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Review

Individual selection, culture and manipulation of higher plant cells*

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1 Summary. Due to the heterogeneity in morphology, physiological and morphogenetical capabilities of higher plant cells in mass culture, the development of methods for individually culturing defined cells seemed to be useful and necessary. Individual cell culture represents a powerful tool for studies on the physiology of different cell types, the analysis of differentiation programs, the genetic manipulation of plant cells and cell-cell interactions. An improved microculture system based on a computer-controlled set-up for the efficient selection, transfer and individual culture of defined higher plant cells until regeneration of whole plants is described. Related experimental approaches for individually manipulating higher plant cells under controlled conditions, such as electrofusion of defined pairs of protoplasts and subprotoplasts, cell reconstruction and intranuclear microinjection of protoplasts and karyoplasts – mainly performed with cells of the crop plant *Brassica napus* L. – are presented.

Key words: Microculture – Electrofusion – Microinjection – Karyoplasts – Cytoplasts – Protoplasts – Plant regeneration – Conditioning – *Brassica napus* – *Nicotiana tabacum*

* This article is dedicated to the memory of H.-G. Schweiger, initiator and mentor of the experimental single cell approach reviewed herein

2 Introduction

The work on unicellular plant model systems including unicellular algae and the availability of plant cell cultures have resulted in a revolutionary increase in the knowledge of plant cell biology. Milestones on the way are the possibility to regenerate whole plants from protoplasts and to genetically manipulate plant cells (Braun 1959; Cocking 1960; Vasil and Hildebrandt 1965; Power et al. 1970; Takebe et al. 1971; Carlson et al. 1972; Melchers et al. 1978; Schweiger and Berger 1979; Gleba and Hoffmann 1980; Schell et al. 1984; Schreier et al. 1985; Schweiger and Neuhaus 1986).

Plant cell culture techniques are mainly based on methods developed for microbiology. These methods include work with high numbers of cells and selection markers.

In addition to the obvious advantages of working on cell populations there is the disadvantage that only indirect conclusions can be drawn with respect to the individual cell as the work is based on the assumption that all cells in a population are identical. If one looks at a population of protoplasts through a microscope it becomes evident that this by no means is true (Fig. 1).

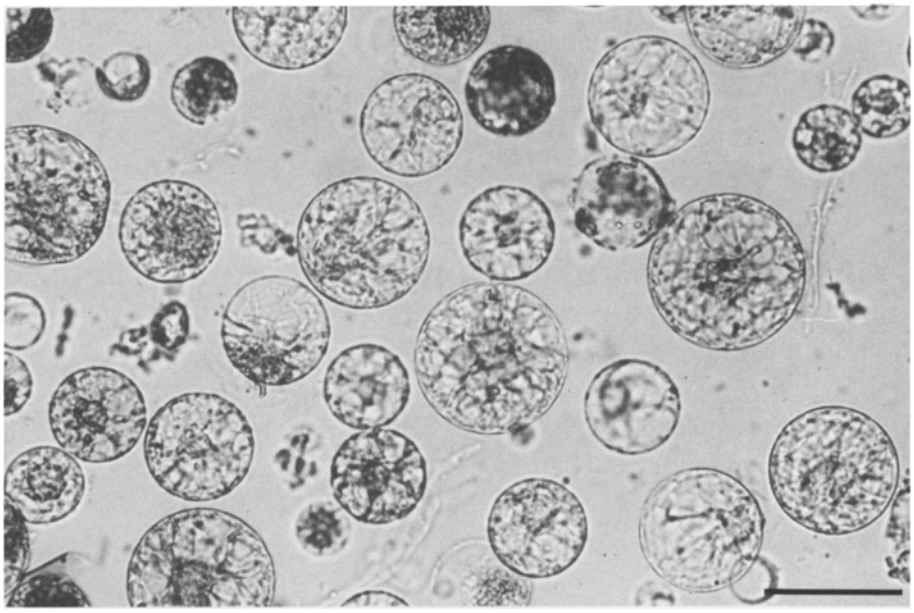

Fig. 1. Hypocotyl protoplasts of *Brassica napus* L. immediately after isolation. Note the heterogenous appearance of the cells. Bar = 100 μm

Such protoplasts are heterogenous in size, subcellular structure and other morphological markers which may also represent different functional, developmental and cell cycle stages.

Working with a high number of cells generally compels one to accept the disadvantages of a heterogenous population. In many cases only a few cells of a population are the really interesting and useful ones and sometimes it is a major problem to identify and enrich or isolate these target cells.

If the cell type wanted represents a particular stage in the cell cycle the problem may be overcome by using synchronized cells which to a certain degree are also available in higher plant cell cultures (Constabel et al. 1977; Cress and Gerner 1979; Amino et al. 1983; Guri et al. 1984). Recently, it has been shown that even with relatively simple and non-hazardous means a sufficient synchronization of plant cell cultures can be achieved (Weber et al. 1986). In general, however, the simplest way to bypass the problem of heterogeneity would be the use of individual cells.

Experimental work on individual plant cells is by no means a novel technique (Haberlandt 1902; Muir et al. 1954; de Ropp 1955; Torrey 1957; Muir et al. 1958; Braun 1959; Bergmann 1960; Jones et al. 1960; Reinert 1963; Vasil and Hildebrandt 1965; Kao 1977; Gleba 1978). Biological experiments have been performed for more than 50 years with the unicellular and uninucleate marine green alga *Acetabularia* (Hämmerling 1932; Hämmerling 1955; Schweiger and Berger 1979). Individual and defined cells routinely were used for studies on the physiology and development of *Acetabularia*. Fragmentation and grafting experiments were of particular importance. These experiments substantially contributed to our knowledge on the role of the cell nucleus, the cytoplasm and their interrelationships (Hämmerling 1955; Schweiger et al. 1967; Berger and Schweiger 1975; Schweiger and Berger 1979; Schweiger et al. 1984). More recently, experiments have demonstrated that *Acetabularia* cells are uniquely qualified for in vivo gene expression and as a model for highly efficient transformation (Cairns et al. 1978a; Cairns et al. 1978b; Cairns et al. 1978c; Neuhaus et al. 1983; Neuhaus et al. 1984; Neuhaus et al. 1986). Starting from these experiments the question arises as to whether similar techniques and experiments can be performed on higher plant cells. This would include separately and individually growing defined cells, fragmenting cells, combining selected cells, subcellular fragments and genetically manipulating such cells by transformation.

In the following, experiments are summarized which have been carried out with cells of higher plants, preferentially of *Brassica napus* L. A crop plant was chosen since a good deal of present day cell biology of higher plants is praxis oriented.

3 Individual culture of cells

3.1 General conditions

In many cases, the establishment of plant cell or protoplast cultures does not present unsurmountable problems. It is, however, a major drawback of these techniques that they produce heterogenous populations of cells (Partanen 1963; Kant and Hildebrandt 1969; Mizukami et al. 1978; Bayliss 1980; Dougall et al. 1980; Constabel et al. 1981; Kinnersley and Dougall 1982; Sree Ramulu 1984; Ellis 1985). The simplest way to avoid this problem is to identify and select an individual cell and to culture it separately from the others.

In order to do so certain prerequisites should be fulfilled: The method should be economical or, in other words, it should not be too laborious, so that a sufficient number of cells can be handled and cultured in a given

time. The identification of the type of cell to be selected should be easily possible. This does not represent a major problem as long as suitable morphological markers are available which can be used for the identification under the light microscope. Identification can be facilitated by the use of a number of methods like vital and fluorescence staining and thereby extending the application of microscopical criteria (Patnaik et al. 1982; Bergounioux et al. 1983; Barsby et al. 1984). Another prerequisite which should be fulfilled by a suitable single cell culture technique is an appropriate method of separating and transferring the identified cell from the population to the microculture chamber. This means that the methods should largely avoid damaging the cell, thereby leading to a high survival rate. Thus the cells should be capable of dividing and finally regenerating whole plants at a high rate.

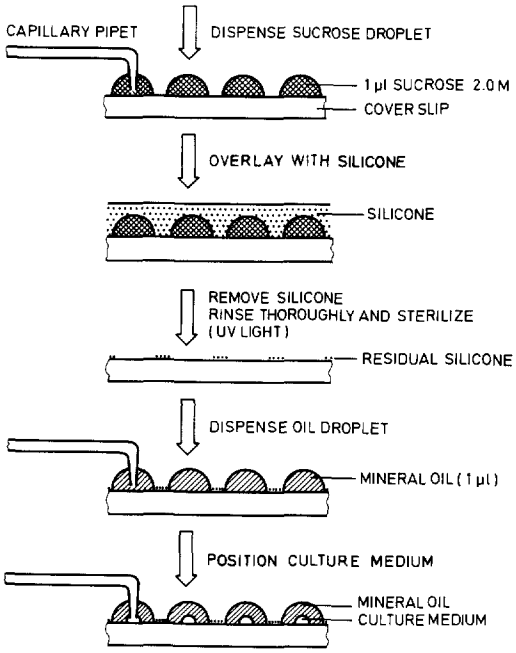

Fig. 2. Preparation of a microculture chamber for individual cell culture. The distances between the centers of the droplets are approximately 3,400 µm (Koop and Schweiger 1985 a)

3.2 Preparation of the microculture chamber

In a set of preliminary experiments it was shown that individual cell culture strongly depends on the features of the medium, in particular on the volume of medium per cell ratio. These experiments have demonstrated that the successful individual culture of a single cell requires an extremely small volume. Since the surface of a small droplet is relatively large the risk of evaporation and a subsequent change in the composition of the medium is high. Therefore the question of how one could reduce evaporation under these conditions is paramount. A simple way to solve this problem is to cover the microdroplet of medium with mineral oil (Jones et al. 1960; Koop et al. 1983 a).

