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Deep-resolution plant phenotyping platform description.

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ABSTRACT

DRPPP (Deep-Resolution Plant Phenotyping Platform) is a combination of protocols for plant tissue preparation, labeling, scanning, and open-source software to visualize and analyze 4D biological datasets. Here we describe a step-by-step procedure, including sample preparation and data analysis.

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KEYWORDS

Phenotyping, gene expression, epigenetics, Segmentation, cell cycle, 3D imaging

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BEFORE STARTING

Sample preparation.

The analysis of whole organs at sub-cellular resolution requires recording multiple tiles (typically 3-5), with a depth-dependent dynamic adaption of recording parameters. Thus, living objects (especially roots) were hard to label and produce high-quality images because of rapid growth and movement. So, although the pipeline does not preclude live imaging, high-quality analyses can only be performed on fixed tissue.

The platform includes four different tissue labeling options or their combinations: cell border labeling, nuclei labeling (including EdU labeling for cell cycle kinetics), gene expression (protein localization), and protein complex detection. All four options can be combined to study cell geometry/cell cycle/gene expression simultaneously. The quality of labeling is crucial for further analysis and can be reached by using our protocols (Pasternak et al., 2015,

2018).

Software:

1. Fiji: https://imagej.net/Fiji including bio-format importer and hdf5 plugin,
2. XUVtools stitching software (version 1.7.0) (http://www.xuvtools.org/doku.php?id=download) (Emmenlauer et al., 2009).
3. (https://lmb.informatik.uni-freiburg.de/lmbsoft/iRoCS/
4. **Manual**: https://lmb.informatik.uni-freiburg.de/lmbsoft/iRoCS/walkthrough.html

1 Image pre-processing pipeline:

1.1 Import .lsm/.tiff file to Fiji: Plugins -> Bio Formats -> Bio-Formats Importer -> select all relevant series -> OK

1.2 Export to HDF5: Plugins -> HDF5 -> Save to HDF5 (new or replace)

1.3 1.Stitching in XUV-tool: (recommended):Add file to the project-> select files you want to stitch-> Run stitcher-> ignore the current coordinates ->Run->Save as .hdf5 (do not use save the project; default saving option is .ims). When you scan simultaneously 2-3 channels, it is highly recommended to save the position of the tiles from the first channel (by click position editor and mention positions of each tile after stitching). When you stitched the second-third channels, use this information for each channel. In the case you stitched all channels automatically, you may have a slight differences in overlapping volume, which will affect your analysis in the future).

1.4 1.Alternatively, one can use Fiji 3D stitching option, but it allows to stitch only 2 tiles simultaneously and require much higher memory resources.

Stitch: Plugins -> Stitching -> deprecated -> 3D Stitching -> select first and second series -> OK, then repeat and select combined series and the third one, repeat until all the series are stitched together.

1.5 Note: we recommended to orientated the root horizontally, with the root tip on the left side.

2 Image analysis (segmentation/nuclei detection).

For the analysis, images must be imported to organ analysis software (https://lmb.informatik.uni-freiburg.de/lmbsoft/iRoCS/).
Channel-> import channel-> Ok
For better visualization, normalize button must be clicked and the signal should be adjusted by changes to gamma, alpha and choosing best color (figure 1 for clarity).
2-3-4 channels can be imported one by one.
After that, the corresponding plugin should be applied: Segmented Root or Detect Nuclei.

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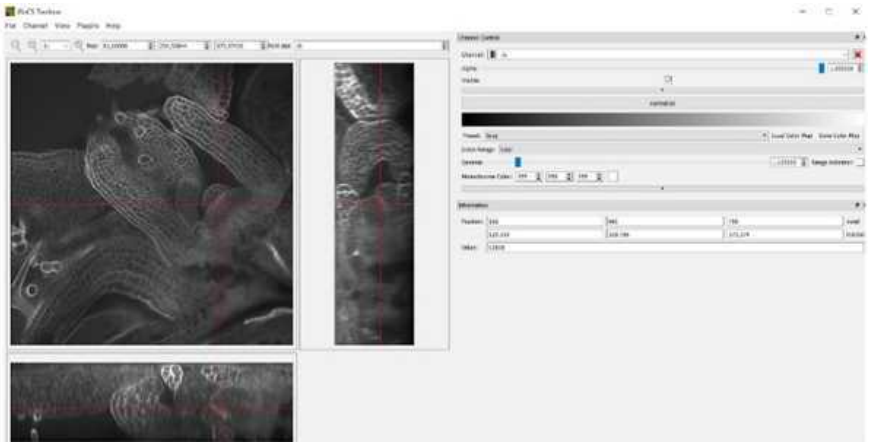

Figure 1. Example of software panel after importing channel with the Arabidopsis shoot.

