**STANDARD OPERATING PROCEDURE**

**NUMBER:** **000/ROCHETTE/22**

**TITLE:** **Auto-measure Perkins Eye Index (PEI) of American Lobster (*Homarus americanus*) embryo using Fiji/ImageJ image analysis software.**

**PURPOSE:** **To determine developmental status of lobster embryos based on morphometric analysis.**

**EFFECTIVE DATE:** **November XX 2022**

**APPROVED BY:**  **\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

**POSITION** **NAME** **SIGNATURE** **DATE**

**Supervisor** **Rémy Rochette** **\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_** **\_\_\_\_\_\_\_\_\_\_\_\_**

**Prepared by** **Lydia White** **\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_** **\_\_\_\_\_\_\_\_\_\_\_\_**

**Materials**:

* Fiji software (Download [**https://fiji.sc**](https://fiji.sc/))

**References:**

**Perkins, H. C. 1972.** Developmental rates at various temperatures of embryos of the northern lobster (*Homarus americanus* Milne-Edwards). *Fish. Bull.* **70**: 95-99

**Helluy, S.M., and Beltz, B.S. 1991.** Embryonic development of the American lobster (*Homarus americanus*): Quantitative staging and characterization of an embryonic molt cycle. *Biol. Bull.* **180**(3): 355-371.

**Fiji/Image J manual** <https://imagej.nih.gov/ij/docs/guide/user-guide.pdf>

**BioVoxxel Toolbox:**

* <https://doi.org/10.5281/zenodo.5986130>
* *Documentation*: <https://imagej.net/plugins/biovoxxel-toolbox>

**SET-UP**

1. **Install Fiji** [**https://fiji.sc**](https://fiji.sc/)

Fiji is considered a “batteries-included” version of ImageJ that comes preloaded with the Plug-Ins you will need to run this macro.

1. **Ensure the BioVoxxel plug-in is running.**

In Fiji, click the red arrow at the right end of the tool bar (looks like >>), you will see “BioVoxxel Toolbox” in the list. Click on it. Once you have you should see a green cube in the tool bar. This confirms BioVoxxel is loaded.

*\*If BioVoxxel is not displayed in your list of plug-ins, refer to Appendix 1 for installation instructions.*

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1. **Set measurements**

In the task bar, click “Analyze” > “Set Measurements…”.

Ensure that Area, Feret’s diameter and Display label are selected.**Graphical user interface, application

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1. **Set the scale**

This is arguably one of the most important steps as your scale will impact the values of your measurements. Ensure that whatever image you are using to set the scale is the same dimensions as the photos you are analyzing.

Open whatever image you are using to set your scale and draw a straight line over the known length of your scale. Then from the tool bar click Analyze > Set Scale.

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In the Set Scale dialogue box put in the Known Distance of your scale (for this example it is 250 um). Additionally, you may write in the units of the scale. Make sure “Global” is selected.

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**IMPORTANT:** If you are measuring Images from the same microscope, and the images are the same size, the scale (value below Global) needs to be identical every time you run the macro. Adjust the values of the Known distance in the Set Scale dialogue box to adjust your scale until it is correct.

As of November 17, 2022, the scales used for the current microscopes (images dimensions 4000 px vs 3000 px) are:

**Leica: 4.0026 pixels/um**

**Olympus: 2.3723 pixels/um**

\*These are subject to change depending on microscope and/or image dimensions. Confirm there are the proper scales before beginning to measure.

***NOTE:*** *Each time you start a new session with Fiji it is recommended to repeat steps 2-4. Sometimes Fiji will save your settings but that is not guaranteed.*

**RUNNING THE MACRO**

**Before running the macro:**

* Avoid using/taking photos where there are light or reflective spots along the perimeter of the eye. The macro will fill in any within the eye area, but those along the perimeter may cause erroneous PEI and/or area estimates.
* The best way to run this macro is to have one folder with the images you want measured. The macro will run all images in that folder. It is recommended to start with a smaller number of images in the folder to ensure the macro is functioning properly and then increasing the number as desired.

