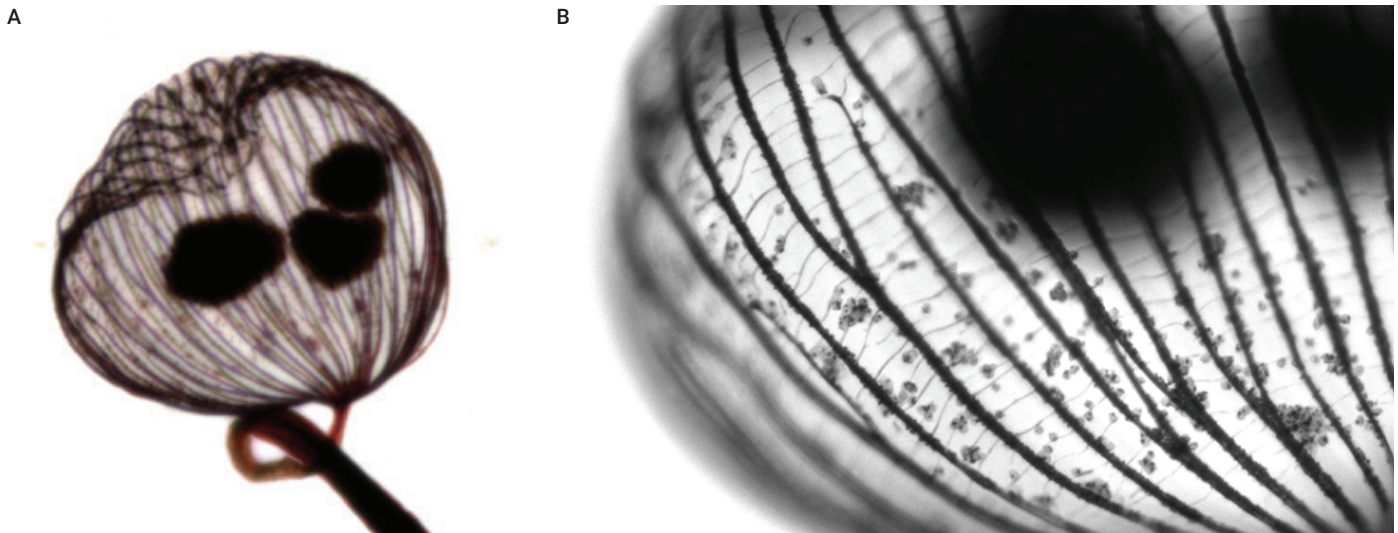


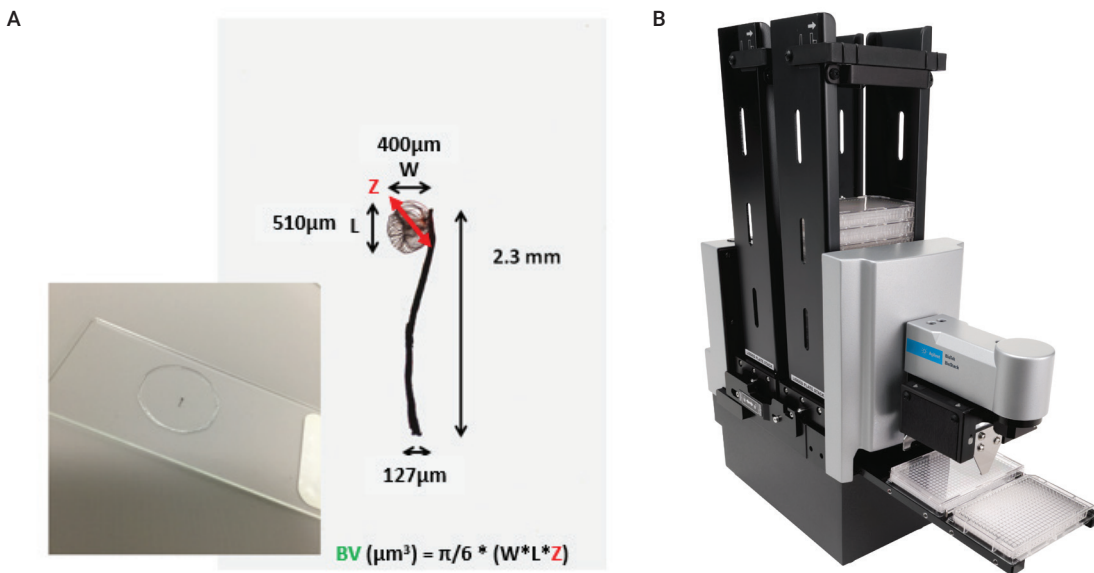
# Automated Image Analysis of *Dictyidium* Sporothecae Biovolume Using Robotics

