Ca^{2+} Signaling During Embryonic Cytokinesis in Animal Systems.

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Abstract

In this Chapter, we provide a review of the literature that describes the role of Ca^{2+} signaling during embryonic cytokinesis in animal systems. We begin with a historic overview (starting with the earliest reports published in the latter part of the 19th Century) that gives some of the first descriptions linking Ca^{2+} with cell division in embryos. This introductory overview also outlines the different techniques that were used and developed over time, from the pre-Ca^{2+} imaging days through to the sophisticated approaches that are available today for directly visualizing Ca^{2+} signals in living cells. In the remainder of the Chapter, we describe the more recent advances in cytokinetic Ca^{2+} signaling research, starting where the introduction finished, in the 1990's. Reports of the various Ca^{2+} signals visualized during cytokinesis in fish, amphibian, echinoderm and insect embryos using both fluorescent and luminescent Ca^{2+} probes are described, as well the investigations carried out to determine both the requirement of elevated Ca^{2+} during cytokinesis and the source of the Ca^{2+} involved in this process. The current hypotheses regarding the possible roles and targets of the different cytokinetic Ca^{2+} signals observed are also briefly discussed.

Keywords

Ca^{2+}, cytokinesis, cleavage furrow, positioning, propagation, deepening, apposition, actomyosin

1. Introduction: A Historical Perspective

There is a vast literature concerning cytokinesis in animal cells and many hypotheses and ideas have been proposed over the years attempting to explain this complex process in a variety of different cell types. These have been nicely reviewed by numerous authors, reflecting the current views that prevailed [1-12]. No one, however, perhaps does so with such insight and eloquence than Rappaport in his excellent book on animal cell cytokinesis [13]. In this Chapter, however, we have attempted to sift through both the past and current literature and focus specifically on reports concerning the possible roles played by Ca^{2+} in initiating and orchestrating the cytokinesis process. We have narrowed the scope of this Chapter even further, by concentrating on the role of Ca^{2+} signaling during embryonic cytokinesis in animal systems, as this is our major research interest. By doing so, we hope to present the reader with a more in-depth review of what we propose to be a distinct form of cytokinesis, and one that has been particularly amenable for visualizing cytokinesis-related intracellular Ca^{2+} transients. There are other previous reviews where the possible roles played by Ca^{2+} during mitosis and the cell cycle have been considered. Although these do not specifically focus on Ca^{2+} and cytokinesis, we would like to draw the readers' attention to four of these in particular for further reference, for the dual reason that they help to place cytokinetic Ca^{2+} signaling into an overall cell cycle signaling context, and the fact that their authors have made significant contributions to our understanding of the Ca^{2+} signaling field. The first is Berridge [14], where he discusses the role played by Ca^{2+} and cyclic nucleotides in the cell division process; the second is by Steinhardt [15] where he considers Ca^{2+} regulation of the first cell cycle of the sea urchin embryo; the third by Hepler [16] that mainly focuses on Ca^{2+} and mitosis, but also contains a section on Ca^{2+} and cell division; and finally the fourth by Whitaker [17] where he considers the regulation of the cell division cycle by inositol trisphosphate (IP_3) and the Ca^{2+} signaling pathway.

The study of cytokinesis in embryonic systems has a significant experimental history that allows one to follow the link between Ca^{2+} and cytokinesis from pre-Ca^{2+}

imaging days, through to the sophisticated techniques we currently possess for visualizing Ca^{2+} signals in living cells. As pointed out by Rappaport [13] with regards to cytokinesis in general - but even more so concerning the possible roles played by Ca^{2+} in this process – that it is quite easy when searching through the early literature to miss important Ca^{2+}-related observations, as they often do not appear prominently in the title of papers, but are consigned to footnotes, figure legends and discussion sections. Thus, it is hard to present a comprehensive review of the literature on this subject. The strategy we adopted, therefore, was to identify what we felt were key publications, where an investigation of the role played by Ca^{2+} during embryonic cytokinesis was the focus of the study, rather than an adjunct observation. We do apologize in advance, however, to those who have published excellent work concerning Ca^{2+} signaling during cytokinesis in non-embryonic situations (such as tissue culture cells and dividing unicellular systems). We suggest that this would make an excellent focus for a separate review. We also believe that the study of embryonic cytokinesis in particular is most relevant to the title of this Volume – *"Calcium, A Matter of Life or Death"* – for without the successful completion of the post-fertilization phase of rapid embryonic cell division, a multi-cellular Blastula Stage would never be attained, resulting in certain embryonic death.

We suggest that our current ideas regarding the possible roles played by Ca^{2+} during embryonic cytokinesis can be traced back to the early mechanistic hypothesis relating to increases in equatorial tension proposed in 1876 by Bütschli [18]. He suggested that cells divide because the tension at the equatorial surface increases, and that the increase somehow results from the action of the mitotic apparatus. The unknowns that lay ahead at this time were the nature of the equatorial contractile apparatus, and how it got positioned, assembled and modulated, as well as the identity of the cellular messengers that orchestrated these processes. In 1937, Mazia [19] was one of the first to suggest that Ca^{2+} might be responsible for triggering cell division after fertilization. However, a direct mechanistic connection between cytokinesis and Ca^{2+} perhaps has its origins in the observations of physiologists in the 1950s, who saw

similarities in the contractile nature of muscle, and the movements of, and within, cells - including cell division [20-22]. At the time, these observations were regarded as being one of the most exciting developments with respect to understanding the biochemical processes involved in animal cell cleavage [2]. Following on from this general view, Tilney and Marsland [23] proposed that the deposition of contractile filaments in the furrow cortex of dividing sea urchin (*Arbacia punctulata*) eggs might be triggered by the mobilization of Ca^{2+} from intracellular stores. They proposed that this store was the interior of the nucleus, and that Ca^{2+} was released on nuclear envelope breakdown (NEB). In addition, they also suggested that polar relaxation (thought at the time to be an essential pre-requisite for successful cleavage) was achieved via localized Ca^{2+} sequestration activity of “relaxing factors” [23]. Thus, they were among the first to suggest that both the release and the re-sequestration of Ca^{2+} from intracellular stores might play important roles in the cytokinesis process.

Following on from these Ca^{2+} related observations, Bluemink [24] reported the presence of a diastema during cleavage in the salamander (*Ambystoma mexicanum*) egg. The most prominent feature of this cytokinetic structure (seen in the large yolky eggs of certain primitive fish and amphibians) he described as being elongated, closed profiles of double stranded membranes, which he termed “cisternae”. Associated with, and presumably originating from the cisternae, Bluemink [24] also described “light vesicles”. He proposed that these helped to polarize and organize the filaments in the contractile ring, and also served as membrane-bound compartments for Ca^{2+} transport in order to stimulate the contraction processes during cytokinesis. Another significant report from this time was that of Gingell [25] who demonstrated that directly injecting Ca^{2+} into the cortex of eggs of the frog (*Xenopus laevis*) resulted in a strong cortical contraction. He also observed that wound-induced surface wrinkling displayed properties similar to early cleavage furrow formation. Furthermore, he demonstrated an absolute requirement for Ca^{2+} in the extracellular medium, which would induce a cortical contraction around a tear in the plasma membrane in order to seal the opening, and that the contraction process involved microfilaments. These observations formed

the basis of the idea that elevated intracellular Ca^{2+} in some way stimulated microfilaments in the cortex to contract and suggested that there might be mechanistic similarities between the contraction processes involved in surface wound healing and cytokinesis.

Soon after, Baker and Warner [26] reported that Ca^{2+} was essential for early embryonic cleavage in *Xenopus* embryos. They could not, however, determine whether changes in Ca^{2+} regulated cytokinesis, as opposed to cytokinesis merely having a requirement for some intracellular free Ca^{2+}. This is an especially significant paper, as Baker and Warner were also among the first investigators to use the Ca^{2+}-sensitive bioluminescent reporter, aequorin, to attempt to directly record temporal Ca^{2+} dynamics in developing *Xenopus* embryos during cytokinesis. They reported that 5 out of 8 aequorin-injected embryos showed an increase in aequorin-generated light output at the time of both the first and second embryonic cleavages. These transient changes were seen in eggs bathed in Ca^{2+}-free solution containing EGTA, which they suggested indicated that the increases in intracellular Ca^{2+} could not have resulted from Ca^{2+} entry across the plasma membrane during cleavage, but resulted from release from intracellular stores. They did, however, suggest that the results from their aequorin-based experiments should be treated with considerable caution. As we will see in subsequent sections, Baker and Warner [26] were being perhaps over-cautious with regards to their seminal observations. However, the early days of recording Ca^{2+} dynamics from living cells were fraught with technical difficulties when gathering data and an understandable caution was displayed when interpreting results.

A second report from the same year by Timourian *et al.* [27], utilizing the Ca^{2+} buffer EDTA, clearly demonstrated that in sea urchin (*Lytechinus pictus*) embryos, Ca^{2+} appeared to play a role in specifically determining the site of the cleavage furrow. They proposed that Ca^{2+} was released from the mitotic centers and via diffusion, attained its highest concentration at the cortex equidistant from the two centers. Furthermore, using frozen longitudinal sections of *L. pictus* eggs prepared during metaphase and anaphase of the first cell division cycle, in conjunction with electron-probe

microanalysis, the same group was able to demonstrate that Ca^{2+} was more concentrated at the furrow region at metaphase, but not anaphase [28].

