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LA RECHERCHE AGRONOMIQUE POUR LE DÉVELOPPEMENT

Scanning protocol for soil invertebrates

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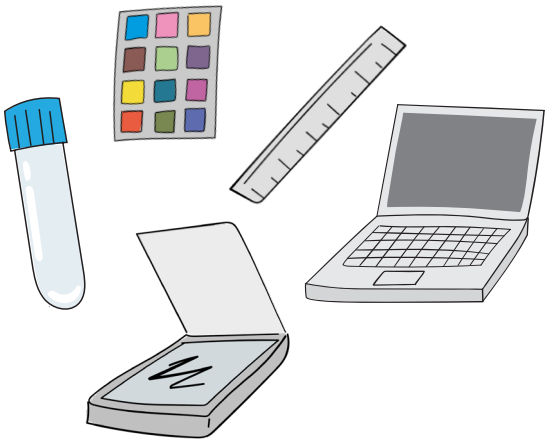
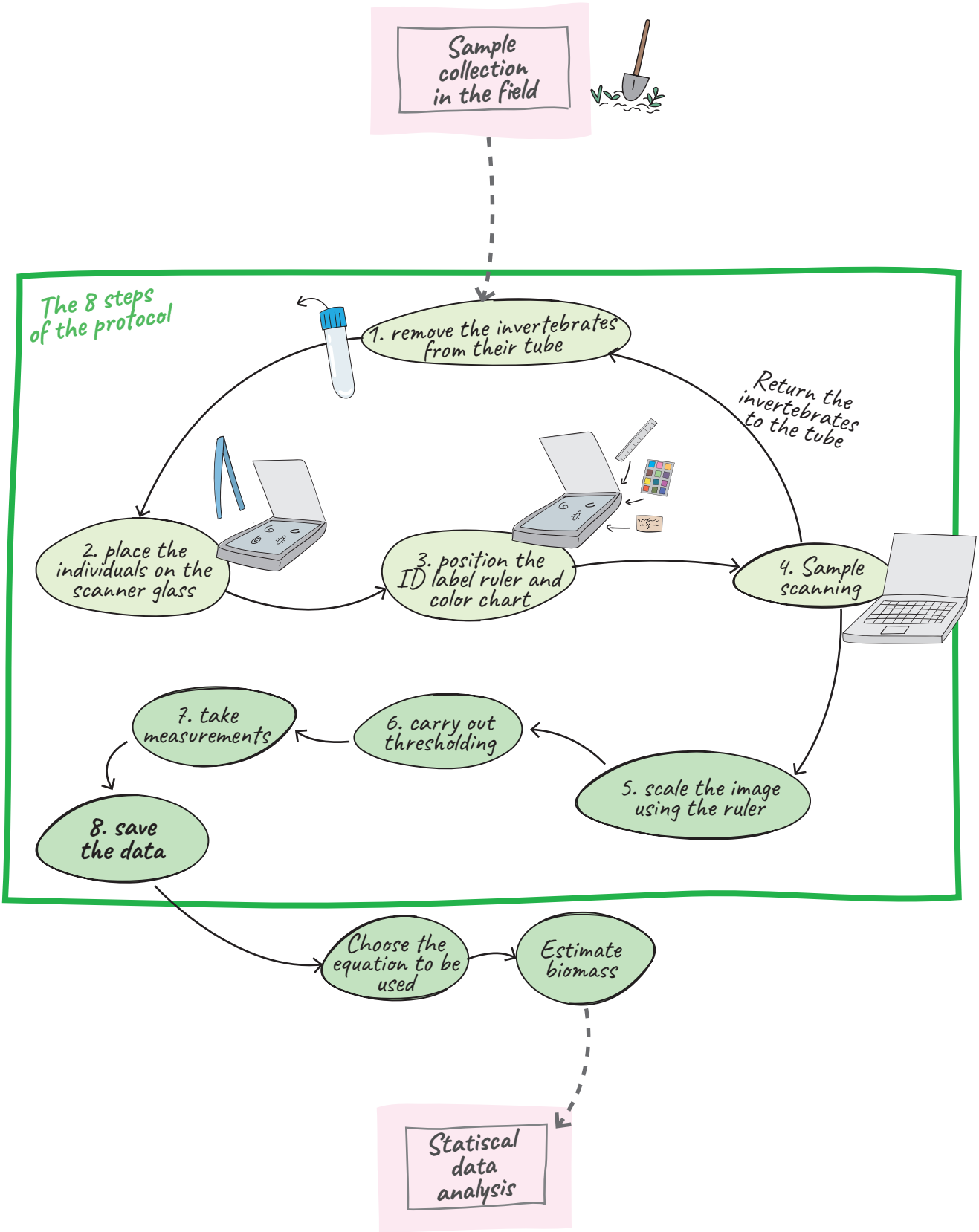


An 8-step method



Principle

THIS PROTOCOL DESCRIBES A SIMPLE WAY OF ESTIMATING THE BIOMASS OF INVERTEBRATES IN SOIL.

