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Variations on a theme: imaging cytokinetic and stable rings in situ using *Caenorhabditis elegans*

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Abstract

Cytokinesis is an essential event in canonical cell division. In multicellular organisms, cells must divide in the context of neighboring cells in intact tissues. Recent studies have shown that tissue architecture can regulate the dynamics of and molecular requirements for cytokinesis. On the other hand, regulated cytokinesis failure occurs in, and is required for the proper function of, certain cell types and tissues including cardiomyocytes, hepatocytes, and germ lines. One way to build our understanding of cytokinesis in diverse cell types is to visualize cytokinesis in intact tissues. The nematode *Caenorhabditis elegans* is a powerful system for such inquiries due to the well-characterized, invariant lineage of each of its cells, the ease of genomic modifications including tagging proteins, and many more advantages. The clear cuticle of *C. elegans* allows for live imaging of intact tissues; however, the worm's motility can confound imaging. Here we introduce two *C. elegans* tissues, an epithelial tissue and the germ line, both excellent systems for the study of cytokinesis in the context of an intact animal. Additionally, we present three protocols for overcoming the challenges of live imaging in *C. elegans*.

INTRODUCTION

Cytokinesis is the final stage of cell division, during which nascent daughter nuclei are compartmentalized into separate cells. Early in animal development, completion of cytokinesis is critical for embryo viability. Success of every division is especially crucial for species with determinate development, wherein failure to create a certain cell can result in loss of an entire tissue. However, as development proceeds, many cell types including cardiomyocytes, hepatocytes, and germ cells are created via regulated cytokinesis failure and require high ploidy for proper function (reviewed by Lacroix & Maddox, 2012). Despite the essential nature of cells that are multinucleated, polyploid, and/or syncytial, our understanding of this source of tissue diversity is only rudimentary. This is partially due to the relative inaccessibility of such tissues.

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SUPPLEMENTARY DATA

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Most of our understanding about the process of cytokinesis has been gleaned from studies of isolated cells and cells in culture; however, in multicellular organisms, cells must divide in the context of surrounding cells within a tissue. Recent studies of cells in intact tissues have shown that certain cellular features, including apical junctions or substrate adhesions, can influence the dynamics of, and molecular requirements for, cytokinesis (Founounou, Loyer, & Le Borgne, 2013; Guillot & Lecuit, 2013; Herszterg, Leibfried, Bosveld, Martin, & Bellaiche, 2013; Morais-de-Sá & Sunkel, 2013; Reyes et al., 2014). For example, the presence of cadherin-based apical junctions in epithelial cells causes polarized furrowing along the apicobasal axis. It is not clear how what we know about cytokinesis from studies of isolated and cultured cells is relevant to the process in these more complex settings. Here we describe three techniques for studying cytokinesis in two intact tissues in the nematode *Caenorhabditis elegans*: an epithelium (Fig. 1A) and the syncytial germ line (Fig. 2A).

MODEL SYSTEM: CAENORHABDITIS ELEGANS

Caenorhabditis elegans was first introduced as a model organism in 1965 by Dr. Sydney Brenner (Riddle, Blumenthal, Meyer, & Priess, 1997). Brenner analyzed a number of potential model systems for his research before ultimately choosing *C. elegans*, which continues to prove itself an excellent model system today. *Caenorhabditis elegans* are small hermaphroditic roundworms measuring only 1 mm in length when fully grown. They can self-fertilize, develop from embryo to adult in 3 days, and have a short life span of only ~2.5 weeks. Moreover, their cuticle is transparent throughout development, which allows for live imaging of intact organisms (Corsi, 2015). Further, early studies in *C. elegans* determined that cell lineages are invariant (Sulston, Schierenberg, White, & Thomson, 1983). The characterization of these lineages allows researchers to identify specific cells at any point throughout development. *Caenorhabditis elegans* has also been improved as a model system by the generation of research tools including fluorescent protein tagging, gene-product knockdown via RNAi, and endogenous genome editing via a CRISPR/Cas9 (Chalfie, Tu, Euskirchen, Ward, & Prasher, 1994; Dickinson, Pani, Heppert, Higgins, & Goldstein, 2015; Fire et al. 1998; McNally, Audhya, Oegema, & McNally, 2006; Miller et al., 1999). Given the advantages described previously and the ease and low cost of maintaining worms in the lab, the *C. elegans* research community has grown and thrived over the past decades. Moreover, this highly active community has generated a number of resources including an RNAi library covering ~87% of the *C. elegans* genome, a repository of strains, and online resources such as *wormbase.org*, *wormbook.org*, *wormatlas.org*, and *Phenobank* (Green et al., 2011; Kamath et al., 2003; Kamath, Martinez-Campos, Zipperlen, Fraser, & Ahringer, 2001; Sönnichsen et al., 2005).

