

DAUGHTER 3T3 CELLS Are They Mirror Images of Each Other?

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ABSTRACT

Using a new technique to visualize the tracks of moving 3T3 cells and combining it with the visualization of actin-containing microfilament bundles by indirect immunofluorescence (Lazarides, E. and K. Weber. 1974. *Proc. Natl. Acad. Sci. U. S. A.* 71:2268–2272), I present experiments which suggest that: (a) 30–40% of the pairs of daughter 3T3 mouse fibroblasts in noncloned cultures have mirror symmetrical actin-bundle patterns. (b) The angle between separating daughter cells is approx. 90° or 180° and seems related to the directions of certain actin-containing bundles. (c) Approximately 40% of separately moving daughter cells which did not collide with any other cell in the culture performed directional changes in a mirror symmetrical way. Both daughter cells entered the next mitosis at approximately the same time.

I suggest that the actin-bundle pattern, the angle of separation, major directional changes during interphase, and the time of the next mitosis are predetermined by the parental cell.

Considering only the contents of two daughter cells produced by normal mitosis in an established cell line, one would expect them to be identical. This identity in content, however, does not make the cells identical twins. Only after also considering the spatial arrangement of the contents may we identify them as identical twins, as mirror images of each other, or as two randomly packed assemblies of those contents.

Judging by the perfect mirror symmetry of the mitotic spindle, one may suspect that during mitosis other cellular components are likewise mirror-symmetrically distributed between the two compartments which are to become the daughter cells (Fig. 1). Such perfection of mirror symmetry in cells which will soon initiate locomotion and pronounced shape changes can hardly be expected to persist for long.

Yet, if not the perfection of symmetry, at least the left- or right-handedness of the arrangements

of the cellular components may persist for a considerable length of time after mitosis. A left-handed three-dimensional cellular arrangement cannot become right-handed by displacements, rotations, or continuous deformations of the cell as a whole. Such operations would merely make it more difficult for the observer to recognize the topological relationship between the cells. The "handedness" can only be destroyed by randomization or the duplication of all components which occurs in cells preparing themselves for the next mitosis. A change to the opposite handedness would require complex individual movements of certain components. In the following, I will use the term "mirror symmetry between daughter cells" in the more general sense of the simultaneous occurrence of one left- and one right-handed cell after mitosis.

If the mirror symmetry between daughter cells would indeed persist for a considerable portion of

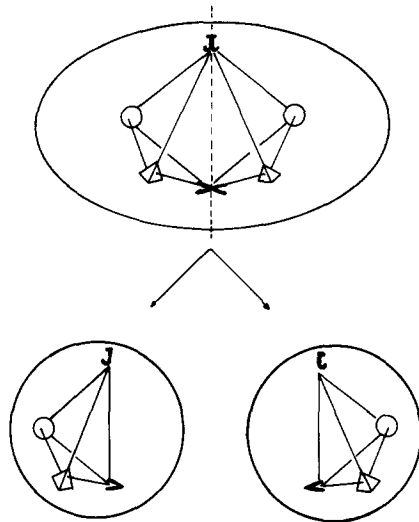


FIGURE 1 Schematic of the way in which mitosis may produce mirror symmetrical intracellular organizations between the two daughter cells. V's and J's symbolize sister chromatids, the small spheres centrioles, and the small tetrahedron represents an unspecified cytoplasmic component. After cleavage, one daughter cell has a right-handed, the other a left-handed, three-dimensional intracellular organization.

their cell cycles, it may have important implications. One may begin to look for a relationship between the universally found bilateral symmetry of organisms and the mirror symmetry between certain daughter cells at an early state of an embryo. Left or right-handedness may also be the simplest way by which nature primes one of the daughter cells, if only one of them is supposed to enter a certain pathway of differentiation.

In this paper, I present arguments for the mirror symmetry between daughter 3T3 cells and then consider a third implication which is based on the following argument: if the two daughter cells after spatial separation and without external cause still perform certain actions in mirror symmetrical (or identical) ways, then those actions, may have been predetermined at mitosis. Using this reasoning, I suggest that locomotion in 3T3 mouse fibroblasts is not so random as it appears (6) but is predetermined in various aspects.

MATERIALS AND METHODS

Cell Cultures

Swiss/3T3 cells (8) were a kind gift from Dr. Howard Green (Massachusetts Institute of Technology, Cambridge, Mass.). The cells were used in the experiments

within 1 mo after thawing. Culture conditions were those described elsewhere (4).

Gold Particle Preparation and Coating of Glass Cover Slips

These methods have been described elsewhere (3, 4).