We were led to develop this method because we found that many studies only measure invertebrate density, to the detriment of biomass, as it often raises a methodological or logistical difficulty.

The method described here does not require any specific laboratory equipment and can be applied under a variety of conditions with limited resources. It is based on image analysis of a scanned sample and can be carried out on invertebrates that have been freshly collected or preserved in fluid (alcohol).

Data are acquired with a conventional desktop scanner. Various measurements are then carried out using **ImageJ** software.

These measurements are used to estimate parameters on an individual scale (specimen) or a community scale (sample). The method enables semi-automated calculation of the abundance (number of individuals per sample) and surface area of individuals. This information is then used to estimate biomass.

This protocol focusses on the first two phases of the method, namely:

> 1. Scanning
> 2. Image analysis with **ImageJ** software.

The third phase (3), involving biomass estimation using an allometric equation, is not described here. Calibration and verification of the equations involves specific work covered in another document.

Equipment

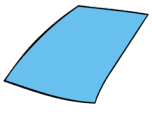
Desktop scanner

Ruler

ID label

70° alcohol

Color chart



Sheet of blue cardboard

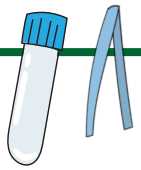
Sheet of transparent PVC

Absorbent paper

Glass dishes (optionel)

Flexible forceps

1. SCANNING



This method involves scanning and measuring a set of individuals in one go. In practice, this generally means all the individuals from the same sample, which are scanned on the same image and analyzed.

It is also possible to divide the individuals of a given sample into different groups (functional group, trophic group, taxonomic group, etc.) prior to scanning to simplify subsequent data processing.

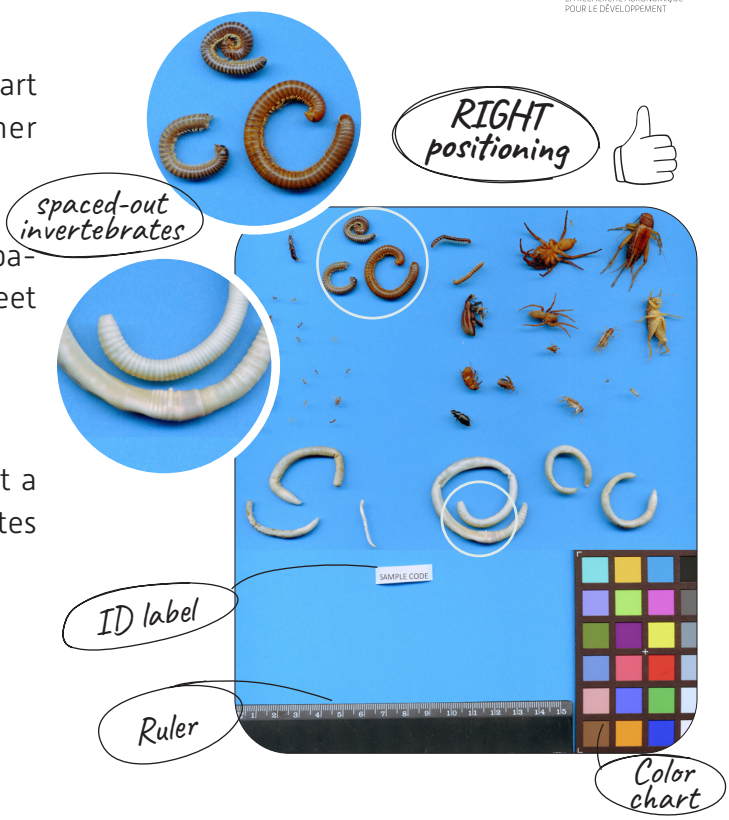
This dividing phase is optional and depends on the aims of the study.

