Title: Protoplasting Brachypodium leaf and viability quantification

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This protocol is modified from
Ortiz-Ramírez, C., Arevalo, E. D., Xu, X., Jackson, D. P. & Birnbaum, K. D. An Efficient Cell Sorting Protocol for Maize Protoplasts. *Curr Protoc Plant Biol* **3**, e20072 (2018)

Buffer preparation

Prepare all solutions using Milli-Q water/autoclaved water. Add the enzymes for the enzymes mix only on the day you do the protocol, not earlier!

Enzyme mix solution (3 ml required for one sample, prepare washing buffer first and then add the enzymes freshly to it at the beginning of the protocol)

Reagents	**Final concentration**	**Amount for 12.5 ml**	**Amount for 3 ml**
D-Mannitol (Roth, Art no:4175.1)	0.4 M	**910 mg**	
MES hydrate (Sigma, Cat no: M8250)	20 mM	**48.5 mg**	
KCL (4M)	20 mM	**62.5 µl**	
Cellulase RS	2.4% (w/v)	**300 mg**	**72 mg**
Macerozyme R-10	0.4% (w/v)	**50 mg**	**12 mg**
Adjust the pH to 5.7 with (1M Tris-HCL, pH8) and add following two reagents			
Calcium chloride (1M$CaCl_2$)	10 mM	**125 µl**	
BSA	0.1% (w/v)	**12.5 mg**	

Washing solution (12.5 ml, for 10X Genomics: around 7 ml required per run, can be prepared beforehand but do not use anymore if it is older than a month)

Reagents	**Final concentration**	**Amount for 12.5 ml**	**Amount for 125 ml**
D-Mannitol (Roth, Art no:4175.1)	0.4 M	**910 mg**	**9.1 g**
MES hydrate (Sigma, Cat no: M8250)	20 mM	**48.5 mg**	**485 mg**
KCL (4M)	20 mM	**62.5 µl**	**625 µl**
Adjust the pH to 5.7 with (1M Tris-HCL, pH8) and add following two reagents			
Calcium chloride (1M$CaCl_2$)	10 mM	**125 µl**	**1.25 ml**
BSA	0.1% (w/v)	**12.5 mg**	**125 mg**

A. Protoplasting /Procedure:

1. Prepare freshly on ice, both the enzyme mix solution and the washing solution.

2. Collect developing leaf zone by carefully pulling out newly developing grass leaves from engulfing sheath with tweezers. Remove the mature zone and keep only ~3-5mm. Use ~15 leaves per sample to yield between 4 and 7 million protoplasts (depends on species and tissue, test this out for your samples). Collect tissue in a small petri dish placed on ice containing 3mL water (cold!!).

3. Perform vacuum infiltration for 5mins. Incubate at room temperature for 45mins with gentle shaking (90rpm) under a lid to keep it dark.

4. Carefully remove water using P1000 pipette.

5. Add 3ml freshly prepared enzyme solution to the petri dish and perform vacuum infiltration for 5mins.

6. Using a razor blade, cut the tissue into smaller fragments (~1mm or smaller (as small as you can)). Incubate 90mins at room temperature with gentle shaking (90rpm) under a lid to keep it dark.

7. After 90 mins, the solution should look a bit cloudy due to the release of protoplasts. **Cut the tip (~3mm) of 1mL pipette tip!!!** and **very gently**, pipette the suspension up and down several times (for about 3 mins). You should see clouds of protoplasts being released.

8. Filter the protoplast suspension through a cell strainer/filter with 20µm mesh filter size for developing cells and 50 µm mesh filter size for more mature cell types (Cat no: 04-004-2325 and 04-004-2327 respectively, Sysmex, Celltrics). Place the cell strainer on top of the 1.5 ml Eppendorf LoBind tube and slowly pass the suspension through the filter using a P1000 pipette **whose pipette tip is cut!!!** Keep on ice.

9. Centrifuge the protoplast solution for 3 mins at 300×g, preferably at 4°C.

10. Carefully remove as much supernatant as possible without disturbing the pellet. Using P1000 pipette, resuspend the pellet with 500µl of **cold washing solution (Cut the pipette tip!!!). Resuspension should be done very gently** as it might **drastically** decrease the viability of protoplasts. **Store the protoplasts on ice!!**

Optional: *instead of resuspending in washing solution 0.01% FDA staining solution can be used instead to test for viability (see Part B below)*

11. Optional additional washing steps (using 1 ml washing buffer) can be performed. Repeat Step 9 and 10 one to three times. If a sample will be used for FACS, no additional washing step is required.

