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Preparation of Drosophila larval and pupal testes for analysis of cell division in live, intact tissue

--Manuscript Draft--

1 **TITLE**
2 Preparation of *Drosophila* Larval and Pupal Testes for Analysis of Cell Division in Live, Intact Tissue
3
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16 **KEYWORDS**
17 *Drosophila*, spermatocyte, testis, mitosis, meiosis, cell division, microtubule, spindle,
18 endoplasmic reticulum
19
20 **SUMMARY**
21 The goal of this protocol is to analyze cell division in intact tissue by live and fixed cell microscopy
22 using *Drosophila* meiotic spermatocytes. The protocol demonstrates how to isolate whole, intact
23 testes from *Drosophila* larvae and early pupae, and how to process and mount them for
24 microscopy.
25

26 **ABSTRACT**
27 Experimental analysis of cells dividing in living, intact tissues and organs is essential to our
28 understanding of how cell division integrates with development, tissue homeostasis, and disease
29 processes. *Drosophila* spermatocytes undergoing meiosis are ideal for this analysis because (1)
30 whole *Drosophila* testes containing spermatocytes are relatively easy to prepare for microscopy,
31 (2) the spermatocytes' large size makes them well suited for high resolution imaging, and (3)
32 powerful *Drosophila* genetic tools can be integrated with in vivo analysis. Here, we present a
33 readily accessible protocol for the preparation of whole testes from *Drosophila* third instar larvae
34 and early pupae. We describe how to identify meiotic spermatocytes in prepared whole testes
35 and how to image them live by time-lapse microscopy. Protocols for fixation and immunostaining
36 whole testes are also provided. The use of larval testes has several advantages over available
37 protocols that use adult testes for spermatocyte analysis. Most importantly, larval testes are
38 smaller and less crowded with cells than adult testes, and this greatly facilitates high resolution
39 imaging of spermatocytes. To demonstrate these advantages and the applications of the
40 protocols, we present results showing the redistribution of the endoplasmic reticulum with
41 respect to spindle microtubules during cell division in a single spermatocyte imaged by time-lapse
42 confocal microscopy. The protocols can be combined with expression of any number of
43 fluorescently tagged proteins or organelle markers, as well as gene mutations and other genetic

44 tools, making this approach especially powerful for analysis of cell division mechanisms in the
45 physiological context of whole tissues and organs.
46
47 **INTRODUCTION**
48 Cell division is often studied using cell lines grown in culture[1]. While we have gained a wealth of
49 invaluable insight and understanding of fundamental mechanisms from these studies[2], cells
50 grown in culture cannot fully recapitulate the physiology of cell division as it occurs in intact,
51 living tissue. For example, in intact tissues and organs, cells must divide at the right place and at
52 the right time so that progeny cells are properly situated within the tissue, so that they can
53 undergo appropriate differentiation or functional programs, and so that cell proliferation is
54 properly coordinated with tissue growth or homeostasis[3]. For cells grown in culture on the other
55 hand, cell division is generally regulated by growth factors in the culture medium[4], and thus we
56 cannot learn from these cells how in vivo environmental factors such as tissue architecture or
57 developmental signaling influence the division process. It is also important to note that many of
58 the cell lines used to study cell division, such as HeLa and U2OS cells, were derived from
59 metastatic tumors[5]. Therefore, many aspects of the basic physiology of these cancer cells, such
60 as cell cycle regulatory mechanisms and chromosomal stability, have likely been altered
61 compared to healthy cells. Complete understanding of cell division physiology, therefore,
62 depends on our ability to study dividing cells in their native, in vivo environments that preserve
63 physiological regulatory mechanisms and tissue architecture.
64
65 Advances in understanding how cell division operates within intact tissues and organs are
66 hampered by difficulties inherent to in vivo or ex vivo analysis. First, it can be difficult or
67 impossible to access dividing cells for microscopic analysis within large organs or thick tissues.
68 Second, it is often difficult to predict when individual cells will divide in vivo. Third, tissue
69 physiology may rapidly deteriorate during ex vivo culture. In this protocol, we describe readily
70 accessible methods for live and fixed analysis of *Drosophila melanogaster* spermatocytes as they
71 undergo meiotic cell divisions within fully intact testes. These cells are ideal for live, ex vivo
72 analysis because they are readily accessible with standard optical methods such as confocal
73 microscopy, they divide at predictable times and locations, and intact testes can be maintained
74 in ex vivo culture for up to about 24 hours. In addition, *Drosophila* spermatocytes are large round
75 cells (approximately 20 - 30 µm in diameter) that do not change shape when they divide, making
76 them ideal for high resolution, time-lapse imaging of cellular components such as the spindle
77 apparatus and cytoplasmic organelles. Although these cells undergo meiosis as opposed to
78 mitosis, many of the essential cell division processes are very similar between these two cell
79 division mechanisms[6]. These advantages, combined with powerful *Drosophila* genetic tools, have
80 made *Drosophila* spermatocytes an extensively used model for ex vivo analysis of essential cell
81 division processes including spindle formation and regulation, cytokinesis, and organelle
82 remodeling and partitioning[7-11].
83
84 Spermatocytes are cells that are at the meiotic stage of spermatogenesis. In *Drosophila*,
85 spermatogenesis begins with a group of germline stem cells that are located in a small region or
86 "hub" at one pole of the testis[12,13]. These cells divide by an asymmetric mitosis, giving rise to a
87 single differentiated spermatogonium. Spermatogonia then undergo four synchronous mitoses

