ZOOSCAN

MANUAL TO SCAN and PROCESS SAMPLES

Quantitative Imaging Platform of Villefranche sur Mer (PIQv)

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SUMMARY

1. IMPORTANT NOTE ON ZOOPROCESS VERSION

- This protocol applies for the Zooprocess 7.27 version and above.
- DO UPGRADE Zooprocess as often as it is updated on the zooscan.com website : http://www.zooscan.obs-vlfr.fr/
- The PIQv will assist you only if you use the latest available update of Zooprocess.

2. ZOOSCAN WORKING PROTOCOL

This working protocol might be adjusted by users according to their experience and needs in order to take advantage of the tools provided by the application. We anyhow recommend to always keep the default settings offered by Zooprocess.

The typical analysis sequence is as follow:
- Fill in Sample metadata (as many sample as useful)
- SCAN Background (on a daily base)
- SCAN sample (one or several in a raw)
- CONVERT and PROCESS samples
- CHECK process
- SEPARATION of touching objects
  - On vignettes (best option)
  - On global image, either B&W or in grey level (old option)
- PROCESS image again to include separation mask and get a better final dataset
- IMPORT in Ecotaxa to predict identification and manually validate or correct the prediction made by the application.

3. CREATE A PROJECT

A projet is a a folder where images and data of a cruise, survey or experiment will be saved.

- Open Image J. Zooprocess starts (or click on the Z icon if ImageJ is already running) and choose the option "CREATE NEW PROJECT" which is at the bottom of the project list.
- Choose the drive.
- Select the scanning option: we recommend choosing the Large frame for images at 2400 dpi resolution. The Narrow frame for images at 4800 dpi resolution can be utilized only if you run a ZooScan V3 or V4 with a Windows 7 pro, 8 or 10 64 bits computer. It does not provide much better resolution and limit the scanning area and the number of organisms in the image.
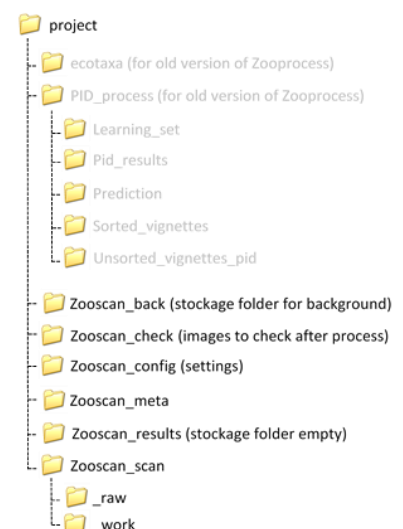
A folder will be created on the selected drive and all files from this project will later be saved in the specific subfolders.

4. Fill in Sample metadata

This operation is no longer connected with the scanning of the samples. You can fill in metadata in advance for several samples. You can even do it on the field and then move your project to the computer which is connected to the Zooscan.

At this point you should have carefully considered the naming convention of your samples! As it not possible to change the name of your sample afterward. And you should carefully check the format of several metadata such as Latitude and Longitude (see the metadata windows)!

Do not use the "d1", "d2" or "tot" extensions in the sample names. These extends will automatically be added to the scan names at the time of the scan (see below).

An excel table named :
"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 you have filled in.
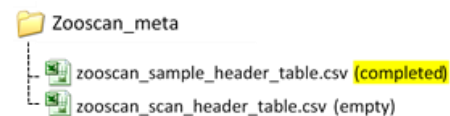
5. EDIT and MODIFY metadata

You can modify metadata (except the sample name) any time between all the steps clicking the option "EDIT and MODIFY metadata".

You can EDIT metadata at any time to correct possible mistakes or complete information on the samples or the scans. Pay attention at these metadata. They will be essential for the later analysis of the results.

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

6. SCAN (CONVERT) Background Image

The background image is a "blank" image that will be used during the image analysis process. It should be made before the samples are scanned and using the same parameters than for the scan of the samples. You have to scan a minimum of two background images (just follow the steps!) that will be combined in a unique "blank" image. You will do it at the beginning of every scanning session when you turn on your ZooScan, so usually every morning before the first sample.

The two background images will be processed into a unique background image and automatically saved in "Zooscan_back" folder.