Preparation of Experimental Cells

1-2 days after the last passage, cells were freshly suspended in phosphate-buffered saline (PBS) solution containing 0.5 mM EDTA and 0.05% trypsin. They were plated onto gold particle-coated glass cover slips (Corning Glass Works, Science Products Div., Corning, N.Y., cover glass no. 1 $\frac{1}{2}$, 22 \times 22 mm²) in 3.5-cm Falcon plastic dishes (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) containing 2 ml of culture medium with 10% calf serum and were left in the incubator for one or several days. After fixation in 3.5% formaldehyde in PBS, the inverted cover slips were placed on a drop of Elvanol (DuPont Instruments, Wilmington, Del.) on a microscope slide and examined in epi-dark-field illumination in a Zeiss photomicroscope II. Pictures were taken on Kodak Plus-X 35 mm film.

Indirect Immunofluorescence

Antibody against gizzard actin was a kind gift from Dr. Keith Burrigge (Cold Spring Harbor Laboratory). Gold particle-coated or plain glass cover slips with cells were treated according to the following protocol: at room temperature, 30-min fixation in 3.5% formaldehyde in PBS, 1 min in PBS, 1 min in a 1:1 acetone/water mixture, 5 min in acetone, 1 min in a 1:1 acetone/water mixture, and 1 min in PBS; and at 37°C, 30-min incubation with 100 μ l of 1:30 diluted antiactin-serum at saturated humidity, five times washing in PBS, and 30-min incubation in 100 μ l of 1:10 diluted fluorescein-isothiocyanate labeled goat-antirabbit serum (37°C; saturated humidity). The cells were observed in epifluorescence in a Zeiss photomicroscope II (objective lens: 63 \times ; NA 1.4). Pictures were taken on Kodak Tri-X 35 mm film.

RESULTS

Actin Patterns of Adjacent Daughter 3T3 Cells

In approaching the question of whether cytoplasmic structures can be mirror-symmetrically distributed in daughter 3T3 cells, I examined the patterns of actin-containing microfilament bundles in indirect immunofluorescence. I chose this technique, which was recently developed by Lazarides and Weber (7), because it reveals complex and characteristic cytoplasmic structures which span the whole cell body and allows a fast screening of a large number of cells. In order to facilitate the

finding of daughter cells, I chose 4–6 day old cultures in which locomotion is greatly reduced, thus leaving many daughter cells close to each other. Similarities in cell shape and in pattern of nucleoli between both cells were further criteria to identify daughter cells.

Examination of 62 such pairs of adjacent

daughter cells showed that 47% of them had identifiable elements of mirror symmetry (Fig. 2*b–d*), 19% of the pairs appeared identical in patterns (Fig. 2*a*), and in 34% I failed to recognize any relationship between their microfilament patterns.

