

3D Cell Monolayer Segmentation and Analysis Tutorial

- Note that this tutorial is an advanced tutorial. We recommend completing the **Beginner Segmentation tutorial** in order to learn principles of image thresholding and segmentation prior to starting this tutorial.
- Appropriately naming the output(s) of each CellProfiler module is important in order to avoid confusion, especially in large and complex pipelines. Throughout this tutorial we will suggest names for each of the outputs, but feel free to use your own.
- This tutorial will guide you through the creation of a complete analysis pipeline. If you wish, you can also find a final version of a similar pipeline in the *3d_monolayer_final.cppipe* file, which should be in the same folder as this tutorial. To load the pipeline, simply drag the file to the left panel on the CellProfiler window.
- Helpful video tutorials are available on the Center for Open Bioimage Analysis YouTube page at https://www.youtube.com/channel/UC_id9sE-vu_i3oBd-skay7Q.
- This tutorial features images of human induced pluripotent stem cells from the Allen Institute of Cell Science. More details are available at the following link: <https://bbbc.broadinstitute.org/BBBCo34>.

Organizing and importing images

Z-stacks as TIFFs

- CellProfiler 3D currently only works with TIFF files. TIFF files can be rather complicated, having hyper-stack structures with all channels and z-planes in a single file. The acceptable CellProfiler format for storing z-stacks is to have a separate TIFF file for each channel.
- CellProfiler can be used to convert from other file formats to individual TIFF files for each channel using the **SaveImages** module.

Importing data in CellProfiler

1. Highlight the **Images** module.
2. Drag-and-drop the images you will analyze into the **Images** module window.
3. Highlight the **Metadata** module.
4. Enter the following regular expression:

```
^(?P<Plate>.*)_xy(?P<Site>[0-9])_ch(?P<ChannelNumber>[0-9])
```

This regular expression will parse the filenames and organize the data.

5. Highlight the **NamesAndTypes** module.
6. Assign a name to “Images matching rules”.
7. Choose “Process as 3D”
8. Populate the fields for “Relative Pixel Spacing”.
 - You can find this information on the image metadata. Open the image in Fiji and go to Image > Show Info... (or use Ctrl + I)
 - Near the end of the metadata you will find the information for the “Voxel size” (x,y,z). You can also

record this metadata when collecting your own images

- For this example, the relative pixel spacing is 0.26 in x and y and 0.29 pixels in z.
- The actual units do not matter, rather their relative proportion. The numbers are unitless and therefore the decimal place does not matter.

9. Create “rule criteria” to identify an image by its color/channel. For example, using the Metadata you just extracted - **Metadata** → **Does** → **Have ChannelNumber matching** → **0** would match the first image.

10. Give the images “variable names” that describe the contents of the image. For example, use the name *dna* to describe an image stained with DAPI.

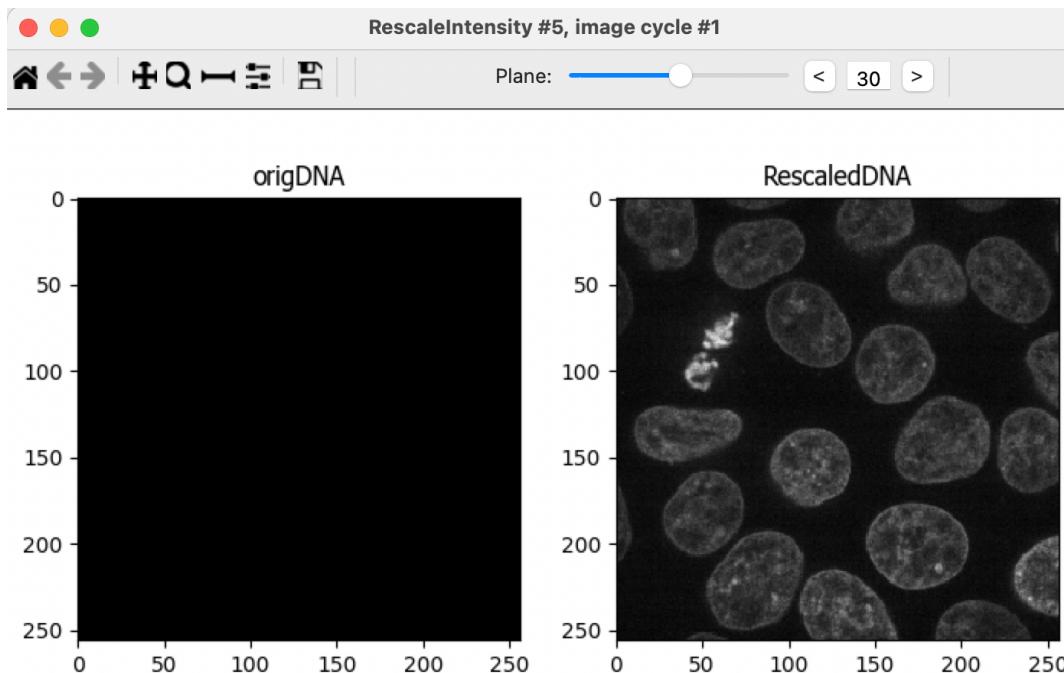
11. Add images with rulesets for the other channels in the experiment. In this case, Channel 0 contains images of the plasma membrane, Channel 1 contains images of mitochondria, and Channel 2 contain images of DNA. You can name them *origMemb*, *origMito* and *origDNA*, respectively.

Find objects: nuclei

Image preparation

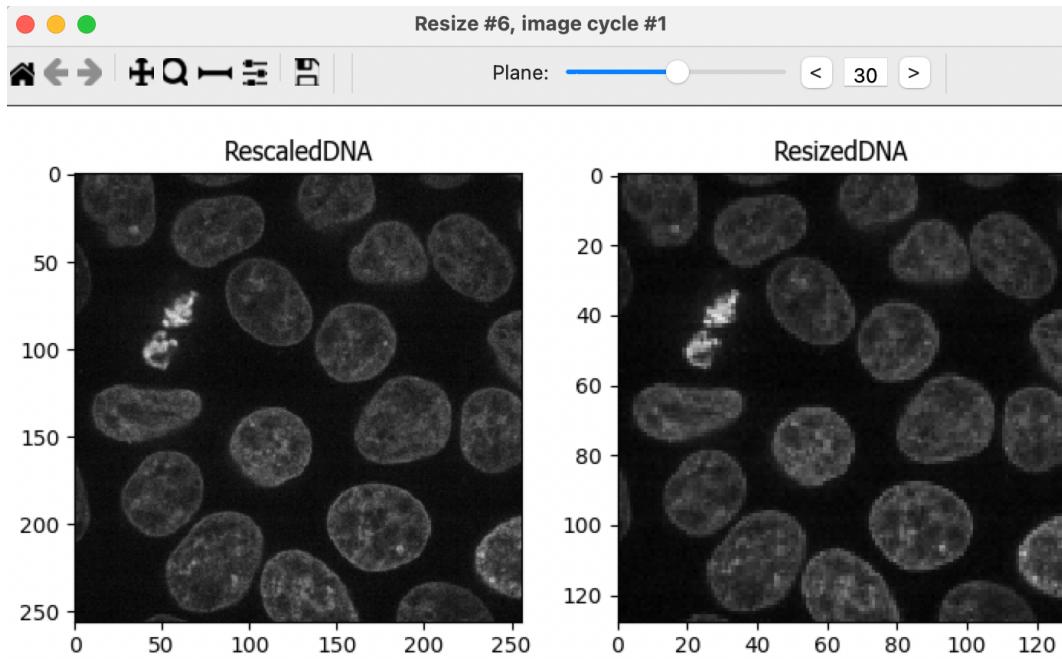
Before attempting to segment the cells in the images, pre-processing the images with filters and various image processing methods will improve the results.

1. Add a **RescaleIntensity** module for the DNA channel. Rescaling the DNA image proportionally stretches the intensity values to the full intensity range, from 0 to 1. In this case, we find that rescaling improves the thresholding and subsequent segmentation of nuclei. When using rescaling in your pipelines, be careful to perform measurements on the original images, not the rescaled images. Name the output *RescaledDNA*.

