Jun 20, 2022

ZooScan Protocol

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LOVComplex IMEV_PIQv

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The ZooScan (HYDROPTIC Inc.) is an imaging system for the measurement and classification of organisms and particles (150 µm to 5 cm) present in a liquid. It is suitable for meso- and macro-planktonic organisms that must be immobile (fixed or anaesthetised).
See also : http://www.hydroptic.com/index.php/public/Page/product_item/ZOOSCAN

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See more here : https://sites.google.com/view/piqv/selected-publications?authuser=0

plankton, imagery, zooscan, zooprocess, ecotaxa, classification

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See also : http://www.hydroptic.com/index.php/public/Page/product_item/ZOOSCAN

- zooscan
- computer
- extractor hood
- sieves
- wash squeeze bottle (fresh water and/or filtered sea water)
- funnel
- cactus sticks
- pipette

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.
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

- install your zooscan on a perfectly straight and stable lab bench
- install the associated software (zooprocess, vuescan... etc):
https://sites.google.com/view/piqv/zooprocess-uvpapp?authuser=0
- collect a plankton sample using a net and use logsheets to record all the metadata necessary to trace your sample
- remove seawater and fixative from the sample

IMPORTANT NOTE about the ZOOPROCESS VERSION

1
- This protocol applies for the Zooprocess 7.27 version and above. The images are issued from versions up to 8.12.
- DO UPGRADE Zooprocess as often as it is updated on the PIQv website.
- The PIQv would assist you only if you use the latest available update of Zooprocess.
- This protocols does not describe the installation of the Zooscan software and drivers on your computer.

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- More ddocumentation available on the PIQv website.

TYPICAL ZOOSCAN WORKING PROTOCOL

2 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**.

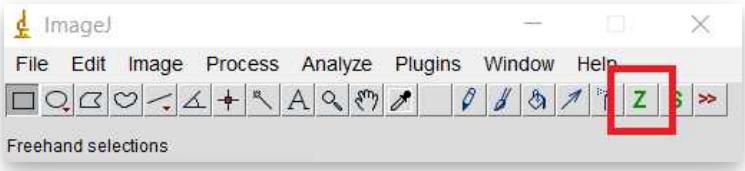
The typical analysis sequence is as follow:

- Fill in Sample metadata (as many sample as useful, can be done at sea...)
- SCAN and CONVERT a Background (on a daily base)
- SCAN a Sample (one or several in a raw)
- CONVERT & PROCESS scanned SAMPLES in batch mode
- CHECK the process using segmented images
- SEPARATION of touching objects on selected vignettes
  - 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.

CREATION of a PROJECT

3 A projet is a folder where images and metadata of a cruise, survey or experiment will be saved. A project thus contains many samples and scans.

- Open ImageJ. Zooprocess or click on the Z icon if ImageJ is already running (**do not open twice !**):



- Choose the option “CREATE NEW PROJECT” which is at the bottom of the project list.

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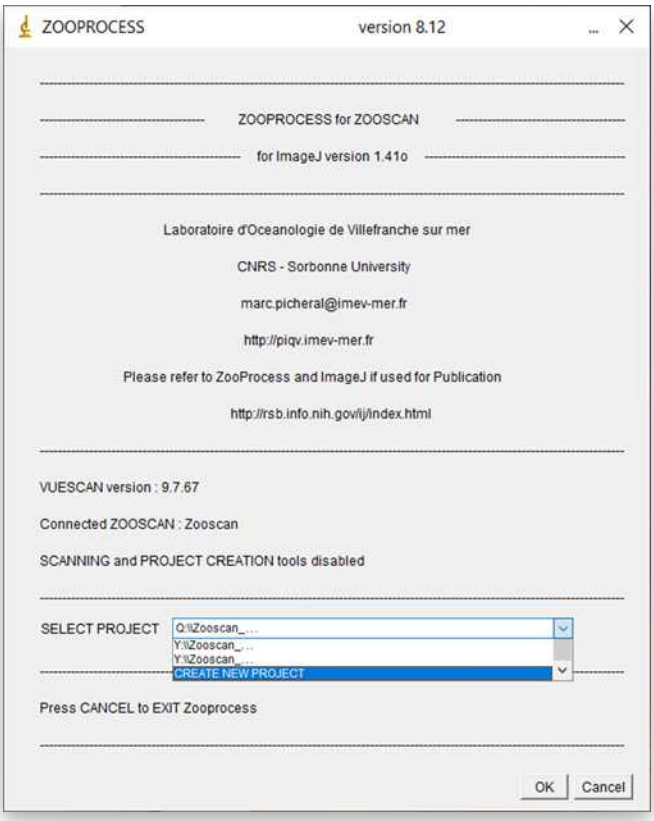
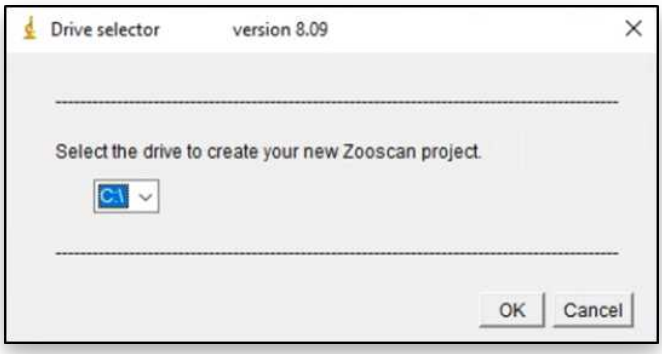
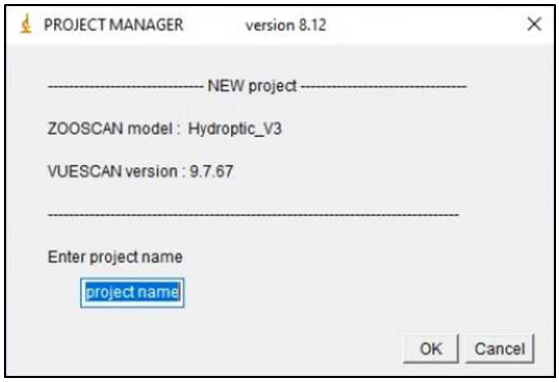


- Choose the drive and enter the name of your project (do not add the "Zooscan_" prefix).





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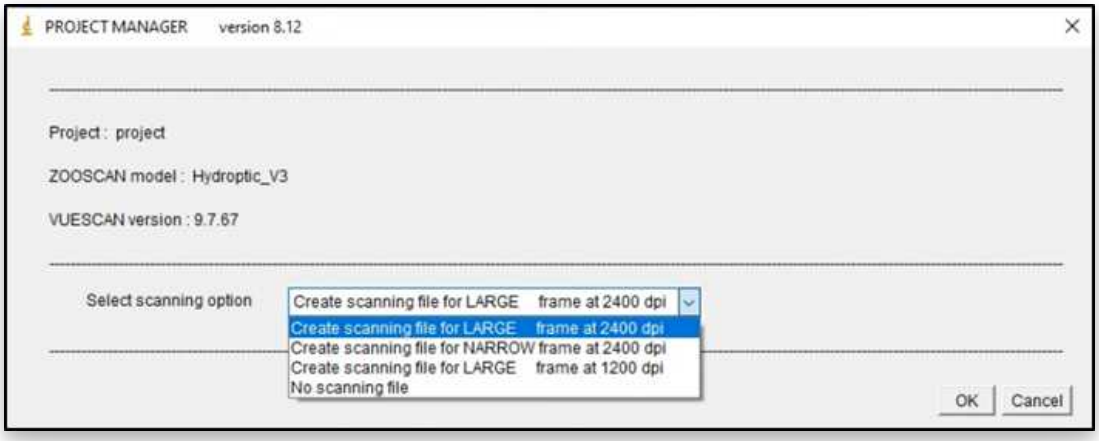
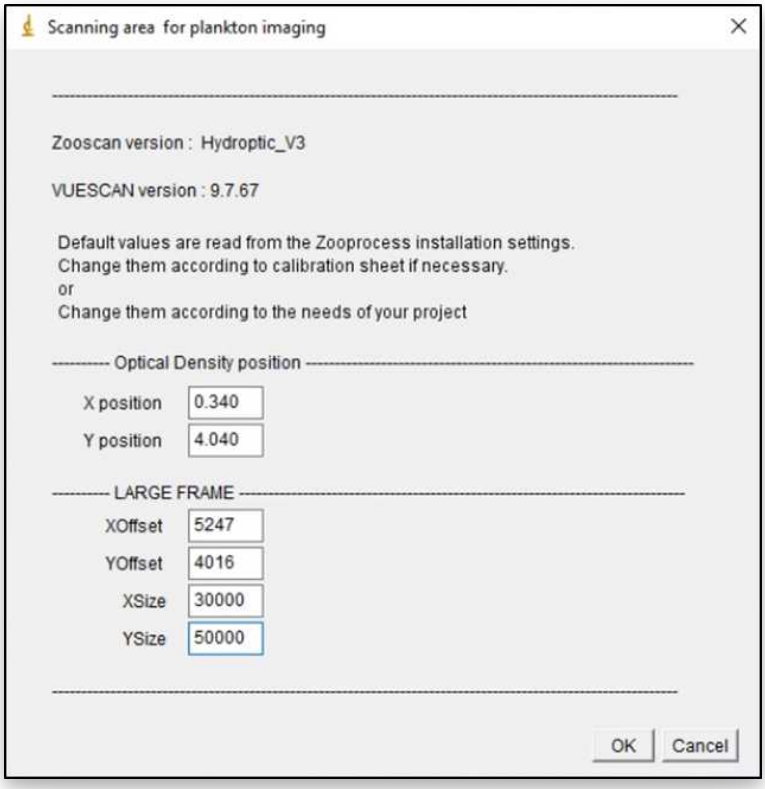
- Select the scanning option: we recommend choosing the Large frame for images at 2400 dpi resolution. The lareg frame permits to scan more objects in a raw. Note that the 4800 dpi resolution is no longer valid as the Zooscan true resolution does not provide true 4800 dpi images.



