

Making photonic structures via cell culture: *Morpho* butterfly scales

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The photonic structures that cause the electric blue colour of some *Morpho* butterflies are desirable within industry. They have been reproduced on very small areas to produce prototype biomimetic structures, yet there is currently no technique to accurately manufacture these structures at a commercial level. Here, tissue and cell culture techniques are explored as a possible method of producing large quantities of the blue scales of *Morpho peliedes*. This represents the first attempt to culture the wing scales of a *Morpho* butterfly, and many problems were encountered. These could be remedied in future studies, but it is concluded that cell culture is probably not a viable technique to provide the *Morpho* structure for industry in the near future. However, tissue culture experiments revealed important results of scale development.

1. Introduction

Animals and plants boast a range of sub-micron photonic devices, such as to produce structural colour.¹ While physicists design their photonic devices to suit the human eye or specific detectors, the devices of animals and plants have evolved to target different but equally precise detectors – eyes – and under specific conditions. Optical biomimetics involves the characterisation of natural photonic devices and the manufacture of artificial analogues. Currently, it exploits modern, sub-micron fabrication techniques, although has to progress yet far beyond the prototype stage. Although the ultimate goal of optical biomimetics is to contribute to commercial products, it may also provide some degree of inspiration for industry.

The most iconic species in the field of natural photonics is the *Morpho* butterfly. There are around 29 species of the genus *Morpho*, inhabiting Central and South America, and most are conspicuous by their electric blue structural colour (although some are pearlescent).² The scales of different species of *Morpho* have been studied for their optical properties, using different methods (e.g. ^{3,4,5,6}); the colour is generated mainly as a result of a quarter-wave stack involving alternating thin layers of chitin (refractive index = 1.56) and air, with around three or four pairs of layers

(Figure 1). Often this colour-generating scale is covered by another scale, which serves to further scatter the light. Beneath the scale system exists (in the electric blue species) a layer of the dark absorbing pigment, melanin.

Analogues of blue *Morpho* butterfly scales have been manufactured.^{7,8} Originally, corners were cut. Although the *Morpho* wing contained two layers of scales, the model copied only the principle.⁷ However, the engineered device closely matched the butterfly wing – the colour observed changed only slightly with changing angle over 180°, an effect difficult to achieve and useful for a broad-angle optical filter without dyes.

A new approach to make the 2D ‘Christmas tree’ structure (a vertical, elongated ridge with several layers of 70-nm thick side branches) has been achieved using focused-ion-beam chemical-vapour-deposition (FIB-CVD).⁶ By combining the lateral growth mode with ion beam scanning, the Christmas tree structures were made accurately. However, this method is not ideal for low-cost mass production of 2D and 3D nanostructures, and therefore the ion-beam-etched Christmas trees are currently limited to high-cost items including nano- or micron-sized filters (such as ‘pixels’ in a display screen or a filter).

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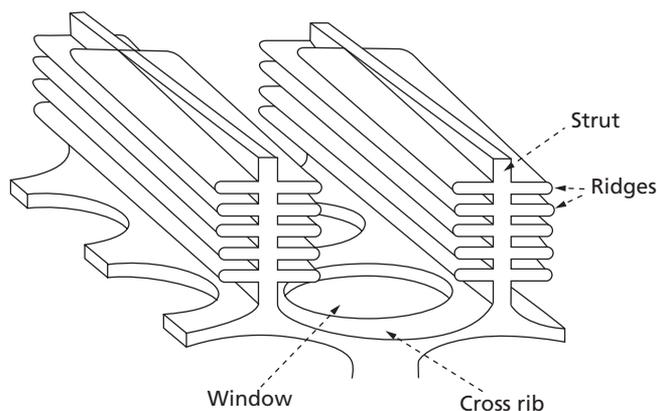


Figure 1. A diagrammatic dorsal (uppermost) section of a general *Morpho* scale that generates strong, blue, structural colour. The ridges, and the air between, form the two layers of a quarter-wave stack (a 'Christmas tree' structure when viewed in cross section), while in some cases elements of the struts cause additional scattering effects

In an attempt to find a cost-effective method for the reproduction of the *Morpho* reflector, we may exploit an aspect of the butterfly other than its architectural design – that the *Morpho* makes its own scales efficiently. Therefore, can we let nature manufacture the devices for us via cell culture techniques?