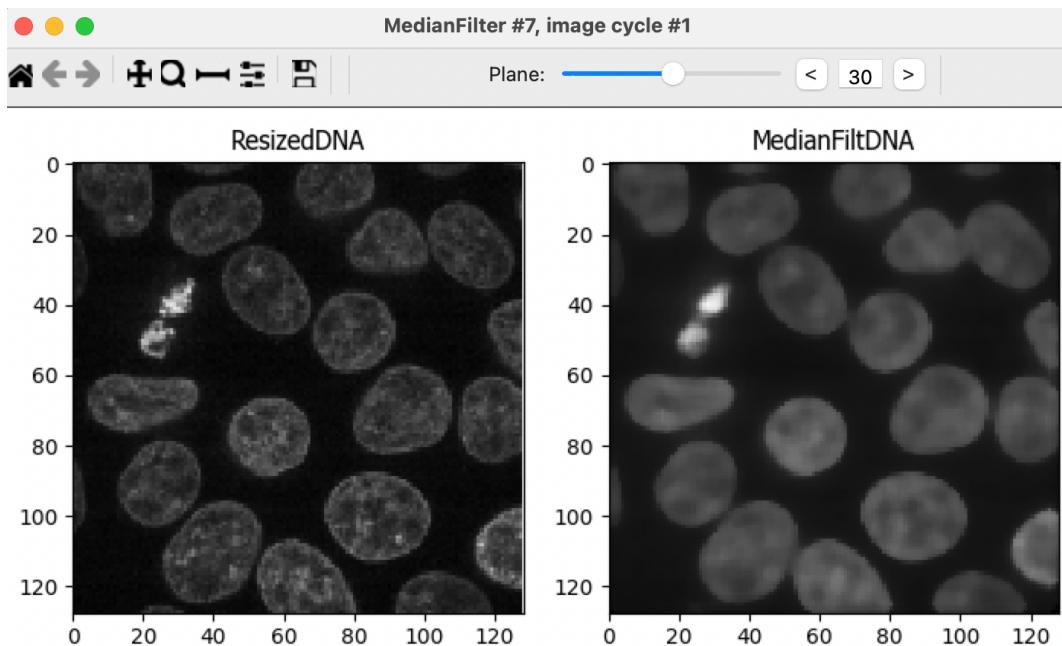


2. Add a **Resize** module. Processing 3D images requires much more computation time than 2D images. Often, downsampling an image can yield large performance gains and at the same time smooth an image to remove noise. Final segmentation results will be minimally affected by downsampling if the objects of interest are relatively large compared to the pixel size. Choose a value of *0.5* for both *X* and *Y*, this will halve each of the XY dimensions, so the resulting image will have a quarter of the area of the

original. *Do not resize Z* (keep the factor at 1), otherwise you will discard images from the Z stack. Name the output *ResizedDNA*.



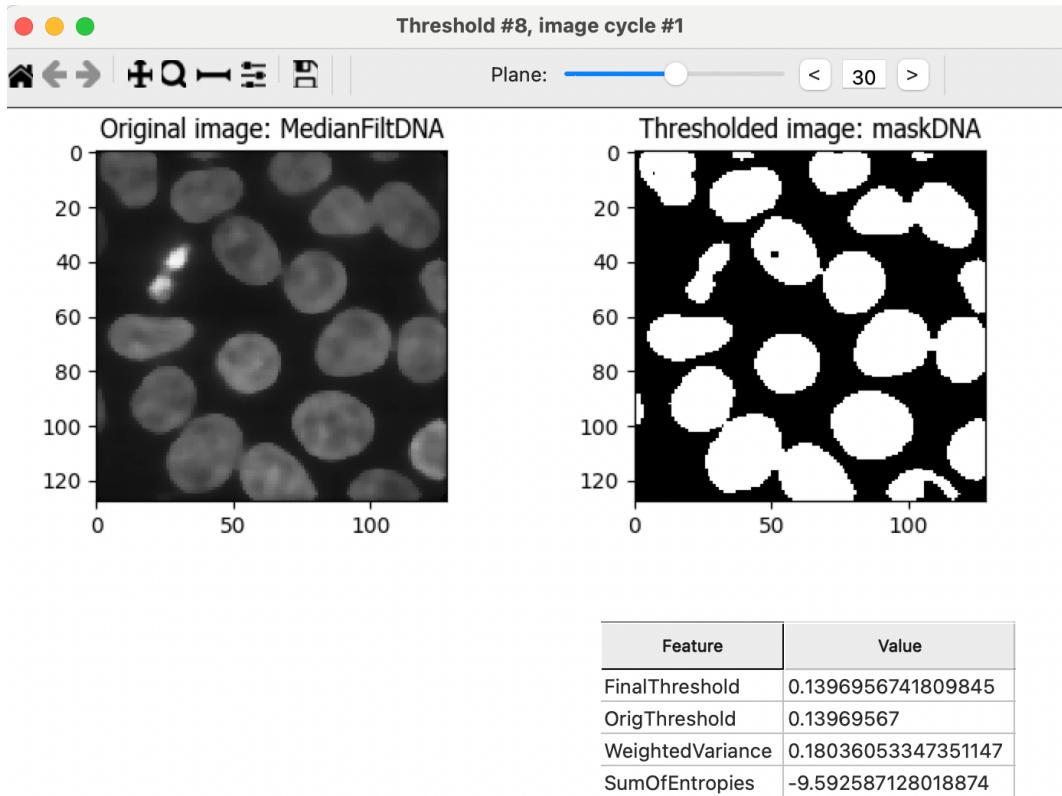
3. Add a **MedianFilter** module. A median filter will homogenize the signal within the nucleus and reduce noise in the background. DNA is not uniformly distributed throughout the nucleus, which can lead to holes forming in the downstream object identification. A median filter will preserve boundaries better than other smoothing filters such as the Gaussian filter. For the example images, choose a filter size of 5. This number was chosen empirically: it is smaller than the diameter of a typical nucleus; it is small enough that nuclei aren't merged together, yet large enough to suppress over-segmentation of the nuclei. Name the output *MedianFiltDNA*.



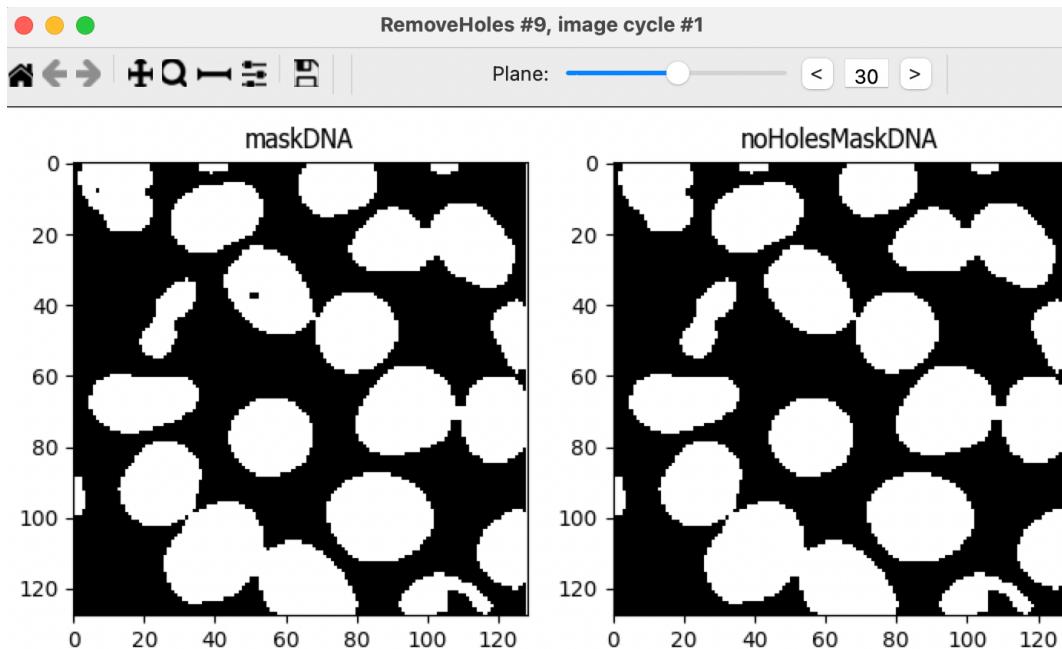
Segmentation

In CellProfiler 4 (and previous versions) the **IdentifyPrimaryObjects** module does not support 3D images. Thus, we will have to use a different strategy to segment the nuclei.

- Add an **Threshold** module. This identifies a pixel intensity value to separate the foreground (nuclei) from the background. Empirically, we've found that a two-class Otsu threshold works well for this data. We encourage you to try other thresholding methods to compare the outputs. Name the output *MaskDNA*.

