Video Article

Extracting Metrics for Three-dimensional Root Systems: Volume and Surface Analysis from In-soil X-ray Computed Tomography Data

Niraj Suresh[1], Sean A. Stephens[1], Lexor Adams[1], Anthon N. Beck[1], Adriana L. McKinney[1], Tamas Varga[1]

[1]Environmental Molecular Sciences Laboratory

Correspondence to: Tamas Varga at Tamas.Varga@pnnl.gov

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Abstract

Plant roots play a critical role in plant-soil-microbe interactions that occur in the rhizosphere, as well as processes with important implications to climate change and crop management. Quantitative size information on roots in their native environment is invaluable for studying root growth and environmental processes involving plants. X-ray computed tomography (XCT) has been demonstrated to be an effective tool for *in situ* root scanning and analysis. We aimed to develop a costless and efficient tool that approximates the surface and volume of the root regardless of its shape from three-dimensional (3D) tomography data. The root structure of a Prairie dropseed (*Sporobolus heterolepis*) specimen was imaged using XCT. The root was reconstructed, and the primary root structure was extracted from the data using a combination of licensed and open-source software. An isosurface polygonal mesh was then created for ease of analysis. We have developed the standalone application *imeshJ*, generated in *MATLAB*[1], to calculate root volume and surface area from the mesh. The outputs of *imeshJ* are surface area (in mm^2) and the volume (in mm^3). The process, utilizing a unique combination of tools from imaging to quantitative root analysis, is described. A combination of XCT and open-source software proved to be a powerful combination to noninvasively image plant root samples, segment root data, and extract quantitative information from the 3D data. This methodology of processing 3D data should be applicable to other material/sample systems where there is connectivity between components of similar X-ray attenuation and difficulties arise with segmentation.

Video Link

The video component of this article can be found at https://www.jove.com/video/53788/

Introduction

Roots, as part of the rhizosphere[2-5], represent an "invisible" part of plant biology since soil makes it difficult to image roots non-invasively [6, 7]. However, studying root growth and interaction within the soil environment is critical to understanding root/plant growth and nutrient cycling, which in turn affect forestation, food security, and climate. X-ray computed tomography (XCT) has proven to be a valuable tool for noninvasive imaging of plant root samples in their local environments [8]. In order to measure root development and dimensional changes under different conditions, and be able to compare data from different datasets/specimens, one needs to extract quantitative information from the tomography data. Segmentation of the root data from that of the surrounding soil, that is, the isolation of the root image from everything else around it (including, for example, a neighboring plant) is a critical step before accurate size analysis can be done. However, a simple thresholding approach is often unfeasible for root data. The challenges associated with imaging plant roots in soil include variations in the X-ray attenuation properties of the root material, and the overlap in attenuation values between root and soil caused by water and organic matter. These issues have been superbly addressed recently by Mairhofer *et al*. in their visual tracking tool RooTrak [7, 9]. The next step after a successful segmentation is the accurate determination of root volume and surface area. The volume may be estimated by counting the number of voxels and multiplying by the voxels' size cubed as shown before[7]. For a more accurate determination of root surface area and volume, the isosurface of the segmented root system can be represented by a mesh of triangles, using an algorithm known as Marching Cubes [10]. The open-source ImageJ[11] can be employed to approximate the root volume based on the Marching Cubes algorithm. To the best of our knowledge, only a limited number of open-source software dedicated to calculating tomography-based volume/surface data for root specimens in the centimeter range and above is currently available [12]. One open-source software we looked at[13] focuses on root growth and is aimed at cellular features enabling quantitative volume analysis at single-cell resolution. Some open-source software dedicated to whole root systems[14] is excellent for small-diameter tubular root systems based on the approximation that their shape is actually tubular. However, some work with 2D images and are unable to handle 3D stacks[14]. Furthermore, the tubular shape approximation may not be valid when root systems with rough surfaces and non-uniform shapes, such as those of trees, are studied. Another approach[15] uses two-dimensional (2D) rotational image sequences innovatively circumventing the need for a costly CT scanner. It measures, records, and displays root system lengths. The software we have tested from those only available commercially[16-18]; one does not appear to be able to handle 3D image stacks[16], the second is a leaf area and root length measurement tool[17], while the third is based on color analysis[18]. Based on this survey, we suggest that a costless option that approximates the surface and volume of the root regardless of its shape from 3D tomography data is desirable.

