Protoplast Preparation and Determination of Cell Death

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[Abstract] The protoplasts assay constitutes a powerful tool that allows an easy uptake of active agents and a precise quantification of cell death induction in different populations. Our study showed that the basal level of cell death in our controls is low and stable throughout the length of our experiments (Danon *et al.*, 2005; Pineau *et al.*, 2013). In addition, the data obtained from the protoplast assay are applicable to intact seedlings, where it is possible to see differences in the intensity of necrotic lesions (Danon *et al.*, 2006) even if those differences are not as easily and clearly quantifiable as with the protoplast assay.

Materials and Reagents

1. Plant materials: *Arabidopsis thaliana* sterile seedlings grown *in vitro* from 1 to 3 weeks in the required conditions
2. Gamborg B5 medium salt and vitamins (Duchefa Biochemie, catalog number: G0210)
3. 2-(N-Morpholino) ethanesulfonic acid (MES)
4. Mannitol
5. Digestion enzymes
   a. Cellulase (Duchefa Biochemie, catalog number: C8001)
   b. Macerozyme (Duchefa Biochemie, catalog number: M8002)
6. Culture medium (see Recipes)
7. Digestion medium (see Recipes)
8. W5A medium (see Recipes)
9. 21% sucrose (see Recipes)
10. 1% Evans blue (see Recipes)

Equipment

1. Light microscope
2. An appropriate slide (*e.g.* Malassez slide)

3. Centrifuge for 15 ml and 50 ml Falcon tubes
4. Sterile pipette
5. Filter (0.45 µm)
6. Hemocytometer (VWR International, catalog number: 631-0975)
7. Cell dissociation sieve (100 µm) (Sigma-Aldrich, catalog number: CD1-1KT) sieve mounted on a sterile 150 ml beaker
8. Micropore tape (VWR International, catalog number: 115-8172)

Procedure

A. Protoplast extraction

1. Harvest whole young seedlings with sterile forceps and put them in a sterile Petri plate.
2. Cover the leaves with the digestion solution (~15 ml for a 10 cm round plate).
3. Close the plates with micropore tape and let digest at room temperature in the dark, overnight (~16 h).
4. Separate the protoplasts from the seedlings by pipetting and releasing delicately the medium using a 3 ml sterile pipette.
5. Filter the medium containing the protoplasts with a sieve of 100 µm, mounted on a sterile 150 ml beaker.
6. Transfer the content of the beaker in a sterile 15 ml tube.
7. Centrifuge 5 min at 180 *x g* at room temperature, eliminate the supernatant and resuspend the pellet in 4 ml of W5A medium.
8. Very slowly deposit the protoplasts on 8 ml of 21% sucrose using a 3 ml sterile pipette (use a 50 ml tube if you have a lot of material, and a 15 ml tube otherwise).
9. Centrifuge 13 min, 720 *x g* at room temperature.
10. Carefully harvest the living protoplast at the surface of the sucrose solution (thin cushion, Figure 1) using a 3 ml sterile pipette and transfer them into a 15 ml sterile tube.

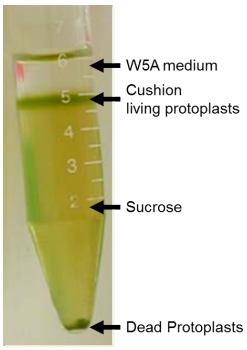

Figure 1. Living and dead protoplasts after centrifugation on 21% sucrose

11. Complete to 15 ml with W5A and calculate protoplast concentration on an aliquot using an appropriate slide (*e.g*. Malassez slide) and a light microscope (20x magnification).
12. Centrifuge 5 min, at 180 *x g* at room temperature.
13. Eliminate the supernatant, and resuspend the pellet in the appropriate volume of culture medium depending on the required concentration.

B. Cell death determination

1. Add 1 µl of 1% Evans blue to an aliquot of 25 µl of protoplast.
2. Using light microscopy and an appropriate slide (*e.g.* Malassez slide), calculate the percentage of dead protoplasts that are stained with Evans Blue (blue protoplast/total protoplast, Figure 2).

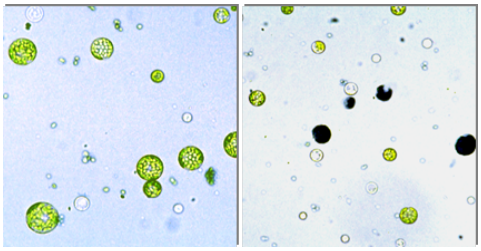

Figure 2. Control protoplast (left panel) and singlet oxygen-treated protoplast. Dark blue protoplasts are dead (Danon *et al.*, 2005).

Recipes

1. Culture medium (500 ml)
   Gamborg B5 medium salt and vitamins: 3.2 g
   0.4 M mannitol: 36.4 g
   0.4 M glucose: 36 g
   0.1% MES: 0.5 g
   Complete to 500 ml with distilled H_2O
   Go to pH 5.7 with KOH
   Sterilize by autoclave and keep at 4 °C
2. Digestion medium (500 ml)
   0.4 M mannitol: 36.4 g
   1% cellulase: 5 g
   0.25% macerozyme: 1.25 g
   8 mM $CaCl_2$: 0.44 g
   0.1% MES: 0.5 g
   Complete to 500 ml with distilled H_2O, homogenise until complete dissolution

Go to pH 5.7 with KOH

Sterilise by 0.45 µm filtration (it is easier to centrifuge before 5 min at 16,000 *x g*)

Aliquot 10 ml and keep at -20 °C

3. W5A medium (2,000 ml)

   5 mM glucose: 1.8 g

   154 mM Nacl: 18 g

   125 mM $CaCl_2$: 27.8 g

   5 mM KCl: 0.76 g

   0.1% MES: 2 g

   Complete to 2,000 ml with distilled H_2O

   Adjust to pH 5.7 with KOH

   Sterilise by autoclave and keep at RT

4. 21% sucrose (100 ml)

   Sucrose: 21 g

   Complete to 100 ml with distilled H_2O

   Sterilise by autoclave and keep at RT

5. 1 % Evans blue (100 ml)

   Dissolve 1 g Evans blue in 100 ml water

Acknowledgments

This work was supported by Sorbonne Universités, UPMC Univ Paris 06 and the Centre National de la Recherche Scientifique.

References

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