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

Work performed January 2013 by:
Mikalai Malinouski, PhD, Union Biometrica, Holliston, MA

Objective

Sorting of fragile plant protoplasts has long been a challenge due to the absence of cell wall. We developed a method for high-throughput analysis and sorting of intact protoplasts for downstream applications including imaging and physiological assays.

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 protoplasts. 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 BioSorter® 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.

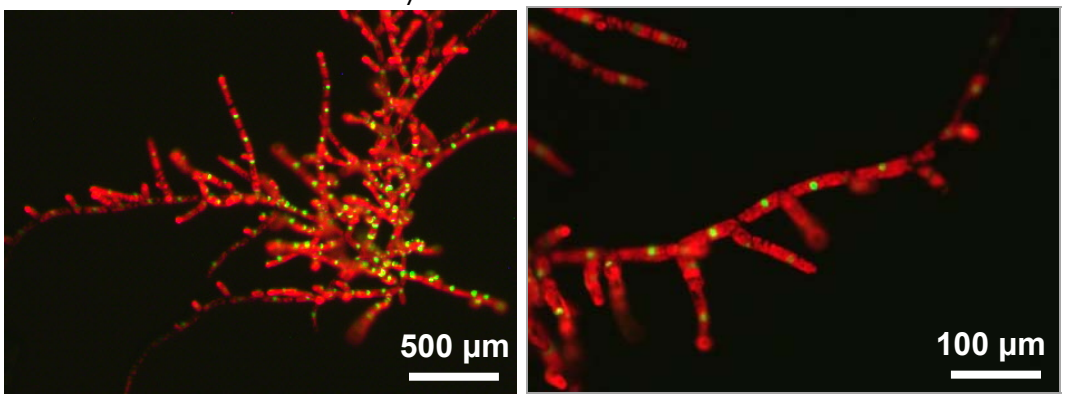

Figure 1. Fluorescence imaging of MNLBF4 (nuclear GFP) moss. Red - chlorophyll fluorescence, Green – nuclear GFP.

Union Biometrica
USA: Tel: + 1(508) 893-3115 Fax: +1 (508) 893-8044
Europe: Tel: +32-(0) 14-570628 Fax: +32- (0) 14-570621
http://www.unionbio.com sales@unionbio.com

UNION BIOMETRICA
Large Particle Flow Cytometry

Preparation 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). 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 250g 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. Protoplasts were resuspended in the protoplast storage buffer (8% mannitol, 8% sucrose in PBS, pH 7.4). Microscopy of freshly prepared protoplasts was also performed (Fig. 2).

The BioSorter® instrument, equipped with 250µm and 500µm FOCAs (Fluidic and Optical Core Assembly) was used for the method. Physical characteristics of size (TOF), optical density (EXT), and 3 different channels of fluorescence signals (FLU) were collected. Objects are passed axially, one by one, through the focus of a laser beam. Relative size is determined by the time of flight (TOF) measurement. The optical density of the object is determined by the extinction (EXT) measurement. Using Profiler II we collected spatial information on fluorescence and extinction for each object passing through the flow cell. A 488 nm solid state laser was used in the experiments. GFP fluorescence was collected using BP510/23 nm filter (PMT1) and chlorophyll autofluorescence using BP 670/20 nm (PMT3).

