Microbead Encapsulation of Living Plant Protoplasts: A New Tool for the Handling of Single Plant Cells

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Source: Applications in Plant Sciences, 4(5)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1500140

Applications in Plant Sciences 2016 4(5): 1500140

PROTOCOL NOTE

MICROBEAD ENCAPSULATION OF LIVING PLANT PROTOPLASTS: A NEW TOOL FOR THE HANDLING OF SINGLE PLANT CELLS[1]

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- *Premise of the study:* Understanding plant cell biomechanics has been hampered by a lack of appropriate experimental tools. Here we introduce a protocol for the incorporation of individual plant protoplasts into precisely sized agarose microbeads. This technology may lead to new ways to manipulate the physical and chemical microenvironment of individual plant cells.
- *Methods and Results:* Living protoplasts obtained from BY-2 tobacco suspension cultures were continuously incorporated into a stream of agarose microdroplets, collected in cooled mineral oil as gelled microbeads, and then transferred into liquid MS medium for culture. In this first report, we show that spherical microbeads containing living protoplasts can be easily generated in quantity and that these encapsulated cells continue to grow and divide.
- *Conclusions:* Microbead encapsulation of protoplasts affords the opportunity to precisely control the physical microenvironment of individual plant cells. Ultimately, this method may help facilitate novel studies in plant biomechanics.

Key words: biomechanics; microfluidics; protoplasts; single cell biomechanics.

Although the likely significance of cell and tissue mechanics in plant development has been appreciated for many years, the study of cellular micromechanics at the level of the individual cell has proven to be problematic. The universal presence of the cellulosic cell wall and the apoplastic continuity that it provides endows plant tissues with a unique level of mechanical coupling. In principle, this makes it possible for plant tissues to transmit stress-mechanical information precisely and instantaneously over multicellular distances. However, the same apoplastic continuity that makes stress-mechanical signaling attractive as a possible developmental effector also makes it difficult to interpret responses and isolate mechanical variables at the level of the individual cell.

With the advent of droplet microfluidics, it is now possible to manipulate individual cells in novel ways, potentially revealing levels of developmental control that have previously been experimentally inaccessible. In this study, we present a reliable procedure for capturing large numbers of individual plant protoplasts in mechanically isotropic hydrogel microbeads, thereby isolating them from the physical influence of neighboring cells and allowing them to regenerate their walls and proceed through cell division in a precisely controlled physical environment. A detailed protocol is provided as Appendix 1.

Microfluidic devices designed to facilitate the handling and analysis of individual cells are now becoming available and have already been used to encapsulate animal cells (Kumachev et al., 2011), but in studies with plant cells they have found only limited use (Agudelo et al., 2012; Ghanbari et al., 2014). Here we show that droplet microfluidic systems are capable of rapidly and efficiently capturing large numbers of individual plant protoplasts in precisely sized spherical hydrogel beads, providing plant scientists with new ways of dissecting the biophysical background of plant development.

METHODS AND RESULTS

***Protoplast isolation*—**Protoplasts were obtained from 4-d-old BY-2 tobacco (*Nicotiana tabacum* L. cv. BY-2) suspension cultures grown at 27°C in Murashige and Skoog (MS) basal salts (Caisson Laboratories, Smithfield, Utah, USA), 3% sucrose (Fisher Scientific, Fair Lawn, New Jersey, USA), 100 mg/L myo-inositol (Alfa Aesar, Ward Hill, Massachusetts, USA), 1 mg/L thiamine (Fisher Scientific), and 0.2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D; Caisson Laboratories). Suspension-cultured cells were removed from the culture medium and then enzymatically stripped of their cell walls in a digestion medium consisting of 0.5% (w/v) Onozuka RS, 0.5% (w/v) Onozuka R-10, 0.1% (w/v) Macerozyme R-10 (all from Yakult Pharmaceuticals Co. Ltd., Kunitachi-Shi, Tokyo, Japan), and 0.1% Pectolyase Y-23 (MP Biomedicals LLC, Solon, Ohio, USA). The digestion medium was made up in 5.2% mannitol (Acros Organics, Morris Plains, New Jersey, USA), 0.05% $MgCl_2$ (Sigma-Aldrich, St. Louis, Missouri, USA), and 0.2% MES buffer (Sigma-Aldrich) at pH 5.8. Suspension culture BY-2 cells were digested for 3 h at 27°C on a shaker table. Protoplasts were separated from the digestion medium by centrifugation at $100 \times g$ for 7 min. After being washed twice in a solution containing 5.5% mannitol and 0.05% $MgCl_2$ at pH 5.8 (Medium 1) protoplasts were suspended in a culture medium containing 0.44% Caisson MS medium (Caisson Laboratories), 3% sucrose, 2.7% mannitol, and 0.2 mg/L 2,4-D and allowed to rest (7°C for 8 h followed by 12 h at room temperature). Before droplet encapsulation, cells were pelleted by centrifugation at $100 \times g$ for 7 min and then resuspended in MS medium for droplet encapsulation.