Important notices:
NOTE1: IRoCS Toolbox is developed inside Freiburg University; hence its binaries are not signed for Windows SmartScreen. However, it is safe to run labelling.exe or other released executables, and their source code can be found on GitHub (https://github.com/lmb-freiburg/iRoCS-Toolbox). If Windows SmartScreen displays an information window when running labelling.exe, saying "Windows protected your PC", you can click "More Info", and then "Run Anyway".
NOTE2: you can visualize every step, View>Log.
NOTE3: make sure that you save your images in every step that you made a modification; the correction can NOT be recovered.
NOTE4: it is recommended to save the file after labeling it with a new name. After channel rename, both channels have been saved, which is significantly increases the file size.

3 Detailed protocol for segmentation step by step:

3.1 **Load HDF5 file**: Channel -> Import Channels -> select file -> select t0/channel0

3.2 **Perform segmentation**: Plugins -> C01 Segment root -> for (THIS TYPE OF ROOT) adjust processing element size to 0.5-0.7 range for reasonable runtime and results. Smaller element size will produce better segmentation but consumes significantly more time and computational resources. Examples of recommended parameters are in Figure 2.

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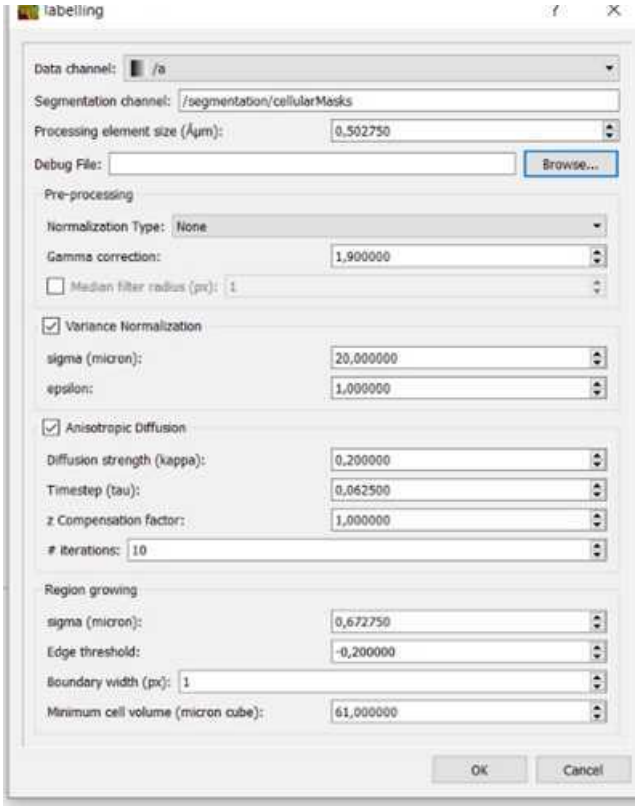

Figure 2. Possible segmentation parameters. Processing element size can be 0.5-0.7; Gamma corrections are dependent from the image quality and can be in the range 1-2.

3.3 **Optionally remove irrelevant segments**: while holding Shift, right-click on black background, then left-click on the segments to remove. Make correction of over-segmented cells: while holding Ctrl, right-click on one fragment, move the cursor to the fragment you want to fuse, and left-click on it. Examples are in figure 3.
NOTE: *This step is important if your images have particles or undesirable elements in your samples. Removing this issue helps to get a faster analysis and clear images. To remove this issue, move the bars and red lines in all the images and make sure to do not have large irrelevant segments.*

