USING LASER CONFOCAL SCANNING MICROSCOPE IMAGES TO RECONSTRUCT THREE-DIMENSIONAL VIEWS OF FRUITING MORPHOGENESIS IN *COPRINUS CINEREUS* AND *PLEUROTUS PULMONARIUS*

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We are developing the use of laser confocal microscopy to establish an accurate data set describing the geometrical arrangement of the hyphal components of fungal tissues. This cannot be done using conventional microscopy because z-axis (vertical) dimensions and internal branch angles cannot be measured. After a range of trials, involving conventional serial sectioning for both light and electron microscopes, we obtained data from serial ***optical*** sections using samples of fungal tissue labelled with fluorescein isothiocyanate labelled lectin to reveal the N-acetyl-beta-D-glucosamine/glucosaminyl groups of hyphal walls. Three-dimensional reconstruction was achieved utilising a standard desktop personal computer, with AVS/Express and Confocal Assistant software. The confocal images are readily converted to red/green anaglyphs (using Confocal Assistant) which provide an easily realised three-dimensional visual sensation. However, the intention is to produce three-dimensional visualisations (using AVS/Express). These are fairly primitive at the moment (though they can be rotated for viewing from various angles), but they hold the promise of development to full 3-D visualisations which can be inspected 'from within' and used to extract geometrical measurements. A number of factors still need to be addressed to make this a routinely useful procedure. Such visualisations will be the source of the accurate 3-D observational data needed to enable tissue structure to be mathematically described. Parameters extracted from the visualisations will enable computer modelling to be extended into 3 spatial dimensions. The aim is a computer model able to simulate the hyphal architecture of mushroom tissues that can be explored to catalogue the structural effects of changes in its parameters. Study of these simulations will reveal morphogenetically important parameters and define experiments to improve knowledge of *in vivo* morphogenetic control very considerably.

The evolutionary separation between the major Kingdoms must have occurred at a stage when the most highly evolved things were single cells. Consequently, each Kingdom has independently evolved ways to organise populations of cells to make the multicellular organisms we now know as mushrooms, mice or marigolds. The three major Kingdoms we see today are very different from one another in ways that determine shape and form.
A key feature during the embryology of even lower animals is the movement of cells and cell populations, so cell migration (and everything that controls it) plays a central role in animal development.

- Being encased in walls, plant cells have little scope for movement and their changes in shape and form are achieved by regulating the orientation and position of the wall which forms when a plant cell divides.
- Fungi are also encased in walls; but their basic structural unit is a tubular cell, called a hypha. It has two peculiarities which result in fungal development being totally different from that in plants. These are that the hypha grows only at its tip and that new walls form only at right angles to the growth axis of the hypha.

Studying ***how*** a mushroom ***makes*** a mushroom is an investigation every bit as deep and meaningful (and difficult!) as studying how a human embryo develops or how a tree is

shaped and sculptured in the forest. Sadly, in the popular imagination mushrooms don't have the same status as human animals or forest trees and, anyway, mycologists have never been very good at thinking in four dimensions.

We need an ***objective*** view of tissue structure, yet the best descriptions available are ***subjective interpretations***.

If we are to understand ***how*** fungal morphogenesis works we need first to find out ***what*** it consists of. We need to catalogue the patterns of hyphal growth and hyphal branching that produce particular shapes and morphologies. We can't do that from 2-dimensional renditions - even when they are combined with educated guesses about how they might be related. Guesses are still only guesses no matter how erudite the guesser! [Don't believe what I ***tell*** you, ***look*** at what I'm showing you!]

We need to "get inside" a 3-dimensional chunk of tissue to ***measure*** growth directions, branching frequencies, branch directions and branch growth kinetics. And not just one chunk of tissue, but hundreds, thousands even, so that we have a library of such descriptions large enough to reveal underlying developmental strategies and show similarities and differences.

Conventional sections for the light microscope (20, 30, 40 μm thick) are not good enough to study hyphal distributions. They are so thick that hyphae disappear within them and even with serial sections you find a totally different population of hyphal profiles exposed at each successive cut surface. Good for tissue morphologies and distribution, maybe, but not for individual hyphae.

Embedding in resins like glycol methacrylate and even Spurr's resin enables very thin serial sections to be cut. But we are left with three problems:

- specimen preparation (fixation, dehydration, embedding) raises the usual question of how much of what is left is ***real***?
- section orientation (on the slide or grid) becomes a major issue; if you remember that you (or your computer) will be trying to marry up the position of the same hyphal wall over a (hopefully large) number of successive sections you will appreciate that those successive sections need to be oriented in *x* and *y* directions to an accuracy ***less than the thickness of the hyphal wall***.
- the wall might be 100 to 200 nm thick; if you are skilled enough to produce sections which are only half a micrometre in thickness, the wall you (or your computer) are trying to follow can extend ***within the plane of one section*** so that hyphal profiles in serial sections lose their spatial relationships.

We have found greatest promise with the confocal microscope. Fresh specimens can be examined, and the observation technique produces digitised optical sections without mechanical disturbance.

It's not free of problems. Specimens can move during observation so an embedding agent to restrict this is necessary - glycerol or low-gelling temperature agarose work well. The microscope depends on laser-excited fluorescence so we need a fluorescence "stain" for chitin. FITC-conjugated wheat-germ agglutinin and calcofluor are effective, but penetration of the "stain" into the block of tissue, uniformity of fluorescence and fading are issues which remain.

Nevertheless data sets have been obtained which can be used to produce anaglyphs that give a good ***visual*** impressions of depth and parallax and consequently go one step further than conventional 2-D images. But these are not suitable for quantitative measurements.

Fortunately, the digitised images which contribute to the anaglyphs can be extracted and prototype ***3D visualisations*** have been produced using AVS, an industry-standard work-station/mainframe software suite that generates 3-dimensional models within the computer.

These are not just images in a 3-D rendition. They are computational models which approach the aim of being virtual tissue segments which can be explored - from any external direction ***and from within***.

So we've shown that the virtual mushroom is attainable.
Of course, we've now run out of funding! Any suggestions? Any offers?!