

# focus on Microscopy & Microtechniques

## Electron Microscopy Reveals Secrets of Nanocrystal-Assembly in Coral Skeletons

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One of the most important examples of mineral forming organisms are corals, in particular reef-building corals, which play central roles in the marine ecology by hosting 25% of marine livestock as well as the economy via coastal protection, tourism and fishery. The mechanical stability of coral colonies is caused by a massive accretion of a calcium carbonate based exoskeleton. We are interested in the relation between growth conditions, such as the chemical composition of seawater, the pH value and the temperature on the microstructure of the mineral deposited by the corals. For this purpose we focused on the details of the microstructure down to the atomic level to understand the patterns of assembly of the nanocrystalline building blocks constituting the skeleton. These studies were undertaken using state-of-the-art electron microscopy available at the University of York and form parts of a collaboration with world-leading research groups in the area of Marine Science at the University of Western Australia and the University of St Andrews.

### Transmission Electron Microscopy (TEM)

#### Instrumentation

TEM samples were predominantly analysed at the York Jeol Nanocentre utilising a JEM 2011 (LaB6 heated filament with an EDX detector for elemental analysis) and JEM 2200 FS (field emission, double Cs aberration corrected with EDX detector for elemental analysis and an in-column omega filter for EELS and EFTEM) TEM operated at 200kV.

Additionally TEM analysis was performed at the Centre for Microscopy, Characterisation and Analysis (CMCA) at the University of Western Australia using a JEM 2100 TEM (LaB6 heated filament with an 11M pixel Gatan Orius digital camera and a Gatan Tridiem energy filter for EELS) operated at 120 and 200 kV and a JEM 3000 F FEGTEM (field emission with a 1M pixel Gatan 694 MSC digital camera and a Gatan GIF2000 energy filter for EELS and EFTEM) operated at 300 kV.

#### Microscope conditions and beam damage; Electron microscopy

During electron microscopy image or data acquisition, beam damage and sample contamination should be considered. Damage caused by elastic interactions can affect the original structure and/or chemistry of the specimen by a variety of phenomena such as the breaking of chemical bonds, the displacement of atoms, creating point defects or simply the heating caused by phonons interacting with the sample [1]. Contamination is caused by the deposition of carbonaceous material due to hydrocarbons that are present in the TEM chamber reacting with electrons from the beam forming hydrocarbon ions and condensing on the irradiated specimen surface [2]. In case of biogenic samples a hydrocarbon organic film is often naturally present on the specimen itself, which greatly enhances this phenomenon. Especially during analytical EM: EDX and EELS, beam damage is problematic as the beam is often condensed on a spot for several tens of seconds' acquisition time. This can cause severe structural damage: removal of material, and re-deposited together with the previously mentioned hydrocarbons on the specimen surface. Beam damage also occurs during high resolution imaging as the high magnification (>400 000 x) acts as a condensed beam on a small surface area, this often results in damage to the crystal lattice fringes (Figure 1).

To reduce beam damage imaging and diffraction is performed using predominantly a LaB6 filament TEM instead of a field emission gun, which has a higher intensity. Furthermore small condenser apertures (40 and 20  $\mu\text{m}$  for condenser aperture n. 3 and 4 respectively JEM 2200 FS, and 20 and 10  $\mu\text{m}$  for the JEM 2011) and small spot size (~2 nm) are used at all times. The disadvantage of these measures is reduced intensity and contrast in the image. Else, the TEM is operated at a standard 200 kV.

