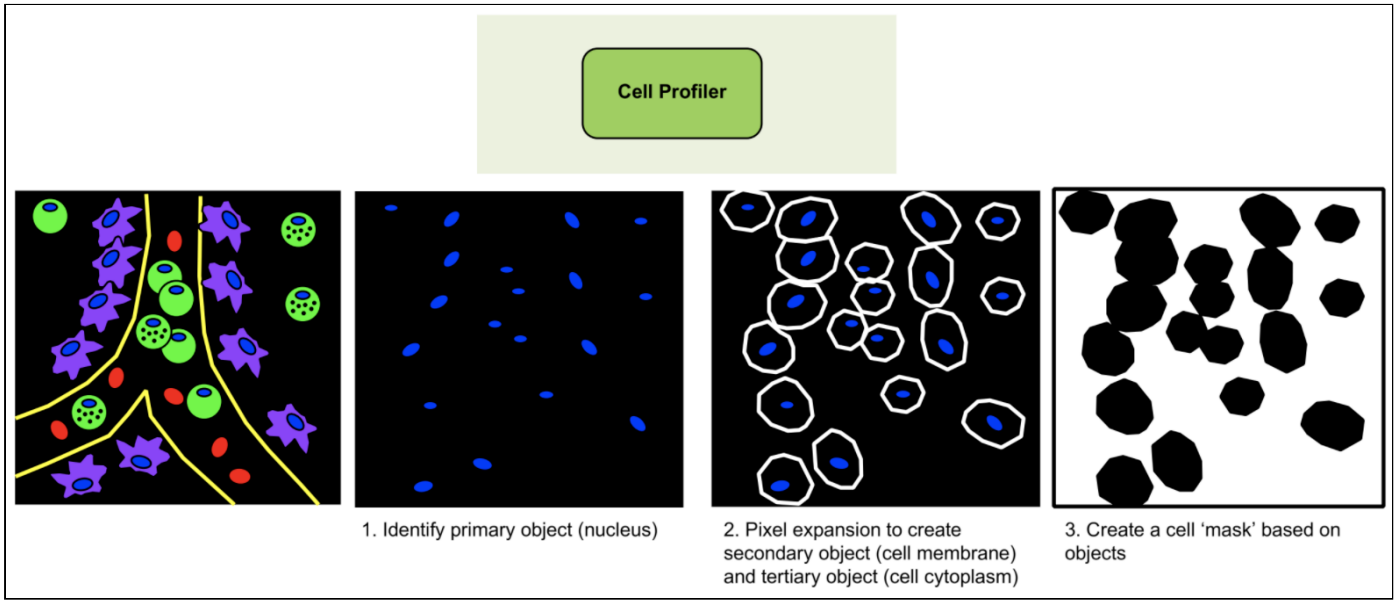
Introduction

This is an analysis pipeline to perform *basic* segmentation of Imaging Mass Cytometry (IMC) images. Essentially, the nucleus in each ROI is identified, and a nuclear 'mask' is generated. Subsequently, the shape of this mask is expanded by a number of pixels, creating a cellular mask, and also a cytoplasmic mask. This is known as 'pixel expansion'. This method of cell segmentation is very simple, and works for many situation, but is not recommended for images with dense cell communities – for these, the intermediate or advanced segmentation pipelines are recommended.



Software installation

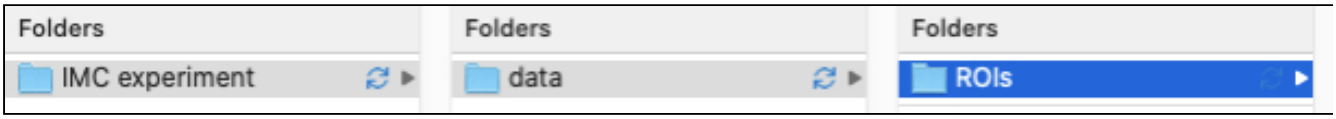
To use this workflow, you will need to install R/RStudio, the Spectre, and CellProfiler (note: you do **not** need the custom IMC modules listed on the linked CellProfiler page).

If you are unfamiliar with using R, RStudio, or Spectre, check out our 'getting started' page.

Setup

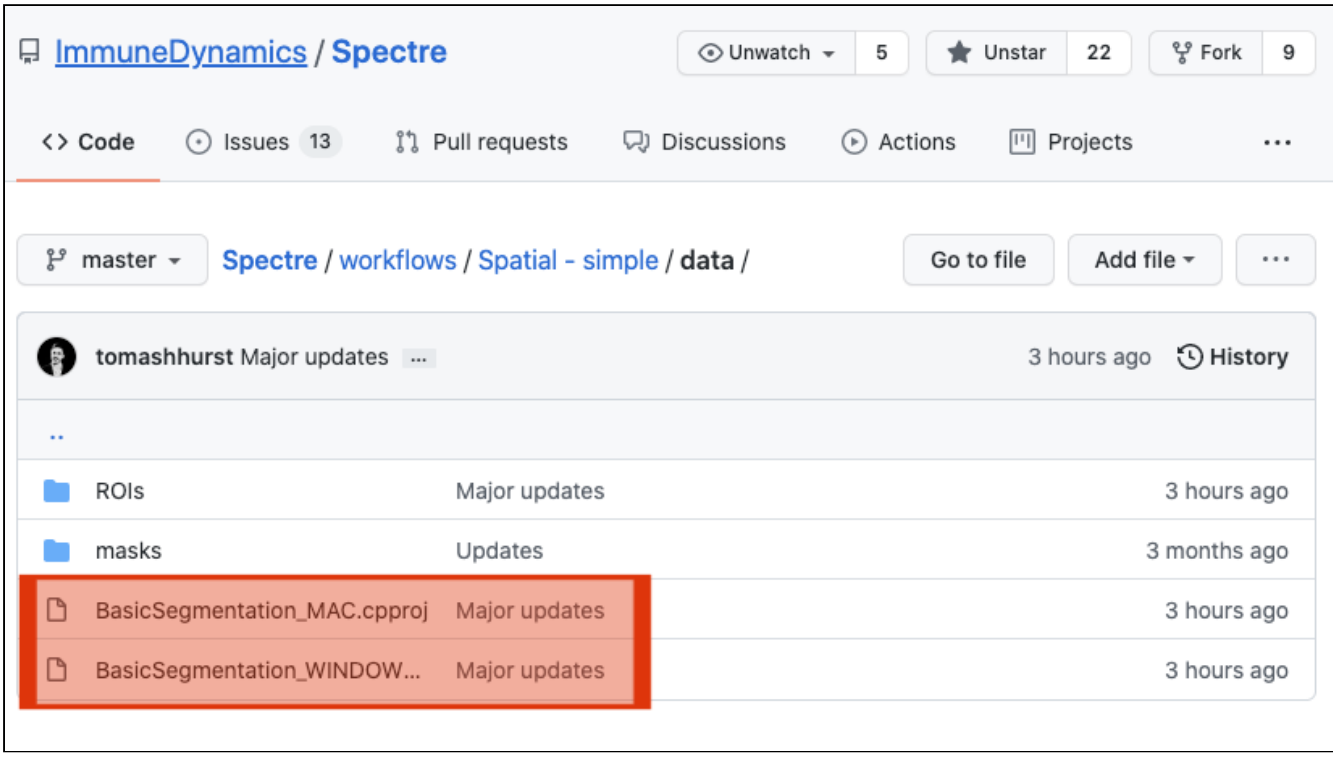
Experiment folder

Create a folder for your experiment, and then create a subfolder called 'data', another subfolder under that one called 'ROIs'.

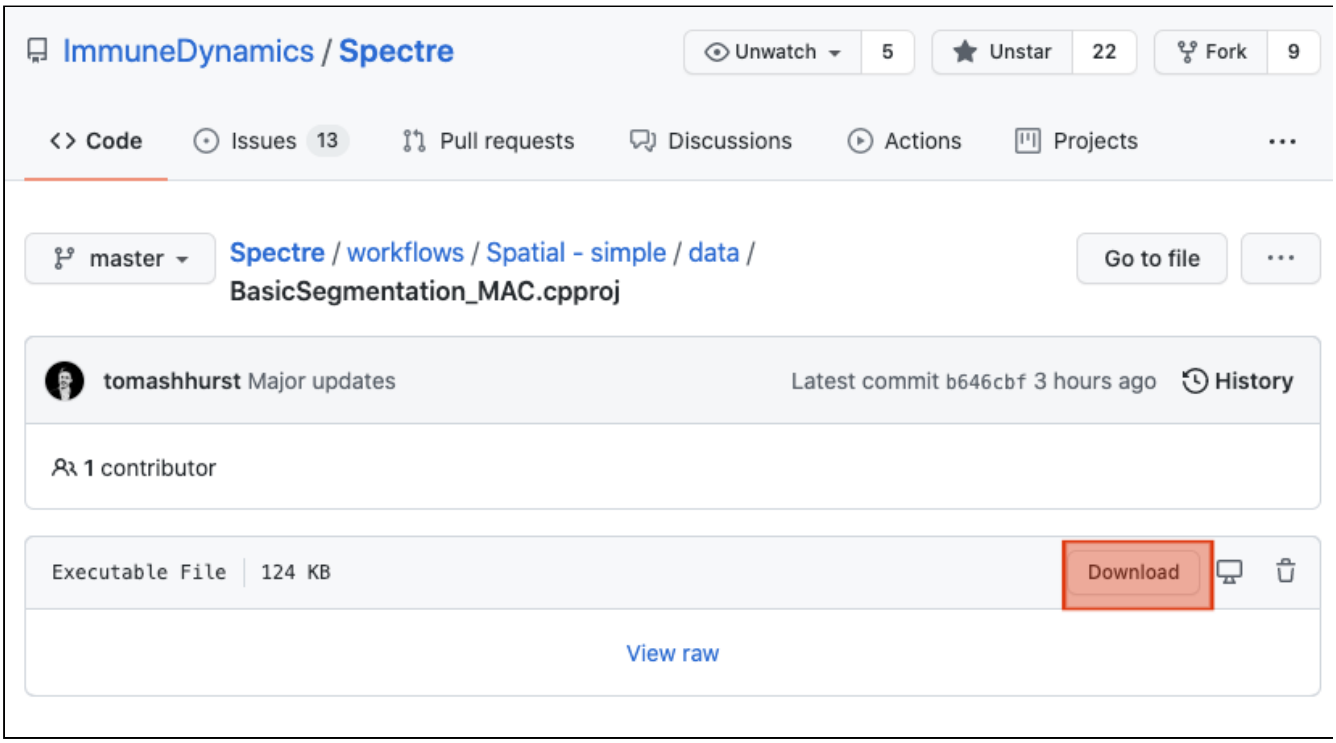


Download CellProfiler template

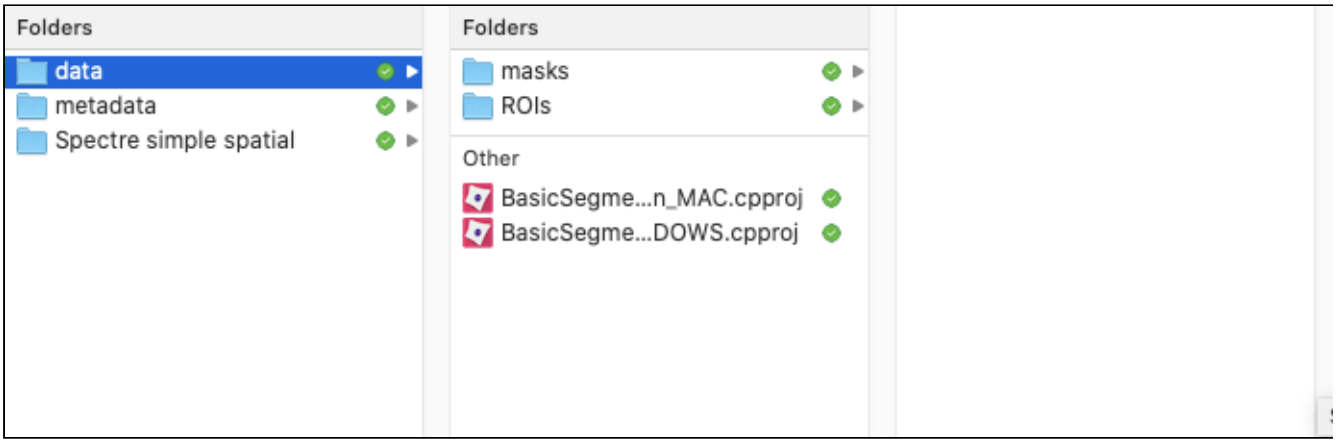
Go to: https://github.com/ImmuneDynamics/Spectre/tree/master/workflows/Spatial%20-%20simple/data and click on either the MAC or WINDOWS version of the CellProfiler (.cpproj) file.



Then click '**Download**'.

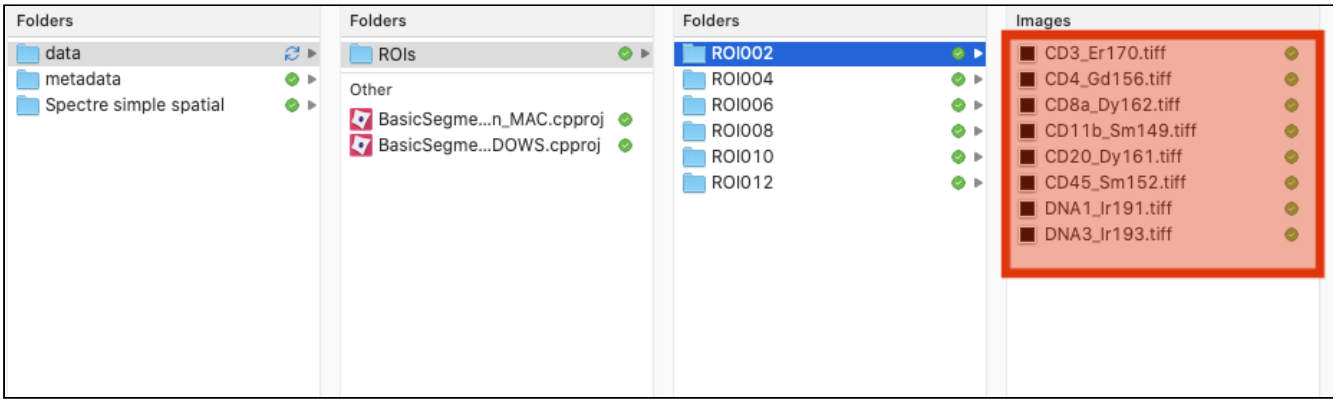


Add the CellProfiler (.cpproj) file to your experiment folder.



