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Fertilization-induced changes in the microtubular architecture of the maize egg cell and zygote – an immunocytochemical approach adapted to single cells.

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Abstract By using single cell micromanipulation techniques, we developed an immunocytochemical procedure to examine subcellular protein localization in isolated and cultured cells. Localization of microtubules was examined in isolated single egg cells and developing zygotes of maize with anti-α-tubulin antibodies. In egg cells, a few cortical microtubules were detected but well organized microtubules were hardly observed. In contrast, distinct cortical microtubules and strands of cytoplasmic microtubules radiating from the nucleus to the cell periphery were observed in developing zygotes. Solely cortical microtubules were observed in zygotes up to 7 h after in vitro fertilization. After this time, additionally radiating microtubules appeared and persisted during zygote development. These results indicate early and pronounced fertilization-induced changes of the microtubular organization in the fertilized egg cell of maize.

Keywords:

Anti-α-tubulin antibody, Egg cell, fertilization, Maize, Microtubule, *Zea mays*, Zygote development

Abbreviations:

BSA: bovine serum albumin · DABCO: 1,4-diazabicyclo [2,2,2] octane · EGTA: ethylene glycol bis (2-aminoethyl ether)-N,N,N’,N’-tetraacid PIPES: 1,4-piperazinediethanesulfonic acid · FITC: fluorescein isothiocyanate · MSB: microtubule-stabilizing buffer

Introduction

A higher plant zygote develops from a single cell to a whole plant through precise control of cell divisions and spatio-temporal coordination of morphogenetic events (reviewed by Goldberg et al. 1994). The coordination of these events immediately after fertilization are also of importance for polar growth of the embryo. Among other fertilization-induced processes, changes in the cytoskeleton architecture in the fertilized egg cell, especially molecular events, are largely unknown.

Redistribution of microtubules occur during embryo sac development in maize (Huang and Sheridan 1994) and in *Arabidopsis thaliana* (Webb and Gunning 1994) as well in egg cells during fertilization and zygote development. Techniques for detailed three-dimensional observations of isolated embryo sacs and egg cells of *Plumbago* were developed (Huang et al. 1990). Microtubules in the egg cell of the synergid-lacking embryo sac of *Plumbago zeylanica* are longitudinally aligned in the micropylar and mid-lateral areas, forming bundles near the filiform apparatus. In the perinuclear cytoplasm they are more randomly aligned. Microtubule distribution changes in the egg cell during pollen tube penetration. Whereas the longitudinal arrays remain conspicuous near the filiform apparatus, they are no longer pronounced in the egg cell. The zonal pattern of microtubule distribution in this cell disappear, whereas a distinct tubulin signal appear around the pollen tube (Huang et al. 1993). A re-orientation of microtubules to a transverse cortical distribution was observed during elongation of the *Arabidopsis* zygote (Webb and Gunning 1991).

The use of isolated gametes and zygotes combined with an in vitro fertilization system leading to zygotic embryogenesis in maize plants (Kranz and Lörz 1993), offer an invaluable system to study such initial - sperm cell induced - changes in the egg cell.

Here we describe a protocol for handling of isolated gametes and zygotes of maize and the adaptation of immunocytochemical procedures to these single cells. To analyze the microtubular architecture in these fragile, individual cells, it was necessary to establish a protocol for localization of proteins which allows to maintain cell integrity throughout the procedure as much as possible.

In the present study, we established a protocol for the detection of microtubules in isolated single egg cells and zygotes with an anti-α-tubulin antibody using indirect immunocytochemistry. Whereas only a few cortical microtubules were detected in egg cells, distinct cortical microtubules and strands of cytoplasmic microtubules radiating from the nucleus to the cell periphery were observed in developing zygotes. These results indicate pronounced fertilization-induced changes of the microtubular organization in the fertilized egg cell of maize.

Materials and methods

Plant material

Maize (*Zea mays* L.) inbred line A188 (courtesy of Dr. A. Pryor, Commonwealth Scientific and Industrial Research Organization, Canberra, Australia) was used for isolation of egg cells, sperm cells and zygotes. Maize plants were grown in the greenhouse under standard conditions.

Isolation and selection of egg and sperm cells

Egg and sperm cells were isolated and selected as described previously (Kranz et al. 1991). Egg cells were isolated by microdissection of ovule pieces with microneedles after enzyme treatment under an inverted microscope (Axiovert 135; Carl Zeiss, Oberkochen, Germany). Sperm cells were isolated after bursting in mannitol solution (650 mosmol kg^{-1} H_2O) by osmotic shock.

Egg and sperm cells were selected under a microscope and transferred into mannitol droplets on coverslips overlaid with mineral oil (paraffin liquid for spectroscopy, Merck, Darmstadt, Germany) using microcapillaries and a computer-controlled micropump (Microlab-M; Hamilton, Darmstadt, Germany).

