A Transient Expression Assay Using *Arabidopsis* Mesophyll Protoplasts

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[Abstract] This method can be used to free and separate the mesophyll cells from *Arabidopsis* leaves. The protoplasts that are generated in this way can be used for transient expression for protein activity and subcellular localization assays.

Materials and Reagents

1. *Arabidopsis* (Ecotype: *Columbia*)
2. Fetal bovine serum (FBS) (Sigma-Aldrich, catalog number: F6178)
3. PEG4000 (Fluka, catalog number: 81240)
4. Mannitol
5. NaCl
6. KCl
7. $CaCl_2$
8. $MgCl_2$
9. 0.5 M MES (pH 5.7)
10. β-mercaptoethanol
11. Cellulase R10 purchased from http://www.yakult.co.jp/ypi/english/frame03.html
12. Macerozyme R10 purchased from http://www.yakult.co.jp/ypi/english/frame03.html
13. Bright-Line/Dark-Line Counting Chambers (catalog numbers: 3100, 3110, 3200, 3500, 1490, 1492, 1475 and 1483)
14. Enzyme solution (see Recipes)
15. PEG solution (see Recipes)
16. W5 solution (see Recipes)
17. MMg solution (see Recipes)
18. Washing and incubation solution (see Recipes)

Equipment

1. IEC clinical centrifuge
2. Chamber Counter (instruction attached)

3. Petri plate
4. Vacuum desiccator
5. Zeiss LSM510
6. Aluminum foil
7. Nylon filters (35-75 µm) (Carolina Biological Supplies, catalog number: 65-2222N)

Procedure

1. Protoplast Isolation

   Arabidopsis Columbia plants are planted on soil, cold treated for 3 d, and then transferred to the growth chamber (10 h Light/14 h Dark, 22 °C day/ 20 °C night, 80-100 µE). Well expanded leaves from 3.5-4.5 weeks old plants (6-8 leaves with elongated petiole) are used to prepare protoplasts.

   Protoplast isolation procedure:

   a. Make 10 ml enzyme solution. This is enough for more than 10 standard transfections. Pour solution into 15 cm petri plate.
   b. Cut 0.5-1 mm leaf strips with fresh razor blades without wounding. Use 2-4 young leaves per plant. Put strips in enzyme solution immediately after cut. 10 ml enzyme solution can hold 40-60 such leaves.
   c. Put the plate into to a vacuum desiccator and apply vacuum for 30 min. Continue the digestion for about 3 h without shaking in the dark (wrapped in Al foil) at room temperature (RT) 22-25 °C.
   d. Use a round-bottom tube (like the one used for *E. coli* culture), filter the enzyme solution containing protoplasts with a 35-75 µm nylon mesh by slowly releasing the cell-containing solution from a 10 ml transfer pipette. Rinse the plate once with 4 ml W5 solution. Combine the filter-through and spin at 100 *x g* to pellet the protoplasts 1.5 min (speed 3 with an IEC clinical centrifuge).
   e. Resuspend protoplasts once in 10 ml W5 solution. Spin at speed 3 for 1.5 min, and resuspend in 2 ml W5 solution. Count the cells using a chamber counter. Add more W5 to a cell density of 2.5×10^5/ml.
   f. Keep the protoplasts on ice (30 min) in W5 solution.
   g. Spin down protoplasts (speed 3 for 1 min) and resuspend in MMg solution (2.5×10^5 /ml) before PEG transfection.

2. PEG Transfection

   All steps are carried out at RT (*e.g.* 23 ˚C)

   a. Coat the 15 ml conical bottom tube with 5% FBS for 1 sec. Spin for 1 min and remove the leftover.

b. Add 60 µl DNA (60-120 µg of plasmid DNA of 5 kb in size. For co-transfection, use each with equal moles, the total remains the same).
c. Add 400 µl protoplasts to a microfuge tube (1 x 10^5 protoplasts), mix well gently with a 2 ml plastic transfer pipet.
d. Add 460 µl of PEG/Ca solution, mix well (handle 6-10 samples each time) gently with a 2 ml plastic transfer pipet. Incubate at 23 °C for 5-30 min.
e. Dilute with 3 ml W5 solution and mix well gently with a 2 ml plastic transfer pipet.
f. Spin at speed 3 in a clinical centrifuge for 1 min, remove supernatant. Resuspend protoplasts gently in 200 µl WI solution by a 2 ml plastic transfer pipet.
g. Wrap the tubes with Al foil and keep at ~23 °C until microscopic observation.
h. Check the cells for fluorescence under microscope. Most cells should be round with flashy red chloroplasts (auto fluorescence) dispersed evenly throughout the cell.

