Advanced segmentation and organelle analysis :
A computer exercise using CellProfiler & CellProfiler Analyst software

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Background information:

The images in this experiment come from the Broad Bioimage Benchmark Collection. They are 240 of 69,120 fields of U2OS cells treated with a panel of 1600 known bioactive compounds and imaged in five channels for a so-called Cell Painting assay- see Gustafsdottir et al, 2013 for more information. The compounds target a wide range of cell pathways, meaning that some cells and organelles will have very different morphologies both from each other and from the mock treated controls. This will give you an opportunity to try to find segmentation parameters that work across a wide range of conditions.

While in the traditional Cell Painting protocol we do not actually segment out any organelles (other than the nucleus), the large number of stained compartments make this an excellent set of images to find subcellular features. Finding the average or count of smaller objects inside a larger 'parent' object is a feature of many pipelines and an important skill to have in setting up a CellProfiler analysis.

Cell Painting generally consists of a few simple segmentation steps followed by adding as many measurement modules as can be reasonably included in a single pipeline; we have found that by doing this we can measure ~1500 features of each cell and from that create a 'morphological profile' that can be used to predict interesting biology including drug mechanisms of action, gene-pathway interactions, and more. See Bray et al 2016 and citations within for more information.

Goals of this exercise:

This exercise will give you practice at finding segmentation parameters that will be robust across whatever variability may exist in your sample. This is not always straightforward, so examining your segmentation across a wide range of images will be necessary.

This exercise will additionally show you some ways to pull out smaller features in your image by segmenting organelles within the cells and nuclei. You will also be shown how to use RelateObjects so that you can study the average counts, distances, and measurements of the smaller organelles inside their larger parent objects.

Materials necessary for this exercise:

These 1200 images (240 sites in 5 channels) represent 120 wells from a single 384 well plate, either mock treated with DMSO or treated with a variety of bioactive compounds. A CSV file containing associated drug treatment information has also been included.

It is additionally expected that you are generally familiar with CellProfiler, preferably after completing the Translocation tutorial or a similar introductory exercise.

1) Load images and metadata

- Start CellProfiler by double-clicking the desktop icon 

- Drag and drop the 'BBBC022_Analysis_Start.cppipe' file into the 'Analysis modules' box. 7 modules should pop up, and almost all of them will show errors. This is the expected behavior.

- Drag and drop the 'BBBC022_20585_AE' folder into the 'File list' box. It should automatically populate. Notice that illumination correction images (with a file extension of '.mat') are included in this data set.

2) Import metadata from the CSV

- In the 'BBBC022_Exercises' folder there will be a CSV called '20585_AE.csv' detailing drug treatment info for each image.

- Three metadata extraction methods should already be present:

    - The first pulls Well, Site, and Channel metadata from all of the image files except for the illumination correction functions
    - The second pulls Plate metadata from the image folder
    - The third pulls Plate metadata from the illumination correction functions

- Add a fourth metadata extraction step by hitting 'Add another extraction method'

    - Select 'Import from file' as the metadata source
    - Use the '20585_AE.csv' file located in the 'BBBC022_Exercises' folder
    - Under 'Match file and image':
        - Match Image_Metadata_PlateID to Plate
        - Match Image_Metadata_CPD_WELL_POSITION to Well
        - Match Image_Metadata_Site to Site

- Click 'Update'. You should now see a number of additional columns in the Metadata window.

3) Examine the channel mappings in NamesAndTypes (optional)

- Two different ways of mapping images to channel names are demonstrated here. There are several others, and often you could create several correct mappings for a given set of images, but these may serve as a helpful example to refer to in your own work.

- The '.tif' image files are assigned a name by the Metadata extracted in the previous module (specifically ChannelNumber)
- The '.mat' illumination correction functions are assigned a name based on a unique string in the name (such as 'IllumER')

As there is only one set of illumination correction functions for each entire plate, the image sets cannot simply be constructed by using 'Image set matching' as 'Order'.