1.1. PREPARING SAMPLES

Use flexible forceps to pick up the invertebrates and position them on the glass.

- For a sample containing relatively few large invertebrates (<50), they can be placed directly on the scanner glass.
- If the sample contains a large number of small invertebrates (>50) it is best to place them in a flat-bottomed glass dish (e.g. a Petri dish) to simplify handling.
- When positioning the invertebrates on the glass, make sure that they do not touch, that they do not curl up, or that certain body parts overlap.
- An example can be found on the next page showing how to rearrange poorly positioned individuals to ensure a suitable image for analysis.

- Place the sample ID label, ruler and color chart face down on the free area of the scanner glass. .
- Carefully place the sheet of semi-rigid transparent PVC over the samples and the blue sheet of cardboard on top.
- Close the scanner cover.
  - > If the cover is heavy, be sure to insert a small wedge to prevent the invertebrates from being crushed by the cover.
- The sample is ready for scanning.



1.2. SAMPLE SCANNING

- Switch on the scanner and do a preview to set the scanning parameters.

Each scanner and its associated software may have its own specific features, but the general principles are as follows:

- > The scan must be in color with sufficient resolution for the subsequent analysis.
- > For large organisms, such as earthworms, a resolution of **600 dpi** can generally be used.
- > If smaller organisms are scanned, a higher resolution of **up to 2000 dpi** is recommended. Trial runs may be needed to choose the right resolution.
- > **Finer calibration** may be needed for some parameters to adjust brightness and white balance, for which the color chart is very useful.

- Start the **scan** and **save the image**, naming the file with **the precise code** of the analyzed sample. This last point is optional but proves very useful later on when processing the scans by **ImageJ**.

1.3. STORING SAMPLES

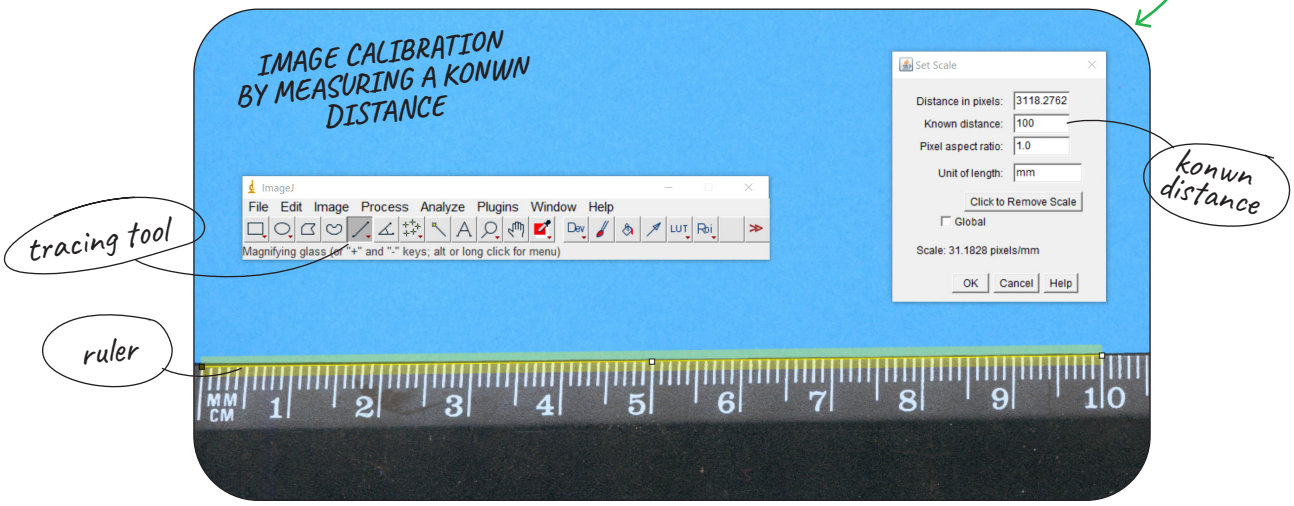
Use the flexible forceps to return the invertebrates and the sample ID label to the tube and add 70° alcohol.

- > The tube can be returned to its storage place and should be kept at least until the image processing and analysis process is completed.

2. IMAGE ANALYSIS

2.1. CALIBRATION

- Start the **ImageJ** software.
- After opening the image, trace a line along the ruler with the "**tracing tool**".
- Then indicate the known distance corresponding to the traced line. To do this, go to the "**Analyse -> Set Scale...**" menu, the measuring unit (mm) can be indicated as a reminder, but this step is not essential.