1. Turn on the ZooScan (biotom, v1 and v2: power button on grey box; v3 or v4: power button behind the instrument), manipulate the ZooScan gently and maintain it (e.g., always hold the tray when you lift it to remove the water or it could fall).

2. Clean and rinse the scan tray and the cover glass using freshwater to remove the dirtiness.
3. Eliminate marks on the glass and the frame and check from time to time if the glass of the ZooScan cover has no mark.
4. Pour some clean freshwater (stored at room temperature) to cover the tray.
5. Place the frame that was defined for your project (NARROW/LARGE). Control that the frame is well placed on the recommended and tagged corner of the scanning tray *(the area of scan is set up to include the frame borders)*. For biotom, v1 and v2 instruments there is a mark on the frame for his placement on the tray. Check the manuel delivered with the ZooScan.
6. Fill the tray with freshwater until the edge of the frame is covered. Check that there is no dust or bubble below the OD (dark circle) position and that the tray and the water are clean : remove dirtiness and bubbles using a plastic pipette or a cactus stick provided with the instrument to avoid scratching the glass tray.

Figure 1 : Filling the Zooscan tray

7. BIOTOM, V1 and V2 models: DON'T FORGET to turn on the light (green button or lever button) just before scanning. V3 and V4 models: Check that the light rotating switch (left side of the Zooscan) is on UPPER position.
8. Click on "SCAN (CONVERT) Background Image" tool. Follow the instructions that pop up on your computer screen. Wait the recommended time (30 sec.) between the preview, scan n°1 and scan n°2 (cf. the instructions window on your computer screen). Don't forget to press OK in the instructions window before the second scan.
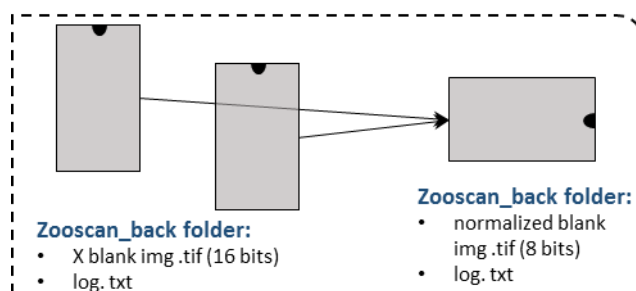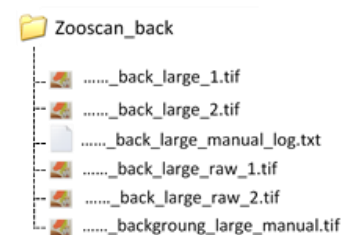

Figure 2 : Process of background images

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7. Sample preparation and scan

7.1 *Sample preparation*

Please do consider that the FORMALIN is a carcinogen and mutagen product. Apply all necessary safety procedure to remove all risks when you remove the formalin from the sample:
- **work in a extractor hood**
- **use gloves, glasses and lab coat**

For safety reasons, we never poor directly the samples containing formalin on the Zooscan tray but we previously remove the seawater and formalin from the sample and use filtered seawater (or fresh water) instead.

