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## Micromanipulation of Chromosomes in Insect Spermatocytes

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| **Author Comments:** | Please find attached our revision of Manuscript JoVE57359 by Lin et al.<br><br>We have substantially revised the manuscript, making changes in response to all Editor requests and nearly all requests from reviewers. We have added several panels to the figures, and substantially modified Figure 6 to show more of what happens in a micromanipulation experiment. We were unable to respond to part of point 1 by reviewer 3 relating to illustrating stiffness and force measurements. We are currently unable to measure stiffness of the needle or provide information on needle calibration. This is a project that we intend to pursue this summer, but at this point, the work of using micromanipulation needles for force measurements is past work done by other, retired, researchers that we do not currently have the ability to demonstrate. Beyond this single point, we have addressed all other points requested by the Editor or Reviewers in the manuscript.<br><br>We thank you for your patience. Unfortunately, due to several deaths and illnesses in my family, the revision period for this manuscript was extended. I apologize for the delay in revising this manuscript. |
| **Additional Information:** | |
| **Question** | **Response** |
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Cover Letter

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Dear Sir or Madam:

Please find the attached invited manuscript, Micromanipulation of Chromosomes in Insect Spermatocytes, by Nicolas K.H. Lin, Ryder Nance, Jane Szybist, Alan Cheville, and Leocadia V. Paliulis. This is the first submission of this work to JoVE, and it is not currently under review by any other journal.

Micromanipulation of chromosomes has been a key method used in pivotal experiments that have illuminated the mechanism for chromosome congression, the spindle checkpoint, and anaphase chromosome movements. Using a small glass needle with a very fine tip, experimenters like Bruce Nicklas detached chromosomes from the spindle, applied forces to attached chromosomes, and repositioned chromosomes within the cell. Micromanipulation was key in revealing the role of tension in the spindle checkpoint (Li and Nicklas, 1995), in stabilizing chromosome attachments to the spindle (King and Nicklas, 2000) and also revealing the forces exerted on the chromosome during cell division (Nicklas, 1983).

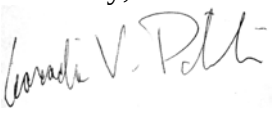
Micromanipulation experiments have been done by a small handful of biologist. One reason for this is that micromanipulators that move chromosomes well are rarely available in the marketplace. We have found that a joystick-controlled piezoelectric micromanipulator controls needle movement with no vibration, drift, or lag between joystick movement and needle movement. The micromanipulators designed by Ellis and Begg (Ellis, 1962; Ellis and Begg, 1981) were ideal for this purpose, but needed to be constructed by the experimenter or someone the experimenter consulted; and essentially required collaboration with engineers or a physics shop. Micromanipulators are currently commercially available and commonly used in electrophysiology, but none are readily useful for micromanipulation of chromosomes, as they often have problems with latency or lag in movement, or have controllers that are not able to accommodate movements required for moving chromosomes. Thus, scientists needed to build their own micromanipulators or modify a commercially available micromanipulator; a difficult and generally prohibitive task.

A group of undergraduate engineering students at Bucknell University, after learning from me about the problems described above, took on the task of constructing a micromanipulator using commercially-available components and 3-D printed parts. The micromanipulator has features not previously available, like adjustable sensitivity and a single joystick positioner for both coarse and fine movements. Plans are freely available via a website cited in our manuscript, and this has now allowed us to make demonstration of the technique available to researchers through JoVE.

While there has been a previously-published article on the technique of micromanipulation (Zhang and Nicklas, 1999), this new article features a new apparatus and approach, and will illustrate the technique much more clearly in video format. We think an article on micromanipulation will be of great interest to readers of JoVE. Micromanipulation experiments have great potential to reveal much more about the internal workings of the cell, and specifically about the movements of chromosomes. Micromanipulation can be combined with the use of small molecule inhibitors, or used in mutants to reveal key features of chromosome movements that have not previously been explored. We believe that this article will lead to some new and very exciting experiments.

Thank you for your consideration.

Sincerely,



Leocadia Paliulis
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**TITLE:**

# Micromanipulation of Chromosomes in Insect Spermatocytes

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**KEYWORDS:**

Micromanipulation, chromosomes, mitosis, meiosis, spermatocytes, grasshoppers, crickets

**SHORT ABSTRACT:**

In this protocol, we describe the selection and preparation of appropriate cells for micromanipulation and the use of a piezoelectric micromanipulator to reposition chromosomes within those cells.

**LONG ABSTRACT:**

The micromanipulation of chromosomes has been an essential method for illuminating the mechanism for chromosome congression, the spindle checkpoint, and anaphase chromosome movements, and has been key to understanding what controls chromosome movements during a cell division. A skilled biologist can use a micromanipulator to detach chromosomes from the spindle, to reposition chromosomes within the cell, and to apply forces to chromosomes using a small glass needle with a very fine tip. While perturbations can be made to chromosomes using other methods such as optical trapping and other uses of a laser, to date, no other method allows the repositioning of cellular components on the scale of tens to hundreds of microns with little to no damage to the cell.