2.2. THRESHOLDING

This step removes the blue background from the image

This step is very important as it will help to distinguish between the different individuals of the scan and will influence their area, so great care must be taken. Rather than using predefined methods, manual thresholding is recommended using the most appropriate color space depending on the case.

The color spaces used for this purpose are either "RGB", "Lab", or "YUV".

Using "Lab" or "YUV" is recommended to avoid shadow problems.

see the steps

2.2. THRESHOLDING

To begin, go to the "**Image" -> Adjust -> Color Threshold...**" menu.

> Then set the three color slider bars to maximum so as to select all the pixels in the image.

Next, gradually move the blue spectrum slider bar so as to "deselect" blue-dominant pixels.

> Shadows can sometimes be a problem.

> Thresholding may also result in "holes" or "jagged contours", which can bias measurements (especially the perimeter, leading to poor biomass estimation).

To ensure successful thresholding, it is advisable to zoom in on a part of the image where thresholding seems tricky (e.g. with a shadow) to select optimum thresholding.

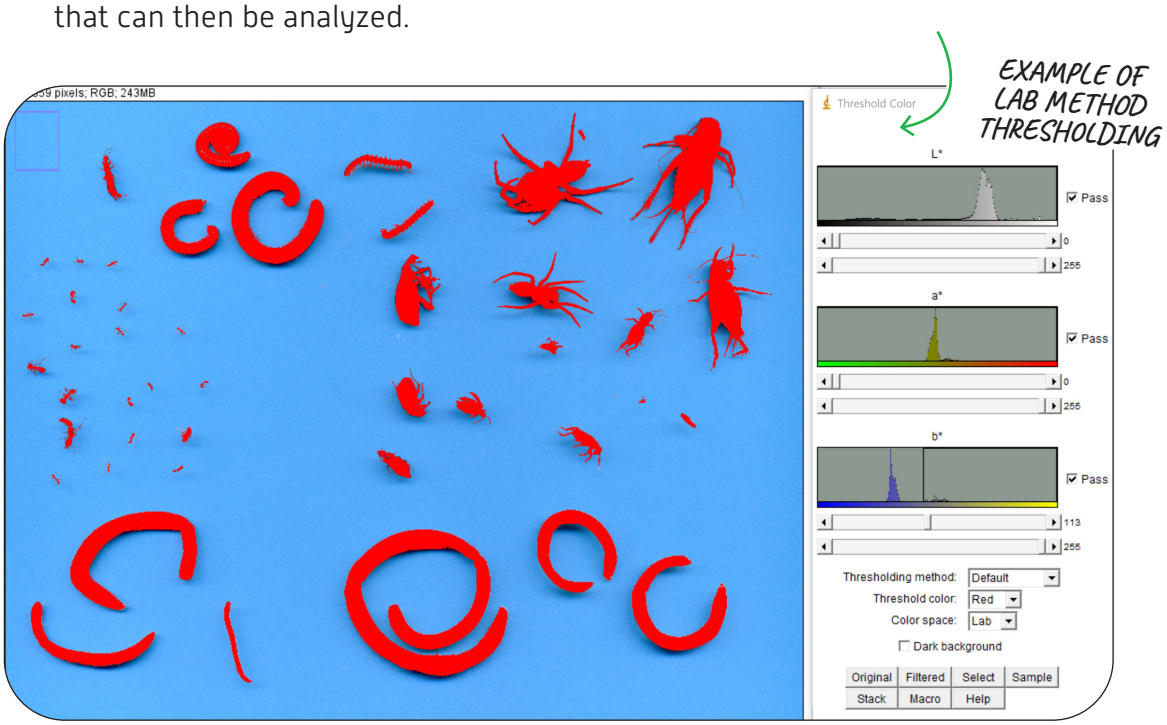
> If a good compromise is not achieved with the selected color space, repeat the operation with another color space.

> If optimum selection of individuals cannot be obtained with these different methods, it is possible to adjust selection with the "**Expand**" or "**Erode**" options to solve the thresholding problems.

> To apply these adjustments to all individuals, we recommend that you do so at this stage.

> However, for specific adjustments on one or more poorly "outlined" individuals, it is preferable to do it later with the ROI Manager (see measuring step page 10).