Building on the freely available RooTrak and ImageJ, we have developed a program, named *imeshJ* (see Supplemental Code File) which processes an isosurface mesh (surface stereolithography file) generated from segmented root data, and calculates the volume and surface area of the root by doing simple geometrical calculations on the mesh triangle index data. Here we report a method that combines the use of XCT imaging, data reconstruction and visualization (software CT Pro 3D and VG Studio), segmentation of the root of the specimen from the soil in the 3D data (open-source software ImageJ and RooTrak), and extraction of the surface and volume information from a triangular mesh (ImageJ and the computer code *imeshJ*).

Protocol

Caution: The operation of an X-ray tomography scanner requires both general radiation training, and instrument-specific radiation safety training. All corresponding procedures relevant to the experimenter's laboratory should be followed.

1. Root Imaging

Note: This step describes the imaging of a grass specimen held in its original soil in a tubular plastic pot (a plastic tube with a diameter of 40 mm, a height of 210 mm, and wall thickness of about 2 mm).

1. Place potted plant on the sample manipulator of the instrument at a distance desired for target magnification. For a plant in a 2-inch diameter holder, the sample to source distance should be about 3 inches (7 cm).
2. Adjust the X-ray scan settings to achieve optimal color (grey level) contrast in detector image. Note: These settings are available in the instrument control software used.
    1. Set X-ray power settings; 85 kV and 190 µA were used in this example.
    2. Set exposure time. Here, a relatively long exposure time of 1 sec was used for better signal-to-noise ratio.
    3. Set number of projections and frames per projection; 4 frames per projection for a total of 3,142 projections is suggested for good data statistics.
    4. Run a shading correction using the measurement conditions set above by selecting the "Shading correction" tab, and clicking "Create". Note: The shading correction compensates for the variation in response of the imaging device's pixels when illuminated with a constant set of X-ray flux. The process takes blank images (with sample removed from beam path) with the X-ray beam turned on, and with the beam turned off. This correction is applied to all images collected.
    5. Select the "Minimize ring artefacts" option (also called "shuttling mode"); the sample will be rotated in angular steps whilst the projection images are acquired. This leads to data acquisition at a slower rate, but helps eliminate ring artefacts.
    6. Start the scan by clicking the "Acquire" button under the Acquisition tab (with the settings outlined above, image collection will take approximately 4 hr).

2. Data Reconstruction

Note: This section describes the reconstruction of 3D volume data from the raw images (radiographs from the CT scan).

1. Load the raw data into the program.
2. Compare first and last image (they should be nearly identical as the last image is taken after a 360^o rotation of the specimen) to make sure the specimen did not move or the scan settings did not change during data acquisition.
3. Calculate the center of rotation (COR) by selecting the "Center of rotation" tab, and clicking "Start"; use options "Automatic" COR finding with "High Quality" accuracy, and "Dual" (upper and lower) slice selection for COR calculation.
4. Select the sample volume to be reconstructed: select the "Volume" tab, and edit the volume selection windows using the thumbnails.
5. Perform reconstruction to create the volume file containing 3D data by clicking "Start".

3. Data Processing / Segmentation

Note: This section describes the steps to be taken to prepare the reconstructed data for further processing in the program RooTrak to track roots as they branch out through soil, and isolate the roots from any surrounding material to produce a stack of binary images of just the root itself.

1. Processing of volume data in ImageJ to prepare a RooTrak processable image stack:
    1. Load the volume file into ImageJ.
    2. Optimize image contrast between root and soil by adjusting brightness and contrast settings (Click Image/Adjust/Brightness/Contrast). When the region of interest within the image is visible and clearly distinguishable, the settings are considered optimized.
    3. Save as an image stack in jpeg, bmp, or png format.

2. Processing in RooTrak to segment the root:
    1. Load image stack into RooTrak (go to "Tools" tab, and press "Tracker").
    2. Set seed points inside root: click several points inside each of the pertinent root sections visible in the top view slice of the volume data.
    3. Set tracker parameters "Smoothness" and "Similarity" to 0.3 and 0.8, respectively.
    4. Run the tracking function. This will follow the root from the top image slice all the way to the bottom slice.
    5. After viewing the volume data, select the number of slices according to the useable data volume; in this case, tracking was stopped at 200 slices, equivalent to a depth of 6.2 mm, where root boundaries became ill-defined (the image of the tracked root started to blend into that of the soil).

Note: The image stack produced will be saved automatically to wherever the output directory was created.

4. Volume and Surface Analysis

Note: This step describes the isosurface mesh generation from the image stack created by RooTrak.