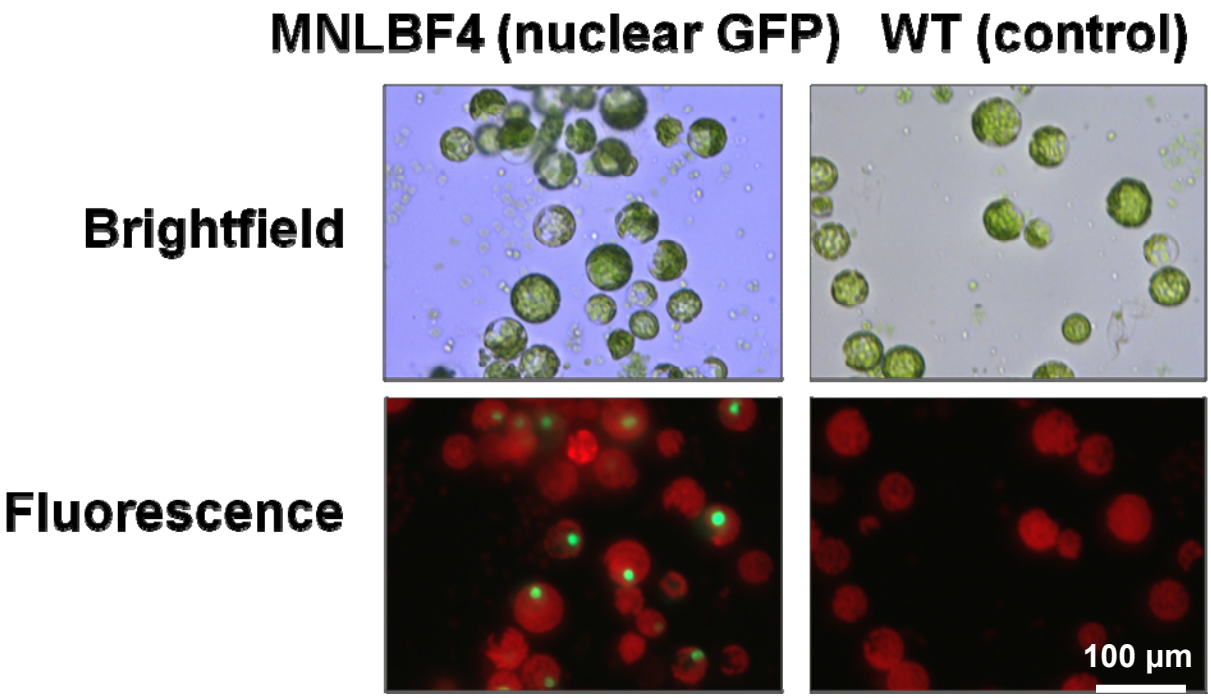

Figure 2. Imaging of freshly isolated moss (*Physcomitrella patens*) protoplasts. Red – chlorophyll fluorescence. Green – GFP.

Results

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 250µm and 500µm flow cells on BioSorter® instrument (Fig. 2). Live protoplasts were selected based on presence of red autofluorescence (670nm filter) from the chlorophyll. Using FlowPilot software we were able to gate-out doubles and debris. Profiler II allowed identifying single cell population (Fig. 3A). Cells were further gated based on the chlorophyll fluorescence and GFP (Fig. 3B).

