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Spatially-resolved thermoluminescence from snail opercula using an EMCCD

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Abstract

In recent years opercula of the snail species *Bithynia tentaculata* have been shown to emit thermoluminescence (TL) signals that can be used to determine equivalent dose, and may be capable of dating events throughout the entire Quaternary period. Concentric growth lines are a notable feature of almost all *Bithynia tentaculata* opercula, but it is not known whether the luminescence emitted by the opercula is influenced by these structures. This study uses a newly developed EMCCD imaging system to measure the TL signals from opercula. A combination of microscopic analysis of the opercula using visible imagery, and measurement of the TL using the EMCCD system has been undertaken. Variations in TL intensity and equivalent dose (D_e) are seen, but the two are not correlated. Changes in TL intensity broadly mimic the concentric growth structures, but the largest variations in intensity are between different margins of the opercula, not individual growth bands. The EMCCD system makes it possible to produce a two dimensional map of the D_e measured from an operculum. Dose recovery experiments give D_e values that are consistent with each other across the whole opercula. Measurement of the D_e arising from irradiation in nature shows significant variability across a single operculum, but at present the reason for this variability is unknown.

Keywords:

Calcite; TL; dating; *Bithynia tentaculata*; imaging

1. Introduction

Calcite and aragonite precipitated as a result of biological processes gives a thermoluminescence (TL) signal in response to irradiation (Carmichael et al. 1994) and it has been suggested that these materials could be used as dosimeters that could form the basis of a Quaternary dating method (Ninagawa et al 1988). Early work focussed on marine shellfish, but Duller et al. (2009) suggested using opercula from the species *Bithynia tentaculata* or slug plates from the *Limacidae* family. These were selected by Duller et al (2009) because of the low organic content of these carbonates, and because both organisms secrete calcite rather than aragonite, thus reducing some of the concerns about recrystallisation. Stirling et al. (2012) continued the work on the TL signals from opercula, and based on analysis of the kinetics suggested that the TL peak observed at ~335 °C when heating at a rate of 0.5 °C/s had a lifetime of 140 Ga at 15°C, of similar magnitude to the value of 640 Ga obtained by Kim and Hong (2014). Coupled with a D_0 value of 3250 ± 163 Gy for growth of this peak, this implies that opercula may be a valuable geochronometer capable of covering the entire Quaternary period (2.6 Ma).

The results presented by Duller et al. (2009) and Stirling et al. (2012) made measurements on whole opercula using blue sensitive photomultiplier tubes (PMTs). However, the TL signal from calcite has its peak emission at ~600 nm (Townsend et al. 1994; Duller et al. 2009), and the blue sensitive PMTs used in these studies of opercula have limited sensitivity in this range. Additionally, the opercula used for analysis exhibit significant structure in their growth, with banding visible on almost all specimens, but PMT systems are unable to provide any

spatially-resolved information, and hence the significance of these growth structures cannot be investigated.

The aims of this paper are to use a newly developed imaging system for the Risø TL/OSL reader based around an electron-multiplying charge coupled device (EMCCD) (Kook et al. these proceedings) to investigate four issues. The first question is practical - opercula may be up to ~1 mm in thickness but to avoid complications arising from crushing calcite (e.g. Khanlary and Townsend 1991) they are measured whole. Because of their size, is there variation in the efficiency with which different parts of an operculum are heated during TL measurement? Secondly, microscopic imaging of opercula shows the presence of growth structures, and it is possible that the incorporation of Mn and other impurities may vary in different parts of the structure. Is there spatial variability in the intensity of the TL signal emitted by opercula? Thirdly, are any silicate contaminants from the matrix in which opercula are collected retained on the surface of the opercula (in spite of extensive cleaning)? Finally, does the equivalent dose obtained from an operculum vary spatially?