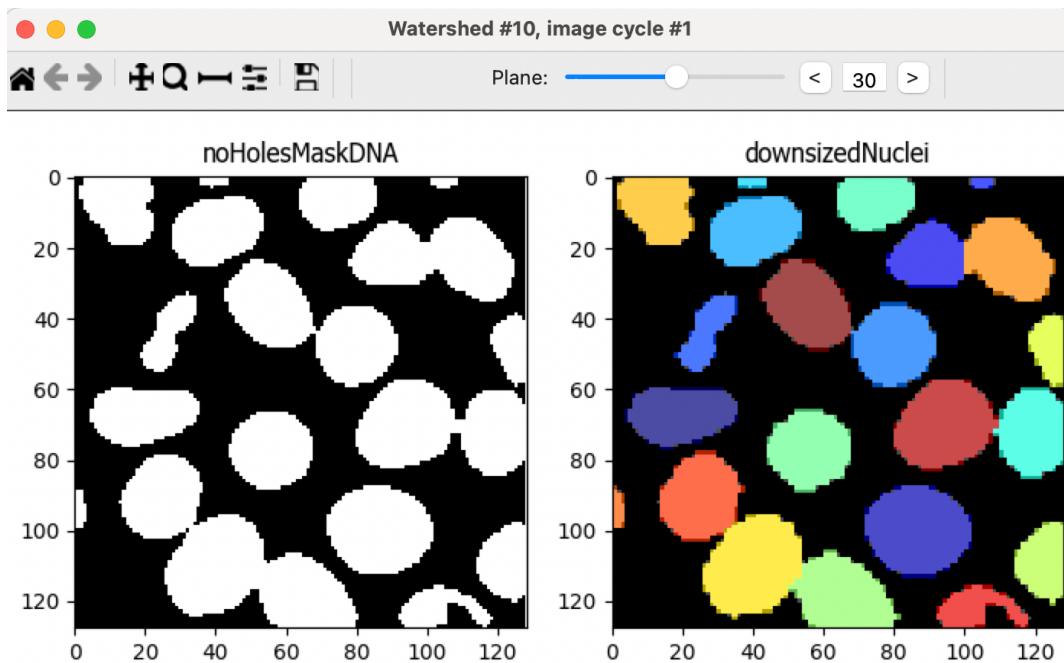


- Add a **RemoveHoles** module. This module implements an algorithm that will remove small holes within the nucleus. Any remaining holes will contribute to over-segmentation of the nuclei. Choose a size of *20*.

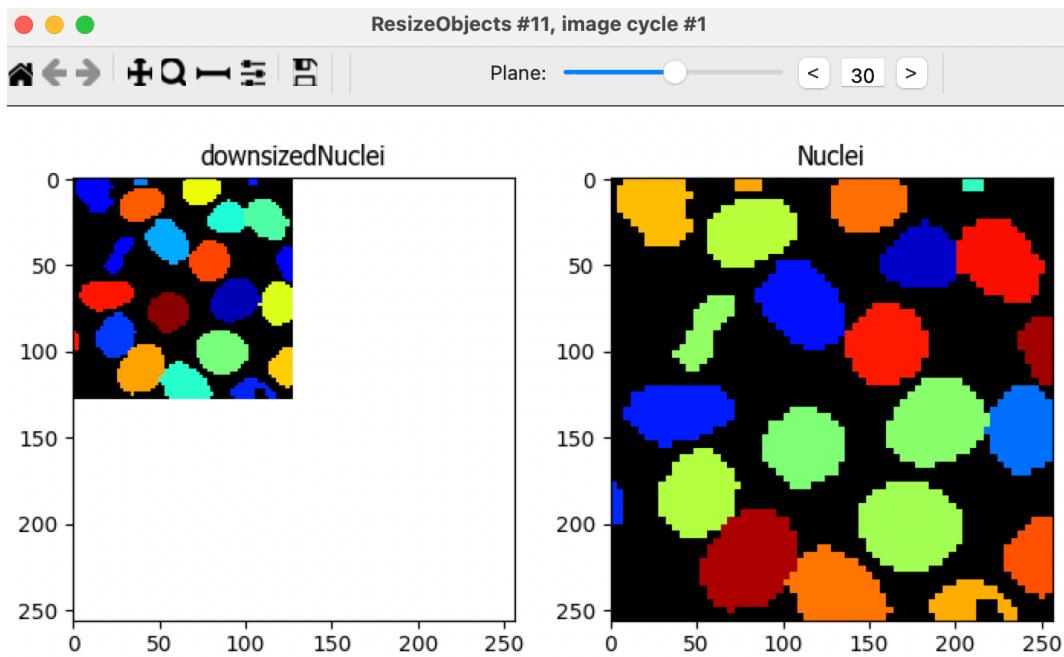


- Add a **Watershed** module. This module implements the watershed algorithm, which will segment the nuclei. Select a Footprint of *10* and Downsample by *2*. Downsampling reduces processing time and decreases noise. For more information on the watershed algorithm refer to this helpful [MATLAB blog](#)

post.



4. Add a **ResizeObjects** module to return the segmented nuclei to the size of the original image. Since the original image was scaled down by 0.5 , it must be scaled up by 2 . The output of this module is the nuclei we are seeking. Name the output *Nuclei*.



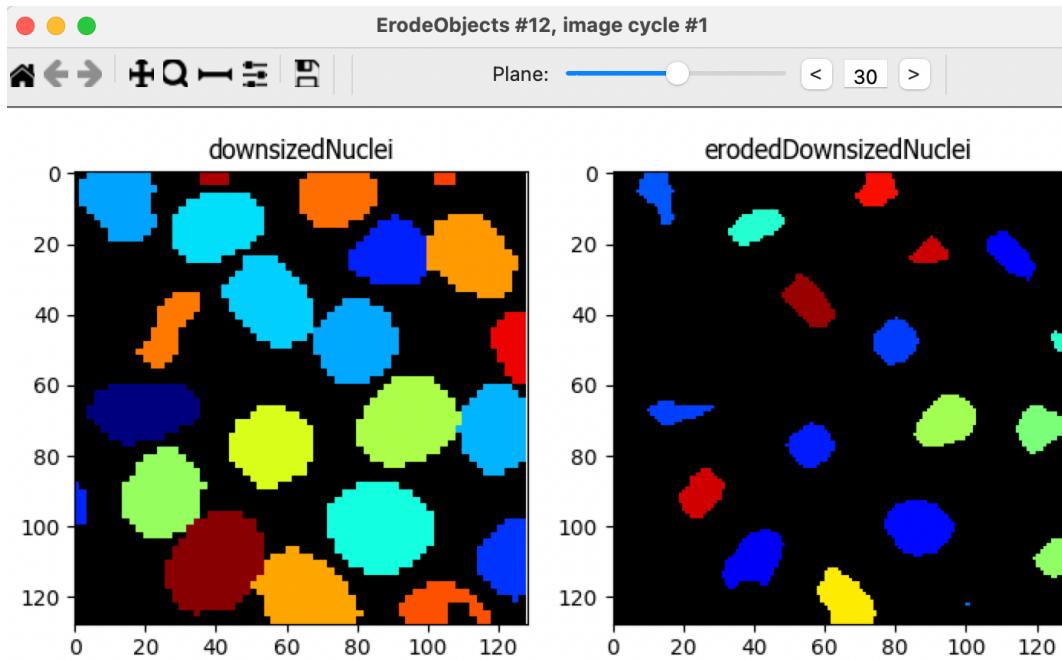
Find objects: cells

Now that we've segmented the nuclei, we want to segment the cytoplasm for each nuclei whose boundaries are defined by the membrane channel. The membrane channel presents more of a challenge because, unlike the nuclei, the membrane signal is variable and the boundaries are connected together in a sort of mesh. However, we can use the location of the nuclei we already found as 'seeds' to guide the **Watershed** module later on to identify regions with cells.