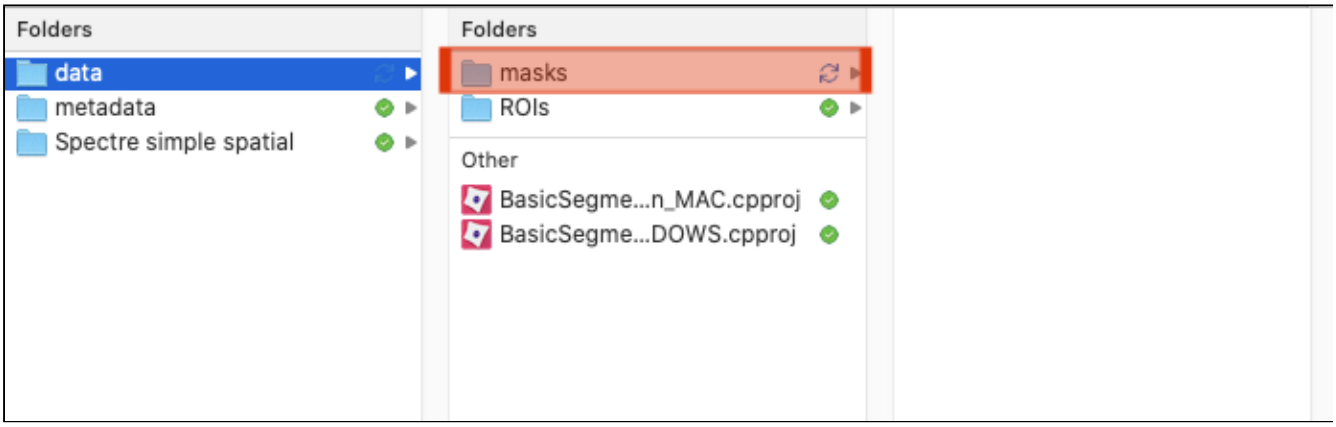
Export TIFF files from the original MCD files

You can do this using either MCD Viewer (on windows) or HistoCat++ (on Mac). Each ROI should be a folder, containing single-page TIFFs (one per channel for that ROI). Under 'ROIs', place your ROI folders



Masks folder

Create a final folder under 'data' called 'masks'.

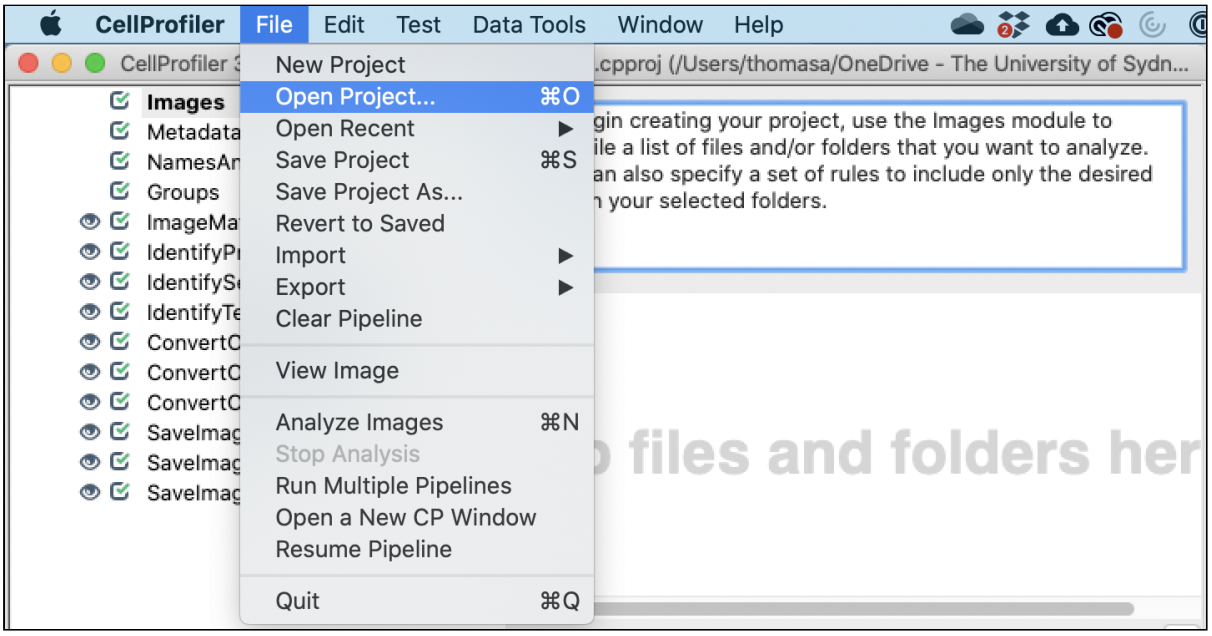


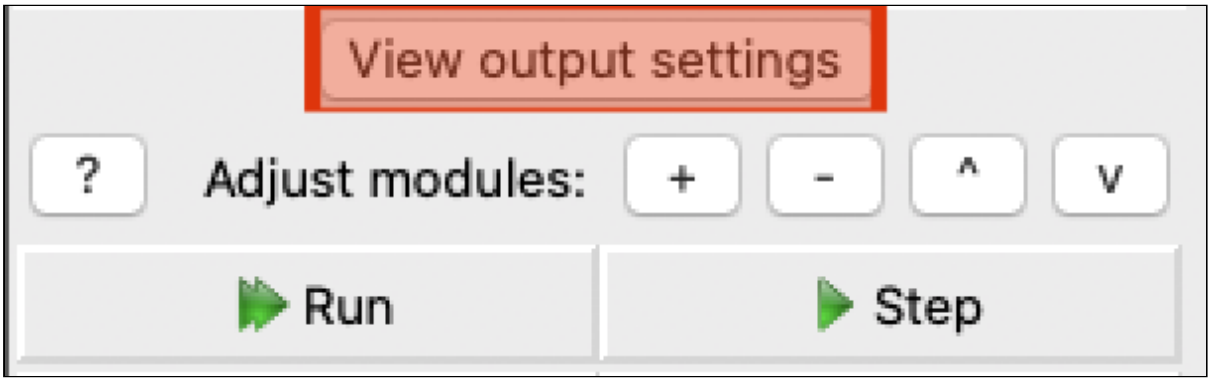
Generating cell masks using CellProfiler (v3.0 or above)

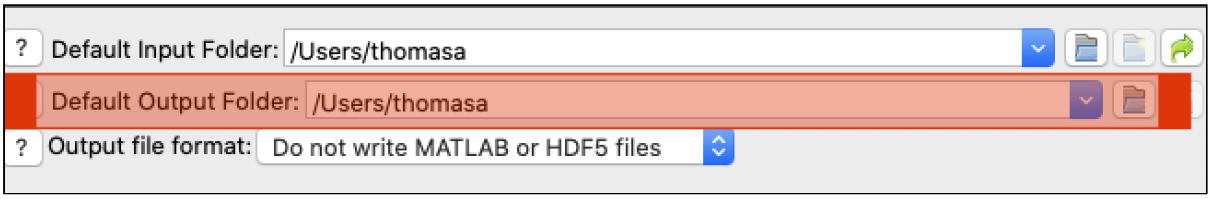
Once we have produced TIFF files, we can import the DNA TIFF files into CellProfiler.

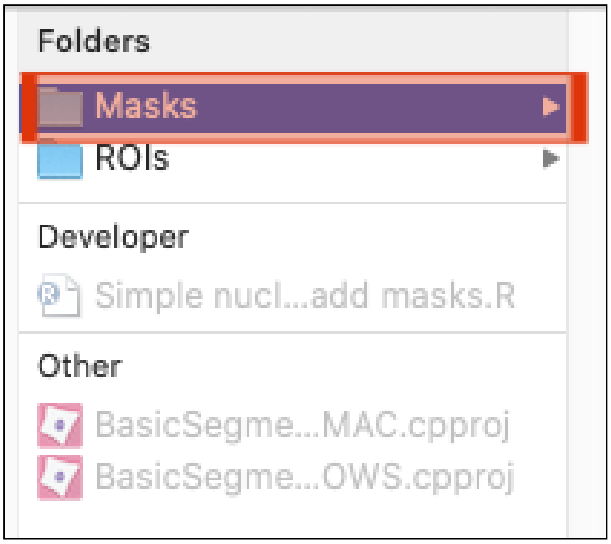
CellProfiler: open CellProfiler and then open the template file

- Open CellProfiler (v3.0 or higher)
- Select File > Open, and open the template file (provided above)









CellProfiler: overview of pipeline

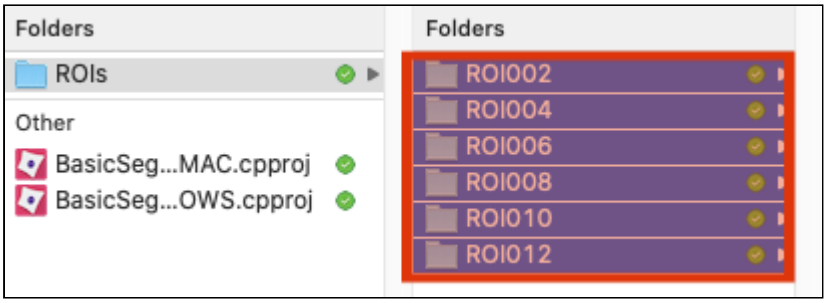
- Images, Metadata, NamesAndTypes, and Groups are effectively settings that relate to the images
- ImageMath combines the two DNA channels and amplifies the signal by a specified factor
- IdentifyPrimaryObjects (nuclei) uses one of a number of methods to identify nuclei
- IdentifySecondaryObjects (cells) uses pixel expansion to estimate the boundaries of the cell, by taking the shape of the nucleus and expanding outwards by a certain number of pixels
- IdentifyTertiaryObjects (cytoplasm) uses the cell object and subtracts the nuclear object, to create a cytoplasm object

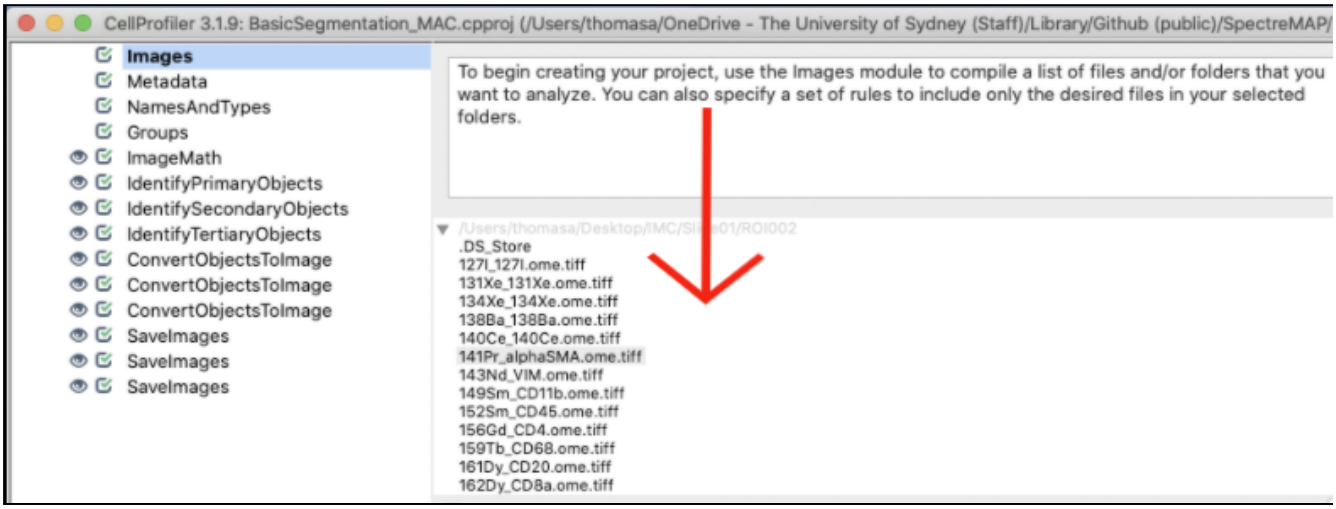
CellProfiler: set default output folder

- Go to File > Preferences
- If Default Output Folder is suitable, then don't change anything
- If you would like output images to be saved elsewhere, change Default Output Folder to a desired destination