12. Generally, between 4-7 million protoplasts can be generated using 15 leaves if the above mentioned experimental set up is used. For FACS, the end yield can be divided directly into 3 FACS tubes (Cat no: 352235, Corning) without counting and **cold washing solution** can be added up to 1000µl to reach the desired concentration of 1-2 million protoplasts/mL for FAC sorting. **Keep the FACS tubes on ice until sorting!!**

B. Staining protoplasts with Fluorescein diacetate (FDA)

1. Dilute the FDA stock with washing solution to obtain the final concentration of 0.01%(v/v). For instance, 33µl FDA stock (Stock = 0.3% in Acetone) in 966.7µl washing buffer.

2. Perform staining by resuspending in 0.01% FDA solution instead of normal washing solution after Step A9 (see above). Remove as much washing solution as possible from the protoplast suspension. Resuspend the protoplasts using 500µl of 0.01% FDA solution. **(Cut the pipette tip!!!)**

3. Incubate the protoplasts with FDA for 30mins in the dark.

4. Quantify GFP positive protoplasts by using either Fuchs Rosenthal counting grid (see Part C below) or FACS.

C. Protoplasts quantification (Fuchs Rosenthal counting grid)

1. Spread 20µl protoplast suspension on the counting grid.
2. Use a 20x objective to visualise the protoplasts.
3. Count the protoplasts in 3 bigger squares (within one counting grid), each on a different row as shown on (Figure 1).
4. Calculate the average and multiply it with 5 to obtain the number of protoplasts /µl as one square corresponds to 0.2 µl.
5. Multiply the number by 1000 to get the total number /mL and in case of a diluted sample, multiply it further with corresponding dilution factor. Example: if the average number of protoplasts was 27 in square 1 and 2 (Figure 1), the total number of protoplasts in 1 mL volume will be 27*5*1000=135000 if the sample was undiluted.

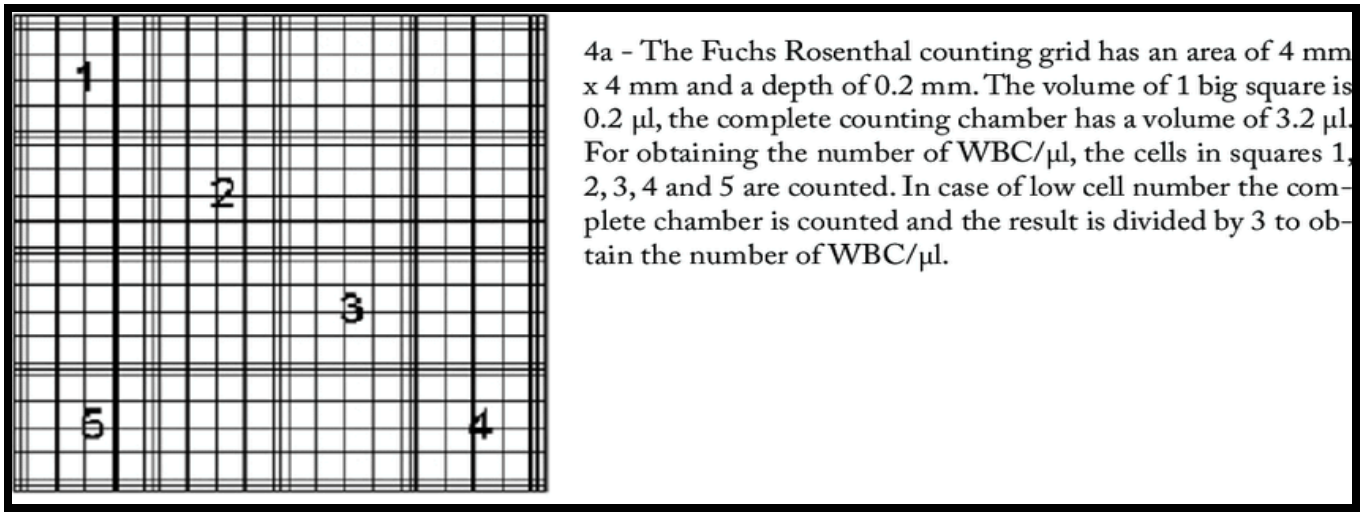

Figure 1: Layout of Fuchs-Rosenthal counting chamber.