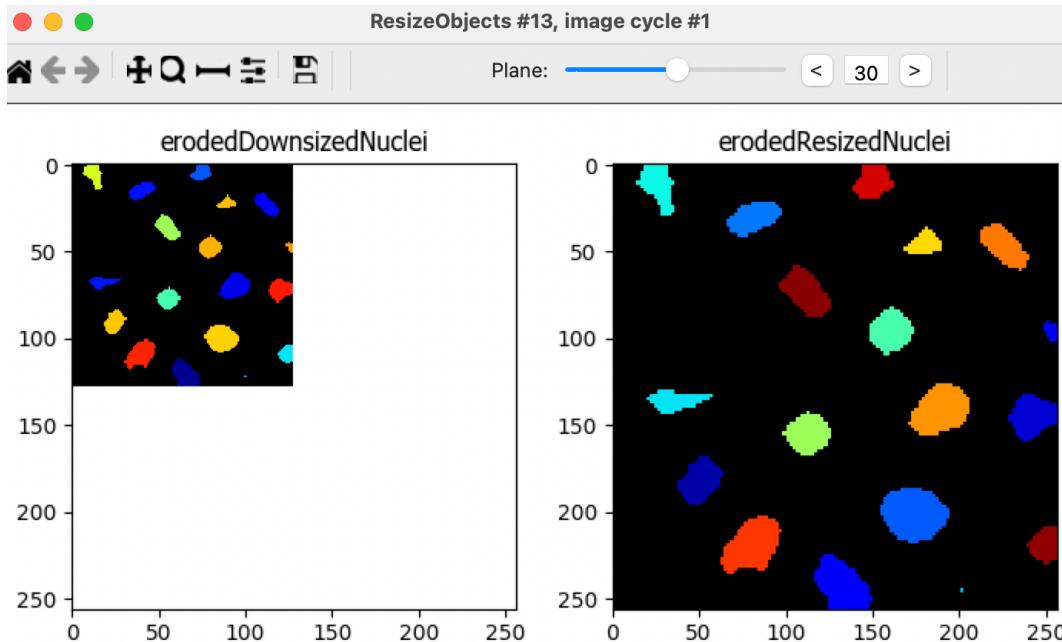
Transform nuclei into ‘seeds’

1. We will start by shrinking the nuclei to make them more seed-like by adding an **ErodeObjects** module. Use the *ball* structuring element with a size of *5*. Select “Yes” for the “Prevent object removal” option in order to avoid losing any nuclei.

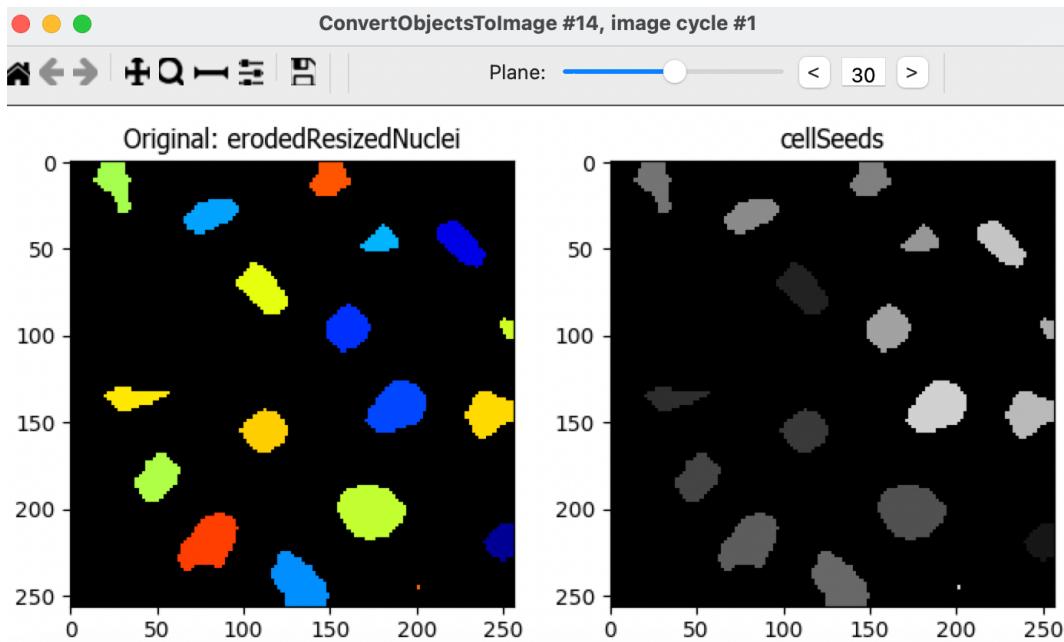
We've found that we can achieve the best results by applying **ErodeObjects** to the output of the **Watershed** module rather than the resized Nuclei that are at the original size (since the **Watershed** module output has been downsampled, the resulting seeds from **ErodeObjects** are smaller and more seed-like). So, select the *downsizedNuclei* object as input. Name the output *erodedDownsizedNuclei*.



2. Resize these seeds using the **ResizeObjects** module with a factor of *2*. Name the output *erodedResizedNuclei*.



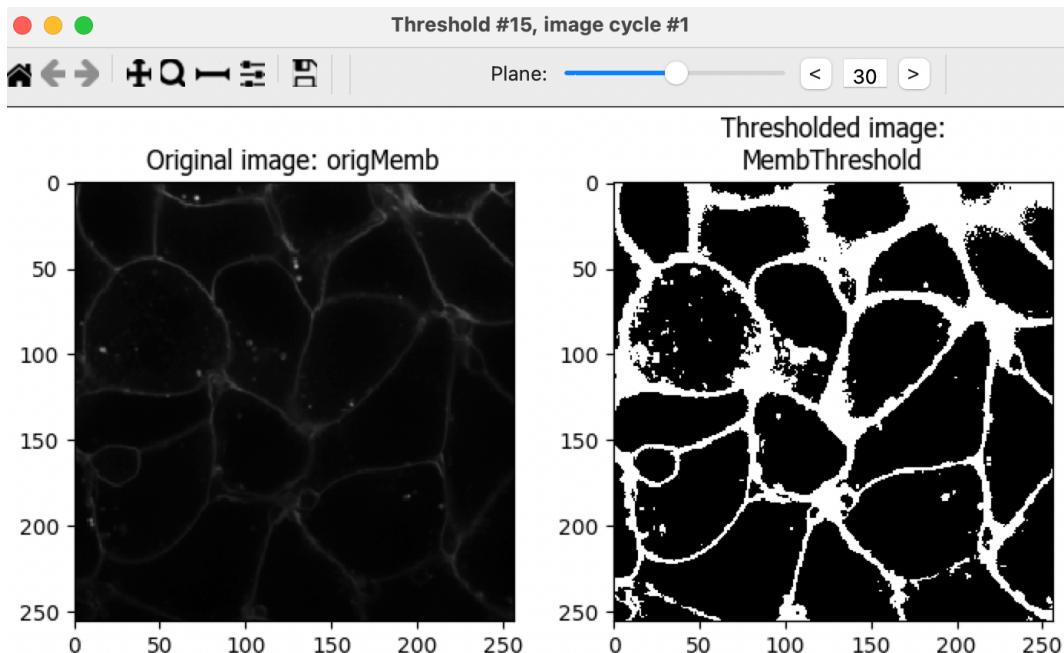
3. Next convert the eroded and resized nuclei to an image using the **ConvertObjectsToImage** module. Select the *uint16* color format. This image will serve as the seeds for segmenting the cells. Name the output *cellSeeds*.



Transform the membrane channel into cytoplasm signal

The **Watershed** module finds objects that have bright signal, so the cytoplasm that will define the cell volume should have bright signal. However, this is not the case in the membrane channel; it must be transformed into an image where the cytoplasm is bright and the boundaries between the cells are dark. Therefore, we will invert the membrane channel to achieve this effect.