VULVAL PRECURSOR CELLS

The vulval precursor cells (VPCs) comprise an elegant model for the study of cytokinesis in an intact tissue (Bourdages, Lacroix, Dorn, Descovich, & Maddox, 2014). These epithelial cells give rise to part of the egg-laying apparatus of the worm and exhibit many behaviors typical of epithelia. This epithelium is superlatively simple: it is a single layer and begins as a single row (Fig. 1A). The VPCs comprise a subset of the hypodermal cells, which are born during embryonic development and remain nonproliferative until the molt that separates the second and third larval stages. Only three hypodermal cells adopt the VPC fates, based on

proximity to the ventral nerve cord and the anchor cell in the somatic gonad, and fate specification signaling among VPCs (reviewed by Schindler & Sherwood, 2013). The Anchor Cell, and thus the VPCs, is centrally located on the worm's anterior-posterior axis (Fig. 1A). The apical surfaces of the VPCs are oriented towards the ventral nerve cord and the basolateral surfaces face a double basement membrane between the VPCs and the somatic gonad. The orientation of VPC divisions is controlled such that the row of three becomes a row of six and then of twelve. During the third round of divisions, certain VPCs divide perpendicular to the worm's long axis, giving the tissue more three-dimensionality. Concurrent with the last round of division, some of the VPCs undergo apical constriction, contributing to the invagination of the tissue (reviewed by Schindler & Sherwood, 2013). Thus, the VPC model allows for study of the three-dimensional challenges associated with cytokinesis in the context of an epithelial tissue.

GERM LINE SYNCYTIIUM

In some tissues, cytokinetic rings are arrested to form stable intercellular bridges that link the cytoplasm of adjacent cells, creating cysts or syncytia. Intercellular bridges are an evolutionarily conserved feature of both the male and female germ line and have also been observed in some somatic tissues (reviewed in Haglund, Nezis, & Stenmark, 2011). The *C. elegans* adult germ line is an emerging model to study syncytiogenesis and this important variation of cytokinesis.

At hatching, the *C. elegans* hermaphrodite germ line consists of two cells called the Z2 and Z3. By adulthood, these two cells have built two syncytial gonad arms, each consisting of nearly 1000 cells organized into an interconnected tubular structure (Fig. 2A) (Hirsh, Oppenheim, & Klass, 1976; reviewed in Hubbard & Greenstein, 2000). In this structure, each of the germ cells is connected to a central pool of cytoplasm, called the rachis, via an intercellular bridge. Bridges are proposed to facilitate communication and coordination among cells and are required for fertility in *C. elegans* and most other systems. Structural and regulatory components of the cytokinetic ring are enriched at intercellular bridges (Amini et al., 2014; Maddox, Habermann, Desai, & Oegema, 2005; Zhou, Rolls, & Hanna-Rose, 2013), supporting the long-standing hypothesis that these structures are stabilized cytokinetic rings generated during regulated cytokinesis failure (also referred to as incomplete cytokinesis). While the mechanisms underlying the arrest of cytokinetic rings and formation of these stable intercellular bridges are not known, a large-scale high-content screen has identified genes that regulate the formation and/or maintenance of these germ line compartments (Green et al., 2011), and recent work is beginning to shed light on how key players, such as scaffold proteins ANI-1 and ANI-2, regulate this process (Amini et al., 2014).