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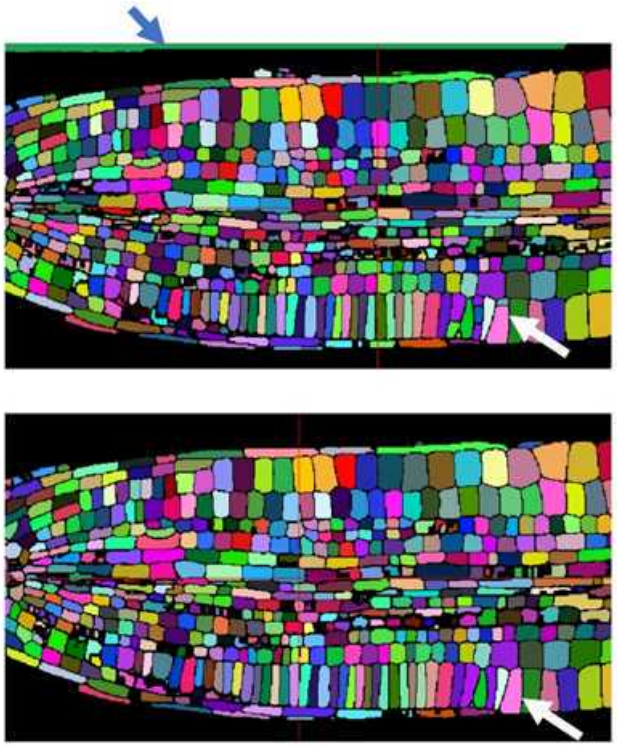

Figure 3. Example of the segmentation corrections. Blue arrow-irrelevant segments; white arrow- correction of the over-segmented cell. This option available only in version 1.2.4.

3.4 **Add QC marker**: Channels -> New annotation channel -> drop point in the place of QC-> optionally rename to QC

3.5 **Attach axis**: Plugins -> C02 Attach iRoCS to segmented cells -> select segmentation/cellular Masks channel as segmentation channel, the previously created channel with the marker as QC channel.

3.6 **Convert mask to a marker** by using the corresponding plugin: choose connected component labeling and choose cells as marker type, start processing.

3.7 **Assign layers***:* For Arabidopsis root: Plugins -> C03 Assign layers to segmented cell -> chose model files (download from website to your folder) Plugins -> OK

For the other root/organs you can do it manually: In the window *channel control,* you can move the channel "/cellularMasks" and after "/annotation/segmented" and select "visible". Then in view type, choose a "label" and operation choose "label marker". In section "marker presets," choose a label marker design or assign a new one and choose the "cell type" for a label.
Every time that you click over you selected cells, they will have a number and the contour of the cell the color that you selected.

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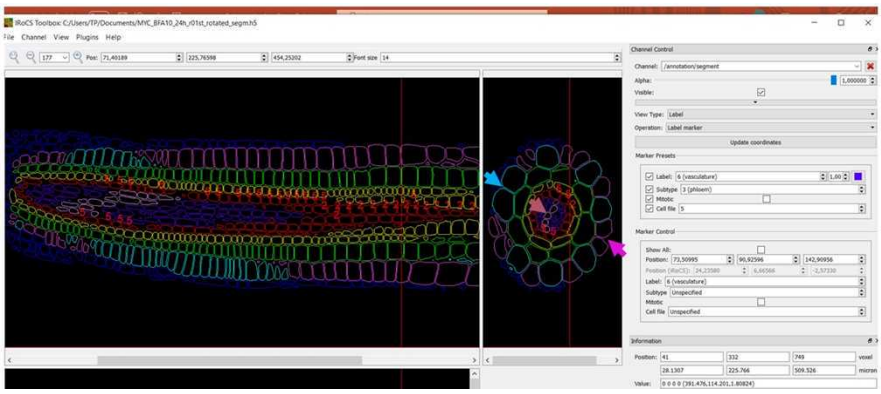

Figure 4. Example of assigned layers. Vasculature labeling with cell sub-type *arraow) and visible root asymmetry in epidermis - blue and magenta arrows. Quantitative geometry of each cells come in step 3.9.

3.8 **Subtype and cell file labels**:In section “marker presets,” choose “subtype” and click over your selected cells. For the cell files, choose “cell file” and click on the cell you wish. Later on, you can recognize these cells in .csv file. See Figure 4 for visualization.

