CHAPTER

17

Using fast-acting temperature-sensitive mutants to study cell division in *Caenorhabditis elegans*

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CHAPTER OUTLINE

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Abstract

Fast-acting temperature-sensitive (ts) mutations are powerful conditional tools for studying transient cellular processes such as cytokinesis. Fast-acting ts cytokinesis-defective mutants are functional at the permissive temperature; yet show a fully penetrant loss-of-function cytokinesis failure phenotype when upshifted to the restrictive temperature. Fast-acting ts mutations thus allow functional tunability and rapid and reversible protein inactivation by simply shifting the temperature at precise times throughout cell division. In this chapter, we describe several techniques and discuss various approaches for harnessing the power of fast-acting ts mutants to study cytokinesis in *Caenorhabditis elegans* using both simple passive heat transfer and more advanced fluidic-based thermal control systems. We also provide detailed protocols for standard dissection, mounting, and imaging of early worm embryos.

INTRODUCTION

Cytokinesis, the physical division of one cell into two (D'Avino, Giansanti, & Petronczki, 2015; Green, Paluch, & Oegema, 2012; Rappaport, 1996), is arguably the most important event in the cell division cycle as without successful cytokinesis, daughter cells do not form and mitosis is bootless. Furthermore, cytokinesis failure is associated with a growing number of human pathologies including neurological disorders, immunological defects, and cancer (Lacroix & Maddox, 2012; Normand & King, 2010; Sagona & Stenmark, 2010).

To ensure each daughter cell inherits a single copy of the genome, cytokinesis is coordinated with nuclear division by both the cell cycle machinery and the mitotic spindle (D'Avino et al., 2015; Green et al., 2012). In animal cells, the mitotic spindle specifies the site of cytokinesis after anaphase onset via signals from the astral and central spindle microtubules, which together specify a zone of active RhoA, a small GTPase, at the division plane (Bement, Benink, & von Dassow, 2005; Jordan & Canman, 2012). Active RhoA then promotes both actin nucleation (via diaphanous formins) and myosin-II motor activation, which form the actomyosin contractile ring. Cytokinesis begins by an initial membrane invagination called the cytokinetic furrow, formed by constriction of the actomyosin contractile ring. Ring constriction proceeds until a dense remnant of the central spindle called the midbody remains at the narrow cytoplasmic bridge between the two daughter cells. The final severing of the cytoplasmic bridge, or abscission, occurs later and is thought to involve the recruitment of microtubule severing proteins, membrane vesicles, and membrane sculpting/transport proteins to the midbody (D'Avino et al., 2015; Green et al., 2012; Mierzwa & Gerlich, 2014).

While traditional forward genetics and RNAi screens have provided a basic understanding of the key genes required for cytokinesis, we still do not understand

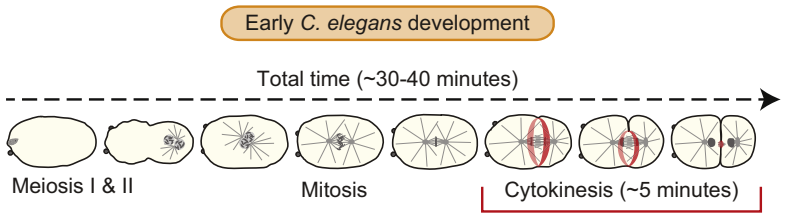

FIGURE 1 Developmental timing of the early *Caenorhabditis elegans* embryo.

The fertilized embryo progresses through meiosis and the first mitotic division in ~30–40 min. As many cytokinesis proteins are required for multiple cellular events, high-resolution temporal control of protein function allows modulation of activity at specific cellular time points.

why the same mutational disruption of a cytokinesis gene does not uniformly affect division of all cell types within a multicellular organism or why some cell types are naturally programmed to fail in cytokinesis during development (Normand & King, 2010). There are two key reasons why traditional gene knockdown is limited in studying the molecular regulation of cytokinesis in vivo: (1) cytokinesis is a dynamic and highly transient process (~5–45 min in most animal cells) and (2) many of the key molecular players in cytokinesis (eg, actin, myosin-II, RhoA) are required at multiple stages of the cell cycle and throughout morphogenesis during organismal development (Fig. 1). To overcome these challenges, researchers have turned to acute protein inactivation to study function specifically during cytokinesis. This has been accomplished with drug treatment, inhibitory antibody injection, dominant-negative protein injection, and fast-acting temperature-sensitive (ts) mutations (Canman et al., 2003; Canman, Salmon, & Fang, 2002; Carvalho et al., 2011; Kurz et al., 2002; Mabuchi & Okuno, 1977; Oegema, Savoian, Mitchison, & Field, 2000; Potapova et al., 2006; Severson, Hamill, Carter, Schumacher, & Bowerman, 2000).

Fast-acting ts mutations are especially powerful because of the ease of functional tunability combined with the power of transient and rapid protein inactivation (~20 s, for example, see Davies et al., 2014). Fast-acting ts mutant proteins are functional enough at the permissive temperature to allow normal development and organismal viability, yet show a fully penetrant loss-of-function cytokinesis failure phenotype when upshifted to the restrictive temperature (Fig. 2). Moreover, ts mutants are "tunable"—that is, use of semirestrictive and semipermissive temperatures can allow varying degrees of functionality. Thus, ts mutants can be used as a sensitized background for synthetic genetic suppression studies by rapid upshift to semirestrictive temperatures at which protein function is weakened but contractile ring constriction is more robust than at fully restrictive temperature (Fig. 2B) (Canman et al., 2008; Jordan et al., 2016; Lewellyn, Carvalho, Desai, Maddox, & Oegema, 2011; Severson et al., 2000). Similarly, synthetic genetic enhancement screening can be done by rapid upshift to semipermissive temperatures at which cytokinesis

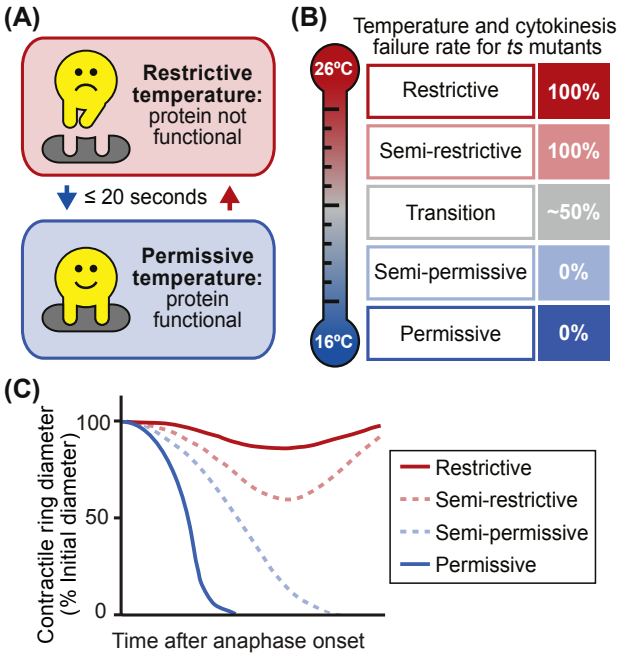

FIGURE 2 Schematic of temperature-sensitive protein activity.

