ZooScan-Zooprocess user manual and procedures at the Quantitative Imaging Platform of Villefranche-sur-Mer (PIQv)

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1. Introduction

1.1 Typical ZooScan working protocol at the PIQv

The ZooScan (HYDROPTIC Inc.), in association with the Zooprocess application, is an imaging system for the analysis of organisms and particles (from 300 µm–5 cm, down to 150 µm–5cm using specific settings) present in a liquid. It is suitable for meso- (0.2–2 cm) and macro- planktonic (2–20 cm) organisms that must be immobile (fixed or anesthetised).
See also: http://www.hydroptic.com/index.php/public/Page/product_item/ZOOSCAN

The Villefranche-sur-Mer Quantitative Imaging Platform has analysed more than 400 projects using ZooScan since its invention. As a result of this long-standing experience, the PIQv has implemented analysis and Quality Control procedures. The aim of this manual is to present these rules and to highlight best practices to guarantee data quality and best use of the associated EcoTaxa application for the annotation of images.

This working protocol may be adjusted by users according to their experience and needs to take advantage of the tools provided by the application. We recommend always keeping the default settings proposed by Zooprocess.

The typical analysis sequence is as follows:

1) Fill in sample metadata (as many samples as convenient)
2) Quality Control for metadata
3) Scan background (on a daily basis)
4) Scan sample (one or several in a row)
5) Convert and process samples
6) Check process
7) Quality Control for acquisition and process
8) Separation of touching objects
9) Process image again to include separation mask and get a better final dataset
10) Import into EcoTaxa to predict identification and manually validate or correct the application's prediction
11) Quality Control for multiple

1.2 Instructions prior to starting

- Install your ZooScan on a perfectly straight and stable (no vibration) lab bench (see Hydroptic manual).
- Install the associated software from the PIQv website.
- Collect a sample and use logsheets (see Appendix 1) to record all the metadata necessary to trace your sample.
- Remove the seawater and fixative from the sample (see Chapter 6).

1.3 Safety warnings

For safety reasons, we never place samples containing formalin directly on the ZooScan tray. We first remove the seawater and formalin from the sample and then use 0.2 µm filtered seawater (or freshwater) instead.

Please note that formalin is a carcinogenic, mutagenic and reprotoxic substance. Follow all necessary safety procedures to avoid all risks when removing formalin from the sample:

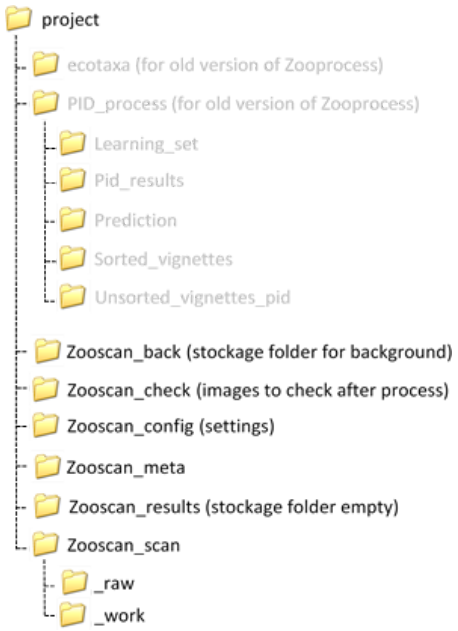
- work in an extractor hood,
- wear gloves, glasses and a lab coat.

1.4 Important note on Zooprocess version

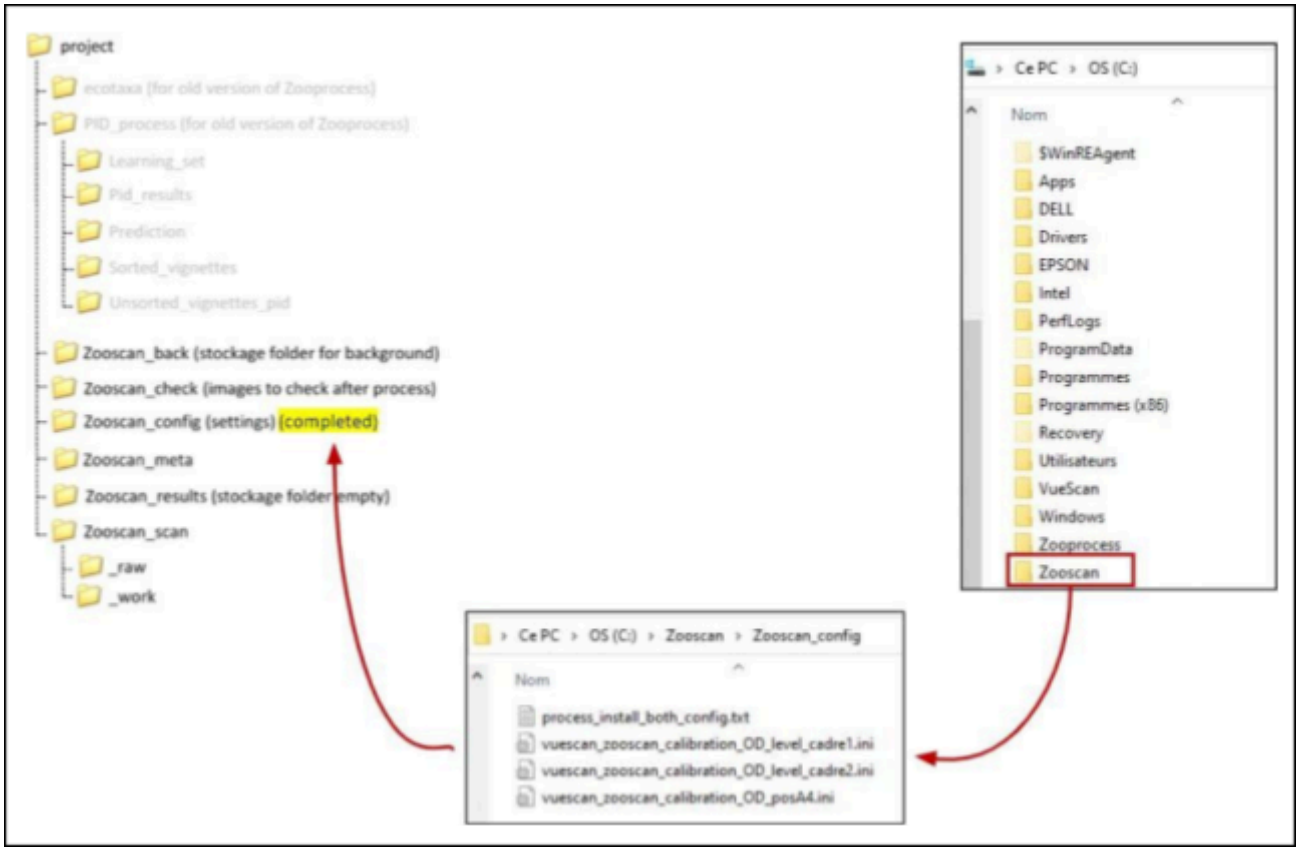
- This protocol applies to Zooprocess version 7.27 and above. The images are issued from versions 8.23.
- **Upgrade** Zooprocess whenever a new update is available on the PIQv website.
- The PIQv will only assist you if you use the latest available update of Zooprocess.
- This protocol does not describe installation of the ZooScan software and drivers on your computer (see the manuals on the PIQv website).
- Additional documentation is available on the PIQv website.

2. Create a new project

A project is a folder where images and data of a cruise, survey or experiment will be saved. A project thus contains many samples and scans.
The project will be created at the root of a selected drive and all files from this project will later be saved in the specific sub-folders.

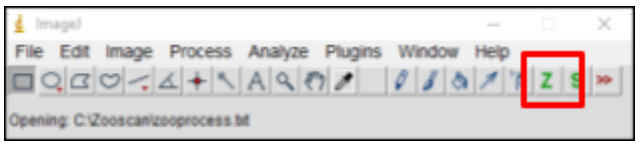


IMPORTANT: Create the project on the computer **to which the ZooScan is connected** because when you install ImageJ on a computer connected to a ZooScan, a "ZooScan" folder is created on the C: disk. This folder contains specific information about the connected ZooScan, such as frame dimensions, which Zooprocess needs to integrate into your project.

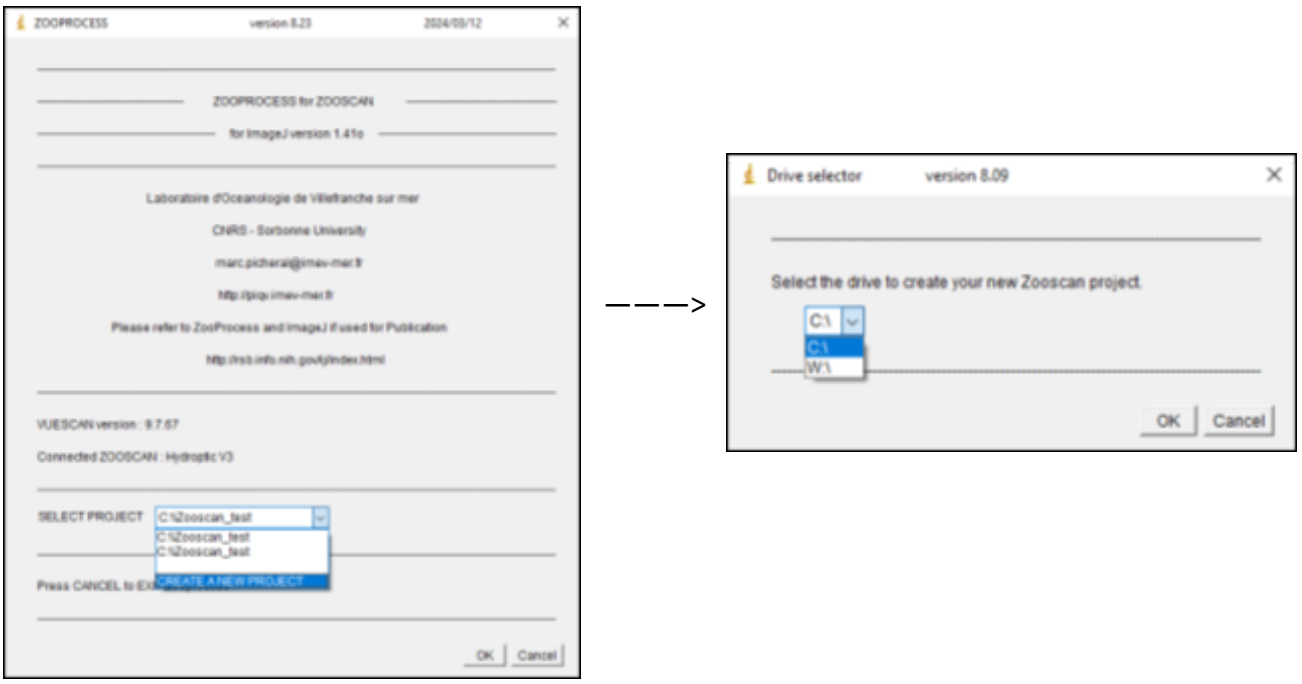


PROCEDURE

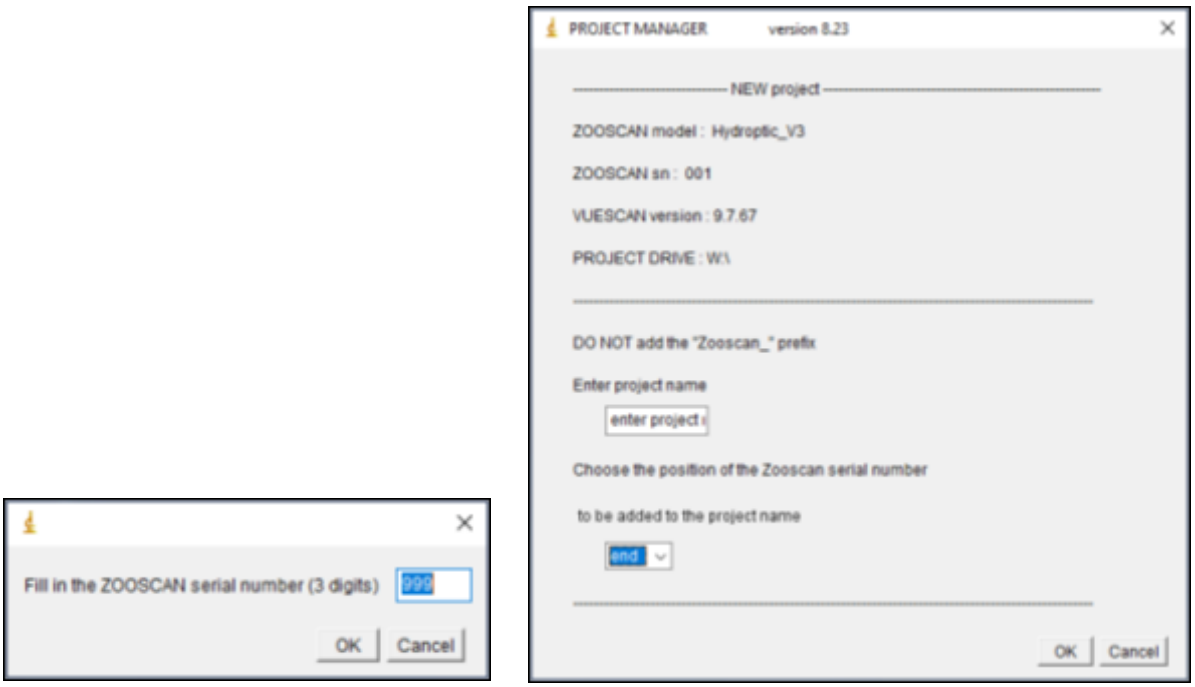
- Open ImageJ/Zooprocess or click on the Z icon if ImageJ is already running. Do not open it twice (if you do, close all ImageJ occurrences and restart it).



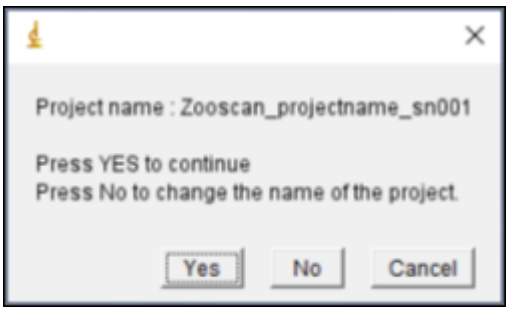
- Select the option “CREATE A NEW PROJECT”, which is at the bottom of the project list, and then select the drive.



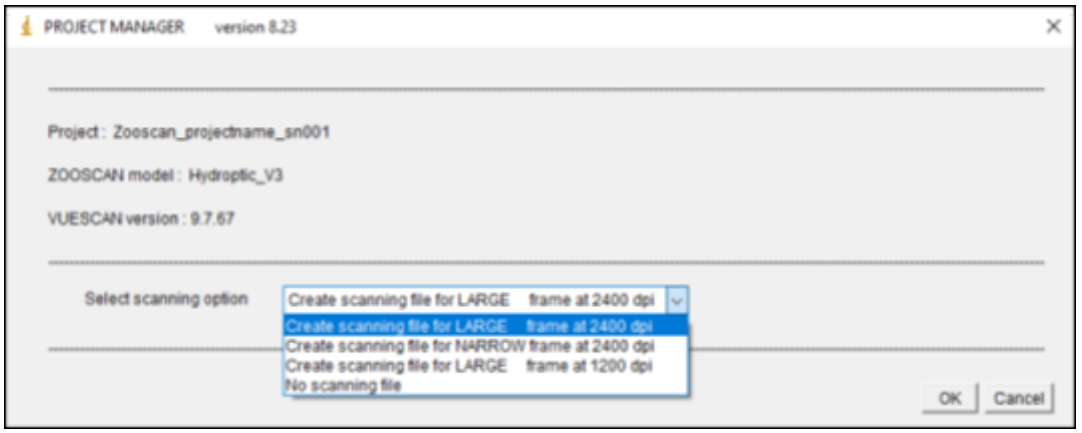
- Enter the serial number of your ZooScan (3 digits) and then choose the name of your project (do not add the "zooscan_" prefix). **Do not use special characters in your project name.** You can decide whether to place the serial number of your ZooScan at the beginning of the project name (in which case, if you have multiple ZooScans, the list of projects will be sorted by ZooScan type) or at the end (in which case, the list of projects will be sorted alphabetically).

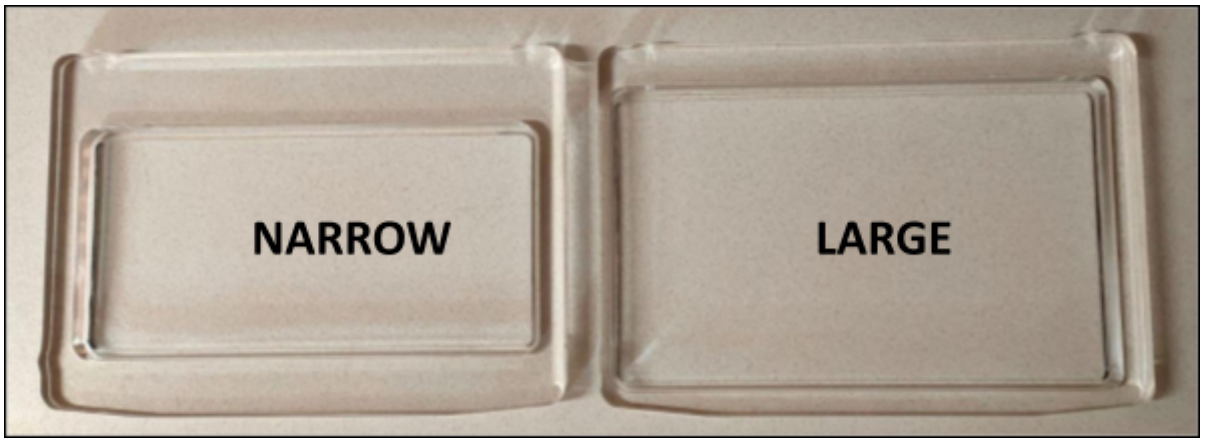


→ Zooprocess shows you a preview of your project name, so take the time to validate it or change it:

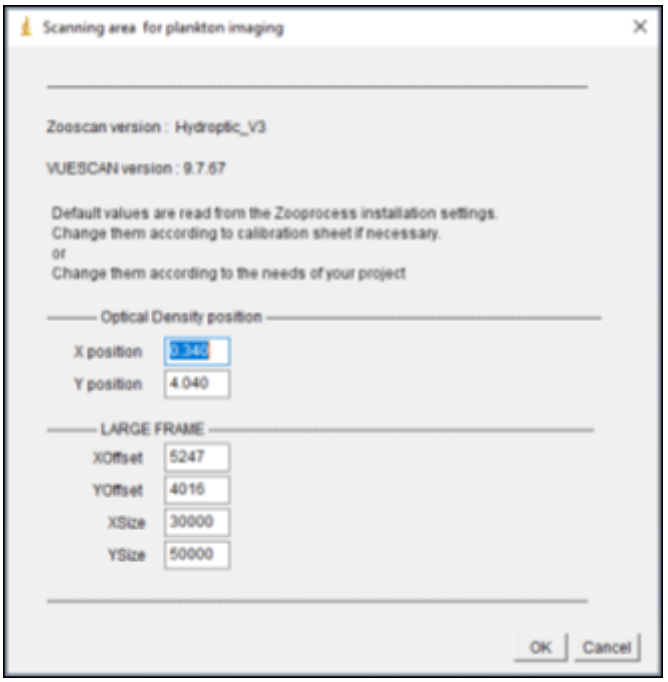


- Select the scanning option: we recommend choosing the **large frame** for images at a resolution of 2400 dpi. The large frame allows you to scan more objects in a row. The narrow frame does not provide significantly better resolution and limits the scanning area as well as the number of organisms in the image; it was useful for old 32-bit computers.