Gamete fusion

Egg protoplasts were fused electrically with isolated sperm protoplasts (Kranz et al. 1991). Collected single egg and sperm protoplasts were transferred to mannitol (650 mosmol kg^{-1} H_2O) droplets, which were overlaid with mineral oil, on a coverslip for electrofusion. Fusion between egg and sperm protoplasts was induced by DC pulses (50 µsec; 1.0 kV cm^{-1}) after dielectrophoretic alignment (1 MHz, 70 V cm^{-1}) on one of the

electrodes for a few seconds with an electrofusion apparatus (CFA 400; Krüss, Hamburg, Germany).

Zygote isolation

For zygote isolation, ears were harvested and pollinated in vitro as described previously (Kranz and Lörz 1990; Kranz and Brown 1992). Briefly, the ear segments were placed on agarose-solidified MS medium (Murashige and Skoog 1962), in 6-cm- diameter -plastic dishes, covered with wet filter papers. These dishes were then placed in larger plastic dishes (diameter 14,5 cm). The silks hung outside the smaller dish into the larger one. Subsequently, pollen grains, which were harvested in the morning and kept in plastic dishes with moistened papers inside of the cover, were sprayed on the silks. The ovule pieces were prepared from these ears 15 to 18 h after pollination. Zygote isolation was performed as egg cell isolation. Zygotes were selected on the basis of synergid degeneration. Fertilization occurred in our system approximately 4 - 5 h after in vitro pollination.

Zygote culture

Zygotes produced by in vitro gamete fusion or in vitro pollination were cultured in ‘Millicell-CM’ -inserts (diameter 12 mm, Millipore, Bedford, USA) placed in the middle of 3.5 cm -diameter -plastic dishes which were previously filled with 1.5 ml and the inserts with 0.1 ml of MS medium (Murashige and Skoog 1962) supplemented with 2.0 mg/l 2,4-D and 600 mosmol glucose. Aggregates of non-morphogenic maize feeder cells were added into the culture medium outside the ‘Millicell-CM’ -insert for co-cultivation (Kranz et al. 1991). Zygotes were incubated in a growth chamber at 26 ± 1°C.

Indirect immunofluorescence analysis

Transfer of individual cells from one solution to another and exchange of media were performed with a microcapillary connected to a micropump via a hydraulic system (Figure 1). To monitor the cells, procedures were performed under an inverted microscope. Isolated egg cells and zygotes, as well as in vitro produced and cultured zygotes were first transferred into‘ Millicell-CM’ -inserts containing 100 µl mannitol solution (650 mosmol kg^{-1} H_2O). The amount of liquid medium inside ‘Millicell-CM’ -inserts was reduced to prevent dilution of fixative during the following step of the procedure. Subsequently, these inserts containing the cells were transferred into another 3.5 cm diameter plastic dishes previously filled with 4 ml freshly prepared fixative. The fixative consisted of 4% (w/v) paraformaldehyde, 0.1% (v/v) glutaraldehyde and 600 mosmolar mannitol in microtubule-stabilizing buffer at pH 6.9 (MSB: Brown and Lemmon 1995; Schliwa and van Belekom 1981 with minor modification), containing 50 mM 1,4- piperazinediethanesulfonic acid (PIPES), 5 mM ethylene glycol bis (2-aminoethyl ether)-N,N,N’,N’-tetraacid (EGTA), 1 mM $MgCl_2$ and 2% (v/v) glycerol. Remaining mannitol solution in ‘Millicell-CM’ -inserts was removed and fixative was added into the inserts with a microcapillary. The semi-permeable membrane of ‘Millicell-CM’ -inserts allow an exchange of fixative between the two compartments. After fixation for 20 min, cells were transferred into another ‘Millicell-CM’ -insert, containing 100 µl MSB. This insert was previously placed in a 3.5 cm -diameter -plastic dish filled with 4 ml MSB. The cells remained in this solution just before use.

Coverslips (24 × 40 mm) were treated with poly-L-lysine [0.1% (w/v) in water; Sigma Diagnostics Inc.] for adhesion of cells to the surface of the coverslip. Onto the

coverslips, walls were built with adhesive tape to keep solutions in place. Fixed cells were mounted in a 50 µl MSB droplet on the coverslips by use of a capillary.

Cells were incubated for 1 h in a 50 µl droplet containing monoclonal anti-α tubulin antibody (DM1A, NeoMarkers, CA, USA) diluted 1:100 in MSB supplemented with 0.25% (w/v) bovine serum albumin (BSA). After three times of washing with MSB, cells were incubated for 1 h with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody raised in goat (Product No. F0257, Sigma Diagnostics Inc.) diluted 1:100 in MSB supplemented with 0.25% (w/v) BSA. Antibodies were incubated at room temperature. Subsequently, cells were washed again three times by exchange of MSB.