1. Select Plugins > Macro > Run …
2. Select the macro file from where it is saved on your computer (see Appendix 2 if you do not have the macro file).
3. Another dialogue box will pop up. Select the folder that contains your embryo images and click Open.
4. One last dialogue box will appear. Select the folder where you would like the ROIs to be saved and click Open. This can be in the same folder as the images, or in a different folder.
5. The macro should be running now. The Results window will open, and the measurement values will be added to it as the macro measures each image.
6. Once the macro has finished measuring all the images, save your Results accordingly.

**VALIDATING MEASUREMENTS**

This macro is not perfect, errors can occur. The following validation steps are recommended to ensure accuracy of the macro measurements on your photos.

1. Look for any duplicate measurements (i.e., more than one measurement for each photo) or any missing measurements (i.e., no measurements for a supplied photo).

Sometimes if the other eye is present (and large enough) in the photo, the macro will pick it up as well. When you have duplicate measures, open that specific image and perform a manual measure on the eye to confirm which measurement is correct.

If you have any missing measurements, open the missing image(s) and perform a manual measurement.

*For manual measurements procedures, refer to SOP 0003/ROCHETTE/18 “Measuring Perkins Eye Index (PEI), membrane area and yolk area of American Lobster (Homarus americanus) embryo using Image J image analysis software.”*

1. Select 50 of your images that were measured by the macro. Ensure that these images encompass as large of a range of eye size as possible (e.g., 300-550 um). For each of the 50 selected images, manually measure the eye size. Look at the correlation between the manually and automatically measured values so assess strength, as well as evidence of bias in the slope and intercept. It is also recommended to explore the residuals to check for any marked outliers.
2. Finally, it is recommended that you take multiple photos of the same embryos to quantify the coefficient of variance in embryo positioning.

APPENDIX:

Appendix 1: Installing BioVoxxel in Fiji

1. Open the Fiji application and navigate to Help > Update…

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1. Select “Manage update sites” and scroll down the list and check off “BioVoxxel”

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1. Click Close on the “Manage update sites” window, and then click “Apply changes” on the “ImageJ Updater” window.
2. For the updates to apply and thus for the BioVoxxel Toolbox to be available, restart Fiji.

Appendix 2: Macro code

1. To create a macro select Plugins > Macros > Record… , and paste the following code:

// Macro to measure lobster embryo eyes

run("Clear Results"); // clear the results table of any previous measurements

// The next line prevents ImageJ from showing the processing steps during

// processing of a large number of images, speeding up the macro

setBatchMode(true);

// Show the user a dialog to select a directory of images

inputDirectory = getDirectory("Choose a Directory of Images");

outputfolder3 = getDirectory("Choose a Directory to Save ROIs");

// Get the list of files from that directory

// NOTE: if there are non-image files in this directory, it may cause the macro to crash

fileList = getFileList(inputDirectory);

for (i = 0; i < fileList.length; i++)

{

if(endsWith(fileList[i], ".tif")) {

processImage(fileList[i]);

}

}

setBatchMode(false); // Now disable BatchMode since we are finished

updateResults(); // Update the results table so it shows the filenames

function processImage(imageFile)

{

// Store the number of results before executing the commands,

// so we can add the filename just to the new results

prevNumResults = nResults;

open(imageFile);

// Get the filename from the title of the image that's open for adding to the results table

// We do this instead of using the imageFile parameter so that the

// directory path is not included on the table

filename = getTitle();

minferetiner="50-Infinity";

roiManager("reset");

run("Set Measurements...", "area feret's display redirect=None decimal=3");

run("8-bit");

run("Auto Local Threshold", "method=Phansalkar radius=600 parameter\_1=0 parameter\_2=0");

run("Extended Particle Analyzer", "min\_feret="+ minferetiner + " show=Outlines redirect=None keep=None display add exclude include");

if (roiManager("Count") > 0){

roiManager("Select", 0);

roiManager("rename", filename);

roiManager("Save", outputfolder3 + "ROI\_lw\_ " + filename + ".zip");

}

// Now loop through each of the new results, and add the filename to the "Filename" column

for (row = prevNumResults; row < nResults; row++)

{

setResult("Filename", row, filename);

}

close("\*"); // Closes all images

}

2. Save the macro by clicking Create and then File > Save. *NOTE: The macro file name has to end in .ijm for Fiji to open it.*