In several cases, photographs had to be rotated

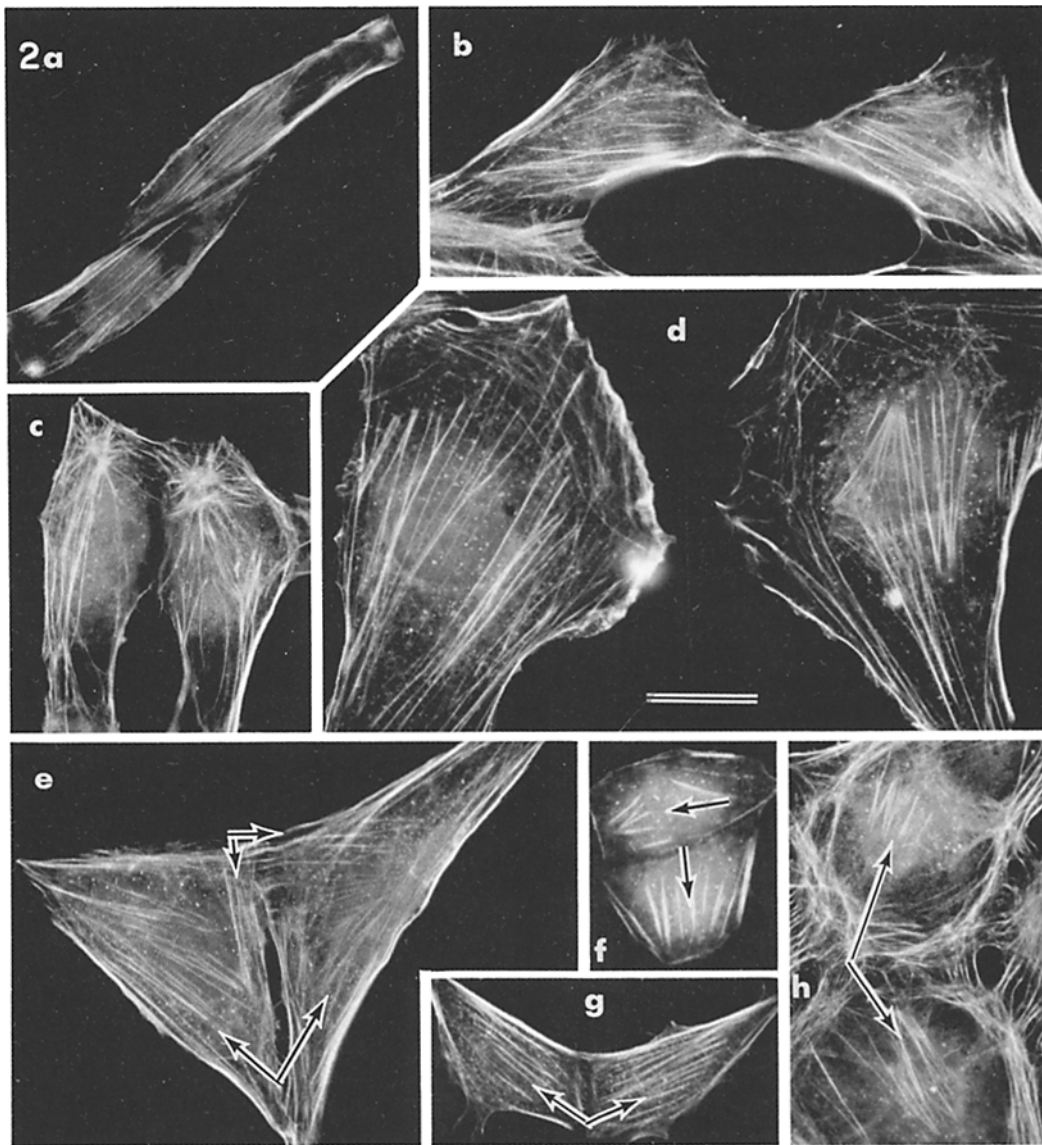


FIGURE 2 Actin patterns in adjacent daughter 3T3 cells. Bar indicates 20 μm . I interpret the patterns in the following way: (*a*) Identical pattern. (*b–d*) Mirror symmetrical patterns. (One cell in (*d*) was rotated by 90° relative to the other.) (*e* and *f*) Cases where a right angle orientation of certain conspicuous bundles (arrows) interferes with the development of mirror symmetry (or identity) of the patterns. (*g* and *h*) Cases where an angle of about 120° between conspicuous bundles (arrows) is compatible with mirror symmetry. Bar, 20 μm .

relative to each other in order to better recognize the topological relationship. It appeared that the necessary angle of rotation was 80°–120° (Fig. 2*d*) or 180° (Fig. 2*a*). These angles were also found to be formed between pronounced fiber bundles of daughter cells which had not yet separated completely. Figure 2*a* and *b* shows examples of a 180° angle between conspicuous bundles, and Fig. 2*e*–*h* shows examples of 80°–120° angles. In cases such as Figs. 2*e* and *f*, the right-angle orientation seemed to interfere with the development of symmetrical patterns, whereas in cases such as Fig. 2*d*, *g*, and *h* this orientation is combined with the mirror symmetry. Fig. 2 also shows that various different patterns of actin-containing bundles can be found in cultures of uncloned cells (see also Fig. 6). Therefore, the occurrence of symmetrical or identical patterns is not trivial in the sense that all cells in such 3T3 cultures have similar patterns anyway.

Phagokinetic Tracks of 3T3 Cells

In this section, I wish to briefly introduce a new assay for cellular locomotion which will be described in detail in a future publication. I will use it in the following sections to add a further aspect to the concept of mirror symmetry between daughter cells.

If freshly suspended cells are plated on top of a gold particle-coated cover slip, they produce various surface protrusions and remove the particles within a ring around each cell during the 1st h (3). This phenomenon can be used to quantitate the motile activity of such protrusions under various extracellular conditions (4). Within the following 10 h, the cells begin to move while cleaning more particles out of their way. The particles become partly internalized and partly accumulate on the cell surface in big clumps (Fig. 3*c*) which are pinched off once in a while and can be found floating in the medium.

In order to distinguish locomotion on plain surfaces from this combination of phagocytosis and cellular displacement, I suggest the use of the term “phagokinetics.” The particle-free tracks (phagokinetic tracks) can be conveniently visualized in darkfield illumination as black lines (Fig. 3*a*).