Cells were finally treated with 4',6-diamidino-2-phenylindole (DAPI) (0.1 µg/ml in PBS) for 15 min to stain DNA. Following three washes by exchange of MSB, antifading solution containing 100 mg/l 1,4-diazabicyclo [2,2,2] octane (DABCO: Sigma Diagnostics Inc.) in MSB was added to the cells. The coverslips were kept above moistened papers in a Petri dish (diameter 12 cm) during incubation periods.

Exceeding a culture period of 20 h, zygotes, produced by in vitro gamete fusion or *in vitro* pollination, were treated with enzyme solution for cell wall digestion after fixation. The enzyme solution consisted of 1.5% (w/v) pectinase (Serva, Heidelberg, Germany), 0.5% (w/v) pectolyase Y-23 (Seishin, Tokyo, Japan), 1.0% (w/v) cellulase Onozuka RS (Yakult Honsha, Tokyo, Japan), 1.0% (w/v) hemicellulase (Sigma) in 570 mosmolar mannitol, pH 5.0, dissolved in MSB. After transfer onto the coverslips, the cells were treated with the enzyme solution for 10 min. After three times of washing with MSB, antibodies were applied as described above.

In the following immunological control experiments no staining was detected: omission of primary antibody, omission of secondary antibody and omission of both antibodies.

Cells were examined with an epifluorescence inverted microscope (Axiovert 35M; Carl Zeiss, Oberkochen, Germany). The fluorescence of FITC and DAPI were detected with filter set no. 9 and no. 1, respectively. Images were taken with a CCD camera (XC003P: Sony, Japan) and processed with image analysis software (Image ProPlus, Media Cybernetics, Silverspring, MD, USA). Autofluorescence was not observed with camera gain and integration settings used.

Results

Establishing a protocol for indirect immunocytochemistry with isolated single egg cells and zygotes

A number of fixative procedures were tested for immunocytochemistry on single cells in order to maintain their shape and to avoid bursting of cells which possess no or only a fragile cell wall. At first, we examined several concentrations of paraformaldehyde. Among the concentrations of paraformaldehyde tested, 1% (w/v) was insufficient to fix microtubules. On the other hand, the single cells had a tendency to burst after treatment with 4% (w/v) paraformaldehyde treatment. To avoid bursting of cells, the fixative with 4% (w/v) paraformaldehyde was supplemented with glutaraldehyde. High concentrations of more than 0.25% (v/v) glutaraldehyde caused autofluorescence. In the end, a fixative which contained 0.1% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde dissolved in modified microtubule stabilizing buffer (MSB) adjusted to 600 mosmol with mannitol was used. This fixative allowed the maintenance of the shape of both egg cells and zygotes, and preserved microtubular structures for immunocytochemistry.

We developed a reliable protocol for indirect immunocytochemistry with isolated single egg cells and zygotes as illustrated in Figure 1. Fixed egg cells and zygotes remained on the poly-L-lysine-treated coverslip. Exchanging solutions were performed gently on the coverslip with a hand-operated microcapillary under an inverted microscope. Microcapillaries connected to a micropump via a hydraulic system were necessary to exchange the tiny amounts of solutions. The process of solution exchange was performed under continuous microscopic observation. Thus it was possible to adjust a suitable fluid flow to keep cells on the coverslip and to preserve them. By using this

procedure, we analyzed protein expression and localization in 154 individual cells at different developmental stages by immunostaining. 88 % of these cells exhibited staining of microtubular structures.

We detected a comparable microtubular organization in both isolated zygotes, which are protoplasts, as well as in cultured zygotes with a well developed cell wall. No detectable effect of the isolation procedure on this sensitive cytoskeletal component was observed.

Microtubular cytoskeleton and cytological characteristics observed in the egg cell and zygotes during development

Microtubules were examined in isolated egg cells, in isolated zygotes and during development of in vitro-produced zygotes of maize. Some cortical microtubules were detected in the egg cell as confirmed by observation of successive focal planes (Fig. 2A, B) but we hardly observed well-organized microtubules as found in zygotes (Fig. 2E). Few microtubules were detected at mid focal plane in egg cells (Fig. 2B). Double labeling with anti-α-tubulin antibodies and DAPI revealed the position of the nucleus in the cells (Figs. 2B, C and F, G). Appearance of the egg cell after fixation and application of antibodies and DAPI is shown in Figure 2D. The fluorescence signal after DAPI-staining was very week in the egg nucleus compared to the signal in the other organelles surrounding the nucleus (Fig. 2C).

