter 5. Working with Projects and Algorithms for instructions.
	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. Also, export unmixing libraries for use by PhenolImager™ HT. See Chapter 8. Preparing Images 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 Chapter 9. Manual Tissue Segmentation 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 Chapter 10. Trainable Tissue Segmentation for instructions. This feature is only available with inForm Tissue Finder.

Button	Name	Description
	Threshold	Use to create and analyze threshold maps. Exported data includes position, shape, and component signal statistics for each thresholded region. See Chapter 11. Threshold 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 Chapter 12. Colocalization 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 Chapter 13. Segmenting Cells 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 Chapter 14. 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, 50bin, and Double Positivity) and to provide histogram data of staining levels. Exported data includes the score for each selected cell compartment. See Chapter 15. Scoring IHC or IF 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 Chapter 16. Counting Objects for instructions. This feature is available with inForm Cell Analysis or higher.

Button	Name	Description
	Export	Use to select the export directory, the images to export, the data to export, and the file name options. See Chapter 18. Exporting the Data 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. You can edit these settings only if you are viewing a single image, and not while viewing images in Gallery mode. See [D. Image Display Area](#).










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. Toolbar button names and descriptions are shown below. (See Table 3.2.)

TABLE 3.2. Toolbar button names and descriptions

Button	Name	Description
	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 on the image with this tool to return magnification to 100%.

Button	Name	Description
	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.



Button	Name	Description
	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 dropdown 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 Chapter 17. Displaying the Extracted Data for more detailed information on viewing your color and unmixed component images.

Button	Name	Description
	View Editor	Click to open the View Editor Window 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 Option		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. (See Chapter 3.1. View Editor) 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 as illustrated in Table 3.3.

TABLE 3.3. Image Display Area modes

Mode	Button
Single	
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.

Context Menu

The following actions are accessible by right-clicking 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 to add images without training regions to the training set. (See [Chapter 3.2](#).)
- **View Editor:** Opens the View Editor to select the display options. (See [Chapter 3.1](#).)
- **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 to select a project or an algorithm for batch processing, select export directory/folder preferences, and select the image/table options. (See [Chapter 19. Batch Processing](#).) Note that this feature is only 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 to combine the data from multiple output files into a single file for detailed and quantitative analysis. The Review/Merge Editor has tools to filter out data from samples that are inadequately prepared, stained, and/or segmented, so that merged data sets are reliable. (See [Chapter 20. Merging the Data](#).) Note that this feature is only available with inForm Tissue Finder.

G. Build Libraries

The Build Libraries 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. (See [Chapter 21. Building Libraries.](#))

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 [Chapter 17. Displaying the Extracted Data.](#) To open the View Editor, click the View Editor button on the image toolbar. (See Figure 3.2.)

3.1.1. Views Available in Viewers

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 3.2. View Editor.

3.1.2. Views Available in Cell Analysis

The views available in cell analysis includes all views in the 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 [Chapter 17.5. Viewing the Tissue Segmentation Data Table](#) 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 [Chapter 17.10. Viewing the Quant Data](#) for detailed information.)

Colocalization Data: These tables are available when an algorithm contains a colocalization step. (See [Chapter 17.8. Viewing the Colocalization Data](#) for detailed information.) There are 3 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 [Chapter 17.6. Viewing the Cell Segmentation Data](#) 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 [Chapter 17.9. Viewing the Count Data](#) 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 [Chapter 17.7. Viewing the Score Data](#) for detailed information.)

3.1.3. Views Available in Tissue Finder

Includes all views in Cell Analysis. Tissue Segmentation data displays in the Tissue Segmentation Table. (See [Chapter 17.6. Viewing the Tissue Segmentation Data](#) 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. (See Figure 3.3.)

To load additional images into the project, click Load Image. 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 then click Remove.

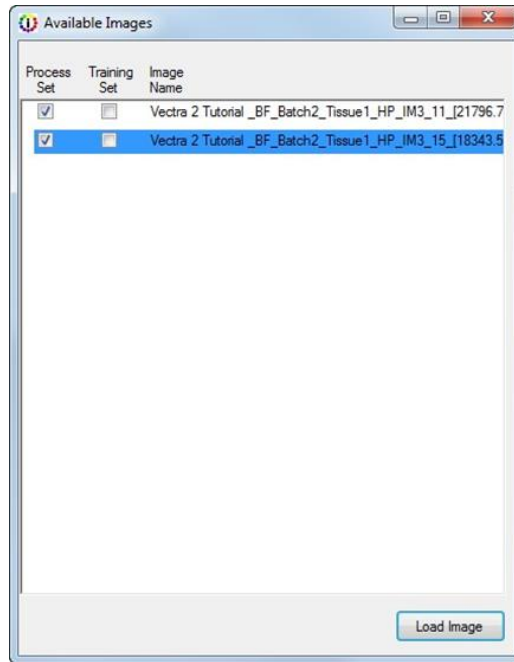


FIGURE 3.3. Available Images window.

3.3. NEW PROJECT WINDOW

Use the New Project Window to select the steps to include in the project. (See Figure 3.4.) Certain steps are only available if a software option has been purchased and installed. For available configurations, see [Chapter 2.2. Software Configurations](#).

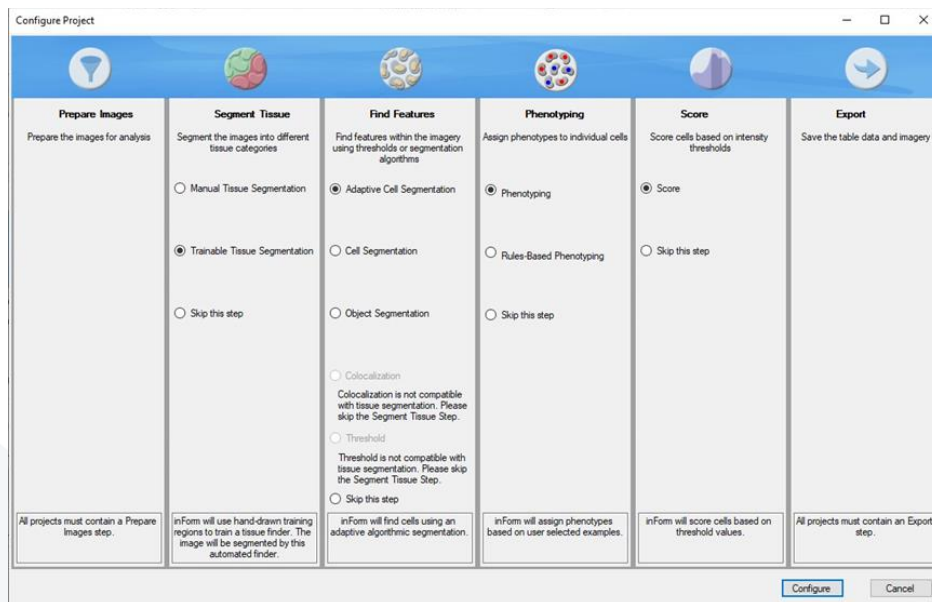


FIGURE 3.4. New Project window.

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 [Chapter 8. Preparing Images](#) for details.

Segment Tissue: Segment the image into specific tissue categories.

- **Manual Tissue Segmentation:** Tissue categories are manually drawn on each image. See [Chapter 9. Manual Tissue Segmentation](#) 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 [Chapter 10. Trainable Tissue Segmentation](#) 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 [Chapter 13. Segmenting Cells](#) for details.
- **Cell Segmentation:** Identifies individual cells or subcellular objects in the image using either thresholded or algorithmic segmentation. See [Chapter 13. Segmenting Cells](#) for details.
- **Object Segmentation:** Identifies objects on an image using either thresholded or algorithmic segmentation. See [Chapter 16. Counting Objects](#) for details.
- **Colocalization:** Identifies regions on an image where multiple unmixed component thresholds overlap. Threshold values for each component are specified individually. See [Chapter 12. Colocalization](#) for details.
- **Threshold:** Identifies regions on an image where a component is above a specific threshold. See [Chapter 11. Threshold](#) 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 [Chapter 5. Scoring IHC or IF](#) 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 [Chapter 18. Exporting the Data](#) 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 View under the Spectral Library pane in the Prepare Images step after selecting a spectral library. (See Figure 3.5.)

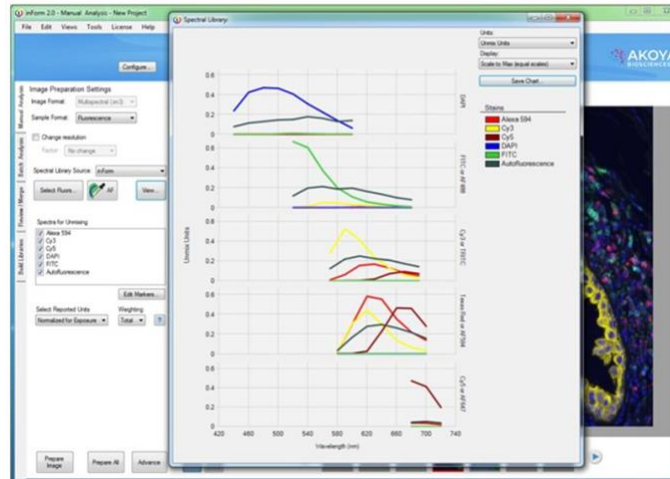


FIGURE 3.5. Spectral Library with multiple filters.

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 range 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. (See Figure 3.6.)

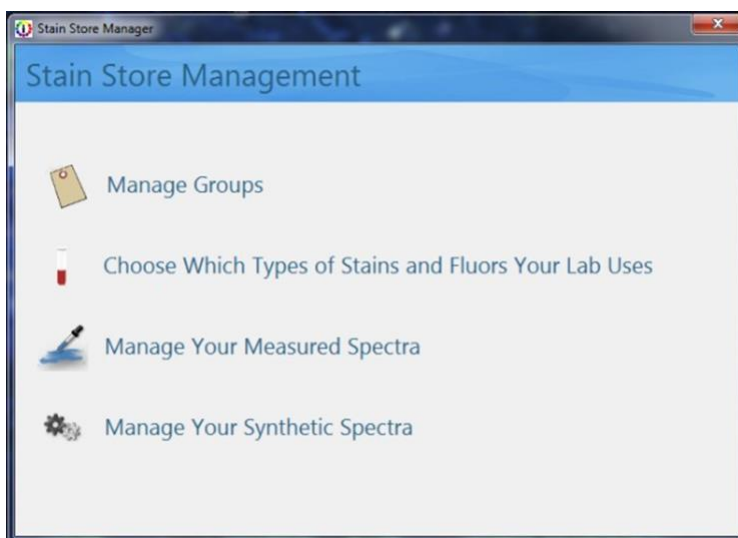


FIGURE 3.6. The home screen for the Stain Store Manager allows you to manage your stains and fluorophores from an intuitive interface, selecting only the stains and fluorophores that you use and allowing you to group them yourself, eliminating clutter and saving valuable time.

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 appear in the Select Fluors list. (See Figure 3.7.)

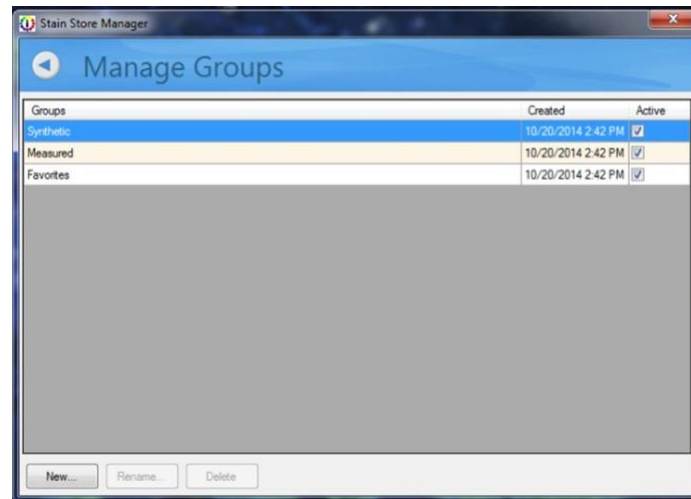


FIGURE 3.7. Manage Groups lets you arrange your stains and fluorophores into convenient Groups.

3.5.2. Choosing Stains and Fluors

Click Choose Which Types of Stains and Fluors Your Lab Uses to select from among a list of common stains and fluorophores grouped into families. (See Figure 3.8. and Figure 3.9.) 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; however, you can add new measured spectra using the New button in the Choose Stains and Fluors window.

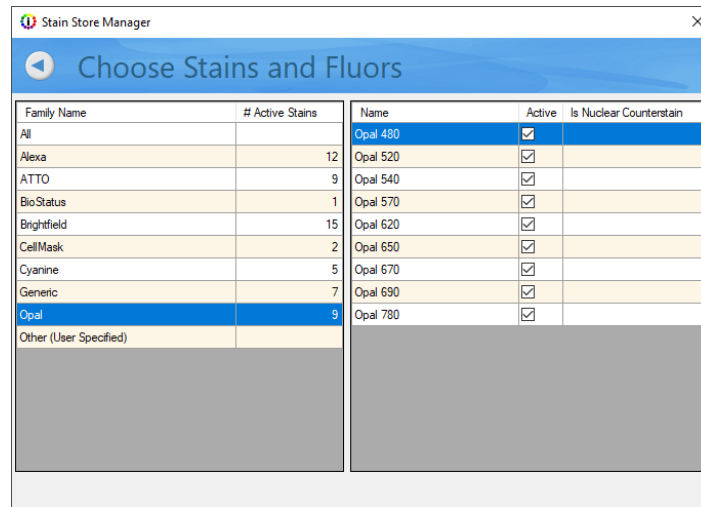


FIGURE 3.8. Stains and fluorophores are arranged by Family or can be seen all at once.

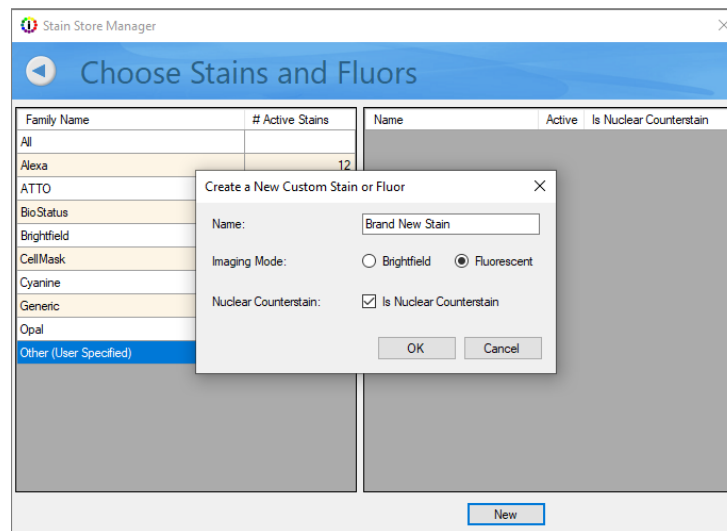


FIGURE 3.9. Adding a new stain or fluorophore can be done from within the Choose Stains and Fluors window.

See [Chapter 21. Building Libraries](#) 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. (See Figure 3.10.) Only checked stains and fluorophores appear in the Stains & Fluors list. Highlighting a particular stain or fluorophore displays 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.

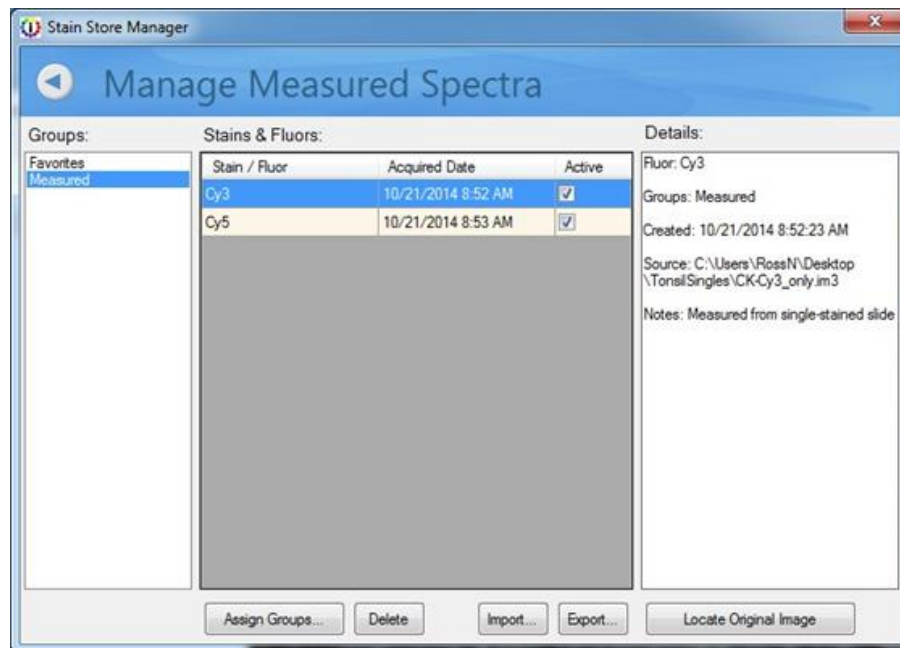


FIGURE 3.10. Manage Measured Spectra lets you select the spectra that you have measured.

You can:

- Assign one or more measured spectra to a group or groups. (See Figure 3.11.) You can assign spectra to existing groups, or you can create a new group if needed. Each spectrum can belong to multiple groups.

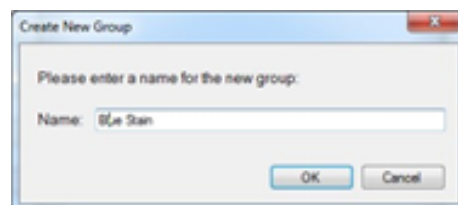


FIGURE 3.11. Assigning a new group.

- 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 Import. Browse to the file containing the appropriate spectra. You can import from algorithms, projects, and .SSE files previously exported. After opening the file, you can preview and select the spectra for import. Selected spectra are added to the Stain Store and are available to your projects.
- Export spectral data files.
To export, click Export, and then select one or more measured spectra. You can save an .SSE file that contains 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 to view a list of all the synthetic spectra that are available for your imaging system. (See Figure 3.12.)

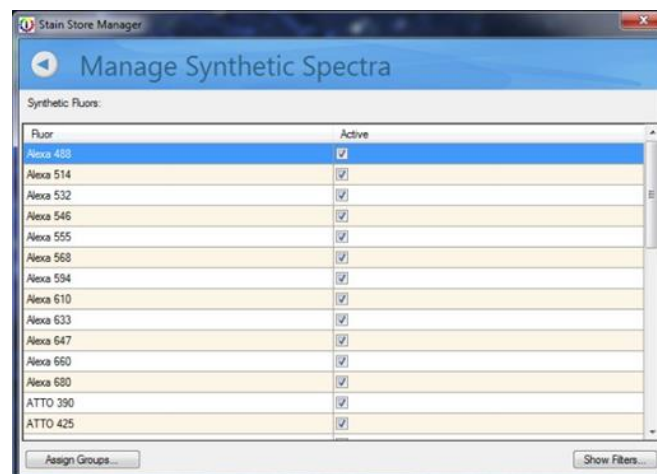


FIGURE 3.12. Choose which Synthetic Spectra will be displayed to choose for a spectral library in the Prepare Images step.

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 Assign Groups to assign a particular Fluor (or a group of Fluors by highlighting more than one) to a new or existing Group. (See Figure 3.13.) Each Fluor can be a member of more than one Group. If you would like to see the filters available to create the Synthetic Spectra, click Show Filter.

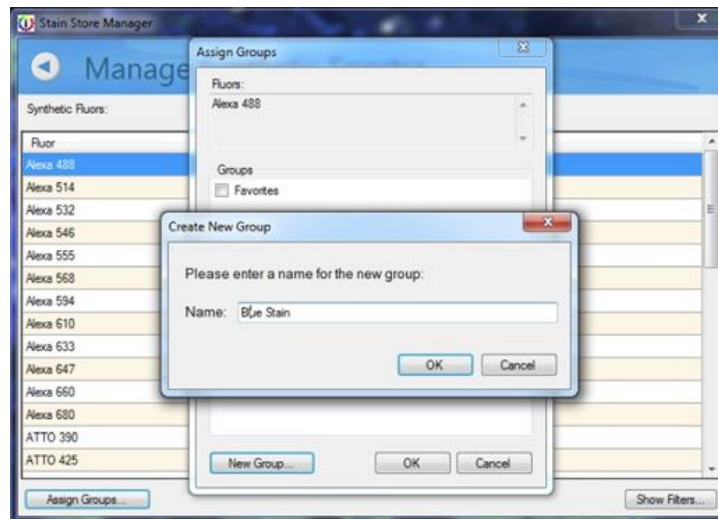


FIGURE 3.13. Assigning a Fluor to a new or existing Group is easy. You can specify the name of a Group and assign stains and fluorophores to multiple groups.

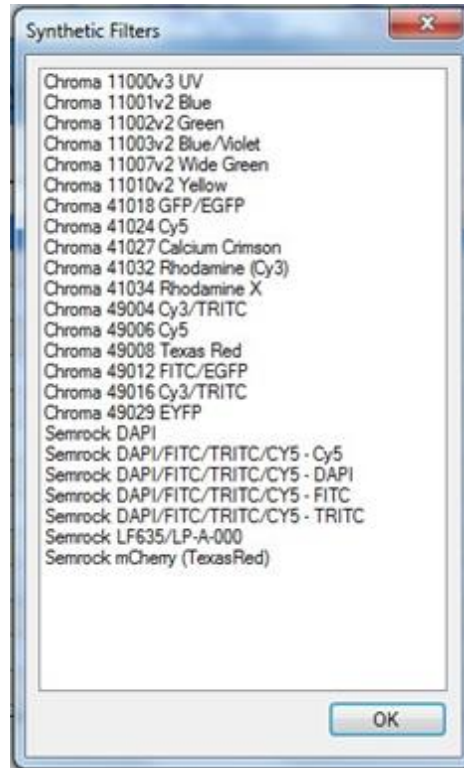


FIGURE 3.14. The list of filters available to generate Synthetic Spectra.

NOTE: Typically, to build up a 'library' of spectra (precise colors that are really unique multispectral profiles of your stains), you capture images from single-stained slides. 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 Phenolmager system. However, because of the variability of stains and the staining process in brightfield IHC, there are no synthetic spectra provided for brightfield microscopy.

CHAPTER 4. COMMON IMAGE ANALYSIS TASKS

Use inForm to create automated image analysis routines to accurately extract data from Akoya images (QTIFF, im3), or conventional RGB images (TIF, BMP, or PNG). (While it is also possible to process compressed JPG images, this is not recommended. These 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.

1. Open a new project. (See [Chapter 5. Working with Projects and Algorithms.](#)) Follow the steps at the top of the window to process the images.
2. Open the appropriate images.
3. Optionally, to use processing regions, draw the necessary regions. (See [Chapter 7. Drawing Processing Regions.](#))
4. Prepare the images for unmixing. (See [Chapter 8. Preparing Images.](#))

5. If you are segmenting tissue, then see either [Chapter 9. Manual Tissue Segmentation](#) or [Chapter 10. Trainable Tissue Segmentation](#); otherwise, continue to Step 6.
6. Refer to the following sections for the remaining project steps:
 - a. Threshold
 - b. Colocalization
 - c. Segmenting Cells
 - d. Scoring ICH or IF
 - e. Counting Objects
7. After you have processed the images, see [Chapter 17. Displaying the Extracted Data](#).

CHAPTER 5. WORKING WITH PROJECTS AND ALGORITHMS

Section	Page
5.1. Creating an inForm Project	41
5.2. Saving an inForm Project	46
5.3. Editing the Step Settings for a Saved inForm Project	47
5.4. Creating and Saving an Algorithm	48

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. An algorithm includes all the steps used in processing an image: image preparation, image analysis, and export. The topics in this section describe how to create and save projects, how to reconfigure the project steps and how to edit the settings for project steps, and how to open, edit, and save an algorithm.

5.1. CREATING AN INFORM PROJECT

Each time inForm starts, a new project is created automatically. A new project contains only the Prepare Images step and the Export step. To create a project with additional steps, you can Create a New inForm Project or Reconfigure a Saved inForm Project.

5.1.1. Create a New inForm Project

The steps that are available when you are creating a new project are determined by the software configuration used to create the project. See [Chapter 2.2. Software Configurations](#) for information on the features available for each software configuration.

To create a new inForm project:

1. Select File > New > Project.

The New Project window opens. Prepare Images and Export are automatically included in the project. (See Figure 5.1.)

NOTE: If an unsaved project is already open, then inForm prompts you to save the current project before the New Project window opens. If you choose No, all unsaved settings are lost.

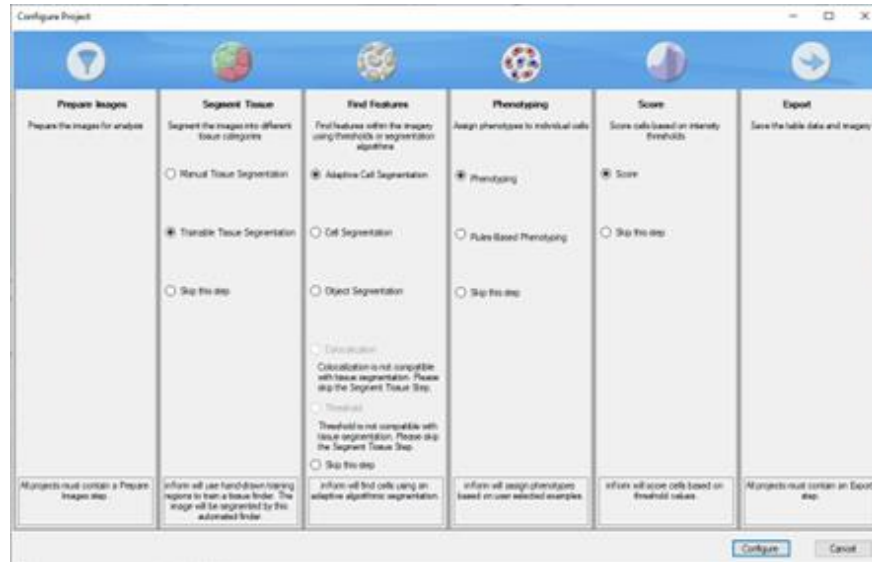


FIGURE 5.1. New Project window.

2. In the New Project text field, enter the name for your new project.
3. Optionally, to segment tissue, do one or both of the following steps; otherwise, click Skip This Step, and then continue to Step 4.
 - a. To manually mark tissue categories on the images, select Manual Tissue Segmentation.
 - b. To train the Tissue Segmenter to automatically identify areas as specific tissue categories, select Trainable Tissue Segmentation.
4. Optionally, to find specific features, do any or all of the following steps; otherwise, click Skip This Step, and then continue to Step 5.
 - a. To identify cellular features such as the nucleus, cytoplasm, and membrane for each cell using an adaptive thresholding algorithm, select Adaptive Cell Segmentation.

- b. To identify cellular features such as the nucleus, cytoplasm, and membrane for each cell, select Cell Segmentation.
 - c. To identify objects of interest, select Object Segmentation.
 - d. To identify areas on the images where selected components are colocated, select Colocalization.
 - e. To identify the areas on the images where the strength of a component at each pixel exceeds a specific threshold, select Threshold.
5. If Cell Segmentation was selected, then do one or both of the following steps; otherwise, continue to Step 6.
 - a. To assign phenotypes to each cell, select Phenotyping.
 - b. To calculate score statistics for the cells, select Scoring.
6. Click Create.
The new project is created with the selected steps.
7. To add the necessary images to the project, see Opening Images.
8. After you have configured the necessary steps and added the images, see [Chapter 5.2 Saving an inForm Project](#).

NOTE: *If you must make adjustments to the step settings after you have saved the project, see [Chapter 5.3. Editing the Step Settings for a Saved inForm Project](#).*

5.1.2. Reconfigure a Saved inForm Project

You can add or remove steps from a saved inForm project. inForm retains as many settings as possible, depending on which steps you add or remove from the project. For example, if you created a project without tissue segmentation and then later decide to include manual tissue segmentation, you can modify the project to add Manual Tissue Segmentation.

When you reconfigure a saved inForm project, any settings that are not affected by the changes are retained, but you might need to adjust other settings that are affected by the changes. You typically make these changes at the time that you are reconfiguring the project, but if necessary, you can make these changes after you save the project.

To reconfigure a saved inForm project:

1. If the saved project is already loaded, then continue to Step 3; otherwise, Select File > Open > Project.

The Open Project dialog box opens.

2. Navigate to the project that you are editing, and then click Open.

The project is loaded and the project images are displayed in Galley mode. (See Figure 5.2.) The View Editor and the Available Images window close if either one was open in the previous project.

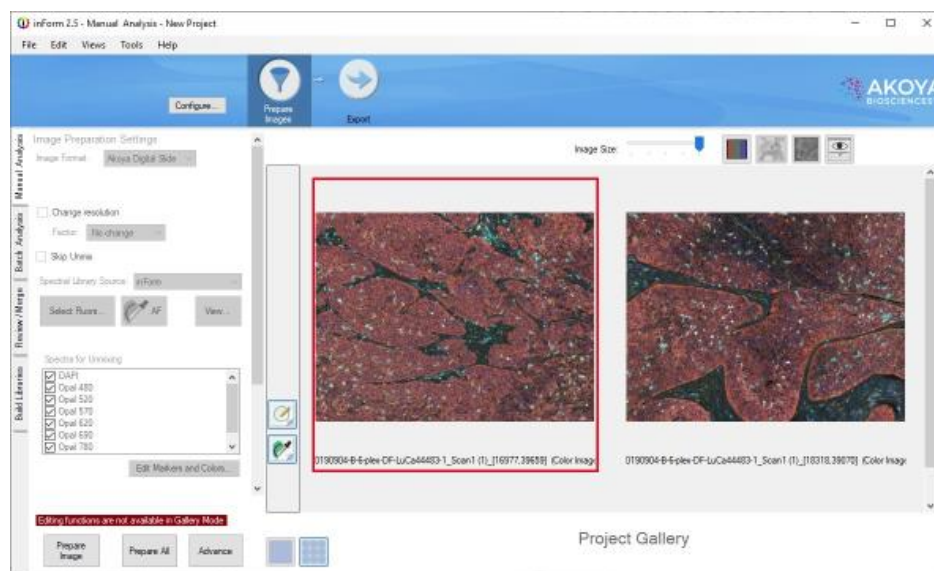


FIGURE 5.2. Galley mode for an inForm project.

3. Select Tools > Configure Project, or click Configure.

The Configure Project window opens. The window displays the same options as the New Project window. (See Figure 5.3.) The loaded project determines the options that are selected when the window opens.

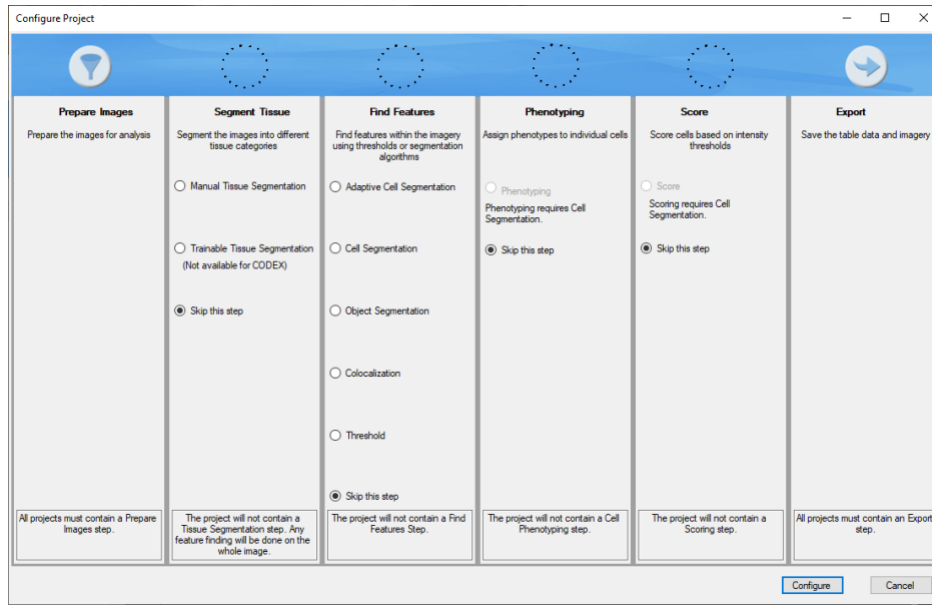


FIGURE 5.3. Configure Project window.

4. Add or remove project steps as appropriate.

NOTE: Changing some steps might require you to reprocess the images for later steps in the project.

5. Click Configure.
inForm reconfigures the open project.
6. Confirm that the new settings are as expected for each step in the project or adjust them as necessary.
7. Save the project. See [Chapter 5.2. Saving an inForm Project](#).

NOTE: If you must make adjustments to the step settings after you have reconfigured and saved the project, see [Chapter 5.3. Editing the Step Settings for a Saved inForm Project](#).

5.2. SAVING AN INFORM PROJECT

A saved inForm project is comprised of the following items: the images, any created composite views, the algorithm for the processing of the images, and any processing regions, training regions, or manual tissue regions.

NOTE: *The following procedure describes how to save a complete inForm project, including the analyzed images. To save only the algorithm that was used for processing the images, and not the images themselves, see [Chapter 5.4. Creating and Saving an Algorithm](#).*

To save an inForm project:

1. Select File > Save > Project.

The Save Project dialog box opens. (See Figure 5.4.)

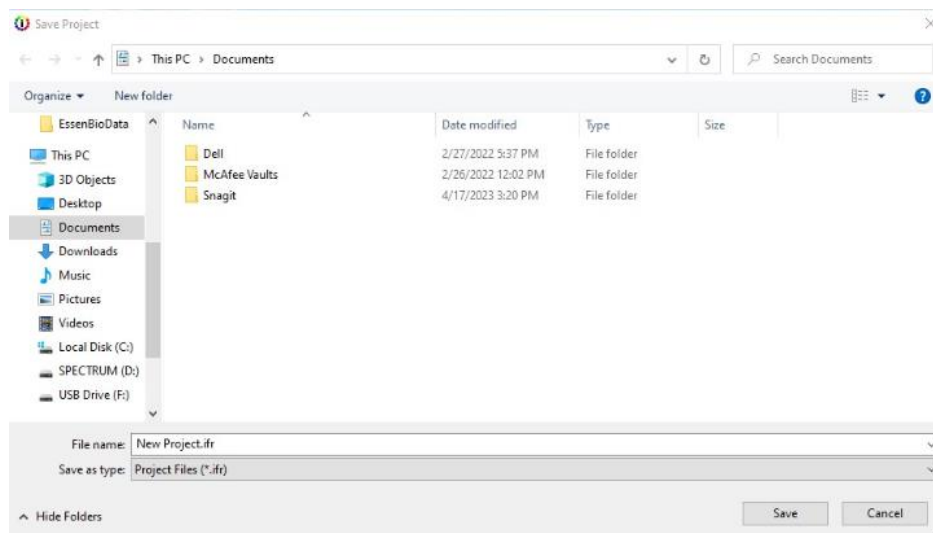


FIGURE 5.4. Save Project dialog box.