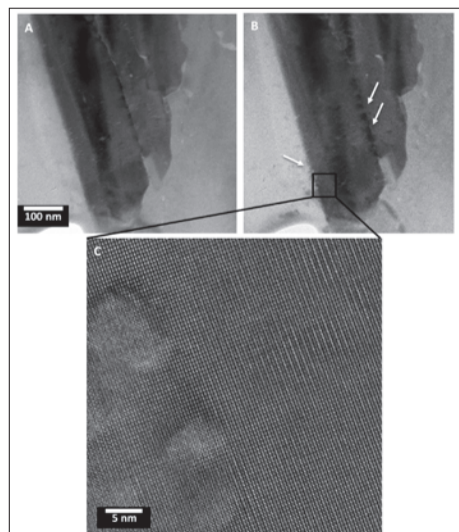


Figure 1. *S. siderea* coral beam damage HRTEM

#### Sample preparation

##### Coral specimens

To investigate the polyp-mineral interface the soft tissue of the polyp consisting of the calciblastic ectoderm, mesoglea and epidermis, is removed by immersion in fresh water, followed by mechanical brushing and drying. Besides adult specimen skeletal material of coral larvae is also investigated. For this purpose new coral recruits were cultivated in filtered seawater at 390 ppm and 750 ppm atmospheric CO<sub>2</sub> at 29°C at the aquaculture facilities, James Cook University, Australia. Corallite cross-sections of approx. 1 x 3 mm in size were cut from the cores perpendicular to the growth direction using a diamond cut-off wheel for TEM analysis (adult specimen only). The cross sections or complete larvae were then immersed in Gatan G1 epoxy resin and allowed to cure at 140°C on a hot-plate for 30 minutes to fill up the coral pores increasing the stability of the samples. The samples were manually polished down to approximately 50  $\mu\text{m}$  thickness using diamond lapping pads (30 - 0.1  $\mu\text{m}$ ) and a tripod holder. Ultimately a bevel is applied, resulting in a sample edge thickness of approximately 20  $\mu\text{m}$ . A molybdenum TEM slot is attached to the sample to provide structural support.

##### Focused ion beam milling

FIB lamellae are created on the thin polished sample edge using a FEI nova 200 Dual Beam FIB/SEM from the Leeds Electron Microscopy and Spectroscopy centre. The lamellae are created by applying the h-bar method: a platinum layer is deposited at the edge of the coral sidewall, perpendicular to the general alignment direction of the acicular crystals related to the targeted COC. A approximately 15 x 30  $\mu\text{m}$  lamella is then excavated with a 30 kV Ga<sup>+</sup> beam. Bulk milling is performed at 20 nA after which cleaning cross sections are applied at 5 nA and 0.30 nA. The final polish is performed with a 10 kV beam at an angle of 3° at 50 pA. The lamella is milled down to a 100 nm thin section and no lift out is performed to retain specimen context as well as structural support (Figure 2).

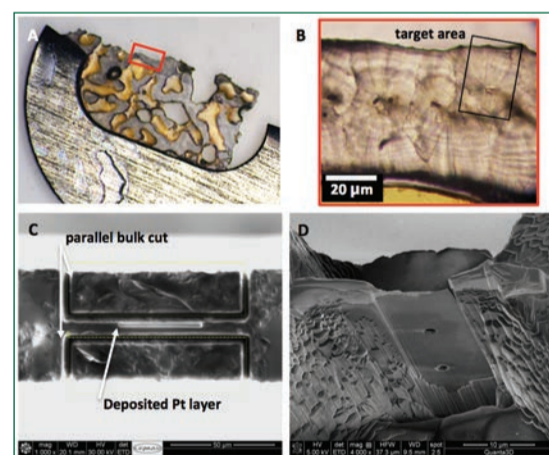


Figure 2. Coral FIB preparation- optical and SEM

### Scanning Electron Microscopy (SEM)

#### Instrumentation

In this study a FEI Sirion S-FEG FESEM (field emission SEM) was used from the York Jeol Nanocentre. This instrument is coupled with a Noran EDX system which uses an Oxford INCA analysis system and a 30 mm 2 light element Silicon-Lithium (SiLi) detector.

### Sample preparation

Specimen preparation for SEM is usually much quicker and less involved as compared to TEM specimen preparation as the samples need not be electron transparent. Since there is a limit to the depth of field and we are interested in the cross section perpendicular to the growth direction of coral skeletons, a bulk cut followed by polishing is performed to a thickness of approximately 100-200  $\mu\text{m}$  using the tripod polishing method, but without applying a bevel. This particular thickness range makes the specimen light transparent, which enables us to combine transmission optical microscope data with surface SEM data.