Comparable to egg cells (52 cells examined), cortical microtubule arrays were observed in zygotes 30 min (5 cells), 1-2 h (11 cells), 5-6 h (3 cells) and 7 h (2 cells) after in vitro fertilization. Cortical microtubules of a zygote, 7 h after in vitro fertilization, are

shown in Figure 2I. DAPI-staining of the same cell is shown in Figure 2J to demonstrate the position of the nucleus.

Microtubule networks comprising cortical microtubules and radiating microtubules were detected in 35 isolated zygotes between 15 and 20 h after *in vivo* fertilization (Figs. 2E and F). Anti-α-tubulin antibodies recognized microtubules throughout the cytoplasm from the cell membrane to the nucleus. Microtubules were particularly concentrated in the perinuclear region. Cortical microtubules were found in a random orientation on the zygote (Fig. 2E). A strong DAPI fluorescence signal was observed in the zygotic nucleus (Fig. 2G). The shape and appearance of the isolated zygote after immunocytochemical procedures are shown in Figure 2H.

Microtubules radiating from the nucleus to the cell periphery emerged in zygotes 27 h after in vitro fertilization are shown in Figure 2K. The position of the nucleus is shown in Figures 2K and L. Abundant microtubules were detected not only radiating from the nucleus to the cell periphery but also in the cortex of these zygotes (data not shown).

Discussion

In this study, we established a reliable procedure for labeling microtubules by indirect immunofluorescence in isolated single egg cells and zygotes. By using this method we found distinct fertilization-induced changes in the arrangement of microtubules in the fertilized egg cell of maize.

More recently, by examination of whole embryo sacs, it was found that microtubules are randomly aligned in the perinuclear cytoplasm, and longitudinally in the micropylar and mid-lateral areas of the synergid-lacking egg cell of *Plumbago zeylanica*, whereas they are organized into bundles near the filiform apparatus (Huang *et al*. 1993). In egg cells of *Arabidopsis thaliana*, microtubules are concentrated around the nucleus, closely positioned to the synergid and found throughout cytoplasm, but not conspicuously arrayed in the cortex (Webb and Gunning 1994). Similarly, we found only a few cortical microtubules in maize egg cells but not in such a pronounced organization as in zygotes.

Microtubules are located mainly in the cortical cytoplasm of non-growing somatic cells and irregular, longitudinally or obliquely positioned (Kost and Chua 2002).
During interphase they remain close to the plasma membrane (Ledbetter and Porter 1963; Newcomb 1969; Seagull and Heath 1980; McClinton and Sung 1997).

In somatic cells, the cytoplasm is generally restricted to a layer of cytoplasm in the cortex and some transvacuolar strands. Other than in somatic cells, many transvacuolar strands pervade the maize egg cell, which is only partially surrounded by a cell wall. We observed in these non-growing cells only a few cortical microtubules. The lack of a dense cortical microtubular network, which control microfibril deposition during interphase (Wymer and Lloyd 1996; Hasezawa and Nozaki 1999) might reflect the limited cell wall

synthesis in egg cells.

We observed only cortical microtubules in zygotes up to 7 h after in vitro gamete fusion. Interestingly, we found additionally radiating microtubules after this time. These radiating microtubules persisted during all further developmental stages in the maize zygote. Changes in microtubular organization also occur in the *Arabidopsis* zygote. Initially, microtubules are located in random orientation in the cytoplasm and mainly in the perinuclear area, followed by a distribution mainly in the cortex and pronounced transverse cortical microtubules organize along with cell elongation (Webb and Gunning 1991). Microtubule distribution was also investigated in growing suspension cultured cells, which were in some cases synchronized in order to analyze association with particular cell cycle events (Kumagai and Hasezawa 2001). It was shown that microtubule arrays are organized mainly in the cell cortex during G1 phase. During early S and G2 phase microtubules form in cytoplasmic strands which radiate from the nucleus to the cell periphery. In G2 phase, microtubules progressively assemble at the preprophase band (PPB), which girdles the cell at the putative site of cell-plate formation (Mineyuki 1999). At telophase, the phragmoplast is formed between the two daughter nuclei. Very similar to this dynamic organization of microtubules in somatic cells, we observed changes in the microtubule network during zygote development and zygotic first cell division. Even though an obvious tight PPB was not seen in our study, parallel cortical microtubule arrays were observed, suggesting formation of a broad PPB.

