# 3D Segmentation of Tumor Cells

**Goal:** The goal is to segment and analyze confocal images of cells and nuclei in 3D. In this example the tumor is not fully imaged, and the cells are not well defined, so we made a best effort to define cells and output cell surface area and volume.

**Images:** Two channel confocal images with 35 Z stacks i.e. each channel as a separate tiff images with 35 Z-stacks (2 multi-frame tiff images).

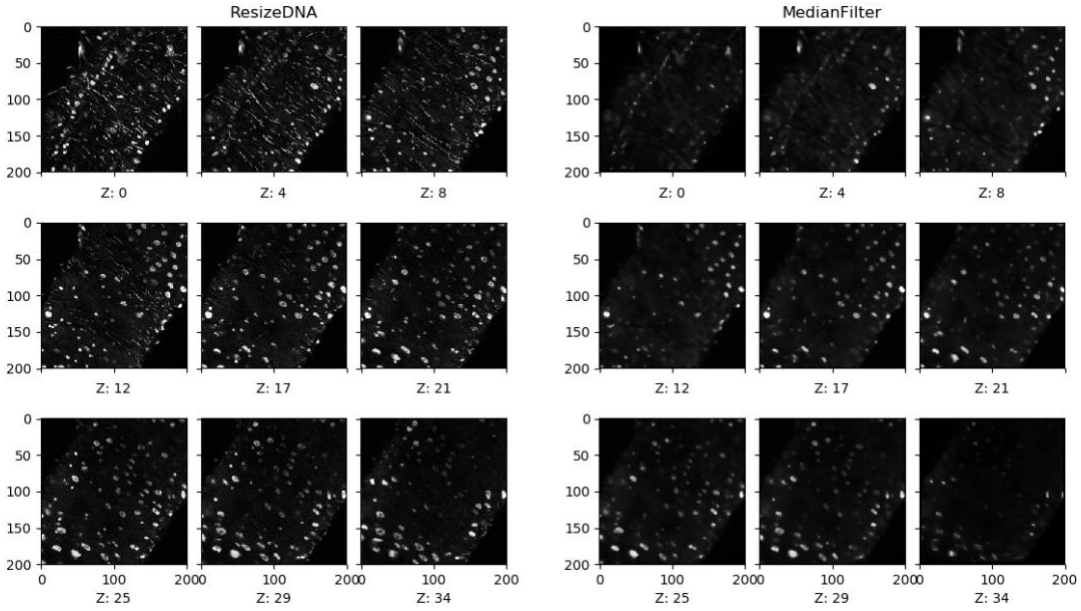
**Pipeline:** This example pipeline shows the segmentation of nuclei and cells in 3D. CellProfiler's standard object processing modules, such as IdentifyPrimaryObjects and IdentifySecondaryObjects do not function for 3D processing, so more basic modules are used in their place, requiring many more steps than a typical 2D pipeline. Visualization within CellProfiler is shown for multiple Z stacks, instead of just one image. The workflow is as follows:

1. Open **CellProfiler**.
2. Click on **Images**. Highlight the two images listed. Right click and Clear File list. Go to the downloaded Input images folder, drag and drop the two Z-stacks images in the appropriate CellProfiler window. The original images maintain the folder structure of the original computer used to create the pipeline. If the images are not reloaded from your computer an error will occur.

