

Protoplast isolation and enrichment using the microfluidic WOLF[®] Cell Sorter

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Introduction

Cell isolation via gentle cell sorting is needed for workflows in plant biology including gene characterization and function, proteomics and transcriptomics, single-cell genomics, improved cell line development through gene engineering/CRISPR-Cas9 editing, and breeding. Sorting plant protoplasts is challenging with conventional droplet cell sorters due to the high sample pressures, shear stress and osmotic changes which damage protoplasts making them unusable for downstream applications. Traditional cell sorters also limit the use of custom sheath buffers, such as plant culture medium, which is critical to support cell viability with protoplasts during and post sort. The microfluidic WOLF cell sorter efficiently enriches for populations of interest using a gentle sorting mechanism (< 2 psi) with low shear stress and supports cell viability by enabling scientists to use culture medium as sheath. Here, we demonstrate protoplast isolation with successful separation of tomato mesophyll protoplast classes with improved post-sort viability.

Methods

Protoplast Isolation

Tomato protoplasts were isolated from leaf fragments using the following methods.^{1,2,3} One gram of mature tomato leaves from nursery grown tomato plants was placed in 12 mL of plasmolysis buffer to prepare two dishes. The leaves in each dish were cut in strips longitudinally with a #23 razor blade attached to a scalpel then chopped horizontally. Chopped leaves remained fully submerged in plasmolysis buffer in the dark at room temperature and stationary for one hour. The plasmolysis buffer was then removed from each plate and replaced with 12 mL of fresh sterile enzyme [1.5% Cellulysin, (Millipore Sigma, # 219466), and 0.4% Macerozyme (Yakult Biochemicals Co. Ltd., Japan)]. The plates were sealed with parafilm and placed in the dark on an orbital shaker (40 rpm) for 18 hours. Protoplast yields and morphology were checked via microscopy in the morning to confirm release and protoplast health.

Protoplast Sieve and Wash

Using the slow speed on an electronic pipet aid (Drummond Scientific, cat# 4-000-105) and a sterile 10 mL wide-bore serological pipet (Cole-Parmer, cat# VV-34567-54), protoplast mixtures were gently sieved successively through a 70 µm

sterile nylon sieve (Corning, cat# 352350 followed by a 40 µm sterile nylon sieve (Corning, cat# 352340) in a petri dish. After filtration, protoplast filtrate was gently transferred to 50 mL sterile conical tubes using 10 mL wide bore serological pipets, taking care to avoid generating bubbles as this causes damage to intact protoplasts. The mixture was then centrifuged for 8 minutes at 46 x g in a swinging bucket rotor. The supernatant was removed without disturbing the pellet and then replaced with 25 mL of wash buffer (W5).¹ The pellets were gently resuspended by tube inversion and centrifuged for 3 minutes at 46 x g. The supernatant was removed and the wash procedure with 25 mL of W5 was repeated. The washed pellet was gently resuspended in 4 mL of Murashige & Skoog basal medium with vitamins (Caisson labs, # MSP09-10LT) supplemented with 0.4M Mannitol (Millipore Sigma, # M1902-500G), 50 µg/mL ampicillin (Millipore Sigma, # A5354) and 0.05% Cellulysin (MS final medium).

Protoplast Counts

A 1:10 dilution of protoplasts in MS medium was made: 50 µL total volume (5 µL protoplasts + 45 µL of MS final medium) in a sterile 1.5 mL microcentrifuge tube. The tube was tapped gently multiple times to mix solution without introducing bubbles. Using a wide-bore micropipette tip, 12 µL of 1:10 dilution was placed in each of the two ends of a hemocytometer. Four squares were counted on each side of the hemocytometer using a light microscope. Final cell counts were calculated using the following formula: average of the 8 squares x dilution factor x 10⁴ x total volume = total number of protoplasts per milliliter of solution.

Microscope Images

10x and 40x bright field images were taken on an inverted light/fluorescence microscope pre and post sort to confirm protoplast intactness and enrichment of classes.

Cell size and distribution: Determine the expected size range and distribution of the protoplasts before attempting to sort. Determining the distribution of protoplast sizes can assist in optimizing pre-sort procedures. In general, plant protoplasts can range from 10-150 µm in size depending on crop and tissue source. If the protoplast population is pushing the upper limit of the sortable size range for WOLF (see normal and left-skewed population examples in Figure 1 below), there are two options: (1) re-filter protoplasts through a specified filter size (e.g., 30-40 µm) to eliminate the largest cell sizes or (2) increase the osmolarity of the sorting media to contract the cells.