(A) A schematic of a hypothetical temperature-sensitive (ts) mutant protein with function at lower temperatures (permissive), but loss of function at higher temperatures (restrictive). (B) At permissive temperatures, ts mutant proteins are functional and support successful cytokinesis. At restrictive temperatures, ts protein function is reduced and cytokinesis fails. At intermediate semipermissive temperature, cytokinesis is successful, whereas at intermediate semirestrictive temperature, cytokinesis fails. The transition is the pivot temperature between cytokinesis success and failure. (C) Graph showing the tunable kinetics of contractile ring constriction overtime in embryos from a hypothetical ts cytokinesis mutant imaged across various temperatures.

is compromised but still successful without additional perturbations (Fig. 2B) (Canman et al., 2008; Jordan et al., 2016).

It is important to note that not all ts alleles are fast-acting or functional enough at permissive temperature to allow normal development. Each specific ts allele must be tested for rapid modulation of function. Generally, there are two classes of ts mutations: slow-acting and fast-acting (O'Rourke, Carter, et al., 2011). Slow-acting alleles tend to result from mutations in the noncoding sequence, which disrupt gene expression or mRNA processing. Thus at the restrictive temperature, the time required for a loss-of-function phenotype after upshift to restrictive temperature is typically several hours to overnight. This prevents rapid and precise control of

protein function. In contrast, fast-acting ts alleles usually result from single amino acid substitutions in the coding sequence that weaken protein structure, such that increased thermal energy (higher temperature) results in a structural change that blocks normal protein folding and function. These ts proteins quickly respond to changes in temperature, allowing rapid control of protein function using the approaches described below.

A collection of *C. elegans* fast-acting ts cytokinesis-defective mutants has been identified in classical forward genetic screens for maternal effect embryonic lethal, sterile, and zygotic lethal mutants (Canman et al., 2008; Encalada et al., 2000; Kemphues, Priess, Morton, & Cheng, 1988; O'Connell, Leys, & White, 1998; O'Rourke, Yochem, et al., 2011; Raich, Moran, Rothman, & Hardin, 1998). *Caenorhabditis elegans* is a powerful genetic system with a rapid generation time (~3–6 days), hermaphroditic self-fertilizing reproduction, optical clarity, and an invariant developmental lineage. Importantly, the core genes required for cytokinesis are conserved from worms to humans. Using fast-acting ts cytokinesis-defective alleles, protein function can be controlled by simply changing the sample temperature either environmentally (with a thermally controlled room or microscope incubation system) or with a thermally precise fluidic control system. In particular, regulating sample temperature with fluidic thermal control allows rapid and reversible modulation of protein function via timed thermal up- and downshifting throughout cell division (Fig. 3).

In this chapter, we describe several techniques for using fast-acting ts alleles to study cytokinesis in the early worm embryo. We describe how to dissect and mount *C. elegans* embryos for live cell imaging and discuss ways to regulate or change sample temperature and thus protein function during imaging. We describe the practical setup required for analysis of any fast-acting ts cytokinesis-defective alleles and several techniques for sample mounting and temperature control, ranging in features and cost. We provide vendors and ordering information for the reagents and equipment we use. Last, we outline a set of criteria to determine if a particular ts allele is suitable to study cell division.

1. MICROSCOPY AND EMBRYO ISOLATION

1.1 MICROSCOPE AND ROOM

To fully harness the power of fast-acting ts mutants, it is ideal to combine temperature control with live cell analysis via fluorescence microscopy. When working with fast-acting ts mutants, it is important to control the temperature of the dissection and imaging room. We use a temperature-controlled room that is allowed to equilibrate at the desired temperature for at least 60 min before imaging (single-zone air conditioner unit with heat pump, eg, Mitsubishi #MSZ-D36NA or

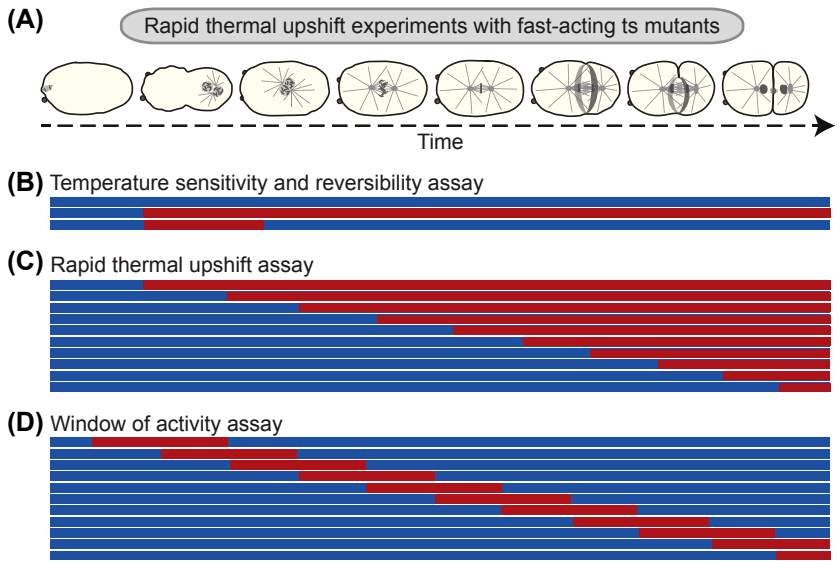

FIGURE 3 Using temperature shifts to study cytokinesis.

(A) Early embryonic development occurs stereotypically from meiosis to cytokinesis. (B–D) Schematic representation of potential experiments combining active temperature control and fast-acting temperature-sensitive (ts) alleles. Upshifts and downshifts between permissive [blue] and restrictive [red] temperatures allow control of protein function at defined stages in the cell cycle. Each line indicates an experiment with an individual embryo at the temperature indicated [red, restrictive; blue, permissive], as it progresses through various cell stages. (B) Upshifting a ts mutant embryo to restrictive temperature immediately before the protein's required function will result in a full loss-of-function (null-like) phenotype if it is fast-acting. Further, a temporary upshift (~3 min) prior to the activity requirement will not cause division failure, if the ts mutant is reversible. (C) Upshifting ts mutant embryos to restrictive temperature at different time points during cell division will indicate when that protein's activity is required during the cell cycle for cytokinesis. Cytokinesis will complete following upshift when the protein function is no longer required. (D) The precise window in which the activity of a specific cytokinesis protein is required can be further determined via temporary upshifts.

