

**Real-time high-resolution optical sectioning suggests biphasic
cytokinetic mechanism in *Dictyostelium discoideum***

Yoshio Fukui

Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL
60611-3008.

(Short Title) Biphasic Cytokinetic Mechanism in *Dictyostelium*

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(Correspondence) Dr. Yoshio Fukui, CMB, W8-132, Northwestern Medical School,
303 East Chicago Avenue, Chicago, IL 60611-3008.

Phone: +1-(312) 503-4234

FAX: +1-(312) 503-7912

Email: y-fukui@nwu.edu

ABSTRACT

Despite its biological significance, much of the mechanism of cytokinesis is not yet resolved. The problems include: (1) signaling mechanism determining the position of the cleavage furrow, (2) molecular and mechanistic nature of the contractile ring, and (3) the origin of forces responsible for cleavage. Using high-resolution imaging technique, the present study analyzes morphometric changes of cytokinesis in wild type (NC4) *Dictyostelium discoideum* amoeba. Sample was prepared by the agar-overlay method, creating 3 μm -thick, nearly two-dimensional cells; and high-resolution image was acquired at 16.7 milliseconds' temporal, 234 nm x, y-, and 100 nm z-axis resolutions. Under this condition, the formation of cleavage furrow initiates at mitotic telophase, and daughter cells separate 18-22 minutes after the furrow initiation. We found that the compression of cells and the room temperature need to be carefully controlled for cytokinesis to proceed in orderly manner. The results demonstrate that the pole-to-pole distance increases by 83% during the initial 5 minutes of cytokinesis, while the distance of equator only decreases by 56%. In contrast, during the subsequent 5 minutes, the pole-to-pole distance only increases by 17%, while the equator distance decreases as much as by 44%. This study indicates that cytokinesis consists of at least two different phases, each of which results from different mechanism. (<http://pubweb.nwu.edu/~yoshifk/fukui.html>).

INTRODUCTION

During cytokinesis, *Dictyostelium* forms a circular microfilament bundle at the equator of cleavage furrow. The ring contains F-actin (Kitanishi-Yumura and Fukui, 1989), myosin I (Fukui et al., 1989), myosin II (Yumura et al., 1984), and other actin-binding proteins (ABPs) (for review, see Fukui, 1993). A recent study, however, suggested a conflicting F-actin distribution; i. e., F-actin was localized only to polar protrusions, but not at the furrow (Neujahr et al., 1997). Although not yet determined, these contradictory evidence are likely results of different fixation protocols. The discrepancy in fixation may have also contributed to the controversial localization of myosin II. The study using picric acid fixation in fact did not identify concentration of myosin II in the furrow, if cells were not compressed (Neujahr et al., 1998). In contrast, Gerald et al. (1998) more recently demonstrated that even in uncompressed cells, myosin II does accumulate into the furrow (see legend of Fig. 6; Gerald et al., 1998). The latter study is in favor of our earlier studies that vitally identified an accumulation of F-actin and myosin II in the furrow (Kitanishi-Yumura and Fukui, 1989; Fukui and Inoué, 1991).

The presence of filamentous ring of F-actin in cleavage furrow (here after called "contractile ring" after Schroeder, 1968) seems prerequisite for cytokinesis; therefore highlights the importance of our capability to preserve these structures for further examination. It is very important to remind, however, that the preservation of F-actin in *Dictyostelium* and other fast-moving amoeboid cells, requires specific conditions. Many of the earlier studies in fact failed to localize F-actin at the furrow while myosin II exhibits a definite accumulation (compare Fig. 3-j and l in Yumura et al., 1984). such discrepancy may be due to the intrinsic property of how actin is organized inside the cell. While actin is found in relatively stable network such as stress fibers, it is also organized

into meshworks that are dynamically controlled (for review about *Dictyostelium* ABPs, see Schleicher et al., 1988). We suspect that these dynamic actin structures are sensitive to slow fixation methods and will disintegrate very rapidly. Originally, this problem was much discussed for myosin II filaments (see Discussion in Yumura and Fukui, 1985).

