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# Programs for the quantitative analysis of leaf surface growth in three dimensions

## General Users Manual

Programs developed using MATLAB 7.5.0 (R2007b) and the Matlab Image processing and Signal processing toolboxes.

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# Getting Started

1. Program names can be found at the RL\_lab website under the tab "Programs - latest versions" (Figure 1). The website is updated regularly to include modifications to the programs.

<https://sites.google.com/site/rlabgroup/Home>

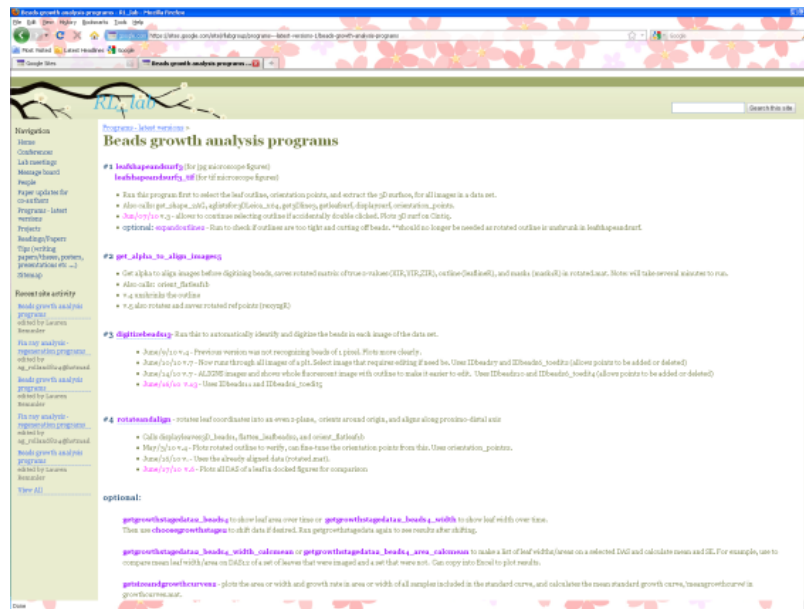


Figure 1. RL\_lab website

2. The folder containing the Matlab programs can be found on the S:\ drive (rech-Rolland-Lagan on 'sci.uottawa.ca\shares\bio' (S:)) under the folder "Matlab" and subfolder "Lauren-programs". The latest "Lauren" folder (without a number) needs to be copied onto the C:\ drive (in the "Matlab" folder) to be accessed by MATLAB 7.5.0 (R2007b).

When opening MATLAB 7.5.0 (R2007b), create a link to the "Lauren" folder.

File -> Set Path... -> Add with Subfolders... -> Select the "Lauren" folder from the C:\ drive -> Save -> Close (Figures 2).

3. Initiate a program in MATLAB 7.5.0 (R2007b) by copying the name into the command Window and pressing "enter" (Figure 3).

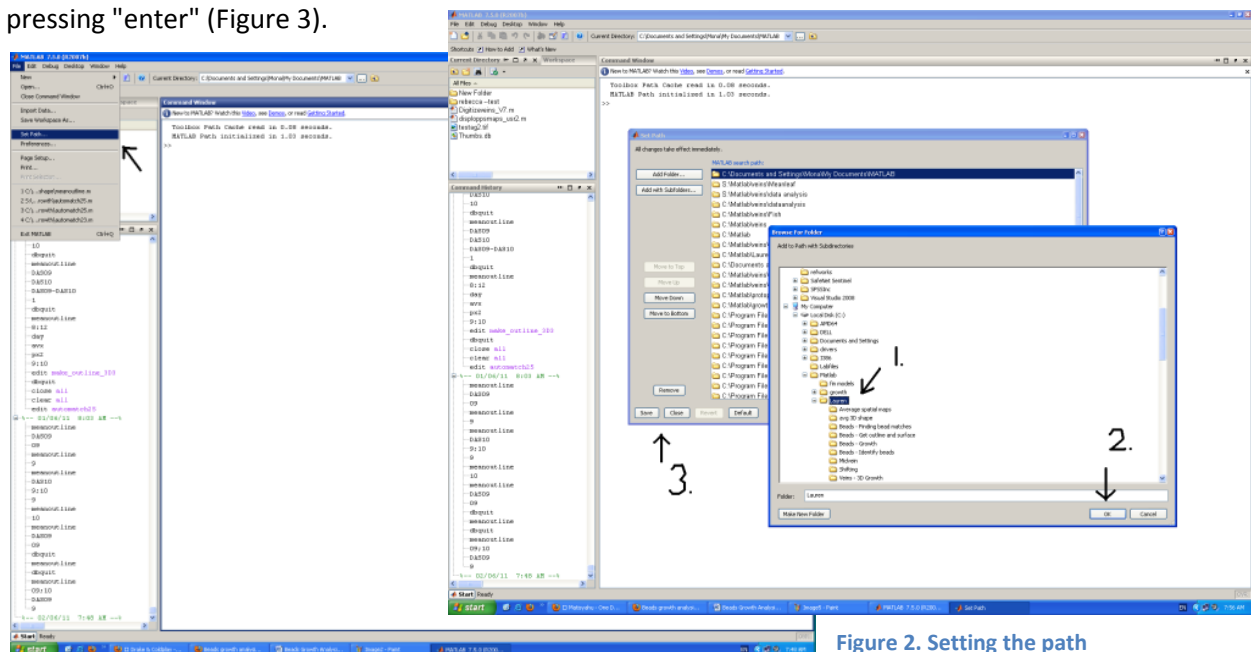


Figure 2. Setting the path

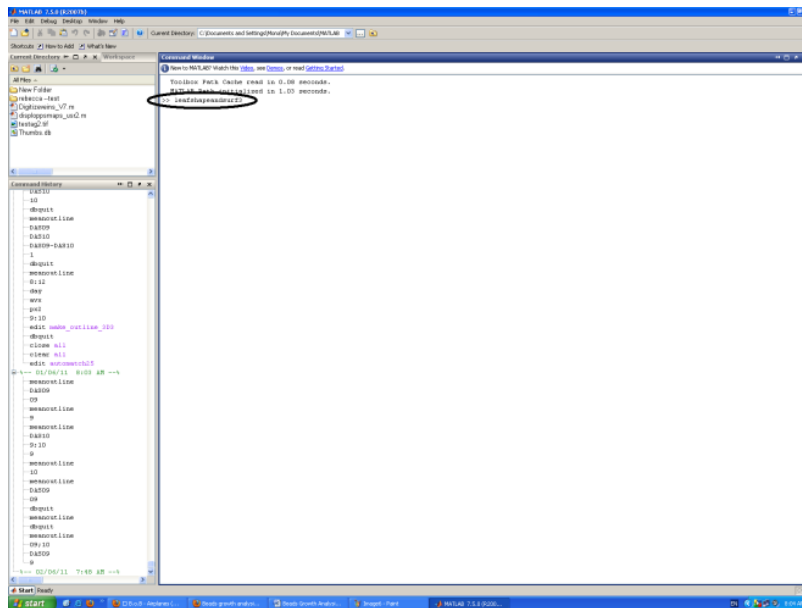


Figure 3. Initiating a program

## Program 1: leafshapeandsurf3

*Run the program to select the leaf outline, the orientation points and to extract the 3D surface of all images in a data set.*

1. When first running the program, start a new data set by pressing "2" and "enter". (If continuing the analysis of a previous data set, press "1" and "enter". Select the results folder previously used for the data set. In the second browser window, select the DAS folder of the PLT from which to continue the analysis)

Create a name for the new results folder (press "enter") and in the browsing window, select the directory in which to store the results folder (Figure 4).