Multiaqua #MHWX-24-H-1). We monitor the temperature by averaging the reading of three digital thermometers, directly affixed to the objective using a glue gun and track three or more thermometers spaced throughout the room. To facilitate in vivo analysis of cytokinesis, we use strains that express fluorescent markers, both on their own (controls) and in addition to the desired ts mutation. Typically, we use markers for the chromatin (Histone2B:mCherry (Audhya, Desai, & Oegema, 2007)) and plasma membrane (Phospholipase-Cδ^{PH}:eGFP (Audhya et al., 2005)) to monitor

the underlying cell cycle and cell shape changes during division, but other fluorescent markers[1] and/or transmitted light can be used as desired.

The precise microscopy setup will vary depending on the application and budget. For simple environmental heat transfer, either an inverted or upright microscope stand may be used. However, the currently available thermally precise fluidic control systems require an inverted microscope stand (see below). We use the following microscope system:

- Nikon Ti inverted microscope with either a 20× 0.75 NA. Plan Apo dry, a 40× 0.95 NA. Plan Apo dry, a 40× 1.3 NA. Plan Fluor oil, or a 60× 1.4 NA. Plan Apochromat oil objective
- Yokagawa CSU-10 spinning disc confocal upgraded with Borealis (Spectral Applied Research)
- Hamamatsu Orca ER camera
- Piezodriven motorized stage (Applied Scientific Instrumentation)
- Perfect Focus auto focus system (Nikon)
- Acousto-optic tunable filter to select the excitation light of two 150 mW lasers for excitation at 488 and 561 nm for eGFP and mCherry, respectively (Spectral Applied Research)
- A filter-wheel for emission wavelength selection (Sutter Instruments)
- Metamorph software (Molecular Devices) to control the microscope components, allowing use of perfect focus, z-sectioning, time-lapse, and multiple stage positions

1.2 WORM CULTURE AND DISSECTION

To obtain healthy single-cell embryos, it is important to ensure a supply of young adults that contain early embryos by chunking the required strains about 2–3 days prior to imaging (older adults will mainly have late-stage embryos). Worms containing ts mutations (and control strains containing the markers alone) should be maintained using standard *C. elegans* husbandry techniques (Brenner, 1974; Riddle, 1997), but grown at permissive temperature to allow growth and propagation of the ts mutant strains. Immediately prior to observation, embryos of the appropriate stage are dissected out of the worm using the following protocol (Fig. 4):

1. Under a dissecting microscope with transmitted illumination in the base (eg, Olympus model SXZ16), pick several young adult hermaphrodites (containing a clear single row of embryos) into a watch glass (Carolina Biological Supply

[1]Note: Many transgenically expressed fluorescent markers are transcriptionally silenced at low temperatures in *C. elegans*. Thus, marker-expressing strains should first be tested for low-temperature silencing by raising them at permissive temperature for several generations. For quantitative analysis of fluorescence intensity, control strains (non-ts mutant) should be similarly maintained at permissive temperature (usually 14–16°C) in parallel for comparison to ts mutant strains.

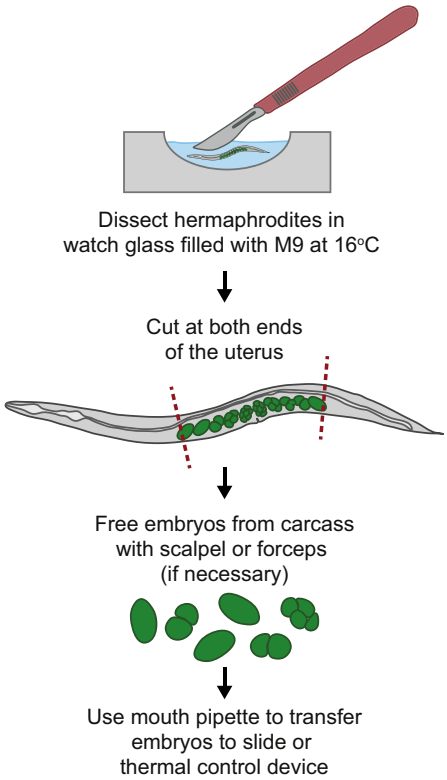

FIGURE 4 Isolating early *Caenorhabditis elegans* embryos for imaging on a light microscope.

Young adults are picked into cold M9 (16°C) and dissected to release embryos by cutting near both ends of the uterus with a scalpel and #15 blade. Embryos of the desired stage can be grouped and transferred to a slide or thermally precise fluidic control system using a mouth pipette.

Company, #742300) containing M9 buffer [85 mM NaCl (Research Products International, #523020), 42 mM Na_2HPO_4 (Research Products International, #523100), 22 mM KH_2PO_4 (Fisher Scientific, #BP362), 1 mM $MgSO_4 \cdot 7H_2O$ (Fisher Scientific, #M63)]. To ensure that the embryos remain at the permissive temperature during dissection, all equipment, M9 buffer, and worm plates should be kept at 16°C prior to use. A small electric wine cooler (Wine Enthusiast #272 02 13) set to 60°F (~16°C) is an inexpensive way to keep worms/watch glass/buffers at the permissive temperature in the microscope room for short periods of time.

a. Alternatively, worms can be directly dissected on the imaging coverslip in a small volume of 16°C M9 (~2–3 μL).

2. To dissect out the embryos, use a #15 scalpel (eg, Bard-Parker #371615) to cut the worm into thirds near both ends of the uterus (red dashed lines, Fig. 4). If multiple embryos are required, dissect up to 10 (or more) worms.
   a. Movement of the worm will eject many embryos from the carcass, usually making further dissection unnecessary, but if embryos remain in the carcass, they can be removed with a scalpel or forceps.
3. Use a mouth pipette to aspirate the collected embryo(s) at the desired developmental stage and transfer to the slide, coverslip, or imaging chamber as described below.
   a. To make the mouth pipette, heat the end of a disposable glass Pasteur pipette (eg, VWR #14673-043) with a flame, and draw the tip out manually, creating a fine ending with ~100 μm diameter. If the diameter of the initial tip is too narrow, the ending can be cut at a wider point. Insert the other (wide) end into a mouth aspirator tube (Sigma Aldrich #A5177).
   b. The shape of the mouth pipette will heavily affect the amount of buffer aspirated with the embryo. Too much buffer can cause the embryos to move, while too little buffer may allow the sample to dry out and/or incorporate air bubbles. With practice, the optimal amount of buffer (and therefore optimal pipette shape) will become clear. Your tongue can be used on the mouth aspirator to minimize suction and control the stop and start of fluid flow.