The Save as type is set to Project Files (*.ifr) and you cannot change this. If you are saving a new project, then the File name is set to the default value of New Project. ifr; otherwise, if you are saving a reconfigured project, then the File name is set to the name of the project.

2. Select the location within the Data File structure to save the project. (See Figure 5.5.) In the File name field:

- a. If you are saving a new project, enter the project name.
 - b. If you are saving a reconfigured project, leave the name as-is, or enter a different name.
3. Click Save.

The Save Project dialog box closes. The inForm project file is saved in the specified location. A folder named <Project Name>Training Images is created in the folder in which the project file was saved. Local copies of all the project images are saved in this Training Images folder for all projects other than Digital Slide projects.

NOTE: Local copies of project images are not stored for Digital Slide projects. If you change the location of the data for a Digital Slide project, then inForm prompts you to find the slide data. When transferring data, please make sure that the study folder and its contents are moved as a unit.

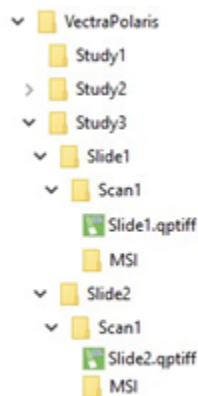


FIGURE 5.5. Data file structure.

5.3. EDITING THE STEP SETTINGS FOR A SAVED INFORM PROJECT

After you create and save a new inForm project, or reconfigure and save an existing inForm project, you might later need to edit the settings for one or more of the project steps. When you open a saved inForm project for editing the settings, the project opens in Galley mode and is locked, which prevents you from making any unintentional changes to the project. To edit the step settings, you must unlock the project by placing it in Single mode.

To edit the step settings for a saved inForm project:

1. Select File > Open > Project.
The Open Project dialog box opens.
2. Navigate to the project that you are editing, and then click Open.
The project is loaded and the project images are displayed in Galley mode as shown in Figure 5.3 above. The View Editor and the Available Images window close if either one was open in the previous project.
3. Click Single.

5.4. CREATING AND SAVING AN ALGORITHM

An algorithm includes all the steps used in processing an image: image preparation, image analysis, and export, but not the images themselves. (Remember, to save the images and all the image processing steps, you must save a project. See Saving an inForm Project.) An algorithm provides you the means of reusing project settings to process compatible images or sets of images without re-selecting the settings for each new project. You can load an algorithm into inForm and then open images for processing, or you can load the images first, and then apply the algorithm. You can use an algorithm to process one image at a time, or you can use an algorithm in [Chapter 19. Batch Processing](#) to automatically process multiple images at the same time using the same analysis settings.

NOTE: If a project is opened for use in Batch Processing, then the algorithm for the project is used to process all the project images.

To create an algorithm *without* images opened first:

1. Select File > Open > Algorithm.
The Open Algorithm dialog box opens.
2. Navigate to the appropriate algorithm, and then click Open.
The Open Algorithm dialog box closes.
3. Open the appropriate images.

4. To prepare the images, click Prepare Image or Prepare All.
5. To review or change the settings in each algorithm step, click Advance to advance through each step, or click each Step button that is displayed at the top of the inForm window.
6. After you satisfied the algorithm development is complete, save the algorithm. See [Chapter 5.4.1. To Save an Algorithm](#).

To create an algorithm *with* images opened first:

1. Select File > Open > Algorithm.
A Data Will be Lost dialog box opens, warning you that loading an algorithm will result in all images being reset to their initial state.
2. Click Yes to close the dialog box and discard all training regions, tissue regions, cell regions, object regions, thresholds, and component images.
The Open Algorithm dialog box opens.
3. Navigate to the appropriate algorithm, and then click Open.
The Open Algorithm dialog box closes.
4. To prepare the images, click Prepare Image or Prepare All.
5. To review or change the settings in each algorithm step, click Advance to advance through each step, or click each Step button that is displayed at the top of the inForm window.
6. After you satisfied the algorithm development is complete, save the algorithm. See [Chapter 5.4.1. To Save an Algorithm](#).

5.4.1. To Save the Algorithm

You can save an algorithm either in an unprotected format, which means that the algorithm is editable, or you can save the algorithm in a locked format, which means that the algorithm is not editable.

1. Do one of the following:
 - a. To save the algorithm in an unprotected format, click File > Save > Algorithm.
The Save Algorithm dialog box opens.
 - b. To save the algorithm in a locked format, click File > Save > Locked Algorithm.
The Save Locked Algorithm dialog box opens.
2. Navigate to the location for saving the algorithm.
3. Enter the name for the algorithm, and then click Save.
The dialog box closes. inForm remains open.

CHAPTER 6. OPENING IMAGES

Section	Page
6.1. Adding Images to an inForm Project	51
6.2. Adding or Removing Images from a Project Processing Set or Training Set	55
6.3. Viewing Images and their Information in a Project	57
6.4. Removing Images from a Project	59

This chapter describes how to add images to an inForm project and the viewing options that are available for the images after you have added them to the project. It also details how to add and remove images from a project and add and remove images from the Processing set and the Training set. The number of images that you can open and view in an inForm project depends not only on the sizes of the images (width and height in pixels), but also on your operating system.

6.1. ADDING IMAGES TO AN INFORM PROJECT

When you add images to a project, you can include the images in the Processing set (the images that the algorithm processes), in the Training set if you are training a Tissue Segmenter, or both. You can add the images to a project using the Open command that is available from the File menu, or you can add images using the Available Images window that is available from the Tools menu.

6.1.1. Add Images to inForm Project from File Menu

1. Ensure that the appropriate project is open in inForm (File > Open > Project), or you can open inForm, which results in the automatic creation of a new project.
2. Select File > Open > Image.

The Select Image dialog box opens. (See Figure 6.1.) By default, the types of image files that you can select is set to All Image Files.

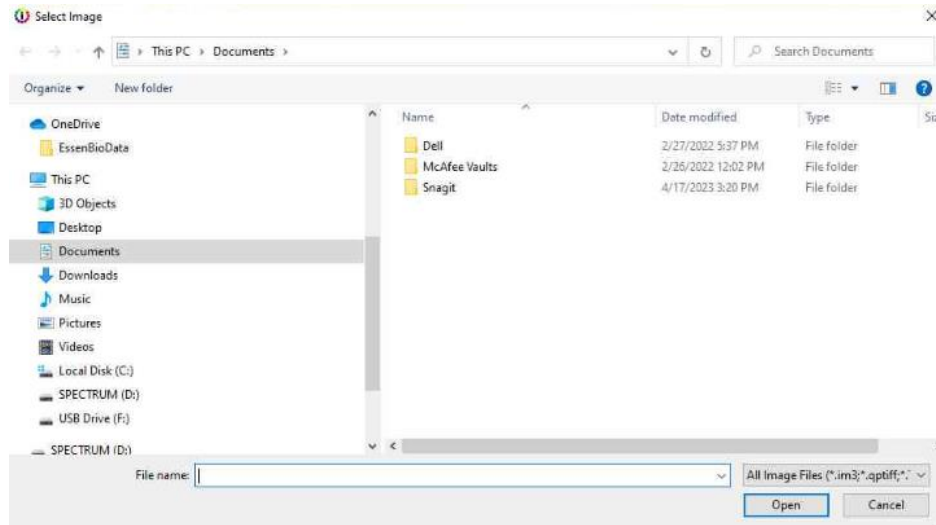


FIGURE 6.1. Select Image dialog box.

3. Leave the image file type set to its default value of all image files, or optionally, select a different file type. (See Figure 6.2.)

Available types are:

- a. Multi-Spectral Files (*.im3).
- b. Akoya Digital Slide (*.qptiff).
- c. Images Files (which are common image file types such as *.TIF, *.TIFF, *.BMP, *.JPG, *.JPEG, and *.PNG for RGB or monochrome images).
- d. Akoya Tiff Extract files (*.qpexport).

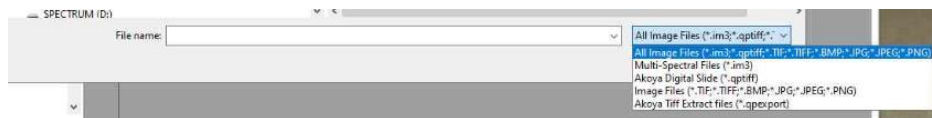


FIGURE 6.2. Available image types.

4. Navigate to the location of the image files that you are adding to the project, and then select one or more image files. Note the following caveats when you are selecting image files to add to a project:

- a. The images that you select must all be the *same* file type (.qptiff, .im3, RGB, or monochrome).

- b. If the images are Akoya Digital Slide images, then inForm loads all stamp annotations that are designated for inForm projects.
5. Click Open.
The Select Image dialog box closes.
 - a. If none of the selected images exceed 16 megapixels (32-bit systems) or 110 megapixels (64-bit systems), then all the selected images are added to the project. By default, the images are also automatically included in the Processing set.
 - b. If one or more images exceed 16 megapixels (32-bit systems) or 110 megapixels (64-bit systems), then the Change Resolution dialog box opens. (See [Chapter 6.1.2.1 Adjust Image Resolution.](#))

6.1.2. Add Images to inForm Project using Available Images Window

The Available Images window displays a list of all the images that have been added to the currently opened project. The window also indicates for each image whether the image is part of the Processing set, the Training set, or both. The window also displays a Load Image option, which you can use to load additional images into the currently opened project. If you use the Load Image option to load additional images into a project, note the following caveats:

- The newly loaded images must be compatible with the Prepare Images settings in the current project.
 - The newly loaded images must be the *same* file type (.qptiff, .im3, RGB, or monochrome) as the images that are already included in the project.
1. Ensure that the appropriate project is open in inForm (File > Open > Project), or you can open inForm, which results in the automatic creation of a new project.
 2. Select Tools > Image List Editor.
The Available Images window opens. (See Figure 6.3.)



FIGURE 6.3. Available Images window.

3. Click Load Image.

The Select Image dialog box opens. The types of image files that you can select for inclusion in the open project is set to that of the images that are already included in the project, for example, Multi-Spectral Files (*.im3).

4. Navigate to the location of the image files that you are adding to the project, and then select one or more image files.
5. Click Open.

The Select Image dialog box closes.

- a. If none of the selected images exceed 16 megapixels (32-bit systems) or 110 megapixels (64-bit systems), then all the selected images are added to the project. By default, the images are also automatically included in the Processing set.
- b. If one or more images exceed 16 megapixels (32-bit systems) or 110 megapixels (64-bit systems), then the Change Resolution dialog box opens. Continue to Adjust Image Resolution.

6.1.2.1. Adjust Image Resolution

When you are adding images to a project, if one or more images exceed 16 megapixels (32-bit systems) or 110 megapixels (64-bit systems), then the Change Resolution dialog box opens with the message that an image is too large to load and prompts you to select an image resolution factor to lower the resolution for the image. The dialog box also displays the number of images of a similar size that can be opened and added to the project at the same time. (See Figure 6.4.)



FIGURE 6.4. Change Resolution dialog box.

Select the appropriate image resolution factor, and then click OK.

The Change Resolution dialog box closes. All the images are added to the project. By default, the images are also automatically included in the Processing set.

6.2. ADDING OR REMOVING IMAGES FROM PROJECT PROCESSING SET OR TRAINING SET

By default, when you add an image to a project, it is automatically added to the Processing set. If you are training the Tissue Segmenter as part of an inForm project, then you can also add an image to the Training set. You always have the option of removing a project image from the Processing set, and if applicable, the Training set as well.

1. Ensure that the appropriate project is open in inForm (File > Open > Project), or you can open inForm, which results in the automatic creation of a new project.

2. Select Tools > Image List Editor.

The Available Images window opens. (See Figure 6.5.)



FIGURE 6.5. Available Images window.

3. Select Tools > Image List Editor.

The Available Images window opens. The window displays a list of all the images that have been added to the currently opened project. The window also indicates for each image whether the image is part of the Processing set, the Training set, or both.

4. Do any of the following:

- a. To remove an image from the project Processing set, clear the Process Set check box for the image.
- b. To add an image to the project Processing set, select the Process Set check box for the image.
- c. To add an image to the project Training set, select the Training check box for the image.

- d. To remove an image from the project Training set, clear the Training check box for the image.
5. Close the Available Images window.

6.3. VIEWING IMAGES AND THEIR INFORMATION IN A PROJECT

After you add images to a project, you have multiple options for viewing the images themselves as well as information about the images. You view images in a project in the Images Display area of the inForm window. You can view images in Gallery mode and you can view images in Single mode, and you can also view an image as a Cube image.

6.3.1. Gallery mode

By default, when you first open a project, all the images in the project are displayed in Gallery mode, which is a display of all the images in the project in the Image Display area. The full name for each image is displayed directly beneath the image. The Gallery mode is useful for comparing all the images in a project at the same time and for quickly locating a specific image. You can always place the image in a project in Gallery mode by clicking the Gallery icon that is displayed below and just to the left of the Image Display area. (See Figure 6.6.)

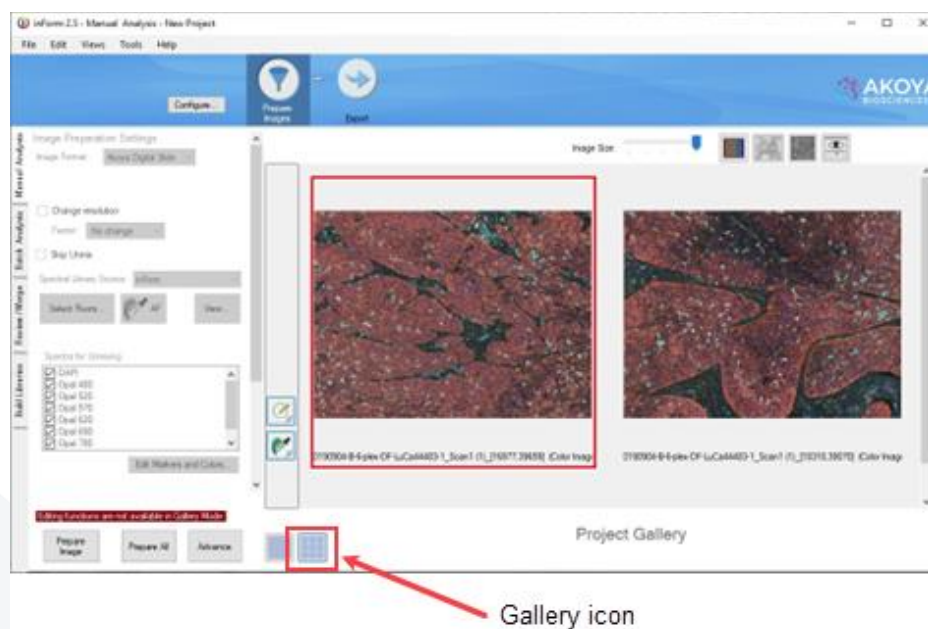


FIGURE 6.6. Image Display in Gallery mode.

The following options are available in Gallery mode:

- To scroll the view of all the images that are displayed, use the vertical scroll bar that is displayed at the far right of the Image Display area.
- To increase or decrease the size of all the images that are displayed at the same time, use the Image Size slider that is displayed at the top of the Image Display area. Move the slider to the left to decrease the image size and increase the number of images that you can view at the same time in the Image Display area without having to use the vertical scroll bar. Move the slider to the right to increase the image size and reduce the number of images that you can view at the same time in the Image Display area. You must then use the vertical scroll bar to view all the images.

NOTE: When you display the project images in Gallery mode, you cannot edit the Step settings for the project.

6.3.2. Single Mode

As the name implies, you use Single mode to display one project image at a time in the Image Display area. To place the project images in Single mode, click the Single mode icon that is displayed below and just to the left of the Image Display area. You use Single mode to zoom in on an individual project image, draw regions on an individual project image, or edit the project algorithm settings. (See Figure 6.7.)

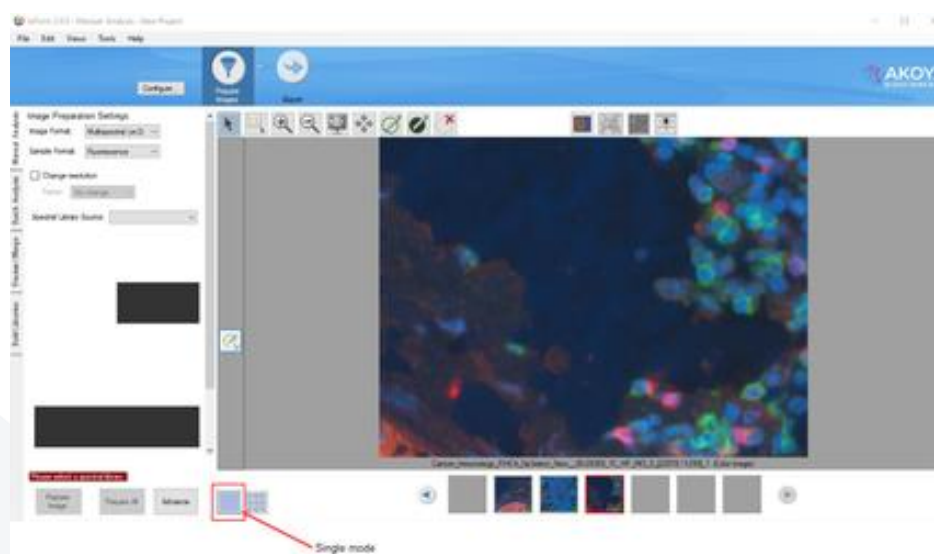


FIGURE 6.7. Image Display in Single mode.

When the project images are displayed in Single mode, thumbnails of all the images are displayed directly beneath the Image Display area. The following options are available in Single mode:

- To scroll through the display of all the project images one at a time, use the Forward and Back navigation arrows that are displayed for the thumbnails.
- To view a specific image in the Image Display area, click the thumbnail for the image.
- To view the name of an image, as well as other information (Processing Set or Training Set), hold your mouse pointer over the thumbnail for the image.

6.3.3. Image Information Dialog Box

You can view the information for an image whether in Gallery mode or Single mode.

- In Gallery mode, select the image, and then on the Tools menu, click Show Image Info.
- In Single mode, click the image thumbnail, and then on the Tools menu, click Show Image Info.

The Image Info dialog box opens and displays detailed information about the image such as the full image name, the image size, the image resolution, exposure times, instrument response data, and so on. The specific information that is displayed depends on how the image was acquired.

6.4. REMOVING IMAGES FROM A PROJECT

You have three options for removing images from an inForm project.

- In Single mode:
 - Click the thumbnail of the image that you are removing to display the image in the Image Display area.
 - Right-click the image in the Image Display area, and then on the Image context menu that opens, click Remove from Project.
- In Galley mode:

- Right-click the image that you are removing, and then on the context menu that opens, click Remove from Project.
- In the Available Images window:
 - Right-click the image that you are removing, and then on the context menu that opens, click Remove from Project.

CHAPTER 7. DRAWING PROCESSING REGIONS

A processing region encloses an area of an image that is processed using the steps in the project or algorithm. You manually draw one or processing regions on an image with the project in Single mode. inForm ignores any areas that fall outside the processing regions on an image and does not include these outside regions in any calculations or statistics. You cannot use processing regions in Batch Processing (See [Chapter 19. Batch Processing](#)).

To draw processing regions on a project image:

1. Ensure that the opened project is in Single mode.
2. Click the image thumbnail.
3. Optionally, use the Zoom in icon and/or the Pan icon to bring the preferred image region into clearer focus.
4. Click the Draw Processing Region icon, and then click and drag your mouse pointer around (encircle) the appropriate image area, making sure to release the mouse button to connect the starting point and ending point of the process region. (See Figure 7.1.)

An ID number is automatically assigned to the processing region, and is displayed in the middle of the region, with "1" being assigned to the first processing region drawn on the image, and incremented sequentially thereafter for each subsequent processing region drawn on the image.

5. Repeat Step 2 through Step 4 for each image in the project.

NOTE: If you draw processing regions on one or more images in a project, and then save the project, the processing regions remain intact. (The images are saved with the processing regions.)

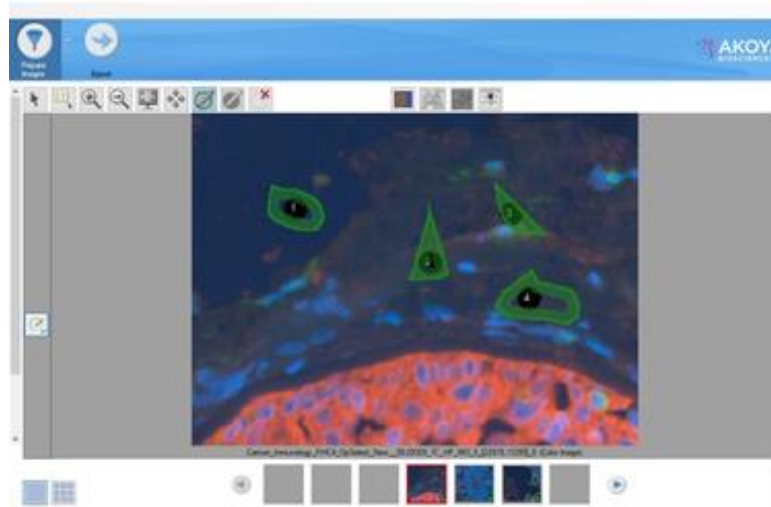


FIGURE 7.1. Image with processing regions drawn on it.

To delete processing regions from a project image:

1. Ensure that the project is opened in Single mode.
2. Click the image thumbnail.
3. To delete one or more processing regions from the image, you have two options:
 - a. Use the Delete Regions button.
 - i. Click the Delete Regions button.
The mouse pointer changes to a set of cross-hairs.
 - ii. Click on each processing region that you are deleting from the image.
 - b. Right-click each processing region that you are deleting, and then on the Region context menu that opens, click Delete Processing Region.

As you delete one or more processing regions from an image, any remaining processing regions that have a higher ID number than the deleted regions are automatically renumbered.

CHAPTER 8. PREPARING IMAGES

Section	Page
8.1. Selecting the Image Format	63
8.2. Choosing a Spectral Library	64
8.3. Viewing and Saving a Spectral Library	71
8.4. Exporting an Unmixing Library	72
8.5. Component Units	73
8.6. Completing the Prepare Images Step	78

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 selecting the image format, choosing a spectral library, selecting the component units, and then completing additional image preparation steps.

8.1. SELECTING THE IMAGE FORMAT

The Image Preparation Settings panel displays the Image Format, Sample Format, and Change Resolution options. (See Figure 8.1.)

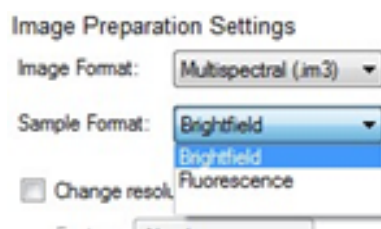


FIGURE 8.1. Image Preparation Settings panel.

8.1.1. Image Format

Before you can begin an analysis, inForm must know the type of images in the project. Available 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 images or Monochrome images.

Additionally, all images that you add to the project must be acquired at the same resolution. If you use HT, imagery that is acquired at a resolution of 0.5um/pixel (20x) is fundamentally different than the imagery that is acquired at 0.5um/pixel (20x binned from 40x) and you cannot process these images within the same project.

8.1.2. Sample Format

All images in the project must be the same sample format. For any RGB images, 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. Images taken with Phenolmager HT and Mantra can vary in their filter order.

If preferred, you can select the image format and the sample format before adding images. In this case, only those images that match the selected image format and sample format can be opened.

HT can create digital slides in three different formats: a Standard 8-bit format, an Extended Range format, and an Unmixed format. All images within a project must be the same format.

8.1.3. Change Resolution

Select Change Resolution 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, then prior to unmixing, lower the image resolution.

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. 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 that is associated with the stain. For example, a fluorescence sample could consist of stain components such as DAPI and FITC. The spectral library can additionally include the color distribution that is associated with tissue autofluorescence.

- For Multispectral Fluorescent Digital Slides in Unmixed format, the data is already unmixed, and there is no need (or option) to select spectral libraries in inForm.
- For Multispectral Fluorescent Digital Slides in Standard or Extended format, the spectral library can be synthetic, or it can be 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 HT or Mantra, inForm can extract the spectrum from singly stained samples. In addition, a synthetic spectral library can be used for fluorescence imagery.
- For RGB images, 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 that is included with inForm can be used if preferred. Spectral libraries are not used for fluorescent PKI TIFF Extracts.

8.2.1. Selecting an InForm Library

If you have previously created spectra by Building Libraries (see [Chapter 21. Building Libraries](#)), you can select those spectra for use with your images. You must have an image loaded to select inForm libraries.

1. Select inForm as the Spectral Library Source. (See Figure 8.2.)



FIGURE 8.2. Selecting inForm as the Spectral Library Source to choose spectra that were created in inForm.

2. Choose the spectra that represent the stains or fluorophores in your sample. (See Figure 8.3.)

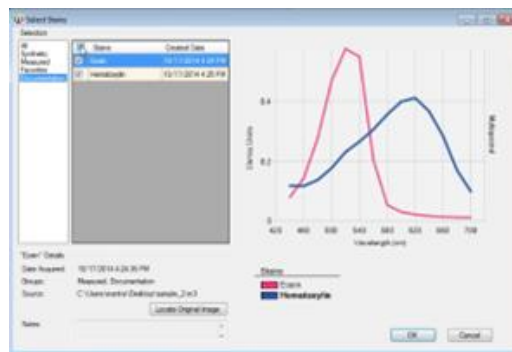


FIGURE 8.3. Available stains can be selected for preparing images.

Note the following:

- While choosing your stains or fluorophores, you can narrow your choices by selecting one or more Groups. Selecting All shows all the spectra that are compatible with your imagery.
- If you must re-select your stains or your fluorophores, click Select Stains/Fluors.
- If this is a fluorescence sample, you have the option to select autofluorescence by drawing on an unstained portion of tissue with the Autofluorescence Picker.

3. Choose the spectra that represent the fluorophores in your sample, and then click OK.

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



Note the following:

- To view the selected Spectral Library, click View, and then see Viewing a Spectral Library.
- For fluorescent images, see Remove Haze for details about removing the background haze before unmixing.

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



- In the Spectra for Unmixing pane, select the components that you are unmixing. (See [Chapter 8.2.3. Choosing Spectra for Unmixing.](#))

8.2.2.1. Editing Markers

You can add the names of your markers to the spectra that are listed in the Spectra for Unmixing pane in the Manual Analysis tab. (See Figure 8.5.)

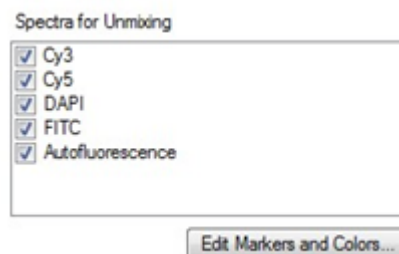


FIGURE 8.5. Spectra for Unmixing pane.

1. Click Edit Markers and Colors...

The Edit Markers and Colors dialog box opens. You use the available options to enter the marker names and change the colors that are associated with your spectra. (See Figure 8.6.)



FIGURE 8.6. Edit Markers and Colors dialog box.

2. Enter your maker names, and optionally, select the default colors that are to be used for spectral unmixing.

For example, if you know that CD4 is your marker for FITC, then in the text field that is displayed to the right of FITC, enter CD4. The fluorophore name is placed in parentheses next to the marker name in Spectra for Unmixing list.

8.2.2.2. Converting to Optical Density

If your goal is to segment cells and measure stain "intensity" (optical density), then you must convert brightfield multispectral and RGB images of IHC-stained samples to optical density. Brightfield multispectral images that are acquired with Mantra or HT are already converted to optical density and therefore conversion is not required as part of Prepare Images. Brightfield multispectral images that were acquired with earlier programs were not converted to optical density, and therefore require conversion as part of Prepare Images.

Selecting a Reference White for Optical Density Conversion

1. Select Convert to Optical Density. (See Figure 8.7.)

2. If the white areas on the image are small, then zoom in on a white area.
3. Click the White Picker button.
Your mouse cursor changes to a white box.
4. Move the cursor over a white region on an image, and then click.
"White Selected" is displayed under Convert to Optical Density.



FIGURE 8.7. Selecting a Reference White for Optical Density.

NOTE: *If the image appears very light after you have selected the White Reference, then you might have clicked an area that was not completely white. You should clear Convert to Optical Density to reset the image to its original state, and then repeat this procedure.*

8.2.3. Choosing Spectra for Unmixing

The Spectra for Unmixing list is populated from the selected spectra or library. Check the stains that you are using to unmix. If you have more than one spectral example from the same stain (for example, multiple DAB spectra) in your list, then you can use only one for unmixing.

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

NOTE: *If you select Scale Views Based on Selected Images in the View Editor (see [Chapter 3.1. View Editor](#)) and then you make changes to the selected Spectra for Unmixing, the image scaling is reset to the default value of Scale Views Equally for All Images in the Project.*

8.2.3.1. Removing the Haze Spectrum

In fluorescence images, image contrast and unmixing accuracy can sometimes be improved if the general haze, or background, 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, then add this spectrum to the spectral library. When the spectral library is loaded, you can select the haze spectrum and remove it from the images.

To remove the haze spectrum:

1. Select Remove Haze.

The Haze spectrum is created.

2. On the Haze Spectrum drop-down list, select Haze. (See Figure 8.8.)

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

3. Click the Prepare Images button.

Your settings are applied and the composite view is displayed.

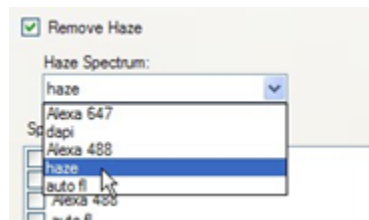


FIGURE 8.8. Removing the Haze spectrum.


8.3. VIEWING AND SAVING 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 spectral library.
2. In the Spectra for Unmixing drop-down list, select the spectra that you are using to unmix the images.
3. Click View.
The Spectral Library window opens.
4. Select the units in which to display the graphs. See the Spectral Library window for a complete description of the graph display.

NOTE: If the selected units are not compatible with the selected spectra, then the message “ “ is displayed instead of the spectral graphs.

5. Select the Display options for the Spectral Library chart.
6. To save an image of the chart with the currently selected Display option, click Save.
7. Enter the name for the image file, select an image format, and then click Save.
8. To close the Spectral Library window, click the Close  button in the upper right corner of the window.

8.4. EXPORTING AN UNMIXING LIBRARY

One important workflow involves exporting an unmixing library from inForm for use by HT so it can acquire whole-slide scans in unmixed format. This is only supported for Multispectral Fluorescent Digital Whole Slide datasets.

To do this, load a representative whole-slide scan in Standard or Extended format. It should have the same bands as the scans you would like HT to acquire in Unmixed format. The exposure times need not be the same, as these are set separately in the HT software. Marker names can be provided now, but they can be overridden in HT so the same library can be used for samples targeting a variety of markers. What is vital is that the dye channels and bands be the same, and the autofluorescence be representative of the samples you wish to scan in HT.

Choose the spectra, which can either be synthetic or measured; and select an autofluorescence signal. Then, go to the menu item Export | Unmixing Spectra, and you will be prompted to save an unmixing library based on your currently selected spectra and marker names (if provided).

8.5. COMPONENT UNITS

The components that are produced by unmixing are calculated using the settings that you select 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. (See Figure 8.9.)



FIGURE 8.9. Reported Units and Weighting options.

8.5.1. Brightfield images versus Fluorescence images

Because of their inherent differences, brightfield images and fluorescence images use different measures schemes.

- 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 a reporter protein.

Consequently, you use optical density for brightfield images and fluorescence counts, either raw or normalized, for fluorescent images.

8.5.2. Select Reported Units

Monochrome (black and white) images and RGB color images produce signals in counts. For multi-spectral images that are not converted to Optical Density, component signal counts are either Raw or Normalized for Exposure.

- Raw reports the measured signal that is associated with a given component, such as DAPI or FITC.
- Normalized for Exposure reports the number of counts that are in the image, after accounting for all the following camera settings that are in use: Gain, Bit Depth, Well Depth, and Exposure Time. When Normalized for Exposure is the selected Reported Units, you can compare images that were taken using different camera settings or exposures, with the assurance that the signal levels indicate the actual brightness at the sample, regardless of the instrument settings.
- Counts that are Normalized for Exposure are calculated according to the following formula:

$$(\text{Counts} \times \text{Relative Well Depth}) / (2^{\text{Bit Depth}} \times \text{Exposure Time} \times \text{Gain} \times \text{Binning Area})$$

where:

- Relative Well Depth is the well depth for the camera sensor relative to nominal Akoya sensor at the chosen gain setting.
- Bit Depth is the bit depth of the imagery, which is typically 8-bit (256 levels of gray) or 12-bit exposure time in seconds.
- Gain the gain setting of the camera.
- Binning Area is 1 for 1 x 1, 4 for 2 x 2, etc.

NOTE: If you are using Mean weighting, then generally, the values are small, and it might be difficult to develop an intuition about what is normal. Select Total weighting to produce larger values.

For brightfield images that are converted to Optical Density, inForm produces unmixed component signals in OD (Optical Density) units, where Optical Density indicates the amount of absorbing material that is present at a given location and is calculated according to the following equation:

$$\text{Optical Density} = -\log_{10} (\text{pixel value} / \text{white reference pixel value}).$$

NOTE: If you select Scale Views Based on Selected Images in the [View Editor](#) and then you make changes to the Reported Units, the image scaling is reset to the default value of Scale Views Equally for All Images in the Project.

8.5.3. Weighting

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

- **Total:** Total weighting is the sum of all signals of the selected component across all wavelengths. Total weighting is available for all images except images that are converted to Optical Density. Total weighting is the default value for fluorescence images.
- **Mean:** Mean weighting is the average of all signals of the selected component across all wavelengths. Mean weighting is available for all units except monochrome image counts. Total weighting is the equivalent of the settings in versions of inForm prior to V2.0.2.
 - 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. For example, 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:** Peak is the peak signal of the selected component across all wavelengths. Peak weighting is available for images converted to Optical Density and for fluorescent multispectral images. Peak weighting is the default value for brightfield images.

The simplified example below explains the differences among the three different weighting options. The example uses two fluor, Fluor A and Fluor B, and eight wavelengths (420, 460, 500, 540, 620, 660, and 700 nm). The example also ignores noise and other sources of error. The spectral library is shown below in Figure 8.10.