Maize egg cell nuclei contain a 1C level of DNA (basic haploid amount) and zygotes are in S phase during the period from 27 to 34 h after pollination which is approximately 3 to 6 h after fertilization (Mogensen et al. 1995). Emergence of radiating microtubules in our zygotes suggest that these cells were in S or G2 phase of the interphase. The

appearance of distinct cortical and radiating microtubules might reflect dramatic metabolic changes which require microtubule-based transport processes (Asada and Collings 1997). Synthetic activities, as for example cell wall formation occur very soon after gamete fusion (Kranz et al. 1995). It is well known that microtubules assist cellulose microfibril deposition (Green 1962; Leadbetter and Porter 1963; Mueller and Brown 1982a; 1982b; Cyr 1994; Burk and Ye 2002). Whether changes in the architecture of the cytoskeleton are involved in nuclear positioning to prearrange the plane of the unequal division has yet to be determined.

Our elaborated protocol will be further used to analyze subcellular localization of specific proteins in isolated single gametes and developing zygotes which might be involved in polar growth processes. Single cell model systems are instrumental to unravel molecular mechanisms involved in cytoskeleton and polar cell growth (Kost and Chua 2002). Recently it was shown that phospholipase D activation is correlated with a reorganization of microtubules in somatic suspension cells (Dhonukshe et al. 2003). Our experimental system using individual cells may contribute to elucidate such processes and underlying mechanisms of reorganization of the cytoskeleton network after external signals, as sperm cell entry into the egg cell.

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References

Asada T, Collings D (1997) Molecular motors in higher plants. Trends in Plant Sci 2: 29-37

Brown RC, Lemmon BE (1995) Methods in plant immunolight microscopy. Methods in Cell Biology 49: 85-107

Burk DH, Ye, ZH (2002) Alteration of oriented deposition of cellulose microfibrils by mutation of a katanin-like microtubule-severing protein. Plant Cell 14: 2145–2160

Cyr RJ (1994) Microtubules in plant morphogenesis: Role of the cortical array. Annu. Rev. Cell Biol. 10: 153–180

Dhonukshe P, Laxalt AM, Goedhart J, Gadella TWJ, Munnik T (2003) Phospholipase D activation correlates with microtubule reorganization in living plant cells. Plant Cell 15: 2666-2679

Green, PB (1962) Mechanism for plant cellular morphogenesis. Science 138: 1404–1405

Goldberg RB, de Paiva G, Yadegari R (1994) Plant embryogenesis: zygote to seed. Science 266: 605-614

Hasezawa S, Nozaki H (1999) Role of cortical microtubules in the orientation of cellulose microfibril deposition in higher-plant cells. Protoplasma 209: 98-104

Huang B-Q, Russell SD, Strout,GW, Mao L-J (1990) Organisation of isolated embryo sacs and eggs of *Plumbago zeylanica* (Plumbaginaceae) before and after fertilization. Amer. J. Bot. 77: 1401-1410.

Huang B-Q, Sheridan WF (1994) Female gametophyte development in maize: microtubular organization and embryo sac polarity. Plant Cell 6: 845-861

Huang B-Q, Pierson ES, Russell SD, Tiezzi A, Cresti M (1993) Cytoskeletal organization and modification during pollen tube arrival, gamete delivery and fertilisation in *Plumbago zeylanica*. Zygote 1: 143-154

Kost B., Chua N-H (2002) The plant cytoskeleton: vacuoles and cell walls make the difference. Cell 108: 9-12

Kranz E, Lörz H (1990) Micromanipulation and *in vitro* fertilization with single pollen grains of maize. Sex. Plant Reprod. 3: 160-169

Kranz E, Brown PTH (1992) Micromanipulation and *in vitro* fertilization with single pollen grains and isolated gametes of maize. In: Cresti M, Tiezzi A (eds) Sex. Plant Reprod. Springer, Berlin, Heidelberg, New York, pp 173-183

Kranz E, Lörz H (1993) *In vitro* fertilization with isolated, single gametes results in zygotic embryogenesis and fertile maize plants. Plant Cell 5: 739-746

Kranz E, Bautor J, Lörz H (1991) *In vitro* fertilization of single, isolated gametes of maize mediated by electrofusion. Sex. Plant Reprod. 4: 12-16

Kranz E, von Wiegen P, Lörz H (1995) Early cytological events after fertilization of cell division in egg cells and zygote development following *in vitro* fertilization with angiosperm gametes. Plant J 8: 9-23

Kumagai F, Hasezawa S (2001) Dynamic organization of microtubules and microfilaments during cell cycle progression in higher plant cells. Plant Biol 3: 4-16

Ledbetter MC, Porter KR (1963) A "microtubule" in plant cell fine structure. J Cell Biol 19: 239–250

McClinton RS, Sung ZR (1997) Organization of cortical microtubules at the plasma membrane in *Arabidopsis*. Planta 201: 252–260

