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BIOMINERAL ELECTRON BACKSCATTER DIFFRACTION FOR PALAEONTOLOGY

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Abstract: Electron backscatter diffraction (EBSD) originated in materials science and has transferred to biomineral research providing insight into fossil and modern biominerals. An electron microscopy technique, EBSD requires a fine polished sample surface where the electron beam diffracts in the first few lattice layers, identifying mineral, polymorph and crystallographic orientation. The technique is particularly well suited for the analysis of modern and fossil calcium carbonate biominerals, where it provides key insight into biological control of mineral formation such as in molluscs and brachiopods. EBSD readily identifies original and secondary mineralogy, which helps to inform our understanding of biomineral evolution such as the identification of original aragonite in Silurian trimerellid brachiopods. As a technique to identify and thus avoid the inclusion of secondary minerals in proxy organisms such as corals, EBSD can be used to ensure accuracy of palaeoproxy data. Even when fossil systems have no modern equivalents, EBSD can provide key data to determine functional mechanisms such as in the lenses of schizochroal eyes of phacopine trilobites. These few examples illustrate that EBSD is proving to be a valuable component of the palaeontology toolkit.

Key words: electron backscatter diffraction, biomineral, crystallography, proxy.

LIVING systems produce mineral structures that perform many functions such as protection, embryonic chambers, locomotion, balance and gravity sensing (Lowenstam and Weiner 1989). These hard biological structures have higher preservation potential than soft tissues, and therefore, biominerals comprise a significant component of the fossil record. Knowledge of the composition and structure of fossil biominerals, or those of their descendants, provides information on the biological process of formation as well as the environment in which the biomineral formed. The wide range of techniques available to study biominerals (DiMasi and Gower 2014) each provide different information such as X-ray diffraction identifying the minerals present, stable isotope measurements of $\delta^{18}O$ providing a means of calculating ambient water temperature and X-ray tomography revealing the 3D structure of biominerals. Electron backscatter diffraction (EBSD) is a technique that originated in materials science for the study of metals for which it is still used extensively (Schwarzer *et al.* 2009). EBSD is an electron microscopy technique, either scanning or, less commonly, transmission, where an electron beam interacts with the first few lattice layers of a polished sample to determine the identity of the metal or mineral, the polymorph and crystallographic orientation at that analysis point. A grid of point analyses essentially provides a map of diffraction intensity, mineral polymorph and crystallographic orientation (Figs 1–3). EBSD has been transferred to biomineral research to identify mineral polymorph and crystallographic orientation *in situ*. This information is essential to understand the biological control exerted on biomineral formation in modern and fossil structures, it helps us understand biomineral function and material properties, and to identify original and secondary minerals even when the secondary mineral alludes to having the same mineral composition as the original; this is important for identifying diagenetic mineralization that could distort palaeoclimate calculations.

BIOLOGICAL CONTROL OF MINERAL FORMATION

To appreciate the importance of biological control exerted during biomineral formation, more information on the process of biomineralization is required. Biologically induced mineralization refers to nucleation on an organic layer or biofilm, such as the highly charged