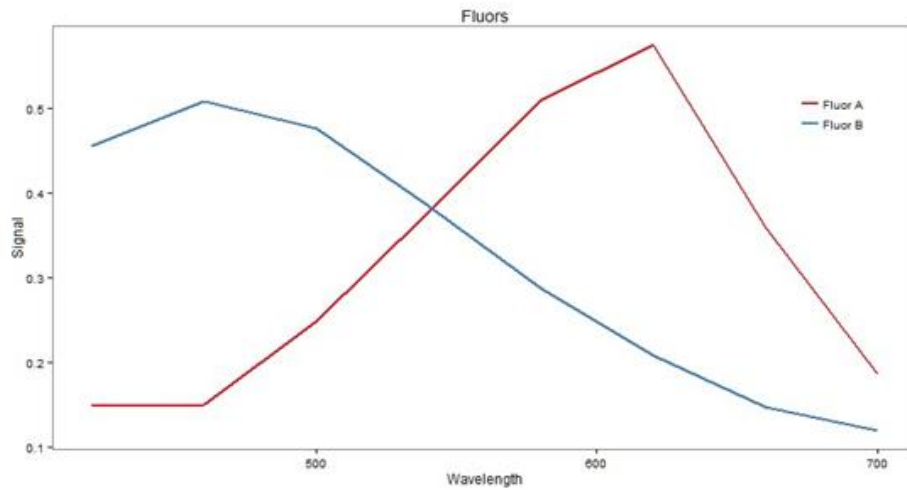


FIGURE 8.10. Spectral Library.

The signal at the image pixel where the pixel expresses one part Fluor A and 3 parts Fluor B is shown below in Figure 8.11.

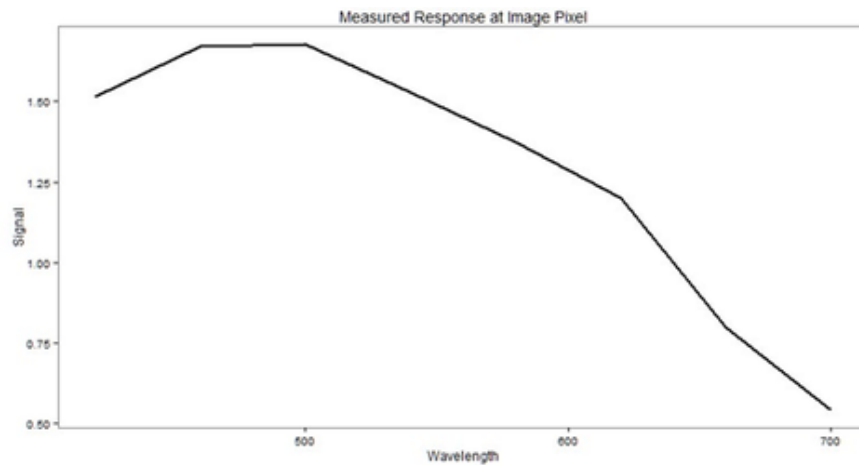


FIGURE 8.11. Signal at image pixel.

Unmixing recovers the original spectra scaled according to the measured expression at the pixel as shown in Figure 8.12.

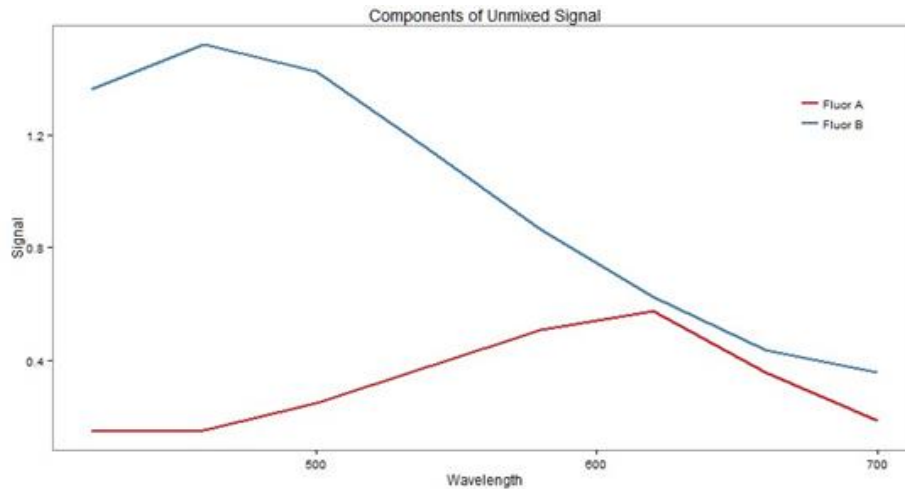


FIGURE 8.12. Original spectra, scaled.

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 and Fluor B = 1.5216.
- Mean weighting represents the spectrum as the average signal across wavelengths. In the example, this gives component values of Fluor A = 0.3189 and Fluor B = 0.9689.
- 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 and Fluor B = 7.7514, where the Total weighting is the Mean weighting multiplied by the number of wavelengths, which is 8 in this example.

The scaled spectra annotated with the weighting values is shown below in Figure 8.13.

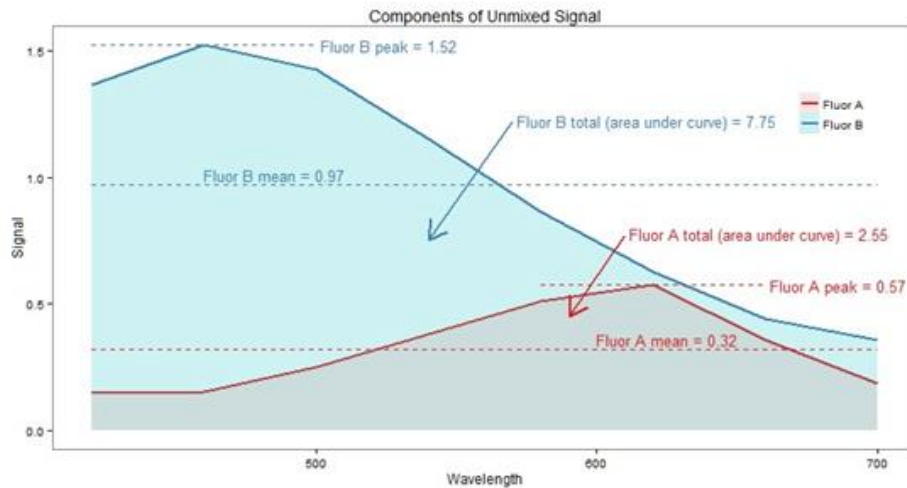


FIGURE 8.13. Scaled spectra.

8.6. COMPLETING THE PREPARE IMAGES STEP

After you have specified all the image preparation settings, you process the images using one of the four options detailed below.

- To apply the selected settings to the currently selected image, click Prepare Image.
- To apply the selected settings to all images that are open in the project, click Prepare All.
- To apply the selected settings to all images that are open in the project and then display the next step in the project, click Advance.
- To process all images in the project using the current settings, click the next step in the project in the Step Bar that is displayed at the top of the inForm window. (See [Chapter 3. Understanding the inForm Work Area.](#)) The settings panel for the next step in the project is displayed.

CHAPTER 9. MANUAL TISSUE SEGMENTATION

Section	Page
9.1. Adding Tissue Categories	79
9.2. Drawing and Deleting Tissue Category Regions	81
9.3. Editing Tissue Category Regions	82

This topics in this chapter describe how to manually draw tissue categories on a project image. Tissue categories divide the image into specific areas that correspond to specific types of tissue (for example, cancer, necrosis, stroma, and so on) or occasionally other structures of interest. The Manual Classification step, which is available in inForm Basic Analysis or higher, provides the options that you can use to draw the appropriate tissue categories over areas of each tissue type on project images. You can then use these tissue categories to generate data or you can use them during the Cell Segmentation step, the Scoring step, or the Object Counting step.

NOTE: For you to manually create tissue categories, you must have included the Segment Tissue step with Manual Tissue Segmentation selected at the time you created the project, or you might need to reconfigure your project. See [Chapter 5.1. Creating an inForm Project](#). If your project algorithm includes a Manual Classification step, then you cannot use this algorithm in Batch Processing.

9.1. ADDING TISSUE CATEGORIES

To manually add tissue categories:

1. If not already, then open the appropriate inForm project.
2. On the Step bar, click the Manual Classification step icon.
The Manual Analysis tab opens. The Tissue Segmentation panel opens on the tab. (See Figure 9.1.)

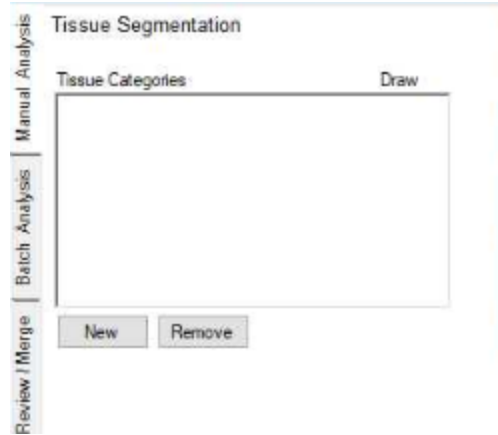


FIGURE 9.1. Tissue Segmentation panel.

3. Click New.

A placeholder with the default name of Category 1 is created for a new manual tissue segmentation category, and a default color is assigned to the category. (See Figure 9.2.)

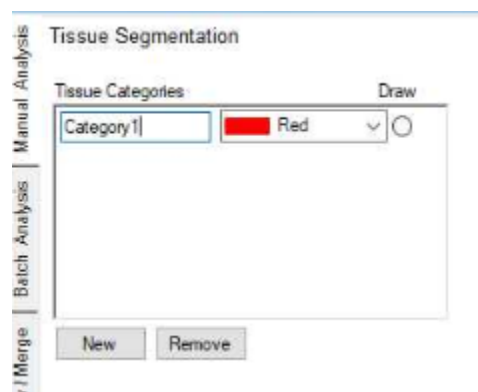


FIGURE 9.2. Tissue Segmentation panel with default category.

4. Change the default name of Category 1 to your preferred category name, and optionally, on the Color drop-down list, select a different color for the category.
5. Repeat Step 3 and Step 4 to create all the necessary tissue categories for the project. By default, every time you create a new tissue category, the Draw option is cleared for the previous category and selected for the new tissue category.

NOTE: If you add a tissue category in error, then to remove it, select the category, and then click Remove. To remove all categories in a single step, click Clear.

6. Continue to Drawing and Deleting Tissue Category Regions.

9.2. DRAWING AND DELETING TISSUE CATEGORY REGIONS

After creating all the necessary tissue category regions, the next step is to draw the regions of these tissue categories on all of the project images that contain one or more of the tissue types. A single region on a project image can belong to only one tissue category type, for example, either tumor or benign, but not both.

To draw tissue category regions on a project image:

1. Ensure that the opened project is in Single mode.
2. Click the image thumbnail.
3. Click the Draw button for the appropriate tissue category, and then click and drag your mouse pointer around (encircle) the appropriate groups of cells, making sure to release the mouse button to connect the starting point and ending point of the tissue category region. For example, if you are drawing the region for a Tumor tissue category, then draw the regions around groups of tumor cells.

Note the following caveats as you manually draw these tissue category regions:

- a. If one region intersects another region in the same tissue category, then the two regions are merged into one region.
- b. If new intersects a region in a different tissue category, the area becomes part of the new tissue category.
- c. As you draw the tissue category regions, a gray mask covers the unselected parts of the image. This may make it difficult for you to see the image clearly enough to draw additional tissue categories. To temporarily hide the existing tissue categories and the gray mask, click the Show/Hide Tissue Segmentation Map button.



After you draw the next tissue category on the image, the tissue segmentation map is again displayed on the image.

4. After you have drawn all the necessary tissue category regions, you can do either of the following:
 - a. Edit the tissue category regions. (See [Chapter 9.3. Editing Tissue Category Regions.](#))
 - b. Continue with the next processing step in the project by clicking Advance or the (next step) button on the Step bar.

To delete tissue category regions from a project image:

1. Ensure that the project is opened in Single mode.
2. Click the image thumbnail.
3. To delete one or more tissue category regions from the image, you have two options:
 - a. Use the Delete Regions button.
 - i. Click the Delete Regions button.
The mouse pointer changes to a set of crosshairs.
 - ii. Click on each tissue category region that you are deleting from the image.
 - b. Right-click each tissue category region that you are deleting, and then on the Region context menu that opens, click Delete Region.

9.3. EDITING TISSUE CATEGORY REGIONS

After you draw tissue category regions on a project image, you can edit the regions. You can add areas to the region, you can remove areas from a region, or you can remove individual pixels from a region.

To add an area to a tissue category region:

1. To refine the image and make it easier for you to add areas to the tissue category region, do any or all of the following:
 - a. Zoom in or zoom out on the image to your preferred magnification.
 - b. Use the Tissue Segmentation Map button to hide the tissue masks.
 - c. If Processing Regions have been defined on the image, then use the Process Regions button to show or hide the process regions mask.
2. Click the option button that is next to the tissue category to which you are adding an area, and then click and drag your mouse pointer around (encircle) the area that you are including, making sure to release the mouse button to connect the starting point and ending point of the area. Defined tissue classification areas can be intersecting. The outline area is assigned to the specific tissue category.

To remove an area from a tissue category region:

1. Click the Select Regions to Unclassify button.



2. Click-and-drag your mouse pointer around (encircle) the area that you are removing, making sure to release the mouse button to connect the starting point and ending point of the area.

The outline area is removed from the tissue category region.

To remove individual pixels from a tissue category region:

1. Zoom in on the image until the individual pixels become visible.
2. Click the Select Pixels to Unclassify button.
3. Remove one or more pixels from the region.

- a. To remove a single pixel from a region, click it.
- b. To remove multiple pixels from the region, click and drag your mouse pointer around (encircle) the pixels that you are removing, making sure to release the mouse button to connect the starting point and ending point of the area.

CHAPTER 10. TRAINABLE TISSUE SEGMENTATION

Section	Page
10.1. Adding Tissue Categories	85
10.2. Drawing Tissue Segmentation Training Regions	88
10.3. Selecting Training Components	88
10.4. Adding Images to Training Set	92
10.5. Editing Masks and Re-Training	93
10.6. Completing Automated Tissue Segmentation Step	95

Tissue categories divide the image into specific areas that correspond to specific types of tissue (for example, cancer, necrosis, stroma, and so on) or occasionally other structures of interest. The Segment Tissue Step, which is available only in inForm Tissue Finder, provides the options that you can use to draw the training regions around representative areas of tissue on images, and then the train the segmenter to automatically locate similar tissue regions in project images.

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

NOTE: For you to carry out trainable tissue segmentation, you must have included the Segment Tissue step with Trainable Tissue Segmentation selected at the time you created the project, or you might need to reconfigure your project. See [Chapter 5.1.1. Creating a Project](#) or [Chapter 5.1.2. Reconfiguring a Saved inForm Project](#).

10.1. ADDING TISSUE CATEGORIES

1. If not already, then open the appropriate inForm project.

2. On the Step bar, click the Segment Tissue step icon.
The Manual Analysis tab opens. The Tissue Segmentation Training panel opens on the tab. (See Figure 10.1.)

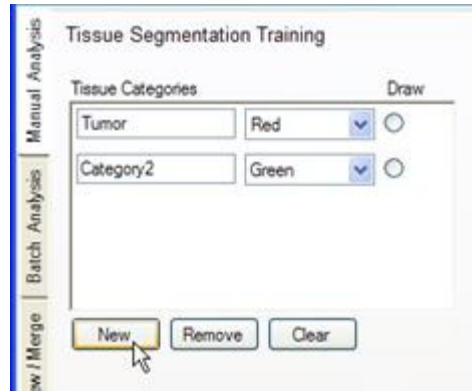


FIGURE 10.1. Tissue Segmentation Training panel.

3. Click New.
A placeholder with the default name of Category 1 is created for a new tissue segmentation training category, and a default color is assigned to the category. (See Figure 10.2.)

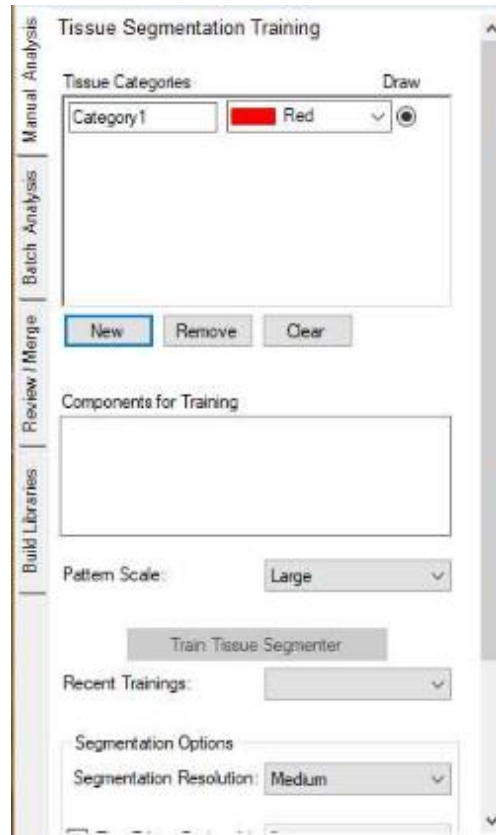


FIGURE 10.2. Tissue Segmentation Training panel with default category.

4. Change the default name of Category 1 to your preferred category name, and optionally, on the Color drop-down list, select a different color for the category.
5. Repeat Step 3 and Step 4 to create all the necessary tissue categories for the project. By default, every time you create a new tissue category, the Draw option is cleared for the previous category and selected for the new tissue category.

NOTE: If you add a tissue category in error, then to remove it, select the category, and then click Remove. To remove all categories in a single step, click Clear.

6. Continue to Drawing the Tissue Segmentation Training Regions.

10.2. DRAWING TISSUE SEGMENTATION TRAINING REGIONS

A training region teaches the tissue segmenter which cells should be included in a specific tissue category. When you are drawing a tissue segmentation training region, you must know what cells or other structures to look for and you must also be able to visually recognize structures that are characteristic of a tissue category. A training region must train on the full range of appearances of the tissue category. For example, if there are tumor cells present in any image that are negative (cells that are not stained), then you must be sure to include both the positive (stained) cells and the negative cells in the training region.

To draw tissue segmentation training regions on each image in a project:

1. Ensure that the opened project is in Single mode.
2. Click the image thumbnail.
3. In the Tissue Categories pane, click the Draw button for a tissue category.
4. Click and drag your mouse pointer around (encircle) the appropriate groups of cells, making sure to release the mouse button to connect the starting point and ending point of the selected tissue category region. For example, if you are drawing the region for a *Tumor* tissue category, then draw the regions around groups of tumor cells.

NOTE: Be sure to capture visual anomalies such as areas slightly out of focus, folds, bubbles, and so on. Also capture stroma regions that have differing qualities of shape or orientation.

5. Repeat Step 3 through Step 4 for each tissue category.
6. After you have drawn all the tissue segmentation training regions on all the project images, continue to the next section.

10.3. SELECTING TRAINING COMPONENTS

You select the training components for tissue segmentation in the Components for Training pane. (See Figure 10.3.)

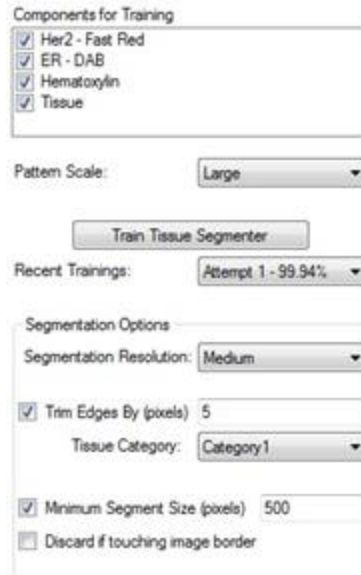


FIGURE 10.3. Components for Training pane.

10.3.1. Training Components Considerations

When you select the training components for the tissue segments, components that provide distinguishing pattern information that is independent of signals to be quantified are preferred. For example, because a counterstain such as DAPI or Hematoxylin 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, you should first try the counterstain component plane for training. For example, if measuring the positivity of cells that were 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.

Conversely, 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. Generally, you should also avoid using components that provide information that is unrelated to the preferred segmentation.

10.3.2. Pattern Scale

Pattern scale refers to the spatial extent of patterns that are measured to support machine learning and image analysis. The recommended approach is to start with the default *Large*

pattern scale. After you have drawn training regions and trained a tissue segmenter, if segmentation accuracy for the training regions is low (<85%), then increasing Pattern Scale might improve accuracy. However, the pattern scale must be compatible with the size of the training regions.

If you select a pattern scale (for example, *Maximum*) that is too large for the drawn training regions, then a gray mask is displayed on training regions that are too small after training and these 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, then reduce the pattern scale (for example, from *Large* to *Medium*), and then retrain. For instance, if you are analyzing tumors in tissue sections in a 20x magnification image, then the architecture is fundamentally on a large scale and you probably should select *Large*. For items that have 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 gray after training.

1. Select the components for training.
2. Select the pattern scale.
3. Click Train Tissue Segmenter.

NOTE: The training process may take some time, so be patient.

After the training is completed, the percent accuracy of the new tissue segmenter is displayed. If, for example, the segmenter was 99% accurate, this would mean that if you applied the new segmenter to all the images that were used in training the segmenter, the segmenter 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.)

4. Because inForm continues to try to improve the accuracy, click Stop 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 is displayed.
5. Click Done to close the Algorithm Training dialog box.

10.3.3. Recent Trainings

The Recent Trainings drop-down lists records a history of segmenters that were trained using the current segmentation settings. For example, the first time you train, the training 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 appropriate training attempt (the segmenter) on this drop-down list. If you change any settings, then the history of segmenters is cleared.

10.3.4. 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 the resolution reduction.

- To segment the images more quickly, select Coarse.
Although the edges of the segmented regions appear pixelated, the segmenter still accurately differentiates structures well, for example, tumor from stroma.
- If the segmented regions require smoother edges, such as images for use in publications, select Fine.

NOTE: Segmenting with Fine resolution takes longer than with Coarse resolution. Also, if you selected a small Pattern Scale, then the Resolution must be set to Medium or finer.

10.3.5. Segmenting the Tissue in the Training Images

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

10.3.6. 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 the region 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 "erodes" a tissue segmentation by removing the specified number of pixels from the periphery of the selected tissue category, making it smaller. This option is useful if the tissue segmenter tends to include pixels that extend beyond the edges of objects or tissue categories of interest (for example, tumor or vessels). For example, if you notice cancer regions that are extending into non-cancerous areas, then select Trim Edges option, select the cancer tissue category, and then enter the number of pixels that are to be trimmed 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 specified value. The tissue segment that is most abundant around the segment that was removed replaces the removed segment.

If Discard if Touching Image Border is selected, then the tissue regions that touch the edge of the image or process region are discarded. If Discard if Touching Image Border is not selected, then all tissue regions are included.

10.4. ADDING IMAGES TO TRAINING SET

When you select images for the training set, Akoya recommends 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 [Chapter 10.2. Drawing Tissue Segmentation Training Regions](#)), inForm automatically adds the image to the training set. You can also add or remove images from the training set manually.

To add images to the training set:

1. Click the Image List Editor button.



The Available Images window opens.

2. For each image that you are adding to the training set, select Training Set.
3. Close the Available Images window.
4. Optionally, to see which project images are in the Processing set and which images are in the Training set, or both, click the Single mode button.

When you switch the project view to Single mode, icons that are displayed on an image thumbnail indicate the sets to which an image belongs. If an image is in the Processing set, then a small blue square is displayed on the image thumbnail. If an image is in the Training set, then a small green square is displayed. (See Figure 10.4.)

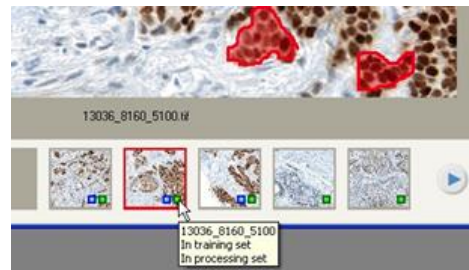


FIGURE 10.4. Processing set and Training set indicators.

5. To add training regions to selected images in the training set, see [Chapter 10.2. Drawing Tissue Segmentation Training Regions](#).

10.5. EDITING MASKS AND RE-TRAINING

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

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

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

To manually edit the tissue segmentation mask:

1. Click the Edit Tissue Segmentation button on the toolbar.
2. On the drop-down list, select the tissue segmentation mask that you are editing. (See Figure 10.5.)
3. Draw around the areas on the image that you are including in the selected Tissue Segmentation mask.

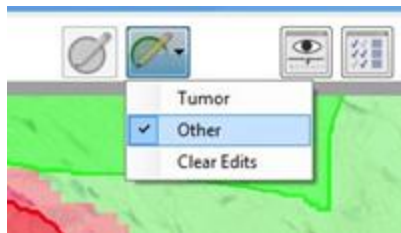


FIGURE 10.5. Editing Tissue Segmentation Masks.

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 carried out before you edited the mask, (see [Chapter 13. Segmenting Cells](#)), then in the Cell Segmentation editor, click the Segment Images button to re-segment the cells in the edited tissue segmentations.

To re-train the tissue segmenter:

1. In the Tissue Segmentation Training panel, select Draw for the tissue category for which you are drawing additional training regions.
2. Draw the training regions around the areas that were incorrectly classified.

3. Re-train the tissue segmenter and then re-segment the image.
4. Verify that the appropriate regions are now correctly classified in the preferred tissue segmentation mask.
5. To see the tissue segmentation on all images, segment all the images.

NOTE: Re-segmenting an image after re-training the tissue segmenter deletes any manual edits that were made to the tissue segmentation mask.

10.6. COMPLETING AUTOMATED TISSUE SEGMENTATION STEP

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

1. Process the images using one of the following methods:
 - a. To segment the currently selected image, click the Segment Image button.
 - b. To segment all the images that are opened in the project, click the Segment All button.
2. Click Advance.
The next step in the project is displayed.

3. Click the next step in the project in the Step Bar (see [Chapter 3. Understanding the inForm Work Area](#)) 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.

CHAPTER 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 for which you are creating a threshold map.
3. You have two options for specifying the preliminary values for the Threshold max and the Threshold:
 - a. To automatically calculate a preliminary value for the Threshold Max and then choose a preliminary Threshold value, click Auto.
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.
 - b. To manually set the values for the Threshold Max and the Threshold value, use the slider option, enter the preferred values, or click the Up/Down arrows.

CHAPTER 12. COLOCALIZATION

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

1. On the project Step bar, click the Colocalization button.



The Colocalization Settings pane opens. (See Figure 12.1.)



FIGURE 12.1. Colocalization Settings pane.

2. For Markers for Colocalization, select the markers for which to view overlap (colocalization) and positivity percentages.
3. For Denominator (Counterstain), select the markers that you are using as the counterstains. To see colocalization of markers throughout the entire image, and not just within a particular counterstain, select All Image Pixels.

For each component that you selected in the Markers or Denominator, a Threshold settings area displays below the Denominator options. A default color is displayed for each marker and counterstain.

4. Use any of the following options to adjust the values for Threshold Max and Threshold for each component:
 - a. To automatically calculate a preliminary value for the Threshold Max and then choose a preliminary Threshold value, click Auto.
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.
 - b. To manually set the values for the Threshold Max and the Threshold value, use the slider option, enter the preferred values, or click the Up/Down arrows.
5. Optionally, to change the default color for a marker or counterstain, click the default color that is displayed, and then select a different color.
6. To view the threshold for each component:
 - a. De-select Visible for all the components except for the component that you are adjusting.
 - b. De-select Visible for Colocalization.
 - c. Adjust the threshold value for the component until all the appropriate areas are marked as Positive.
 - d. If there are small areas that are marked as Positive that should not be included, or if smaller regions should be marked as Positive and are not, click More, and then adjust the value for Minimum Connected Pixels as appropriate.

CHAPTER 13. SEGMENTING CELLS

Section	Page
13.1. Adaptive Cell Segmentation	99
13.2. Cell Segmentation	109

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

inForm contains two approaches for segmenting cells:

- Adaptive Cell Segmentation uses an adaptive thresholding algorithm to identify nuclear pixels and can use both nuclear and membrane signals to guide nuclear splitting. It performs best on fluorescence imagery, especially imagery that contains membrane staining. (See [Chapter 13.1. Adaptive Cell Segmentation](#).)
- 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 [Chapter 13.2. Cell Segmentation](#).)

NOTE: Cells are an assembly of associated cellular components (nucleus, cytoplasm, and membrane). If you are segmenting tissue, some cells might straddle two segmented tissue regions. If a cell is not entirely contained within a single region, then the “containing region” is undefined, and the Region ID in the Data tables is #N/A. (See [Chapter 17. Displaying the Extracted Data](#).)

13.1. ADAPTIVE CELL SEGMENTATION

Adaptive Cell Segmentation uses 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 [Chapter 13.1.1. Selecting Segmentation Components](#).)
2. Detect nuclear pixels using image planes associated with one or more nuclear stains. (See [Chapter 13.1.1. Selecting Segmentation Components](#).)
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 [Chapter 13.1.2.1. Splitting Nuclear Clusters Using Nuclear Component Images](#).)
 - b. (Optional). Image planes associated with assisting cytoplasmic or membrane stains (splits occur at ridges of positive membrane or cytoplasmic signal). (See [Chapter 13.1.2.2. Splitting Nuclear Clusters Using Membrane or Cytoplasm Component Images](#).)
4. Refine nuclear shapes. (See [Chapter 13.1.3. Refining Nuclear Shapes](#).)
5. (Optional) Define cytoplasm and membrane around all nuclei. (See [Chapter 13.1.4. Defining Cytoplasm](#) and [Chapter 13.1.5. Defining Membrane](#).)

13.1.1. Selecting Segmentation Components and Detecting Nuclear Pixels

1. Choose which cellular compartments to segment: Nuclei, Cytoplasm, and/or Membrane. (See Figure 13.1.)



FIGURE 13.1. Segment options.

NOTE: *Adaptive Cell Segmentation can detect cells only with nuclear signal, so you cannot clear Nuclei. Although you can select Cytoplasm, and/or Membrane, we recommend that you wait to add Cytoplasm and/or Membrane options until you have optimized Nuclei detection and splitting settings. This reduces visual*

clutter in the image. Adding those two options later does not alter any parameter adjustments to Nuclei settings.

2. To add the image components that are to be used for segmentation, click Add, and then select the image component name on the drop-down list. (See Figure 13.2.)

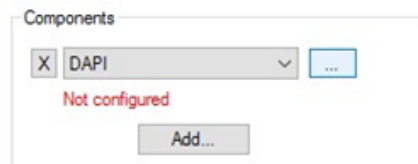


FIGURE 13.2. Adaptive Cell Segmentation components.

When adding the image components, 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. Add only those components that show strong, specific staining. Adding components with weak or non-specific 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.

NOTE: Priority is given to nuclear splitting lines that are attributed to image components higher up in the list of components. As a result, we recommend you add your strongest and most specifically stained components first in this list.

3. Click the “...” button to open a Component Configuration dialog box and then configure each component. (The options on the dialog box change depending on the location where the component is located.)
 - a. Select the compartment where the component is located.
 - b. For nuclear components, use the Typical Intensity slider to adjust the threshold used to detect nuclear pixels.
 - c. If a component belongs to the cytoplasm or membrane, select Use this signal to assist in nuclear splitting. When using this signal to assist in nuclear splitting is

selected, signals in the associated image plane are used to generate potential split lines within clusters of nuclei. We recommend selecting this option only for image components that have strong, specific signals. See dialog box examples below in Figure 13.3.

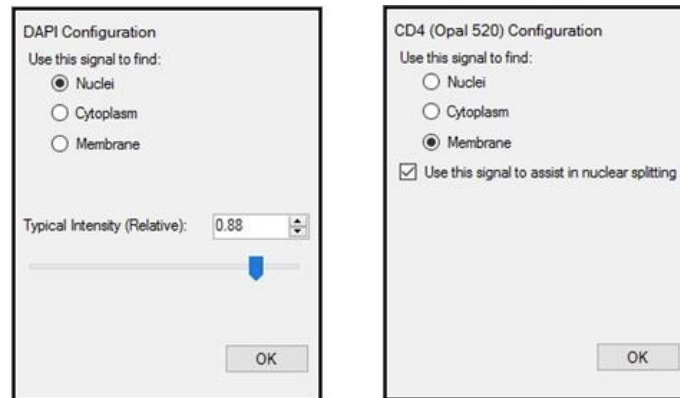
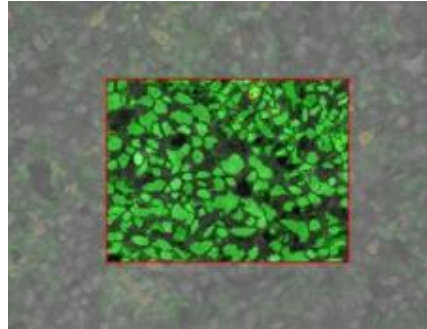


FIGURE 13.3. Component Configuration dialog box examples.

NOTE: For the Component Configuration dialog box on the left, the 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 is addressed in subsequent steps). Focus only on whether the correct pixels are being detected as nuclear.

NOTE: All nuclear component planes are used to assist nuclear splitting even when cytoplasmic or membrane components have been selected. We recommend you do not select this option until after you first optimize nuclear splitting for nuclear component planes. You might also want to assess the changes to the segmentation results after each signal is individually selected so the added value of each component is more obvious.

After at least one image component has been configured, a live preview region appears in the current image. This preview provides live feedback as you adjust settings such as the Typical Intensity. You can click-and-drag the region to preview other areas in the image. You can click-and-drag the image outside the preview region to drag the entire image. (See Figure 13.4.)



Hide Preview

FIGURE 13.4. Live Preview with Hide Preview option.

13.1.2. Splitting Nuclear Clusters into Individual Nuclei

Clusters of nuclear pixels are split into individual nuclei in one of two ways:

- From nuclear components.
- From image planes from assisting membrane and cytoplasm components.

13.1.2.1. Splitting Nuclear Clusters Using Nuclear Component Images

You use the options that are available in the Nuclear Component Splitting panel to adjust the splitting parameters that are used for image planes that are associated with the nuclear components. The settings in this panel are applied to all nuclear components. (See Figure 13.5.)

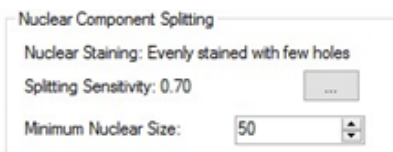


FIGURE 13.5. Nuclear Component Splitting panel.

1. Click the “...” button to open the Nuclear Splitting Settings dialog box. (See Figure 13.6.)

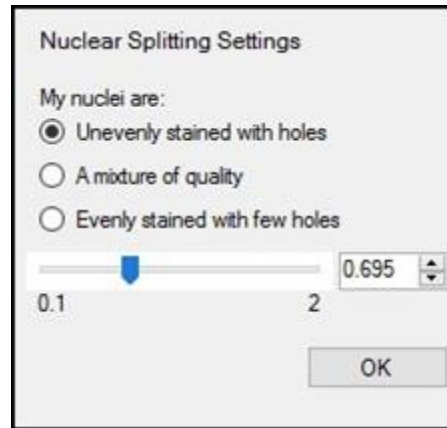


FIGURE 13.6. Nuclear Splitting Settings.