2. USING FAST-ACTING TEMPERATURE SENSITIVE MUTANT EMBRYOS TO STUDY CYTOKINESIS

Once isolated as described earlier, embryos undergoing cell division can be imaged either: (1) on a standard microscopy slide rapidly shifted to a consistent environmental temperature; or (2) on a fluidic control system that allows precisely timed active temperature shifts. The technique used depends on the type of experiment planned, and each has its own strengths and budget.

2.1 DETERMINING THE PERMISSIVE AND RESTRICTIVE TEMPERATURES FOR A TEMPERATURE-SENSITIVE MUTANT

Throughout this methods paper, we refer to permissive temperature as 16°C and restrictive temperature as 26°C; however, this should be individually determined for each mutant allele. At permissive temperature, the ts mutant strain should show no (or very minor defects) relative to control embryos at the same temperature. This depends on the individual screen and allele, but usually falls within 14–16°C. At restrictive temperature, the ts mutant should show a full loss-of-function phenotype (usually 24–26°C). However, restrictive temperature should not be above 26.5°C, as even control embryos lacking any ts mutations start to show

spindle defects at, or above, 27°C—likely due to thermally induced changes in microtubule dynamics.

2.2 ENVIRONMENTAL TEMPERATURE REGULATION

Environmental temperature regulation is relatively inexpensive and can be as simple as moving the slide from an incubator set at permissive temperature to a room or stage incubator set to restrictive temperature. This has the advantage of requiring little specialized equipment but depends on the ability to control the temperature of the room and/or microscope. Moreover, the ability to time the temperature upshift is limited due to the time required to mount the embryos on the microscope, and the sample cannot be imaged during the upshift. If the environmental approach is used, the embryos are mounted on a standard microscope slide (Fig. 5) and kept at 16°C on an ice bucket covered in bubble wrap (Fig. 6) or in an incubator (or electric wine cooler) prior to shifting to the desired temperature by thermal equilibration with the room and microscope set to the desired temperature. Here, we outline two different procedures for mounting

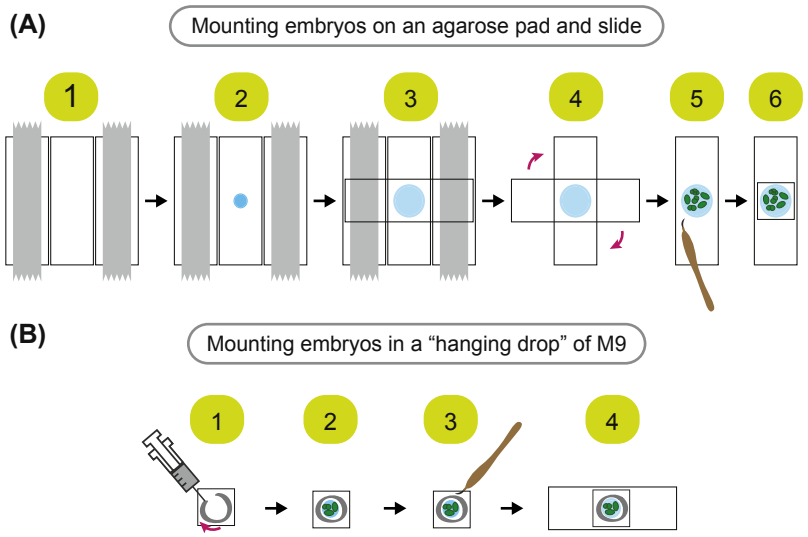

FIGURE 5 Mounting embryos on a slide for environmental temperature upshifts.

(A) To use the agarose pad method, first align three slides side by side and tape down the outer two slides. Next, add a small drop of 2% agarose and cover with a perpendicularly placed slide to make the agarose pad. Once cooled, twist slides relative to each other to expose the pad, add embryos, and top with a coverslip. (B) To mount embryos with the hanging drop method, use a syringe to make a ring of Vaseline on the coverslip. Next, use a mouth pipette to transfer early embryos, cluster embryos with an eyelash tool, and carefully cover with a slide.

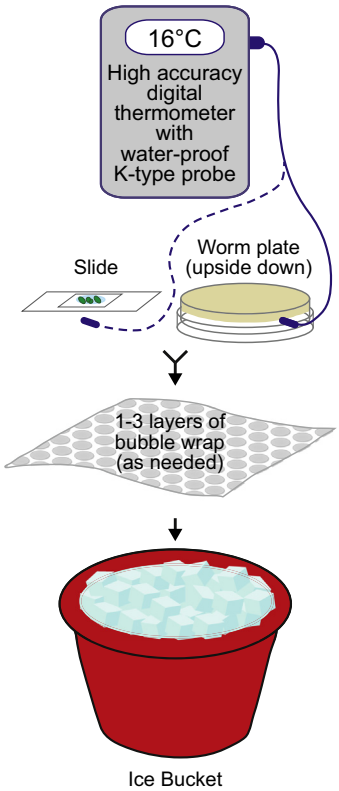

FIGURE 6 An inexpensive method to maintain samples at 16°C.

Worm plates and/or slide-mounted embryos can be maintained at 16°C by placing them on top of an ice bucket covered with bubble wrap, separated from the ice. It is important to monitor the temperature by placing the probe of a digital thermometer as close to the samples as possible on top of the bubble wrap. Depending on the specific bubble wrap, the number of layers may vary, but once you find the correct thickness, this bubble wrap can be saved for future experiments.

embryos on slides for environmental heat transfer, each having its own advantages and disadvantages.

2.2.1 Slide mounting

Traditionally, *C. elegans* embryos have been imaged on standard microscope slides with a 2% agarose pad and a coverslip (Fig. 5A). This has the advantage of being easy to assemble and requiring no special equipment. Moreover, it is possible—with the use of an eyelash tool or ultrafine deer hair—to group embryos together, allowing simultaneous imaging of multiple embryos. Additionally, the process of sandwiching the embryo between the coverslip and the agarose pad results in a slight

compression of the embryos, which can improve imaging of fluorescently tagged proteins. However, the geometric compression can rescue mild polarity defects (Tsou, Ku, Hayashi, & Rose, 2003) and may have unexpected consequences on other cell processes. Furthermore, cellular processes that occur perpendicular to the coverslip (such as the division of the ABa and ABp cells in the four cell stage embryo) are difficult to image under compression conditions, as the embryos tend to divide in a more uniform orientation.

An alternate method termed "hanging drop" traps the embryos in a drop of M9 buffer held between the slide and coverslip with a Vaseline petroleum jelly spacer (Fig. 5B). This prevents compression and results in the embryos being more randomly oriented relative to the plane of the slide. This inconsistency in orientation ensures, at least in a subset of the embryos, that imaging occurs at the optimum viewing angle for most cells during embryonic development.