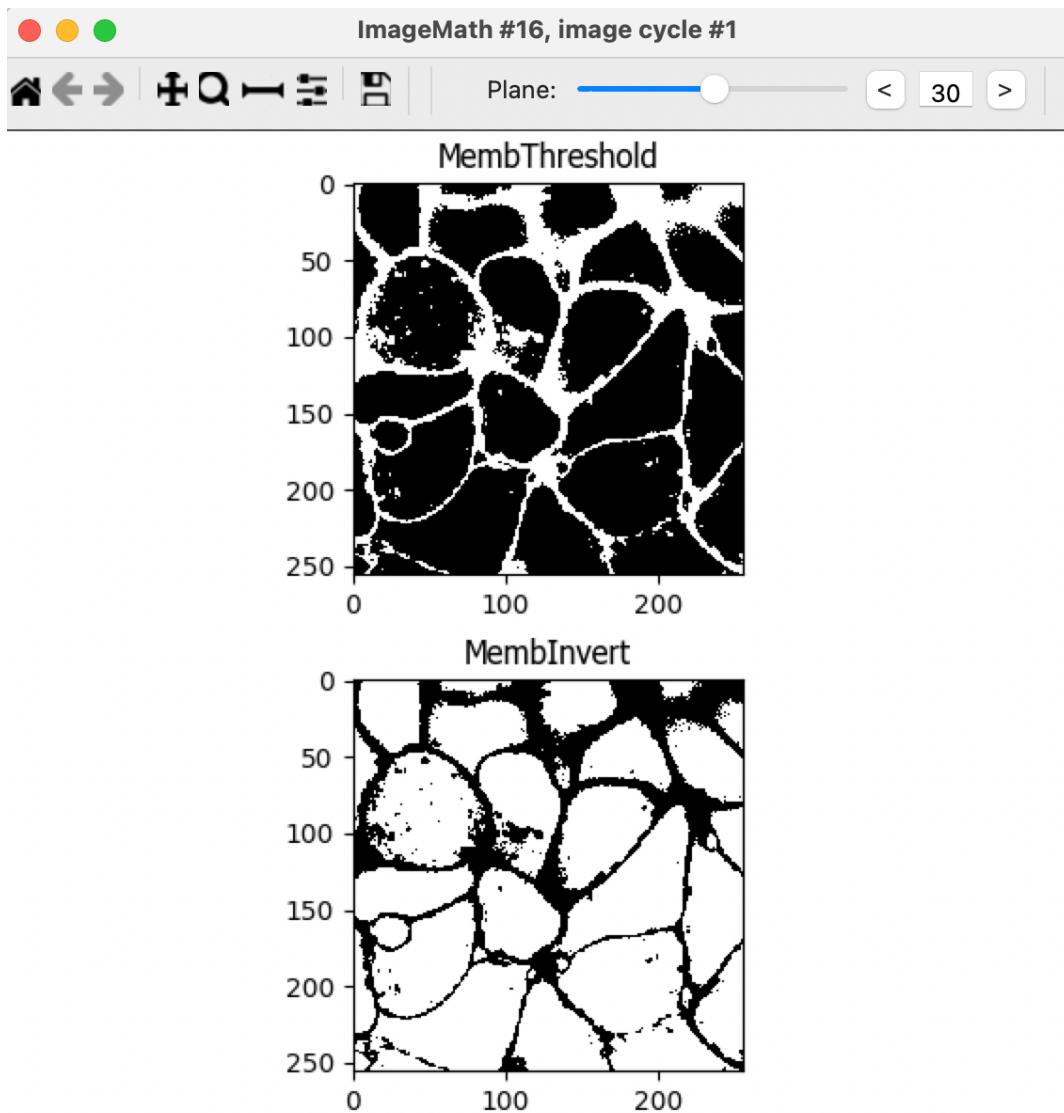
1. Add a **Threshold** module and threshold the original membrane image (*origMemb*). We find that the *Otsu three-class* method with middle intensity pixels assigned to the foreground works well, but feel free to try others. Name the output *MembThreshold*.



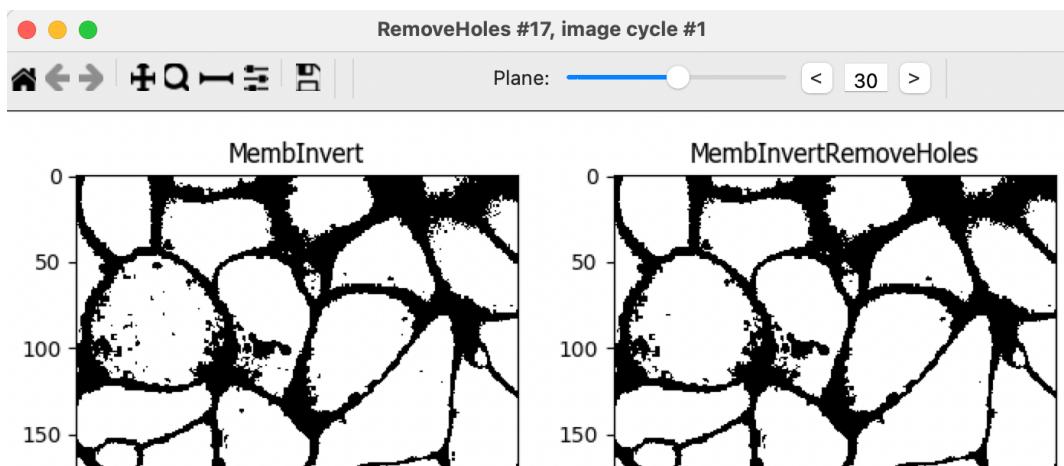
Feature	Value
FinalThreshold	0.0031306028831750154

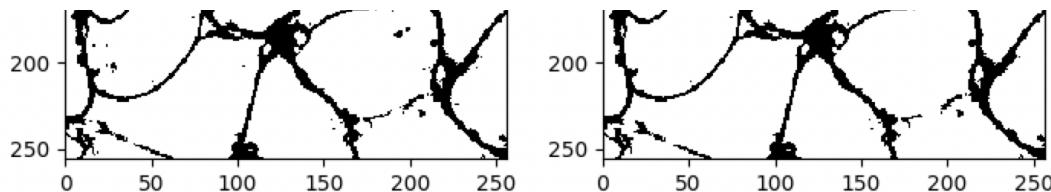
Orig Threshold	0.0031306029
WeightedVariance	0.044483434460683216
SumOfEntropies	-13.468971985758973

2. Add an **ImageMath** module. Within the **ImageMath** module choose the *Invert* operation, and invert the thresholded membrane. Name the output *MembInvert*.

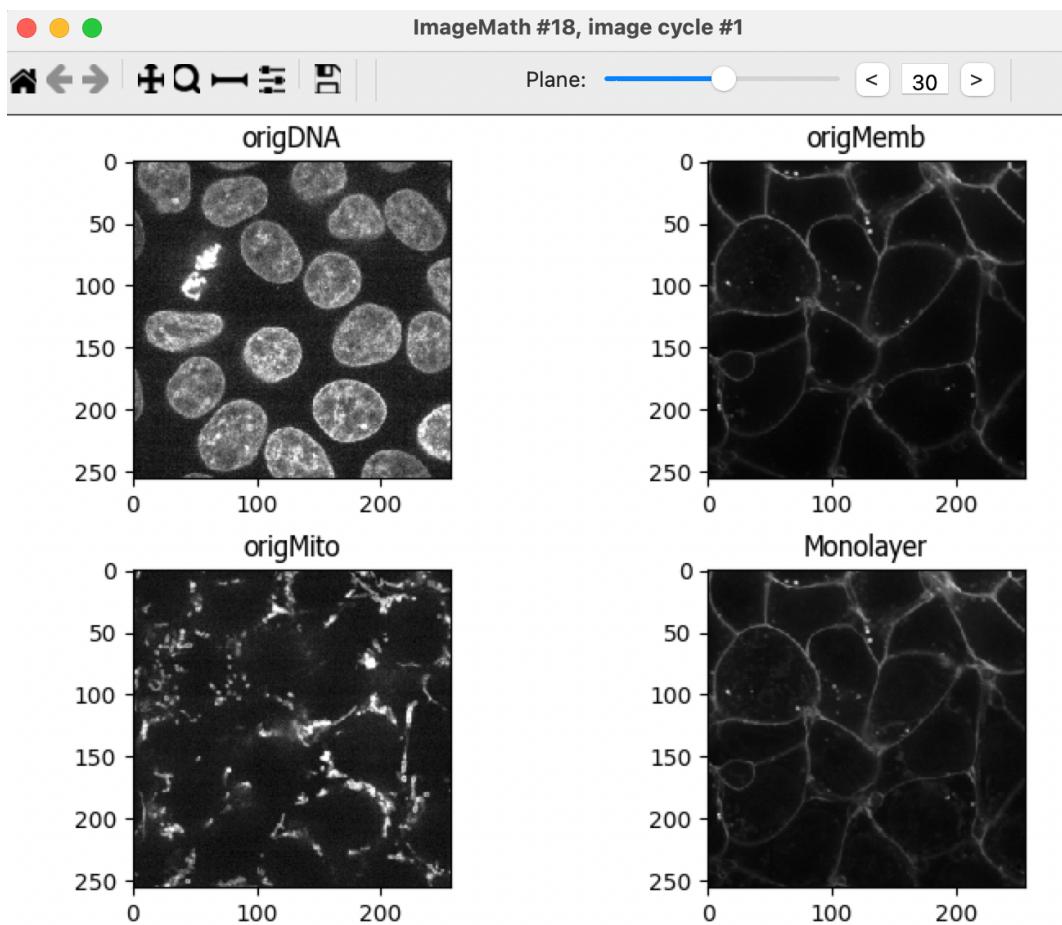


3. Add a **RemoveHoles** module to remove the small holes in the segmentation of the cell interior. This helps to prevent the cells from being split during the Watershed segmentation. Choose a size of *20*. Name the output *MembInvertRemoveHoles*.