88   to produce a group of 16 cells that remain closely associated within a single cyst. At this stage,
89   the cells transition from a mitotic to a meiotic cell cycle and are referred to as spermatocytes.
90   Spermatocytes spend about two to three days in an extended G2 stage of the cell cycle, during
91   which they grow dramatically and undergo cytological changes in preparation for the two meiotic
92   divisions and subsequent spermiogenesis[13,14]. The entire group of 16 spermatocytes within a
93   single cyst then enter the first meiotic division at the same time. Thus, several meiotic
94   spermatocytes can be imaged simultaneously as they proceed through cell division. The first
95   meiotic division proceeds for approximately 1.5 hours and is followed almost immediately by the
96   second meiotic division, yielding 64 total spermatids that go on to differentiate into mature
97   spermatozoa.
98
99   A unique advantage of using spermatocytes to study cell division in live, intact tissue is that
100   groups or cysts of cells constantly progress through the different stages of spermatogenesis, and
101   cells at all stages of spermatogenesis can usually be identified in any given testis (see **Figure 3A**).
102   Therefore, it is relatively easy to find meiotic cells in whole testes. We generally focus our
103   analyses on the first as opposed to second meiosis because these cells are much larger and more
104   amenable to high resolution imaging, but the entire process encompassing both meiotic divisions
105   can be successfully imaged. It should also be noted that the general protocol for testis
106   preparation and culture can be used to analyze other processes of spermatogenesis as well, such
107   as the earlier mitotic stem cell or spermatogonial divisions or the cytological changes that occur
108   as spermatids mature into spermatozoa[15]. Many of these aspects of spermatogenesis are highly
109   conserved between *Drosophila* and humans[16].
110
111   *Drosophila* spermatocytes begin to reach the meiotic phase of spermatogenesis during the third
112   instar larval stage of *Drosophila* development[13]. Therefore, testes isolated from lifecycle stages
113   beginning with third instar larvae and including pupae and adults can be used for analysis of
114   dividing spermatocytes. Several excellent protocols are available for extraction of testes from
115   adult male flies for live and fixed analysis of spermatogenesis[17-19]. These protocols may be
116   preferable for studying late stages of spermatogenesis or if genetic markers that are only visible
117   in adults must be used. The protocol focuses instead on testis preparation from larvae and early
118   pupae, because testes at these stages have several advantages that are specifically pertinent to
119   cell division analysis by high resolution, time-lapse microscopy. First, testes from larvae and
120   pupae are smaller than those from adults, and the cells within the organ are less crowded.
121   Because of this, dividing spermatocytes can often be imaged near the outer surface of larval
122   testes, without having to penetrate through multiple layers of light scattering tissue. Second,
123   adult testes move rhythmically due to contractions of the attached accessory organs, and these
124   movements make time-lapse imaging of individual cells challenging. And third, larval testes are
125   advantageous when studying gene mutations that cause pupal or adult lethality. Our methods
126   are optimized for long-term culture of testes on the microscope stage, allowing for imaging of
127   multiple rounds of cell division or the progression of individual cells through multiple stages of
128   spermatogenesis in the same preparation. We also describe a protocol for fixation and
129   immunostaining of whole testes. Overall, our protocols are particularly useful for those
130   interested in studying cell division in intact tissue, and the ability to combine spermatocyte

131 analysis with highly tractable *Drosophila* genetic tools makes this an especially powerful
132 approach.
133
134 **PROTOCOL**
135
136 **1.   Prepare Animals, Tools, and Media for Dissection**
137
138 1.1.   Cross male and female flies to obtain progeny of the desired genotype. Useful transgenic
139 markers for identification and staging of meiotic spermatocytes include GFP-tubulin to label the
140 meiotic spindle and RFP-histone 2A to label chromsomes[8]. Use five to ten females per cross and
141 maintain crosses on standard fly food in vials in a 25 °C incubator until third instar larvae begin
142 crawling up the sides of the vial (4-5 days). Early white pupae will begin forming one day later.
143
144 1.2.   Obtain two pairs of straight forceps with fine tips. Ensure that the tips are sharp, even in
145 length, and straight (**Figure 1A**). Use these forceps only for dissections, and cap with 10 µL pipette
146 tips when not in use.
147
148 1.3.   Prepare a scalpel tool.
149
150 1.3.1.   Insert the blunt end of a black anodized steel insect pin into a pin holder, and twist the
151 screw of the holder to secure the pin in place. Using a pair of forceps and under a dissecting
152 microscope, grasp the insect pin in the middle and bend it to form an internal angle of 135°
153 (**Figure 1B**). The sharp end is for cutting tissue, while the edge is used for transferring testes
154 between drops of media.
155
156 1.3.2.   Cap with a 1,000 µL pipette tip when not in use.
157
158 1.4.   Working in a tissue culture hood, prepare 5 mL aliquots of Schneider's media and store at
159 4 °C until the time of use. When ready to begin dissections, warm one 5 mL media aliquot to
160 room temperature. Then transfer the warmed dissection media to a 10 mL syringe and attach a
161 0.22 µm syringe filter.
162
163 **2.   Dissection of Testes from Larvae and Early Pupae**
164
165 2.1.   Use the media filled filter syringe to expel three drops of media (50 µL each) at the top,
166 middle and bottom of a glass slide.
167
168 2.2.   Use a dissecting probe to transfer five to ten third instar larvae or early pupae (pupae that
169 are still white or yellow) to the top drop. Gently agitate the larvae and pupae in the media with
170 the dissecting probe to remove any food or debris.
171
172 2.3.   Under a dissecting microscope, turn a single larva or pupa on its side with a dissecting
173 probe to identify the two bilateral testes if present, which appear as oval shaped translucent

structures in the posterior third of the body (**Figure 2A, B**). Animals that lack testes are female and should be discarded.

2.4.   When a male larva or pupa is found and is clean, move it to the second drop of media. Repeat until 3 or 4 male larvae and/or pupae have been transferred to the second drop of media.

2.5.   Working in the second drop of media, grasp a single male larva or pupa with a pair of forceps at its mid-region, just anterior to the testes. Use the second pair of forceps to gently tear the animal in half.

2.6.   Hold the posterior end of the larva or pupa down on the glass slide with a pair of forceps. Starting just next to the forceps, push down on the cuticle using the edge of the scalpel tool and move the scalpel toward the cut end of the animal. This will expel the animal's internal organs, which include the guts, fat bodies, and testes.

2.7.   Gently tease apart the testes from the rest of the organs. The testes are clear, oval shaped organs embedded in ribbons of fat body (**Figure 2C**).

2.8.   Transfer the testes one at a time to the third drop of media. To accomplish this, insert the edge of the scalpel under the fat body, lift the tissue out of the media, and quickly move it to the third drop.

2.9.   Alternatively, if there is not enough fat body still attached to the testes to successfully lift the tissue, gently transfer the tissue using a glass Pasteur pipette that has been pre-wetted with dissection media.

