Shell area analysis using ImageJ

Introduction

ImageJ is a well known image processing and analysis program. It was produced by NIH, and it is maintained by a large group of researchers. Versions are available for computers running Linux, Microsoft Windows, and Apple macOS operating systems. An easy to install package containing ImageJ with commonly used plugins can be found at https://fiji.sc. This package, called FIJI, is recommended.

The protocol described in this document provides an efficient way to measure the areas of 2D images of shells. An image of one or more shells is modified to contain a numbered overlay of the measured area of each shell of interest and a numbered list of those areas. The numbered list of areas can be easily entered into a spreadsheet for further analysis

The following figures indicate original (left) and modified (right) images for a single shell. The number “7” in the modified image corresponds to the seventh measured area in the resulting list of measurements. **IMPORTANT NOTE:** The overlays (area list number and yellow shell outline) are visible only when the saved .tif formatted file is opened in the ImageJ program.

Standard Operating Procedure

Protocol instructions, such as **EDIT > OPTIONS > COLORS: FOREGROUND = BLACK,** represent successive selections and options from the ImageJ menus. For example: select **Edit** from the main menu, then **Options** from the submenu, then the **Colors** option, and set the foreground color to **Black**.

A series of menu option selections can often be accomplished more easily by using a keyboard shortcut. Such shortcuts are represented in brackets. For example **{ctrl+o}** can be used to open the working directory of files.

Configure IMAGEJ options:

- Set colors so “Eraser Tool” color is white and selected area outline color is yellow: **EDIT > OPTIONS > COLORS: FOREGROUND = BLACK, BACKGROUND = WHITE, SELECTION = YELLOW**
- Set the color and size of the area-number label: **IMAGE > OVERLAY > LABELS, COLOR = WHITE, FONT SIZE = 12**
- Set to display the area-number label: **ANALYZE > SET MEASUREMENT >** check **AREA, ADD OVERLAY, DISPLAY LABEL**
- Set to display the “Eraser Tool”: **>>** (icon at the far right of the icon bar) CHECK **DRAWING TOOLS**
- Configure the “Eraser Tool” width: double click the **ERASER TOOL** icon and set the width to 5.
- Preset the tolerance of the **WAND TOOL**.
    - Double-click the **WAND TOOL** on the icon menu, then set the tolerance to 60. This is a good starting value that will generally select the entire shell when using the wand tool.

Create working files using the TIFF format so analysis overlays can be saved and reviewed using Imagej.

This protocol alters the working image, so do not use original data images.

- Create a working directory using your system’s file browser.
- Use ImageJ to run a batch process to create working files (copies of originals): **PROCESS > BATCH > CONVERT:** enter or select the names of the input

(original images) and output (working images) directories, ensure that TIFF is selected as the output format, then press the **CONVERT** button. Use the TIFF formatted files for subsequent steps in this protocol.

Some useful keyboard shortcuts when working with images

- zoom in **{+}**
- zoom out **{-}**
- zoom with mouse **{ctrl+mouse wheel}**
- scan vertically in a zoomed image **{mouse wheel}**
- scan horizontally in a zoomed image **{spacebar+mouse wheel}**
- scan using mouse **{spacebar+left mouse button}**

Measurement Calibration:

ImageJ does not retain calibration information from image to image. For that reason, when many images having the same scaling are being analyzed, it will be most efficient to not calibrate each image in ImageJ. Instead, a calibration value obtained from a single representative image can be measured, then used by a spreadsheet program to convert raw values to calibrated values. Two calibration options are listed below. The first obtains a calibration value that will be used for spreadsheet calculation of calibrated areas, the second process results in calibrated areas being output directly from the ImageJ program

Spreadsheet calibration: ImageJ outputs areas in uncalibrated units of square-pixels

- Open a representative image file containing a measurement scale.
- Select the **LINE TOOL** from the icon menu, then draw a line parallel to a specific length on the scale, e.g., of 100 mm.
- Type the “m” key to measure the image length in pixels of the line. The square of the resulting value will be used to convert raw areas to calibrated areas in a spreadsheet program.