Mineyuki Y (1999) The preprophase band of microtubules: its function as a cytokinetic apparatus in higher plants. International Review of Cytology 187: 1-49

Mogensen HL, Leduc N, Matthys-Rochon E, Dumas C (1995) Nuclear DNA amounts in the egg and zygote of maize (*Zea mays* L.*).* Planta 197: 641-645

Mueller SC, Brown RMJ (1982a) The control of cellulose microfibril deposition in the cell wall of higher plants. I. Can directed membrane flow orient cellulose microfibrils? Indirect evidence from freeze-fractured plasma membranes of maize and pine seedlings. Planta 154: 489–500

Mueller SC, Brown, RMJ (1982b) The control of cellulose microfibril deposition in the cell wall of higher plants. II. Freeze-fracture microfibril patterns in maize seedling tissues following experimental alteration with colchicine and ethylene. Planta 154: 501–515

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plantarum 15: 473-497

Newcomb EH 1969 Plant microtubules. Annu. Rev. Plant Physiol 20: 253–288

Schliwa M, van Blerkom J (1981) Structural interaction of cytoskeletal components. J. Cell Biol. 90: 222-235

Seagull RW, Heath IB (1980) The organization of cortical microtubule arrays in the radish root hair. Protoplasma 103: 205–229

Webb MC, Gunning BES (1991) The microtubular cytoskeleton during development of the zygote, proembryo and free-nuclear endosperm in *Arabidopsis thaliana* (L.) Heynh. Planta 184: 187-195

Webb MC, Gunning BES (1994) Embryo sac development in *Arabidopsis thaliana* II The cytoskeleton during megagametogenesis. Sex Plant Reprod 7: 153-163

Wymer C, Lloyd C (1996) Dynamic microtubules: implications for cell wall patterns. Trends Plant Sci 1: 222-228

Figure 1 Protocol for indirect immunofluorescence analysis with isolated single cells. Isolated egg cells, zygotes or in vitro fertilized zygotes are transferred into mannitol solution in 'Millicell-CM'- inserts, which are placed into a plastic dish previously filled with mannitol. The amount of mannitol solution in the inserts is reduced by a microcapillary and the inserts containing isolated single cells are transferred to freshly prepared fixative into another plastic dish. Remaining mannitol solution in the inserts is removed and fixative is added into inserts with a microcapillary. The fixative allows penetration through a semi-permeable membrane of the bottom of the 'Millicell-CM'-inserts. After 20 min incubation, fixed cells are transferred into MSB and kept just before use. After that, fixed cells are mounted in a 50 µl MSB droplet on a coverslip (24 × 40 mm) treated with poly-L-lysine for cell adhesion. Onto the coverslip, walls are built with adhesive tape to keep solutions in place. The coverslip is kept above moistened papers in a Petri dish (diameter 12 cm) during incubations. Exchange of solutions for washing steps, applying antibodies and DAPI- solutions are performed on the coverslip with a hand-operated micropump which is connected via a small tube to a microcappillary. Procedures are performed under an inverted microscope.

Figure 2 Microtubule distribution and DAPI staining of isolated maize egg cell (A-D), zygote isolated 20 h after in vitro pollination (approximately 15 h after fertilization) (E-H), in vitro-produced zygote 7 h after in vitro fertilization (I,J) and of in vitro-produced zygote 27 h after in vitro fertilization (K,L).

(A) Some cortical microtubules were detected on the egg cell. (B) Few microtubules were detected at mid focal plane in the egg cell. (C) Epifluorescence micrograph of the egg cell stained with DAPI. Arrow indicates the position of the nucleus. (D) Light

micrograph of the egg cell. Arrow indicates the nucleolus. (E) Cortical microtubules were largely observed in random orientation on the isolated zygote. (F) Strands of cytoplasmic microtubules radiating from the nucleus to the cell periphery of the zygote. (G) Epifluorescence micrograph of the zygote stained with DAPI to indicate the position of the nucleus of the zygote (arrow). (H) Light micrograph of the zygote. (I) Few cortical microtubules were detected at the periphery of the in vitro-produced zygote 7 h after in vitro gamete fusion. (J) Light/epifluorescence micrograph of the same zygote as in (I) but stained with DAPI. (K) Strands of cytoplasmic microtubules radiating from the nucleus to the cell periphery of the zygote, produced 27 h after gamete fusion. (L) Epifluorescence micrograph of the same zygote as in (K) but stained with DAPI. Images B, C, F-H were taken at the mid focal plane. Bar indicates 20 μm for all images.