Animal cells are in the order of 10 μm in size and plant cells up to about 100 μm , and hence suitable for nanostructure production. The success of cell culture, though, depends on the species and on the type of cell from that species. Insect cells, for instance, can be cultured at room temperature, whereas an incubator is required for mammalian cells. Cell culture is not a straightforward method, however, since a culture medium must be established to which the cells adhere, before they can be induced to develop to the stage where they make their photonic devices.

The aim of this paper is to explore the possibility of utilising tissue and cell culture techniques for the purpose of the future manufacturing of *Morpho* reflectors, through culturing the cells that manufacture the structurally coloured scales in the blue butterfly *Morpho peleides* (a species accessible for this study through a local insect supplier (Stratford Butterfly Farm)). Although Ghiradella⁹ and Overton¹⁰ have studied butterfly scale morphogenesis in general, this represents the first study on cell culture in the iconic *Morpho* species. As part of this study, the methodology required experimentation, and the final procedure used is itself an important part of the results.

The Peleides Blue *Morpho* (*Morpho peleides*) is an iridescent tropical butterfly found in Mexico, Central America, northern South America, Paraguay and Trinidad. It uses its electric blue colour to frighten away predators, by flashing its wings rapidly. Its wingspan ranges from 7.5 to 20 cm, and its entire life cycle, from egg to adult,

is only 115 d; adults live for approximately 1 month; the chrysalis stage will last from a week to a few months. Weather plays a huge role in the duration of the chrysalis stage, as the chrysalises synchronise their emergence with the arrival of seasonal rains. In places where rain is extremely common, *Morpho peleides* can mate year round and the chrysalis stage is short.² The precise photonic structure of the structural blue scales has been reported (Figure 2).⁶ In particular, the windows in the scales have extended to the edges of the struts, leaving only narrow cross-ribs that join adjacent struts. In this study, the wing tissues from *Morpho peleides* chrysalises are examined up to 22 d of development in culture.

2. Methods and results

Butterfly specimens were obtained as chrysalises. Through experimentation, the following methodology was performed, in order to obtain cultures of the scale-forming cells.

2.1 Chrysalis preparation and structure

The chrysalises were allowed to hang in order for the wings to form properly, whereby they were glued to a horizontal support at their abdomens. They were kept at approximately 20°C–25°C during the day and 18°C at night. The humidity was kept high by keeping damp capillary matting at the bottom of the cage. Once emerged the butterflies were found to eat rotting fruit, preferably banana or orange.

Where delay in emergence was required for practical reasons, this was achieved by keeping the chrysalises at 18°C, which delayed emergence for 3–4 d. However, the percentage viability was greatly reduced since they lacked the strength to emerge.

The chrysalis is a waxy green structure around 3 cm in length that could be cut with a sharp knife and dissecting scissors. When dissecting, the internal structure was anticipated from the grooves on the external surface of the chrysalis. The insect head lies at the opposite end to the 'cap and stalk' by which the chrysalis is suspended. The cap end contains a large amount of white/yellow fatty fluid.

In order to obtain undamaged wings, the first step was to slice off the cap. This also resulted in the death of the insect. The two sets of wings lay on either side of the grooves that are apparent from the external surface. The fore and hind wings lay on the top of each other. It was found best to cut open the chrysalis from the opposite side to the wings; this side has faint horizontal grooves.

