Stable intercellular bridges in development: the cytoskeleton lining the tunnel

Douglas N. Robinson
[illegible]

A wide variety of intercellular junctions that are involved with cell adhesion or signal transduction have been described in recent years. A widespread but less well-characterized type of intercellular junction is the stable intercellular bridge. Several organisms use stable intercellular bridges as cytoplasmic connections, probably to allow rapid transfer of information and organelles between cells. Here, the authors take a detailed look at the assembly of intercellular bridges called ring canals in the Drosophila germline and discuss how examination of mutants that disrupt Drosophila ovarian ring canal assembly indicates that these bridges are required for intercellular transport of cytoplasm.

In multicellular organisms, cells coordinate their activities to perform various developmental tasks. Proliferation, migration, differentiation and morphogenesis are orchestrated by a variety of intercellular signals that are interpreted by signal-transduction cascades. These signals come in several forms ranging from diffusible ligands to contact-mediated interactions. Cell-adhesion molecules can also mediate interactions between cells as they come in contact with each other, as in the case of interactions between T cells and antigen-presenting cells.

The most intimate form of communication between cells is direct sharing of cytoplasm. Skeletal muscle cells and the syncytial *Drosophila* embryo are two extreme examples; however, these are actually single, multinucleated cells. There are many examples where discrete, singly nucleated cells share their cytoplasm with their mitotically derived sister cells. Organisms ranging from plants to mammals have found ways to arrest cleavage furrows during mitotic divisions of certain cell types in order to convert those cleavage furrows into stable intercellular bridges (Table 1). These are distinct from the transient intercellular bridges seen before the completion of cytokinesis in most cell types[25]. The term 'intercellular bridge' in this review refers to the stable bridges. In contrast to tiny gap junctions[26], which allow passage of only small molecules and peptides (<1–2 kDa), some intercellular bridges are large enough to allow movement of organelles between the cells. Although there are a number of examples of intercellular bridges in somatic cells, germline cells make perhaps the most widespread use of intercellular bridges during their development. Both female and male germline cells from species ranging from insects to humans spend some part of their lifetime in syncytia of mitotically related cells. This review gives examples of where intercellular bridges are found and concentrates on germline intercellular bridges, which are the best characterized to date. Initial observations of most intercellular bridge types were made by electron microscopy. Recently, genetic and immunocytochemical approaches have begun to bring our understanding of intercellular bridge structure to a molecular level.

The authors are at the Dept of Genetics, Yale University School of Medicine, 333 Cedar St, New Haven, CT 06510, USA.
E-mail: lynn.cooley@yale.edu

Somatic intercellular bridges

Somatic intercellular bridges are found in at least three kingdoms and range from plasmodesmata in plants to septal pores in filamentous fungi and gonadotropin-releasing hormone (GnRH) neurons in mammals. Although the structures of the somatic intercellular bridges are varied, a common theme of coordinated cell behaviour is apparent. Bridges are found in cells that display synchronization of one or more of the following: cell division, migration, differentiation or hormone release (Table 1). The presence of intercellular bridges probably facilitates synchronization of these behaviours by allowing free passage of signals that initiate them or, in the case of cell migration, tethering the cells together so that they must comigrate.

Drosophila uses intercellular bridges at several stages in development. Small cytoplasmic bridges form between sister cells in the imaginal disc epithelia, in the follicular epithelia of egg chambers (Fig. 1a) and transiently in the cellular blastoderm and extended germ band embryo. These bridges have diameters of 0.25 to 1 μm. The proteins PEANUT (Ref. 11) and SEP1 (Ref. 12) associate with the bridges connecting the newly cellularized embryonic epithelium with the yolk sac (Table 1). PEANUT (Ref. 11), SEP1 (Ref. 12) and SEP2 (Ref. 27), which are fly homologues of the yeast septins[25,28,29], have been isolated as a protein complex that forms filaments *in vitro*[27]. The yeast septins were discovered as cell-division-cycle mutants (*CDC3*, *CDC10*, *CDC11* and *CDC12*) and are required for cytokinesis[28]. Mutations in the *peanut* gene are pupal lethal[11]; third instar mutant larvae have underdeveloped imaginal discs with a large number of polyploid cells containing 2 to 6 nuclei per cell. A similar defect is seen in the follicle cells in the ovary. Other than this, the cells appear to undergo normal mitoses, suggesting that septins in *Drosophila* are also required for cytokinesis[11].