Based on these considerations the following technique was developed. The target cell is individualized, selected, transferred to and kept in a microdroplet of medium. In order to avoid evaporation of the medium the microdroplet is covered with a layer of mineral oil (Koop et al. 1983 a). Exchange of substances between neighbouring cells is prevented by covering every microdroplet of medium with individual separated droplets of mineral oil (Koop and Schweiger 1985 a).

Since the microdroplets are placed on a coverslip one must take precautions to prevent the microdroplets from merging during handling. This is solved by an appropriate preparation of the coverslip supporting the microdroplets. In a first step 50 1 µl droplets of a 2.0 M sucrose solution are dispersed on a thoroughly cleaned coverslip (Fig. 2) 3,350 µm apart from each other in rows of 10 with a distance of 3,400 µm between the rows (Fig. 3 a, b). In a second step the coverslip is dipped into a silicon solution so that the whole coverslip is siliconized except for the circular areas occupied by the sucrose droplets. Subsequently the sucrose droplets are removed by carefully rinsing the coverslip with water. Afterwards the coverslip is dried and UV-sterilized (Fig. 2). The coverslip is now ready for positioning the 1 µl droplets of mineral oil exactly in the center of the

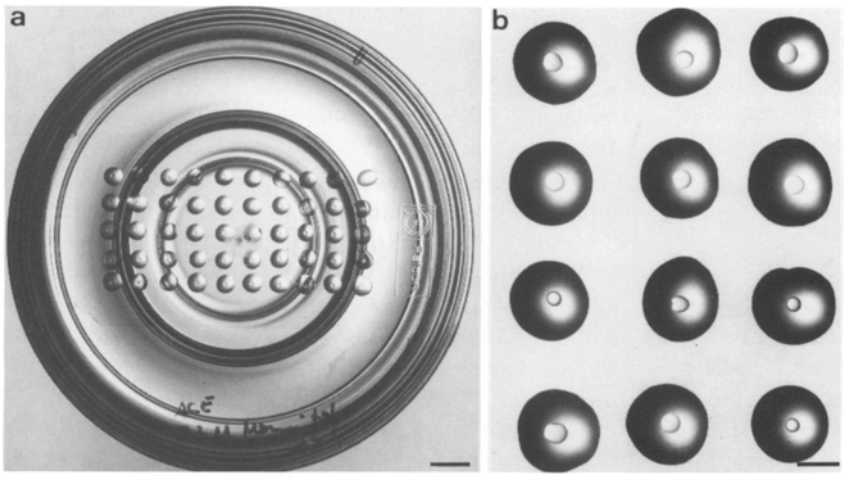

Fig. 3 a, b. Microculture chamber for individual culture of selected cells. **a** Microculture chamber in a moist chamber. Bar = 5 mm. **b** Detailed view of part of a microculture chamber. Each 1 µl droplet of mineral oil contains 30 nl of culture medium. Bar = 1 mm

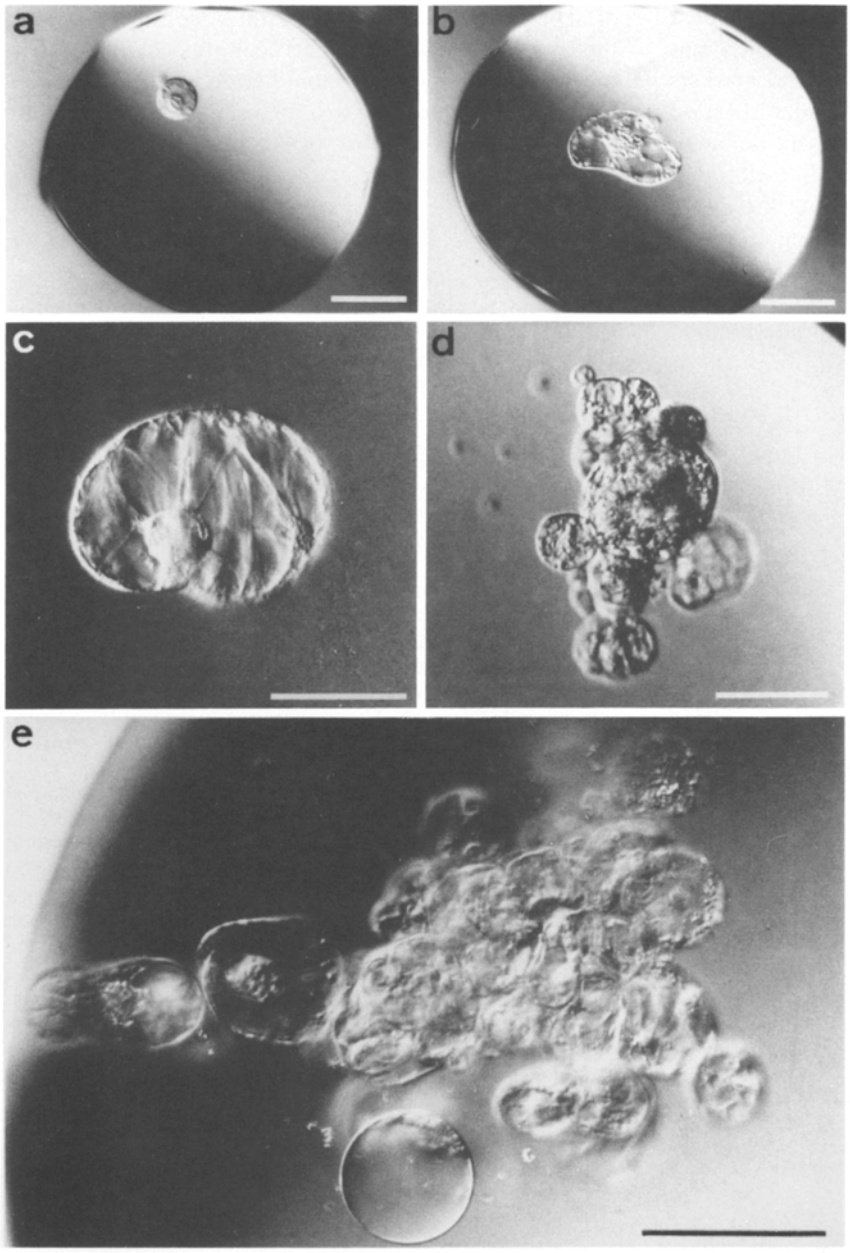

Fig. 4a–e. Development of individually cultured hypocotyl protoplasts of *B. napus*. Bars: 100 µm (Spangenberg et al. 1986a). **a** Protoplasts after transfer into a microdroplet of culture medium. **b** Regenerated cell. **c** Cell division after one day of individual culture. **d** 6 day-old microcallus. **e** 14 day-old microcallus

nonsiliconized circular areas originally occupied by the sucrose droplets. Finally 15 to 100 nl microdroplets of culture medium are injected into the mineral oil droplets. The coverslip prepared in this way is kept in a two compartment Petri dish whose outer compartment is filled with 2 ml 0.2 M mannitol. The Petri dish serves as a moist chamber (Fig. 3a) (Koop and Schweiger 1985a).

3.3 Individual cell culture

In the case of protoplasts from *B. napus* it has proven to be advantagous to use a volume of 50 nl for the microdroplets of culture medium, a volume corresponding to 2×10^4 cells per ml in mass culture. Under these conditions a protoplast will be capable of regenerating cell wall within hours after transfer. Furthermore it will undergo cell division and form calli (Spangenberg et al. 1986a).