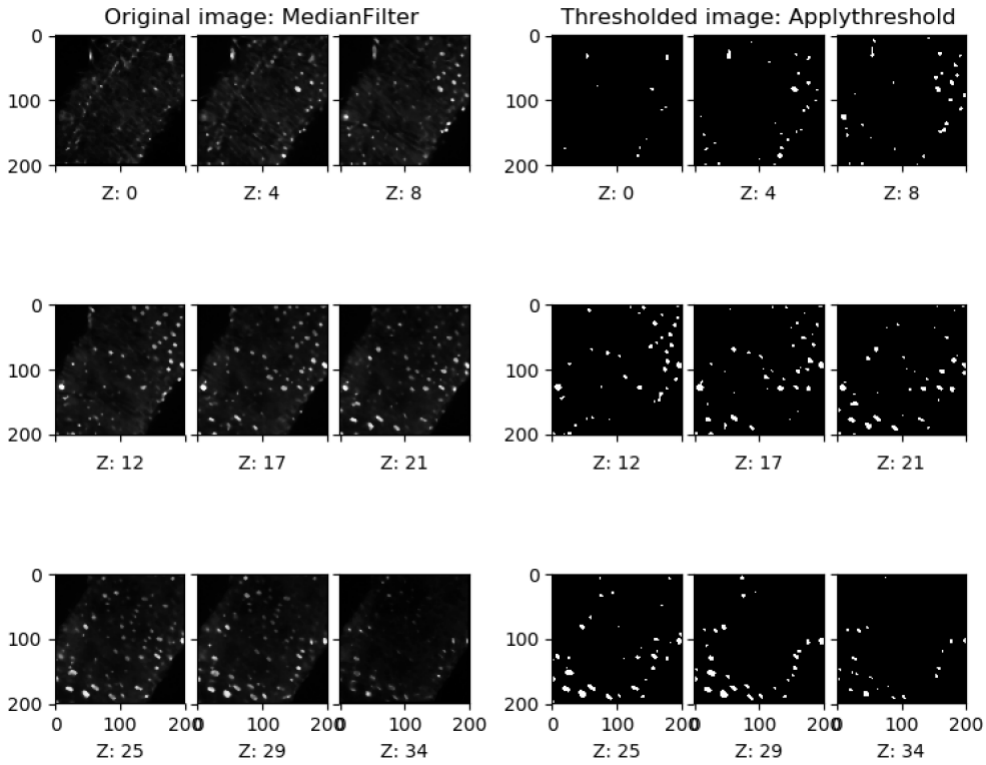
   **Tip:** In case the Z-slices are separate tiff files, one must drag and drop the folder containing all the Z slice images. Since the interest is 3D analysis, the Z-plane information should be extractable from the image **Metadata**. Using the Metadata information, the individual Z slices should be grouped using CellProfiler's **Groups** section in Input modules.
3. The **Metadata** has an expression designed to extract various information, but only file pattern matching is used in this pipeline in the subsequent step.
4. **NamesAndTypes** is used to setup the 3D parameters and set rules to designate DNA images from Cell Images.

a. **Relative pixel spacing** for the X, Y, and Z distances are automatically imported from the image metadata which states a voxel size of 0.4369x0.4369x0.4139 micron^3 for these images. This information can be extracted by pressing the Ctrl + I key when you open the image in Fiji/ImageJ. If CellProfiler did not automatically import these values, they must be calculated and input manually.
b. **Rule Criteria** was set to assign the image from Channel # 1 as DNA and Channel #2 as cell.

5. **RescaleIntensity** is not always necessary but was used in this case to pre-process both the DNA and cell image. This rescales the intensity values, increasing contrast, and aids in thresholding the pixels of interest. In cases where signal to background is large the RescaleIntensity step can be skipped.
6. **Resize** is used on the Rescaled DNA image in order to process the image faster. After the segmentation is completed, the image has to be resized back to its original size before quantification.
7. **MedianFilter** is applied on the Resized DNA image to emphasis bright pixels, helping to highlight the true nuclear signal and aids in nuclear segmentation.

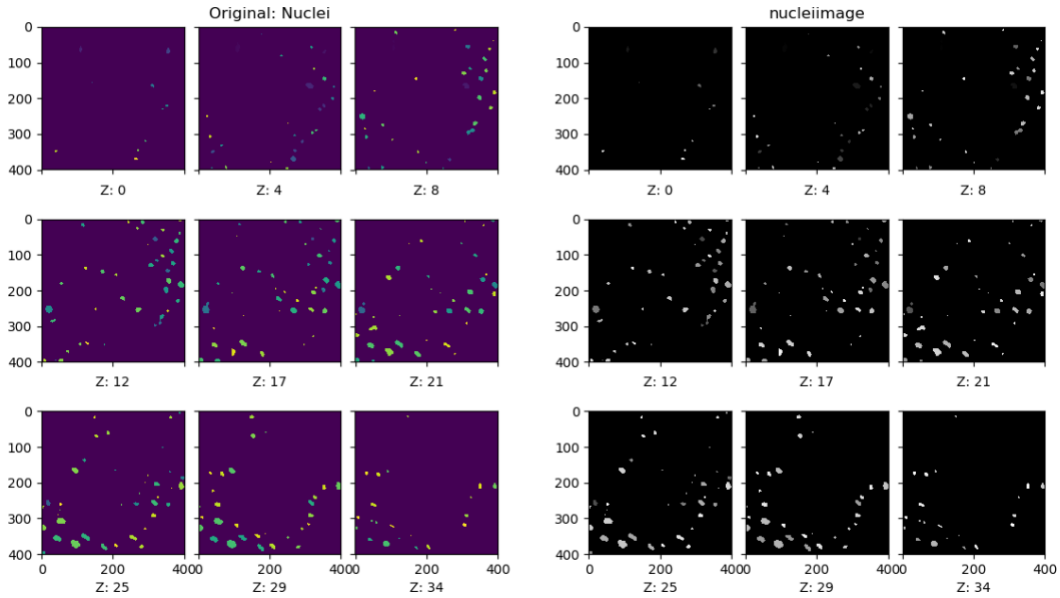


8. **Threshold** is set on the filtered (Resultant image from the MedianFilter module) DNA image to separate the nuclei from non-nuclear signal.