2.10.   Use the sharp end of the scalpel tool to gently slice away excess fat body from the testes, leaving just a small rim of fat body around the edges (Figure 2C). It is not necessary to remove all of the fat body, and attempting to do so will likely result in damaging or rupturing of the testes.

2.11.   Repeat the above steps for all of the male larvae on the glass slide.

2.12.   Proceed either to step 3 or step 5.

3.   Mounting Testes for Live Microscopic Imaging

NOTE: This mounting procedure was adapted from a recently published protocol for imaging of *Drosophila* larval brain neuroblasts[20]. Additional details can be found in this reference.

3.1.   Use the filter syringe to deposit a single drop of Schneider's media (about 30 µL) onto the center of a 50 mm gas-permeable culture dish.

3.2.   Use a pre-wetted Pasteur pipette to transfer the prepared testes to the drop on the gas-permeable dish. The testes will generally float to the surface of the drop.

218

219 3.3.   Use the scalpel tool to gently push the testes down onto the gas-permeable membrane.
220 Take care not to rupture the gas-permeable membrane with the sharp point of the scalpel. Push
221 all of the testes to the center of the drop.
222

223 3.4.   Use a 1 mL syringe filled with halocarbon oil to make four drops of oil, about 30 µL each,
224 on the gas permeable membrane. Space the drops of oil around the drop of media containing
225 the testes to correspond to the four corners of a 22 mm glass coverslip.
226

227 3.5.   Line up the corners of a 22 mm glass coverslip with the four drops of halocarbon oil and
228 gently lower the coverslip onto the media and oil. Allow the coverslip to settle and for the media
229 containing the testes to spread between the drops of oil.
230

231 3.6.   Remove excess media to allow the coverslip to settle onto the surface of the testes. While
232 observing the testes under a dissecting microscope, insert the corner of a delicate task wipe into
233 the media under the coverslip to wick away a small amount of liquid. Wick away enough media
234 until the glass coverslip just makes contact with the surface of the largest testis. It is critical not
235 to remove too much media and lower the coverslip too far, as this will exert pressure on the
236 testes and cause them to rupture (see **Figure 5**).
237

238 **4.   Live Imaging of Meiotic Spermatocytes**
239

240 NOTE: Spermatocytes can be imaged using a laser scanning or spinning disk confocal microscope.
241 The system must have adequate speed and sensitivity to avoid photobleaching of fluorescent
242 proteins or photodamage of the tissue.
243

244 4.1.   Place a drop of immersion oil on the glass coverslip just above the testes. Flip over the
245 dish and place it on the microscope stage and move the microscope objective (40x or 60x) into
246 the oil until it is just below the testes. Use transmitted light to find a single testis and bring it into
247 focus.
248

249 4.2.   While capturing fast images with the confocal, move the testis around to examine the
250 organization of the cells. One end of the testis will have many small, densely packed cells. This
251 end contains the germline stem cells and spermatogonia. Moving toward the other end of the
252 organ, identify progressively larger cells organized into discrete clusters or cysts. These large cells
253 are the spermatocytes (**Figure 3A**).
254

255 4.3.   If imaging GFP-tubulin, identify spermatocytes that will commence meiosis within 30 - 60
256 min by the presence of two bright microtubule asters (i.e., centrosomes) located at the cortex of
257 the cell (**Figure 3B, C**).
258

259 4.4.   Once a cyst of meiotic spermatocytes has been identified, set up acquisition parameters
260 to capture z-stacks of images over time. Typically, acquisition of 15 – 20 images spaced 1.5 µm

261 apart in the z-dimension is sufficient to capture the full volume of several spermatocytes within
262 the same cyst.

263

264 4.5.   Acquire full z-stacks every 2 min if imaging the entire first meiotic division, which takes
265 about 1.5 hours. It is possible to use faster acquisition rates, particularly if only a specific event
266 of meiosis is to be analyzed. However, take care not to cause photobleaching or photodamage
267 due to repeated exposure of the sample to laser excitation.

268

269 4.6.   Use a continuous, automatic focus control system to prevent focal drift over the long
270 time-course of acquisition. Temperature control of the sample on the microscope stage generally
271 is not necessary, assuming room temperature of approximately 22 – 23 °C. If temperature control
272 is available, maintain the sample at 25 °C on the microscope stage.

273

274 4.7.   If meiotic spermatocytes are not found, store the sample for several hours or overnight
275 in a 25 °C incubator and image again.

276

277 **5.   Fixation and Immunostaining**

278

279 5.1.   From step 2.10 above, use a glass Pasteur pipette to transfer testes to a well in a 9-well
280 glass dissecting dish containing 0.5 mL of 8% paraformaldehyde in PBSTx (phosphate buffered
281 saline with 0.3% Triton X-100). Ensure that the testes are laying on the bottom of the dish.

282

283 NOTE: Paraformaldehyde is toxic and should be handled with gloves in a fume hood.

284

285 5.2.   Fix the testes for 20 min at room temperature.

286

287 5.3.   Transfer the testes to a well containing 0.5 mL PBSTx. Wash for 5 min with gentle
288 agitation. Repeat the wash step in new wells with fresh PBSTx two more times.

289

290 5.4.   Dilute primary antibody to the desired concentration in 0.5 mL PBSTx containing 5%
291 bovine serum albumin (BSA) in a 1.5 mL microcentrifuge tube. Transfer testes from the 9-well
292 glass dissecting dish to the tube of diluted primary antibody and incubate overnight at 4 °C with
293 gentle rocking or nutation.

294

295 5.5.   Wash the testes in 0.5 mL of PBSTx for 5 min in a well of a 9-well glass dissecting dish.
296 Repeat the wash step with fresh PBSTx two more times.

297

298 5.6.   Dilute secondary antibody in 250 µL of PBSTx containing 5% BSA and dispense into a clean
299 well of a 9-well glass dissecting dish. If desired, add DAPI to stain DNA. Transfer testes to the well
300 containing secondary antibody, cover with plastic wrap, and incubate in the dark with gentle
301 agitation for 4 hours at room temperature.

302

303 5.7.   Transfer the testes to a clean well filled with 0.5 mL of PBSTx. Wash with fresh PBSTx twice
304 for 5 min and a third time for 30 min.