1. Convert image stack from RooTrak into a binary image format in ImageJ. Select "Process", then "Binary", then "Make Binary".
2. Use the open-source ImageJ plugin, BoneJ, to create the triangular mesh; in ImageJ select "Plugins", then "BoneJ", then "Isosurface".
3. Set "Resampling" and "Threshold" to 6 and 120, respectively (Default settings). Check "Show surface", and press the "OK" button.
4. On the "3D viewer" click on the File tab, then on "Export surfaces", then save as "STL (binary)".
5. Open imeshJ, select the STL file and enter voxel size in microns. Click "Calculate Surface Area" to acquire total sample root surface area in mm^2. Similarly, click "Calculate Volume" to obtain total sample root volume in mm^3.

Representative Results

The specimen consisting of two stems of the native grass Prairie dropseed (*Sporobolus heterolepis*) and the original soil around it was taken from a residential area and placed in a small tube-shaped holder seen in **Figure 1**. The reconstructed data voxel size was approximately 31 µm x 31 µm x 31 µm. The reconstructed volume file was used to create a stack of images from a selected orientation (top view) using the open-source image-processing program ImageJ 1.6[11]. The volume data was also brightened in this program to increase contrast between root and soil values. From the reconstructed data, it was clear that the root and some components of the soil, most likely organic matter, have very similar X-ray attenuation factors resulting in little to no grey-scale contrast in the images (**Figure 2**).

RooTrak, the program used for segmentation, is an open source program developed at the Center for Integrative Plant Biology at the University of Nottingham[7]. It is specifically designed to track roots as they branch out through soil, and isolate the roots from surrounding material to produce a stack of binary images. RooTrak has been shown to produce segmentations better than simple thresholding for root data[7, 9]. A seed point was selected inside each of the pertinent root sections visible in the top slice of the volume data (**Figure 3**) then the tracking function of the software was run. The RooTrak parameters "Smoothness" and "Similarity" were set to 0.3 and 0.8 respectively. This range constantly provides good grey value separation and isolates the region of interest well. RooTrak successfully segmented the selected 200 slices of volume data (**Figure 4**), which was equivalent to a depth of 6.2 mm. See segmentation of the root in the animation file (Rootvideo.mov).

ImageJ was used to generate a triangular mesh, isosurface, of the 3D volume (approximating the surface of the isolated root) from the data produced by RooTrak (**Figure 5**). Default settings employed for "Resampling" and "Threshold" in ImageJ plugin BoneJ (see 4.3 in Protocol) were selected because they produce a detailed isosurface in a relatively quick manner. Adjusting the resampling level will affect the amount of time it takes to render the isosurface. The mesh was saved in the STL format, and *imeshJ* was used to compute the surface area and volume of the mesh. For the reconstructed volume in the present study, the calculated surface area was 351.87 mm^2, and the volume 47.27 mm^3 (see **Figure 6**).

Figure 1. The live specimen used in the study. The grass sample was imaged in its native soil in a 7" tall, 1.5" diameter plastic pot. Please click here to view a larger version of this figure.

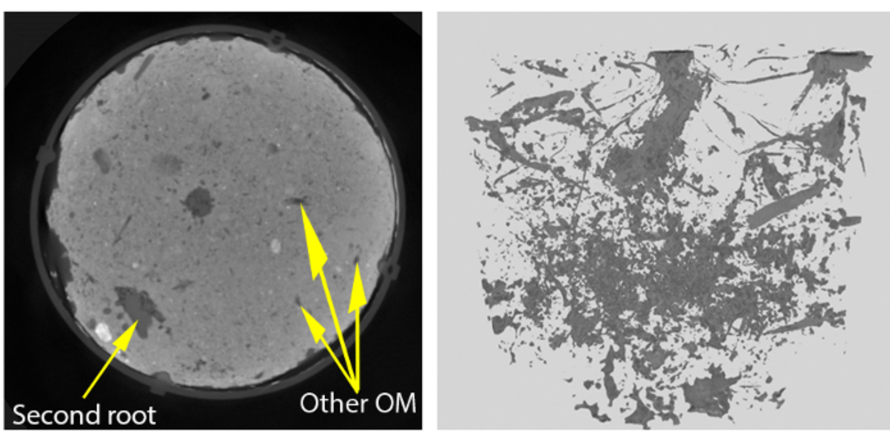

Figure 2. The Segmentation Problem. *Left:* Top view of a horizontal slice of the sample showing organic matter (OM) components of similar gray level to that of the root. *Right:* 3D rendering of the data showing all components that are of similar grey level to the root. Please click here to view a larger version of this figure.