2.2.1.1 Agarose pad method

1. Align three microscope slides (VWR #16004-430) next to each other side by side. Tape down the two outer slides with two pieces of lab tape per slide (VWR #89098), resulting in a double layer of tape acting as a spacer as shown in Fig. 5A.
2. Place a small amount (~25 μL) of molten 2% agarose onto a glass slide using a disposable plastic transfer pipette.
   a. 2% agarose: Dissolve 2 g agarose (Calbiochem #2125) in 100 mL of ddH_20 by heating in microwave. Aliquot into 1.5 mL microcentrifuge tubes for daily use with a heat block near the dissection scope.
3. Immediately place a second slide on top, oriented perpendicularly to the other slides, and allow the agarose gel to set (~20 s).
4. When the agarose has set, carefully separate the two glass slides by sliding them apart; one of the slides should maintain the agarose pad.
5. Transfer the embryos to the pad using a mouth pipette as described earlier. If imaging multiple embryos, carefully cluster embryos together on the coverslip or agarose pad using an eyelash or hair tool.
   a. Eyelash tools are made by attaching a fine eyelash to a toothpick with a small amount of nail polish, or an ultrafine deer hair tool can be purchased (Ted Pella #119).
   b. The appropriate size of each cluster will depend on the magnification used, as increasing the magnification will decrease the number of embryos possible to fit in the field of view. Additional clusters of embryos on the same slide can be used if the imaging parameters allow multiple stage positions. Care must be taken to regulate the volume of buffer used to transfer the embryos as transferring too much buffer will make it difficult to cluster the embryos together.
6. Gently place a coverslip (No. 1.5, 18 × 18 mm, Corning #2850-22) on top of the pad. Lowering the coverslip at an angle, so that one side goes down before the other, will help to prevent air bubbles from being trapped underneath.

a. Invert the slide and locate the embryos using the dissecting microscope. Using a lab marker or sharpie (eg, VWR #52877), carefully draw a circle around the embryos on the slide. It is important not to mark the coverslip side, especially when using an oil objective, as the marker ink could destroy the optics. Hold the slide at an angle on the dissecting scope to prevent squishing the coverslip while marking the position of the embryos on the slide.

2.2.1.2 Hanging drop method

1. Using a syringe and an 18 G needle (eg, BD #309604 and BD #305195), carefully deposit Vaseline petroleum jelly in a ring with approximately 10−15 mm diameter on a coverslip (No. 1.5, 18 × 18 or 22 × 22 mm) (Fig. 5B).
2. Transfer the embryos to the coverslip using a mouth pipette as described earlier.
   a. It is important to cotransfer a sufficient volume of M9 buffer (∼5−10 μL) to ensure contact between drop and slide.
   b. Alternatively, worms can be directly dissected on the imaging coverslip in a small volume of M9 and then encircle the drop with a ring of Vaseline.
3. Cluster the embryos using an eyelash tool. With practice, it is possible to rotate the embryos to increase the likelihood that they are in the desired orientation.
4. Carefully place a microscope slide on top of the coverslip, such that the drop of M9 containing the embryo and the Vaseline ring make contact with the slide. In this case, marking the position of the embryos is not required, as embryos are much easier to locate within the confined drop.

2.2.2 Environmental temperature regulation for slide-mounted embryos

To increase the temperature of the slide-mounted embryo, the microscope and room (or microscope incubator) should be preequilibrated to the desired restrictive temperature (usually 26°C). Once the embryos are mounted on the microscope, the temperature of the embryo will quickly match that of the microscope especially when using an oil objective[2]. In many cases, however, the embryo(s) may not yet be at the desired developmental stage for temperature upshift. In this case, it may be useful to let the embryos continue to develop at permissive temperature prior to mounting on the heated microscope. This can be accomplished by maintaining the prepared slide at 16°C in a refrigerating incubator, an inexpensive wine cooler, or an ice bucket topped with a layer of insulating bubble wrap at a thickness to maintain the top of the wrap at permissive temperature (Fig. 6). By periodically making brief observation of the embryo(s) under a relatively high-zoom dissecting scope, the developmental progress can be observed with only momentary increases in

[2]Note: A heating collar attached to an oil immersion objection may be sufficient to maintain sample temperature at restrictive temperature. However, we do not have direct experience with the accuracy of objective collars, and collar installation often requires the manual removal of the outer objective cover provided by the manufacturer. This makes the collar difficult to use with multiple objectives or any objective with a correction collar.

temperature. Once the desired stage is reached, mount the slide on the microscope, which will cause the temperature of the embryo to increase.

An alternative approach is to dissect out the embryo and prepare the slide in a separate room or in a specific dissecting chamber that is cooled to the permissive temperature (eg, Liu, Maduzia, Shirayama, & Mello, 2010; Severson et al., 2000). This allows the embryo to be observed continually on a microscope without disrupting protein function. Once the desired cell cycle stage is reached, the slide is rapidly taken to another room containing the imaging microscope that is at the restrictive temperature. This technique has the advantage that the embryo can be continually monitored at low resolution with no fluctuation in temperature prior to moving to the imaging microscope. However, it requires the use of two thermally isolated and controllable rooms that are located close to each other and does not allow imaging during the upshift.

2.3 THERMALLY PRECISE FLUIDIC CONTROL SYSTEMS

Thermally precise fluidic control systems are more sophisticated and actively control the temperature of the sample throughout the experiment via direct heat transfer to the specimen holder. There are two commercially available devices that we have used: (1) The Therminator (Figs. 7 and 8) and (2) CherryTemp (Fig. 9), both of which allow the sample temperature to be rapidly changed while simultaneously imaging on a light microscope to monitor the effect on cytokinesis.

2.3.1 Therminator (Bioptechs, Inc.)

We worked with Bioptechs, Inc. to develop a device that we call the Therminator. This device enables rapid shifts ($\sim$17 s) from permissive to restrictive temperature while simultaneously imaging on an inverted spinning disc confocal microscope (or other light microscope) (Davies et al., 2014). The Therminator imaging unit consists of a sample chamber for embryo mounting on top of the fluid flow chamber separated by a thin layer of glass for rapid heat transfer (Fig. 8). The fluid flow chamber is constantly fed by one of two water baths, each of which can be held at defined temperatures (5−40°C but usually 16°C and 26°C). A mixture of 10% 2-propanol and water is used as the heat conducting fluid to prevent microbial contamination. A switch mechanism determines which water bath feeds the fluid flow chamber and maintains the sample temperature (Fig. 7). The system is constructed to be fully compatible with the imaging light path and with the Perfect Focus (Nikon) autofocusing system. In our hands, high-resolution DIC is not possible with this system, as the device obstructs the required condenser position for Köhler illumination; however, decent transmitted light images can be acquired. The Therminator is designed to accommodate the traditional agarose pad technique, but spacers can be added for noncompressive imaging, if required (Fig. 8A). The imaging unit of the Therminator is composed of two parts: (1) the specimen mounting and fluid flow chamber and (2) the coverslip holder (30 mm diameter, No. 1.5, Bioptechs #30-1313-0319) with a plastic extension and securing latches to hold the entire