***Microbead production*—**Agarose microbeads were generated using a microfluidic droplet system manufactured by Dolomite Microfluidics (The Dolomite Centre Ltd., Royston, United Kingdom). Water-based (agarose) droplets were formed as the discontinuous phase in a 2-reagent, 4-channel, glass microfluidic junction chip. The continuous phase was a light mineral oil (Sigma-Aldrich M5310) with 4% Span 80 (Fluka Analytical, St. Louis, Missouri, USA) added to prevent droplet coalescence. Flow rates of the three component fluids

[1]Manuscript received 17 December 2015; revision accepted 19 April 2016.

The authors thank Dr. R. A. Oldinski (Department of Mechanical Engineering, University of Vermont) for her continued assistance and encouragement.

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doi:10.3732/apps.1500140

were adjusted with three dedicated Dolomite P-Pumps independently controlled by proprietary Dolomite software. The fluids were fed into the droplet chip through microbore polytetrafluoroethylene (PTFE) tubing. The two outer channels were reserved for the mineral oil continuous phase (Fig. 1). The two central channels were functionally separated, with one channel reserved for live protoplasts at an approximate density of 5.0×10^6/mL in MS medium containing 0.44% Caisson MS medium, 3% sucrose, 2% mannitol, and 0.5% high-molecular-weight dextran sulfate. The second channel was fed from a warmed reservoir of 1.5% low-gel-temperature agarose (Sigma-Aldrich) made up in 0.44% Caisson MS medium, 3% sucrose, and 2% mannitol, and maintained at a temperature of 34°C. All reagents were sterile or prefiltered through 0.22-µm syringe filters.

With appropriate flow control, a stream of monodisperse agarose droplets forms at the chip junction where the aqueous and oil phases intersect (Video 1; Fig. 1). Droplet diameter can be increased or decreased by adjusting the flow rates of the continuous and discontinuous phases. Liquid droplets exit the chip and stream downward into a cooled mineral oil bath (Sigma light mineral oil with 4% Span 80), where they solidify into gelled beads (Fig. 1).

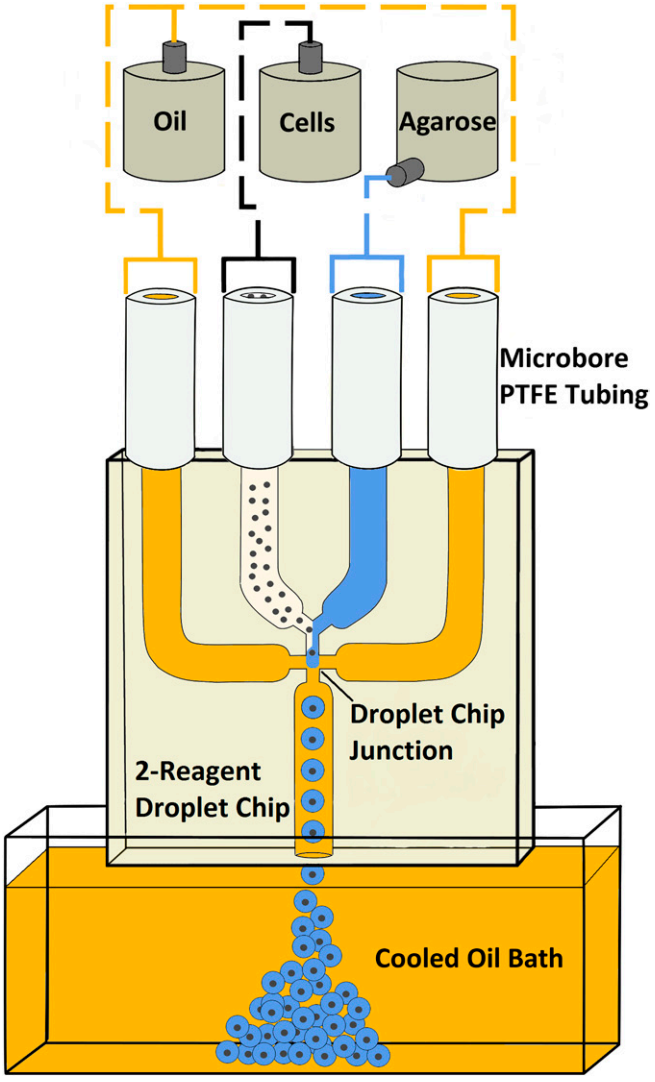
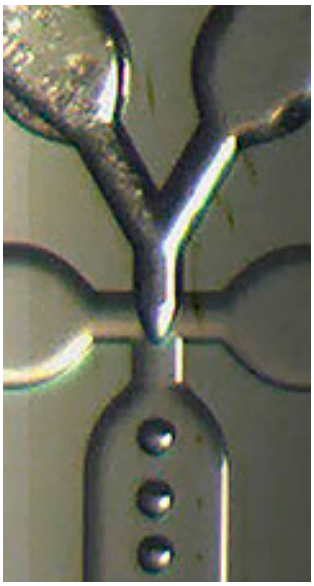