I examined various different cell lines in this assay and found quite different track patterns. Fig. 3*a* and *b* illustrates the difference in patterns between 3T3 cells and the poorly moving epithe-

lial BSC-1 cells. The nontoxicity of the exposure of 3T3 cells to the gold particles has been mentioned in previous publications (4, 5).

Phagokinetic Tracks of Daughter

3T3 Cells

Branching isolated tracks with the cells located at the ends of each branch indicate mitosis and subsequent separate movement of both daughter cells (Fig. 4). There is a slight chance that one cell's crossing the beginning of the track of another cell produces a similar branching pattern. However, in sparse cultures this event is very unlikely.

More than 48 h after plating of the cells, I found branching tracks with further branching at each end (Fig. 4*a*, *b*, *c*, and *m*), showing the four descendants of the cell which started the track. In cultures which are dense enough to allow cell division at all, the occurrence of such tracks is rare, because it is unlikely that none of the four second-generation cells will collide with another cell or cross another cell's track. Therefore, I found only 12 such cases in our preparations. In two cases, only one of the first-generation daughter cells had divided a second time. The observed simultaneous occurrence of all four second-generation cells (cf. also Fig. 4*d* and *e*) suggests that in cells which did not collide with or cross the track of others, the length of the cell cycle is the same in both daughter cells.

I examined 53 first-generation mitotic tracks of cells which did not collide with or cross the track of another cell. The various tracks were quite different in shape (see Fig. 4). Thus, any observed symmetry relationships between the two branches of a mitotic track cannot be called trivial, arguing that most tracks would look alike anyway.

I found the following results concerning the symmetry of tracks and the split angle between the branches. 40% of such tracks showed mirror symmetry in the sense that one daughter cell made directional changes opposite to the other at approximately the same positions of their respective tracks (see Fig. 4). In the following, I will call them “symmetrical tracks.” 14% of the tracks were identical in the sense that both daughter cells made the same directional changes relative to their direction of movement (Fig. 4*e* and *i*). I will call them “identical tracks.” The remaining 47% could not be determined, because the branches either showed no directional change (Fig. 4*g*) or were overly distorted (Fig. 4*m*).