Common filter settings (on Zeiss LSM510):

	Excitation (nm)	Emission (nm)
GFP	488	BP505-530
RFP	543	BP560-615
Chlorophyll	488	LP650

Recipes

1. Enzyme solution (10 ml)

stock	volume	final conc.
1 M mannitol	4 ml	0.4 M
1 M KCl	0.2 ml	20 mM
0.5 M MES (pH 5.7)	0.4 ml	20 mM
cellulase R10	100-150 mg	1-1.5%
macerozyme R10	20-40 mg	0.2-0.4%

Heat the enzyme solution at 55 °C for 10 min (to inactivate proteases and enhance enzyme solubility) and cool it to RT before adding.

1 M $CaCl_2$	0.1 ml	10 mM
β-mercaptoethanol	4 µl	5 mM
10% FBS	0.1 ml	0.1%

The enzyme solution is light brown but clear (passed through a 0.45 µm filter).

2. PEG solution (40%, w/v) 10 ml

PEG4000 **Very Important!!	4 g	40% w/v
1 M mannitol	2 ml	200 mM
1 M $CaCl_2$	1 ml	100 mM
H_2O	3.5 ml	

3. W5 solution (50 ml)

1 M NaCl	7.7 ml	154 mM
1 M $CaCl_2$	6.25 ml	125 mM
1 M KCl	0.25 ml	5 mM
0.5 M MES-K (pH 5.7)	0.2 ml	2 mM

4. MMg solution (5 ml)

1 M mannitol	2 ml	0.4 M
0.3 M $MgCl_2$	0.25 ml	15 mM
0.5 M MES-K (pH 5.7)	40 µl	4 mM

5. Washing and incubation solution (WI) 10 ml

stock	volume	final conc
1 M mannitol	5 ml	0.5 M
0.5 M MES (pH 5.7)	80 µl	4 mM
1 M KCl	0.2 ml	20 mM

6. Directions for Chamber Counter

http://www.hausserscientific.com/

Bright-Line / Dark-Line Counting Chambers

Catalog Numbers: 3100, 3110, 3200, 3500, 1490, 1492, 1475 and 1483

Usage: Cell Counts

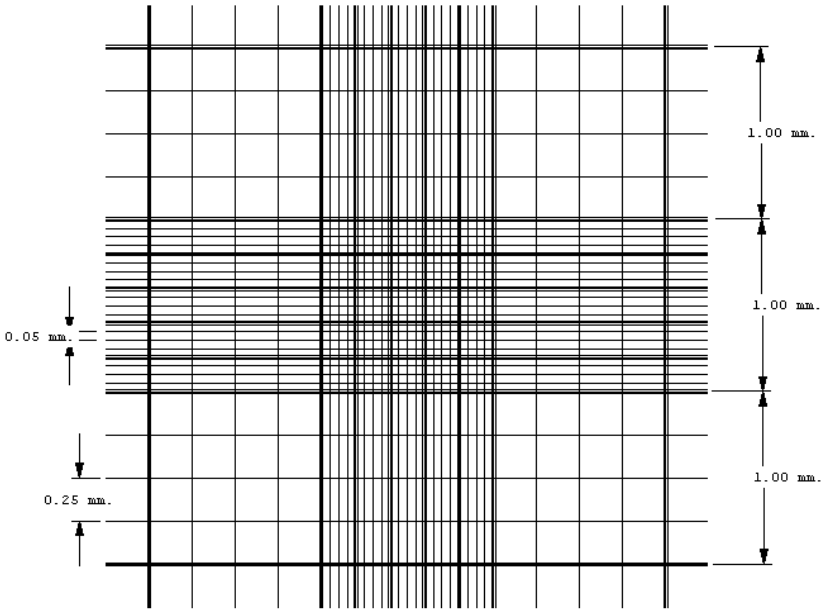
Cell Depth: 0.100mm +/- 2% (1/10 mm)

Volume: 0.1 Microliter

Ruling Pattern: Improved Neubauer, 1/400 Square mm

Rulings cover 9 square millimeters. Boundary lines of the Neubauer ruling are the center lines of the groups of three (these are indicated in the illustration below). The central square millimeter is ruled into 25 groups of 16 small squares, each group separated by triple lines, the middle one of which is the boundary. The ruled surface is 0.10 mm below the cover glass, so that the volume over each of the 16 small squares is.00025 cubic mm.

The number of cells per milliliter = Number of cells counted per square millimeter X dilution (if used) X 10,000



Neubauer Ruling

Acknowledgments

This protocol is consolidated from Jen Sheen's protocol and Inhwan Hwang's protocol. For references please go to the following websites for their publication lists:
http://genetics.mgh.harvard.edu/sheenweb/
http://www.postech.ac.kr/center/cpit/professor.html

References

1. Li, X., Chanroj, S., Wu, Z., Romanowsky, S. M., Harper, J. F. and Sze, H. (2008). A distinct endosomal Ca^{2+}/Mn^{2+} pump affects root growth through the secretory process. *Plant Physiol* 147(4): 1675-1689.