6. Mounting Fixed Testes

6.1.   Transfer the testes from the last wash onto a glass microscopy slide using a Pasteur pipette. If necessary, use the edge of the scalpel tool to push the testes down onto the surface of the slide.

6.2.   Use the corner of a delicate task wipe to wick away excess liquid from the testes. Remove as much liquid as possible.

6.3.   Apply a 30-50 µL drop of microscopy mounting medium to the testes.

6.4.   Place a 22 mm coverslip onto the drop of mounting medium and allow the coverslip to settle and the mounting medium to spread under the coverslip. Apply gentle pressure to the coverslip if necessary to squeeze out excess mounting medium and allow the coverslip to rest on the surface of the testes.

6.5.   Use nail polish to seal the edges of the coverslip.

REPRESENTATIVE RESULTS

When this protocol is successfully executed, testes will remain fully intact for imaging by confocal microscopy or other fluorescence microscopy methods. As seen in **Figure 3A**, the cellular organization of the testes is preserved, and the progression of cell differentiation from one end of the testis to the other including spermatogonia, spermatocytes, and haploid spermatids is visible. GFP-tubulin is a useful marker for identifying dividing spermatocytes and for live imaging of the progression of the cells through meiosis. As shown in **Figure 3B**, spermatocytes about the begin meiosis can be identified by their two prominent microtubule asters. By metaphase the large meiotic spindles are beautifully labeled by GFP-tubulin (**Figure 3C**).

Drosophila spermatocytes' large size and their relative slow progression through meiosis make them ideal for analysis of the dynamics and reorganization of cellular components such as cytoplasmic organelles during cell division[8,11,21]. **Figure 4** and **Video 1** show live imaging analysis of the dynamic reorganization of the endoplasmic reticulum (ER) with respect to microtubules through the course of meiosis. As described above, during late interphase just prior to the onset of meiosis, two centrosomes located at the cortex of the cell are clearly visible by GFP-tubulin fluorescence. At this stage, ER-targeted RFP (RFP-KDEL) fluorescence reveals that the ER is evenly distributed throughout the cytoplasm. The centrosomes then begin migrating to opposite sides of the nuclear envelope to establish the two spindle poles. During this time, the ER rapidly accumulates around the centrosomal microtubules and is progressively cleared from the rest of the cytoplasm. Nuclear envelope breakdown (NEB) is revealed by the appearance of GFP-tubulin fluorescence within the nuclear region. It should be noted that *Drosophila* cells divide by semi-open mitosis or meiosis, meaning that nuclear envelope components break down and disperse and large cytoplasmic molecules enter the nuclear region, but an envelope of ER-derived membranes surrounds the spindle and chromatids throughout cell division[22-24]. By the time of

349   NEB and progressing through metaphase, the ER is nearly completely associated with the astral
350   microtubules that surround the two spindle pole centrosomes. These two astral microtubule
351   accumulations of the ER then partition to the two daughter cells during anaphase, ensuring
352   proper ER inheritance by the newly formed cells[8,11].
353
354   **Figure 5** shows the effects of testis rupture on live imaging of spermatocytes. As described in the
355   protocol, great care must be taken not to lower the coverslip too far onto the surface of the
356   testes during the mounting steps, as this will apply pressure to the testes and cause them to
357   burst. As seen in **Figure 5** and **Video 2**, cysts of spermatocytes rapidly flow out of the ruptured
358   testis, and this movement of the cells makes live, time-lapse imaging extremely difficult.
359
360   **FIGURE AND VIDEO LEGENDS**
361
362   **Figure 1. Tools required for successful dissection.** (**A**) Forceps used for dissection must be
363   straight and have sharp, unbroken tips (indicated by green check mark). Do not use forceps with
364   bent or broken tips (red X marks), as they will be ineffective at grasping larvae and teasing apart
365   tissues. (**B**) To prepare the scalpel tool, bend a black anodized steel insect pin to an internal angle
366   of approximately 135°. The sharp point is used as a knife to cut through tissue, and the straight
367   edge is used for moving tissues.
368
369   **Figure 2. Identification of larval and pupal testes.** (**A**) Images of male and female larvae. The
370   testes are identifiable in the male as the circular or oval translucent structures in the posterior
371   third of the animal (arrowhead). Note the lack of a similar structure in the female. (**B**) Image of
372   an early male pupa, with the two translucent testes indicated by arrowheads. (**C**) Testes with
373   attached fat bodies after dissection. The two testes on the left have excessive fat bodies still
374   attached that need to be trimmed away. The two testes on the right have been properly trimmed
375   of their fat bodies and are ready for imaging.
376
377   **Figure 3. Identification of meiotic spermatocytes in a successfully prepared testis.** (**A**) Confocal
378   image of a successfully prepared live, intact testis expressing GFP-tubulin. The hub of stem cells
379   is located toward the lower tip of the testis, though it is not identifiable in this preparation.
380   Several layers of small spermatogonia are indicated, and these are followed by numerous cysts
381   of spermatocytes. A cyst of spermatocytes in the first meiotic division is identifiable based on the
382   large size of the cells (approximately $20-30$ μm in diameter) and presence of GFP-tubulin labeled
383   meiotic spindles. Spermatocytes in the second meiotic division similarly have spindles, but these
384   cells are approximately half the size meiosis I cells. Post-meiotic spermatids are also visible, as
385   are elongating spermatozoa. (**B**) Spermatocytes that will begin meiosis within $30-60$ min can be
386   identified by their two large, very bright microtubule asters (indicated by arrow heads) labeled
387   by GFP-tubulin. The cyst of pre-meiotic cells is delineated by the dashed yellow line. (**C**) Cells in
388   meiosis are identifiable by their large meiotic spindles that are easily visualized with GFP-tubulin.
389   The cyst of meiotic cells is delineated by the dashed yellow line.
390
391   **Figure 4. Time-lapse imaging of meiosis in a single spermatocyte.** A single spermatocyte co-
392   expressing GFP-tubulin and RFP targeted to the ER (RFP-KDEL) was imaged every two minutes

