Teaching Guide: Computational Image Processing in Microscopy

Overview
The age of big data includes sophisticated imaging datasets. Computational image processing is essential for extracting quantitative information from these large image datasets. Computer scientists have been working for decades to build image analysis tools. It is critical for biologists to understand the concepts in image processing so that they can communicate with computer scientists in designing image processing pipelines and applying these tools to their own images. We focus on microscopy images, but the principles apply to other types of images as well. Furthermore, it is important to understand what manipulations are appropriate in preparing images for publication, what manipulations must be disclosed in the methods and the figure legends, and what manipulations are unacceptable. Here we introduce computational image analysis concepts and terms and illustrate them with Fiji and the COSTANZA (COnfocal STack ANalyZer) plugin. We provide a step by step, hands-on workshop with a sample image so that students can try some of these functions themselves.

Learning Objectives
By the end of this module the student should be able to:
1. Develop the vocabulary to communicate about image analysis.
2. Identify properties of images that make them amenable to computational image analysis.
3. Understand how confocal microscopes generate images.
4. Apply the COSTANZA image analysis tools to analyze microscopy images.
5. Discuss the advantages and limitations of these image analysis tools.
6. Distinguish between unacceptable image manipulation and quantitative image analysis.

Teaching Strategies:
There are multiple strategies to teach computational image analysis using the materials provided in this teaching tool including traditional lecture, paper discussion, and flipped classroom. In the end of the lecture slides and lecture notes, we provide a hands-on workshop, a sample image, and an answer key to the questions in the workshop. Steps 1-4 of the workshop (excluding the section on trying your own image) take about 1 hour to complete.

In preparation for a paper discussion students can read a short paper on image manipulation:

http://jcb.rupress.org/content/166/1/11

For students interested in further reading, “A computational image analysis glossary for biologists” provides an in-depth discussion of computational analysis terms that goes beyond the concepts and terms presented in this teaching tool.

http://dev.biologists.org/content/139/17/3071
For eager students, additional references illustrating the application of image processing to exciting questions in plant biology have been provided in the Recommended Reading and References section of the lecture notes.

### 1 day lecture plus paper discussion

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<tr>
<th>Before class</th>
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| Class 1      | 1. Lecture on computational image processing.  
2. Discuss image manipulation versus image processing.  
3. Assign the workshop for homework. |

1. Read image manipulation paper.

### 2 Day partially flipped classroom

<table>
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| Class 1      | 1. Discuss image manipulation versus image processing.  
2. Lecture on image processing concepts using lecture slides. |

1. Read image manipulation paper.

| Class 2      | 1. Work with classmates in groups to optimize the parameters in COSTANZA and detect cells/nuclei/features in the images the students brought to class (bring laptops or use computer lab).  
2. Class discussion of what types of images worked and what type did not and why. |

1. Install ImageJ and COSTANZA software.  
2. Complete workshop to analyze images provided with COSTANZA.  
3. Bring your research image to class (found online).

Before the first day, the students read the paper on image manipulation detailing what is not allowed for scientific publications. In class, the instructor lectures on image processing concepts using the lecture slides 1-36. The students discuss improper image manipulation based on the paper figures. Before the second class, the students apply their new knowledge of image processing by completing the workshop (lecture slides 37-56 with accompanying lecture notes) using Fiji and the COSTANZA plugin, which they download to analyze a sample image provided. In class, the students work together in groups to attempt to analyze scientific images they found on the web or the additional sample images provided. The students learn about how to optimize parameters in the COSTANZA plugin for different images, and that even with optimized parameters some types of images cannot be successfully analyzed.

**Study / exam questions** (understanding and comprehension)

1. Describe a typical computational image processing pipeline.
2. Explain why resampling an image to increase the DPI while keeping the print size constant is not allowed for scientific images.
3. You have two tomato species with different size meristems. You want to know how many cells are in each meristem. You have a DNA specific fluorescent stain and a
clearing solution that will let you take confocal images capturing the whole tissue. How
would you rapidly determine how many cells are in each meristem?
4. You notice when you grow plants with nitrogen fertilizer they produce bigger leaves than
when you grow them on low nitrogen soil. You want to know whether the bigger leaves
have more cells than the smaller leaves. Design an experiment to rapidly determine the
total number of cells in 20 leaves grown on each growth condition.
5. Why is denoising a useful pre-processing step for computational image processing?
6. Give one example each of an image manipulation that is acceptable for publication and
one that is not.

Discussion questions (engagement and connections)

1. What are the advantages of computational image processing?
2. What characteristics make an image amenable for computational image processing?
3. How do you reconcile image manipulation policies with computational image
   processing?
4. What are the advantages and disadvantages of each segmentation algorithm:
   thresholding, gradient ascent, watershed, and edge detection.
5. For what type of image would you want to display the maximum intensity projection and
   for what type of image might you want to display a single optical section?

Lecture synopsis

Overview and general background (1-3)
Scientists are generating vast imaging datasets which require computational processing to
extract quantitative information. Here we focus on the Fiji platform and its extension through
plugins for detecting features such as nuclei and cells in plant microscopy images.