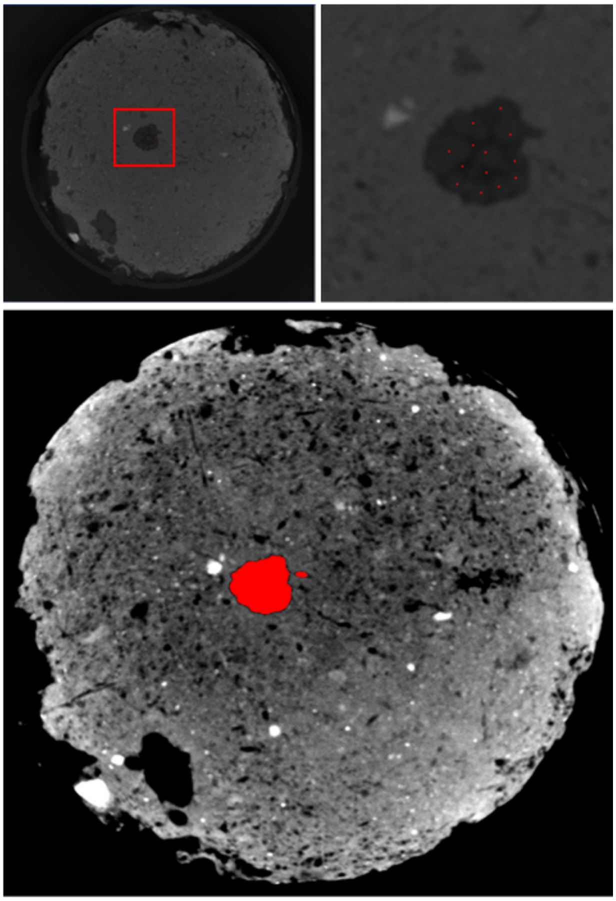

Figure 3. Starting point in RooTrak. *Top left*: top view slice of the volume data where seeding is started; *Top right*: augmented view of the slice are marked by a red square in the left figure, seed points are selected inside the pertinent root cross section; *Bottom*: The root saturated in red color is selected for segmentation. Please click here to view a larger version of this figure.

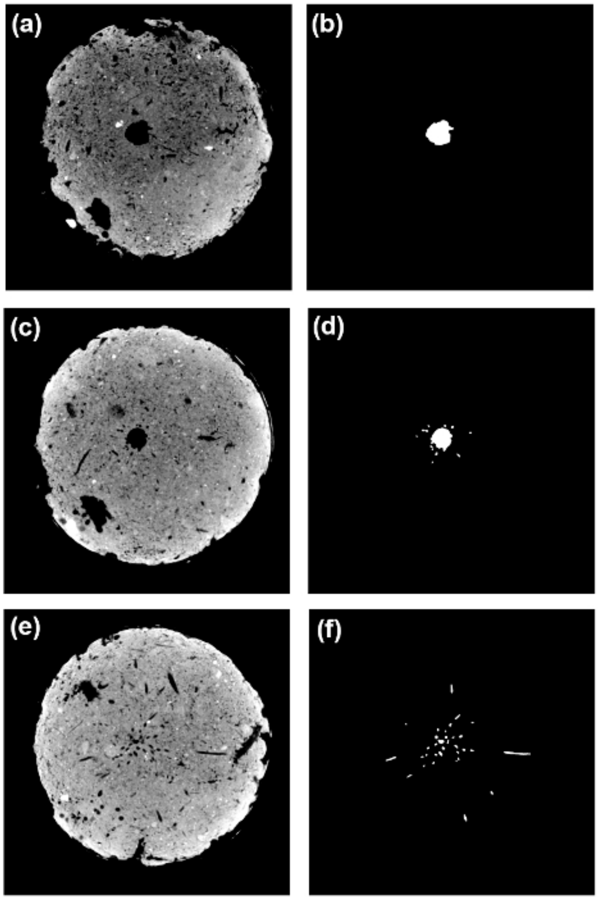

Figure 4. Comparison of slices from RooTrak. Three representative pairs of top view slices from different "heights" showing the root segmented by RooTrak. (**A**-**B**): top slice and the corresponding slice of the segmented stem; (**C**-**D**): mid region slice and the corresponding slice of the segmented root; (**E**-**F**): bottom region slice and the corresponding slice of the segmented root. Please click here to view a larger version of this figure.