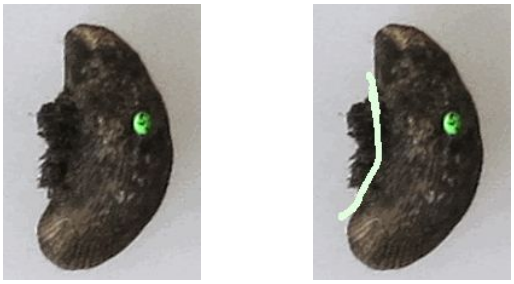
Calibration of each image: Imagej outputs areas in calibrated units, e.g. square-mm

- Open an image file containing a measurement scale.

- Select the **LINE TOOL** from the icon menu, then draw a line parallel to a representative length, e.g., 100 mm.
- Calibrate ImageJ: **ANALYZE > SET SCALE > KNOWN DISTANCE =** representative length, e.g. 100mm; **UNIT LENGTH =** mm.

Image Analysis:

- Open image file***:*** **FILE > OPEN {ctrl-o}**. NOTE: the next image in sequence can be obtained easily by **FILE > OPEN NEXT {ctrl+shift+o}**
- Adjust window size and zoom as needed.
- **Digitally clear shell images** to remove areas of attached barnacles, byssi, and debris extending outside the area of interest and to digitally separate two shells. Use the **ERASER TOOL** to remove image regions extending outside the area of the shell of interest. The most efficient way of doing this is to move the tool parallel to the shell margin, ensuring that the erasure begins and ends in the background. The figure below illustrates before (left) and after (right) images showing an erasure that will exclude the byssi when the shell area is analyzed.



- ***Select the shell of interest*** using the **WAND TOOL.** It may be necessary to touch the wand to several regions of the shell until the resulting selection includes the entire area of the shell. The following figure illustrates an original image on the left and a well-selected shell area on the right, with intervening images illustrating inaccurate selections made when the Wand Tool selection overlapped with the color/density properties of the adjacent background (2nd and 3rd images from the left) or did not sufficiently overlap with other regions of the selected shell. If necessary, the tolerance setting of the wand tool can be adjusted to optimize the selection tolerance by double-clicking the tool icon.

- ***Measure the selected area using the "m" key on the keyboard.*** This will cause the program to calculate the selected area and to add the calculated area to a running list of measurements.
    - Analyze additional shells on the same image file by selecting a new shell image and repeating the measurement step.
- **Save the modified image file** using the keyboard shortcut **{CTRL-S}**, or save with a new filename/format with **FILE > SAVE AS**.
- **Save the RESULTS list** by right-clicking the RESULTS window and selecting the **Save As** option.

Typical Shell Image Analysis Problems

- Tag numbers are illegible:
    - Images are out of focus.
        - Every image must be reviewed to ensure it is in focus.
    - Some tag color combinations are better than others.
        - Numbers on white, green, or yellow backgrounds are much more legible than ones on red or blue backgrounds
        - Yellow tag color is difficult to differentiate from green tag color when photographed under fluorescent illumination.
        - Black text is more legible than white.
    - Excessive glue can make a tag illegible.
        - Because cyanoacrylate glue is repelled by waxes, tag legibility could be improved by waxing the front surfaces of tags.
            - A round toothpick dipped in a paste wax or a slightly molten mixture of beeswax and lanolin could be used to coat and manipulate tags.
            - A dry spray lubricant such as Sailkote might be used to selectively coat the front surfaces of tags.
    - A tag number might be illegible because of ambient light glare.
        - Minimize glare by using a polarizing filter in front of the camera lens.
        - Minimize glare by using a “copy box” to better control ambient illumination.
- Data entry entails a multistep process of back and forth comparisons between two images. One image has readable shell tags while the other has shell images that contrast with the background. The first is made with ambient illumination; the second is made by using a typical laboratory light box to backlight the shells placed on it.
    - It would be substantially more efficient if a single image contained both readable tags and shell images that the ImageJ program can reliably distinguish from the background.
        - Shells must be front-lighted so tags are readable.
        - In order to differentiate shells from the background, the background quality must be substantially different from the dark, almost black, mussel shells, and the off-white portions of oyster (and clam) shells. The upper surface of a typical oyster shell is problematic