Part 1. Image processing pipeline (4)
Designing an image processing pipeline involves optimizing the microscopy settings to obtain
an image good for processing, pre-processing the image to remove noise, automated
computational segmentation of the image, post-processing and often hand correction of the
segmentation, and finally analysis of the data.

Step 1. Imaging (5-19)
There are a number of considerations in imaging and saving the image to optimize it for
subsequent image processing. Most importantly simple images where the object to be detected
are uniformly colored and the background is black work best. Confocal microscopes produce
3D images that are stacks of individual optical sections. These slices can be combined into a
single 2D image through projection. It is critical to save enough pixels in the original image,
which affects the size at which the image can be displayed. Finally, when saving compression
can reduce the size of the file, but be cautious because lossy compression also removes data.

Step 2. Pre-processing (20-21)
Before trying to detect the objects in the image, it often is useful to run a denoising filter. In
addition, pre-processing can take advantage of other properties know about the objects to be
detected, such as their minimum intensity.

Step 3. Segmentation (22-27)
Segmentation is the detection of objects in the image. Segmentation partitions the image into the objects of interest and the background. There are many different segmentation algorithms including thresholding, gradient ascent/descent, watershed, and edge detection. Different algorithms are optimized for different images.

**Step 4. Post-processing (28-33)**
Post processing can take advantage of knowledge about the objects in the image to improve the segmentation. Often a scientist intervenes by hand to correct obvious mistakes of the automated segmentation in a semi-automated approach, which can be part of the validation process. To further track the segmented objects in time, the images are first registered (aligned) and then corresponding features are identified.

**Part 2. Image manipulation versus image processing (34-36)**
Changing the original images so that they no longer accurately represent the data is not acceptable in science. Journals such as Plant Cell have specific image manipulation policies detailing what types of manipulations are not allowed. Generally linear changes such as brightness and contrast are acceptable as long as they are applied to the whole image and do not obscure anything in the original image. In contrast, removing or adding a part of the image including background is not allowed. Groupings of images should be explicit. Images, such as controls, cannot be reused in multiple figures without disclosure in the figure legend. Image processing often violates these rules, so it must be carefully detailed in the methods and disclosed in the figure legends. Often the original image and the processed image can both be displayed.

**Part 3. Computational Image Processing Workshop (37-57)**
The best way to learn about image processing is with hands-on experience. These slides go with the workshop exercises at the end of the lecture notes that give step by step instructions for installing and exploring the Fiji image analysis software package and the COSTANZA plugin. These exercises are to be used with an example image given before trying other images.

Step 1: Install Fiji and COSTANZA (41-44)
Step 2: Exploring a confocal stack in Fiji (45-46)
Step 3: Measure objects by hand in Fiji (47-48)
Step 4: Segment nuclei using COSTANZA (49-54)
Scientific conclusion (55-56)
Step 5: Try your own image (57)

**Lecture slide concepts**

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Computational Image Processing Workshop Answer Key

Part 3, Step 2: Exploring a confocal stack:

*How many z slices are in the image? (Hint look at the information at the top.)*
There are 134 z slices.

*Pick a large nucleus (c slider =2). For how many consecutive z-slices is the nucleus in view (i.e. from the bottom to the top)?*
Answers may vary slightly based on which nucleus is chosen, but should be about 15-20 slices.
(For the nucleus at x=111, y=309, the nucleus is visible from z=76 to z=96 for a total of 21 slices. For the nucleus at x=186, y=358, the nucleus is visible from z=67 to z=86 for a total of 20 slices.)

*When you are reading a research paper, how do you know what the colors in a confocal image mean?*
In order to know, the authors have to specify what the colors mean in the figure legend. The colors themselves are not detected by the microscope’s photomultiplier tube (PMT); colors are added afterwards and can be changed. Usually the color seen in the image and that of the fluorophore match, as a matter of convention, but not always.

*Using the fire LUT and the calibration bar, what is the approximate range of intensities of the pixels in the nuclei (c =2)?*
I see dark purple nuclei, that appear to be half way between 0 (black) and 64 (light purple), so I would estimate these are around 32. The brightest spots I see within nuclei are in the mid orange range around 150. Note you can check your estimates by hovering the cursor over the pixel and checking the value that appears on the bottom of the main Fiji toolbar.

*What can you see in the projection that was not clear from the stack? What information that is included in the stack is lost in the projection?*
The projection shows the whole surface of the sepal, which is not visible in a single z-slice because the sepal is curved (not flat in the z-dimension). Spatial information and curvature are lost, ie. it cannot be known from the maximum intensity projection whether two nuclei that appear to be touching are in the same plane or whether one is higher or lower (in z) in the sample. Additionally, information from cells under other cells that fluoresce more brightly will be lost, although that does not happen much in this sample.

*Is the mCitrine-ATML1 signal in the epidermis? Mesophyll?*
mCitrine-ATML1 (initially green nuclei) is only in the epidermis or the outer layer of cells.