Durham [29] also considered the possible role of Ca^{2+} in positioning and stimulating the embryonic cleavage furrow in an attempt to unify the then current ideas and suggestions regarding the control of actin and myosin in non-muscle movement. He proposed that what he described as the “ion permeability barrier” would be reduced in the maximally stretched region of the egg surface (due to the expanding mitotic apparatus) and that a resulting local Ca^{2+} influx would thus enhance actomyosin contraction. In this fashion the contractile ring would be assembled and stimulated to contract. Thus, the idea that a localized elevation in intracellular Ca^{2+} might play a crucial role in positioning the cleavage furrow became an area of considerable experimental interest. To further explore this possibility, Schroeder and Strickland [30], using the recently developed Ca^{2+} ionophore A23187, reported that furrow-like deformations resulted from ionophore-induced increases in intracellular Ca^{2+} concentrations in frog (*Rana pipiens*) eggs, and as a result suggested that A23187 might be a good experimental tool for investigating the role of Ca^{2+} during cytokinesis. Subsequently, Arnold [31] demonstrated that the application of A23187 to the surface of squid (*Loligo pealei*) embryos could precociously induce the appearance of furrows. Furthermore, application of the ionophore while cleavage furrow progression was underway, speeded up this process and resulted in an extension of the furrows beyond their normal meroblastic boundaries. Both these effects were reported to occur in Ca^{2+}-free medium, suggesting that the ionophore was releasing Ca^{2+} from internal stores. Following up on the observations of Gingell [25], Hollinger and Schuetz [32] demonstrated that microinjection of Ca^{2+} into frog (*R. pipiens*) oocytes after NEB, resulted in a cleavage-like constriction and the formation of an embryonic structure that resembled a two-cell embryo. Furthermore, the location of the microinjected Ca^{2+} determined the orientation of the resulting cleavage furrow. They suggested that Ca^{2+} diffused away from the injectate bolus towards the cortex and at some specific, critical concentration of Ca^{2+} it activated some contractile elements (which they proposed

might be microfilaments) in the embryonic cortex. The following year, Conrad and Davis [33] reported that the microiontophoretic injection of Ca^{2+} resulted in rapid shape changes in fertilized eggs of the gastropod *Ilyanassa obsoleta* that resembled, in some cases, cleavage furrows. The same group subsequently demonstrated, via the use of Ca^{2+} and calmodulin antagonists, that Ca^{2+} may be necessary for cytokinesis (and polar lobe formation) in *I. obsoleta*, and that the Ca^{2+} involved was released from intracellular, sequestered stores rather than being derived from exogenous sources [34].

1977 also saw the first report suggesting that the early cleavages in teleost embryos may also be accompanied by transient elevations in intracellular Ca^{2+}. Although mainly focused on the large Ca^{2+} transient associated with egg activation, Ridgway *et al.* [35] - using aequorin-loaded medaka (*Oryzias latipes*) eggs and a photomultiplier tube (PMT) - reported that in one experimental embryo, small transient increases in intracellular Ca^{2+} appeared to correlate with the first two cell divisions. When considered along with Baker and Warner's 1972 [26] report from dividing *Xenopus* embryos, the data presented from these first attempts to directly measure changes in intracellular Ca^{2+} associated with cell division in live embryos, is somewhat equivocal. Subsequent experiments, however, carried out on both of these embryonic systems [36, 37] using improved intracellular Ca^{2+} reporters in combination with better imaging technology (and discussed in following sections) clearly support and vindicate these early reports. The investigators responsible for these early experiments thus deserve appropriate acknowledgement for their pioneering attempts to advance our understanding of the role played by Ca^{2+} in the cytokinetic process.

The 1980s saw a dramatic step forward in our understanding of cytokinetic Ca^{2+} signaling, through the development and use of reliable fluorescent-based intracellular Ca^{2+} reporters such as fura-2, which had the advantage of improved sensitivity as well as easier handling and loading into cells [38]. These were used to describe, for the first time, temporal changes in intracellular Ca^{2+} during cell cycle progression in individual sea urchin (*L. pictus*) eggs, where Ca^{2+} transients recorded after mitotic events were

reported to be associated with cytokinesis [39]. In addition, through the use of inserted Ca^{2+}-sensitive microelectrodes, Shantz [40] reported that in one experimental medaka embryo, during two successive cleavages, Ca^{2+} rose transiently four-fold above the original resting level in synchrony with each cell division. During this period, however, contradictory results with regards to the role played by Ca^{2+} were also reported. For example, Yoshimoto *et al.* [41], like Ridgway *et al*. [35], used a PMT to measure the light output of single aequorin-injected medaka eggs. They, however, reported that the intracellular concentration of Ca^{2+} (judged by the luminescent output from the whole egg) appeared to be lowest at the time of furrowing. They also reported similar results from the eggs of several echinoderm species, including those of the sea urchin, *Hemicentrotus pulcherimus*. However, in the case of the echinoderms, because they could not detect any post-fertilization light emitted from single aequorin-loaded eggs, they devised a method where they suspended a population of eggs in a solution of Ca^{2+}-free sea water (plus EGTA), then both aequorin and the Ca^{2+} ionophore A23187 were added to the suspension medium to measure the total amount of Ca^{2+} that leaked out of the eggs during successive cell division cycles. They reasoned that the total Ca^{2+} efflux induced by the ionophore would reflect, at least qualitatively, changes in the intracellular Ca^{2+} concentration within a population of synchronously diving echinoderm eggs. Eggs were removed from the measuring chamber at regular intervals in order to check on cytokinetic progression. Like the temporal data they collected from individual medaka eggs, their echinoderm-based results suggested that the times of furrowing always corresponded with temporal troughs in a cyclic pattern of luminescent output [41]. The indirect method used to try and measure subtle intracellular Ca^{2+} dynamics, combined with the assumptions that were made with respect to interpreting the data, serve as a good example to illustrate the difficulties involved in attempting to measure changes in intracellular Ca^{2+} in dividing cells. One of the keys to addressing this problem would be the development of Ca^{2+} reporters and imaging devices with the resolution and sensitivity to visualize small, localized changes within individual, healthy, dividing cells. In addition to these attempts to directly (or indirectly) measure Ca^{2+}

changes during cytokinesis, investigators continued to report experimental results demonstrating that the direct injection of Ca^{2+} into eggs such as those of *Xenopus*, could initiate precocious pseudo-cleavage formation [42].

Thus, there is a considerable body of evidence from the older literature to support the suggestion that positioning, assembly and stimulation of the cell division machinery may indeed involve localized regions of elevated intracellular Ca^{2+}. The technical challenges associated with measuring changes in localized intracellular Ca^{2+} levels during cytokinesis have in the past, and still continue to, generate uncertainties with regards to the timing, location and even the direction of Ca^{2+} changes. The development, however, of non-disturbing, intracellular Ca^{2+} reporters has greatly improved our understanding of the Ca^{2+} signaling events that occur throughout the cytokinetic process, and these will be discussed in the following sections of this Chapter. It must be stressed and acknowledged, however, that much of what we currently know about Ca^{2+} signaling during cytokinesis has its origins in the series of elegant experiments and careful observations made by individuals over the past few decades, who did not have access to the techniques and equipment we sometimes take for granted today.

2. The sequential stages of embryonic cytokinesis.

In animal cells, cytokinesis begins with the invagination of existing plasma membrane as a result of the contraction of an actomyosin ring or arc [6,13] and is complete with the permanent separation of the daughter cells. How this cleavage process is achieved, varies depending on both the size and geometry of the cells involved. In what we would classify as ‘large’ embryos (for example, zebrafish, with a diameter of ~600 µm and *Xenopus*, with a diameter of ~1200 µm), we suggest that there are several distinct basic processes that contribute to cytokinesis. These include an initial positioning of the furrow within the cell cortex; the propagation (without significant deepening) of the furrow across the surface; this is then followed by furrow deepening (i.e., furrow ingression), which results in two daughter cells that are

separated by a distinct groove, and finally furrow apposition, where the daughter cells 'zip' up together. In small embryos, (for example, echinoderms, with a diameter of ~60 µm) the first two components (i.e., furrow positioning and propagation) may be indistinguishable as separate entities, as the furrow appears almost simultaneously in the equatorial cortex of the late anaphase cell. In addition, in small somatic and tissue culture cells, as well as dividing unicellular organisms, the final stage of cytokinesis may involve the abscission of the final intercellular connection between the prospective daughter cells, which results in complete cell separation [13, 43-45]. During embryonic cell division, however, the cells are faced with two opposing requirements: daughter cells must be separated from one another, while at the same time the embryonic blastoderm must be held together. Thus, following the process of furrow deepening that separates the daughter nuclei, the respective daughter cell plasma membranes do not separate from one another but undergo a process of apposition that holds the cells together. Cleavage furrow apposition, therefore, represents the final step in this type of distinct embryonic cytokinesis [36, 46]. Due to the large size of their embryos, and thus the fact that the different cytokinetic components, (positioning, propagation, deepening and apposition) are separated both temporally and spatially, the first few meroblastic cleavages of teleost embryos and the early holoblastic cleavages of amphibian embryos thus provide a unique opportunity to explore both the Ca^{2+} signaling pathways and the mechanisms specific to each stage in the embryonic cell cleavage process.