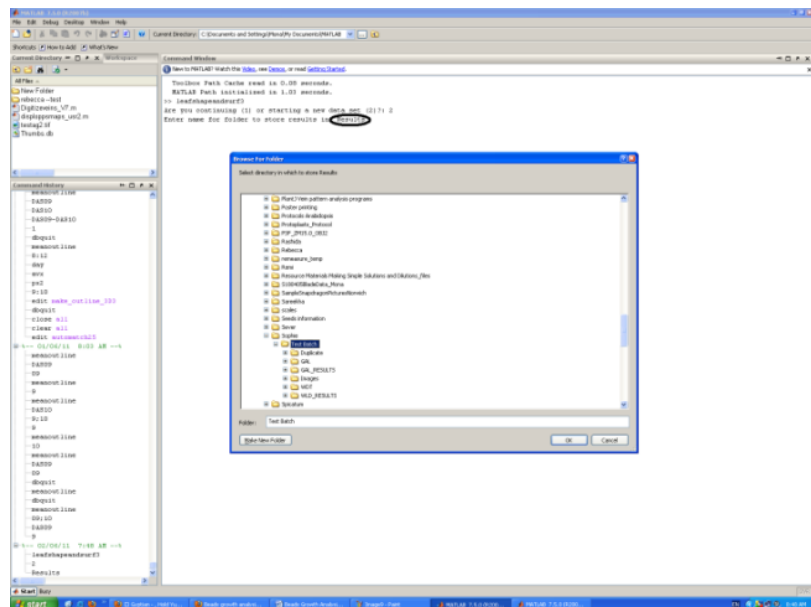


Figure 4. Creating the results folder

2. In a second browsing window, select the folder containing all the images for the data set.

3. Evaluate the quality of the confidence map (Figure 5). Press "y" (yes) for a map of sufficient quality or "n" (no) for a map of insufficient quality. In the latter case, the program will skip to the next image of the data set. Delete all images of insufficient quality (Note: Verify the confidence map during imaging to avoid this problem. i.e. black corresponds to 0% confidence in the depth map and accuracy and white corresponds to 100%, so make sure regions of the leaf surface are not too dark. Note that in Figure 5 the leaves are just emerging and it may not be possible to do better, but we would prefer to avoid having the dark values at the base of the leaf).

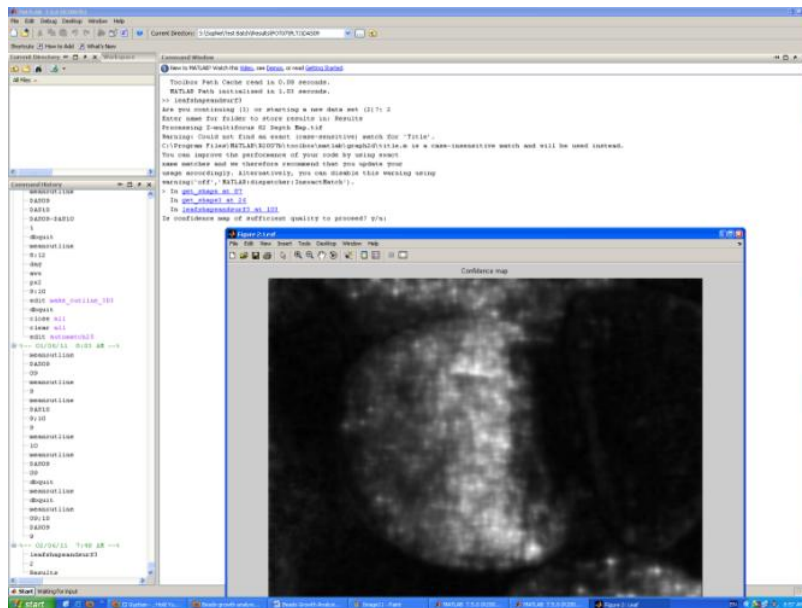


Figure 5. Confidence map

the sides of the leaf in question are not touching other leaves; the automatic outline extraction algorithm will run and display the predicted outline on the brightfield image for the user to accept or reject. Otherwise, trace the leaf outline using the mouse (left click) or the tracer pen\* on a Cintiq monitor (Figure 6). Trace the outline a few mm outside the actual leaf boundary (because during processing it will be shrunk a little). Double click or right click once the selection is complete (will appear in blue).

To restart the outline, press "n". If satisfied with the selected area, press "y".

\*Note that the functions of the pen can be customized by opening the Wacom Tablet Properties program. For example, different buttons on the pen can indicate right and left click, and/or the buttons on the tablet could be set to 'y', 'n', 'enter', or other frequently used keys.

**5. Selecting the orientation points:** Select the orientation points using the mouse (left click)

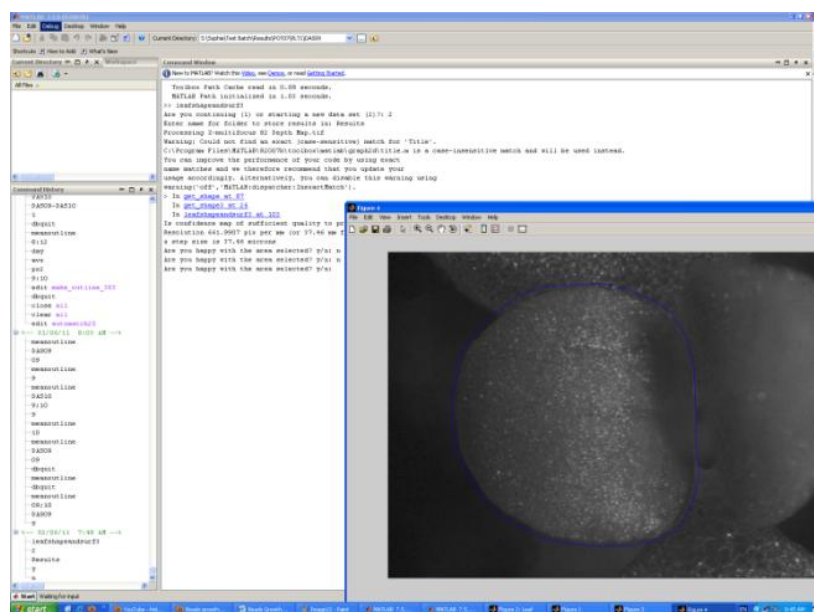


Figure 6. Tracing the outline

or the tracer pen on a Cintiq monitor (Figure 7). In order (counter clockwise), select: the leaf tip, the left side of the petiole and the right side of the petiole.

To redo the orientation points, press "n". If satisfied with the selected points, press "y".

6. The program will analyse the 3D shape of the leaf (Figure 9). Click and move the mouse around on the figure to rotate the leaf. If satisfied with the given 3D shape, press "n" to continue to the next image.

Pressing "y" will allow for modification of the 3D shape. Improve the 3D shape of the leaf by editing problem areas or adjusting the window smoothing.

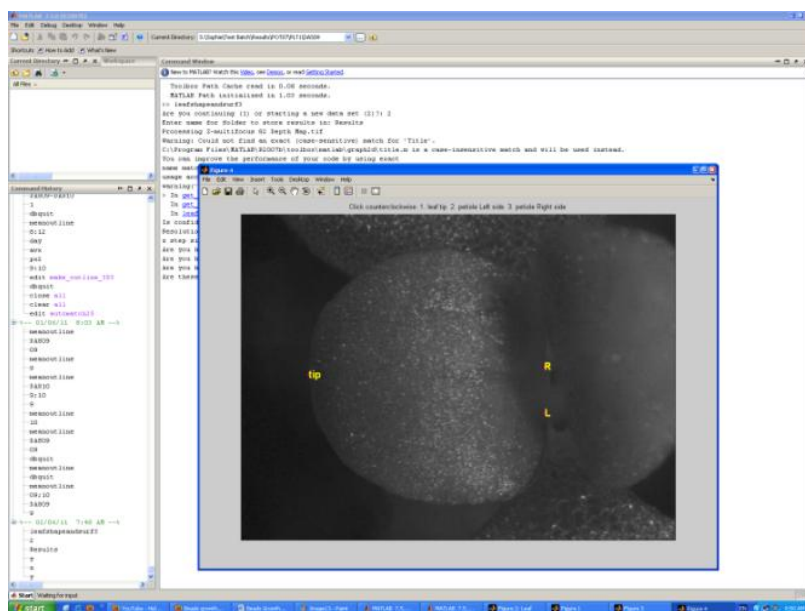


Figure 7. Selecting the orientation points

#### Editing problem areas: Problem

areas can be removed from the 3D leaf shape. Encircle a problem area with the mouse (left click) or the tracer pen on the Cintiq monitor (Figure 8). Right click once the selection is complete (will appear in green).

To edit more problem areas press "y", or press "n" when the selection is complete. Trichomes are an example of areas that generally require editing.