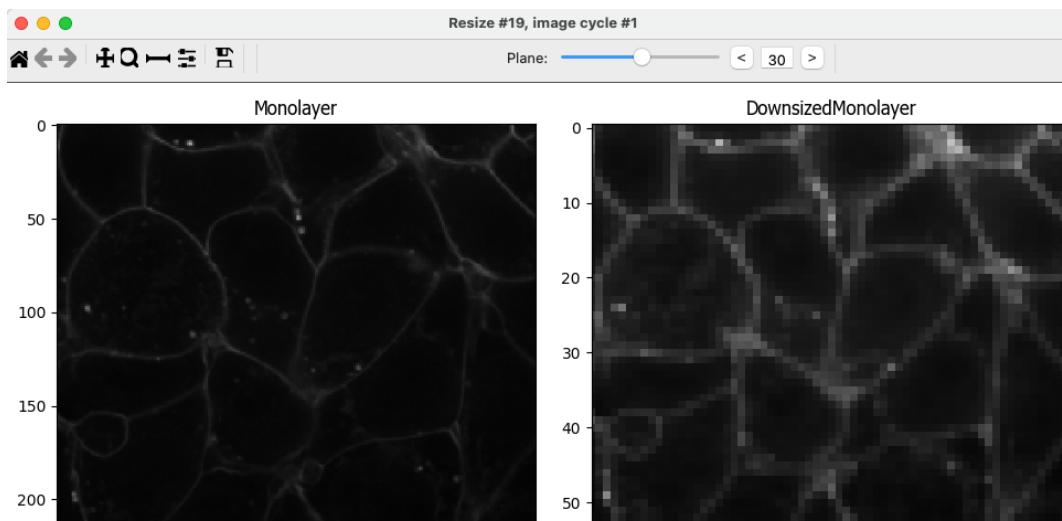


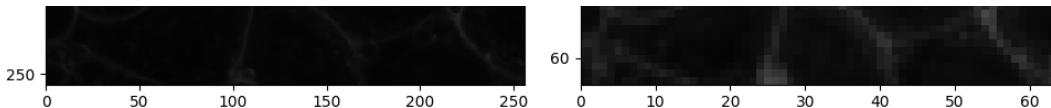


4. Add another **ImageMath** module. Add all of the original images together. This creates a composite image that will be used in the following steps to define where cells are present and the background above and below the cells. Name the output *Monolayer*.

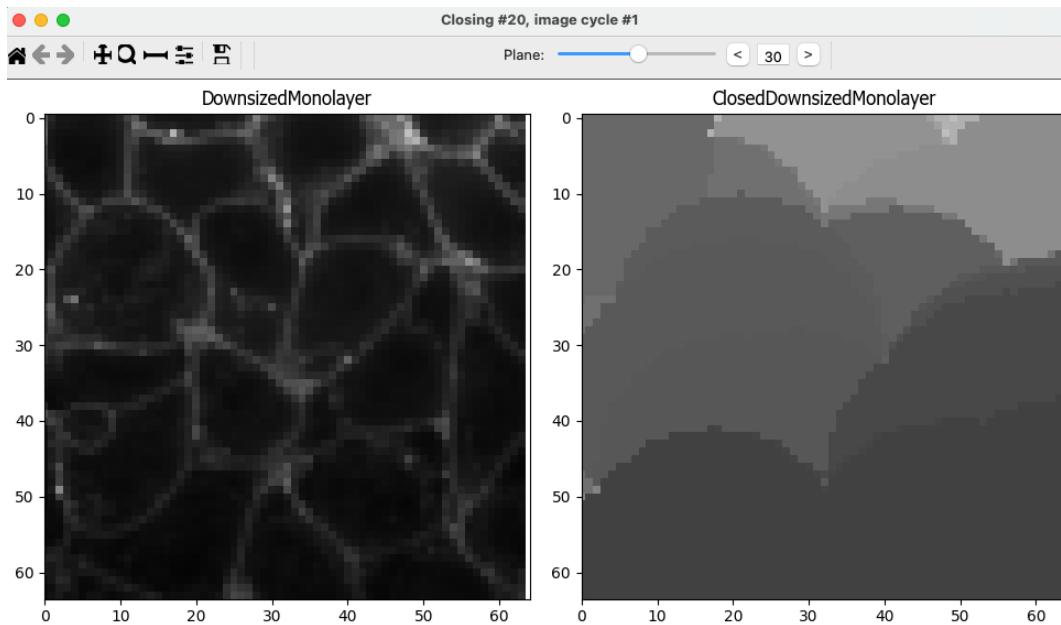


5. Add a **Resize** module to resize the Monolayer with a *Resizing factor* of *0.25* for X and Y (keep a factor of *1.0* for Z). Downsampling the image makes processing faster and decreases noise. Name the output *DownsizedMonolayer*.

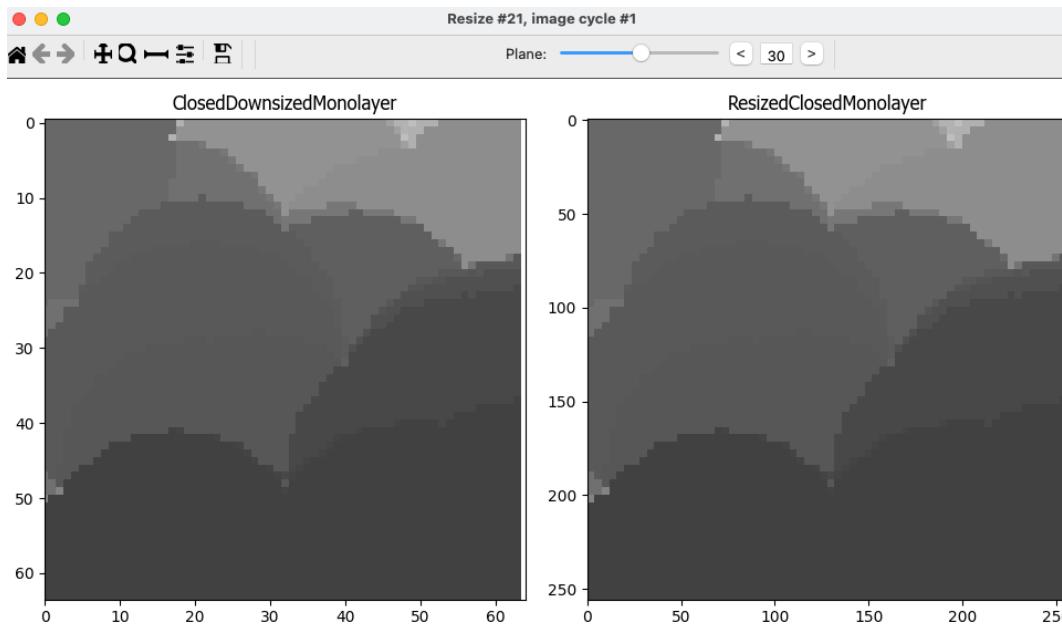




6. Add a **Closing** module. Choose a size of **17** to blend the signal together. The result should look like a cloud of signal where the monolayer resides. Name the output *ClosedDownsizedMonolayer*.



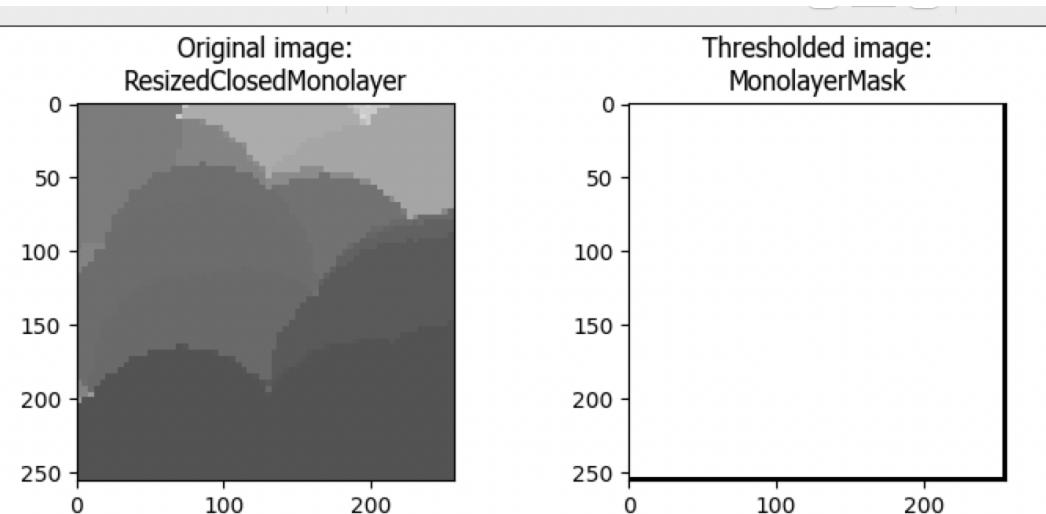
7. Add a **Resize** module to resize the closed Monolayer back to its original size, *Resizing factor of 4* for X and Y (keep a factor of *1.0* for Z). Name the output *ResizedClosedMonolayer*.