3. Recent advances in cytokinetic Ca^{2+} signaling research.

The following sections review more recent reports regarding cytokinetic Ca^{2+} signaling, starting from the early 1990's (i.e., where the Introduction finished). The first Ca^{2+} imaging studies were reported in the 90's, using both luminescent (i.e., aequorin) and fluorescent (e.g. calcium green-1) Ca^{2+} reporters, in combination with custom-built photon imaging microscopy [36] and time-lapse confocal microscopy [47], respectively. Furthermore, a combination of these improved Ca^{2+} imaging techniques, along with the intracellular Ca^{2+} modulation procedures that were developed previously (e.g., using

Ca^{2+} ionophores and chelators, as well as Ca^{2+} channel agonists and antagonists) provided researchers with the tools required to explore the significance and function of Ca^{2+} signaling in the cytokinetic process.

3.1 Cytokinetic Ca^{2+} signaling in fish embryos.

3.1.1. Visualization of Ca^{2+} transients that accompany cytokinesis.

Using the bioluminescent reporter aequorin, Fluck *et al.* [36] were the first to show that slow Ca^{2+} waves accompanied the progression of furrows across blastomere surfaces in medaka embryos, during the first few meroblastic cell divisions. Two successive Ca^{2+} waves, of ~0.5 µm/sec, were observed, the first, relatively narrow wave, was reported to accompany furrow extension (i.e., propagation) while the second, which was ~3 to 7 times wider than the first, accompanied the subsequent furrow deepening then apposition of the daughter cells.

The first reports describing Ca^{2+} transients during the early embryonic cleavages in zebrafish (*Danio rerio*), yielded conflicting results. Reinhard *et al.* [48] reported no Ca^{2+} activity during the early cleavage cycles, whereas Chang and Meng [49] observed that localized elevations in free Ca^{2+} were associated with cytokinesis. Furthermore, Chang and Meng [49] clearly showed that intracellular Ca^{2+} was elevated 'not only in the "right" place, but also at the "right" time', and suggested that such a signal played a role in determining the position of the furrowing plane. Both groups used the fluorescent Ca^{2+} reporter, calcium green-1 dextran, the only apparent difference between the two investigations being the size of the dextran that the reporter was conjugated to. These conflicting reports from the same embryonic system only served to illustrate the challenges involved in visualizing cytokinetic Ca^{2+} transients.

This apparent contradiction in dividing zebrafish embryos was subsequently addressed using bioluminescent aequorin-based imaging [50]. Using this method, several distinct Ca^{2+} signals were observed to accompany the sequential stages of cytokinesis. The first was a clear localized elevation of intracellular Ca^{2+} (subsequently given the name the 'furrow positioning signal'), which was observed to precede the first

appearance of the furrow arc at the blastodisc surface. As the leading edges of the arc progressed outward toward the margins of the blastodisc, they were accompanied by two sub-surface slow Ca^{2+} waves (called the 'furrow propagation signal') moving at ~0.5 µm/sec. **Figure 1A** shows a representative animal pole view of an *f*-aequorin-loaded zebrafish embryo demonstrating the changes in intracellular free Ca^{2+} during positioning and propagation of the first cell division cycle. As these propagation wave fronts approached the edge of the blastodisc, another Ca^{2+} signal appeared in the central region where the positioning signal had originally appeared. Like the propagation signal, it likewise extended outward to the margins of the blastodisc at ~0.5 µm/sec, but in this case it also moved downward (at ~0.1 µm/sec), accompanying the deepening process that separates the daughter cells. This Ca^{2+} signal was given the name the 'furrow deepening signal'. The region of localized elevated Ca^{2+} persisted throughout the furrow apposition process, and only returned to the resting level once the cleavage furrow was fully apposed [46]. This signaling sequence was also observed during the second cell division cycle [50]. The results obtained in zebrafish loaded with aequorin thus both supported and also extended further the observations of Chang and Meng [49], by demonstrating that localized increases in Ca^{2+} were associated with furrow positioning, propagation, deepening and apposition during the first two cleavages in these embryos. Subsequently, other reports confirmed that such localized increases in Ca^{2+} do occur in zebrafish embryos during the different phases of cytokinesis using both aequorin [51] and fluorescent Ca^{2+} reporters [52]. The accumulated data suggest that the positioning, propagation and deepening transients are generated exclusively by Ca^{2+} released from an IP_3-sensitive store, i.e., the ER [46,49,52-54]. The apposition transient, on the other hand, may rely on Ca^{2+} derived from this same common store as well as a contribution from extracellular sources, due to the fact that furrow apposition fails for proceed normally when embryos are bathed in Ca^{2+}-free medium [36,50,55].

Apart from the reports from medaka and zebrafish described above, little else has been published regarding the Ca^{2+} signaling events during cytokinesis in other fish

species. However, here we present new data, which suggest that a sequential series of Ca^{2+} transients might be a conserved feature of embryonic cytokinesis in fish. **Figure 2** (panels A and B) illustrates the Ca^{2+} transients that accompany cytokinesis of the first cell division cycle in embryos of the rosy barb (*Puntius conchonius*; **Fig. 2A**), and mummichog (*Fundulus heteroclitus*; **Fig. 2B**). It appears, therefore, that localized Ca^{2+} transients are a conserved feature during embryonic cytokinesis within at least three orders of teleosts: Cypriniformes (zebrafish and rosy barb), Beloniformes (medaka) and Atheriniformes (mummichog). Beloniformes and Atheriniformes are acanthopterygians, whereas Cypriniformes are ostariophysans [56]. As both of these groups are within the Eutelostei, cytokinetic Ca^{2+} signaling during development is likely an ancestral trait at this taxonomic level and may well be present in more basal teleosts and other actinopterygians.

3.1.2. Determination of the requirement of elevated Ca^{2+} for cytokinesis.

In addition to direct visualization, there is also good indirect evidence (for example, from injecting Ca^{2+} chelators such as BAPTA-type buffers [57] at various times during the cell cycle) to indicate that Ca^{2+} signaling plays a required role in cleavage of both medaka [58] and zebrafish [46, 49--51, 54] embryos.

In order to understand more clearly what effect Ca^{2+} chelators (such as BAPTA buffers) have on the generation of a particular transient, and the developmental significance of blocking or modulating that transient, the timing of their introduction as well as their rate of spread within an early embryo is crucial. Taking this into account, Webb *et al.,* [50] focused on the propagation transient, and waited until the furrow had been positioned on the blastodisc surface (by observing either the appearance of the furrow on the surface or the Ca^{2+} transient associated with this event) before introducing the buffer. These experiments clearly indicated a Ca^{2+}-requirement for furrow propagation in cleaving zebrafish embryos. Subsequently, and again by careful timing of the introduction of the Ca^{2+} buffer, it has more recently been shown that a

localized elevation of Ca^{2+} is also essential for both furrow deepening [46] and furrow positioning [54] in zebrafish embryos.

3.1.3. Determination of the source of the Ca^{2+} generating the various cytokinetic transients.

Zebrafish embryos have also been treated with antagonists of the various Ca^{2+} release channels in order to explore the Ca^{2+} stores responsible for the generation of the cytokinetic Ca^{2+} transients. Chang and Meng, [49] demonstrated that the cytokinetic Ca^{2+} signal that they observed using calcium green-1 dextran, could be blocked via the introduction of heparin, an antagonist of IP_3 receptors (IP_3Rs), but was not affected by ryanodine (a ryanodine receptor antagonist), nifedipine and La^{3+}, (inhibitors of plasma membrane Ca^{2+} channels) or the removal of Ca^{2+} from the external medium. The authors concluded that the cytokinetic Ca^{2+} transient arose from internal stores through the release of Ca^{2+} via IP_3Rs [49].