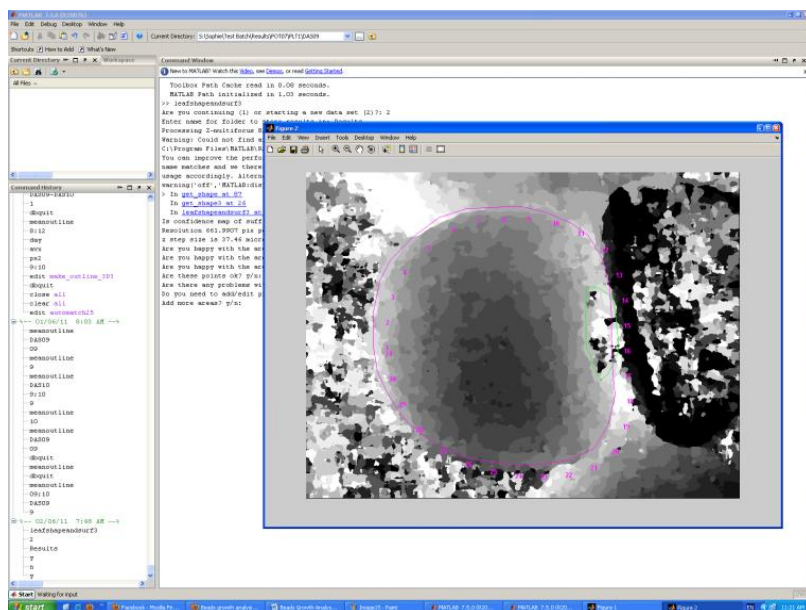


Figure 8. Editing the problem areas

#### Modifying 3D leaf shape

**parameters:** The scaling factor for the border average mask should remain at the default setting (0.05). Enter the scaling factor and press "enter". If the leaf shape is dipping down at the edges, increasing the scaling factor may fix this, but probably indicates that there is a problem with the

depth map or the outline (you went too far from the edge of the leaf).

Increasing the window smoothing will give a more uniform 3D leaf shape by spreading the high and low spots over a greater area. Subsequently, decreased window smoothing will result in a more rugged leaf. The default window smoothing is 100. Enter the new window smoothing and press "enter".



Be cautious when modifying the window smoothing. Only increase it if you see a step-like shape to the leaf. 3D leaf shapes produced with high smoothing window may not be representative of the actual data. Do NOT use the smoothing window to smooth out problem areas – crop them out using the previous option.

Modifications to the 3D shape can be repeated multiple times until satisfactory.

After completing a sample, press "enter" to continue with the following image of the data set. To quit the program, press "q". To redo the sample, press "r".

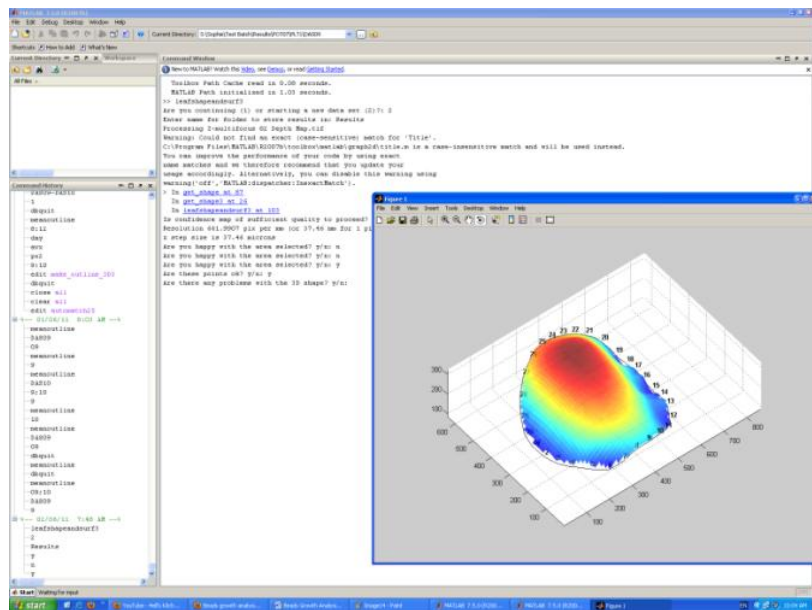


Figure 9. 3D leaf shape

## Program 2: get\_alpha\_to\_align\_images5

*Run the program to align the images before digitizing beads. The program saves the rotated matrix of true z-values.*

1. In the browsing window, select the results folder for the data set.
2. In the second browsing window, within the results folder, select the DAS directory of the PLT with which to begin the analysis.
3. The program aligns the images automatically.

## Program 3: digitizebeads13

*Run the program to automatically identify and digitize the beads in each image of the data set.*

1. In the browsing window, select the results folder for the data set.
2. In the second browsing window, within the image folder, select the DAS directory of the PLT with which to begin the analysis.

To proceed with the first image, press "n". Press "y" to skip to the next image.

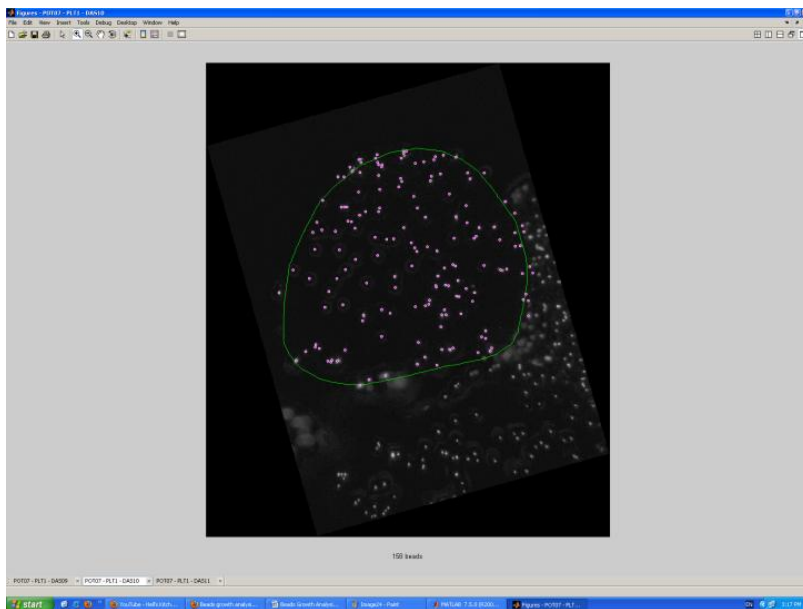
4. Improve bead identification or correct mistakes by modifying the minimum bead size, changing the minimum bead brightness or adding/deleting beads manually. If satisfied with the automatic bead identification, press "cont".

**parameters:** To modify the minimum bead size, press "s". Increasing this parameter will limit the detection of beads to specks of larger size, and is useful when you have image artefacts, such as random individual pixels that are brighter, and are

To modify the minimum bead brightness, press "b". No default setting is associated to the minimum bead brightness (the optimal setting is automatically chosen by the program for every image). Decrease

## Adding/deleting beads

When using the mouse, left-click to add beads and right-click to delete beads. When using the tracer



The screenshot displays the ROSETTA software interface. At the top, the menu bar includes File, Edit, View, Insert, Tools, Debug, Desktop, Window, and Help. Below the menu bar is a toolbar with various icons for file operations, editing, and visualization. The main window shows a 3D visualization of a protein-ligand complex. The protein is represented as a green mesh, and the ligand is shown as a stick model. The interface includes a top menu bar, a toolbar, and a status bar at the bottom. The status bar at the bottom shows the current selection: "ROSETTA: PTL1-04308" and "ROSETTA: PTL1-04310".

### Figure 11. Manually adding/deleting beads



pen on the Cintiq, press directly on the screen (or use the upper button) to add beads and use the lower button to delete beads (commands for the tracer pen can be modified).

Modify only one DAS at a time. Press "x" to exit before changing DAS or zooming in/out (Zooming tools are on the upper left corner of the window; Figure 12). It is not necessary to be exact with deleting beads – the program will delete the bead nearest to where you clicked. For adding beads, however, be precise. Zoom in to make sure you are defining the x,y-coordinate correctly (sometimes the placement of a dot on the unzoomed image looks fine, but is not quite right when you zoom in, and this inaccuracy will lead to errors in the growth parameters). Press "m" again to resume the manual application or deletion of beads.

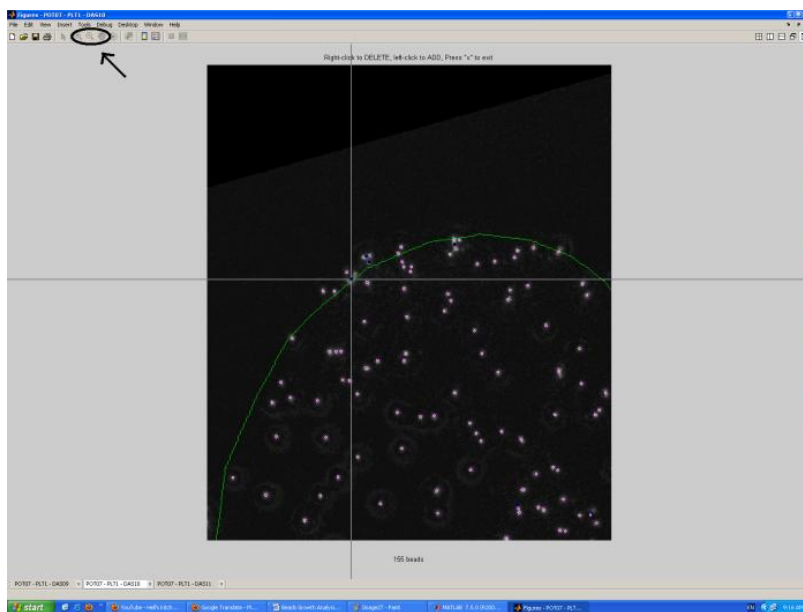


Figure 12. Zooming in

Remove beads outside the leaf boundary or in undistinguishable clusters. If you have good bead coverage, you don't need to worry about identifying every single bead in a cluster. Just track one, or none. Note that this is why all of the DAS's are displayed at once, on different tabs – if you are omitting beads, omit the same ones across all time points.