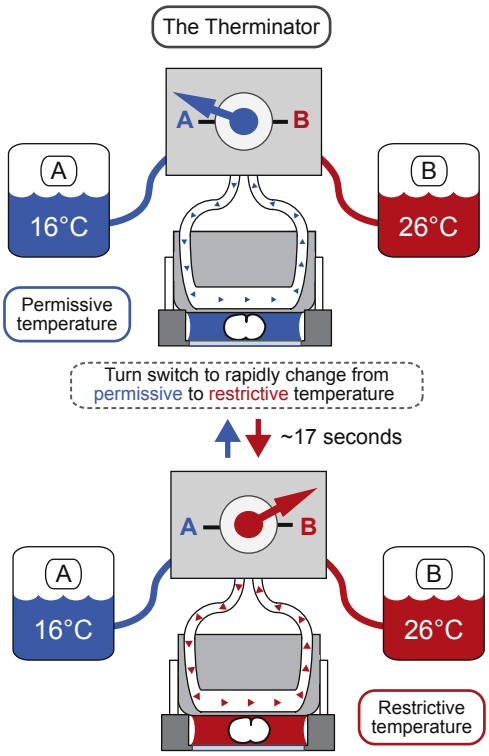

FIGURE 7 Schematic of the Therminator system.

Two water baths, A and B, feed the fluid flow chamber directly above the specimen chamber. By switching between baths A and B (each set to different temperatures), it is possible to rapidly change sample temperature in ~17 s.

imaging unit together (Fig. 8). The fluid flow chamber with mounted specimen is inserted into the coverslip holder so that the specimen is sandwiched between the agarose pad and the glass coverslip. The use of a precisely regulated temperature control system facilitates not just rapid temperature changes but also helps to stably maintain the specimen at a constant temperature with little to no interference from the room temperature.

2.3.1.1 Mounting embryos on the Therminator (Fig. 8B)

1. Prepare the 2% agarose as described earlier (Section 2.2.1.1), and add ~35 μL of molten agarose onto the imaging area of the specimen mounting and fluid flow unit.
   a. Before mounting embryos, ensure that the water bath set to the initial temperature (usually permissive temperature or 16°C) is feeding the flow chamber of the fluid flow unit.

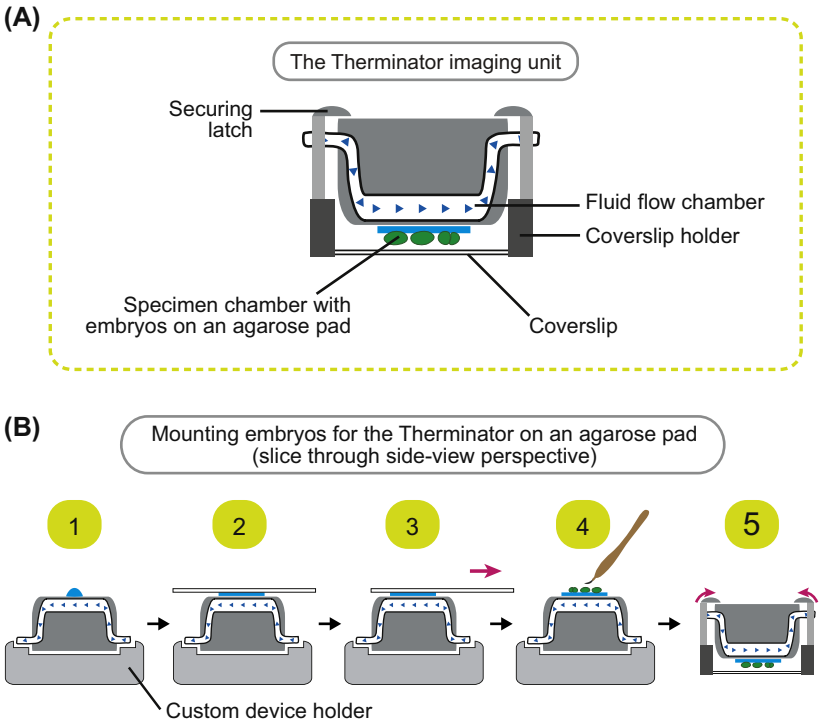

FIGURE 8 Mounting embryos on the Therminator imaging unit.

(A) Schematic diagram of the Therminator imaging unit. Note: Embryos are mounted directly adjacent to the fluid flow chamber allowing for rapid temperature change by convective heat transfer to the glass on which the embryos are mounted. (B) Schematic of protocol for mounting *Caenorhabditis elegans* embryos on an agarose pad for the Therminator. Invert imaging unit onto custom device holder to make the agarose pad. Add molten agarose and use a glass slide to flatten into a pad, allow agarose to solidify, then slide off glass slide carefully so the agarose pad remains on top of the fluid flow chamber. Use a mouth pipette to mount embryos and cluster them with an eyelash tool. Remove the fluid flow chamber from the custom device holder and attach the coverslip holder containing a round coverslip (30 mm). Turn latches to secure the entire imaging unit together.

2. Quickly place a glass slide on top to create a thin agarose pad while the agarose is still molten. Allow the agarose to solidify (~20 s).
3. Carefully slide off the glass slide, leaving the agarose pad on the imaging area of the specimen mounting unit.
4. Transfer the embryos to the agarose pad using a mouth pipette as described earlier. If imaging multiple embryos, carefully cluster embryos together using an eyelash or ultrafine hair tool.

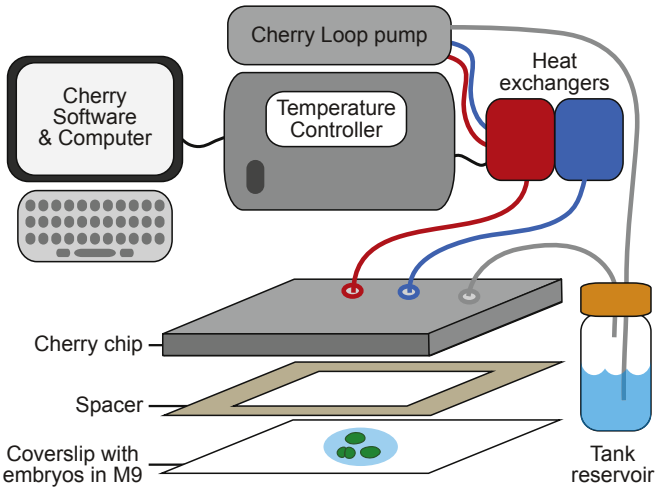

FIGURE 9 Schematic of the CherryTemp system with imaging unit.