For the individual cell culture of *B. napus* protoplasts PBN 7 medium was found to be suitable for the first 6 days (Fig. 4) (Spangenberg et al. 1986a), followed by the addition of 30 nl of a 1 : 1 mixture of PBN 7 and CBN 7 from day 6 onwards. This is repeated every third day until after 25–30 days microcalli of a diameter of 300 µm have developed (Fig. 5a). The microcalli are transferred together with 500 nl of a 1 : 1 mixture of PBN 7 and CBN 7 to 1,000 nl microdroplets positioned in wells on a polycarbonate plate. During the following

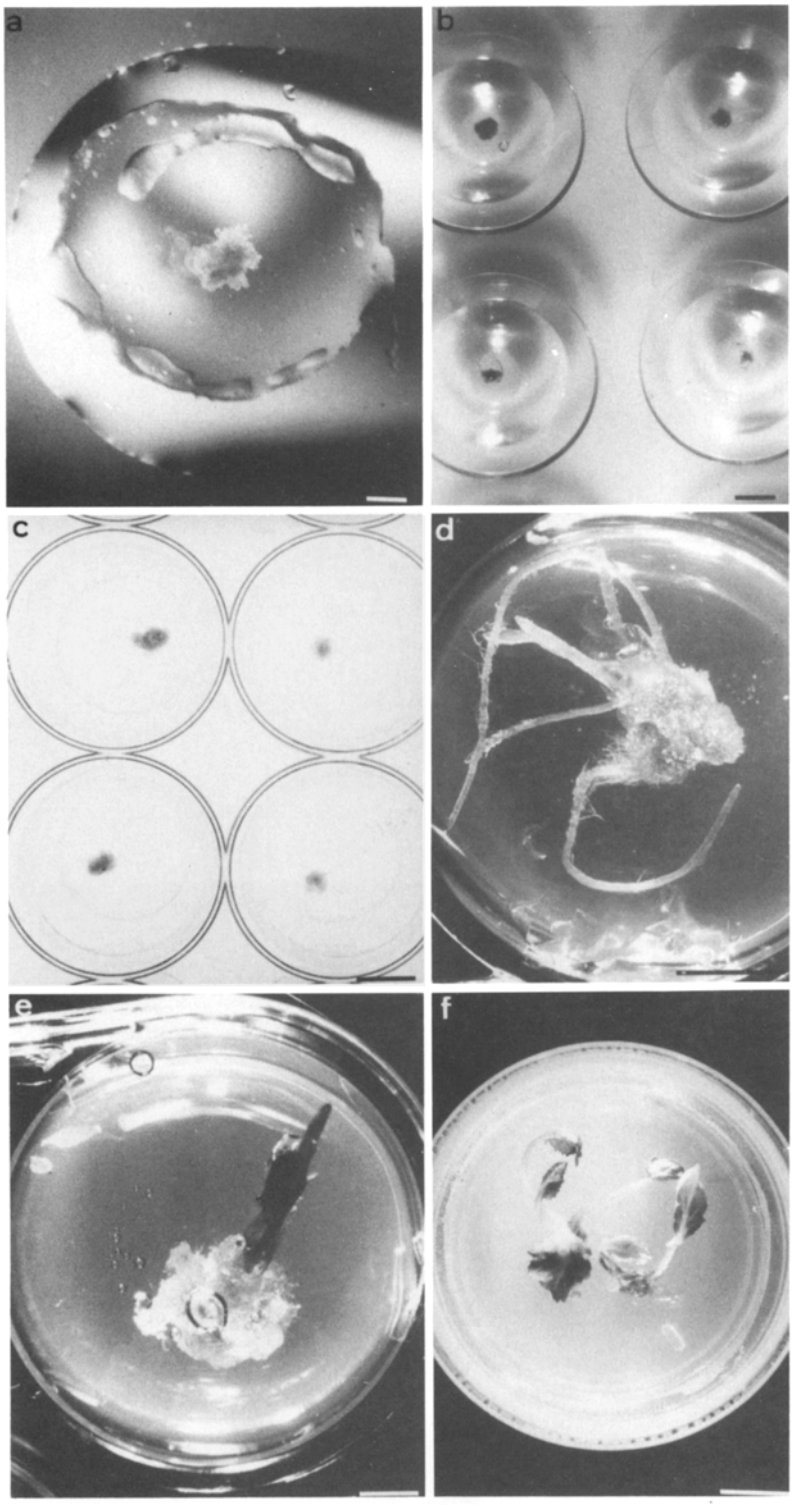

Fig. 5a–f. Plant regeneration from individually selected and microcultured hypocotyl protoplasts of *B. napus* (Spangenberg 1986). **a** Microcallus 30 days after start of individual culture of a single protoplast. Bar = 100 µm. **b** Microcalli growing in Terasaki dishes. Bar = 1 mm. **c** Calli growing in Costar dishes. Bar = 5 mm. **d** Root formation on a callus. Bar = 5 mm. **e** Shoot morphogenesis. Bar = 2.5 mm. **f** Regeneration of whole plants. Bar = 1 cm

30 days 500 nl of the PBN 7–CBN 7 (1 : 1) culture medium are added every week until the microcalli have reached a diameter of 1 mm. At this time the microcalli are transferred to Terasaki dishes (Fig. 5 b). Usually they need another 30 days to reach a diameter of 2 mm. The 2 mm microcalli are transferred to 125 µl solidified MS II medium in wells of a microtiter system 96 well-dish. After another 30 days the calli are transferred to 1 ml solidified culture medium K3 on Costar 24 dishes and passaged every second week using the same medium until they form shoots (Fig. 5 c–f). Rooting is achieved by transferring the shoots onto MS medium containing NAA and activated charcoal. This treatment results in regeneration of whole plants (Figs. 6 and 7).

The yields obtained with this method naturally strongly depend on the species (Koop and Schweiger 1985 a; Spangenberg et al. 1986 a). From 100% selected protoplasts of *Nicotiana tabacum* 55% would directly regenerate whole plants, while with *B. napus* approximately 1% would regenerate calli and eventually roots, shoots and whole plants. The significantly lower yield with *B. napus* is mainly due to the last steps of the culture technique; i.e. during formation of microcalli and calli (Table 1).

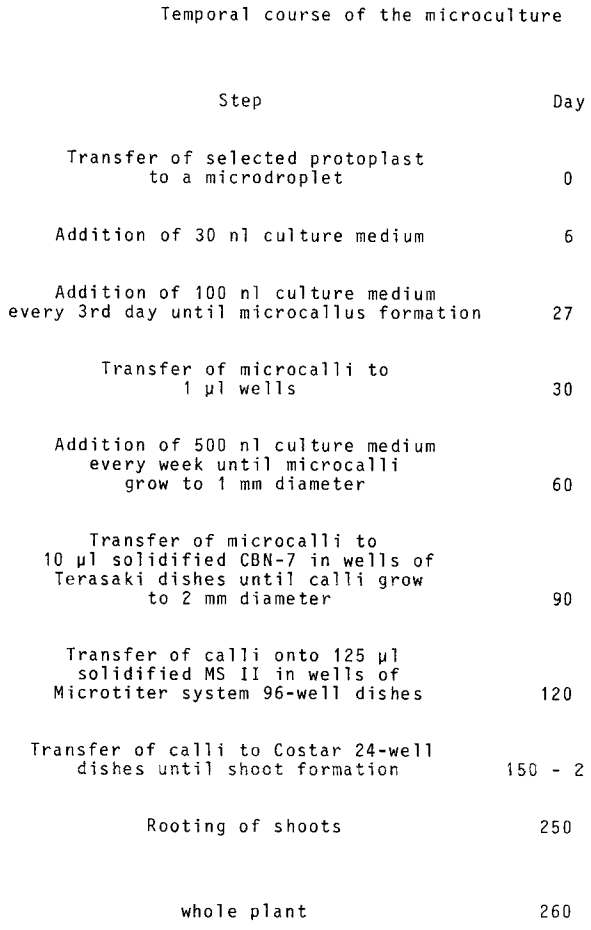

Fig. 6. Temporal course of the microculture

3.4 Applications

The single cell culture technique is highly advantagous for solving a number of problems in cell biology. This

Table 1. Regeneration starting from individually cultured protoplasts

Percentage of:	*Nicotiana tabacum* (%)	*Brassica napus* (%)
Selected protoplasts	100	100
Surviving protoplasts (after 8 days)	85	80
Cells undergoing one division (after 8 days of microculture)	80	60
Formation of microcalli (multiple divisions after 8 days)	70	15
Formation of calli (after transfer to solid medium)	65	2
Calli exhibiting morphogenetic activity	55	1

Fig. 7. Plant regenerated from one individually microcultured rapeseed protoplast

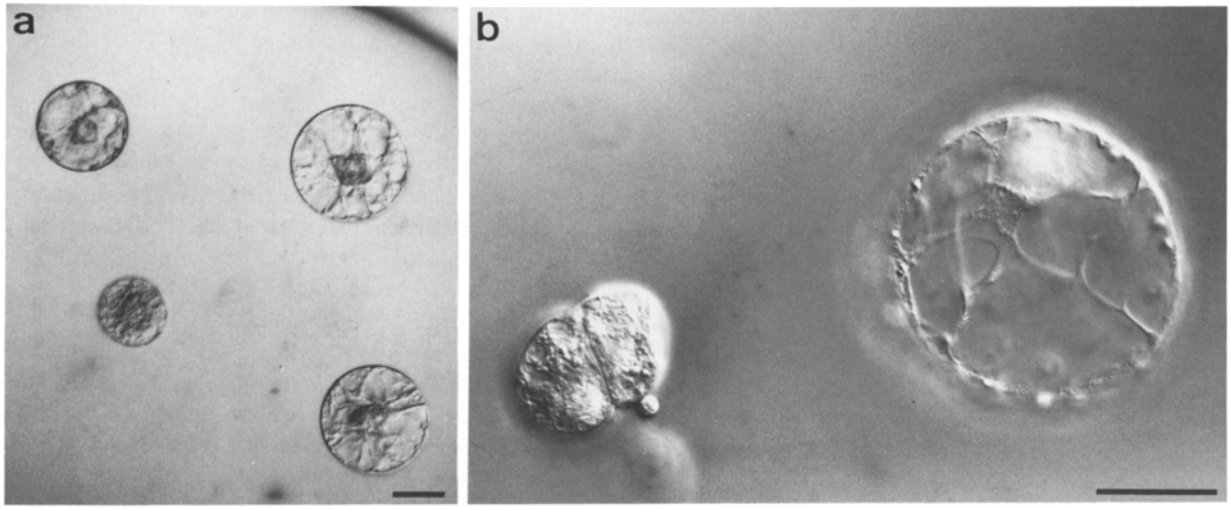

Fig. 8a, b. Microculture of hypocotyl protoplasts of *B. napus* under different conditions (Spangenberg et al. 1985). **a** On type A and three type B protoplasts immediately after transfer to a microdroplet. **b** A type A cell after division and a non-dividing type B protoplast after one day of coculture. Bars = 50 µm. Type A: small, 25–60 µm diameter, unvacuolated protoplast. Type B: large, 80–115 µm diameter, highly-vacuolated protoplast