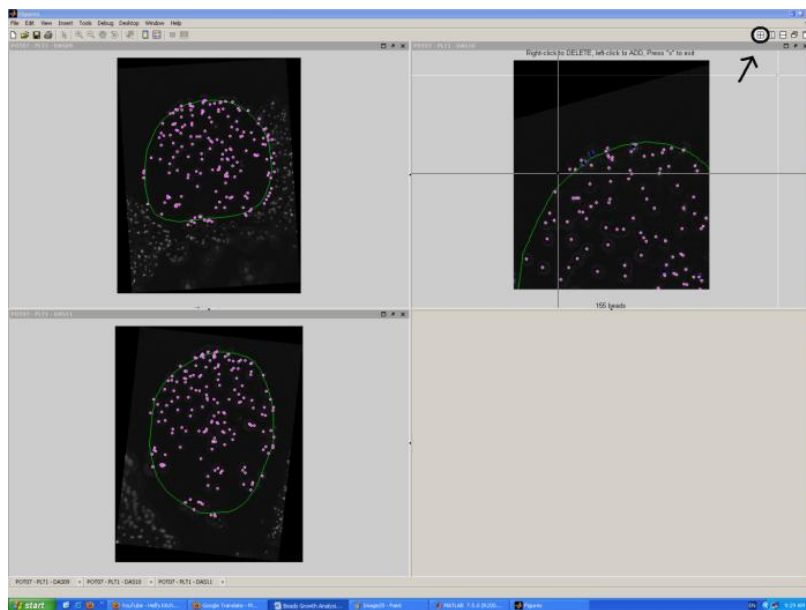


Figure 13. Display tools

To place multiple DASs in a single window, use the icon on the upper right corner (Figure 13).

## Program 4: rotateandalign

*Run the program to rotate leaf coordinates into an even z-plane, orient around the origin and align along the proximo-distal axis.*

1. In the browsing window, select the results folder for the data set.
2. In the second browsing window, from the results folder, select the DAS directory of the PLT with which to begin the analysis.
3. Verify the proper orientation of the leaf shape. To adjust the orientation of the leaf, press "enter". If satisfied with the orientation, press "k" and proceed to the next DAS.

**Adjusting the orientation of the leaf shape:** Select three orientation points using the mouse (left click) or the tracer pen on a Cintiq monitor (Figure 14). In order (counter clockwise), select: the leaf tip, the left side of the petiole and the right side of the petiole.

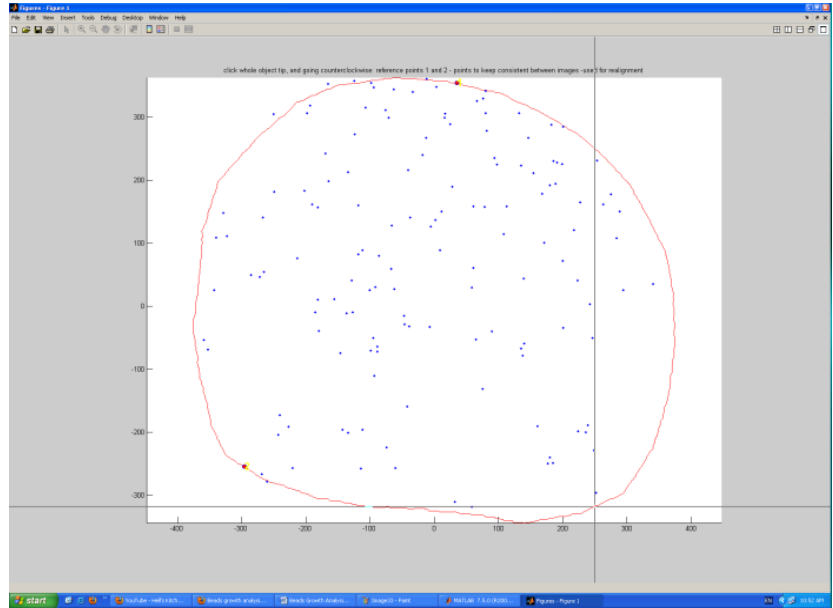


Figure 14. Adjusting the leaf orientation

To readjust the orientation of the leaf, press "enter". If satisfied with the orientation, press "k".

Orient all DASs of one plant in the same way. This is important for proper alignment of the mean maps and mean leaf outline, and particularly important for the semi-automatic bead tracking program to work.

## Program 5: automatch25

*Run the program to automatically find bead matches.*

1. In the browsing window, select the folder containing all the images for the data set.
2. In the second browsing window, from the results folder, select the DAS directory of the PLT with which to begin the analysis.
3. The program matches beads automatically for two DASs of a PLT (Figure 15).

4. If not all beads are matched automatically, modify the matching parameters to increase the number of matches. Press "y" to change parameters. Press "n" if satisfied with the automatic matches.

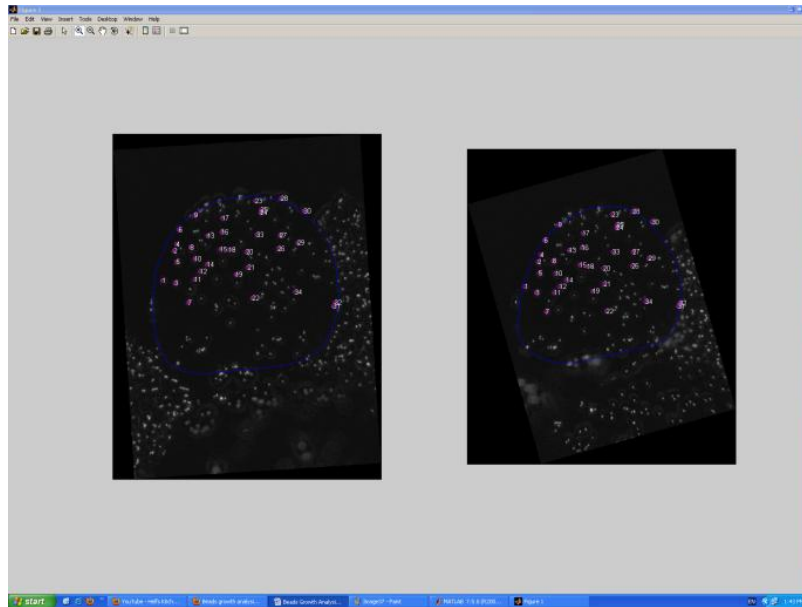


Figure 15. Automatic bead matching

Increasing the length difference authorized between beads can allow for more matching. However, too much of an increase will affect the accuracy of the matching. The percent difference allowed for the relative lengths should therefore remain under 0.60. The default percent difference allowed for the relative lengths is 0.30.

Like for the previous parameter, increasing the difference in radians allowed between neighbour angles can allow for more matching. Here again, too much of an increase will affect the accuracy of the matching. The parameter should therefore remain under 0.45. The default difference in radians allowed between neighbour angles is 0.15. Note that early time points will likely require the default parameters to be increased, since there is more significant growth and deformation in the first few days. Later time points on the other hand should rarely require any adjustments.

