Synchronisation of BY-2 cell culture

Introduction

- Nicotiana tobacum cell suspension culture Bright Yellow 2 (BY-2)
- Synchronisation with aphidicolin to achieve up to 70% synchrony
- Aphidicolin arrests cells in S-phase, after wash out cells enter G2 and M – maximum mitotic index (when most cells are in M phase) is dependent on cell culture
- Adapted from: Graumann K and Evans DE, (2014), "Dynamics of the Plant Nuclear Envelope during Cell Division", Methods in Molecular Biology

Materials

Synchronising BY-2 suspension culture cells with aphidicolin

CAUTION: Aphidicolin is very toxic and should be handled with great care. It should be handled wearing gloves and skin contact should be avoided; wear lab coat and eye protection. Inhalation of dust should be avoided. Disposal of aphidicolin should be according to local regulations.

1. Two 100 ml conical flasks with 50 ml liquid BY-2 medium, sterile (for larger cultures 100 ml in 250 ml flasks)
2. Liquid BY-2 medium for washing (for 50 ml culture use 500 ml media for washing)
3. Sterile dH_2O (50 ml)
4. Aphidicolin (5 mg/ml, Sigma A0781) in dimethyl sulfoxide, observe manufacturer's instructions. Add suitable filter-sterilized antibiotics, for transformed BY-2 cells only.
5. Wild-type or transformed stationary-phase BY-2 cells (7-d old cultures) grown in suspension
6. Retort stand
7. Autoclaved sintered glass funnel (G3 [porosity 3], Borosilicate glass; Fisher 11922148; either 80 ml or 250 ml)
8. Shaking incubator at 130 rpm, 25°C
9. Liquid waste container
10. Laminar flow hood
11. Rubber tubing and Hoffman clamp if required
12. Sterile pipette and pipette tips

Determining mitotic index

20% Triton X-100 (1 µl Triton X-100 in 5 µl dH_2O)

DRAQ5 (Biostatus; http://www.biostatus.com) used according to the manufacturer's instructions.

Hoechst stain (100 µl stock): 1 µl 10 mg/ml (Sigma Bisbenzimide Ca B-2883) 2 µl Triton X-100 and 97 µl of distilled water stored at 4°C.

Other nuclei stains such as DAPI or ethidium bromide can also be used.

Other materials needed:

1. 1.5 ml microcentrifuge tube
2. Sterile pipette tips
3. Microscope slides
4. Coverslips
5. Lens oil (if required)
6. Confocal or fluorescence microscope

Confocal Imaging of live cells

1. Confocal microscope
2. Microscope slides
3. Coverslips
4. Solid BY-2 medium (0.7% w/v agarose)
5. Sterile pipette tips
6. Laminar flow hood

Methods

Synchronising BY-2 suspension culture cell division with aphidicolin

1. Use a 7 days old BY-2 suspension culture (either wild type or stable transformed).
2. In a laminar flow hood, transfer 7 ml of the 7 days old culture into 50 ml fresh medium (in 100 ml conical flask, sterile).
3. Add 50 µl of 5 mg/ml aphidicolin and incubate culture for 24 h in a shaking incubator at 130 rpm, 25°C with no light.
4. After the incubation, remove aphidicolin by washing 10 times with 50 ml sterile BY-2 medium, in a laminar flow hood. The wash steps should be carried out as follows.

5. In the laminar flow hood, fasten an autoclaved sintered glass funnel (for 50 ml culture use 80 ml funnel) in a retort stand and place a beaker for collecting waste.
6. Rinse the funnel with 50 ml autoclaved dH_2O.
7. Once the water has completely drained, transfer the BY-2 cell suspension culture into the funnel and wait for the cells to settle at the bottom and most of the medium to drain off. It is important that the cells do not dry out but a small meniscus of medium remains to cover the cells.
8. Add 50 ml of fresh BY-2 medium and allow it to drain through the funnel. Repeat this wash step 9 more times. During the wash, cells can be gently resuspended with a wide-bore pipette.
9. If the flow rate of the washing medium is faster than 50 ml/ min, attach rubber tubing to the base of the funnel and a Hoffman clamp to the tubing. Set the clamp so that the flow through is lowered to less than 50 ml/ min.
10. After the last wash, resuspend the cells in 50 ml of fresh BY-2 medium and transfer the culture to a new, sterile 100 ml conical flask.
11. Return culture to incubator and remove 1 ml samples every hour to check mitotic index. Depending on BY-2 cell line, maximum mitotic index can be observed approximately 8 h after aphidicolin wash out.

If larger, 100 ml cultures are used or the culture is very thick and funnel flow through is very slow, use a larger, 250 ml funnel instead.