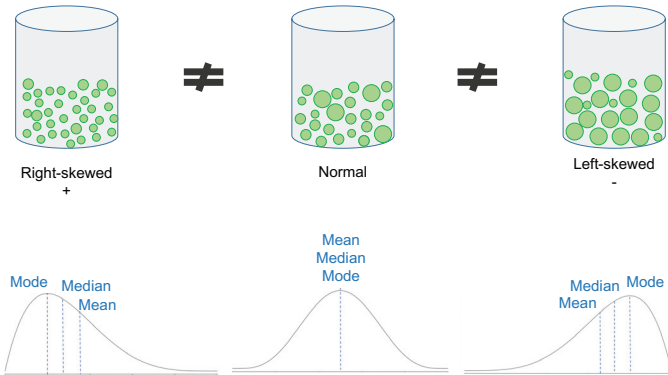


Figure 1. The distribution of protoplast size: The above images of samples represent protoplast populations within the 30-50 μm size range, but these populations differ greatly in their distribution and behavior through a sorter. Right-skewed and normal distributions tend to sort successfully on WOLF with minimal to no culture media modifications; but left-skewed populations may benefit from increasing the osmolarity of the culture media.

Sorting Protoplasts with the WOLF

Protoplasts were diluted to a concentration of 2.5×10^5 cells per milliliter and kept in 100 x 25 mm petri dishes. When ready to sort, 3.0 mL of protoplasts were transferred to a sterile 5.0 mL FACS tube. The WOLF was primed and calibrated with sterile-filtered, distilled water and then re-primed with sterile-filtered protoplast MS final medium prior to analyzing and sorting protoplasts. PBS was not used as sheath buffer to sort protoplasts because the osmolarity is too low and leads to cell lysis. Sorting parameters are summarized in Table 1.

Plant protoplast classes were identified using chlorophyll autofluorescence and cell size estimated from scatter (Figure 4). These were then successfully separated via gentle sorting (Figure 5). All events shown above the 10^3 decade are considered positive for chlorophyll and therefore likely plant cells and organelles. The parent gate encompasses chlorophyll-positive events (top left), and the child plot (top right, gated to chlorophyll positive events) shows the three independent gates for each of the three chlorophyll containing populations that were used for cell enrichment.

NOTE: It is suggested to pause the sort and invert the FACS tube gently every 15 minutes for the duration of the sort to keep protoplasts in suspension and to prevent cartridge clogging. Plant mesophyll protoplasts are denser than the sample buffer and sink rather quickly.

Parameter	Setting/voltage (GAIN)
Laser Configuration	488 nm (blue)
Trigger Channel	FSC
FSC	10,000
FL1 (GRN)	200
FL2 (YLW)	200
FL3 (RED)	200

Table 1. Parameters and PMT detector gain settings for tomato mesophyll protoplasts on a WOLF cell sorter.

Processes and optimization steps for protoplasts:

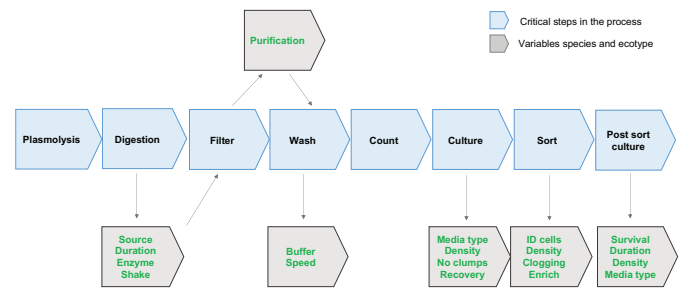


Figure 2. Process map for protoplast sorting. Blue boxes represent critical steps in the process, gray boxes represent variables in the process that are dependent on plant crop/species and ecotype.

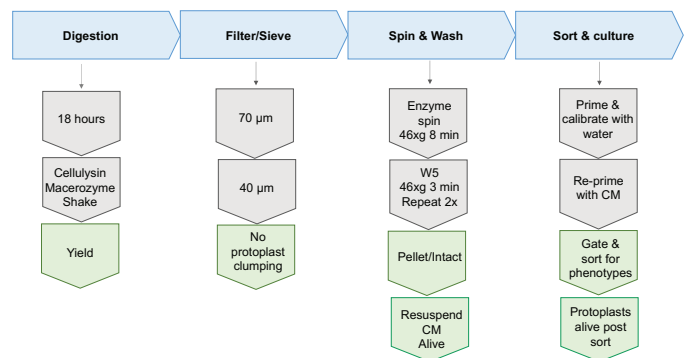


Figure 3. Workflow for tomato mesophyll protoplast isolation and culture. Blue boxes represent critical steps in the process, gray boxes represent variables in the process that are dependent on plant crop/species and ecotype, and green boxes represent outcomes in the process for each critical step.

Results

The successfully sorted populations are shown by microscopy in Figure 5. Size ranges of the high- and low-chlorophyll protoplast populations are the same: 30-50 μm ; while the cell-free chloroplasts are substantially smaller $\sim 10 \mu\text{m}$.