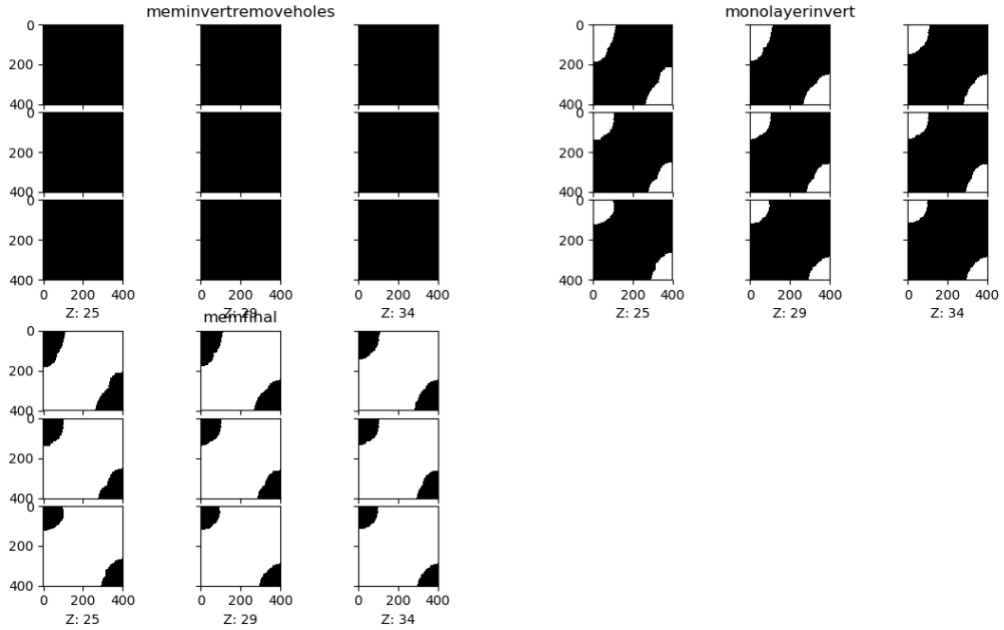

9. **RemoveHoles** is used on the Thresholded DNA image as a post-processing step to remove any holes remaining in the detected objects.
10. **Watershed** is applied on the resultant DNA image from the RemoveHoles module to declump/segment adjacent nuclei.
11. **ResizeObjects** is now used on the declumped DNA image to resize the nuclei back to the original size before it is converted to an image that can be saved for use in a presentation.
12. **ConvertObjectsToImage** is used to convert the segmented Nuclei objects to images that can be saved in a later step.

13. **Erosion** is used to shrink the nuclei objects. The small nuclei objects are used later watershed step below as markers of the approximate center of the cells.




14. **Threshold** is used on the Cell image to separate the cell signal from non-cell signal.
    a. **Otsu** was used with two class because there is a clearly distinguished foreground and background.
15. **ImageMath** is used on the Thresholded Cell image to invert the cell objects. This will be used later to create an area in the empty region of the image which is not needed for quantification.
16. **ImageMath** is used to combine the original rescaled DNA and Cell images from Step 5 into a single image. This will be used to make a mask of the tumor which will be used to facilitate thresholding later in the process.
17. **Resize** is used on the combined image to improve processing speed.
18. **Closing** is used on the combined image to fill in hole and dilate objects. In this case it is the first step in creating a mask of the tumor image.
19. **Resize** is now used on the combined image to bring it back to its original size
20. **Threshold** is used on the combined image to completely binarize the image into black and white or 0 and 1.
21. **ImageMath** is used to invert the combined image and create a mask of the tumor.
22. **RemoveHoles** is used to remove any holes in the inverted cell object created in step 15.

23. **ImageMath** is used to subtract the mask of the tumor from the black images.




24. **Watershed** uses the image above as a mask and the small nuclei objects created in Step 13 to create segmented cells.
25. **ConvertObjectsToImage** is used on the DNA image to convert the cell objects to images that can be saved in a later step.
26. **SaveImages** saves the cell and nuclei images to your hard drive.
27. **MeasureObjectInensity** saves intensity values for the cell and nuclei objects.
28. **MeasureObjectSizeShape** saves size and shape values for the cell and nuclei objects.
29. **ExportToSpreadsheet** exports all calculated values for each object as separate .csv files.

Below is the 3D visualization for the segmented nuclei created by the 3D viewer in Fiji using the segmented nuclei.