## Introduction

*Dictyidium cancellatum* is a myxomycete (slime mold) of the order *Liceales* found mostly in wet, dark forest environments where it decays organic matter. A distinguishing feature of *dictyidium* is the sporotheca, a bulbous container at the head of the organism that produces and contains the spore mass of the fruiting body, and as such plays an important reproductive role in the *dictyidium* life cycle. *Dictyidium* sporangia have been described as a cage, net, basket, or more commonly a Chinese lantern because of the unique shape created by thin strands of hyphae that make-up the peridial net. Biovolume measures cubic space of an object, and as such is one way to investigate changes to the sporotheca that could indicate a loss of reproductive viability of the organism, for example from stressors brought about by disruptions to ecological climate homeostasis. In this case study, whole mount *dictyidium* samples were used as a model to compare biovolume of the sporotheca for multiple specimens. This was accomplished by capturing digitalized microscopy images using a z-stacking method, whereby multiple images were taken at a constant focal height interval resulting in a depth value. A final z-projected image was then rendered from a range of in-focus z-stacks, and a primary mask was applied to the final region of interest to obtain length and width values. One way to study *dictyidium* is by using a wet mount on standard microscopy slides, a format that can limit the efficiency of acquiring and analyzing large data sets. A robotic arm was therefore used to automate slide imaging at increased throughput, a solution that could enable larger population studies.



**Figure 1.** (A) A color brightfield z-projected image shows large spore aggregates within the peridial net of a *dictydium* sporotheca. (B) A digitally zoomed brightfield image at one focal height shows individual and small clusters of spores among the hyphae. Both images captured at 10x on an Agilent BioTek Cytation 5 cell imaging multimode reader.