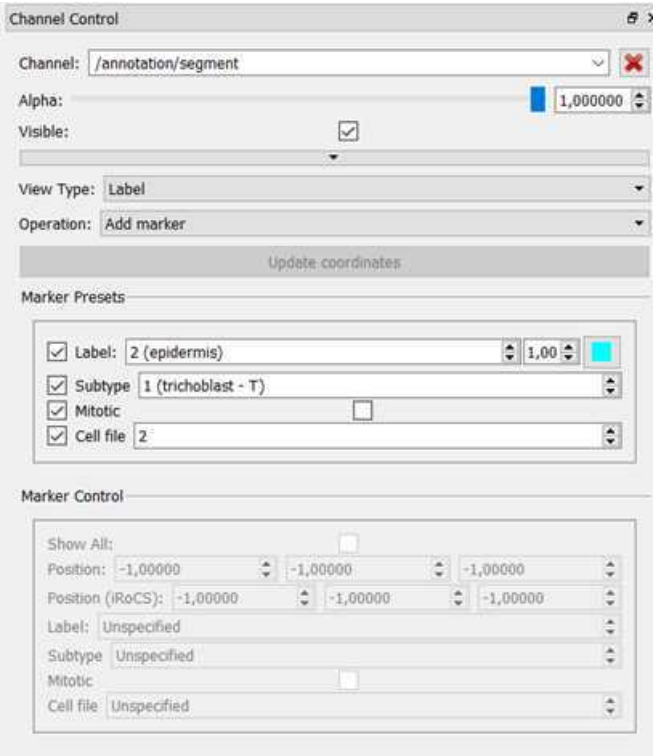

Figure 5. Panel “Channel control” example.

3.9 **Export results.** Channel>Export annotation channel to CSV.

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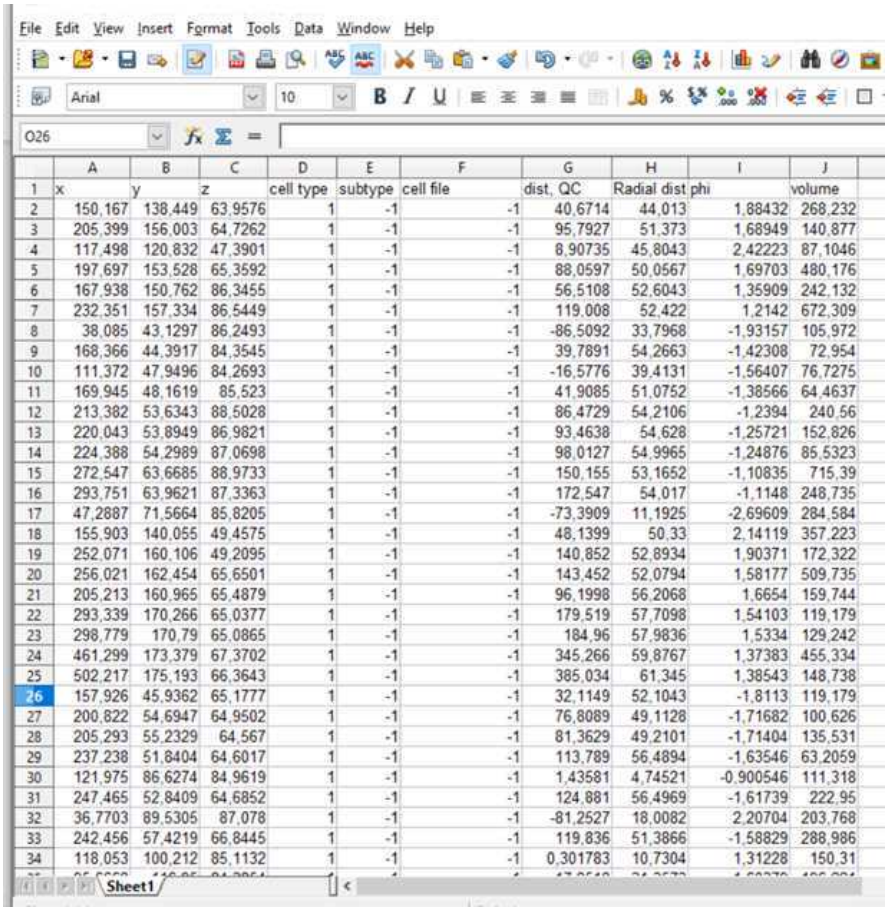