In the adult gonad, oocytes progress developmentally from mitosis through the stages of meiotic prophase as they advance spatially from the distal tip to the proximal gonad, adjacent to the spermatheca (Fig. 2A). This linear arrangement makes it possible to examine all stages of oogenesis in a single sample gonad. Throughout germ cell development intercellular bridges are stably open and only close shortly before oocytes become fully mature (reviewed by Hubbard & Greenstein, 2000). Oocyte pseudocells in the distal gonad

have relatively stable bridges, and as cells enter the proximal region of the gonad, their intercellular bridges dynamically expand and cytoplasmic flows from more distal cells inflate the oocytes (Wolke, Jezuit, & Priess, 2007). When oocytes are full sized, the bridges close, resulting in cellularization of the now mature oocyte. Proper regulation and maintenance of intercellular bridges is required for *C. elegans* fertility (Green et al., 2011). Premature bridge closure blocks oocyte inflation making oocytes nonfunctional. Despite the essential nature of germ line intercellular bridges in *C. elegans* and throughout phylogeny, little is known about their structure and dynamics. The methods outlined here will facilitate studies of these open questions.

1. METHODS

1.1 IMAGING CYTOKINESIS OF VULVAL PRECURSOR CELLS

A technical challenge confounding all in situ imaging of *C. elegans* is the mobility of the worms. *Caenorhabditis elegans* were named for their “elegant” sinusoidal movement, but this movement confounds the imaging of probes that require long exposure times or processes that occur over extended periods of time. To properly image *C. elegans*, the worms must be immobilized. Techniques to physically inhibit worm movement have been developed and include adhering worms to substrates, the use of microfluidic chambers to clamp down worms, and using agarose and polystyrene beads to block worm movement (Goodman, Hall, Avery, & Lockery, 1998; Hulme, Shevkoplyas, Apfeld, Fontana, & Whitesides, 2007; Kerr et al., 2000; Kim, Sun, Gabel, & Fang-Yen, 2013; Rohde, Zeng, Gonzalez-Rubio, Angel, & Yanik, 2007). Additionally, anesthetics are commercially available that temporarily arrest worm movement. Tetramisole hydrochloride, for example, is an alkaline phosphatase inhibitor that acts as a nicotinic acetylcholine receptor agonist. Treatment of *C. elegans* with tetramisole (or levamisole; Acros Organics #187870100) causes prolonged muscle contraction ultimately leading to paralysis (Lewis, Wu, Levine, & Berg, 1980). We recommend combining several of these techniques to image cytokinesis in intact tissues.

Materials:

22 × 22 mm coverslips

95% ethanol

Poly-L-lysine 0.1%

0.01% tetramisole solution in M9 buffer

M9 buffer (to make up 1 L, add 5.8 g Na₂HPO₄, 3.0 g KH₂PO₄, 0.5 g NaCl, and 1.0 g NH₄Cl to dH₂O and autoclave)

5% agarose in M9

VaLaP (1:1:1 Vaseline, lanolin, and paraffin)

Glass slides

Custom nanofabricated silica plate with 20- to 80-μm-wide pyramidal ridges

Mouth pipette

Eyelash tool (eyelash mounted onto a handle such as a toothpick or pipette tip)

1.1.1 Preparation of poly-L-lysine-coated coverslips

1. Dip 22 × 22 mm coverslips in 95% ethanol.
2. Dry them quickly over a flame.
3. Bathe coverslips in 0.1% poly-L-lysine for 4 h to overnight, on a 2D shaker.
4. Let coverslips dry completely before use.