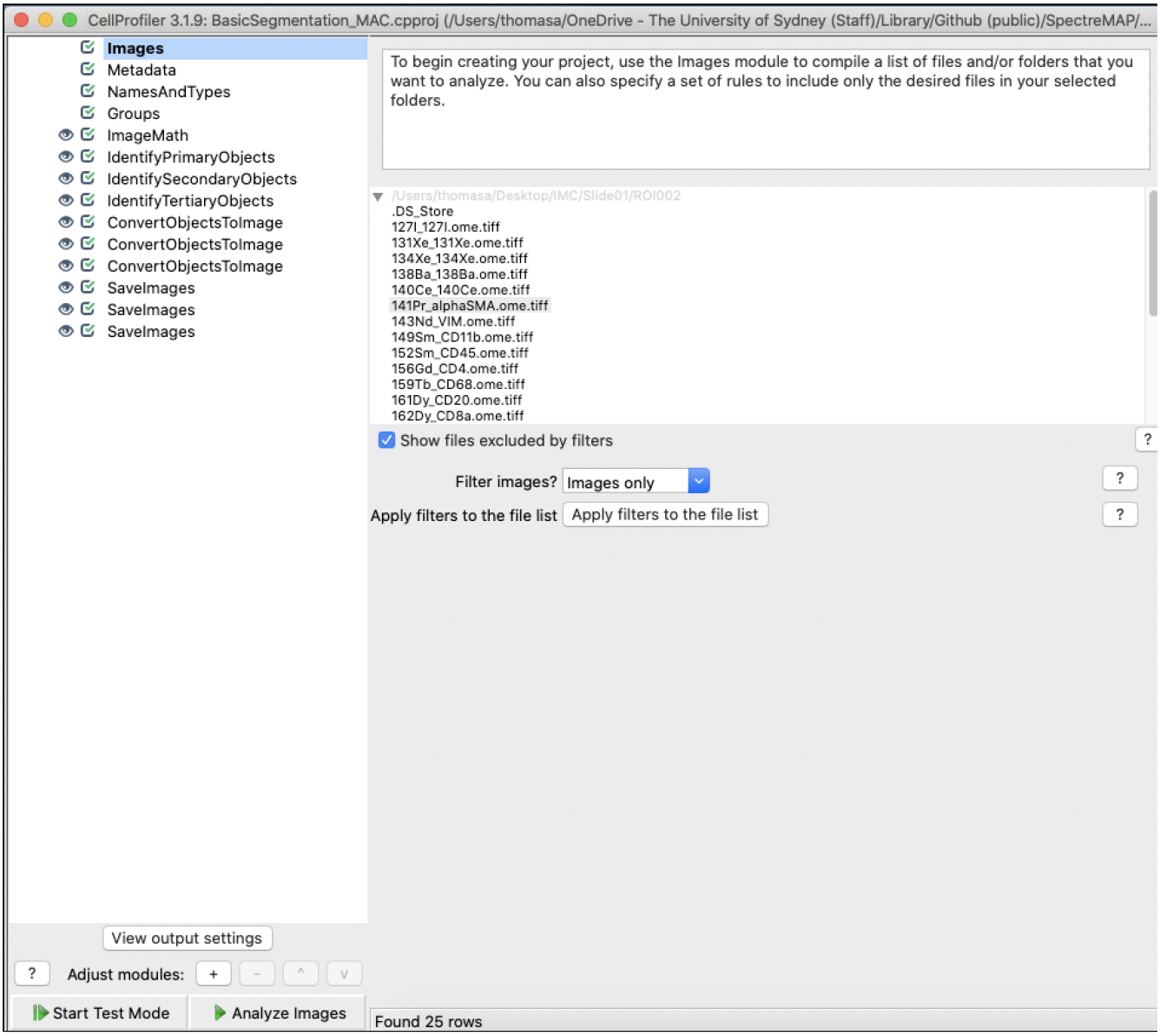
CellProfiler: load images

- Drag and drop the ROI folders into the area shown





- Each folder containing TIFFs represents one ROI, and the TIFFs within that folder represent different signals measured in that ROI.
- In this example: ROI was a particular 1 mm^2 area of bone marrow, and each TIFF was a different metal-conjugated antibody measured on that area.



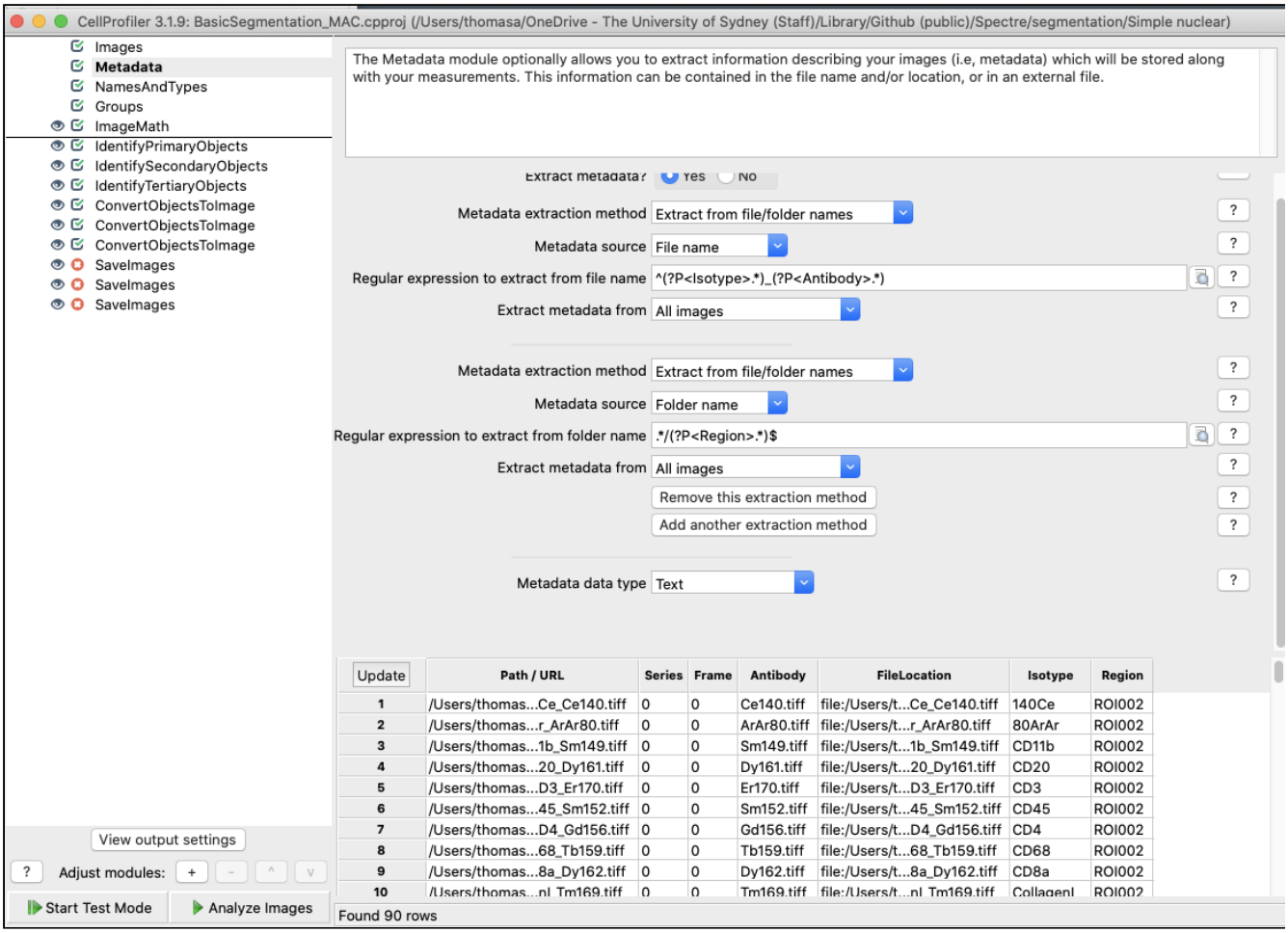
CellProfiler: metadata

- The Metadata module uses regular expression patterns in the way the TIFF files are named to determine the **metal** and **antibody** names represented by each TIFF file
- Additionally, regular expression are used to determine the **Slidename** (i.e. the MCD file) and the **Region** (i.e. the ROI).

Important

The default (PC) template uses "\\" to denote folder locations for metadata. It appears twice in the 'Slidename' and 'Region' regular expression bar (see image on right). If you are using a Mac, you should either use the Mac template file (provided above), or change both instances of the "\\" to "/".

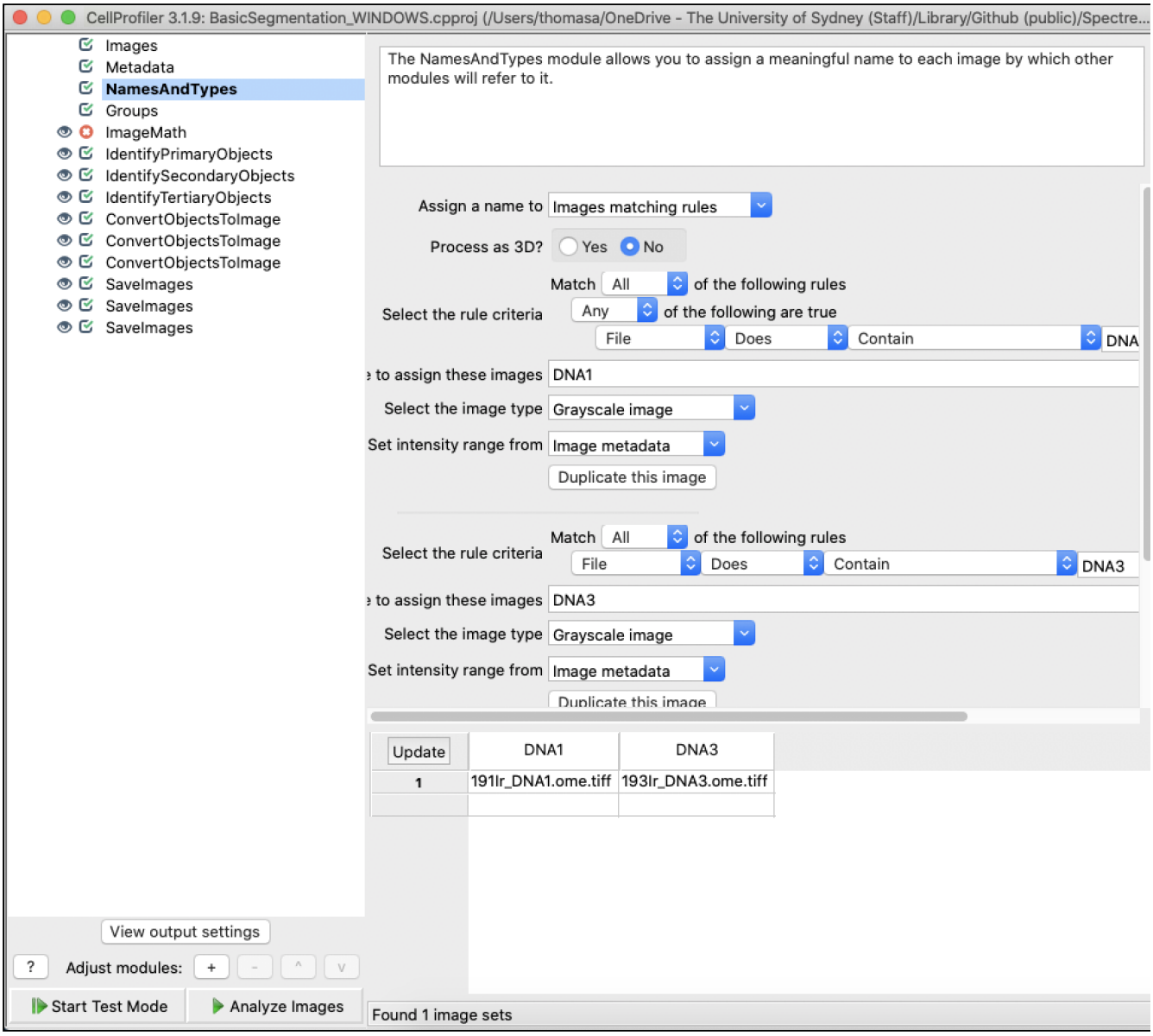
- The rules that govern how these regular expressions are read can be modified if required.
- Press UPDATE to scan through the TIFF files using these rules to view the Metadata output



	Path / URL	Series	Frame	Antibody	FileLocation	Isotype	Region
1	/Users/thomas...Ce_Ce140.tiff	0	0	Ce140.tiff	file:/Users/t...Ce_Ce140.tiff	140Ce	ROI002
2	/Users/thomas...r_ArAr80.tiff	0	0	ArAr80.tiff	file:/Users/t...r_ArAr80.tiff	80ArAr	ROI002
3	/Users/thomas...1b_Sm149.tiff	0	0	Sm149.tiff	file:/Users/t...1b_Sm149.tiff	CD11b	ROI002
4	/Users/thomas...20_Dy161.tiff	0	0	Dy161.tiff	file:/Users/t...20_Dy161.tiff	CD20	ROI002
5	/Users/thomas...D3_Er170.tiff	0	0	Er170.tiff	file:/Users/t...D3_Er170.tiff	CD3	ROI002
6	/Users/thomas...45_Sm152.tiff	0	0	Sm152.tiff	file:/Users/t...45_Sm152.tiff	CD45	ROI002
7	/Users/thomas...D4_Gd156.tiff	0	0	Gd156.tiff	file:/Users/t...D4_Gd156.tiff	CD4	ROI002
8	/Users/thomas...68_Tb159.tiff	0	0	Tb159.tiff	file:/Users/t...68_Tb159.tiff	CD68	ROI002
9	/Users/thomas...8a_Dy162.tiff	0	0	Dy162.tiff	file:/Users/t...8a_Dy162.tiff	CD8a	ROI002
10	/Users/thomas...pl_Tm169.tiff	0	0	Tm169.tiff	file:/Users/t...pl_Tm169.tiff	Collagenl	ROI002

CellProfiler: NamesAndTypes

- For this basic segmentation pipeline, the two DNA channels are named 'DNA1' and 'DNA2' by looking for the metal name (from Metadata) that corresponds to those channels.
- On our instrument, the metal name is repeated with an underscore in between (e.g. 191Ir_191Ir). This can be modified for datasets where the metals are named differently.
- If you aren't sure what the metal names are, have a look in the metadata module (or check the name of the TIFF file).
- Press UPDATE to see the labels and which images they are applied to.