	A	B	C	D	E	F	G	H	I	J
1	x	y	z	cell type	subtype	cell file	dist, QC	Radial dist	phi	volume
2	150,167	138,449	63,9576	1	-1	-1	40,6714	44,013	1,88432	268,232
3	205,399	156,003	64,7262	1	-1	-1	95,7927	51,373	1,68949	140,877
4	117,498	120,832	47,3901	1	-1	-1	8,90735	45,8043	2,42223	87,1046
5	197,697	153,528	65,3592	1	-1	-1	88,0597	50,0567	1,69703	480,176
6	167,938	150,762	86,3455	1	-1	-1	56,5108	52,6043	1,35909	242,132
7	232,351	157,334	86,5449	1	-1	-1	119,008	52,422	1,2142	672,309
8	38,085	43,1297	86,2493	1	-1	-1	-86,5092	33,7968	-1,93157	105,972
9	168,366	44,3917	84,3545	1	-1	-1	39,7891	54,2663	-1,42308	72,954
10	111,372	47,9496	84,2693	1	-1	-1	-16,5776	39,4131	-1,56407	76,7275
11	169,945	48,1619	85,523	1	-1	-1	41,9085	51,0752	-1,38566	64,4637
12	213,382	53,6343	88,5028	1	-1	-1	86,4729	54,2106	-1,2394	240,56
13	220,043	53,8949	86,9821	1	-1	-1	93,4638	54,628	-1,25721	152,826
14	224,388	54,2989	87,0698	1	-1	-1	98,0127	54,9965	-1,24876	85,5323
15	272,547	63,6685	88,9733	1	-1	-1	150,155	53,1652	-1,10835	715,39
16	293,751	63,9621	87,3363	1	-1	-1	172,547	54,017	-1,1148	248,735
17	47,2887	71,5664	85,8205	1	-1	-1	-73,3909	11,1925	-2,69609	284,584
18	155,903	140,055	49,4575	1	-1	-1	48,1399	50,33	2,14119	357,223
19	252,071	160,106	49,2095	1	-1	-1	140,852	52,8934	1,90371	172,322
20	256,021	162,454	65,6501	1	-1	-1	143,452	52,0794	1,58177	509,735
21	205,213	160,965	65,4879	1	-1	-1	96,1998	56,2068	1,6654	159,744
22	293,339	170,266	65,0377	1	-1	-1	179,519	57,7098	1,54103	119,179
23	298,779	170,79	65,0865	1	-1	-1	184,96	57,9836	1,5334	129,242
24	461,299	173,379	67,3702	1	-1	-1	345,266	59,8767	1,37383	455,334
25	502,217	175,193	66,3643	1	-1	-1	385,034	61,345	1,38543	148,738
26	157,926	45,9362	65,1777	1	-1	-1	32,1149	52,1043	-1,8113	119,179
27	200,822	54,6947	64,9502	1	-1	-1	76,8089	49,1128	-1,71682	100,626
28	205,293	55,2329	64,567	1	-1	-1	81,3629	49,2101	-1,71404	135,531
29	237,238	51,8404	64,6017	1	-1	-1	113,789	56,4894	-1,63546	63,2059
30	121,975	86,6274	84,9619	1	-1	-1	1,43581	4,74521	-0,900546	111,318
31	247,465	52,8409	64,6852	1	-1	-1	124,881	56,4969	-1,61739	222,95
32	36,7703	89,5305	87,078	1	-1	-1	-81,2527	18,0082	2,20704	203,768
33	242,456	57,4219	66,8445	1	-1	-1	119,836	51,3866	-1,58829	288,986
34	118,053	100,212	85,1132	1	-1	-1	0,301783	10,7304	1,31228	150,31

Figure 6. Example of extracted data in .csv file.

4 Detailed protocol for nuclei analysis step by step:

4.1 **Load HDF5 file**: Channel -> Import Channels -> select file -> select t0/channel0 channel

4.2 **Detect nuclei**: Plugins -> 01 Detect Nuclei -> as a model use corresponding file from web-site

4.3 **Label epidermis:** Plugin -> 02 Label Epidermis -> as a model, use file from the website (Arabidopsis only) or label manually at least 50 randomly chosen epidermis cells.