1.1.2 Worm mounting (Fig. 1B)

1. Pick 10–20 worms at the desired larval stage and place them in a drop of 0.01% tetramisole solution prepared with M9 buffer.
2. Soak worms for a minimum of 10 min to ensure they are completely anesthetized, while ensuring that the worms do not dry out.
3. While the worms soak, melt VaLaP and 5% agarose at 95°C.
4. Place a drop of melted agarose on a microscopy slide positioned between two parallel slides each covered with one piece of lab tape. After allowing the drop to cool slightly, place another clean slide orthogonal to the first two slides, over the drop, compressing it into a flat pad. Alternatively, we use custom nanofabricated silica plates with 20- to 80- μ m-wide pyramidal ridges, to create grooves in the agarose.
5. Allow time for the agarose to solidify.
6. Remove the slide or silica plate carefully so as not to break the pad.
7. The agarose pad can be trimmed with a scalpel to ensure it is smaller than the coverslip to be used. When using the silica plate, we ensure that worms will be aligned orthogonally to the microscope stage by creating grooves parallel to the long axis of the slide.
8. Use a mouth pipette to transfer a few anesthetized worms on the agar pad, being careful to transfer only a small amount of the tetramisole solution (not more than 10 μ L).
9. Using an eyelash tool, gently arrange the worms, and align them into the grooves if available. During this period, additional tetramisole solution can be added to prevent the worms from drying.
10. Using a mouth pipette or small piece of Whatman paper, remove excess liquid.
11. Place a poly-L-lysine-coated coverslip over the pad. The sticky coating contributes to immobilizing the worms.
12. Carefully inspect the preparation to ensure that the pad is not too dry (a good contact throughout the surfaces of the coverslip and pad indicates proper

moisture level), since worm survival is dependent on moisture. However, too much liquid will displace worms upon addition of the coverslip.

13. To seal the coverslip, place a small drop of melted VaLaP on each corner of the coverslip using a 20–200 μ L pipette tip.
14. Add more tetramisole solution to fill the entire cavity beneath the coverslip by pipetting the solution at the edge of the coverslip and allowing capillary action to spread it across the sample. Introduce the liquid slowly so as not to disturb the aligned worms.
15. Seal all sides of the coverslip with VaLaP to minimize evaporation.
16. The slide is now ready for imaging. We recommend waiting 10–30 min before beginning imaging, since worms are more likely to remain still during imaging if they have done so during this short delay following slide preparation.
17. VPCs can be identified by transmitted light. They occupy the ventral surface of the worm, midway along the animal's antero–posterior axis, thus centered under the germ line (Fig. 1A). From early L3, almost until the L3/L4 molt, the VPCs form a single row. The anchor cell within the developing somatic gonad may be visible as unusually flared against the paired basement membranes that lie between the somatic gonad and the VPCs; the position of the anchor cell corresponds to the center of the row of VPCs. VPCs in interphase have prominent nuclei and nucleoli. The lateral membranes between VPCs are invisible by transmitted light.

1.2 IMAGING OF THE SYNCYTIAL GERM LINE

Attempts to monitor the germ line during the course of development have been technically challenging. In adults, this tissue is $\sim 50 \mu\text{m}$ thick, necessitating a high-resolution microscope (confocal, two-photon) for optimal imaging. Mitotic division, and thus cytokinesis of developing oocytes, occurs at the distal tip of the germ line in adult worms, which has a complex three-dimensional structure and can lie at different depths within the body (Fig. 2B and C; Movie 1). Additionally, the *C. elegans* gut, which often covers and obscures the germ line, is autofluorescent in the green channel, obscuring weakly expressed fluorescent probes. Lastly, as elaborated previously, worms are highly mobile. Despite these difficulties, some progress is underway toward longitudinally tracking germ line development, using microfluidic chambers to trap animals (R. Green, E. Gutierrez, unpublished data). Presently, several groups have developed methods for imaging cortical dynamics, incomplete cytokinesis events, and intercellular bridge regulation at a single developmental stage (Amini et al., 2014; Green et al., 2011; Maddox et al., 2005; Wang et al., 2015; Wolke et al., 2007; Zhou et al., 2013). Two techniques for imaging intact *C. elegans* germ lines are outlined here. The first method allows for live imaging of dividing cells in the distal germ line, while the second allows for analysis of stable cytokinetic rings throughout the germ line/germ cell development.

1.2.1 Live imaging in the distal germ line—*Materials*:

Unseeded NGM plate

Bleach (5%)

M9

2% agarose in M9

Tetramisole hydrochloride (TMHC; Sigma L9756)

Glass slides

Coverslips

Depression slide

Pick or mouth pipette

10 cm petri dish

1.2.1.1 Live imaging protocol

1. Prepare 0.1 mg/mL of tetramisole hydrochloride freshly from powder, in M9 (1X TMHC).
2. Place a depression slide inside a 10 cm petri dish. Pipette a pool of 1X TMHC into a depression slide.

Note: A 2X mixture will anesthetize faster, but may be toxic to younger worms.

