

NOT TO BE CITED. A manual by Eneko Bachiller and Jose A. Fernandes based on Azti procedures and ZooImage manuals: <http://www.sciviews.org/zooimage/>

Note: It is a specific manual that only uses some of the functionality of ZooImage and adapted for specific purposes. For more information consult ZooImage manuals and website.



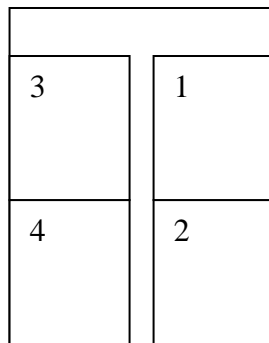
PREPARATION OF PLANKTON SAMPLES TO SCAN

- Remove the sample smoothly and spill it in a test tube.
- Note the initial volume.
- Take three aliquots (6 ml each) in three labelled recipients.
- Scan the samples.



SAMPLE SCANNING

- Spill the aliquot in the sieve (mesh size: 150 µm); wash the sample with water (use the siphon) and then spill it in a plate (take care of not having bordered plates, use preferably new ones), with **temperate** water. The sample should cover the whole surface of the plate; we have to use the minimum amount of water for that.
- Place the four plates (four samples) in the scanner like this:



❖ COMPUTER:

- Switch on the scanner (EPSON PERFECTION 4990 PHOTO).
- Open "vuescan 8.4.17"
- "Load options" → load predetermined options depending on what World we like to do. There are four options: *Grey2400*, *Grey4800*, *Stained2400* and *Stained4800*.
- After scanning them, save the created files in a new folder and change their name. In this way we can maintain the original folder empty and the next four samples will take the names of *p-0001.tif*, *p-0002.tif*, *p-0003.tif* and *p-0004.tif* again, making our image classification much easier.

❖ STAINING :

- Once all the samples are scanned, we can stain them with **Eosin (5gr/L)**.
- Add 0.5ml of Eosin to each sample.
- Wait at least one day to scan stained samples.



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- **With original samples: Grey scale scanning** (16 bit Gray) in two resolutions: 2400dpi and 4800dpi.
- **With stained samples: Colour scale scanning** (24 bit RGB) in two resolutions: 2400dpi and 4800dpi.



USE OF ZOOIMAGE

Getting ready with the Spread Sheet

- Prepare an empty folder on the hard disk.
- Open Zooimage.
- Select the active directory.
Options → Change active dir...
Select the folder just created.
- Copy the "SpreadSheet-example" file in our new folder. It can be downloaded from the Zooimage website. It contains:
ImportTemplate.zie
Zooimage-example.txt
Zooimage-example.xls
10 images (examples) (that should be deleted or replaced with our previous scanned images)

Exporting the table from Microsoft Excel

- Open *Zooimage-template.xls* into Microsoft Excel. Now it has to be modified depending on our samples.
- We can add new columns, but...
Never change order or name of original columns in blue and orange!!
- **Make sure there is no empty lines at the end of the file!!**

Resumed advices

- Give names like p-0001.jpg to your samples instead of p-1.jpg. There is some automatic renamers out there.
Be careful with missing stations!!

- Remove stations where there is no image from the Spread Sheet.

- Use the current date format (yyyy-mm-dd): 2005-12-22.

- Use "." notation for decimal number.

- Do not change order or names of columns in the excel file.

- Random forest can not have empty folders in the training set, neither only one item (at least two).

- SubPart should have at least four digits of decimal precision.

- Vollni should have at least three digits of decimal precision.

- Save the file as "Text (Tab delimited) (*.txt)" in the same folder where the original .xls file is located. It is possible that we will have to replace the existing .txt file by the new.



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- Finally, our folder should have:
 - Zooimage-template.xls* (It is **VERY IMPORTANT** to KEEP the excel's COLUMN FORMAT UNTOUCHED!)
 - Zooimage-template.txt* (Be sure to have **NO EMPTY LINES AT THE END**)
 - ImportTemplate.zie* (check it with a plain text editor for possible modifications needed)All our scanned images, with optimized format names.



Importing images

- Click on *Analyze* → *Import images...*
- Open the .txt file we have just created from the original excel (*Zooimage-template.txt*).

We can leave the computer unattended since it will give us an informative message about possible problems!!

- At the end, we should have:
 - .zie file (original: *ImportTemplate.zie*)
 - .txt file
 - .xls file
 - .zim files (one per each station, for example)
 - .jpg files (or .tif); **Depending on Zooimage version, we have to change the extension of our scanned images manually to “.jpg” in this folder!!**
 - *_raw* and *_work* subdirectories:
 - Original images (renamed and as .jpg or .tif, depending on the version)
 - New created .zie file (*Import_Zooimage-template.zie*)

Depending on the Zooimage version, these two folders are used during processing.



Processing images

- Click on *Analyze* → *Process images...*
- “ImageJ” will open.
- Click on *Plugins* → *ZooPhytoImage* → *Scanner Color* OR *Scanner Gray16*.
- Since we have got one .zim file for each image (or station), we have to select only the first .zim file to process **all** images of our folder. All images are associated with their own .zim file.
- “Zooimage1 Image Processor” window will open. Select these options:
 - *Process all items in this directory*
 - *Analyze particles*
 - *Make vignettes*
 - *Sharpen vignettes*

This process will slow down all the computer for quite a long time so that do not use any other application while this process is executing.



Creating ZID files

- Click on *Analyze* → *Make .zid files...*
- Click *OK* (check if we want to update comments of our zipped files from *_raw* folder).
- Select the folder where all our created .zim files and other folders (like *_raw* and *_work*) are located



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(f.ex. *Tintadas2400*).

Make sure there is no error reported once the compression is done!!