We describe below the general procedure in use at the Plateforme d'Imagerie Quantitative de Villefranche sur Mer for 200µm mesh size nets.
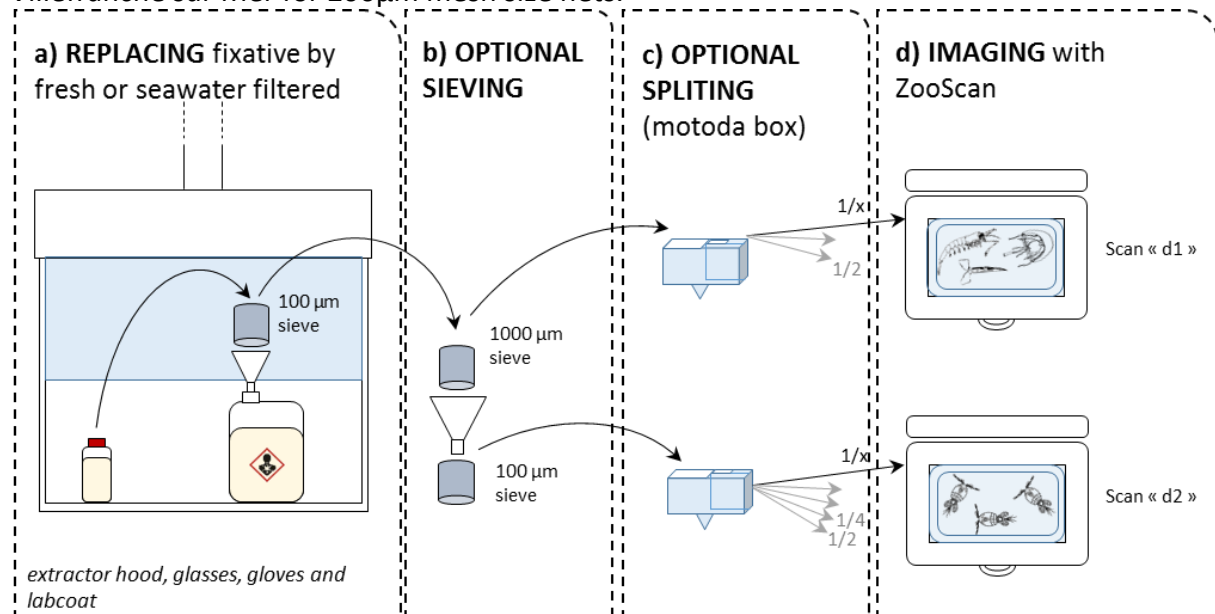

Figure 3: Procedure to prepare a sample collected using a WP2 net (200µm) : a) removal of the seawater and formalin, b) the sample is optionally sieved in two fractions, c) each of them is optionalloy splited in order to get convenient numbers of organisms and d) scan of both fractions ("d1" and "d2")

The sample is sieved under the extractor hood to remove the preservative and the sea water. You can keep it to re-fill the sample afterwards.

In order to prevent the underestimation of large and rare organisms and depending on the mesh size of the net utilized to collect the sample, the sample is then sieved through a 1000 µm mesh and 100 µm mesh to get 2 size fractions.

The two fractions are then splited before scanning to obtain a proper number of organisms: a large one (>1000 µm) and a small one (100µm-1000µm).

NOTE:
- You can control that it is usefull to sieve the sample in two size fractions by comparing the splitting ratio necessary to obtain a good image or a correct quantity of organisms for the two fractions. **This ratio must be higher for the lower fraction.**

- By experience we scan 500-1000 organisms for the large fraction and 1000-1500 organisms for the the lower fraction. These numbers can be adapted according to user experience and care. The number of touching organisms must be as limited as possible to avoid spending much time to later separate them from the images.
- You can decide to split the whole sample instead of using two size fractions (net mesh size > 500µm for example).
- In order to avoid mistakes during the splitting procedure using the Motoda box, we recommend to keep all successive remaining fractions in pre-labelled jars (1/2, 1/4, 1/8, 1/16….).

## 7.2 *STEP BY STEP SCANNING PROCEDURE*

NOTES:
- The METADATA must be previously filled (see above)
- The BACKGROUND must be scanned (see above)

1. Pour some water (freshwater or filtered seawater) on the scanning tray until the glass is totally covered (don't cover the edges of the frame at this step as the addition of the sample will then cover the edge). In order to prevent condensation and bubbles to appear on the ZooScan tray (because of the differents temperatures between the working room and the water in the pipes), we recommend to anticipate your water usage and store few liters of water in the same room of the instrument.
2. Place the frame. The frame size (NARROW or LARGE) depends on your choices at step 2 "Create new project"). Check that the frame is well placed, touching the recommended and tagged corners of the scanning tray as for the scan of the background (see above).
3. Clean water droplets or marks on the frame and remove the dirtiness in you water with pipette and the cactus sticks.
4. Pour the sample from the last fraction of the Motoda box.
5. Add water (if necessary) until all the edges of the frame are covered as for the background. If you pour too much water, the floating organisms will be out of focus. You thus have to struggle betwenn : enough water to cover the edges and an insuficiant level to avoid out of focus organisms.
6. Allow 5-15 minutes to separate touching organisms with the cactus sticks. No organisms on the edge and along the edge! Pay attention to the separation of the objects from each other. Make a compromise between the time spent for this task and the quality of the image. After the process of the image, you can separate touching objects on the final image with the separation tool (see below) in Zooprocess but you may loose 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 pouring the extra water on conglomerated organisms of the tray to "dissolve" them.
    - **Place the larger individuals in the center** of the tray because the image will be cropped on the edges of the frame. If some organisms are floating, try to sink them by little pushes with the cactus sticks (the size measurements of floating organisms are biased, and their image captions are blurred). If they do not sink

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and are very few, the best option is to move them apart of the image (e.g. placing them on the edge of the frame). This step is critical to have good data quality.