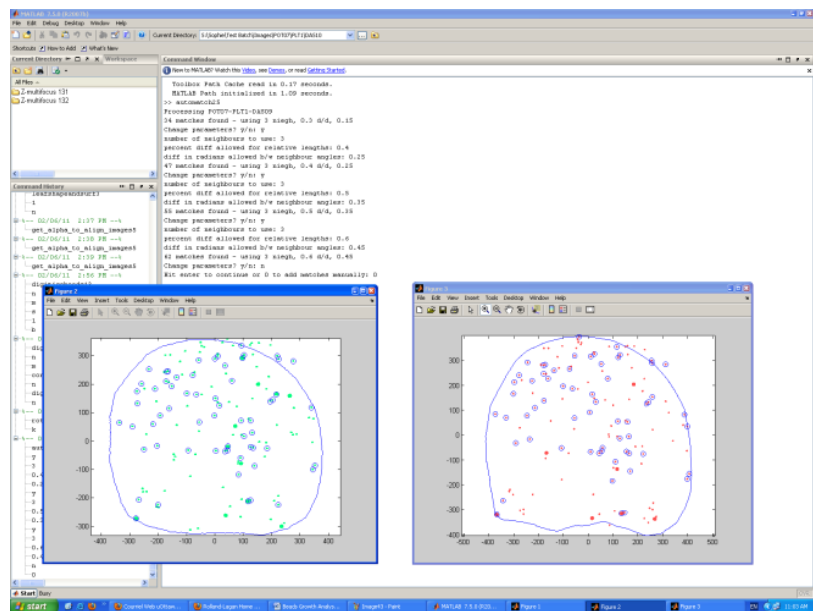


Figure 16. Manually matching beads

5. If satisfied with the matching, press "enter" to continue. To manually add beads left unmatched, press "0".

**Adding matches manually:** Note that this is only necessary if the previous program did not find a reasonable number of well-dispersed beads – you do NOT need to have every single bead matched in this step. Just make sure there are matches well dispersed across the leaf, and you may want to have a few extras in areas where beads are crowded.

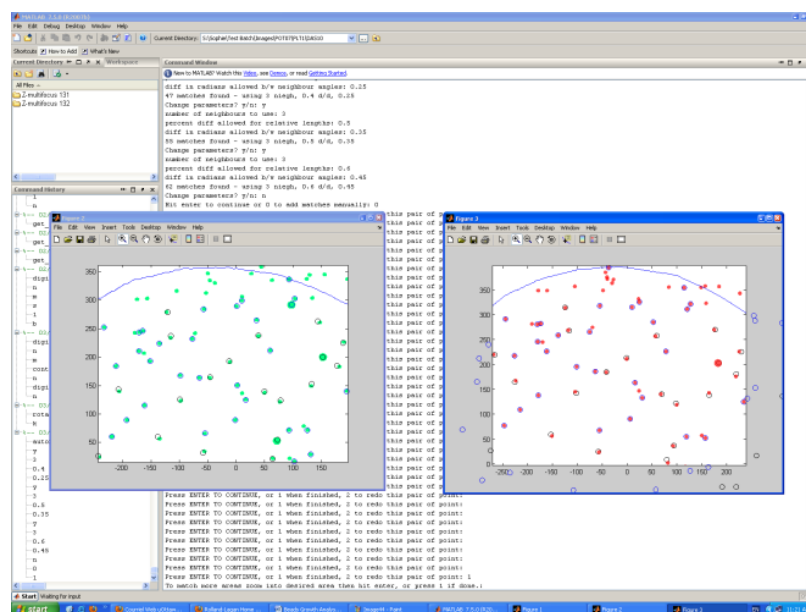


Figure 17. Zooming in

Using the mouse (left-click) or the tracer pen on the Cintiq, encircle a green bead from the first figure (earlier DAS) and pair it to a red bead in the second figure (later DAS). If an error occurs, redo the match by pressing "2". Again, the exact position when clicking is not

important – the program is finding the nearest bead coordinate (Figure 16).

To create another match, press "enter". Add the matches that are visible without zooming in and finish by pressing "1". Pressing enter between circling the green and red bead will result in a program error.

If necessary, zoom in and press "enter" to add more difficult matches (Figure 17). Zooming tools are located at the upper left corner of the window. When satisfied, press "1" to continue to the next step.

6. The program will use the matches generate to warp the beads from one time point (green) to the next (red) on one figure. The colours are somewhat transparent, so overlapping beads will appear brown (Figure 18).

To perform further matching, press "0". Match from red to green. If no additional bead pairs can be detected from this image, press "enter" to continue.

## 7. Modifying matching

**parameters:** Preset matching parameters can be modified by pressing "0". They will not typically require modification if the dispersion of matches found in the previous step is good. Press "enter" to continue to the next step.

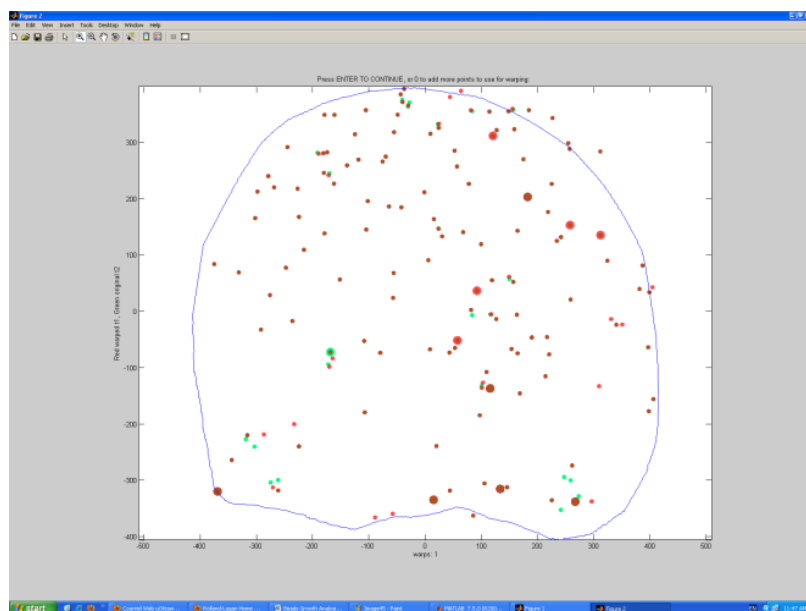


Figure 18. Automatic warping of beads

Two beads will be matched if positioned within the set maximum distance. Modification to the "Maximum distance between points in order to be considered a match" (increase when sparse and decrease when concentrated) can optimize warping according to bead dispersion. The default setting is 5.

Two beads at similar positions will not be matched if a second potential match is found in close proximity (does not surpass the set minimum distance). If bead dispersion is dense, increasing the "Minimum distance allowed between top two matches for it still to be considered a match" will raise the accuracy of the matching. The default setting is 5.

Two beads will be matched (even if not within the set maximum distance) if no other feasible match is found in proximity (set minimum distance). If bead dispersion is sparse, decreasing the minimum separation can allow for more matching. However, too much of a decrease could affect the accuracy of the matching. The default setting is 50.

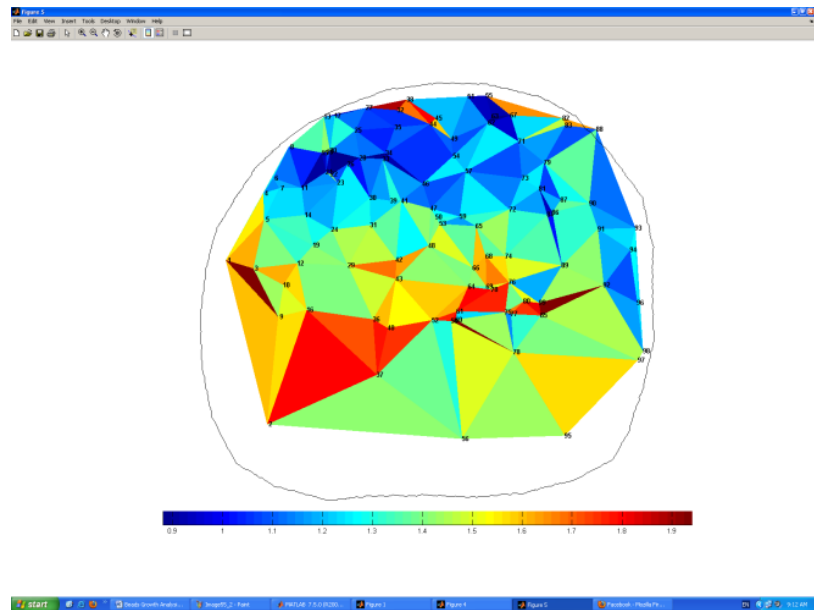


Figure 19. Color gradient on the growth map

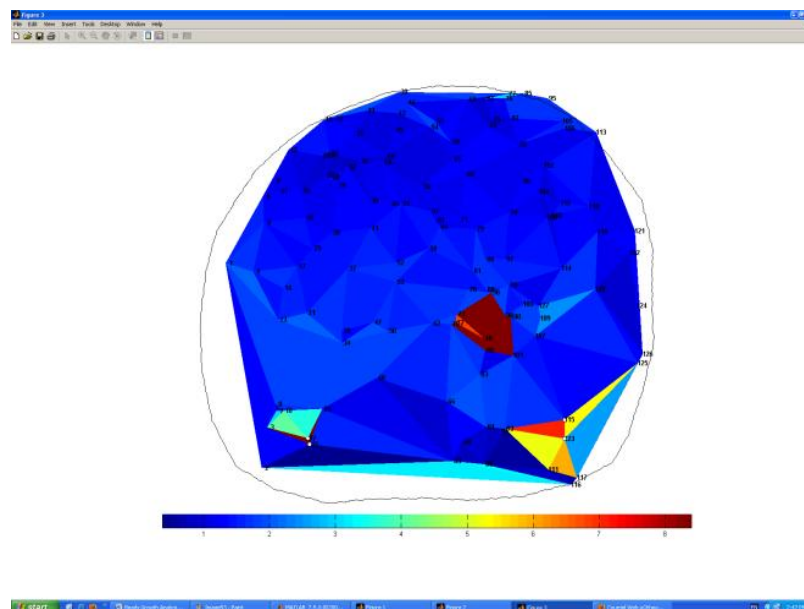


Figure 20. Inappropriate color triangle

Parameters can be modified multiple times until satisfactory. If satisfied, press "enter" to continue to the next step.

