Protocol: Protoplasting for rice roots

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Protocol brief description:

This protocol is for generating protoplasts from rice root tips. The protoplasts can be used for single-cell RNA sequencing experiments and also transient gene expression test.

The experiment described in this protocol takes about 5 hours:

Solution preparation: 0.5 hour

Root harvest and chopping: 0.5 hour

Digestion: 3 hours

Pelleting and cleaning: 0.5 hour

Protoplast checking and counting: 15 minutes

Key words:

Rice roots, Protoplast, single-cell experiments

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Chemicals needed for this protocol:

Specialized reagents and materials:

1. Cellulase RS:

    Supplier: Duchefa

    CAS number: 9012-54-8

    Product link: https://www.duchefa-biochemie.com/product/details/number/C8003

2. Macerozyme R10:

    Supplier: Duchefa

    CAS number: 9032-75-1

    Product link: https://www.duchefa-biochemie.com/product/details/number/M8002/name/macerozyme-r-10

3. Pectolyase:

    Supplier: Sigma Aldrich

    Catalog number: P5936-100MG

    Product link: https://www.sigmaaldrich.com/US/en/product/sigma/p5936

4. Razor blades:

    Supplier: Fisher

    Catalog number: 12-640

5. 70 and 40 μm filter:

    Supplier and product name: Fisherbrand™ Sterile Cell Strainers

    Catalog number: 70 μm, 22-363-548; 40 μm, 22-363-547

6. C-ChIP (Disposable Hemocytometer):

    Supplier: INCYTO

    Catalog number: DHC-F01 (Fuchs Rosenthal)

    Product link: http://www.incyto.com/shop/item.php?it_id=2

Common reagents:

Mannitol,

MES hydrate,

1 M KCl solution,

1 M Tris solution (pH 8),

1 M $CaCl_2$ solution,

MACS® BSA Stock Solution (10%),

β-mercapoethanol,

1X PBS solution [Diluted from Invitrogen 10X PBS (pH 7.4, RNase-free, Catalog number AM9625); when diluted to a 1X working solution with RNase free water, contains 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4]

To prepare the fresh stock solution:

*For 50 mL

-400 mM mannitol (3.65 g)

-20 mM MES hydrate (195 mg)

-20 mM KCl (1 mL of 1 M KCl)

-H_2O up to 50 mL

-Adjust pH to 5.7 with 1 M Tris pH 8.

Filter sterilized and kept at 4°C

Prepare the protoplast digestion solution (15 mL for 3 reactions, need ~5 ml per sample, to be used at Steps 1–4):

*For 15 mL

- Add:

-4% Cellulase RS (600 mg)

-1.5% Macerozyme R10 (225 mg)

-0.24% Pectolyase (36 mg)

https://www.sigmaaldrich.com/US/en/product/sigma/p5936

Also Add:

-5 mM $CaCl_2$ (75 µL of 1 M $CaCl_2$)

-0.1% BSA (150 µL)

-2.69 µL of β-mercapoethanol (Add in hood).

*Vortex vigorously and filter through a 70 µm strainer to remove clumps of enzyme/BSA.

Also make the wash buffer (10 mL for 3 reactions):

*For 10 mL

Add:

-0.1% BSA (100 µL)

-1.79 µL of β-mercapoethanol (Add in hood)

*Aliquot 1 ml for cell resuspension (To be used at Step 13)

Then Add:

-5 mM $CaCl_2$ (45 µL of 1 M $CaCl_2$) (To be used at Step 8)

Step-by step experiments

* Take notes: (1) Total root numbers: _____ OR (2) Total root weight:_____.

1. Get one clean square petri dish (Genesee, Cat # 26-275) and add 1 ml protoplast digestion solution at the center

2. Get rice seedlings out of the growth media, rinse them in the 1X PBS solution (Diluted from Invitrogen 10X PBS, Cat# AM9625) and briefly dry them with Kimwipes or paper towels. Then cut the 1cm primary root tips with Fisherbrand™ Razor blades (Cat# 12-640) from the rice seedling and place them into the digestion solution.

3. Gently chop the tissue with the same razor blade for ~1 minute then use a small pestle (the marble one working together with a Mortar) to gently squeeze the chopped tissues.

4. Use P1000, wide-bore pipette tips, add more digestion solution into the petri dish and then transfer the digestion solution with roots into a 6 cm round petri dish. The final volume should be 4 ml.

5. Put the 6 cm petri dish onto the square petri dish, rotate with covering by Aluminum foil. The digestion was incubated on an 85-rpm shaker at 28°C for 2.5 hours with additional pipette mixing every 30 minutes.

(**Important: At this step, make sure to reserve Centrifuge 5810 R, or an appropriate centrifuge you choose to use, before the experiment!** Cells cannot be left unprocessed for too long)

6. Filter through 40 μm filter (Falcon™ Cell Strainers Cat# 08-771-1) and collect the filter-through with a new 50 ml falcon tube; then filter through 40 μm filter again and collect the filter-through into another new 50 ml falcon tube.

7. Transfer to small culture tubes (Corning, Falcon® 5 mL Round Bottom Tube, Product number 352052) and spin down at 500 g for 5 minutes at room temperature

8. Gently remove the supernatant and rinse the pellet with 2 ml wash buffer (Gently mix the wash buffer with pellet with Pipette)

9. Spin down at 500 g for 3 minutes.

13. Resuspend in 50 – 150 µl wash buffer without Calcium. A 50-µM volume would be good to decrease the amount of mannitol etc. put into the 10X genomics reaction.

14. Load 20 µl resuspended solution (can do a 4X dilution) to C-ChIP [Incyto, DHC-F01 (Fuchs Rosenthal)], http://www.incyto.com/shop/item.php?it_id=2), then use Dissect Microscopy or Axio Zoom to count the cell numbers.

Note: Count cells in 1 big square (4X4). For undiluted sample, the number timed by 5 is the cell number in 1 microliter. Ideally the concentration should be 1000-2000 cells per microliter (at least 50 µl).