Protocol

This protocol describes the black and white version. Notes on running the colour version as well as adapting the protocol to new species and sites can be found below.

Things to do before first use on a device:

- Get the folder “Seed_Analysis” with all the necessary files and save it to the desktop. Create an empty folder inside it called "Scans".
- Make sure all necessary programs are installed:
  - Canon IJ Scan Utility for CanoScan LiDE 220
  - Fiji/ ImageJ
  - R
  - R Studio will help to run R

1) Seed preparation

a. Start by separating the seeds from litter. A sieve with 1mm mesh size can be helpful here.

b. If seeds are to be distinguished between filled and unfilled ones, this has to happen before the analysis. Follow b.1 and b.2 for this, otherwise go to c.

1) Two of our species (THPL and CANO) cannot be easily tested for filled or unfilled. For those two seed species, manually assess if they are filled or unfilled. Separate then from the rest of the seeds and label them properly (Site name, Trap n°, date of collection, Species name, Status: filled or unfilled; ie. AE10, T9, 06.07.2023, THPL, unfilled).

2) For other species: Use a beaker wide enough to fit a tea strainer, lable it (Site name, Trap n°, date of collection, Species name, Status: filled or unfilled; ie. AE10, T9, 06.07.2023, THPL, unfilled) and soak the seeds in water for at least 72h. Stir once or twice a day. The ones that float will be considered as unfilled and the sunken ones as filled. Collect the floating seeds with a tea strainer, place in a plastic container and dry as described below. Label the plastic container (Site name, Trap n°, date of collection, Species name, Status: filled or unfilled; ie. AE10, T9, 06.07.2023, THPL, unfilled). Sieve the remaining water and seeds and apply the same method as for the unfilled seeds.

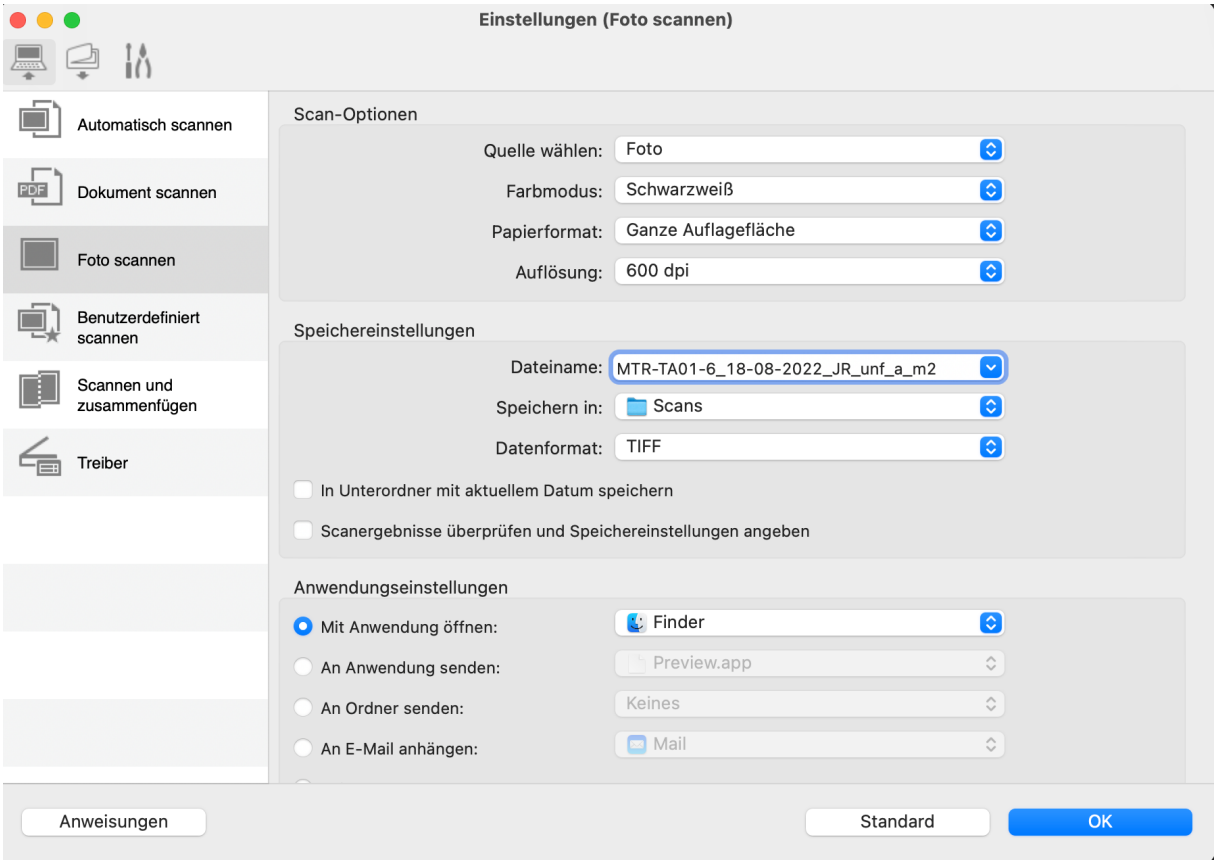
c. Dry the seeds for 24 hours at 40° C.