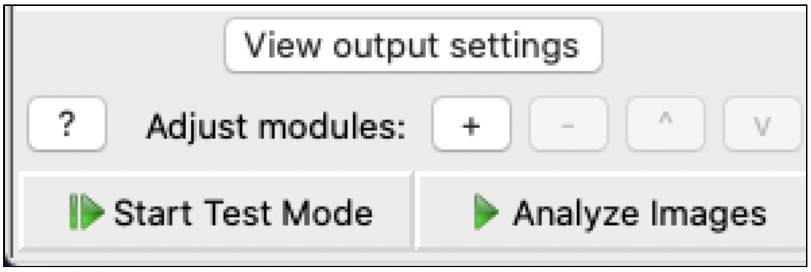


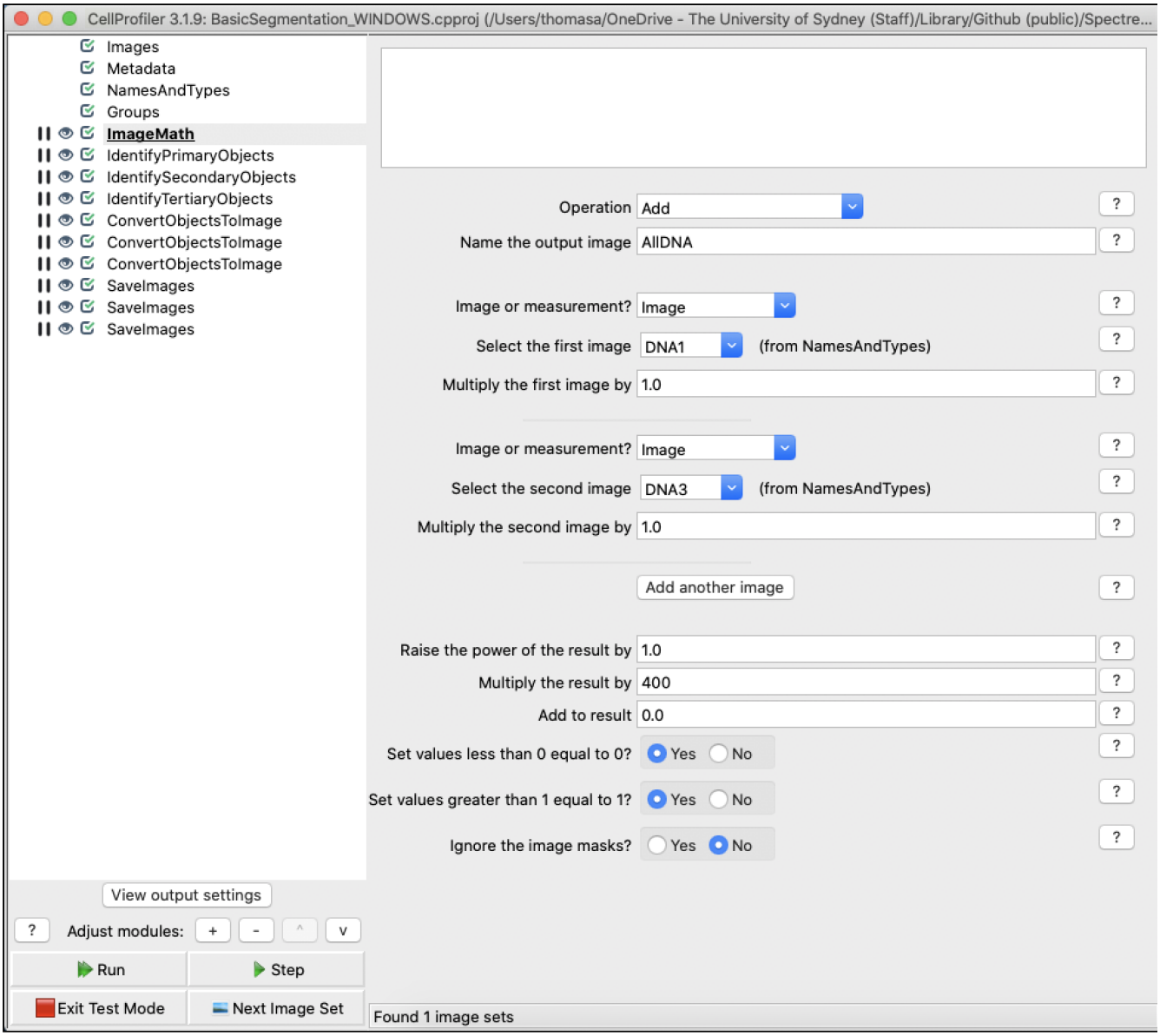
CellProfiler: Groups

- The Groups module isn't used in this workflow

CellProfiler: start TEST mode

By using test mode, you can step through each of the modules – which will be applied to the first set of images in your dataset. This way you can check that each component of the pipeline is working correctly.





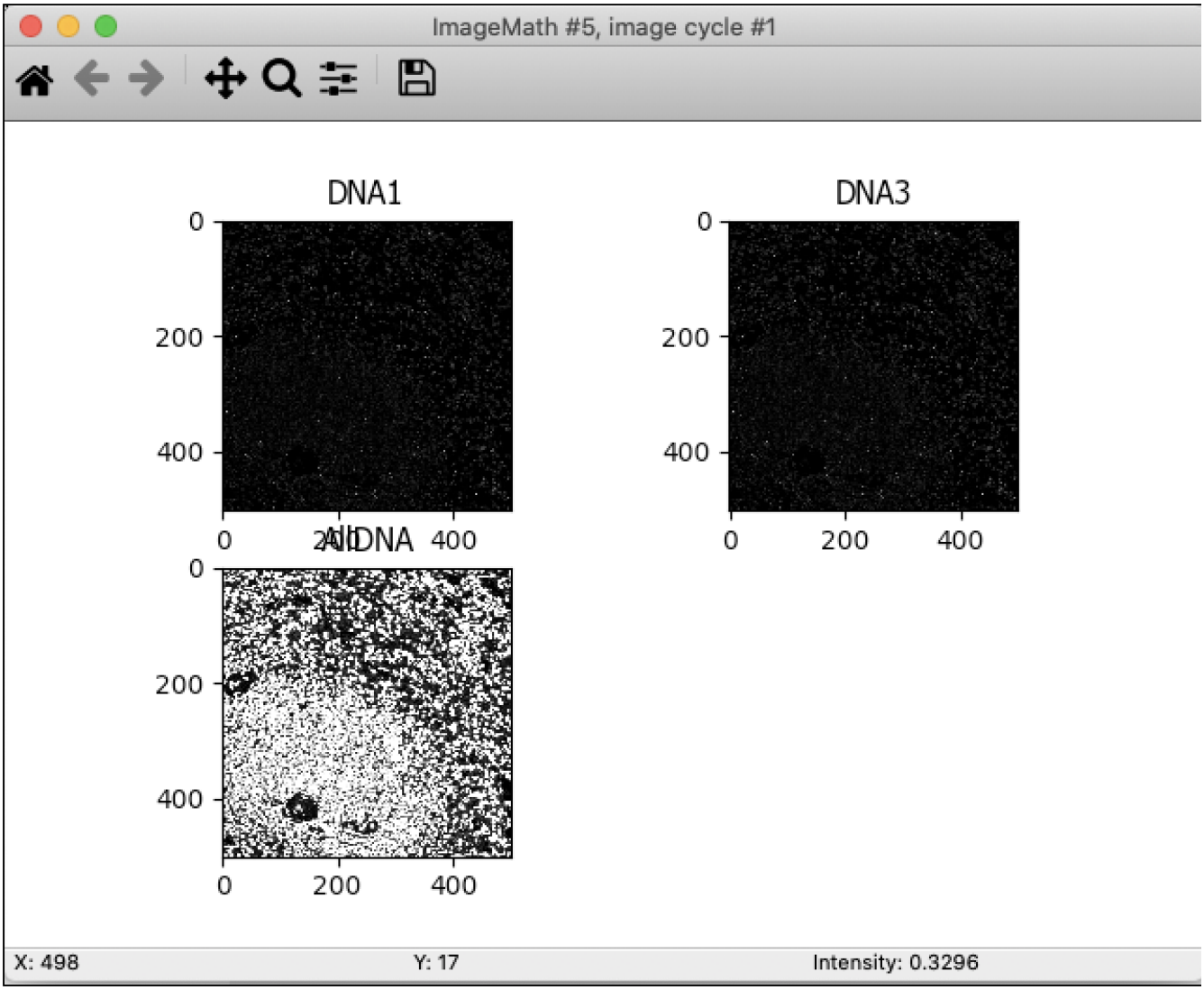
- Press 'Start Test Mode'.
- You can see which module the process is up to, as it will be underlined.
- Press 'Step' to execute the current module and visualise the output (if a visualisation step is included).
- Press 'Step' again to proceed to the next module, or modify the parameters of the existing module if required (if a parameter is modified, pressing 'Step' will re-run the current module).

Use test mode for the first run-through of each of the following modules. Then the entire dataset can be analysed automatically using 'Analyze Images'.

CellProfiler: ImageMath

This step merges the two DNA channels together and multiplies that signal by a specified factor and calls this output 'AllDNA'

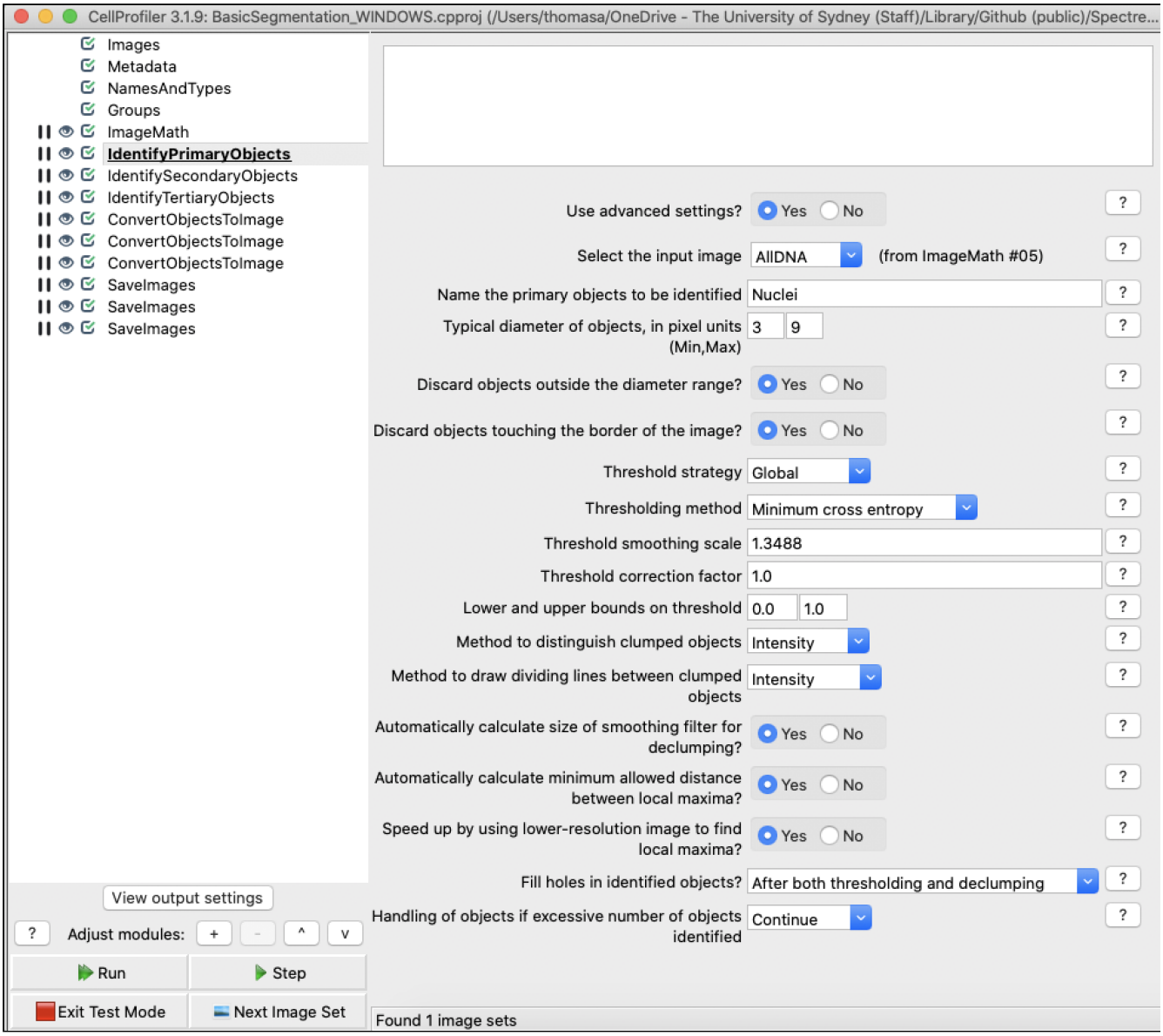
- Press 'Step'
- View the output
- Use the zoom tool (magnifying glass) to zoom into an area of the image (on any image, the zoom will be applied the same), and determine if the DNA signal is clear and bright
- If the signal is too bright or too dim, the multiplication factor can be changed from the default, and 'Step' can be clicked to run ImageMath again.