4.4 **Add QC marker**: Channels -> New annotation channel -> drop point in the place of QC-> optionally rename to QC

4.5 **Attach axis**: Plugins -> 03 Attach iRoCS -> select annotation channel as annotation/detector and as QC previously created a channel with the marker as QC channel.

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4.6 **Assign layers**: Plugins -> 04 -> select model file (corresponding model from website), "Layer assignment " file (direct link https://lmb.informatik.uni-freiburg.de/lmbsoft/iRoCS/wt-model.rf.h5)

4.7 **Export results.** Channel>Export annotation channel to CSV.

5 Corrections of the layers after segmentations and nuclei labeling.

Models for segmentation, nuclei detection, and layers labeling were created for high-quality images for the root of 4 days old Arabidopsis plants. If you image quality or age of the plants (species) are different, you must use manual corrections for the layers.
Nuclei corrections: choose annotation channel, label marker, and changes manually layer label.

6 3D-rendering.

3D rendering allows you to visualize organ structure as layers by layers by animation. View>shown open GL rendering.
For the segmented object, version 1.2.1 is optimal, while for the nuclei, version 1.2.4 is only abundant.

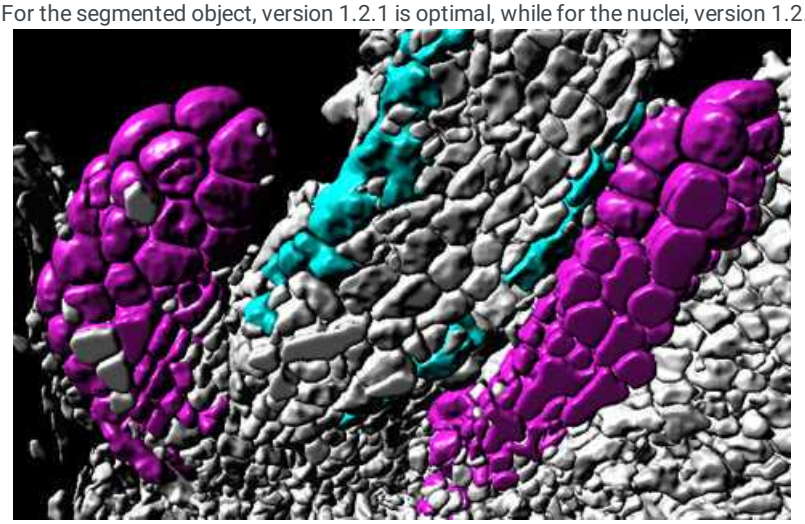

Figure 7. Example of the stipule rendering

7 Extraction of 3D cell structure for 3D printer.

File>export as analyze and STL
This plugin converts your data to the .stlfile. Cell with the same label convert in the same file. 3D structure of each cell is available.

8 Data extraction/presentation.

After corrections, you need to export annotation channels to .csv and process them in excel. In an excel file.

From the segmentation, you will have X, Y, Z as a position of center mass of the cells in the whole frame; label as the cell file number; distance from QC in µm, radial distance in µm, subtype; mitotic; cell file and cell volume.

From the nuclei labeling, you will have: X, Y, Z as a position of the nuclei in the whole frame; label as the cell file number; distance from QC in µm, radial distance in µm, nuclei radius; subtype; mitotic; cell file.

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In the case of the root analysis, each cell/nuclei have three basic coordinates: distance from QC, radial distance, and radians (un-rolled organ). We would suggest using in the graphical presentation un-rolled root, e.g., radians on the x-axis and distance from QC as the y-axis. Volume can be embedded as third coordinates for the cells as well as for nuclei.

9 Statistical analysis.

The platform allows us to extract at least all parameters from 5000 cells from Arabidopsis and 30000 cells for large roots (tobacco, tomato, millet). In the commonly used method, authors analyzed maximal 100 cells per root. The platform allows to collect more detailed information, which helps to get deeper insight of developmental processes as well as helps better understanding of plant responses to environmental stimuli.

10 Literature.

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