Temperature is controlled via the Cherry software, which regulates the heat exchangers and fluid flow into the Cherry chip. Embryos are mounted on a coverslip that fits into the Cherry insert. The Cherry chip is placed on top of the coverslip with a spacer sandwiched in between to avoid compressing the embryos.

5. Prepare the imaging unit sandwich and close the latches to secure the coverslip holder in place. Care must be taken to ensure that a nice seal is established between the coverslip and the agarose pad while confirming that the embryo is not subjected to undue compressive pressure. Mount the assembled device on the microscope stage. It can be secured using stage clips designed for a 35 mm culture dish or manually using small balls of modeling clay (eg, Prang #00740).

Imaging is performed as described earlier to monitor embryonic development. When a change in temperature is experimentally desired, turn the switch to change the source water bath flowing to the fluid flow chamber above the specimen.

2.3.2 CherryTemp (Cherry Biotech; Fig. 9)

CherryTemp is a computer-assisted thermally precise microfluidic control system that maintains sample temperature with constant specimen-level thermal feedback (Fig. 9). The CherryTemp platform is comprised of the CherryTemp dual channel temperature controller box, the CherryLoop fluid pump, the Peltier heat exchangers, the Cherry insert for specimen mounting, and reusable Cherry chips. The platform is controlled through the Cherry software. Using the CherryTemp design, embryos are mounted in M9 buffer sandwiched between a coverslip and the Cherry chip, a microfluidic flow chamber fed by the two Peltier heat exchangers (the agarose pad method is not yet compatible with CherryTemp). The Cherry chip is constructed from transparent polymers that are fully compatible with microscopic imaging methods, such

as transmitted light, DIC imaging, phase contrast, or any fluorescent imaging technique. Each heat exchanger can be set and maintained at a specific temperature ranging from 5°C to 45°C by the CherryTemp controller using the Cherry software. The thermalization liquid contained in a glass bottle is continuously pumped to the heat exchangers, then to the Cherry chip, and then recycled back to the bottle in a closed-loop configuration. Before using the CherryTemp platform, all parts of the system must be properly connected, the microscope stage adaptor for the Cherry insert must be installed in place of your regular stage insert, and a Cherry chip should be connected to the heat exchangers. The CherryTemp system has the added safety feature of automatic flow stop if any leak is detected in the system.

2.3.2.1 Mounting embryos on CherryTemp (Fig. 9)

1. Place a coverslip on the Cherry insert (24× 60 mm, No. 1.5). Dissect worms in a watch glass and transfer embryos on the coverslip using a mouth pipette (or dissect directly on the coverslip). Place the provided spacer on the coverslip. The spacer will prevent embryo compression by the Cherry chip and will avoid evaporation of the sample buffer.
   a. Before mounting the embryos, ensure that the temperature has equilibrated in the heat exchangers using the Cherry software and that liquid at the permissive temperature is flowing to the Cherry chip.
2. Place the Cherry insert with the coverslip on the microscope stage adaptor. Install the Cherry chip on top of the spacer so that the drop of medium that contains the embryos is sandwiched between the imaging coverslip and the Cherry chip. Lock the chip in place using the two slide clasps (not shown).
3. Using the Cherry software interface, control the temperature by activating the flow from one heat exchanger to the other (fast shift) or by entering a new set point for the currently flowing heat exchanger (slower shift) as desired throughout the experiment.

3. CHARACTERIZING TEMPERATURE SENSITIVE ALLELES

As described earlier, not all ts alleles behave similarly, as some are fast- and some are slow-acting. Before using a ts allele to investigate when specific protein functions are required during cytokinesis, it is important to check that the allele fulfills specific criteria and is truly a fast-acting ts mutant (Fig. 10). We use the following criteria to determine if a ts mutant is appropriate for rapid upshift experiments:

1. Loss-of-function phenotype
   It is important to determine whether ts mutant embryos behave like their wild-type counterparts at the permissive temperature but show thermal sensitivity and undergo a gradual transition with increasing temperature to mimic full loss of function at the restrictive temperature (eg, Fig. 2). The loss-of-function phenotype may be confirmed by another method (such as RNAi). This is

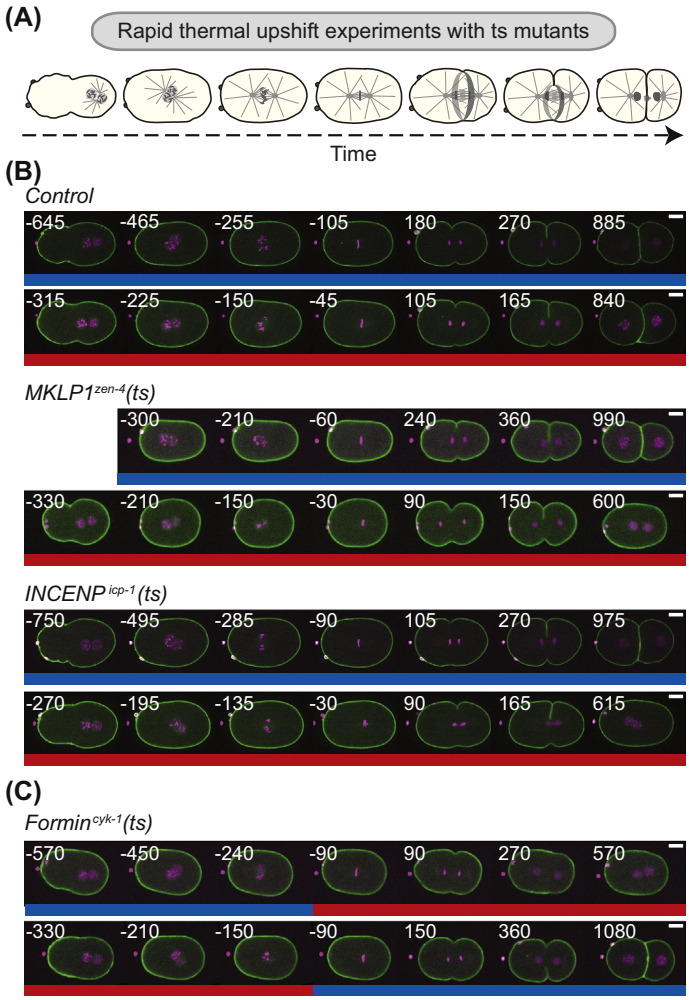

FIGURE 10 Characterizing fast-acting temperature-sensitive (ts) cytokinesis-defective *Caenorhabditis elegans* alleles.