2. Configure the splitting sensitivity, making sure to select the option that best describes the staining quality of all the selected nuclear planes. (You might need to experiment to verify your selection.)
3. Adjust the splitting threshold, either by using the slider bar, manually enter the value, or using the Up/Down arrows.
4. Click OK to close the dialog box and return to the Nuclear Component Splitting pane.
5. Set the value for the Minimum Nuclear Size (in number of pixels). Splitting lines that generate nuclei smaller than this size are ignored. Any isolated nuclei that are smaller than this value are discarded.

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

13.1.2.2. Splitting Nuclear Clusters Using Membrane or Cytoplasm Component Images

You use the options that are available in the Assisting Component Setting panel to adjust the parameters for cytoplasmic images, membrane images, or both. This pane is visible only if you selected at least one assisting component. The settings in this panel are applied to all

cytoplasmic components and membrane components that were selected with the Use this signal to assist in nuclear splitting option. (See Figure 13.7.)

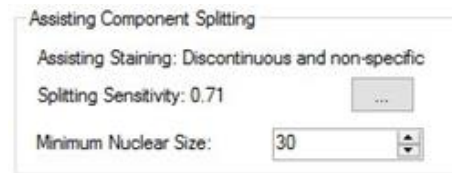


FIGURE 13.7. Assisting Component Splitting panel.

1. Click the “...” button to open the Assisting Component Splitting Settings dialog box. (See Figure 13.8.)

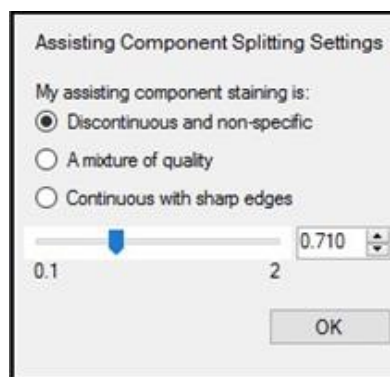


FIGURE 13.8. Assisting Component Splitting Settings dialog box.

2. Configure the splitting sensitivity, making sure to select the option that best describes the staining quality of the assisting membrane and cytoplasmic planes. (You might need to experiment to verify your selection.)
3. Adjust the splitting threshold, either by using the slider bar, manually enter the value, or using the Up/Down arrows.
4. Click OK to close the dialog box and return to the Nuclear Component Splitting panel.
5. Set the value for the Minimum Nuclear Size (in number of pixels).
The system ignores splitting lines that generate nuclei smaller than the indicated size.

If you are more confident in the splitting lines that the assisting components generated than the nuclear components generated, or, if the cells that have membrane signals tend to be smaller than those that do not, you can want to set this value to be smaller than the Minimum Nuclear Size that you specified in the Nuclear Component Splitting panel.

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

13.1.3. Refining Nuclear Shapes

After you have optimized the settings for detection of nuclei and the splitting of nuclei, optionally, you can use the settings in the Other Settings panel to further refine your results. (See Figure 13.9.)

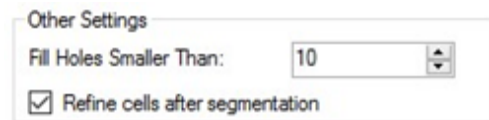


FIGURE 13.9. Other Settings panel.

You can adjust the value for one or both settings:

- If you find that some nuclei contain empty “holes,” then increase the value for the Fill Holes Smaller Than setting until all these empty holes are filled.
- If you find that the detected membrane shapes are more jagged or angular than you prefer, then select Refine cells after segmentation to compact the cells into rounder shapes.

NOTE: The following sections, 13.1.4 Defining Cytoplasm and 13.1.5 Defining Membrane, are presented as two independent topics with independent settings panes; however, if you selected both cytoplasm and membrane, then a single combined settings panel, Membrane and Cytoplasm Settings, is displayed. (See Figure 13.10.) You can set the values in this combined settings panel just as you would in the independent panes that are discussed in these sections.

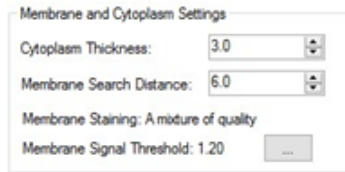


FIGURE 13.10. Membrane and Cytoplasm Settings panel.

13.1.4. Defining Cytoplasm

You use the option that is available in the Cytoplasm Settings panel to segment cytoplasm. (See Figure 13.11.)

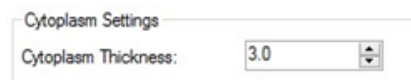


FIGURE 13.11. Cytoplasm Settings panel.

To define the distance, in pixels, from the boundary of each cell's nucleus to the outer boundary of its cytoplasm, adjust the value for Cytoplasm Thickness. For each cell, all pixels that are between the nuclear boundary and the cytoplasm outer boundary are defined as cytoplasm. You should adjust this distance based on the visual inspection of the results, so that cytoplasm segmentation approximately matches the average size of the cells.

NOTE: Cytoplasm segmentation does not account for any signals that are from cytoplasm component images.

13.1.5. Defining Membrane

You use the options that are available in the Membrane Settings panel to segment membrane. (See Figure 13.12.)

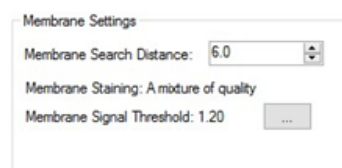


FIGURE 13.12. Membrane Settings panel.

1. To define the distance, in pixels, from the boundary of each cell's nucleus to search for membrane signals, adjust the value for the Membrane Search Distance.

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

- a. If positive signals are detected between the nucleus boundary and the Membrane Search Distance, then membrane is drawn where the positive signal is detected.
- b. If no positive signal is detected between the nucleus boundary and the Membrane Search Distance, then the membrane is drawn at the nearest of three different stop points:
 - Membrane Search Distance.
 - A neighboring membrane.
 - Cytoplasm Thickness (if cytoplasm segmentation was selected).

NOTE: If the membrane is drawn at positive membrane signals that are found at a distance between the Cytoplasm Thickness and the Membrane Search Distance, then the resulting cytoplasm spans the entire area between the nucleus and the membrane, even if this results in a cytoplasm that is thicker than the selected Cytoplasm Thickness value.

2. Click the “...” button to open the Membrane Settings dialog box. (See Figure 13.13.)

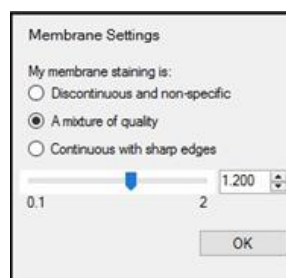


FIGURE 13.13. Membrane Settings dialog box.

3. Configure the sensitivity of the membrane signal detection, making sure to select the option that best describes the staining quality of the configured membrane components. (You might need to experiment to verify your selection.)
4. Adjust the membrane detection threshold, either by using the slider bar, manually enter the value, or using the Up/Down arrows. Lower values result in the detection of weaker signals.

NOTE: These settings are applied to all components that are configured as “membrane.”

5. Click OK to close the dialog box and return to the Membrane Settings pane.

13.2. CELL SEGMENTATION

You use the options that are available in the Cell Segmentation Settings panel to set the parameters for segmenting cells into their respective categories so that you can extract the cellular data and analyze the data on per-cell basis. (See Figure 13.14.)

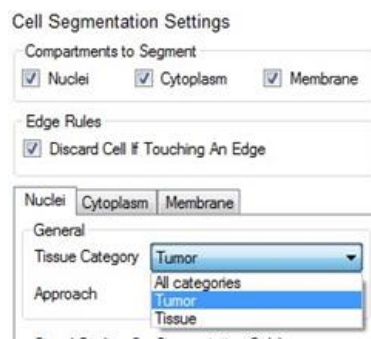


FIGURE 13.14. Cell Segmentation Settings panel.

To segment cells:

1. Select the cell components to segment: Nuclei, Cytoplasm, and Membrane. (Cytoplasm and Membrane are available for selection only after Nuclei is selected.)
2. To discard any cell that has a cell component that touches the edge of the image, or if applicable, a process region, or tissue region, select Discard Touching an Edge.

Only the cells that are contained entirely within the image, a process region (if applicable), and the selected tissue category (if applicable) are included in the segmentation map. If you do not select this option, then the 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, then the size of the nucleus or cytoplasm inside the region might be smaller than the specified Minimum Size.

3. Do one or more of the following, as appropriate:
 - a. If you selected Nuclei, then open the Nuclei tab and select the parameters. Use the Preview window to determine the optimal parameters for segmentation. See Segmenting Nuclei for a description of the available parameters.
 - b. If you selected Cytoplasm, then open the Cytoplasm tab and select the parameters. Use the Preview window to determine the optimal parameters for segmentation. See Segmenting Cytoplasm for descriptions of the parameters.
 - c. If you selected Membrane, then open the Membrane tab and select the parameters. Use the Preview window to determine the optimal parameters for segmentation. See Segmenting Membrane for a description of the parameters.
4. To segment the current image using the selected parameters, click the Segment Image button.
5. Review the segmentation of the current image, and then do one of the following:
 - a. If you are satisfied with the results, then click the Segment All button to segment all the project images.
 - b. If you are not satisfied with your results, then iteratively repeat Step 3 through Step 5 until you are satisfied with the results, then click the Segment All button.

13.2.1. Segmenting Nuclei

1. In the Compartments to Segment pane, select Nuclei.

2. Optionally, if you intend to find cytoplasm and membrane, then you can select Cytoplasm and Membrane also.
3. Open the Nuclei tab, and then select the values for the following options:
 - a. Tissue Category: The option to select a specific Tissue Category or all Tissue Categories in which to find the cell nuclei is displayed for segmenting nuclei only if tissue has been segmented, either manually or automatically. Examples of tissue categories that might of be interest when segmenting nuclei are tumor regions in oncology samples or islet cells in pancreatic samples. Cells that are outside the chosen category are ignored.
 - b. Approach: Select one of two options:
 - i. Pixel-Based (Threshold): As the name implies, this approach is purely pixel-based. Select this approach when there is a reliable nuclear counterstain, and nuclear pixels can be found by applying a simple threshold. You can also use the pixel-based 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.
 - ii. Object-Based: 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 required to detect nuclei.
4. Specify the signal scaling settings for the selected segmenting approach. (See Figure 13.15.)



FIGURE 13.15. Signal Scaling Settings for Segmentation.

- a. Auto Scale: If you select Auto Scale, then before inForm carries nuclei segmentation it automatically scales each nuclear counterstain component plane on an

- individual basis. Auto Scale is a good starting point for nuclei segmentation and it is useful if nuclear counterstain signals vary widely. Also, Auto Scale is sufficient for fluorescence applications.
- b. Fixed Scale: If you require better nuclear segmentation performance and counterstain signals are consistent and reliable then select Fixed Scale. (Counts is the unit for Fixed Scale signal scaling.) If you select Fixed Scale, you must manually enter a scaling value. To determine the value that you should enter, use the View Component Data tool and then move your mouse pointer over the brightest nuclei in the image to see the signal levels of the component that is being used for nuclear segmentation. Every object that is in a nuclear segmentation, which is indicated by a green region, should be nuclei.
 - i. If the nuclear segmentations are smaller than the nuclei, or if no nuclear segmentations occur when the segmenter is finished, then reduce the value for the maximum OD or Counts.
 - ii. If the nuclear segmentation is too sensitive and it is finding many objects that weakly stained and are not nuclei, then increase the scale until the unwanted regions are not detected.
5. Optionally, to further add to what you consider to be nuclear pixels, in the Components for Nuclear Segmentation pane, select other components in the underlying signal. See [Chapter 13.2.1.1. Selecting Additional Components in the Underlying Signal](#).
 6. In the Size Range pane, set the value (in pixels) for the minimum size for the segmented nuclei. (See Figure 13.16.)



FIGURE 13.16. Size Range pane for nuclear segmentation.

Note the following when you set the value for Minimum Size:

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

Note the following when you set the value for Maximum Size:

- a. A value for maximum size sets an upper limit on nuclear segmentations (in number of pixels). Nuclear segmentations that are larger than this value are ignored. Setting a value for the nuclei maximum size can be useful for ignoring large clusters of nuclei, or if there are other objects in the classified region of interest that are not nuclei.
 - b. If you intend to select the Refine Splitting clean-up discussed in the next step (Step 8), then you must set a value for the maximum size.
8. If your initial segmentation results are not as expected or satisfactory, then optionally, you can specify values for specific “Clean-up” parameters. (See Figure 13.17.)



FIGURE 13.17. Clean-up parameters for nuclei segmentation.

- a. **Fill Holes:** A common effect in tissue sections is to have some of the nuclei in the image segmented with “holes” in them. To counter this effect and have all holes of

all sizes in all nuclei automatically filled, select Fill Holes. To limit the filling of nuclei holes to only those holes that are smaller than a specified size, select Max. Hole Size, and then enter the value for the maximum hole size in pixels. Nuclei with holes that are larger than this value are not filled.

- b. **Refine Splitting:** Select Refine Splitting to have nuclei that are touching each other split into separate nuclei instead of being detected as one large nucleus. Be aware, however, that if you select Refine Splitting, cell segmentation can take considerably longer than without Refine Splitting selected.
- c. **Roundness: Minimum Circularity:** To find only nuclei of a defined roundness, select Roundness: Minimum Circularity, and then enter a value. The allowed values are decimal values that fall within the range of 0-1, inclusive, where a value of 0 returns any shape, and the maximum value of 1 restricts selections to the most round objects that are found in the image.

13.2.1.1. Selecting Additional Components in the Underlying Signal

Optionally, when segmenting nuclei, you can further add to what you consider to be nuclear pixels and select other components in the underlying signal. (See Figure 13.18.)

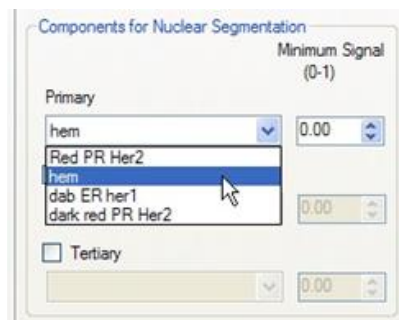


FIGURE 13.18. Components for Nuclear Segmentation pane.

1. At a minimum, select an individual component as the Primary signal, and then enter a minimum signal value for the component.
2. Optionally, select up to two additional components, one as the Secondary component and one as the Tertiary component, and then enter a minimum signal value for each component.

Note the following when you are specifying the signal value for each additional component:

- a. In Auto Scale mode, the signal range for or each component is 0 to 1. If you selected Fixed Scale, then 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, if you switch between Auto Scale and Fixed Scale, then the values are reset to zero.
- b. Only pixels that are above the minimum signal value are counted as nuclei. Adding secondary and tertiary components is primarily useful in brightfield IHC applications where nuclear IHC stains are dark or 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.
- c. After you have selected at least one component, and the selected segmentation settings are valid, a live preview is displayed in the current image. This preview provides live feedback as you adjust settings. Optionally, you can:
 - i. Click-and-drag the region to preview other areas in the image.
 - ii. Click-and-drag the image outside the preview region to drag the entire image.
 - iii. Click Hide Preview to hide the preview. Click Show Preview to show the preview again. (See Figure 13.19.)

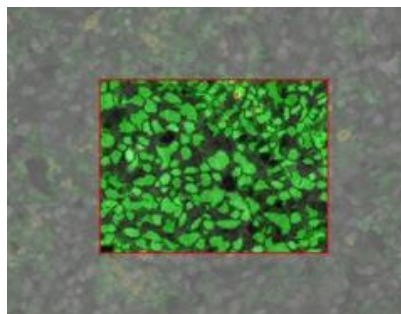


FIGURE 13.19. Live Preview image.

13.2.2. Segmenting Cytoplasm

Because inForm searches for cytoplasm only where nuclei have been found, you must segment the nuclei in addition to segmenting cytoplasm.

1. In the Compartments to Segment pane, confirm that Nuclei is selected, and then select Cytoplasm.
2. Open the Cytoplasm tab (see Figure 13.20), and then select the values for the following options:

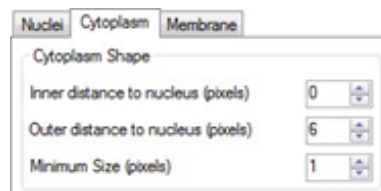
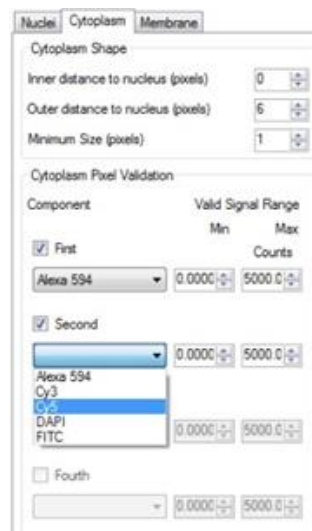


FIGURE 13.20. Cell Segmentation Settings panel, Cytoplasm tab.

- a. Inner distance to nucleus (pixels): The Inner distance to nucleus is the distance, in pixels, that is from the edge of nuclear segmentations to the inner edge of the cytoplasm annular regions that are 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.
- b. Outer distance to nucleus (pixels): The Outer distance to nucleus is the distance, in pixels, that is 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 preferred.
- c. Minimum Size (pixels): The Minimum Size is the minimum cytoplasm sample size, in pixels. If cytoplasm segmentations for detected nuclei have fewer pixels than this number, then these cells are excluded from the analysis. This situation 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, then 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.
3. Optionally, when segmenting cytoplasm, you can further add to what you consider to be “valid” cytoplasm and select other components in the underlying signal. You have three options:
 - a. At a minimum, select an individual component as the First signal, and then enter a minimum signal value for the component, and then optionally, you can select up to three additional components (Second, Third, and Fourth), and then enter a minimum signal value for each component.
 - b. Select Every Component, and then enter a signal value.
 - c. Select Any Component, and then enter a signal value. (See Figure 13.21.)



The screenshot shows the 'Cytoplasm Pixel Validation' dialog box. It has three tabs: 'Nuclei', 'Cytoplasm', and 'Membrane'. The 'Cytoplasm' tab is active. Under 'Cytoplasm Shape', there are three input fields: 'Inner distance to nucleus (pixels)' with value 0, 'Outer distance to nucleus (pixels)' with value 6, and 'Minimum Size (pixels)' with value 1. Under 'Cytoplasm Pixel Validation', there is a table with columns 'Component', 'Valid Signal Range', 'Min', and 'Max'. The 'Valid Signal Range' column is further divided into 'Min' and 'Max' sub-columns. The 'Component' column has a dropdown menu. The 'Min' and 'Max' columns have input fields with values 0.0000 and 5000.0 respectively. The 'Component' column has four rows: 'First' (checked), 'Second' (checked), 'Third' (unchecked), and 'Fourth' (unchecked). The 'First' component is 'Alexa 594'. The 'Second' component dropdown is open, showing a list of components: 'Alexa 594', 'Cy3', 'Cy5', 'DAPI', and 'FITC'. The 'Third' component is 'DAPI' and the 'Fourth' component is 'FITC'.

Component	Valid Signal Range	
	Min	Max
<input checked="" type="checkbox"/> First		
Alexa 594	0.0000	5000.0
<input checked="" type="checkbox"/> Second		
Alexa 594	0.0000	5000.0
Cy3		
Cy5		
DAPI	0.0000	5000.0
FITC		
<input type="checkbox"/> Fourth		
	0.0000	5000.0

FIGURE 13.21. Other cytoplasm signal components.

Only those pixels that fall within the given signal range are counted as cytoplasm, where the signal range varies based on the image type.

13.2.3. Segmenting Membrane

Because inForm searches for cytoplasm only where nuclei have been found, you must segment the nuclei in addition to segmenting membrane.

1. In the Compartments to Segment pane, confirm that Nuclei is selected, and then select Membrane.
2. Open the Membrane tab. (See Figure 3.22.)

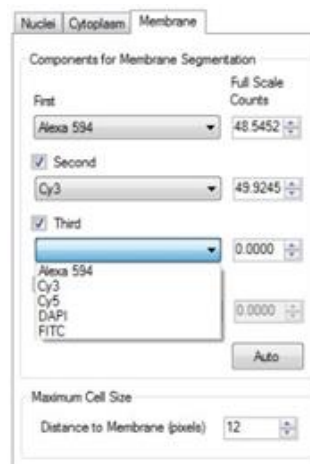


FIGURE 13.22. Cell Segmentation Settings panel, Membrane tab.

3. Select the First component signal for membrane segmentation.
4. Optionally, select up to three additional component signals for membrane segmentation: Second, Third, and Fourth.
5. For each component, do the following to set the Full Scale:
 - a. Select an image that is typical of all the images that are to be analyzed.
 - b. Click Auto to find the brightest pixels in the image for each of the selected components.

The brightest value for each component is displayed in the associated Full Scale field. The units can be Counts or OD.

In the Maximum Cell Size pane, specify the value for Distance to Membrane (pixels), where 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.

The value that you specify here limits how far from the nucleus the algorithm is permitted to go when segmenting the cell 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 too large, then try reducing the value. Conversely, if the algorithm is finding a membrane too small, then try increasing the value.

CHAPTER 14. PHENOTYPING CELLS

Section	Page
14.1. Adding, Deleting, or Renaming Phenotyping Schema	120
14.2. Adding and Deleting Phenotype Classifiers	122
14.3. Training a Phenotype Classifier	124

NOTE: Before you can phenotype cells, you must have first carried out accurate cell segmentation.

After you have carried out cell segmentation, you can proceed with phenotyping of the cells in an image. Just like the Tissue Segmentation feature, inForm's phenotyping feature is trainable.

14.1. ADDING, DELETING, OR RENAMING A PHENOTYPING SCHEMA

1. Open a project in inForm.
2. Configure the project to include the Phenotyping step.
Carry out accurate cell segmentation.

3. Advance to the Phenotyping step.

The Phenotyping Settings panel opens on the Manual Analysis tab. inForm creates an Unnamed Phenotyping Schema on the Phenotyping Schema drop-down list with a Standard Associated View. Two default phenotypes are also created within the phenotyping schema: "a" with a default color of Red and "b" with a default color of Green. (See Figure 14.1.)

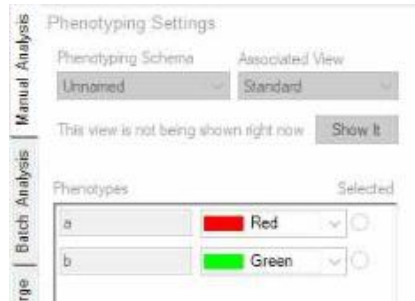


FIGURE 14.1. Default Phenotyping Schema placeholder.

4. On the Phenotyping Schema drop-down list, select Edit. (See Figure 14.2.)



FIGURE 14.2. Phenotyping Schema for an inForm project with two Phenotyping classifiers and the Edit button.

The Edit Phenotype Schema dialog box opens. The first time that this dialog box opens, the dialog box displays only the Unnamed Phenotyping Schema. (See Figure 14.3.) Going forward, it displays the all the Phenotyping Schemas that have been added for the project. You use the options in this dialog box to add, delete, or rename a phenotyping schema.

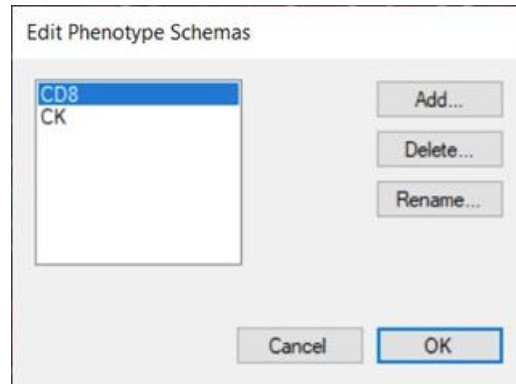


FIGURE 14.3. Edit Phenotype Schemas dialog box.

5. Do any of the following options:
 - a. To add your first phenotyping schema to a project, select the unnamed phenotyping schema, and then click Rename. Enter the new name for the phenotyping schema, and then click OK. (You can also use the Rename option to rename any existing Phenotyping Schemas, not just the default Unnamed schema.)
 - b. To add more phenotyping schemas to a project, click Add. Enter the name for the new phenotyping schema, and then click OK.*
 - c. To delete a phenotyping schema, select the schema that you are deleting in the Phenotyping Schema pane, and then click Delete. Click OK to confirm the deletion.

NOTE: You cannot click *Phenotype All* to apply a trained classifier to all images in a project unless you have first trained classifiers for all existing Phenotyping Schemas. As a result, we recommend that you successfully train the classifiers for your first schema before you add more schemas. See [Chapter 14.3. Training a Phenotyping Classifier](#).

14.2. ADDING AND DELETING PHENOTYPE CLASSIFIERS

You can add multiple classifiers to a Phenotyping Schema, you can delete individual classifiers from a phenotyping schema, and you can delete all classifiers from a Phenotyping Schema in

a single step. You carry out these actions in the Phenotyping Settings panel on the Manual Analysis tab.

To add a classifier to a Phenotyping Schema:

1. On the Phenotyping Schemas drop-down list, select the schema to which you are adding the classifiers.
2. Click Add.
The Create New Phenotypes dialog box opens.
3. Enter the name for the new phenotype, and then select its color.
4. Click OK.

The Create New Phenotype dialog box closes. The newly added phenotype and its color are displayed in the Phenotypes pane. (See Figure 14.4.)

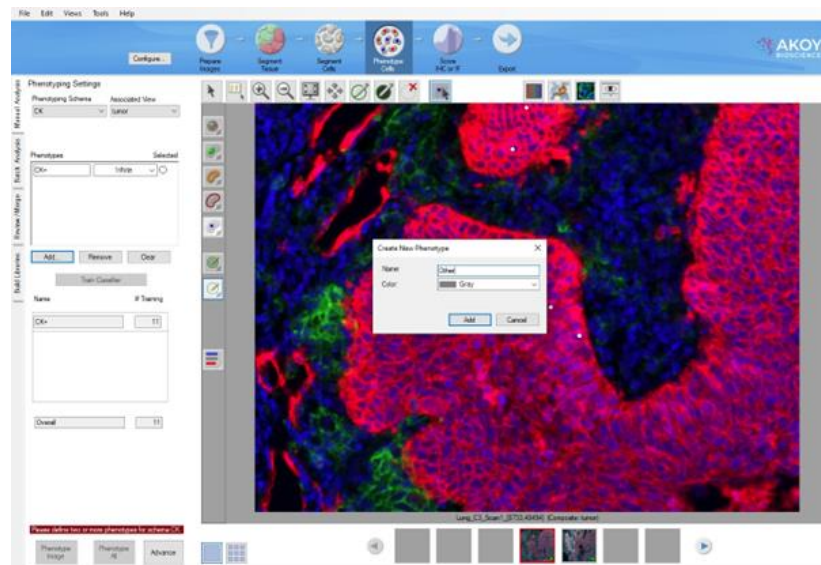


FIGURE 14.4. Phenotyping step in a project. A new phenotype is being added to a Phenotyping Schema named CK. The new phenotype is named Other and has been assigned the color gray.

After you have added a Phenotyping Schema, note the following:

When you select a Phenotyping Schema, an Associated view is automatically selected for the schema. You can select an available Composite View from the Associated View drop-down list.

If you switch to a different view, then inForm alerts you. You can click Show It to quickly return to the Associated view. (See Figure 14.5.)

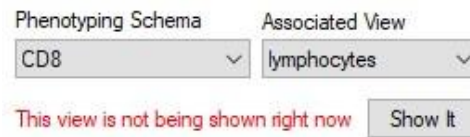


FIGURE 14.5. Switching to a view other than the Associated View for a Phenotyping schema.

NOTE: *Only those channels that are included in the Associated View are used to train the classifiers for a Phenotyping Schema. See [Chapter 14.3. Training a Phenotyping Classifier](#).*

To delete a classifier from a Phenotyping Schema:

1. On the Phenotyping Schemas drop-down list, select the schema from which you are deleting the classifier.
2. Click the Select option that is displayed to the right of the classifier that you are deleting.
3. Click Remove.

To delete all classifiers from a Phenotyping Schema:

1. On the Phenotyping Schemas drop-down list, select the schema from which you are deleting all the classifier.
2. Click Clear.

14.3. TRAINING A PHENOTYPING CLASSIFIER

When you are carrying out the Phenotyping step in an inForm project, you can train multiple classifiers. Each classifier is described by a Phenotyping Schema, which includes a list of possible phenotypes and an Associated View. You can analyze a maximum of 50 channels in inForm.

Note the following caveats when you are training a phenotyping classifier:

- You can choose cells across multiple images if, for example, you cannot find many cells of a particular phenotype in one image. Ideally, 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 a classifier, then you must start training again.
- The first time you train a classifier, the training step might take longer than subsequent steps because the features that inForm has been trained to recognize are cached in memory for use in training steps subsequent steps.
- You might find that turning off the visualization of the nuclei allows you to better select the cell phenotypes that you recognize.
- You can also toggle back and forth between the different viewing modes to help identify a cell phenotype.
- To improve phenotyping accuracy, Akoya Biosciences recommends assigning multiple classifiers when you are phenotyping cells that are identifiable as “double positive” or “triple positive,” such as CD3+/CD8+/PD-1+.

To train a phenotyping classifier:

1. Open the inForm project.
2. Ensure initially that the project is in Single mode.
3. Select the training image.
4. Ensure that the correct Phenotyping Schema is displayed on the Phenotyping Schema drop-down list in the Phenotyping Settings panel.
5. Click the Edit Phenotypes button.



The phenotypes are now available to assign to the cells in the image.

- Click a cell in the image to open a drop-down list of all the available phenotypes for the selected Phenotyping Schema, and then select the phenotype for the cell. (See Figure 14.5.)

NOTE: You need to identify at least 5 cells in each phenotype. For optimal results, Akoya Biosciences recommends choosing 25 or more cells of each phenotype. You might start with a small number of cells, and then add mis-classified cells to the training set by correcting their phenotypes.

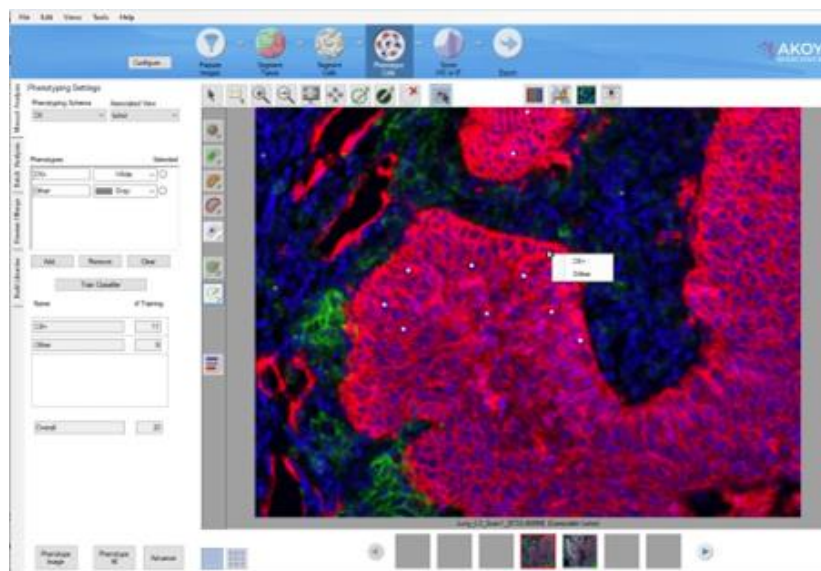


FIGURE 14.5. Selecting a phenotype for given cell.

- Repeat Step 6 for as many cells in the image as is appropriate.
- After you are finished with selecting the training cells for a given Phenotyping Schema, click Train Classifier.
After the classification process is complete, the following information is available for the training image:

- a. A Training table opens at the bottom of the Phenotyping Settings panel. The table lists each phenotype that you selected for training purposes, the number of cells of each phenotype that you selected for training purposes, and the total number of cells that you have selected for training purposes.
- b. You can hold your mouse pointer over any particular cell in the training image to open a tooltip that displays the phenotype that inForm assigned to the cell as well as the confidence level in the classification. An entry is displayed for each Phenotyping Schema with a trained classifier.

NOTE: You can also view the phenotype and confidence data for each cell in the training image in the Cell Segmentation Data table. (See Figure 14.6.)

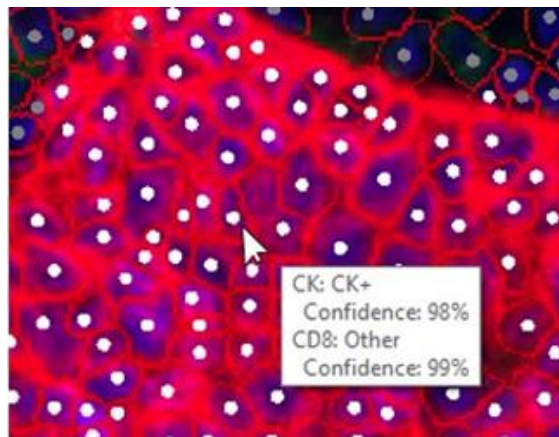


FIGURE 14.6. Viewing the training data for a classifier.

- c. You can view the parameters for classification in the inForm log file. (To access the log file, on the inForm main menu, click Help > View Log.) inForm logs the parameters after training and you can view these parameters in the Log file that is date-stamped with the training date. The log shows the normalized non-zero coefficients that were used in the linear classifier for each phenotype. Normalized coefficients are scaled by the standard deviation of the associated feature, which results in all the coefficients being put on the same scale and allows for the comparison of 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](#) for a detailed

description of the phenotyping algorithm and the parameters that are contained in the log file.

14.3.1. Editing the Training Results

You can correct errors in classification and you can remove cells from a training set. You can also add correctly classified cells to a training set. After you make any edits to a completed training set, the Training table display is automatically updated to reflect the new training information.

To correct errors in classification:

1. Ensure that the correct training image is opened in Single mode in the Image Display area.
2. Click the Edit Phenotypes button.
3. In the training image, click the mis-classified cell, and then on the drop-down list that opens, select the correct phenotype for the cell. inForm then re-classifies the cell at a 100% confidence for the selected phenotype and adds the cell to the training set.

To remove classified cells from a training set:

1. Ensure that the correct training image is opened in Single mode in the Image Display area.
2. Click the Edit Phenotypes button.
3. In the training image, click the appropriate cell, and then on the drop-down list that opens, select Remove from training. (See Figure 14.7.)



FIGURE 14.7. Reclassifying the phenotype for a training cell.