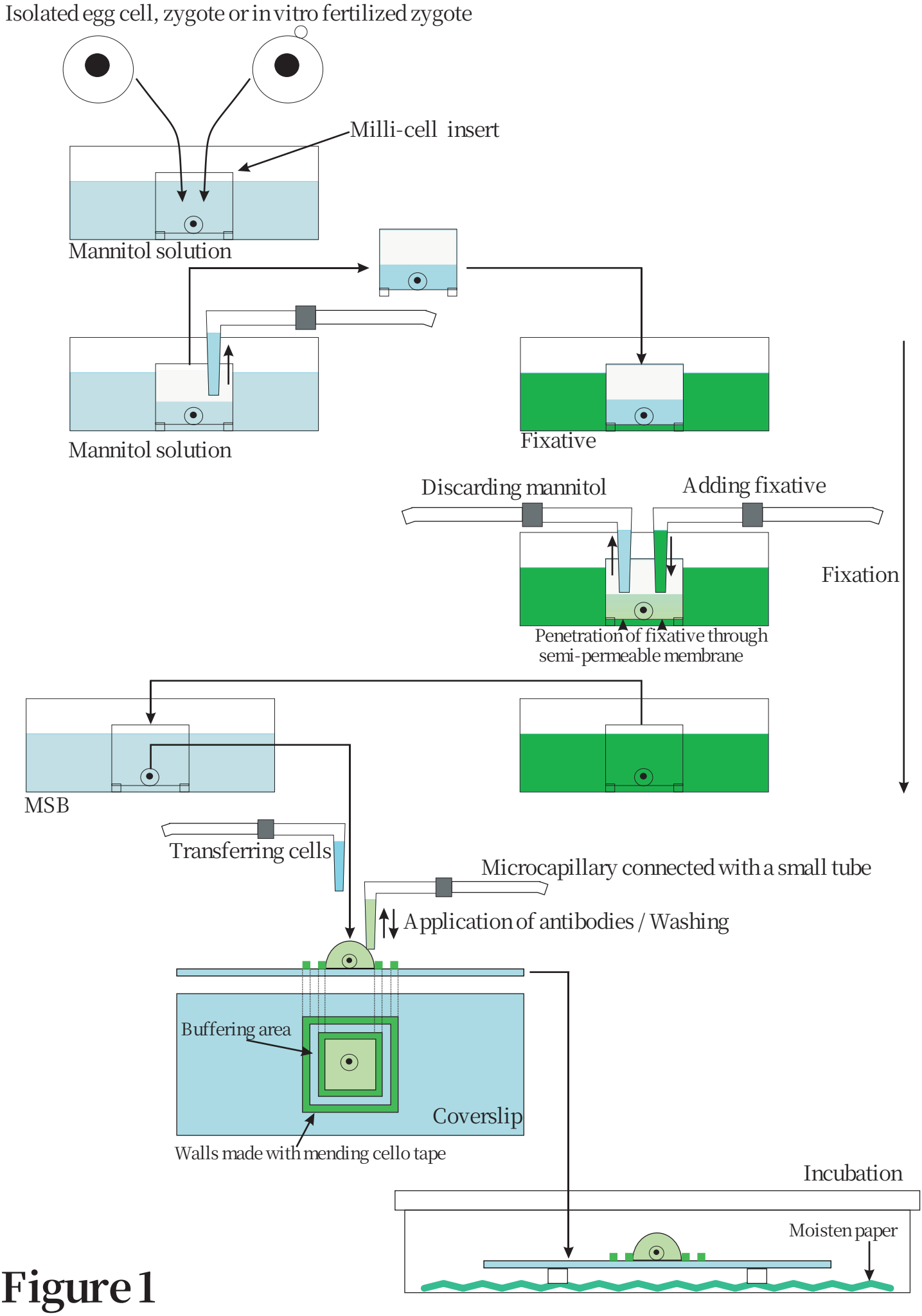
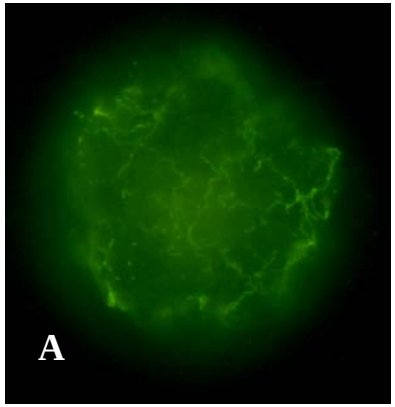

Isolated egg cell, zygote or in vitro fertilized zygote
Milli-cell insert
Mannitol solution
Mannitol solution
Fixative
Discarding mannitol
Adding fixative
Fixation
Penetration of fixative through
semi-permeable membrane
MSB
Transferring cells
Microcapillary connected with a small tube
Application of antibodies / Washing
Buffering area
Coverslip
Walls made with mending cello tape
Incubation
Moisten paper