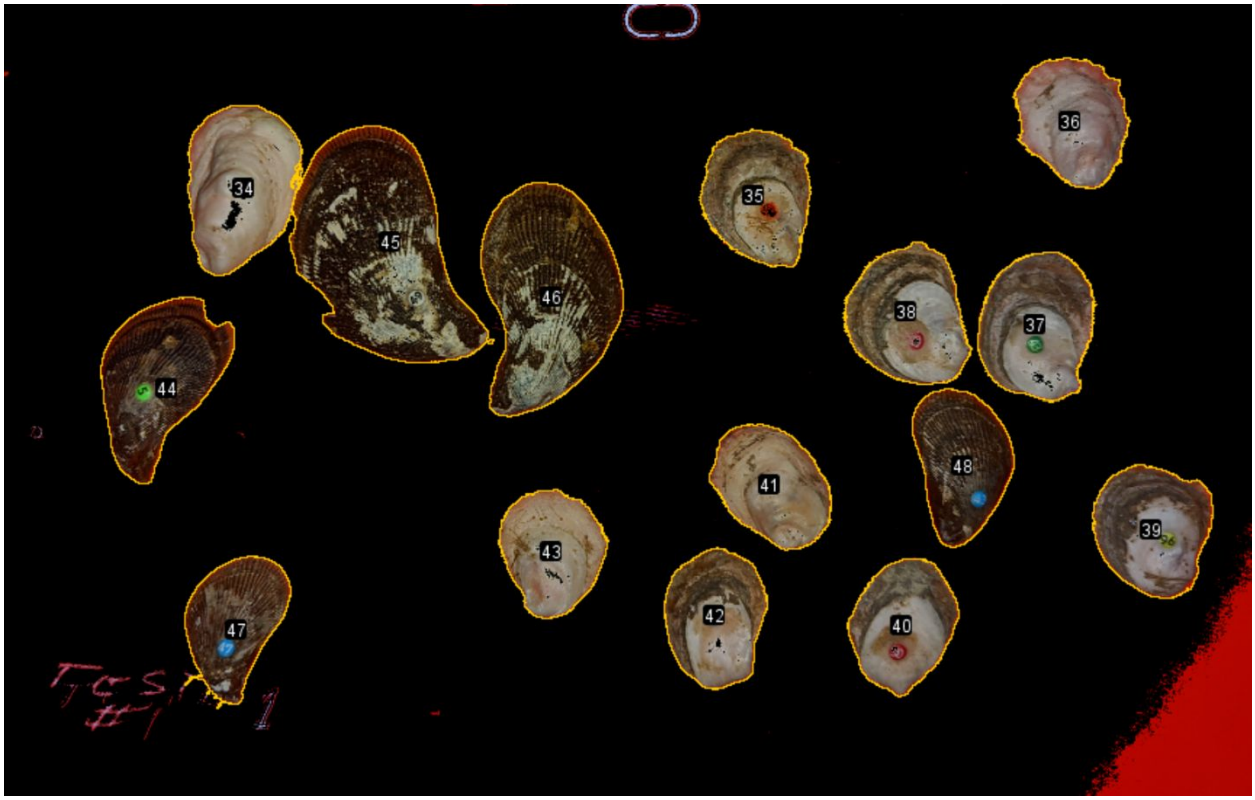
because it contains an off-white “bill” region that differs from the tan coloration of the rest of the shell; the bottom shell is more uniformly colored.

- Front and back lighting should be uniform across the field of view to minimize shadows and to facilitate shell selection with the Wand Tool .