To date, the precise molecular mechanism of cytokinesis is poorly understood (for review, see Fishkind and Wang, 1995; Wolf et al., 1999). We are still at the stage of identifying key components within the cleavage furrow. Albeit a dominant mechanochemical transducer, how the motor activity of myosin II is harnessed to facilitate cleavage furrow contraction remains to be fully elucidated (Clarke and Spudich, 1974; Mabuchi and Okuno, 1977).

Complete removal of myosin II from *Dictyostelium* unequivocally demonstrated that the mutants are still capable of dividing, but *only when* they are attached to the substratum (Manstein et al., 1989). The myosin II null (*mhcA*⁻) mutants, however, are unable to divide when they are cultured in suspension or on hydrophobic surface, indicating that cytokinesis is not a single process (Zang et al., 1997). It is clear that there exist myosin II-dependent and nondependent mechanisms responsible for cytokinesis (Neujahr et al., 1997, Fukui et al., 1999b). This myosin II-independent (and *probably* anchorage-dependent) cytokinesis most likely utilizes forces originating from F-actin and its binding proteins including coronin and cortexillins (Fukui et al., 1999a; Weber et al., 1999). For localization of other ABPs, see review by Fukui (1993).

In this article, I document an original work in which I morphometrically analyzed normal cytokinesis of wild type cells prepared for two-dimensional shape by applying a gentle pressure by agarose overlay. The original image was recorded in real-time using Inoué's Universal Polarizing Light Microscope with highest spatial resolution available (Inoué and Spring, 1997). The results indicate the presence of two distinctive morphometric phases in *Dictyostelium* cytokinesis.

MATERIALS AND METHODS

Cells and culture

A wild type *Dictyostelium discoideum* (NC4: Raper, 1935, 1984) was cultured with *Escherichia coli* (B/r) on agar plates made of 2% Bacto-agar (Difco Laboratories, Detroit, MI; #0140-01), 2% α -lactose (Sigma Chemical Company, St. Louis, MO; #L-3625) and 2% Bacto-peptone (Difco Laboratories; #0118-8) (modified from LP-medium by Bonner, 1947). NC4 strain has been cultured under this condition in our laboratory since 1972, and the author has not noticed any changes in growth and development. Stock culture of NC4 is transferred onto 2% agar plates containing 1% glucose, 1% peptone, and 17 mM Sørensen's K/Na-phosphate buffer (pH 6.5) (N-medium: Bonner, 1947), and the spores are preserved in sterile silica gel or frozen in 100% glycerol. The stock culture of *E. coli* is transferred every 3 weeks onto N-medium. All cultures are made at 22 °C.

Microscopy

For live cell observation, the samples were prepared and recorded by the same method as described previously (Fukui and Inoué, 1991, 1992). Succinctly, the image

was recorded into sVHS video tapes in real-time and laser disks by time lapse mode through Universal Polarizing Light Microscope (Inoué and Spring, 1997). The microscope was equipped with 100x, N. A. 1.4 oil-immersion objective and a N. A. 1.4 oil-immersion condenser (Nikon Inc., Melville, NY). The sample was illuminated with 546 nm monochromatic light from custom xenon-mercury arc (Hamamatsu Photonics, K. K., Hamamatsu City, Japan). Under this condition, x-y resolution is 234 nm (Inoué and Spring, 1997), and z-axis resolution is about 100 nm (Inoué, personal communication).

Double or triple fluorescence staining was made using rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR; #R-415), a rabbit polyclonal anti-myosin I ^{*1)} (Fukui et al, 1989), a mouse monoclonal anti-myosin II (DM2) ^{*2)} (Yumura et al, 1984), and 4', 6-diamidino-2-phenylindole ^{*3)} (DAPI: Sigma, #D-9542) (Kitanishi-Yumura and Fukui, 1989).

Morphometric analysis

Nineteen cytokinetic sequences were analyzed using an Integrated Image Acquisition and Analysis System ("MetaMorph" Universal Imaging Corporation, West Chester, PA). Briefly, single field images were frozen by using FOR-210 digital time base corrector (FOR.A, Tokyo, Japan) and saved into hard drive. The time resolution of single field is 16.7 milliseconds (Inoué and Spring, 1997), and the distance was calibrated using an objective micrometer (Carl Zeiss, Inc., Thornwood, NY; #473390). Morphometric analysis was performed as described previously (Chu and Fukui, 1996; Fukui et al., 1999).

