

A new flow cytometry method enabling rapid purification of diatoms from silica-rich lacustrine sediments.

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Abstract

Sub-fossil diatoms represent an important archive of past environmental change in both terrestrial and marine settings. In recent years the isotopic analysis of diatom frustules has developed into an important area of paleo-environmental research. The extraction and concentration of diatoms from marine and lake sediments has proven difficult, particularly in silica rich samples. Here we present a new method for the rapid extraction of diatom frustules from lacustrine sediment using flow cytometry. This new technique produces samples suitable for geochemical and isotope based research with a high degree of purity over a much shorter time frame than existing techniques, hours rather than days.

Introduction

Sub-fossil diatoms represent an important archive of past environmental change in both terrestrial and marine settings (Mackay et al 2003; Leng & Swann 2010). A key advance in diatom analysis in recent years has been the application of stable isotope geochemistry to the four main elements (oxygen, carbon, nitrogen and silicon) that combine to form the diatoms frustule (Leng and Barker 2006). In lacustrine and marine diatoms the ratios of oxygen and carbon isotopes have been used to investigate past changes in temperature (Schmidt et al. 1997; Crespin et al. 2010) ocean circulation, relative sea level and fresh/melt water input (Malin and Swann 2006) and the carbon cycle (Hurrell et al. 2011). Similarly, silicon and nitrogen have provided an insight into silicon (Leng et al. 2009) and nitrogen cycling (Kalansky et al. 2011). However, obtaining pure diatom samples has proven problematic, with contamination from biogenic and mineral sources of silica a well-documented problem (Lamb et al. 2007; Morley et al. 2004; Swann and Leng 2009).

A range of techniques has been developed to separate and concentrate the frustules from clastic and organic material that comprises the bulk of a lake or marine core sediment sample. Floatation and density separation techniques (Schrader 1973; Ellwood and Hunter 1999) have been successfully used on a range of sample types but can potentially introduce additional chemical contaminants to the sample. Split-flow thin fractionation (SPLITT) (Rings et al 2004) offers an alternative approach, separating the diatoms and clastic particles under laminar flow according to their density and hydrodynamic properties. The technique can be very efficient but is often a very time consuming process.

Flow cytometry (FCM) is a method that was developed for analysing, counting and sorting fluorescently tagged cells and microscopic particles (Fulwyler 1965). FCM has been shown previously to sort a variety of phytoplankton from aquatic environments (Cellamare et al. 2009; Hofstraet et al. 1994; Phinney and Cucci 1989) and we believe it can be applied to diatom analysis.

Materials and Methods

We aimed to develop a method, which would allow for the purification of diatoms from clastic rich sediments. The methodology presented in this paper was developed using sub-samples from a core taken at Baularvallavatn, a small freshwater lake in Iceland (66°55' 12"N; -22°052' 60W) (Langdon et al. 2008). The core (Baul3) was known to have a well-preserved diatom record, but has a very clastic-rich matrix containing an abundance of tephra. Previous attempts to extract diatoms from the record for isotopic analysis had proven problematic due to the difficulty in separating the diatoms from the tephra (Caseldine pers. commun.). The data presented here are from a single sample (65 – 65.5 cm) that was known to contain a high concentration of both diatom frustules and tephra shards.

Sample Preparation

0.5 g of dry weight sediment samples were resuspended in deionised H₂O, vortexed and passed through a 100-µm sieve (BD Falcon™ 352360) to remove any larger particles that could block the flow cytometer.

The samples were then ready for analysis by flow cytometry.

Flow Cytometry

FCM is a technique that allows for the rapid, individual analysis of microscopic particles (Fulwyler 1965). Particles, known as events, are transported and aligned in a stream of phosphate buffered saline (PBS) solution. When each event passes through a laser beam, corresponding detectors generate a signal pulse. The width of this pulse is determined by the length of time the particle takes to pass through the laser beam. A larger pulse width is indicative of a larger event. The height of the pulse is indicative of the strength of the signal caused by the event. The values for the pulse height, width or area for each event from the detectors can be displayed in a variety of plots. By selecting particular regions of these plots, events that fall within these areas can be sorted away from the sample for collection. Sorting is achieved by applying a variable electrostatic field across the stream. Events can be sorted into a collection tube by applying the electrostatic field and deflecting the targeted events away from the stream, leaving the unwanted detritus to be routed to the waste.