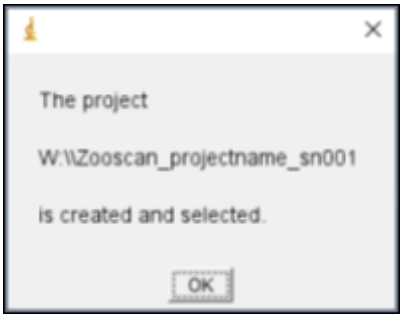




- Do not change the values. Click on “OK”. If necessary, these values are indicated in the instrument documents provided by Hydroptic on the associated USB key. The Optical Density position is useless in recent versions of the ZooScan.



- The project is now created:



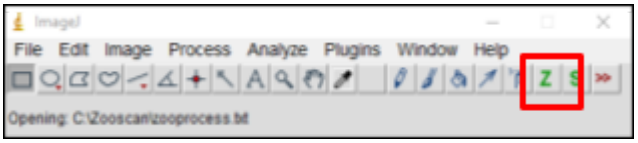
3. Fill in Sample metadata

This operation is no longer linked to scanning of the samples. You can fill in metadata for several samples in advance. You can even do this in the field and then transfer your file to the project on the computer connected to the ZooScan.

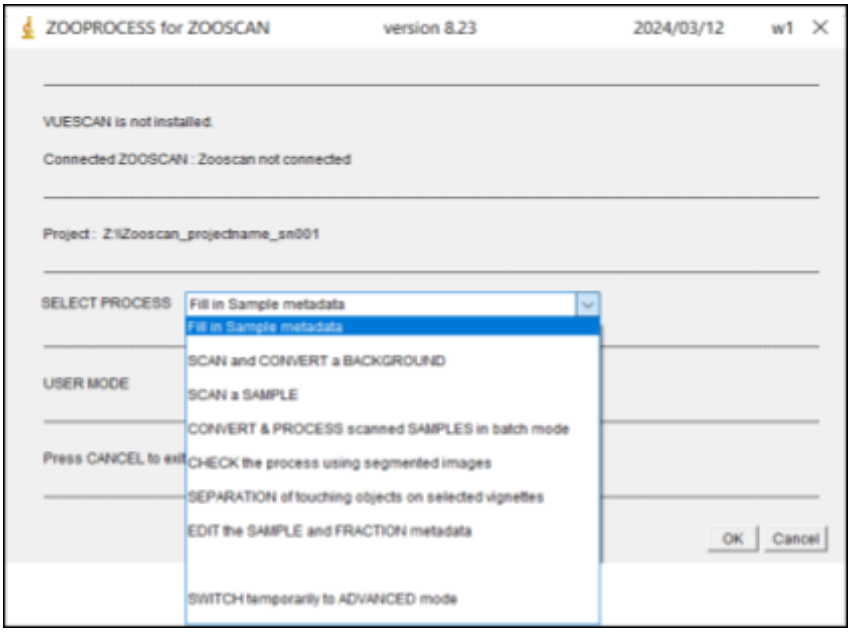
Zooprocess saves a backup copy of the previous sample and scans tables in the “archives” sub- folder every time these tables are modified (see Chapter 4).

PROCEDURE

- Open ImageJ/Zooprocess or click on the Z icon if ImageJ is already running. Do not open it twice (if you do, close all ImageJ occurrences and restart it).

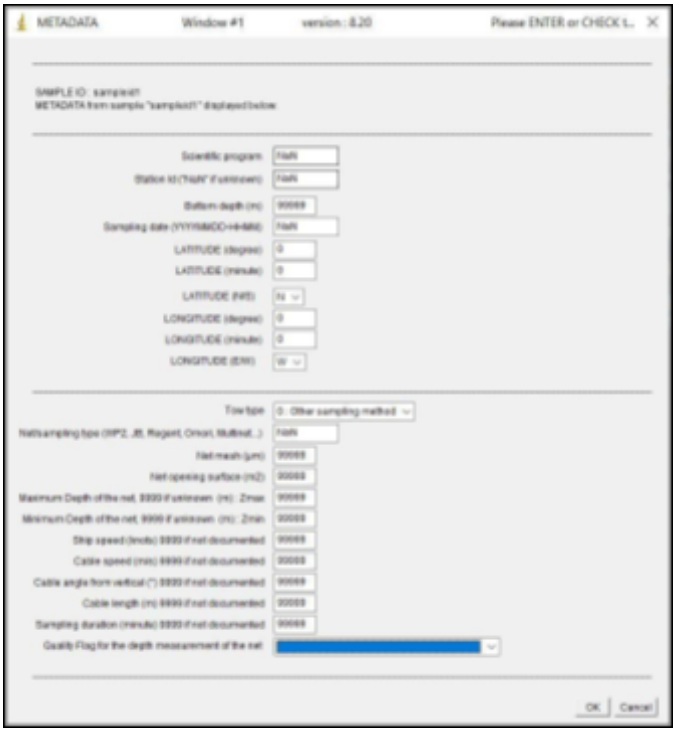


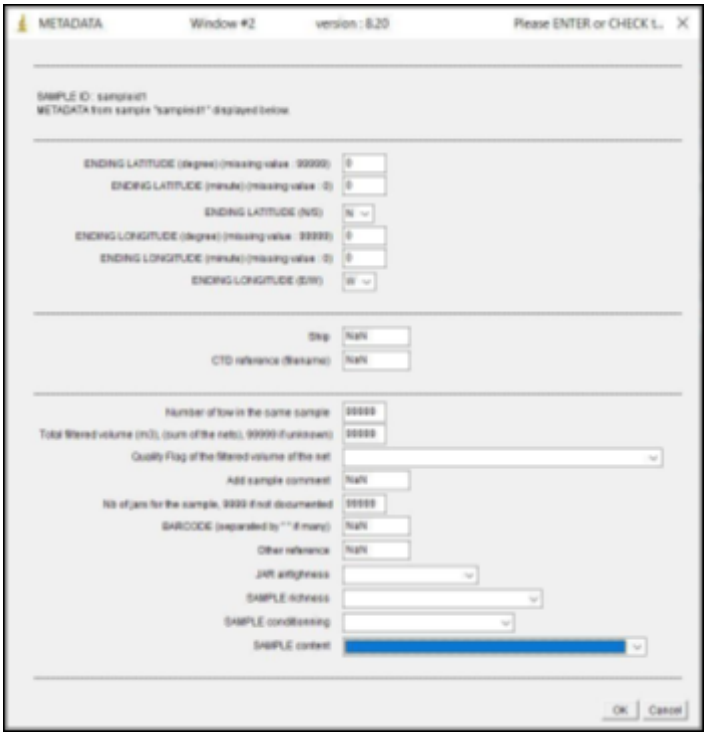
Select your project and choose "Fill in Sample metadata".



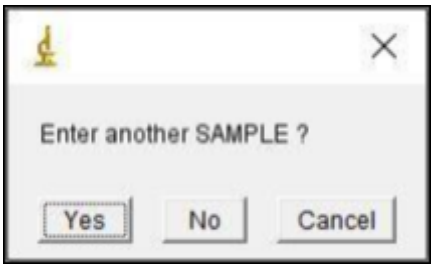
- Choose the sampleID. **At this point, you should have carefully considered the naming convention of your samples**, as it is **not possible to change the name of your sample** afterwards. Do not use the extensions “d1”, “d2” or “tot” in the sample names. These extensions will automatically be added to the scan names during the scanning step (see Chapter 6.3).

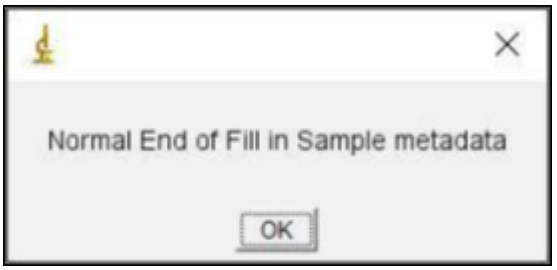
- Fill in the forms. **Do not use special characters**.
  ATTENTION: Even though these metadata can be edited and corrected later, you must be very careful when filling in the metadata such as sampleID, depth>zmax, lat and lon start ≠ Lat and lon end for horizontal nets, consistency between net type and net mesh, nb of jars vs nb of barcodes, consistency of volume vs distance of net, consistency and format of GPS points, among others.





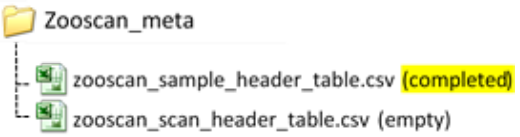
- You can enter several samples. At the end, you have a “Normal End” message.





ADDITIONAL INFORMATION

“zooscan_sample_header_table.csv” will be created by the application in the “Zooscan_meta” folder of your project. This table lists all the samples and associated metadata that you have filled in.



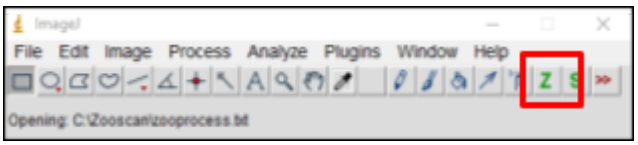
4. Edit the sample and fraction metadata

You can edit and modify metadata at any time throughout all steps to correct possible mistakes or complete information on the samples or scans. Pay attention to these metadata, as they will be essential for the subsequent analysis of the results.

The tool changes the metadata in the tables of the meta folder and inside all files related to the sample throughout the project.

PROCEDURE

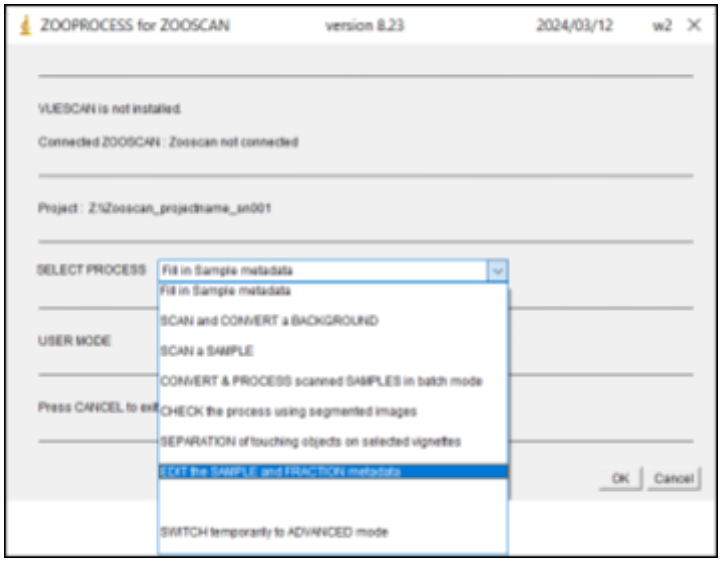
- Open ImageJ/Zooprocess or click on the Z icon if ImageJ is already running. Do not open it twice (if you do, close all ImageJ occurrences and restart it).



There are two ways to edit and modify metadata:

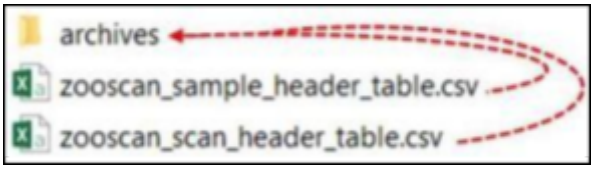
1) <u>One by one (fully safe)</u>

- Select “EDIT the SAMPLE and FRACTION metadata” tool, then select the sample. Zooprocess saves a backup copy of the previous sample and scan tables in the “archives” sub-folder every time these tables are modified.



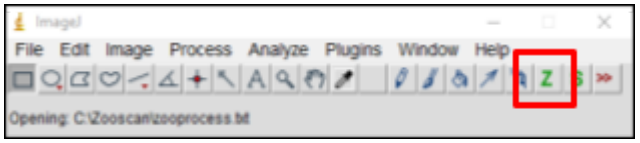
2) <u>In batch mode (not recommended, be very careful)</u>

- Copy/Paste your .csv tables into the “archives” folder. This operation is performed automatically when you edit a single sample using the Zooprocess interface:

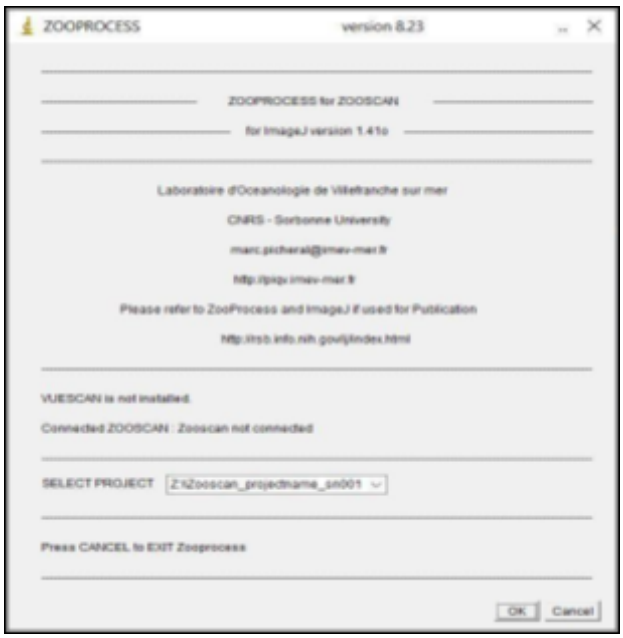


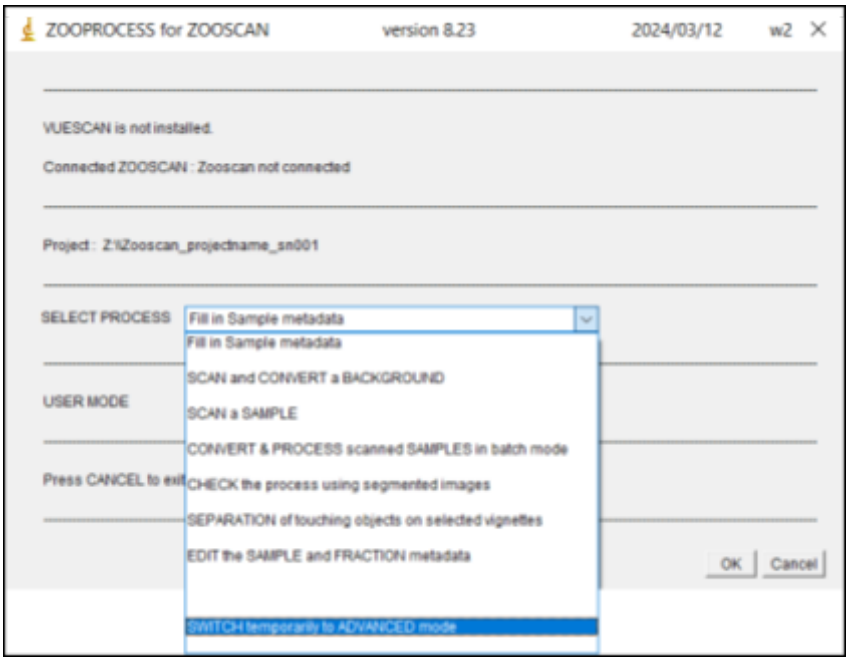
- Modify the .csv tables directly and then save them: **PAY ATTENTION TO THE METADATA FORMATS.**

- Open ImageJ/Zooprocess or click on the Z icon if ImageJ is already running. Do not open it twice (if you do, close all ImageJ occurrences and restart it).

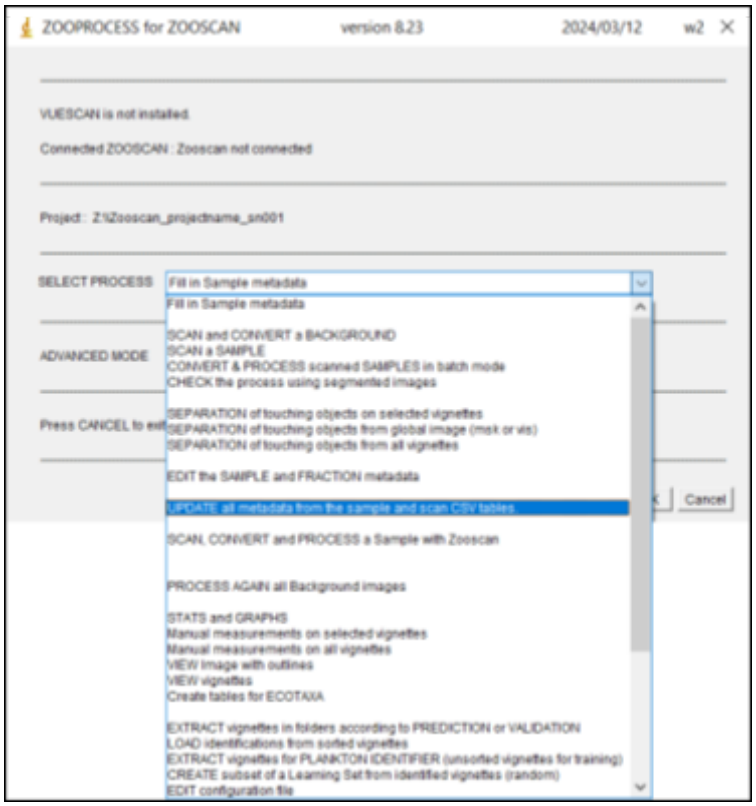


- Select your project and choose “SWITCH temporarily to ADVANCED mode”.

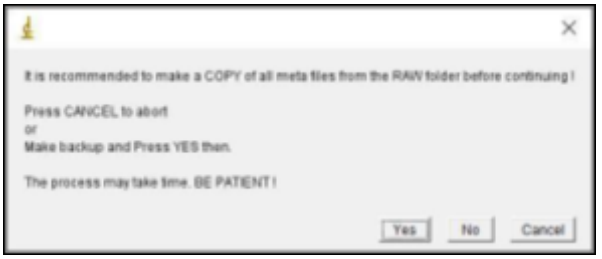




- Select “UPDATE all metadata from the sample and scan CSV tables”. A message will appear; click the “Yes” button.



→



5. Scan and convert a background

The background image is a “blank” image that will be used during the image analysis process. It should be created before the samples are scanned, using the same parameters as those used for scanning the samples. You have to scan a minimum of two background images that will be combined into a single “blank” image. This should be done at the beginning of every scanning session when you turn on your ZooScan, typically every morning before scanning the first sample.