Webb *et al.*, [50] confirmed with aequorin that zebrafish embryos could generate a regular series of cytokinetic Ca^{2+} transients and divide normally (for at least the first few cell division cycles) in Ca^{2+}-free medium, thus supporting Chang and Meng's [49], observation that extracellular Ca^{2+} is not involved in generating these transients. More recently, Lee *et al*., [46, 54] investigated the source of cytokinetic Ca^{2+} in further detail by carefully timing the introduction of the various antagonists in order to focus specifically on the deepening and positioning Ca^{2+} transients. They showed that the introduction of heparin or another IP_3R antagonist, 2-aminoethoxydiphenylborate (2-APB), at the appropriate time to challenge only the deepening transient, blocked the Ca^{2+} signal and resulted in an inhibition of furrow deepening. On the other hand, antagonists of the ryanodine receptor and NAADP-sensitive channel had no effect on either furrow deepening or on the deepening Ca^{2+} transient [46]. They also demonstrated that the endoplasmic reticulum (ER) and IP_3R are both localized on either side of the cleavage furrow during the deepening process and thus provided additional evidence for the possible intracellular Ca^{2+} store and

release mechanism for the deepening Ca^{2+} transient. Most recently, Lee *et al*., [54] demonstrated that the positioning Ca^{2+} transient is also generated by Ca^{2+} release via IP_3Rs. They also showed that this transient is a required component in positioning the cleavage furrow at the blastodisc surface, and has a distinct rising phase, which clearly distinguishes it from the subsequent propagation transient. **Figure 1B** shows a series of line-graphs to illustrate the dynamics of the positioning (panels a-ii to a-iv) and propagation (panels a-v to a-vii) Ca^{2+} transients that were obtained from the embryo shown in **Figure 1A.**

With regards to exploring the upstream events that might be involved in organizing the furrow positioning Ca^{2+} transient, evidence has recently been presented to demonstrate that a dynamic array of microtubules may be involved. This array, which originates from the mid-zone of the mitotic spindle, and then expands both upward and outward toward the surface of the blastodisc, may play a role in mechanistically linking (in both a spatial and temporal manner) mitotic and cytokinetic events [53]. Lee *et al*., [53] reported that this 'pre-furrowing microtubule array' (or pf-MTA) localised together with a zone of corticular ER and IP_3Rs in the blastoderm cortex just prior to the morphological appearance of the cleavage furrow at the blastodisc surface. The authors suggested that the pf-MTA might be involved in organising the ER and IP_3Rs, required to generate the Ca^{2+} signals that are essential for cleavage furrow formation in zebrafish embryos. **Figure 1Ca** shows a single confocal section taken from an animal pole view through the middle of the blastodisc of a representative zebrafish embryo during the metaphase/anaphase transition of the first cell division cycle. The microtubules (red) were labelled by immunohistochemistry and the DNA (green) labeled with SYTOX Green. This image illustrates that the pf-MTA appears to arise from the mid-zone spindle. **Figure 1Cb** (panels i and ii) are schematic representations of a blastodisc from a facial view to show (i) the approximate location of the confocal scan in **Figure 1Ca** and (ii) the co-localisation of the pf-MTA, ER, IP_3Rs and Ca^{2+}. In addition, representative examples of the localisation patterns of these four elements before the morphological appearance of the

cleavage furrow at the start of the second cell division cycle are shown in **Figure 3**. **Figure 1C** is a hypothetical model to illustrate how Ca^{2+} released via the activation of IP_3Rs in the ER might generate the furrow positioning and propagation transients during the first cell division cycle via a blip/puff/wave Ca^{2+} signaling cascade [59-61].

3.2. Cytokinetic Ca^{2+} signaling in amphibian embryos.

3.2.1. Visualization of Ca^{2+} transients that accompany cytokinesis.

In amphibians, much of the research on Ca^{2+} signaling during cytokinesis has been done using *Xenopus* embryos, although there are a few reports suggesting a possible role of Ca^{2+} during cleavage in other amphibian species. These include the older reports on the salamander and other frog species that are described in the Introduction, as well as more recent publications from the newt, *Cynops pyrrhogaster* [62, 63]. The role of Ca^{2+} in amphibian cytokinesis has always been a somewhat controversial issue. As mentioned earlier (see the Introduction), Baker and Warner [26], using aequorin in conjunction with a PMT, reported that in *Xenopus* embryos, transient changes in Ca^{2+} were sometimes detected during the first and second cell cleavages. On the other hand, Rink *et al*., [64], who measured the free Ca^{2+} in *Xenopus* embryos with inserted Ca^{2+}-selective microelectrodes, concluded that cell division is not accompanied by a change in the level of free Ca^{2+}. More recent reports were also contradictory regarding the role of Ca^{2+} in cleavage in these embryos. In 1991, for example, Grandin and Charbonneau [65], again using Ca^{2+}-selective microelectrodes demonstrated that periodic Ca^{2+} oscillations do accompany cell division in *Xenopus* embryos. They showed that these oscillations reached a peak just a few minutes after the start of a membrane hyperpolarization [65], which is reported to correspond to the onset of cleavage and the production of new plasma membrane in the forming blastomeres [66]. These observations during embryonic cleavage in *Xenopus* were supported by those of Aimar and Grant, [67], who reported that the injection of Ca^{2+} into enucleated newt (*Pleurodeles waltl*) embryos stimulated the formation of pseudo-furrows on the egg surface. However, two other independent

reports that were published in the early 90's appeared to contradict these findings. Using semi-synthetic aequorin in conjunction with a PMT, Kubota *et al*. [68] and Keating *et al*. [69] reported that whereas the Ca^{2+} oscillations that occurred had the same frequency as cytokinesis, cleavage actually began when Ca^{2+} was at a minimum, with the level of Ca^{2+} reaching maximum just before the onset of mitosis. Furthermore, both these groups demonstrated that the Ca^{2+} oscillations continued even when cleavage was blocked with microtubule disrupting drugs, such as colchicine or nocodazole. A potential problem that might result from measuring the total light output from an embryo during cytokinesis using a non-spatial photon counting system such as a PMT, is that it does not enable one to distinguish Ca^{2+} dynamics within localized regions (i.e., in the cleavage furrow) of the dividing embryo. Once again, direct visualization using a spatially sensitive detection system was required to help resolve this controversy in amphibian systems.

Thus, using a combination of calcium green-1 (or calcium green-1-dextran, 10 kD) and confocal microscopy, Muto *et al*. [37] reported that a series of highly localized Ca^{2+} waves propagated along the cleavage furrows in *Xenopus* embryos. Furthermore, they reported that these localized Ca^{2+} waves could be distinguished from the sinusoidal oscillating Ca^{2+} waves identified previously by other groups [68,69]. These cleavage furrow waves were reported to begin a few minutes after the completion of the furrow propagation process (i.e., after furrows had spread from the animal to the vegetal pole) and they then propagated along the deepening furrows at a velocity of ~3 μm/s. The waves lasted for ~10 mins, after which the newly formed blastomeres, which at this stage were separated from each other by a distinct groove, 'zipped' back up together. These data thus suggested that localized Ca^{2+} transients might be associated with furrow deepening and apposition in *Xenopus* embryos.

Most recently, Noguchi and Mabuchi [70], again using calcium green-1-dextran (10 kD) and confocal microscopy, reported the presence of not one, but two distinct Ca^{2+} waves during the first cleavage in *Xenopus* embryos. Like Muto *et al.*, [37], Noguchi and Mabuchi did not detect any type of Ca^{2+} signal associated with either

furrow positioning (i.e., as reported in zebrafish) or furrow propagation (as observed in zebrafish and medaka). They suggested that the first Ca^{2+} wave might be the counterpart of the furrow deepening transient observed in zebrafish and medaka, and that the second might be involved in the apposition of the two daughter blastomeres after furrow deepening has been completed. However, as the waves (that lasted for only ~5 mins), did not propagate into the vegetal region of embryos and were not localized in the region of the contractile band, the authors concluded that Ca^{2+} was not specifically required for either the formation of the cleavage furrow or for its deepening during cytokinesis in *Xenopus* embryos [70].

To add to the controversy, **Figure 2** (panel C) illustrates some preliminary imaging data to show a Ca^{2+} transient that accompanies cytokinesis of the first cell division cycle in an aequorin-injected *Xenopus* embryo, (Lee *et al*., unpublished results). In this vegetal pole view of the embryo, two waves of Ca^{2+}, which last for ~20 mins, can be seen to propagate from the equator of the embryo (possibly arising at the animal pole) and then converge on the vegetal pole. These preliminary data, therefore, suggest that in *Xenopus* embryos, propagating waves of intracellular Ca^{2+} release perhaps accompany the progression of the leading edges of the holoblastic cleavage furrow. When considering all the reported data, however, it would appear that we are still far from fully understanding the possible roles played by Ca^{2+} during cytokinesis in amphibian embryos.

3.2.2. Determination of the requirement of elevated Ca^{2+} for cytokinesis.

The function and developmental significance of the Ca^{2+} transients observed during cytokinesis in *Xenopus* embryos has been explored once again by introducing Ca^{2+} chelators such as BAPTA and EGTA. As described earlier (see section 3.1.2), the timing of the injection of the buffer is critical. For example, it has been shown by injecting BAPTA-type buffers into dividing *Xenopus* embryos that if the buffer is introduced too early then it blocks karyokinesis rather than cytokinesis, and the embryos fail to divide [71,72]. If, however, it is introduced immediately after the

completion of karyokinesis, but before the onset of cytokinesis, then furrow positioning is affected [71]. Indeed, Miller *et al.*, [71] demonstrated that the position of a buffer-induced ectopic furrow always lay on a meridian passing through the animal pole, and they suggested that this might be due to the disruptive action of the buffer on a central spindle (carrying positional information to the cell cortex) rather than on the mitotic asters. Thus, they did not suggest that Ca^{2+} itself was the primary positioning signal, but that it might play a key role in the formation and/or subsequent stability of the central spindle. If on the other hand, buffer was injected after furrow propagation had begun the buffer quickly arrested furrow extension and eventually resulted in its regression [71]. These results thus suggest that Ca^{2+} is required to position, extend and maintain the cleavage furrow in *Xenopus* embryos.

As mentioned previously, Noguchi and Mabuchi [70] challenged the proposal that elevated Ca^{2+} was required for furrow formation and deepening. This was supported by data demonstrating that the use of dibromo-BAPTA (or EGTA) at concentrations sufficient to suppress the two Ca^{2+} waves they visualized, had no effect on cleavage process in *Xenopus* embryos. This once again clearly indicates that further work is required in order to settle these seemingly contradictory findings with regards to the specific roles, if any, played by Ca^{2+} transients during embryonic cleavage in amphibians.