The selection and preparation of appropriate cells for the micromanipulation of chromosomes, specifically describing the preparation of grasshopper and cricket spermatocyte primary cultures for the use in live-cell imaging and micromanipulation, are described here. In addition, we show the construction of a needle to be used for moving chromosomes within the cell, and the use of

a joystick-controlled piezoelectric micromanipulator with a glass needle attached to it to reposition chromosomes within dividing cells. A sample result shows the use of a micromanipulator to detach a chromosome from a spindle in a primary spermatocyte and to reposition that chromosome within the cell.

**INTRODUCTION:**

Micromanipulation has revealed parts of the mechanism for a chromosome congression, the spindle checkpoint, and anaphase chromosome movements. The earliest publication describing the results of micromanipulation experiments was by Robert Chambers[1]. Chambers used a mechanical micromanipulator with an attached glass needle to probe the cytoplasm of a number of different cell types. Unfortunately, contrast methods that allowed the visualization of chromosomes and many other cellular components in living cells were not available at the time, so Chambers' experiments could not show the effects of repositioning such cellular components. Early micromanipulations that altered the chromosome position used the Chambers apparatus to sweep the spindle midzone in anaphase cells, showing that such manipulations could alter the position of chromosome arms in anaphase grasshopper neuroblasts[2]. Nicklas and his collaborators were the first to perform fine micromanipulations of chromosomes, stretching the chromosomes[3], detaching them from the spindle and inducing a reorientation[3-4], stabilizing a malorientation by applying tension to the chromosomes[5-7], and measuring the forces produced by spindles in anaphase[8-9]. Other work by the Nicklas lab showed that cytoplasmic granules could also be manipulated[10] and that centrosomes could be repositioned by micromanipulation[11]. Micromanipulation is not just useful for moving chromosomes and other cellular components. A micromanipulation needle can cleanly cut through a mitotic spindle in demembranated cells[12] or can be used to dissolve the nuclear envelope[13]. In addition, adjacent cells can be fused by micromanipulation[14-15].

With such a wide variety of interesting experiments that can be done using micromanipulation, it is at first glance surprising that micromanipulation experiments have been done by very few chromosome biologists. One reason for this deficiency is that the mitotically-dividing cultured cells that are derived from vertebrate tissues and are commonly used for studying chromosome movements are extremely difficult to micromanipulate. These tissue culture cells generally have a cortical cytoskeleton that "gets in the way" of the micromanipulation needle, and chromosomes either cannot be reached by the needle or the needle grinds through the cell, leading to a cell rupture and death. We, and other experimenters who use micromanipulation, have found arthropod cells to be amenable to micromanipulation. Arthropod spermatocytes are easily spread under a layer of halocarbon oil and appear to have a much less robust cortical cytoskeleton underlying the cell membrane during a cell division. Thus, arthropod testes provide a good source of meiotically-dividing cells (spermatocytes) and mitotically-dividing cells (spermatogonia) with easily accessible chromosomes for micromanipulation. A serial sectioning of a grasshopper spermatocyte fixed during a manipulation revealed that the needle never penetrates the cell membrane; the cell membrane deforms around the needle (Nicklas R. B., personal communication). Spermatocytes from a number of insect and spider taxa have been micromanipulated successfully, including grasshoppers, praying mantids, fruit flies, crane flies, crickets, spittlebugs, moths, black widow spiders, cellar spiders, and orb-weaving spiders[3,7,17-22].

Cultured, mitotically-dividing cells from insects can be micromanipulated. For example, the chromosomes in grasshopper neuroblasts in a primary culture have chromosomes that can be readily micromanipulated[2,23]. We suspect that the available cultured lines derived from *Drosophila* and other insects will also be micromanipulatable, though we have not tested the technique with these cells. We will show how dividing cells from grasshoppers and crickets can be prepared for a micromanipulation. Crickets are easy to obtain from most pet stores at any time of the year. Grasshoppers are only easily obtainable in the summer unless the researcher has access to a laboratory colony, but the species used (*Melanoplus sanguinipes*) has easily flattened cells, and long, easy-to-manipulate chromosomes.

Another reason why micromanipulation experiments have been done by a small handful of biologists is that micromanipulators that move chromosomes well are rarely available in the marketplace. We have found that a joystick-controlled piezoelectric micromanipulator controls the needle movement with no vibration, drift, or lag between the joystick movement and the needle movement, but other types of manipulators can also successfully push chromosomes around in the cell. The micromanipulators designed by Ellis and Begg[25,26] are ideal for the micromanipulation of chromosomes, though they use older technology. Piezoelectric micromanipulators are currently available and commonly used in electrophysiology; however, these micromanipulators are not typically joystick-controlled. Joystick control is key to the smooth movements required for a successful micromanipulation, and so a custom joystick should be constructed to make the currently-available piezoelectric micromanipulators work for a chromosome micromanipulation. The joystick-controlled piezoelectric micromanipulators that work best have direct position control, in which the movement of the joystick translates directly to a needle movement.

A newly-designed piezoelectric micromanipulator can be constructed from commercially-available parts that can be easily replaced and from some small 3-D printed components, and it works well for chromosome micromanipulation[24]. The micromanipulator has adjustable sensitivity, manual coarse positioning, and no vibration, drift, or lag in the needle movement, and direct position control of the needle. Scientists can construct the micromanipulator using instructions available online[24]. Below are the methods for preparing a primary spermatocyte cell culture and for micromanipulating the chromosomes within the cells in that culture.