In addition to a role in cytokinesis, the septin complex might be required to maintain stable intercellular bridge structure. The yeast bud neck, whose structural components are probably the septins[27,28], might well be compared with intercellular bridges. The septin

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TABLE 1 – STABLE INTERCELLULAR BRIDGES IN DEVELOPMENT

Structure	Organism	Known components	Known or proposed functions	Refs
Plasmodesmata	Plants; some filamentous fungi	Proteinaceous specialization of ER	Transport of molecules <1–2000 Da; size-exclusion limit increased by cellular proteins and viruses	1–4
Septal pore	Filamentous fungi (e.g. ascomycetes and basidiomycetes)	Chitin R glucan (basidiomycetes)	Regulate cellular differentiation; allow transport of cytoplasm, organelles and nuclei; 0.05–0.5 μm diameter	1,2,5,6
Somatic bridges:				
Bridges called cell-division remnants	*Caenorhabditis elegans*	Actin	Regulate embryonic divisions and, possibly, spindle reorientation; approx. 1 μm diameter	7
Intercellular bridges	*Hydra*	Unknown	Synchronize cnidoblast differentiation into nematocysts; approx. 0.5 μm diameter	8
Bridges in cellular blastoderm and germ band extension embryo	*Drosophila*	Actin, septins	Mediate interaction between yolk sac and epithelium during gastrulation and germ band extension; 0.5 μm diameter	9–12
Bridges in imaginal disc cells and ovarian follicle cells	*Drosophila*	Probably septins	Maintain clonal association of cells; coordinate cell behaviour; 0.25–0.5 μm diameter	11–13
Bridges between GnRH neurons	Mammals	None	Coordinate pulsatile release of GnRH; may or may not be mitotically derived; 0.1–0.5 μm diameter	14
Germline bridges:				
Bridges between spermatocytes and spermatids	Mammals	Actin	Coordinate development and entry into meiosis; transcript sharing; 1–1.5 μm diameter	8,15–18
Bridges between oogonia	Mammals; chicken	None	Coordinate development, entry into meiosis, and/or atresia; 1–1.5 μm diameter	19–22
Bridges between spermatocytes and spermatids	*Drosophila*	Phosphotyrosine proteins, ANILLIN, septins	Coordinate development and entry into meiosis; 1–1.5 μm diameter	23
Ovarian ring canals	*Drosophila*	Phosphotyrosine proteins, actin, HTS, KELCH, DSRC64	Facilitate transport of components between nurse cells and oocyte; 0.5–10 μm in diameter, depending on stage of development	24, –[a]

[a]S. Dodson and M. Simon, pers. commun.

ring is noncontractile and delineates a bridge of ~1 μm that provides a passageway for the daughter cell nucleus into the bud[28]. In addition, at least two cell types, imaginal discs and follicle cells, reported to be affected by mutations in *peanut* form stable intercellular bridges. A defect in the formation or maintenance of this structure could produce the multinucleate cells observed in *peanut* mutants. Finally, intercellular bridges between *Drosophila* male germ cells may rely upon septins for structural integrity (see later section).

Mammalian germline intercellular bridges

Both male and female gametogenesis begin with mitotic proliferation of the germline. In the male, this continues throughout adult life, whereas, in the female, mitosis is restricted to early foetal ovarian development. In both cases, cytokinesis is incomplete, giving rise to syncytia of interconnected cells. Each cell in male syncytia enters meiosis, ultimately producing four sperm. The fate of female sibling cells is unknown; only a small fraction of the female germline survives programmed cell death (atresia) to become individual oocytes surrounded by granulosa cells. Thus, females are born with a finite number of oocytes.