3.2.3. Determination of the source of the Ca^{2+} generating the various cytokinetic transients.

Evidence suggests that in *Xenopus* (as for zebrafish and medaka), the cytokinetic Ca^{2+} signals arise from internal stores through the release of Ca^{2+} via IP_3Rs. For example, Han *et al.* [73] demonstrated that when one blastomere of a 2-cell stage *Xenopus* was injected with heparin or an antibody to PIP_2 (an upstream component of the phosphatidylinositol (PI) pathway), when the first cleavage furrow had formed, then the second cleavage of this blastomere was blocked. In addition, Muto *et al*. [37] reported that both the progression of the Ca^{2+} wave that they observed and also the

morphological process of furrow deepening could be blocked by the injection of heparin, and thus concluded that IP_3-mediated Ca^{2+} mobilization played a role in the propagation of the furrow deepening Ca^{2+} wave.

It has also been reported that the microinjection of Ca^{2+} store-enriched microsomal fractions (derived from mouse cerebella and CHO cells) into dividing newt (*C. pyrrhogaster*) eggs, can induce extra cleavage furrows at the site of injection [62]. When these microsomal fractions were co-injected with heparin or an IP_3R antibody, however, cleavage furrow induction was suppressed. These authors concluded that in the newt, the cleavage furrow is induced via IP_3-mediated Ca^{2+} release. Thus, Ca^{2+} release via IP_3Rs might also play a key role in positioning as well as deepening these embryonic cleavage furrows. There is also evidence from amphibian embryos that, like in zebrafish [53], microtubules are involved in the initiation of cleavage. For example, Mitsuyama and Sawai [63] demonstrated that when Ca^{2+} store enriched microsomal fractions that expressed IP_3Rs were injected into newt embryos, these stores moved toward the next cleavage furrow, in a microtubule-dependent manner.

Thus, in spite of the various contradictory reports that have been published, evidence is slowly accumulating to suggest that localized, elevated levels of intracellular Ca^{2+}, released via IP_3Rs in the ER (which are in some way organized in the cleavage furrow via microtubules) are a conserved feature of cytokinesis both in amphibians and in fish.

3.3 Cytokinetic Ca^{2+} signaling in echinoderm embryos.

As mentioned in the Introduction, Poenie *et al*. [39] were the first to demonstrate that a Ca^{2+} transient was generated during cleavage in embryos of the sea urchin *L. pictus*. They reported that the Ca^{2+} transient, which rises to ~3-fold above the resting level, is initiated just before the furrow appears and then accompanies the developing furrow during cleavage. More recently, Ciapa *et al*. [74] demonstrated that in the fura-2-dextran-loaded embryos of another sea urchin (*Paracentrotus lividus*), a broad, 200 nM Ca^{2+} transient accompanies cleavage. In addition, they showed that this transient

coincides with an increase in IP_3 levels. Suzuki *et al*., [75] subsequently presented indirect evidence to suggest that Ca^{2+} might play a role in cytokinesis in sea urchin embryos by demonstrating that when *Lytechinus variegatus* embryos were treated with the Ca^{2+} ionophore, A23187, shortly after the furrow appeared, then progression of the furrow stopped followed by regression. The authors suggested that the ionophore might inhibit the furrowing process in these embryos by causing a global elevation of Ca^{2+} and thus disrupt the localized rise in Ca^{2+} required for successful cleavage furrow formation [75]. A nice aspect of these experiments was the fact that the effects of the A23187 were reversible. Thus, when the ionophore was washed away, cytokinesis resumed.

A broad peak of Ca^{2+} was also shown to occur in sand dollar (*Echinaracnius parma*) embryos during cytokinesis [76]. When one of the two blastomeres in two-cell stage embryos were injected with aequorin, and then the Ca^{2+} dependent luminescence measured with a microscope photometer, a broad peak of luminescence was observed in the injected blastomere, that correlated with the 10-12 minute duration of cytokinesis [76].

Starfish embryos have also been shown to undergo transient rises in their intracellular free Ca^{2+} during cleavage [47]. Using calcium green dextran, loaded into *Asterina miniata* and *Pisaster ochraceus* oocytes, in conjunction with time-lapse confocal microscopy, a series of repetitive Ca^{2+} oscillations were imaged, which began within ~90 min following fertilization. Some of these Ca^{2+} transients were reported to precede the formation of the cleavage furrow. Furthermore, treatment with heparin both blocked the Ca^{2+} oscillations and inhibited cleavage, thus suggesting that the Ca^{2+} release was once again mediated by IP_3Rs [47].

There are a variety of technical challenges associated with attempting to visualize cytokinetic Ca^{2+} signals from echinoderm embryos. These are currently being overcome (for example, see [47]) and thus this classic animal group, historically used for studying embryonic cytokinesis, will hopefully provide much additional new data in the future.

3.4 Cytokinetic Ca^{2+} signaling in insect embryos.

By imaging *Drosophila melanogaster* embryos that had been loaded with aequorin, Créton *et al.* [77] showed that Ca^{2+} is elevated in the ventral region of the embryo during early development (stages 1 to 4) and oscillates with the cell cycle. However, as the earliest stages of *Drosophila* development are characterized by nuclear division without cytokinesis, these Ca^{2+} signals are more likely to be associated with some aspect of the nuclear cycle and not with embryonic cell cleavage. Ca^{2+} levels were also shown to increase (this time largely on the dorsal side of the embryo), during cell formation, which starts at stage 5 and lasts for ~60 min. This increase in intracellular Ca^{2+} on the dorsal side resulted in a dorsal-to-ventral Ca^{2+} gradient, which was suggested to play a role in dorsal-ventral axis formation [77]. This, to date, is the only report suggesting that elevated Ca^{2+} might play some role in the cell cycle and cellularization process in *Drosophila* embryos, but no data was presented to link either of these elevated Ca^{2+} events to a specific cytokinetic process.

On the other hand, however, Ca^{2+} has been shown to play a key role in cytokinesis in *Drosophila* spermatocytes [78]. Using indirect methods, for example, by treating the spermatocytes with BAPTA-AM or the Ca^{2+} ionophores A23187 and ionomycin, these investigators showed that Ca^{2+} is required to stabilize the cleavage furrow during deepening, but is not required for the initiation of cleavage. They also demonstrated that PIP_2 is localized in the plasma membrane and in the cleavage furrows of dividing *Drosophila* spermatocytes and, like Ca^{2+}, is not needed for the initiation of cytokinesis but is required for continued furrow deepening. They concluded, therefore, that in these particular cells, Ca^{2+} released from intracellular stores via the PI pathway is required for the normal progression of cytokinesis.

In spite of the paucity of Ca^{2+} imaging data reported from *Drosophila* embryos, this system - with its powerful genetics - represents a potent model in which to explore the interaction between Ca^{2+} signaling, cell division and development.

4. Possible targets of the cytokinetic Ca^{2+} signals.

It has been well documented from studies in both embryonic (see Introduction) and tissue culture cells, that cleavage involves the contraction of an actomyosin band (or ring) that lies at the base of the cleavage furrow, via a mechanism comparable to the contraction of smooth muscle (reviewed by Satterwhite and Pollard, [6]). In addition, it was suggested that an increase in Ca^{2+} in the furrow region might (1) induce the recruitment of microfilaments into the contractile band [36]; (2) regulate the binding of myosin to the actin filaments [75,79] and/or (3) trigger actomyosin contraction via the activation of the Ca^{2+}-sensitive myosin light chain kinase (MLCK; [80,81]). Indeed, a current proposition that was discussed at length at the 2004 ASCB Summer Symposium on cytokinesis (see conference report [82]), was that furrow positioning is closely associated with myosin regulation via the activation of MLCK. This might mechanistically link the localized region of elevated Ca^{2+} with a cascade of molecular events that results in the assembly of the contractile apparatus, followed by its activation (see **Figure 4)**. In newt (*C. pyrrhogaster*) embryos, for example, bundles of actin filaments were identified in surgically isolated cleavage furrows. These isolated furrows were induced to contract by the addition of ATP and Ca^{2+} [83]. In addition, it has been demonstrated that in embryos of the sand dollar (*Clypeaster japonicus*) and several sea urchin species (i.e., *Pseudocentrotus, Strongylocentrotus* and *Hemicentrotus* sps), the phosphorylation of myosin by MLCK is required for the formation of the contractile ring [84]. In addition, a more recent report suggests that in *Drosophila* syncytial blastoderm embryos, F-actin and myosin II are transported along microtubules and co-localize in areas where pseudocleavage furrows form [85]. Taken together, these results thus suggest that many of the components of the contractile mechanism required for successful cytokinesis are a conserved feature of many animal cells, as might be the suggested modulatory role played by Ca^{2+}. Furthermore, more recently, Field *et al*., [86] have demonstrated that PIP_2 is required for the adhesion of the contractile ring to the plasma membrane in tissue culture cells, thus providing

evidence for another possible role of cytokinetic Ca^{2+} signaling in the formation of the cleavage furrow.