2. Equipment and sample

An Evolve™ electron-multiplying charge coupled device (EMCCD) with an array of 512 by 512 pixels was used for measurement of luminescence emissions. The EMCCD system was mounted on a Risø TL/OSL DA-20 reader in place of the normal photomultiplier tube. This system is ideally suited to measuring the TL signal from opercula since it has high sensitivity at the peak emission of calcite (~600 nm). The details of the system are described by Kook et al (these proceedings). A system of lenses was used to focus emissions from the sample onto the surface of the EMCCD and gave a magnification of 0.8. Individual pixels on the detector are 16 μm by 16 μm , and after magnification this gives an effective pixel size at the sample of 20 μm , and a maximum image size of 10.24 mm by 10.24 mm. Schott BG-39 glass filter (4 mm thick) was used to suppress the black body radiation at high temperatures.

Opercula of *Bithynia tentaculata* were used for all measurements described in this paper. The opercula were collected from the site of Purfleet in the south-east of the United Kingdom using an opaque plastic tube driven horizontally into a freshly cleaned face of the sedimentary section. Under subdued red lighting in the laboratory, opercula were obtained by wet sieving the sediment using a 300 μm sieve and subsequently hand picking. After isolation of the opercula they were washed in distilled water using an ultrasonic bath for 5 minutes to displace any adhering materials, and then placed in 13% NaOCl (bleach) for 48 hours to remove any organic residues. At the end of this process the opercula were washed in distilled water three times and dried.

Individual opercula were mounted on stainless steel planchettes and an EMCCD image was collected every 1°C during heating. Duller et al. (2009) suggested using a low heating rate (0.5 °C/s) so that the TL peaks that are of interest for dating are observed at lower temperatures than would be the case at higher heating rates. Stirling et al. (2012) found that the TL peak at ~335 °C (using 0.5 °C/s heating rate) was likely to be the most appropriate for dating. A typical TL glow curve, heated to 400°C is shown in Figure 1a. Prior to this measurement the operculum had received a dose of 1680 Gy from the beta source and had been preheated to 320 °C at a heating rate of 5 °C/s. During measurement of the TL signal EMCCD images were collected every two seconds, meaning that each image represents 1°C in the TL glow curve. After measuring the TL signal, the planchette is cooled to room temperature and then heated a second time to measure the black body radiation. In Figure 1a an area of 40 by 40 pixels has been integrated, and black body radiation of 645 counts per second is observed at 400 °C (0.4 counts per second per pixel). A number of spikes are clear in the data in Figure 1a; these arise from cosmic rays passing through the EMCCD device (Kook et al., these proceedings) and are unavoidable. They may be effectively removed by applying a median filter and this procedure was used for all data analysis in this paper.

3. Spatial uniformity of heating of opercula

Thermal lag between the temperature of a hotplate and the temperature of a sample mounted on a holder is a common issue in TL measurements (Betts et al. 1993; Jain et al 2007). Opercula from *Bithynia tentaculata* are commonly 3-4 mm in length, 2-3 mm in width and 500 – 1000 μm in thickness. A concern for such large objects is whether they are heated uniformly during TL measurements. In all the measurements reported here, two litres per minute of oxygen free nitrogen is pumped through the chamber to ensure effective transfer of energy from the hotplate to the sample by convection, and a slow heating rate (0.5 $^{\circ}\text{C/s}$) is used to reduce the likelihood of thermal lag.

Using the EMCCD system spatially-resolved TL glow curves can be obtained across the surface of an operculum. The data shown in Figure 1a is for a single 40 by 40 pixel area of an operculum. The data were analysed in greater detail by calculating TL glow curves for 168 separate 10 by 10 pixel areas (200 μm by 200 μm). The data for each glow curve was smoothed using a five point running mean and the position of the peak TL intensity was determined. A histogram of these peak positions is shown in Figure 1b. The temperature at which the TL peak is observed is highly reproducible across the entire operculum, with a standard deviation of less than 3 $^{\circ}\text{C}$ in spite of differences in thickness of the operculum.