Determining mitotic index

- DRAQ5- for use with fluorescence or confocal microscope

1) To 500 µl cells add 1 µl 20% Triton X-100 (1 µl Triton X-100 in 5 µl dH_2O) and 0.2 µl of 5 mM DRAQ5 for a working concentration of 10 µM.

2) Incubate for 1-2 min before imaging.

3) Mount 60 µl on microscope slide, add coverslip and, if required, lens oil (or water).

4) Excitation with any laser from 488 nm upwards; capture excitation in LP650

- Hoechst Staining for use with a fluorescence microscope

Work with a Hoechst stock containing Triton X-100 to enhance uptake of the stain. To make 100 µl stock, use 1 µl Hoechst (Sigma, Bisbenzimide, CaB-2883), 2 µl Triton X-100 and 97 µl dH2O; stock can be stored at 4°C.

1) Take 100 µl of cells from the culture flask with a wide bore sterile pipette.
2) Place the cells in a 1.5 ml Eppendorf tube and add 5 µl of Hoechst stock
3) Place 20 µl of cells on a slide, add coverslip and view with a fluorescence microscope (excitation 350 nm, emission 460 nm). Score for mitotic index by counting mitotic cells as a percentage of total number of cells.

5. Confocal imaging of live cells

For time lapse imaging (keeping cells for more than 25 min on slide and imaging) cover the microscope slide first with solid BY-2 medium. This is useful to keep cells alive for longer time lapses such as imaging one whole mitotic division (Graumann and Evans, 2011). To keep medium sterile, prepare these slides in flow hood.

1. Prepare solid BY-2 medium as above but use 0.7% w/v agarose.
2. Cover microscope slide evenly with approximately 1 ml of warm, liquefied medium and allow to set. Prepare slides fresh before use.
3. Mount cells (approximately 60-100 µl), add cover slip and, if required, lens oil or water.

For confocal imaging, use settings appropriate for fluorescent probe. For longer time lapse imaging, keep laser output and transmission as low as possible to avoid bleaching. Time lapse imaging has previously been used to visualize the dynamics and relationships of nuclear and mitotic membranes as well as chromatin throughout an entire mitotic division as well as specific mitotic phases (Irons et al., 2003; Evans et al., 2011; Graumann and Evans, 2011).

In addition to tracking the localisation of a protein throughout the cell cycle, confocal imaging can also be used to analyse protein mobility and protein interactions. Cook et al. (2013) have used BiFC to report protein interactions of nuclear kinase WEE1 with SKIP1 of the 26S proteasome system. Analysis of protein mobility by FRAP and protein interactions by apFRET requires a more elaborate setup but similar to FRAP or FRET using other plant tissue (Graumann et al., 2010; Graumann and Evans, 2011; Sparkes et al., 2011; Graumann, 2014). In addition to interphase structures, however, FRAP and apFRET can also be carried out on mitotic structures such as the mitotic spindle membranes, phragmoplast and cell plate. Graumann and Evans (2011) used FRAP to observe the mobility of NE proteins AtSUN1 and AtSUN2 during NE breakdown and reformation, in mitotic spindle membranes and at the cell plate. Similarly, mobility and turnover of tubulin and microtubules at the phragmoplast and cell plate have been recorded (Smetenko et al., 2011).

Additional Reading

Kumagai-Sano, F., Hayashi, T., Sano, T. and Hasezawa, S. (2007). Cell cycle synchronization of tobacco BY-2 cells. Nature Protocols **1**, 2621 - 2627

Nagata, T., Nemoto, Y., and Hasezawa, S. (1992). Tobacco BY-2 cell line as the “HeLa” cell in the cell biology of higher plants. Int. Rev. Cytol. **132**,1-30.

Graumann, K. and Evans, D.E. (2011). Nuclear envelope dynamics during plant cell division suggest common mechanisms between kingdoms. Biochemical Journal **435**, 661-667

Sparkes, I.A., Graumann, K., Martiniere, A., Schoberer, J., Wang, P. And Osterrieder, A. (2011). Bleach it, switch it, bounce it, pull it: using lasers to reveal plant cell dynamics. Journal of Experimental Botany **62**, 1-7

Waadt, R., Schmidt, L.K., Lohse, M., Hashimoto, K., Bock, R. and Kudla, J. (2008). Multicolor bimolecular fluorescence complementation reveals simultaneous formation of alternative CBL/CIPK complexes in planta. The Plant Journal **56**, 505–516.

Walter, M., Chaban, C., Schutze, K., Batistic, O., Weckermann, K., Nake, C., Blazevic, D., Grefen, C., Schumacher, K., Oecking, C., Harter, K. and Kudla, J. (2004). Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. The Plant Journal **40**, 428–438.