3. Add worms to the pool of TMHC using standard picking techniques, and swish worms off the pick until well separated. Worms will thrash for several minutes. Cover the petri dish to minimize evaporation.
4. Anesthetize for 15–30 min.

Note: Younger worms require less time (15 min), while adults require longer time (30 min).

5. Periodically check for anesthetization by tapping the depression slide. Worms will not thrash at all when fully anesthetized.
6. During this incubation period, prepare an agarose pad. Place a dime-sized drop of melted 2% agarose on a glass slide, let it cool for 5–10 s and place a second slide on top perpendicular to the slide below, using a single layer of lab tape as a spacer.
7. When worms are anesthetized, remove the top slide to expose the agarose pad and place it on the stage next to the depression slide.
8. Place a 3 μ L drop of anesthetic on the agarose pad. Using a pick, transfer the worms one at a time to the agar pad.

Note: Small worms may be more easily transferred using a mouth pipette, but can get stuck to the walls of the glass capillary; transfer method is a matter of preference.

9. Using an eyelash tool, group the worms together in the center of the pad and cover with a coverslip.

Note: To improve the chances of finding a worm at the ideal orientation for imaging, mount several worms at a time, since positioning of the worm is random. Select the worms whose germ line distal tip is closest to the coverslip.

10. We recommend imaging a small z-stack (5–10 μm total thickness) with a time interval of 20 s to 1 min to visualize the dynamics of mitotic events at the distal tip.

The mitotic index in the distal tip of younger worms is higher than that in older worms and thus can yield more mitotic events in a given imaging field (approximately 6–9). In addition, since the germ line is not as thick or architecturally complex in young larvae as in adults, it is easier to track divisions in larvae. The ease of identifying worms at L4 stage is another advantage of this strategy. L2–L3 worms offer less complexity still and also have an enhanced number of mitotic cells, but can be harder to stage. This problem can be circumvented by using synchronized worms (described in the next section). Worms of any stage (L2–adult) can be anesthetized via the protocol described here, though the younger worms are more susceptible to toxicity from overanesthetization.

1.2.1.2 Synchronization of young worms

1. Bleach 10–20 adult worms by placing them in a 5 μL droplet of bleach on a small unseeded NGM plate. The bleach will destroy the adult bodies but soak and dilute into the plate by the time the embryos hatch. Alternatively, use bleach to wash adult worms off a small plate, transfer to a 2 mL tube, vortex and pulse spin, remove the supernatant containing bleach and adult carcass parts, and wash the embryo pellet two times with M9.
2. Allow bleached embryos to hatch overnight, and arrest at the first larval stage (L1) by culturing them in the absence of food for 18 h (either on an unseeded plate or in M9 solution).
3. Transfer hatched worms to a plate containing food to allow worms to progress to L2 (24 h) or L3 (36 h).

1.2.2 Static imaging of intercellular bridges in the germ line—*Materials:*

Worm plates

Glass slides

Poly-L-lysine solution (0.1% w/v aqueous)

18 $\times$ 18 mm glass coverslips

M9

25G 5/8 needle

Eyelash tool

Vaseline

1.2.2.1 Preparing worms

1. Identify L4 worms by the ventral white semicircular appearance of the developing vulva.
2. Using standard picking techniques move 15–30 L4s to worm plates seeded with either OP50 bacteria or RNAi bacteria depending on the experiment.
 - a. For information on how to conduct RNAi, see Ahringer (2006) lab protocol.
3. Allow worms to develop overnight into young adults.

1.2.2.2 Preparing poly-L-lysine-coated coverslips

1. Apply three 10 μ L drops of poly-L-lysine solution (0.1% w/v aqueous) to a glass slide.
2. Place an 18 $\times$ 18 mm coverslip over each drop.
3. Heat on heating block set to 125°C until liquid has evaporated and coverslips are dry and appear slightly darker.
4. Let coverslips come to room temperature.