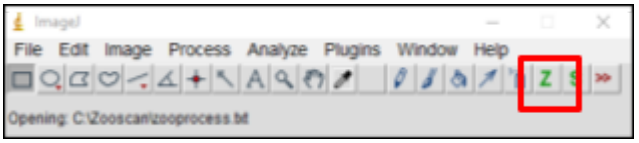
PROCEDURE

- Turn on the ZooScan and handle it gently. Clean and rinse the scan tray and the cover glass using fresh water to remove any dirt. Eliminate marks on the glass and the frame and periodically check to ensure that the ZooScan glass cover remains clear.

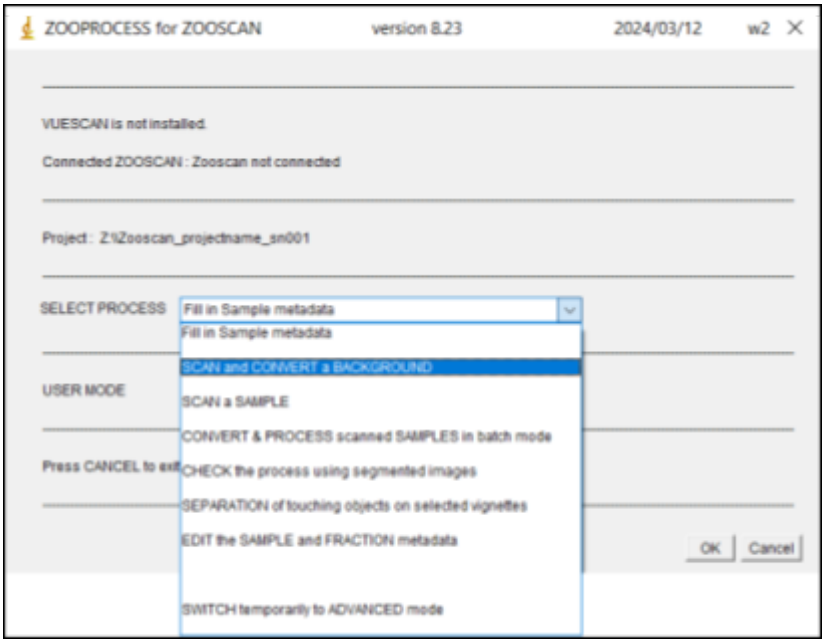
- Pour some clean, fresh water stored at room temperature into the tray until it is covered. Place the frame designated for your project (Narrow or Large) onto the tray. Ensure that the frame is well placed on the recommended and tagged corner of the scanning tray, so that the area of scan is set up to include the frame borders. Refer to the Hydroptic manual provided with the ZooScan for further guidance.

- Fill the tray with fresh water until the edge of the frame is covered. Check that there are no bubbles or dust and that both the tray and the water are clean: remove any dirt and bubbles using a plastic pipette or a cactus stick provided with the instrument to avoid scratching the glass tray. Lateral view:

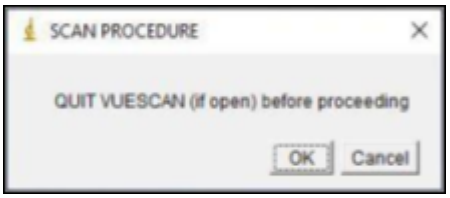
- Open ImageJ/Zooprocess or click on the Z icon if ImageJ is already running. Do not open it twice (if you do, close all ImageJ occurrences and restart it).



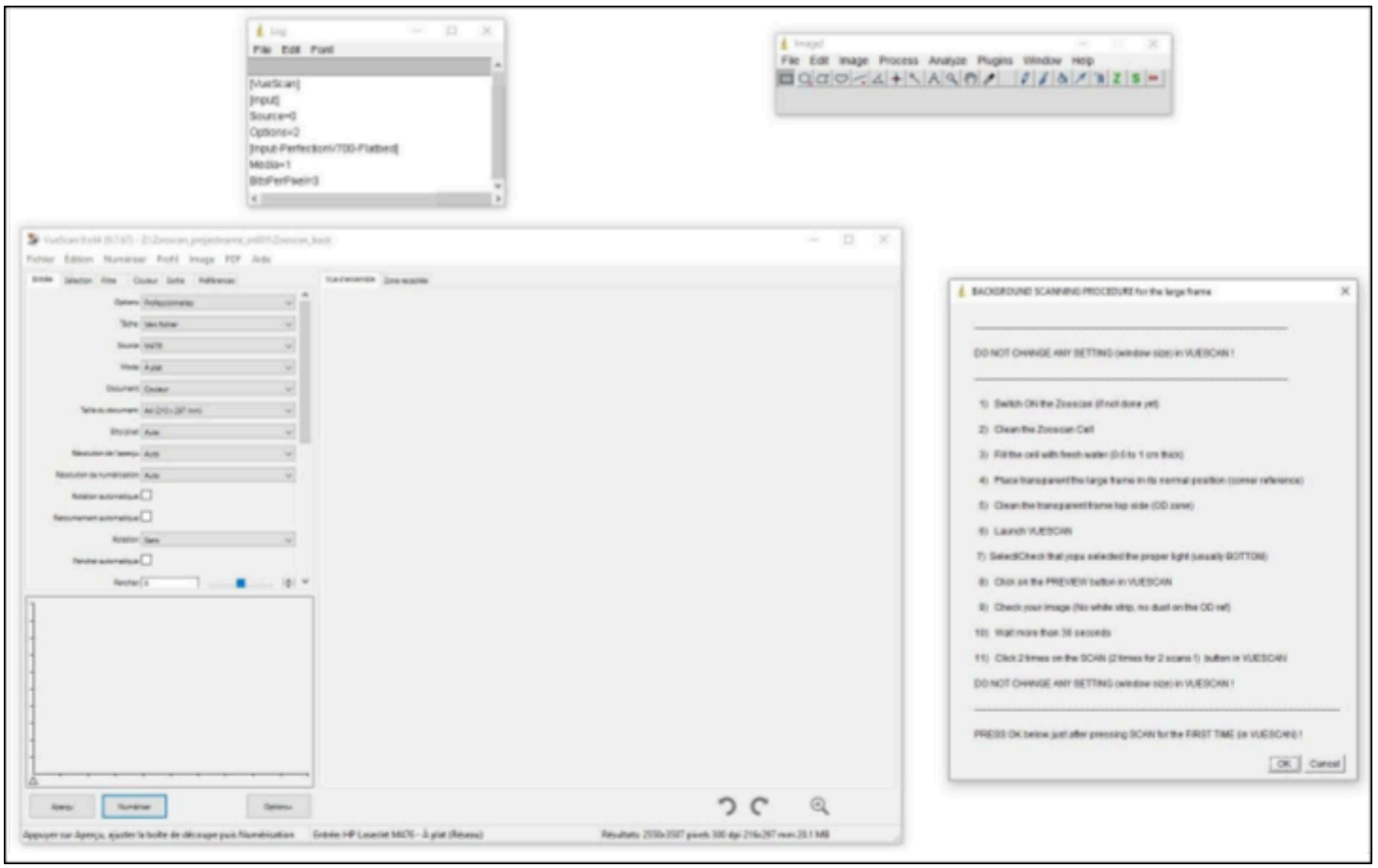
- Select your project and select “SCAN and CONVERT a BACKGROUND” tool.



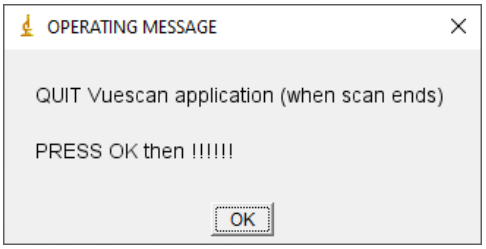
- Follow the instructions that pop up on your computer screen.



→ **Pay attention to this message**

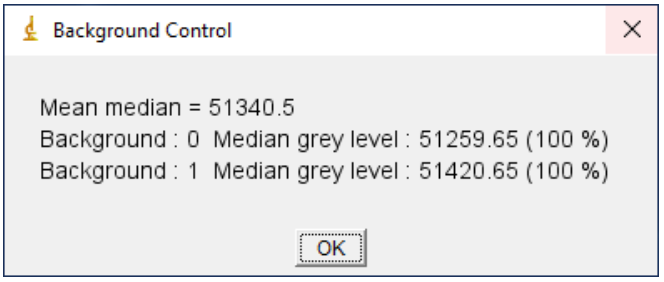


→ Wait for the recommended time (30 seconds) between the preview, scan no. 1 and scan no. 2 (see the instructions window on your computer screen). Do not forget to press OK in the instructions window immediately after the second scan.



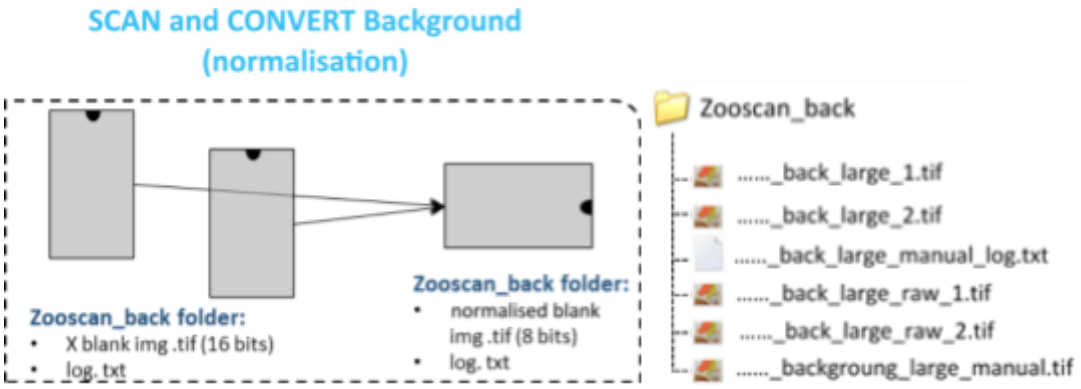
→ Pay attention to this message

- At the end, you have the median grey level for your two backgrounds and the average of the two medians. The two values must be similar unless a warning is displayed.

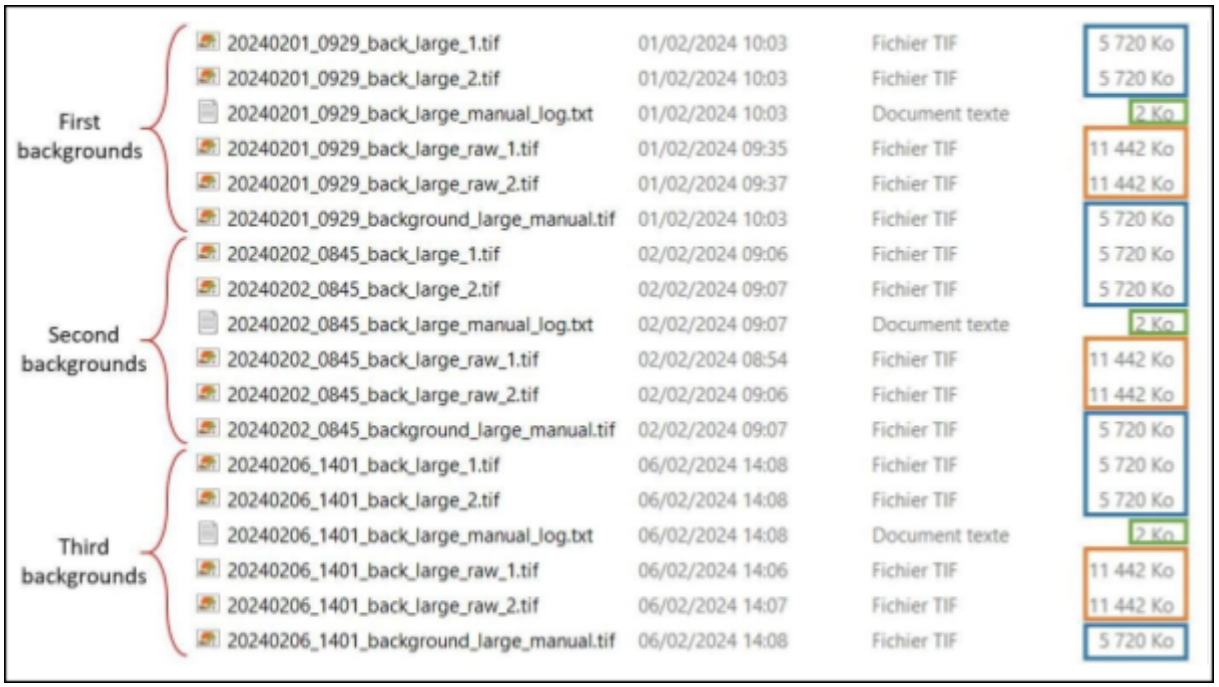


ADDITIONAL INFORMATION

The two background images will be processed into a single low-resolution background image and automatically saved in the “Zooscan_back” folder.



To find out the quality of your background, you can check the weight: it must be the same for all backgrounds.



6. Sample preparation and scanning

6.1 Safety warnings

For safety reasons, we never pour samples containing formalin directly onto the ZooScan tray. Instead, we first remove the seawater and formalin from the sample and use filtered seawater (or fresh water) instead.

Please note that formalin is a carcinogenic, mutagenic and reprotoxic product.
Apply all necessary safety procedures to minimise risks when removing formalin from the sample.

- work in an extractor hood,
- wear gloves, glasses and a lab coat.

6.2 Sample preparation

Below, we describe the general procedure in use at the Plateforme d'Imagerie Quantitative de Villefranche-sur-Mer. This procedure is the result of years of experience with the analysis of plankton samples using ZooScan and Zooprocess.

As plankton abundance is usually very high in a sample, only a fraction of the sample is scanned. For this, a motoda box (splitter) is used: it allows the sample to be split into two equal aliquots. However, to avoid underestimating large and rare organisms when using the motoda box, the sample is first divided into two **size** fractions depending on the mesh size of the net. As a result, there is one scan per size fraction.
Indeed, for a **mesh net of 300 µm or smaller**, the sample is usually divided into two size fractions to get sufficient specimens of large organisms:

- fraction named "**d1**": large organisms > 1000 µm,
- fraction named "**d2**": small organisms 100 µm–1000 µm.

For **a mesh net larger than 300 µm**, the sample is usually not divided and is referred to as "**tot**".

PROCEDURE

The procedure described below is for a 200 µm net (example for WP2) requesting two size fractions.

- The sample is sieved under the extractor hood to remove the preservative and the seawater. You can keep the removed liquid to refill the sample afterwards (see step "a" on the image below), but we prefer to use well-controlled seawater with buffered formaldehyde.
  We always use a sieve of lower mesh than that of the net to avoid any loss of organisms.

- The sample is sieved through a 1000 µm mesh and a 100 µm mesh to obtain two size fractions, d1 and d2 (see step "b" on the image below).

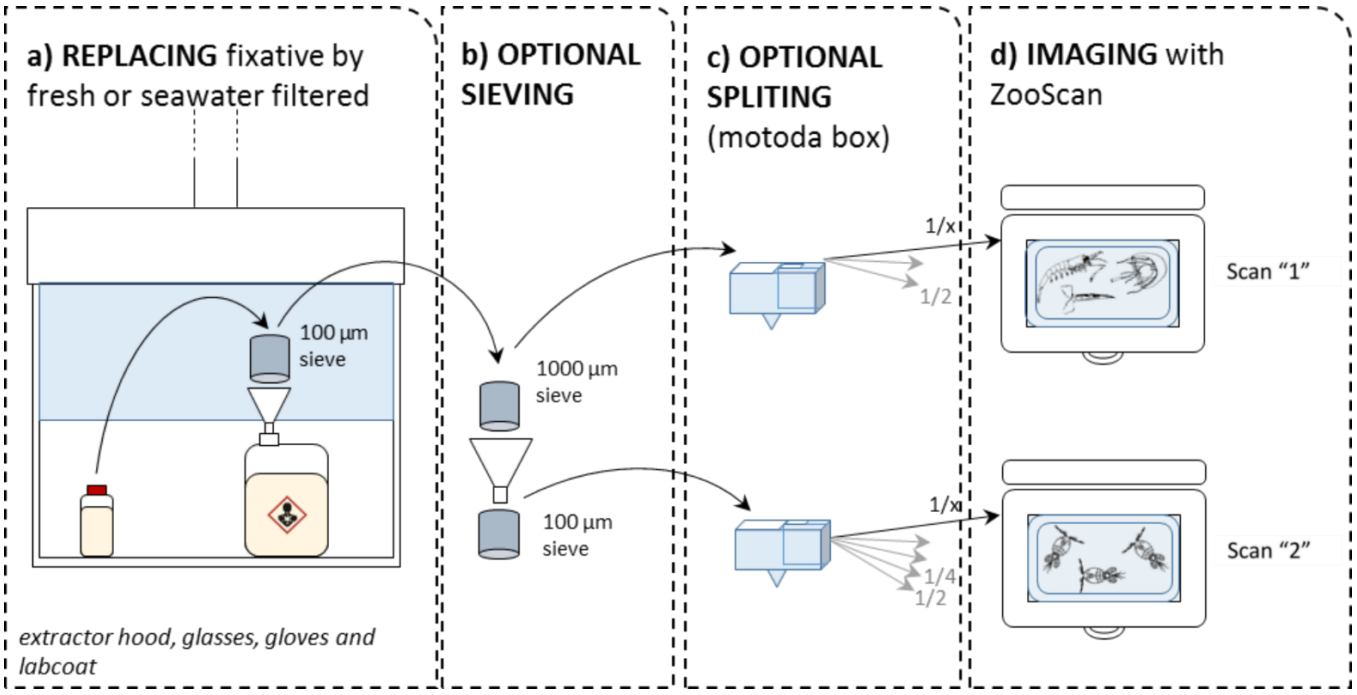
- The two fractions are then split with a motoda box before scanning to obtain a proper number of objects*.

The method of splitting is as follows:

1) Tilt the box so that the open portion (without the cover) is facing up.
2) Use a squeeze bottle filled with seawater to pour plankton from the sieve into the box.
3) Add a small amount of seawater into the box **just before the septum**.
4) To homogenise the sample, vigorously move the box back and forth about **twenty** times, tilting it from left to right. Ensure the edges of **the box touch the table** during this process.
5) After homogenisation, tilt the box to the opposite side. The septum will split the sample into two subsamples, with no exchange of water or plankton between them.
6) Turn the box over to pour one subsample into a jar through the open corner of the cover. The other subsample will remain inside, blocked by the cover.
7) Return the box to its normal position.
8) Rinse the sides of the box to remove any remaining plankton.
9) Repeat steps 3–8 for the second subsample.

To avoid errors during the splitting procedure with the motoda box, we recommend storing all subsequent fractions in pre-labelled jars (1/2, 1/4, 1/8, 1/16 etc.) (see step “c” on the image below).

- Each fraction, d1 and d2, is scanned (see step “d” on the image below).



***IMPORTANT**

- You can verify the usefulness of sieving the sample into two size fractions by comparing the splitting **ratio** necessary to obtain an adequate number of organisms for the two fractions. **This ratio must be higher for the lower fraction (d1) except if there is a bloom of large organisms (e.g. salps): must be noted in the sample and scan comments.**

- The number of touching organisms must be as low as possible to avoid spending excessive time later separating them in the images, while still ensuring that there are enough objects to be representative of the sample.