4. Growth structures and distribution of TL emission

Crystallisation of calcite by *Bithynia* is mediated by biological processes within the organism. A clear result of this is the concentric rings visible in the opercula (**Figure 2a**). It is not known whether the biochemical changes that result in these visible structures have any impact either upon the distribution of Mn, which is thought to be involved in the recombination centre responsible for the main TL signal from calcite, or upon other aspects of the crystal that may influence the intensity of the TL signal emitted. The operculum shown in **Figure 2a** received a 504 Gy beta dose and the TL signal was measured shortly afterwards at a heating rate of 0.5 $^{\circ}\text{C/s}$. An intense TL peak is observed at 105 $^{\circ}\text{C}$ and the image in **Figure 2b** was produced by summing the TL emissions from 5 EMCCD images spanning the

temperature interval 105-110 °C. TL emission varies across the operculum by a factor of 5 or more, and this variation does follow the growth structures. However, the fine structure seen in the visible microscope image is not seen in the TL image, although this may result from the lower resolution of the EMCCD system. A striking feature of the TL image is the difference between different areas of the operculum, with much brighter emissions on the left hand side and dimmer ones on the right. The pattern of TL emission from opercula varies from one individual to another, but it is common for some concentric patterns to be visible, reflecting the growth structure. It is also common for there to be a 'bright' edge and a 'dim' edge to the opercula.

In early work trying to look at biogenic materials, Huntley and Johnson (1976) worked on deep ocean cores and thought that the TL signals that they measured arose from radiolaria, siliceous microfossils. However Wintle and Huntley (1979) concluded that the TL signal originated from detrital silicates which had not been removed from the surface of the radiolarian, rather than from the biogenically precipitated material. **Figure 2b**, and similar images of other opercula, exhibits smooth changes in TL emission, and does not give any indication of contaminants adhering to the surface. This was also checked at higher magnifications using visible microscopy undertaken after TL measurements and this confirmed that no silicate grains remained on the opercula.

5. Assessing the reproducibility of TL measurements on opercula

The use of imaging devices to construct dose response curves presents a number of challenges. One of the greatest of these is the need to register the images in relation to each other so that a specific pixel in the image collected from one measurement is giving the signal from the same part of the sample as in other images (Duller et al. 1997; Clark-Balzan and Schwenninger 2012). Minor movement of the sample from one measurement to another is common and needs to be corrected for.

In the system used here, the sample was briefly illuminated at the end of each luminescence measurement using a low power yellow LED (since replaced by an IR LED; Kook et al. these proceedings) and this illumination is used to take an image of the sample at the end of each TL acquisition. Features on the surface of the operculum are then identified on each image, and any movement of these features from one image to another is corrected for by rotating or translating the position of the image.

To assess whether this image registration software is working effectively, an operculum that had previously been repeatedly heated to 400°C during TL measurements was given a 210 Gy dose with the beta source. A single aliquot regenerative dose type procedure was then applied. This procedure (Table 1) has been developed following on from the data presented by Stirling et al. (2012) and involves using a preheat of 320 °C heating at 5°C /s prior to the main TL measurement from room temperature to 400°C which uses a heating rate of 0.5 °C/s. The high heating rate used in the preheat (step 2, Table 1) is designed to force as much sensitivity change in the material as possible prior to measurement of the signal used for dating. The use of a low heating rate for measurement of the TL signal used for dating (step 3 Table 1) means that the peak occurs at a lower temperature than it would when using a high heating rate, and this reduces the contribution from black body radiation.

Initially the signal from the whole operculum was analysed by integrating a region 231 pixels in width and 164 pixels high centred on the operculum (**Figure 3a**). A series of TL glow curves were obtained from this region and are shown in **Figure 3b**. The TL peak visible in this data was integrated by summing the signal from 250 to 370 °C and these values were used as the basis for calculating a sensitivity corrected dose response curve (**Figure 3c**). This gave a measured dose of 209 ± 15 Gy, indistinguishable from the given dose of 210 Gy.