**PROTOCOL:**

## 1. Preparation of Primary Insect Spermatocyte Cell Culture for Micromanipulation

### 1.1. Slide preparation

1.1.1. Obtain a 75 mm x 25 mm glass slide with a 20 mm diameter circular hole cut out in the center of the slide.

Note: These were cut from a single sheet of window glass to be the size of a glass slide with a hole in the center.

1.1.2. Run a 25 mm x 25 mm #1.5 coverslip through a Bunsen-burner flame for 2 s.

1.1.3. Apply vacuum grease around the edge of the hole in the glass slide

1.1.4. Place the coverslip on the hole as in **Figure 1**. Press it and make sure to form a tight seal.

1.1.5. Flip the glass slide and fill the dissection well with halocarbon oil.

### 1.2. Spermatocyte culture preparation for viewing on microscope

Note: Here, we describe the culture preparation method using grasshopper or cricket cells. Testes contents of other arthropods can be cultured using a similar method.

1.2.1. Obtain subadult male crickets (or grasshoppers). Place a living insect in a refrigerator for approximately 2 min to cold-anesthetize it. Using dissecting scissors, quickly cut through the dorsal surface parallel to the long axis of the abdomen directly behind the wing buds. Manually squeeze the abdomen gently to push the testes through the cut in the exoskeleton (**Figure 2**).

1.2.2. Using forceps, place the isolated testes in a dissection well containing halocarbon oil.

1.2.3. If needed, view the testes under a dissecting microscope. Divide them into smaller pieces using fine-pointed forceps, removing any fat covering the testes, and use fine-pointed forceps to break up the testes (**Figures 3A** and **3B**). Spread the testes contents under the oil, on the surface of the coverslip (**Figure 3C**).

1.2.4. Spread the testes contents until the spread portion of the testis is barely noticeable to the eyes (**Figure 3C**). Use additional oil-loaded dissociation wells if needed.

## 2. Micromanipulation

### 2.1. Production of the microneedle

2.1.1. Place one end of a (0.85 mm in outer diameter, 0.65 mm in inner diameter) glass tube in the flame of a Bunsen burner. Pull the end of the glass tubing in such a way that a 150° angle is formed, and an extended area of narrow glass tubing is created. Break the tubing in the thin region so that the thin region extending from the angle is approximately 10 mm long (**Figure 4A**).

2.1.2. Using a microforge (either custom-made according to Powell[27] or commercially available—see **Table of Materials**), touch the tip of the glass needle to the hot platinum wire to melt the glass at the tip. Form an approximately 45° angle between the needle and the platinum wire of the microforge (**Figure 4B**). Pull the glass away from the hot wire, while shutting off the heat to the wire, forming a thin tip of ≤ 1.5 mm at the end of the glass needle.

Note: The tip diameter will be difficult to measure, but a tip of this length is likely to produce a firm but flexible needle that will be appropriate for micromanipulation. Alternatively, a micropipette puller could be used to create glass tubing with an appropriate tip diameter (the experimenter will have to test different pulling recipes to produce an appropriately firm but flexible microneedle for the job). The needle could be placed in a holder that is shaped similarly to the manipulation needle created according to the method described above.

## 2.2. Positioning the micromanipulator

2.2.1. Place the prepared slide on the stage of an inverted, phase-contrast microscope. Find the dividing cells and center them in the field of view. Focus on the cells using the lowest magnification possible.

Note: We used a 16X phase-contrast objective.

2.2.2. Place the microneedle in the needle holder on the micromanipulator.

2.2.3. Manually position the microneedle in the light path of the microscope so that the tip of the needle is illuminated by the light (**Figure 5B**).

2.2.4. Focus the microscope several focal planes above the plane in which the cells lie.

2.2.5. Reposition the microneedle using the joystick controller at a low sensitivity several times to find the shadow of the needle in the X- and Y-axes. Continue to readjust the position until the position of the tip is visible. Adjust the position of the needle along the Z-axis until the needle tip is in focus.

2.2.6. Refocus on the cells, then focus the microscope above the cell plane so that cells are just out of focus and invisible. Readjust the position of the needle so that its tip is in focus in this focal plane.

2.2.7. Adjust the magnification of the microscope as needed.

Note: We used a 100X 1.4 numerical aperture (NA) phase-contrast objective for the micromanipulation.

2.2.8. After focusing on the cells using the higher-magnification objective, again focus the microscope above the cell plane so that the cells are just out of focus and invisible. Using a higher sensitivity setting, readjust the position of the needle so that its tip is in focus and centered in the focal plane above the plane in which the cells lie.

2.2.9. Refocus on the cells, adjusting their position so they remain in the center of the field of view. The needle is now ready for the micromanipulation.

**2.3. Micromanipulation of chromosomes**

2.3.1. Using the same sensitivity setting as for the fine positioning at a high magnification, use the joystick to control the microneedle and push the chromosomes around inside the cell. Keep the needle tip above the plane of the cells.

2.3.2. To pull on or move a chromosome, focus on a chromosome near the top of the cell.

Note: These chromosomes are easier to manipulate—manipulating chromosomes close to the coverslip makes it likely that the needle will grind into the coverslip, bursting the cell and ending the experiment.

2.3.3. Adjust the Z-axis on the joystick to bring the needle tip into focus, and then move the needle tip with the joystick in X and Y. Place the tip of the needle directly on the chromosome to be manipulated and push it in the desired direction to allow the manipulator to reposition the chromosome.