- Scroll to the bottom of the 'NamesAndTypes' to see how the image sets are constructed

  - 'Image set matching' is set to 'Metadata'
  - Each image channel is set to 'Plate->Well->Site'.
  - Each illumination correction function is set to 'Plate->(None)->(None)'

- Metadata based matching can be useful in any circumstance where a larger group of images needs to be mapped with a smaller one, such as every plate in an image set having its own illumination correction function or every movie in a series of timelapse movies being matched to its own unique cropping mask.

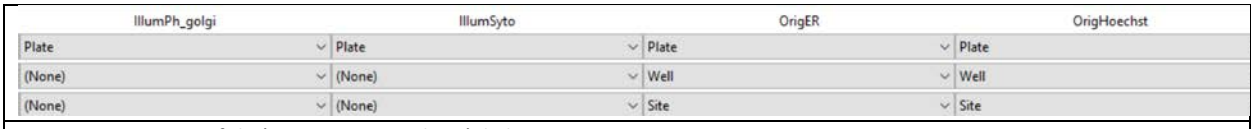


Figure 1: A section of the' Image set matching' dialog

4) Examine the output of the CorrectIlluminationApply module (optional)

- Enter test mode and hit 'Step' to run the CorrectIlluminationApply module.

- Briefly examine the output of the CorrectIlluminationApply module- you can see that the illumination correction functions show significant heterogeneity across the field of view.

  - These functions were created by averaging and smoothing all 3456 images from this plate, indicating the image captured is consistently dimmer in those regions for nearly all images.

- Also note that while the illumination correction functions for each channel are similar, they aren't identical; each channel in your own experiments should therefore be illumination corrected independently.

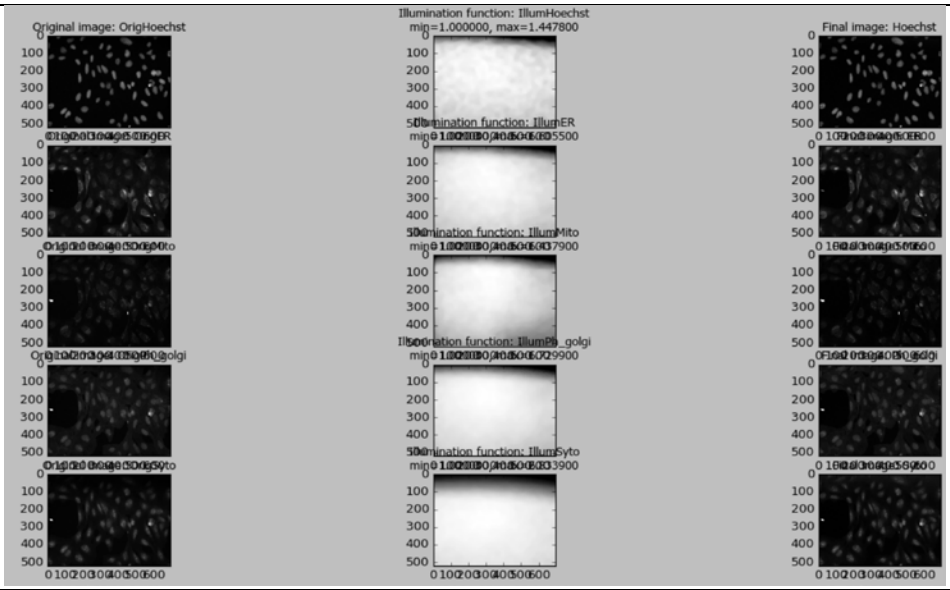


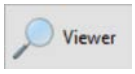
Figure 2: Application of the illumination correction functions

5) IdentifyPrimaryObjects- Nuclei

- **After** the CorrectIlluminationApply module but **before** any others, add an IdentifyPrimaryObjects module (from the 'Object Processing' module category).

- Create objects called Nuclei by segmenting on the Hoechst channel. Hit 'Step' to run the module.

6) Use the WorkspaceViewer to examine the nuclear segmentation

- Next to the 'Step' button, press the button marked 

. This will bring up the WorkspaceViewer. This a tool that allows you to examine multiple images and segmentations simultaneously.