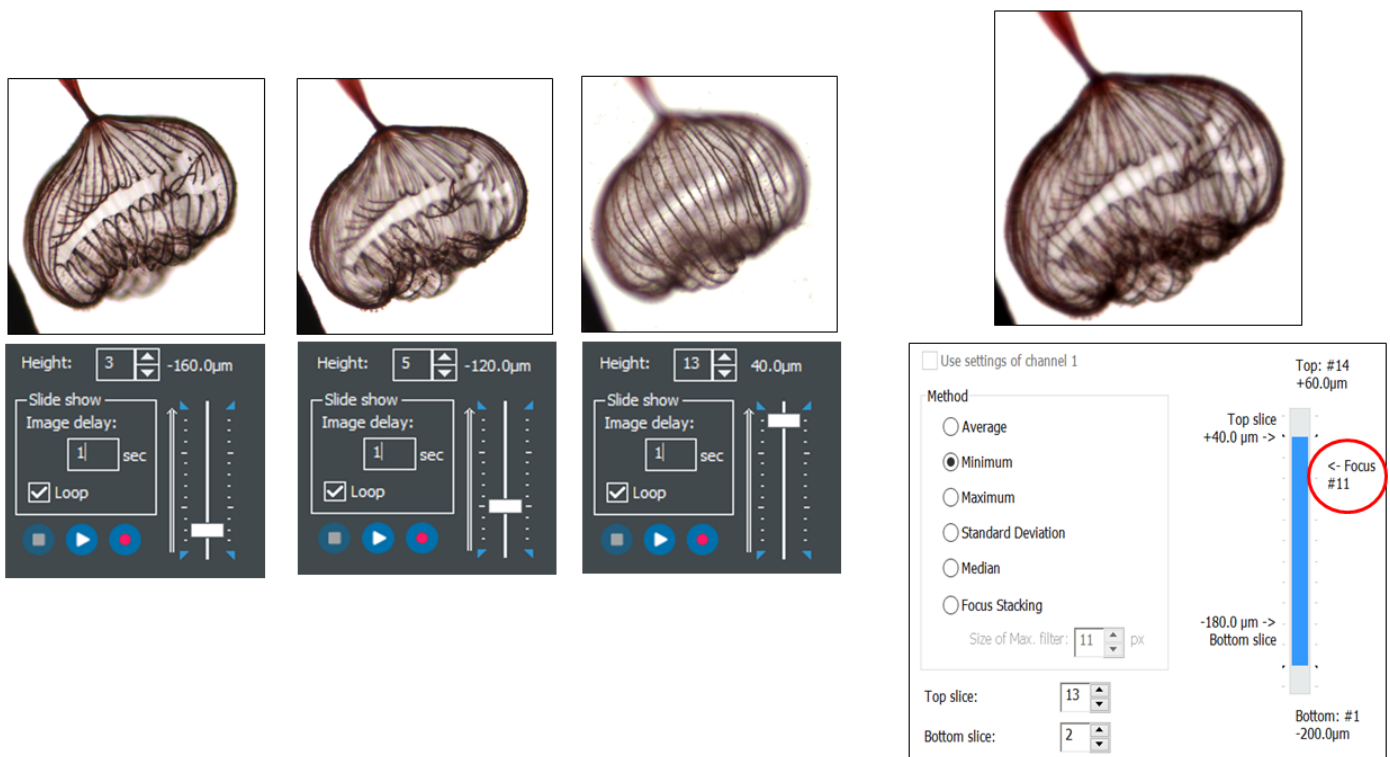
**Figure 2.** (A) Dimensions of a typical mature *dictydium* were manually calculated from the microscopy slide shown inset and overlaid on a stitched 4x color brightfield image of the same slide. Biovolume (BV) calculation on multiple organism sporangia was automated using an Agilent BioTek BioStack 4 robot (B), an Agilent BioTek Cytation 5 imager, and Agilent BioTek Gen5 multimode reader and imager software. A z-stacking feature within Agilent BioTek Gen5 data analysis software allowed calculation of the depth parameter of the sporotheca (Z). Primary masking on a final z-projection provided length (L) and width (W) values.