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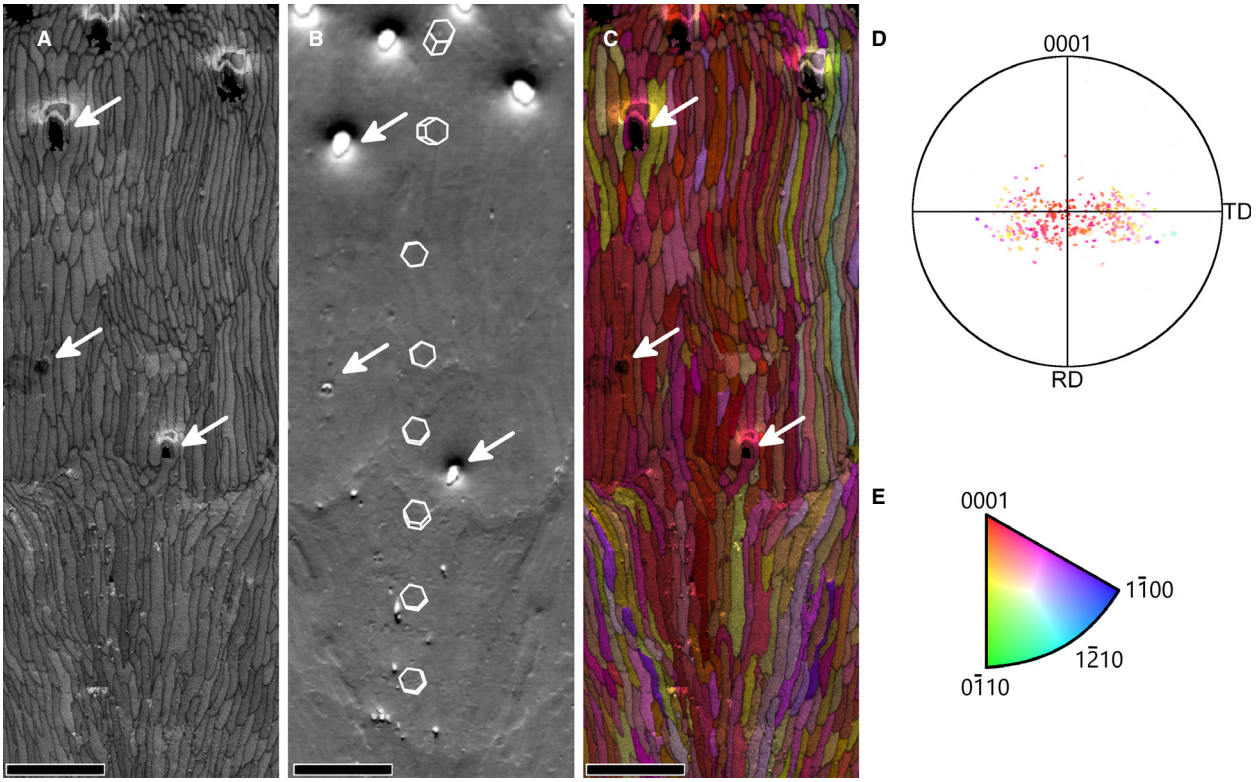

FIG. 1. Electron backscatter diffraction (EBSD) analyses of a modern *Terebratulina retusa* brachiopod shell. *T. retusa* shell embedded in life position in epoxy resin, ground and polished to remove the primary layer and investigated by EBSD as in Pérez-Huerta and Cusack (2009). A–C, area of shell analysed, with some of the punctae indicated by white arrows; A, diffraction intensity map; B, secondary electron image with wire frames indicating the orientation of the calcite *c*-axis; C, crystallographic orientation map, overlain on diffraction intensity map of A, with the reference direction normal to the plane of the page. D, pole figure indicating the crystallographic orientation of the calcite fibres in A–C. E, colour key indicating the crystallographic planes corresponding to the colours in C and D. Scale bar in A–C represents 50 μm.

polysaccharide films of cyanobacteria that result in calcium carbonate deposition in stromatolite formation (Macintyre *et al.* 2000). Biologically induced mineralization (Mann 2001) does not exert genetic control other than in the production of polymers that induce nucleation. In contrast to this essentially passive process, biomineralization usually refers to biologically controlled mineral formation (Mann 2001) that is under strict biological control where biology controls the shape, dimensions and even the polymorph of the mineral components with resultant species-specific structures.