2.3.4. Depending on how far the chromosome is pushed, either apply tension to the chromosome or apply sufficient tension to detach a chromosome from the spindle. Once a chromosome is removed from a spindle, place it anywhere within the cell.

**REPRESENTATIVE RESULTS:**

**Figure 6** shows a sample micromanipulation of 2 adjacent grasshopper primary spermatocytes in several examples of the possible uses of micromanipulation. This experiment was done using an inverted, phase-contrast microscope. The 0:00 (times shown are in min:s) image shows both cells prior to the manipulation. One chromosome in the bottom cell is shown under tension applied by the micromanipulation needle (0:05; black arrow) and then completely detached from the spindle (0:10; black arrow). One chromosome in the bottom cell (6:25; yellow arrow) is pushed toward 1 spindle pole of that cell (7:05; yellow arrow) and then moved outside of the main spindle area (7:10; yellow area). Both manipulated chromosomes (yellow and black arrows) in this experiment are kept from reattaching to the spindle by being continually nudged with the micromanipulation needle to prevent a formation of new spindle attachments. The shadow cast by the micromanipulation needle is visible in some images, but the tip of the needle is rarely visible. These images show that it is possible to reposition, apply tension to, and detach a chromosome from the spindle using micromanipulation. Chromosomes can be detached from a spindle in their prometaphase, metaphase, and anaphase, though anaphase chromosomes are extremely difficult to detach.

**FIGURE LEGENDS:**

**Figure 1**. **Glass slide with coverslip attached.** A 25 mm x 25 mm, flame-treated coverslip is attached to a 75 mm x 25 mm glass slide with a 20 mm in diameter circular hole cut in the center and sealed with vacuum grease. Note the lack of visible bubbles or gaps in the vacuum grease under the coverslip.

**Figure 2**. **Removal of testes from cricket and grasshopper**. Testes (arrow) can be removed from a male cricket (*Acheta domesticus*—top) or a grasshopper (*Melanoplus sanguinipes*—bottom) after an incision is made on the abdomen of the insect, directly behind the wing buds (arrowheads).

**Figure 3**. **Preparation of cell spread**. (**A**) The testes bulk of a cricket is shown by the arrow. The testes segment used for a single slide is shown by the arrowhead. The bar = 1 mm. (**B**) Grasshopper testes are composed of a number of tubules. Typically, we used 4 tubules from a juvenile male grasshopper for each slide, as shown in this image. The bar = 1 mm. (**C**) For either species, the testes are broken using forceps, and the contents of the testis segments are spread in halocarbon oil. The spread testes contents under oil are shown. The bar = 10 mm.

**Figure 4**. **A micromanipulation needle formed from glass tubing**. (**A**) The general shape of the needle is a long, unmodified segment of glass tubing (≥ 10 cm), with a bend of approximately 150° approximately 10 mm from the end (formed by stretching and bending the glass tubing in a Bunsen burner flame). (**B**) The panel shows the needle placed in the microforge. The needle (arrowhead) forms an approximately 60° angle with the platinum wire filament (arrow) prior to heating it. (**C**) The tip of the needle (arrowhead) is formed after the bent glass tube is touched to the hot platinum wire filament (arrow) in the microforge and pulled away.

**Figure 5**. **Placement of needle in microscope light path**. (**A**) This panel shows a view of the micromanipulator. (**B**) This panel shows a close-up view of the micromanipulation needle placed in the micromanipulator needle holder, which is a piece of 3-D printed plastic. (**C**) A needle tip positioned in the light path of an inverted, phase-contrast microscope.

**Figure 6**. **Micromanipulation of chromosomes in two adjacent grasshopper primary spermatocytes.** The times are shown in min:s. The 0:00 image shows both cells prior to the micromanipulation. Tension is applied on the chromosome (shown by the black arrow) using the micromanipulation needle toward the top spindle pole of that cell (0:05). The chromosome is detached from the spindle and pushed toward the bottom spindle pole (0:10), and eventually moved outside of the main spindle area (6:25, 7:05, and 7:10). A chromosome in the adjacent cell (6:25, yellow arrow) is pushed toward the top spindle pole in that cell (7:05, yellow arrow) and then moved outside the main spindle mass (7:10, yellow arrow). The bar = 10 μm.

**DISCUSSION:**

With practice, moving chromosomes around in the cell can become second nature. Needles that are both sufficiently stiff and sufficiently thin-tipped are difficult to “get the knack of” fabricating, but this ability also comes with practice. Needles that are so fine that they deform when moved in the halocarbon oil will not be useful for pushing chromosomes in the cell. Needles that are so blunt that their tips are visible and as large as 1/3 of the width of a chromosome (or larger) are very likely to kill the cell. The creation of a needle that is sufficiently fine to push chromosomes but not so blunt as to kill the cell is the crucial step of the protocol.

Success typically results when healthy organisms are used for making spermatocyte preparations, and the chromosomes near the top surface of the cell are selected for micromanipulation. Attempts to manipulate chromosomes near the coverslip-surface of the cell often result in grinding the micromanipulation needle into the coverslip and puncturing the cell, thus ending the experiment. If the cell is not punctured or otherwise unhealthy, the cell should survive micromanipulation. Cell death is easily diagnosed, as the chromosomes very rapidly clump together. Micromanipulated cells typically survive through the anaphase and cytokinesis, and we have successfully conducted 6 h long micromanipulation experiments.