As soon as the chrysalis was dissected, the tissue and fluids rapidly turned black (in seconds to minutes). This was due to the presence of phenyloxidase in the haemolymph. The activity of the phenyloxidase can be inhibited with phenylthiourea (PTU). PTU works by binding strongly to divalent cations such as manganese, and magnesium, to inhibit enzyme activity. Samples

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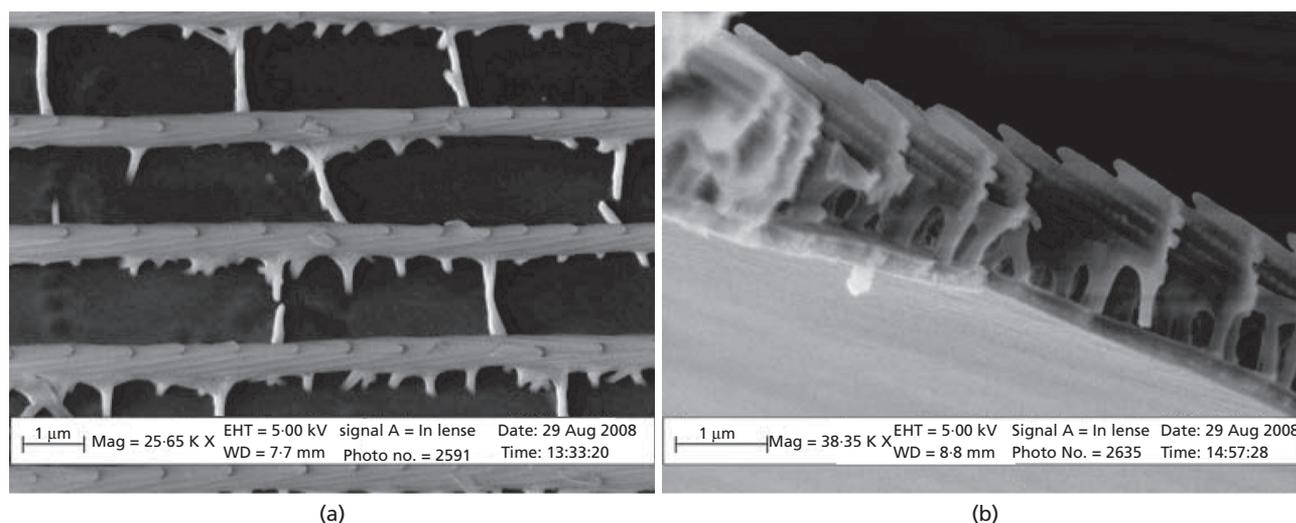


Figure 2. Scanning electron micrographs of a section of a blue colour-generating scale of *Morpho peleides*; (a) dorsal view and (b) cross section. Reproduced with permission from the authors (Ref. 6)

were routinely dissected into Graces media supplemented with 40 micromolar N-phenylthiourea antibiotic/antimycotic from Sigma (Poole, UK).

2.2 *In vitro* growth of *Morpho peleides* pupal wings

Pupal wings were carefully dissected in their entirety from the chrysalis directly into phosphate-buffered saline (PBS) and phenylthiourea. At $t = 0$, the sample was placed into 70% ethanol, and then into a fresh 70% ethanol solution, and then stored in 100% ethanol. The remaining wings were incubated individually in small petri dishes with Graces media supplemented with antibiotic/antimycotics at 27°C. The dishes were taped with micropore tape to limit evaporation while allowing gas exchange. The media was changed approximately every 5 d. Samples were examined by scanning electron microscopy (SEM) (Figure 3).

Wing tissues examined, before preparation for SEM, showed that pigment maturation had occurred, indicating that the enzymatic processes necessary for pigment production were active *in vitro* (Figure 4). However, the structural blue colour remained absent throughout.

2.3 *In vitro* culture of *Morpho peleides* cells

Once the wings had been excised, the cells were isolated. This was achieved to some degree by mechanical disruption, but further enzymatic breakdown was required. Here, the enzymes trypsin and dispase were tested; dispase emerged as less harmful to the tissue. Dispase is a bacillus-derived metal protease that works by cleaving fibronectin, collagen IV, and to a lesser extent collagen I (it cannot cleave collagen V or laminin). The dispase was stored as a 50x stock solution (1x is approximately equal to 0.6 U/ml), obtained from Roche Applied Sciences Cat. No. 10 210 455 001. The

reaction with dispase was performed in Dulbecco's PBS (Sigma, D8537) overnight at room temperature.

Cells from *Morpho peleides* were cultured and shown to produce projections, as is usual in the initiation of a cell line. Here, pupal wings were excised and dissected into small (1 mm²) segments. The segments were incubated with trypsin overnight, and disrupted further mechanically, then incubated in Graces medium, 5% fetal calf serum (FCS).

