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Thresholding Images for Rhizovision Root Trait Analysis

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We use this protocol and it's working

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Abstract

Members of the Tumber-Dávila Lab developed this protocol for preparing and analyzing archival scans of fragmented roots. The protocol provides directions to compile, crop, and clean scans in Fiji (ImageJ) and analyze scans. Refer to the Tumber-Dávila Lab Workspace for related protocols on scanning roots.

Before start

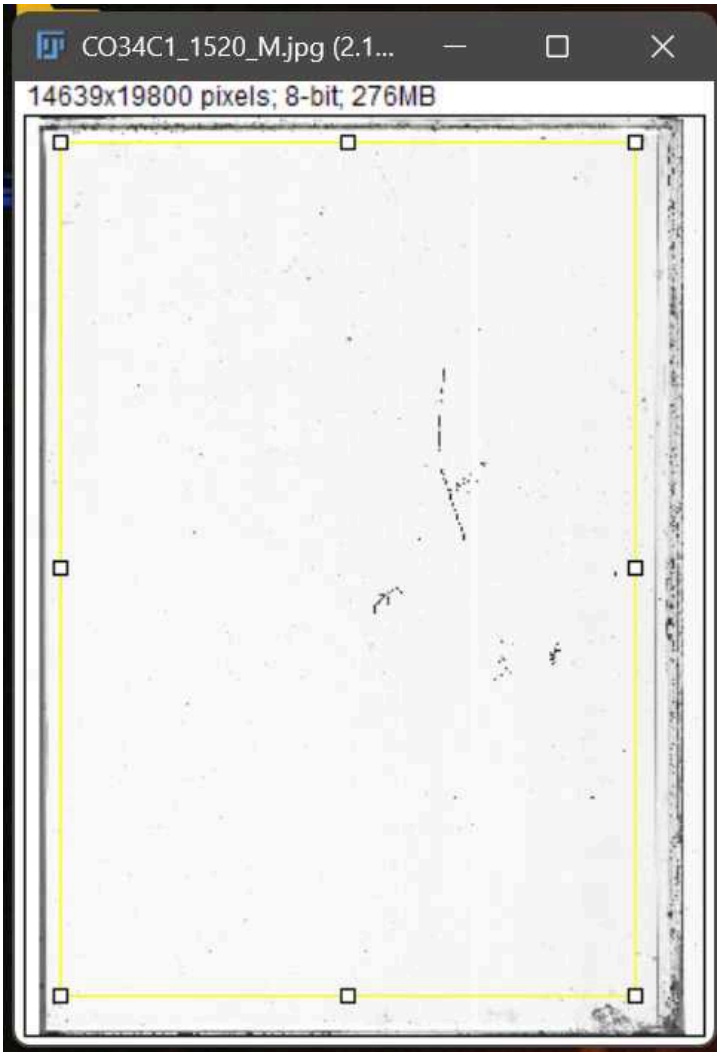
Download Fiji. (https://imagej.net/software/fiji/downloads)
Download Rhizovision (https://www.rhizovision.com/installation)

Removing the borders (using Fiji)

1 Open Fiji (Schindelin et al. 2012).

2 Import the images that need cropping: File→ Import→ Image Sequence. Once a white box pops up, choose the folder with the images and check the box for virtual stack (the scans are big, and Fiji doesn't have enough space for them) → OK.

3 Use the rectangle feature (top left corner) to delineate the area that needs to be cleared by dragging the rectangle to an appropriate position that removes the edges of the image.



Note

There are arrows on the bottom of the box so you can see how the rectangle fits the different images and you can adjust the rectangle accordingly.

4 Get its coordinates: Edit→ Selection→ Properties (check the box that lists the coordinates) → OK. You will get x, y coordinates for the four corners of your rectangle.

Note

Be careful not to crop any roots in the clearing process.