The functions and components of the bridges between the mammalian gametes are not understood. However, it is known that, in mammalian spermatids, gene products are shared between connected cells. Mice hemizygous for a transgene expressed only postmeiotically produce haploid sperm that all contain the transgene product[17]. Transcript sharing has also been detected by generating aneuploid sperm in mice heterozygous for two Robertsonian translocations and examining the distribution of postmeiotic transcripts from the aneuploid region[18]. All sister sperm were equivalent for the transcripts produced in spite of the inequality of the genomes. A possible general

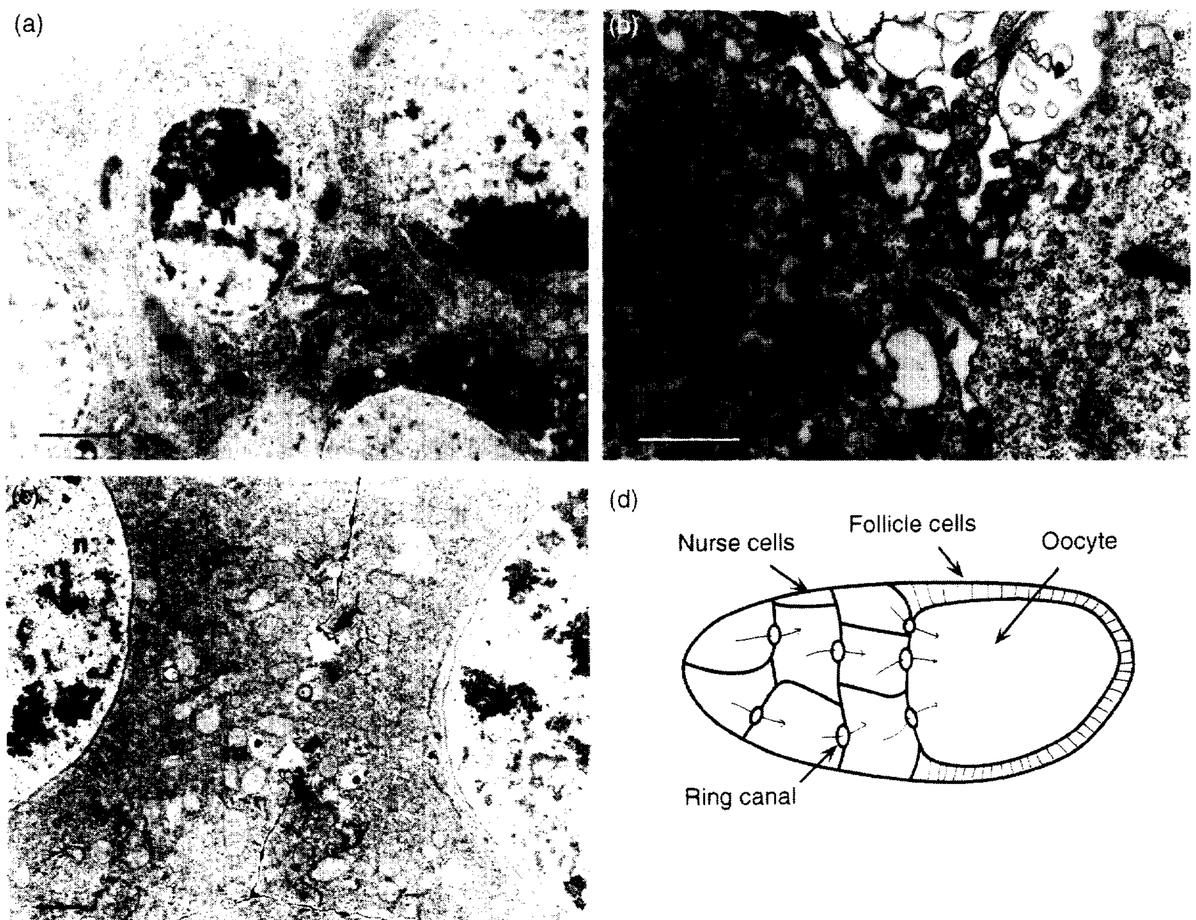


FIGURE 1

Ultrastructure of *Drosophila* and mammalian intercellular bridges. (a) Electron micrograph of columnar epithelial follicle cells in a *Drosophila* egg chamber showing an intercellular bridge connecting the two cells. The bridge consists of a thickening of the plasma membrane (arrow) and a region of lower electron density that forms the inner rim (arrowhead). The structure of this intercellular bridge is very similar to the structure of imaginal disc intercellular bridges described by Poodry and Schneiderman[13]. n, nucleus; m, mitochondrion. Bar, 1 µm. (b) Electron micrograph of foetal mouse ovary revealing an intercellular bridge connecting two primary oocytes. These bridges have only the thickening of the plasma membrane (arrow) with no apparent associated inner rim. Bar, 1 µm. (c) Electron micrograph showing a ring canal connecting two *Drosophila* nurse cells. The ring canal consists of an outer rim (arrows) with a thickening of the plasma membrane and an inner rim (arrowheads), which is a region of lower electron density. n, nucleus; m, mitochondrion. Bar, 1 µm. (d) A *Drosophila* egg chamber shown schematically consists of one oocyte and 15 nurse cells encapsulated by a layer of somatically derived follicle cells. Ring canals connecting nurse cells to each other and the oocyte mediate the flow of cytoplasm into the oocyte.

role for intercellular bridges might be in the normal development of sperm bearing the Y chromosome, enabling this class of sperm to receive X chromosome gene products from interconnected sister cells.