The simple dose response curve shown in **Figure 3c** involved 20 separate TL measurements (including measurement of the black body radiation). To ascertain whether

the registration of these images had successfully corrected for any movement, a more detailed analysis of the dataset was undertaken constructing glow curves and dose response curves for smaller regions of interest (Figure 4). Regions of the opercula 200 μm by 200 μm in size were analysed (10 by 10 pixels) following the same method used to construct **Figure 3b** and c. The average of the 24 values of equivalent dose along this transect was 203 ± 3 Gy (compared with the given dose of 210 Gy) and there is no indication of systematic patterns of increase or decrease at the margins of the operculum (Figure 4). Such artefacts are visible if analysis is undertaken on images that are not registered due to small movement of the operculum between measurements for the natural signal and the dose response curve. As well as undertaking a single transect across the operculum, a 20 by 16 grid of regions of interest (ROI) was analysed. Equivalent doses were accepted if the uncertainty on the test dose was less than 10%, and if the signal resulting from the test dose was at least three times larger than the standard deviation of the signal from 250-370 $^{\circ}\text{C}$ from the black body radiation. Of the 320 ROIs analysed, 229 gave equivalent dose values that passed these criteria. The average of these values was 202 ± 1 Gy with zero overdispersion.

Processing of data from the EMCCD to remove noise spikes using a median filter, and registration of images on the basis of the images collected using artificial illumination at the end of each luminescence measurement, are able to produce data of sufficient quality that a dose of 210 Gy can be recovered both through analysis of the signal from the whole operculum and when undertaking more detailed spatial analysis. The next section applies this same method to an operculum that retains its natural dose, and explores whether spatial variations in equivalent dose are observed.

6. Spatially-resolved measurements of equivalent dose from an operculum retaining its natural signal

The dose recovery experiment in the previous section was undertaken on an operculum that had been heated to 400 °C prior to the experiment, and only the response to laboratory irradiation was studied. That experiment was designed to evaluate the performance of the EMCCD system and the data manipulation and analysis software. The same measurement and analytical procedure was then applied to an operculum that retained the signal that it had acquired during burial at the site of Purfleet, Essex, UK which is thought to date to ~315 ka (Bridgland et al. 2013) and would therefore be expected to have absorbed a dose of ~290 Gy since burial.

Growth bands are visible in the structure of the operculum (Figure 5a) and there is variation in the intensity of the TL emitted (Figure 5b). As seen in **Figure 2b**, one side of the operculum is brighter than the other. In this case the upper right hand edge of the operculum has TL counts consistently in excess of 5500, whilst the remainder of the operculum shows dimmer emissions. The equivalent dose calculated for each 200 by 200 μm region of the operculum also shows some variation across the surface (Figure 5c), but these changes in D_e do not correlate with variations in TL intensity. The average value obtained from the 228 D_e determinations shown in Figure 5c is 324 ± 3.9 Gy, similar to the value of 314 ± 9 Gy obtained by integrating the TL signals from the entire area.

The D_e across much of the surface in **Figure 5c** is similar, with high values seen just in the lower left hand side. The cause of this area of high D_e values is not clear, but it could relate to small scale dosimetry variations, due to variations in either the external or internal dose rate. Further spatially-resolved measurements are required to see whether such variations are common, and whether the magnitude of the variations could be explained by the occurrence of potassium rich feldspar grains, or other minerals which would lead to 'hotspots' in the radiation field.

7. Conclusions

Spatially-resolved measurements of the TL emitted by snail opercula using an EMCCD system demonstrate that the intensity of the signal does vary significantly from one part of the sample to another (at least by a factor of five), and that these changes are influenced by the growth structures visible under a microscope. However the largest variations are not related to the growth bands, but seem to occur from one edge of the opercula to another. The reason for these variations in brightness is not known, but they do not appear to have significant impact upon the equivalent dose that is measured.