8. Add a **Threshold** module and threshold the smoothed monolayer image. The idea is to end up with a 3D mask of the region where the cells of the monolayer exist in. We found that using a global Otsu method with three classes (middle class identified as foreground) works well for this example. This will define what is and is not monolayer. Name the output *MonolayerMask*.

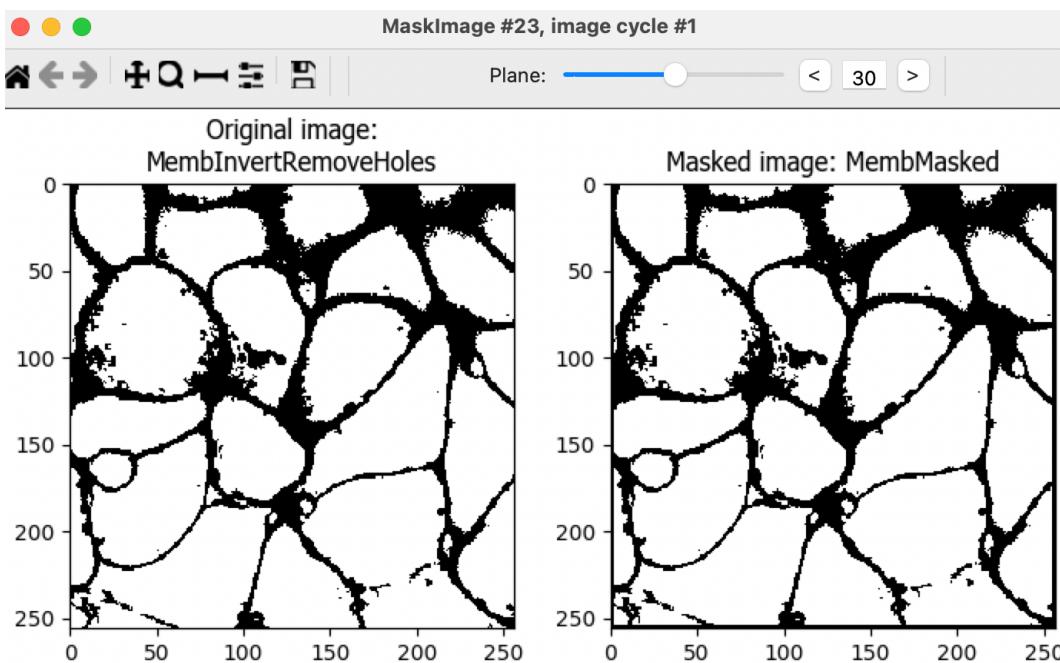
Note that most of the middle planes of the stack should be completely white (part of the monolayer), while the regions above and below are primarily black (not part of the monolayer).





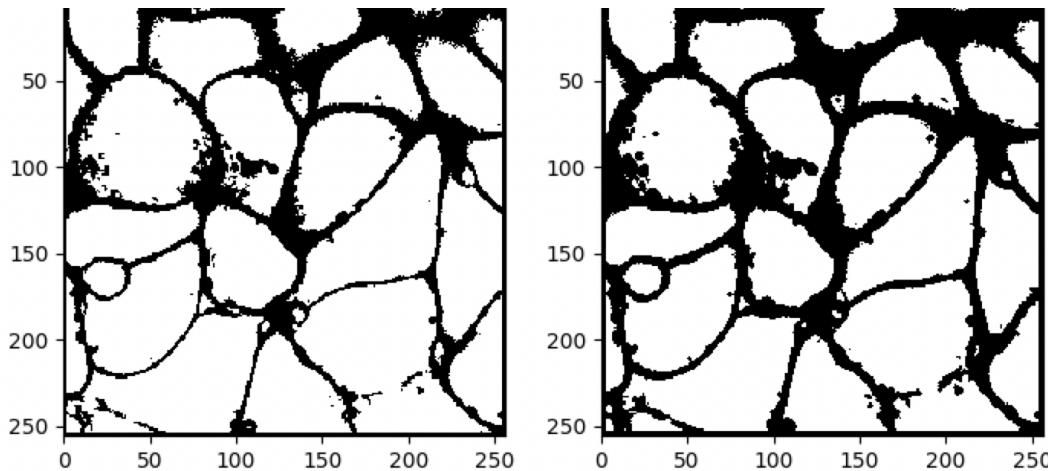
Feature	Value
FinalThreshold	0.007245768327265978
OrigThreshold	0.0072457683
WeightedVariance	0.0214677868231233
SumOfEntropies	-13.105850939422261

9. Add a **MaskImage** module. You will use an *Image* as a mask (the MonolayerMask image generated in the previous step). In this case, the mask does not need to be inverted. Note that the planes on the bottom and top of the z-stack are black in the masked image. Name the output *MembMasked*.

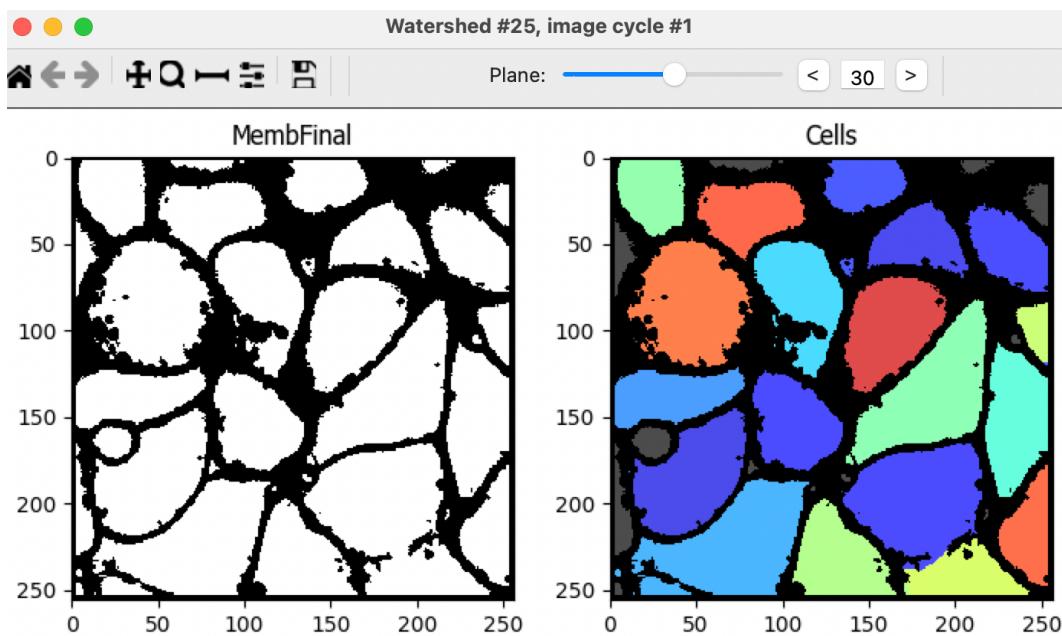


10. Add an **ErodeImage** module. We will use this module to erode the membrane image generated in the previous step. Eroding using a *ball* of size 1 improves the separation between individual cells in the Watershed segmentation (the next step). Name the output *MembFinal*.