RESULTS

1. Spatio-temporal relations between spindle and contractile ring

Cytokinesis is a process that is spatially and temporally coupled with mitosis (review, Rappaport, 1996; Oegema and Mitchison, 1997). It has been established in cellular slime mold that the nuclear envelope does not entirely break down and the central spindle fenestrates through holes during anaphase to telophase (Roos, 1975; McIntosh et al., 1985). It has been also established that the actomyosin and microtubule systems manifest dynamic reorganization during mitotic cleavage (Kitanishi-Yumura and Fukui, 1989). These studies examined the components in fixed cells by electron or fluorescence microscopy. The live dynamics of the spindle and actomyosin systems have been also studied by high-resolution and high-extinction polarization microscopy (Fukui and Inoué, 1991).

In the present study, the three-dimensional relations between the spindle and the nucleus were determined by through-focus polarization microscopy (Fig. 1). Under our experimental condition, estimated z-axis resolution was 100 nm (see Materials and Methods). As shown in panel a - d, the fenestration of nuclear envelope (pointed with four arrows) was first identified during metaphase (a), and the hole elongates as the spindle stretches in anaphase (c, d). Plane of the optical section in a - d is shown by a dotted horizontal line in panel a' - d'. The central microtubules manifests as a dark-

contrasted birefringence rod and passes through nuclear envelope through fenestration (c, d), confirming previous ultrastructural studies (Roos, 1975; McIntosh et al., 1985).

A diagram in cross-sectioned view (Fig. 1; a' – d') illustrates a structural relation between nucleus, spindle and chromosomes (Fig. 1; panel a' – d'). In this diagram, chromosomes (1), chromosomal (2), pole-to-pole (3), and astral microtubules (4) are illustrated based on previous electron, fluorescence and video microscopic studies (Roos, 1975; McIntosh et al., 1985; Kitanishi-Yumura and Fukui, 1987; Fukui and Inoué, 1991). A 3-D architecture of nucleus, spindle, spindle-pole bodies (SPBs'), and asters is illustrated in panel a'' – d''. Our previous electron microscopic study determined the SPB as a 210 nm-wide, 370 nm-long, 180 nm-high cuboid, made of electron dense and opaque micro pads that are layered 15 times (Omura and Fukui, 1985). Note that, during anaphase, orientation of the spindle is in fact not fixed relative to the cell contour, but dynamically *oscillates* at an angle of 25 – 75 degrees as demonstrated by previous video microscopic study (Fukui and Inoué, 1992). The oscillation of the spindle subsides at telophase, when the contractile ring encompasses the equator of the furrow.

2. Localization of F-actin, myosin I and myosin II during cytokinesis

We have previously demonstrated that F-actin is organized into three distinctive structure during cytokinesis (Fukui and Inoué, 1991). As shown in Fig. 2A-a, F-actin organizes into (1) contractile ring (large arrows), (2) axial filament bundles (small arrows), and (3) meshworks in polar lamellas (arrowheads). In contrast, myosin II associates only with the contractile ring (Fig. 2A-b). The mechanism of localization of myosin II into the contractile ring is an interesting issue yet to be elucidated (see Uyeda et al. in this issue).

As we have previously demonstrated, myosin I and myosin II are differentially localized during cytokinesis (Fukui et al., 1989). Interestingly, a triple staining of myosin I-B/D, myosin II, and DNA (Fig. 2B) indicates that there is a substantial concentration of myosin I-B/D into the furrow (double arrows in b). Accumulation of myosin I into the cleavage furrow appears obvious in our previous study (Fig. 3c in Fukui et al., 1989), but its implication was not fully appreciated. Myosin I undoubtedly exhibits highest accumulation into polar lamellas manifesting protrusion-retraction (Fig. 2b: arrowheads), leading us to suggest that it may embrace protruding lamellas (Fukui et al., 1989).

The accumulation of myosin I into the furrow (Fig. 2B-b) implies a contribution of this and other unconventional myosins in constriction of the furrow. Recent study, however, demonstrated that a triple knock-out mutant that does not express myosin IA (myoA), IB (myoB), and myosin II are still capable of divide if attached to substratum (Kitayama et al., 1998).