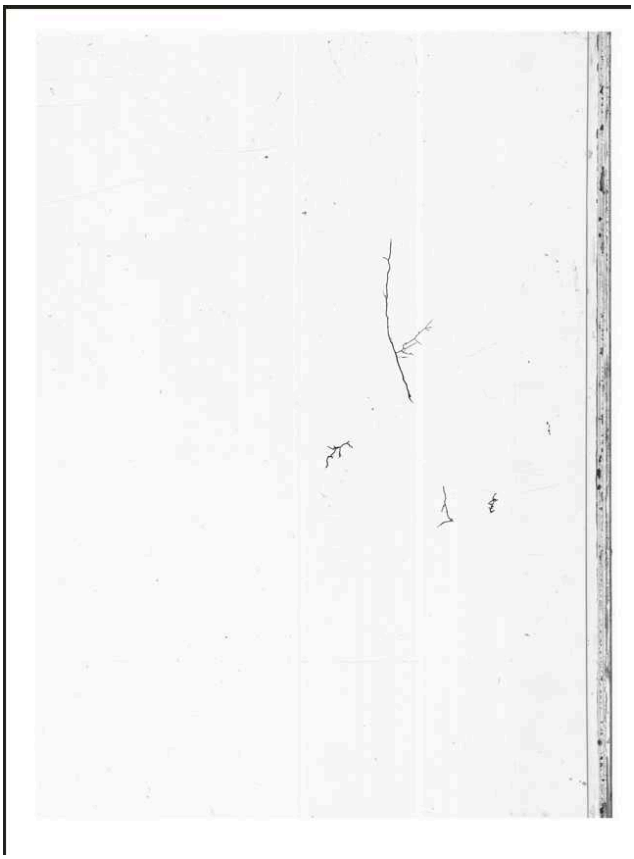
5 Once you have the coordinates, go to Process→ Batch→ Macro. Choose your input and output folders. Write the following code in the box and hit process (Images will save to the output folder).

```
// Set background color to white
setBackgroundColor(255, 255, 255);  #When you clear the borders,
it will replace the space with white instead of black, which is
the default color.

// Create rectangle selection
makeRectangle(x, y, width, height); # width = x2-x1, height= y2-y1

// Clear everything outside the rectangle
run("Clear Outside");
```

6 Go through all the images to make sure all the borders are clear and identify shadows/stains; some borders will still be visible in the images because not all the images have the borders in the exact same place.



7 To remove stains and remaining borders, individually clear the images: File→ Open (select the image you want). Use the rectangle feature to select the part you wish to clear: Edit→ Clear. This will clear what's inside the box. Save the image: File→ Save as→ JPEG.

8 Once all the borders and stains are cleared, the images are ready for RhizoVision!

Thresholding of images in RhizoVision Explorer

9 Open RhizoVision Explorer (Seethepalli and York, 2020).

10 **Image pre-processing:**

Note

You should open a few images to try out different settings before committing to any particular settings. To do this go to File-> Open-> Image. Try a couple of different settings and hit the “Run analysis” button to see the output image and the features. Adjust as needed.
To test the settings, we selected 3 images of each species. To make sure that the settings work in all scenarios, test one images with a few small roots, one that is noisy (e.g., lots of shadows), and another with a lot of roots. You can also manually count the root tips to see if they match the output value.

10.1 Choose the broken roots analysis mode.

10.2 Choose to convert pixels to physical units and select the DPI of the images (We used 1200 DPI).

10.3 For the image thresholding level, find a number that removes noise/specs without removing small roots. In our case, the 140-thresholding level worked well. This thresholding level is not universal, and it might need adjustment depending on the DPI of the images being analyzed.

10.4 Choose to remove non-root objects smaller than 1 mm.

10.5 Choose to enable root smoothing: Thresholding Level 2.

11 **Feature extraction:**

11.1 Choose to enable root pruning to erase false root tips. In our case, Threshold Level 5 was enough.

12 **Running the analysis:**

12.1 Once you have your settings, go to File→ Batch Analysis and choose the input and output folders. Check the box to save processed images and click the start button to run the analysis.

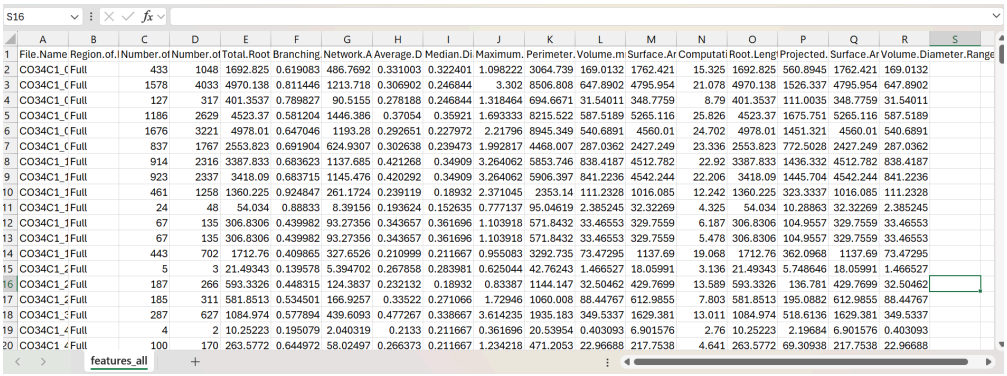
12.2 Let RhizoVision work its magic.

Note

The duration of this process will vary based on the number of scans and may take a couple of hours.