Currently, only actin has been identified as a component of intercellular bridges in developing mammalian spermatocytes[16]. Additionally, *in vitro* treatment of squirrel interconnected spermatocytes with the microfilament-depolymerizing agent cytochalasin D caused the intercellular bridges to open up, producing multinucleate cells. This experiment highlights the importance of the actin cytoskeleton in maintaining the spermatocyte shape and the interconnecting bridge[16].

Early in mammalian oogenesis, developing oocytes are also interconnected[19–21]. The bridges have a thickened plasma membrane-derived rim (Fig. 1b). A function of interconnected development in the female may be to mediate survival through waves of atresia. It is not known whether every cell within an interconnected cluster dies and only certain clusters survive to undergo meiosis or whether a subset of the cells within a cluster is selected for atresia. In either case, it seems likely that the interconnections can facilitate the regulation of this decision and the process to remove certain cells from the meiotic pool. Coordinated entry into meiosis might also be facilitated by intercellular bridges since all oocytes must enter meiosis during foetal development but be arrested in metaphase II. The identification of specific intercellular bridge components, followed by their genetic disruption, could allow the dissection of the function of these intercellular bridges during mammalian gametogenesis.

Drosophila ovarian ring canals

The intercellular bridges in *Drosophila* oogenesis are better characterized than those in mammalian cells. At the beginning of oogenesis[30] (Fig. 2a), a stem cell divides to produce two unequal daughters. One

daughter cell remains a stem cell while the other daughter cell (cystoblast) undergoes four mitotic divisions with incomplete cytokinesis to generate a 16-cystocyte cluster. One of these cells becomes the oocyte while the other 15 become nurse cells, and all 16 germline cells become encapsulated by somatically derived follicle cells to make an egg chamber (Fig. 1d). The arrested mitotic cleavage furrows are then transformed into intercellular bridges called ring canals (Fig. 1c). The ring canals allow the transfer to the oocyte of cytoplasmic components such as mRNAs, proteins and organelles synthesized in the nurse cells[31]. In contrast to male germ cells and somatic syncytia, the egg chamber is one situation where the function of intercellular bridges is clear: to allow directional transport of cytoplasm between cells.