- Do not change the values, click on “OK”. (If necessary, these values are indicated in the instrument documents provided by Hydroptic in the associated USB key.



A folder will be created on the selected drive and all images and files from this project will later be saved in the specific subfolders.

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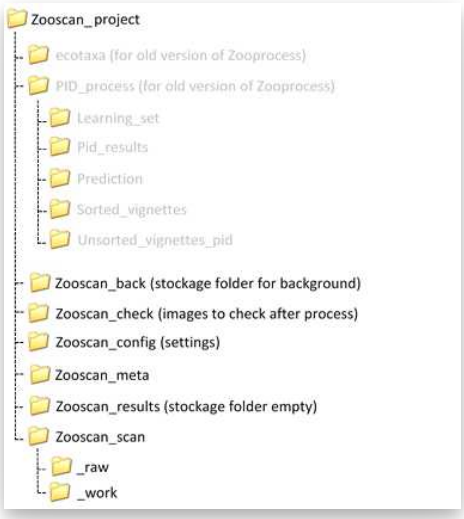
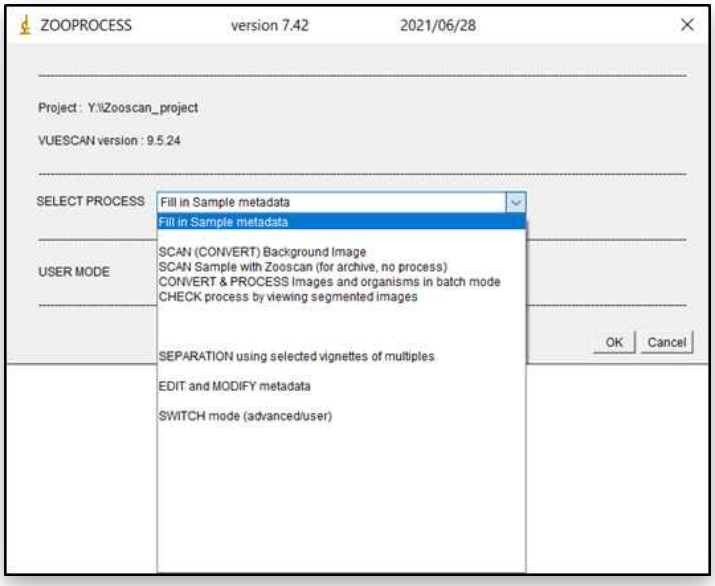


FILL IN the SAMPLE METADATA

4 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.

Open Image J. Zooprocess or click on the Z icon if ImageJ is already running, choose your project then choose "Fill in Sample metadata" :



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**.

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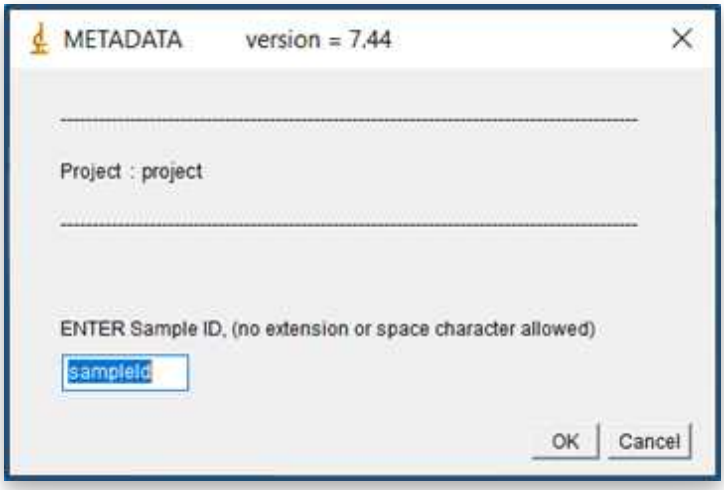
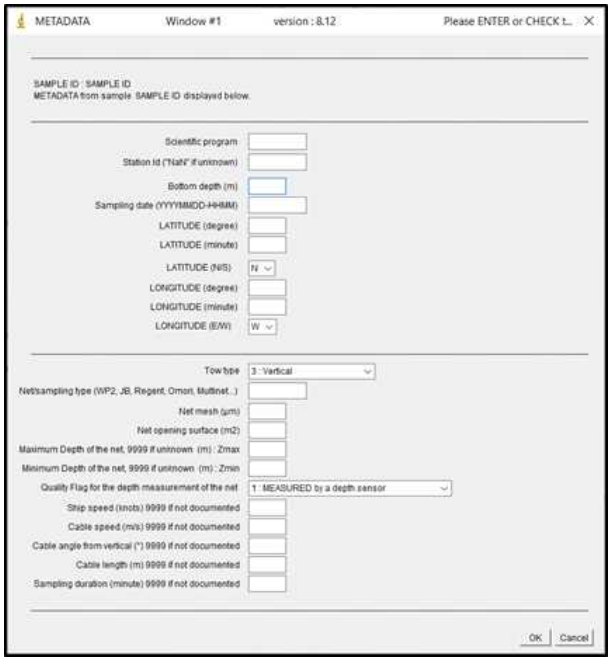
Do not use the “d1”, “d2” or “tot” extensions in the sample names. These extensions will automatically be added to the scan names at the time of the scan (see below).



Fill in the form and you should carefully check the format of several metadata such as latitude and longitude (see the metadata windows)!



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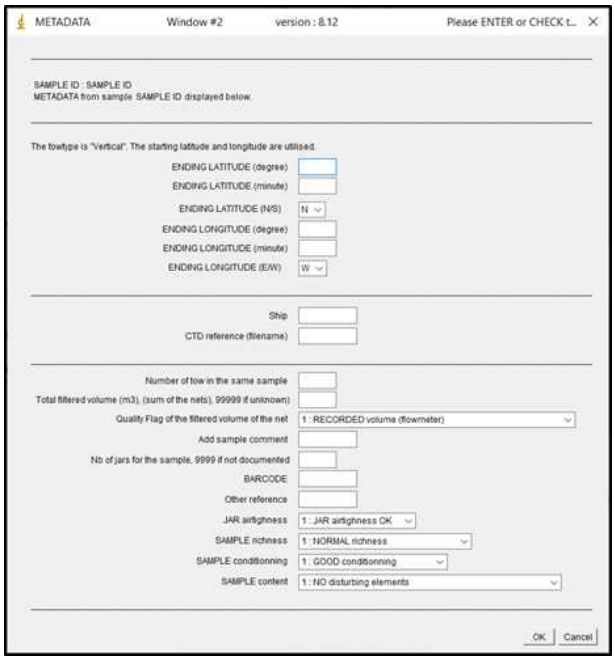
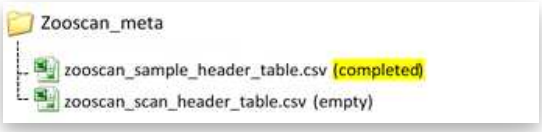


An CSV (excel) table named : “zooscan_sample_header_table.csv” will be created by the application in the “Zooscan_meta” folder of your project. This table lists all the samples and associated metadata you have filled in.



In your folder

EDIT AND MODIFY the METADATA

5 You can check/modify the 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.

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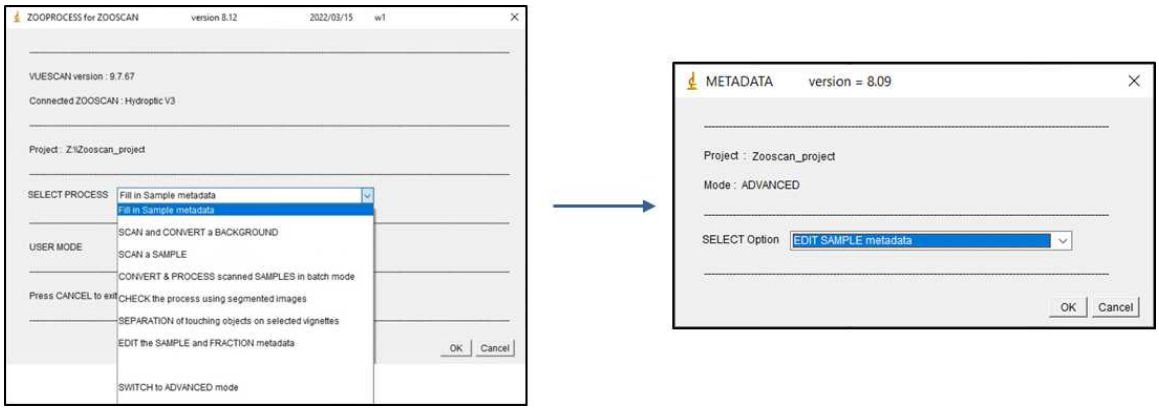
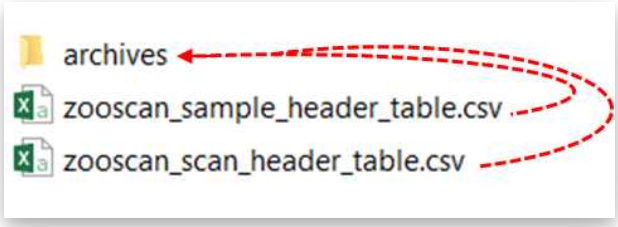
To edit and modify metadata, you have two ways:

1) One by one (fully safe !)