Proposed Cure All for Shell Area Imaging Problems

- Place shells on top of a digital tablet or computer screen displaying a bright, primary color for the backlighting.
    - Depending on the background device and its software, it may be possible to use a digital pen to write group information on the screen.
- Photograph images with a tablet computer that has a high-resolution, rear-facing camera and flash, thereby producing front-lighting with the flash, and making it possible to easily monitor image quality.
- When analyzing images in ImageJ, color thresholding will be used to isolate objects and background, with the black “threshold” color option being used when light objects, e.g., oyster shells, using the Wand Tool, and the white color “threshold” color option being used when selecting dark objects, e.g., mussel shells.
- Incidental issues:
    - Tablet/computer screens can be protected from water damage by placing the shells on a thin glass plate positioned just above the screen.
    - Older generations of Apple i-pads, Android tablets, and 2 in 1 computers, e.g., the Asus Flip Chromebook, would work well. A diagonal screen size of ten inches might be ideal as it is large enough to contain 15-25 small samples, such as those in Sarah’s study, but small enough to limit the image field to a size that would result in adequate resolution of tag numbers.
- Proof of concept:

    The following pages contain pairs of original and processed images. Front lighting was produced by the camera flash. The background was generated by a Samsung Tab S4 tablet and a drawing program that permitted background color selection. Photographs were made using a handheld Google Pixel 4XL camera set to always use the camera flash.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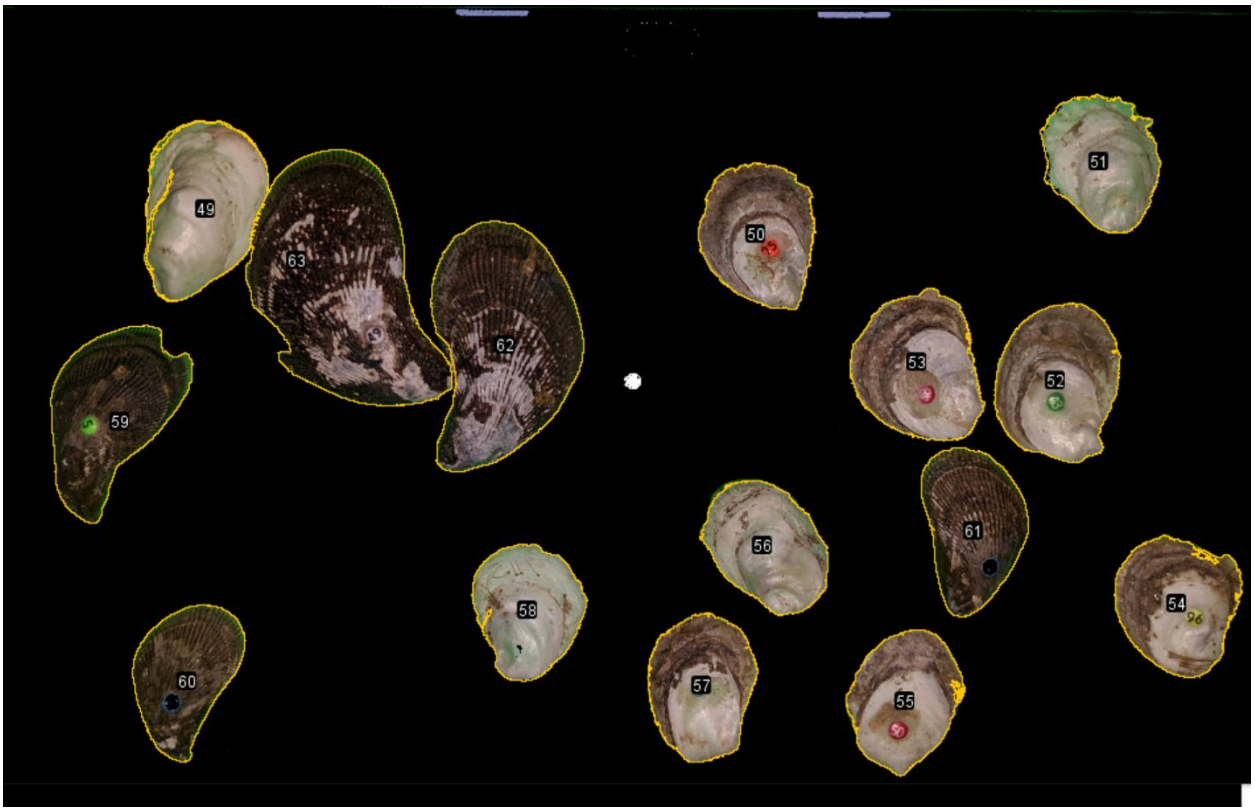
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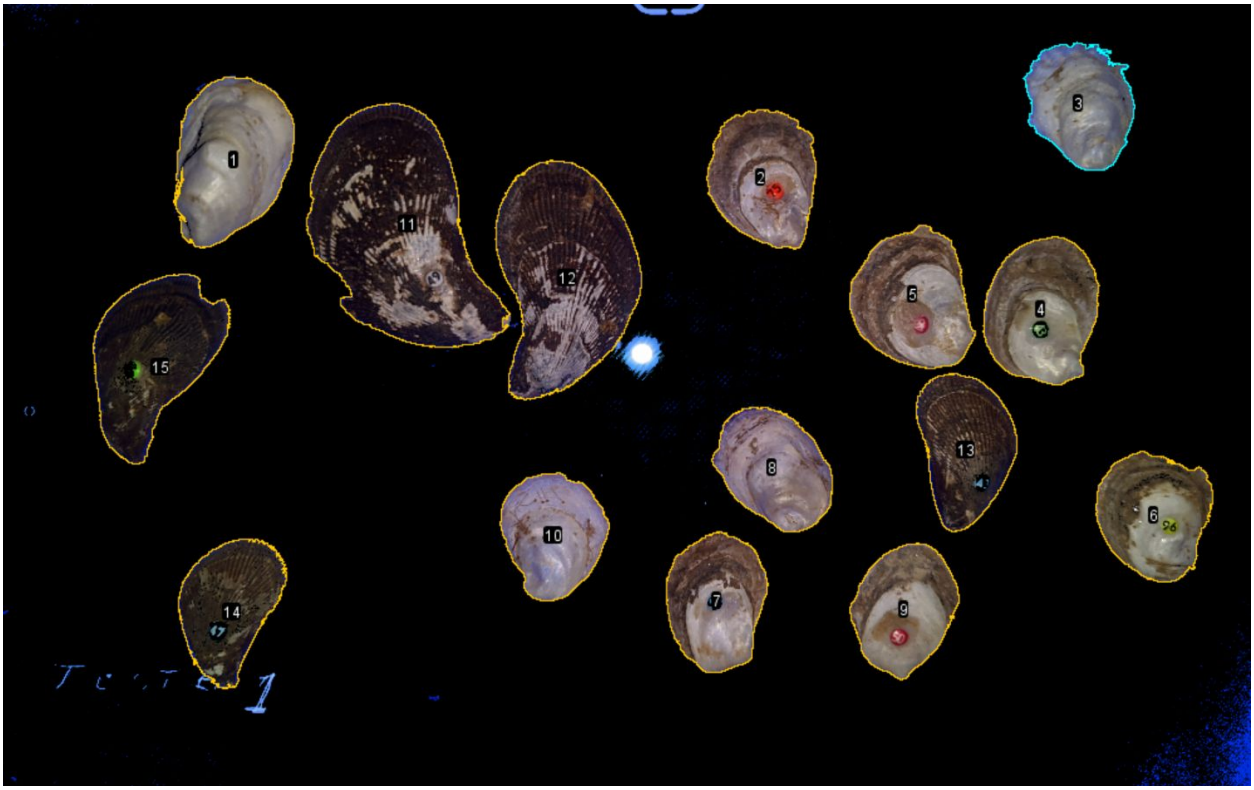
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