While there are a steep learning curve and a requirement to acquire the equipment in gearing up to micromanipulate, there is a great deal of value in being able to manually reposition cellular components in a precise manner. As stated above, there is no other method currently available that allows such precise repositioning of large cellular structures. There is a long history of using micromanipulation to move structures in the cell. Examples, with beautiful figures of the process, of using micromanipulation to measure forces exerted on anaphase I chromosomes[8] and apply tension to chromosomes[5] are available in the literature. In addition, many examples of how micromanipulation can also be used to alter the position of other structures in the cell-like microtubules[28-29], disrupt other structures in the cell like the nuclear envelope[13], or fuse adjacent cells[14-15] are illustrated in the literature.

More micromanipulation experiments need to be done, as the ability to manually reposition chromosomes and other structures, to apply forces to structures, and to measure forces on chromosomes in different stages of the cell division will lead to a better understanding of cellular processes, and the creation of accurate, predictive mathematical models of cellular processes. Future applications will allow an easy measurement of the forces on chromosomes in all stages of the cell division.

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**DISCLOSURES:**
The authors have nothing to disclose.

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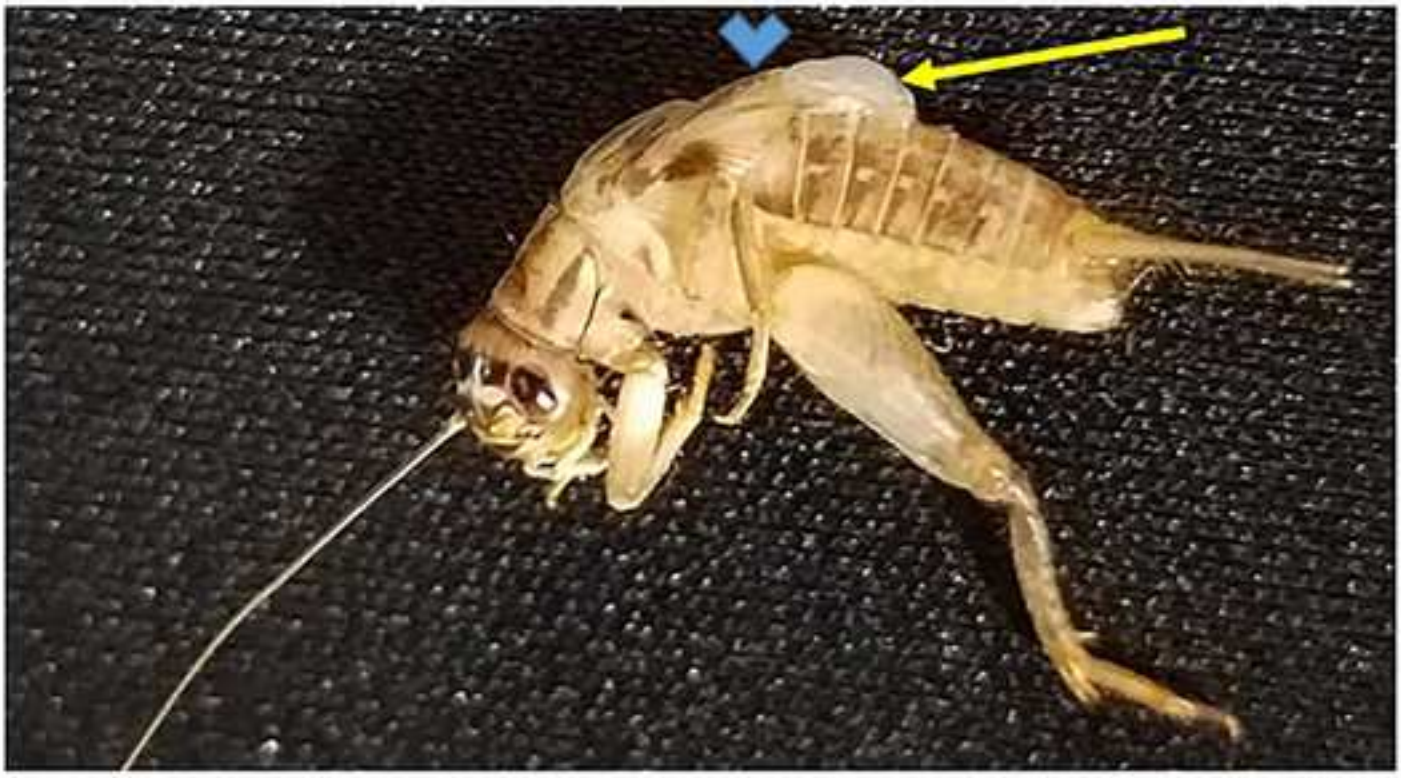