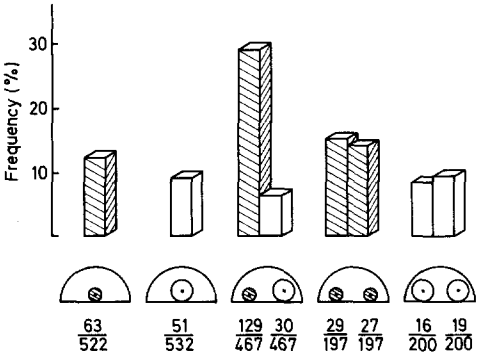

Fig. 9. Microcallus formation from different types of protoplasts of *B. napus*. Small type A protoplasts (dashed bars) and large type B protoplasts (empty bars) were individually cultured and cocultured. Microcallus formation frequency was measured after 8 days of culture. Ratios represent number of microcalli formed per number of selected protoplasts from at least 4 independent experiments (Spangenberg et al. 1985)

can be convincingly illustrated by a single example. One may ask whether two cells belonging to morphologically distinguishable cell types can mutually influence their growth and cell division. A suitable morphological marker is, e.g., the size of the protoplasts. Large, highly vacuolated and small, unvacuolated *B. napus* protoplasts can easily be distinguished. The small protoplast exhibits a more pronounced readiness for division than the large one (Fig. 8). A first finding is that when two protoplasts are co-cultured in a microdroplet a small one does not affect another small one and a large one does not affect another large one (Fig. 9). The situation, however, is completely different when a large and a small cell are co-cultured in one microdroplet.

Table 2. Conditioning capability of different cell compartments. Microcallus formation of individual type A protoplasts in microdroplets conditioned by preincubation with different cell compartments for 48 h was measured after 8 days of culture

Preincubation with	Microcallus formation (%)
Karyoplasts	32
Cytoplasts	29
Protoplasts (type B)	26
Cells (type B)	25
Vacuoles	6
Control (without preincubation)	7

Under these conditions the readiness to divide is increased in the small cell (Figs. 8 and 9). This may have to do with conditioning of the medium, since in other experiments not described here, it was shown that medium which had already been used for the culturing of another protoplast or subprotoplast exerts a similar stimulating effect (Table 2). In addition, co-culturing with more than one large protoplast exerts an even stronger effect on a small protoplast (Spangenberg et al. 1985).

4 Manipulation of cells

4.1 Electrofusion: aims

Among the different techniques suitable for manipulating cells of higher plants fusion plays an important role (for a review see Evans et al. 1983; Gleba and Sytnik 1984). Beyond the intention of combining cells of the

same or different species it is a major aim to fuse defined types and numbers of cells or, in other words, to perform fusion under controlled conditions (Koop et al. 1983 b; Koop and Schweiger 1985 b; Spangenberg and Schweiger 1986). It is obvious that a fusion method based on the single cell microculture technique would be appropriate if a sufficient yield can be achieved with the fusion method. Ideally one would like to have a fusion method which allows the successful fusion of each selected pair of cells. In addition, it would be a most interesting and useful application if subcellular fragments could efficiently be fused under controlled conditions. For these purposes, among the different possibilities, electrofusion seems to be the most promising one (for a review see Zimmermann 1982).

4.2 Electrofusion: set-up

In contrast to other electrofusion methods working on a multiplicity of cells, it is a characteristic of the individual cell fusion method that the cells are not brought to the electrodes but rather that microelectrodes are brought to the preselected cells (Koop et al. 1983 b). The individual cell fusion method is carried out in a microdroplet. For the electrofusion at least two protoplasts or subcellular fragments, respectively, are transferred into one microdroplet containing low ionic strength fusion medium (Fig. 10). Normally in one experiment a set of several fusions are performed using several microdroplets on one coverslip overlaid with a common layer of mineral oil. This layer is kept in the pregiven area on the coverslip by siliconizing the edges of the coverslip.

On the individual cell level electrofusion is performed by means of a pair of electrodes made of platinum wire with a diameter of 50 µm and a length of 10 mm. The distance between the electrodes can be adjusted. The electrodes are fixed to an electrode support mounted under the condensor of an inverted microscope (Fig. 10).

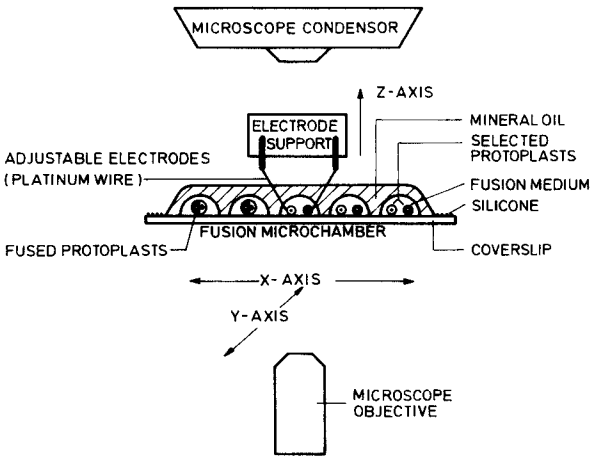

Fig. 10. Set-up for fusing individually selected protoplasts and subprotoplasts (Spangenberg 1986)

For electrofusion, protoplasts are selected and transferred to the microdroplet of fusion medium. After the microelectrodes are properly positioned the electrofusion process itself is performed (Fig. 11). Immediately after fusion the achieved fusion products are transferred to a microculture chamber with microdroplets containing PBN 7 culture medium (Fig. 12). The method described here has been used to fuse protoplasts from different strains of the same species and has been used with protoplasts from different species (Koop et al. 1983 b; Koop and Schweiger 1985 b; Spangenberg and Schweiger 1986). In a number of cases whole plants were regenerated from the fusion products.

4.3 Cell fragmentation

Recently it has been shown that the electrofusion method is suitable not only for fusion of protoplasts but also for the fusion of subcellular fragments like karyoplasts and cytoplasts (Spangenberg and Schweiger 1986). The importance of this type of manipulation, for example, lies in the fact that in this way it is possible to combine the cytoplasmic and nuclear genomes originating from different strains or even species. Subcellular fragments are obtained by gradient centrifugation of protoplasts treated with cytochalasin B and DMSO (Wallin et al. 1978; Spangenberg et al. 1985). From the resulting karyoplast and cytoplast populations individual fragments are selected and transferred into microdroplets of the single cell culture system. A karyoplast and a cytoplast but also a cytoplast and a protoplast or a karyoplast and a protoplast can be fused without major difficulties (Table 3). About 40% of the fused subcellular fragments will survive and undergo cell divisions (Fig. 13) (Spangenberg and Schweiger 1986).

Table 3. Electrofusion of individually selected protoplasts and subprotoplasts. Type A and B: protoplasts

Fusion partners		Yield (%)[a]
a	b	
Type A	type A	60 ± 13
Type A	type B	45 ± 18
Type B	type B	60 ± 18
Karyoplast	protoplast	32 ± 9
Cytoplast	protoplast	34 ± 3
Karyoplast	cytoplast	30 ± 11