Here are the rules applied at the PIQv:
When 'sample_net_mesh' >= 500 and 'motoda_frac' strictly equals 1:
→ The number of .jpg images in the '_work' sub-folder must not exceed 1500.
When 'sample_net_mesh' >= 500 and 'motoda_frac' is strictly above 1:
→ The number of .jpg images in the '_work' sub-folder must be between 500 and 1500.
When 'sample_net_mesh' < 500 and 'FracID' = d1 and 'motoda_frac' strictly equals 1:
→ The number of .jpg images in the '_work' sub-folder must not exceed 1500.
When 'sample_net_mesh' < 500 and 'FracID' = d1 and 'motoda_frac' is strictly above 1:
→ The number of .jpg images in the '_work' sub-folder must be between 500 and 1500.
When 'sample_net_mesh' < 500 and 'FracID' = d1+N or 'FracID' = tot or 'FracID' = plankton and motoda_frac strictly equals 1:
→ The number of .jpg images in the '_work' sub-folder must not exceed 2500.
When 'sample_net_mesh' < 500 and 'FracID' = d1+N or 'FracID' = tot or 'FracID' = plankton and motoda_frac is strictly above 1:
→ The number of .jpg images in the '_work' sub-folder must be between 1000 and 2500.

These rules are applied using the ZooScan Quality Control (ZQC) application available on the PIQv website, which we recommend running on the projects at various different stages of the work.

6.3 Scanning procedure

The metadata **must have been** previously completed (see Chapter 2).
A background **must have been** scanned (see Chapter 4).

PROCEDURE

- Pour some water (either fresh water or 0.2 µm filtered seawater) onto the scanning tray until the glass is totally covered. Do not cover the edges of the frame at this step, as the addition of the sample will then cover them. To prevent condensation and bubbles appearing on the ZooScan tray due to temperature differences between the working room and the water in the pipes, we recommend storing a few litres of water in the same room of the instrument in advance.

- Place the frame. The frame size (Narrow or Large) depends on your choices in Chapter 1 "Create a new project". Ensure that the frame is properly positioned, touching the recommended and tagged corners of the scanning tray as indicated for scanning the background (see Chapter 5).

- Wipe off any water droplets or marks on the frame and remove any dirt from the water using a pipette and cactus sticks.

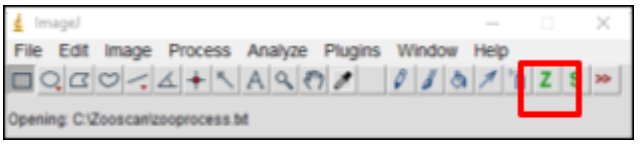
- Pour the sample from the last fraction of the motoda box.

- Add water if necessary, until all the edges of the frame are covered, as was done for the background. Be cautious not to pour too much water, as this can cause the floating organisms to become out of focus. You thus need to find a balance between having enough water to cover the edges and maintaining a sufficient water level to keep the organisms in focus. Lateral view:

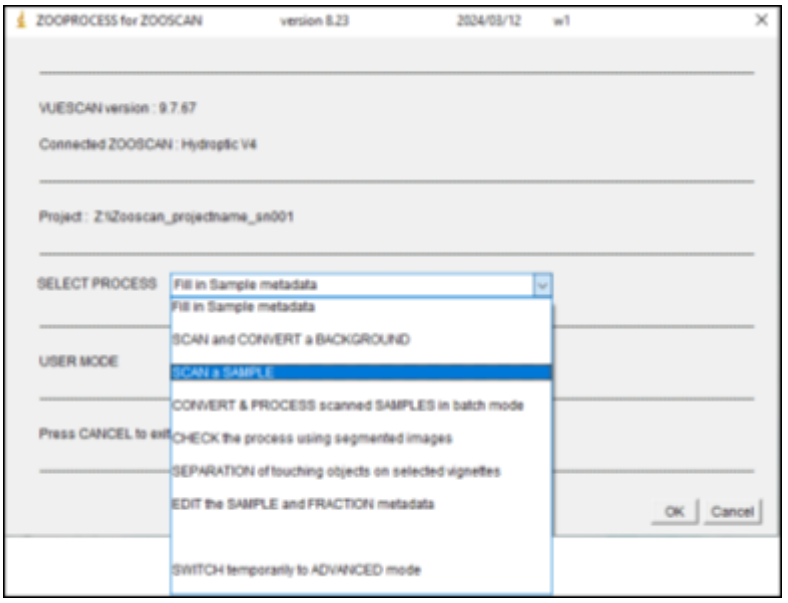
- Allow 15–20 minutes to separate touching organisms with the cactus sticks. **Ensure no organisms are on the edge or along the edge.** Pay attention to the separation of the objects from each other. Make a compromise between the time spent on this task and the quality of the image. After processing the image, you can separate touching objects in the final image using the separation tool in Zooprocess (see Chapter 10). However, you may lose details on the organisms and they may be truncated.
  You can accelerate the separation of organisms by pouring the sample homogeneously on the tray and by adding extra water on conglomerated organisms of the tray to space them out.
  Place the larger individuals in the centre of the tray, as the edges of the frame will be cropped in the image. If some organisms are floating, try to sink them by gently pushing them with the cactus sticks. Floating organisms can result in biased size measurements and blurred image captions, which can hinder correct identification.
  → **for this step, please read Chapter 6.4 carefully**

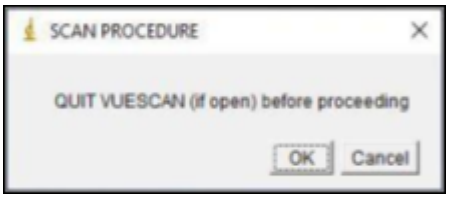
- Ensure that there is no condensation on the glass of the ZooScan cover.

- Open ImageJ/Zooprocess or click on the Z icon if ImageJ is already running. Do not open it twice (if you do, close all ImageJ occurrences and restart it).



- Select your project and choose “SCAN a SAMPLE”.

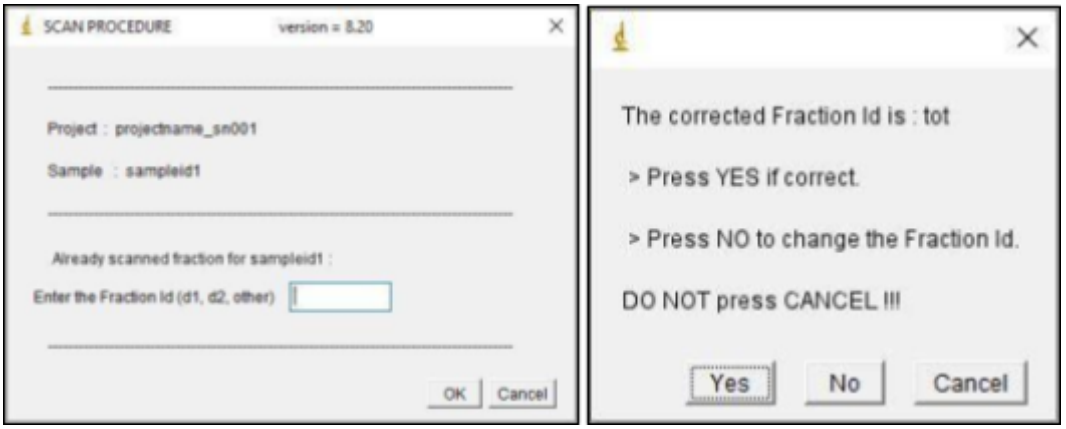




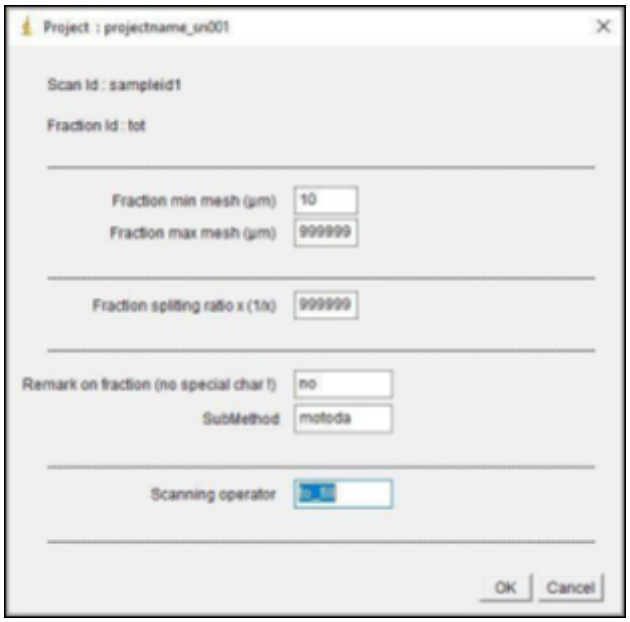
→ Pay attention to this message

- Select the sample name. If your sample is barcoded and you have a connected reader, you can use the dedicated option at the end of the sample list.

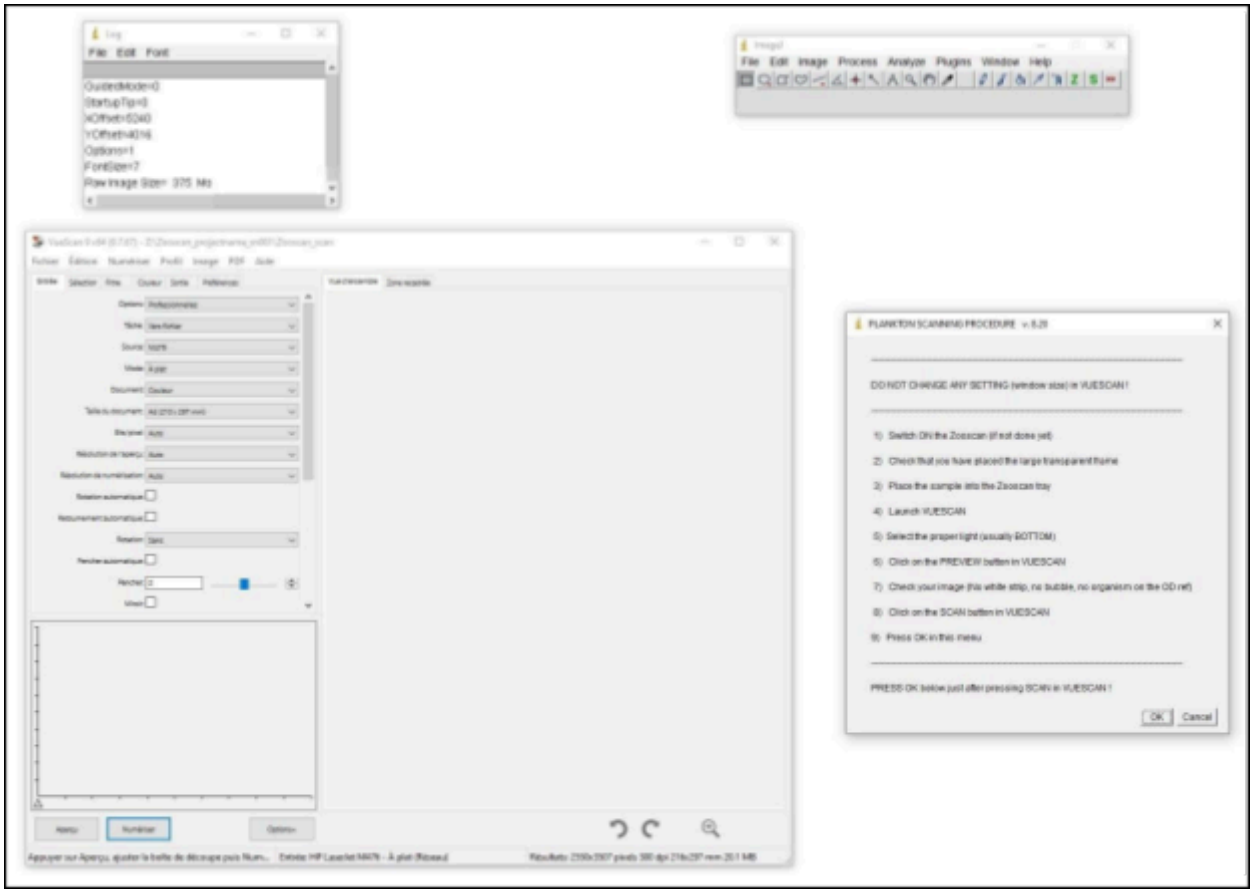
- Then, follow the instructions that pop up on the screen. In Zooprocess, type in the proper fraction name “d1”, “d2”, “tot” or user defined according to the fraction when prompted.



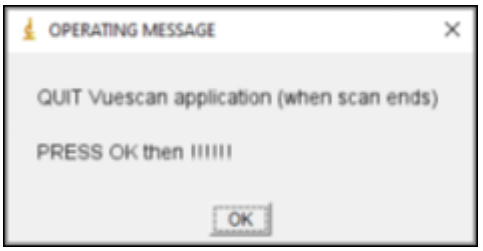
- Fill in the form. Do not forget to fill in any useful comments for the analysis. Do not use special characters. For “scanning operator”, fill in “firstname_lastname”.



- **Carefully follow the instructions in the Zooprocess window to start the scan of the sample (one scan only).**

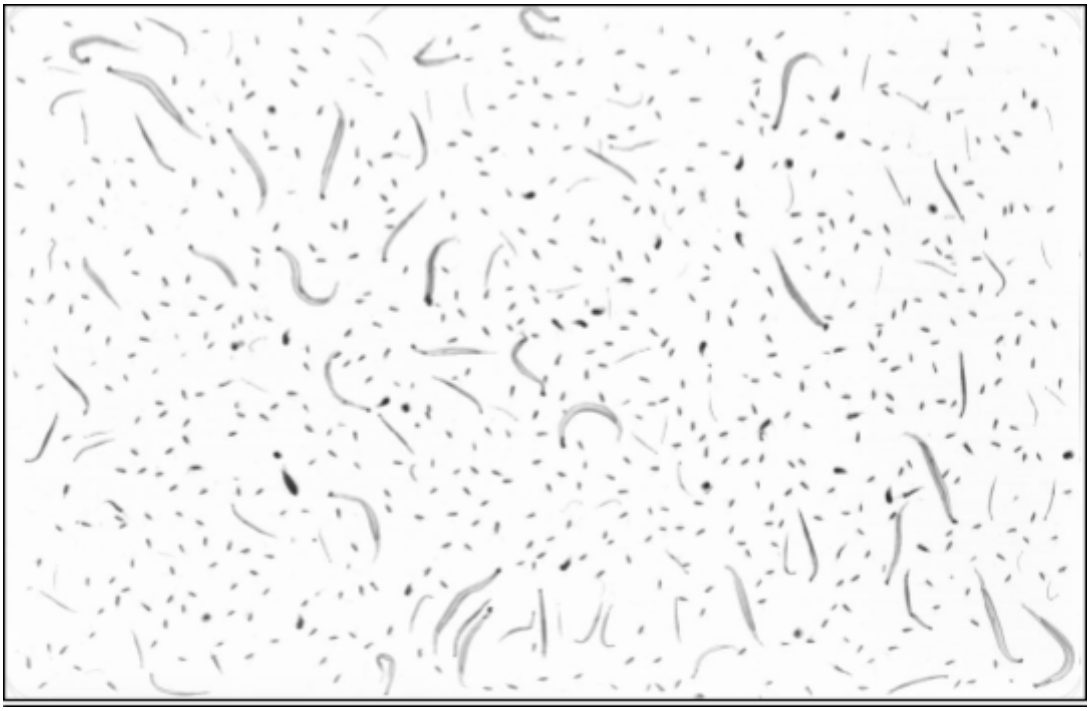


→ Wait 30 seconds between the preview and the scan. Do not forget to press OK in Zooprocess after pressing scan in Vuescan. Do not touch any key of the keyboard during the scan to avoid modifying the scan settings. Avoid any vibrations on the ZooScan that may disturb the water surface on the tray.

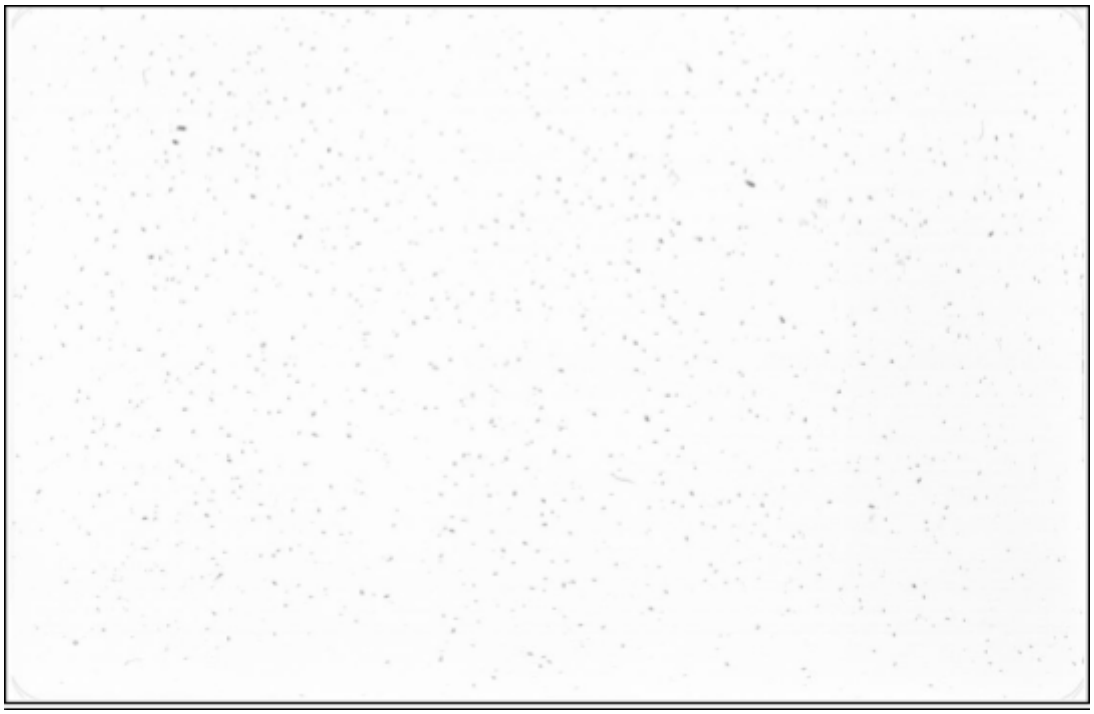


→ Pay attention to this message

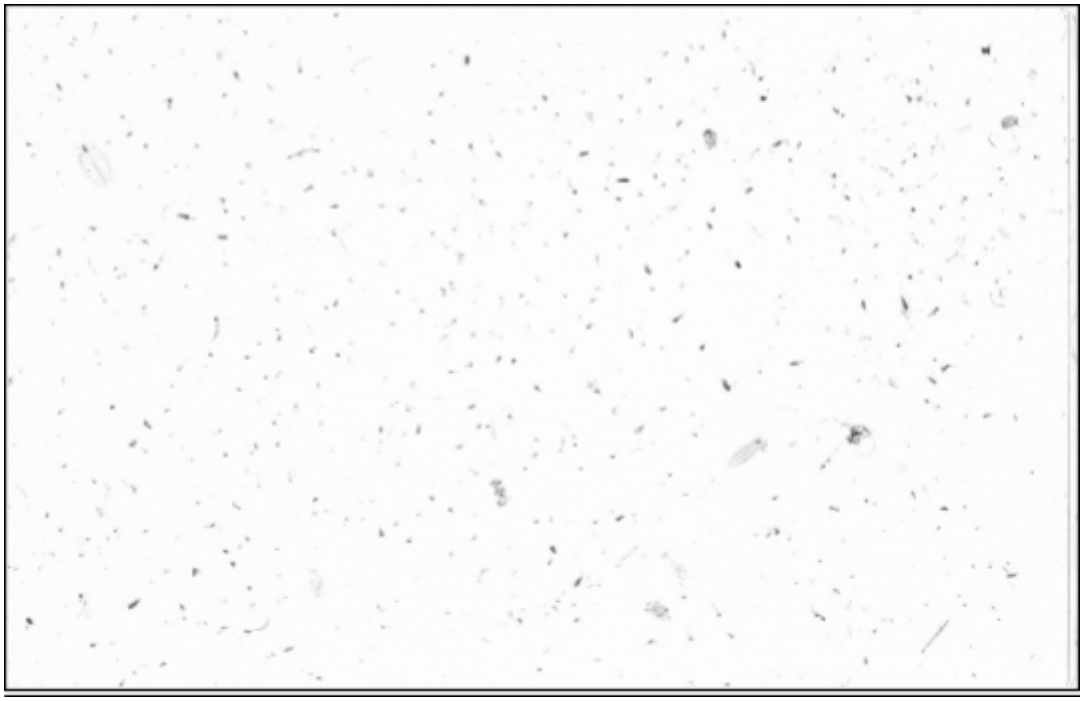
Example of “d1” scan for ≤ 200 µm net mesh (large fraction > 1000 µm)