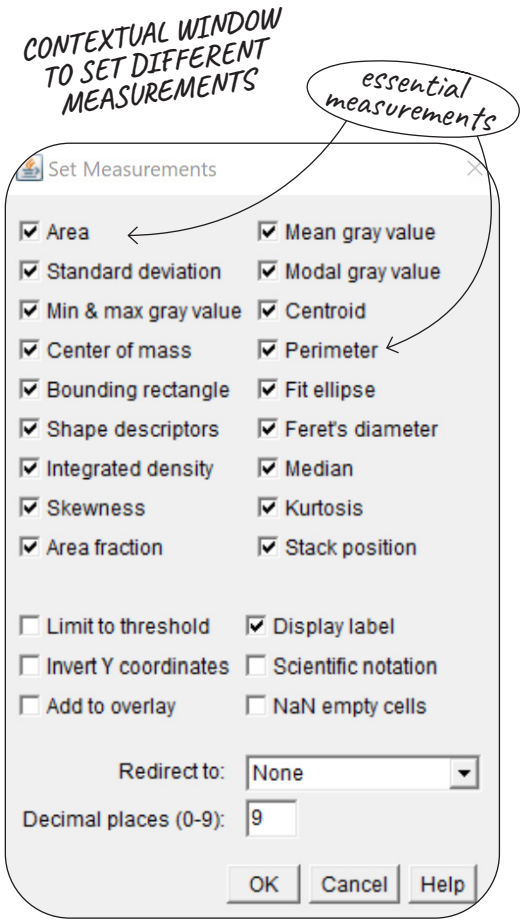
> Finish this step by pressing "**Select**" then "**Sample**". This gives a binary image that can then be analyzed.



2.3. MEASURING

- **Select the zone to be measured** so as to include all the specimens, while excluding items you do not want to measure, such as the ruler, color chart and ID label.
  - > For this step to go smoothly, it is important for the operation to have been well organized during the scanning phase.
  - > If the individuals are grouped in dishes use the "**circular selection tool**"; otherwise, use the "**rectangular selection tool**".

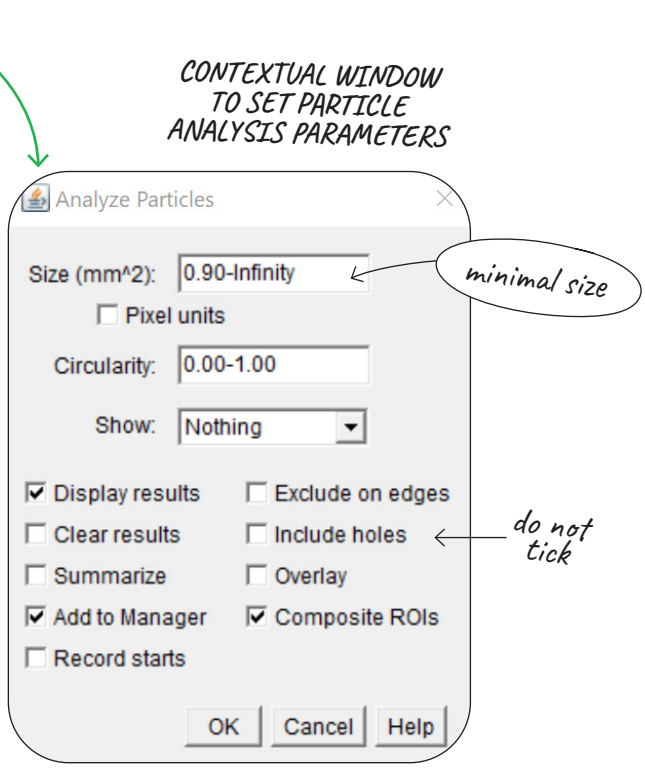
- Prior to measuring, make sure that the desired parameters will be measured by going to "**Analyse" -> Set Measurements...**".
  - > The main two measurements sought are the perimeter and the area.
  - > It is also worth measuring other variables that might be used for subsequent analyses. We therefore advise you to **tick all possible measurements**.
  - > It is also important to select the "**Display label**" box so that the file name is indicated on each measurement line, which is important for later data management.