Fig. 1. Schematic of the microdroplet system. Three pumps store solutions in pressure-controlled chambers and drive fluid flow during droplet formation (top). Microbore PTFE tubing (dotted lines) carries fluids from the pressure pumps to the 2-reagent droplet chip. Droplet production occurs at the droplet chip junction where the channels intersect. Agarose (blue) and cells (black) meet immediately before being cleaved into droplets by continuous oil flow (orange). Liquid microdroplets exit the microchip into a cooled mineral oil bath where they solidify.



Video 1. The production of uniformly sized microdroplets at the junction of the droplet chip. Also seen is the problematic coalescence of droplets caused by occasional cell clumps. This video is an MPEG file (.mpg) and can be viewed here with QuickTime or Windows Media Player, or it can be viewed from the Botanical Society of America's YouTube channel.

Microbeads were separated from the mineral oil by centrifugation into a sublayer of liquid MS culture medium and then filtered through an 88-µm nylon mesh to remove coalesced droplets. Finally, agarose microbeads containing live protoplasts were suspended in liquid culture medium containing 0.44% Caisson MS medium, 3% sucrose, 1.6% mannitol, and 0.2 mg/L 2,4-D (pH 5.8). The growth medium was supplemented with filtered medium from a rapidly growing BY-2 cell suspension culture. New cell wall synthesis was confirmed by adding a drop of 0.1% Calcofluor (Fluorescent Brightener 28; Sigma-Aldrich) to a microbead slide preparation.

Consistently sized (ca. 60 µm), spherical hydrogel microbeads were successfully generated at a rate of approximately 130 beads per second. Individual protoplasts were successfully encapsulated with good viability as determined by cytoplasmic streaming activity shortly after encapsulation (Fig. 2A). Cellulose staining with Calcofluor White showed that 32 h after protoplast release and 20 h after cells were encapsulated all living cells had begun to regenerate a thin cell wall (Fig. 2D). Living cells proceeded to elongate and divide, eventually bursting the agarose microbead in which they had been encapsulated (Fig. 2B). Immediately following droplet formation, approximately 25% of generated droplets contained protoplasts.

CONCLUSIONS

In order for the study of plant biomechanics to keep pace with rapid advances in our understanding of the molecular controls underlying plant development, we need to be able to isolate mechanical and physical inputs at the cellular level. Studies conducted at the tissue level are inherently limited by the stresses and strains that pervade and organize all multicellular plant tissues. With the technique described here, it is possible to capture individual plant protoplasts in physically isotropic, hydrogel microbeads whose mechanical properties can be controlled.