2) In batch mode (for GURU users, be very carefull)

- Copy/Paste your .csv tables in “archives” folder (this operation is automatic when you edit a single sample using the Zooprocess interface) :



- Modify directly the .csv tables than save them

PAY ATTENTION TO THE METADATA FORMATS !

- Open ImageJ/Zooprocess and choose “SWITCH to ADVANCED mode”, then “UPDATE all metadata from the sample and scan CSV tables.”, you have a message: click on “Yes”

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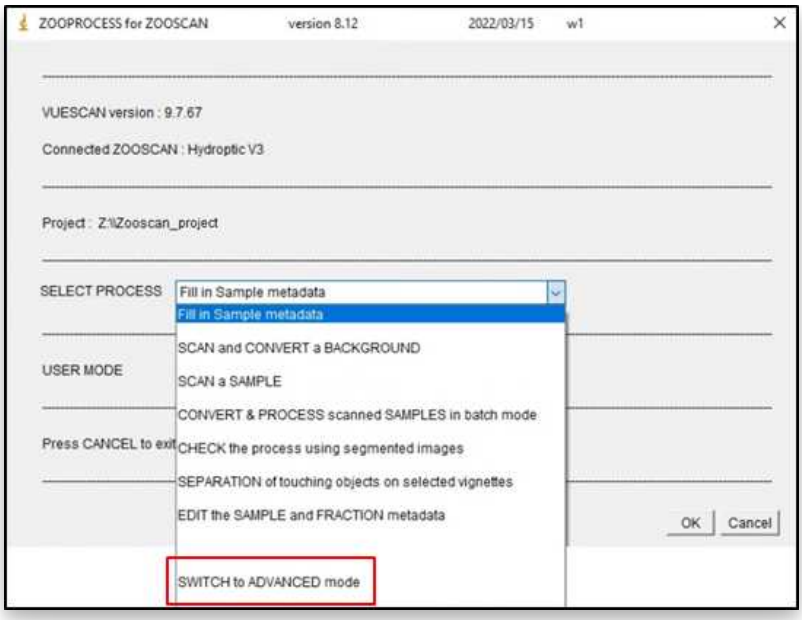

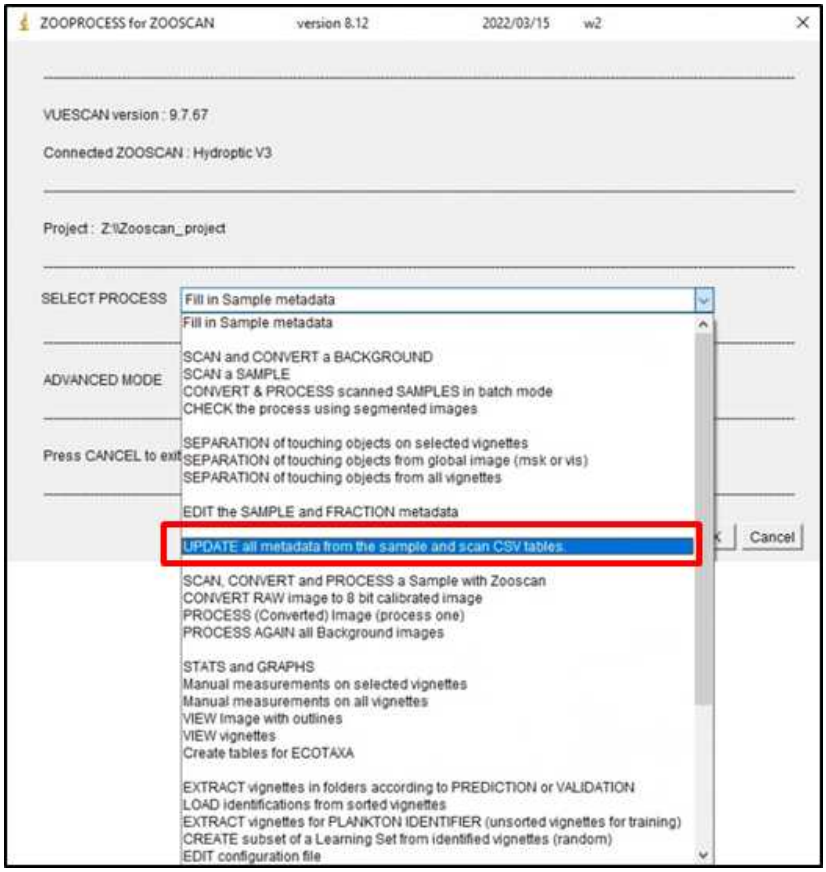

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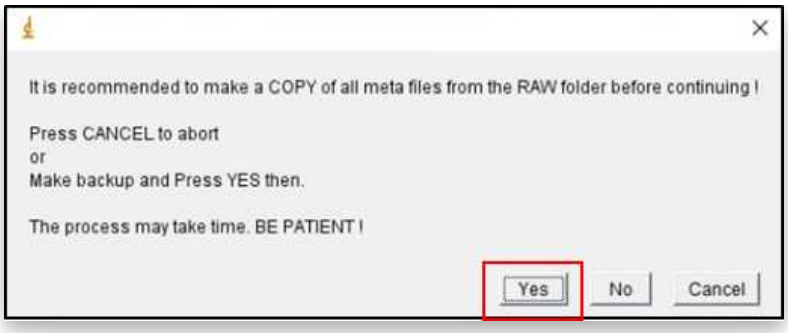

ZIP and archive all META.TXY files from the RAW folder prior pressing YES.

These tools uses the sample and scan tables to correct the metadata in the tables of the meta folder and inside all files related to the sample anywhere in the project.

SCAN (CONVERT) a BACKGROUND IMAGE

6 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 averaged into a unique background image and automatically saved in “Zooscan_back” folder.

6.1 Clean and rinse the scan tray and the cover glass using freshwater to remove the dirtiness.

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6.2 Eliminate marks on the glass and the frame and check from time to time if the glass of the ZooScan cover has no mark.

6.3 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.

6.4 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 (Zooscan V1 and V2) 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.

Filling the Zooscan tray (lateral view)

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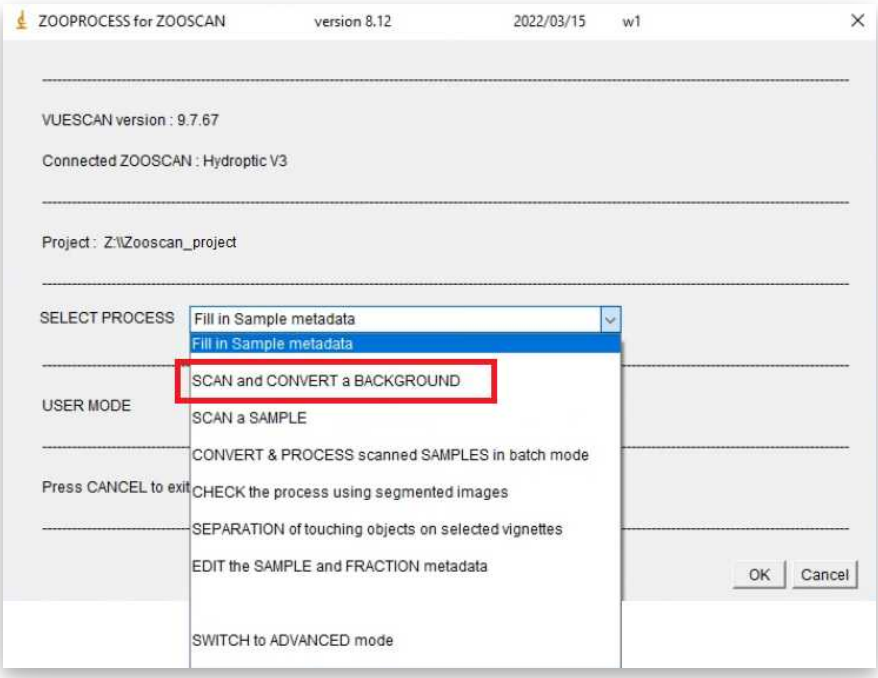
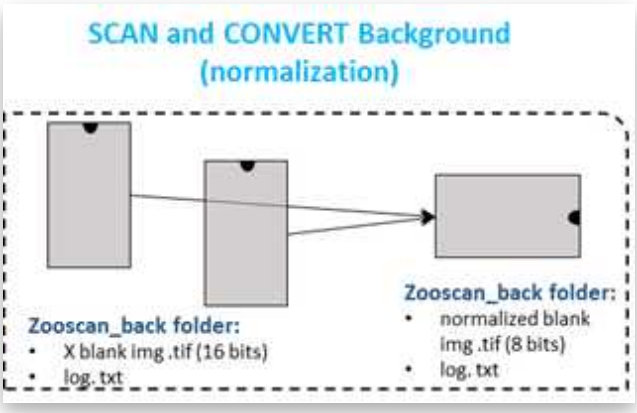
6.5 Click on “SCAN (CONVERT) Background Image” tool.