To add correctly classified cells to a training set:

1. Ensure that the correct training image is opened in Single mode in the Image Display area.
2. Click the Edit Phenotypes button.
3. In the training image, click the appropriate cell (a cell that was *not* used to train the classifier), and then on the drop-down list that opens, select the correct phenotype for the cell.

CHAPTER 15. SCORING IHC OR IF

Section	Page
15.1. Scoring Types	132

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. A Histogram scoring type is used to assess the intensity of each label. Five histogram scoring types are available. When you score images, the results are displayed at the bottom of the Score IHC or IF Settings panel. (See Figure 15.1.)



FIGURE 15.1. Score IHC or IF Settings panel example.

The table below describes the options that are available in the Score IHC or IF Settings panel, regardless of the selected Scoring Type. Other panel options that are displayed depend on the selected Scoring Type and are defined in the Scoring Type section.

TABLE 15.1 Options available in Score IHC or IF Settings panel

Option	Description
Tissue Category	This option is displayed only if tissue has been segmented and only those tissue categories in which cells have been segmented are available. Select the tissue category to use for scoring. Cells that are outside the chosen tissue category are ignored.
Scoring Type	A Histogram scoring type that is used to assess the intensity of each label. Five histogram scoring types are available. See Scoring Types for a description of the Histogram scoring types.
Compartment	Select the compartment to score. You can select Nuclei, Cytoplasm, or Membrane. Optionally, you can score the nuclei, and then score the cytoplasm and the membrane together, or score the cytoplasm and the membrane separately. The scoring data results from the binning of a histogram of all pixels within the selected compartment.
Marker Settings: Some Scoring Types have only a single marker while others have multiple markers. Regardless, all markers have the same three options detailed below.	
Component	Select the component to use for calculating the scoring data.
Threshold Max	Click Auto to calculate the value for the Threshold Max for the selected component. The Threshold Max specifies the maximum value allowed for the Threshold Slider. If necessary, use the slider to adjust the value (increase or decrease). For OD converted images, the maximum possible value is 3.0.
Positivity Threshold	The Positivity Threshold specifies the threshold values for the Scoring Type. You can drag the slider, manually enter a value in the Positivity Threshold field, or click the Up/Down arrows to set the values. See Scoring Types for a description of each threshold.
Score Results (Percent)	
Show Score Colors	If you select Show Score Colors, then the score colors are shown on the image and around the Negativity/Positivity values. For an example with the negativity value is outlined in blue and positivity value is outlined in red, see Figure 15.1.
Change Color	Click Change Color to open the Change Color dialog box, and then select the colors for each Histogram bin or restore the default colors for the bins.
Histogram Score results	Different score results are displayed depending on the selected Scoring Type.

15.1. SCORING TYPES

Five histogram scoring types are available for scoring IHC or IF images: Positivity, Double Positivity (2x2 bin), D 0-3 + (4-bin), Ten-bin, and Fifty-bin.

15.1.1. Positivity

The Positivity Scoring Type divides the spectrally unmixed nuclear signals, cytoplasm signals, or membrane signals into two bins, negative and positive, with a single threshold. The data is provided in percentages. If you decrease the Positivity Threshold, then the percent positivity increases. The Histogram Score results that are displayed at the bottom of the IHC and IF Settings panel show the negativity and positivity percentages of the cell nuclei, cytoplasm, or membrane. (See Figure 15.2.)



FIGURE 15.2. Score IHC or IF Settings panel, Positivity Scoring Type.

15.1.2. Double Positivity (2 x 2 bin)

Select the Double Positivity (2 x 2) bin 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. (See Figure 15.3.)

Score IHC or IF Settings (research use only)

Tissue Category:

Scoring:

First Marker Settings

Compartment: Nuclei Cyto Membrane

Component:

Threshold Max:

Positivity (Optical Density)

0.0 0.2

Negativity **36.77 %** Positivity **63.23 %**

Second Marker Settings

Compartment: Nuclei Cyto Membrane

Component:

Threshold Max:

Positivity (Optical Density)

0.0 0.2

Negativity **100.00 %** Positivity **0.00 %**

Histogram Results (Percent)

Show Score Colors

Single Positive 1	Double Positive
63.23 %	0.00 %
Double Negative	Single Positive 2
36.77 %	0.00 %

FIGURE 15.3. Score IHC or IF Settings panel, Double Positivity (2 x 2 bin) Scoring Type.

Double Positivity contains the same options as Positivity as well as the addition of Second Marker settings. Just as you did with the First Marker settings, you must select the Compartment, Component, Threshold Max, and Positivity Threshold settings for the Second Marker.

- For the First Marker, the Single Positive 1 color indicates the positive regions. The Double Negative color indicates the negative regions.
- For the Second Marker, the Single Positive 2 color indicates the positive regions. The Double Negative color indicates the negative regions.
- The Double Positive color indicates the double positive (First Marker + Second Marker) regions.

15.1.3. D 0-3 + (4-bin)

The 0-3 + (4-bin) Scoring Type divides the spectrally unmixed signals in nuclei, membrane, or cytoplasm into four bins. Select this Scoring Type to calculate H scores with nuclear stains.

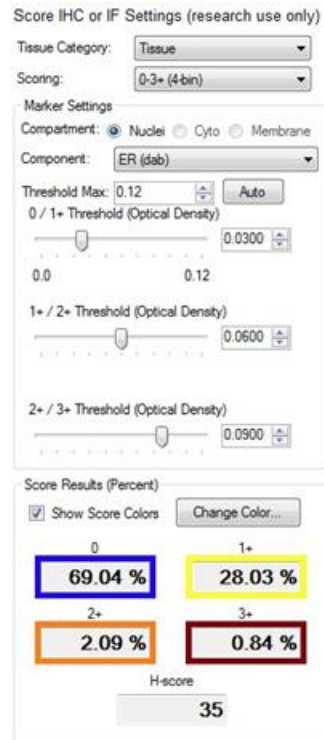


FIGURE 15.4. Score IHC or IF Settings panel, 0-3 + (4-bin) Scoring Type.

- 0/1+ Threshold: Cellular objects that fall below the 0/1 threshold are placed in the 0 bin.
- 1+/2+ Threshold: Cellular objects that are above the 0/1+ threshold but fall below the 1+/2+ threshold are placed in the 1+ bin.
- 2+/3+ Threshold: Cellular objects that are above the 1+/2+ threshold but fall below the 2+/3+ threshold are placed in the 2+ bin. Cellular objects that are above the 2+/3+ bin are placed in the 3+ bin.

The Histogram Score results show the percentage positivity of the cell nuclei, membrane, or cytoplasm within each bin. The H-score is calculated using the percentages that are found in each bin. The H-Score ranges from a value of 0 to 300. (See Figure 15.4.)

15.1.4. Ten-bin

The Ten-bin Scoring Type divides the data into ten bins for more quantitative work. The 10th Bin Nominal Level slider specifies the lower threshold for the ninth bin lower. The other eight thresholds are equally spaced between zero and the ninth threshold. The Histogram Score results boxes display the percentages of pixels that fall into each bin as determined by the position of the 10h Bin Nominal Level slider. (See Figure. 15.5.)

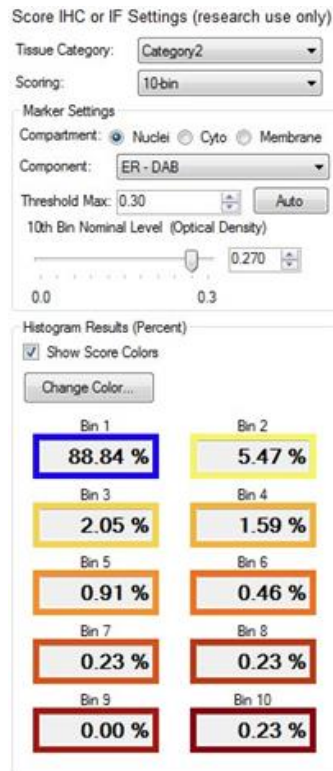


FIGURE 15.5. Score IHC or IF Settings panel, Ten-bin Scoring Type.

15.1.5. Fifty-bin

The Fifty-bin Scoring Type divides the 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 0 and the 50th bin threshold. For the Histogram Score results, display, the 50 values are summed into 10 values, with each value being the sum of 5 bins. The actual values for all 50 individual bins are displayed in the Score table. (See Figure 15.6.)

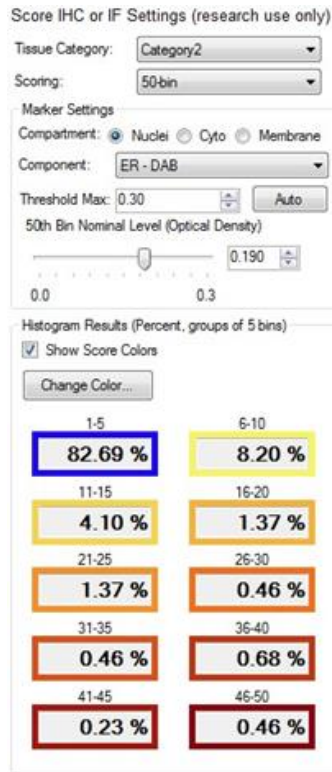


FIGURE 15.6. Score IHC or IF Settings panel, Fifty-bin Scoring Type.

CHAPTER 16. COUNTING OBJECTS

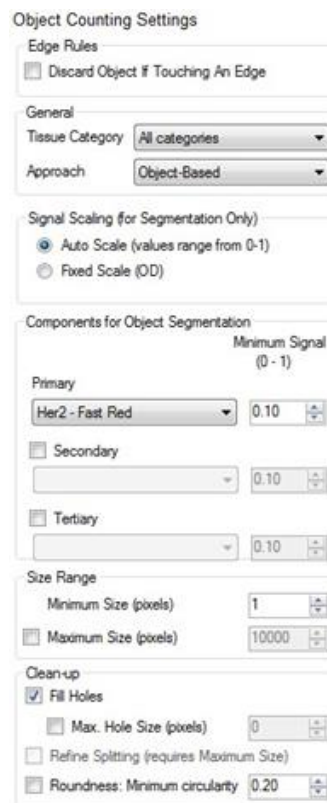
The Count Object function locates individual, irregularly shaped objects within an image field or, if the tissue has been segmented, within a selected tissue category.

To count objects:

1. On the Step bar, click the Counting Objects button.



The Object Counting Settings panel opens on the Manual Analysis tab. The Tissue Category option is displayed in the panel only if the tissue has been manually or automatically segmented. (See Figure 16.1.)



Object Counting Settings

Edge Rules

Discard Object If Touching An Edge

General

Tissue Category: All categories

Approach: Object-Based

Signal Scaling (for Segmentation Only)

Auto Scale (values range from 0-1)

Fixed Scale (OD)

Components for Object Segmentation

Minimum Signal (0 - 1)

Primary: Her2 - Fast Red 0.10

Secondary: 0.10

Tertiary: 0.10

Size Range

Minimum Size (pixels): 1

Maximum Size (pixels): 10000

Clean-up

Fill Holes

Max. Hole Size (pixels): 0

Refine Splitting (requires Maximum Size)

Roundness: Minimum circularity 0.20

FIGURE 16.1. Object Counting Settings panel.

2. Optionally, to exclude any objects that are touching the edge of the image, process region, or tissue region, select Discard Object If Touching an Edge.
3. If the tissue has been segmented, then select the category of tissue in which to find the objects. (Objects that are found outside the selected tissue category are ignored.)
4. Select the approach to use to identify objects: Object-Based or Pixel-Based (Threshold).
 - a. Select Object-Based if the stain does not provide consistent and specific staining of objects, and more advanced morphometry-based approaches are required.
 - b. Select Pixel-Based (Threshold) if the stain is reliable or consistent and object pixels can be found by applying a simple threshold. (As the name implies, this approach is purely pixel-based.)
5. Select the signal scaling method.
 - a. Auto Scale: The Auto Scale method automatically scales each component plane individually before carrying out segmentation.
 - b. Fixed Scale: The Fixed Scale method provides for better segmentation performance if the stain signals are reliable and consistent.
6. On the Primary component drop-down list, select the primary component for object segmentation.
7. Adjust the value Minimum Signal value for the primary component as appropriate.
8. Optionally, do any or all of the following:
 - a. Select Secondary and Tertiary components, and then set the appropriate minimum signal values.
 - b. To exclude objects that are smaller than a specified number of pixels, increase the value for the Minimum Size (pixels) option. (The default value is one.)

- c. By default, Fill Holes is selected, which results in all holes in all objects being automatically filled, regardless of the size of the hole. To limit the size of holes in objects that are automatically filled, leave Fill Holes selected and then select Max. Hole Size (pixels) and specify the size of the largest holes that are to be filled. If you clear Fill Holes, then no holes of any size in object are filled.
- d. To detect objects that touch other objects as individual objects, instead of as one single object, select Refine Splitting.

NOTE: This option is enabled only if Max. Hole Size (pixels) is selected and a legitimate value provided.

- e. To exclude objects based on the roundness of the object, select Roundness, and then specify the value for Minimum Circularity. The allowed value ranges from 0-1, inclusive, with 0 returning any object shape and the maximum value of 1 restricting object selection to only the most round objects identified.
9. After processing is complete, use the View Editor to view the resulting Count data table. You can also advance to the Export step and export the Count data table.

CHAPTER 17. DISPLAYING EXTRACTED DATA

Section	Page
17.1. Viewing the Color Image	141
17.2. Viewing the Component Image	142
17.3. Viewing the Composite Image	146
17.4. Viewing Data Tables	149
17.5. Viewing the Tissue Segmentation Table	149
17.6. Viewing the Cell Segmentation Table	151
17.7. Viewing the Score Data Table	155
17.8. Viewing the Colocalization Data Table	157
17.9. Viewing the Count Data Table	159
17.10. Viewing the Quant Data Table	161
17.11. Image Options	163
17.12. Component Stats	167
17.13. TMA Core Info	167
17.14. Slide Info	168
17.15. Position Stats	168
17.16. Shape Stats	169

After you analyze your project images, you can use the options in the View Editor to customize the view of the images and extracted data. (See [Chapter 3.1. View Editor](#).) The options that are available in the View Editor do not change the underlying images, they only change how the images are displayed.

NOTE: *This chapter discusses all the options that are available in the Data Displayed list. The options that are available to you depend on the software configuration that is installed and which steps you included in your project.*

17.1. VIEWING THE COLOR IMAGE

Selected option: Color Image. (See Figure 17.1.)

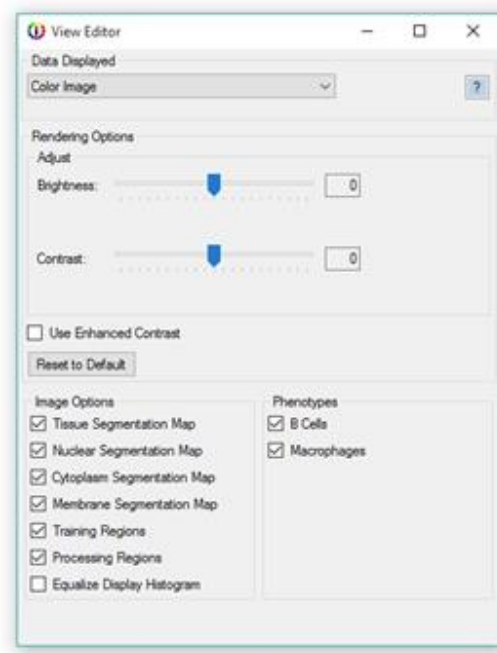


FIGURE 17.1. View Editor with Color Image options.

TABLE 17.1. Rendering Options for Color Image and descriptions

Option	Description
Rendering Options	
Brightness	Manually enter a value to change the brightness of the image. Use the Brightness Percentage slider to change (lighten or darken) the image.
Contrast	Manually enter a value to change the contrast between the light and dark portions of the image. Use the Contrast Percentage slider to change (increase or decrease) the contrast.
Use Enhanced Contrast	Select this option to artificially increase the vividness of the image.
Use Equal RGB Color Weighting	Applicable only for fluorescence images. Select to distribute the wavelength range equally among the red, green, and blue components. If this option is not selected, then image is displayed as it looks to the human eye.
Reset to Default	Click to reset the image Rendering Options to their default values.
Image Options: See Chapter 17.11. Image Options .	

17.2. VIEWING THE COMPONENT IMAGE

Selected option: Component: < >, where < > is a specific component such as Her2: Fast Red.
(See Figure 17.2.)

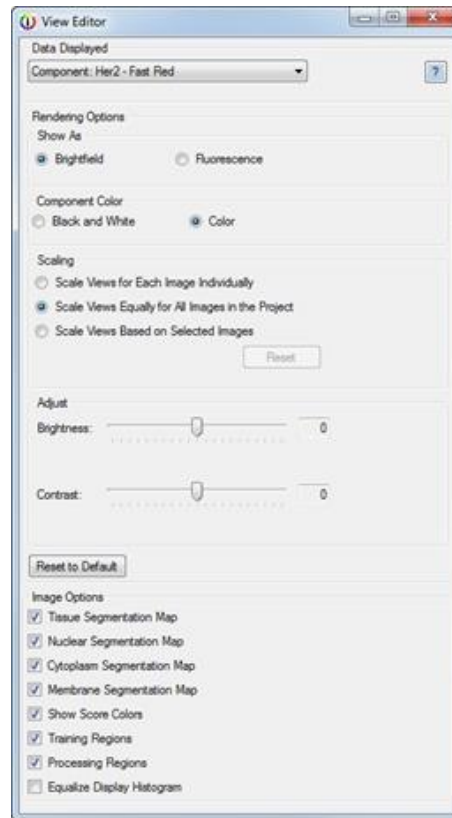


FIGURE 17.2. View Editor with Component Image options.

TABLE 17.2. Rendering Options for Component Image and descriptions

Option	Description
Rendering Options	
Show As:	
Brightfield	Select to display the component as if it were conventionally stained along with a counterstain. Stains are displayed as DAB, autofluorescence is displayed as eosin, and DAPI is displayed as hematoxylin. This option is available when inForm can identify a hematoxylin or DAPI counterstain. If no counterstain is apparent, then inForm displays the component signal only
Fluorescence	Select to display only the component signal on a black background.
Component Color: Applicable when a component is shown without its counterstain. Select an option to display the color for the component.	
Black and White	Select to show the component as a white signal against a black background.

Option	Description
Color	Select to show the component in color. This color is calculated from the spectral shape of the component, which is 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 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	<p>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.</p> <p>NOTE: If this option is selected and changes are made to the selected Spectra for Unmixing or to the Reported Units in the Prepare Images step, then the image scaling is reset to the default value of Scale Views Equally for All Images in the Project.</p>
Reset	Click open the Scaling Image Selection Window to change the images. See Scaling Based on Selected Images for the suggested workflow.
Adjust	
Brightness	Manually enter a value to change the brightness of the image. Use the Brightness Percentage slider to change (lighten or darken) the image.
Contrast	Manually enter a value to change the contract between the light and dark portions of the image. Use the Contrast Percentage slider to change (increase or decrease) the contrast.
Reset to Default	Click to reset the image Rendering Options to their default values.
Image Options	See Image Options.

Scaling Based on Selected Images

You should use images with bright signals in each component, such as positive controls or positively expressing images, to scale all the images that are in a project. When Scale Views

Equally for All Images or Scale Views Based on Selected Images is selected, then the scaling limits are saved with the algorithm and the project. If the algorithm or project is used in Batch mode, then all images that are process in the batch job use the same saved scaling limits.

1. Load only the images of the positive controls.
2. Process the images as preferred.
3. Open the View Editor, and then select any component in the Data Displayed drop-down list.
4. Select Scale Views Based on Selected Images.
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. Load the algorithm or project in the Batch tab to use the batch processing settings.
7. If the positive controls change, then open the project, and then on the View Editor, click Reset.
8. To set the new scaling limits, select the new positive controls in the [Scaling Image Selection Window](#).

Scaling Image Selection Window

Use the Scaling Image Selection Window to select the images that are to be used to scale the brightness of all images in the project. (See Figure 17.3.) 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 is displayed at the bottom of the list and Select for Scaling is not selected.

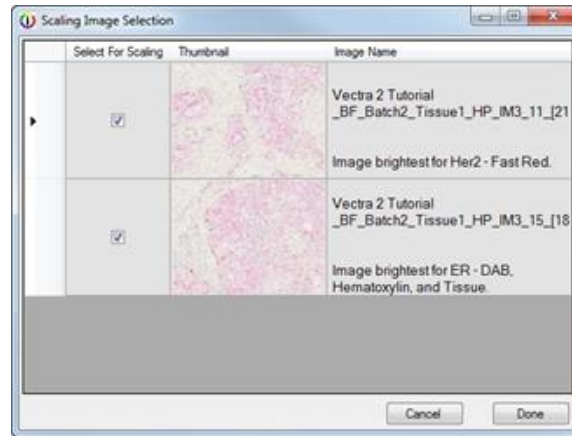


FIGURE 17.3. Scaling Image Selection window.

- **Select for Scaling:** The scaling limits are calculated using all selected images. These scaling limits are saved and used when processing images in Batch mode.
- The Thumbnails and Image Names identify the project images. The Image Name also identifies the brightest image for each component.
- **Cancel:** Click to close the Scaling Image Selection window without re-calculating any image scaling limits. The original limits remain unchanged.
- **Done:** Click to re-calculate the image scaling limits using the selected images, and then closes the Scaling Image Selection window.

17.3. VIEWING THE COMPOSITE IMAGE

Selected option: Composite. (See Figure 17.4.)



FIGURE 17.4. View Editor with Composite Image options.

TABLE 17.3. Rendering Options for Composite Image and descriptions

Option	Description
Rendering Options	
Show As:	
Brightfield	Select to display the composite image as if it were conventionally stained along with a counterstain. Stains are displayed as DAB, autofluorescence is displayed as eosin, and DAPI is displayed as hematoxylin. This option is available when inForm can identify a hematoxylin or DAPI counterstain. If no counterstain is apparent, then inForm displays the component signal only
Fluorescence	Select to display only the composite image signal on a black background.

Option	Description
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 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	<p>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.</p> <p>NOTE: If this option is selected and changes are made to the selected Spectra for Unmixing or to the Reported Units in the Prepare Images step, then the image scaling is reset to the default value of Scale Views Equally for All Images in the Project.</p>
Reset	Click open the Scaling Image Selection Window to change the images. See Scaling Based on Selected Images for the suggested workflow.
Component Display: The following options are displayed for all selected components.	
Display Intensity:	
True	Select to display each component in the composite image at the original intensity. For example, if Component A is dimmer than Component B, then are displayed this way in the composite.
Adjustable	Select to display each component in the composite image at the intensity that selected on the associated Intensity slider. (The Intensity slider for is displayed only when Adjustable is selected.)
Display Color:	
True Color	Select to display each selected component in the calculated color from the spectral shape of the component.
False Color	Select to display each component in the False color that is shown in the Color field for the component. (You can click the Color field for a component and open a drop-down list of colors and pick a different color.) The False Color field is displayed for a components only when False Color is selected.

Option	Description
Reset to Default	Click to reset the image Rendering Options to their default values.
Image Options	See Image Options.

17.4. VIEWING DATA TABLES

The project steps determine the data tables that are available for viewing. To view a data table, select the table in the Data Displayed drop-down list. Note the following caveats about viewing a data table:

- If you change the selections in the View Editor, then the data that is displayed in the selected data table is immediately updated to reflect the selections.
- To sort the data in the data table, click a column header. The arrow that is displayed in the column header indicates the sort order of the data: Up indicates that the table data is sorted in ascending order based on the data in the selected column while Down indicates that the table data is sorted in descending order based on the data in the selected column.
- Summary rows are always displayed at the top of the table and remain displayed at the top of the table even if you change the sort order of the data.

17.5. VIEWING THE TISSUE SEGMENTATION DATA TABLE

Selected option: Tissue Segmentation Data. (See Figure 17.5.)



FIGURE 17.5. View Editor with Tissue Segmentation Data options.

The Tissue Segmentation Data Table is available when an algorithm contains a manual tissue segmentation or trainable tissue segmentation step. The data table provides information on the manually or automatically segmented tissue regions.

NOTE: During image analysis, segmented tissue categories are assigned ID numbers. These numbers are used in the data tables to identify the tissue category for statistics such as area (pixels), area (percent), and so on.

TABLE 17.4. Rendering Options for Tissue Segmentation Data Table and descriptions

Option	Description
Table Contents	
Components	
<ul style="list-style-type: none"> All Components < > Individual components such as DAPI 	Select All components or individual components for which to display the component statistics. Each selected component creates one column for each component statistic that is selected. If there is no data for component, then the component is not displayed in the list of available components.

Option	Description
Tissue Categories	
<ul style="list-style-type: none"> • All Categories • < > Individual categories such as Tumor 	Select All categories or individual categories for which to display the data. An entry row is created for each Region ID that is in the tissue category.
Component Stats	Select the values to display for each component signal. See Chapter 17.6. Component Stats for a description of each option.
Position Stats	Select which values to display for each segmented object. See Chapter 17.9. Position Stats a description of each option.
TMA Core Info	Select the options to include in the data table. See Chapter 17.7. TMA Core Info for a description of each option.
Shape Stats	Select the options to include in the data table. See Chapter 17.10. Shape Stats for a description of each option.
Coordinate Units	Select the unit of measure for the data table statistics: microns or pixels. NOTE: This option is available only for images taken with Mantra or Phenolmager HT.

17.6. VIEWING THE CELL SEGMENTATION DATA TABLE

Selected option: Cell Segmentation Data. (See Figure 17.6.)

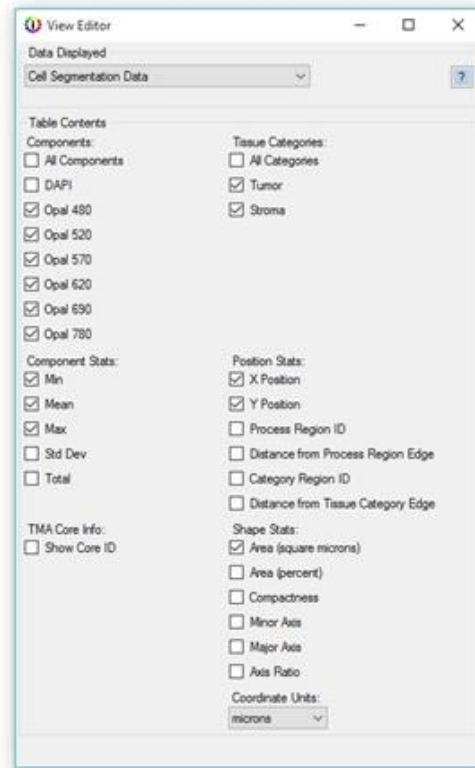


FIGURE 17.6. View Editor with Cell Segmentation Data options.

The Cell Segmentation Data Table is available when an algorithm contains a cell segmentation step. The data table displays statistics for only those cell compartments that have been segmented (nuclei, cytoplasm, membrane). For example, if cytoplasm is not segmented, then the Cytoplasm column is not included in the table. The Cell Segmentation Data table always displays the following data columns, regardless of any other options that are selected:

- Cell ID: Displays a unique ID number for each cell, based on the location of the nuclei and if tissue has been segmented within each tissue category.
- Total Cells: The total number of cells that were scored in the image.
- Tissue Category Area (Pixels): If tissue has been segmented, either automatically or manually, then this column displays the area of the selected tissue category, measured in the number of pixels. If the project did not have a tissue segmenting step, then this column displays the total number of pixels that are in the whole image.
- Cell Density (per megapixel):

- o If tissue has been segmented, either automatically or manually, then this column displays the cell density for each tissue category, defined as:

(The number of cells in the tissue category area) / (the number of pixels in the tissue category area / 1,000,000)
- o If the project did not have a tissue segmenting step, then this column displays the cell density for the entire image, defined as:

(Total Cells) / (the number of pixels in the image / 1,000,000)
- Phenotype and Confidence: If the algorithm contains a Phenotyping Cells step, then cell phenotypes and confidence levels are automatically added to the table. If the project had more than one Phenotyping Schema, then there is a Phenotype and Confidence column for each schema where the schema name is appended to the end of the column name.

TABLE 17.5. Rendering Options for Cell Segmentation Data Table and descriptions

Option	Description
Table Contents	
Components	
<ul style="list-style-type: none"> • All Components • < > Individual components such as DAPI 	Select All components or individual components for which to display the component statistics. Each selected component creates one column for each component statistic that is selected. If there is no data for component, then the component is not displayed in the list of available components.
Tissue Categories	
<ul style="list-style-type: none"> • All Categories • < > Individual categories such as Tumor 	Select All categories or individual tissue categories for which to display data. If selected this option is selected, then a Tissue Category column is displayed in the Cell Segmentation table to specify the Tissue Category for each cell. If there is only one Tissue Category defined or if there is no Segment Tissue step in the project, then this option is not displayed. NOTE: Rarely, the tissue segmentation step does not classify small areas of tissue. Cells that are found within these unclassified areas are not reported in the data table.

Option	Description
Component Stats	Select the values to display for each component signal. Each selection adds one column for each segmented cell component and one column for the entire cell. See Entire Cell Statistics for information about calculating the statistics for the entire cell. See Chapter 17.12. Component Stats for a description of each option.
Position Stats	Select which values to display for each segmented object. See Chapter 17.9. Position Stats a description of each option.
TMA Core Info	Select the options to include in the data table. See Chapter 17.7. TMA Core Info for a description of each option.
Shape Stats	Select the options to include in the data table. See Chapter 17.10. Shape Stats for a description of each option.
Coordinate Units	Select the unit of measure for the data table statistics: microns or pixels. NOTE: This option is available only for image that was taken with Mantra or Phenolmager HT.

Entire Cell Statistics

Statistics are calculated according to the following parameters:

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

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, where:

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.

17.7. VIEWING THE SCORE DATA TABLE

Selected option: Score Data. (See Figure 17.7.)

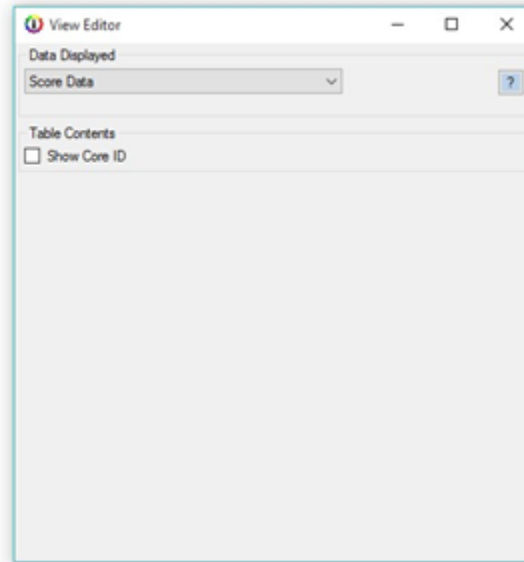


FIGURE 17.7. View Editor with Score Data options.

The Score Data Table is available when an algorithm contains a Score step. The selected scoring type determines the data that is available for inclusion in the table. The Score Data table always displays the following data columns, regardless of any other options that are selected:

- **Tissue Category:** If tissue has been segmented, either automatically or manually, then this column displays the tissue category that was used for scoring. If the project did not have a tissue segmenting step, then this column is blank.
- **Tissue Category Area (Percent):** If tissue has been segmented, either automatically or manually, then this column displays the area of the selected tissue category. If the project did not have a tissue segmenting step, then this column displays 100% (the whole image).
- **Number of Cells:** The number of cells that were scored in the image.
- **(First) Cell Compartment:** The cell compartment that was used in scoring.

- **(First) Stain Compartment:** The stain that was used to create the threshold for scoring.

For other data table contents and a description of each option, see [Chapter 17.13. TMA Core Info](#).

If Double Positivity (2 x 2) scoring is selected, then the Score Data table also displays the following data columns:

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

If 0-3+ (4-bin) scoring is selected, then the Score Data table also displays the following data columns:

- **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+:** Click Auto to set this value to 1/4 of the Threshold max. You can change this value.
- **Threshold 1+/2+:** Click Auto to set this value to 1/2 of the Threshold max. You can change this value.
- **Threshold 2+/3+:** Click Auto to set this value to 3/4 of the Threshold max. You can change this value.

If 10-bin or 50 bin scoring is selected, then the Score Data table also displays the following data column:

- **Bin X** (where X ranges from 1-10 or 1-50 per scoring type): These columns show the percent of scored values that fall in each scoring bin. You set the lower threshold for the last bin. The last bin is the percent of cells whose component strength is greater than the threshold that you set. The thresholds for the remaining bins are spaced evenly from 0. For example, the 49th bin has a lower limit of $(48 * \text{threshold}) / 49$.

17.8. VIEWING THE COLOCALIZATION DATA TABLE

Selected option: Colocalization Data. (See Figure 17.8.)



FIGURE 17.8. View Editor with Colocalization Data options.

The Colocalization Data tables are available when an algorithm contains a Colocalization step.

TABLE 17.6. Rendering Options for Colocalization Data Table and options

Option	Description
Colocalization Table: Shows how much the numerator components are colocalized 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 the statistics for the whole image and the colocalization region.	
Components	

Option	Description
<ul style="list-style-type: none"> All Components < > Individual components such as DAPI 	Select All components or individual components for which to display the component statistics. One column per numerator component is displayed.
Component Stats	Select the values to display for each component signal. See Chapter 17.12. Component Stats for a description of each option.
Position Stats	Select which values to display for each segmented object. See Chapter 17.9. Position Stats a description of each option.
TMA Core Info	Select the options to include in the data table. See Chapter 17.7. TMA Core Info for a description of each option.
Shape Stats	Select the options to include in the data table. See Chapter 17.10. Shape Stats for a description of each option.
Coordinate Units	Select the unit of measure for the data table statistics: microns or pixels. NOTE: This option is available only for image that was taken with Mantra or Phenolmager HT.

17.9. VIEWING THE COUNT DATA TABLE

Selected option: Count Data. (See Figure 17.9.)



FIGURE 17.9. View Editor with Count Data options.

The Quant Data Table is available when an algorithm contains either a Threshold step or a Colocalization step.