The development of ring canals begins with an assembly phase during which the arrested cleavage furrows are modified by the addition of several proteins (Fig. 2b). Phosphotyrosine epitopes[32] become detectable as 0.5–1 μm rings by the end of the third mitosis. The appearance of phosphotyrosine epitopes suggests that initial ring canal development might be controlled by specific kinases. One candidate for such a regulator is the DSRC64 kinase since mutants for *Dsrc64* have reduced amounts of phosphotyrosine staining and produce small ring canals (S. Dodson and M. Simon, pers. commun.). After the fourth mitosis, additional bipolar[33] actin filaments called inner rim actin and one product from the *hu-li tai shao* (*hts*) gene become localized simultaneously to the ring canals[32]. A few hours later in egg chamber development, products from the *kelch* locus[34] begin to be localized to the ring canals. By the time KELCH reaches all of the ring canals, they have grown to a diameter of 3–4 μm and the contractile ring component, ANILLIN (Ref. 35), is no longer visible. This marks the end of ring canal assembly, after which the ring canals continue to grow to a final diameter of ~10 μm.

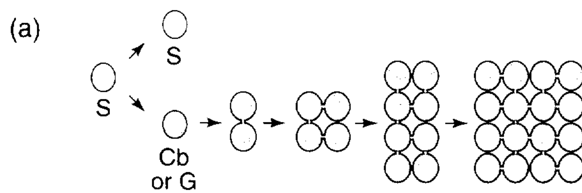
As the ring canal is assembled, the actin filaments of the inner rim must be anchored to the outer rim. This process depends on two genes: *cheerio* (D. N. Robinson, T. Smith-Leiker and L. Cooley, unpublished) and *hts* (Ref. 36). Flies harbouring severe mutations in these two genes localize ANILLIN and phosphotyrosine epitopes. However, in *cheerio* mutant egg chambers, actin, HTS and KELCH proteins are present but do not accumulate into the inner rim of ring canals (D. N. Robinson, T. Smith-Leiker and L. Cooley, unpublished). As a result, the ring canals fail to grow normally and the transport of cytoplasm into the oocyte is severely compromised. Females carrying *cheerio* mutations are sterile, producing very small oocytes. The *hts* mutant phenotype is more complicated since this gene is required for cystocyte divisions as well as ring canal assembly[36]. Egg chambers are produced with too few germline cells, often lacking an oocyte, and ring canals that rarely accumulate actin, HTS and KELCH proteins. The *hts* locus produces several products, some of which are probably required for the cystocyte divisions and one of which is required for ring canals. The HTS ring canal protein is derived from the C-terminal portion of the ovary-specific *hts* cDNA[32], possibly by proteolysis of a precursor protein or by internal translational initiation. Although this region of HTS is not similar to known actin-binding proteins, the strict colocalization of actin and HTS early in ring canal establishment suggests that HTS may directly or indirectly bind actin filaments in the inner rim.

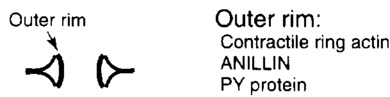
Following assembly, the ring canals increase in diameter 2.5 times from 3–4 μm to 10 μm. The number of actin filaments in the inner rim remains constant at ~720 during this phase of ring canal growth[33], implying that the filaments increase in length rather than number. The organization of actin filaments might be maintained by crosslinking proteins that break and reform contacts as the filaments elongate. Based on the density of actin filaments in the inner rim, the concentration of actin is ~6–7 mM. If the crosslinking proteins have molar ratios with actin of between 1–5 and 1–10, then the concentrations of these proteins would be of the order of a few hundred micromolar to 1–2 millimolar. Low-affinity interactions could exist between these proteins given their potentially high concentrations within the annulus of the ring canal inner rim. These associations could be formed, broken and reformed during expansion of the ring canal.