The differences between biominerals and their non-biogenic counterparts are well established. The intimate association of organic and mineral components (Smith *et al.* 1999), the hierarchy of biomineral structures (Weiner and Wagner 1998; Aizenberg *et al.* 2005) and controlled crystallographic alignment (Pérez-Huerta *et al.* 2007*a*) results in material properties that are highly advantageous in biogenic structures with, for example, many marine shells being light and strong and able to resist crack propagation. The rules of classical crystal growth that result in well-faceted crystals with sharp edges do not apply to biomineral formation where mineral nanoparticles with associated organic components are packed, often in crystallographic register to produce mesocrystals that are effectively single crystals (Cölfen and Mann 2003; Cölfen and Antonietti 2005). EBSD can be employed to examine this crystallographic control in biominerals that serve different functions in a range of phyla, throughout ontogeny in order to understand the extent of crystallographic control in different biological and environmental settings.

There are at least 64 minerals in the biosphere (Lowenstam and Weiner 1989; Knoll 2003; Weiner and Dove 2003) in which the general dichotomy is that calcium phosphate forms internal vertebrate skeletons and silica and calcium carbonate are employed by invertebrates to produce external structures in what is sometimes termed the 'Bone/Shell Divide' (Cusack and Freer 2008). The abundance of calcium carbonate biominerals in the marine realm, their major contribution to the fossil record and their tendency to diffract well explains why the majority of biomineral EBSD studies focus on marine calcium carbonate biominerals. EBSD has enhanced our understanding of several such systems such as brachiopods (Goetz *et al.* 2007;