8. Following modifications of the matching parameters, the program allows again for the manual matching of points. To improve the automatic matching and manually add matches, press "0". To continue to the next step, press "enter".

9. The program will construct a growth map for the pair of DASS based on the relative change in distance and angle between matched beads. Triangles of red color indicate regions of strong growth, where as triangles of dark blue color indicate regions of weak growth. A gradient from red to blue is expected starting at the leaf base (Figure 19).

10. **Editing growth maps:** Inappropriate color triangles (ex: a red triangle surrounded by blue triangles) is the indication of a matching error and can be edited by pressing "0", and click on it to delete the bead match (Figure 20). If satisfied with the growth map press "123" to proceed to the next pair of DASs.

To redo the entire matching process, press "321". To edit current matches, press "789".

To delete certain matches, press "456". Encircle the beads to delete using the mouse (left-click) or the tracer pen on the Cintiq. Press "enter" to delete more beads or "2" to redo a deletion.

Deleting beads can completely change the growth map (Figures 21). Try only deleting beads at the junction of a red and blue triangle. Also delete very few beads at a time and frequently verify the state of the growth map.

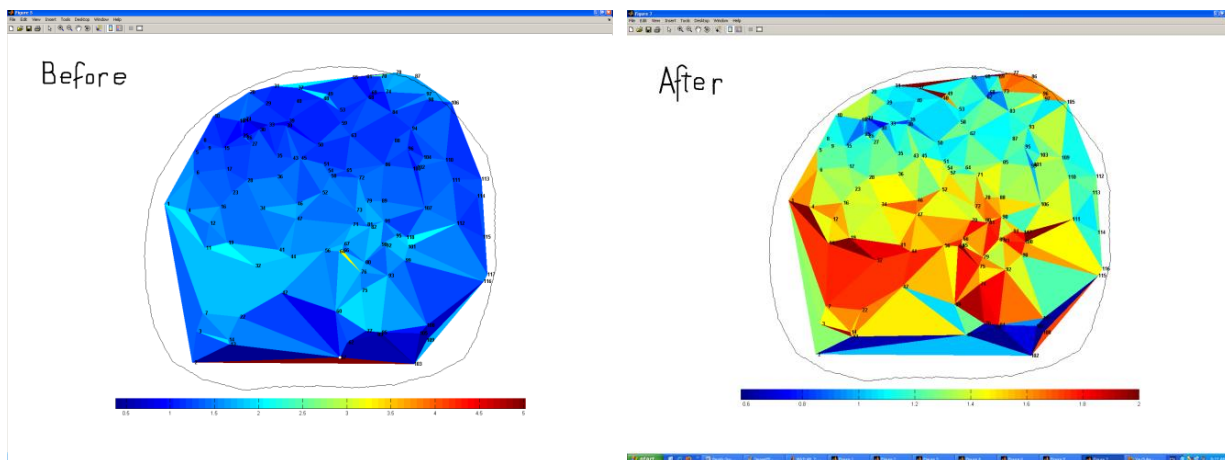


Figure 21. Before and after deleting beads

Press "1" when done deleting beads.

11. Once bead matches are completed between all DASs of a PLT, growth programs may be run. To do so, press "enter". To exit the program, press "0".

Display the relative growth rate maps by pressing "1" (Figure 22).

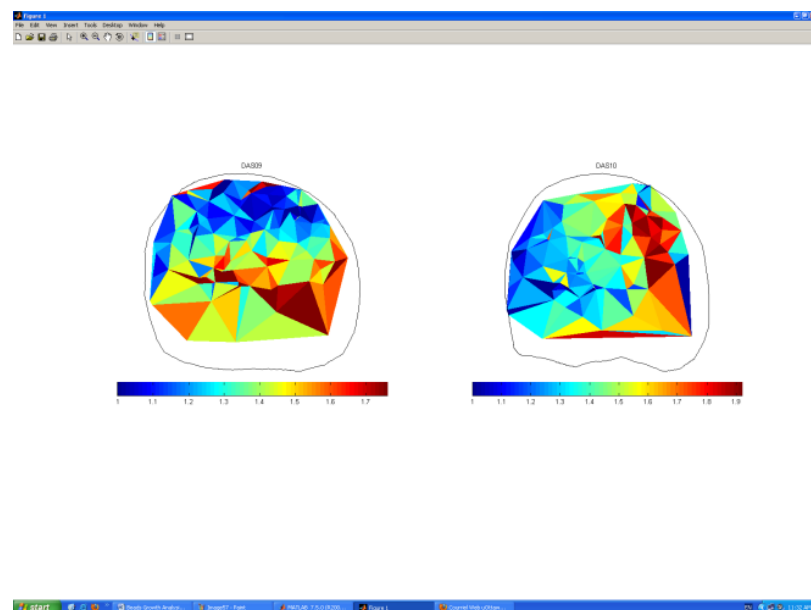


Figure 22. Relative growth rate maps



## Program 6: processallgrowth3D5

Run the program to calculate 3D growth strain parameters based on all of the points that were matched up (includes manually and automatically matched).

1. In the browsing window, select the results folder for the data set.
2. The program processes the result folder automatically. To display growth maps as the results are analysed, press "1".

The program pauses at each step to allow for visualisation of growth maps (Figure 23). Press any key to continue.

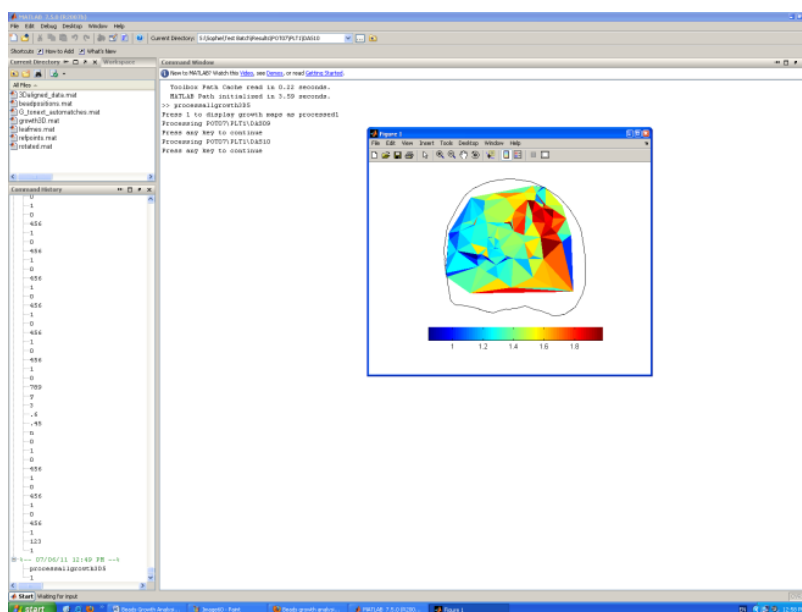


Figure 23. Calculating 3D growth strain parameters

## Program 7: meanoutline

Run the program to create "NewBladeLine" for each leaf and calculate the average leaf outline for each time point.

1. In the browsing window, select the results folder for the data set.
2. Enter the DAS numbers to process. Separate the first and last DAS by a colon (ex. 07:10). The colon replaces the word "to".

The program automatically calculates the average leaf outline for each DAS (between all PLTs).

In the browsing window, select the directory in which to store the mean outlines (separate from the results folder; Figure 24) and create a name for the file. Do not include spaces in the name, use "underscores" instead.