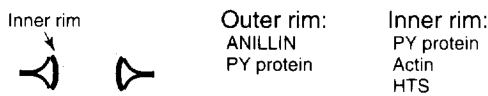
KELCH proteins facilitate the proper organization of the inner rim of the ring canal during expansion[32,33]. *kelch* mutants produce ring canals in which all of the known ring canal proteins are present except for KELCH. However, during the growth phase, the ring canal inner rim proteins (actin, HTS and phosphotyrosine-containing proteins) become disorganized. In particular, the actin-filament bundles extend into the lumen of the canal, although the outer diameter of the ring canal remains similar to that of the wild-type. This disorganization results in partial occlusion of the ring canal lumen to a width of 1–2 μm (Ref. 32). Although there is sufficient space between the actin filament bundles to allow passage of organelles[33], *kelch* mutant females have a severe transport defect that results in small oocytes and female sterility. Therefore, the disorganized ring canals probably cannot accommodate the bulk flow of cytoplasm necessary to maintain normal egg chamber development[32].

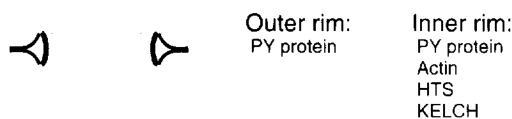
Ring canals during *Drosophila* spermatogenesis

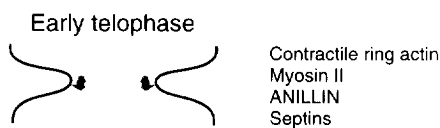
Recent characterization of ring canals between *Drosophila* male germ cells has uncovered striking similarities and differences between the sexes regarding ring canal development. In spermatogenesis[37], a stem cell divides producing a daughter stem cell and a daughter gonial cell (Fig. 2a). The gonial cell is functionally similar to the cystoblast in oogenesis and undergoes four mitotic divisions giving rise to a cluster of 16 primary spermatocytes that are interconnected by ring canals. The interconnected spermatocytes then undergo synchronized meiosis that produces a cluster of 64 interconnected haploid spermatids. ANILLIN, PEANUT, SEP1, SEP2 and phosphotyrosine epitopes are localized to mature postmitotic and postmeiotic ring canals[23] (Fig. 2c). Throughout spermatogenesis, all of these proteins are detectable on the ring canals, which are maximally 1–2 μm in diameter. Actin filaments and ovarian ring-canal-specific HTS

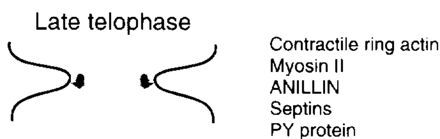


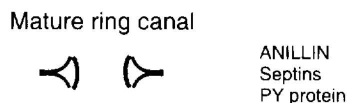












and KELCH proteins are not found on male ring canals – highlighting one significant difference between male and female germline ring canals[23, 32]. However, the presence of the septins on male ring canals may mean that they bear the structural burden that actin filaments, HTS and KELCH provide for the female ring canals. It may also mean that male ring canals are structurally more similar to the yeast bud neck, which is rich in septin filaments.

Conclusions

The formation of structurally sound intercellular bridges occurs during normal development of a wide range of cell types and species. Molecular studies of some of these bridges are well under way. In nearly all cases, the formation of intercellular bridges is coupled closely with altered cytokinesis; indeed, proteins present in cleavage furrows sometimes persist in stable bridges. The elaborate ring canals in *Drosophila* egg chambers may reflect the unique structural burden that these ring canals incur during the rapid phase of global cytoplasm transport. As intercellular bridge components of other cell types, including mammalian germline cells, are identified, new experiments can be designed to determine the functional significance of maintaining intercellular junctions. Sharing of nutrients and regulatory molecules between sister cells is probably one common function of intercellular bridges. The role of intercellular bridges in the regulation of cell division and cell behaviour in other developmental systems should become clearer in the future as researchers continue to explore these fascinating structures.