When analyzing the pre-sort sample, free chloroplasts are visible, which is an indication of cell lysis (Figure 4). However, after sorting, the sample contains undamaged protoplasts (Figure 5B and C). The low pressures in the fluidics ($< 2 \text{ psi}$) paired with the use of protoplast culture medium as sheath provides an environment for a successful sort.

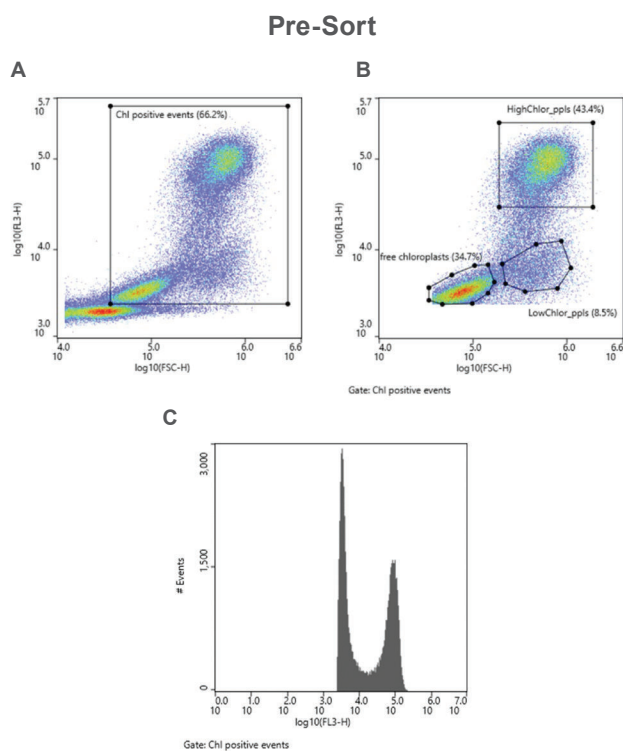


Figure 4. Plant protoplast sorting strategy. Protoplast classes were separated using chlorophyll autofluorescence (FL3, 665LP) and estimated cell size (FSC) both excited by the 488nm laser. **A.** Parent gate (chlorophyll positive events), **B.** Subsequent child gate showing the three populations identified and sorted, which include a high chlorophyll-containing class, a low chlorophyll-containing class, and free chloroplasts from lysed pre-sorted cells. **C.** Is a histogram representation of the chlorophyll positive events within the parent gate.

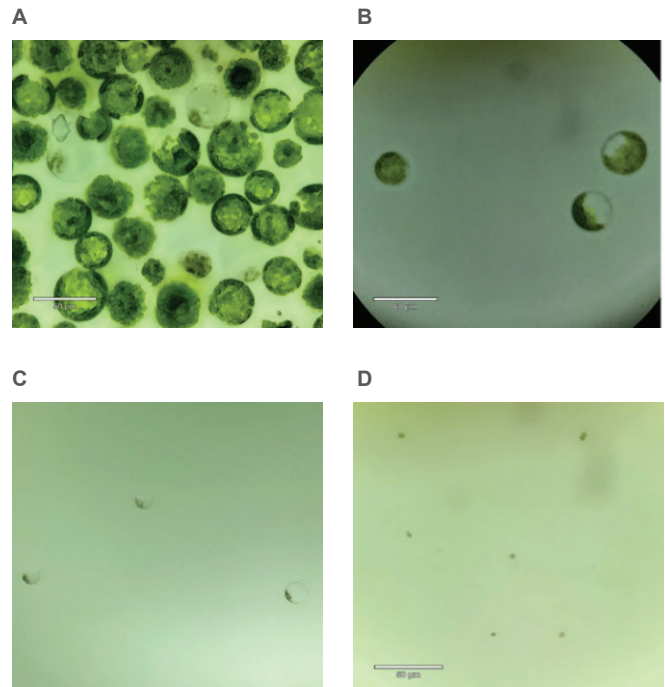


Figure 5. Pre- and post-sort images of chlorophyll containing classes. These classes include **A.** Unsorted mixed protoplast classes, **B.** Sorted high chlorophyll-containing protoplasts, **C.** Sorted low-chlorophyll-containing protoplasts, and **D.** Sorted chloroplasts. Images were taken on an inverted light/fluorescence microscope using a 40x objective and a zoom function (ECHO Labs). 50 μm scale bars are provided and plant mesophyll protoplasts range from 30-50 μm in diameter.

Conclusion

Enriching plant protoplasts can be challenging due to their variation in size, fragility, and sensitivity to shear stress and osmotic changes. A successful sort requires optimizing pre- and post-sort cell culture medium to support protoplast populations throughout the workflow (Figures 2 and 3). Results from these experiments demonstrate that the WOLF Cell Sorter can enrich plant protoplasts up to 50 μm in diameter and keep them intact and viable post sort (Figure 5). In addition, the WOLF was able to successfully identify and separate protoplast classes with varying chlorophyll levels, which is key for enriching different cell types that exist in plant leaves.

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References

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