Protoplast Isolation and Cell Wall Regeneration.

1. Cell Suspension Culture

1.1 Maintenance of Cell Suspension

Materials:
Suspension-cultured Alex cells of *Arabidopsis thaliana* L. (Heynh.), ecotype Columbia. The cultured cells were established by Mathur et al. (1998).
Suspension culture medium (pH5.8, 1L). 4.33g of Murashige and Skoog Plant Salt Mixture (Wako Co. Ltd., Japan), 3.0% sucrose, 10mg thiamine-HCl, 1mg nicotinic acid, 1mg pyridoxin, 1mg glycine, 100mg myo-inositol, 0.5mg dichlorophenoxyacetic acid (2,4-D).

1. Transfer 15 mL of 7-day-old cell suspension into 35 mL suspension culture medium in 300-mL Erlenmeyer flask.
2. Place the flask on a rotary shaker at 120 rpm under dark conditions at 23°C.
3. Culture the cell suspensions every 7 day.

2. Protoplast Isolation and Regeneration

2.1 Protoplast from Cell Suspension

Materials:
5-day-old suspension-cultured Alex cells.
0.45M mannitol solution (pH5.8).
Protoplasting enzyme solution (pH5.8): 1.0% Cellulase Onozuka RS (Yakult Pharmaceutical Ind. Co. Ltd., Tokyo, Japan) and 0.1% Pectolyase Y-23 (Kyowa Hakko Kyogo Co. Ltd., Tokyo, Japan) dissolved in mannitol solution.
Modified cell wall regeneration medium (pH5.8, 1L): 3.2g Gamborg’s B-5 Basal Medium with Minimal Organics (Sigma-Aldrich), 1.0M glucose, 0.25M mannitol, 1µM NAA.

1. Collect 50 mL of Five-day-old suspension-cultured cells in a sterile Falcon tube at 190×g for 10 min. Remove the supernatant
2. Wash the cells with 0.45M mannitol solution and centrifuge them at 190×g for 5min.
3. Remove the supernanant and resuspend the pelleted cells in a four to eightfold volume of enzyme solution.

4. Incubate the cells for >1 h at 37°C (Fig. 1).

5. Collect the protoplasts at 70×g for 10 min and washed twice with 40 ml of 0.45M mannitol solution.

6. Resuspend the purified protoplasts gently in cell wall regeneration medium at a final density of about 10^5 protoplasts ml⁻¹, and incubated at 25°C to regenerate cell walls.

![Fig. 1. Alex cells treated with protoplasting enzyme solution for 0, 15, 30, 45 min.](image)

3. Ultrastructural and Hisochemical Analysis of Cell Wall Regeneration

3.1 Scanning Electron Microscopic Observation

Material:

2% glutaraldehyde solution (pH 6.0): 2% glutaraldehyde solution with 120 g L⁻¹ sorbitol adjusted to pH 6.0.

0.2% (v/v) ruthenium tetroxide (RuO₄).

Ethanol solution series (50%, 60%, 70%, 80%, 95%, 100%): For the 100% ethanol solution, use 100% bulk ethanol that has Molecular Sieves in the bottom of the bottle.

1. Fix the protoplasts in 2% glutaraldehyde for 16 h.

2. Remove 2% glutaraldehyde and for an additional fixation, incubate 0.2% (v/v) ruthenium tetroxide for 30 min at room temperature.

3. Remove 0.2% ruthenium tetroxide and replace with 50% ethanol. Incubate for 30 min.

4. Repeat for the following ethanol solutions: 60%, 70%, 80%, 95%, 100%.

5. Remove the 100% ethanol, replace with the

![Fig. 2. Protoplast regenerating cell wall for 1h.](image)
100 ethanol and incubate for 30 min.
6. Remove as much as possible for the 100% ethanol, replace with n-butyl alcohol.
8. Sputter-coating the specimens with silver (E-1030, Hitachi Science Systems Ltd.), and Observe the sputter-coated specimens with an SEM (S-4100, Hitachi Science Systems Ltd.) at 3 kV (Fig.2).

3.2 Staining with Calcofluor White
Calcofluor White can be used to detect cell wall matrix polysaccharide because it readily binds to cellulose and other β-linked glucans.

Material:
1% glutaraldehyde solution (pH 6.0): 1% glutaraldehyde solution with 120 g L⁻¹ sorbitol adjusted to pH 6.0.
0.001% Calcofluor White: 0.001% Calcofluor White M2R (Sigma, St Louis, MO, USA) with 120 g L⁻¹ sorbitol adjusted to pH 6.0.
1. Fixed the protoplasts in 1% glutaraldehyde.
2. Remove 1% glutaraldehyde, rince three times with sorbitol solution, and incubate with 0.001% Calcofluor White.
3. Remove Calcofluor White, rince three times with sorbitol solution
4. Observe the specimens under a fluorescence microscope equipped with a UV fluorescence filter set (excitation filter, 350 nm; barrier filter, 430 nm) (Fig.3).

Fig.3. Protoplasts stained with Calcofluor White M2R observed under a differential interference contrast microscope (a–d) and an epifluorescent microscope (e–h) (Kown et al. 2005). Native suspension-cultured cells (a and e), fresh protoplasts (b and f), protoplasts regenerated for 1 h (c and g) and those regenerated for 3 h (d and h).
3.3 Staining with Aniline Blue

Aniline blue is used to stain the β-1,3-glucan callose (but not β-1,4-glucans). Callose is known to be accumulated on the surface of protoplasts in the early stages of cell wall regeneration.

Material:
1% glutaraldehyde solution (pH 6.0): 1% glutaraldehyde solution with 120 g L⁻¹ sorbitol adjusted to pH 6.0.
0.005% Aniline Blue: 0.005% Aniline blue fluorochrome 100-1 (Biosupplies Australia), 120 g L⁻¹ sorbitol in 0.15M K₂HPO₄ (pH8.6).

1. Fixed the protoplasts in 1% glutaraldehyde.
2. Remove 1% glutaraldehyde, rinse three times with sorbitol solution, and incubate with 0.005% Aniline Blue.
3. Observe the specimens under a fluorescence microscope equipped with a UV fluorescence filter set (excitation filter, 395 nm; barrier filter, 495 nm).

References