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Microtubule organization and stability change dramatically during the cell cycle, and little is known about the mechanisms involved in these changes. Understanding of spindle assembly requires that one understands how microtubule dynamics is regulated during the cell cycle and which factors are required for the changes in microtubule dynamics at the onset of mitosis. To answer these questions, one needs an in vitro system from which factors can be fractionated and purified and depleted. Xenopus egg extracts are currently the in vitro system of preference for these studies. Microtubule dynamics can be followed by light microscope, Nomarski, which keeps the extract in native conditions (Nomarski, or Allen Video Enhanced Contrast Digital Interference Contrast=AVEC-DIC). However, the presence of vesicles makes the observation of microtubules by AVEC-DIC impossible in concentrated extracts. Fluorescence microscopy is therefore used to study microtubule dynamics in Xenopus egg extracts. The drawback of fluorescence microscopy is that the presence of fluorochromes causes photodamage via the formation of free radicals. It is therefore necessary to include anti-fading systems for fluorescence microscopy studies.

Materials

Purified centrosomes from KE37 cells (Moudjou and Bornens, 1998)
Stored at -80 degree.

Xenopus egg extracts (Murray, 1991)

BRB80
K-Pipes pH6.8, 1mM EGTA, 1mM MgCl2

Rhodamine labelled tubulin (Hyman et al, 1991)
1 µl aliquot kept at -80 degree

Hemoglobin
saturated solution (30-40 mg/ml; take a small spatula's worth of hemoglobin, put in a small Eppendorf tube and add 50 µl of water) dissolved in water and spun 2-5 minutes at 15000 rpm (leave the aggregated pellet at the bottom of the tube, and pipette thereafter from the supernatant). Fresh every day.

Catalase
10 mg/ml in BRB80 - 50% glycerol. Store for about a week maximum at -20 degree.

Glucose oxidase
10 mg/ml in BRB80 - 50% glycerol. Store for about a week maximum at -20 degree.

Glucose
1M solution in H2O stored at -20 degree.

Slides
washed in ethanol, rinsed in water and dried, kept in boxes away from dust.

Coverslips
store in ethanol

Preparation of sample for fluorescence microscopy

In the lab

1. Make up fresh the hemoglobin solution.
2. Dilute 1 µl of rhodamine tubulin with 2.5 µl of BRB80. Spin 5 minutes at 30 PSI in the airfuge in the cold room. Cut the tube and take out the supernatant.

In the microscope room

1. Make up the anti-fade solution.

   5 µl of catalase (10mg/ml) + 5 µl of glucose oxidase (10mg/ml) + 5 µl of glucose (1M)

   Mix and keep at room temperature for 30-45 minutes maximum. Pipette thereafter from the bottom of the tube, thus minimizing oxygen exchange.

2. Start NIH image. Open the AAA- macro (in videomicroscopy folder). Open the folder where you want to store your images. Define the timelapse ([F8]). Make ROI ([Z] on keyboard).

3. With the Argus 10, average 8 images.

4. Deep the slide in ddH2O, and dry it with Kimwipes (red box).

5. Rinse a coverslip with ddH2O and dry it with the coverslip centrifuge. The upper side is going to be in contact with the extract.

6. Do on ice (to prevent microtubule polymerization).

7. For 10 µl of extract, add 1 µl of centrosomes, 0.5 µl of hemoglobin, 0.4 µl of rhodamine tubulin, 0.32 µl of anti-fade mix.

8. Mix, using hemoglobin as a tracer for mixing, and keep on ice.

9. On the stage put the clean slide, drop 2 µl of extract containing all the different reagents, and carefully put the coverslip on top of the extract. Verify that the extract spreads evenly under the coverslip; you may gently tap the coverslip with a pair of forceps to help the fluid to even out. Start the timer of the argus.

10. Use the 100x lens. Focus by closing the diaphragm. You are on the right focal plane when you can clearly see the edges of the diaphragm.

11. Look for asters you want to record. Close shutter.

12. Start recording ([F15]).

13. Record for not more than 5 minutes following the start of the timer.

14. For stopping simultaneously press apple mark and ">" key.

Principles of the anti-fade system

Photodamage is caused by the formation of free radicals through the breakage of double-bonds in the flluorochrome. Free radicals react with molecular dioxygen causing the formation of superoxide anions (O2-). O2- reacts with a proton forming HO2-. HO2- disproportionates through the formation of hydrogen peroxide. The following reaction scheme is followed:

$O_2 + e^-$ (from free radical formation) --> O_2^-
$O_2^- + H^+$ --> $H_2O_2 + O_2$
Catalase breaks down H_2O_2
H_2O_2 --> $H_2O + 1/2\ O_2$
and glucose oxidase oxidizes glucose ($C_6H_{12}O_6$) into gluconic acid and H_2O_2
$C_5H_{11}O_5\text{-}COH + O_2 + H_2O$ --> $C_5H_{11}O_5\text{-}COOH + H_2O_2$

The role of hemoglobin in the anti-fade mix is to bind molecular dioxygen (O_2).

The net effect of hemoglobin and the anti-fade system is to keep the concentration of molecular dioxygen low and to degrade by-products from free radical formation.

Analyzing movies

1. Put all the images in a stack and save them as a stack.
2. Open the macro 'MT macro' (in videomicroscopy folder).
3. For each movie, always start by pressing [S], set scale.

   Then define the initial time and timelapse [T].
4. Put the arrowhead where you want your origin to be and press [Z] 'Define origin'.

   You can move from one image to the other in the stack by pressing [X] 'Next image' or [C] 'Previous image'.

   For measuring a microtubule, put the arrowhead at the + end of the microtubule and press [V] 'Measure'. For the last measurement of a microtubule press twice [V].

   You can measure several microtubules one after another this way.
5. Copy the measurements.
6. Open the Excel file 'MT dynamics-final'

   Paste into sheet 2. The first column is the MT length, the second is the time and the third is the frame number. Copy the 2 first column and paste them in sheet 1. Then fill down in order to have the MT dynamics parameters.

References

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Moudjou, M. and Bornens, M. (1998) Method of centrosome isolation from cultured animal cells. In Cells, J.E. (ed.), Cell Biology, a Laboratory Handbook, Second Edition, Academic Press Inc., San Diego, Vol. 2, pp.111-119.

Murray, A.W. (1991) In Kay, B.K. and Peng, H.B. (eds.), Xenopus laevis: Practical uses in cell and molecular biology. Academic Press Inc., San Diego, Vol. 36. pp.581-605.