[a] Mean values ± standard error from 4 independent experiments are given

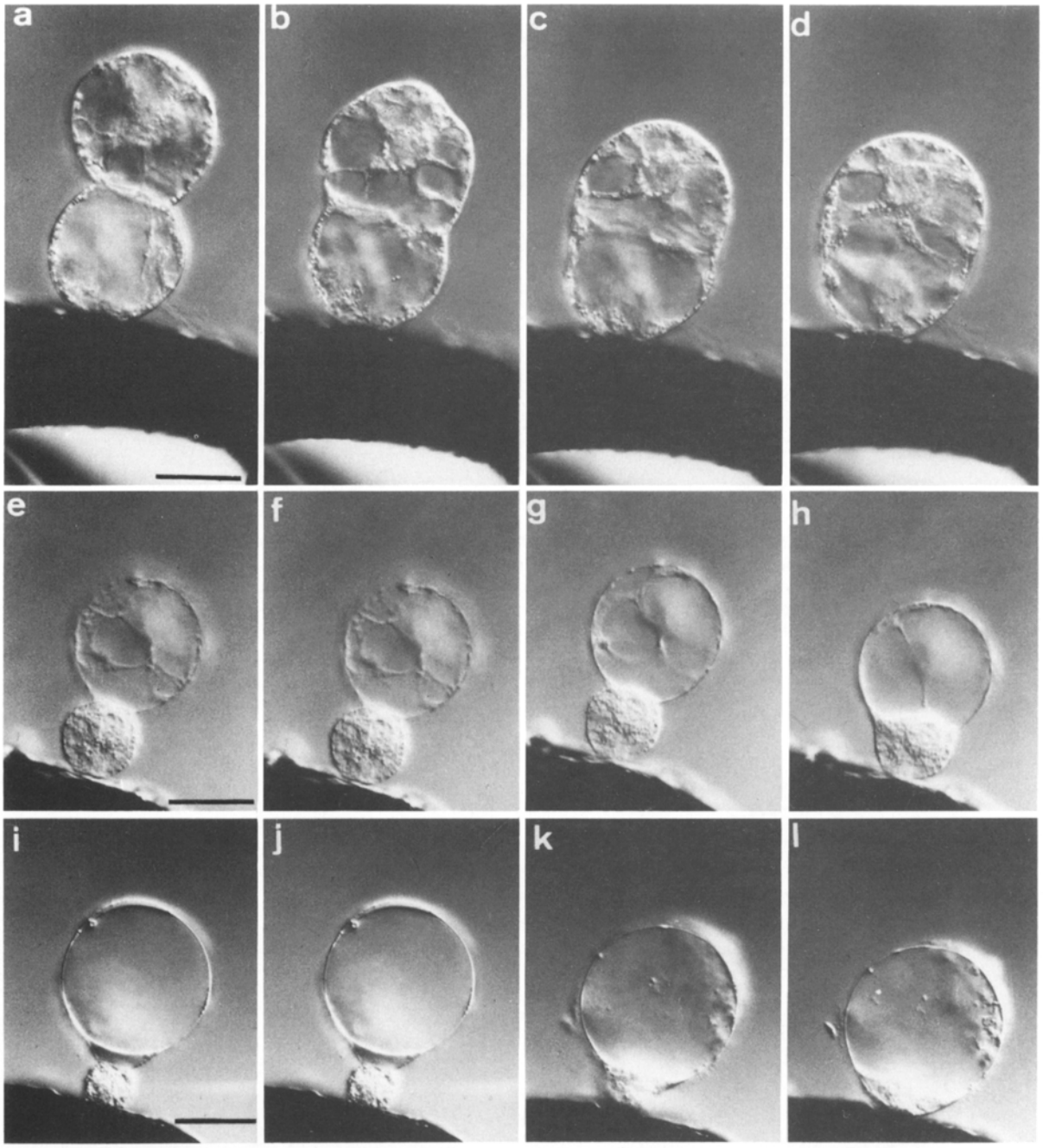

Fig. 11 a–l. Electrofusion of *B. napus* protoplasts and subprotoplasts (Spangenberg and Schweiger 1986). **a–d** Fusion of two type B protoplasts. Bar = 50 μm. **e–h** Fusion of a type A and a type B protoplast. Bar = 50 μm. **i–l** Fusion of a karyoplast and a cytoplast (cell reconstruction). Bar = 20 μm

4.4 Microinjection

A particularly interesting aspect of the method described above is that an isolated karyoplast can be microinjected prior to fusion (Figs. 14–16). This means that genes and gene constructions as well as macromolecules like RNA or proteins can be introduced into the cell nucleus under controlled conditions. Usually a high percentage of karyoplasts will survive microinjection. In a number of microinjection experiments with *B. napus* karyoplasts the yield was about 80%. If microinjected karyoplasts were electrofused with cytoplasts approximately 30% of the fusion products survived (Table 4). More recently, it was found that survival does not absolutely depend on fusing the microinjected karyoplast with a cytoplast (Figs. 14 and 16). In fact, the microin-

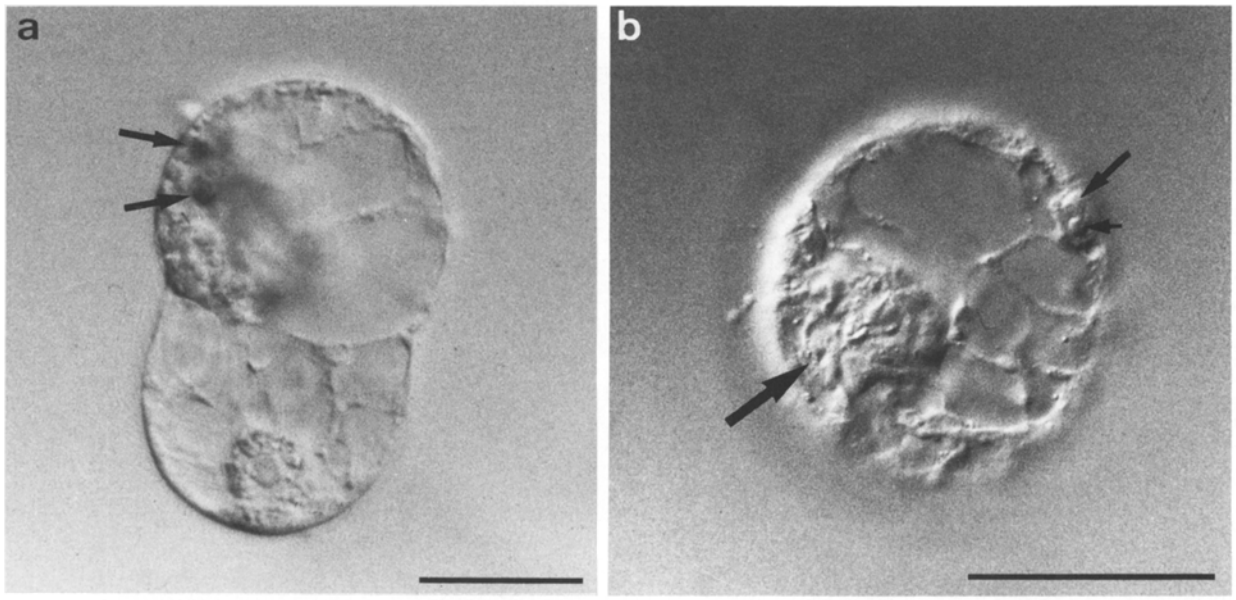

Fig. 12a, b. Products of electrofusion (Spangenberg and Schweiger 1986). **a** Fusion product of two type B protoplasts. One from *B. napus* cv. 'Tower ATR' (lower) and the other from *B. napus* cv. 'Bronowski Ag 903' (upper). Arrows indicate the presence of anthocyanoplast. **b** Fusion product of a type B protoplast from *B. napus* cv. 'Tower ATR' and a karyoplast from *B. napus* cv. 'Bronowski Ag 903'. Large arrow indicates where the nuclear region of the protoplast is. The medium and the small arrow indicate the position of the nucleus and the nucleolus of the karyoplast, respectively. Bars = 50 μm

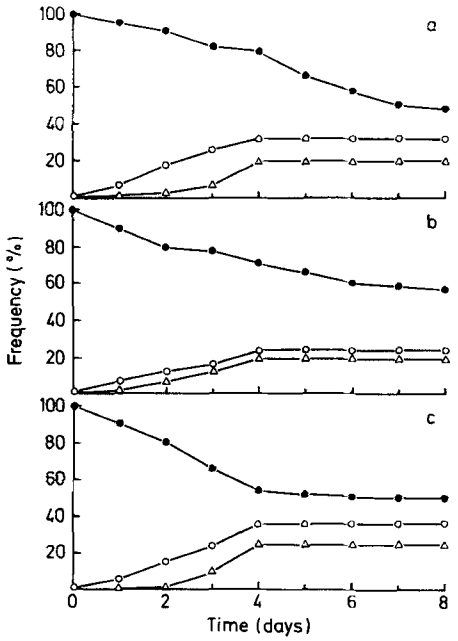

Fig. 13a–c. Time course of the development of fusion products. Pairs of defined protoplasts and subprotoplasts were selected for electrofusion (Spangenberg and Schweiger 1986). Survival (●——●), single division (○——○), microcallus formation (△——△). **a** Control; individually cultured hypocotyl protoplasts. **b** Individually cultured fusion products of a type A and a type B protoplast. **c** Individually cultured cells reconstructed from selected pairs from defined karyoplasts and cytoplasts

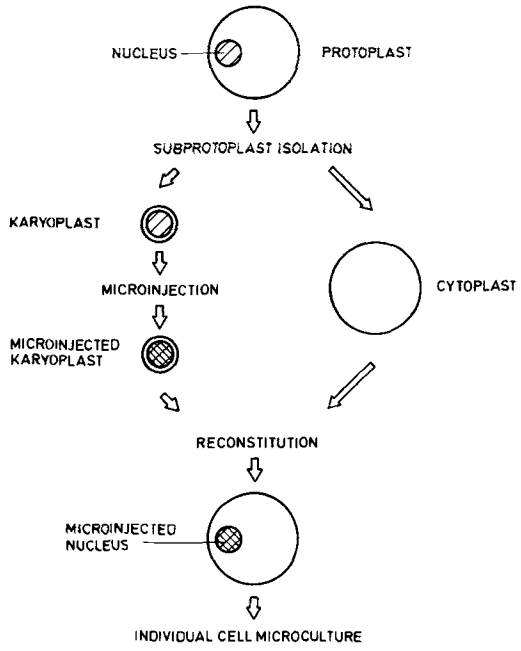

Fig. 14. Electrofusion mediated cell reconstruction from a cytoplast and a microinjected karyoplast (Spangenberg 1986)