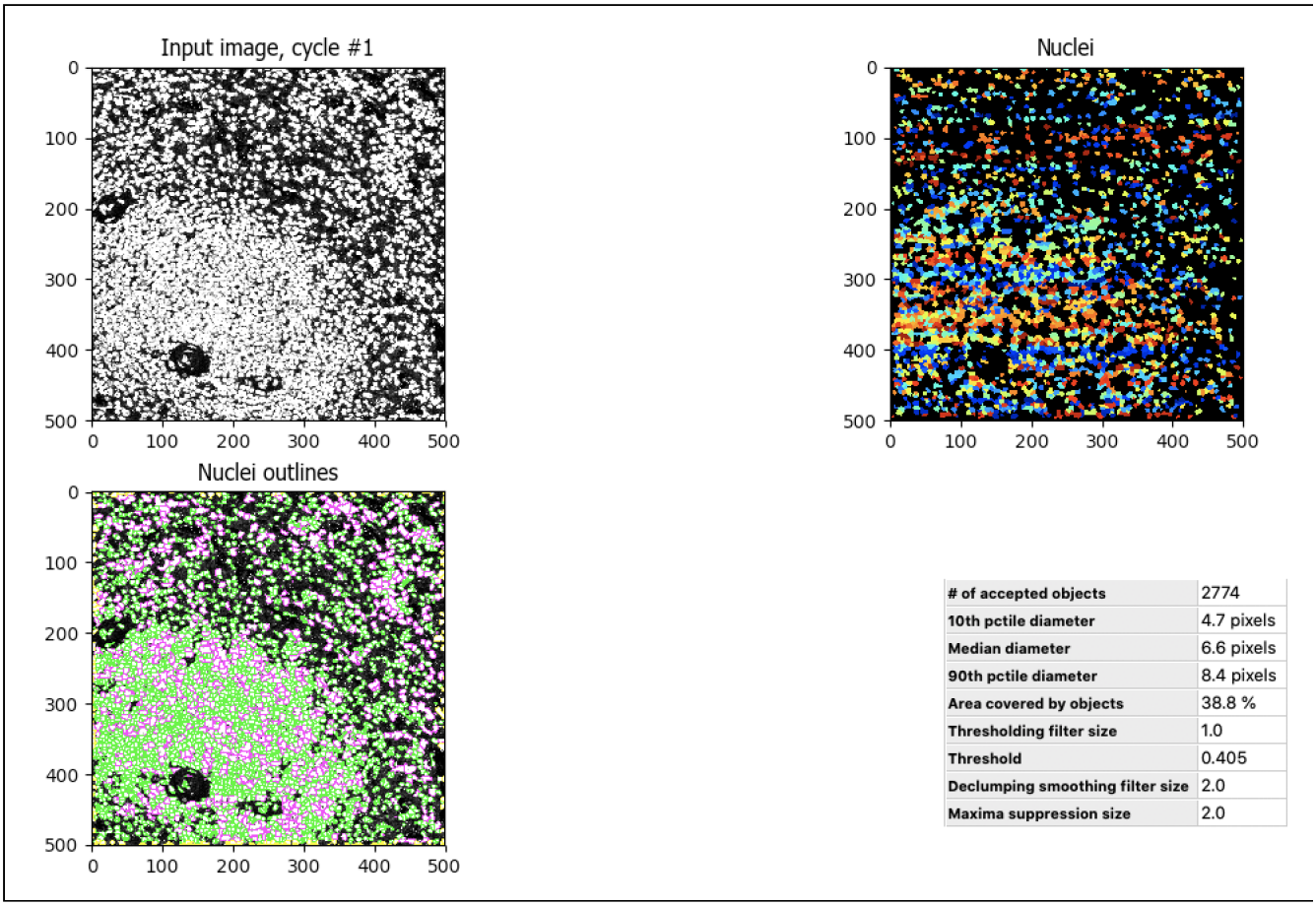


CellProfiler: Primary Objects

In this step, CellProfiler attempts to identify the nuclei in the image.

- Press 'Step' and view the output (coloured nuclei 'object', and nuclei outlines)
- Use the zoom tool to zoom in on an area of the image
- Green boundaries are the proposed boundaries of nuclei objects, purple outlines are possible nuclei that were excluded because the did not fall within the required parameters for a nucleus (e.g. too large, too small, etc)
- If the creation of nuclei objects was not suitable, then modify the parameters (usually changing the 'Typical diameter of objects to by identified) minimum and maximum values.


CellProfiler 3.1.9: BasicSegmentation_WINDOWS.cpproj (/Users/thomasa/OneDrive - The University of Sydney (Staff)/Library/Github (public)/Spectre...
Images
Metadata
NamesAndTypes
Groups
ImageMath
IdentifyPrimaryObjects
IdentifySecondaryObjects
IdentifyTertiaryObjects
ConvertObjectsToImage
ConvertObjectsToImage
ConvertObjectsToImage
SaveImages
SaveImages
SaveImages
View output settings
Adjust modules:
Run
Step
Exit Test Mode
Next Image Set
Use advanced settings? Yes No
Select the input image AllDNA (from ImageMath #05)
Name the primary objects to be identified Nuclei
Typical diameter of objects, in pixel units (Min,Max) 3 9
Discard objects outside the diameter range? Yes No
Discard objects touching the border of the image? Yes No
Threshold strategy Global
Thresholding method Minimum cross entropy
Threshold smoothing scale 1.3488
Threshold correction factor 1.0
Lower and upper bounds on threshold 0.0 1.0
Method to distinguish clumped objects Intensity
Method to draw dividing lines between clumped objects Intensity
Automatically calculate size of smoothing filter for declumping? Yes No
Automatically calculate minimum allowed distance between local maxima? Yes No
Speed up by using lower-resolution image to find local maxima? Yes No
Fill holes in identified objects? After both thresholding and declumping
Handling of objects if excessive number of objects identified Continue
Found 1 image sets

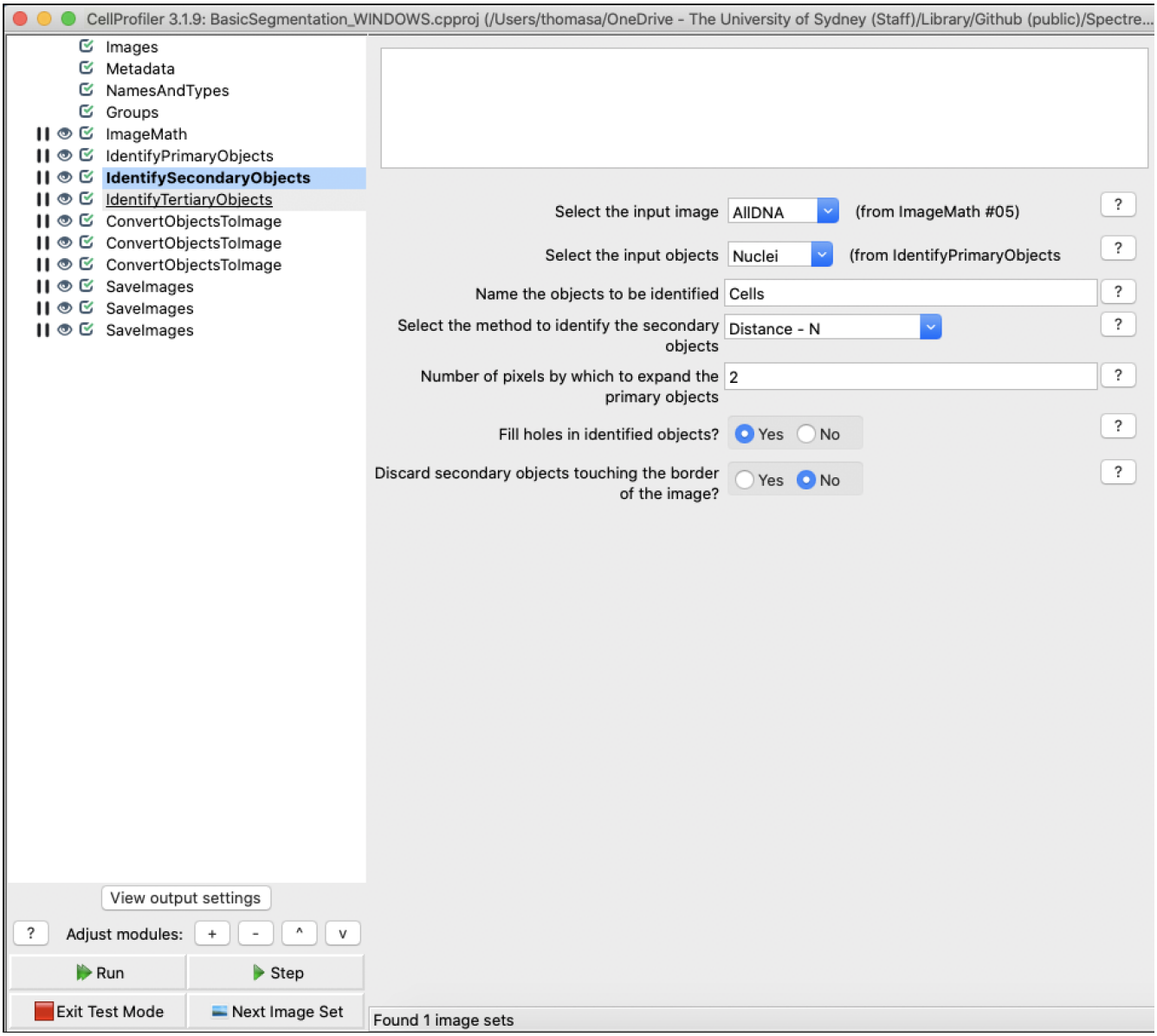


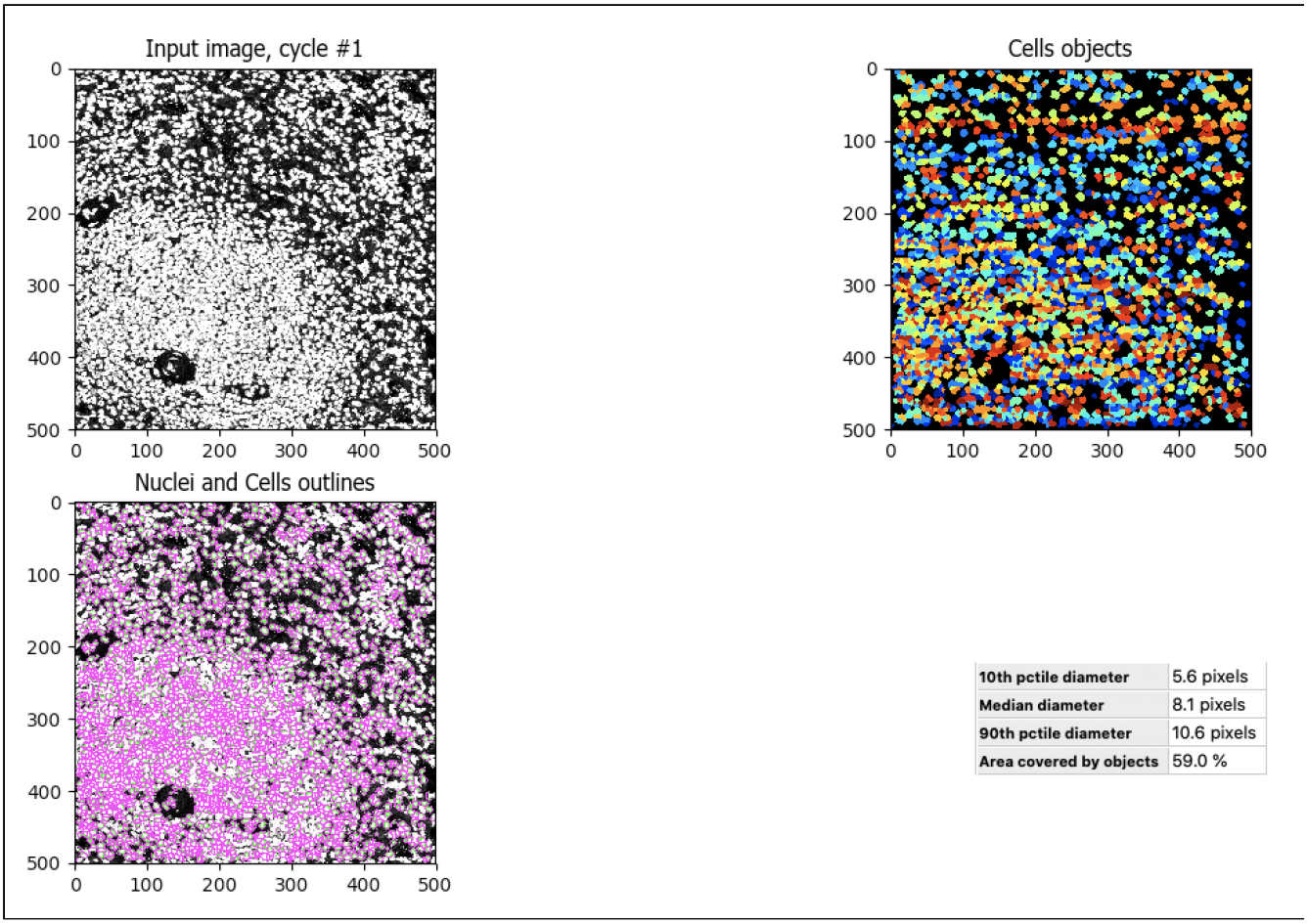
# of accepted objects	2774
10th pctile diameter	4.7 pixels
Median diameter	6.6 pixels
90th pctile diameter	8.4 pixels
Area covered by objects	38.8 %
Thresholding filter size	1.0
Threshold	0.405
Declumping smoothing filter size	2.0
Maxima suppression size	2.0

CellProfiler: Secondary Objects

This step attempts to generate a cell 'object' by taking the shape of the nucleus, and expanding that shape out in all directions by a certain distance. This is a very simple method of cell segmentation, as it uses very few signals as input, but as a result it will perform poorly when cells are packed closely together.