3. Biphasic morphometric changes during cytokinesis

Our previous study demonstrates that F-actin is organized into axial (“pole-to-pole”) filament bundles and the ring structure, that are oriented perpendicular to each other (Fukui and Inoué, 1991). Although a function of axial F-actin bundles are unknown, their 3-D organization is currently investigated using an analytical polarization microscope (Fukui, Y. and Oldenbourg, R., manuscript in preparation). On the other hand, the ring structure unquestionably manifests the “contractile ring” (Schroeder, 1968, 1973; Schroeder and Otto, 1988).

Statistical analysis of 19 cytokinetic sequences resulted in an interesting morphometric property in compressed *Dictyostelium* cell. As shown in Fig. 3, both pole-to-pole distance (P: shaded circle) and the length of equator (E: open circle) exhibit sigmoid mode of elongation or shortening, respectively. The ratio of those values (P/E ratio), however, increases exponentially (solid circle), indicating that the elongation and constriction occur in unique kinetics. On average, 83% of the pole-pole elongation occurs in the first 5 minutes, while constriction of the furrow occurs more uniformly (56% and 44% in the first and second 5 minutes).

It should be pointed out that the results of the present study represents the cytokinetic mechanistics of progeny of the original wild type strain (NC4: Raper, 1935). Most recent studies on *Dictyostelium* are performed using axenic strains that are actually mutants resulting from two mutations (Williams et al., 1974; Williams, 1976). Experimental results from those axenic strains must be carefully interpreted, since those mutants do exhibit motile properties different from NC4 (Kayman and Clarke, 1983; review, Clarke and Kayman, 1987). The cytokinetic property of NC4 revealed in the present study hopefully provides a rudimental information for future studies on various mutant strains.

DISCUSSION

As stated in Introduction, recent studies by Neujahr et al. (1997, 1998) raises a question about a role of myosin II in cytokinesis. Their technique, however, does not seem to perfectly preserve F-actin. In contrast, other studies unquestionably demonstrate positive localization of myosin I, myosin II, and F-actin (Kitanishi-Yumura and Fukui, 1989; Fukui and Inoué, 1992; Fukui et al., 1989; Gerald et al., 1998). Therefore, it is premature to rule out the current dogma; i. e., myosin I and myosin II are involved in generation of protrusive or contractile forces, while F-actin serves as the architectural foundation (Fukui et al., 1989; review, Fukui, 1993). Note that any of those studies by no means underestimates potential roles of ABPs in cytokinesis.

Despite contribution of myosin II to cytokinesis is unquestionable, it seems a long way to go to determine exact forces responsible for perfect cytokinesis (Mabuchi and Okuno, 1977, Fishkind and Wang, 1995). Studies in fibroblasts and *Dictyostelium* indicate that other F-actin-based forces may also play essential roles in cytokinesis (Cao and Wang, 1990; Fukui et al., 1999b). Determining exactly what their contributions are, and how the cells can manage to divide under adverse conditions are vital. Our hope is, once these mechanisms are elucidated, the information should help unraveling the mechanism of cell transformation into cancer and other cell division-related biological problems.

In our recent study, we showed that F-actin accumulates into the cleavage furrow, assembled into the contractile ring, and remains assembled until cleavage is completed (Fukui et al., 1999b). This evidence was achieved in live *Dictyostelium* cells by recording fluorescence phalloidin that was non-invasively injected into NC4 cells. The architectural transformation of the contractile ring into cortical microfilament bundle in daughter cells was also demonstrated previously by high-extinction polarization microscopy (Fukui and Inoué, 1991, 1992).

The observed F-actin flow towards the cleavage furrow has been proposed playing a significant role in the generation of traction forces (Fig. 4A). This model assumes that: (1) F-actin is polarized such that the “actin treadmilling” (Wang, 1985) occurs centripetally, and (2) F-actin is laterally bound to the plasma membrane. Albeit much of this model is only hypothetical, the directional flow into the furrow deserves critical investigation for testing this hypothesis.

F-actin also moves rearwards in migrating cells, and this flow is thought to generate traction forces for the amoeboid locomotion (Fukui et al., 1999b). In analogy, the F-actin flow into the cleavage furrow may generate a traction force, which causes elongation of dividing cell (Fig. 4A). These considerations lead us to suggest that the first phase of morphometric changes identified in the present study (i.e., elongation of pole-to-pole distance) may be executed by the F-actin flow.