9

393   through the course of meiosis. Shown are representative images of the spermatocyte at specific
394   stages of meiosis, beginning in late interphase and progressing through telophase. Times are in
395   minutes. Each image is a single optical plane from a stack of fifteen optical slices acquired at each
396   time point. This figure was modified from Karabasheva and Smyth, 2019[8].
397
398   **Figure 5. Representative images of a testis before and after rupturing.** The image on the left,
399   labeled "Intact", shows a GFP-tubulin expressing testis just before rupturing. The image on the
400   right shows the same testis after rupturing. Cysts of spermatocytes can be seen streaming out of
401   the ruptured testis. The arrowheads indicate a cyst of meiotic spermatocytes; note the significant
402   movement of these spermatocytes after the testis ruptures, making imaging these
403   spermatocytes over time extremely challenging.
404
405   **Video 1. Full meiotic division of a single spermatocyte.** Shown is the full time-lapse of the GFP-
406   tubulin and RFP-KDEL expressing spermatocyte in **Figure 4**. Images were captured every two
407   minutes, and the playback rate is three frames per second. This video was modified from
408   Karabasheva and Smyth, 2019[8].
409
410   **Video 2. Ruptured testis.** Time-lapse of a testis before and after rupturing. The rupture is evident
411   due to the sudden streaming of cysts of cells through the lower left corner of the testis. Images
412   were captured every two minutes, and the playback rate is three frames per second.
413
414   **DISCUSSION**
415   We have described a protocol for the preparation of larval or early pupal *Drosophila* testes,
416   optimized for long-term, live imaging of spermatocyte cell division. This is a powerful method for
417   analysis of cell division in the physiological context of intact tissue. The power of this method is
418   further expanded when combined with *Drosophila* genetic tools, such as specific gene mutations,
419   tissue-specific RNAi-mediated suppression, and fluorescently labeled protein and organelle
420   markers. In addition, the small size of larval and pupal testes, and the ability to image dividing
421   cells very close to the glass coverslip, make the method ideal for high resolution imaging including
422   super-resolution approaches. The versatility of this experimental system is demonstrated by the
423   representative results describing the dynamic reorganization of the ER during cell division.
424   *Drosophila* spermatocytes are particularly useful for such analysis of organelle dynamics during
425   cell division due to these cells' large size and relatively slow transit through meiosis. Further, the
426   same cells can be imaged post-meiotically to understand how changes in organelle dynamics or
427   cell division mechanisms affect subsequent events of spermatogenesis. Thus, there is great
428   potential with this experimental approach to understand the physiology of cell division within
429   the larger context of tissue development and cell differentiation, outcomes that are unattainable
430   when studying cells grown in culture.
431
432   The protocol includes several elements that facilitate long-term ex vivo culture of testes for up
433   to about 24 hours. First, Schneider's media, which was developed for maintenance of *Drosophila*
434   tissue culture cells, contains readily available energy sources and is optimized to maintain
435   physiological pH in atmospheric air. Second, the gas-permeable membrane used for mounting
436   testes allows for rapid gas exchange. An important advantage of long-term culture is that if cells

437 at the desired stage of development or meiosis are not found immediately after sample
438 preparation, the sample can be stored and imaged again at a later time. We have verified that
439 testes maintained in a 25 °C incubator overnight, for a total of 16-24 hours, are viable and appear
440 healthy based on the routine presence of cells actively undergoing meiosis and progressing
441 through later stages of sperm differentiation. However, precautions should be taken when
442 culturing testes for longer than 2-3 hours to ensure that tissue physiology is not adversely
443 affected. Most importantly, testes continue to grow in culture as cells proliferate and maturing
444 sperm elongate. This may cause the testes to enlarge to the point that they burst due to the
445 restrictive space between the membrane and coverslip, rendering the testes unsuitable for live
446 imaging for reasons described below. In addition, if long-term cultures are to be routinely
447 employed in experiments, it is advisable to test whether lab-specific extended culture conditions
448 result in meiotic abnormalities such as prolonged meiotic durations or increased frequencies of
449 lagging chromosomes or cytokinesis failure.
450
451 In order to image individual cells over long time-courses, it is important that cells do not move
452 significantly once the prepared testes are on the microscope stage. Thus, it is essential when
453 executing the protocol that the testes remain intact and undamaged, because cells spill out of
454 damaged testes and all the cells within the organ move as a result (see **Figure 5** and **Video 2**). It
455 may still be possible to image spermatocytes in damaged testes if the cells eventually settle and
456 stop moving, though by the time this occurs the stages of meiosis that one intends to analyze
457 may have already occurred. In addition, effects of tissue damage on the cell division process itself
458 cannot be discounted. Damage to the testes can occur when clearing away fat bodies following
459 removal of the testes from larvae. Great care must therefore be taken to avoid piercing or tugging
460 on the organs during this step of the protocol. In fact, it is often not necessary to remove very
461 much of the fat bodies, so long as they do not obscure visualization of the testis. More commonly,
462 testis damage is the result of removing too much culture medium following placement of the
463 coverslip during the mounting procedure for live imaging (step 3.6). As medium is removed, the
464 coverslip falls lower and exerts pressure on the testes, and excess pressure will cause the testes
465 to rupture. Thus, while it is important for the coverslip to be as close as possible to the testes to
466 facilitate optimal imaging of the spermatocytes, some practice is necessary to avoid removing
467 too much medium and lowering the coverslip too far. It is also helpful to point out that for some
468 applications, such as acute exposure of spermatocytes to drugs or other small molecules, it can
469 be advantageous to disrupt the testes and disperse the cysts of spermatocytes for analysis.
470 Several excellent protocols are available for the preparation and culture of dispersed
471 spermatocyte cysts[18,25].
472
473 An important consideration when using spermatocytes for cell division analysis is that these cells
474 execute meiotic as opposed to mitotic divisions. Importantly, many of the essential aspects of
475 cell division mechanics, such as spindle architecture and cytokinesis, are similar between male
476 meiosis and mitosis[6]. Thus, mechanistic insights gathered from studying spermatocyte meiosis
477 can be broadly applicable to general mechanisms of animal cell division[7]. However, depending
478 on the mechanistic questions being addressed, important differences between spermatocytes
479 and mitotic cells in processes such as chromosomal dynamics and cell cycle regulation may need
480 to be considered[26]. At the same time, *Drosophila* spermatocytes present the unique opportunity