The potential for this technique to be used as a means of manipulating the mechanical forces acting on individual plant cells in culture suggests that it could facilitate novel studies. Additionally, with ongoing rapid development in the fields of microfluidics and hydrogel engineering, it seems that these techniques

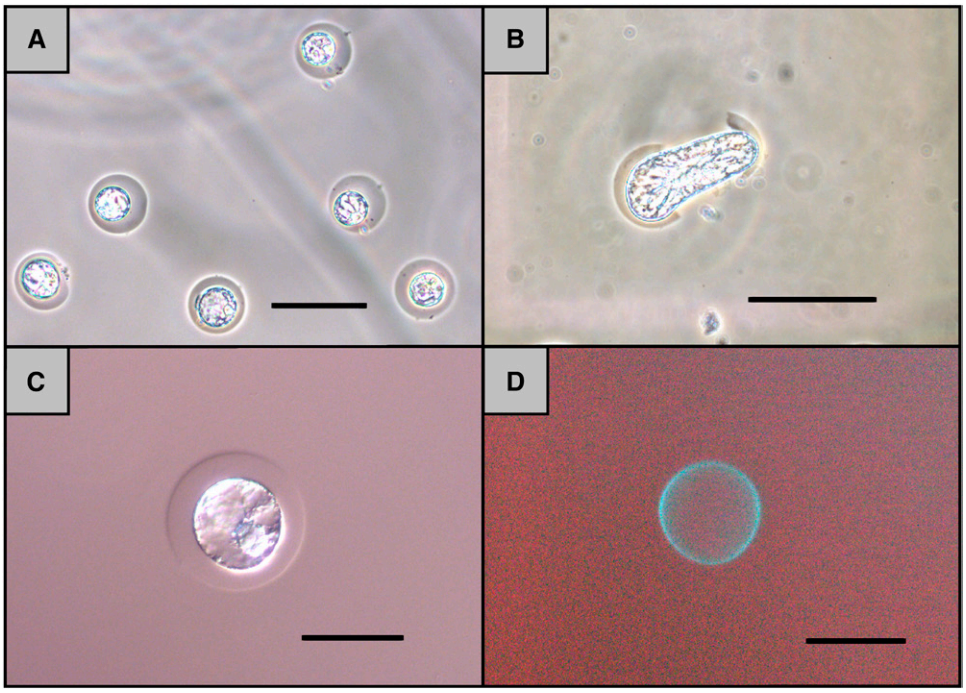


Fig. 2. BY-2 plant cells in various stages following encapsulation in agarose microbeads. (A) Phase-contrast image of BY-2 protoplasts in solidified microbeads suspended in MS medium immediately after encapsulation. (B) Phase-contrast image of a BY-2 cell breaking through the agarose microbead 6 d after encapsulation. (C) Nomarski differential interference contrast image of an individual BY-2 cell 20 h after encapsulation and 32 h after the removal of its cell wall. (D) Calcofluor White fluorescence of the respective cell in Fig. 2C showing regeneration of a thin surrounding cell wall. Scale bars = 100 µm (2A, 2B) and 50 µm (2C, 2D).

are poised to improve over time. We anticipate that future work in these areas will lead to improvements in the capture efficiency of live cells, and to the investigation of other hydrogel environments with different material properties.

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Appendix 1. Supply list and protocol sheet.

A. Solutions (All aqueous solutions pH 5.8)

1. Cell Wall Digestion Medium (342 mOsM)
   - 5.2% mannitol
   - 0.05% $MgCl_2$
   - 0.2% MES buffer
   - 0.5% Onozuka R-10 cellulase
   - 0.5% Onozuka RS cellulase
   - 0.1% Macerozyme R-10
   - 0.1% Pectolyase Y-23

2. BY-2 Suspension Culture Medium (207 mOsM)
   - MS basal salts
   - 3% sucrose
   - 100 mg/L myo-inositol
   - 1 mg/L thiamine
   - 0.2 mg/L 2,4-D

3. Protoplast Wash Medium (325 mOsM)
   - 5.5% mannitol
   - 0.05% $MgCl_2$

4. Mineral oil for droplet formation and collection
   - Light mineral oil
   - 4% Span 80

5. Protoplast Culture Medium 1 (326 mOsM)
   - MS basal salts
   - 3% sucrose
   - 2.7% mannitol
   - 0.2 mg/L 2,4-D

6. Protoplast Culture Medium 2 (265 mOsM)
   - MS basal salts
   - 3% sucrose
   - 1.6% mannitol
   - 0.2 mg/L 2,4-D
   - Conditioned medium from a BY-2 suspension culture

7. Cell Solution for Droplet Formation (300 mOsM)
   - MS basal salts
   - 3% sucrose
   - 2% mannitol
   - 0.5% high-molecular-weight dextran