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.



Process of background images

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In your folder

6.6 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).

6.7 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.

SAMPLE PREPARATION

7

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.
Please do consider that the FORMALIN is a carcinogen and mutagen product. Apply all necessary safety procedure to avoid any risk when you remove the formalin from the sample:

- **work in a extractor hood**
- **use gloves, glasses and lab coat**

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.

For a mesh size net of 200 µm or less, in order to prevent the underestimation of large and rare organisms, the sample is sieved through a 1000 µm mesh and 100 µm mesh to get 2 size fractions.
The two fractions are then splited with a motoda box before scanning to obtain a proper number of objects. By experience we scan 500-1000 objects for the large fraction **that we**

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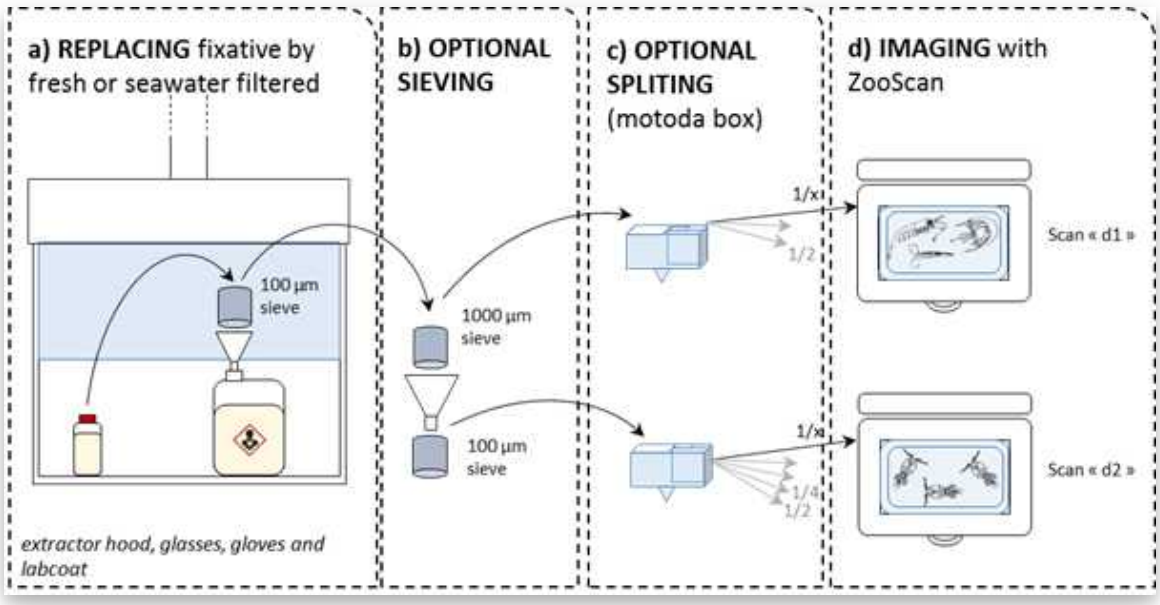
recommend to name "d1" and 1000-2000 objects for the the lower fraction **that we recommend to name "d2"**. These numbers can be adapted according to user experience and care. The number of touching organisms must be as limited as possible to avoid spending much time to later separate them from the images.

For > 200 µm mesh size net, the fractions d1 and d2 are not necessary. The entire sample is directly splited with the motoda box and he fraction ID for scan is "tot". In this case, the proper number of ojects is 1000 to 2000 and replicates can be done if wanted.

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



Procedure to prepare a sample collected using a WP2 net (200 µm) : 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")

- We use only MOTODA splitting system at PIQv. Our recommendation are thus applicable for this system.
- 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.
- 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….).

STEP BY STEP SCANNING PROCEDURE

Citation: LaÃÂ«titia Jalabert, Marc Picheral, Corinne Desnos, Amanda Elineau ZooScan Protocol https://dx.doi.org/10.17504/protocols.io.yxmvmk8j9g3p/v1

8

- The METADATA must be previously filled (see chapter 4)
- The BACKGROUND must be scanned (see chapter 6)

8.1 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 than the instrument.

8.2 Place the frame. The frame size (NARROW or LARGE) depends on your choices at chapter 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 chapter 7).

8.3 Clean water droplets or marks on the frame and remove the dirtiness in you water with pipette and the cactus sticks.

8.4 Pour the sample from the last fraction of the Motoda box.

8.5 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 between : enough water to cover the edges and an insuficiant level to avoid out of focus organisms.

8.6 Allow 15-20 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 chapter 11) 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

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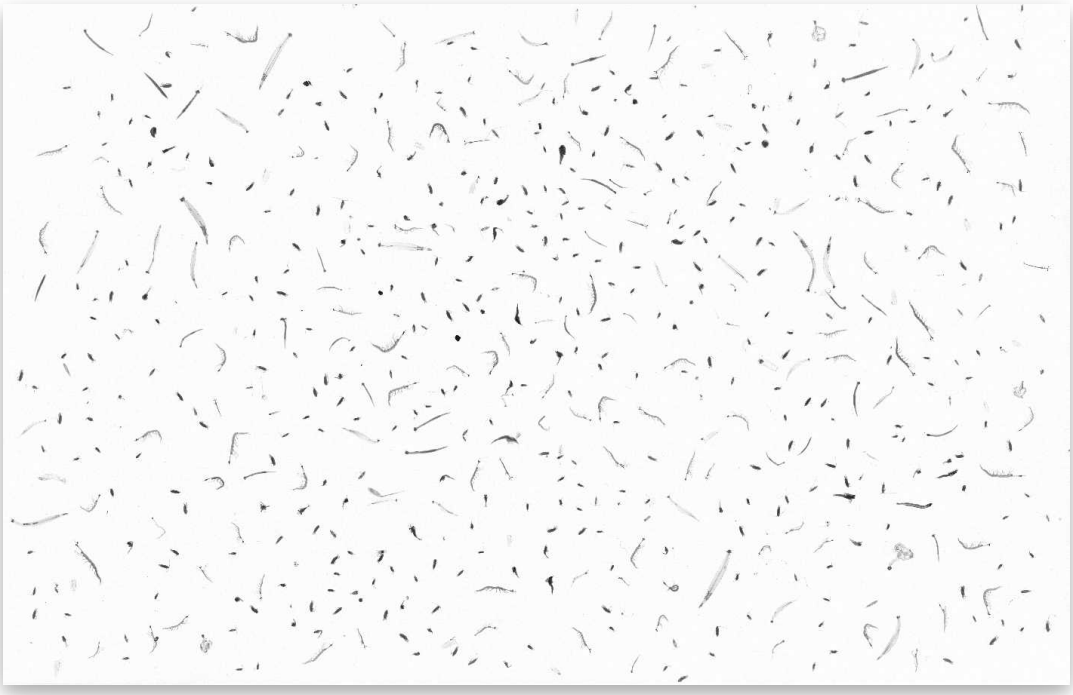
organisms of the tray to “dissolve” them.

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 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.