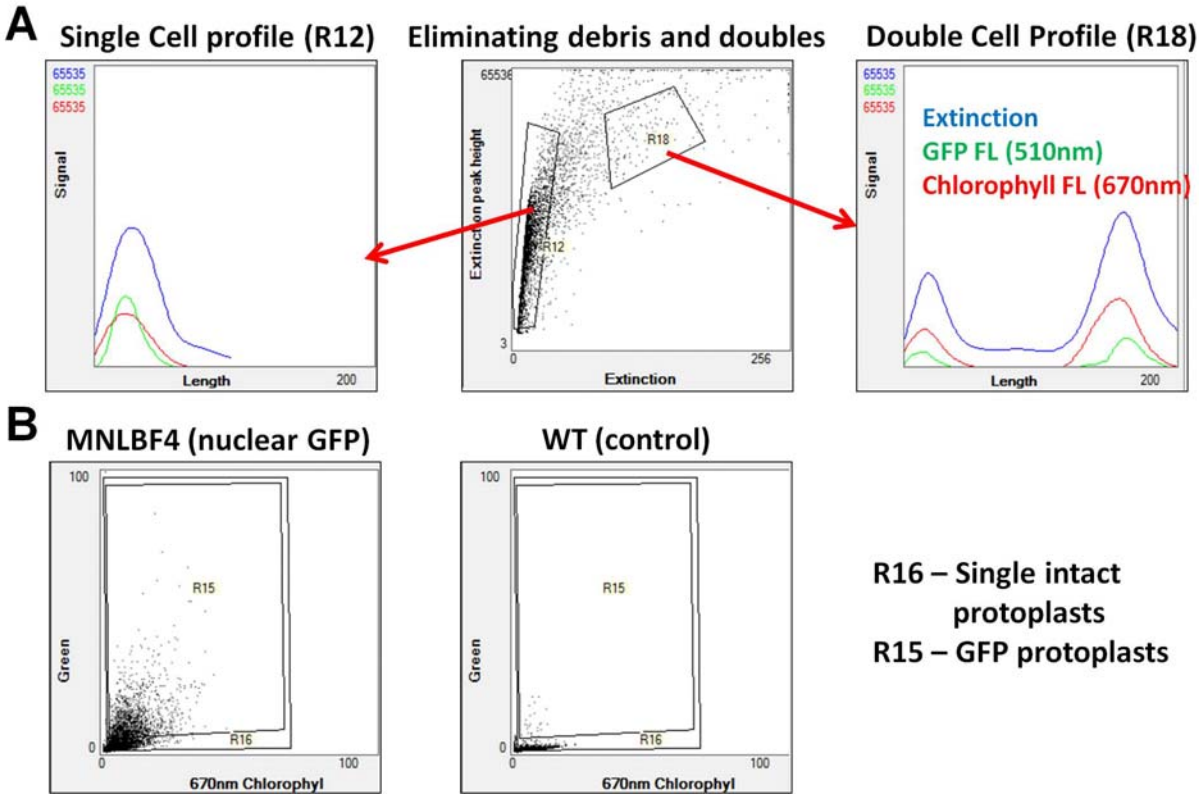

Figure 3. Flow cytometry analysis of protoplasts. A. Selecting single cell population. B. Selecting intact protoplast expressing GFP (only R12 population is shown). R16 was set based on the chlorophyll fluorescence to include only intact single cells.

Sorting of protoplasts

For successful transformation and other experiments it is important to get reliable and reproducible protoplast populations. Protoplasts can easily be contaminated with other undifferentiated cell types. Several groups have reported using conventional flow cytometers for high-throughput protoplast isolation. However, in these experiments cells are often sorted directly to the lysis buffer and are not suitable for transformation and propagation. We used the BioSorter instrument with its gentle sorting mechanism. Protoplasts in R15 region (Fig. 3B) 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. At least 100 cells were analyzed per condition. Four different buffer conditions were tested (Table 1, Fig. 4).

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.