Output

13 An example of the output file is shown below:



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1	File.Name	Region.of.I	Number.of	Number.of	Total.Root	Branching.	Network.A	Average.D	Median.Di	Maximum.	Perimeter.	Volume.m	Surface.Ar	Computati	Root.Leng	Projected.	Surface.Ar	Volume.Diameter.Range	
2	CO34C1_0	Full	433	1048	1692.825	0.619083	486.7692	0.331003	0.322401	1.098222	3064.739	169.0132	1762.421	15.325	1692.825	560.8945	1762.421	169.0132	
3	CO34C1_0	Full	1578	4033	4970.138	0.811446	1213.718	0.306902	0.246844	3.302	8506.808	647.8902	4795.954	21.078	4970.138	1526.337	4795.954	647.8902	
4	CO34C1_0	Full	127	317	401.3537	0.789827	90.5155	0.278188	0.246844	1.318464	694.6671	31.54011	348.7759	8.79	401.3537	111.0035	348.7759	31.54011	
5	CO34C1_0	Full	1186	2629	4523.37	0.581204	1446.386	0.37054	0.35921	1.693333	8215.522	587.5189	5265.116	25.826	4523.37	1675.751	5265.116	587.5189	
6	CO34C1_0	Full	1676	3221	4978.01	0.647046	1193.28	0.292651	0.227972	2.21796	8945.349	540.6891	4560.01	24.702	4978.01	1451.321	4560.01	540.6891	
7	CO34C1_0	Full	837	1767	2553.823	0.691904	624.9307	0.302638	0.239473	1.992817	4468.007	287.0362	2427.249	23.336	2553.823	772.5028	2427.249	287.0362	
8	CO34C1_1	Full	914	2316	3387.833	0.683623	1137.685	0.421268	0.34909	3.264062	5853.746	838.4187	4512.782	22.92	3387.833	1436.332	4512.782	838.4187	
9	CO34C1_1	Full	923	2337	3418.09	0.683715	1145.476	0.420292	0.34909	3.264062	5906.397	841.2236	4542.244	22.206	3418.09	1445.704	4542.244	841.2236	
10	CO34C1_1	Full	461	1258	1360.225	0.924847	261.1724	0.239119	0.18932	2.371045	2353.14	111.2328	1016.085	12.242	1360.225	323.3337	1016.085	111.2328	
11	CO34C1_1	Full	24	48	54.034	0.88833	8.39156	0.193624	0.152635	0.777137	95.04619	2.385245	32.32269	4.325	54.034	10.28863	32.32269	2.385245	
12	CO34C1_1	Full	67	135	306.8306	0.439982	93.27356	0.343657	0.361696	1.103918	571.8432	33.46553	329.7559	6.187	306.8306	104.9557	329.7559	33.46553	
13	CO34C1_1	Full	67	135	306.8306	0.439982	93.27356	0.343657	0.361696	1.103918	571.8432	33.46553	329.7559	5.478	306.8306	104.9557	329.7559	33.46553	
14	CO34C1_1	Full	443	702	1712.76	0.409865	327.6526	0.210999	0.211667	0.955083	3292.735	73.47295	1137.69	19.068	1712.76	362.0968	1137.69	73.47295	
15	CO34C1_2	Full	5	3	21.49343	0.139578	5.394702	0.267858	0.283981	0.625044	42.76243	1.466527	18.05991	3.136	21.49343	5.748646	18.05991	1.466527	
16	CO34C1_2	Full	187	266	593.3326	0.448315	124.3837	0.232132	0.18932	0.83387	1144.147	32.50462	429.7699	13.589	593.3326	136.781	429.7699	32.50462	
17	CO34C1_2	Full	185	311	581.8513	0.534501	166.9257	0.33522	0.271066	1.72946	1060.008	88.44767	612.9855	7.803	581.8513	195.0882	612.9855	88.44767	
18	CO34C1_3	Full	287	627	1084.974	0.577894	439.6093	0.477267	0.338667	3.614235	1935.183	349.5337	1629.381	13.011	1084.974	518.6136	1629.381	349.5337	
19	CO34C1_4	Full	4	2	10.25223	0.195079	2.040319	0.2133	0.211667	0.361696	20.53954	0.403093	6.901576	2.76	10.25223	2.19684	6.901576	0.403093	
20	CO34C1_4	Full	100	170	263.5772	0.644972	58.02497	0.266373	0.211667	1.234218	471.2053	22.96688	217.7538	4.641	263.5772	69.30938	217.7538	22.96688	

features_all

Note

The output file includes features like number of root tips, total root length, average diameter, perimeter, volume, etc.

14 **Calculation of root traits:**

14.1 Calculate Specific Root Length (SRL) by dividing Total Root Length (listed in the output file) by the root dry weight.

14.2 Calculate Root Tissue Density (RTD) by dividing root dry weight by Volume (listed in the output file).

Protocol references

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