Making the Training Set

- Copy *bioman.zic* (OR *Pairovet_JUV06.zic*) into our main folder. We can change our future groups by adding names with a plain text editor. Nevertheless, it is not so important since this file will be modified as we create new folders during our training set.
- Click on *Analyze* → *Make training set...*
- “*Select a .zic file*” window will open. Select our *.zic* file (*Pairovet_JUV06.zic*).
- Select the folder where we want to place the training set.
- Give a name to the folder (by defect: “*_train*”).
- Select the *.zid* files with the vignettes that are going to be used for making the training set.



XnView will open.

- “*_*” named folder will contain all the images (vignettes) that are not going to be taken into account for the training set. All the images will be there at the beginning.
- Move particles or vignettes to the corresponding folder.
- New folders can be created inside the “*_train*” folder. The program will automatically take into account those new folders adding them to the original *.zic* file.
- Finally, we will have: - As many folders as classified groups
- All *.RData* files from original source of training set images



Reading the Training Set

- Click on *Analyze* → *Read training set...*
- Select the folder where our training set has been created (“*_train*”).
- Give a name to the object that will be created in **R** (by defect: “*ZItrain*”).

After loading, statistics of the Zooplankton classes are shown.



Making classifier

- Click on *Analyze* → *Make classifier...*
- It is recommended to choose “*random forest*” option to make the classifier.
- Choose the object created on the last step (*ZItrain*).
- Give a name for the *ZIClass* object to create (by defect: “*ZIClass*”).



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Analyzing classifier

- This is a confusion matrix to evaluate how good is the classifier. If the error is big or there is a lot of confusion, the training set has to be done.
- The diagonals are the instances well classified.



How to improve the Training Set

- At least 50 individuals per each specie have to be caught in different recipients.
- Scan all samples of pre-classified species.
- Copy one .zim file created before in previous processes and rename it with exactly the same name as the image file name obtained from the scanner. As much .zim files as image files have to be copied and renamed. One .zim file for each image has to be created in the same folder.
- **Take note** that the .RData files have to be taken from previous processes taking into account the source, copying a grey scale process .RData file for grey scale training set and a colour scale process .RData for colour scale training set. In case of colour scale image processing, all images have to be renamed as .jpg instead of .tif, otherwise zooimage will not recognise the file format.
- Open the ZooImage.
- Click on *Analyze* → *Process images* (ImageJ will open)
- Click on *Plugins* → *ZooPhytoImage* → *Scanner Gray 16* (in case of **Grey scale processing**)
→ *Scanner Color* (in case of **Colour scale processing**)
- Select the first .zim file of the list → *open*
- Activate all options of the next screen (select “wide spectrum” in particle size selection option) in both options, such in grey scale as in colour scale.
- Then, .zid files have to be created. The process is the same as the one done with original files.
- Follow instructions of the chapter “Making the Training Set”
- Follow instructions of the chapter “Reading the Training Set”
- Follow instructions of the chapter “Making the classifier”
- Finally, obtained images have to be introduced into appropriate destination folders of previous built Training Set.
- ❖ **Balance-Disbalance process:** In order to improve the training, a second duplicated training set could be done taking into account the % of apparition of different groups to choose the optimum quantity of “model individuals” per class for training. Finally, two training sets will be unified in a single one; After loading two training sets in R, the following code must be typed, f.ex.: `ZltrainDef<-rbind(ZltrainDef,ZltrainDef_duplicated)` (so that “ZltrainDef” will include “ZltrainDef_duplicated”).
- ❖ **Ranking Classes:** This process is done when it is useful to make new groups in order to minimize the classification error %.



Process Samples

- The following files will be necessary to continue with this process:
 - **ZIRes.r** (it has been modified in order to use the minor diameter of particles for their classification; on the other hand, biomass conversion formula (Alcaraz *et al.*, 2003) is used to calculate the biomass from images).
 - **.zis** file (f.ex.: *DescripcionAbreviada.zis*)
 - **Conversion.txt** (it is necessary to distinguish biomass adding particles and non-biologic particles)
 - **.zid** files (one per station, obtained from the original image's folder)
- Once the **training set** has been **read** and the **classifier made**, the final sample processing can be done. If the training set has been improved, loading of those two steps will be necessary.

- Take the file *ZISres.r* from the source folder and charge it in R (dragging it into R screen).
- Click on *Analyze* → *Process samples...*
- Select the source for *.zis* file (f.ex: *StainedSamples2400Zids\DescriptionAbreviada.zis*)
- Activate the “*Save individual calculations*” option.
- Choose our classifier –previously chaged in R- (f.ex.: *Zlclass_Balanced_mejorado*)
- *Object* → *Save* → f.ex.: “*Zl.res*” as *Zlresultados*

If we would like to obtain all results in appropriated format for Excel, we have to write the following sentence in R:

```
write.table(Zlres, "resultados.csv", sep=";",)
write.table(attr(Zlres, "spectrum"), "spectrum.csv", sep=";",)
```

- Obtained files: f.ex.: *Spectrum.csv*
Resultados_transposed.csv



RESULTS

- Two types of files will be obtained from the sample processing:
 - **.csv** files (f.ex.: *Spectrum.csv*)
 - **.txt** files (f.ex.: *JUV.2006-9-28.P0009_Bio.txt*). One file per each station will be saved.
- **ABUNDANCES: number of species per m³**
 - Abundances per species
 - Abundances per particle size (minor diameter)
 - Abundances per species and particle size (minor diameter)
- **BIOMASS: mg per m³**
 - Biomass per species
 - Biomass per particle size (minor diameter) –method from Alcaraz *et al.*, 2003.
 - Biomass per species and particle size (minor diameter)
- ❖ Particle-size classification intervals: $(x_1, x_2]$
- Individual Biomass of particles: **µg**