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FIGURE 2

Schematic of *Drosophila* female and male ring canal formation. (a) Female and male gametogenesis begin with the proliferation of the germline. A stem cell (S) undergoes one division that produces a daughter stem cell and a differentiated cystoblast (Cb) in the female or gonial cell (G) in the male. The cystoblast or gonial cell undergoes four mitotic divisions characterized by incomplete cytokinesis. In the female, the 16 cystocytes differentiate into 15 nurse cells and one oocyte. In the male, all 16 spermatocytes are equivalent and undergo meiosis to produce a cluster of 64 interconnected spermatids. The 64 spermatids separate upon maturation. (b) During the mitotic divisions of the ovarian cystocytes, contractile ring actin and ANILLIN are visible. It is probable that myosin II is also present in the contractile ring, although it is not readily detectable. After the third mitotic division, phosphotyrosine-containing proteins (PY protein) become detectable in the outer rim (red) of the arrested cleavage furrow. After the completion of the fourth mitosis, the thick inner rim (yellow) containing actin, HTS and additional PY proteins becomes apparent. Several hours later in development, KELCH is localized to the inner rim. Once KELCH is put into place and ANILLIN is removed, the contractile ring has completed the transition to a ring canal. Mutations in *hts* or *cheerio* block the addition of the inner rim components. (c) During early telophase of meiosis, the male meiotic cleavage furrows (green) contain contractile ring actin, myosin II, ANILLIN and septins (PEANUT, SEP1 and SEP2). By late telophase, anti-phosphotyrosine epitopes (PY protein) begin to accumulate in the ring canal. Finally, in the mature ring canal, ANILLIN, septins and PY protein are localized[23].

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Pictures in cell biology

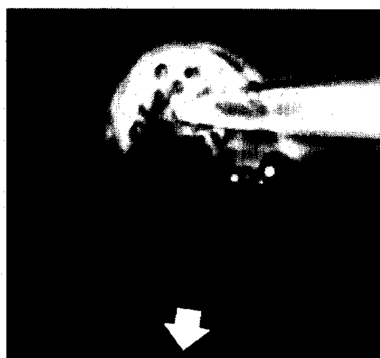
Keratocytes roll!

FIGURE 1

Fluorescent beads picked up by the cell rotate within the cell body as the cell advances. Arrow shows the direction of cell body rolling.

FIGURE 2

Mechanical manipulation of the cell body. In this picture, the cell body is being restrained from moving forward by pressure from the needle, but the lamellipodium continues to protrude at the normal rate. Arrow shows the direction of cell movement.



The actin-dependent crawling of metazoan cells involves protrusion at the cell front and retraction of the rear. Fibroblasts move sluggishly because retraction of the trailing cell body is poorly coordinated with protrusion of the cell front. By contrast, the fish keratocyte seems to glide over the substrate, at velocities an order of magnitude greater than those of the fibroblast (up to 20 μ min^{-1}), keeping its cell body tucked tightly behind its protruding lamellipodium. The secret of the keratocyte's motile efficiency has been revealed by recent studies [Anderson, K. I., Wang, Y-L. and Small, J. V. (1996) *J. Cell Biol.* 134, 1209–1218] that show that the cell body of the keratocyte rolls, rather like a steam roller, behind the advancing lamellipodium. Fluorescent beads picked up by the cell can be seen rotating with the cell body as the cell advances. The forces behind cell body traction appear to arise from lateral tension developed across the cell body, extending into the flanks of the lamellipodium. The authors manipulate the keratocyte cell body with microneedles and show that lamellipodium advance is not affected by disturbance of or pressure on the rest of the cell, putting the final nail in the coffin of the old idea that contraction at the rear of the cell drives lamellipodium protrusion. The complete video sequences used to prepare these figures are available for downloading in two formats, NIH Image stacks and QuickTime movies, at: `http://imb.oeaw.ac.at/kahli/JCB-movies.htm` It is difficult to appreciate the movement from static pictures, and the movies are definitely worth the effort!

These images were kindly provided by Kurt Anderson and Vic Small of the Austrian Academy of Sciences, Salzburg, Austria.

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