481 to compare mitotic and meiotic cells within the same tissue and germline lineage. For example,
482 the same testis preparation can be used to analyze germline stem cells or spermatogonia
483 undergoing mitosis and spermatocytes undergoing meiosis. We typically do not attempt to image
484 germline stem cell or spermatogonial mitoses in live testis preparations because the timing of
485 these divisions is difficult to predict. However, we have fortuitously captured these divisions in
486 our experiments, demonstrating that the general protocol can be applied to analysis of mitotic
487 cells within testes as well.
488
489 The methodology presented focuses on testis preparation for live imaging of spermatocyte
490 meiosis, as this allows for real-time analysis of cellular and molecular dynamics. We also provide
491 a method for fixation and immunostaining of intact testes, as this approach may be preferable if
492 required fluorescently labeled expression constructs are not available or to avoid confounding
493 effects of protein overexpression. An important consideration though is that fixation does not
494 always faithfully preserve native tissue and cellular architecture. For example, we and others
495 have found that fixation often results in fragmentation or disruption of the ER[11,27]. Some of these
496 problems can be alleviated by using different fixatives or tissue preparation methods, and some
497 troubleshooting may therefore be necessary to identify the optimal fixation protocol for
498 preservation of a particular cell component or protein organization. Fortunately, a number of
499 additional protocols for *Drosophila* spermatocyte fixation are also available[18,28,29].
500
501 In conclusion, we have described versatile methods for the preparation of *Drosophila* testes for
502 live and fixed cell analysis of spermatocyte meiosis. Specific strengths of this experimental
503 approach include analysis of cell division in intact tissue, high resolution imaging of large cells,
504 and integration with *Drosophila* genetic tools. Experiments employing the methodology have the
505 potential to make important contributions to our understanding of how cell division integrates
506 with complex mechanisms of tissue development and homeostasis.
507