7. Check that there is no condensation on the glass of the ZooScan cover.
8. Launch Zooprocess, select your project and click on the option: "SCAN sample with Zooscan (for archive, no process)".
9. Select the sample name. Then, follow the instructions that pop up on the screen (Zooprocess Instructions window). In Zooprocess, select the proper fraction name "d1", "d2", "tot" or user defined according to the fraction (100µm<"d2"<1000µm, d1 > 1000µm, "tot" if no sieving) when asked.
10. **Follow carefully the instructions in the Zooprocess windows to start the scan of the sample (one scan only). Do not forget to press OK in Zooprocess after pressing SCAN in Vuescan. Do not touch any key of the keyboard during the scan ! Do not make any vibration on the Zooscan that may perturbate the water surface on the tray!** Biotom, v1 and v2 Zooscan versions : do not forget to turn on the green light of the Zooscan before launching the scan in Vuescan. WAIT 30 seconds between the preview and the actual scan. v3: select the UPPER light!

ADDITIONNAL INFORMATION:

- *Three files will be created in the "_raw" folder of the project. The filename is composed of the sample name plus the fraction name (sample_d1.*)*
    - o *TIF image (16 bits) will be processed later when using "CONVERT & PROCESS Images and organisms in batch mode" option in the Zooprocess main menu.*

    - o *META file gives information 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 summarizes the information contained in the sample and scan tables from the meta folder of the project.*
    - o *LOG file records information on the scanning method (parameters).*
- *An additional scan_header.csv file records all scanning information in the Zooscan_meta folder*
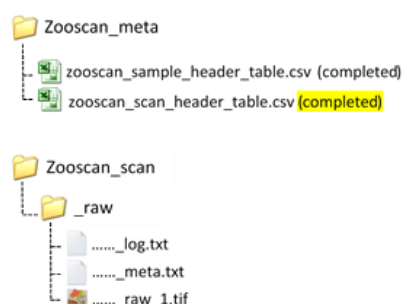
8. RECOVER sample

1. Remove and rinse the transparent frame with a squeeze bottle above the scanning tray to recover all specimens that may be stuck on it.
2. Remove the sample from the tray: lift the ZooScan gently and maintain it (e.g., always hold the tray when you lift it to remove the water or it could fall).
3. Clean the tray with a squeeze bottle of freshwater or filtered seawater to avoid contamination between the differents samples.

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

9. CONVERT & PROCESS Images and organisms in batch mode

The image process can be performed just after the scan if you use a powerful computer or during night or lunch time.

It is very important to process the scanned images as soon as possible after their acquisition or at least every day. The process permits to rapidly check (see below) that the background and the samples have been correctly acquired.