Example of “d2” scan for ≤ 200 µm net mesh (small fraction 100–1000 µm)



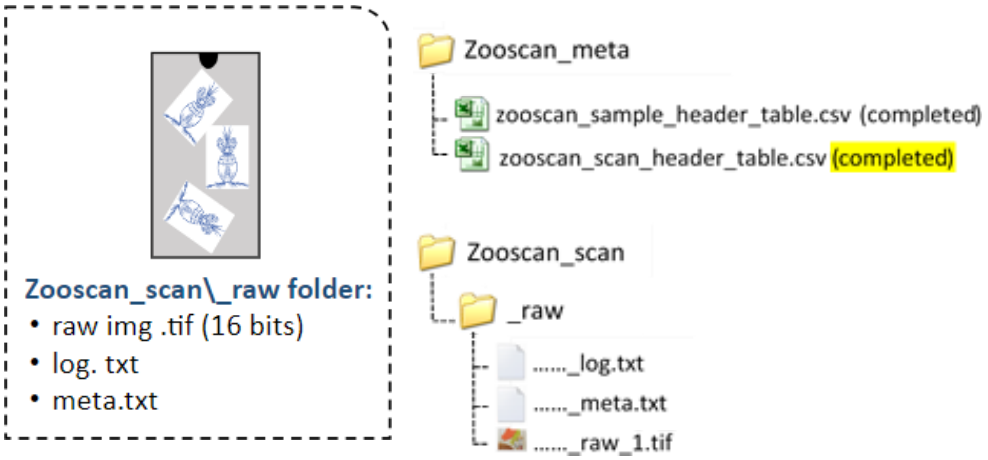
Example of “tot” scan for > 200 µm net mesh



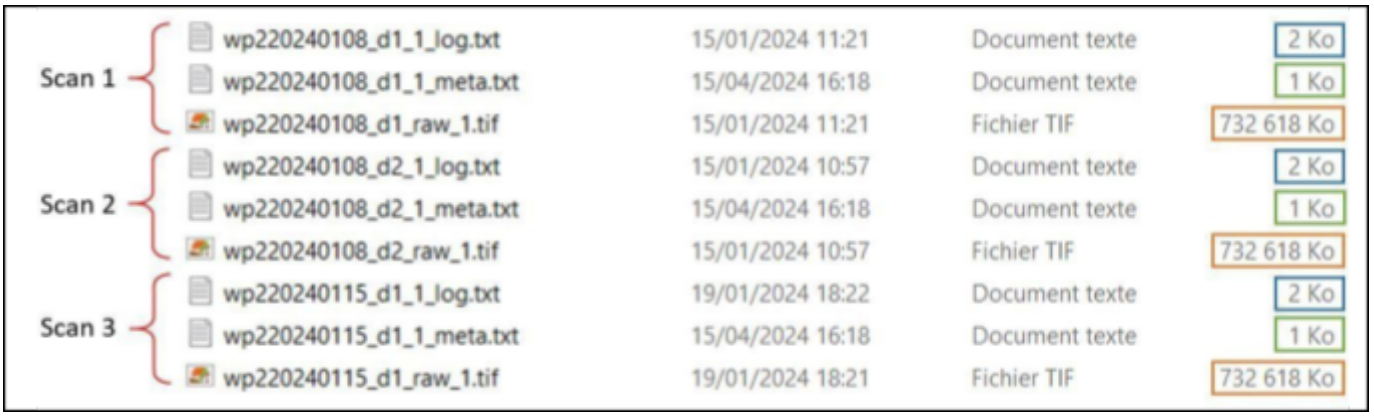
ADDITIONAL INFORMATION

- Three files will be found in the “_raw” folder of the project at the end of the scan. The filename consists of the sample name followed by the fraction name (sample_d1.*).
    - The .TIF image (16 bits) will be processed later using the “CONVERT & PROCESS scanned SAMPLES in batch mode” tool in the Zooprocess main menu (see Chapter 8).
    - The meta.txt file provides information (metadata) on the sampling method (e.g. sampling site, net dimensions, tow, volume) and on the sample preparation for the Zooscan (e.g. pre-filtering and subsampling ratio). It summarises the information contained in the sample and scan tables stored in the meta folder of the project.
    - The log.txt file records information on the scanning method (parameters).

- The scan_header.csv file records all scanning information in the Zooscan_meta folder.



To evaluate the quality of your scan, you can check the weight of the image files, which must be equal for all scans.

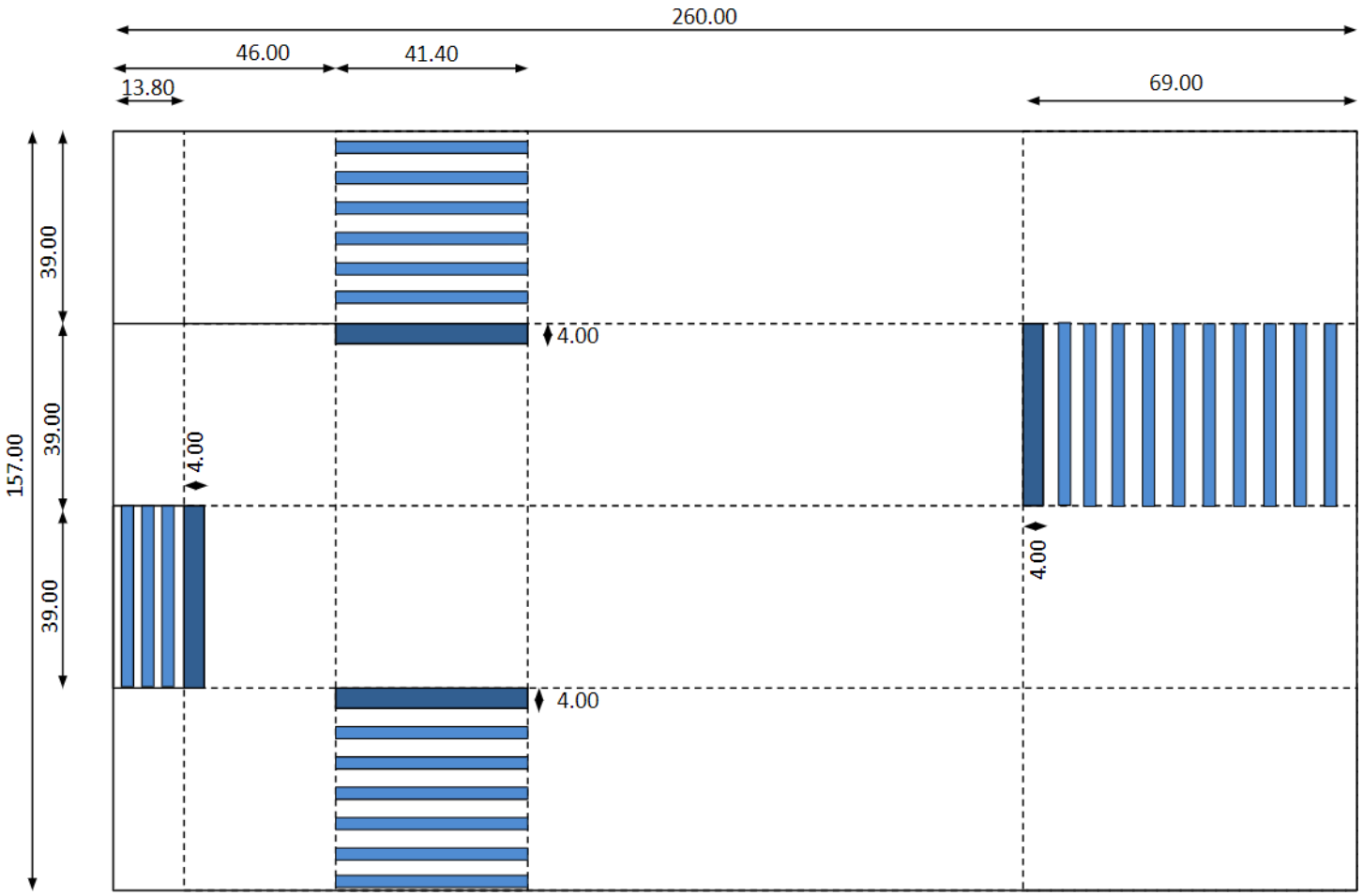


6.4 How to place objects correctly on the ZooScan

During the convert and process (see Chapter 8), there are several stages, including detection of the edges of the frame and segmentation of the objects. These two stages are presented briefly here to explain the consequences of the positioning of the objects.

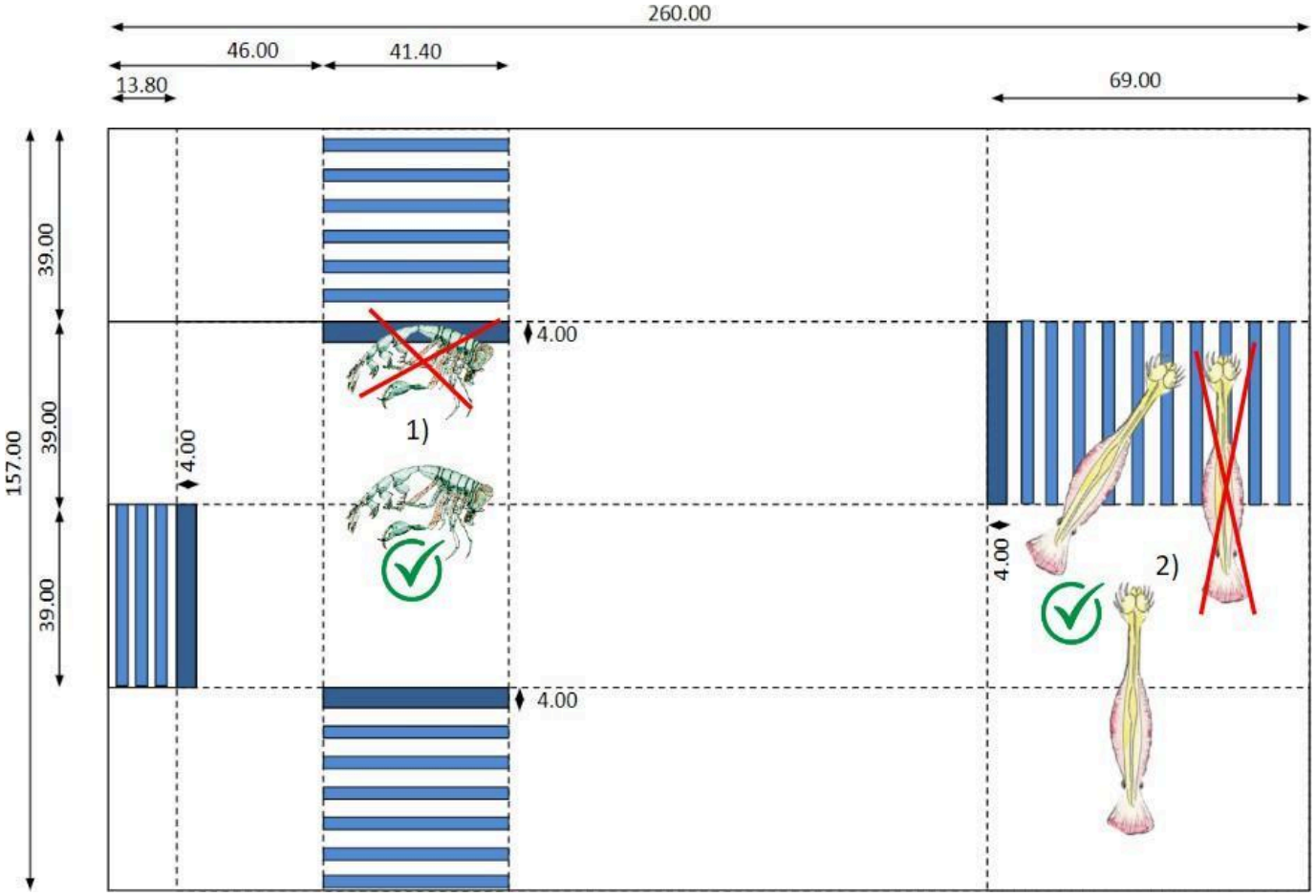
6.4.1 Frame edge detection

This processing is carried out after normalisation and before background subtraction (see Chapter 8). What Zooprocess does: in each of the 4 dark blue areas, it calculates the grey level averages which become its 4 references. It then measures the mean grey level in the smaller light blue rectangles. When the values are less than 5% of the grey level reference, Zooprocess considers that it has detected the edge of the image.



For these reasons, **you should not place:**

1) **large** organisms in dark blue areas, as this can distort detection of the limits of the frame due to a truncated reference measurement
2) **long** organisms parallel to the blue rectangles in their zone, as they may be confused with the edges of the frame

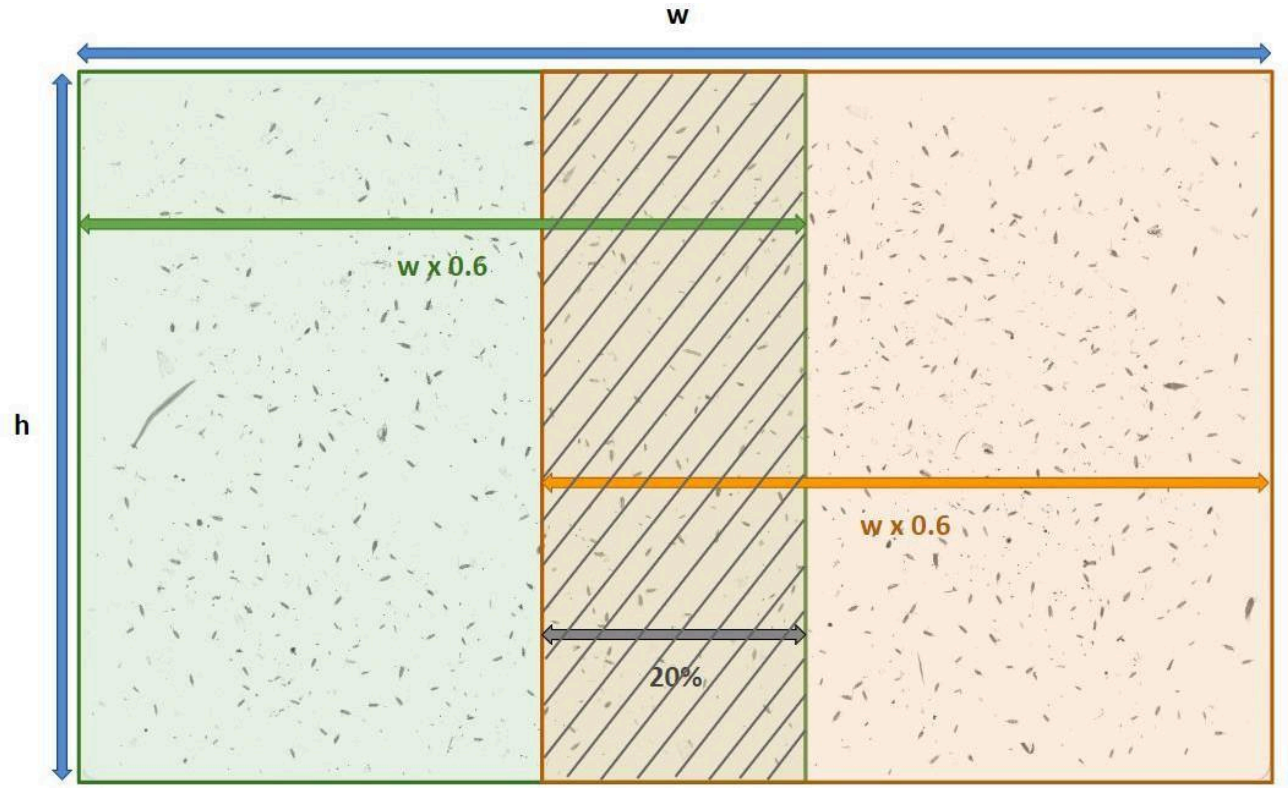


We do recommend that you print this target (Appendix 2) to scale on transparent paper to check the placement of your objects before scanning.

6.4.2 Image segmentation

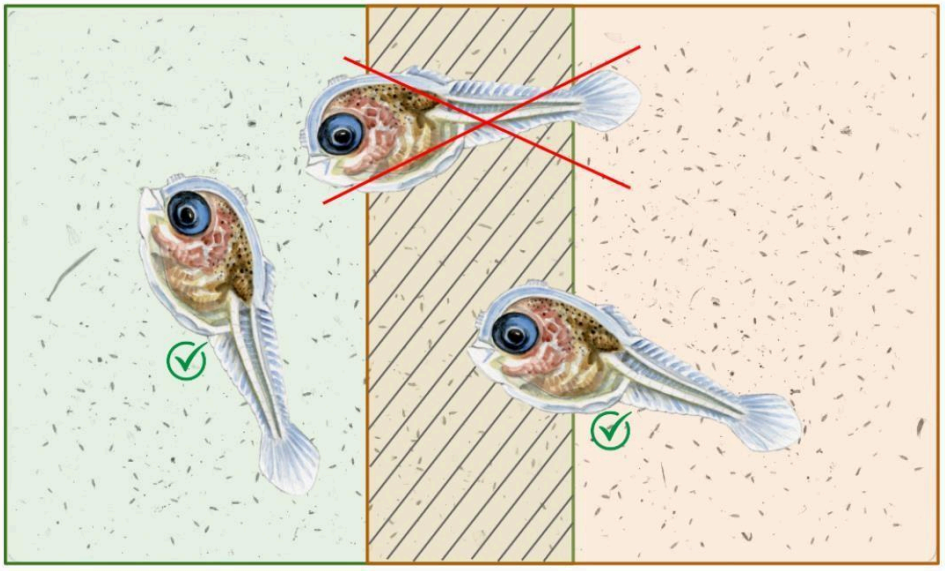
When Zooprocess was created, the computers did not have enough RAM to process the entire scan image during segmentation. A split method was therefore applied. Zooprocess considered 60% of the width (in green) and then detected the objects in this area. It then emptied the RAM and considered the other 60% on the other side of the scan (orange) and detected the objects. This means that there is an area (the hatched area: superimposition of the green and orange areas) corresponding to 20% of the scan where the objects are counted twice. Zooprocess detected the duplicated objects automatically and removed them from the analysis.