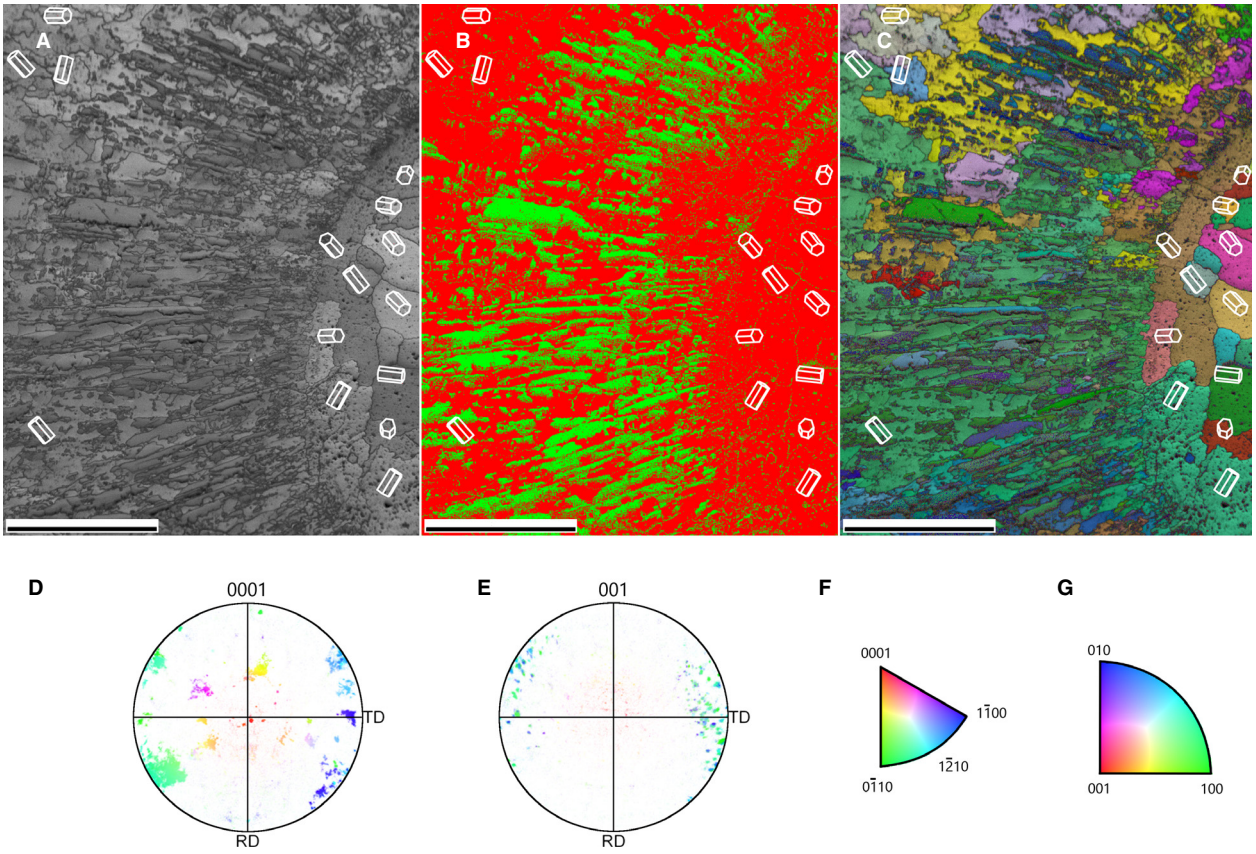

FIG. 2. Electron backscatter diffraction (EBSD) analyses of a trimerellid brachiopod shell. EBSD analyses of a polished block of *Trimerella* spp. PMU 1234 from the Middle Silurian of Gotland. A–C, area of shell analysed with wire frames indicating the crystallographic orientation of calcite; A, diffraction intensity map; B, phase map with calcite depicted in red and aragonite in green; C, crystallographic orientation map, overlain on diffraction intensity map of A, with the reference direction normal to the plane of the page. D–E, pole figures depicting crystallographic orientation of: D, calcite; E, aragonite; colours correspond to those on the crystallographic orientation map C which uses the colour key for calcite (F) and aragonite planes (G). Scale bars in A–C represent 200 μm.

Cusack *et al.* 2008*a*) molluscs (Checa *et al.* 2013; Cusack *et al.* 2013), corals (Cusack *et al.* 2008*b*; Vielzeuf *et al.* 2010; Dalbeck *et al.* 2011), echinoderms (Moureaux *et al.* 2010), arthropod cuticles and mandibles (Seidl *et al.* 2012; Huber *et al.* 2015), fish otoliths (Schulz-Mirbach *et al.* 2013), earthworm granules (Lee *et al.* 2008), eggshells (Dalbeck and Cusack 2006; Grellet-Tinner *et al.* 2012) and trilobite eyes (Torney *et al.* 2014). Although not exhaustive, this review provides a flavour of some of the diverse biomineral systems for which EBSD has provided key information leading to a better understanding of biomineral materials and their structure.

MODERN AND FOSSIL BRACHIOPODS

With a long, continuous fossil record and stable low-Mg calcite in the rhynchonneliformean subphylum (Williams *et al.* 1996), brachiopods are a rich source of palaeoclimate information. The secondary (inner) fibres of rhynchonelliform brachiopods are formed in isotopic equilibrium with ambient seawater and therefore record seawater temperature via calcite $\delta^{18}O$. In contrast, primary (outer) layer calcite is isotopically light and would result in the calculation of erroneously high temperatures (Carpenter and Lohmann 1995; Auclair *et al.* 2003; Parkinson *et al.* 2005; Cusack *et al.* 2012). This difference in isotopic composition between primary and secondary layers is likely to result from kinetic differences with the primary layer being deposited more quickly (Parkinson *et al.* 2005) and possibly with less biological control than the secondary layer.

The primary layer of calcite-shelled brachiopods lacks structural detail in contrast to the exquisite ultrastructure of the secondary layer in rhynchonelliform and craniiform brachiopods. The differences in structure would support there being less biological control on primary layer formation.

By way of example (Fig. 1), a crystallographic map of the secondary later fibres of *Terebratulina retusa* indicates that each fibre is a single crystal (Cusack *et al.* 2008*a*).