Figure 1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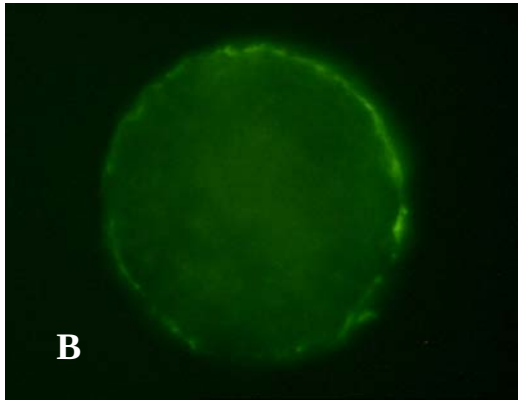
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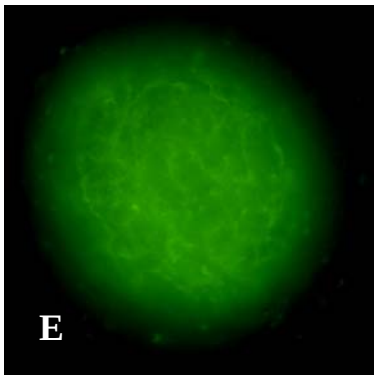
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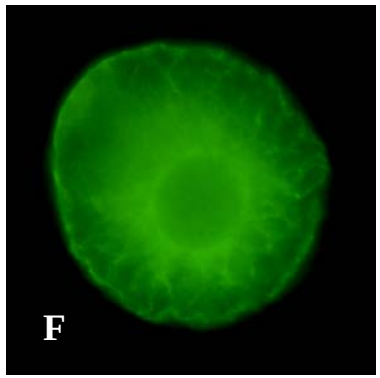
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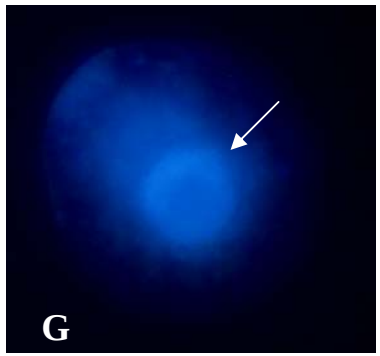
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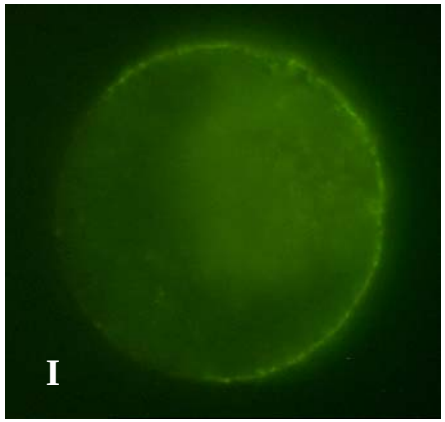
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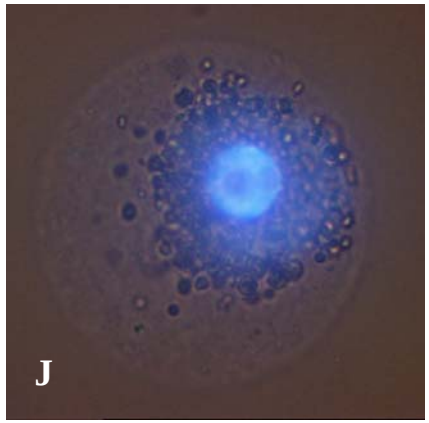
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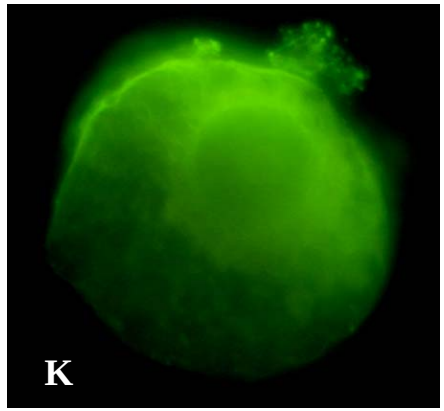
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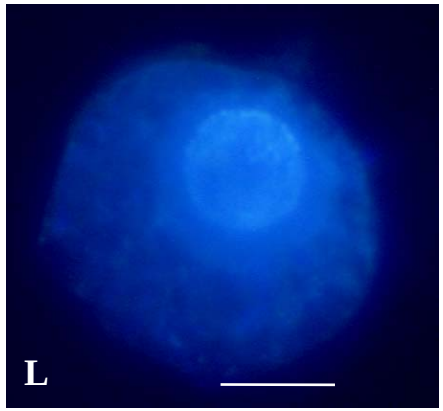
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