**ESM III Technical instructions for installation of FIJI, the required plugins and the presented macros and for first use of the macros.**

This document provides technical instructions for the use of two macros, “Draw a mask” and “Measure masks”, presented in the paper “A new method for morphometric analysis of opal phytoliths from plants” (Out et al). The macros can be run in the open-source software program FIJI. The macros were developed for morphometric phytolith analysis but can also be applied to measure other objects. The macro “Draw a mask” isolates a phytolith from a photograph by manually making a digital drawing, thus obtaining its mask. The macro “Measure masks” allows automatic collection of measurements of multiple variables of size and shape of various phytolith masks at the same time. Please refer to the original paper when using the macros or referring to them.

Technical support for the installation of the program, macros and plugins.

1) Install FIJI (Schindelin et al., 2012). Versions suitable for various operating systems can be downloaded for free from the website http://fiji.sc/Fiji. After installation, update the program via *Help*, *Update ImageJ*. FIJI basically consists of the program ImageJ combined with JAVA that can also be downloaded for free. Instructions, user guides and user forums for both ImageJ and FIJI are available on the Internet. To use the macros for measuring phytoliths or other objects, a general understanding of FIJI is recommended.

2) Download the bundle of plugins Morphology (Landini, 2013) from the website http://www.dentistry.bham.ac.uk/landinig/software/software.html. The bundle is provided behind the text: “Download the full set as a single zip file from here.”.

Unzip the files in the FIJI plugin folder. From this package you will use the plugin Particles\_8. Read the information about the plugin on the above-mentioned website to understand the variables and the methodological details.

3) Download the plugin Measure Roi (Dougherty, 2005) from the website [http://www.optinav.com via *ImageJ plugins*, *Measure Roi*](http://www.optinav.com/Measure-Roi.htm). Download the latest version of the Java file: Measure\_Roi\_Curve.java. Place the plugin in the FIJI plugin folder or go in FIJI to *Plugins*, *Install*, select the file Measure\_Roi\_Curve.java and save it in the FIJI plugin folder. Read the information about this plugin on the relevant website.

4) Open or restart FIJI. You will now see the package Morphology and the plugin Measure ROI provided in the Plugins menu.

5) Copy the macros “Draw a mask” and “Measure masks” from ESMs 1 and II and save them in the FIJI macros folder. To do so, first open the FIJI script editor by going to *File*, *New*, *Script*. Then copy the complete script of “Draw a mask” (ESM I) into the editor and select the language by going to *Language*, *ImageJ Macro*. Save the file in the FIJI macros folder as “Draw a mask.ijm”. Open a new file in the script editor and repeat this for “Measure masks” (ESM II); save this second file (ESM II) as “Measure masks”. It is important to use these names precisely since they will be used afterwards.

6) To assure consistent collection of measurements with three digits behind the comma, also when running the macro “Measure masks” repetitively during a single FIJI session, adapt the script of the plugin Particles\_8. To do so, open the FIJI Script editor by going to *File*, *New*, *Script* (if not still open). Then open Particles\_8.java by going to *File*, *Open, FIJI, Plugins, morphology* and select the file there. Search for the line

“public void run(ImageProcessor ip) {”

(“ and ” excluded, as is the case with further programming lines below), add a new line afterwards by pressing enter, type the text “rt.setPrecision (3);” in this new line and save. Close the script editor and restart FIJI afterwards. When working with a Mac, possible problems with opening the FIJI folder may be solved by setting the Finder window to a mode other than the column mode.

7) To run the macro’s “Draw a mask” and “Measure masks” in FIJI, choose *Plugins*, *Macro’s*, *Install*, and choose one macro. When you now choose this route again, the just installed macro appears at the bottom end of the same menu (*Plugins, Macros*). Select the macro to open it. By installing the macros in this way, the program enables the installation of only one macro at a time (see also 9 for a more convenient installation procedure). With the minimal required preparations presented above, you are now ready and can start using the macros.

8) Optional: to have the two macros installed automatically every time you open FIJI, the macros can be added to a file StartupMacros.txt. To do so, open the file StartupMacros.fiji.ijm that is located in the macros folder by going to *File*, *New*, *Script* and by going in the script editor to *File*, *Open*, *FIJI, Macros* to select the StartupMacros file. Also open the macros “Draw a mask” and “Measure masks” in the script editor by going to *File*, *Open*, and selecting the files in the FIJI macro folder. There are now three files opened in the script editor. In the file StartupMacros.fiji.ijm, first type

“macro "Draw a mask" {“

at the very end of the script. Then copy the text of the macro “ Draw a mask” underneath and type ”}” on a new line below. Then repeat this with the macro “Measure masks”. Thus, go again to the (new) end of the file StartupMacros.fiji.ijm, type

“macro "Measure masks" {“

at the very end of the script, copy the text of “Measure masks” underneath and type ”}” on a new line below. Save the Startup file in the FIJI macros folder as a text file with the name StartupMacros.txt. Close the script editor. When restarting FIJI, the two macros can be installed both at the same time by going to the FIJI tool bar, clicking on the menu *More tools* (pictogram on the right: >>), and selecting *Startup Macros*. To open one of the macros afterwards, go to *Plugins, Macros* and click on the name of one of the macros on the bottom of the menu.

