VI. Counting Organisms Growing on a Disc Using ImageJ (Manual): Photomicrographs

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This tutorial will help you analyse pictures taken under the stereomicroscope. The object we will analyse here is a rectangular substrate overgrown with the tube-dwelling polychaete, *Polydora ciliata* (picture below).

As always, it is important to take a photo of a reference scale under the same magnification as the pictures you are going to analyse (cf. tutorial **Microscopy and ImageJ**). Here, both the reference scale and the disc were immersed in water to minimise the effects of refraction on apparent size. Take at least 10 photos of random points on the substrate.

The rectangular substrate overgrown with the polychaete

A close-up of the polychaete

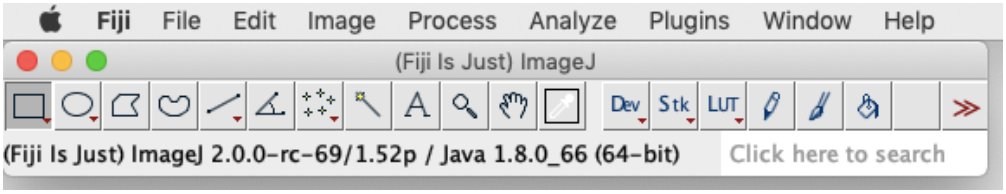
We are going to estimate how many polychaetes are growing on the entire surface of the substrate.

(Note: in this tutorial you will only practice the method with one single photograph and then we will proceed to explain how the results from many photos are analysed.)

1. Download the version of Fiji ImageJ which is compatible with the operating system of your computer from this website: https://imagej.net/Fiji/Downloads

   (The following description refers to Version 2.0.0 of ImageJ as of 2018-12-04 (check in Menu **Fiji - About ImageJ**). If a different version is used, some modifications may have to be made, but in principle the procedure should still be applicable.)

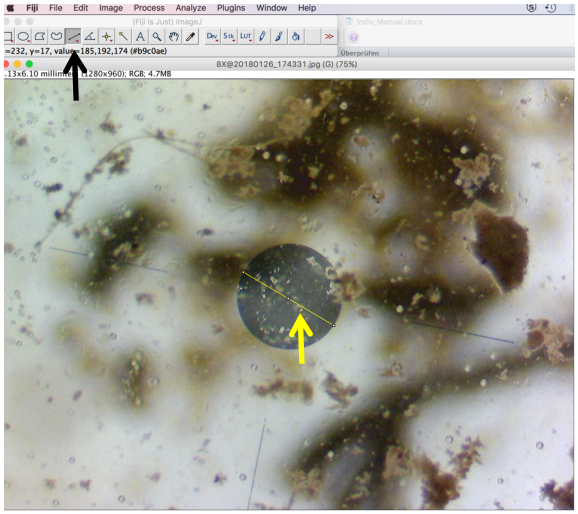
2. Open the program Fiji and you will see the tool selection bar under the menu bar. (Figures in this tutorial are from the Mac version. Under Windows or Linux, details may look different).



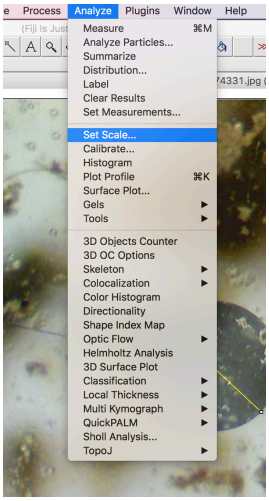
(If you are working with a freshly installed version of Fiji, no adjustments need to be made. If you have used this version of Fiji before and changed some settings in **Edit - Options**, you should go to **Edit - Options - Reset,** confirm the reset and restart Fiji to make sure you are using the default settings required in this tutorial.)

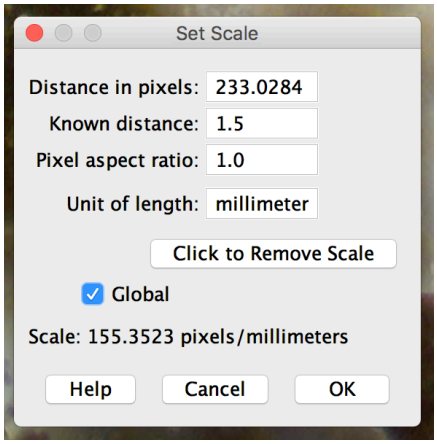
3. Set the scale. Load the picture of your reference scale by dragging the file and dropping it on the tool selection bar. In our example (file 8X@20180126_174331.jpg in the collection of practice images) we have a dot with a known diameter of 1.5 mm from a Motic 4-dot calibration slide.