- Under 'Images', add the 'Hoechst' image.

- Under 'Objects', add the 'Nuclei' objects. Click the checkbox under 'Show'. How does your segmentation look?

- Use the magnifying glass at the top of the window to zoom in on an area that was segmented poorly, then update some of your parameters in IdentifyPrimaryObjects and hit 'Step' to rerun the segmentation. You'll notice that unlike the IdentifyPrimaryObjects window, the WorkspaceViewer's zoom does not reset each time you re-run the analysis but stays in the same place, allowing you to more easily check how changing a module's settings affects the segmentation of a given area.

- Adjust the segmentation parameters until you feel you're ready to move on to identifying the cells around the nuclei; as you will test the parameters for robustness later, however, the identification should be good but doesn't need to be perfect before you move on.

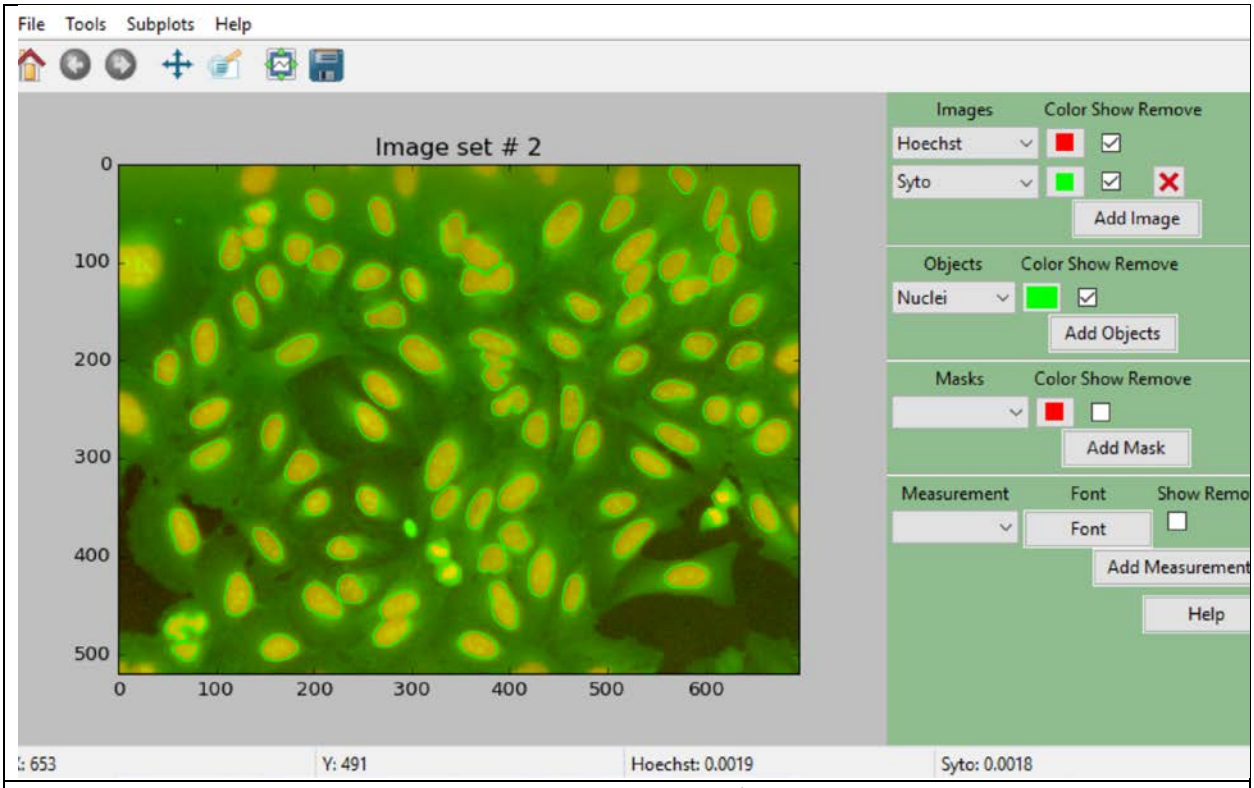


Figure 3: The WorkspaceViewer allows you to view multiple channels and/or objects at one time.