1. Start Zooprocess if necessary, click once on the Z icon and select the tool "CONVERT & PROCESS Images and organisms in batch mode" and then "Convert AND Process Image AND particles (images in RAW folder)". It is recommended to keep the default settings for the process.
2. At the end of the process, a Zooprocess window appears with a "NORMAL END…" message.
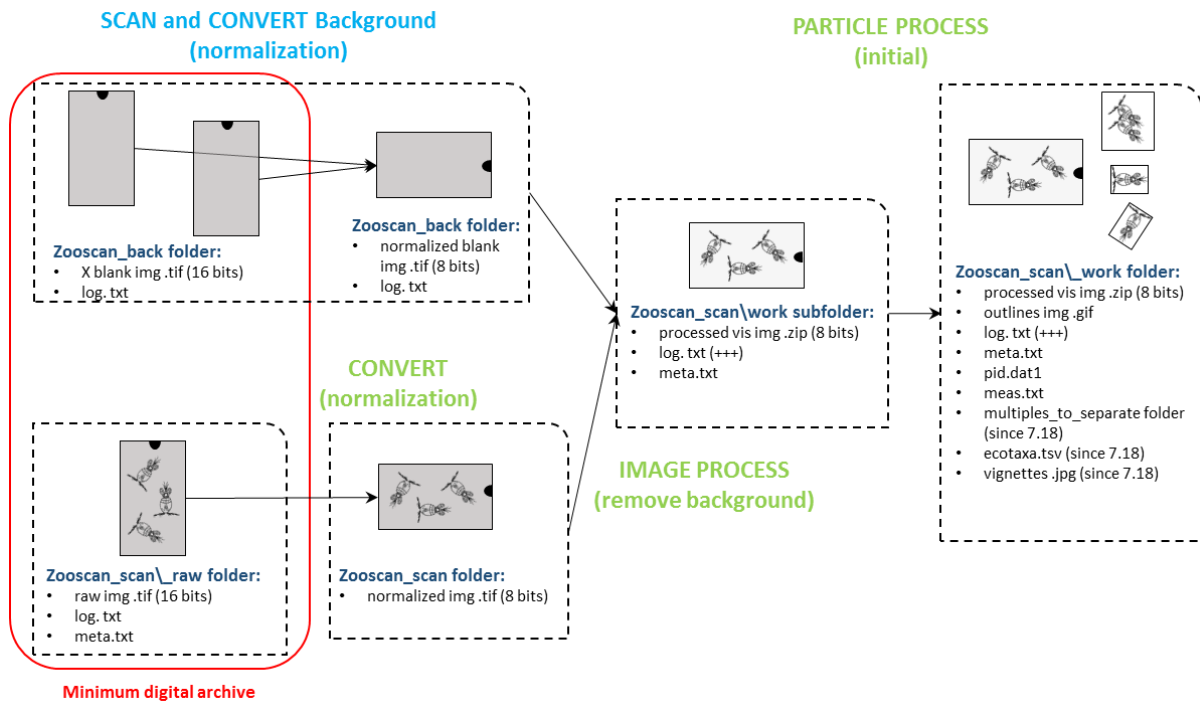
FOR YOUR INFORMATION :

Figure 4 : Description of the standard initial background and image processes. The operations CONVERT, IMAGE PROCESS and PARTICLE PROCESS are all included in the "Convert AND Process Image AND particles (images in RAW folder) » tool.

- *The resulting files are saved in the _work and Pid_results folders.*
- *The .pid file is a single file that concatenates the log.txt, the meta.txt, the processing functions applied and the meas.txt (table containing all objects (rows) and their measurements (columns)). The measurements that might be utilized to compute the size for the data analysis are Area, Major and Minor (major and minor axes of an ellipse that have the same area of the object measured). Other measurements correspond to variables of shape and texture used for automatic recognition, and of position in the*

tray. Note that the PID file is automatically copied in the PID_Results folder of your project.

- *Since the Zooprocess 7.22 version: vignettes of scanned objects are extracted by default and an "ecotaxa_*.tsv" file is created. They are both imported into the ECOTAXA (http://ecotaxa.obs-vlfr.fr/) application to predict and classify 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 to continue to use them. You can contact us in order to get assistance to move your already classified samples/projects into ECOTAXA.*
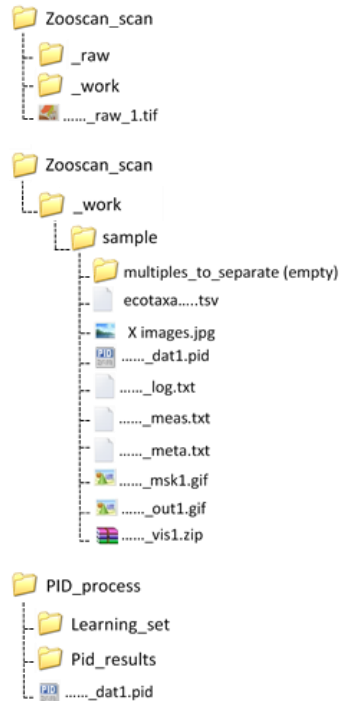
10. CHECK process by viewing segmented images