Specimens are subsequently lightly etched with deionised water (coral larvae) and dilute acetic acid-water mixture (adult coral specimen) to reveal the microstructure (Figure 3).

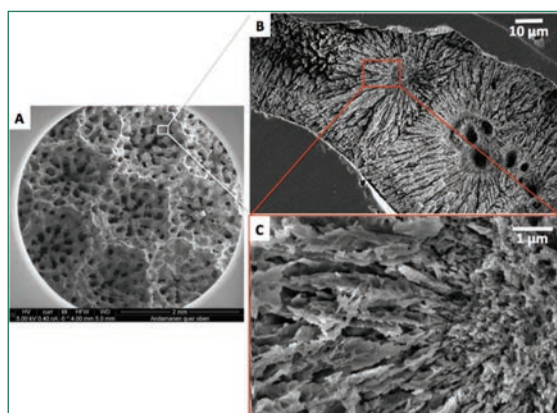


Figure 3. *P.lobata* coral SEM series

### Summary of Conclusions (as published in recent paper\*)

In this study we show that new insights can be gained on the nano- and microstructure of corallites by TEM investigation of large-scale (15 x 30  $\mu\text{m}$ ) FIB lamellae from adult and juvenile scleractinian coral skeletal specimens. By leaving the FIB prepared lamella within the coral skeletal context (no lift out) the lamella is mechanically more stable and durable while being directly comparable to the larger scale (several tens of microns) not ion-milled skeletal areas by optical analysis. Thus we could identify a crystallographic evolution from a centre of

calcification outward over acicular and granular (daily) bands. In the adult *Porites lobata* specimen, a succession of randomly orientated nanocrystals with high porosity, followed by partly aligned nanocrystals with high porosity, to dense acicular crystals of several microns in length, was observed. A new layer of nanocrystals followed by the same succession is then repeated, in correlation with the contrast banding seen in transmission light images. Both the partly-aligned nanocrystals and the densely packed acicular bundles are preferentially oriented in the [001] crystallographic growth direction (Pm3n space group notation), (Figure 4).

