Geometrical Modelling for 3D Laser Microdissection

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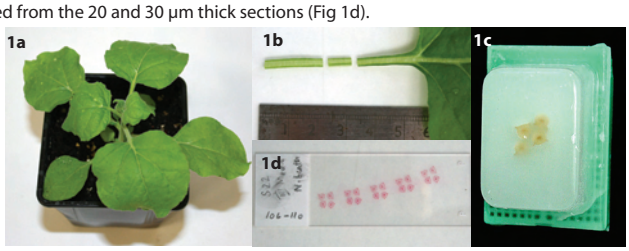
Introduction

Phloem tissue, part of the plant vascular system, is responsible for trafficking many molecules throughout the plant, including signaling molecules and intracellular pathogens such as viruses. Phloem is deeply embedded in ground tissue and is therefore difficult to study. Collaboration between the Scottish Crop Research Institute, Invergowrie, UK, the Fraunhofer Institute, Magdeburg and Molecular Machines and Industries, Munich, Germany, aims to produce a 3-dimensional laser microdissection instrument capable of sampling such tissues. Geometrical models of *Nicotiana benthamiana* petioles were created to test whether they could be used to guide a laser to allow 3D laser dissection of phloem tissue. Each process of the procedure was optimized to increase the quality and efficiency of modeling from plant sample to 3D model utilising as much computer-automated segmentation as possible.

Results

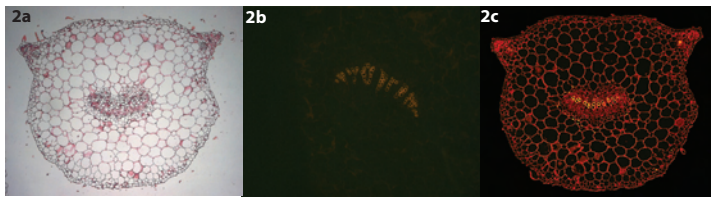
1. Tissue Preparation

Samples of three different petiole sizes were collected from 31, 38 and 45 days old *Nicotiana benthamiana* plants (Fig 1a and Fig 1b). The samples were fixed, embedded in wax (Fig 1c) and sectioned using a microtome. Medium sized samples from the 38 day old plants were found to be the most appropriate to create geometrical test models as larger samples did not allow data to be collected as single images, while smaller petioles were more fragile and easily damaged. Sections of 10, 20 and 30 µm thicknesses were tested and models were created from the 20 and 30 µm thick sections (Fig 1d).



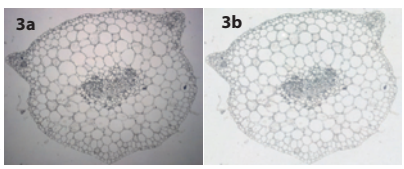
2. Imaging and Staining

27 different stains in combination with Bright field (2a), fluorescence (2b) and confocal (2c) imaging methods were tested, but confocal and bright field images stained using a safranine-O and eosin stain combination gave best contrast to distinguishing the phloem from other tissues. Confocal microscopy using 488 and 568nm excitation produced an overlay image in which xylem tissue and certain other regions of endodermis (Fig 2c shown in yellow) could be discriminated from other tissue types (Fig 2c shown in red). Bright field and confocal image datasets of serial sections were collected using their respective optimal conditions.



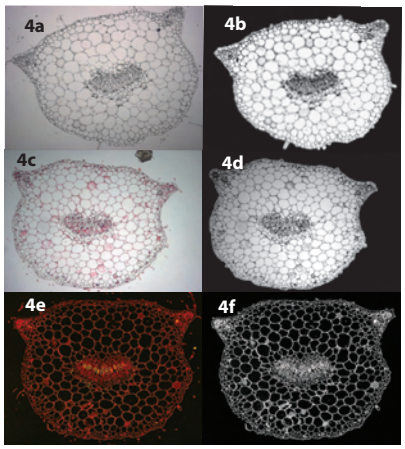
3. Background Correction

Bright field images had a non-uniform background (3a) due to the reduced quality of low magnification lenses and non-uniform lighting across a large field of view. This was rectified by subtracting corresponding blank images from each image (3b).