2.3. MEASURING

To measure, go to:

- "**Analyse -> Analyze Particles...**". A window opens in which you need to specify certain parameters prior to measuring.
- Firstly, indicate a minimum size of the objects to be measured; this is done to exclude impurities present on the scanner.
- To correctly choose this parameter, it can be worth doing a few tests to determine empirically the size of the smallest individual on the scan.
- Be careful not to tick the "include holes" box, otherwise the area of the holes will be measured, which happens when two insect legs touch (see page 4).
- To manage measurement masks, also tick the "**Add to Manager**" and "**Composite ROIs**" boxes.
- Lastly, the "**Display results**" box will allow you to take the measurements and display them in a new window.



2.3. MEASURING

If a measurement is not suitable for certain individuals, due for example to thresholding, each individual can be selected separately.

> The selection editing tool can be used for this purpose.

> Choose the individual selection mask in the ROI Manager, then change its shape.

For example, in the event of a reflection leading to incorrect thresholding, use the "**Enlarge**" function, indicating either plus or minus values.

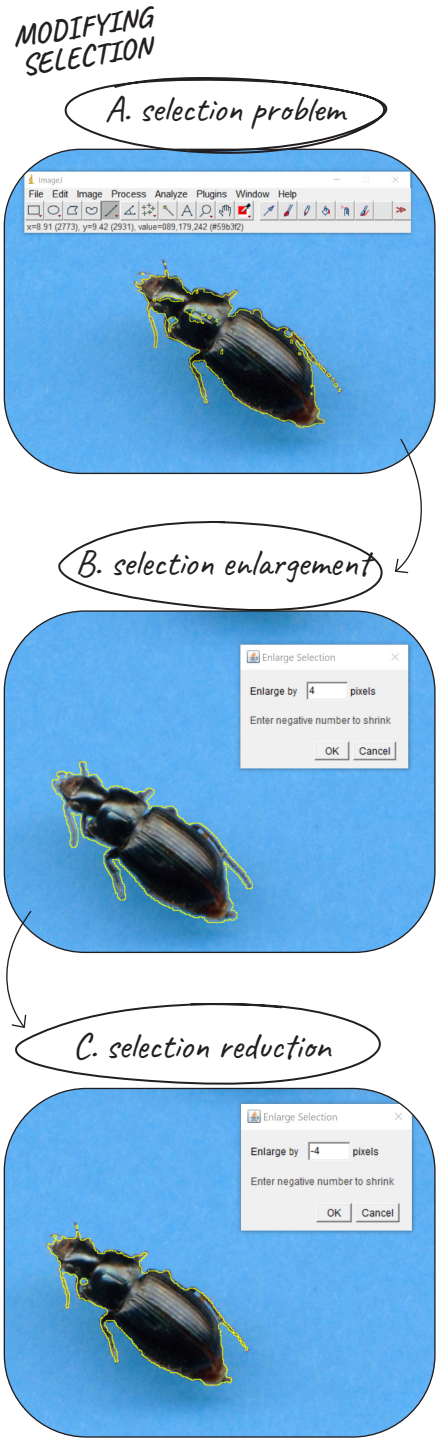
> This will fill any holes in the selection.

> The modified selection should then be saved in the ROI, and the measurements carried out again.

The thresholding and measuring steps can be carried out separately.

> Once the ROIs have been saved, they can be reopened and overlaid on the original image for visual feedback prior to measurement.

> In this case, measurements are taken directly from the ROI Manager. Selection corrections and label modifications can also be done at this stage.



2.4. DATA SAVING AND MANAGEMENT

NEARLY THERE !

Saving masks

> After measuring, it is strongly recommended to save the contour masks used to take the measurement.

> This operation takes up very little space on the hard disk and offers the possibility of repeating the measurement in the event of a problem, thus improving the quality and reliability of the results.

> In addition, masks can be reworked and/or renamed directly without needing to repeat the thresholding step, using the selection editing tools.

> To do this, make sure you have not selected the mask of a single individual; press "**Deselect**" to avoid this.

> To save, click on the "**MORE**" button in the ROI Manager window to access a drop down window where the "**Save**..." button is available.

MASK SAVING

Recording measurements

> After measuring, the data are temporarily stored in the **ImageJ** window called "**Results**".

> These data can be exported in CSV format. You can save your measurements either for each sample in a separate file, or add together the measurements of different samples in the "**Results**" window and combine the results in a single file.

> The "**Label**" column is then used to identify the measurements and is filled in with the name of the image file analyzed.

> It is therefore advisable to name the images with the codes used for the study to facilitate subsequent data processing.