Cells from explant were spherical at the initiation of the culture. Projections (Figure 5) were first seen after 4 d.

However, yeast in the culture rapidly colonised the culture and by around 8 d prevented further initiation of a cell line. This was despite the addition of antimycotics.

Cleaning of the external surfaces of the chrysalis with benzalkonium chloride was attempted. Even when the surface was cleaned, however, it was probable that the inner tissues had yeast present, which could not be removed without destroying the tissue. This could possibly be remedied if the butterflies were reared under sterile conditions rather than in glasshouses at the Stratford Butterfly Farm. Also, it is possible that this problem could be overcome with more sophisticated tissue culture facilities.

3. Discussion

In this promising study, tissue comprising an entire forewing from a *Morpho peleides* chrysalis was successfully cultured to the stage of producing melanin (background) pigment, and near complete development of the photonic scale structures (Figure 3). The tissue

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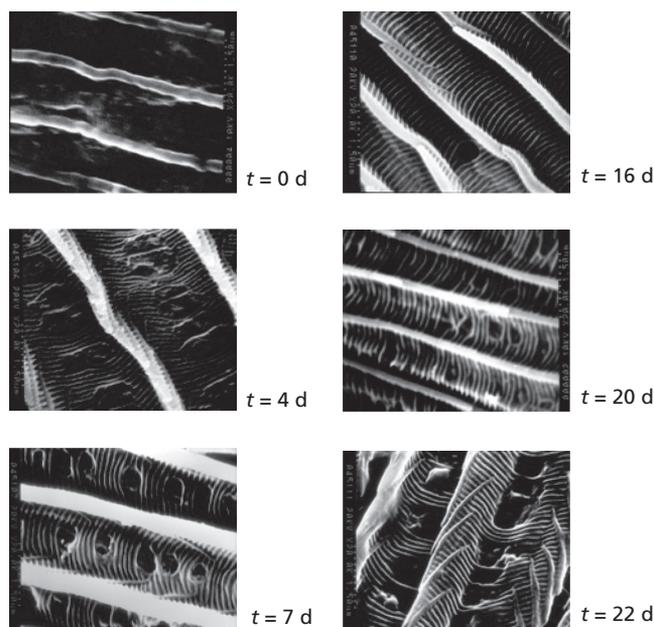


Figure 3. Scanning electron micrographs of sections of a single developing scale after different stages of development (dorsal views). The struts (large, parallel 'bars') are spaced by about 1.0–1.6 μm

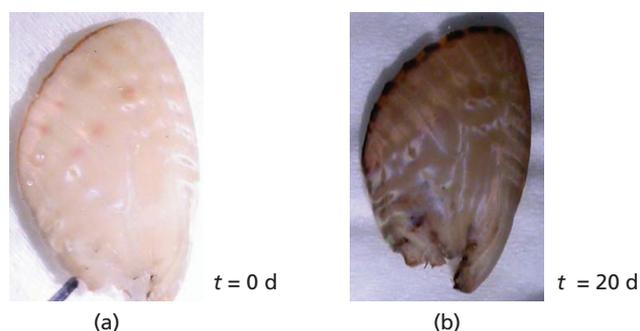


Figure 4. Excised pupal forewings, on immediate removal from the chrysalis (a) and after 20 d incubated in growth medium (b). The wing is shown to develop pigment after 20 d, an enzymic reaction

was excised from the chrysalis at the stage where only the basis of struts had developed. After 4 d in culture, further definition in the scales was evident, in the form of transverse striations. Windows began to open up and further develop from 4 to 22 d, and by 22 d they had reached the near mature stage of becoming almost complete open spaces; cross-ribs remain as the borders of the windows, gradually decreasing in number.⁶ Also, after 4 d, the origins of ridges could be observed, which became increasingly prominent through to 22 d, although did not reach the full mature stage by that time. The striations (which appear much reduced on