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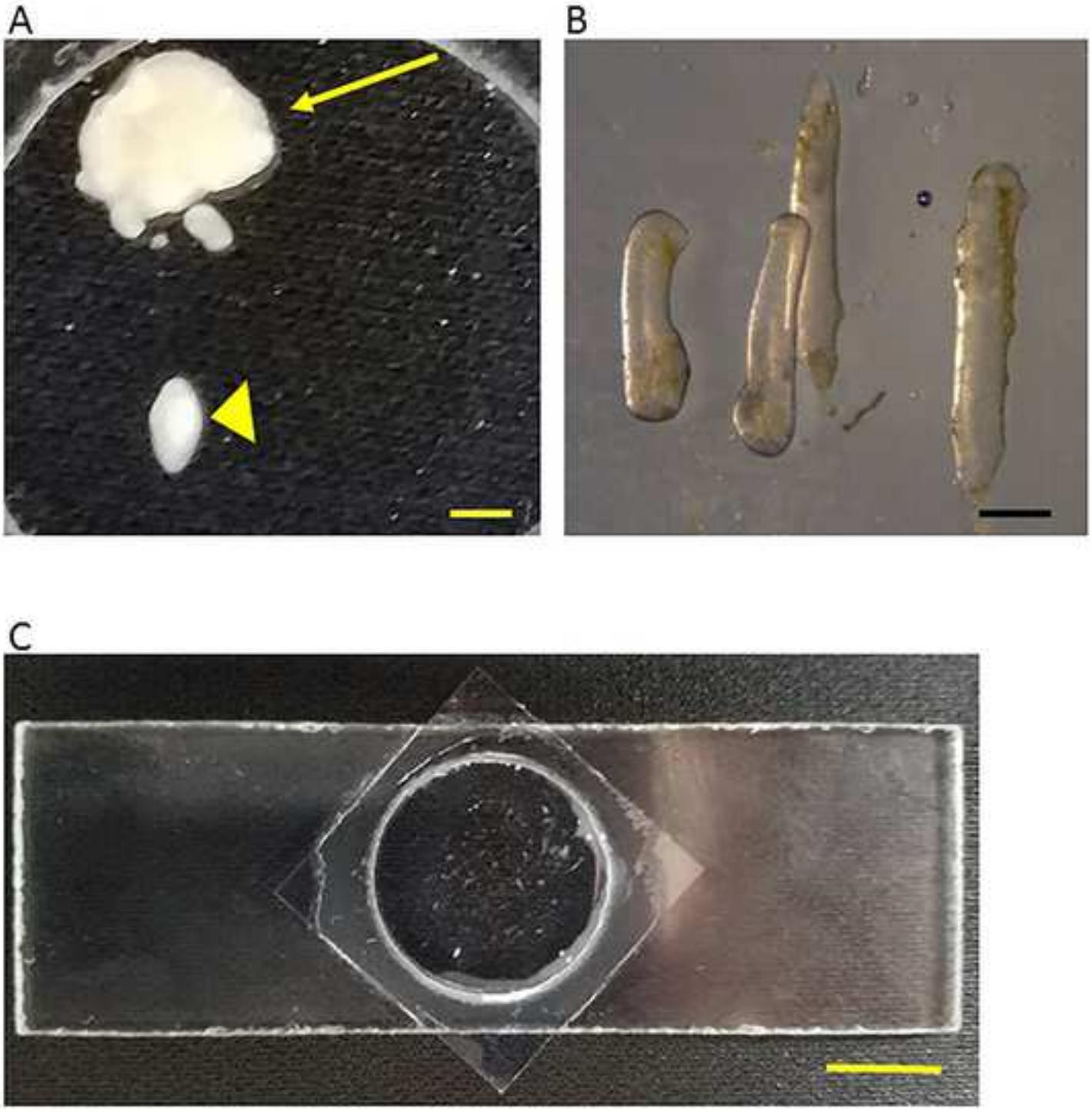