After any image process, you must check that the background was well subtracted from the sample image using the dedicated checking tool: "CHECK process by viewing segmented images". The opened image ("sample_msk1.gif") shows if the background was properly extracted from your image, (i.e., no saturated areas, with many dots). You can also check on this image the degree of aggregated organisms.
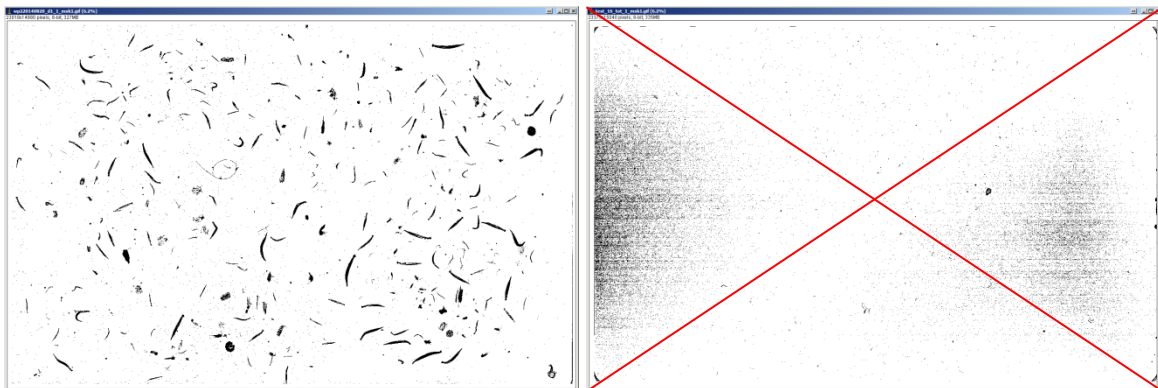

*Figure 5 : Specimen of image "masks" : correct and incorrect*

Some basic reasons for bad quality images can be :
- Too many organisms
- Too many very large organisms or badly positionned
- Bad background (dirty, condensation, insufisant quantity of water) => all samples of the same day (processed using the same background) present similar defaults
- Bad sample (dirty, condensation, insufisant quantity of water)
- Insufficient delay between the Preview of the sample and its scan

11. CHECKING "Multiples" and SEPARATION using selected vignettes of multiples

If you are not satisfied with the manual separation that you performed on the scanning tray (too many objects touching in the image) and you notice that too many vignettes contains touching objects, you should perform an additional separation on the vignettes.