Example of "d1" scan (large fraction > 1000µm)

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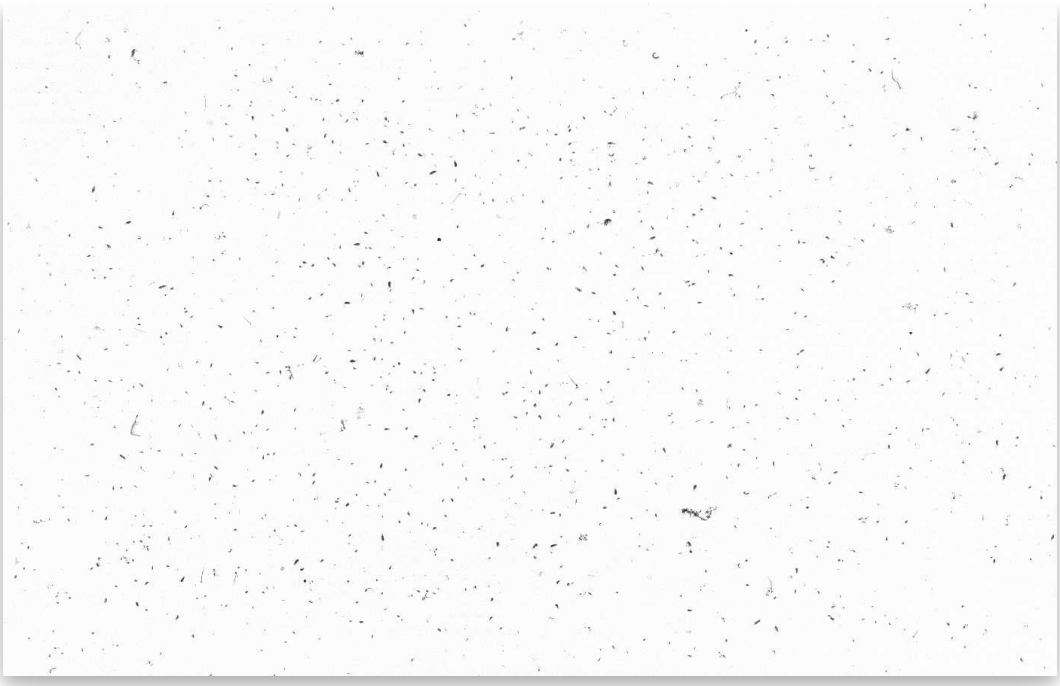


Example of "d2" scan (100 - 1000µm)

8.7 Check that there is no condensation on the glass of the ZooScan cover.

8.8 Launch Zooprocess, select your project and click on the option: “SCAN sample with Zooscan (for archive, no process)”.

8.9 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.

8.10 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! For 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!

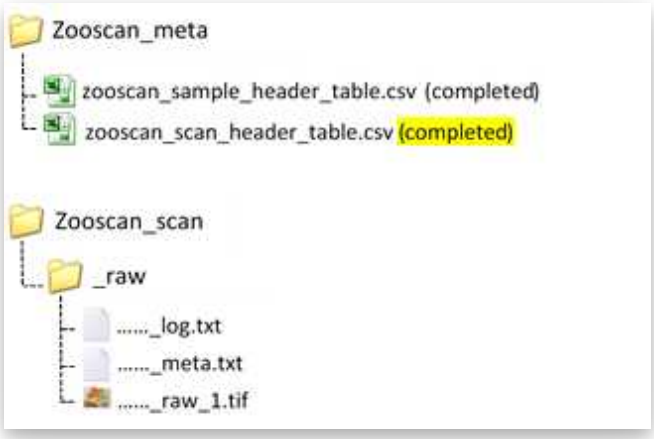
8.11

Citation: LaÃÂ«titia Jalabert, Marc Picheral, Corinne Desnos, Amanda Elineau ZooScan Protocol
https://dx.doi.org/10.17504/protocols.io.yxmvmk8j9g3p/v1

- 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) :

1. The TIF image (16 bits) will be processed later when using "CONVERT & PROCESS Images and organisms in batch mode" option in the Zooprocess main menu.
2. The 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 (*.csv) from the meta folder of the project.
3. The LOG file records information on the scanning method (parameters).

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



In your folders

RECOVERING the SAMPLE

9

9.1 Remove and rinse the transparent frame with a wash squeeze bottle above the scanning tray to recover all specimens that may be stuck on it.

9.2 Remove the sample from the tray: lift the ZooScan gently and maintain it (i.e., always hold the tray when you lift it to remove the water or it could fall).

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9.3 Clean the tray with a wash squeeze bottle of freshwater or filtered seawater to avoid contamination between the differents samples.

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

CONVERT AND PROCESS IMAGES AND ORGANISMS IN BATCH MODE

10 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 chapter 11) that the background and the samples have been correctly acquired.

Start Zooprocess if necessary or click once on the Z icon if Zooprocess is already opened and select the tool “CONVERT & PROCESS scanned SAMPLES in batch mode” :

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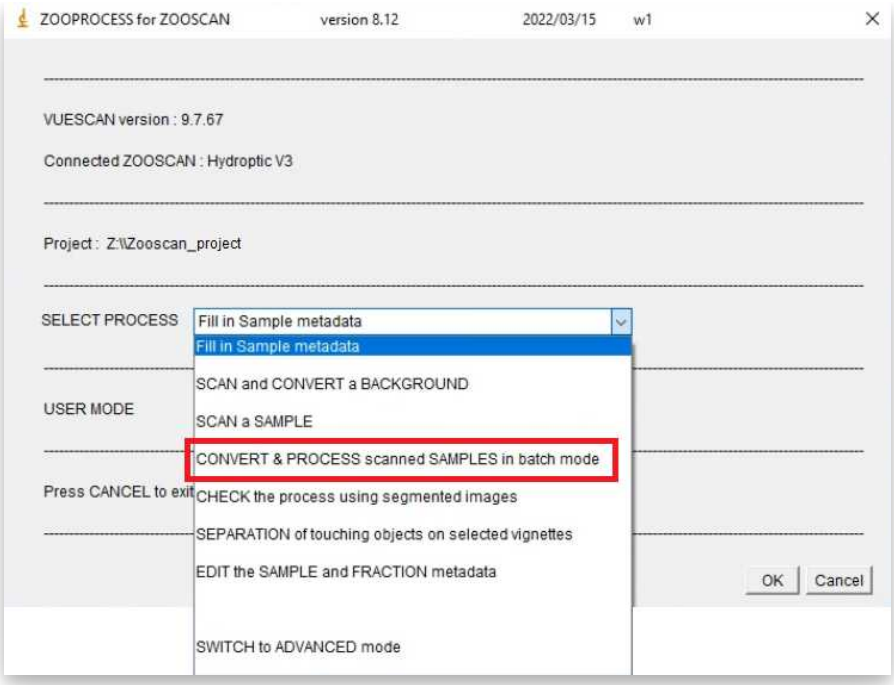
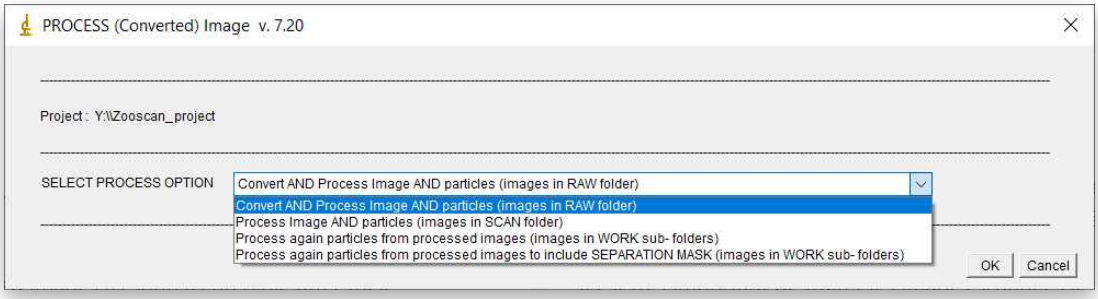
https://dx.doi.org/10.17504/protocols.io.yxmvmk8j9g3p/v1



Then, select “Convert AND Process Image AND particles (images in RAW folder)”.



It is recommended to keep the default settings for the process. Click on "ok" :

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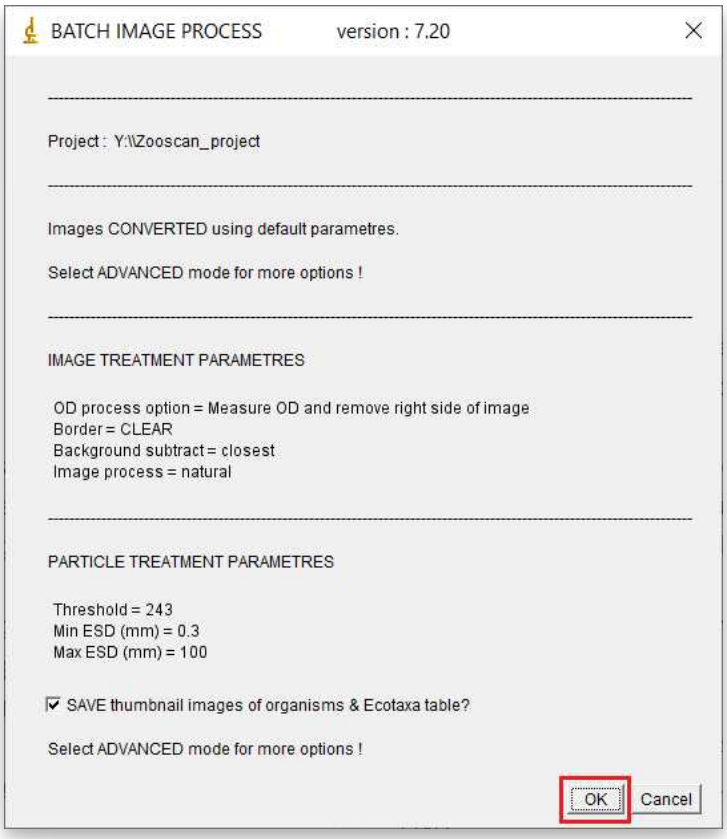
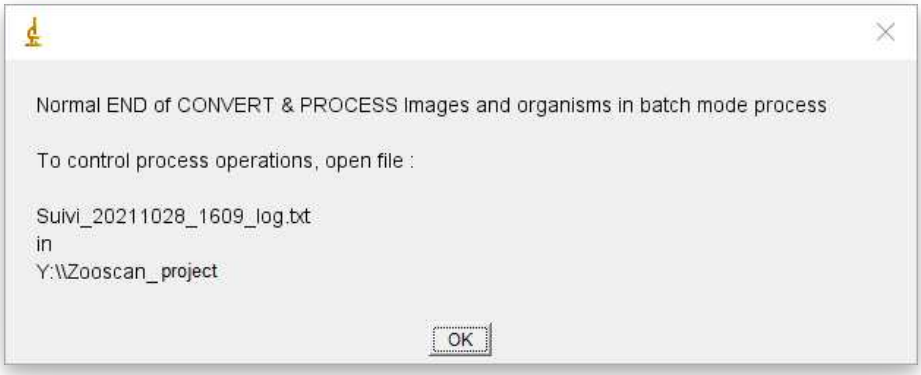
https://dx.doi.org/10.17504/protocols.io.yxmvmk8j9g3p/v1