A dose recovery experiment demonstrated that it was possible to obtain measurements of the equivalent dose for regions measuring 200 by 200 μm across an operculum, and that within uncertainties no variation in D_e was seen across the surface. However, measurement of the D_e from a specimen from a Quaternary site thought to have been deposited ~315 ka did exhibit variations in D_e beyond experimental uncertainties, and this may reflect variations in dose rate to different parts of the operculum. The EMCCD system described here has produced high quality data that have enormous potential to unlock the complexities of opercula as dosimeters that could be used to date the Quaternary period.

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References

- Betts, D.S., Couturier, L., Khayrat, A.H., Luff, B.J., Townsend, P.D. 1993. Temperature distribution in thermoluminescence experiments .1. Experimental results. *Journal of Physics D-Applied Physics* 26: 843-848.
- Bridgland, D.R., Harding, P., Allen, P., Candy, I., Cherry, C., George, W., Horne, D.J., Keen, D.H., Penkman, K.E.H., Preece, R.C., Rhodes, E.J., Scaife, R., Schreve, D.C., Schwenninger, J.-L., Slipper, I., Ward, G.R., White, M.J., White, T.S., Whittaker, J.E. 2013. An enhanced record of MIS 9 environments, geochronology and geoarchaeology: data from construction of the High Speed 1 (London-Channel Tunnel) rail-link and other recent investigations at Purfleet, Essex, UK. *Proceedings of the Geologists Association* 124: 417-476.
- Carmichael, L.A., Sanderson, D.C.W., Ni Riain, S. 1994. Thermoluminescence measurement of calcite shells. *Radiation Measurements* 23: 455-463.
- Clark-Balzan, L., Schwenninger, J.-L. 2012. First steps toward spatially resolved OSL dating with electron multiplying charge-coupled devices (EMCCDs): System design and image analysis. *Radiation Measurements* 47: 797-802.
- Duller, G.A.T., Bøtter-Jensen, L., Markey, B.G. 1997. A luminescence imaging system based on a charge coupled device (CCD) camera. *Radiation Measurements* 27: 91-99.
- Duller, G.A.T., Penkman, K.E.H., Wintle, A.G. 2009. Assessing the potential for using biogenic calcites as dosimeters for luminescence dating. *Radiation Measurements* 44: 429-433.
- Huntley, D.J., Johnson, H.P. 1976. Thermoluminescence as a potential means of dating siliceous ocean sediments. *Canadian Journal of Earth Sciences* 13: 593-596.
- Jain, M., Bøtter-Jensen, L., Murray, A.S., Essery, R. 2007. A peak structure in isothermal luminescence signals in quartz: origin and implications. *Journal of Luminescence* 127, 678-688.
- Khanlary, M.R., Townsend, P.D. 1991. TL spectra of single crystal and crushed calcite. *Nuclear Tracks and Radiation Measurements* 18: 29-35.
- Kim, K.B., Hong, D.G. 2014. Kinetic parameters, bleaching and radiation response of thermoluminescence glow peaks separated by deconvolution on Korean calcite. *Radiation Physics and Chemistry* 103: 16-21.
- Kook, M.H., Lapp, T., Murray, A.S., Thomsen, K.J., Jain, M. (these proceedings) A luminescence imaging system for the routine measurement of single-grain OSL dose distributions. *Radiation Measurements*

- Ninagawa, K. , Takahashi, N. , Wada, T. , Yamamoto, I. , Yamashita, N., Yamashita, Y.
1988. Thermoluminescence measurements of a calcite shell for dating. *Quaternary Science Reviews* 7: 367-371.
- Stirling, R.J., Duller, G.A.T., Roberts, H.M. 2012. Developing a single-aliquot protocol for measuring equivalent dose in biogenic carbonates. *Radiation Measurements* 47: 725-731.
- Townsend, P.D., Luff, B.J., Wood, R.A. 1994. Mn²⁺ transitions in the TL emission spectra of calcite. *Radiation Measurements* 23: 433-440.
- Wintle, A.G., Huntley, D.J. 1979. Thermoluminescence dating of a deep-sea sediment core. *Nature* 279: 710-712.