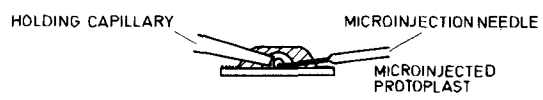

Fig. 15. Microinjection of individually selected protoplasts or karyoplasts

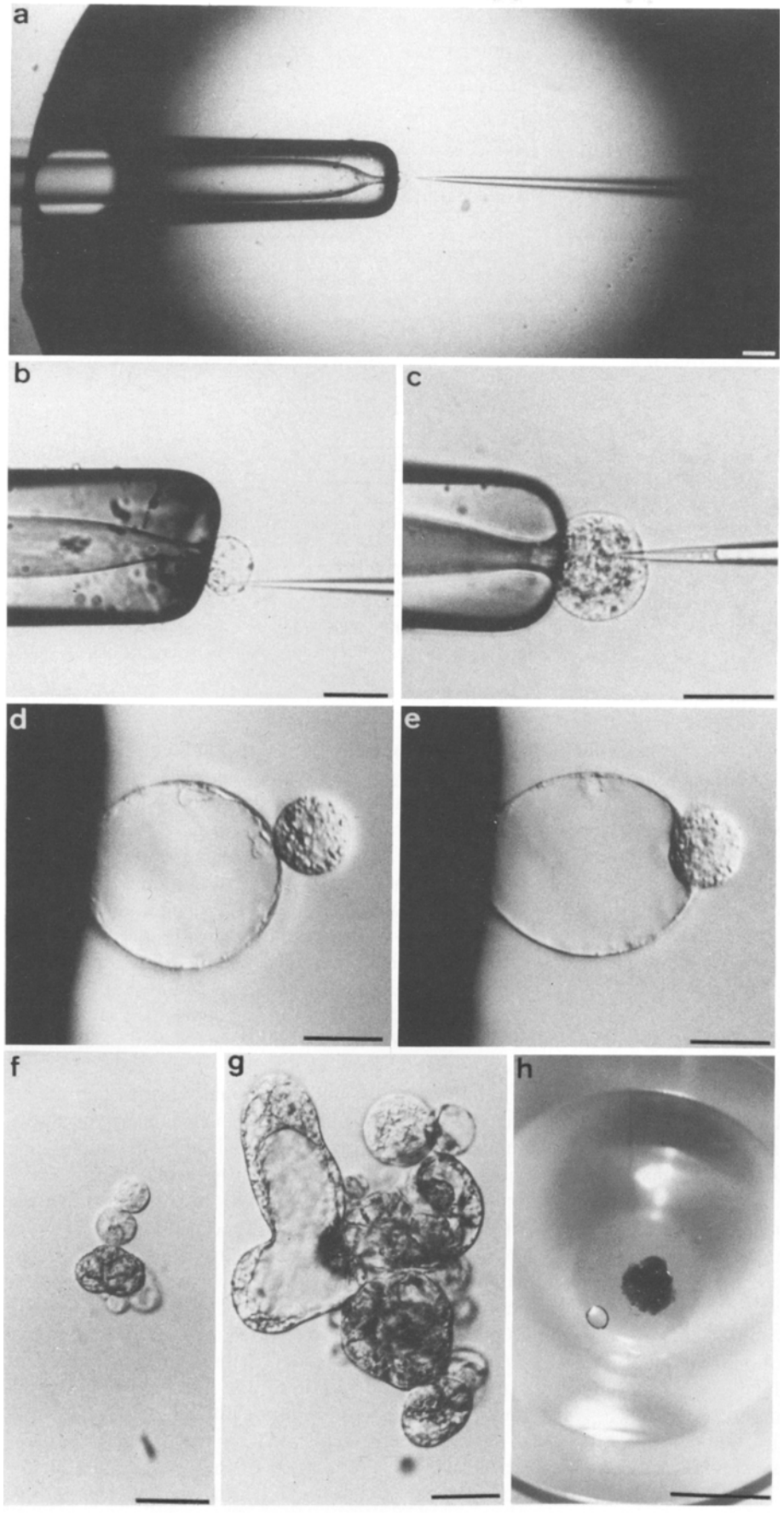

Fig. 16 a–h. Microinjection, electrofusion mediated cell reconstruction and callus formation (Spangenberg 1986). **a** A microinjection needle and a holding capillary fixing a selected hypocotyl protoplast of *B. napus* by negative pressure. Bar = 200 μm. **b** Intranuclear microinjection of a hypocotyl protoplast. Bar = 100 μm. **c** Microinjection of a karyoplast. Bar = 10 μm. **d, e** Electrofusion mediated cell reconstruction from a microinjected karyoplast and a cytoplast. **d** Before application of the membrane breakdown dc-pulse. **e** Beginning of the rounding off after dc-pulse application. Bars = 10 μm. **f–h** Development of microinjected reconstructed cells in microculture. **f** Multiple divisions after 6 days of individual culture. Bar = 100 μm. **g** 2 week-old microcallus formed in microcultured. Bar = 50 μm. **h** 1 month-old microcallus growing in a Terasaki dish. Bar = 1 mm

Table 4. Microinjection of individually selected protoplasts, karyoplasts and cell reconstructions with microinjected karyoplasts

Microinjected cell or fragment	Survivors (%)[a]
Protoplast	82±6
Karyoplast	83±8
Karyoplast + cytoplast	22±9

[a] Mean values ± standard error from 8 independent experiments are given

jected karyoplast is capable of regenerating cytoplasm and cell wall rather soon after microinjection and also of undergoing cell division and therefore behaves like a microinjected protoplast (Figs. 15 and 16). The major difference is that intranuclear microinjection into a karyoplast is much easier than into a protoplast (Spangenberg et al. 1986 b).

5 Computer-aided control of the set-up

Depending on the different types of manipulation and cells the overall yield, i.e. the percentage of manipulated cells that are regenerated into morphogenetically active calli, is in the range of 1 to 50% or more (Koop et al. 1983 a; Koop et al. 1983 b; Koop and Schweiger 1985 a, b; Spangenberg et al. 1985; Spangenberg and Schweiger 1986; Spangenberg et al. 1986 a; Spangenberg et al. 1986 b). This means that under unfavourable conditions at least 100 trials must be performed in order to attain one or more successful events. At first glance this low rate does not seem to justify applying this method since the individual cell microculture technique is rather laborious.

To extend the applicability of the set-up to events with a yield range of 10^{-3}, or even less, efforts were made to automate the set-up (Figs. 17 and 18) (Spangenberg 1986). This was achieved in the following way. The microculture chambers are prepared by means of a computer-directed x/y-axis motor driven microscope stage. An Apple II microprocessor is programmed so that any given position and sequence of steps can be adjusted. This means that on the coverslip, for examples, 50 microdroplets are placed automatically at predetermined positions in 5 rows with 10 microdroplets in one row. Moreover, the whole set-up is constructed in a way that positions not only on one but on 6 such coverslips can be adjusted successively without any intervention by the experimentalist.

A microcapillary integrated into the set-up can be moved up and down along the z-axis and is also driven by a computer-controlled motor. The microcapillary is

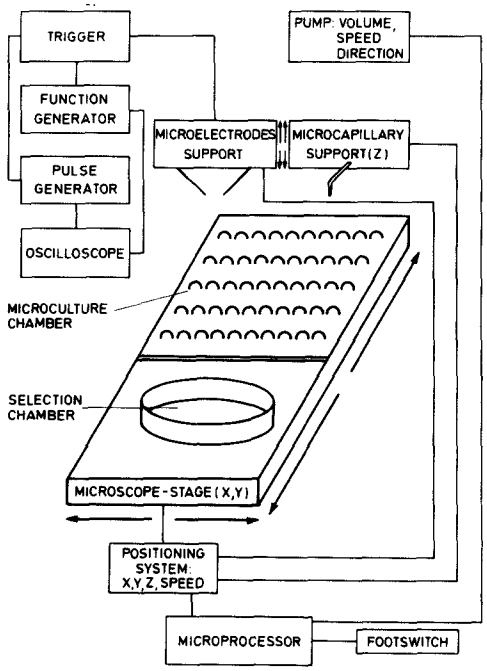

Fig. 17. Set-up for microprocessor-controlled selection, transfer and electrofusion of protoplasts (Spangenberg 1986)

combined with a computer-directed pump which can be programmed with respect to volume, speed and direction of the pumping. With these features the set-up is capable of performing automatically all the different steps involved in preparing the microculture chamber. The set-up can, for example, be programmed to adjust a given position, lower the microcapillary, make the pump release a predetermined volume with a given speed, lift the microcapillary and adjust the microcapillary to the next position. This means that the set-up can automatically place droplets of sucrose solution, mineral oil and medium in predetermined positions on the coverslips.