Although the FACS (Fluorescence Activated Cell Sorter) was developed to sort fluorescent particles, diatoms do not naturally fluoresce. Instead we are able to use the forward and side scatter detectors which allow us to measure the size and internal complexity of particles (Fulwyler 1965). Size (forward scatter) is estimated by measuring the amount of light being refracted by the particle when entering the laser beam. Forward scatter is an indication of size. Particles of the same size will have the same forward scatter measurement. Size difference is displayed respectively to the size measurement given by the machine. The maximum sized particle a flow cytometer is able to identify is machine dependant, but tends to be in the region of 1 µm to 100 µm. Internal complexity (side scatter), indicative of a particles internal and surface structure, can be measured from the amount of light scattered by the particle as it passes through the laser beam. Similar to forward scatter, particles with same structure will give the same side scatter measurements.

The Flow cytometer used in this study was a BD FACS Aria II (Becton Dickinson, San Jose, USA), 488 nm excitation laser, Size (Forward scatter) detector and Internal Complexity (Side scatter) detector. PBS (pH 7.4) was used for analysis and sorting of all samples. 10,000 events from Baul3 were recorded using a flow cytometer and forward and side scatter plots were analysed for populations of interest.

Results

The recorded events were initially displayed on a size, pulse width vs. internal complexity, pulse width plot (Fig. 1a) to differentiate the larger and longer events, such as diatoms (Fig. 1b) from the unwanted detritus. The largest items were selected and gated as P1 (Fig. 1a). This population was then analysed on a separate plot (Fig. 1c), using the pulse height vs. pulse width from the internal complexity detector. We are able to identify events with similar signal intensity (i.e. events of similar type) by using the pulse height, whilst maintaining that we are identifying events that have a longer shape by using the pulse width. The P2 gate (Fig. 1c) selects the main population that has similar pulse height values, excluding any outliers, which may not be diatoms. The P2 population is then displayed on a size vs. internal complexity, pulse area plot (Fig. 1d), as a final way of selecting an area for sorting. We yielded superior purity by selecting an area of suspected detritus, P3 (Fig. 1d) and then sorting the events that didn't fall within that particular area. The P3 gate was positioned based on a trial and error method. The gate was placed and events falling outside the P3 gated area were sorted. The resulting sample was checked by microscopy until purity was achieved. 10,000 events were sorted at a rate of 100 events s^{-1} from the finalised non-gated area and collected. These were centrifuged for 5 minutes at 1500 rpm and then imaged (Fig. 1e).

Discussion

As can be seen in Figs. 1 b and 1e, using FCM we were able to successfully sort diatoms from the Baul3 lake sample with negligible contamination. The sample used for this method development was an extreme, with large amounts of silica contamination. A huge advantage of this technique is the rapid nature at which diatoms are analysed and sorted. The speed at which diatoms can be sorted is proportional to the concentration they are within the sediment sample. The concentration of the diatoms within the Baul3 sample was comparatively high, at 0.0036 g g^{-1} of dry weight sediment. A limitation of this technique is that if an unwanted particle has identical forward and side scatter properties as that of a desired particle, it is not possible to differentiate between these two particles. This also means that diatoms, which have the same optical characteristics as detritus cannot be sorted, as they are indistinguishable by the flow cytometer. As a result not all diatoms present within the sample will be sorted. Although the purity of the sorted sample was near perfect, total purity cannot be guaranteed. The inclusion of a final phase of density separation may be required. The successful application of FACS to diatom analysis opens the possibility for its application to other microfossil studies that require pure samples.