7) IdentfiySecondaryObjects- Cells

- **After** the IdentifyPrimaryObjects module but **before** the EnhanceOrSuppressFeatures module, add an IdentifySecondaryObjects module.

- Create an object called Cells that is seeded on the Nuclei primary objects that you just created; use the Ph_golgi image.

- For the purposes of this exercise, you need not worry about excluding cell bodies that touch the edge of the image.

- Examine the segmentation in WorkspaceViewer, adding any additional channels you like under 'Images' and adding 'Cells' under 'Objects'. Click 'Show' after each new addition.

- Adjust the segmentation parameters until you feel you're ready to test them on another image; they need not be perfect before you move on.

8) Test the robustness of your segmentation parameters across multiple compounds

- Go to Test->Choose Image Set to bring up a list of the images in your experiment.

- Look at the column titled 'Image_Metadata_SOURCE_COMPOUND_NAME' to see what chemical was used in each well of the experiment. You may click on the column to sort the whole table by the values in it if you so desire.

- Choose a row where 'Image_Metadata_SOURCE_COMPOUND_NAME' is blank- this will be a mock treated well. Press the 'OK' button, then run that image in test mode for your first 3 modules (through your IdentifySecondaryObjects step). Examine the output in workspace viewer – did your nuclear and cellular segmentation hold up compared to the first images you looked at? Once your segmentation is good, try it on one additional mock treated image.

- Test your segmentation on images from a few different compounds- you may choose ones you've worked with before, random ones, or some combination therein; if possible avoid using multiple compounds you KNOW have the same mechanism of action, though it's alright if they occasionally do. Update your segmentation parameters until they work well on a few different compound wells, then go back to a mock treated well to make sure it still works well there.

    - You're encouraged to explore the compound list on your own, but if you find yourself consistently ending up with images that look similar you can try adding images from the following list of wells- B18, C7, D6, D19, D22, E3

9) IdentifyTertiaryObjects- Cytoplasm

- **After** the IdentifySecondaryObjects module but **before** the EnhanceOrSuppressFeatures module, add an IdentifyTertiaryObjects module.

- Create an object called Cytoplasm using the Cell and Nuclei objects you've created; 'Shrink smaller object prior to subtraction?' and 'Retain outlines of tertiary objects?' should both be set to 'No'.

10) Examine the steps used to segment the Nucleoli

- The next 3 modules have to do with the creation of the Nucleoli objects. Look at the output from each to see how the image is transformed to aid in segmentation.

    - EnhanceOrSuppressFeatures is a module that helps enhance particular parts of an image- in this case, punctate objects or 'Speckles'. By specifying the feature size, you can enhance different parts of the object. As we are looking for nucleoli, we apply this to the RNA channel (Syto) image, and call the output 'FilteredRNA'

- MaskImage allows you to create a version of the 'FilteredRNA' image called 'SytoNuclei' where all of the pixels except the ones you specify are set to an intensity of 0- in this case, we set to 0 any pixel not inside a nucleus. By doing this, we can decrease the likelihood of detecting the cytoplasmic RNA dots.
- IdentifyPrimaryObjects is used to find the Nucleoli- this is a Primary object segmentation because we are not using another object as a seed to grow around, but only segmenting based off the intensity in our 'SytoNuclei' image.
  - You should check the nucleolar segmentation in the Syto channel in the WorkspaceViewer to assure yourself that the segmentation not only matches the speckle-enhanced 'SytoNuclei' image, but also looks accurate on the unprocessed image as well.

- Now that you've seen an example of how to segment an organelle, you will do so for Mitochondria in the following steps

11) Mask the Mito image by the Cytoplasm object

- **After** the IdentifyPrimaryObjects module for Nucleoli but **before** the RelateObjects modules, add a MaskImage module (from the Image Processing module category).