The set-up also has additional features helpful for selecting and transferring cells. This is achieved under optical control by optionally connecting the microscope with a TV-camera which transmits the microscopical image to a screen. In combination with the screen the selection chamber can be adjusted with a joystick under optical control and focussed at a selected cell on crosshairs. The adjustment of the microscope stage does not only mean that the selected cell can be moved to the crosshairs position on the screen, but also, that in this position the microcapillary is just above the cell. This can be moved up and down and in connection with the pump it can be used for taking up and releasing a cell in a computer-controlled way. Similar to the steps described for the preparation of the microculture chamber, the x/y-axis-positioning can alternatively be per-

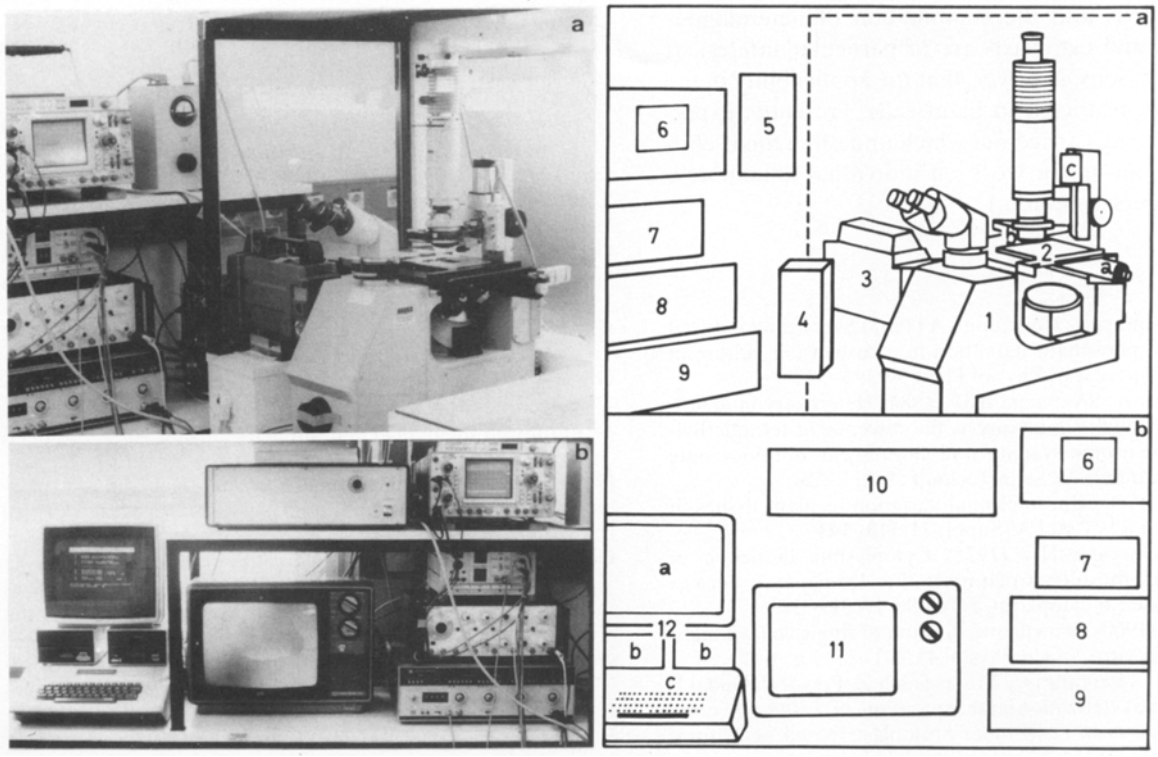

Fig. 18a, b. Components of the set-up. 1) Inverted microscope; 2) computer-controlled microscope stage with x, y, z-stepmotors (a, b, c); 3) colour-video-camera; 4) computer-controlled pump; 5) power supply for microscope lamp; 6) oscilloscope; 7) trigger-electronics for electrofusion-set up; 8) sinewave-generator 9) pulse-generator; 10) controll-electronics for positioning-system; 11) video-colour-monitor; 12) microprocessor Apple II (a) monitor; (b) disk drives; (c) keyboard (Spangenberg 1986)

formed by computer-directed movements of the microscope stage to a preprogrammed position. After the cell preselected on the screen is taken up by the microcapillary, the microscope stage automatically moves the microculture chamber to a predetermined position, the microcapillary still under computer control is lowered and the pump is activated so that the cell is injected into the correct microdroplet. In the next computer-controlled step the microcapillary is lifted and the stage is moved back to the selection chamber where the whole process is restarted again (Figs. 17 and 18).

In order to speed up the fusion process the electrofusion set-up is also integrated into the computer-directed system. In a first step the distance betweeen the microelectrodes is manually adjusted for the type and size of cells or fragments to be fused. Under the control of the TV-monitor the cells are brought into the cross-hairs labeled position. The support for the microelectrodes is lowered along the z-axis by computer control, the ac-field for dielectrophoresis is switched on and the membrane breakdown dc-pulse is manually triggered. After the electrofusion step is finished the support for the microelectrodes is lifted, the stage is moved to the next position and the set-up is ready for fusing a new pair of cells or fragments (Spangenberg 1986).

With this set-up it is possible to handle a high number of cells or subcellular fragments within a short time. To give an idea of what the speed is with which all these processes are performed it should be mentioned that the pretreatment of the coverslips with the sucrose solution needs less than 20 min for 100 droplets. Approximately the same time is needed for preparing the microdroplets of mineral oil and injecting the culture medium into them. All these steps are performed automatically with a stand-alone function of the system. Preselected pairs of protoplasts in microdroplets can be fused at 10 sec intervals, while protoplasts or karyoplasts can be microinjected with a rate of 2/min.

6 Synopsis

This article intends to review the methodology of the single cell microculture and related techniques. This methodology has proven to be extremely useful for approaching a number of problems of plant cell biology. Among others, studies on the mutual influence of cells with different morphological features on cell division, the microinjection of karyoplasts and the fusion of a karyoplast and a cytoplast in order to obtain transfor-

mation of cells and the combination of heterologous karyoplasts and cytoplasts are of particular interest. It should be obvious, however, that the applicability of the set-up is not restricted to plant cells. Presently, experiments are being carried out which underline the usefulness of the set-up for work on individual animal cells (O. Walla, pers. commun.).

References

Amino S, Fujimura T, Komamine A (1983) Synchrony induced by double phosphate starvation in a suspension culture of *Catharanthus roseus*. Physiol Plant 59:393–396

Barsby T, Yarrow SA, Shepard JF (1984) Heterokaryon identification through simultaneous fluorescence of tetramethylrhodamine isothiocyanate and fluorescein isothiocyanate labelled protoplasts. Stain Technol 59:217–220

Bayliss MW (1980) Chromosomal variation in plant tissues in culture. Int Rev Cytol A (Suppl) 11:113–144

Berger S, Schweiger HG (1975) Cytoplasmic induction of changes in the ultrastructure of the *Acetabularia nucleus* and perinuclear cytoplasm. J Cell Sci 17:519–529

Bergmann L (1960) Growth and division of single cells of higher plants in vitro. J Gen Physiol 43:841–851

Bergounioux C, Perennes C, Tabaeizadeh Z, Prevot P, Gadal P (1983) Characterization and separation of *Petunia hybrida* and *Lycopersicon esculentum* protoplasts based on natural fluorescence or FITC added to label antibodies in immunoreaction with the protoplast membrane. In: Potrykus I, Harms CT, Hinnen A, Huetter R, King PJ, Shillito RD (eds) Protoplasts, experienta supplementum, vol 5. Birkhäuser, Basel, pp 262–263

Braun AC (1959) A demonstration of the recovery of the crowngall tumor cell with the use of complex tumors of single-cell origin. Proc Natl Acad Sci USA 45:932–938

Cairns E, Sarkar S, Schweiger HG (1978a) Translation of tobacco mosaic virus RNA in *Acetabularia* cell cytoplasm. Cell Biol Int Rep 2:573–578

Cairns E, Gschwender HH, Primke M, Yamakawa M, Traub P, Schweiger HG (1978b) Translation of animal virus RNA in the cytoplasm of a plant cell. Proc Natl Acad Sci USA 75:5557–5559

Cairns E, Doerfler W, Schweiger HG (1978c) Expression of a DNA animal virus genome in a plant cell. FEBS Lett 96:295–297

Carlson PS, Smith HH, Dearing RD (1972) Parasexual interspecific plant hybridization. Proc Natl Acad Sci USA 69:2292–2294

Chu YE, Lark KG (1976) Cell cycle parameters of soybean (*Glycine max* L.) cells growing in suspension culture: Suitability of the system for genetic studies. Planta 132:259–268

Cocking EC (1960) A method for the isolation of plant protoplasts and vacuoles. Nature 187:962–963

Constabel F, Kurz WGW, Chatson KB, Kirkpatrick JW (1977) Partial synchrony in soybean cell suspension cultures induced by ethylene. Exp Cell Res 105:263–268