510
511 **DISCLOSURES**
512 The authors have nothing to disclose.
513

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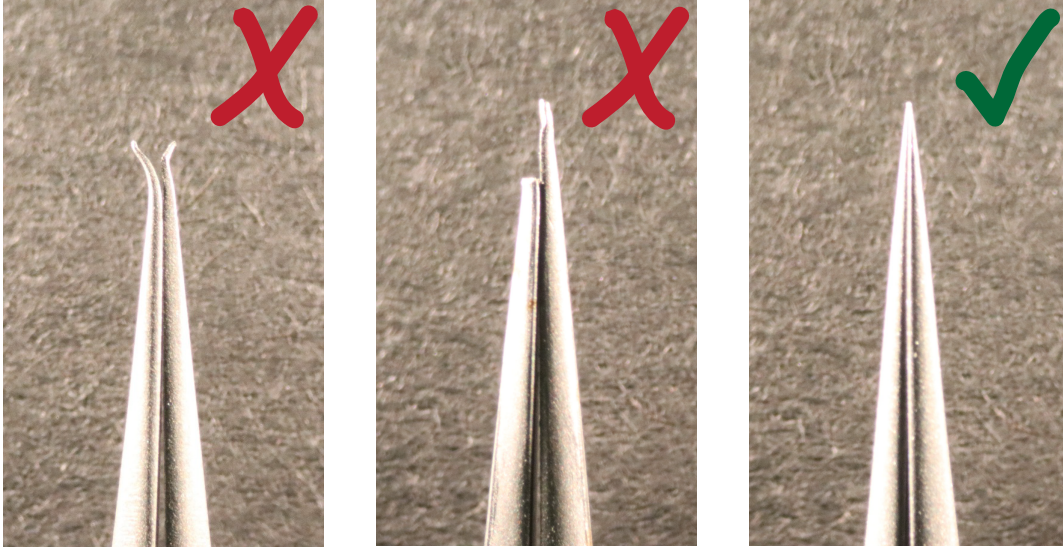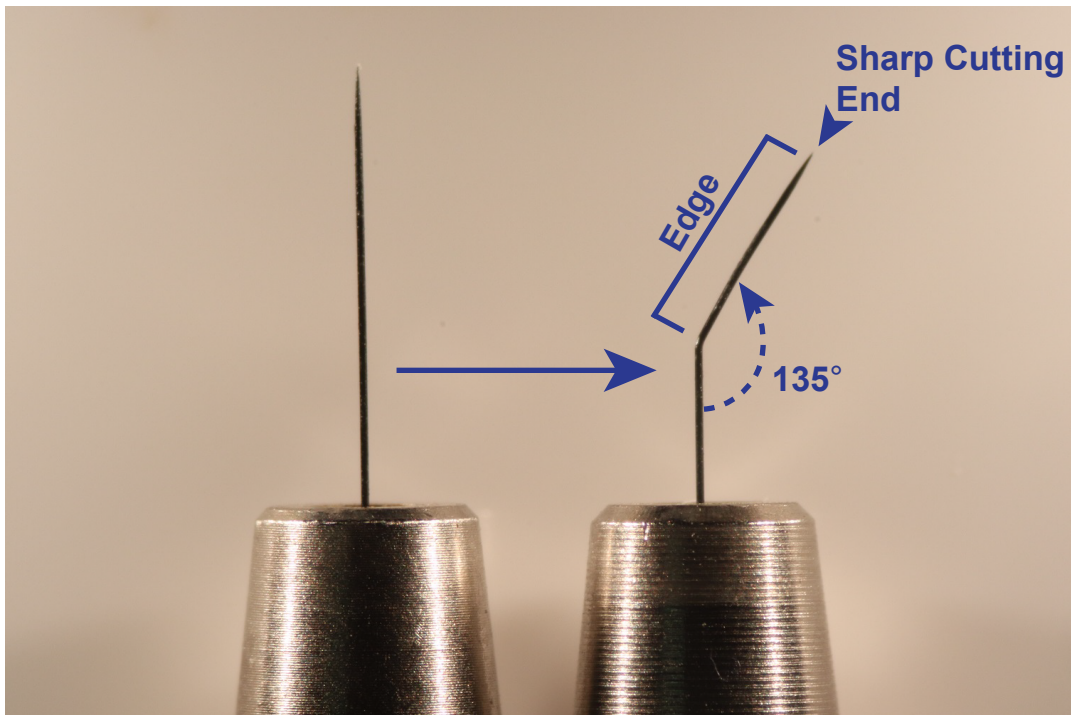
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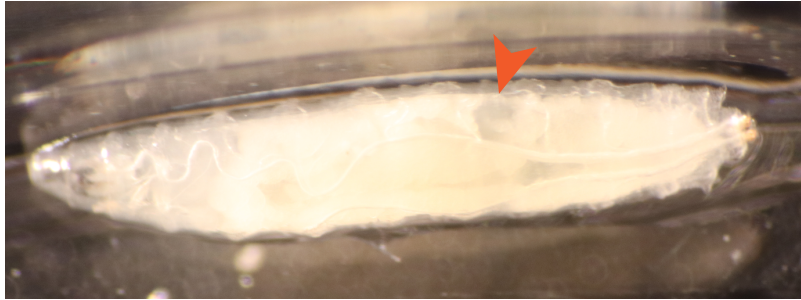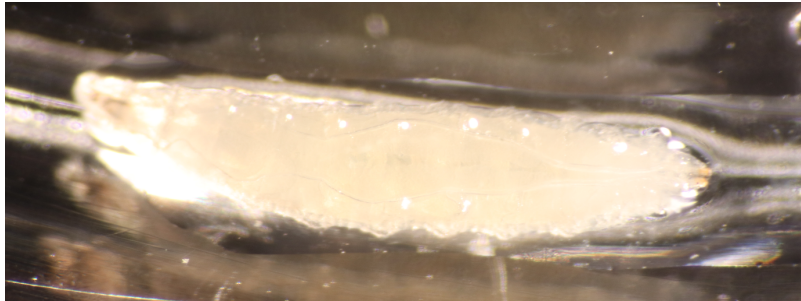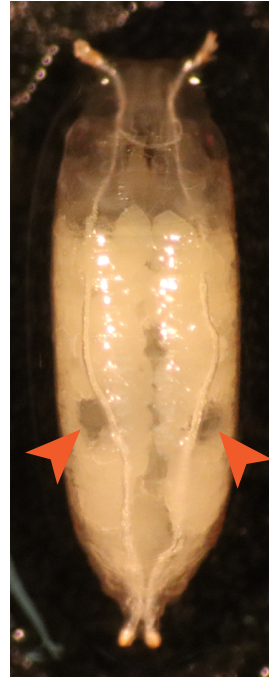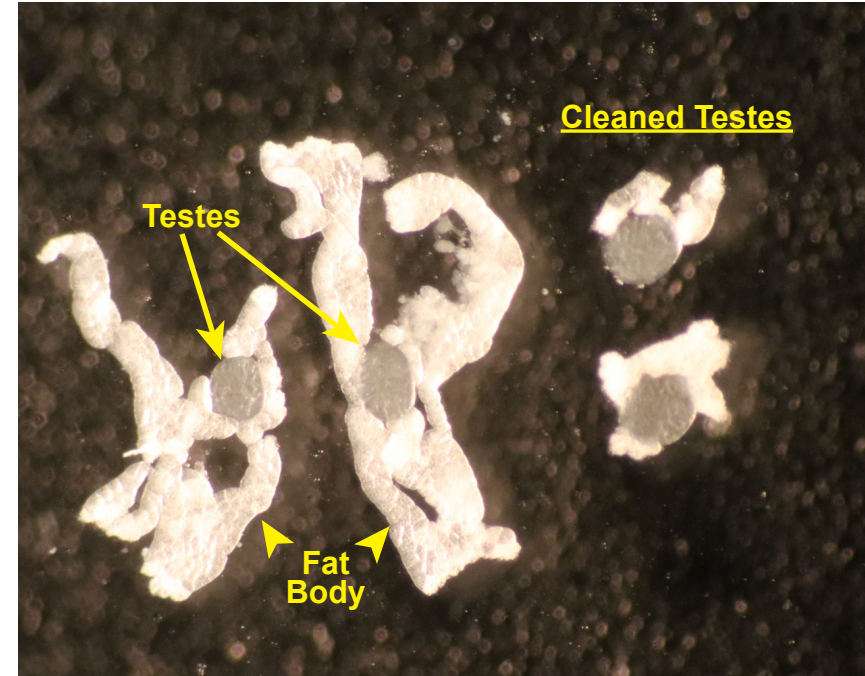
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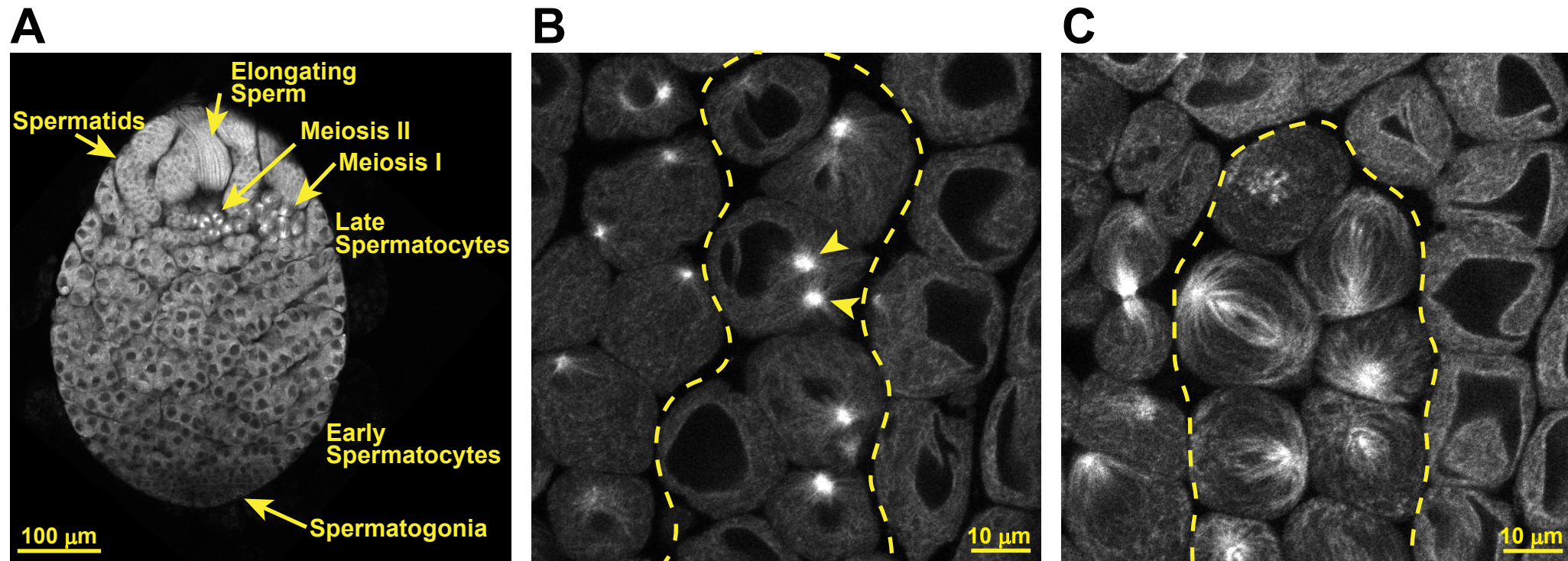
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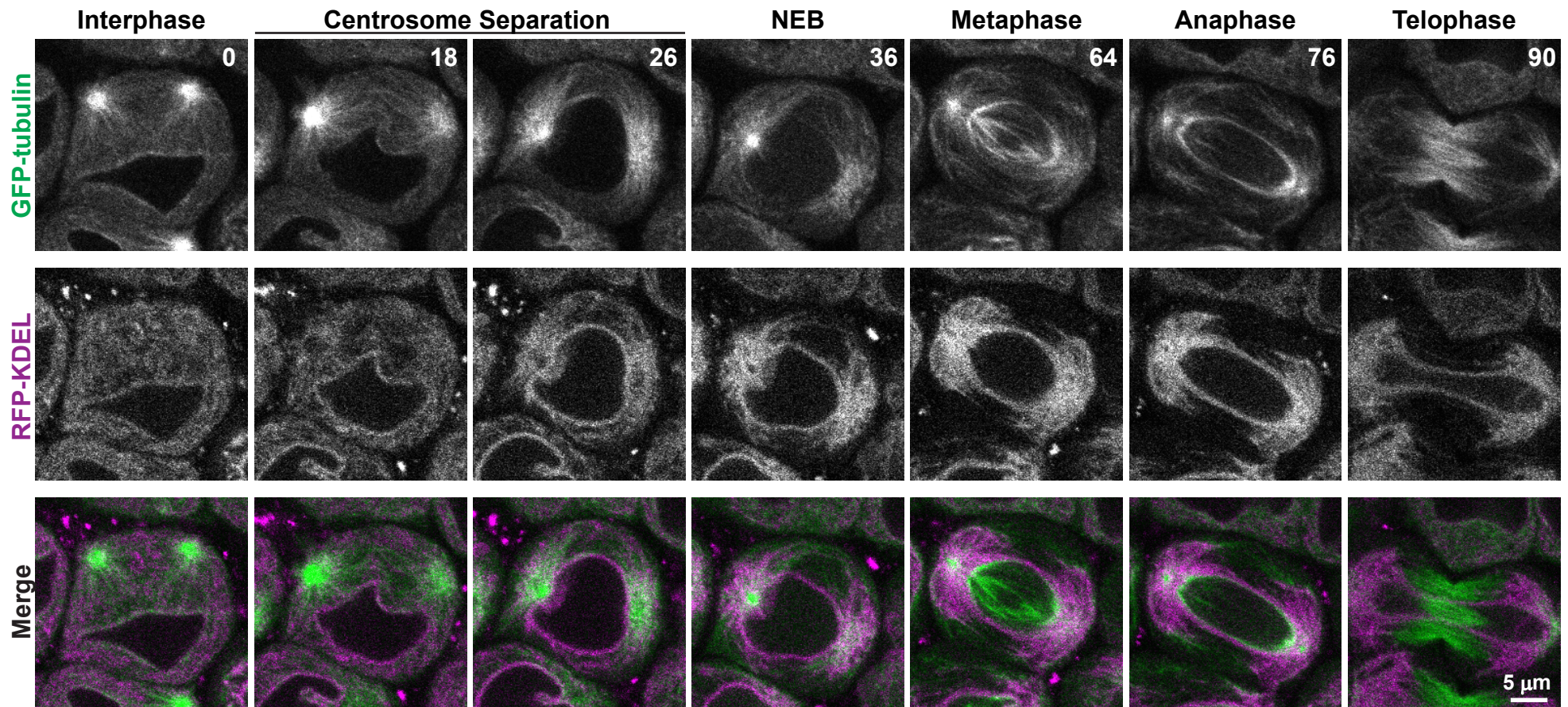
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576