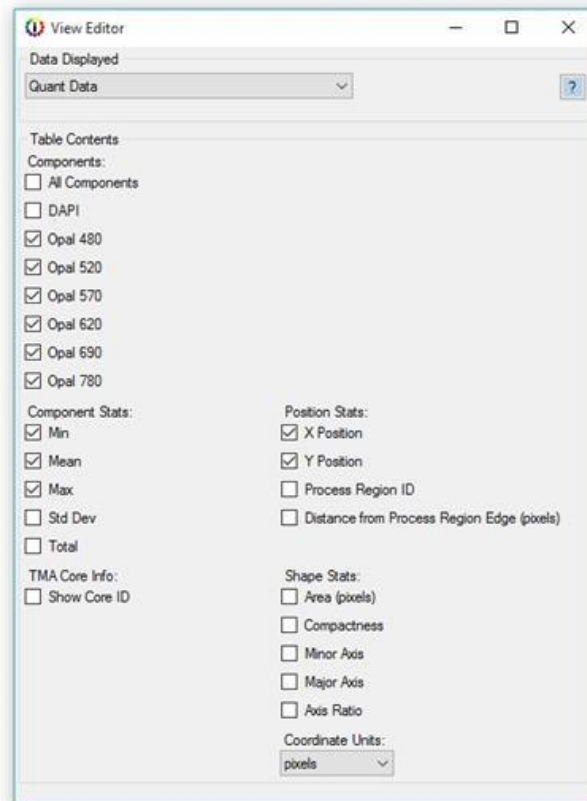
TABLE 17.7. Options for Count Data Table and descriptions

Option	Description
Components	
<ul style="list-style-type: none"> All Components < > Individual components such as DAPI 	Select All components or individual components for which to display the component statistics. Each selected component creates one column for each component statistic that is selected. If there is no data for component, then the component is not displayed in the list of available components.
Component Stats	Select the values to display for each component signal. Each selection adds one column for each segmented cell component and one column for the entire cell. See Entire Cell Statistics for information about calculating the statistics for the entire cell. See Chapter 17.12. Component Stats for a description of each option.

Option	Description
Position Stats	Select which values to display for each segmented object. See Chapter 17.9. Position Stats a description of each option.
TMA Core Info	Select the options to include in the data table. See Chapter 17.7. TMA Core Info for a description of each option.
Shape Stats	Select the options to include in the data table. See Chapter 17.16. Shape Stats for a description of each option.
Coordinate Units	Select the unit of measure for the data table statistics: microns or pixels. NOTE: This option is available only for image that was taken with Mantra or Phenolmager HT.

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 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 for descriptions of each option.
- **TMA Core Info:** Select the options to include in the data table. See TMA Core Info.
- **Position Stats:** Select which values to display for each segmented object. See Position Stats.
- **Shape Stats:** Select the options to include in the data table. See Shape Stats.
- **Coordinate Units:** Select the unit of measure for your table statistics, microns, or pixels. This option is only available for imagery taken with Mantra, the legacy Vectra 3 system, and the PhenolImager HT.

17.11. IMAGE OPTIONS

When you select an image on the Data Displayed drop-down list on the Image Editor, the following image options are available. (These same options are also displayed as buttons on the left side of the image display area.)

TABLE 17.8. Image Options and descriptions

Option	Description
Tissue Segmentation Map	Select this option to display the Tissue Segmentation map on the image. This option is available only for projects that include a Manual Classification step or a Tissue Segmentation step.
Nuclear Segmentation Map	Select this option to display the Nuclear Segmentation map on the image. This option is available only for projects that include a Segment Cells step.
Cytoplasm Segmentation Map	Select this option to display the Cytoplasm Segmentation map on the image. This option is available only for projects that include a Segment Cells step.
Membrane Segmentation Map	Select this option to display the Membrane Segmentation map on the image. This option is available only for projects that include a Segment Cells step.
Show Score Colors	Select this option to display the Score Colors map on the image. This option is also accessible from the Score Settings panel, and it is available only for projects that include a Score IHC step or Score IF step. .
Object Segmentation Map	Select this option to display the Object Segmentation map on the image. This option is available only for projects that include a Count Objects step.
Training Regions	Select this option to display the Training regions on the image. This option is available only for projects that include a Segment Tissue step.
Processing Regions	Select this option to display the Processing regions on the image.
Equalize Display Histogram	Select to this option to map the pixels so that 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 this option is not selected, then 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, all of which prevents a few bright or saturated pixels from skewing the display. This option is accessible only from the Image Editor.

Shortcuts are also available for the image views (Color, Component, and Composite) as buttons on the image toolbar. (See Figure 17.10.) To toggle across the displays, click the buttons as appropriate.



FIGURE 17.10. Image View shortcut buttons (from left to right, Color, Component and Composite).

Simulated Brightfield Views (Pathology Views)

When you are viewing fluorescence imagery, you use the Component button to display the image components in a simulated brightfield mode, which is referred to as Pathology Views. Pathology Views renders fluorescence images in a manner that is familiar to anyone who is trained in histopathology, displaying the images as simulated H&E images or as simulated DAB/Hematoxylin images. The image components are displayed as if you were looking at serial sections that are single-stained. However, the difference is that Pathology Views displays multiplexed stains on a single tissue section with high contrast and perfect registration.

- **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 among the different image components:

1. Click the Component button.
A drop-down list of all the available image components opens. (See Figure 17.11.)
2. Select the component that is to be displayed.
The legend shows the currently selected component.

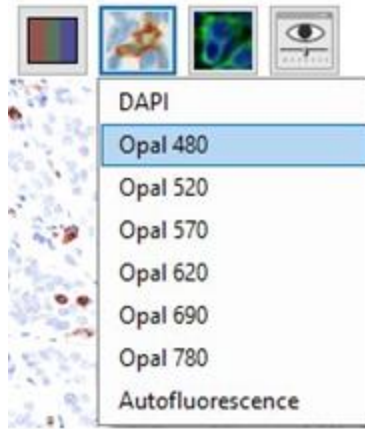


FIGURE 17.11. Component button.

Tip: You can also use the *Up* and *Down* arrows on your keyboard to move through the image components.

Composite Views

Although you can open an image in inForm that contains up to 50 components, it can display only 10 channels at time. When you first open an image in inForm, it opens in the Standard View, which is the default composite view. This view shows the first 10 components that were designated in the scan protocol. You can use the Composite button to add new Composite views, editing existing Composite views, and select which components to display per Composite view. (See Figure 17.12.) After you have added or edited existing Composite views, you can then select any of these views for viewing the project images. You can save a view as part of a project or algorithm.



FIGURE 17.12. Composite button.

NOTE: inForm cannot open views that were created using the Phenochart software. To open such an image in inForm, you must use inForm to create a new image.

To use the Composite Editor:

1. Click the Composite button, and then on the drop-down list that opens, click Edit. The Composite Views dialog box opens. (See Figure 17.13.)

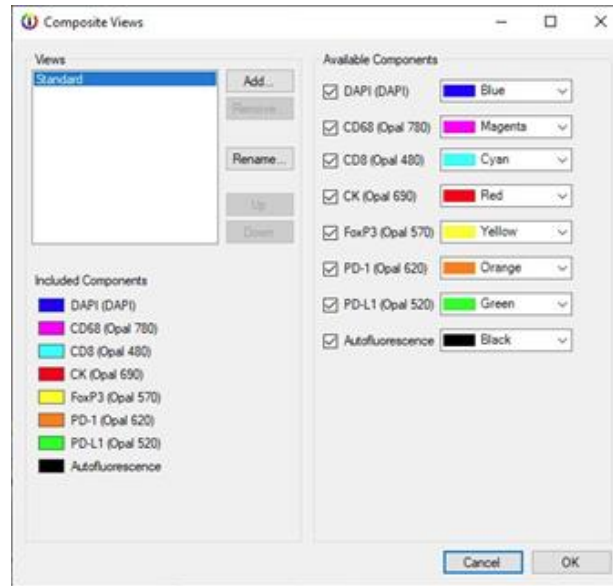


FIGURE 17.13. Composite Views dialog box.

2. Do any of the following as appropriate for adding, editing, and maintaining your Composite views, including the Standard view:
 - a. To change the name of a Composite view, select the view, and then click Rename.
 - b. To change the components that are displayed for a view, select the view, and then select or clear the Available Components as appropriate.
 - c. To change the color for a view component, select the view, select the component, and then click the current preset color to open a drop-down list from which you can select another preset color, or click Custom to create a custom color.
 - d. To add a new Composite view, click Add, and then name the view and add the view components as prompted. You can add an unlimited number of Composite views. (See Figure 17.14.)

- e. To change the order in which the views are displayed in the Composite views drop-down list (the list that is displayed after you click the Composite button), select a view, and then click Up or Down as appropriate.
- f. To delete a view, select the view, and then click Remove.

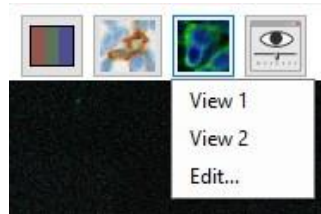


FIGURE 17.14. Example of available options from the Composite button after adding/renaming views.

17.12. COMPONENTS STATS

The following options are available for Component Stats:

- **Min:** If this option is selected, then one column that contains the minimum value of the component in each region is displayed in a data table. For the Cell Segmentation data table, one column for each cell compartment that was segmented is displayed, and one column for the entire cell is displayed.
- **Mean:** If this option is selected, then the mean of the component in the region is displayed in a data table. 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:** If this option is selected, then the maximum value of the component that is in the region is displayed in a data table.
- **Std Dev:** If this option is selected, then the sum of the value of the component across all the pixels in the region is displayed in a data table.

17.13. TMA CORE INFO

The following option is available for TMA Core Info:

- **Show Core ID:** If the image is of a core on a TMA slide that was scanned with an Akoya scanner, then the Sector, Row, Column and Field of the core are displayed. If there is no core information, then a zero (0) is displayed in each of these table columns.

17.14. SLIDE INFO

Exported data tables contain the Lab ID, the Slide ID, and the Field ID for the appropriate data formats. These fields are displayed in the image name rather than in the data tables in an inForm project.

17.15. POSITION STATS

The following options are available for Position Stats:

- **X Position** and **Y Position:** If this option is selected, then the X and Y locations of the center of the bounding box for the region are displayed.
- **Process Region ID:** If process regions are defined for the image and this option is selected, then the ID number of the process region that contains the tissue category is displayed in a data table. If an object spans multiple regions, then #N/A is displayed instead.
- **Distance from Process Region Edge (Pixels):** If this option is selected, then the distance, in pixels, from the center of this region's bounding box to the nearest edge of the containing process region is displayed in a data table. If the distance cannot be determined, then #N/A is displayed instead.
- **Category Region ID:** If tissue is segmented and this option is selected, then the Region ID of the tissue category that contains the segmented object is displayed in a data table. This option is available only in the Cell Segmentation and Count Data Tables and only if tissue has been segmented.
- **Distance from Tissue Category Edge (Pixels):** If this option is selected, then the distance, in pixels, from the segmented object to the nearest edge of the tissue category is displayed in a data table. If the distance is reported as #N/A, then 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 might 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. This option is available only in the Cell Segmentation and Count Data Tables and only if tissue has been segmented.

17.16. SHAPE STATS

The following options are available for Shape Stats:

- **Area (pixels):** If this option is selected, then the size, in pixels, of the region is displayed in a data table.
- **Area (percent):** If this option is selected, then the region size as a percent of the total processed area is displayed in a data table. If there are no processing regions, then the total image area is displayed instead. Otherwise, the total area of the processing regions is displayed.
- **Compactness:** If this option is selected, then the compactness of the region is displayed in a data table, where the Compactness is defined as the ratio of the area of the region to the area of a circle (the most compact shape) that has 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:** If this option is selected, then the smaller of the two axes of the minimum area bounding box that encloses the region is displayed in a data table.
- **Major Axis:** If this option is selected, then the larger of the two axes of the minimum area bounding box that enclosed the region is displayed in a data table.
- **Axis Ratio:** If this option is selected, then the ratio of the major axis/minor axis of the minimum area bounding box that encloses the region is displayed in a data table.

CHAPTER 18. EXPORTING 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. (See Figure 18.1.)
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.) Outputs containing multiple image layers will be exported as TIFFs even if you select JPEG. The outputs that may contain multiple layers are labeled as “multi-image TIFF”.
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.
 - a. The background for the Composite with Tissue Segmentation Map, Composite with Cell Segmentation Map, Composite with Scoring Map, and Composite with All Segmentation Maps images will be the most recently viewed Composite View.
 - b. The Phenotype Map and Composite with Phenotype Map are color images with one dot representing each phenotyped cell. These images will have a separate layer for each Phenotype Schema. The Composite with Phenotype Map image will include the Associated View for each schema as a background image. (See [Appendix F. Composite Image and Phenotype Map TIFF Specification.](#))

Export Settings

Export Directory
C:\ Browse...

Image export options
Image Output Format: JPEG

Images to export:

- RGB
- Composite with Tissue Segmentation Map
- Composite with Cell Segmentation Map
- Composite with Phenotype Map *
- Composite with Scoring Map
- Composite with All Segmentation Maps
- Composite Image *

Legend: Omit

- Pathology Views
- Component Images *

Maps to export:


- Segmentation Maps *
- Phenotype Map *

* multi-image TIFF file

Tables To Export

- Tissue Segmentation Data
- Cell Segmentation Data
- Score Data

Table Fields To Export

Export all fields Use view settings 

File name options

In case of file name conflict:

- Overwrite existing files
- Rename new files

Directory Structure:

Copy level(s) of directory structure from source image

FIGURE 18.1. Export Settings.

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.](#))
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.](#))

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.

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 [Chapter 17. Displaying the Extracted Data.](#))
10. Select File Name Options: If files are already saved in the selected export directory, select how to name the new files. You can:
 - a. Overwrite existing files if the new file names match.
 - b. 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.

A sample list of exported files is shown below in Figure 18.2.





















Name	Type	Size
 Lung_C3_Scan1_[8733,48494].jpg	JPG File	381 KB
 Lung_C3_Scan1_[8733,48494]_Autofluorescence_path_view.jpg	JPG File	412 KB
 Lung_C3_Scan1_[8733,48494]_binary_seg_maps.tif	TIF File	2,857 KB
 Lung_C3_Scan1_[8733,48494]_CD8_path_view.jpg	JPG File	431 KB
 Lung_C3_Scan1_[8733,48494]_CD68_path_view.jpg	JPG File	414 KB
 Lung_C3_Scan1_[8733,48494]_cell_seg_data.txt	Text Document	10,273 KB
 Lung_C3_Scan1_[8733,48494]_cell_seg_data_summary.txt	Text Document	39 KB
 Lung_C3_Scan1_[8733,48494]_component_data.tif	TIF File	68,585 KB
 Lung_C3_Scan1_[8733,48494]_composite_image.tif	TIF File	15,892 KB
 Lung_C3_Scan1_[8733,48494]_DAPI_path_view.jpg	JPG File	399 KB
 Lung_C3_Scan1_[8733,48494]_FoxP3_path_view.jpg	JPG File	406 KB
 Lung_C3_Scan1_[8733,48494]_image_with_all_seg.jpg	JPG File	948 KB
 Lung_C3_Scan1_[8733,48494]_image_with_cell_seg_map.jpg	JPG File	929 KB
 Lung_C3_Scan1_[8733,48494]_image_with_phenotype_map.tif	TIF File	10,995 KB
 Lung_C3_Scan1_[8733,48494]_image_with_score_map.jpg	JPG File	447 KB
 Lung_C3_Scan1_[8733,48494]_image_with_tissue_seg.jpg	JPG File	314 KB
 Lung_C3_Scan1_[8733,48494]_PD-1_path_view.jpg	JPG File	446 KB
 Lung_C3_Scan1_[8733,48494]_PD-L1_path_view.jpg	JPG File	430 KB
 Lung_C3_Scan1_[8733,48494]_phenotype_map.tif	TIF File	313 KB
 Lung_C3_Scan1_[8733,48494]_Tumor_path_view.jpg	JPG File	458 KB

FIGURE 18.2. Exported Data files.

CHAPTER 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.

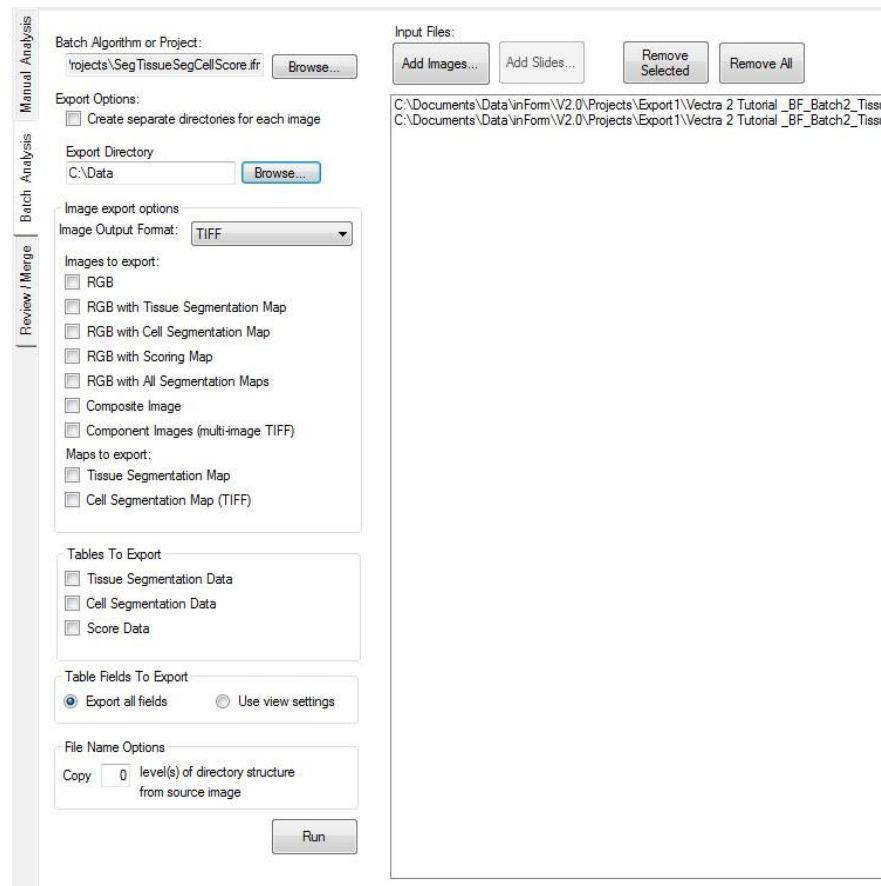


FIGURE 19.1. Batch Analysis tab.

1. Click the Batch Analysis tab to configure the batch settings and select images for processing. You are prompted to save the open project. (See Figure 19.1.) 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 [Chapter 18. Exporting the Data](#).
6. Click the Add Images button to select images to process. Click the Add Slides button to select slides scanned by PhenolMager HT; 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 a Mantra, legacy Vectra 3 system, PhenolMager HT, or Fusion, hold the control button down before clicking Add Slides. If you need to change the 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 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.

CHAPTER 20. MERGING 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:
 - a. Select or clear the check marks in the list to include or exclude specific data sets.
 - b. Click the Include All button to select all data sets in the list.
 - c. 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:
 - a. 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.
 - b. If the imagery contains areas that you would like to exclude, you can draw regions of disinterest using the toolbar.

- c. If you would like to limit the data to a specific area instead, you can draw processing regions.
 - d. Once your regions are drawn, click Re-process to re-process the imagery using the new regions.
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.

The Review/Merge tab is shown below in Figure 20.1.

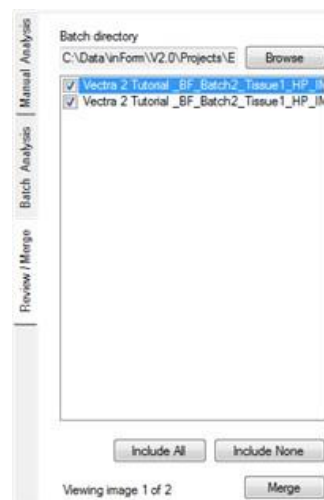


FIGURE 20.1. Review/Merge tab.

CHAPTER 21. BUILDING LIBRARIES

Section	Page
21.1. Singly Stained Tissue Sections	179
21.2. Extracting a Stain or Fluorophore	180
21.3. Extracting a Custom Stain or Fluorophore	181
21.4. Extracting a Fluorophore with a Custom Filter	185
21.5. Extracting a Stain from a Color Image	185
21.6. Using Processing Regions	187

IMPORTANT: This feature works with images taken with images taken with a Mantra, legacy Vectra 3 system, PhenImager HT, or Fusion. It will not work with fluorescence Nuance images.

There are 3 ways to extract the spectra from a stain or fluorophore using the Build Libraries tab:

- 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.
- 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.
- 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.

21.1. 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 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. (See Figure 21.1.)

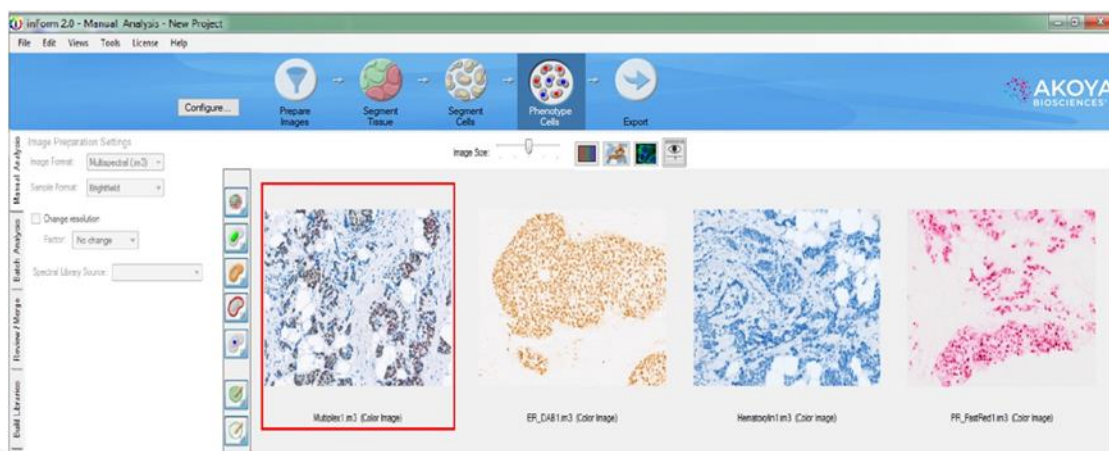



FIGURE 21.1. You should strive to utilize measured and extracted signals from singly stained slides that you can store for later use. Left-to-right: multiplexed IHC stained tissue section, DAB-only stained tissue section, hematoxylin-only stained tissue section, and Fast Red-only stained tissue section.

21.2. 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 the cursor over the image to 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.

An open DAPI image prior to stain extraction is shown below in Figure 21.2.

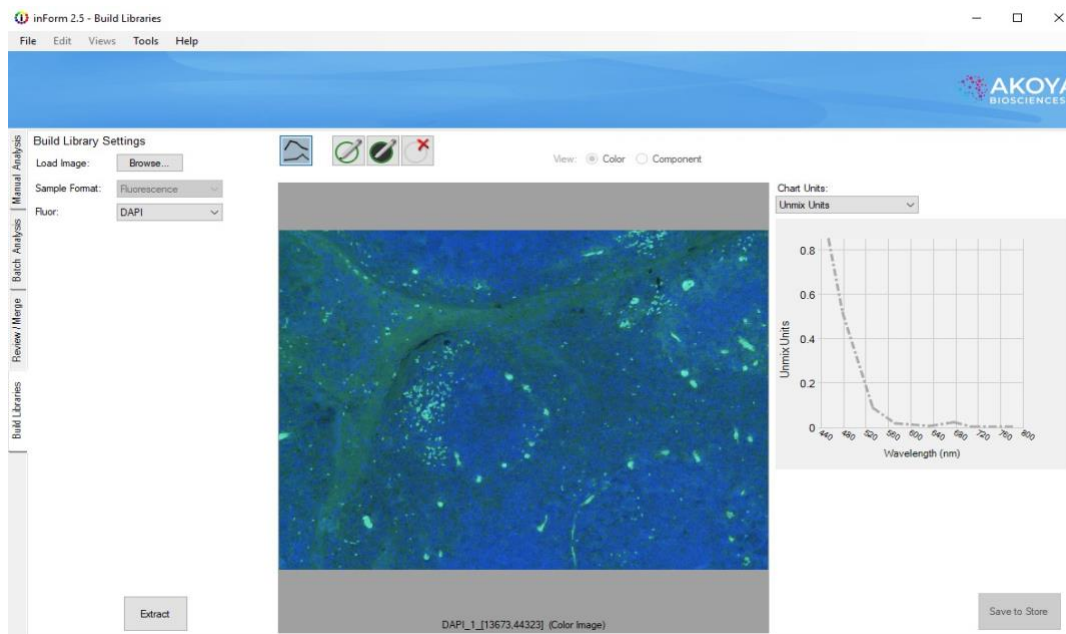


FIGURE 21.2. An opened DAPI image before the stain has been extracted.

Select the display color, add notes, and choose groups for your extracted stain or fluorophore before saving it. (See Figure 21.3.)

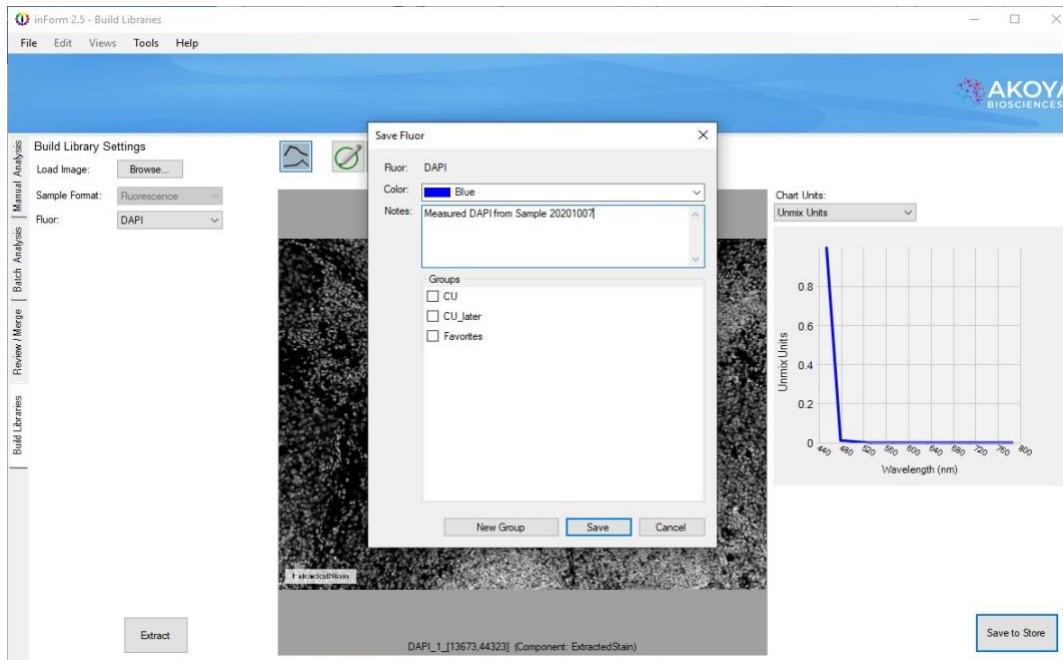


FIGURE 21.3. Clicking Extract allows you to extract and save a measured single stain to the Stain Store, which serves as your library of spectra for different stains and fluorophores.

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 singly stained sample.

21.3. 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. 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. (See Figure 21.4.)

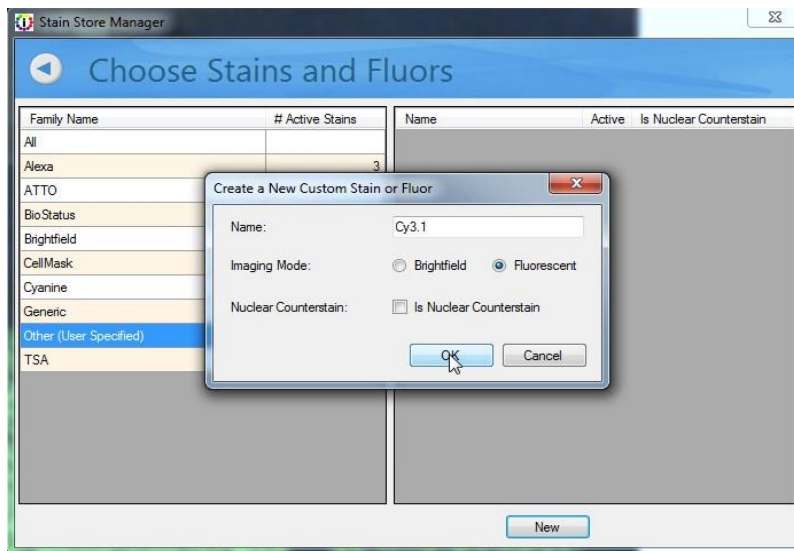


FIGURE 21.4. If you have a new stain or fluorophore that does not appear in the Stain Store, you can create a new listing for one.

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. (See Figure 21.5.)

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 corresponding to each of your epifluorescence filters.

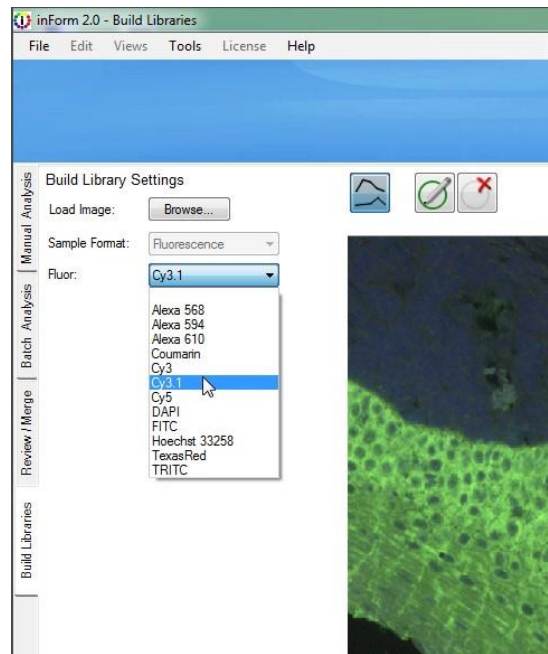


FIGURE 21.5. In the Build Libraries tab, you can select your new/custom stain or fluorophore in the Stain/Fluor drop-down menu.

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 14.6 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. (See Figure 21.6.) Then click the Extract button.

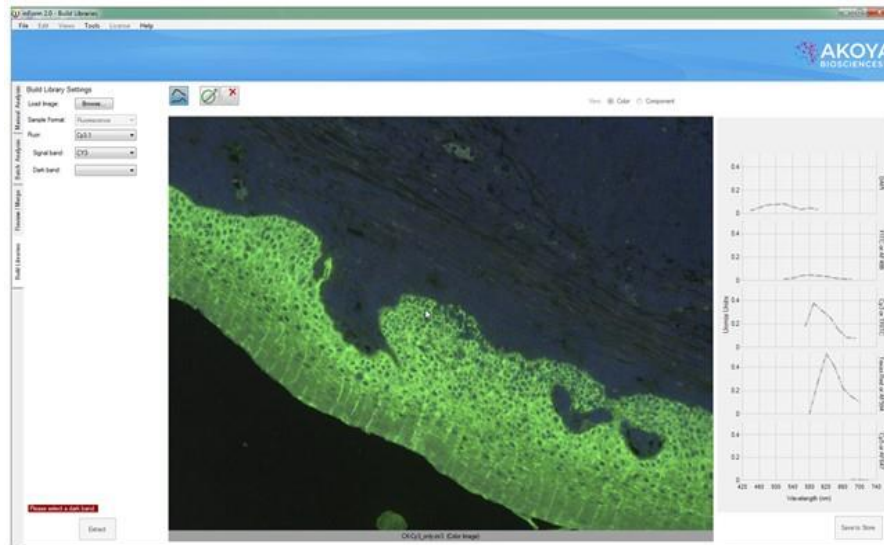


FIGURE 21.6. You can use the curves to the right of your image that display the spectra under your cursor to select the “Signal Band” where the majority of the signal of your new/custom fluorophore appears, and also the “Dark Band” where there is little or no signal.

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. (See Figure 21.7.)

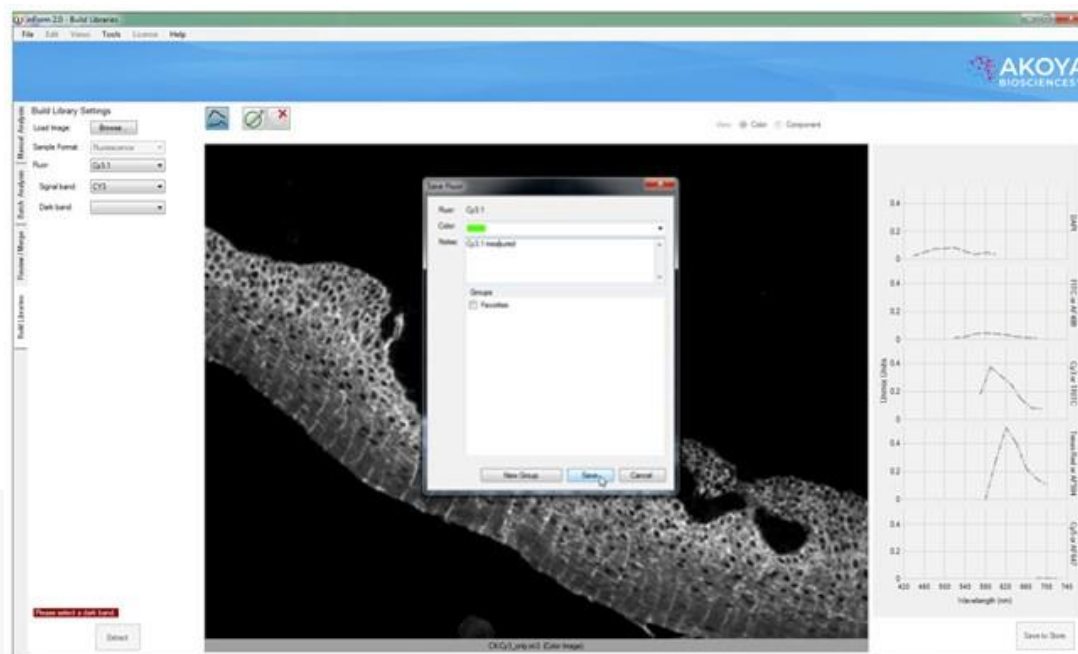


FIGURE 21.7. Once the spectra from your new/custom stain or fluorophore has been extracted, you can save it to the Stain Store.

21.4. EXTRACTING A FLUOROPHORE WITH A CUSTOM FILTER

inForm has built-in settings for many manufacturers' epifilter 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. (See Figure 21.8.)

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.

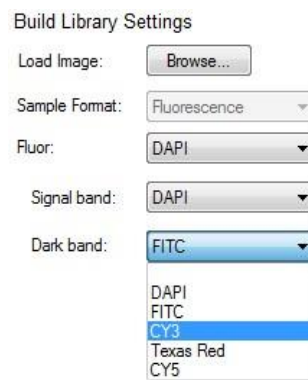


FIGURE 21.8. Dark Band drop-down list.

21.5. 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. (See Figure 21.9.)

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 choose the name of the stain in the Stain drop-down menu.