## Materials and methods

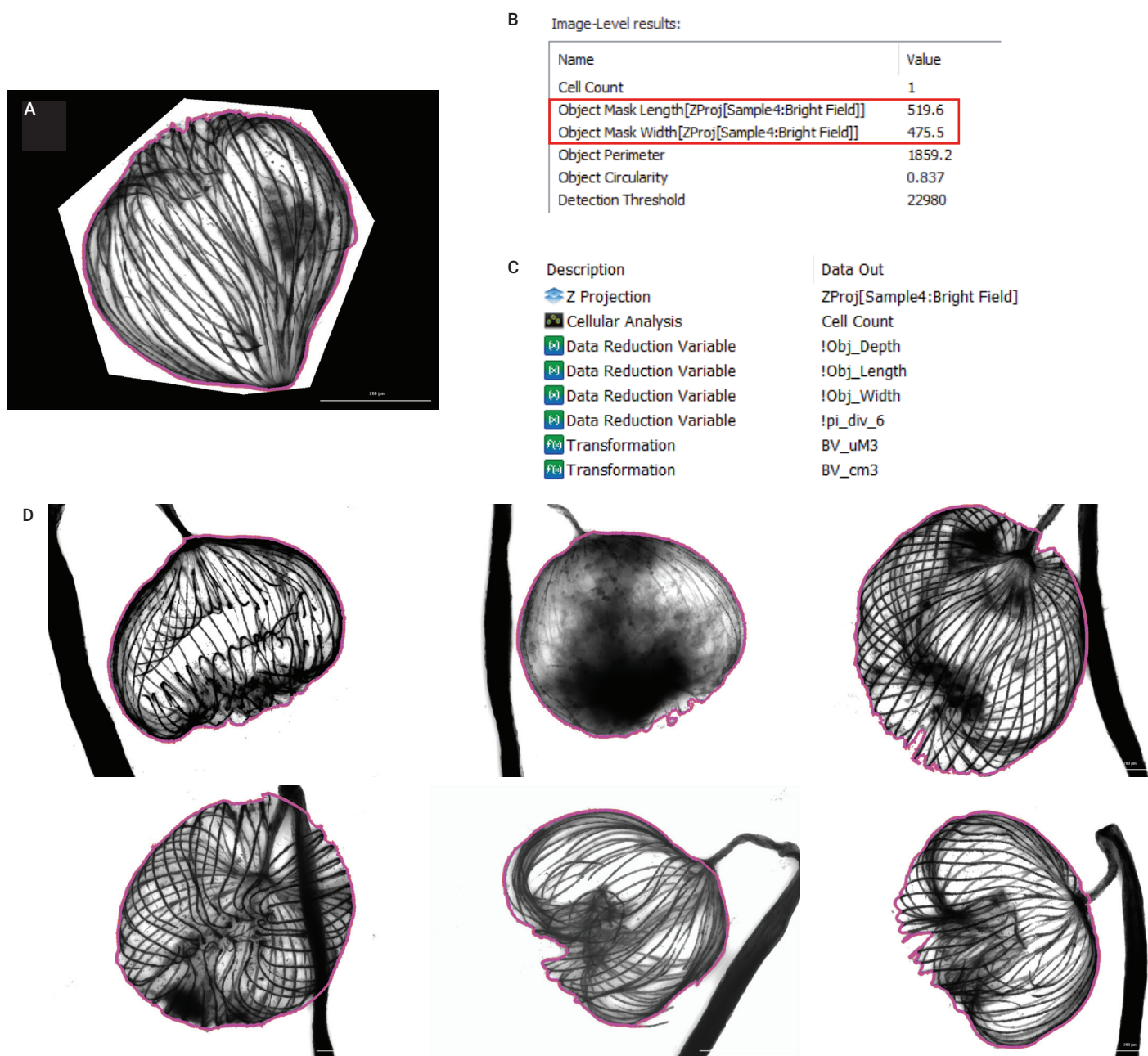
Ten whole wet mount *dictyidium* slides, part number 29-7328, from Carolina Biological Supply Co. (Burlington, NC) were loaded coverslip down into five Agilent BioTek slide holders (part number 1220548). Slide holders were loaded into a 10-plate supply tower of an Agilent BioTek BioStack 4 linked to an Agilent BioTek Cytation 5 cell imaging multimode reader using an integration kit (part number 7310053). Under the control of Agilent BioTek Gen5 software, slides were robotically transferred from the BioStack to the Cytation 5 and images were captured in brightfield and color brightfield modes. For one batch, multiple images of each slide were captured using a 2 × 3 montage with a 4x objective (part number 1220519). In the second batch, average slide horizontal and vertical offset values were used to image each slide using a 10x objective (part number 1320516). Z-stacking values of 14 slices, taken 20 µm apart with 10 images

requested below a fixed focal height of 600 µm, resulted in a total focal range of 400 to 660 µm at 10x. A z-stack slide viewer and slide show feature were used to review all 14 image slices, and a z-projection on the optimal focal range was performed using a method of 'minimum' (Figure 3). The final depth of each sample was calculated as the total focal range (µm) of the final z-projection and was entered into Gen5 data reduction using a custom variable. A polyline plug shape was applied to isolate the sporotheca perimeter on each image (Figure 4). Using the 10x brightfield image on a light background, a primary mask with a detection threshold of 22980 and a selection to fill holes in the mask and include primary edge objects was applied to an object size selection of 100 to 1,000 µm (Figure 4). Gen5 calculates object length and width measurements using an ellipse overlay correction, and therefore an ellipse equation<sup>1, 2, 3</sup> was defined in Gen5 data reduction to solve for biovolume (Figure 4).