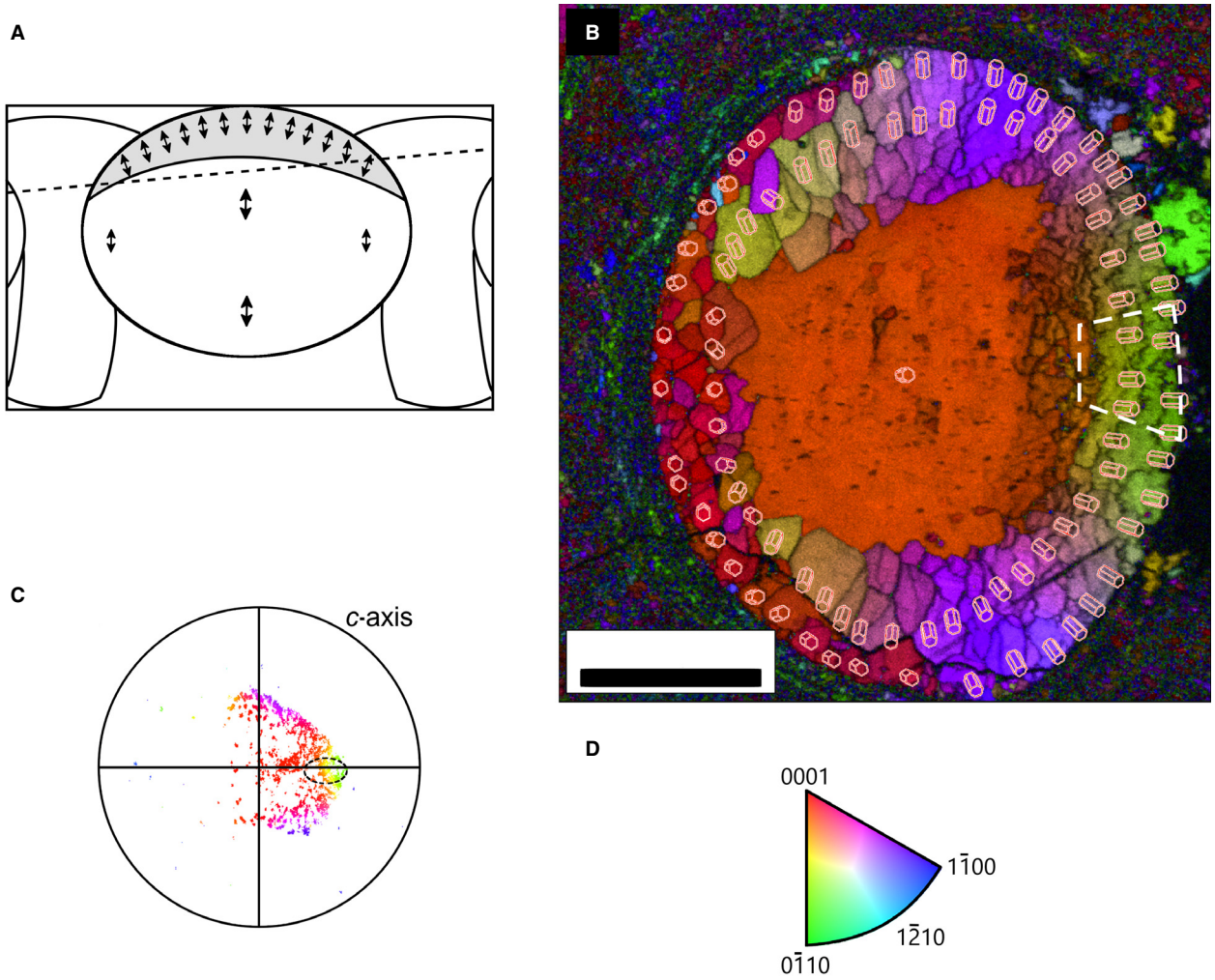

FIG. 3. Electron backscatter diffraction (EBSD) analysis of the schizochroal lens of phacopid trilobite eyes. EBSD analysis of a thin section of a lens of *Geesops schlotheimi* (Bronn; GLAHM 152335/1, 2). A, diagram showing the orientation of the thin section (indicated by dashed line) in B which is 73° to the lens axis; double-headed arrows indicate the orientation of the calcite *c*-axis in the lens. B, crystallographic orientation map overlain on a diffraction intensity map crystallographic orientation map with the reference direction normal to the plane of the page; wire frames indicate crystallographic orientation of calcite; scale bar represents 100 μm. C, pole figure showing the orientation of the *c*-axis in B; dashed black outline indicates those data points from the radial fringe within the white dashed outline in B. D, colour key of calcite planes used in B and C. Figure extracted from Torney *et al.* (2014, fig. 8).

The single colour of each fibre in this EBSD analysis indicates that each fibre is effectively a single crystal. These fibres can twist around the punctae (Fig. 1) that permeate the shell (Pérez-Huerta *et al.* 2009), while maintaining regular crystallographic orientation.

Electron backscatter diffraction analysis of modern rhynchonelliform brachiopods has confirmed that the calcite *c*-axis of the secondary layer fibres is perpendicular to the fibre axis (Fig. 1) and shell exterior (Schmahl *et al.* 2004*a*, *b*, 2009; Cusack *et al.* 2007, 2008*a*; Goetz *et al.* 2007; Griesshaber *et al.* 2007). While brachiopod shells are less well constrained crystallographically than bivalve molluscs (Cusack *et al.* 2007), the crystallographic control within a single calcite fibre is absolute, as indicated in Figure 1 with each fibre being a single crystal. The overall crystallographic orientation is the *c*-axis perpendicular to the fibre axis although each fibre may have a slightly different crystallographic orientation to its neighbours (Fig. 1), and thus, the overall brachiopod shell crystallography may be less well constrained than in bivalve molluscs (Cusack *et al.* 2007). Although the primary layer diffracts more poorly than the secondary layer, the general crystallographic orientation of primary and secondary layers are the same in rhynchonellifrom brachiopods (Cusack *et al.* 2010; Goetz *et al.* 2011) and craniiform brachiopods (Cusack *et al.* 2010). Craniiform brachiopods are also less well constrained crystallographically than bivalve molluscs (England *et al.* 2007). In craniformean brachiopods, the *c*-axis of