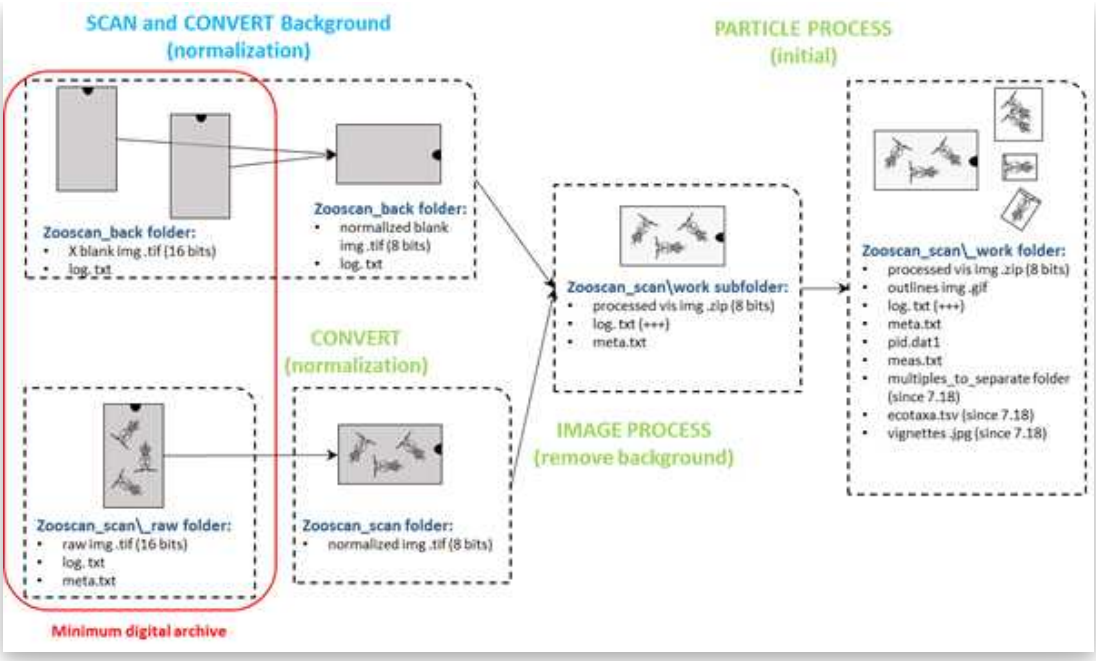


At the end of the process, a Zooprocess window appears with a “NORMAL END…” message :



For your information:

Citation: LaÃÂ«titia Jalabert, Marc Picheral, Corinne Desnos, Amanda Elineau ZooScan Protocol
https://dx.doi.org/10.17504/protocols.io.yxmvmk8j9g3p/v1



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 (*.jpg and *.tsv) 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 recommend to swich to EcoTAXA which is much more efficient**. You can contact us in order to get assistance to move your data already classified in PKId samples or projects into EcoTAXA : piqv@imev-mer.fr

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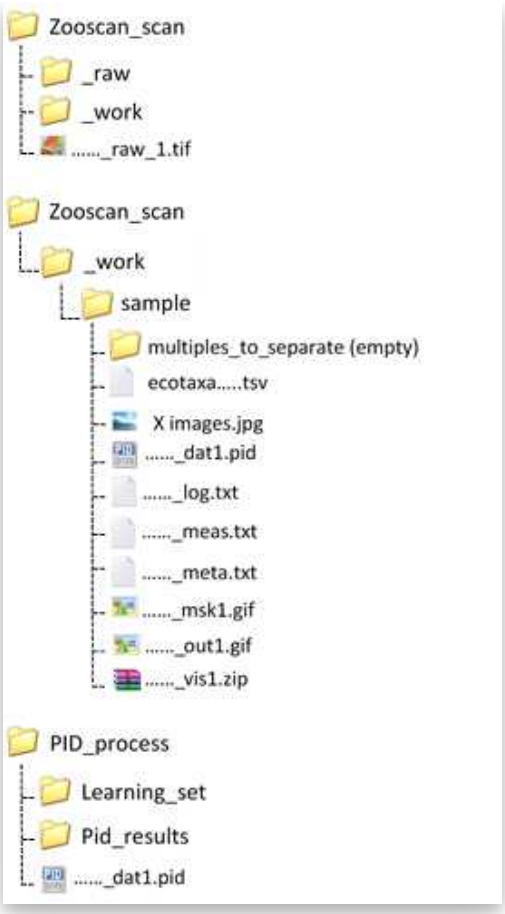
https://dx.doi.org/10.17504/protocols.io.yxmvmk8j9g3p/v1



In your folders

CHECK the PROCESS BY VIEWING SEGMENTED IMAGES

11 **After any image process**, you must check that the background was well subtracted from the sample image using the dedicated checking tool: “CHECK the process using 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.

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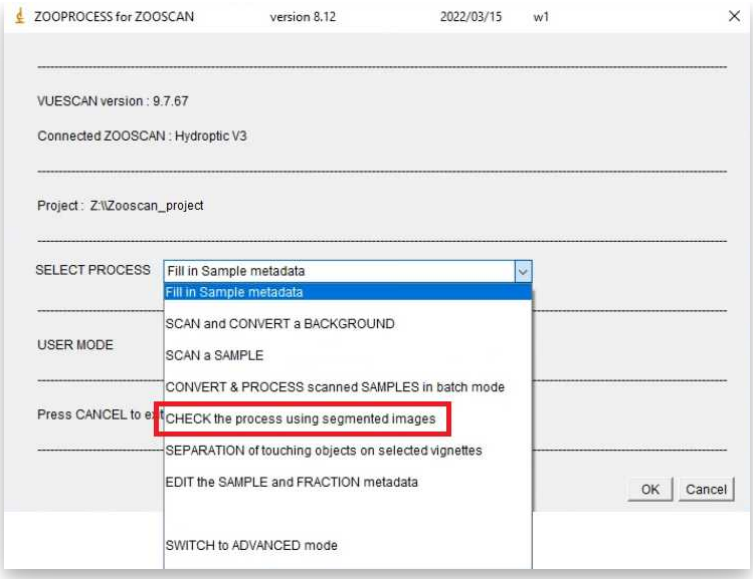
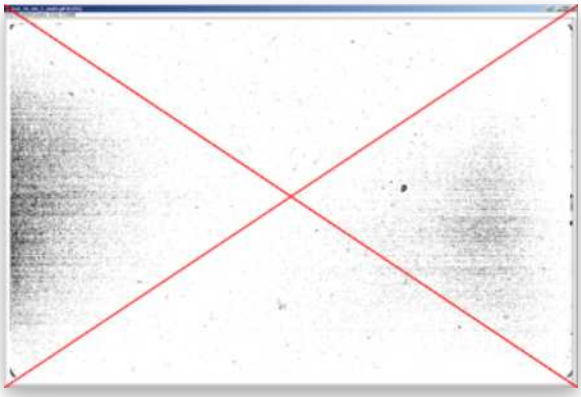
https://dx.doi.org/10.17504/protocols.io.yxmvmk8j9g3p/v1



Example of images "masks":

correct



incorrect

Citation: LaÃÂ«titia Jalabert, Marc Picheral, Corinne Desnos, Amanda Elineau ZooScan Protocol
https://dx.doi.org/10.17504/protocols.io.yxmvmk8j9g3p/v1