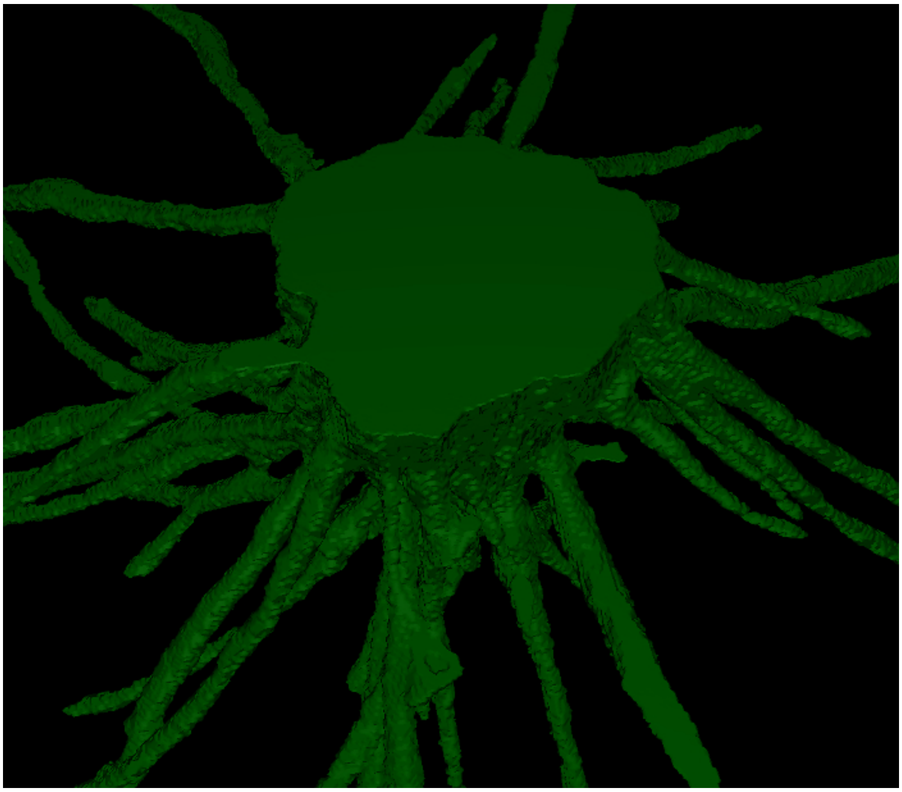

Figure 5. Isosurface of the root as captured from ImageJ. The root surface was approximated by a triangular mesh. Please click here to view a larger version of this figure.

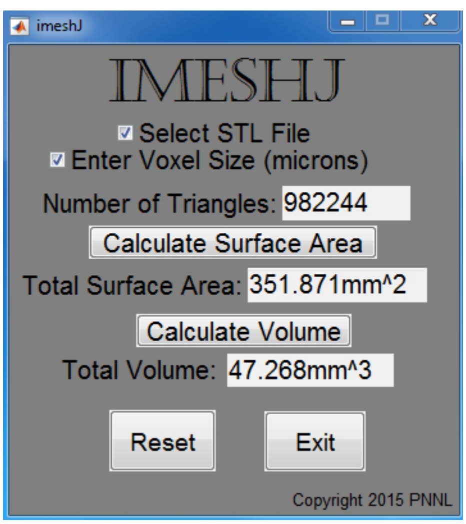

Figure 6. The imeshJ interface. The graphical user interface of *imeshJ* showing the actual results of the calculation. Please click here to view a larger version of this figure.

Discussion

A combination of X-ray computed tomography and several open-source programs proved to be a powerful combination to noninvasively image plant root samples, segment root data, and extract quantitative information (surface area and volume) from the 3D data. Our ability to visualize and measure features is always limited by scan resolution, as well as by limitations of the RooTrak software. However, scan resolution was sufficient to capture the majority of the features of the sample in this study, and RooTrak was able to successfully segment a significant portion of the root. The version of RooTrak employed in this work did not track upward traveling root segments (upward branching was not present to a significant extent in the specimen studied here); a newer version of the program addresses this problem[9].