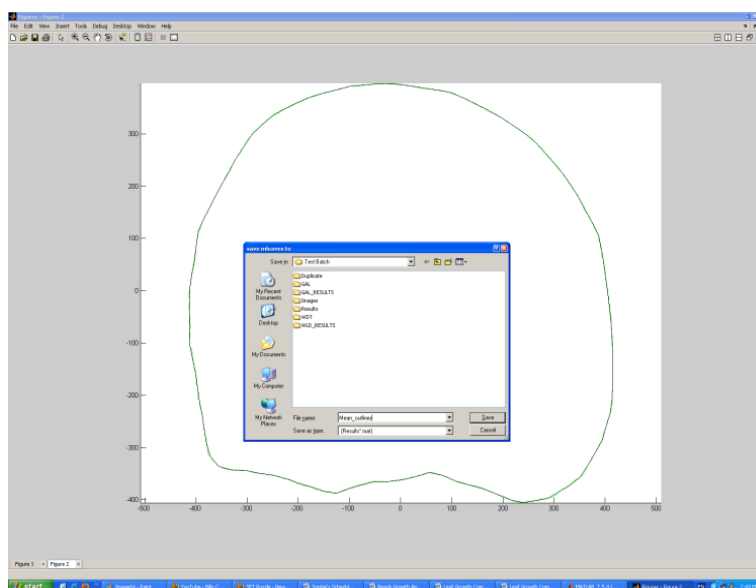


Figure 24. Calculating the average leaf outline

## Program 8: warpleaves\_3D\_dbs9

*Run the program to warp leaf outline to the average leaf outline for the DAS and then use the transformation to warp the triangles to the average leaf outline as well.*

When first entering the program name in the Matlab command window, include in brackets (separated by a comma): the figure display command ("1" to display, "0" not to display) and the range of days to warp.

For example, `warpleaves_3D_dbs9(1, 7:10)`.

As indicated previously, DASs are paired. Thus, only indicate the second last DAS when including the range of days to warp. It is recommended to display the "warp" figure.

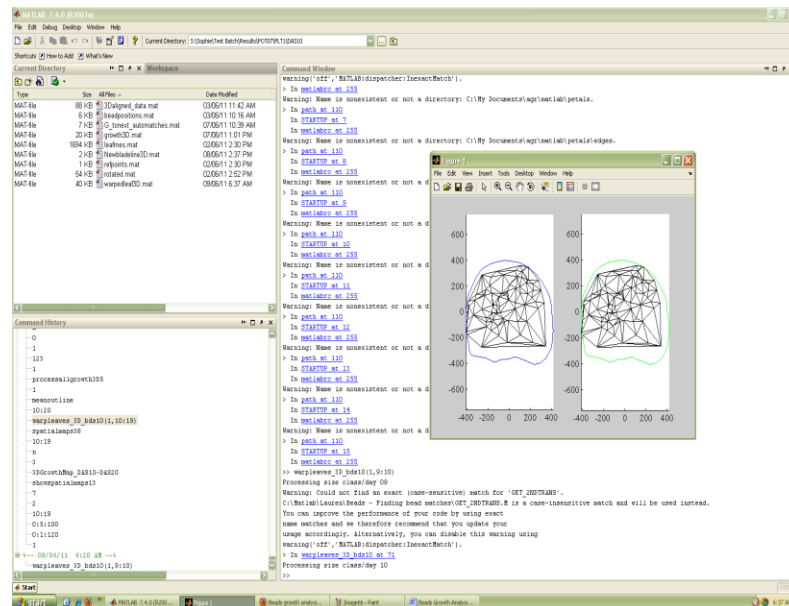


Figure 25. Automatic warping of the leaf outline and triangles to the average leaf outline

1. In the browsing window, select the results folder for the data set.

2. In the second browsing window, select (load) the average leaf outline file.

3. The program automatically warps the leaf outline and triangles to the average leaf outline for every DAS (Figure 25).

Figures are displayed during the process if directed (indicated "1" as the figure display command).

## Program 9: spatialmaps38

*Run the program to compute and/or display mean growth maps, standard error maps, sample number maps, before and after removing outliers.*

1. Indicate the DAS or range of DASs (ex. 7:10) to be computed.
2. Press "n" to maintain the outliers (data more than 500% from the median).

3. Press "3" to obtain 3D spatial maps. ("1" will display growth maps from warped points and "2" will display 2D maps, which you are unlikely to generate).

4. In the browsing window, select (load) the average leaf outline file.

5. In the second browsing window, select the results folder.

6. In the browsing window, create and name a new folder (ex. Figures) in which to save spatial map data. Use the "Make New Folder" button in the lower left corner of the window (Figure 26).

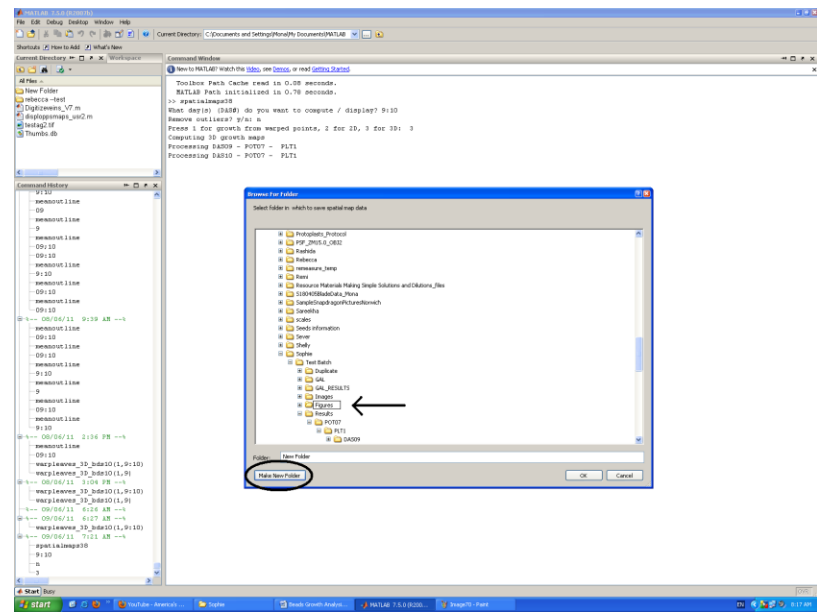


Figure 26. Creating a new folder

Create a file name for the 3D growth maps (press "enter").

7. The program automatically saves the spatial data. To display the spatial maps, run the showspatialmaps program.

## Program 9: showspatialmaps13

*Run the program to plot results from the previous program (spatialmaps38).*

1. In the browsing window, select the file containing the spatial map data.

2. In the second browsing window, select the folder in which to save the images (or create a new folder using the "Make New Folder" bottom on the lower left corner of the window).

3. The program can display 24 matrixes (Figure 27):

(1) mean\_aniso: Mean anisotropy

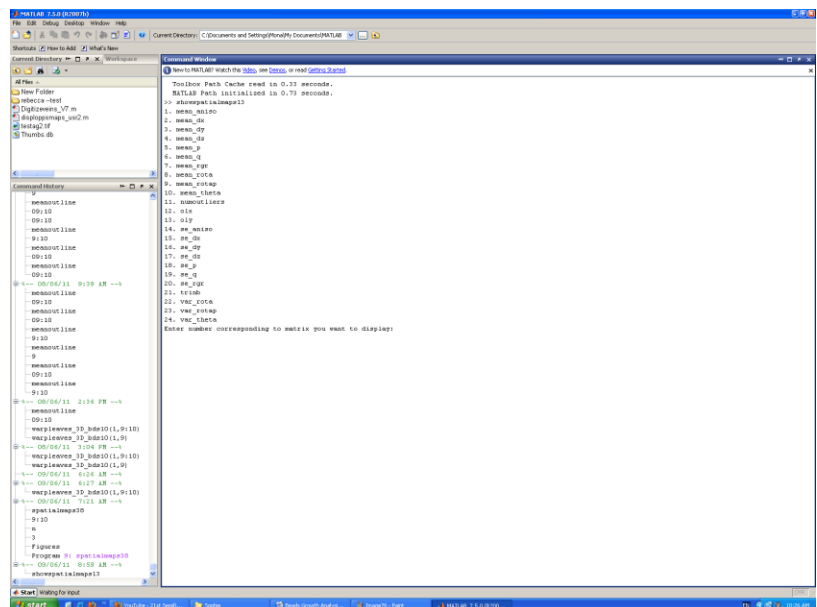


Figure 27. Choosing a matrix

~~(2) mean\_dx~~

~~(3) mean\_dy~~

~~(4) mean\_dz~~

(5) mean\_p: Mean scaling factor in the max direction of growth

(6) mean\_q: Mean scaling factor in the min direction of growth

(7) mean\_rgr: Mean relative growth rate (%)

(8) mean\_rota: Mean rotation

~~(9) mean\_rotap~~

(10) mean\_theta: Mean direction of growth

(11) numoutliers: Number of outliers in the data

~~(12) olx~~

~~(13) oly~~

(14) se\_aniso: Standard error of anisotropy

~~(15) se\_dx~~

~~(16) se\_dy~~

~~(17) se\_dz~~

(18) se\_p: Standard error of scaling factor in the max direction of growth

(19) se\_q: Standard error of scaling factor in the min direction of growth

(20) se\_rgr: Standard error of relative growth rate (%)

(21) trinb: Number of triangles

(22) var\_rota: Circular variance of rotation

~~(23) var\_rotap~~

(24) var\_theta: Circular variance of direction of growth

Matrix 2, 3, 4, 9, 12, 13, 15, 16, 17 and 23 (crossed out) are not used.