In addition to its assembly and contractile-related activities, another suggested downstream function of the furrow deepening and apposition Ca^{2+} transients, is the recruitment followed by the exocytosis of vesicles at the ingressing furrow membrane [46]. Several lines of evidence indicate that membrane trafficking plays a key role in membrane remodeling during cytokinesis in animal embryos [87,88]. For example, the addition of new membrane has been found to be a common feature of cytokinesis in many species including *Xenopus, Drosophila*, zebrafish and various sea urchin species [87,89-91]. In addition, an inhibition of cytokinesis has in some cases been shown to be associated with the lack of new membrane formation [92]. It has been suggested that membrane addition may be due to a mobilization of membranes from internal stores [55,93] with the Golgi being the prime candidate [93]. How the furrow membrane is restructured as well as the identity and precise function of the trafficking molecules involved in this process, are currently areas of intense interest. We propose that membrane-bound t-SNARE complexes, that possess a Ca^{2+}-sensitive element, may be one of the targets of the deepening and apposition Ca^{2+} transients. Thus, vesicles, decorated with the appropriate v-SNARE could be moved to the ingressing furrow membrane along microtubule arrays [53,55], where docking and fusion might be stimulated via these Ca^{2+} transients (see **Figure 5**).

5. Conclusions

In all the embryonic systems studied so far, even though a close relationship has been shown to exist between cytokinetic Ca^{2+} transients and various morphological events during the cytokinesis process - be it furrow positioning, propagation, deepening or apposition (see **Tables 1 and 2**) - the precise roles of these transients still remain obscure. Several hypothetical models have been proposed over the years, however, that attempt to link these Ca^{2+} transients to specific cytokinetic events [36,46,50,53,54]. We have also attempted to do so in this Chapter, and this is illustrated in Figures 1C, 4

and 5. It is obvious that a great deal of additional effort needs to be spent in order to fully understand the specific, and perhaps multiple, functions of each of the cytokinestic Ca^{2+} transients. We suggest that this work will be greatly aided through the development of even more sensitive imaging devices and intracellular Ca^{2+} reporters, as well as the extension of these studies into different animal systems, especially those of invertebrate species that are particularly suited to imaging studies.

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Tables

Animal	Embryonic System	Ca^{2+} Reporter	Cytokinetic events where Ca^{2+} signals were observed.	Reference
Fish	Medaka (*Oryzias latipes*)	Aequorin	Furrow extension, deepening and apposition.	[36]
	Zebrafish (*Danio rerio*)	Calcium-green dextran (10 kDa)	Determination of the plane of furrow formation.	[49]
		Aequorin	Furrow Positioning, Propagation and Deepening.	[50]
		Aequorin	Furrow Propagation and Deepening.	[51]
		Calcium-green dextran	Furrow Positioning, Propagation and Deepening.	[52]
		Aequorin	Furrow Positioning, Propagation, Deepening and Zipping.	[46,54]
	Rosy barb (*Puntius conchonius*)	Aequorin	Furrow Positioning, Propagation and Deepening.	Webb *et al*., Unpublished data
	Mummichog (*Fundulus heteroclitus*)	Aequorin	Furrow Positioning, Propagation and Deepening.	Chan *et al*., unpublished data
Amphibians	African Clawed Frog (*Xenopus laevis*)	Calcium green-1	Furrow deepening.	[37]
		Calcium green-1 dextran (10 kDa)	Not involved in cytokinesis.	[70]
		Aequorin	Furrow propagation and deepening.	Lee *et al*., unpublished data
Echinoderms	Starfish (*Asterina miniata* and *Pisaster ochraceus)*	Calcium-green dextran (10 kDa)	Cleavage furrow formation.	[47]
	Sand dollar (*Echinaracnius parma)*	Aequorin	Cytokinesis.	[76]
	Sea urchin (*Lytechinus pictus)*	Fura-2	Starts just before and accompanies the developing furrow during cleavage.	[39]
	Sea urchin (*Paracentrotus lividus*)	Fura-2-dextran	Accompanies cleavage.	[74]

Table 1. Summary of the reports that describe Ca^{2+} transients during cytokinesis, indicating the system under examination and Ca^{2+} reporter used.

Animal	Embryonic System	Ca^{2+} chelator / antagonist / agonist	Ca^{2+} dependent cytokinetic event(s) affected.	Reference
Fish	Medaka (*Oryzias latipes*)	Dibromo-BAPTA	Cytokinesis blocked	[58]
	Zebrafish (*Danio rerio*)	BAPTA, Heparin	Cytokinesis blocked	[49]
		Dibromo-BAPTA	Furrow propagation	[50]
		BAPTA	Cleavage blocked	[51]
		Dibromo-BAPTA Heparin 2-APB	Furrow deepening blocked	[46]
		Dibromo-BAPTA 2-APB	Furrow positioning blocked	[54]
				[52]
Amphibians	Frog (*Xenopus laevis*)	EGTA	Onset of cleavage slowed/blocked	[26]
		Heparin, Anti-PIP_2 antibody	Cleavage inhibited	[73]
		BAPTA buffers	Furrow propagation (blocked) and apposition (delayed).	[71]
		BAPTA buffers	Cleavage delayed	[72]
		Heparin	Onset and extension of cleavage furrow delayed.	[37]
		Dibromo-BAPTA	No effect on cleavage	[70]
Echinoderms	Starfish (*Asterina miniata* and *Pisaster ochraceus)*	Heparin	Cleavage furrow formation	[47]
	Sea urchin (*Lytechinus pictus)*	EDTA	Furrow positioning	[27]
	Sea urchin (*Lytechinus variegatus)*	A23187	Blocked furrow progression	[75]
Mollusks	Squid (*Loligo pealei*)	A23187	Promoted cleavage formation	[31]
Insects	Fruit fly (*Drosophila melanogaster*) spermatocytes	BAPTA-AM, 2-APB U73122,	Furrow deepening blocked	[78]

Table 2. Summary of the reports outlining possible roles played by Ca^{2+} in the cytokinesis process determined via modulation of the cytokinetic Ca^{2+} transient. The system under examination and Ca^{2+} modulating agent used, are indicated.

Figure Legends

Figure 1. Cleavage furrow positioning and propagation in zebrafish zygotes. (A) Representative animal pole (AP) view of an *f*-aequorin-loaded zebrafish embryo demonstrating the changes in intracellular free Ca^{2+} during positioning and propagation of the first cell division cycle. (Aa) A schematic illustration of the embryo to show the location and extension of the cleavage furrow as well as the position of a Linescan analysis (the results of which are shown in (B)). The luminescent images (pseudocolor panels, labeled a-i to a-vii) represent 10 s of accumulated luminescence and were obtained at the following time intervals: (a-i) 30 s before the appearance of the positioning transient; (a-ii) during the initial appearance of the positioning transient (see arrow); then (a-iii) 30 s, (a-iv) 60 s, (a-v) 90 s, (a-vi) 120 s and (a-vii) 150 s after the initiation of the positioning transient. (B) Analysis of luminescence along the first cleavage furrow, before (b-i), and then during the positioning (b-ii to b-iv) and propagation (b-v to b-vii) transients. The profiles represent the average grey level of light emission from the region of the blastodisc shown in (Aa) and were measured using the Linescan function in the Metamorph (v.6.1) image analysis software. 0 µm represents the location on the blastodisc where the positioning signal was first detected and corresponds to the mid-point of the furrow. (Ca) A pre-furrowing microtubule array (pf-MTA) appears to arise from the mid-zone spindle and grows upward and outward to the blastodisc cortex where it precedes the appearance of the furrow on the surface of the blastodisc. This representative embryo was fixed during the metaphase/anaphase transition of the first division cycle and the microtubules (MTs; red) and DNA (green) labeled. In this single optical section through the blastodisc, the pf-MTA is located between the separating chromosomes (green arrowheads) and originates from the remnants of the mitotic spindle (white arrowheads). The MTOCs (asterisks) at the spindle poles are also shown. (Cb-i) A schematic representation of the blastodisc from a facial view to show the approximate location of the single confocal section in (Ca). (Cb-ii) We propose that the pf-MTA transmits positional information to the blastodisc cortex, which results in the localized release of Ca^{2+} via the activation of IP_3Rs in the

ER, and subsequently the correct positioning of the cytokinetic contractile apparatus. (Cc) Hypothetical model showing an axial view of a zebrafish blastodisc to illustrate how Ca^{2+} released via the activation of IP_3Rs in the ER might generate the furrow positioning and propagation transients during the first cell division cycle via a blip/puff/wave Ca^{2+} signaling cascade. Scale bars represent 200 µm (in A) and 20 µm (in Ca). Reproduced with kind permission from Zygote [53,54].