(A) Schematic of early embryonic worm development from pronuclear migration to the end of the first mitotic division. (B) Ideal ts alleles will divide normally at the permissive temperature [16°C, blue], but show the full loss-of-function (null-like) phenotype when rapidly upshifted to 26°C [red], as seen here for *MKLP1^{zen-4}(ts)* and *INCENP^{icp-1}(ts)*. Control embryos divide normally at both permissive and restrictive temperatures. (C) The reversible nature of these ts alleles allows protein inactivation at defined times in the cycle. *Formin^{cyk-1}(ts)* mutant embryos were upshifted to the restrictive temperature either from metaphase until the end of cytokinesis, or from pronuclear meeting until metaphase at which point they were returned to permissive temperature. Inactivation of formin^{CYK-1} prior to metaphase did not disrupt cytokinesis, whereas inactivation after metaphase during cytokinesis blocked successful division. Thus, the *formin^{cyk-1}(ts)* allele is both fast-acting and reversible (See also Davies et al., 2014). Time is in seconds relative to Anaphase Onset (t = 0). Scale bars = 10 μm.

illustrated in Fig. 10B by two different alleles. INCENP^{ICP-1} is a component of the chromosomal passenger complex (CPC) while MKLP1^{ZEN-4} is the kinesin component of the centralspindlin complex. At the restrictive temperature, ts alleles of these proteins [respectively, *INCENP^{icp-1}(or663ts)* and *MKLP1^{zen-4}(or153ts)*] show their well characterized full loss-of-function cytokinesis failure phenotypes and, in the case of *INCENP^{icp-1}(or663ts)*, chromosomal segregation defects (Fig. 10B) (Davies et al., 2014; Severson et al., 2000). Importantly, control embryos lacking a ts mutation divide successfully at both permissive and restrictive temperature (Fig. 10B).

2. Fast-acting mutation
   Does upshifting the embryo to the restrictive temperature cause an immediate loss-of-function phenotype? Determining this depends on the gene, phenotype, and cell process being observed. In the case of cytokinesis, we define alleles as fast-acting if temperature upshift in early mitosis causes cytokinesis failure, indicating that function must be lost within minutes (Fig. 3). For example, formin^{CYK-1} is required for polymerization of contractile ring actin. The *formin^{cyk-1}(or596ts)* allele is fast-acting, as upshifting the mutant embryo to the restrictive temperature at metaphase prevents the formation and ingression of the contractile ring which would normally occur just minutes later (Fig. 10C). If cytokinesis does not fail upon rapid upshift or if longer shifts are required to see a loss-of-function phenotype, then the ts mutant would not be deemed fast-acting and/or would be considered a hypomorphic allele.
3. Phenotypic reversibility
   As described previously, fast-acting ts alleles likely lead to temperature-dependant changes in protein structure rather than protein degradation or other long-term effects. Because of this, many ts alleles are reversible and multiple temperature shifts can be used to inactivate and then reactivate function throughout development (Fig. 3). This allows determination of the functional cell cycle window for a protein during cytokinesis with high temporal precision. Function can be blocked during specific cell cycle stages and then reactivated at a later stage in the division cycle. For example, using the *formin^{cyk-1}(or596ts)* allele, we found that the actin nucleating protein diaphanous formin^{CYK-1} is not essential during earlier stages in mitosis prior to anaphase onset (Fig. 10C) (Davies et al., 2014). That is, *formin^{cyk-1}(or596ts)* mutant embryos can be maintained at restrictive temperature early in development without disrupting cytokinesis as long as they are returned to permissive temperature prior to anaphase onset. Upon returning these ts mutant embryos to permissive temperature, formin^{CYK-1} activity is restored and the cell can divide successfully (Fig. 10C) (Davies et al., 2014).

4. CHOOSING A TEMPERATURE CONTROL METHOD

Here we have described current methods to control the temperature of *C. elegans* embryos, allowing acute conditional inactivation of fast-acting ts alleles. Both the

Table 1 Table Comparing the Feature of Different Temperature Control Methods

Upshift Method	Passive Heat Transfer	Therminator	CherryTemp
Temporal resolution of thermal shift	~60 s	~17 s	~7 s
Simultaneous imaging and thermal shift	No	Yes	Yes
Rapid reversibility	No	Yes	Yes
Cost in USD (excluding microscope)	<$1000	~$15000	~$25000
Supplier	–	Bioptechs, Inc. Butler, PA	Cherry Biotech Rennes, France

environmental and the thermally precise fluidic control methods of temperature regulation have distinct advantages and disadvantages, as outlined below. The key advantage of a thermally precise fluidic control system is the ability to simultaneously image the embryo while precisely modulating the protein function with temperature as well as the rapidity and reversibility of temperature changes. This has allowed us to determine the temporal requirements for key cytokinetic proteins with high resolution (Davies et al., 2014). Furthermore, the ability to simultaneously monitor the cell using a light microscope while abrogating protein function allows continual measurement of cellular parameters (such as chromosome position or contractile ring diameter) in a way that would not be possible if switching between a "cool" and "warm" microscope. Additionally, the ability to shift the temperature of the sample multiple times in a single experiment exploits the reversibility of protein inactivation at defined periods of time during the cell cycle, enabling determination of the functional window of activity required for cytokinesis (Fig. 3D) (Davies et al., 2014). However, the financial investment in a device such as the Therminator or CherryTemp system for sample temperature regulation is much greater than that required for environmental temperature regulation (Table 1).

CONCLUSIONS

In this review, we have outlined ways to use fast-acting ts mutants to study cell division in *C. elegans*. The complexity and cost of the approaches outlined earlier vary considerably; however, each allows rapid inactivation of ts mutants for any lab interested in studying cytokinesis (or other complex cellular event). Although environmental temperature change methods have been used successfully (eg, Liu et al., 2010; Severson et al., 2000) and are undoubtedly useful and inexpensive, the ability to reversibly switch temperatures while simultaneously imaging provides a key advantage. For this reason, thermally precise fluidic control systems are likely to

be important in the future, both in further study of cytokinesis, but also in examining other dynamic cell processes or developmental events where rapid control of protein function is required. The current limiting factor in using this technology is the availability of fast-acting ts alleles. Previous forward genetic screens for ts alleles have provided a range of useful fast-acting ts mutant strains (Canman et al., 2008; Encalada et al., 2000; Kemphues et al., 1988; O'Connell et al., 1998; O'Rourke, Yochem, et al., 2011; Raich et al., 1998). With the advent of genome engineering techniques such as CRISPR, it now seems possible to design ts alleles of desired proteins. This is complicated by a lack of understanding of the mechanism(s) by which ts mutations give rise to a ts phenotype. However, many ts alleles contain mutations in amino acid residues conserved within protein families and between species, which could guide the design of ts alleles.

The approaches described previously allow dissection of cell processes with high temporal resolution. However, they are limited by lack of spatial specificity, as the whole embryo is subjected to the same temperature changes. Future work will combine ts alleles with techniques that control temperature changes at a subcellular level.

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