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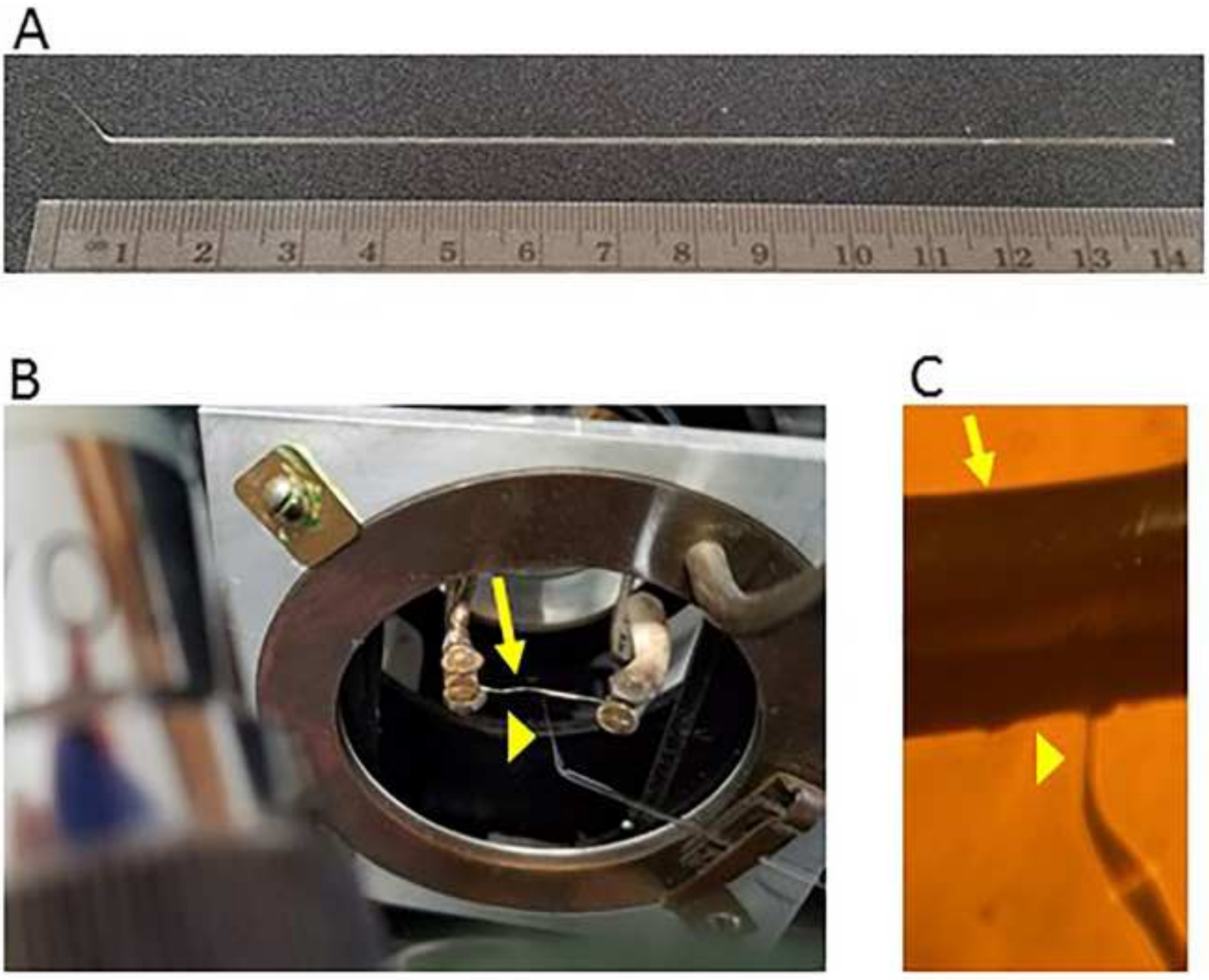

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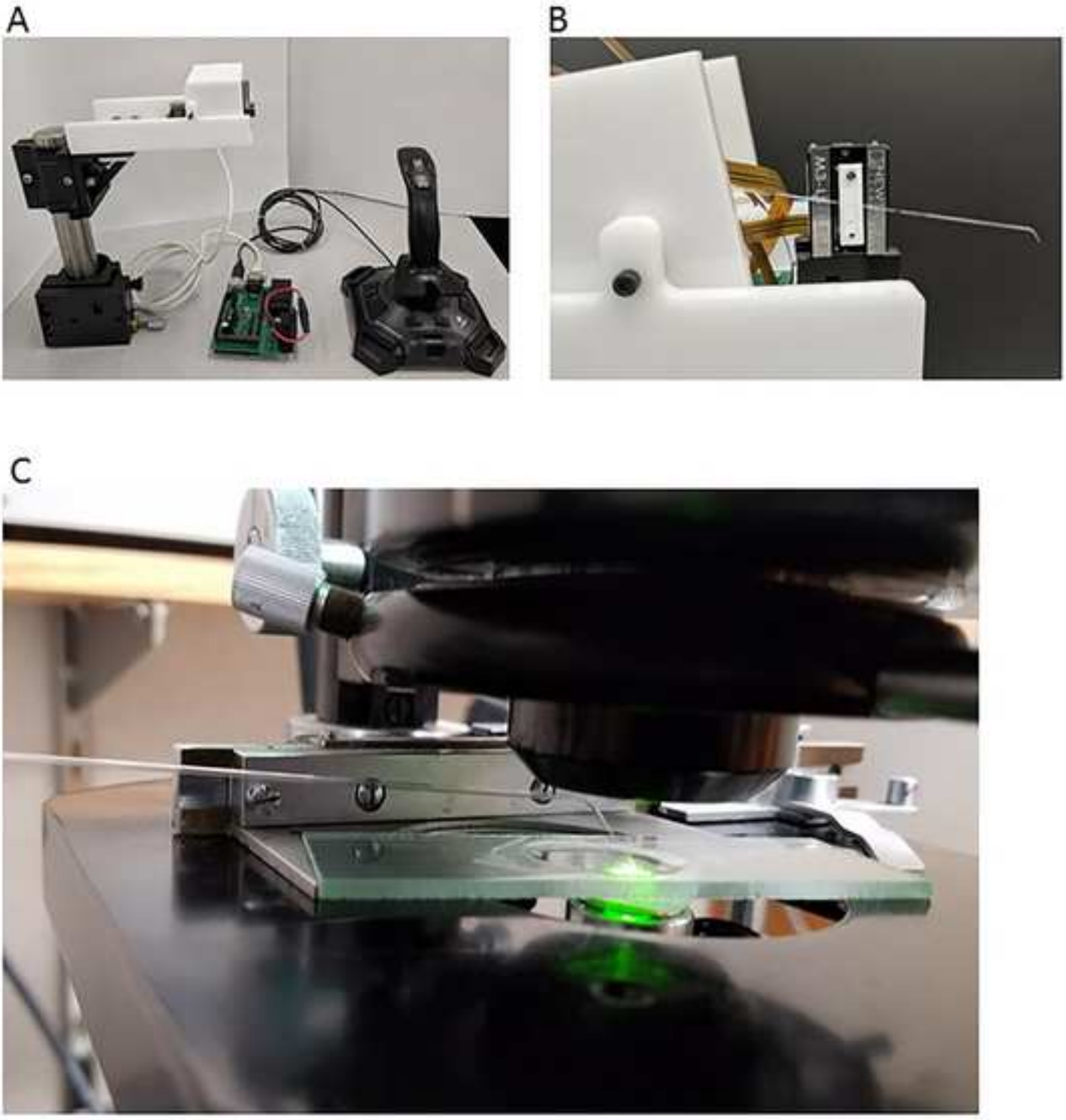