2) Scanning the Seeds

Scan seeds; use the “CanoScan LiDE 220” scanner and the Canon IJ Scan Utility Program. Connect the scanner to the computer, open the software and choose the right scanner (CanoScan LiDE 220, not 120). Seeds from one trap can be distributed over several scans, but one scan must only contain seeds from the same trap and collecting season.

a) Distribute seeds on the scanner surface. They should not touch each other or the edge. When the sample has a large number of seeds, separate them into multiple scans (labelling according to 2.c). For each scan, repeat this sequence twice, mixing up the seeds in between (see e).

b) In the Canon IJ Scan Utility program go to Einstellungen > Foto scannen > match the specifications to the screenshot below: The folder Scans for storing the images is a subfolder of “Seed Analysis” and should not contain images that have already been analysed as seen in step 3).



c) Naming system (to be entered under “Dateiname”):

Project-Site-TrapNumber_Date of collection_Operator_filled/unfilled_part of sample_mix n°

Example: MTR-TA01-6_18-08-2022_JR_unf_a_m2

Project: MTR for Mt-Rainier and LWF for the Swiss sites

Site and Trap number: on the bag with the seeds; stand: 4 characters; trap number 1: number

Date of collection: dd-mm-yyyy please remember that the MTR samples will be labelled in mm/dd/yyyy on the bag and remember to adjust them to the dd-mm-yyyy order.

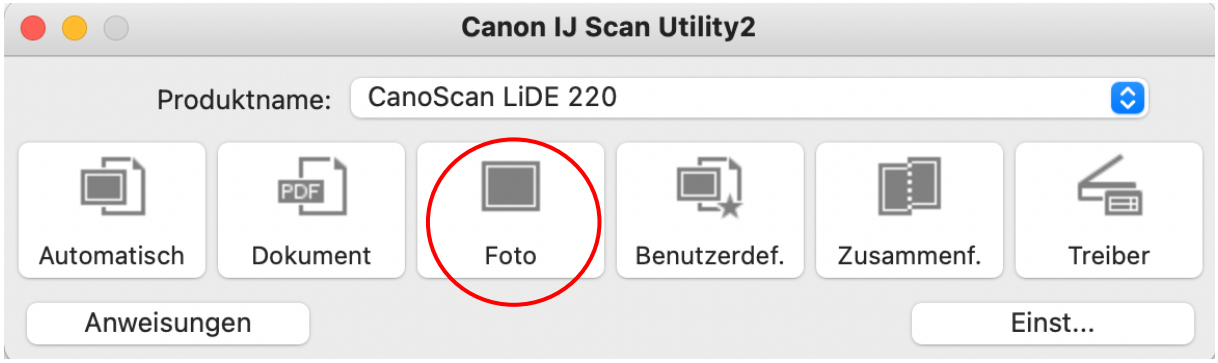
Operator: Two-letter code marking who scanned the seeds; first letter of first name, first letter of last name

Filled/ unfilled: 3 letters: unf for unfilled seeds, fil for filled seeds

Part of sample: A sample with a large number of seeds may have to be split into several scans as per 2.a. The first scan is always labelled a, if more are needed proceed with b, c,…

Mix: start with m1 when scanning a batch for the first time. When the seeds are scanned the same batch will be scanned again (see e)). The name will be the same except that the mix will be designated m2.

d) Click OK, then click the icon “Foto”; make sure the lid of the scanner stays closed.



e) When opening the lid, make sure no seeds get lost. Move them around on the scanner surface, altering their placement, orientation, and which side is facing upwards. Scan them again (the name should now end in m2). A minimum of 2 scans per sample should be done.