calcite semi-nacre follows the undulations of the laminae and is generally parallel with the shell exterior (England *et al.* 2007). Cheilostome bryozoans also have semi-nacre (Weedon and Taylor 1995), but here, the *c*-axis is perpendicular to the laminae which compares to molluscan aragonite nacre where the *c*-axis is perpendicular with the nacre tablets and therefore the shell exterior (England *et al.* 2007).

Brachiopod palaeontology benefits from our understanding of modern brachiopods, where it has been demonstrated that the crystallographic orientation corresponds to the original crystallographic arrangement as in fossil craniids (Pérez-Huerta *et al.* 2007*b*). A key example of this approach enabled a long-standing question in brachiopod research to be answered. Trimerellid brachiopods display poor preservation compared to other co-located brachiopods or molluscs, and this had led to the suggestion that their shells were composed of aragonite (Jaanusson 1966). However, over geological time, aragonite will tend to dissolve and re-precipitate as calcite (Cherns and Wright 2009), making it difficult to identify shells with an original aragonite composition. The large, thick-shelled trimerellids were characteristic of Ordovician–Silurian tropical shallow-water environments (Webby and Percival 1983; Percival and Webby 1996; Popov *et al.* 1997). EBSD analysis reveals that, encased within the thick calcite shells, there are elongated aragonite crystals with common crystallographic orientation (Balthasar *et al.* 2011; Fig. 2). The uniformity of aragonite crystallographic orientation contrasts with that of the encasing calcite where large blocky calcite crystals grow at different orientations to their neighbours, indicating an absence of biological control in the formation of this calcite (Fig. 2). Raman spectroscopy also confirms the aragonite composition of these crystals. Energy dispersive spectroscopy (EDS) reveals that, relative to the surrounding calcite, these aragonite crystals lack magnesium and are enriched in strontium, which is in keeping with the ease with which strontium inhabits the aragonite lattice and magnesium the calcite lattice. These EBSD analyses extend the range of identification of original aragonite back by more than 130 million years. Before the study of Balthasar *et al.* (2011), the oldest original aragonite shells were from the Pennsylvanian (Brand 1989) with indications of relic aragonite in microstructural textures of Devonian shells (Carter and Tevesz 1978). This multitechnique approach that investigates aragonite effectively encased and preserved within calcite offers a strategy for the identification of original biogenic aragonite structures in the fossil record.