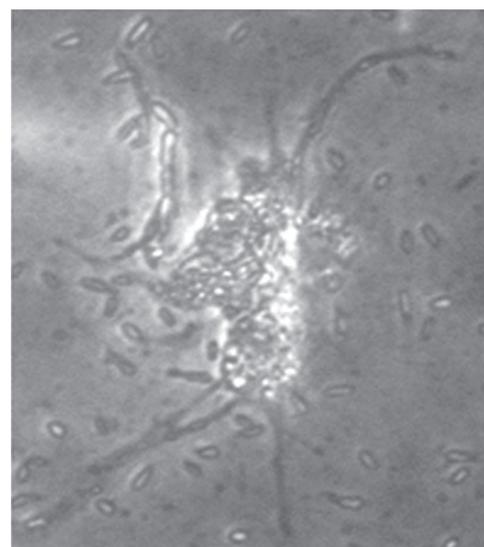


Figure 5. Transmission light micrograph of a cell from *Morpho peleides* after 8 d of incubation, revealing projections that are indicative of the start of a cell culture line. Magnification: $\times 400$

an adult scale)⁶ remained equally prominent on the struts, and this lack of a completely mature stage was also evident through the absence of the blue structural colour.

In the next experiment, the initial stage of the start of a cell culture line was reached, but restrictions in the methodology prevented further development of the cells towards the production of wing scales. While the main problems with establishing a cell line were due to yeast contaminations of the cultures, it still remains a major obstacle that any cell line produced would most likely be basal wing tissue. The growth of a scale from this tissue would probably require a complex biochemical signalling pathway, for example, Bombyxin II. Furthermore, the wing is not composed of only one type of cell that is what would be created in a cell culture line. On the wing, the scale cell is held by specialised socket cells within a general lamina of cells.^{9,10} Cell lines would therefore possibly need to be derived from wing imaginal discs. However, published cell lines from imaginal discs still do not produce scales.

In butterflies in general, it is known that, during larval development, epithelial cells in the imaginal discs (developing wings) segregate and undergo two rounds of cell division. First, a scale precursor cell divides and produces two daughter cells, one of which dies. The second cell differentiates from the surrounding epithelial. This first round of cell division occurs around 15 h after pupation. The surviving cell then increases in size and becomes polyploid. This cell then divides again around 24–26 h after pupation. The resulting cells are either socket-building cells or scale-building cells. Seventy-two hours after pupation, the scales begin to be secreted through the sockets.^{9,10} Therefore,

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to further these experiments it would probably be necessary to have a culture comprising both socket- and scale-building cells and for them to know how to self-organise. After the initiation of a scale, the cell would then need to secrete cuticle: scales finally result from the covering of precisely arrayed cell structures with chitin.^{9,10}

In general, it is clear that *Morpho* crystalises are not the ideal candidates for cell culture studies; they are far from model species. Most problematic, however, is the concept that each scale on the *Morpho* wing results from the sacrifice of a living cell: the scale cell. Although it is possible that the socket cells, which make the scale cells (and ultimately the scales), could exist in a cell line to produce replenishing batches of scales, probably much work is needed to discover whether this ultimate aim of optical biomimetics will ever be possible. The next stage in this research, however, should perhaps continue with a more detailed mapping of the development of the scale cells in a complete wing tissue.

In terms of manufacturing optical devices found in nature through cell culture techniques (for industrial application), a far simpler task emerges where the iridescent organism is single-celled. Diatoms, for example, carry an added advantage of exponential growth in numbers – each individual can give rise to 100 million descendants in a month.¹¹ Unlike most manufacturing processes, diatoms achieve a high degree of complexity and hierarchical structure under mild physiological conditions. Fuhrmann *et al.*¹² showed that the presence of pores in the silica cell wall of the diatom *Coscinodiscus granii* means that the frustule can be regarded as a photonic crystal slab waveguide. Furthermore, they present models to show that light may be coupled into the waveguide and give photonic resonances in the visible spectral range. Such iridescent structures may be suitable to provide structural colour in paints and cosmetics, for example, and certainly provide an ease of culture that, at present, makes them more suitable than *Morpho* butterflies as industrial photonic devices that can be produced by cell culture techniques.

Acknowledgement

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