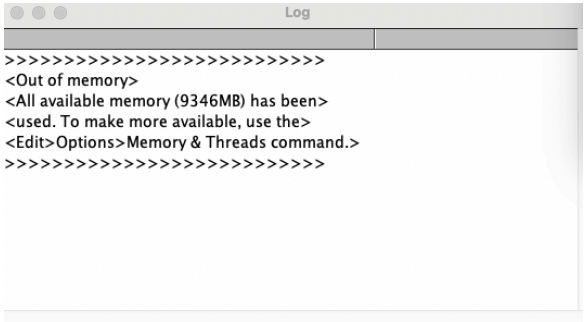
3) Analysing the Scans

Use imageJ for measurement (seed species identification). How many images from the “Scans” folder can be run through the following code at once depends on the allocated RAM of the computer, though 60-80 is a guideline. Step c) explains how to check if all scans could be analysed.

a) Open the ImageJ/ Fiji macro “measuring_bw.ijm”. If asked with which program to open the file choose Fiji.

b) Click on “Run”, then choose the folder “Scans” containing the scans. Should there be an error with opening the file, save the folder “Scans” to the desktop and try again.

c) Make sure the program could finish running through all scans and says command finished, not aborted or out of memory. Out of memory would result in a log report like this:



You can also check the file name of the last scan that was analysed and the last scan in the folder. In case it could not run through all scans, repeat the process on the left-over ones (run code on a folder that only contains those).

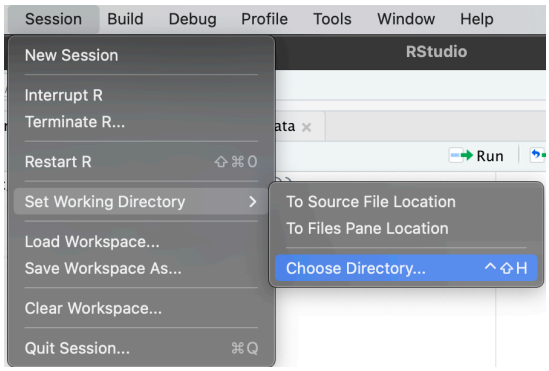
d) The csv file with the measurements will be in the same folder as the scans.

e) To close the open Scans, quit ImageJ entirely and **do not save the changes**!

f) Drag the Measurements.csv file from the subfolder “Scans” to the general folder “Seed_Analysis”.

4) Open the R file “Seed_Classification.R” in Rstudio.

a) Set your directory to the correct folder containing the Measurements.csv file (“Seed_Analysis”). You can either type it out in the first row or go via session > set working directory > choose directory as per the image below.



b) Run the entire code (press control+shift+enter or select all of it and click “run”).

c) Retrieve the file “summary.csv” from the folder “Seed Analysis” and save the it.

d) Retrieve the file “Measurements.csv” from the folder “Seed Analysis” and save it.

e) Remove the scans from the folder “Scans” and save them.

Notes on Running the color version:

For the colored analysis, ‘Macro_color_padded_com.ijm’ has to be run first in order to cut the scan into images with one seed on each. The measurements can then be obtained with ‘Measurements_from_padded_color_com.ijm’ and the color statistics with ‘Whole_Color_Macro_com.ijm’. Analysis can be done with the R file ‘Seed_Classification_Color_com.R’.

Notes on applying this to a different dataset:

Ensure that enough samples of every species as well as all locations are present in the training dataset. Location names need to be written in exactly the same way (letters and numbers) in the training dataset as in the scan file names.