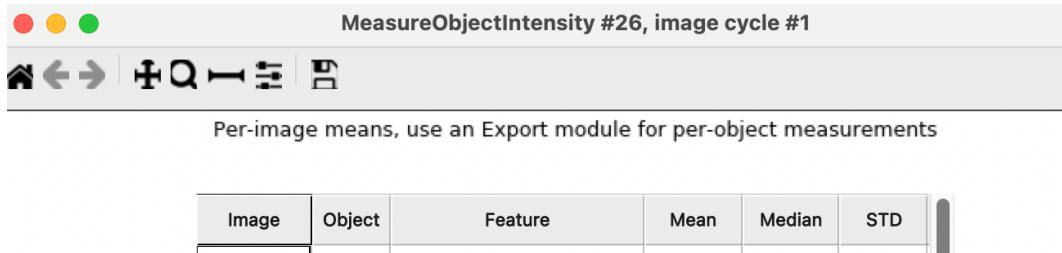
11. Add a **Watershed** module. The input is the result of the previous **ErodeImage** module, referred to here as the *MembFinal*. Change the *Generate from* option to *Markers*. The Markers will be the *cellSeeds* image, which is the output of the **ConvertObjectsToImage** module. Finally, set the Mask to also be the *MembFinal*. This will help preserve the cell boundaries. Name the output of this module *Cells*.



Making measurements

Now that the nuclei and cells have been segmented in this monolayer, measurements can be made using modules from the **Measurements** category.

1. Add any desired measurements modules. For example, you might choose to **MeasureObjectIntensity** and/or **MeasureObjectSizeShape**. When applying these measurements, be careful to measure the original images, not rescaled images.

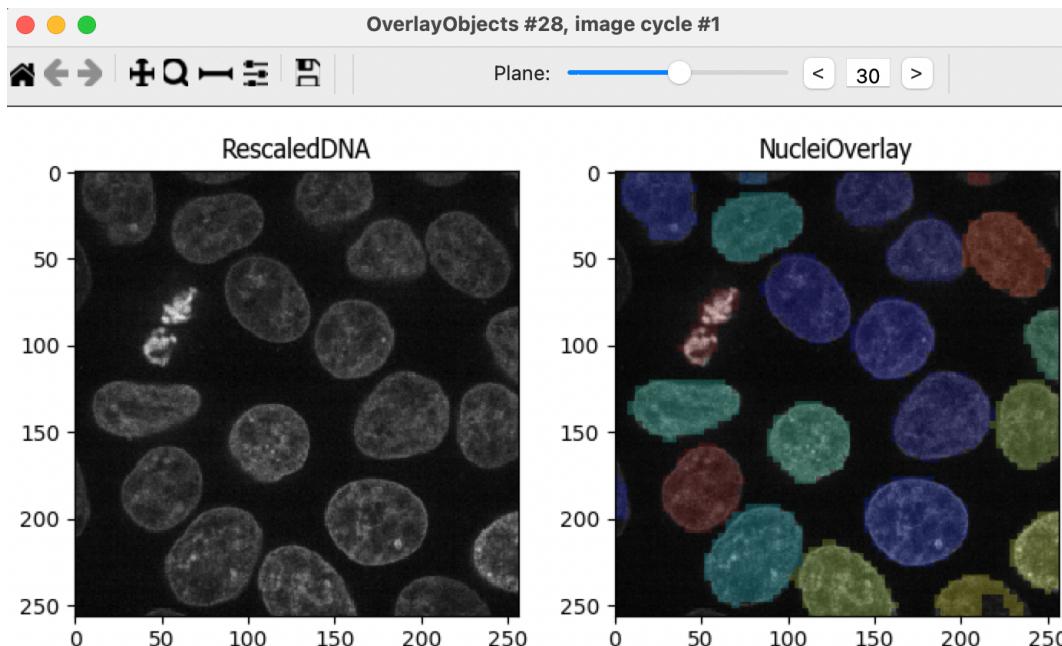


origDNA	Nuclei	IntegratedIntensity	55.636	61.926	26.401
origDNA	Nuclei	MeanIntensity	0.002	0.002	0.0
origDNA	Nuclei	StdIntensity	0.0	0.0	0.0
origDNA	Nuclei	MinIntensity	0.002	0.002	0.0
origDNA	Nuclei	MaxIntensity	0.002	0.002	0.0
origDNA	Nuclei	IntegratedIntensityEdge	9.48	10.896	3.941
origDNA	Nuclei	MeanIntensityEdge	0.002	0.002	0.0
origDNA	Nuclei	StdIntensityEdge	0.0	0.0	0.0
origDNA	Nuclei	MinIntensityEdge	0.002	0.002	0.0
origDNA	Nuclei	MaxIntensityEdge	0.002	0.002	0.0
origDNA	Nuclei	MassDisplacement	0.063	0.059	0.034
origDNA	Nuclei	LowerQuartileIntensity	0.002	0.002	0.0
origDNA	Nuclei	MedianIntensity	0.002	0.002	0.0
origDNA	Nuclei	MADIntensity	0.0	0.0	0.0
origDNA	Nuclei	UpperQuartileIntensity	0.002	0.002	0.0
origDNA	Nuclei	CenterMassIntensity_X	135.929	140.875	75.07
origDNA	Nuclei	CenterMassIntensity_Y	118.349	118.695	78.989
origDNA	Nuclei	CenterMassIntensity_Z	34.808	34.403	2.18
origDNA	Nuclei	MaxIntensity_X	138.227	142.5	75.452
origDNA	Nuclei	MaxIntensity_Y	121.727	125.0	78.065

Creating visuals

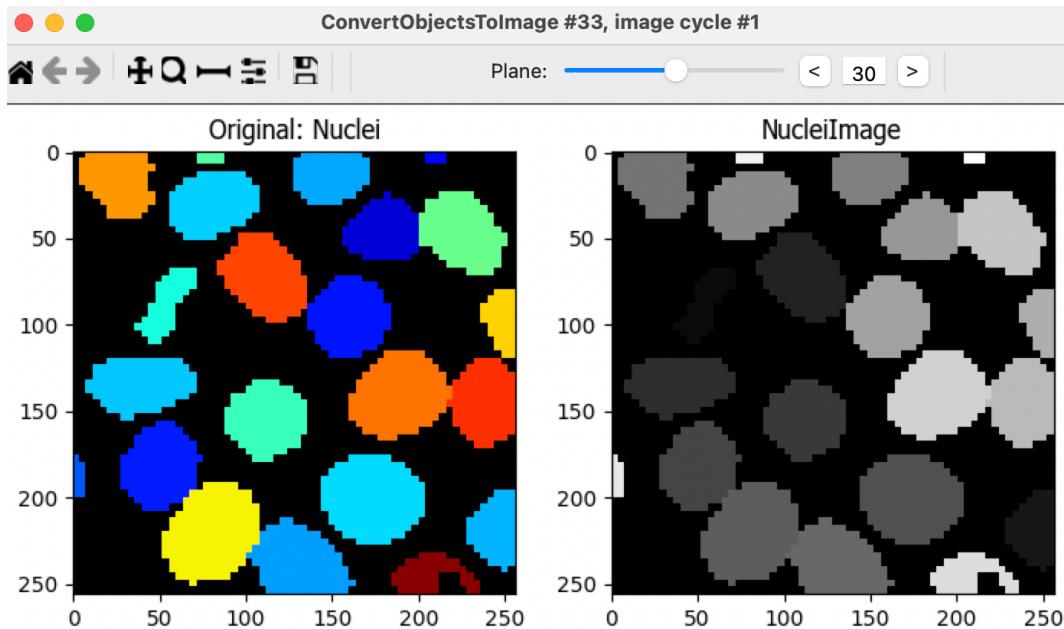
Congratulations! The nuclei and cells have been segmented and measured in this monolayer. Visuals that reveal the details of the segmentation can be also be created within CellProfiler. The following steps will walk you through two different options to visualize your CellProfiler segmentations.

1. The **OverlayObjects** module will overlay the objects as colored masks on the image. We recommend overlaying onto rescaled images, which will be easier to visualize outside of CellProfiler. For example, you can choose the *Nuclei* as the objects and the *RescaledDNA* as your image. Name the output *NucleiOverlay*. You can try and do the same for the cell bodies, overlaying the *Cells* object onto a rescaled version of the *origMemb* image. Name the output *CellsOverlay*.



2. You can also convert the objects to images using the **ConvertObjectsToImage** module and then save the output using **SaveImages**. This option will allow you to visualize the segmentations directly

in Fiji and use them as masks for further processing.



Export measurements

1. Save the output of the measurements modules using **ExportToSpreadsheet** or **ExportToDatabase**.

It's good practice to place all export modules at the end of your pipeline. CellProfiler automatically calculates execution times for each module that was run before the export module. By placing your export modules at the end of your pipeline, you will have access to module execution times for each module in your pipeline.

Thank you for completing the 3d monolayer tutorial!