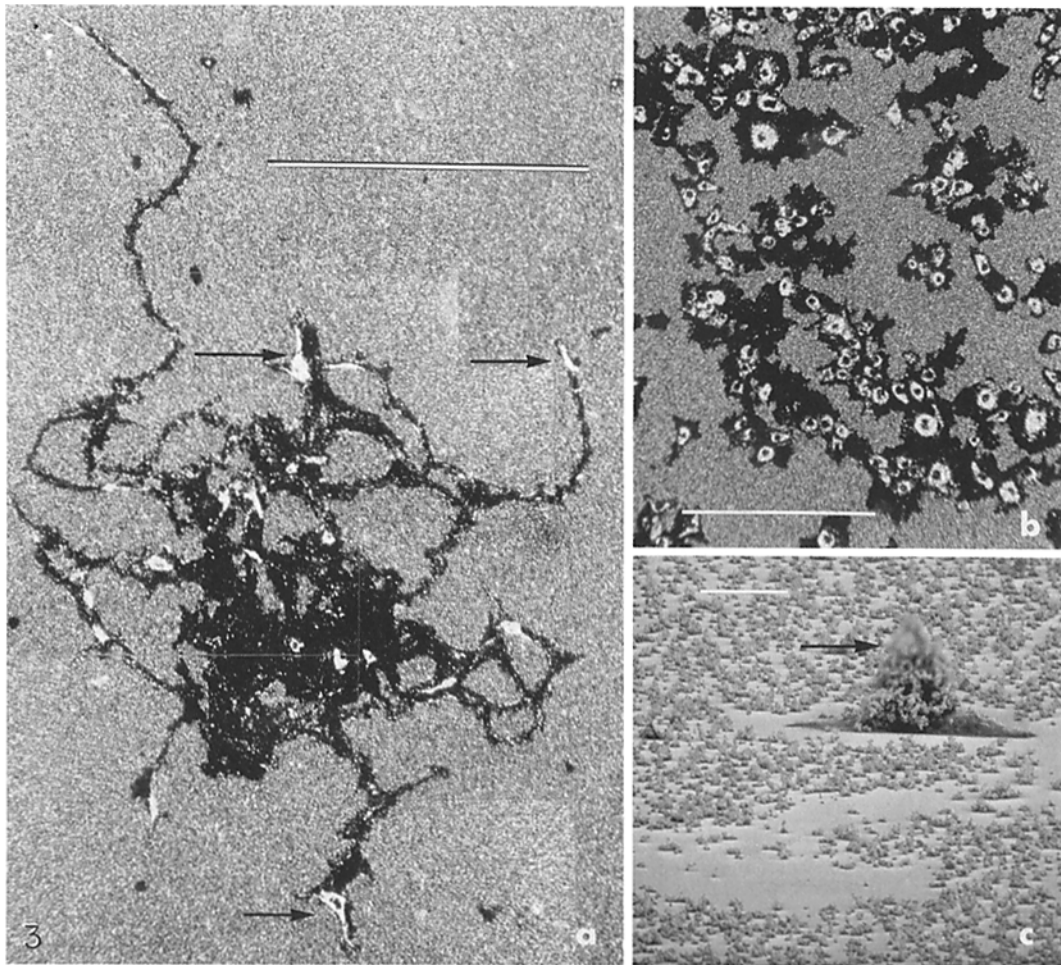


FIGURE 3 Phagokinetic tracks. (a) Tracks which 3T3 cells (arrows point to the clusters of accumulated gold particles inside and on top of the cells) developed during 9 days in a very sparsely plated culture. (Darkfield illumination; composite of several frames). Bar, 1 mm. (b) Phagokinetic tracks produced within 48 h by the nonmoving epithelial BSC-1 cells. The particles around the cells are mostly cleared away by surface protrusions even during the 1st h after plating. Bar, 0.5 mm. (c) Scanning electron micrograph of a 3T3 cell and its phagokinetic track 48 h after plating. The particles which are cleared out of the way by the moving cell are partly phagocytosed, partly accumulated on the cell's surface (arrow). Those clumps will be pinched off once in a while. Tilt angle: 81° . Bar, $20 \mu\text{m}$.

As to the split angle between the branches, again I found the two ranges of 80° – 120° (Fig. 4 *a–h* and *k*) or 180° (Fig. 4 *i* and *l–n*). Figure 5 shows a histogram of the split angles assorted in 10° intervals. About two-thirds of the observed angles were between 80° and 120° . This finding raises the suspicion that the angle between certain microfilament bundles of the two daughter cells may be related to the angle under which they move away

from each other.

The results suggest that in the majority of cases both daughter cells performed directional changes in mirror symmetrical or identical ways, even after spatial separation, provided the cells did not encounter any other cells. Furthermore, the split angle seems to assume one of only two possible values, and both daughter cells enter the next mitosis at the same time. Therefore, I feel that the