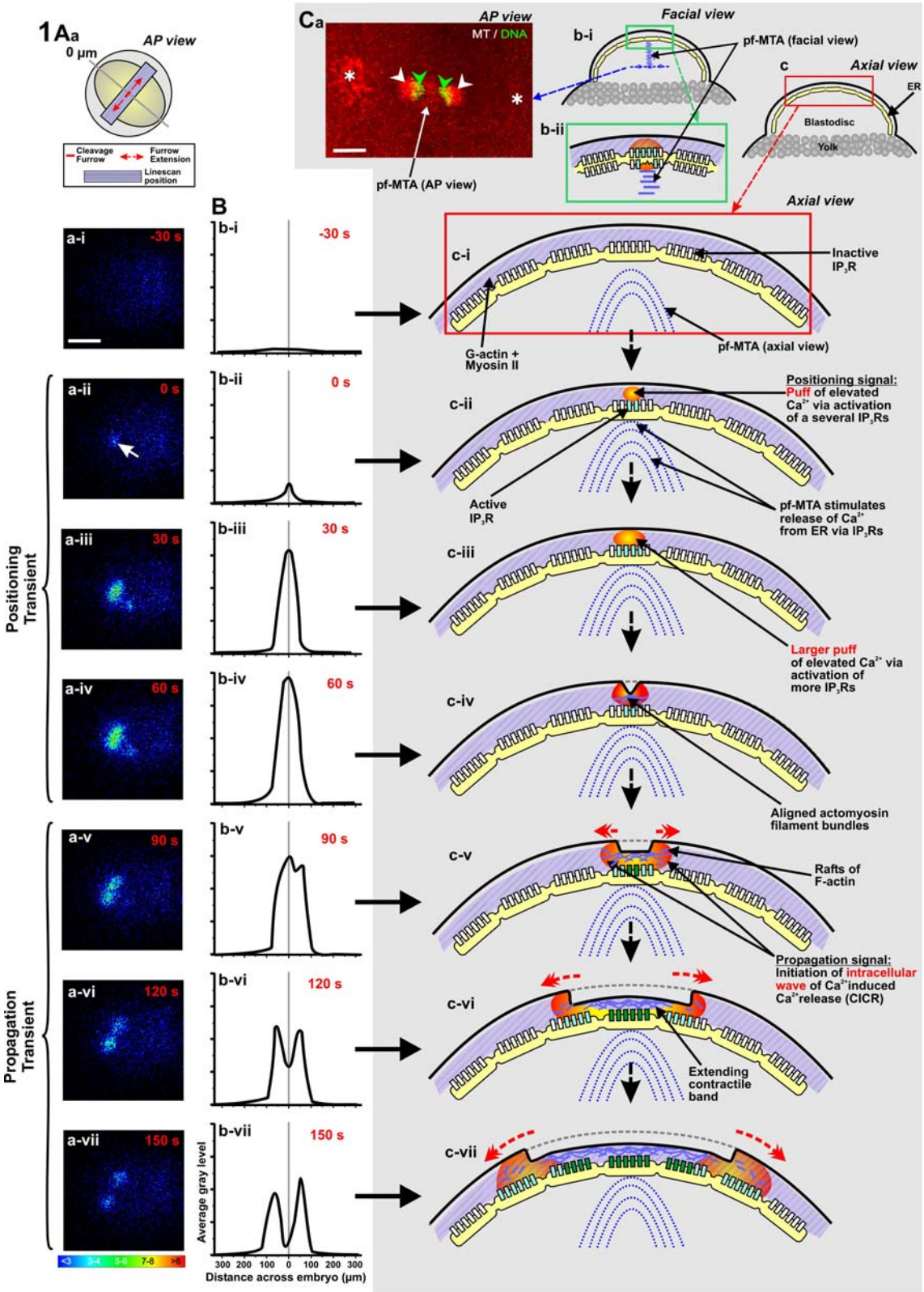
Figure 2. Representative examples of the changes in intracellular-free Ca^{2+} that occur during the first cell division cycle in *f*-aequorin-loaded (A) *Puntius conchonius*, (B) *Fundulus heteroclitus* and (C) *Xenopus laevis* embryos. In (A), the luminescent images (pseudo-colored panels, labeled i-b to viii-b) represent 30 s of accumulated luminescence with a 2-min gap between each image, in (B) the luminescent images (i-b to vii-b) represent 30 s of accumulated luminescence with an 8-min gap between each image and in (C) the luminescent images (i to vii) represent 120 s of accumulated luminescence with a 4-min gap between each image. In (A) and (B), corresponding bright-field images (in A labeled i-a to viii-a and in B labeled i-a to vii-a) were grabbed immediately after the respective luminescent images. The superimposed luminescent and bright-field images (shown in A, panels i-c to viii-c and in B, panels i-c to vii-c) show the location of the Ca^{2+} signals more clearly. Color scale indicates luminescent flux in photons/pixel. Scale bars represent 200 µm. (A) (B) and (C) are Webb *et al.*, Chan *et al*., and Lee *et al*., unpublished results, respectively.

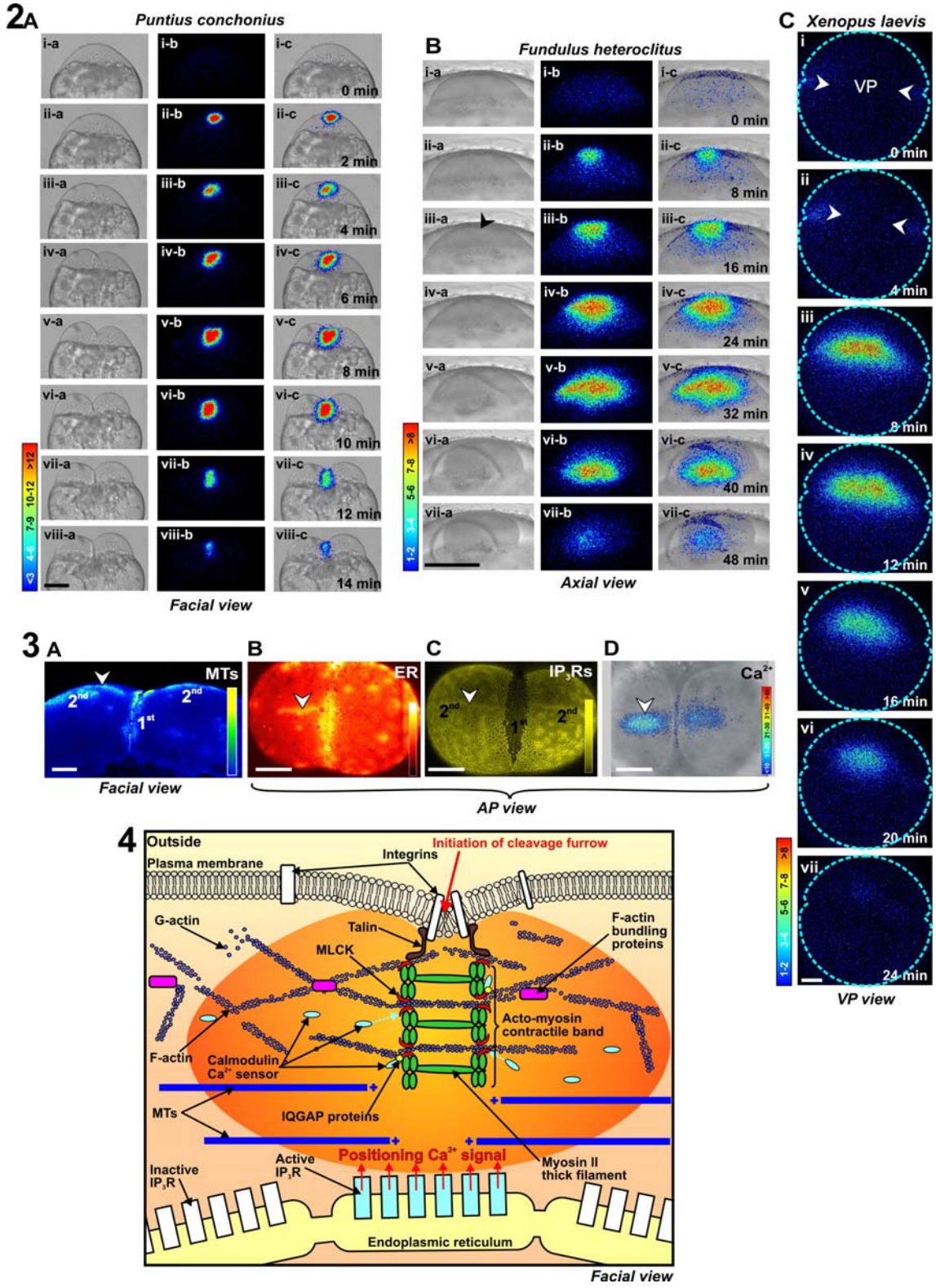
Figure 3. Localization of microtubules, endoplasmic reticulum, IP_3 receptors and Ca^{2+} before the first morphological appearance of the second cleavage furrow. These representative examples of embryos viewed from (A) a facial and (B-D) animal pole views, show the co-localization (see arrowheads) of (A) the microtubules (MTs), (B) the endoplasmic reticulum (ER), (C) IP_3Rs and (D) Ca^{2+} at the site of the future furrows of the second cell division cycle. (D) The aequorin-generated image (in pseudocolor) is superimposed on the appropriate bright-field image of the embryo