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References

- Cellamare M, Rolland A, Jacquet S (2009) Flow cytometry sorting of freshwater phytoplankton. *Journal of Applied Phycology* 22 : 87–100
- Crespin J, Sylvestre F, Alexandre A, Sonzogni C, Paille's C, Perga M-E (2010) Re-examination of the temperature-dependent relationship between $\delta^{18}\text{O}_{\text{diatoms}}$ and $\delta^{18}\text{O}_{\text{lake water}}$ and implications for paleoclimate inferences. *Journal of Paleolimnology* 44 : 547 - 557
- Ellwood MJ, Hunter KA (1999) Determination of the Zn/Si ratio in diatom opal: a method for the separation, cleaning and dissolution of diatoms. *Marine Chemistry* 66 : 149-160
- Fulwyler MJ (1965) Electronic separation of biological cells by volume. *Science* 150 : 910–911.
- Hofstraat JW, van Zeijl WJM, de Vreeze MEJ, Peeters JCH, Peperzak L, Colijn F, Rademaker TWM (1994) Phytoplankton monitoring by flow cytometry. *Journal of Plankton Research* 16: 1197–1224.
- Hurrell ER, Barker PA, Leng ML, Vane CH, Wynn P, Kendrick CP, Verschuren D, Street-Perrott FA (2011) Developing a methodology for carbon isotope analysis of lacustrine diatoms. *Rapid Commun. Mass Spectrom* 25 : 1567–1574.
- Kalansky JF, Robinson RS, Popp BN (2011) Insights into nitrogen cycling in the western Gulf of California from the nitrogen isotopic composition of diatom-bound organic matter. *Geochemistry Geophysics Geosystems* 12 : Q06015, 14 PP.doi:10.1029/2010GC003437
- Lamb AL, Brewer TS, Leng MJ, Sloane HJ, Lamb HF (2007) A geochemical method for removing the effect of tephra on lake diatom oxygen isotope records. *Journal of Paleolimnology* 37 : 499–516.
- Langdon PG, Holmes N, Caseldine CJ (2008) Environmental controls on modern chironomid faunas from NW Iceland and implications for reconstructing climate change. *Journal of Paleolimnology* 40 : 273 - 293.
- Leng MJ, Swann GEA, Hodson MJ, Tyler JJ, Patwardhan SV, Sloane HJ (2009) The Potential use of Silicon Isotope Composition of Biogenic Silica as a Proxy for Environmental Change. *Silicon* 1: 65 - 77.
- Leng MJ, Swann GEA (2010) Stable Isotopes from Diatom Silica. In: Smol JP, Stoermer EF (eds) *The Diatoms: Applications for the Environmental and Earth Sciences*, 2nd ed. Cambridge University Press, Cambridge, UK, pp 127–143
- Leng MJ, Barker PA (2006) A review of the oxygen isotope composition of lacustrine diatom silica for palaeoclimate reconstruction. *Earth Science Reviews* 75 : 5 - 27.
- Mackay AW, Jones VJ, Battarbee RW (2003) Approaches to Holocene climate reconstruction using diatoms. In : Mackay AW, Battarbee RW, Birks HJB, Oldfield F (eds) *Global Change in the Holocene*. Arnold, London, 294-309.
- Malin MA, Swann GEA (2006) Isotopes in Marine Sediments. In: Leng MJ (ed) *Isotopes in Palaeoenvironmental Research*. Springer, pp 227–273
- McCormick PV, Cairns J (1994) Algae as indicators of environmental change. *Journal of Applied Phycology* 6 : 509–526.

Morley DW, Leng MJ, Mackay AW, Sloane HJ, Rioual P, Battarbee RW. Cleaning of lake sediment samples for diatom oxygen isotope analysis. *J Paleolimnol.* 31: 391–401. Phinney DA, Cucci TL (1989) Flow cytometry and phytoplankton. *Cytometry* 10: 511–521

Schmidt M, Botz R, Stoffers P, Anders T, Bohrmann G (1997) Oxygen isotopes in marine diatoms: A comparative study of analytical techniques and new results on the isotope composition of recent marine diatoms. *Geochemica et Cosmochimica Acta* 61: 2275–2280

Schrader, HJ (1973) Proposal for a standardized method of cleaning diatom-bearing deep-sea and land-exposed marine sediment. *Nova Hedwigia/Beihefte. Lehre* 45: 400-409

Swann GEA, Leng MJ (2009) A review of diatom $\delta^{18}\text{O}$ in palaeoceanography. *Quaternary Science Reviews*, 28: 384–398

Figure caption

Figure 1 : 1a: Size – Pulse Width vs. Internal Complexity - Pulse Width dot plot displaying all events, selected population P1 coloured red. 1b: Brightfield image of sample before analysis and sorting by FCM. 1c: Internal Complexity – Pulse Height vs. Internal Complexity –Pulse Width dot plot displaying events from P1, selected population P2 coloured blue. 1d: Size – Pulse Area vs. Internal Complexity – Pulse Area dot plot displaying events from P2, detritus selected in P3, the unselected events are sorted. 1e: Brightfield image of sorted sample by FCM. The sample in this instance was dominated by *Aulacoseira* spp. Scale bar = 10µm.