Choose the **"Straight"** tool (black arrow) from the tool selection bar and draw a straight line across the diameter of the dot (yellow arrow).



4. Click on **Analyze – Set Scale** in the menu bar and fill in the information on **"Known distance"** (1.5) and **"Unit of length"** (mm). Select **Global** to use this scale for all the images you are going to analyse. Confirm with **OK**. Close the image of the reference scale.

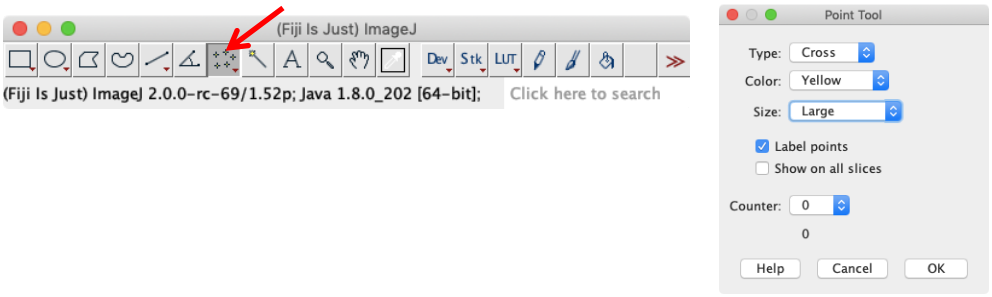




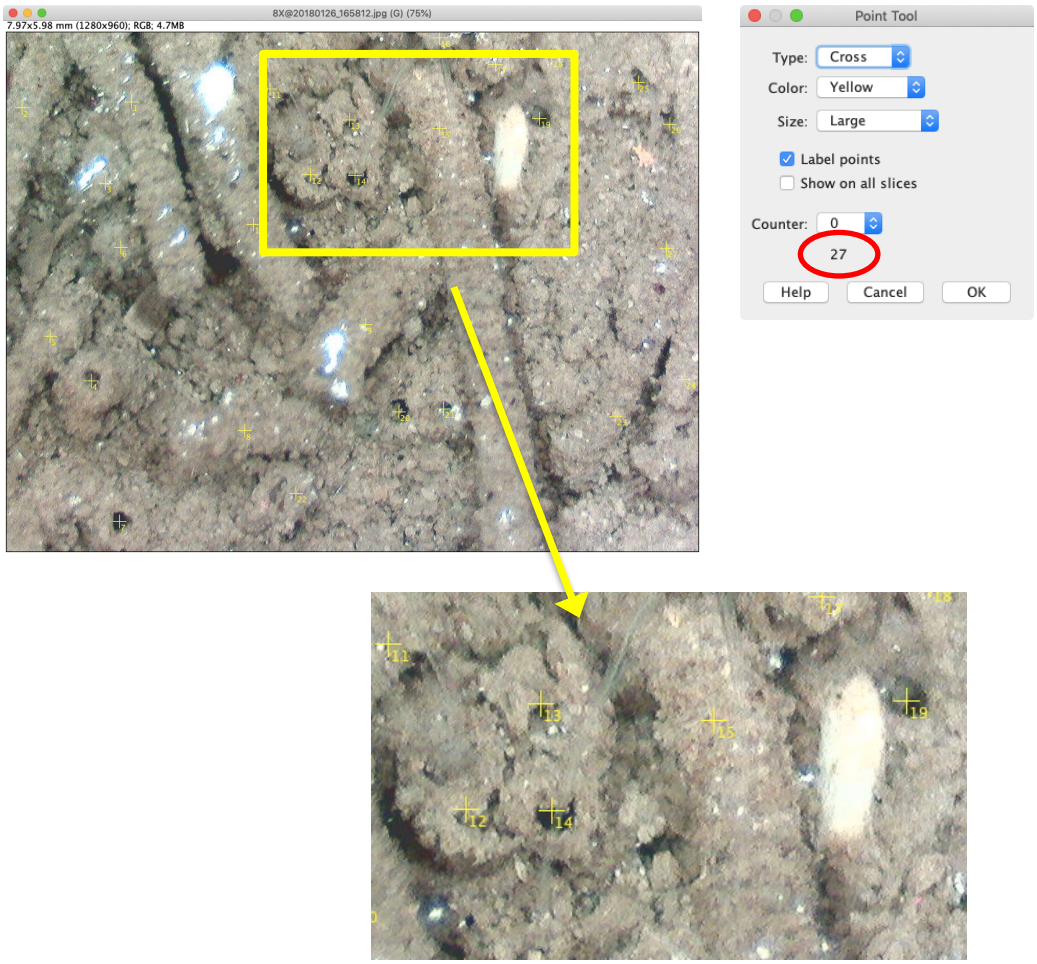
5. Open the first image by clicking on **File – Open** and choosing the appropriate file. Alternatively, you can also open an image in ImageJ by dragging the file directly onto the tool selection bar.