8. Agarose Solution for Droplet Formation (292 mOsM)
   - MS basal salts
   - 3% sucrose
   - 2% mannitol
   - 1.5% low-gel-temperature agarose

For autoclave sterilization:
1. BY-2 Suspension Culture Medium
2. Protoplast Wash Medium
3. Protoplast Culture Medium 1 and 2

For filter sterilization:
1. Cell Wall Digestion Medium
2. Mineral oil
3. Cell Solution for Droplet Formation
4. Agarose Solution for Droplet Formation

B. Equipment

- 3 Dolomite Remote Basic P-Pumps (The Dolomite Centre Ltd., Royston, United Kingdom)
- Dolomite Flow Control Centre software (The Dolomite Centre Ltd.)
- Dolomite “heating rig” for agarose bead synthesis (The Dolomite Centre Ltd.)
- SCILOGEX MS-H-Pro Circular Hotplate (Scilogex LLC, Rocky Hill, Connecticut, USA)
- 4-channel 2-reagent Dolomite microdroplet chip: 100-µm etch depth (The Dolomite Centre Ltd.)
- PTFE microbore tubing: 250-µm and 500-µm internal diameter (The Dolomite Centre Ltd.)
- California Air Tools Ultra Quiet Air Compressor (California Air Tools, San Diego, California, USA)

C. Procedure

1. Protoplast digestion
   i. Pellet approximately 10 mL of 4-d-old BY-2 tobacco suspension culture by centrifugation and remove the supernatant.
   ii. To the roughly 5-mL pellet of cells add 25 mL of Cell Wall Digestion Medium.
   iii. Digest the cells for 3 h at 27°C on a shaker table.
   iv. After 3 h, pellet the protoplasts by centrifugation at 100 × *g* for 6 min.
   v. Remove the supernatant and resuspend the pellet in Protoplast Wash Medium.
   vi. Centrifuge the cells again at 100 × *g* for 6 min and decant the supernatant. Repeat.
   vii. Resuspend the remaining protoplasts in a small amount of Protoplast Culture Medium 1.
   viii. Allow protoplasts to rest for ~20 h (8 h at 7°C and 12 h at room temperature).
2. Microfluidic system setup
   i. Connect the three Dolomite P-Pumps to the air compressor and computer.
   ii. Place the "heating rig" on the hotplate and set the hotplate to 34°C.
   iii. Cut pieces of microbore tubing to carry fluids.
      a. 500 µm diameter (agarose)
      b. 500 µm diameter (protoplasts)
      c. 250 µm diameter (mineral oil)
   iv. Connect the piece of tubing carrying mineral oil into a T-junction to split the flow into two separate lines.
   v. Connect all tubing lines to their corresponding P-Pump chamber on one end, and to the microdroplet chip through a Dolomite 4-way Connector on the other end.
3. Droplet formation
   i. Prepare all three solutions for droplet formation.
   ii. Add molten agarose to the "heating rig" reservoir maintained at 34°C.
   iii. Add the mineral oil solution to a 10-mL beaker and place it inside the pressure chamber of one of the Remote Basic P-Pumps.
   iv. Pellet the protoplasts prepared from the digestion by centrifugation.
   v. Remove supernatant and resuspend protoplasts in the Cell Solution for Droplet Formation at an approximate density of 5×10^6.
   vi. Using the flow control software, run fluids through the 4-channel 2-reagent droplet chip with the following pressure settings:
      a. Agarose reservoir (~95 mBars)
      b. Protoplasts (~95 mBars)
      c. Mineral oil (~375 mBars)
   vii. Collect agarose microdroplets in a mineral oil bath significantly cooler than the gelling point of the agarose.
   viii. Run the system for ~2 h to produce droplets in quantity.
   ix. Once the droplet run is completed, pipette gelled microbeads out of the mineral oil bath and layer them on top of 8 mL of Protoplast Culture Medium 2 in a 15-mL centrifuge tube.
   x. Centrifuge at 150 × *g* for 5 min. Microbeads should pellet down to the bottom of the aqueous culture medium and separate away from the mineral oil.
   xi. Drain microbeads from the bottom of the centrifuge tube by creating a small hole with a hot needle.
   xii. Once droplets have been removed from the oil, filter them through an 88-µm nylon mesh to remove larger coalesced droplets.
   xiii. Suspend microbeads in Protoplast Culture Medium 2 to allow for cell growth.