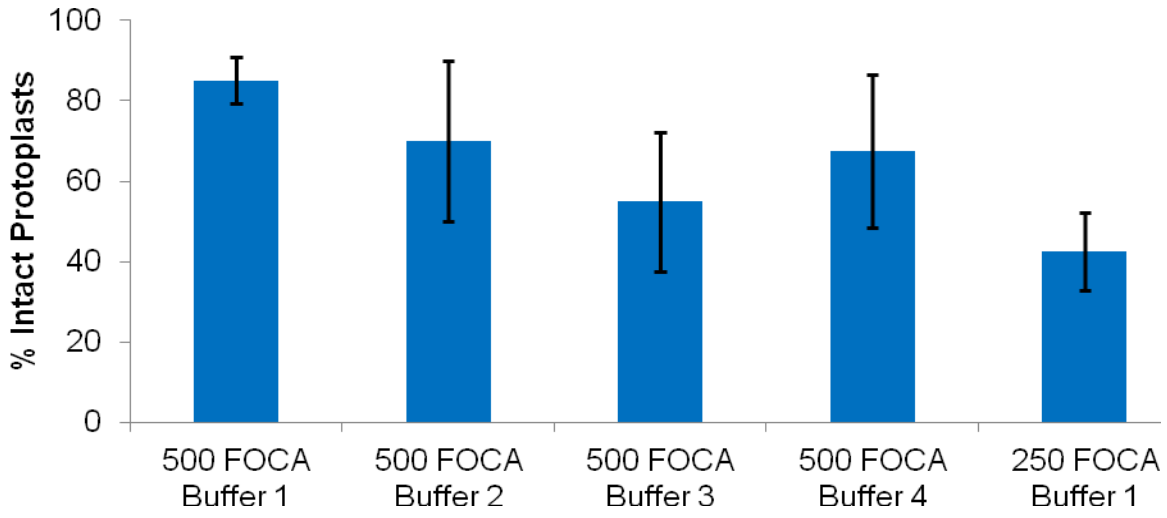

Figure 4. Sorting of moss protoplasts prepared in different buffers. Protoplasts were sorted to the slides and counted under the microscope. At least 100 protoplasts were counted for each condition.

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 (Fig. 5).

We tested two sizes of flow cell, one with the 250 micron-wide microfluidics channel (250 FOCA) and the other having a 500 micron-wide microfluidics channel (500 FOCA). For all conditions tested, the larger 500 FOCA was better than the 250 FOCA as measured by the percentage of sorted intact protoplasts.

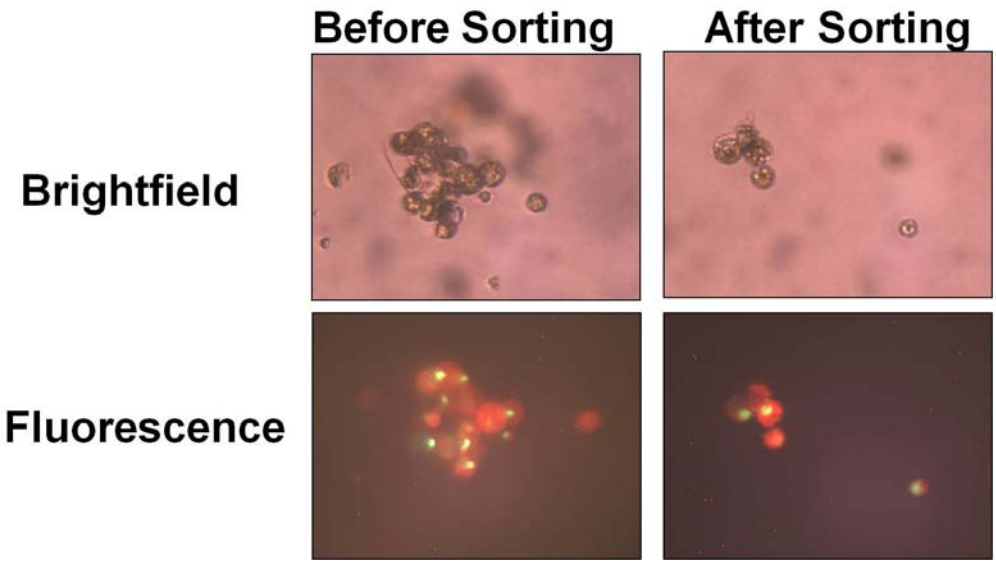

Figure 5. Representative images of intact moss protoplasts before and after sorting. High chlorophyll fluorescence indicates that cells are not damaged during sorting.

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 (100-200 events/sec) of moss protoplasts using the BioSorter. This method allowed sorting of freshly prepared (3h) protoplasts using conditions were greater than 85% of these protoplasts remain intact. We determined that the larger microfluidics channel of the 500 FOCA was better for collecting intact protoplasts than the 250 FOCA with its narrower microfluidics channel. This is likely due to the lower pressures needed and the reduced stream velocity and shear forces. The BioSorter provides an unbiased approach to analysis and sorting of plant protoplasts.

Acknowledgments

We thank Dr. Luis Vidali and Dr. Fabienne Furt from Department of Biochemistry, Worcester Polytechnic Institute for the help preparing protoplasts.