- Press 'Step' and view the output
- Zoom into a specific region of the image
- The green outline at the nuclei objects, and the green outlines at the proposed cell objects
- Cells outlines/objects are not ideal, then modify the parameters (usually changing the distance used for pixel expansion, default = 2)


CellProfiler 3.1.9: BasicSegmentation_WINDOWS.cpproj (/Users/thomasa/OneDrive - The University of Sydney (Staff)/Library/Github (public)/Spectre...
Images
Metadata
NamesAndTypes
Groups
ImageMath
IdentifyPrimaryObjects
IdentifySecondaryObjects
IdentifyTertiaryObjects
ConvertObjectsToImage
ConvertObjectsToImage
ConvertObjectsToImage
SaveImages
SaveImages
SaveImages
Select the input image
AllDNA
(from ImageMath #05)
Select the input objects
Nuclei
(from IdentifyPrimaryObjects
Name the objects to be identified
Cells
Select the method to identify the secondary objects
Distance - N
Number of pixels by which to expand the primary objects
2
Fill holes in identified objects?
Yes
No
Discard secondary objects touching the border of the image?
Yes
No
View output settings
Adjust modules:
Run
Step
Exit Test Mode
Next Image Set
Found 1 image sets

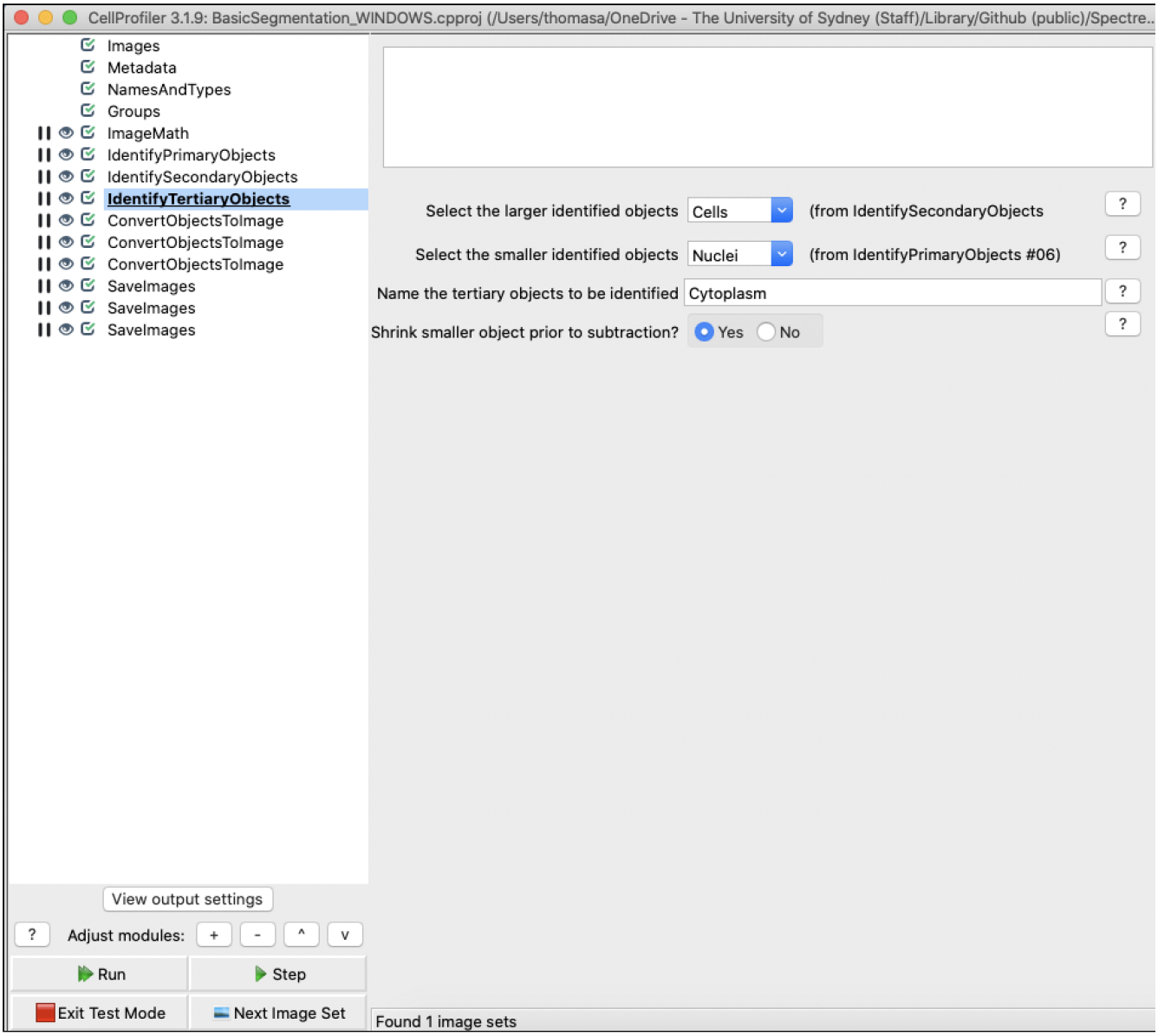


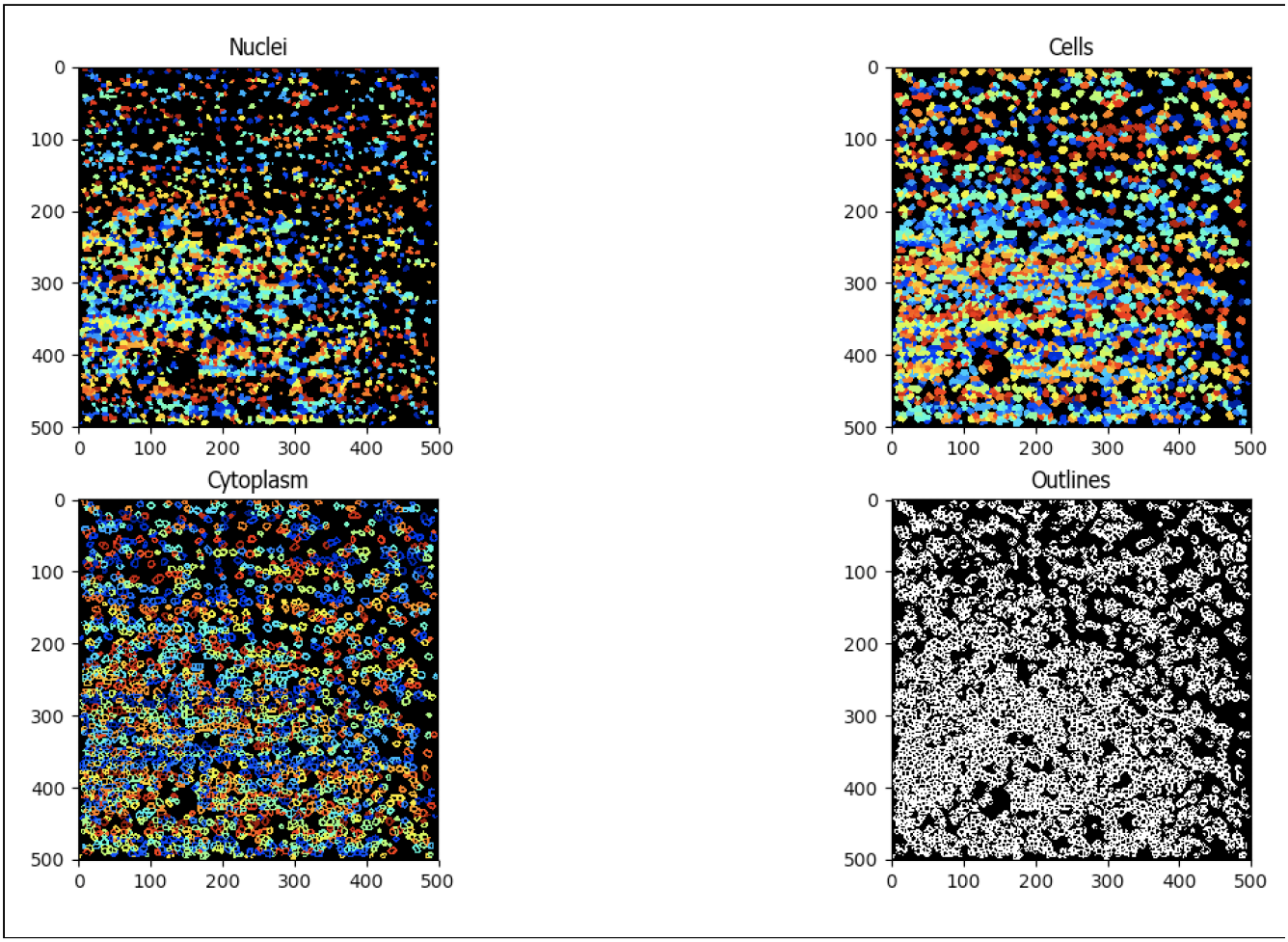
10th pctile diameter	5.6 pixels
Median diameter	8.1 pixels
90th pctile diameter	10.6 pixels
Area covered by objects	59.0 %

CellProfiler: Tertiary Objects

This step takes the cell object and subtracts the corresponding nuclear object, leaving a 'cytoplasmic' mask.

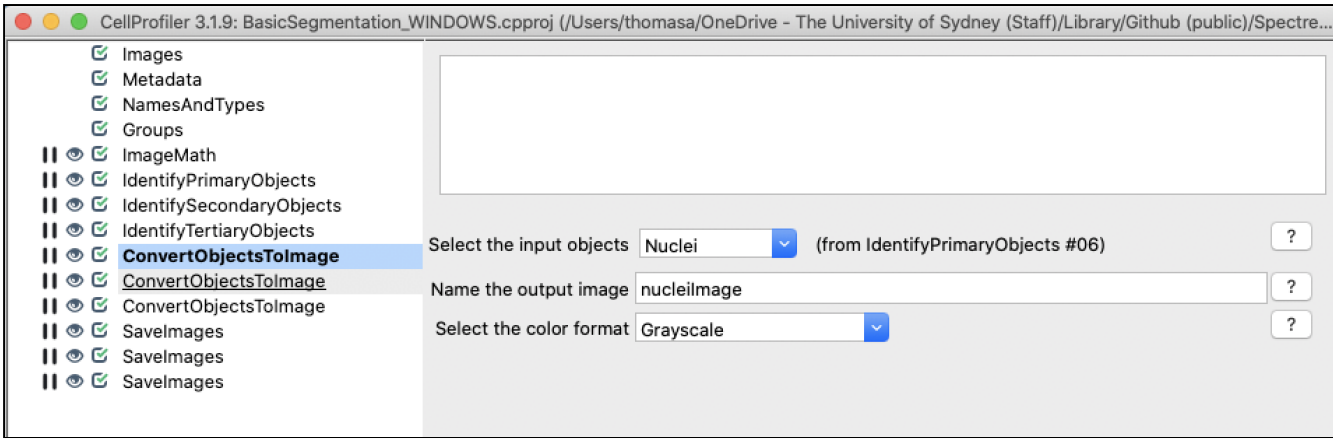
- Press 'Step' and view the output
- This module has no moving parts (i.e. no tuneable parameters)

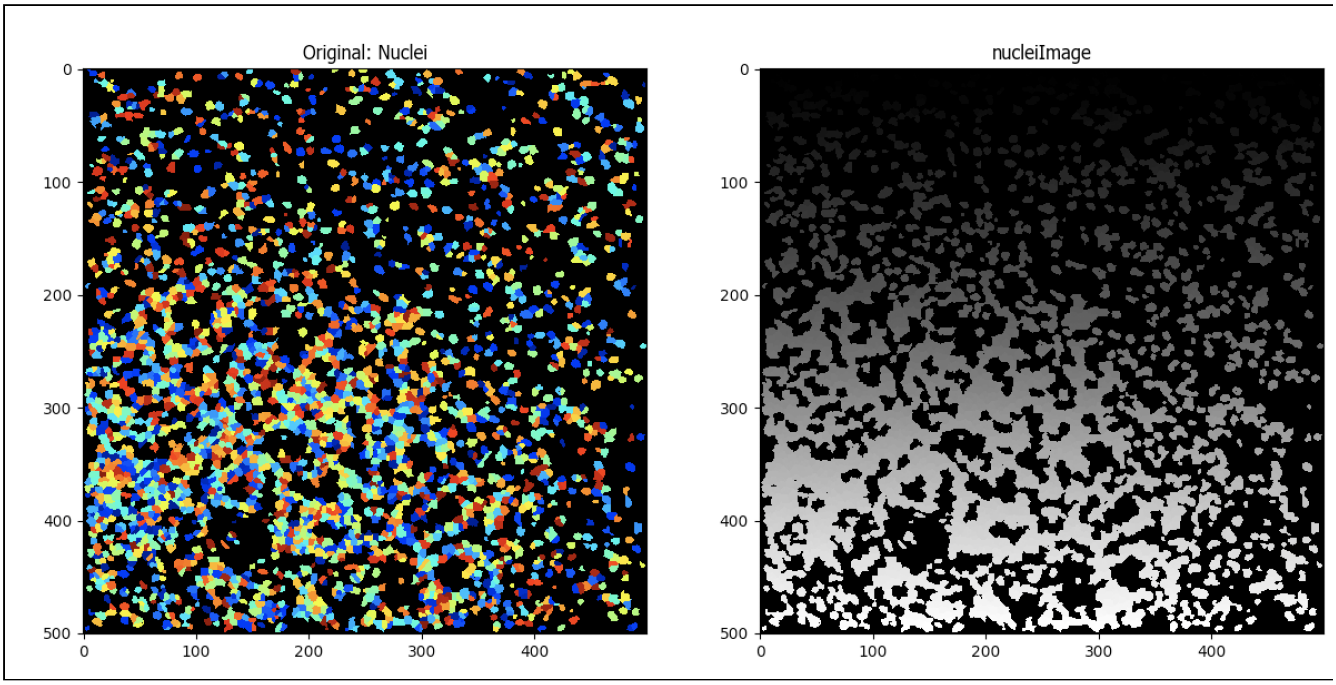

CellProfiler 3.1.9: BasicSegmentation_WINDOWS.cpproj (/Users/thomasa/OneDrive - The University of Sydney (Staff)/Library/Github (public)/Spectre..
Images
Metadata
NamesAndTypes
Groups
ImageMath
IdentifyPrimaryObjects
IdentifySecondaryObjects
IdentifyTertiaryObjects
ConvertObjectsToImage
ConvertObjectsToImage
ConvertObjectsToImage
SaveImages
SaveImages
SaveImages
Select the larger identified objects
Cells
(from IdentifySecondaryObjects
Select the smaller identified objects
Nuclei
(from IdentifyPrimaryObjects #06)
Name the tertiary objects to be identified
Cytoplasm
Shrink smaller object prior to subtraction?
Yes
No
View output settings
Adjust modules:
Run
Step
Exit Test Mode
Next Image Set
Found 1 image sets

