Method for High-Throughput Analysis and Sorting of Moss Protoplasts (*Physcomitrella patens*)

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UNION BIOMETRICA
Large Particle Flow Cytometry

Introduction

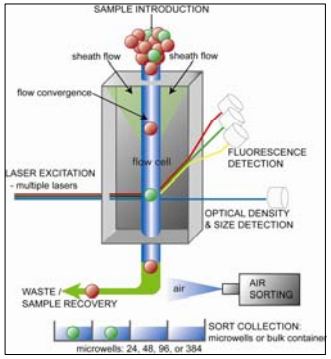
Plant protoplasts possess several features that make them an ideal single cell system for applying the techniques of modern biology. Protoplasts can be gently isolated from cell wall-enclosed plant cells and handled much like other cell types – grown in culture, transformed with exogenous DNA. However, other technologies have some difficulties when applied to protplasts. For example, protoplasts are very fragile and they are often quite large, making them a challenge as a sample for flow cytometry. Their large size results in the need for a flow cytometer with a wide microfluidic channel, and their delicate nature requires lower pressures, lower shear forces, and a gentle sorting mechanism. Here we describe the development and optimization of a method for analyzing plant protoplasts of the moss, *Physcomitrella patens*, using large particle flow cytometer (BioSorter® from Union Biometrica, Inc.) and sorting transgenic GFP-expressing cells. These cells remain intact after sorting. We believe this capability of analyzing and sorting intact protoplasts is not broadly available with most other flow cytometers.

The BioSorter is able to analyze particles with diameters of 1-1500µm in a continuously flowing stream at a rate up to 30000 objects/second and measure physical properties such as object size, optical density and intensity of fluorescent markers.

FOCA	Object Size	Applications
250 µm Forward Scatter	1-40 µm	- Cells - Pollen
250 µm	20-150 µm	- *C.elegans* - Stem Cells (Clusters)
500 µm	40-300 µm	- Fragile cells (adipocytes, plant protoplasts) - *D. melanogaster* embryos
1000 µm	200-700 µm	- pancreatic islets - Seeds (*A. thaliana*, tobacco)
2000 µm	500-1500 µm	- Zebrafish (embryos, larvae) - Plant cell clusters (calli)

Fluidics and Optics Core Assembly (FOCA)

The BioSorter has interchangeable fluidics and optics core assemblies (FOCAs), allowing the instrument to analyze and dispense objects across a large size range. For the plant protplasts, we used the 250 and 500 FOCAs which have a 250µm-wide and 500µm-wide microfluidics channels respectively.



Sorting Mechanism Diagram

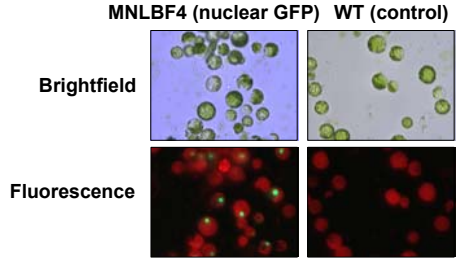
A gentle pneumatic device located after the flow cell is used for sorting. This approach avoids damaging or changing the fragile samples. This makes the instrument suitable for handling live biological materials or sensitive chemistries. The fluid pressures of the instrument (up to 6 psi) are also significantly lower than those of traditional flow cytometers, resulting in lower shear forces and less damage to the samples.

Growth Conditions and Prep of Protoplasts

Moss (*Physcomitrella patens*) strains were grown on PPNH4 plates at 27° C. Seven day old plants were used for protoplast isolation. Plants were collected into Petri dish containing 8% mannitol and 2% driselase enzyme (Sigma, D9515-25G). Plate was incubated at room temperature with gentle shaking for 1 hour. Crude extract was filtered through 100 µm mesh (BD Falcon, 352350). Filtered suspension was centrifuged at 250 g for 5 minutes. Protoplasts were gently resuspended in 8% mannitol and washed two more times. 10 µL suspension of protoplast solution was counted in a hemocytometer to determine protoplast yield.

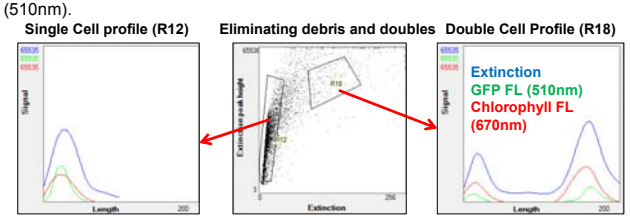
Analyzing Protoplasts

Moss (*Physcomitrella patens)* is a widely used model for studies on plant development and physiology. We tested the ability of BioSorter® to analyze and sort moss protoplasts. Two strains of moss (wild type, MNLBF4 (nuclear GFP)) were analyzed using 500µm flow cell on BioSorter® instrument. Protoplasts were selected based on presence of red autofluorescence (670nm filter) from the chlorophyll, a distinguishing feature of protoplast in this sample.