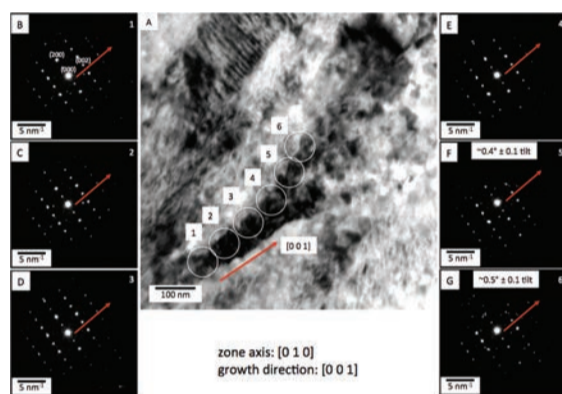


Figure 4. *P.lobata* coral SAD series nanocrystals

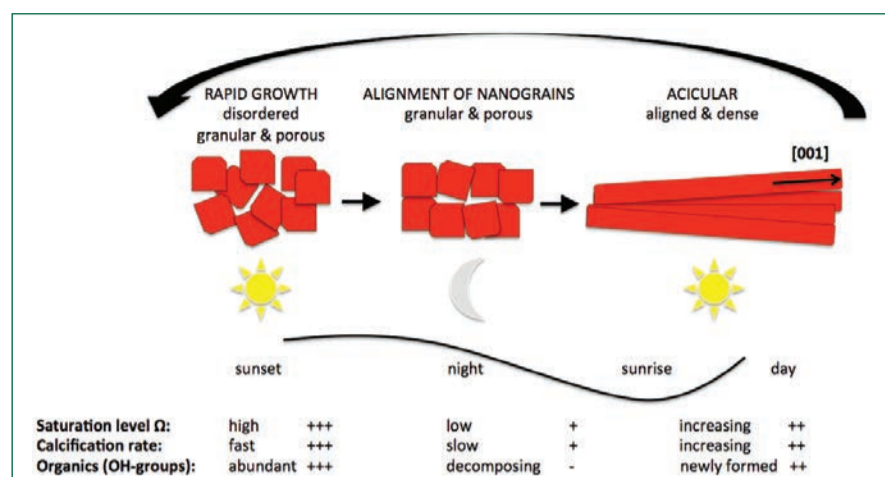


Figure 5. Schematic Diurnal cycle

We draw a parallel to the diurnal photosynthetic cycle of the zooxanthellae in symbiotic corals that control the levels of oxygen and carbon availabilities, both recognised as significant drivers of coral calcification processes. The transport of large amounts of glycerol by zooxanthellae and its potential impact on coral calcification was also discussed. This process could play a role in the specific alignment of the aragonite crystals as was demonstrated by synthetic experiments with OH-group containing additives by Sand et al. (2011) and the TEM investigation of these precipitates by the authors. The juvenile *Acropora millepora* specimen also showed the large acicular crystals interrupted by thin porous bands, but lacked the nanocrystalline phase, which may be linked to the absence of zooxanthellae and thus the typical daily cycle. But at this stage a comparison is not possible as we are dealing with different species and growth rates and morphologies are also likely to play an important role (Figure 5).

Finally, we conclude that our data do not support a growth mechanism via self-assembly of submicron-sized units associated with a mesocrystalline morphology, as the nanocrystalline areas are found to be polycrystalline on the micrometer scale with no extended co-orientation. We therefore conclude that the coral mineralisation follows a classical crystallisation pathway.

### References

- [1] D. B. Williams, C. B. Carter, *Transmission electron microscopy: a textbook for materials science*, Springer, 2009.  
 [2] S. Horiuchi, T. Hanada, M. Ebisawa, et al., *ACS nano* 3 (2009), pp. 1297-1304.

\*Paper: Microstructural evolution and nanoscale crystallography in scleractinian coral spherulites Renée van de Locht et al, *Journal of Structural Biology* 183 (2013) pp.57-65



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## RISE Microscopy for Correlative Raman-SEM Imaging Launched at Analytica 2014

Tescan Orsay Holdings and WITec GmbH jointly launched RISE Microscopy at Analytica 2014.

RISE Microscopy is a novel correlative microscopy technique which combines confocal Raman Imaging and Scanning Electron (RISE) Microscopy within one integrated microscope system. This unique combination provides clear advantages for the microscope user with regard to comprehensive sample characterisation. The RISE Microscope enables for the first time the acquisition of SEM and Raman images from the same sample area and the correlation of ultra-structural and chemical information with one microscope system.

Both analytical methods are fully integrated into the RISE Microscope. Between the different measurements an extremely precise scan stage automatically transfers the sample inside the microscope's vacuum chamber and re-positions it. The integrated RISE software carries out the required parameter adjustments and instrument alignments. The acquired results can then be correlated and the Raman and SEM images overlaid. "RISE Microscopy enables unprecedented opportunities for the most comprehensive ultra-structural and molecular sample analyses," explained Dr Olaf Hollricher, CEO and Director R&D at WITec. "The novel RISE Microscope is another striking example of WITec's enormous innovative strength. It fulfils all requirements of an outstanding, correlative microscopy technique and will convince the Raman as well as the SEM community."

TESCAN and WITec arranged worldwide sales and after-sales cooperation for the RISE Microscope to take advantage of the synergy effects of both companies.

The RISE Microscope provides all functions and features of a stand-alone SEM and a confocal Raman microscope. Both SEM and Raman are high-resolution imaging techniques with sub-nanometer and diffraction limited 200 - 300 nanometer resolution, respectively. In Raman imaging mode the sample can be scanned through a range of 250  $\mu\text{m}$  x 250  $\mu\text{m}$  x 250  $\mu\text{m}$ . RISE Microscopy pairs ease-of-use with exceptional analysing benefits and is therefore suited to a large variety of applications such as nanotechnology, materials science, and life science.

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