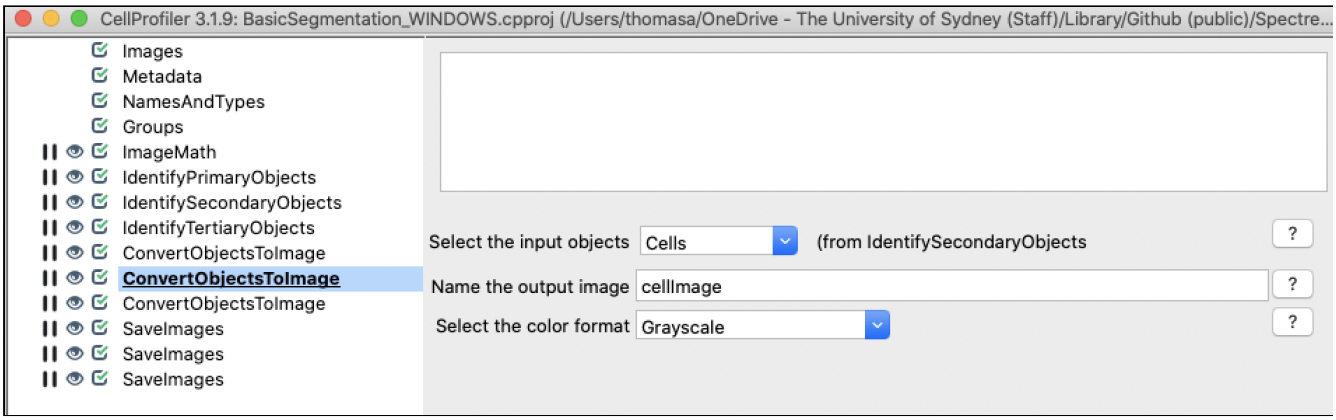


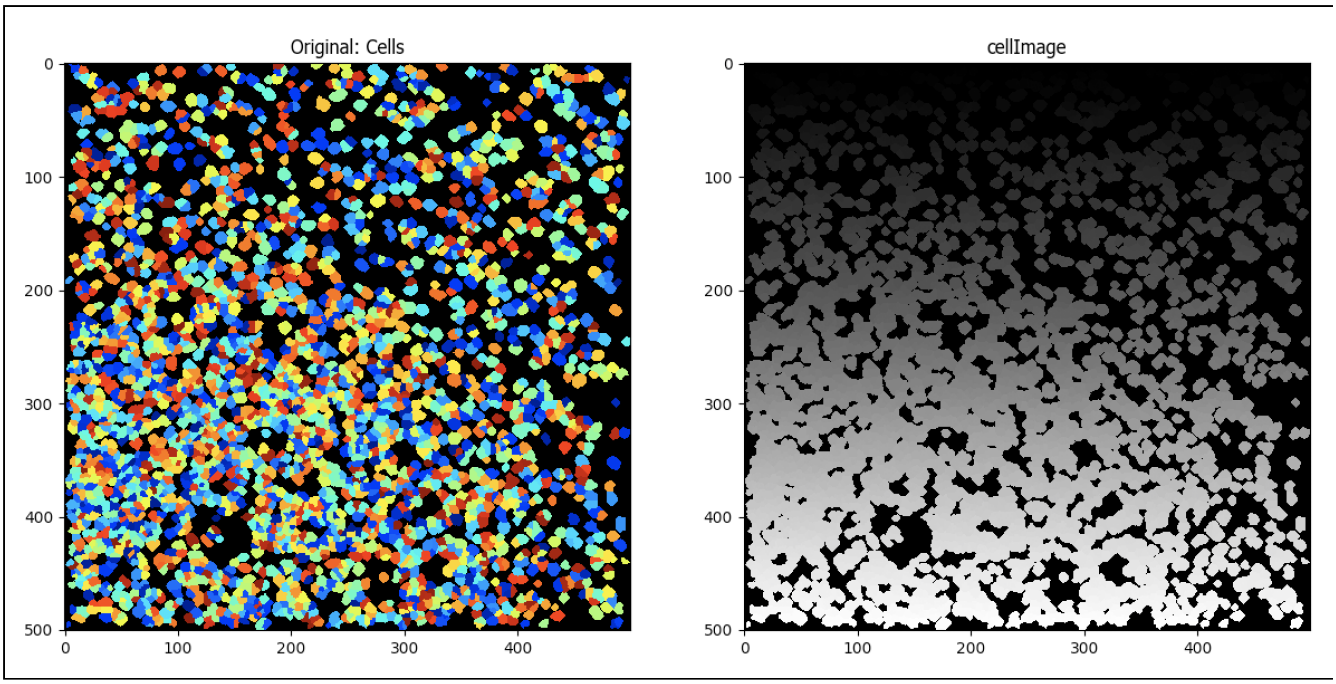
CellProfiler: conversions

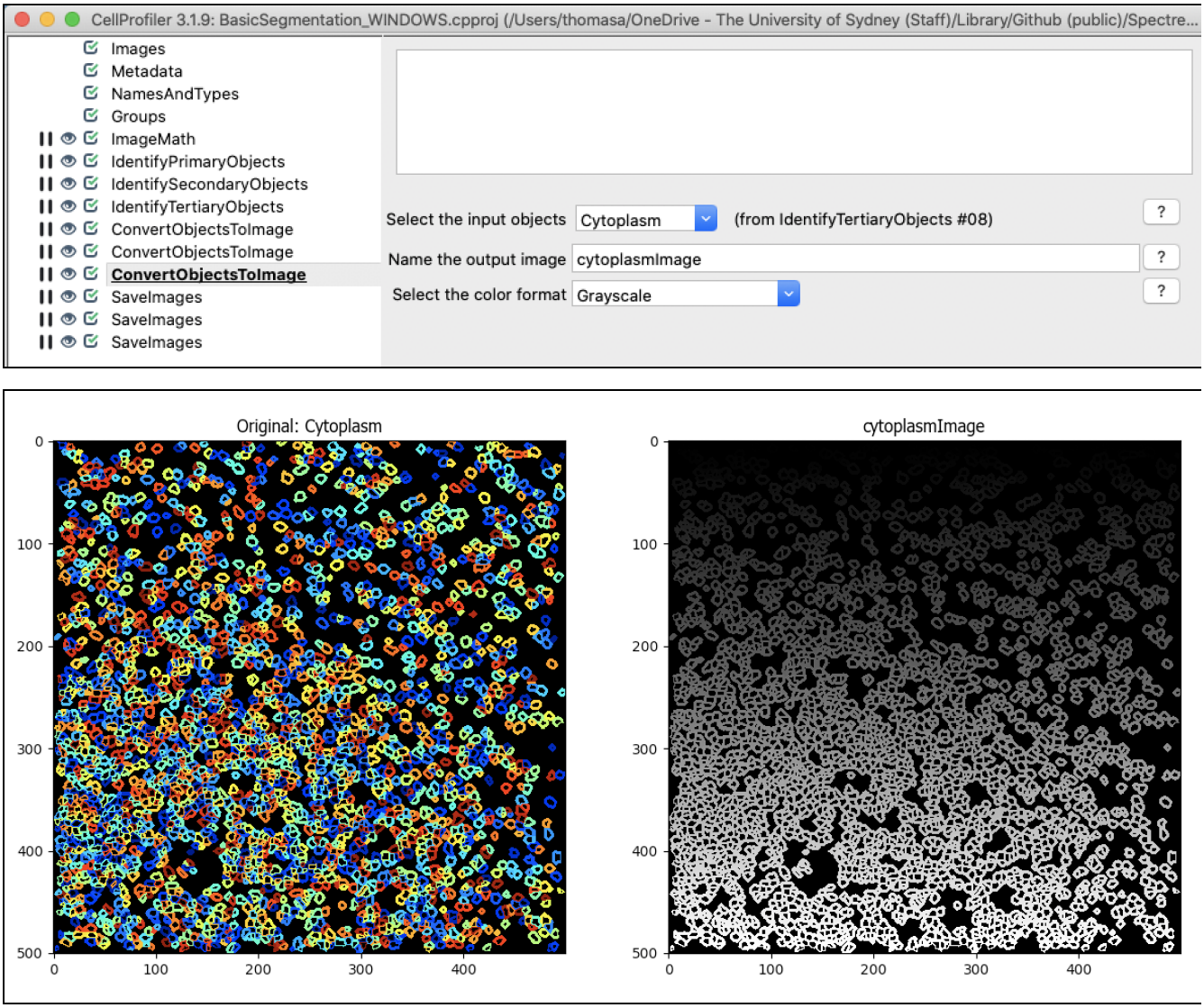
- 'ConvertObjectsToImage' converts the nuclei, cell, and cytoplasm objects into images.




Original: Nuclei
nucleiImage
0
100
200
300
400
500

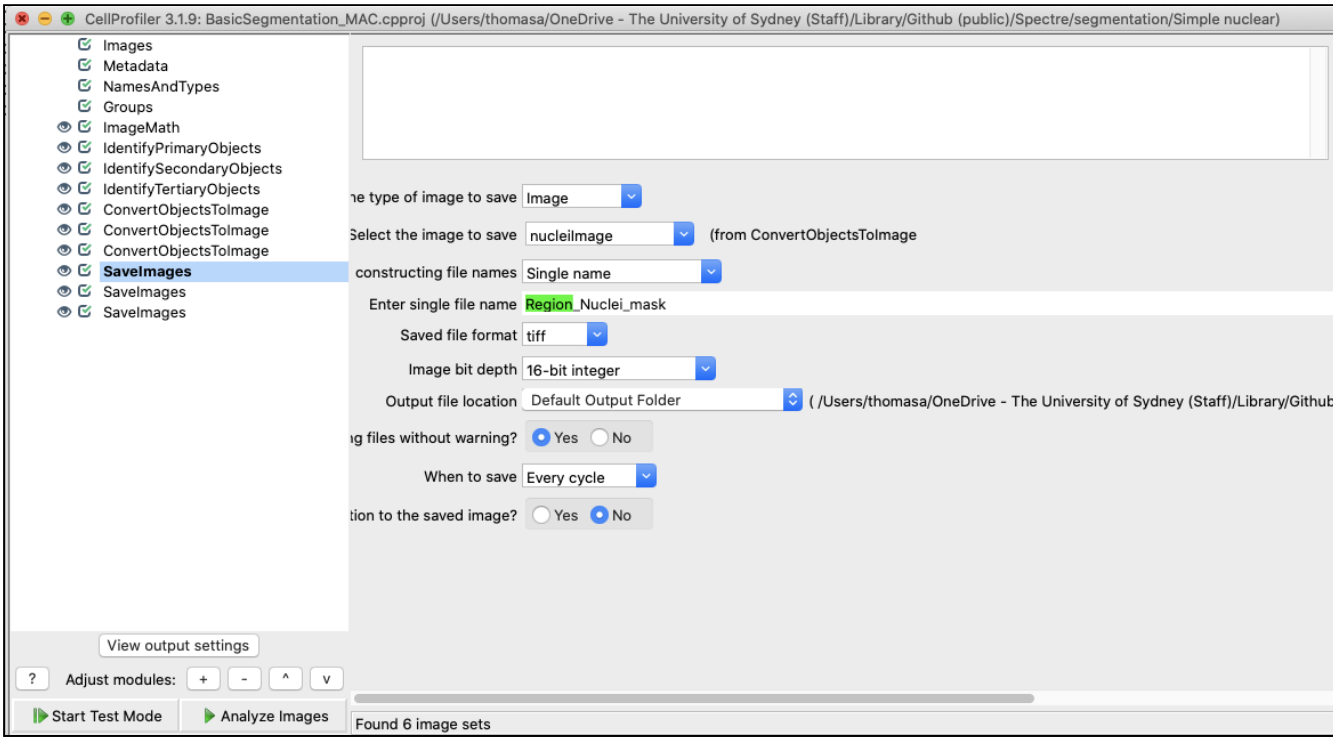

CellProfiler 3.1.9: BasicSegmentation_WINDOWS.cpproj (/Users/thomasa/OneDrive - The University of Sydney (Staff)/Library/Github (public)/Spectre...
Images
Metadata
NamesAndTypes
Groups
ImageMath
IdentifyPrimaryObjects
IdentifySecondaryObjects
IdentifyTertiaryObjects
ConvertObjectsToImage
ConvertObjectsToImage
ConvertObjectsToImage
SaveImages
SaveImages
SaveImages
Select the input objects
Cells
(from IdentifySecondaryObjects
Name the output image
cellImage
Select the color format
Grayscale