1. Use XnView or any image browser application to check the sample folder (sub folder of the _work folder) for vignettes containing touching objects and move (cut or copy) them into the "multiples_to_separate" subfolder.
2. Use the tool "SEPARATION using selected vignettes of multiples" to separate the touching organisms by drawing lines between them. The latest version of the tool (Zooprocess 7.25) permits to draw many lines on a single vignette in a batch.
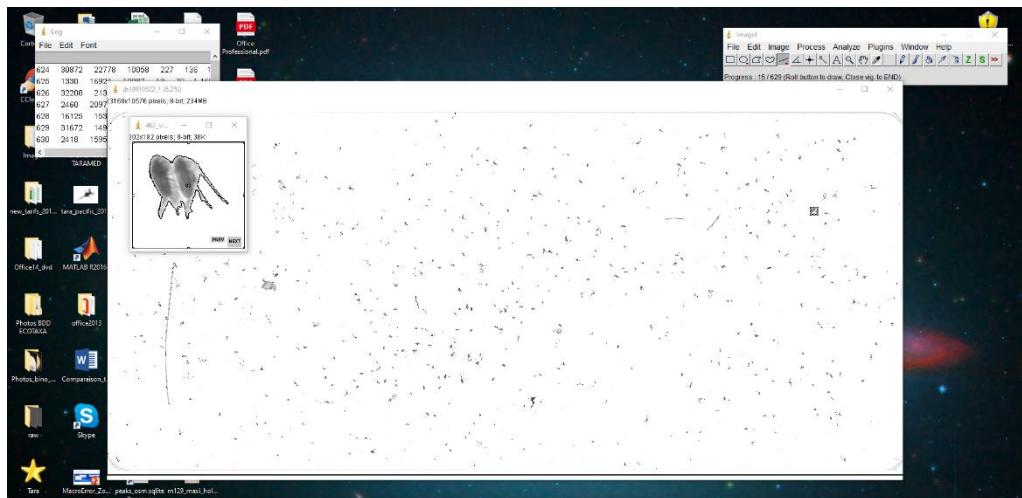
With the mouse, click on the first image to be separated ①. Then, click for a moment on the scroll of the mouse to make a black dot appear in the upper left corner of the thumbnail ②. The mouse turns into a cross that will allow you to create separation lines ③. Then click on the "Next" button.
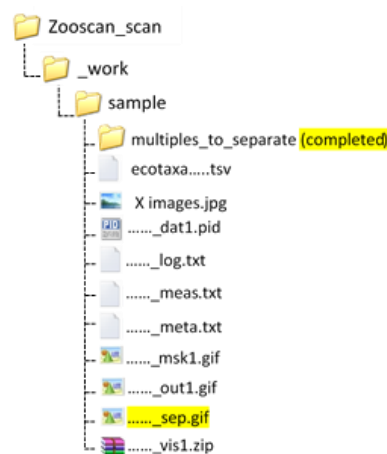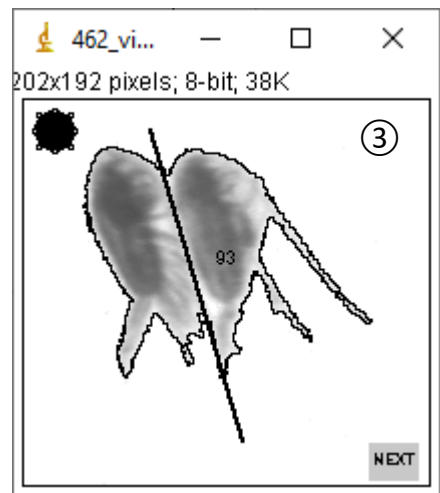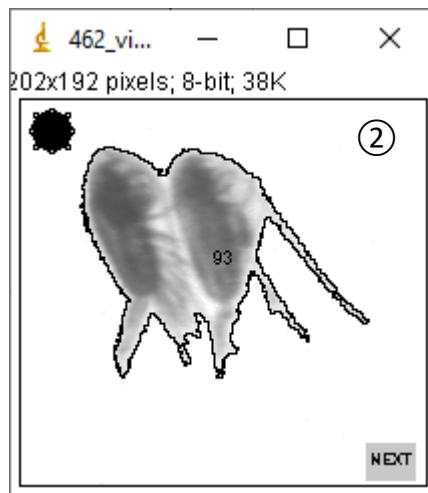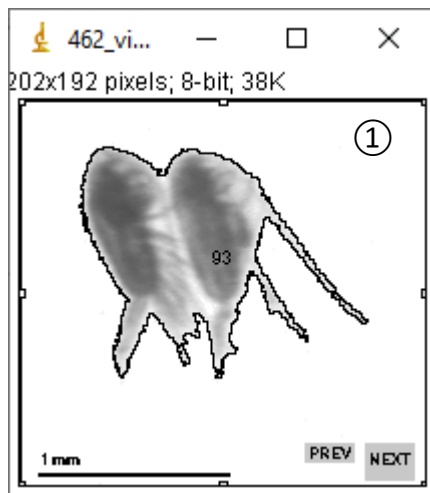
12.RE-PROCESS after separation