Interestingly, a similar F-actin flow occurs in myosin II null (*mhcA*⁻) cells during migration and cytokinesis (Fukui et al., 1999b). This *myosin II-independent* F-actin flow is thought to represent a mechanism responsible for the migration and cytokinesis of *mhcA*⁻ mutant. We propose that this F-actin flow is the mechanism underlying defective motility of *mhcA*⁻ mutant, which cannot complete cytokinesis in suspension. The traction forces driven by F-actin flow, however, are probably *not* robust enough to complete constriction of the cleavage furrow. Consequently, the mutant cells frequently fail to divide even on substratum.

The second phase appears to require myosin II (Fig. 4B). This process also seems providing critical forces for cytokinesis when cells are not anchored to substratum. Under this condition, the cortical F-actin is not cross-linked to substrate and thereby the *myosin II-independent* traction forces cannot be created. As a result, the cell fails to separate, becoming multinucleated. It is very likely that the division of *mhcA*⁻ cells depends greatly on these F-actin-based, myosin II-independent forces that manifest as the observed F-actin flow into the cleavage furrow. Exact nature of concentration of myosin I in the furrow and its possible function in cytokinesis is a significant issue to be determined.

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FOOTNOTES

- *1) The antiserum was made by Dr. Thomas Lynch at Dr. Edward Korn's laboratory in NIH.
- *2) The hybridoma was isolated in collaboration with Dr. Hiroshi Mori at Fukui's laboratory in Osaka University.
- *3) A DNA-binding fluorescence probe, used at 1 $\mu\text{g/ml}$.

FIGURE LEGENDS

- Figure 1.** Architectural organization of microtubule system during mitosis in wild type (NC4) *D. discoideum*. (a – d) Image sequence of an optical section cut at the middle of central spindle. Polarizing microscope image. Arrows: fenestration of nuclear envelope. The central spindle manifests as dark-contrasted shaft (b – d). (a' – d') A diagram illustrating cross-sectioned view of chromosomes (1), central spindle (2), chromosomal (3), and astral microtubules (4). The dotted line indicates the plane of optical section of the image (a – d). (a'' – d'') An artist's sketch illustrating the nucleus, central microtubules, astral microtubules, and spindle pole bodies.
- Figure 2.** Fluorescence images demonstrating distribution of the major actomyosin components during cytokinesis in wild type (NC4) *D. discoideum*. (A) Double fluorescence staining showing F-actin (a) and myosin II (b). Myosin II is primarily associated with F-actin in the contractile ring. Large arrows: cleavage furrow. Arrowheads: polar lamellas. Thin arrows: axial (pole-to-pole) F-actin bundles. (B) Triple fluorescence staining showing phase-contrast (a), myosin I-B/D (myoB and myoD) (b), myosin II (c) and DNA (d). Note that, in addition to the polar lamellas (arrowheads in b), myosin I-B/D are rich in the furrow (double thin arrows in b). Rod-shaped fluorescence structures in d demonstrate mitochondria.
- Figure 3.** Morphometric changes of dividing wild type (NC4) *D. discoideum*. (A) A representative DIC image at the mid-cytokinesis. (B) Graph showing the morphometric changes. The data represents a summary of nineteen sequences. E: equator width (open circle), P: pole-to-pole distance (shaded circle), P/E ratio: ratio between pole-to-pole distance and width of equator (solid circle). See text for detail.
- Figure 4.** A diagram illustrating a biphasic cytokinetic model in wild type (NC4) *D. discoideum*. (A) First phase that represents *myosin II-independent* traction forces by centripetal flow of F-actin and its associating components. Large right-angled arrows: hypothetical traction forces. Beads-on-a-string: F-actin with speculative membrane anchoring components. Shade: speculative association of F-actin to the equatorial cortex. For detail, see Fig. 7D, E of Fukui and Yumura (1999). (B) Second phase representing *myosin II-dependent* contractile forces (right-angled arrows). Beads-on-a-string; F-actin, with anti-parallel orientation. Rod with geranium seed-like appendix: bipolar myosin II filament.