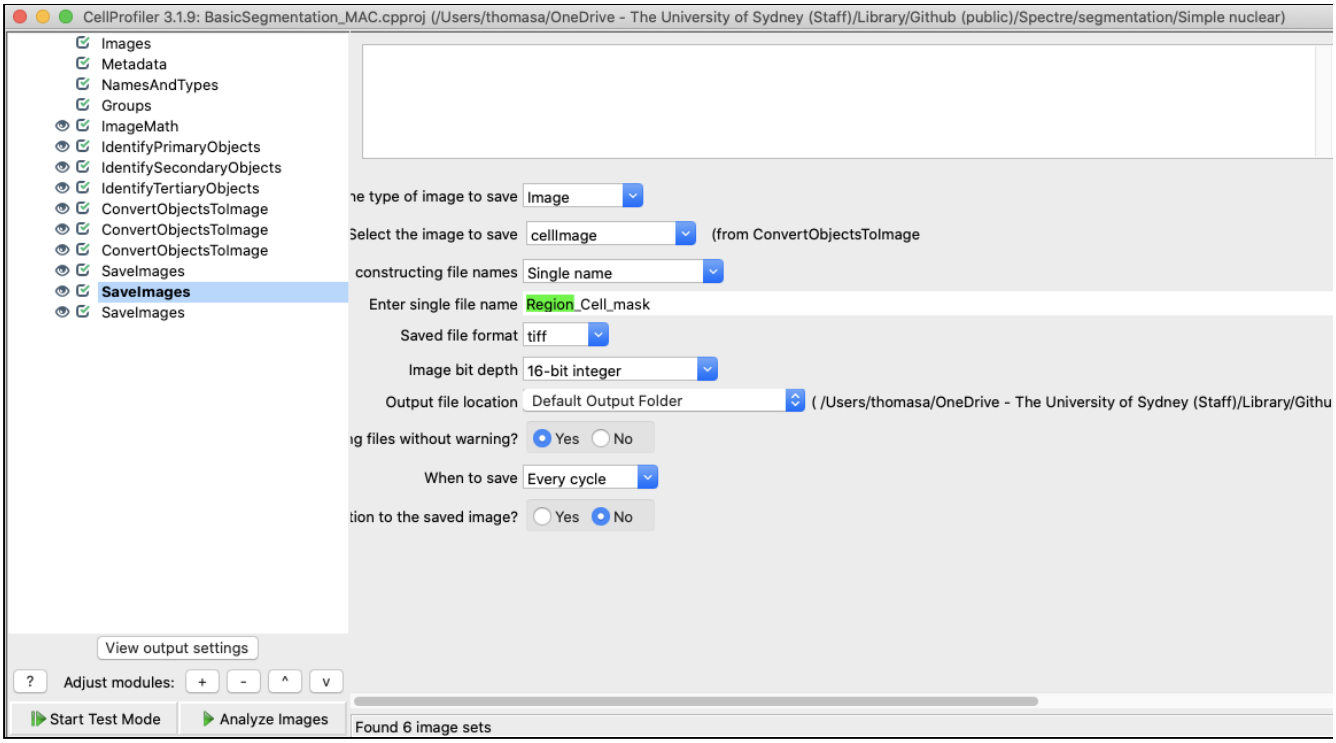

Original: Cells
cellImage
0
100
200
300
400
500

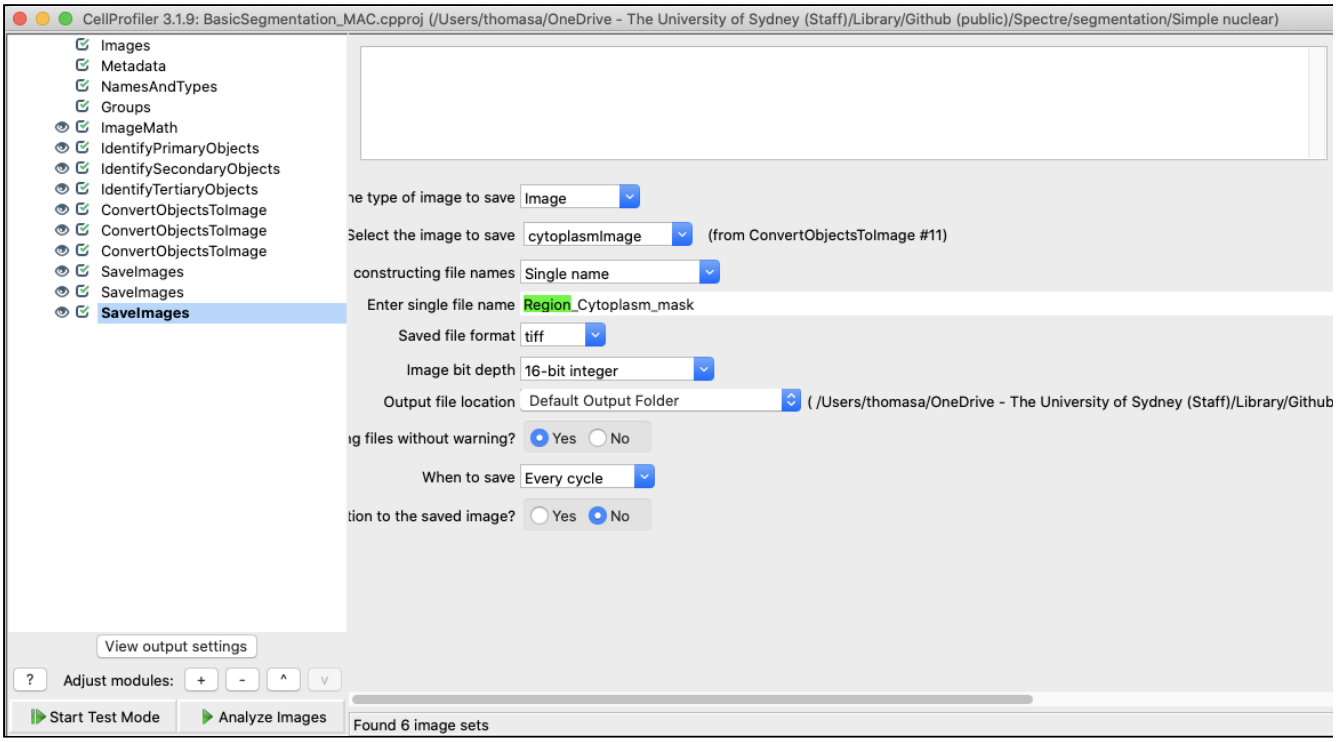


CellProfiler: save images

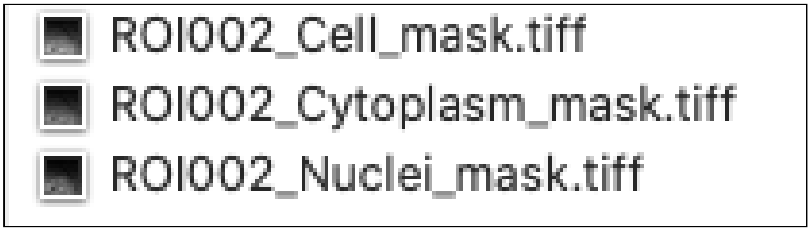
- 'SaveImages' saves the nuclei, cell, and cytoplasm images as TIFFs (these will be used as 'masks' in HistoCat. The 'Slidename' and 'Region' extracted in the Metadata module are used to name each image.


CellProfiler 3.1.9: BasicSegmentation_MAC.cpproj (/Users/thomasa/OneDrive - The University of Sydney (Staff)/Library/Github (public)/Spectre/segmentation/Simple nuclear)
Images
Metadata
NamesAndTypes
Groups
ImageMath
IdentifyPrimaryObjects
IdentifySecondaryObjects
IdentifyTertiaryObjects
ConvertObjectsToImage
ConvertObjectsToImage
ConvertObjectsToImage
SaveImages
SaveImages
SaveImages
ne type of image to save Image
Select the image to save nucleiImage (from ConvertObjectsToImage
constructing file names Single name
Enter single file name Region_Nuclei_mask
Saved file format tiff
Image bit depth 16-bit integer
Output file location Default Output Folder (/Users/thomasa/OneDrive - The University of Sydney (Staff)/Library/Github
g files without warning? Yes No
When to save Every cycle
tion to the saved image? Yes No
View output settings
Adjust modules:
Start Test Mode
Analyze Images
Found 6 image sets


CellProfiler 3.1.9: BasicSegmentation_MAC.cpproj (/Users/thomasa/OneDrive - The University of Sydney (Staff)/Library/Github (public)/Spectre/segmentation/Simple nuclear)
Images
Metadata
NamesAndTypes
Groups
ImageMath
IdentifyPrimaryObjects
IdentifySecondaryObjects
IdentifyTertiaryObjects
ConvertObjectsToImage
ConvertObjectsToImage
ConvertObjectsToImage
SaveImages
SaveImages
SaveImages
ne type of image to save Image
Select the image to save cellImage (from ConvertObjectsToImage
constructing file names Single name
Enter single file name Region_Cell_mask
Saved file format tiff
Image bit depth 16-bit integer
Output file location Default Output Folder (/Users/thomasa/OneDrive - The University of Sydney (Staff)/Library/Github
g files without warning? Yes No
When to save Every cycle
tion to the saved image? Yes No
View output settings
Adjust modules:
Start Test Mode
Analyze Images
Found 6 image sets

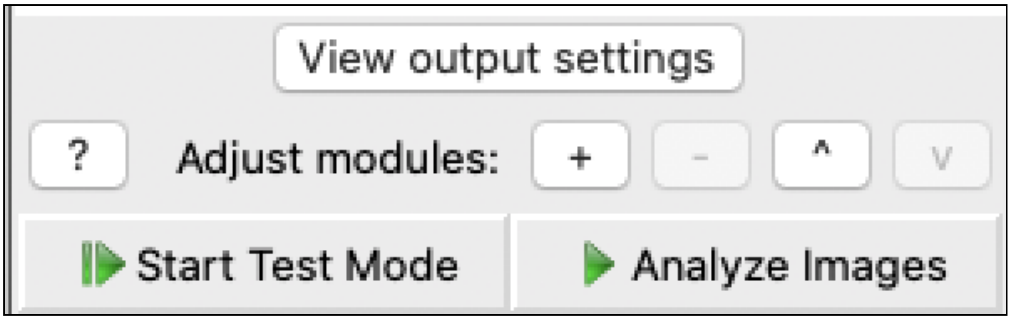


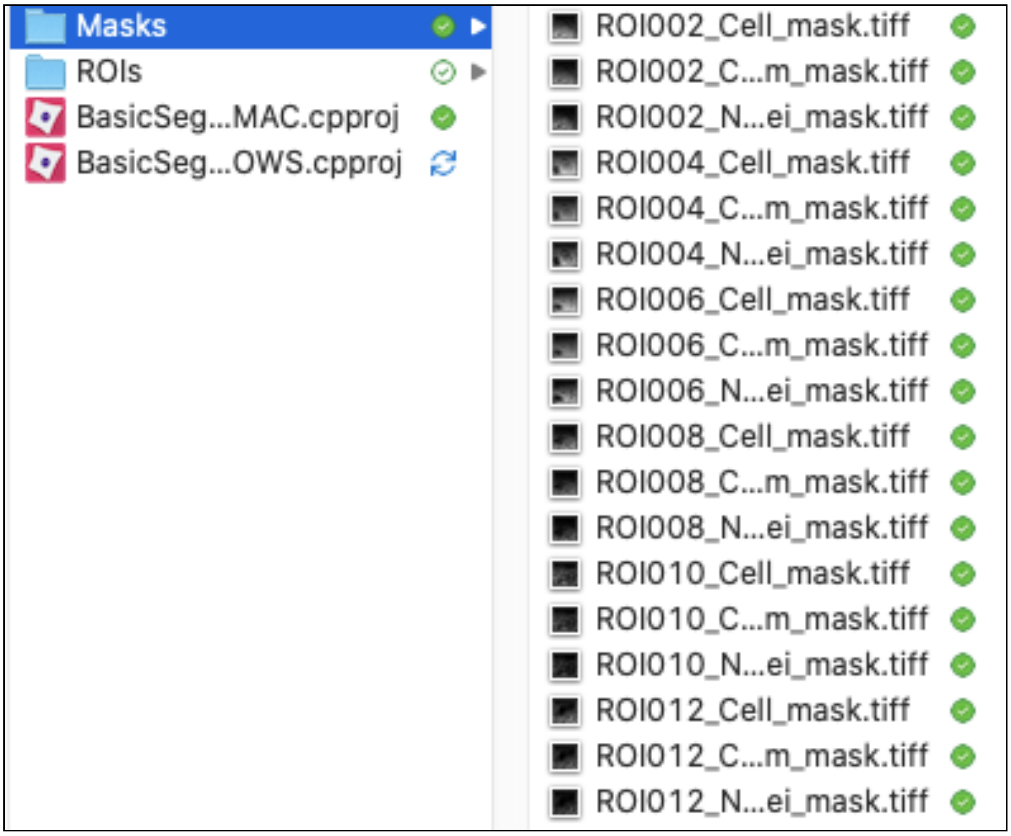
Output:



CellProfiler: Analyze Images

- Once the test mode run has been completed, press 'Analyze Images' to run the pipeline on all image sets




Masks
ROIs
BasicSeg...MAC.cpproj
BasicSeg...OWS.cpproj
ROI002_Cell_mask.tiff
ROI002_C...m_mask.tiff
ROI002_N...ei_mask.tiff
ROI004_Cell_mask.tiff
ROI004_C...m_mask.tiff
ROI004_N...ei_mask.tiff
ROI006_Cell_mask.tiff
ROI006_C...m_mask.tiff
ROI006_N...ei_mask.tiff
ROI008_Cell_mask.tiff
ROI008_C...m_mask.tiff
ROI008_N...ei_mask.tiff
ROI010_Cell_mask.tiff
ROI010_C...m_mask.tiff
ROI010_N...ei_mask.tiff
ROI012_Cell_mask.tiff
ROI012_C...m_mask.tiff
ROI012_N...ei_mask.tiff