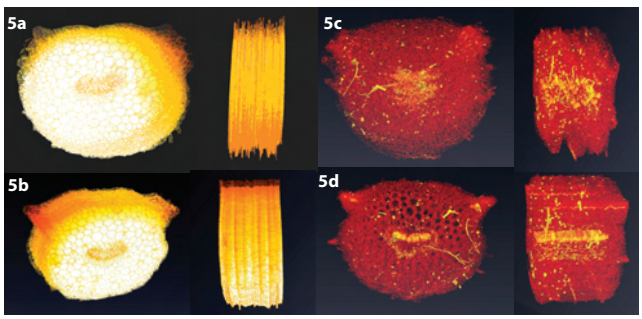


4. Masking

Raw confocal (4e) and bright field images (4a, 4c) contain aberrations such as background noise and debris surrounding the sections. These artefacts were removed by masking protocols to prevent interference with the classification of voxels during automatic segmentation. The images were also masked for luminance to ensure uniform background levels (Fig 4b, 4d, 4f).



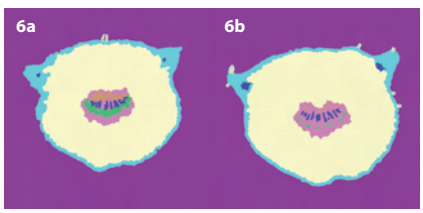
5. Registration and Aligning



The images of serial sections were registered to regain the 3D structural coherence by re-aligning adjacent sections. (Fig 5a, 5b, 5c, 5d).

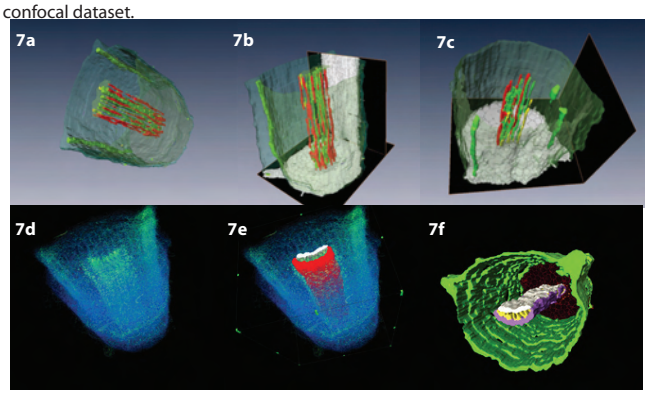
6. Segmentation

Images were segmented into labelfields, with each labelfield representing a different tissue type: inner and outer phloem, xylem, vascular parenchyma, cortex, epidermis and background (Fig 6a and 6b). Manual segmentation of images is time consuming but necessary to teach the computer tissue recognition for automated segmentation. For this purpose, reference datasets with varying levels of manual segmentation were tested to find the minimum number of manual references required to obtain high quality models. Models were created using datasets where one in every ten images was manually segmented using Amira software.



7. Modelling

The processed images were resampled to obtain iso-tropic voxel sizes of histological volumes. Models were generated using specific algorithms for surface extraction, surface reduction and finally visualized using Amira software. Models based on three different datasets were made. Two of them were developed from bright field datasets of 20 µm thicknesses while one was developed from a confocal dataset of 30 µm thickness. Figure 7a-b shows the model of individual phloem bundles from 20 µm bright-field images, Fig 7c of 30 µm bright-field images and Fig 7d-f shows the model of phloem regions obtained from the confocal dataset.



Conclusions & Future Work

- Three dimensional models were successfully generated for specific sizes of *Nicotiana benthamiana* petioles.
- Sections of 20 and 30 µm thicknesses were sufficient to generate quality models.
- Unstained sections yielded good bright field images that allowed individual phloem bundles to be discriminated, while a combination of safranin-O and eosin staining gave optimal confocal images.
- The models produced should be of sufficient quality to guide a laser for 3D-microdissection of phloem tissues.

The quality of the models may be improved in future by implementing further modifications. Future tests will include:

- Using osmium tetroxide staining of tissue to increase image contrast and improve computer-automated segmentation.
- Embedding tissue in resin to allow thinner serial sections to be cut using an ultramicrotome (to produce images with greater cellular clarity).
- Test modeling efficiency using a lower frequency of manually-segmented images.