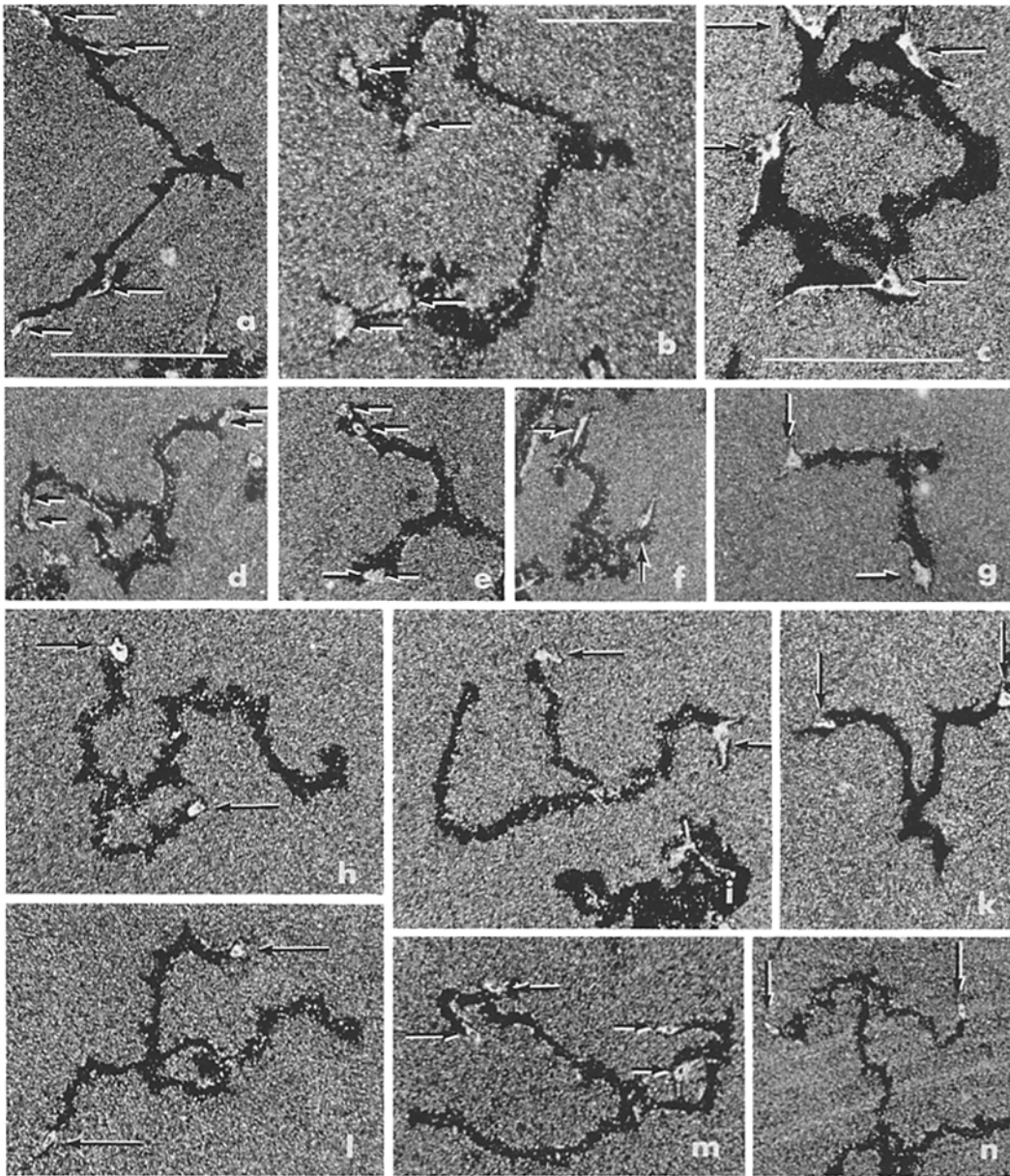


FIGURE 4 Phagokinetic tracks of mitotic 3T3 cells (48–72 h after plating in culture medium with 10% or 20% calf serum) suggesting mirror symmetrical (*a, b, c, d, f, h, k, l, and n*) or identical (*e and i*) directional changes of the cells even after spatial separation. Locations of the cells are indicated by arrows. Second-generation cells are shown in panels *a–e*, and *m*. Cases of indeterminable tracks are illustrated in panels *g* and *m*. Bar in panel *a* indicates 0.5 mm (*d–g, m, and n* have the same magnification); bar in panel *b* indicates 0.2 mm; bar in panel *c* indicates 0.5 mm (*h–l* have the same magnification).

future behavior of a cell is largely predetermined at mitosis. The accidental collision with another cell may influence future directional changes and the time of the next mitosis. Studies of “post-collision” tracks will have to decide this question.

Actin-Patterns of Daughter 3T3 Cells in Phagokinetic Tracks

The branching tracks of mitotic cells allow an easy identification of daughter cells, even after

spatial separation. Therefore, I examined daughter pairs by indirect immunofluorescence after they had formed branching phagokinetic tracks. These experiments were independent of those described above and, therefore, can be considered partly as an internal control.

The staining procedure did not affect the particle coat, nor did the particles adsorb any fluorescent antibody. In contrast to cells on gold particle-free substrates, such prepared cells showed some nuclear fluorescence, and larger numbers of elongated cell shapes were observed (see Fig. 4*a* and *f*, and Fig. 6*c* and *d*). I found a strong resemblance in the complex bundle patterns and shape characteristics of the daughter cells as shown in Fig. 6. This finding suggests that these patterns are likely to be predetermined by the parental cell.

As to the symmetry relationships between the patterns, in 42 daughter cell pairs examined, I found 34% symmetrical (Fig. 6*a* and *b*), 24% identical (Fig. 6*c*, *d*, *e*, and *f*), and 42% unrelated actin-bundle patterns. These percentages fit reasonably well the percentages observed in adjacent daughter cells.

One obvious question at this point is whether the symmetry relationship between the two branches of a mitotic track correlates with the symmetry relationship between the actin patterns of the cells at the end of each branch. Table I shows my attempt to answer this question by assorting each of the 42 examined cases according to its quality of tracks and actin pattern. Due to the cases of unrelated patterns and indeterminable tracks, the result is inconclusive. However, I did not find a case where a symmetrical actin-pattern was associated with an identical track or vice versa.

DISCUSSION

The reader will have noticed that in presenting my results I appealed to his or her visual symmetry perception and willingness to allow for deformations and omissions in the patterns to be compared. I also argued implicitly that some of the pairs of rather complex actin patterns or tracks are unlikely to be produced by accidental coincidences. I am aware that both the visual perception and feelings for probabilities are very deceptive. Yet, in presenting these data I hope to stimulate more discussion about a potentially relevant concept in animal cell biology.