9) Optional: the user always defines the scale of the photographs and masks when opening the macro “Draw a mask”, during the analysis of the first photograph only. When always working with photographs of the same scale and when processing many photographs, removing the step of defining the scale from the script may be relevant to prevent scaling errors and reduce unnecessary repetition. This can be done by collecting the calibration data of your photographs once, de-activating the script lines that result in setting the scale after opening the macro and instead adding lines that define the automatic application of a standard scale.

To collect the calibration data that will be applied to all photographs, run the macro “Draw a mask” as normal. When you see the window “Set scale”, write down the “Distance in pixels” of the drawn line, the known length of the scale bar (the length indicated by your camera software, not the length provided by FIJI) and the unit of length.

To adapt the script of the macro “Draw a mask”, open the script editor by going to *File*, *New*, *Script*. Then open the script of “Draw a mask” by going to *File*, *Open*, and select the file.

To de-activate the option to actively set the scale while running the macro, add “//” in front of each of the following lines (//if (loop==0) { //setTool("line"); //waitForUser ("Calibration:…"); //run("Set Scale...") //}). Save the adapted file.

To activate the automatic application of a standard scale whenever opening the macro, delete the provided numbers and fill in your earlier collected calibration data in the line // run("Set Scale...", "distance=256 known=20 pixel=1 unit=µm global");, whereby distance = the distance of the drawn line in pixels, known = the known length of the scale bar, and unit= the unit of length. Delete “//” in front of this line to active the line. Save the adapted file.

Technical support for running the macros.

“Draw a mask”:

-At 1) “Action: select a photograph showing at least one phytolith in focus and select “Open” ”: using photographs automatically numbered by a computer and a counter will help selecting each time the correct next photograph.

-At 2) “*To calibrate*”: when the scale bar has side bars, include only one side bar in your measurement.

At 3) “*Set scale”*: the options provided in the text box but not mentioned in this paper can be left out of consideration.

-At 6) “*Zoom in*”: the enlargement of the photographs is arbitrary but should be consistent within a study and ideally also between studies.

If your object of interest does not fit in the window provided, you can zoom out instead of in with pressing *Control* or the *Command Key* and *–* at the same time. If you want to zoom out systematically, you can adapt the script of the macro “ Draw a mask” by replacing the line “setTool("zoom");” with “run("Out [–]");” (In FIJI, go to *File, New, Script,* and in the script editor go to *File, Open*, to select “Draw a mask”. Save the file afterwards). To zoom out multiple times, add this line multiple times. The text box “Zoom in...” can be ignored, or removed from the script by“//” in front of this line.

-At 7) “*Make a drawing of your object of interest*”: users testing the application and not yet applying a drawing pen but the mouse instead can use the polygon tool from the FIJI toolbar. For ergonomic reasons, precise measurements and optimal comparison of phytolith measurements between investigators, a drawing pen of any brand and the freehand selections tool are strongly recommended as the standard option.

-At 7) “*Make a drawing of your object of interest*”: when using a drawing pen and the freehand selections tool, the functions ALT and SHIFT allow adjusting drawings when a preliminary drawing is already available. Start drawing outside the existing drawing and press ALT to erase part of the drawing and start inside the existing drawing and press SHIFT to expand the drawing. When make an adjustment, it may be helpful to return to the starting point of the adjusting line before releasing the mouse or drawing pen. In case of an unsuccessful drawing, a completely new drawing on the same image can be initiated with a double click (the existing drawing is erased completely). Drawing high-quality masks as well as adjusting them takes some practise in the beginning but will soon be quick and easy. When using a mouse and the polygon tool instead of a drawing pen, drawings can be adapted without using ALT and SHIFT but by repositioning the individual points instead.

-At 9) “*Drag the rectangle around your drawing*”: to customize the size of the rectangle, change the numbers in the line of the script containing the command “makeRectangle”. When applying the macro to objects of interest of variable size (e.g. objects other than phytoliths), it may be wise to search for the largest object of interest before starting with drawing the masks, and base the size of the rectangle on the size of that object.

To visualise and/or print a series of masks, you can run the macro “Measure masks” in ImageJ, which can be downloaded from the website <http://rsb.info.nih.gov/ij/>. This program is highly similar to FIJI. To see a window with all masks located in a folder, install and run the macro “Measure masks” as done in FIJI (described under 7 above), selecting the relevant folder with masks. The macro will give an error message and therefore not close the montage window. Save the montage window as an image (it can be saved as a pdf file in other image software programs). White masks on a black background can be changed into printer-friendly black masks on a white background by selecting *Process, Binary, Make binary*.

In case of the error message:

-an x-bit image is required: it may be helpful to convert jpeg files into tiff files. This can be done for a large number of files automatically by using an online file converter or by using FIJI again. The macro was successfully tested with tiff files, jpeg files and pdf files.

-a white mask appears on a black background: this may be solved by running the macro another time.

“Measure masks”:

-At 1) “*Select a folder*”: select the folder “Masks” created with the first macro “Draw a mask”. The macro runs automatically afterwards; the length of the process may depend on the speed of your computer and the number of masks in the folder. If the macro does not run automatically after selecting the folder “Masks”, select a random mask file in the folder “Masks” and try again. After running the macro, a data folder with two output files is available in the folder “Masks”.