MOLLUSCS – WHERE TWO POLYMORPHS MEET

As an abundant, widespread and diverse phylum, it is not surprising that the biominerals of the phylum Mollusca have received much attention that includes analyses by EBSD. The occurrence of the two major polymorphs of calcium carbonate, calcite and aragonite, as a common feature of many molluscan shells, is another reason for the application of EBSD to investigate the formation of these two polymorphs. The remarkable material properties of aragonite nacre (Jackson *et al.* 1989, 1990) attracts much research interest, with EBSD being used to determine the overall orientation with the *c*-axis of aragonite perpendicular to the shell exterior as in the Pterioida (Checa and Rodriguez-Navarro 2004). The interface between calcite prisms and nacre in the marine bivalve *Mytilus edulis* (Dalbeck *et al.* 2006; Griesshaber *et al.* 2013) and between aragonite prisms and the inner nacreous layer of freshwater molluscs has been investigated by EBSD in *Anodonta anatina* and *A. cygnea* (Freer *et al.* 2010). The prisms themselves have also been investigated such as calcite prisms of *Pinctada fucata* (Okumura *et al.* 2010) and aragonite prisms of *Entodesma navicular* (Harper *et al.* 2009).

While nacre and prisms have received much attention, other fabrics are commonplace such as calcite folia which is fairly widespread among bivalves, and the *c*-axis of these platy calcite crystals is consistently perpendicular to the growth direction in oysters and scallops at a high angle to the platy calcite (Checa *et al.* 2007). In both valves of the oyster *Crassostrea gigas*, the *c*-axis of calcite is perpendicular to the shell exterior throughout the prismatic layer, folia and chalky lenses (MacDonald *et al.* 2010). While the *c*-axis of calcite of prisms of oysters and scallops is perpendicular with the shell exterior, the *a*-axis becomes more aligned with neighbouring prisms as prisms grow (Checa *et al.* 2009). EBSD has been used to advance our understanding of the formation of the bosses on the shells of modern and fossil trigonioid bivalves (Checa *et al.* 2014).

Gastropods have not been neglected with investigations into limpet shells (Suzuki *et al.* 2010) providing detailed knowledge of each shell layer. In abalone, EBSD has been employed to gain insight into the infill of apertures in what is effectively a natural repair mechanism (Cusack *et al.* 2013). While modern cephalopods have only the shell of *Nautilus* as a true shell, our understanding of cephalopod biomineralization has been advanced by studies of nacre in *Nautilus* shells (Checa *et al.* 2013) as well as other cephalopod biomineral structures such as the paper nautilus, *Argonauta nodosa*, shell (Wolfe *et al.* 2013) and the cuttlebone (Cusack and Chung 2014).

TRILOBITES WITHOUT DOUBLE VISION

Fossils with no modern day equivalent present a greater challenge to our understanding of the fossil record.