## Results and discussion

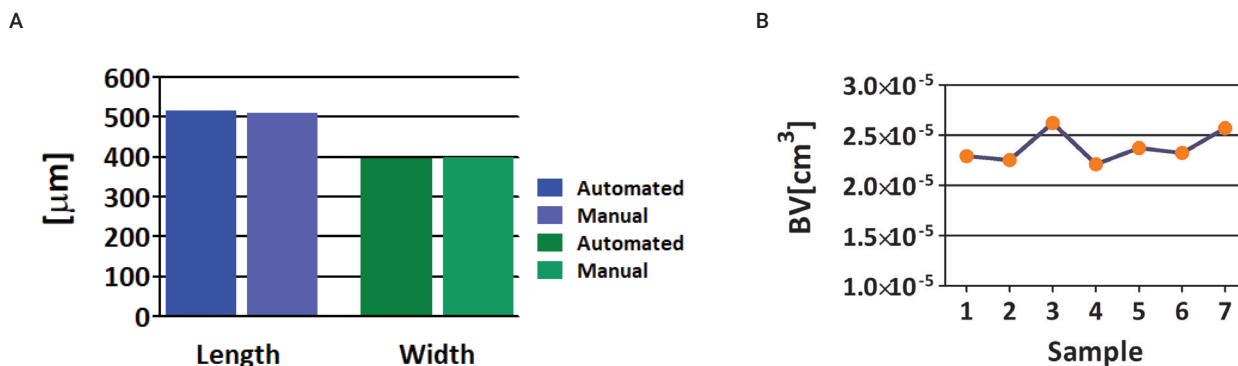


**Figure 3.** (Left) Three of fourteen z-stack images captured from a *dictyidium* sample. Minimum and maximum focal points for the object of interest can be determined using a slide show feature. The final z-projection for this specimen was rendered on stacks 2 to 13 (right). Focus slice #11 (circled) was fixed at 600 µm for all samples. The z-projection shown resulted in a total sporotheca depth of 220 µm obtained by multiplying the number of slices in the projection (11) by the focal interval between each slice (20 µm).



**Figure 4.** (A) A polyline plug is used to isolate the *dictydium* sporotheca on the z-projected image. A primary mask (pink) was defined on the object perimeter to obtain length and width parameters used in the Agilent BioTek Gen5 data analysis software data reduction steps that generate the biovolume calculation (B and C). A montage of representative samples displaying the applied primary mask criteria in full pixel view is shown (D).





**Figure 5.** (A) For the sample shown by Figure 2, length and width values measured manually by an independent party using a micron caliber are compared to those for the same sample obtained from automated image analysis. Length and width values each differed by less than 1% of the mean. (B) Automated z-stack imaging has the advantage of also generating a depth value, facilitating the ability to quantitate biovolume of an object, as shown for the seven randomly selected dictyidium samples in Figure 4D. Biovolume units ( $\mu\text{m}^3$ ) were converted to  $\text{cm}^3$  by applying a factor of 10 to 12. Although visually distinct, *dictyidium* exhibit homogenous sporotheca biovolume.

## Conclusion

Z-stacking, z-projection, masking, and data reduction tools available within Agilent BioTek Gen5 multimode reader and imager software can be used to obtain physical dimensions of 3-D samples that can be applied to calculate and quantitate biovolume, as demonstrated using a *dictyidium* model organism. To increase accuracy when interpreting very low calculated values, it is suggested to apply scientific notation with maximum significant digits when viewing results in the Agilent BioTek Gen5 data analysis software. Biovolume, or derivatives thereof, could also be applied to investigate and quantitate a range of other 3-D samples, for example, cancer cell spheroid models, small mammal and insect organs, tissue sections, or micro environmental growth monitoring such as biofilm formation. Applied robotics facilitated higher throughput microscopy slide imaging, but is also compatible with many vessel types, enabling larger data sets to be acquired without manual intervention. The Agilent BioTek BioStack 4 robotic arm is available with three tower sizes, accommodating from ten to fifty vessels in a single batch. When imaging slides at higher throughput, it is recommended to integrate both montage and z-stack imaging steps to cover an imaging area large enough to compensate for variations in mounting location between slides.

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Printed in the USA, April 1, 2021  
5994-2562EN