- Call your output image 'MaskedMito'.

- As you saw above with the Nucleoli example, mask the image via Objects, and use the Cytoplasm objects to create the mask.

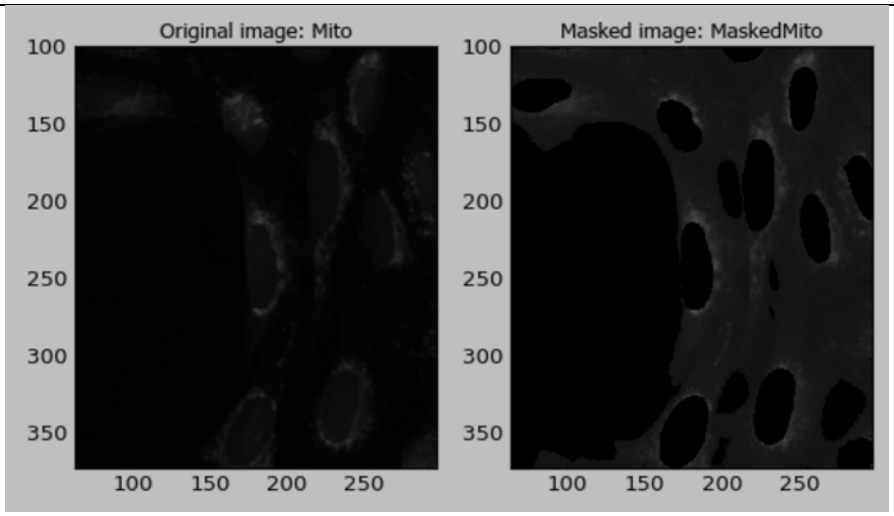


Figure 4: The MaskedMito image contains only the regions of interest.

12) IdentifyPrimaryObjects- Mitochondria

- **After** your MaskImage module but **before** the RelateObjects modules, add an IdentifyPrimary Objects module to identify Mitochondria from your MaskedMito image.

- You should consider using a wide range of pixel sizes here; 2-20 is a reasonable first place to start.

13) Add measurement modules to your pipeline

- **After** your segmentation of the mitochondria but **before** the RelateObjects modules, add as many object measurement modules as you would like.

- Some suggested modules to add- MeasureObjectSizeShape, MeasureObjectIntensity, MeasureGranularity, MeasureObjectNeighbors.

    - Which objects do you think would be valuable to measure with each of these modules? Which channels would you measure your objects in?
    - For a typical Cell Painting experiment you would add as many measurements as possible, but that isn't necessary here; however, do make sure every object gets at least some measurements.

- While MeasureCorrelation, MeasureTexture, and MeasureObjectIntensityDistribution can produce valuable data for downstream profiling, they can be memory-intensive and/or slow so should not be added for this example pipeline in the interest of pipeline run time. MeasureNeurons is not well suited for this pipeline.

14) Examine the settings of RelateObjects

- **After** your Measurement and **before** your Export modules you should find two RelateObjects modules. One relates Nucleoli to Nuclei, while the other relates Mitochondria to Cells.

- Relating the objects allows you to create per-parent means (ie, for this cell what is the average size of an individual mitochondrion) and calculate distances from the child objects to the edge and/or the center of the parent (ie how far is each nucleolus from the center of the nucleus).

15) Run the pipeline (optional)

- If you have time and/or if you'd like to play with the data in CellProfiler Analyst later, exit test mode, close the eyes next to each module, and run the pipeline

- The pipeline will create a database called BBBC022.db, containing the output of all of the measurements you have added to your pipeline

- Because you have different object counts for some of your different types of objects (the counts of Nuclei, Cells, and Cytoplasm will be the same, but the counts of Mitochondria and Nucleoli will not be),

you will not be able to export the objects as a single data table but must instead use a different data table for each object. This will not affect the actual outcome of the experiment, but will mean that each object will get its own properties file and that you can only look at the measurement for one object at a time in CellProfiler Analyst.