I consider the symmetry relationship between the phagokinetic tracks of daughter 3T3 cells as

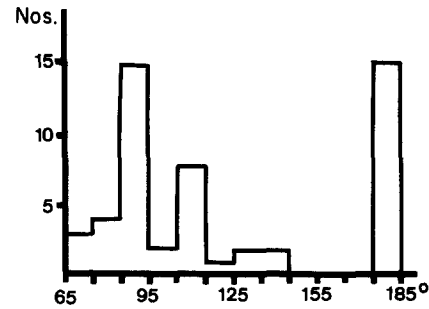


FIGURE 5 Histogram of the angles of separation between daughter 3T3 cells based on 53 measurements on branching phagokinetic tracks.

TABLE I

Actin patterns	Phagokinetic tracks		
	Symmetrical	Identical	Indeterminable
Symmetrical	10 (24)	0	4 (10)
Identical	0	5 (12)	5 (12)
Unrelated	6 (14)	2 (4)	10 (24)

Numbers (percentages are shown in parentheses) of daughter pairs of 42 examined cases where a certain symmetry relationship between the actin patterns of the daughter cells coincided with a certain symmetry relationship between the branches of the corresponding phagokinetic track.

the central subject of this paper. If the reader agrees that there are such relationships between the branches of mitotic tracks, then these relationships would be sufficient by themselves to imply predetermination of certain major cellular movements. On the other hand, one may take these observations as hints that in general the intracellular organization of one daughter 3T3 cell may be mirror imaged by the other. Seeking evidence for this suspicion, I examined the actin patterns of daughter cells.

Although I found numerous cases of mirror symmetry, there were also cases of identity and unrelatedness among the examined tracks and actin patterns. Consistently, I found about twice as many cases of mirror symmetry as of identity among the examined patterns and tracks. Therefore, if any of the two relationships between daughter cells holds in general, it is more likely the symmetry. The additional occurrence of identity and unrelatedness may not be surprising.

Both the tracks and actin-pattern images are merely two-dimensional expressions or projections of processes and structures inside the three-