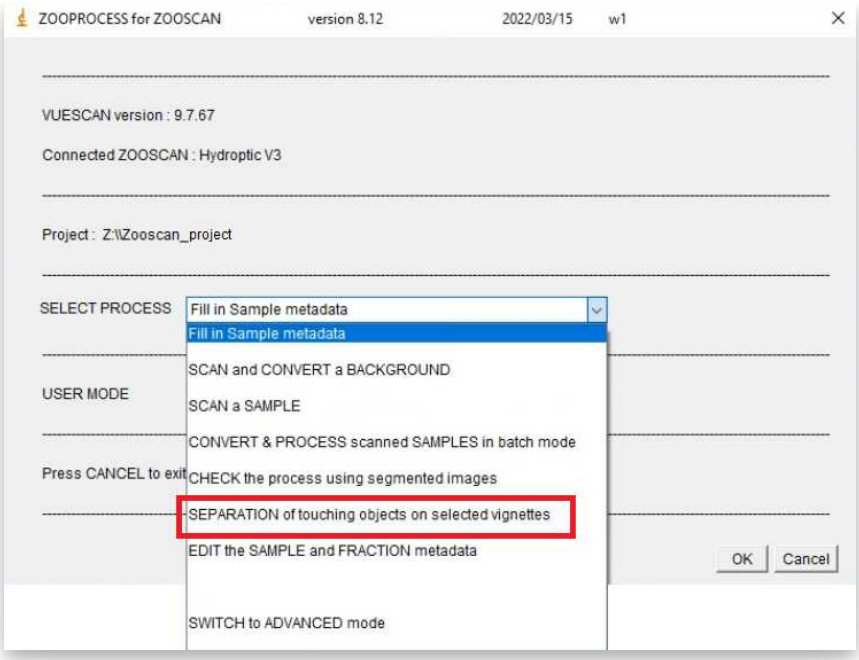
CHECKING "MULTIPLES" AND SEPARATION USING SELECTED VIGNETTES OF MULTIPLES

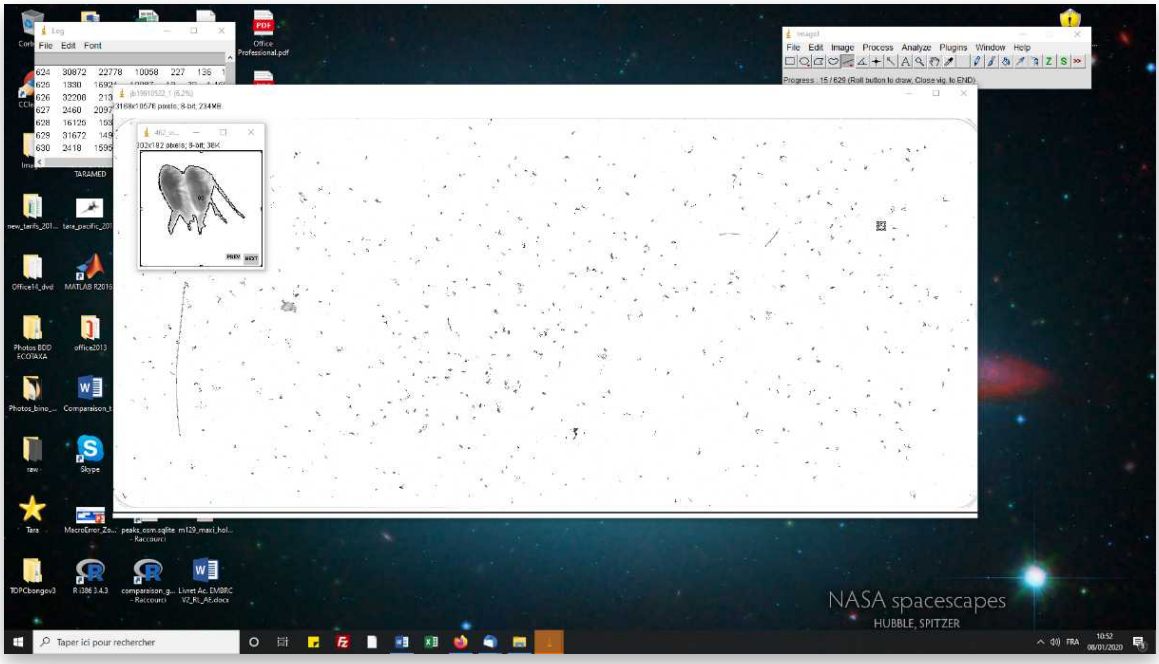
12 If you are not satisfied with the manual separation that you performed on the scanning tray (too many objects touching in the image) and you notice that too many vignettes contains touching objects, you should perform an additional separation on the vignettes. Note that the same procedure should be applied on all samples of the same project for data consistency.

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.

Use the tool “SEPARATION of touching objects on selected vignettes” to separate the touching organisms by drawing lines between them. The tool permits to draw many lines on a single vignette in a batch.

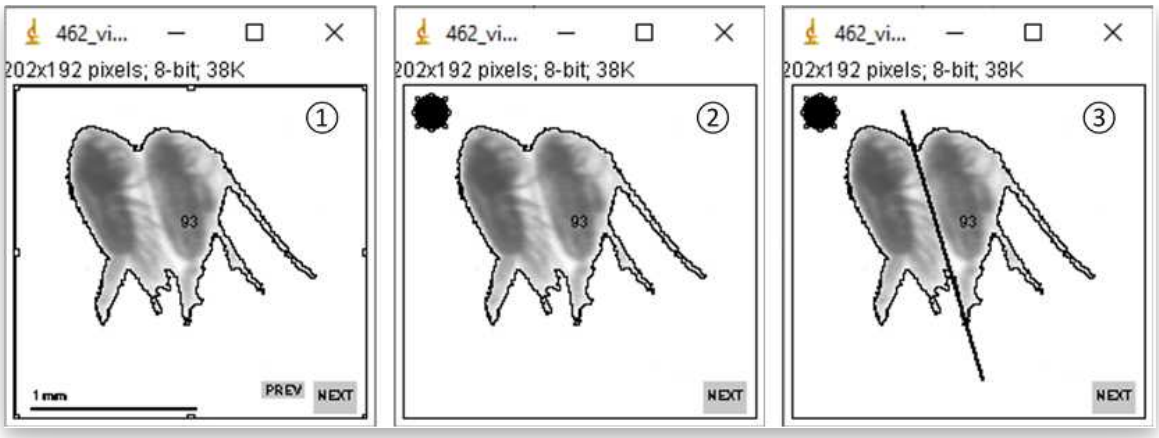
Citation: LaÃÂ«titia Jalabert, Marc Picheral, Corinne Desnos, Amanda Elineau ZooScan Protocol https://dx.doi.org/10.17504/protocols.io.yxmvmk8j9g3p/v1



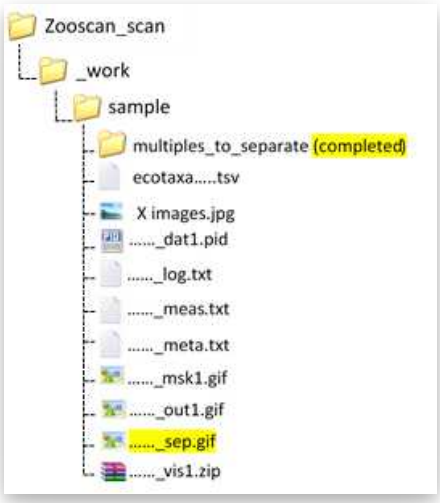


With the mouse, click on the first image to be separated ①. Then, click for a moment on the scroll of the mouse to get a black dot in the upper left corner of the thumbnail ②. The mouse turns into a cross that will allow you to create one or more separation lines ③. Then click on the “Next” button.

Citation: LaÃÂ«titia Jalabert, Marc Picheral, Corinne Desnos, Amanda Elineau ZooScan Protocol https://dx.doi.org/10.17504/protocols.io.yxmvmk8j9g3p/v1



When all the images of the sample have been separated, a new *_sep.gif file is created in the sample file :