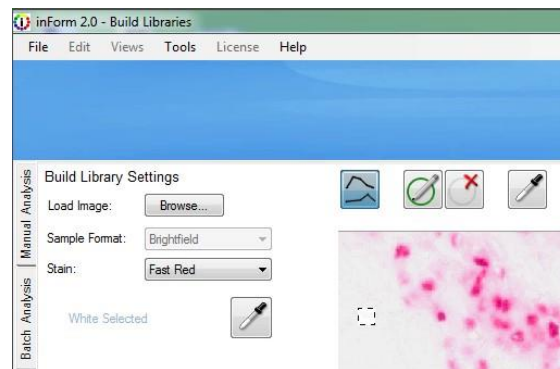


FIGURE 21.9. When you open a color (RGB) image of a singly stained slide, you need to select a white area in order to help the software convert the image to optical density, which is necessary in order to properly quantify stain intensity.

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 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. (See Figure 21.10.)

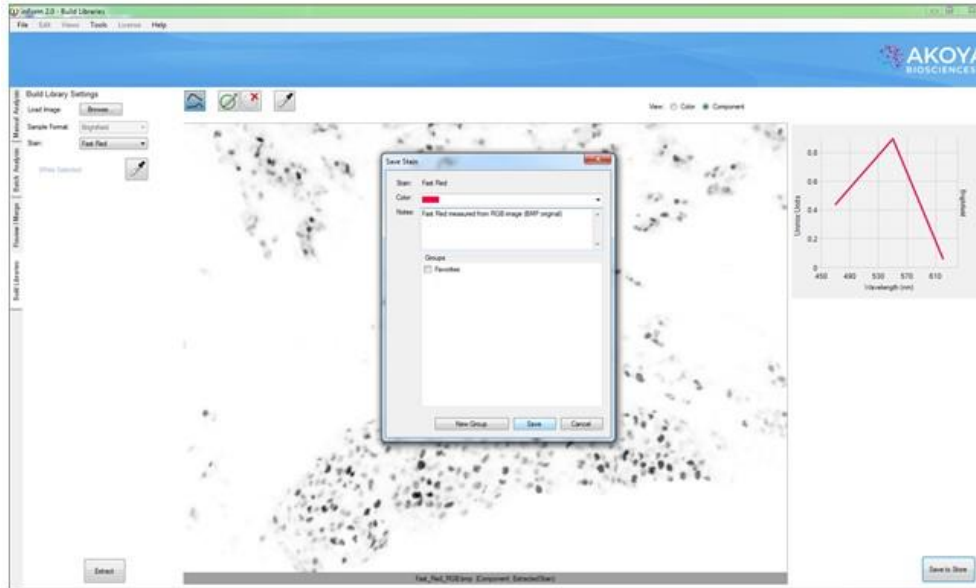



FIGURE 21.10. You can extract a pure signal from a color (RGB) image after it is converted to optical density, but the resulting spectra will not be as detailed as the pure signal extracted using a multispectral .IM3 file. Note the lack of a smooth spectral curve representing the Fast Red stain. Instead of information from many wavelengths, an RGB image only contains information for three broadly overlapping regions in the visible wavelength range.

21.6. 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 to select areas with both the primary signal and autofluorescence for fluorescence images. (See Figure 21.11.)

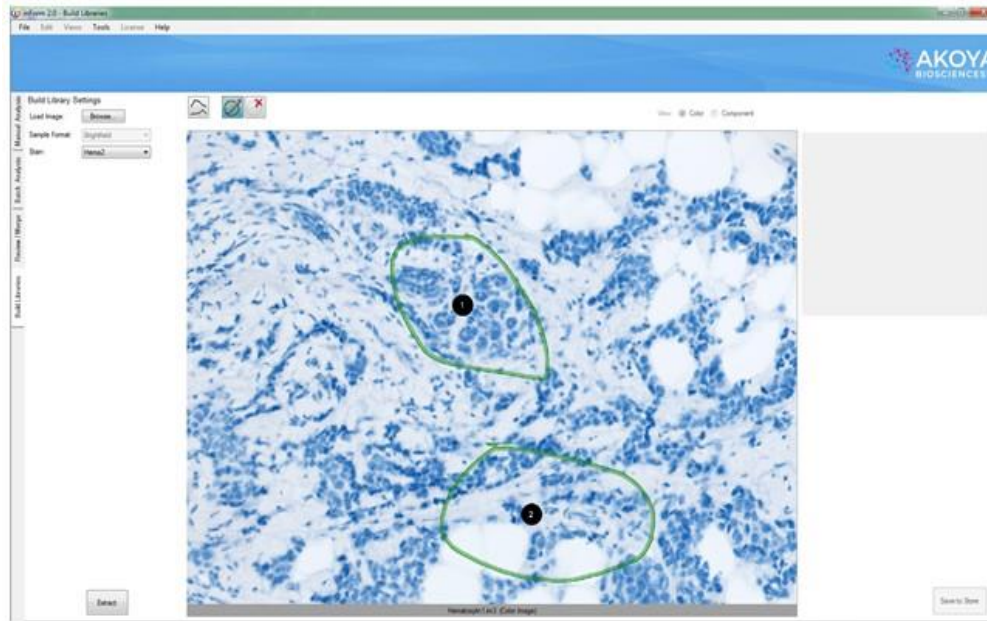



FIGURE 21.11. You may have a slide that contains small areas of your stain or fluorophore, and clicking the Extract button results in an error. Click the ROI button (diagonal pencil over a green circle) and select areas to assist the inForm extraction algorithm. You may need to select regions based on 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. An image containing a large amount of eosin and a much smaller amount of hematoxylin is illustrated in Figure 21.12. You can draw a region or regions around the tissue where just the eosin is present, ignoring the rest of the image.

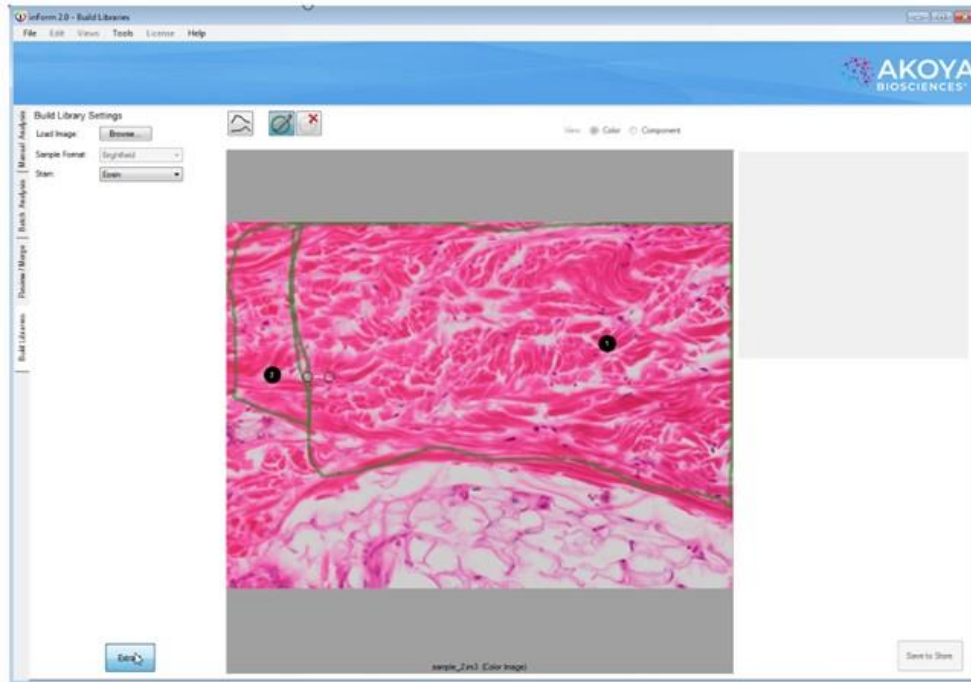


FIGURE 21.12. In the Build Libraries tab, you can select regions within your specimen that contain reasonably pure spectra from a single stain. In this case pink eosin, avoiding areas where there are very small amounts of blue hematoxylin present.

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. (See Figure 21.13.)

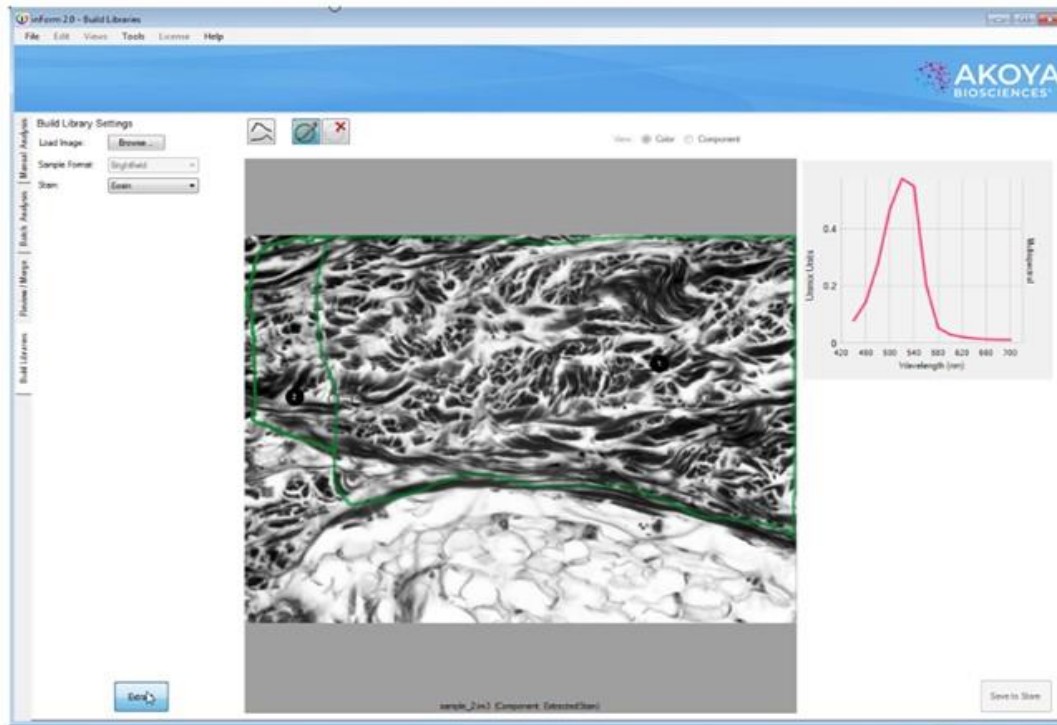


FIGURE 21.13. If there is enough of the pure spectra present for the stain or fluorophore that you chose from the drop-down menu, inForm will display the spectral curve that it has extracted and a black-and-white image representing the presence of that stain or fluorophore.

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.

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 legacy Vectra systems.

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 images taken with a Mantra, legacy Vectra 3 system, Phenomager HT, or Fusion.

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

CREATE A PIVOTTABLE TO COMPLETE 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 dropdown 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.

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. (See Figure B.1.)

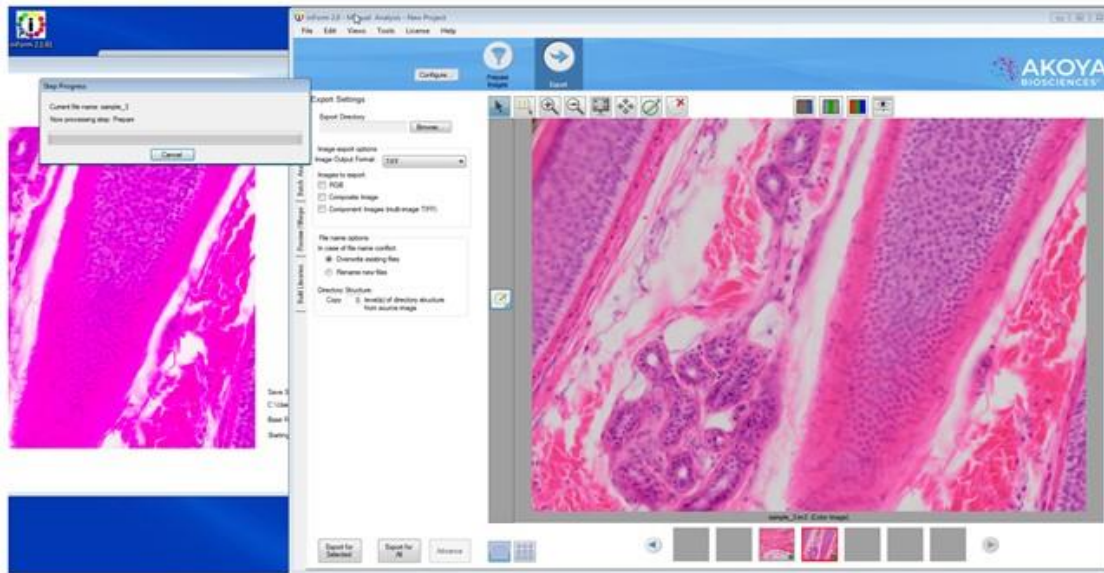


FIGURE B.1. A Mantra image was used to develop an inForm algorithm and then a second image taken on the Mantra workstation. Immediately after clicking the Snap button, the second image has automatically been opened in inForm and the open algorithm is being applied to the image.

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 glmnet¹ package from R² creates the machine learning classifier. While the creation of the classifier is complex, the result is a relatively simple multinomial logistic regression classifier³ with softmax output. The classifier is applied to the features to assign phenotypes to cells.

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).
5. 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 [Chapter 14. Phenotyping Cells](#). 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 $n_{\text{compartment}} > 1$ compartments and $n_{\text{component}}$ components, the number of cell segmentation features will be approximately

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

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-Occurrence Features

Grey-level co-occurrence features⁴ 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 regularization⁶ 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 simple. For each of the K phenotypes in the training set, `glmnet` constructs a linear predictor $f_i(\mathbf{X}) = \mathbf{X} * \beta_i$ from the available features \mathbf{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 \mathbf{X} belongs to phenotype i is given by the softmax function:

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

The phenotype for a cell is then taken to be the one with the highest probability, i.e., $\mathop{\text{argmax}}_i P(i | \mathbf{X})$. The confidence reported by inForm is the probability of the winning phenotype, $P(i | \mathbf{X})$, 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 $f_i(\mathbf{X})$ and a set of features, it is straightforward to compute the $f_i(\mathbf{X})$ and $P(i | \mathbf{X})$ 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.

-
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, 267-288. <http://www-stat.stanford.edu/~tibs/lasso/lasso.pdf>.

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 are stored in strips rather than in tiles.

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. Note that the thumbnail can be used for qualitative sample identification, but should not be used to assess relative brightness of different images. 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 illustrated below in Table D.1.

TABLE D.1. Images

Description	RGB / mono	Tile / 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	Strip	~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	Strip	~2000 x 4000	Required for whole-slide scans Optional for simple RGB images and extracted components
Label image	RGB	Strip	~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 D.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

TIFF Tag	Optional?	Description of contents
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 <PerkinElmerQPI-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 D.3. Image Description tags

Tag	Optional?	Contents
DescriptionVersion		Version of the image description field itself, a single number. This document describes version 4 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.
SampleDescription	Y	Text description of the sample.
OperatorName	Y	Name of the operator as reported by the operating system running the acquisition software.
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: <ul style="list-style-type: none"> • 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).

Tag	Optional?	Contents
SignalUnits		<p>A byte <i>www ttt</i> where the <i>ttt</i> nibble indicates the signal unit type from the following:</p> <ul style="list-style-type: none"> • raw counts • normalized (counts/second/gain/fullscale/binning) • OD (optical density) • dark-corrected counts
		<p>and the <i>www</i> nibble indicates how the signal is weighted across the spectral bands (or colors):</p> <ul style="list-style-type: none"> • average across all bands • total summed signal across all bands • peak signal in highest-valued band <p>For example, a value of 68 (hex 44) encodes OD units with peak-signal weighting.</p>
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. This does not imply a specific pixel resolution – see the XResolution and YResolution standard TIFF tags instead.
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.

Tag	Optional?	Contents
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: <ul style="list-style-type: none"> • Unknown (default) • PriorLumen200 • PriorLumen220 • XCiteMultiBandLed • PriorMimicLed • SolaLed • Colibri7
InstrumentType	Y	String with the instrument type, one of the following: <ul style="list-style-type: none"> • Unknown (default) • Keyence700 • Keyence800 • AxioObserver • Dmi8 • Phenolmager HT

Tag	Optional?	Contents
CameraType	Y	String with the camera sensor type, one of the following: <ul style="list-style-type: none"> • 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 below.
Band		Description of a band with child tags 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.

Tag	Optional?	Contents
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 QTIFF file with 8-bit resolution).
Orientation		String containing one of the following values: <ul style="list-style-type: none"> • '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 Phenolmager HT and unmixed FL multispectral images originating from Phenolmager HT.

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. Their contents are described in Table D.4.

TABLE D.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 ^{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>Full Resolution</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>
```

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 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>
```

APPENDIX F. COMPOSITE IMAGE AND PHENOTYPE MAP TIFF SPECIFICATION

inForm exports Composite Images, Composite with Phenotype Map images, and Phenotype Map images as multi-image TIFFs where all images are 8-bit RGB images. The Composite Image has one layer for each Fluorescence Composite View. The Composite with Phenotype Map and Phenotype Map images have one layer for each Phenotyping Schema.

Metadata about each image layer is stored as XML within the image's ImageDescription tag. The XML structure for the Image Description is:

```
<?xml version="1.0" encoding="utf-16"?>
<ImageDescription>
  <Version>1</Version>
  <PhenotypeSchema>
    <Name>{phenotyping Schema name}</Name>
    <Phenotypes>
      <Entry>
        <Name>{phenotype name}</Name>
        <Color>{alpha},{red},{green},{blue}</Color>
      </Entry>
      { ...other entries }
    </Phenotypes>
  </PhenotypeSchema>
  <Composite>
    <Name>{composite view name}</Name>
    <Components>
      <Entry>
        <Marker>{marker name}</Marker>
        <Color>{alpha},{red},{green},{blue}</Color>
      </Entry>
      { ...other components }
    </Components>
  </Composite>
</ImageDescription>
```

The XML contains one <Entry> element in the <Phenotypes> block for each phenotype possibility in that Phenotyping Schema, and one <Entry> element in the <Components> block for each component included in that Fluorescence Composite View. Colors are specified as four decimal integers in the range 0-255.

If an image has no composite view, only the PhenotypeSchema block is included; if it has no phenotype map—only the Composite block is included.

APPENDIX G. 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

Select the Adaptive Cell Segmentation radio-button in the Find Features column. (See Figure G.1.)

NOTE: The classic Object-based cell segmentation algorithm is still available using inForm 2.4, but the Counterstain-base option is no longer available.

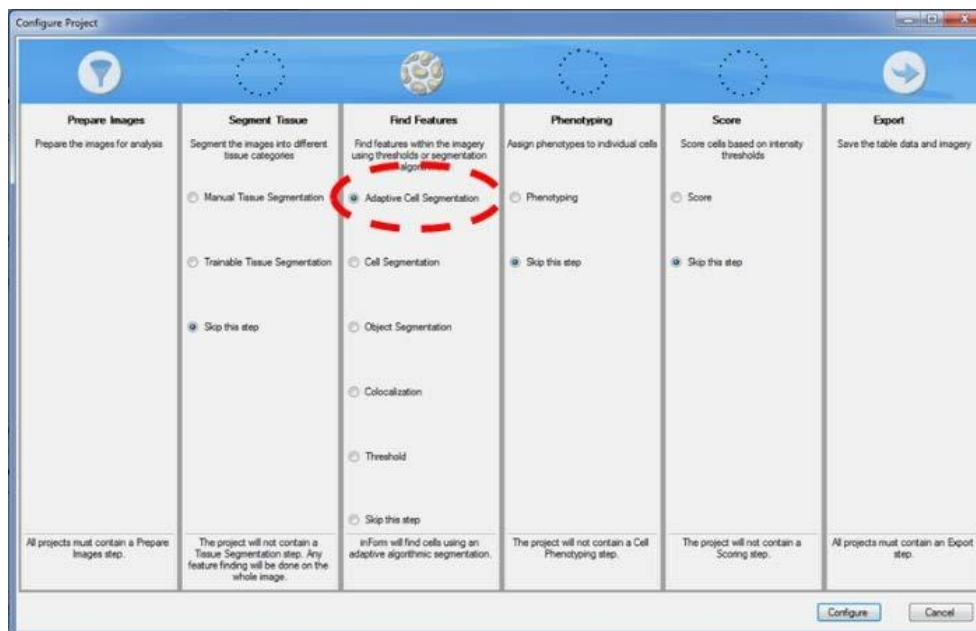


FIGURE G.1. Configure Project – Adaptive Cell Segmentation.

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.
 - a. 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.
 - b. 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.
 - c. The Live Preview Window provides immediate feedback as segmentation parameters are adjusted with each step. (See Figure G.2.) The dotted red circle indicates where to choose which cellular compartments to segment. Red arrows indicate required selections for DAPI configuration.

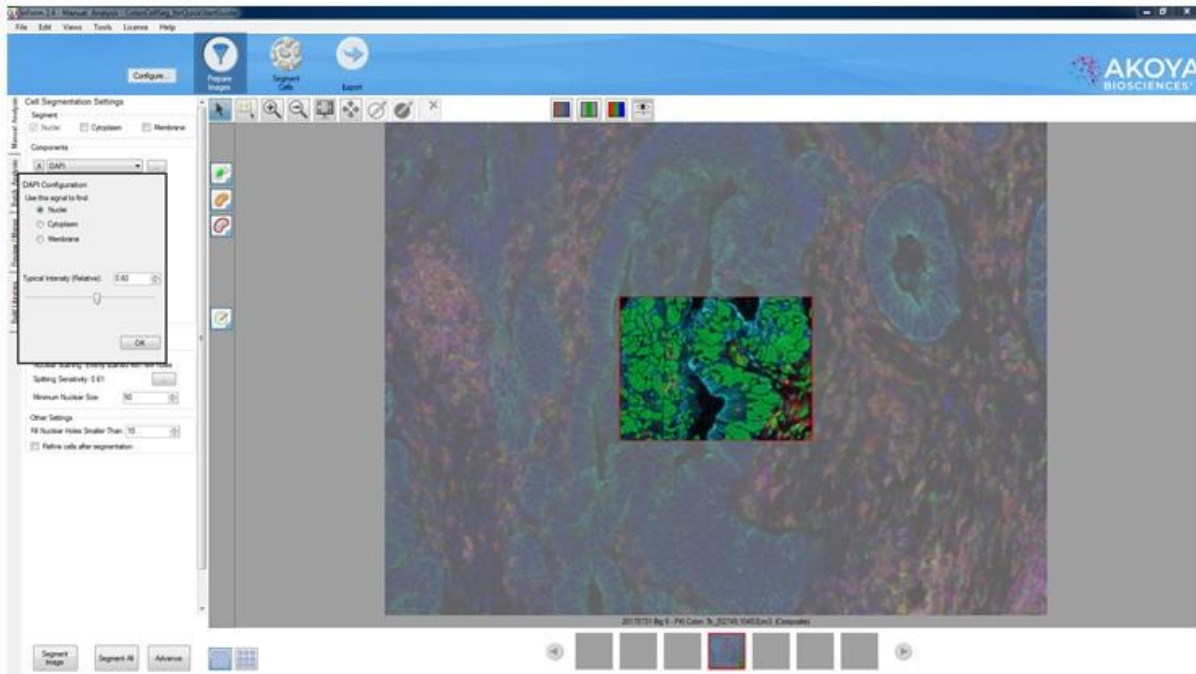


FIGURE G.2. Live Preview - DAPI Configuration Window.

3. 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 14.3.) Moving slider right (higher value) collects less signal. (See Figure 14.4.) 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.

- a. Moving the slider left (lower value) collects more signal from faint nuclei. (See Figure G.3.)



FIGURE G.3. Slide Left - Lower Value - Settings Requiring Configuration.

- b. Moving the slider right (higher value) collects less signal from faint nuclei. (See Figure G.4.)

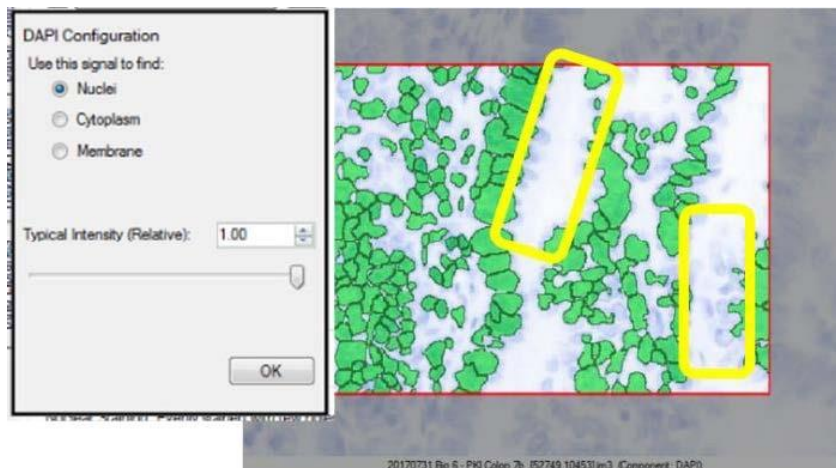


FIGURE G.4. Slider Right - Higher Value.

- 4. 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. (See Figure G.5.)

- a. Use the Typical Intensity (Relative) Slider to adjust the threshold until you have detected all pixels expressing nuclear signals.
 - i. Moving the slider right (higher values) reduces the amount of splitting.
 - ii. Moving the slider left (lower values) increases the amount of splitting.

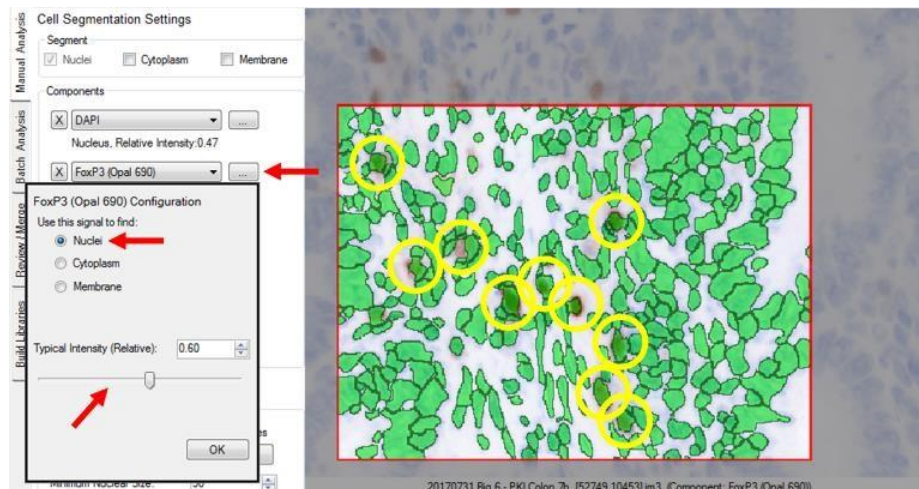


FIGURE G.5. Multiple Nuclear Stains.

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 14.7.) Moving the slider left (lower value) increases the amount of splitting. (See Figure 15.1.) Red arrows indicate the settings requiring configuration for nuclear splitting.

- a. Use the Nuclear Splitting Settings Slider to adjust the splitting threshold:
 - i. Moving the slider right (higher values) reduces the amount of splitting. (See Figure G.6.)

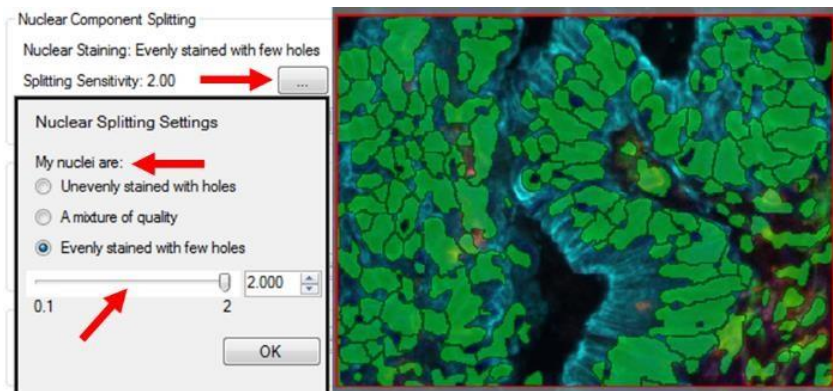


FIGURE G.6. Slider Right - Higher Values.

- ii. Moving the slider left (lower values) increases the amount of splitting. (See Figure G.7.)

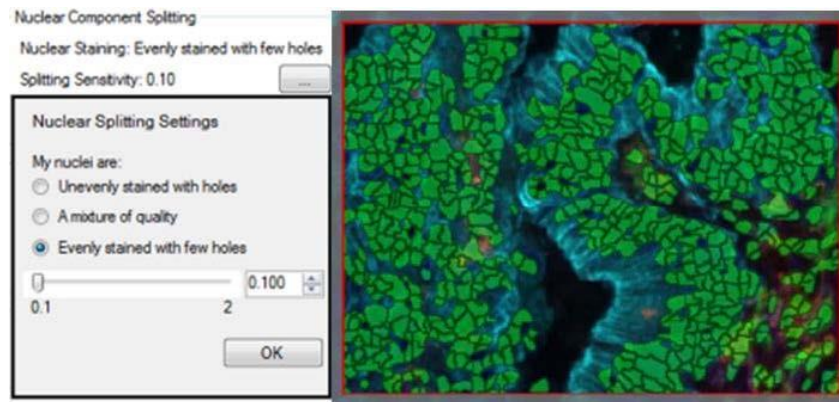


FIGURE G.7. Slider Left - Lower Values.

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

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.

NOTE: *With adaptive cell segmentation, having a 'pan immune marker' such as CD45 may result in better identification of all immune cells.*

NOTE: *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](#).

3. Repeat for other added stains.

Split Using Membrane or Cytoplasm Stains

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.

- Use the Assisting Component Splitting Settings Slider to adjust the splitting threshold:
 - Moving the slider right (higher values) reduces the amount of splitting. (Figure G.8.)

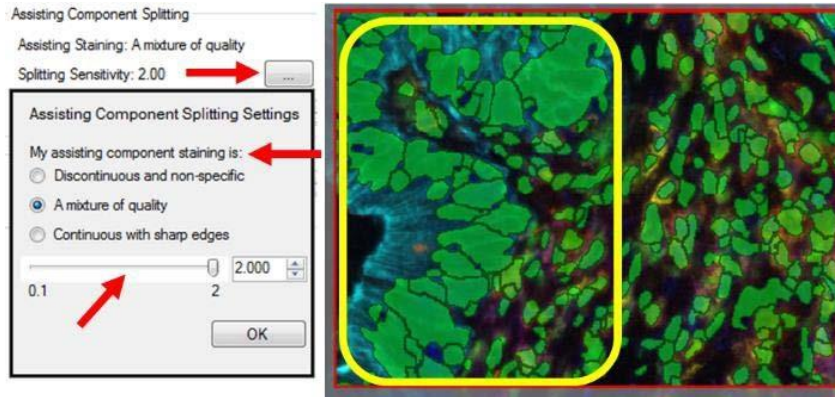


FIGURE G.8. Slider Right - Higher Value.

- o Moving the slider left (lower values) increases the amount of splitting. (See Figure G.9.)

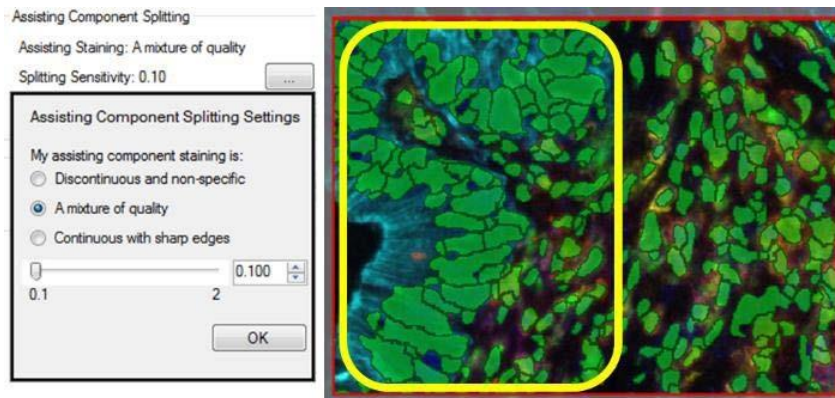


FIGURE G.9. Slider Left - Lower Values.

NOTE: Adjustment of the splitting sensitivity will affect all markers you have selected to assist in nuclear splitting.

Further Refinement of Nuclear Shapes

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

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.
 - b. 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:
 - Membrane Search Distance.
 - Neighboring Cell Membrane.
 - 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 with those that 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. (See Figure G.10.)

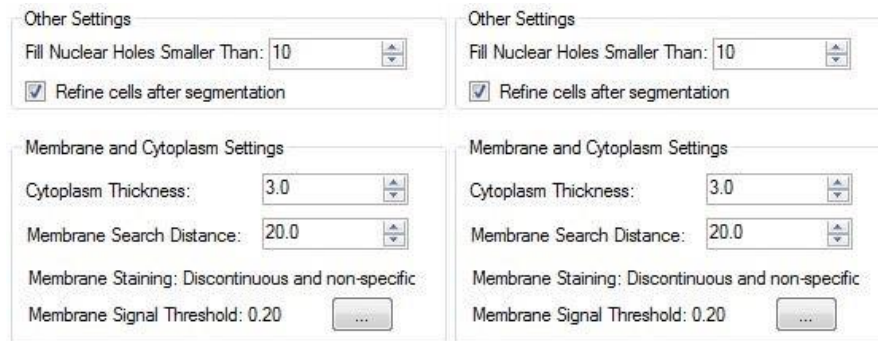


FIGURE G.10. Membrane Search Distance and Cytoplasm Thickness.

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:
 - i. Moving the slider to the right (higher value) collects less signal.

- ii. Moving the slider to the left (lower value) collects more signal from weaker membrane signals. (See Figure G.11.)

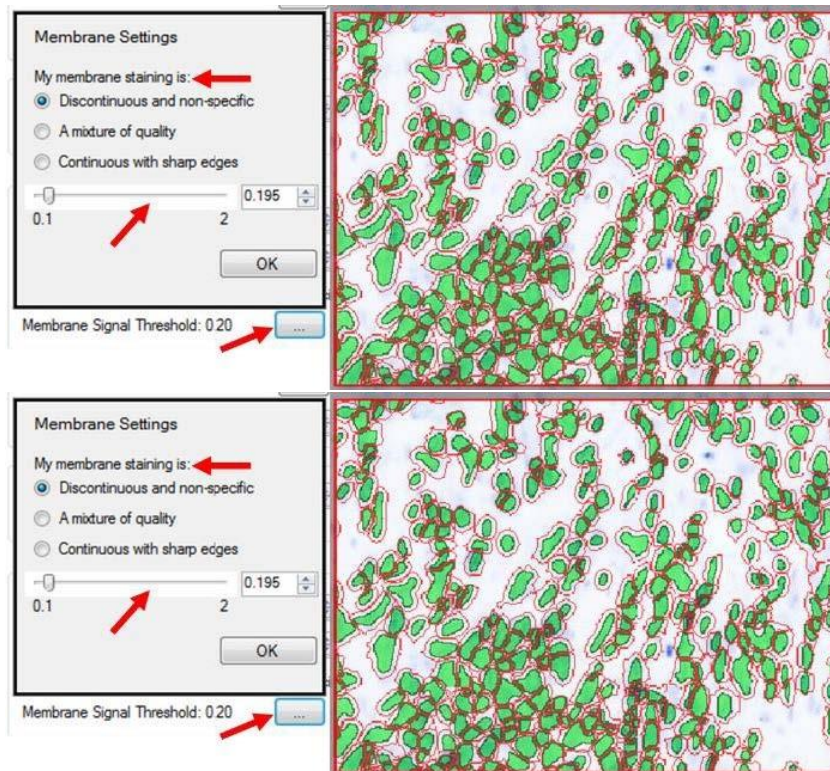


FIGURE G.11. Membrane Detection Threshold.