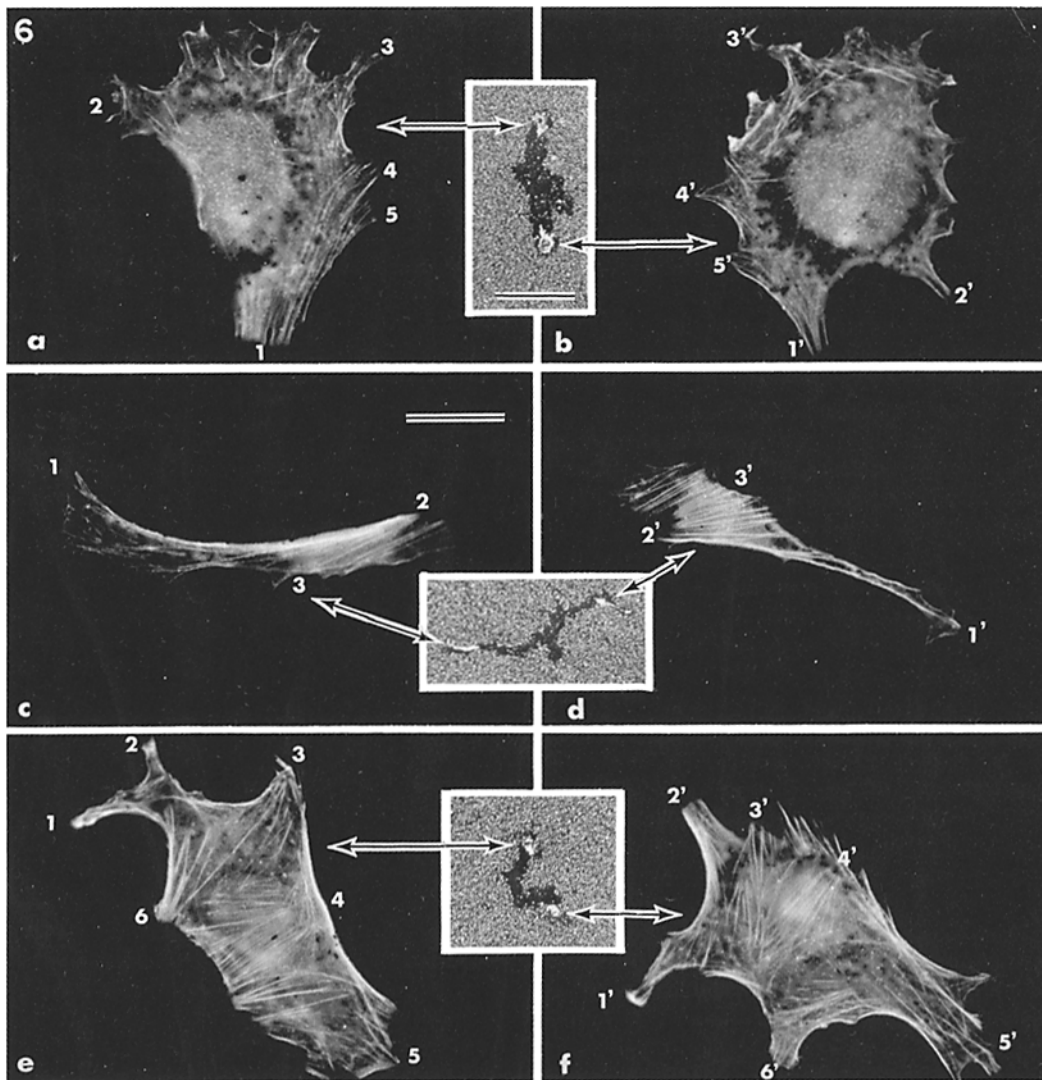


FIGURE 6 Actin patterns of daughter 3T3 cells in phagokinetic tracks. (Symmetry relationships between daughter cells after spatial separation.) The *insets* show the tracks formed by the same cells which are shown in indirect immunofluorescence. Bar in the *inset a* and *b* indicates $250\ \mu\text{m}$; bar in panel *c* indicates $20\ \mu\text{m}$. With the exception of the cell on panel *b*, which was rotated by 180° , the cells in the fluorescence micrographs are shown in the same orientation as on the *insets*. The numbers around the cell's periphery refer to shape characteristics which can be correlated in the daughter cells. They can also help the reader to follow corresponding fluorescent bundles inside the cells. My interpretation as to the symmetry is as follows: (*a* and *b*) symmetrical patterns; (*c* and *d*) identical patterns in elongated cells; and (*e* and *f*) identical patterns.

dimensional cells. A simple flipping over of one of the daughter cells during or after cleavage will change any two-dimensional projection of a cellular structure into its mirror image, although it cannot change the handedness of the three-dimensional cellular organization. Such flipping over

may occur during the action of the contractile ring. It is conceivable that the narrowing of the ring produces a torque. If one of the daughter cells is not well anchored to the substrate, e.g., it forms on top of the other daughter cell, the torque may rotate it. A flipped-over cell may then develop an

actin-pattern whose two-dimensional projection looks identical to that of the other daughter cell. Likewise, it may later perform directional changes identical to that of the other cell. The cases of unrelated patterns and tracks may be explained either by incomplete flipping over or by unknown influences on only one of the cells, e.g., by microscopic inhomogeneities of the substrate. Therefore, I feel that my results support the idea that the intracellular organizations of daughter 3T3 cells form mirror images of each other.

I do not know as yet how long the handedness of cells lasts, although it cannot last longer than to the next prophase, when all duplicated cellular components begin to separate. The mirror symmetric changes of the phagokinetic tracks seem to persist to about 75% of the track (see Fig. 4*b*) which suggests that the left- or right-handedness of the cells persists at least for about 18 h. The biological significance of the difference in handedness is not known either, although one may speculate about it as I did in the introduction to this paper.

The occurrence of split angles around both 90° and 180° raises the question of which of them is fundamental, i.e., preprogrammed. Unless both angles are equally permissible or characterize two different subpopulations, it seems that the 90° angle is more likely to be fundamental. If one or both daughter cells attempt to move at right angles into the fluid medium, adhesion and gravity may force them back to the substrate, thus opening the right angle into a 180° angle. The biological meaning of this right angle is not known, either. However, one may suspect that mitotic cells are programmed to move one of the daughter cells vertically out of a cell layer or a string of cells.

If cellular movements are indeed preprogrammed, the program does not seem to be a list of mandatory instructions to the moving cell. I have recently presented evidence suggesting that 3T3 cells are equipped with organs to explore the vicinity of the cell. These organs induce lamellar extensions upon contact with a favorable object (2), and the cells move towards this object. Therefore, if there are movement instructions for a cell, they can be overruled by an extracellular stimulus. The cellular movements during an accidental collision of two cells (1, 9, 10) seem to present another case where those instructions can be overruled and replaced by another sequence of possibly again preprogrammed movements.

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