To display a matrix, enter the corresponding number.

Press "1" to omit areas where the number of samples is low. Enter "3" as the minimum number of samples allowed. The option to omit areas of high standard error was removed from the program.

Enter the DAS or range of DASS to display.

**Selecting the color scale range:** Enter the color scale range (min:step:max). The best starting point is (0:5:100). The optimal range will vary with each data set. Trial and error is necessary to find the appropriate minimum and maximum.

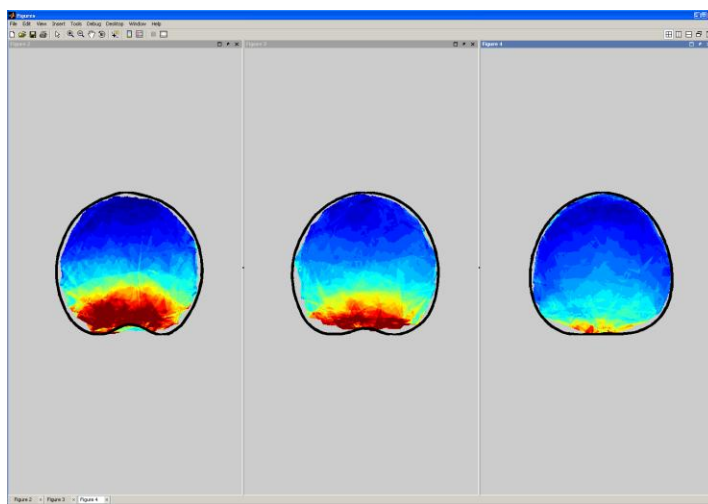


Figure 28. Mean spatial maps of relative growth rates for three time points

A higher step size permits for a faster image upload. Wide ranges will take a long time to make maps for, so you can "test out" a range with a bigger step, e.g. 0:50:300 to quickly check that it's an appropriate range. Once the optimal range is identified, the step size should be reduced to "1".

The program automatically creates a name for the image file (identifying the selected DAS range and color scale range). A tag (ex. \_1) can be added.

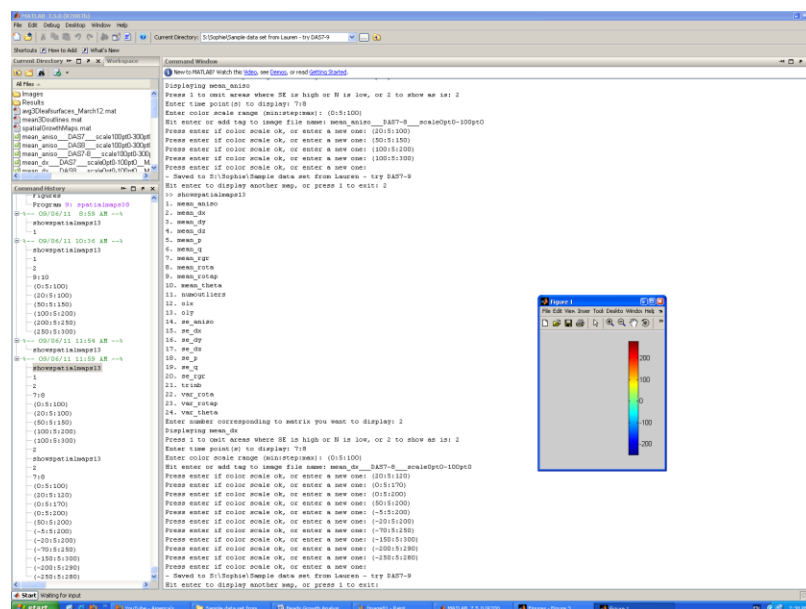


Figure 29. Scale bar

step to "1". Press "enter" to save the scale bar (Figure 29).

To display another map, press "enter". Press "1" to exit the program.

## Program 11: showanglemaps2

Run the program to display angle maps with lines oriented in the direction of the angle (scaled or not by anisotropy).

Press "enter" to continue.

To find the optimal color scale range, balance the colors in the figure (Figure 28). Large areas of dark blue or red indicate problems with the minimum and maximum.

To reduce the amount of dark blue in the figure, decrease the minimum. The color scale range may include negative number. To reduce the amount of dark red in the figure, increase the maximum.

Modify the color scale range until satisfied Remember to change the

3. The program can display 3 different angle maps (Figure 30):

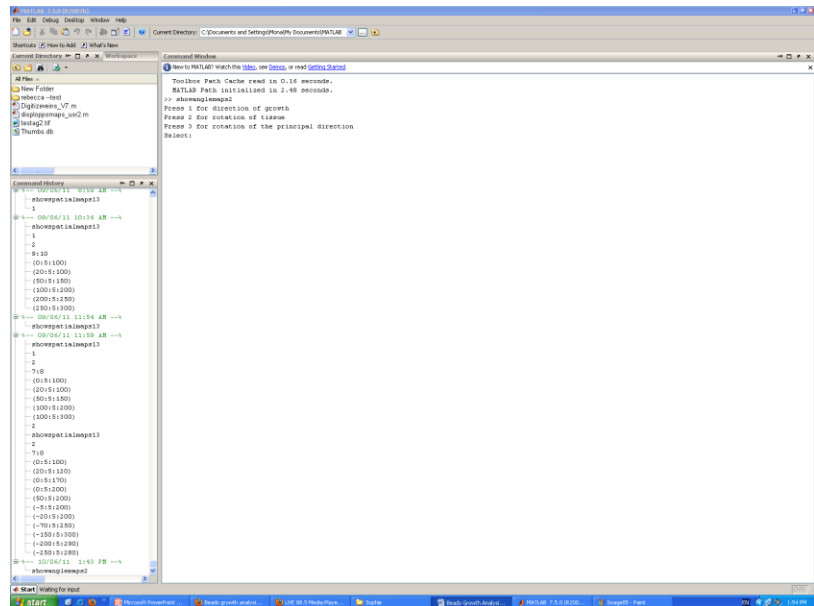
(1) Direction of growth

(2) Rotation of tissue

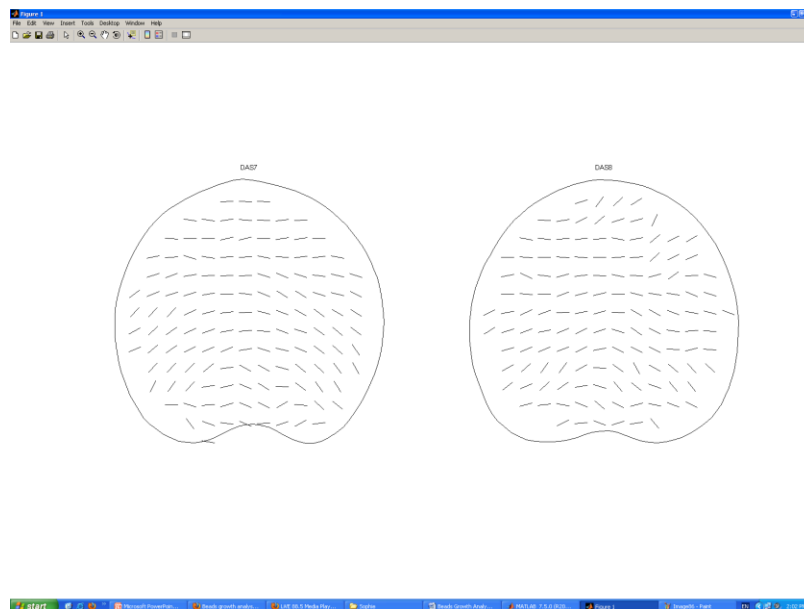
### (3) Rotation of the principal direction

To display a map, enter the corresponding number.

4. Enter the DAS or range of DASs to display.



### Figure 30. Choosing an angle map



**Figure 31. Angle map**

The program displays angle maps for the indicated DASs (Figure 31).

The program automatically creates a name for the image file (identifying the selected DAS range). A tag (ex. `_1`) can be added.

Press "enter" to save the image  
and continue.

To display another map, press "enter". Press "1" to exit the program.