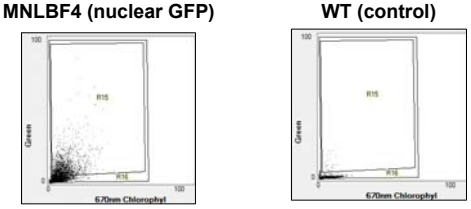


Freshly Isolated Moss Protoplasts

Using FlowPilot software we were able to gate-out doubles and debris. Profiler II allows identifying cell shape and localizing fluorescence. Protoplasts were then gated based on high chlorophyll fluorescence (670nm) and GFP fluorescence (510nm).



Flow Cytometry Analysis of Protoplasts



R16 – All protoplasts
R15 – Protoplasts expressing nuclear GFP

Protoplasts in R15 region represent a subset of all protoplasts and were sorted using BioSorter. Protoplasts were dispensed to slides and counted under the microscope to verify identity and accuracy of sorting. Higher numbers of intact protoplasts were achieved after 48h incubation due to the cell wall recovery on the cells.

Optimization of Sorting Conditions

Fresh protoplasts are very fragile and can be disrupted by mechanical impact. For successful transformation and certain other experiments it is important to get reliable and reproducible protoplast populations. In other applications protoplasts from one tissue type could be sorted away from cells of other tissue types. Several groups have reported using conventional flow cytometers for high-throughput protoplast analysis and isolation. However, in these experiments cells are often sorted for sample preparation and not isolated intact. We used the BioSorter instrument with its gentle sorting mechanism. Cells were prepared in four different buffers and sorted using two different flow cells (250 and 500µm widths). Viability was assessed manually by observing chlorophyll autofluorescence in sorted cells.

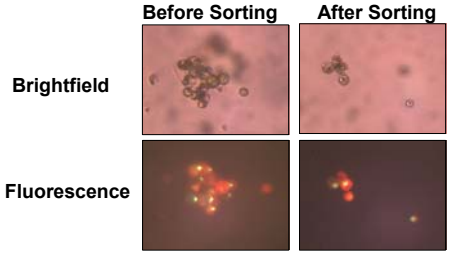
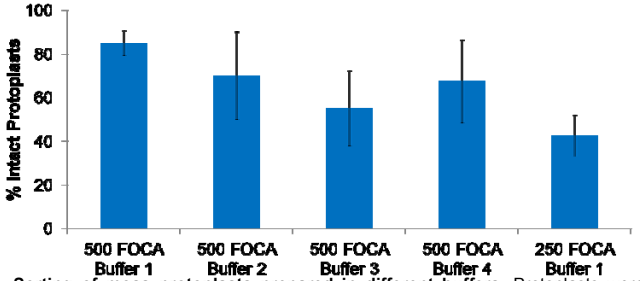


Table 1. Protoplast Buffers Tested

Buffer	Content	Reference
Buffer 1	8% mannitol, 8% sucrose in PBS (pH 7.4)	Modified from J Vis Exp. 2011 Apr 19;(50)
Buffer 2	2mM MES (pH 5.7), 154mMNaCl, 125mM CaCl2 and 5 mM KCl	Nat Protoc. 2007;2(7):1565-72
Buffer 3	8% mannitol in PBS (pH 7.4)	J Vis Exp. 2011 Apr 19;(50)
Buffer 4	8% mannitol, 8% sucrose in PBS (pH 7.4) 460 mg l1 ammonium tartrate	Biotechnol Adv. 2005 Mar;23(2):131-71.



Sorting of moss protoplasts prepared in different buffers. Protoplasts were sorted to the slides and counted under the microscope. At least 100 protoplasts were counted for each condition.

We achieved the highest numbers of intact protoplasts (85%) prepared in 8% mannitol, 8% sucrose in PBS (pH 7.4) buffer and sorted using 500 µm flow cell.

Conclusions

We demonstrate high speed gentle sorting of fragile cells, specifically fresh protoplasts from *Physcomitrella patens*. We developed and optimized a new method for high-throughput analysis and subsequent sorting (200-300 events/sec) of moss protoplasts using the BioSorter. This method allowed sorting of freshly prepared (3h) protoplasts using conditions where greater than 85% of these protoplasts remain intact. The BioSorter provides an unbiased approach to analysis and sorting of plant protoplasts.

Acknowledgements

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