Figure and Table Captions

Figure 1: TL glow curve constructed by integrating the signal from a region of interest (ROI) 40 by 40 pixels from 400 EMCCD images collected while heating the operculum to 400°C at a heating rate of 0.5 °C/s. The operculum had received a dose of 1680 Gy and had been preheated to 320 °C at 5 °C/s prior to this TL measurement. (a) Raw data for the first heating and for the second heating taken to assess the contribution from black body radiation. Spikes due to cosmic rays are apparent in the data; these can be removed by applying a median filter. (b) Peak temperature calculated for 168 regions across the operculum, each 10 by 10 pixels (200 by 200 μm). A normal curve is shown fitted to this data.

Figure 2: (a) Image of an operculum through a visible microscope. The operculum is ~3.8 mm along its longest axis. Concentric growth structures can be seen clearly. (b) TL signal (105-110°C) emitted by the operculum following a 504 Gy beta dose.

Figure 3: (a) Image of an operculum taken using artificial illumination, showing the area used to define the 231 by 164 pixel region of interest (ROI) for the TL glow curves shown in (b). (b) Background subtracted TL glow curves obtained following different laboratory irradiations, and (c) dose response curve for the operculum, showing the dose recovered. Inset shows the change in sensitivity during the sequence of measurements.

Figure 4: TL signal intensity (shown in red) and equivalent dose (shown in black) along a transect across an operculum. The horizontal dashed line shows the given dose (210 Gy). Each point represents data from a 200 by 200 μm (10 by 10 pixel) area. The position of the transect is shown in the upper image.

Figure 5: An operculum from Purfleet showing variation in the intensity of the natural TL signal and variation in the equivalent dose.

Table 1: Measurement procedure used for equivalent dose determination

FIGURES

Figure 6: TL glow curve constructed by integrating the signal from a region of interest (ROI) 40 by 40 pixels from 400 EMCCD images collected while heating the operculum to 400°C at a heating rate of 0.5 °C/s. The operculum had received a dose of 1680 Gy and had been preheated to 320 °C at 5 °C/s prior to this TL measurement. (a) Raw data for the first heating and for the second heating taken to assess the contribution from black body radiation. Spikes due to cosmic rays are apparent in the data; these can be removed by applying a median filter. (b) Peak temperature calculated for 168 regions across the operculum, each 10 by 10 pixels (200 by 200 μm). A normal curve is shown fitted to this data.

Figure 7: (a) Image of an operculum through a visible microscope. The operculum is ~3.8 mm along its longest axis. Concentric growth structures can be seen clearly. (b) TL signal (105-110°C) emitted by the operculum following a 504 Gy beta dose.

Figure 8: (a) Image of an operculum taken using artificial illumination, showing the area used to define the 231 by 164 pixel region of interest (ROI) for the TL glow curves shown in (b). (b) Background subtracted TL glow curves obtained following different laboratory irradiations, and (c) dose response curve for the operculum, showing the dose recovered. Inset shows the change in sensitivity during the sequence of measurements.

Figure 9: TL signal intensity (shown in red) and equivalent dose (shown in black) along a transect across an operculum. The horizontal dashed line shows the given dose (210 Gy). Each point represents data from a 200 by 200 μm (10 by 10 pixel) area. The position of the transect is shown in the upper image.

Figure 10: An operculum from Purfleeet showing variation in the intensity of the natural TL signal and variation in the equivalent dose.

Table 2: Measurement procedure used for equivalent dose determination

Step		Signal
1	Dose	
2	Preheat to 320°C at 5 °C/s	
3	*TL to 400 °C at 0.5 °C/s	L_x
4	Test dose (245 Gy)	
5	Preheat to 320 °C at 5 °C/s	
6	*TL to 400 °C at 0.5 °C/s	T_x

Note:

* All TL measurements included a second heating to measure the black body radiation so that this signal could be subtracted