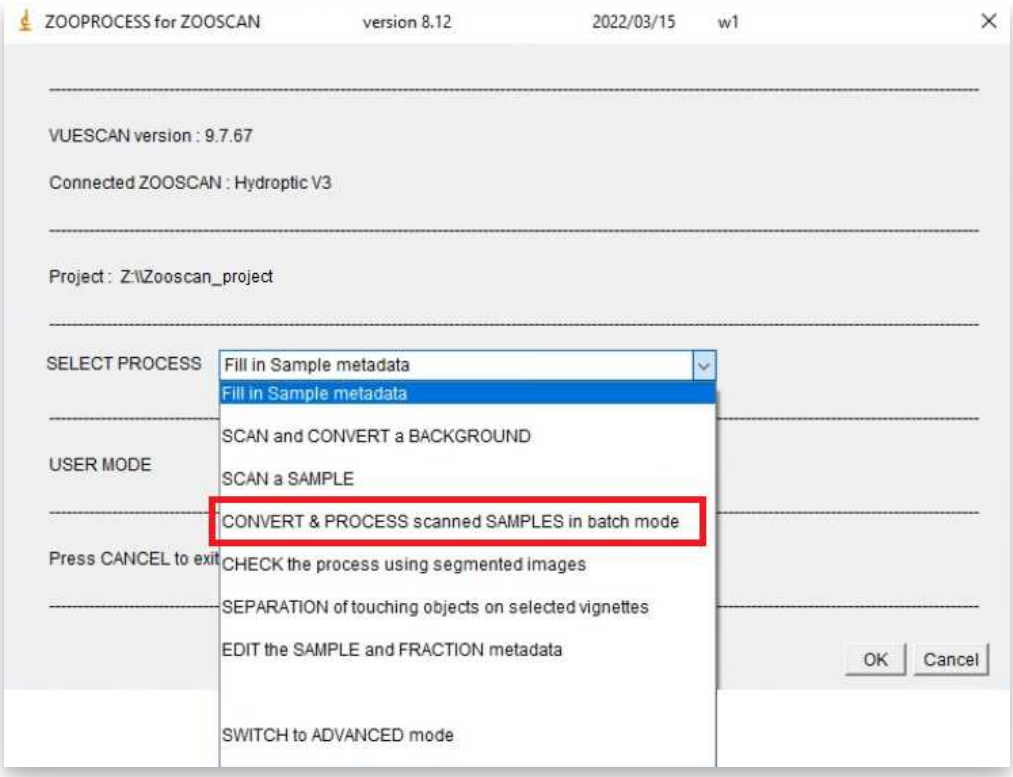


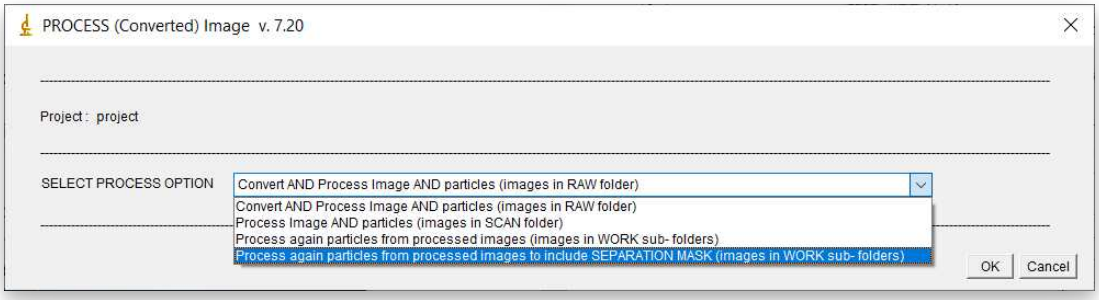
In your folders

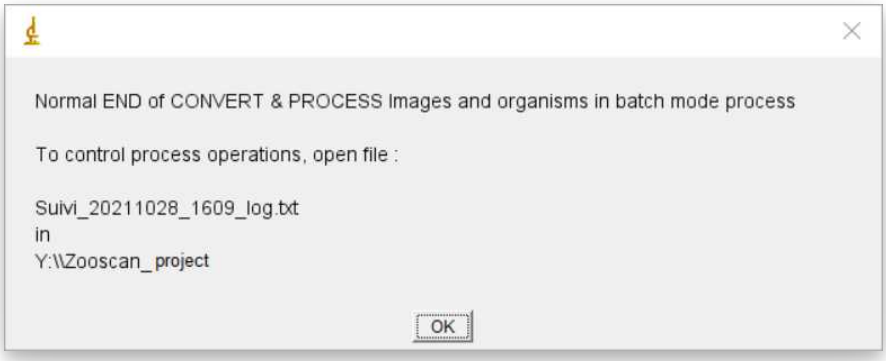
RE-PROCESS of the scan AFTER SEPARATION

13 You must redo the “CONVERT & PROCESS scanned SAMPLES 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”.

Citation: LaÃÂ«titia Jalabert, Marc Picheral, Corinne Desnos, Amanda Elineau ZooScan Protocol
https://dx.doi.org/10.17504/protocols.io.yxmvmk8j9g3p/v1







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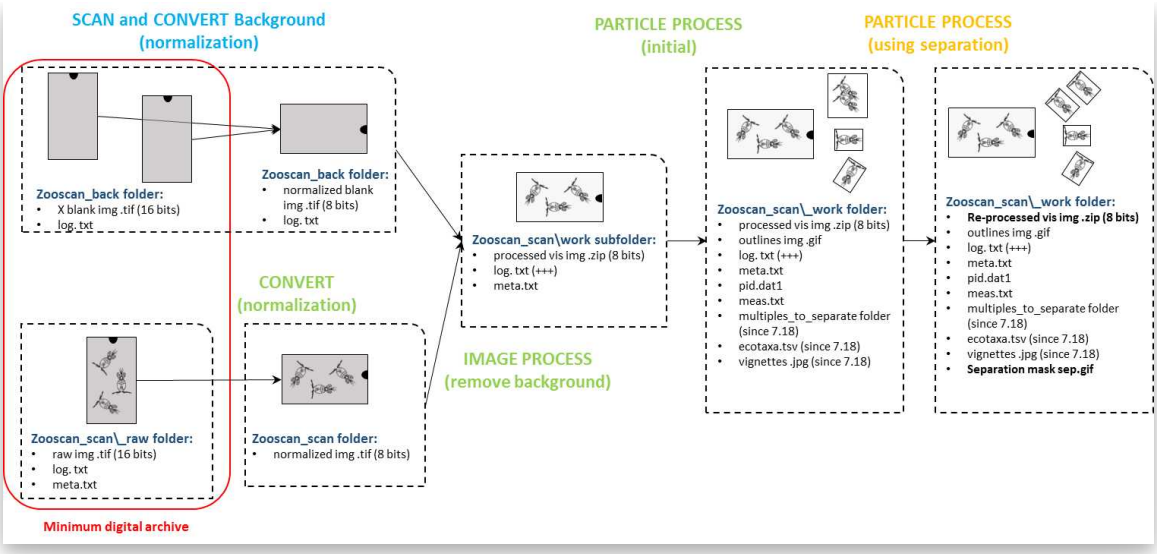
https://dx.doi.org/10.17504/protocols.io.yxmvmk8j9g3p/v1



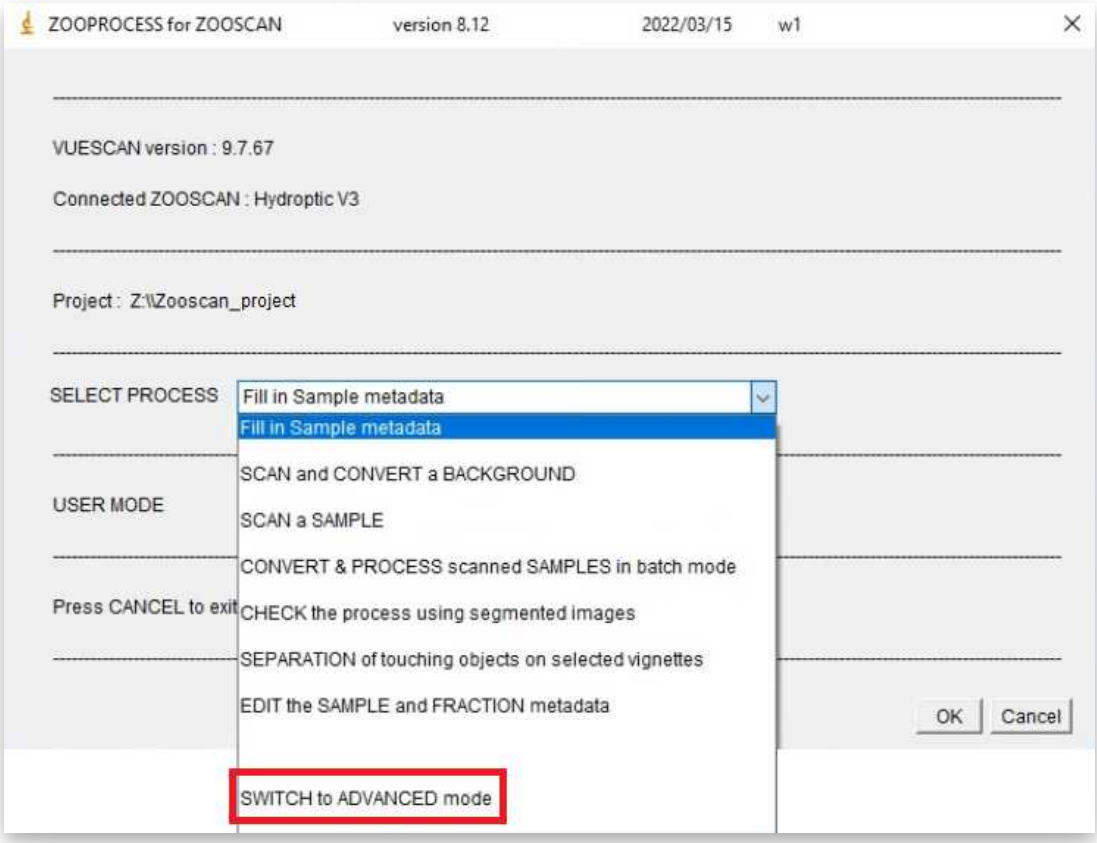
Standard processes plus additional separation of touching objects

In your folders

USER/ADVANCED MODE FOR A PROJECT

Citation: LaÃÂ«titia Jalabert, Marc Picheral, Corinne Desnos, Amanda Elineau ZooScan Protocol
https://dx.doi.org/10.17504/protocols.io.yxmvmk8j9g3p/v1

14 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 to ADVANCED mode” tool, wich is at the bottom of the options list. Zooprocess will return to USER mode automatically.



Citation: LaÃÂ«titia Jalabert, Marc Picheral, Corinne Desnos, Amanda Elineau ZooScan Protocol
https://dx.doi.org/10.17504/protocols.io.yxmvmk8j9g3p/v1