This method is still used today because we want to be able to reprocess the old scans and get exactly the same results.



The consequences are that if a large object is located through these three zones, it will not be detected as it touches the sides of both right and left images.

It is thus recommended to place the large objects **in the middle of the ZooScan halves**.



7. Recover the sample after the scan

PROCEDURE

- Remove and rinse the transparent frame with a squeeze bottle above the scanning tray to recover all specimens that may be attached to it.

- Remove the sample from the tray by gently lifting the ZooScan. Clean the tray with a squeeze bottle of fresh water or filtered seawater to prevent losing specimens and avoid contamination between the different samples.

- Prepare another sample or clean and **dry** the scanning tray using fresh water if you end a scanning session.

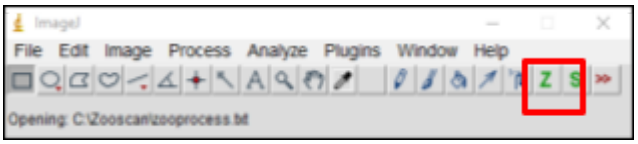
8. Convert & process scanned samples in batch mode

Image processing can be performed immediately after the scan if you use a powerful computer or it can be done during downtime such as at night or during lunch, as it takes about 10 minutes per scan.

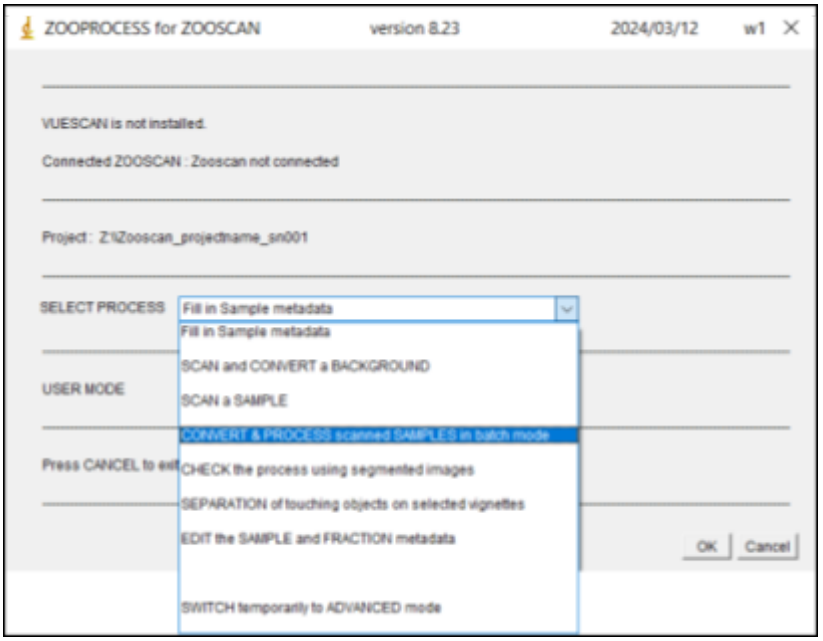
It is very important to process the scanned images as soon as possible after their acquisition or at least once a day. This process allows you to quickly check (see Chapter 9) that the background and samples have been correctly acquired, helping to avoid discovering problems later and having to rescan many of them.

PROCEDURE

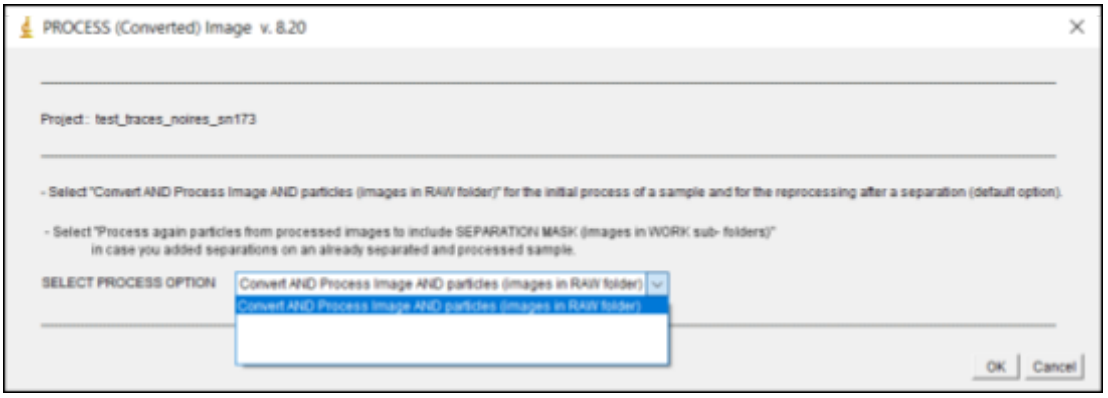
- Open ImageJ/Zooprocess or click on the Z icon if ImageJ is already running. Do not open it twice (if you do, close all ImageJ occurrences and restart it).

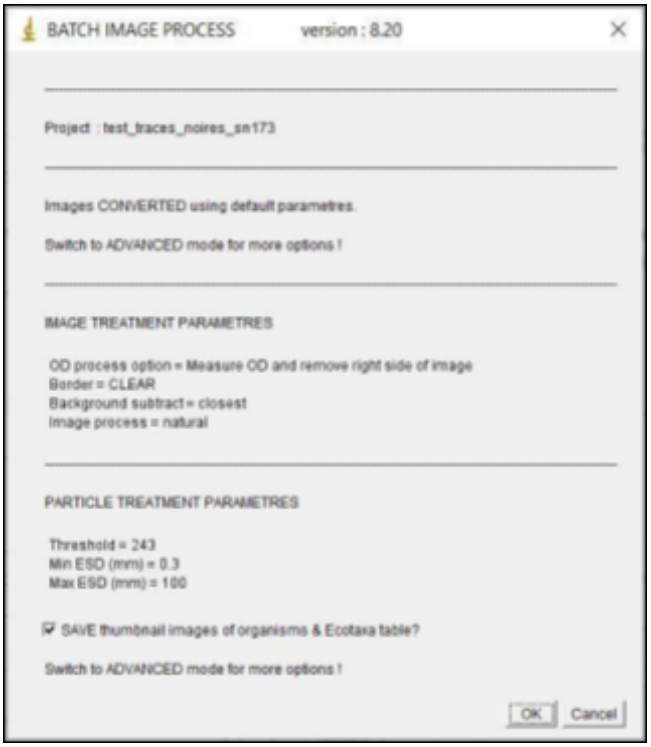


- Select your project and choose the “CONVERT & PROCESS scanned SAMPLES in batch mode” tool.

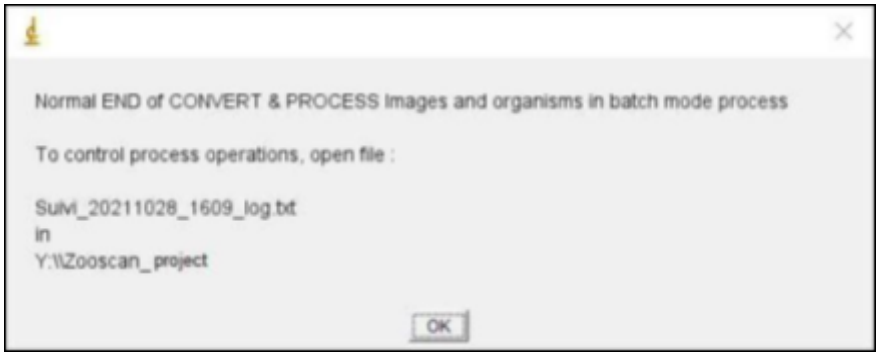


- Select “Convert AND Process Image AND particles (images in RAW folder)”. It is recommended to keep the default settings for the process: click on the “OK” button.





- At the end of the process, a Zooprocess window appears with a “Normal END…” message. Click on the “OK” button and the main menu is displayed.



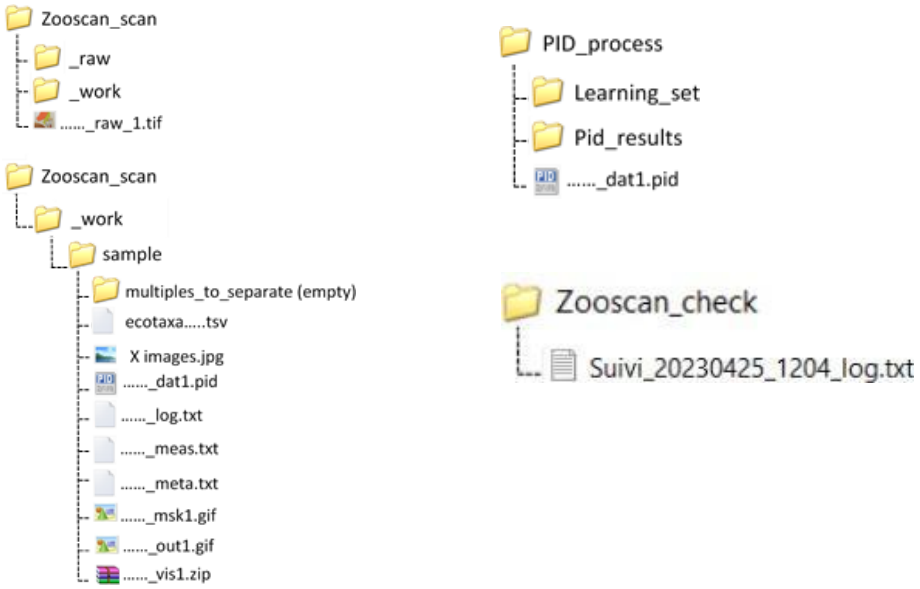
ADDITIONAL INFORMATION

- The resulting files are saved in the _work and Pid_results folders.

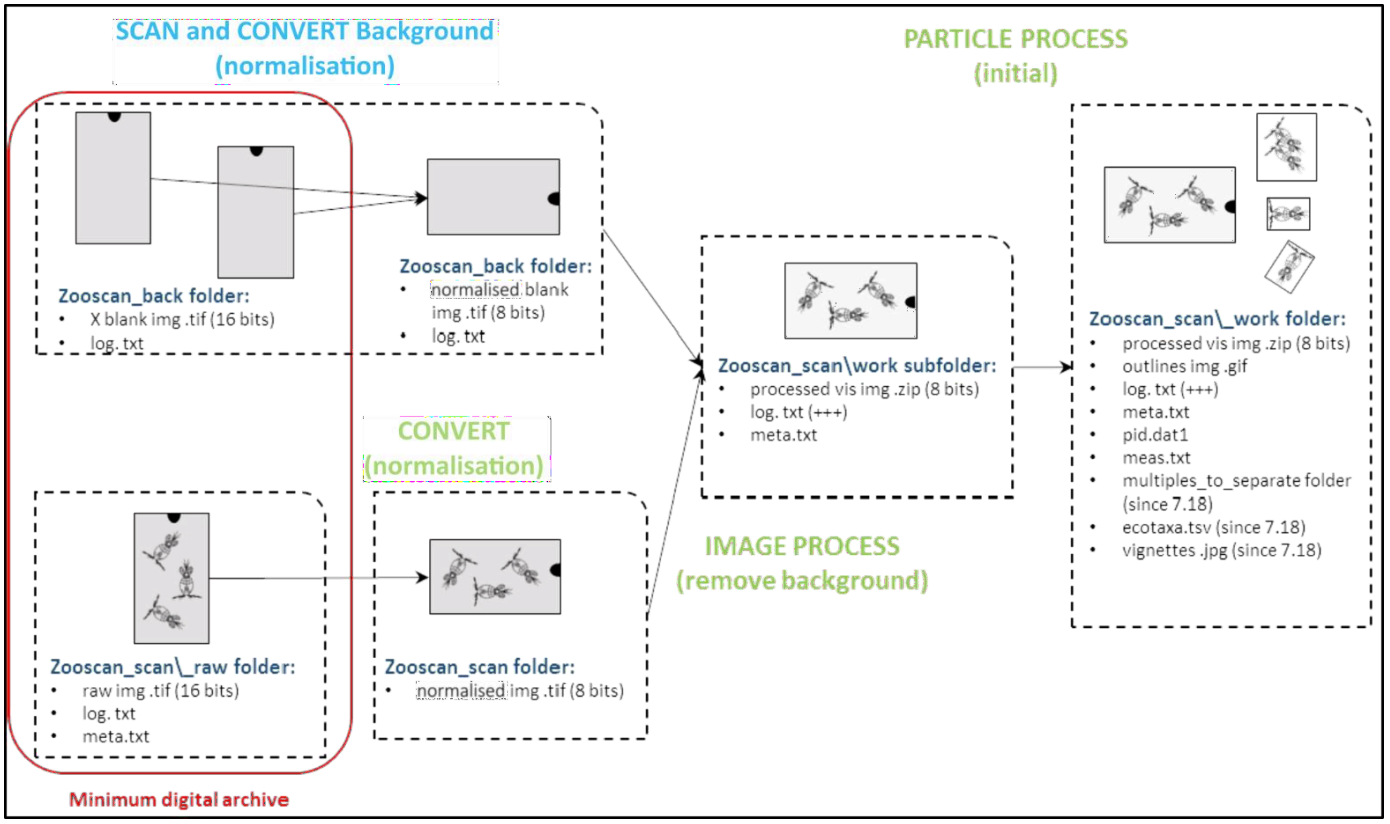
- The *.pid file is a unique file that concatenates log.txt, meta.txt and meas.txt (a table containing all objects as rows and their measurements as columns). The measurements that might be used to compute the size of the objects are Area, Major and Minor (where major and minor refer to the axes of an ellipse with the same area of the object measured). These measurements are in pixels and the pixel size documented in the *.pid file. Other measurements correspond to variables of shape and texture used for automatic recognition, as well as the position in the tray. Note that the .PID file is automatically copied to the PID_Results folder of your project.

- Since Zooprocess version 7.22, vignettes of scanned objects are extracted by default, and an “ecotaxa_*.tsv” file is created. Both the vignettes and the .TSV file are imported into the EcoTaxa (http://ecotaxa.obs-vlfr.fr/) application for predicting and classifying the organisms. The .TSV file contains all data and all useful metadata from the .PID file (see below).

- The resulting files remain compatible with the old Plankton Identifier and XnView tools but **we DO NOT recommend continuing to use them**. You can contact us for assistance with importing your existing classified samples and projects into EcoTaxa.



Description of the standard initial background and image processes



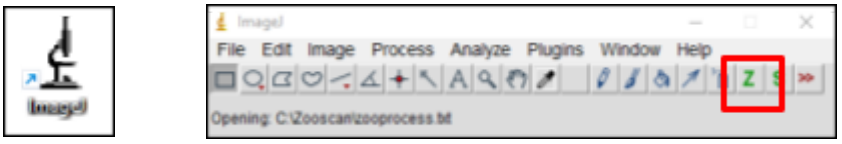
→ The operations “CONVERT”, “IMAGE PROCESS” and “PARTICLE PROCESS” are all performed by the “Convert & Process scanned samples in batch mode” tool.

9. Check the process using segmented images and verify the number of images

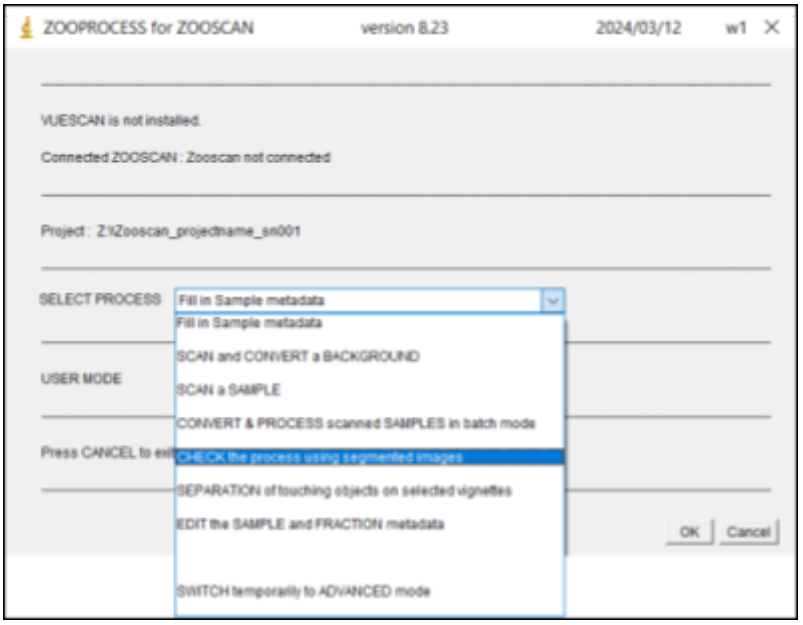
After any image processing, you **must check** your process using the dedicated checking tool: “Check process using segmented images”.

PROCEDURE

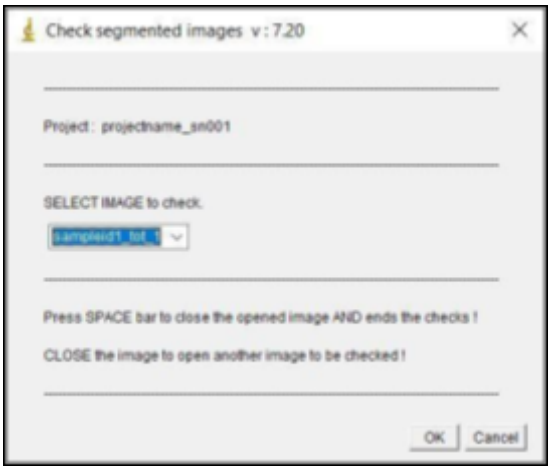
- Open ImageJ/Zooprocess or click on the Z icon if ImageJ is already running. Do not open it twice (if you do, close all ImageJ occurrences and restart it).



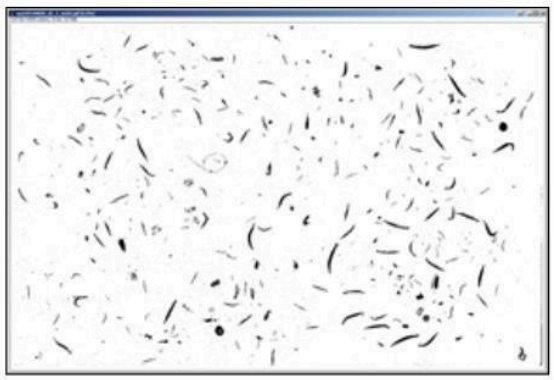
- Select your project and select "CHECK the process using segmented images".