Trilobite eyes are an example of such a challenge that has attracted research interest (Clarkson and Levi-Setti 1975; Fortey 1997; Clarkson *et al.* 2006) in part because they are composed of calcite which, as a birefringent mineral, is not an immediately obvious choice for an optical system. Calcite is used by other organisms such as brittle stars for the microlenses of their light sensing system (Aizenberg *et al.* 2001) rather than a full visual system. Many studies aim to elucidate the mechanism of function of trilobite calcite eyes (Clarkson and Levi-Setti 1975) and mode of life (McCormick and Fortey 1998). The model for the mechanism of function of the schizochroal eye presented by Clarkson and Levi-Setti (1975) involved a difference in refractive index between the upper lens unit and lower intralensar bowl. EDS analyses of schizochroal lenses revealed the mechanism by which the difference in refractive index was achieved with differences in magnesium concentration providing the chemical contrast (Lee *et al.* 2007). EBSD analyses indicated that the calcite *c*-axis of trabeculae within a lens was oriented parallel to the lens and the crystallographic orientation is tightly constrained (Lee *et al.* 2007). More detailed EBSD analyses of schizochroal eyes confirm that the calcite *c*-axis of the trabeculae is in the plane of the lens and that neighbouring trabeculae differ in terms of their *a*-axis orientation (Torney *et al.* 2014). While the trabeculae in the centre of the lens each have *c*-axis parallel to lens axis, in the radial fringe the *c*-axis fans out away from the lens axis (Fig. 3). This fanning of the *c*-axis towards the lens centre enables the construction of a biconvex lens from solid crystalline material (Torney *et al.* 2014). This detailed knowledge of the crystallography of the lenses along with knowledge of the chemical composition is a prime example of EBSD being used to test hypotheses in palaeontology and to assign the mechanisms of function.

CONCLUSIONS

As well as providing information about original mineralogy in fossil biominerals such as trilobite lenses (Lee *et al.* 2007; Torney *et al.* 2014), EBSD is a very powerful technique for the identification of secondary mineralogy that may be present in quantities that are below X-ray diffraction detection yet sufficient to distort palaeoproxy data. Replacement of the fine dissepiments in aragonite scleractinian corals with calcite structures that mimic the original ultrastructure is clear in EBSD (Dalbeck *et al.* 2011). If included in $\delta^{18}O$ calculations of seawater temperature, the replacement calcite dissepiments would have little effect on the calculated temperature. However, Sr/Ca ratios would be distorted resulting in significant increases in calculated temperatures, which emphasizes the importance of combining EBSD with multiproxy approaches to ensure accurate palaeoproxy measurements (Dalbeck *et al.* 2011). Importantly, EBSD analyses can readily identify secondary minerals even when the secondary mineral is the same mineral as the original such as secondary aragonite in corals (Cusack *et al.* 2008*b*). Inclusion of such secondary mineral components that are formed at a different time and in different conditions from the original is an obvious problem for palaeoproxy work that EBSD screening can help avoid.

Combining EBSD with Raman and EDS to investigate fossil biominerals provides a strategy for investigating original mineralogy, understanding in detail the diagenetic processes that alter the original mineralogy but often still leave clues as to the original mineralogy and ultimately for the identification of original mineralogy of biominerals. This approach may lead to the discovery of other aragonite-shelled brachiopods for example, or aragonite-shelled members of other phyla. It is possible that the strategy may discover bimineralic brachiopods that had shells of both calcite and aragonite, a feature that is so common in molluscs but has not been considered in brachiopods, perhaps because of the poorer preservation potential of aragonite over calcite. EBSD is an incisive tool that is being applied to the study of many more organisms that can be addressed in detail here such as the calcite plates of coccolithophorids (Saruwatari *et al.* 2011; Hoffmann *et al.* 2014), fossil and modern corals (Floquet and Vielzeuf 2012; Coronado *et al.* 2015) and conodonts (Pérez-Huerta *et al.* 2012). This widespread applicability demonstrates the great potential for EBSD to provide information of great value to palaeontology. EBSD is a well-recognized analytical technique in the field of materials science, and it is now well established in biomineral research. The current examples in the literature demonstrate the value of including EBSD in the palaeontologist's toolkit.

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