1.2.2.3 Preparing sample (Fig. 3)

1. Remove poly-L-lysine-coated coverslips prepared within one day from slide and place with coated side facing upward on a glass slide.
2. Pipette 10 μ L of M9 buffer onto the center of the coverslip.
3. Transfer ~5 young adult worms to the droplet using a worm pick (Fig. 3A).
4. Using a 25G 5/8 needle, cut the worms just posterior to the pharynx, removing the head of the worm (Fig. 3A). This should allow the front gonad to be extruded from the worm (Fig. 3B).
5. Use an eyelash tool to position the gonad against the coverslip and away from the dark gut that was likely also extruded. The gut can also be cut away (Fig. 3C).

Note: If one or more worm is still moving, remove them so that the gonads are not disturbed during imaging.

6. Apply a drop of Vaseline to each corner of the coverslip (Fig 3D).
7. Flip the coverslip sample side down onto a glass slide. While monitoring the sample through a stereoscope, press the coverslip down gently so that the gonad is only slightly compressed (Fig. 3E).
8. We recommend imaging through the half of the gonad closes to the coverslip, with 0.5 μ m z-steps. A maximum intensity projection of the z-stack will allow *en face* visualization of intercellular bridges (Fig. 3F). Transverse sections of many bridges will also be visible in optical sections.

CONCLUSIONS

To fully understand the regulation and execution of cytokinesis in diverse cell types, we must study it in complex settings. The nematode *C. elegans* has provided a setting for great experimental strides in many areas of research. The three protocols we present for visualizing cytokinesis in the context of intact tissues in *C. elegans* will help continue this trend. Studying cytokinesis in these systems will inform not only how cytokinesis is different among distinct cell types and tissues, but will also provide insight into the general principles of cytokinesis, as well as how this process can be misregulated leading to diseases such as cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1. Worm mounting and imaging of the vulval precursor cells (VPCs) and cells of the somatic gonad in situ

(A) Cartoon of a worm at the third larval stage, showing the imaging region encompassing the VPCs [green] and somatic gonad [blue]. Images of worms expressing GFP-tagged nonmuscle myosin II in the VPCs (left) and cells of the somatic gonad (right). *Dotted lines* encircle all VPCs (left) and the somatic gonad (right). Myosin II-GFP is enriched in the contractile ring of dividing VPCs [green arrow] and somatic gonad cells [blue arrow]. Below: schematics corresponding to the images above of the VPCs (left) and cells of the somatic gonad (right). (B) Schematic of the assembled microscopy slide ready for imaging. The agarose pad (light gray), with grooves 20–80 μm wide containing worms is mounted on the slide (dark gray), overlaid with a poly-L-lysine-coated coverslip (black). The coverslip is sealed with VaLaP [yellow]. The bottom panel shows a top view of a worm properly aligned in the grooves of the agarose pad.

FIGURE 2. Imaging incomplete cytokinesis in the distal tip of the *Caenorhabditis elegans* adult germ line

(A) Schematic of the *C. elegans* adult germ line. *Boxed area* depicts imaged region of strain expressing mCherry-Histone, mCherry-PH, and GFP-tubulin (OD2127). (B) The surface of the germ line distal tip was imaged with five optical sections separated by 1 μm , at 20 s intervals using 90 $\times$ magnification (60 $\times$ objective with 1.5 $\times$ optivar) on spinning disk confocal microscope (Andor). A single confocal slice is shown. *White box* marks region shown in C. (C) 2 $\times$ magnification of the *boxed white region* in B at select time points. Grayscale images (bottom) highlight the changes in cell shape that occur during germ line cytokinesis. Scale bar: 10 μm .

FIGURE 3. Extrusion and mounting of an intact adult gonad

(A–D) Top: lower magnification top-view schematics of dissections. Bottom: higher magnification views of boxed regions above. (A) The cutting edge [purple] of a 25G $\times$ 5/8 needle as well as the optimal cut site for gonad extrusion. (B) Expected results following cutting. Both the gonad and gut have been extruded from the worm body. (C) Sample after the body and autofluorescent gut have been cut away. (D) Vaseline [yellow] applied to the coverslip. (E) Side-view schematic depicting the complete sample with the isolated gonads close to the coverslip and the Vaseline droplets acting as spacers to prevent compression of the gonads. Note: Figures *not* to scale. (F) Stitched DIC and fluorescence images of a gonad extruded from a worm expressing GFP-tagged ANI-2^{anillin}. Scale bar: 50 μ m.