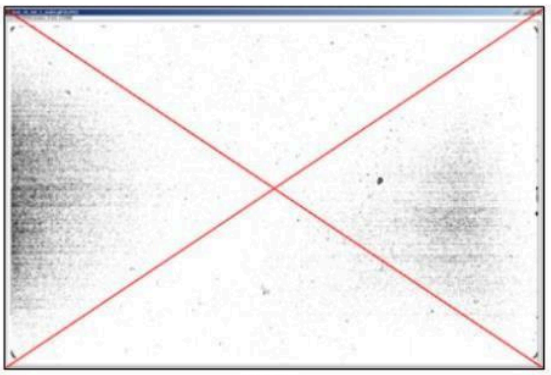
- Choose the scan you want to check.



- The opened image (“sample_msk1.gif”) shows whether the background was properly extracted from your image, (i.e. no saturated areas, with many dots). You can also evaluate the number of aggregated organisms in this image.



OK

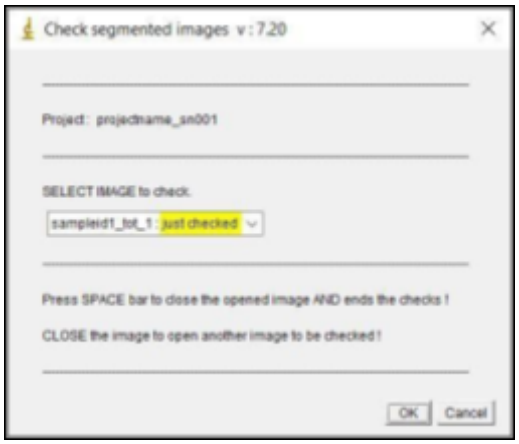


NOK

Some typical reasons for poor-quality images:

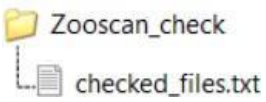
- ➔ Too many organisms
- ➔ Too many very large organisms or poorly positioned
- ➔ Poor background (e.g. dirty, condensation, insufficient water) => in this case, all samples of the same day (processed using the same background) present similar issues
- ➔ Poor sample quality (e.g. dirty, condensation, insufficient water)
- ➔ Insufficient delay between the preview and the scan of the sample.

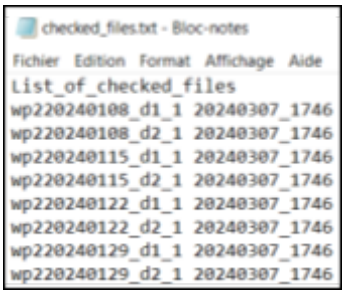
- After checking, this window appears indicating that your scan has been “just checked”. You can either close the window or choose another scan.



ADDITIONAL INFORMATION

The list of scans that have been checked can be found here
All scans must be checked.





IMPORTANT

The number of images (individual vignettes of organisms) created indicates the quality of the fraction chosen with the motoda for scanning: samples with insufficient or excessive number of splits are identified. Therefore, please check the number of images in the work folder after the process.

Here are the rules applied at the PIQv:

When 'sample_net_mesh' >= 500 and 'motoda_frac' strictly equals 1:
→ The number of .jpg images in the '_work' subdirectory must not exceed 1500.
When 'sample_net_mesh' >= 500 and 'motoda_frac' is strictly above 1:
→ The number of .jpg images in the '_work' subdirectory must be between 500 and 1500.

When 'sample_net_mesh' < 500 and 'FracID' = d1 and 'motoda_frac' strictly equals 1:
→ The number of .jpg images in the '_work' subdirectory must not exceed 1500.
When 'sample_net_mesh' < 500 and 'FracID' = d1 and 'motoda_frac' is strictly above 1:
→ The number of .jpg images in the '_work' subdirectory must be between 500 and 1500.
When 'sample_net_mesh' < 500 and 'FracID' = d1+N or 'FracID' = tot or 'FracID' = plankton and motoda_frac strictly equals 1:
→ The number of .jpg images in the '_work' subdirectory must not exceed 2500.

When 'sample_net_mesh' < 500 and 'FracID' = d1+N or 'FracID' = tot or 'FracID' = plankton and motoda_frac is strictly above 1:
→ The number of .jpg images in the '_work' subdirectory must be between 1000 and 2500.

If the number of images is too high, the **only valid** justifications that we use at the PIQv with the ZQC tool are:

- ➔ algae_soup
- ➔ borax
- ➔ sediment for muddy, sandy environments (estuaries, freshwater etc.)
- ➔ bloom of a particular species that have a tendency to aggregate (e.g. genus *Coscinodiscus*), and small transparent species (e.g. Noctilucaceae).

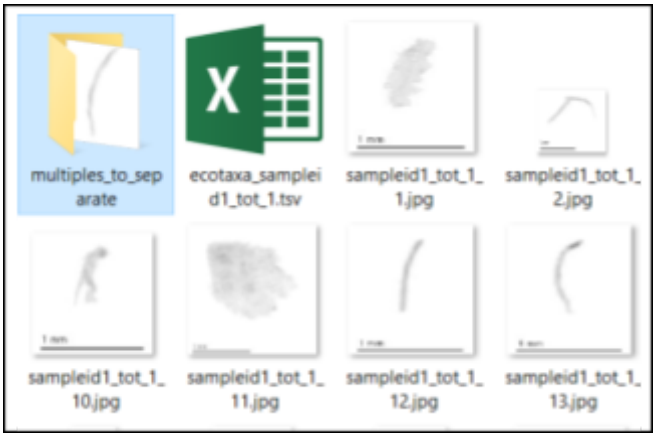
If the number of images is not correct, you need to delete the scan and redo it.

10. Sort “multiples” and separation using selected vignettes of multiples

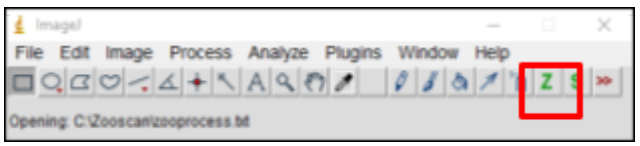
So-called “multiple” images are images containing touching objects with an organism and another organism or non-living object. These images can impact the resulting abundances and biovolumes. This is why further manual separation on the image using Zooprocess is necessary.

PROCEDURE

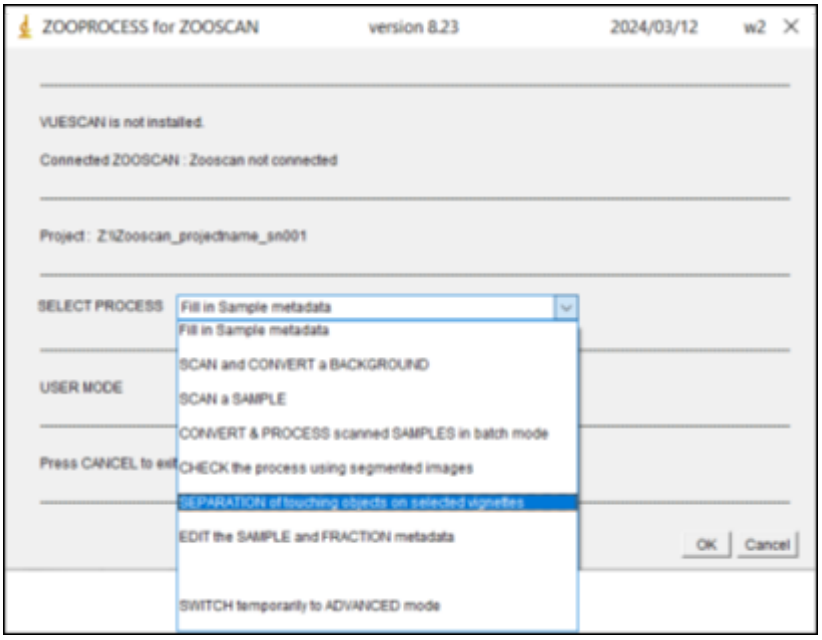
- Use File Explorer to check the sample folder (sub-folder of the _work folder) for vignettes containing touching objects and **move** them into the “multiples_to_separate” sub-folder (cut and paste).



- Open ImageJ/Zooprocess or click on the Z icon if ImageJ is already running. Do not open it twice (if you do, close all ImageJ occurrences and restart it).



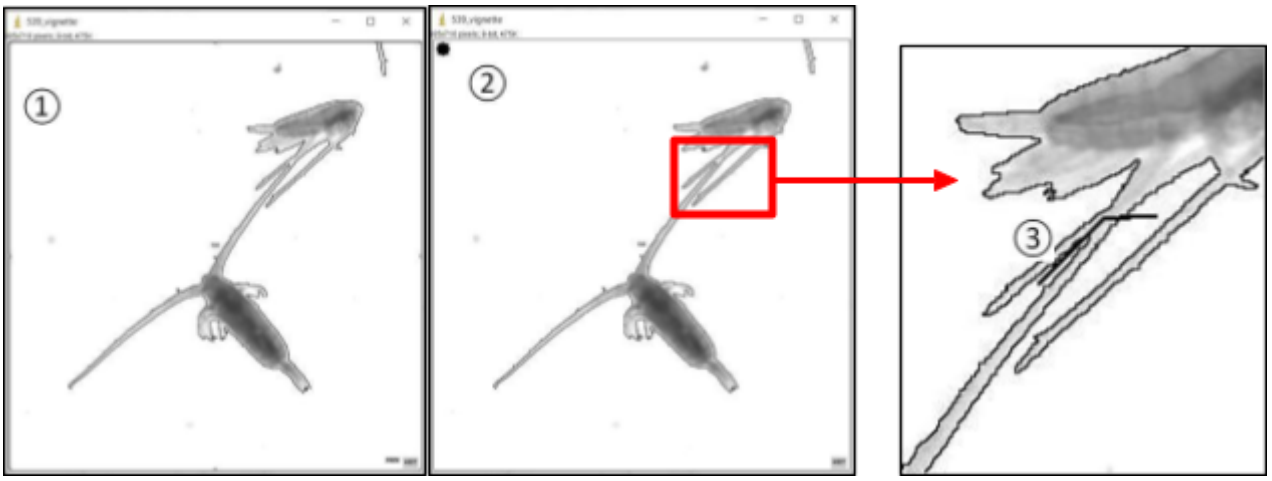
- Select your project and choose the "SEPARATION of touching objects on selected vignettes" tool.



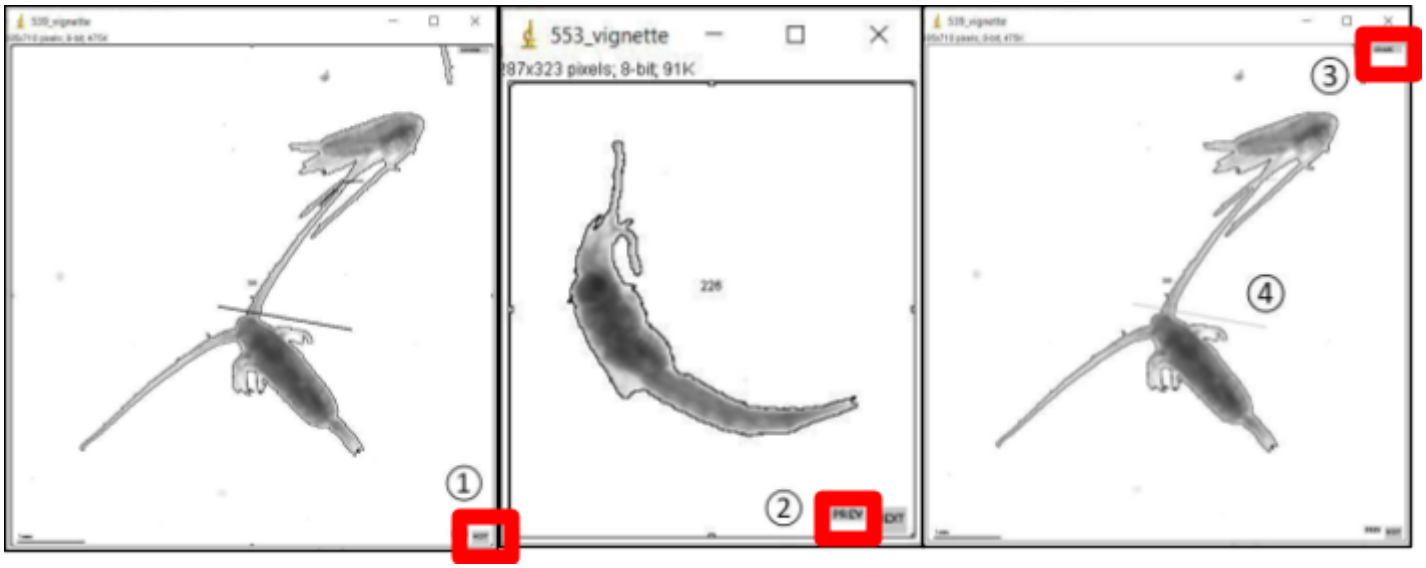
- Choose the scan you want to separate.

- Your scan and the first image to be separated appear.

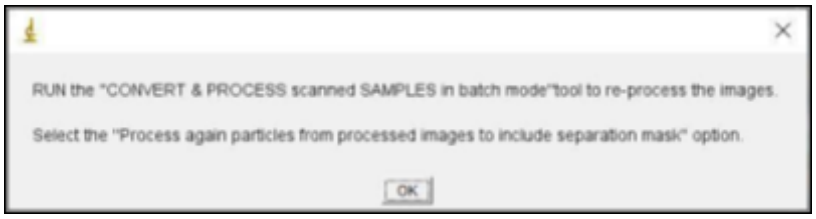
- ① With the mouse, click on the first image to be separated. ② Then, click and hold the scroll wheel of the mouse to make a black dot appear in the upper left corner of the image. ③ You can zoom in or out using the + and - keys on your keyboard. The mouse pointer turns into a cross, allowing you to create separation lines. Finally, click on the “Next” button.



- If you have made a mistake and want to delete it, ① click on the “Next” button, the next image appears, ② click on the “Preview” button, the “Erase” button appears, ③ click on the “Erase” button: ④ **all** your lines become grey as they are erased.

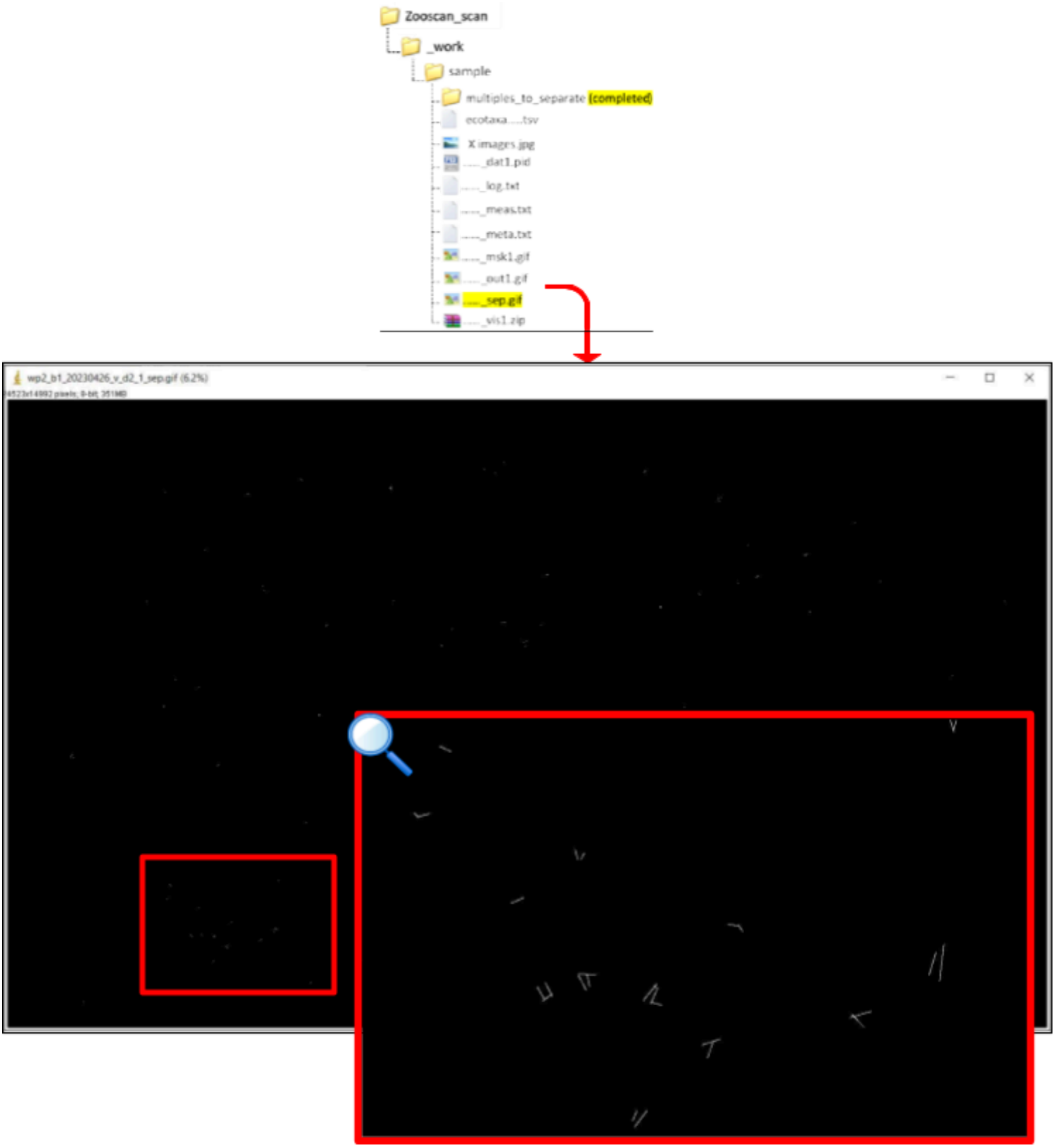


- Once the last image has been separated, these messages appear: click the “OK” button.



ADDITIONAL INFORMATION

In your project, the “multiple_to_separate” folder contains the vignettes to be separated. After the separation process is complete, you obtain a “sep.gif” file in your scan folder.



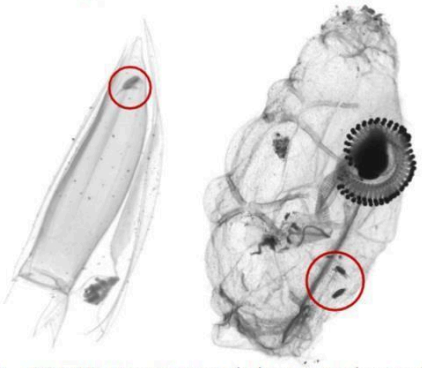
The “sep.gif” file is a separation mask that shows all the lines drawn between the objects that were touching each other in the images. This mask will be superimposed with the scanned image in the next re-processing.

ATTENTION

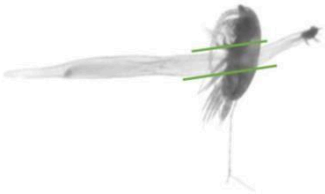
Even if there are no multiples in your scan, **place at least one image in the multiple_to_separate** folder and draw a line in the blank area when separating. This will allow the creation of a **sep.gif** and will indicate that the entire procedure for this scan has been followed.