You must redo the "CONVERT & PROCESS Images and organisms in batch mode" and select "Process again particles from processed images to include SEPARATION MASK (images in WORK sub-folders)" of samples to take benefit of the separation and get results for the resulting objects. At the end of the sample processing a zooprocess window appears with "NORMAL END".
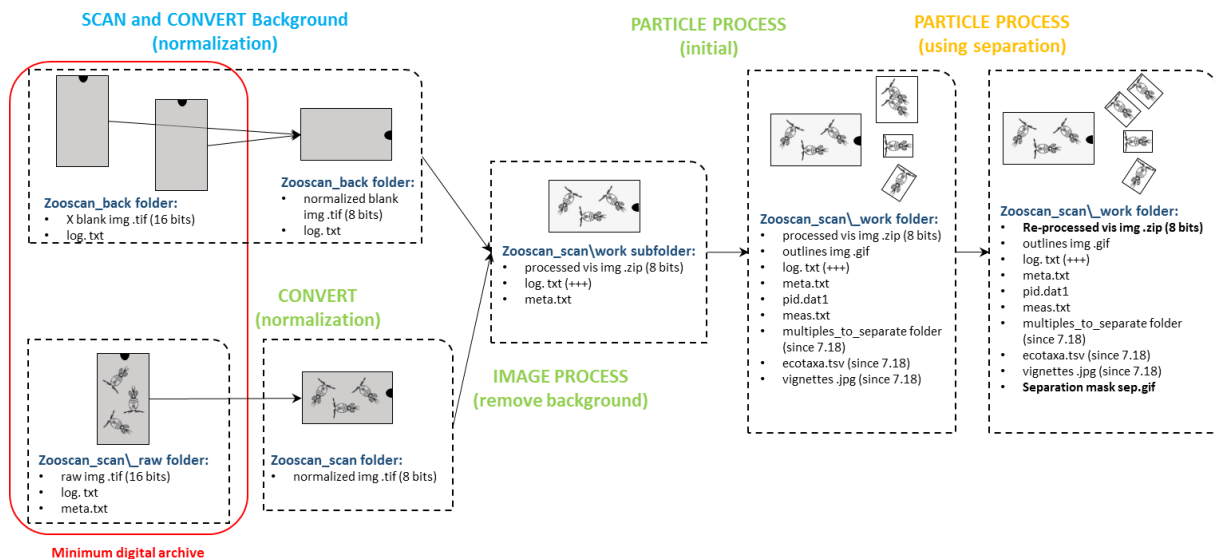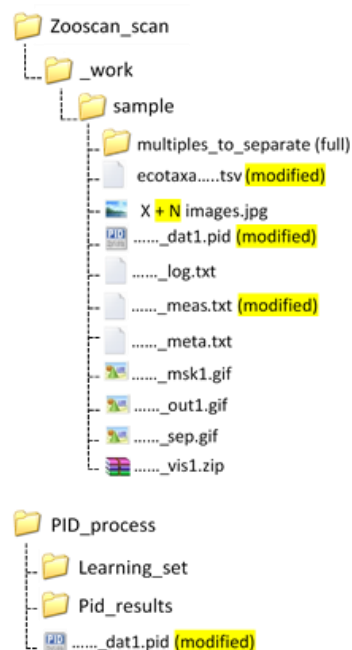
SCAN and CONVERT Background
(normalization)

Zooscan_back folder:
- X blank img .tif (16 bits)
- log. txt

Zooscan_back folder:
- normalized blank img .tif (8 bits)
- log. txt

CONVERT
(normalization)

Zooscan_scan_raw folder:
- raw img .tif (16 bits)
- log. txt
- meta.txt

Zooscan_scan folder:
- normalized img .tif (8 bits)

IMAGE PROCESS
(remove background)

Minimum digital archive

Zooscan_scan\work subfolder:
- processed vis img .zip (8 bits)
- log. txt (+++)
- meta.txt

PARTICLE PROCESS
(initial)

Zooscan_scan_work folder:
- processed vis img .zip (8 bits)
- outlines img .gif
- log. txt (+++)
- meta.txt
- pid.dat1
- meas.txt
- multiples_to_separate folder (since 7.18)
- ecotaxa.tsv (since 7.18)
- vignettes .jpg (since 7.18)

PARTICLE PROCESS
(using separation)

Zooscan_scan_work folder:
- **Re-processed vis img .zip (8 bits)**
- outlines img .gif
- log. txt (+++)
- meta.txt
- pid.dat1
- meas.txt
- multiples_to_separate folder (since 7.18)
- ecotaxa.tsv (since 7.18)
- vignettes .jpg (since 7.18)
- **Separation mask sep.gif**

Figure 6 : Standard processes plus additional separation of touching objects

13.USER/ADVANCED modes for a project

The USER mode is set as default when you create a project. It simplifies the daily work limiting the options the user can access, and preventing most of possible manual errors.
After entering a project, you can switch to the ADVANCED mode to get access to the configuration tools and all other options selecting the "SWITCH Mode" tool, wich is at the bottom of the options list.