As hinted in the Introduction, a survey of the literature suggested that a costless software option to calculate root volume/surface area from 3D tomography data on specimens in the centimeter range and above was highly desirable. The significance of the approach reported here is that, after the root is segmented, it works with a 3D data format created by the widely used program ImageJ. The heart of the analysis is the calculation of the volume and surface area of the root from its image approximated by a triangular mesh. The triangular mesh generated by ImageJ in the .stl format, is represented by the indices of the triangles that make up the surface approximation. *imeshJ* extracts the indices from the binary file format and is able to calculate the surface area from the indices (which is simply half the magnitude of the cross product between vectors joining points [1,2] and points [1,3].) The volume is calculated by constructing a tetrahedron between the triangle points and the origin, and finding the scalar triple-product between the position vectors of the triangle. Although there are several resources for such calculations, the challenge was to efficiently compute the surface area and volume for meshes that contained upwards of 10 million triangles (as may be the case in larger root structures.) By eliminating loop structures from the code, which would process the triangles one by one, and implementing several methods that allow simultaneous processing of multiple data points, we were able to run the calculations on a 6 million-triangle mesh in under 5 sec. With regards to sample size, a potted plant in a 20-ounce container would cover the whole detector range under the same magnification and would result in a 2,000-slice image stack as dataset. If we extrapolate the speed of the calculation from a 200-slice image stack (the sample size of the processed root in this study, 6.2 mm) processed in 5 sec to a 2,000-slice image stack, *imeshJ* should still process that under 1 min.

The products of this method are the surface area and volume of the root sample, which were determined without removing the plant from the soil, or disturbing it in any way. The calculated surface area is 351.87 mm^2, the volume is 47.27 mm^3. Other, intermediate, products are a 3D visualization of the root structure, and the triangular mesh of the roots (**Figure 6**).

The critical steps in the present protocol are the noninvasive tomographic imaging of the specimen to provide data with sufficient density contrast (step 1 in Protocol), the segmentation of the root portion of study from the rest of the sample (step 3), and the calculation of the root volume and surface area from the triangular mesh, isosurface (step 4). To achieve optimal density contrast, the X-ray power settings, 85 kV and 190 µA, were chosen based on detector response for the present sample; lower X-ray power would have produced inferior color contrast, while higher power would have saturated the detector. The grey level histogram feature of the data collection software guides the user in deciding what power settings to use. In general, soil-plant samples with significant organic content tend to require lower (>100 kV) X-ray voltage settings.

The accuracy of the calculated surface area is dependent on the assumption that the isosurface produced by ImageJ is a reasonable approximation to the actual surface of the root. This assumption is reasonable for large root segments, but may prove lesser so when roots with dimensions comparable to the voxel size are imaged. For the sample in this study, the size of the whole root and its segments were magnitudes greater than the voxel size. The default ImageJ/BoneJ settings (see "Threshold" parameter in 4.3 of Protocol) resulting in 3.6×10^{-4} mm^2 triangles *versus* the 9.6×10^{-4} mm^2 image pixel size must have provided an accurate estimate of the root surface area. For specimens with smaller features requiring greater detail, the number of triangles approximating the surface may be increased (triangle size decreased) by lowering the "Threshold" value. This will result in longer computational time. For root segments with dimensions approaching the voxel size, the instrumental resolution becomes the bottleneck, since the voxel representation becomes less accurate. The accuracy of the calculated volume figure is also dependent on the above assumption, but to a smaller degree because smaller-diameter roots contribute proportionally less to the volume of the root than the surface area of the root. However, a smaller cross section of the root can be scanned just as effectively if distance from the X-ray source is minimized. In other words, root segments requiring higher-resolution data, would need to be re-scanned at a higher magnification, and more accurate metrics may be obtained. The accuracy of the surface area calculation may also be affected by imperfect segmentation of the root from the soil. While our ability to distinguish the root from the soil depends on the color (grey level) contrast achieved in the imaging step, improvement of the segmentation step would help reduce any error in the calculation. Development of a new code that would improve the RooTrak segmentation process is underway. The calculation done by *imeshJ* was verified by comparing the output of the program for simple, single-component specimens of known volume and surface area in our laboratory.

This methodology of processing 3D data should be applicable to other material/sample systems where there is connectivity between components of similar X-ray attenuation and difficulties arise with sample segmentation. *imeshJ* will work on 3D data from any sources (PET, MRI scans) on any object of interest as long as an image stack is created from the data, which is used to create an isosurface, and the STL file that *imeshJ* uses. The calculated surface area and volume figures should be compared to values obtained on the same sample by other means (to be developed) to assess the accuracy of these calculations. Such comparison will be important for our ability to further refine the *imeshJ* code. Future plans include the development of a new root tracking tool and the *imeshJ* code for high-throughput imaging of plant root samples.

Disclosures

We have nothing to disclose.

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