Here are the rules applied at the PIQv:

Always favour the rarest objects

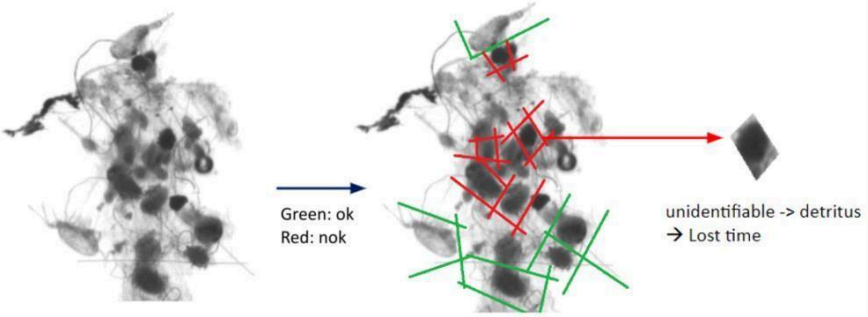


→ DO NOT separate copepods, keep nectophore and Salpida

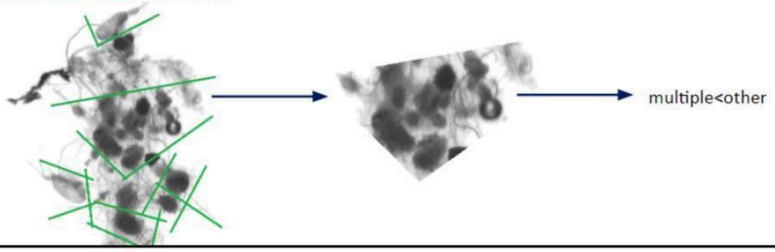


→ Separate copepod, keep Chaetognatha

DO NOT separate large masses, separate only objects at the ends



BUT : you can reduce the biovolume of your multiples to have a good proportion for the QC

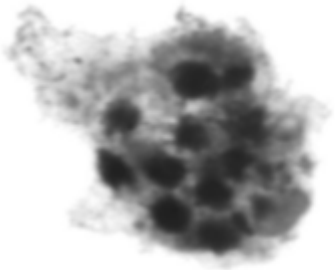


DO NOT separate tiny detritus/bubble

→ This is NOT a multiple

→ This is a multiple

DO NOT separate colony or chain



→ This is colony<Phaeodaria

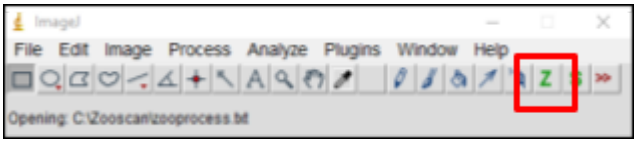
→ This is chain<Salpida

11. Re-process of the images to include the separation mask

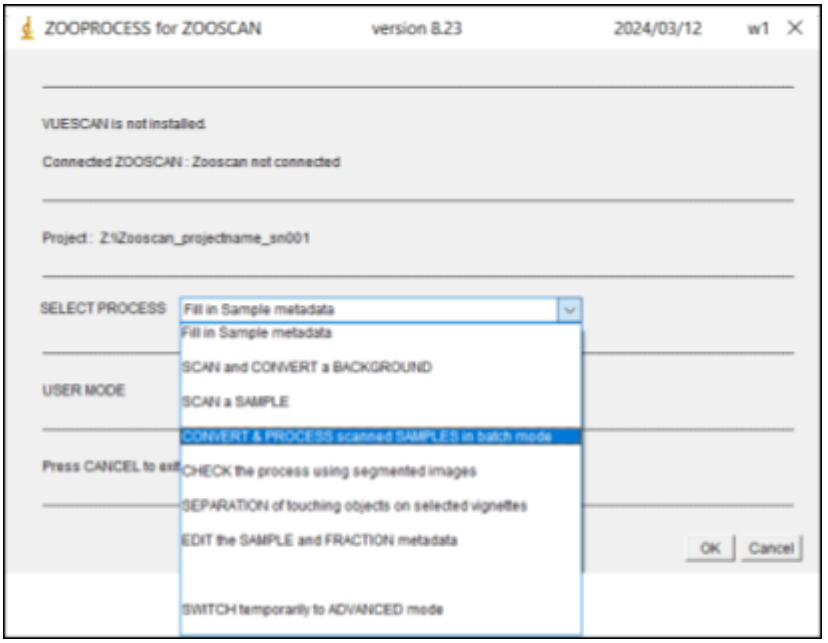
It is necessary to restart a process to include the "sep.gif" separation mask to create the new separated images and calculate their morphometric measurements.

PROCEDURE

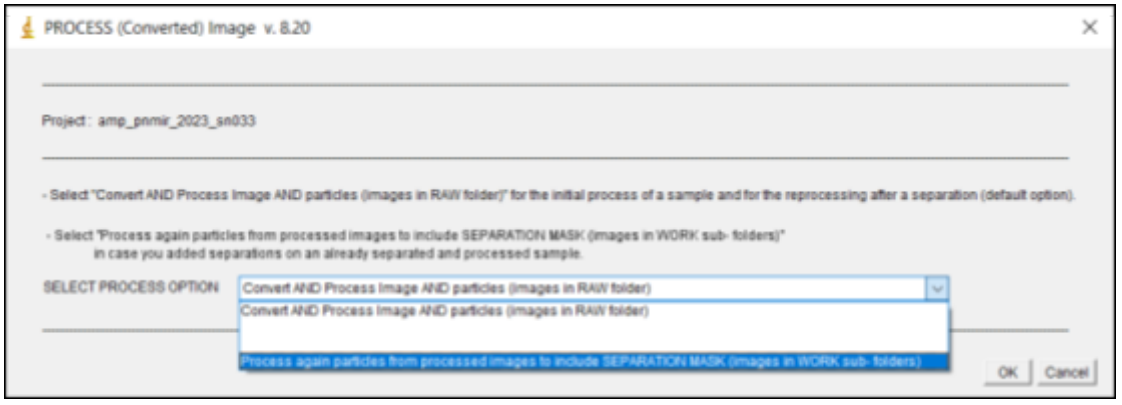
- Open ImageJ/Zooprocess or click on the Z icon if ImageJ is already running. Do not open it twice (if you do, close all ImageJ occurrences and restart it).



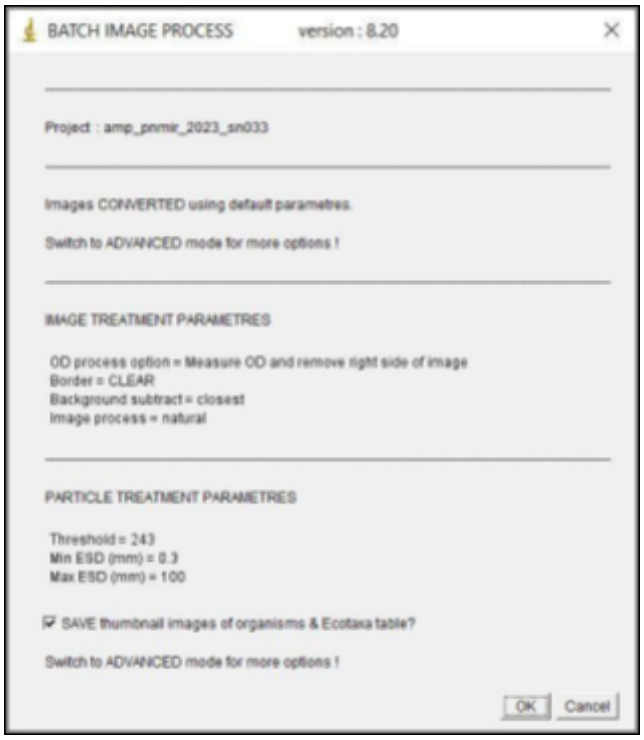
- Choose your project and select "CONVERT & PROCESS scanned SAMPLES in batch mode"



- Choose the second option “Process again particles from processed images to include SEPARATION MASK (images in WORK sub-folders)”.

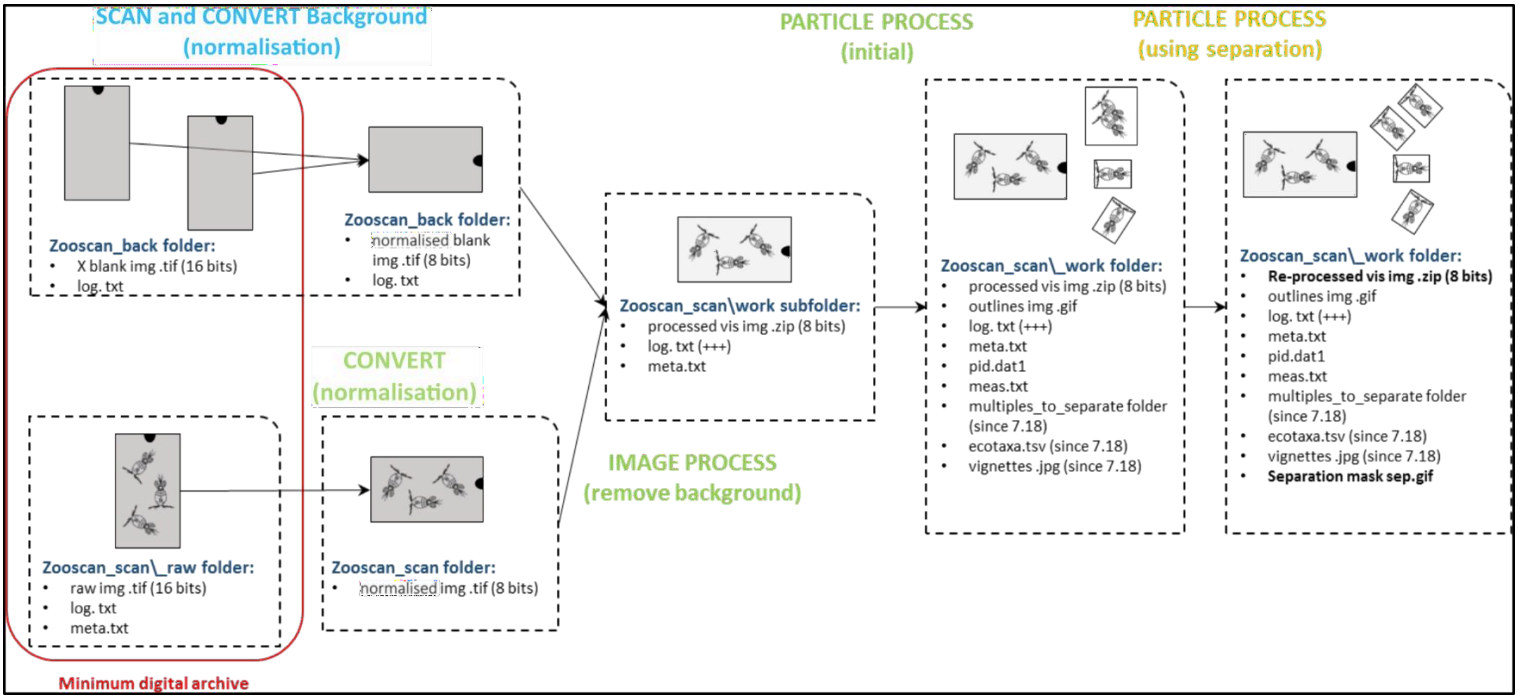


- A window appears: click the “OK” button. Once the process is complete, a Zooprocess window shows a “NORMAL END…” message. Click on the “OK” button and the main menu displays.

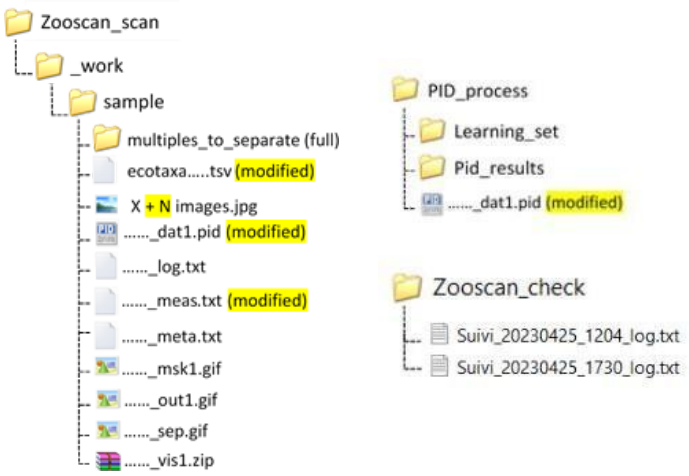


ADDITIONAL INFORMATION

The “PARTICLE PROCESS (using separation)” step allows the separation mask sep.gif to be included, new separated images to be created and their morphometric measurements to be calculated.

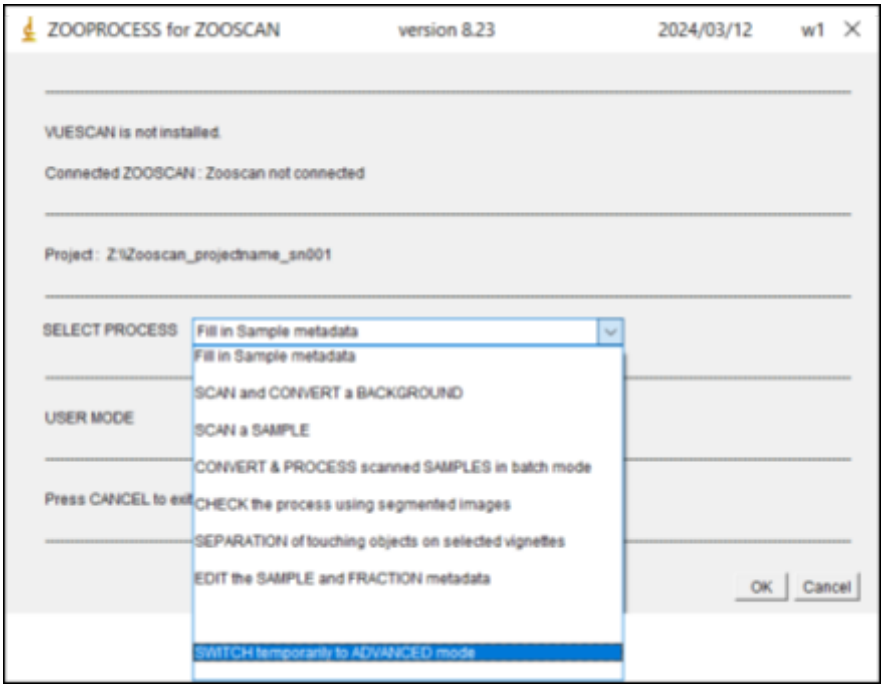


N new *.jpg images are created and all files containing information on the images are modified and updated.



12. User/Advanced modes for a project

“User mode” is set as the default when you create a project. It simplifies daily work by limiting the options the user can access and preventing most possible manual errors.
After entering a project, you can switch to “advanced mode” to access the configuration tools and all other options by selecting the “SWITCH temporarily to ADVANCED mode” tool, which is at the bottom of the options list.



13. Final Quality Control before importing into EcoTaxa

It is necessary to carefully check the sampling metadata, acquisition data and process data before importing into EcoTaxa.

Below, we present most of the criteria checked by the ZQC application available on the PIQv website.

13.1 Quality Control of sampling metadata

- GPS points (for horizontal net, ensure that the GPS start point ≠ GPS end point)
- Sampling date and time: Record the sampling date and time accurately
- Zmax and Zmin of net: ensure Zmax > Zmin
- Substrate depth: ensure Depth > Zmax
- Verify the consistency between the net type and net mesh size
- Verify the consistency between the volume filtered and the distance covered by the net
- Ensure the number of jars equals the number of barcodes.

13.2 Quality Control of acquisition data

- Weight of background must be the same for all of them (see Chapter 5)
- The number of sub-folders in work should be 3 times the number of files in raw
- Ensure consistency between the maximum and minimum sieve mesh sizes for d1/d2/tot
- Fraction motoda: follow the power of 2 rule and ensure d1 < d2, except in cases of bloom of large organisms (include a comment in such cases)
- Verify the spelling for scan_operator and submethod
- Ensure the number of images is accurate.

Reminder of the rules applied at the PIQv (Chapter 6.2)

When 'sample_net_mesh' >= 500 and 'motoda_frac' strictly equals 1:
→ The number of .jpg images in the '_work' sub-folder must not exceed 1500.
When 'sample_net_mesh' >= 500 and 'motoda_frac' is strictly above 1:
→ The number of .jpg images in the '_work' sub-folder must be between 500 and 1500.
When 'sample_net_mesh' < 500 and 'FracID' = d1 and 'motoda_frac' strictly equals 1:
→ The number of .jpg images in the '_work' sub-folder must not exceed 1500.
When 'sample_net_mesh' < 500 and 'FracID' = d1 and 'motoda_frac' is strictly above 1:
→ The number of .jpg images in the '_work' sub-folder must be between 500 and 1500.
When 'sample_net_mesh' < 500 and 'FracID' = d1+N or 'FracID' = tot or 'FracID' = plankton and motoda_frac strictly equals 1:
→ The number of .jpg images in the '_work' sub-folder must not exceed 2500.
When 'sample_net_mesh' < 500 and 'FracID' = d1+N or 'FracID' = tot or 'FracID' = plankton and motoda_frac is strictly above 1:
→ The number of .jpg images in the '_work' sub-folder must be between 1000 and 2500.

13.3 Quality Control of process data

- Ensure the weight of raw is the same for all of them (see Chapter 6.3)
- Verify that the same frame type is used for all scans
- Check whether the process was carried out after acquisition (see Chapter 8)
- Check whether the check process was carried out after processing (see Chapter 9)
- Check whether the separation was carried out
- Check whether the process after separation was carried out
- Number of images = number of lines in ecotaxa.tsv.

Appendix 1 - Example of Logsheet

Point B - RadeZoo

Operator name	.....
Date (DD/MM/YYYY)	..... / ..... / .....

Weather	sea state *(0-9B)*	wind speed *(kn)*	wind dir. *(°)* .....

Net type	**WP2**	**Juday-Bogorov**	**Régent**
Sample ID	**wp2.....**	**jb.....**	**rg.....**

Depth recorded	..... m	..... m	..... m
Time *(UTC hh:mm)*	..... :	..... :	..... : .....
Flowmeter START	.....	.....	.....
GPS	lat. ..... ° .... ' (N/S)	lat. ..... ° .... ' (N/S)	lat. ..... ° .... ' (N/S)
	lon. ..... ° .... ' (E/W)	lon. ..... ° .... ' (E/W)	lon. ..... ° .... ' (E/W)
Cable	speed *(m/s)*	speed *(m/s)*	speed *(m/s)* .....
	lenght *(m)*	lenght *(m)*	lenght *(m)* .....
	angle *(°)*	angle *(°)*	angle *(°)* .....
Flowmeter END	.....	.....	.....

Net type	WP2	Juday-Bogorov	Régent
Barcodes	* * *	* * *	* * *

* Each jar must have its own barcode, even if there are several jars for the same net (e.g. when there is too much plankton in the cod end for just one jar).

General comments

Appendix 2 – Edge detection scan zone