Part 3 Step 3: Measuring objects in images by hand

*What is the average nuclear area? What is the minimum and maximum that you measured? Are all of the nuclei about the same size?*
Example measurements: mean: 42.2 µm²; SD: 17.8 µm²; min: 31.2 µm²; max: 73.3 µm². The nuclei vary substantially in size. (This depends on which nuclei were chosen, so a wider variety of answers are possible.)

*The integrated density gives the total fluorescence in the nucleus. Do all the nuclei have the same integrated density?*
No, there is a wide range (e.g. min: 18295 – max: 72215) in the integrated density values.

**Part 3 Step 4: Segmentation: Detecting objects in images**

*Is each nucleus correctly identified? Are there obvious mistakes? Are nuclei missing, or divided in 2?*

Most nuclei are correctly identified, but there are a few errors. There are some dim nuclei missing near the left side of the sepal.

*How many nuclei are detected?*

108 (0 to 107 in the results window). (If you are getting different results be sure you have the correct scaling entered in set scale and the correct parameter settings.)

*Looking at the results, are all nuclei the same size (BOA volume)?*

No, there is a lot of variation ranging from 21 to 1519.

*How many nuclei are detected in the projection? Which is more accurate in terms of nuclei detected?*

161 nuclei are detected. The inclusion of many dim nuclei that were omitted from the COSTANZA analysis of the stack indicates that the analysis on the maximum intensity projection might be more accurate in this particular image. However, more nuclei are also divided into two. We would need manually segmented ground truth to be sure which is more accurate.

*What change did you make in the parameters and is the nuclear detection more accurate, or are there more mistakes?*

Many possibilities. Increasing the background extraction intensity threshold (to 30) led to the exclusion of many previously identified BOAs, making the analysis less accurate. Decreasing it to a value of 1 led to the detection of almost the same number of nuclei as when the value was 10 (threshold 1 detects 159, threshold 10 detects 160), but the areas of the BOAs are largely overestimated.

*Looking at the results and the third stack, how does mCitrine-ATML1 fluorescence compare between nuclei? Does ATML1 concentration differ between cells?*

Looking at the Basins of attractions (BOA)-intensity reveals variation in intensity values. Checking with the results window shows a range from about 20 to 40 in mean cell intensity. Yes, ATML1 concentration varies in the cells of the sepal.

**Part 3 Step 5: Testing your own image**

We have provided a couple of additional sample images and parameters that work for these images.

**Nuclear segmentation example**

pAR180 8 day 1st leaf ab 10X 1A.lsm

Image of nuclei in the epidermis of the first rosette leaf developing between the petioles of the cotyledons in Arabidopsis.
Set Scale in FIJI (maybe imported automatically from .lsm file):
Distance in pixels: 1.3249
Known distance 1.00
Pixel aspect ratio 1.0
Unit of length: µm

To detect the nuclei, before processing the image, use Image>Color>Split Channels to separate the two channels. Then use Image>Color>Channels Tool>More to change the image to greyscale. If you are getting errors from COSTANZA most likely the channels are not split or the image is not grayscale.

Example parameters that work to segment nuclei:

General:
- Use extended box neighborhood
  - Mark intensity plateau with a single maximum
  - Use secondary stack to measure intensity levels
  - Mark cell centers. Marker pixel radius: 3
  - Display basins of attractions (BOA).
  - Display basins of attractions according to measured intensity.
  - Display internal working stack.

Pre-processor:
- Processor queue:
  - Background extraction
    - Intensity threshold: 50.0
  - Mean filter
    - Radius: 0.5
    - Number of times: 10

Post-processor:
- Processor queue:
- BOA remover
  - Size threshold 5.0
  - Intensity threshold 10
BOA merger
Radius 3.0
Scaling
✓ use ImageJ stack calibration

Note that with these parameters still a few of the nuclei are split into two.
Example segmenting cells outlined with a plasma membrane marker
pAR169 cauline leaf abaxial 20x 1C slice.tif

Single confocal slice image of a plasma membrane marker outlining the cells in the abaxial epidermis of an Arabidopsis cauline leaf.

Scale:
Distance in pixels: 0.9635
  Known distance 1.00
  Pixel aspect ratio 1.0
  Unit of length: \( \mu \text{m} \)

Example parameters that work to segment cells:
General:
  ✔ Use extended box neighborhood
  ✔ Mark intensity plateau with a single maximum
    Use secondary stack to measure intensity levels
  ✔ Mark cell centers. Marker pixel radius: 3
  ✔ Display basins of attractions (BOA).
  ✔ Display basins of attractions according to measured intensity.
    Display internal working stack.
Pre-processor:
  Processor queue:
  Invert Image
  Background extraction
    Intensity threshold: 70.0
  Mean filter
    Radius: 0.5
    Number of times: 2
Post-processor
  (none)
Scaling
  ✓ use ImageJ stack calibration

Note: the cells in the bottom left and top right are still not well segmented with these parameters. This image may require a different segmentation algorithm (e.g. watershed) to achieve good results.