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 nuclei 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.

APPENDIX H. AKOYA SOFTWARE VERSION COMPATIBILITY

Some files are read by multiple Akoya acquisition and analysis software programs to facilitate unmixing and analysis workflows. Due to major updates in the combined release of Phenolmager HT 2.0.0, Phenochart 2.0.0, and inForm 3.0.0 software, outputs from these versions are not backward compatible with previous versions of software. However, files from previous versions of software are forward compatible. These compatibility considerations are detailed in Table H.1.

TABLE H.1. Akoya software compatibility considerations

	VectraPolaris 1.x.x	Phenolmager HT 2.x.x	Phenochart 1.x.x	Phenochart 2.x.x	inForm 2.x.x	inForm 3.x.x
Scanning protocols created in VectraPolaris 1.x.x can be opened by	Yes	Yes	N/A	N/A	N/A	N/A
Scanning protocols created in Phenolmager HT 2.x.x can be opened by	No	Yes	N/A	N/A	N/A	N/A
QTIFF images acquired with VectraPolaris 1.x.x can be opened by	N/A	N/A	Yes	Yes	Yes	Yes
QTIFF images acquired with Phenolmager HT 2.x.x can be opened by	N/A	N/A	Some *	Yes	No	Yes
IM3 images acquired with VectraPolaris 1.x.x can be opened by	N/A	N/A	N/A	N/A	Yes	Yes
IM3 images acquired with Phenolmager HT 2.x.x can be opened by	N/A	N/A	N/A	N/A	Yes	Yes
Annotations created by Phenochart 1.x.x can be opened by	Yes	Yes	Yes	Yes	Yes	Yes
Annotations created by Phenochart 2.x.x can be opened by	No	Yes	No	Yes	No	Yes

	VectraPolaris 1.x.x	Phenolmager HT 2.x.x	Phenochart 1.x.x	Phenochart 2.x.x	inForm 2.x.x	inForm 3.x.x
Algorithms created by inForm 2.x.x can be opened by	N/A	N/A	N/A	N/A	Yes	Yes
Algorithms created by inForm 3.x.x can be opened by	N/A	N/A	N/A	N/A	No	Yes
Projects created by inForm 2.x.x can be opened by	N/A	N/A	N/A	N/A	Yes	Yes **
Projects created by inForm 3.x.x can be opened by	N/A	N/A	N/A	N/A	No	Yes
Spectra extracted in inForm 2.x.x can be exported as an unmixing library for HT 2.x.x by	N/A	N/A	N/A	N/A	No	Yes
Spectra extracted in inForm 3.x.x can be exported as an unmixing library for HT 2.x.x by	N/A	N/A	N/A	N/A	No	Yes

* Standard 8-bit QTIFFs can be opened for viewing, but not all annotation features are available. Extended Range or Unmixed QTIFFs cannot be opened.

** Two considerations:

1. Due to slight changes to the assignment of cell IDs, phenotyping training sets from inForm 2.x.x cannot be used for re-training phenotyping classifiers in inForm 3.x.x. In this case, inForm 3.x.x will post a message that you can run the algorithm's phenotype step with its current training, or use inForm 2.8 or earlier to add training cells or revise phenotyping.
2. For projects created in inForm 2.8 with imagery that has 10+ channels, phenotyping computation may become unusably slow in inForm 3.x.x. It is recommended that inForm 2.8 projects with imagery that has 10+ channels are only run in inForm 2.8. New projects created in inForm 3.0 do not have this issue.

APPENDIX I. INFORM EULA

The following is an agreement (the "Agreement") between you and Akoya Biosciences, Inc., 100 Campus Drive, 6th Floor, Marlborough, MA 01752 ("Akoya") for software known as Research Use Only inForm®, including any updates, upgrades or enhancement provided by Akoya and any and all accompanying documentation (collectively, the "Software").

AKOYA IS WILLING TO LICENSE THE SOFTWARE TO YOU ONLY UPON THE CONDITION THAT YOU ACCEPT ALL OF THE TERMS CONTAINED IN THIS AGREEMENT. PLEASE READ THE TERMS CAREFULLY BEFORE USING THE SOFTWARE, AS INSTALLING AND/OR USING THE SOFTWARE WILL INDICATE YOUR AGREEMENT TO THEM. IF YOU DO NOT AGREE TO ALL OF THE TERMS AND CONDITIONS IN THIS AGREEMENT, AKOYA IS NOT WILLING TO LICENSE THE SOFTWARE TO YOU, AND YOU (1) MAY NOT USE THE SOFTWARE AND (2) MUST CONTACT AKOYA WITHIN THREE (3) DAYS OF RECEIPT TO ARRANGE FOR THE RETURN OF THE SOFTWARE. BY INSTALLING AND/OR USING THE SOFTWARE, YOU ALSO REPRESENT AND WARRANT THAT YOU HAVE THE AUTHORITY TO AGREE TO AND BE BOUND BY THE TERMS OF THE AGREEMENT.

1. Single Use License. The Software is licensed to you and not sold, and you acknowledge that there is no transfer of title or ownership of the Software to you. Subject to the terms and conditions of this Agreement and your payment of the license fees for the Software, Akoya hereby grants to you a restricted, non-exclusive, non-transferable, non-assignable, non-sublicensable and revocable single-use license to use, for your internal research purposes only, the executable code version of the Software and the accompanying documentation in hard copy or electronic format. Akoya grants no other licenses hereunder, except for the Research Use Only inForm® Software set forth above. Nothing in this Agreement shall be construed as a general internal use license. For the avoidance of doubt, no right to use inForm® in Diagnostic Applications is granted under this Agreement. Akoya RESERVES ALL RIGHTS NOT EXPRESSLY GRANTED BY THIS AGREEMENT.

2. Specific Restrictions. You may use the Software only on a single computer. You may make only one (1) copy of the Software, solely for backup purposes, and such copy must reproduce and include all copyright notices indicating Akoya's ownership of the Software. Except as expressly permitted by applicable law or Akoya, you agree that neither you nor a third party acting on your behalf, directly or indirectly, shall: (i) decompile, disassemble or reverse engineer the Software or otherwise attempt to derive the source code of the Software or underlying ideas, techniques or algorithms, including the review of data structures or similar materials produced by the Software; (ii) modify, alter, translate, or create derivative works of the Software; (iii) lease, rent, loan, commercially share or otherwise use the Software in any manner to provide service bureau, commercial time-sharing or other computer services to third parties; (iv) transmit the Software or provide its functionality, in whole or in part, over the Internet or other network; (v) use the Software for any purpose other than solely for your internal research; (vi) use the Software to provide any analytics or diagnostics or otherwise for the benefit of any third party; (vii) sell, distribute, publish, rent, lease, sublicense, assign possession, allow use of or otherwise transfer the Software or any copy of the Software to a third party; (viii) create any computer software program or documentation which is similar to or competitive with the Software; (ix) encumber, disclose or otherwise make available the Software to any third-party; or (x) remove, modify or obscure any copyright, trademark, or other proprietary rights notice in or on the Software. This Agreement may not be assigned in whole or in part, except that either party may assign this Agreement without the other party's prior written consent in the event of a merger with an affiliate or other reorganization with an affiliate. Any attempted assignment contrary to the terms of this Agreement shall be void.

3. Ownership of Software. As between the parties, Akoya owns all right, title and interest, including all existing or future copyrights, trademarks, tools, know-how, and processes, in and to the Software and any copies, modifications, alterations or derivative works thereof. You shall have no title to or ownership of the Software. As between the parties, Akoya shall retain all rights to market, license and/or sell the Software. The Software (including accompanying documentation) constitutes the unpublished confidential information

and trade secrets of Akoya. You (i) acknowledge, and agree not to contest, Akoya's rights in the Software; and (ii) agree not to disclose and to take reasonable steps to protect any and all confidential information of Akoya regarding the Software or that is otherwise disclosed to you in connection with this Agreement. You agree that you are responsible for ensuring your employees and agents are prohibited from taking any action otherwise prohibited to you under this Agreement and to notify Akoya promptly and in writing of the circumstances surrounding any possession, use or knowledge of the Software or any part thereof known to you which is prohibited hereunder. You recognize that the covenants contained in this Agreement are reasonable and necessary to protect the legitimate interests of Akoya, that Akoya would not have entered into this Agreement in the absence of such covenants, and that your breach or threatened breach of such covenants shall cause Akoya irreparable harm and significant injury, the amount of which shall be extremely difficult to estimate and ascertain, thus making any remedy at law or in damages inadequate. Therefore, you agree that Akoya shall be entitled, without the necessity of posting any bond or security, to the issuance of injunctive relief by any court of competent jurisdiction enjoining any breach or threatened breach of such covenants and for any other relief such court deems appropriate. This right shall be in addition to any other remedy available to Akoya at law or in equity.

4. Termination. The license granted in Section 1 above is effective until terminated. This Agreement is conditioned upon your continued compliance with the terms and conditions hereof and will terminate automatically without notice from Akoya if you fail to comply with any term or condition of this Agreement. Furthermore, Akoya may terminate this Agreement at any time upon thirty (30) days' notice. Upon termination of this Agreement, your license to use the Software or any part thereof shall immediately terminate, and you shall immediately destroy all copies of the Software (including all accompanying documentation) and any other confidential and proprietary information you have received during or in connection with this Agreement. Termination of this Agreement by Akoya shall not entitle you to a refund of any fees paid for the license of the Software.

5. Limited Warranty. The sole and exclusive warranty applicable to software and firmware products provided by Akoya for use with a processor is as follows: Akoya warrants that such software and firmware will substantially conform to Akoya's documentation and program manuals current at the time of shipment to you when properly installed on the processor. The limited warranty set forth above shall not apply if the claimed problem or issue is caused by: the malfunction of computer hardware or software not provided by Akoya; your negligence or fault; your failure to follow the instructions set forth in the documentation; changes in the hardware or other software not authorized by Akoya; modifications or changes to the Software not made or suggested by Akoya; or your failure to implement and maintain a proper backup and recovery system. Your exclusive remedy under this Section 5 shall be, at Akoya's option, the replacement or correction of the problem or issue causing the non-conformance with the above warranty as promptly as possible following notice of such non-conformity, or a refund of the price paid for the Software.

6. No Other Warranties. EXCEPT FOR THE LIMITED WARRANTY STATED IMMEDIATELY ABOVE AND TO THE MAXIMUM EXTENT PERMITTED BY APPLICABLE LAW, THE SOFTWARE IS PROVIDED "AS IS" WITHOUT WARRANTY OF ANY KIND, AND AKOYA EXPRESSLY DISCLAIMS ANY AND ALL IMPLIED WARRANTIES, INCLUDING WITHOUT LIMITATION ANY WARRANTY OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE, OR NON-INFRINGEMENT OF THIRD PARTY RIGHTS. AKOYA DOES NOT REPRESENT OR WARRANT THAT THE RESULTS OR THE USE OF THE SOFTWARE WILL BE CORRECT, ACCURATE OR RELIABLE, WILL MEET YOUR REQUIREMENTS, OR THAT THE SOFTWARE WILL OPERATE UNINTERRUPTED OR ERROR-FREE, OR THAT DEFECTS IN THE SOFTWARE WILL BE CORRECTED. Some jurisdictions do not allow the exclusion of implied warranties, so the above disclaimer may not apply to you. YOU ASSUME ALL RISK ASSOCIATED WITH THE USE, RESULTS, AND PERFORMANCE OF THE SOFTWARE.

7. Limitation of Liability. IN NO EVENT SHALL AKOYA, ITS AFFILIATES OR SUPPLIERS, OR THEIR RESPECTIVE EMPLOYEES, OFFICERS OR AGENTS, BE LIABLE FOR ANY DAMAGES ARISING OUT OF THE DELIVERY, INSTALLATION, PERFORMANCE, OR USE OR INABILITY TO USE THE SOFTWARE, INCLUDING WITHOUT LIMITATION INCIDENTAL, SPECIAL, CONSEQUENTIAL, PUNITIVE, EXEMPLARY, INDIRECT OR DIRECT DAMAGES (INCLUDING WITHOUT LIMITATION DAMAGES FOR LOSS OF PROFITS, LOSS OF DATA, RE-RUN

TIME, INACCURATE INPUT, WORK DELAYS, BUSINESS INTERRUPTION OR ANY OTHER COMMERCIAL DAMAGES OR LOSSES), WHETHER IN AN ACTION IN CONTRACT, TORT (INCLUDING NEGLIGENCE AND STRICT LIABILITY) OR OTHERWISE, AND EVEN IF AKOYA HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES, AND REGARDLESS OF WHETHER ANY REMEDY FAILS OF ITS ESSENTIAL PURPOSE. AKOYA'S AGGREGATE LIABILITY ARISING OUT OF OR RELATING TO THIS AGREEMENT, FOR ANY AND ALL DAMAGES THAT YOU MIGHT INCUR (REGARDLESS OF THE FORM OF ACTION GIVING RISE TO SUCH LIABILITY, WHETHER IN CONTRACT, TORT OR OTHERWISE) SHALL NOT EXCEED THE PRICE PAID BY YOU FOR THE LICENSE OF THE SOFTWARE. Some jurisdictions do not allow the exclusion or limitation of liability for consequential or incidental damages. In such jurisdictions, our liability is limited to the greatest extent permitted by law.

8. U.S. Government End Users. The Software qualifies as commercial computer software for purposes of the Federal Acquisition Regulations (FAR) §52.227-19 and the Department of Defense Supplement to the FAR (DFARS) §52.227.7013. If the Software is acquired by a civilian government agency, it is furnished with only the minimum Restricted Rights provided by FAR 52.227-19. If the Software is acquired by a military agency, it is furnished with only the minimum Restricted Rights provided by DFARS 52.227-7013(c)(1)(ii).

9. Miscellaneous. This Agreement contains the entire agreement of the parties with respect to the subject matter hereof and supersedes any proposal or prior agreement, written or oral, and any other communications between the parties relating to the subject matter hereof. No modification or waiver of any provision of this Agreement shall be effective unless in writing and signed by the parties. No delay or failure on the part of any party in exercising any right under this Agreement shall impair any such right or any remedy of such party, nor shall it be construed to be a waiver of any continuing breach or default under this Agreement. No action, regardless of form, arising out of the transactions contemplated herein may be brought by either party more than one (1) year after the cause of action has accrued. In the event any provision of this Agreement is held to be unenforceable, the remaining provisions of this Agreement will remain in full force and effect. This Agreement shall be governed by the laws of the State of Delaware without regard to principles of conflicts of laws. Any disputes relating hereto shall be adjudicated only in any court of competent jurisdiction in the State of Delaware, and you hereby consent to the exclusive jurisdiction of those courts. This Agreement shall not be governed by the United Nations Convention on Contracts for the International Sale of Goods, the application of which is expressly excluded. You may not assign or otherwise transfer this Agreement or any of your rights or obligations therein without the prior written consent of Akoya. You may not use the Software for any unlawful purpose nor export or re-export the Software except as authorized by law.

10. TIFF module: This software uses the LibTIFF 4.0.6 library (<http://www.libtiff.org/>)

The TIFF module used is Copyright © 1988-1997 Sam Leffler Copyright © 1991-1997 Silicon Graphics, Inc.

Permission to use, copy, modify, distribute, and sell this software and its documentation for any purpose is hereby granted without fee, provided that (i) the above copyright notices and this permission notice appear in all copies of the software and related documentation, and (ii) the names of Sam Leffler and Silicon Graphics may not be used in any advertising or publicity relating to the software without the specific, prior written permission of Sam Leffler and Silicon Graphics.

THE SOFTWARE IS PROVIDED "AS-IS" AND WITHOUT WARRANTY OF ANY KIND, EXPRESS, IMPLIED OR OTHERWISE, INCLUDING WITHOUT LIMITATION, ANY WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE.

IN NO EVENT SHALL SAM LEFFLER OR SILICON GRAPHICS BE LIABLE FOR ANY SPECIAL, INCIDENTAL, INDIRECT OR CONSEQUENTIAL DAMAGES OF ANY KIND, OR ANY DAMAGES WHATSOEVER RESULTING FROM LOSS OF USE, DATA OR PROFITS, WHETHER OR NOT ADVISED OF THE POSSIBILITY OF DAMAGE, AND ON ANY THEORY OF LIABILITY, ARISING OUT OF OR IN CONNECTION WITH THE USE OR PERFORMANCE OF THIS SOFTWARE.

11. This software uses the **Vigra 1.6.0 library** (<http://hci.iwr.uni-heidelberg.de/vigra/>)

This software includes machine-executable object code generated by a source language processor from the Vigra libraries covered by the VIGRA license. These libraries have not been modified by Akoya and all rights are reserved by the copyright holder. This software is provided "as is" without express or implied warranty, and with no claim as to its suitability for any purpose.

The Vigra library is Copyright © 2008, Ulrich Kothe, Heidelberg Collaboratory for Image Processing

The VIGRA License

=====

(identical to the MIT X11 License)

Permission is hereby granted, free of charge, to any person obtaining a copy of this software and associated documentation files (the "Software"), to deal in the Software without restriction, including without limitation the rights to use, copy, modify, merge, publish, distribute, sublicense, and/or sell copies of the Software, and to permit persons to whom the Software is furnished to do so, subject to the following conditions:

The above copyright notice and this permission notice shall be included in all copies or substantial portions of the Software.

THE SOFTWARE IS PROVIDED "AS IS", WITHOUT WARRANTY OF ANY KIND EXPRESS OR IMPLIED, INCLUDING BUT NOT LIMITED TO THE WARRANTIES OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE AND NONINFRINGEMENT. IN NO EVENT SHALL THE AUTHORS OR COPYRIGHT HOLDERS BE LIABLE FOR ANY CLAIM, DAMAGES OR OTHER LIABILITY, WHETHER IN AN ACTION OF CONTRACT, TORT OR OTHERWISE, ARISING FROM, OUT OF OR IN CONNECTION WITH THE SOFTWARE OR THE USE OR OTHER DEALINGS IN THE SOFTWARE.

12. This software uses the **Boost 1.68.0 library** (<http://www.boost.org/>)

This software includes machine-executable object code generated by a source language processor from the Boost libraries covered by the Boost license. These libraries have not been modified by Akoya and all rights are reserved by the copyright holder. This software is provided "as is" without express or implied warranty, and with no claim as to its suitability for any purpose.

The Boost license is reproduced below:

Boost Software License - Version 1.0 - August 17th, 2003

Permission is hereby granted, free of charge, to any person or organization obtaining a copy of the software and accompanying documentation covered by this license (the "Software") to use, reproduce, display, distribute, execute, and transmit the Software, and to prepare derivative works of the Software, and to permit third-parties to whom the Software is furnished to do so, all subject to the following:

The copyright notices in the Software and this entire statement, including the above license grant, this restriction and the following disclaimer, must be included in all copies of the Software, in whole or in part, and all derivative works of the Software, unless such copies or derivative works are solely in the form of machine-executable object code generated by a source language processor.

THE SOFTWARE IS PROVIDED "AS IS", WITHOUT WARRANTY OF ANY KIND, EXPRESS OR IMPLIED, INCLUDING BUT NOT LIMITED TO THE WARRANTIES OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE, TITLE AND NON-INFRINGEMENT. IN NO EVENT SHALL THE COPYRIGHT HOLDERS OR ANYONE DISTRIBUTING THE SOFTWARE BE LIABLE FOR ANY DAMAGES OR OTHER LIABILITY, WHETHER IN CONTRACT, TORT OR OTHERWISE, ARISING FROM, OUT OF OR IN CONNECTION WITH THE SOFTWARE OR THE USE OR OTHER DEALINGS IN THE SOFTWARE.

13. This software uses the **CommandLine 1.6.0.0 library** (<https://github.com/commandlineparser/commandline>)

This software makes use of the CommandLine libraries. These libraries have not been modified by Akoya and all rights are reserved by the copyright holder. This software is provided "as is" without express or implied

warranty, and with no claim as to its suitability for any purpose. The libraries are covered by the MIT License, which is reproduced below:

The MIT License (MIT)

Copyright (c) 2005 - 2012 Giacomo Stelluti Scala

Permission is hereby granted, free of charge, to any person obtaining a copy of this software and associated documentation files (the "Software"), to deal in the Software without restriction, including without limitation the rights to use, copy, modify, merge, publish, distribute, sublicense, and/or sell copies of the Software, and to permit persons to whom the Software is furnished to do so, subject to the following conditions:

The above copyright notice and this permission notice shall be included in all copies or substantial portions of the Software.

THE SOFTWARE IS PROVIDED "AS IS", WITHOUT WARRANTY OF ANY KIND, EXPRESS OR IMPLIED, INCLUDING BUT NOT LIMITED TO THE WARRANTIES OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE AND NONINFRINGEMENT. IN NO EVENT SHALL THE AUTHORS OR COPYRIGHT HOLDERS BE LIABLE FOR ANY CLAIM, DAMAGES OR OTHER LIABILITY, WHETHER IN AN ACTION OF CONTRACT, TORT OR OTHERWISE, ARISING FROM, OUT OF OR IN CONNECTION WITH THE SOFTWARE OR THE USE OR OTHER DEALINGS IN THE SOFTWARE.

14. This software uses the NLog libraries (<https://github.com/NLog/NLog>)

This software makes use of the NLog libraries. These libraries have not been modified by Akoya and all rights are reserved by the copyright holder. This software is provided "as is" without express or implied warranty, and with no claim as to its suitability for any purpose. The libraries are covered by the BSD license, which is reproduced below:

The BSD for NLog license:

Copyright (c) 2004-2021 Jaroslaw Kowalski <jaak@jkwalski.net>, Kim Christensen, Julian Verdurmen. All rights reserved.

Redistribution and use in source and binary forms, with or without modification, are permitted provided that the following conditions are met:

- * Redistributions of source code must retain the above copyright notice, this list of conditions and the following disclaimer.
- * Redistributions in binary form must reproduce the above copyright notice, this list of conditions and the following disclaimer in the documentation and/or other materials provided with the distribution.
- * Neither the name of Jaroslaw Kowalski nor the names of its contributors may be used to endorse or promote products derived from this software without specific prior written permission.

THIS SOFTWARE IS PROVIDED BY THE COPYRIGHT HOLDERS AND CONTRIBUTORS "AS IS" AND ANY EXPRESS OR IMPLIED WARRANTIES, INCLUDING, BUT NOT LIMITED TO, THE IMPLIED WARRANTIES OF MERCHANTABILITY AND FITNESS FOR A PARTICULAR PURPOSE ARE DISCLAIMED. IN NO EVENT SHALL THE COPYRIGHT OWNER OR CONTRIBUTORS BE LIABLE FOR ANY DIRECT, INDIRECT, INCIDENTAL, SPECIAL, EXEMPLARY, OR CONSEQUENTIAL DAMAGES (INCLUDING, BUT NOT LIMITED TO, PROCUREMENT OF SUBSTITUTE GOODS OR SERVICES; LOSS OF USE, DATA, OR PROFITS; OR BUSINESS INTERRUPTION) HOWEVER CAUSED AND ON ANY THEORY OF LIABILITY, WHETHER IN CONTRACT, STRICT LIABILITY, OR TORT (INCLUDING NEGLIGENCE OR OTHERWISE) ARISING IN ANY WAY OUT OF THE USE OF THIS SOFTWARE, EVEN IF ADVISED OF THE POSSIBILITY OF SUCH DAMAGE.

15. This software uses the bzip2 program and libbzip2 libraries (<http://www.bzip.org>)

This software makes use of the bzip2 program and associated libbzip2 libraries. The program and libraries have not been modified by Akoya and all rights are reserved by the copyright holder. This software is provided "as is" without express or implied warranty, and with no claim as to its suitability for any purpose.

The libraries are covered by the bzip2 license, which is reproduced below:

This program, "bzip2", the associated library "libbzip2", and all documentation, are copyright (C) 1996-2006 Julian R Seward. All rights reserved.

Redistribution and use in source and binary forms, with or without modification, are permitted provided that the following conditions are met:

1. Redistributions of source code must retain the above copyright notice, this list of conditions and the following disclaimer.
2. The origin of this software must not be misrepresented; you must not claim that you wrote the original software. If you use this software in a product, an acknowledgment in the product documentation would be appreciated but is not required.
3. Altered source versions must be plainly marked as such, and must not be misrepresented as being the original software.
4. The name of the author may not be used to endorse or promote products derived from this software without specific prior written permission.

THIS SOFTWARE IS PROVIDED BY THE AUTHOR ``AS IS" AND ANY EXPRESS OR IMPLIED WARRANTIES, INCLUDING, BUT NOT LIMITED TO, THE IMPLIED WARRANTIES OF MERCHANTABILITY AND FITNESS FOR A PARTICULAR PURPOSE ARE DISCLAIMED. IN NO EVENT SHALL THE AUTHOR BE LIABLE FOR ANY DIRECT, INDIRECT, INCIDENTAL, SPECIAL, EXEMPLARY, OR CONSEQUENTIAL DAMAGES (INCLUDING, BUT NOT LIMITED TO, PROCUREMENT OF SUBSTITUTE GOODS OR SERVICES; LOSS OF USE, DATA, OR PROFITS; OR BUSINESS INTERRUPTION) HOWEVER CAUSED AND ON ANY THEORY OF LIABILITY, WHETHER IN CONTRACT, STRICT LIABILITY, OR TORT (INCLUDING NEGLIGENCE OR OTHERWISE) ARISING IN ANY WAY OUT OF THE USE OF THIS SOFTWARE, EVEN IF ADVISED OF THE POSSIBILITY OF SUCH DAMAGE.

Julian Seward, Cambridge, UK.

jseward@bzip.org

bzip2/libbzip2 version 1.0.4 of 20 December 2006

16. This software uses the libjpeg-turbo 1.5.0 library (<http://libjpeg-turbo.virtualgl.org>)

This software makes use of the libjpeg-turbo library. This library has not been modified by Akoya and all rights are reserved by the copyright holder. This software is provided "as is" without express or implied warranty, and with no claim as to its suitability for any purpose. The library is covered by the libjpeg-turbo License. Additionally, libjpeg-turbo incorporates parts of libjpeg which is covered by the BSD license.

The libjpeg-turbo license is reproduced below:

Redistribution and use in source and binary forms, with or without modification, are permitted provided that the following conditions are met:

- Redistributions of source code must retain the above copyright notice, this list of conditions and the following disclaimer.
- Redistributions in binary form must reproduce the above copyright notice, this list of conditions and the following disclaimer in the documentation and/or other materials provided with the distribution.
- Neither the name of the libjpeg-turbo Project nor the names of its contributors may be used to endorse or promote products derived from this software without specific prior written permission.

THIS SOFTWARE IS PROVIDED BY THE COPYRIGHT HOLDERS AND CONTRIBUTORS "AS IS", AND ANY EXPRESS OR IMPLIED WARRANTIES, INCLUDING, BUT NOT LIMITED TO, THE IMPLIED WARRANTIES OF MERCHANTABILITY AND FITNESS FOR A PARTICULAR PURPOSE ARE DISCLAIMED. IN NO EVENT SHALL THE COPYRIGHT HOLDERS OR CONTRIBUTORS BE LIABLE FOR ANY DIRECT, INDIRECT, INCIDENTAL, SPECIAL, EXEMPLARY, OR CONSEQUENTIAL DAMAGES (INCLUDING, BUT NOT LIMITED TO, PROCUREMENT OF SUBSTITUTE GOODS OR SERVICES; LOSS OF USE, DATA, OR PROFITS; OR BUSINESS INTERRUPTION) HOWEVER CAUSED AND ON ANY THEORY OF LIABILITY, WHETHER IN CONTRACT, STRICT LIABILITY, OR

TORT (INCLUDING NEGLIGENCE OR OTHERWISE) ARISING IN ANY WAY OUT OF THE USE OF THIS SOFTWARE, EVEN IF ADVISED OF THE POSSIBILITY OF SUCH DAMAGE.

The BSD for libjpeg license is reproduced below:

The authors make NO WARRANTY or representation, either express or implied, with respect to this software, its quality, accuracy, merchantability, or fitness for a particular purpose. This software is provided "AS IS", and you, its user, assume the entire risk as to its quality and accuracy.

This software is copyright (C) 1991-2010, Thomas G. Lane, Guido Vollbeding.

All Rights Reserved except as specified below.

Permission is hereby granted to use, copy, modify, and distribute this software (or portions thereof) for any purpose, without fee, subject to these conditions:

(1) If any part of the source code for this software is distributed, then this README file must be included, with this copyright and no-warranty notice unaltered; and any additions, deletions, or changes to the original files must be clearly indicated in accompanying documentation.

(2) If only executable code is distributed, then the accompanying documentation must state that "this software is based in part on the work of the Independent JPEG Group".

(3) Permission for use of this software is granted only if the user accepts full responsibility for any undesirable consequences; the authors accept NO LIABILITY for damages of any kind.

These conditions apply to any software derived from or based on the IJG code, not just to the unmodified library. If you use our work, you ought to acknowledge us.

Permission is NOT granted for the use of any IJG author's name or company name in advertising or publicity relating to this software or products derived from it. This software may be referred to only as "the Independent JPEG Group's software".

We specifically permit and encourage the use of this software as the basis of commercial products, provided that all warranty or liability claims are assumed by the product vendor.

17. This software uses the zlib 1.2.8 library (<http://www.zlib.net>)

This software makes use of the zlib library. This library has not been modified by Akoya and all rights are reserved by the copyright holder. This software is provided "as is" without express or implied warranty, and with no claim as to its suitability for any purpose. The library is covered by the zlib License.

The zlib 1 license is reproduced below:

(C) 1995-2012 Jean-loup Gailly and Mark Adler

This software is provided 'as-is', without any express or implied warranty. In no event will the authors be held liable for any damages arising from the use of this software.

Permission is granted to anyone to use this software for any purpose, including commercial applications, and to alter it and redistribute it freely, subject to the following restrictions:

1. The origin of this software must not be misrepresented; you must not claim that you wrote the original software. If you use this software in a product, an acknowledgment in the product documentation would be appreciated but is not required.
2. Altered source versions must be plainly marked as such, and must not be misrepresented as being the original software.
3. This notice may not be removed or altered from any source distribution.

18. This software may use the RServeCLI2 library (<https://github.com/SurajGupta/RserveCLI2>)

Original work Copyright (c) 2011 Oliver M. Haynold and other contributors.

Modified work Copyright (c) 2013 Suraj Gupta

All rights reserved.

Redistribution and use in source and binary forms, with or without modification, are permitted provided that the following conditions are met:

* Redistributions of source code must retain the above copyright notice, this list of conditions and the following disclaimer.

* Redistributions in binary form must reproduce the above copyright notice, this list of conditions and the following disclaimer in the documentation and/or other materials provided with the distribution.

* Neither the name of the copyright holder(s) nor the names of its contributors may be used to endorse or promote products derived from this software without specific prior written permission.

THIS SOFTWARE IS PROVIDED BY THE COPYRIGHT HOLDERS AND CONTRIBUTORS "AS IS" AND ANY EXPRESS OR IMPLIED WARRANTIES, INCLUDING, BUT NOT LIMITED TO, THE IMPLIED WARRANTIES OF MERCHANTABILITY AND FITNESS FOR A PARTICULAR PURPOSE ARE DISCLAIMED. IN NO EVENT SHALL THE COPYRIGHT HOLDER(S) BE LIABLE FOR ANY DIRECT, INDIRECT, INCIDENTAL, SPECIAL, EXEMPLARY, OR CONSEQUENTIAL DAMAGES (INCLUDING, BUT NOT LIMITED TO, PROCUREMENT OF SUBSTITUTE GOODS OR SERVICES; LOSS OF USE, DATA, OR PROFITS; OR BUSINESS INTERRUPTION) HOWEVER CAUSED AND ON ANY THEORY OF LIABILITY, WHETHER IN CONTRACT, STRICT LIABILITY, OR TORT (INCLUDING NEGLIGENCE OR OTHERWISE) ARISING IN ANY WAY OUT OF THE USE OF THIS SOFTWARE, EVEN IF ADVISED OF THE POSSIBILITY OF SUCH DAMAGE.

19. This software uses Newtonsoft.Json library (<https://www.newtonsoft.com/json>)

This software makes use of the Newtonsoft JSON libraries. These libraries have not been modified by Akoya and all rights are reserved by the copyright holder. This software is provided "as is" without express or implied warranty, and with no claim as to its suitability for any purpose. The libraries are covered by the MIT license, which is reproduced below:

The MIT License (MIT)

Copyright (c) 2007 James Newton-King

Permission is hereby granted, free of charge, to any person obtaining a copy of this software and associated documentation files (the "Software"), to deal in the Software without restriction, including without limitation the rights to use, copy, modify, merge, publish, distribute, sublicense, and/or sell copies of the Software, and to permit persons to whom the Software is furnished to do so, subject to the following conditions:

The above copyright notice and this permission notice shall be included in all copies or substantial portions of the Software.

THE SOFTWARE IS PROVIDED "AS IS", WITHOUT WARRANTY OF ANY KIND, EXPRESS OR IMPLIED, INCLUDING BUT NOT LIMITED TO THE WARRANTIES OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE AND NONINFRINGEMENT. IN NO EVENT SHALL THE AUTHORS OR COPYRIGHT HOLDERS BE LIABLE FOR ANY CLAIM, DAMAGES OR OTHER LIABILITY, WHETHER IN AN ACTION OF CONTRACT, TORT OR OTHERWISE, ARISING FROM, OUT OF OR IN CONNECTION WITH THE SOFTWARE OR THE USE OR OTHER DEALINGS IN THE SOFTWARE.

Should you have any question concerning this Agreement, you may contact Akoya by writing to Akoya Biosciences, 100 Campus Drive, 6th Floor, Marlborough, MA 10752. You may also call +1 855.896.8401

To learn more visit AKOYABIO.COM or email us at INFO@AKOYABIO.COM

For Research Use Only. Not for use in diagnostic procedures.

© 2023 Akoya Biosciences, Inc. All rights reserved. All trademarks are the property of Akoya Biosciences unless otherwise specified.

SW-000001 Rev K