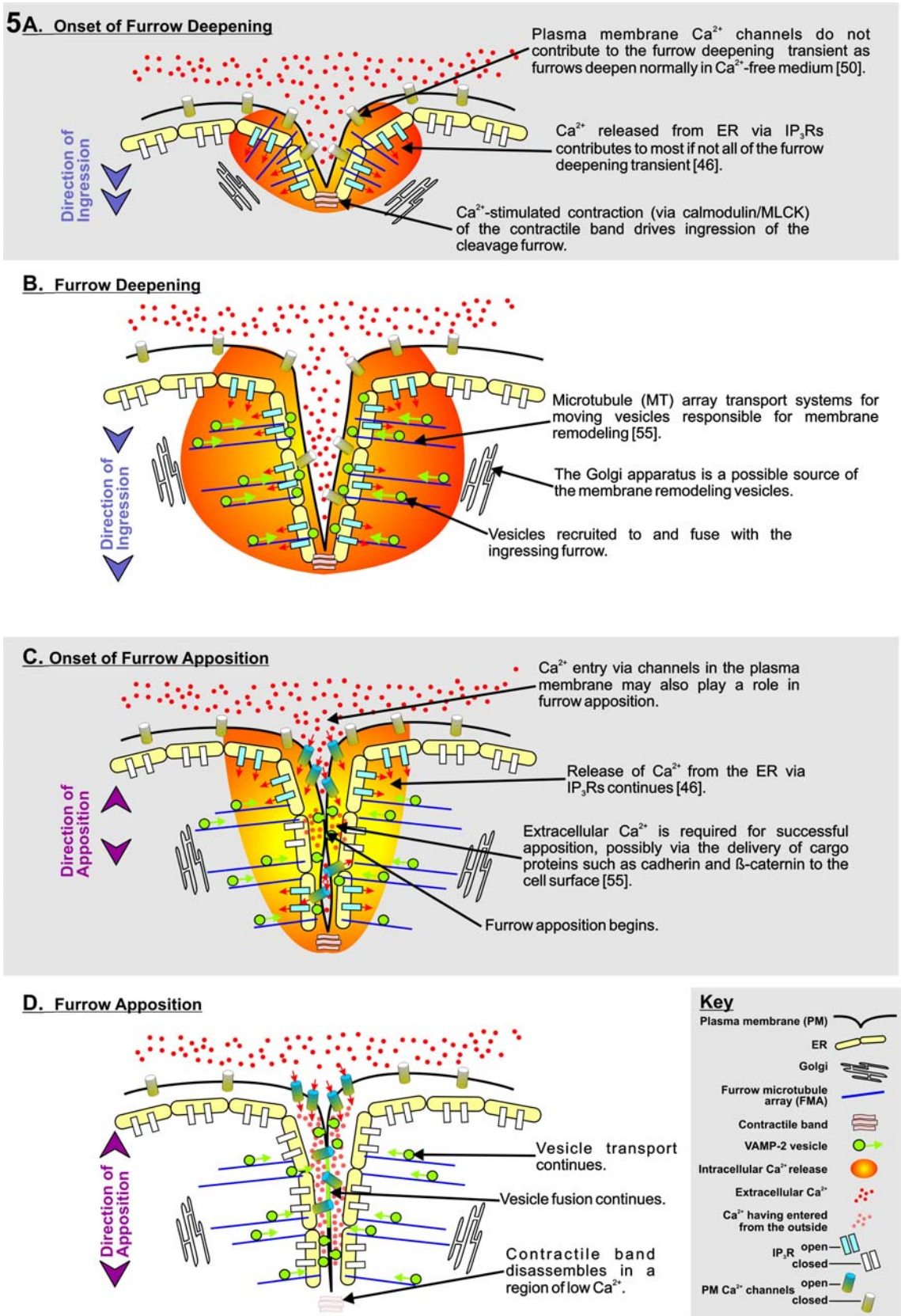
(acquired just prior to the aequorin image) to show the position of the Ca^{2+} signal more clearly. This panel represents 60 s of accumulated luminescence. Color scale indicates luminescent flux in photons/pixel. Scale bars represent 100 μm. The first and second cleavage furrows are labeled in panels (A) and (C). Reproduced with kind permission from Zygote [53].

Figure 4. The possible cytoskeletal targets of the localized release of Ca^{2+}, as well as other molecular elements reported to be involved in cytokinesis. Modified from Figure 2 in [9]. The main Ca^{2+}-sensitive target is the calmodulin Ca^{2+} sensor, which acts via MLCK to organize the actomyosin-based contractile band.

Figure 5. Possible roles of Ca^{2+} signaling during cleavage furrow deepening and apposition in zebrafish zygotes. Hypothetical model showing a facial view of a zebrafish blastodisc to illustrate how Ca^{2+} released via the activation of IP_3Rs in the ER might generate the furrow deepening transient and how Ca^{2+} entering from the outside of the embryo through plasma membrane Ca^{2+} channels might generate the furrow apposition Ca^{2+} transient. The model also suggests that one of the possible downstream functions of these Ca^{2+} transients is to regulate vesicle recruitment and fusion during ingression and apposition. It is suggested that vesicle recruitment might be mediated by cognate v- and t-SNARE partners located on the vesicles and ingressing furrow membrane, respectively. It is also proposed that vesicles are transported to the deepening furrow by an array of perpendicular microtubules [55], that may have developed from the pf-MTA.


1Aa
AP view
0 µm
Cleavage Furrow
Furrow Extension
Linescan position
Ca
AP view
MT / DNA
pf-MTA (AP view)
Facial view
b-i
pf-MTA (facial view)
b-ii
c
Axial view
ER
Blastodisc
Yolk
Axial view
B
a-i -30 s
b-i -30 s
c-i
Inactive IP3R
G-actin + Myosin II
pf-MTA (axial view)
a-ii 0 s
b-ii 0 s
c-ii
Positioning signal: Puff of elevated Ca2+ via activation of a several IP3Rs
Active IP3R
pf-MTA stimulates release of Ca2+ from ER via IP3Rs
a-iii 30 s
b-iii 30 s
c-iii
Larger puff of elevated Ca2+ via activation of more IP3Rs
a-iv 60 s
b-iv 60 s
c-iv
Aligned actomyosin filament bundles
Positioning Transient
a-v 90 s
b-v 90 s
c-v
Rafts of F-actin
Propagation signal: Initiation of intracellular wave of Ca2+induced Ca2+release (CICR)
a-vi 120 s
b-vi 120 s
c-vi
Extending contractile band
a-vii 150 s
b-vii 150 s
c-vii
Propagation Transient
Average gray level
<3 3-4 5-6 7-8 >8
300 200 100 0 100 200 300
Distance across embryo (µm)


2A
Puntius conchonius
i-a
i-b
i-c
0 min
ii-a
ii-b
ii-c
2 min
iii-a
iii-b
iii-c
4 min
iv-a
iv-b
iv-c
6 min
v-a
v-b
v-c
8 min
vi-a
vi-b
vi-c
10 min
vii-a
vii-b
vii-c
12 min
viii-a
viii-b
viii-c
14 min
Facial view
B
Fundulus heteroclitus
i-a
i-b
i-c
0 min
ii-a
ii-b
ii-c
8 min
iii-a
iii-b
iii-c
16 min
iv-a
iv-b
iv-c
24 min
v-a
v-b
v-c
32 min
vi-a
vi-b
vi-c
40 min
vii-a
vii-b
vii-c
48 min
Axial view
C
Xenopus laevis
i
VP
0 min
ii
4 min
iii
8 min
iv
12 min
v
16 min
vi
20 min
vii
24 min
VP view
3
A
MTs
2nd
2nd
1st
Facial view
B
ER
C
IP3Rs
2nd
1st
2nd
D
Ca2+
AP view
4
Outside
Plasma membrane
Integrins
Initiation of cleavage furrow
G-actin
Talin
MLCK
F-actin bundling proteins
Acto-myosin contractile band
F-actin
Calmodulin
Ca2+ sensor
MTs
IQGAP proteins
Positioning Ca2+ signal
Active IP3R
Inactive IP3R
Myosin II thick filament
Endoplasmic reticulum
Facial view


5A. Onset of Furrow Deepening
Plasma membrane Ca2+ channels do not contribute to the furrow deepening transient as furrows deepen normally in Ca2+-free medium [50].
Ca2+ released from ER via IP3Rs contributes to most if not all of the furrow deepening transient [46].
Ca2+-stimulated contraction (via calmodulin/MLCK) of the contractile band drives ingression of the cleavage furrow.
Direction of Ingression
B. Furrow Deepening
Microtubule (MT) array transport systems for moving vesicles responsible for membrane remodeling [55].
The Golgi apparatus is a possible source of the membrane remodeling vesicles.
Vesicles recruited to and fuse with the ingressing furrow.
Direction of Ingression
C. Onset of Furrow Apposition
Ca2+ entry via channels in the plasma membrane may also play a role in furrow apposition.
Release of Ca2+ from the ER via IP3Rs continues [46].
Extracellular Ca2+ is required for successful apposition, possibly via the delivery of cargo proteins such as cadherin and ß-caternin to the cell surface [55].
Furrow apposition begins.
Direction of Apposition
D. Furrow Apposition
Vesicle transport continues.
Vesicle fusion continues.
Contractile band disassembles in a region of low Ca2+.
Direction of Apposition
Key
Plasma membrane (PM)
ER
Golgi
Furrow microtubule array (FMA)
Contractile band
VAMP-2 vesicle
Intracellular Ca2+ release
Extracellular Ca2+
Ca2+ having entered from the outside
IP3R open
closed
PM Ca2+ channels open
closed