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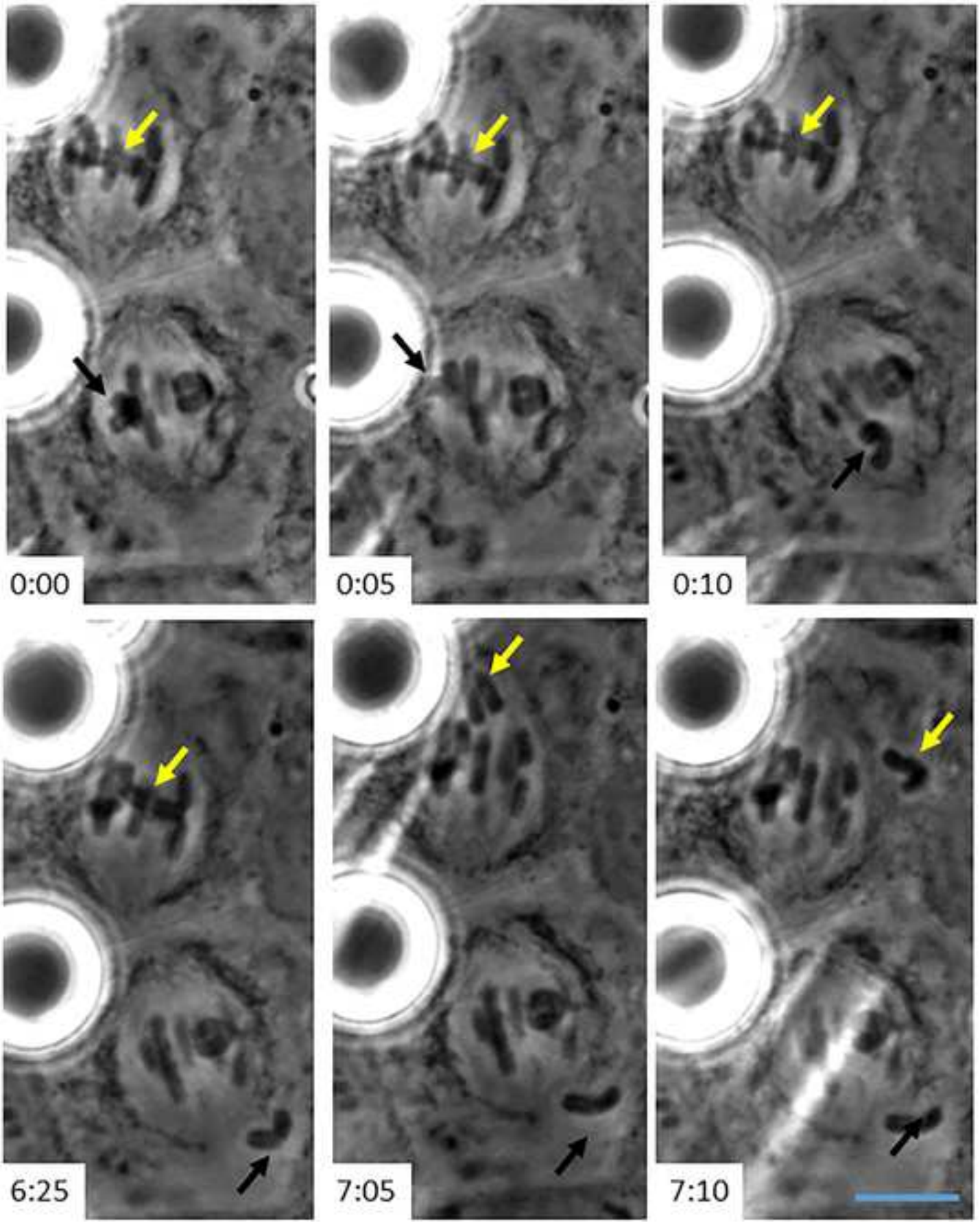

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| Dow Corning High Vacuum Grease | VWR | AA44224-KT | |
| KEL-F Oil #10 | Ohio Valley Specialty Chemical | 10189 | |
| Microdissecting Scissors, Stainless Steel | Sigma-Aldrich | S3271-1EA | |
| Dumont #5 fine foreceps | Fine Science Tools | 11254-20 | |
| 0.85 mm outer diameter, 0.65 mm | Drummond Scientific | Custom order--call to request | |
| Inverted, Phase contrast microscope with 10X or 16X low magnification objective and 60X or 100X high magnification objective | Any brand | | |
| microforge | either custom built or Narashige | MF-900 | |

| | | |
|---|---|---|
| micromanipulator | either custom built or Burleigh PCS-6000 with custom piezo-controlling joystick | PCS-6300 |

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