Constabel F, Rambold S, Chatson KB, Kurz WGM, Kutney JP (1981) Alkaloid production in *Catharanthus roseus* (L.) G. Don. 6. Variation in alkaloid spectra of cell lines derived from one single leaf. Plant Cell Rep 1:3–5

Cress AE, Gerner EW (1979) Hydroxyurea inhibits ODC induction, but not the G1 to S-phase transition. Biochem Biophys Res Commun 87:773–780

De Ropp RS (1955) The growth and behaviour in vitro of isolated plant cells. Proc R Soc London, Ser B 144:86–93

Dougall DK, Johnson JM, Whitten GH (1980) A clonal analysis of anthocyanin accumulation by cell cultures of wild carrot. Planta 149:292–297

Ellis BE (1985) Characterization of clonal cultures of *Anchusa officinalis* derived from single cells of known productivity. J Plant Physiol 119:149–158

Evans DA, Bravo JE, Gleba YY (1983) Somatic hybridization: fusion methods, recovery of hybrids, and genetic analysis. Int Rev Cytol (Suppl) 16:143–159

Gleba YY (1978) Microdroplet culture – tobacco plants from single mesophyll protoplasts. Naturwissenschaften 65:158–159

Gleba YY, Hoffmann F (1980) *"Arabidobrassica"*: a novel plant obtained by protoplast fusion. Planta 149:112–117

Gleba YY, Sytnik KM (1984) Protoplast fusion – Genetic engineering in higher plants. In: Shoeman R (ed) Monographs on theoretical and applied genetics, vol 8. Springer, Berlin Heidelberg New York Tokyo

Glimelius K (1984) High growth rate and regeneration capacity of hypocotyl protoplasts in some Brassicaceae. Physiol Plant 61:38–44

Gould AR, Everett NP, Wang TL, Street HE (1981) Studies on the control of the cell cycle in cultured plant cells. 1. Effects of nutrient limitation and nutrient starvation. Protoplasma 106:1–13

Guri A, Zelcer A, Izhar S (1984) Induction of high mitotic index in Petunia suspension cultures by sequential treatment with aphidicolin and colchicine. Plant Cell Rep 3:219–221

Haberlandt G (1902) Kulturversuche mit isolierten Pflanzenzellen. Sitzungsber Kaiserl Akad Wiss Wien. Math.-Naturwiss. Kl. 111, Abt 1:69–91

Hämmerling J (1932) Entwicklung und Formbildungsvermögen von *Acetabularia mediterranea*. 2. Das Formbildungsvermögen kernhaltiger und kernloser Teilstücke. Biol Zentralbl 52:42–61

Hämmerling J (1955) Neuere Versuche über Polarität und Differenzierung bei *Acetabularia*. Biol Zentralbl 74:545–554

Jones LE, Hildebrandt AC, Riker AJ, Wu JH (1960) Growth of somatic tobacco cells in microculture. Am J Bot 47:468–475

Kant U, Hildebrandt AC (1969) Morphology of edible plant cells and tissues in vitro. Can J Bot 47:849–852

Kao KN (1977) Chromosomal behaviour in somatic hybrids of soybean-*Nicotiana glauca*. Mol Gen Genet 150:225–230

Kinnersley AM, Dougall DK (1982) Variation in nicotine content of tobacco callus cultures. Planta 154:447–453

Koop HU, Schweiger HG (1985a) Regeneration of plants from individually cultivated protoplasts using an improved microculture system. J Plant Physiol 121:245–257

Koop HU, Schweiger HG (1985b) Regeneration of plants after electrofusion of selected pairs of protoplasts. Eur J Cell Biol 39:46–49

Koop HU, Weber G, Schweiger HG (1983a) Individual culture of selected single cells and protoplasts of higher plants in microdroplets of defined media. Z Pflanzenphysiol 112:21–34

Koop HU, Dirk J, Wolff D, Schweiger HG (1983b) Somatic hybridization of two selected single cells. Cell Biol Int Rep 7:1123–1128

Melchers G, Sacristan MD, Holder AA (1978) Somatic hybrid plants of potato and tomato regenerated from fused protoplasts. Carlsberg Res Commun 43:203–218

Mizukami H, Konoshima M, Tabata M (1978) Variation in pigment production in Lithospermum erythrorhizon callus cultures. Phytochemistry 17:95–97

Muir WH, Hildebrandt AC, Riker AJ (1954) Plant tissue cultures produced from single isolated cells. Science 119:877–878

Muir WH, Hildebrandt AC, Riker AJ (1958) The preparation, isolation and growth in culture of single cells from higher plants. Am J Bot 45:589–597

Neuhaus G, Neuhaus-Url G, Schweiger HG, Chua NH, Broglie R, Coruzzi G, Chu N (1983) Expression of higher plant genes in the green alga Acetabularia mediterranea. Eur J Cell Biol (Suppl) 2:30

Neuhaus G, Neuhaus-Url G, Gruss P, Schweiger HG (1984) Enhancer-controlled expression of the Simian virus 40 T-antigen in the green alga *Acetabularia*. EMBO J 3:2169–2172

Neuhaus G, Neuhaus-Url G, de Groot E, Schweiger HG (1986) High yield and stable transformation of the unicellular green alga *Acetabularia* by microinjection of SV40 DNA and pSV2neo. EMBO J 5:1437–1444

Partanen CR (1963) Plant tissue culture in relation to developmental cytology. Int Rev Cytol 15:215–243

Patnaik G, Cocking EC, Hamill J, Pental D (1982) A simple procedure for the manual isolation and identification of plant heterokaryons. Plant Sci Lett 24:105–110

Power JB, Cummins SE, Cocking EC (1970) Fusion of isolated protoplasts. Nature 225:1016–1018

Reinert J (1963) Growth of single cells from higher plants in synthetic media. Nature 200:90–91

Schell J, Van Montagu M, Willmitzer L, Leemans J, Deblaere R, Joos H, Inze D, Wöstemeyer A, Otten L, Zambryski P (1984) Transfer of foreign genes to plants and its use to study development processes. In: Beers RF Jr, Bassett EG (eds) Cell fusion: gene transfer and transformation. Raven Press, New York, pp 113–128

Schreier PH, Seftor EA, Schell J, Bohnert HJ (1985) The use of nuclear-encoded sequences to direct the light-regulated synthesis and transport of a foreign protein into plant chloroplasts. EMBO J 4:25–32

Schweiger HG, Berger S (1979) Nucleo cytoplasmic interrelationships in *Acetabularia* and some other Dasycladaceae. Int Rev Cytol (Suppl) 9:12–44

Schweiger HG, Neuhaus G (1986) Induction of expression in and stable transformation of an algal cell by nuclear microinjection with naked DNA. Plant Gene Res, vol IV, Springer, Wien (submitted)

Schweiger HG, Masters RWP, Werz G (1967) Nuclear control of a cytoplasmic enzyme in Acetabularia. Nature 216:554–557

Schweiger HG, Bannwarth H, Berger S, de Groot E, Neuhaus G, Neuhaus-Url G (1984) Interactions between compartments in Acetabularia during gene expression. In: Wiessner W, Robinson D, Starr RC (eds) Compartments in algal cells and their interactions. Springer, Berlin Heidelberg New York Tokyo, pp 28–35

Spangenberg G (1986) Manipulation individueller Zellen der Nutzpflanze *Brassica napus* L. mit Hilfe von Elektrofusion, Zellrekonstruktion und Mikroinjektion. Thesis, Universität Heidelberg (FRG)

Spangenberg G, Schweiger HG (1986) Controlled electrofusion of different types of protoplasts including cell reconstitution in *Brassica napus* L. Eur J Cell Biol 41:51–56

Spangenberg G, Koop HU, Schweiger HG (1985) Different types of protoplasts from *Brassica napus* L.: analysis of conditioning effects at the single cell level. Eur J Cell Biol 39:41–45

Spangenberg G, Koop HU, Lichter R, Schweiger HG (1986a) Microculture of single protoplasts of *Brassica napus*. Physiol Plant 66:1–8

Spangenberg G, Neuhaus G, Schweiger HG (1986b) Expression of foreign genes in a higher plant cell after electrofusion-mediated cell reconstitution of a microinjected karyoplast and a cytoplast. Eur J Cell Biol 42:236–238

Sree Ramulu K, Dijkhuis P, Röst S, Bokelmann GS, de Groot B (1984) Early occurrence of genetic instability in protoplast cultures of potato. Plant Sci Lett 36:79–86

Takebe I, Labib E, Melchers G (1971) Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. Naturwissenschaften 58:318–320

Torrey JG (1957) Cell division in isolated single cells in vitro. Proc Natl Acad Sci USA 43:887–891

Vasil V, Hildebrandt AC (1965) Differentiation of tobacco plants from single, isolated cells in microcultures. Science 150:889–892

Wallin A, Glimelius K, Eriksson T (1978) Enucleation of plant protoplasts by cytochalasin B. Z Pflanzenphysiol 87:333–340

Weber G, de Groot E, Schweiger HG (1986) Synchronization of protoplasts from *Glycine max* (L.) Merr. and *Brassica napus* (L.). Planta 168:273–280

Zimmermann U (1982) Electric field-mediated fusion and related electrical phenomena. Biochim Biophys Acta 694:227–277