The number in the left upper corner of the image (arrow) displays the dimensions of the image. From this you can calculate the area of the substrate captured in your image. In our example: 8.14 mm x 6.10 mm = 49.65 mm^2.

6. Count the organisms. Double click on the **"Multi-Point"** tool in the tool selection bar (arrow). A window will appear. Enter your choices. For our example the settings are: Type: **Cross**; Color: **Yellow** and Size: **Large.** Activate **Label points**. **Do not** click **"OK"** because you still need the window to show your counts.



7. Start counting by clicking on the individuals on the image. Count the holes and the tubes (if the holes are not visible). For each click a crosshair with a number will appear on the image. This will prevent you from counting the same individual twice. In the Point Tool window, your total count (red oval) is shown below the counter number.



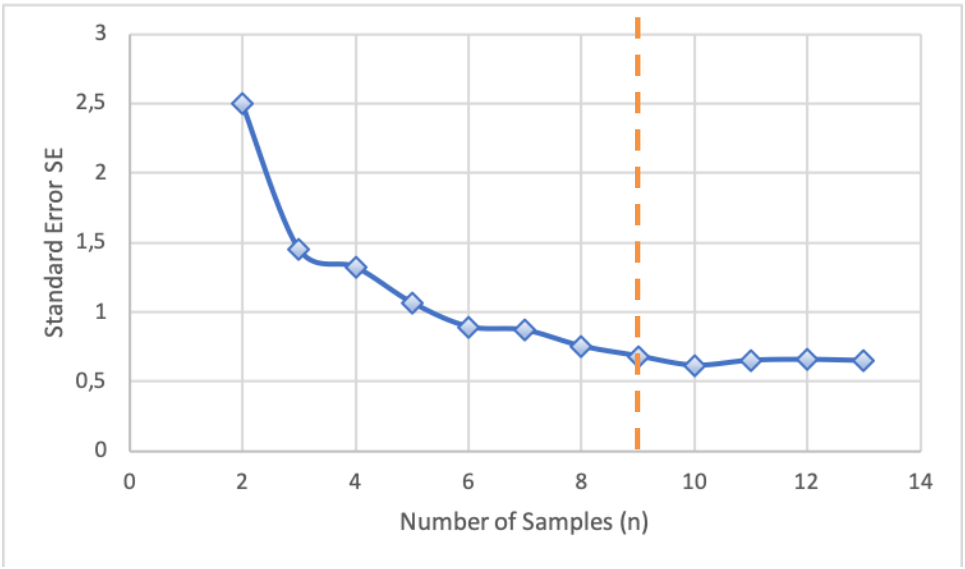
8. Analyse the other images. After finishing one image, note down the count and enter it in a table. You can then close the finished image. The counter will reset with a new image.

9. Load the next image and proceed as in Steps 7 and 8.

10. Determine if your sample size is sufficiently large for stable results. (See tutorial on **"Determining Sample Size"**). If the values you obtain are close to each other, it may suffice to count 10 images. For our example, we counted 13 images. The values obtained are given in the table below:

Image Number / Sample Number (n)	Count (Individuals)	Cumulative Average	Cumulative Standard deviation (s)	Standard Error (SE) = s/√n
1	27			
2	22	24.50	3.54	2.50
3	24	24.33	2.52	1.45
4	21	23.50	2.65	1.32
5	25	23.80	2.39	1.07
6	25	24.00	2.19	0.89
7	27	24.43	2.30	0.87
8	24	24.38	2.13	0.75
9	23	24.22	2.05	0.68
10	25	24.30	1.95	0.62
11	28	24.64	2.16	0.65
12	28	24.92	2.27	0.66
13	28	25.15	2.34	0.65
Total individuals	327			

11. Plotting SE against n, we obtain the curve below. For our example, we should count at least 9 images for a reliable sample size.



12. Calculate the total number of individuals on the sample substrate.

- Total area analysed **(TAA)** = 49.65 mm^2 x 13 = 645.45 mm^2 = 6.45 cm^2 (see Step 5)
- Total number of individuals in the 13 images **(TN)** = 327 individuals
- Extrapolate the data for the total area of the substrate **(AS):**

  We have a substrate with an area of **AS** = 5.5 cm x 10.0 cm = 55.0 cm^2

  -> Total number of organisms growing on the substrate

  = (TN ÷ TAA) x AS

  = (327 individuals ÷ 6.45 cm^2) x 55 cm^2

  = 2788 individuals