Figure 1

A

B

Sharp Cutting End

Edge

135°

Figure 1

Figure 2

A <u>Larvae</u>

Male

Female

B <u>Pupa</u>

C

Cleaned Testes

Testes

Fat Body

Figure 2

Figure 3

A

Spermatids

Elongating Sperm

Meiosis II

Meiosis I

Late Spermatocytes

Early Spermatocytes

Spermatogonia

100 μm

B

10 μm

C

10 μm

Figure 3

Figure 4

Figure 4

Figure 5

Intact

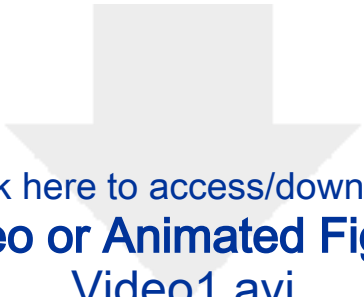**Ruptured**
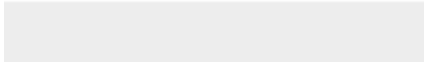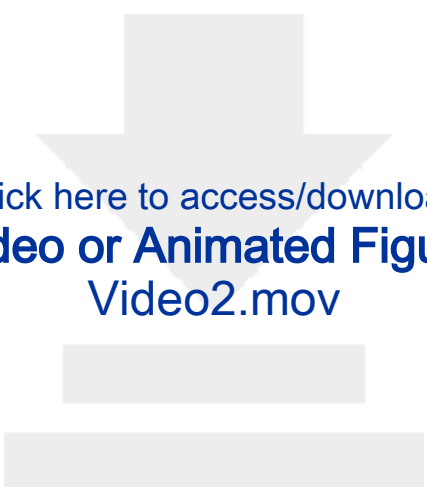
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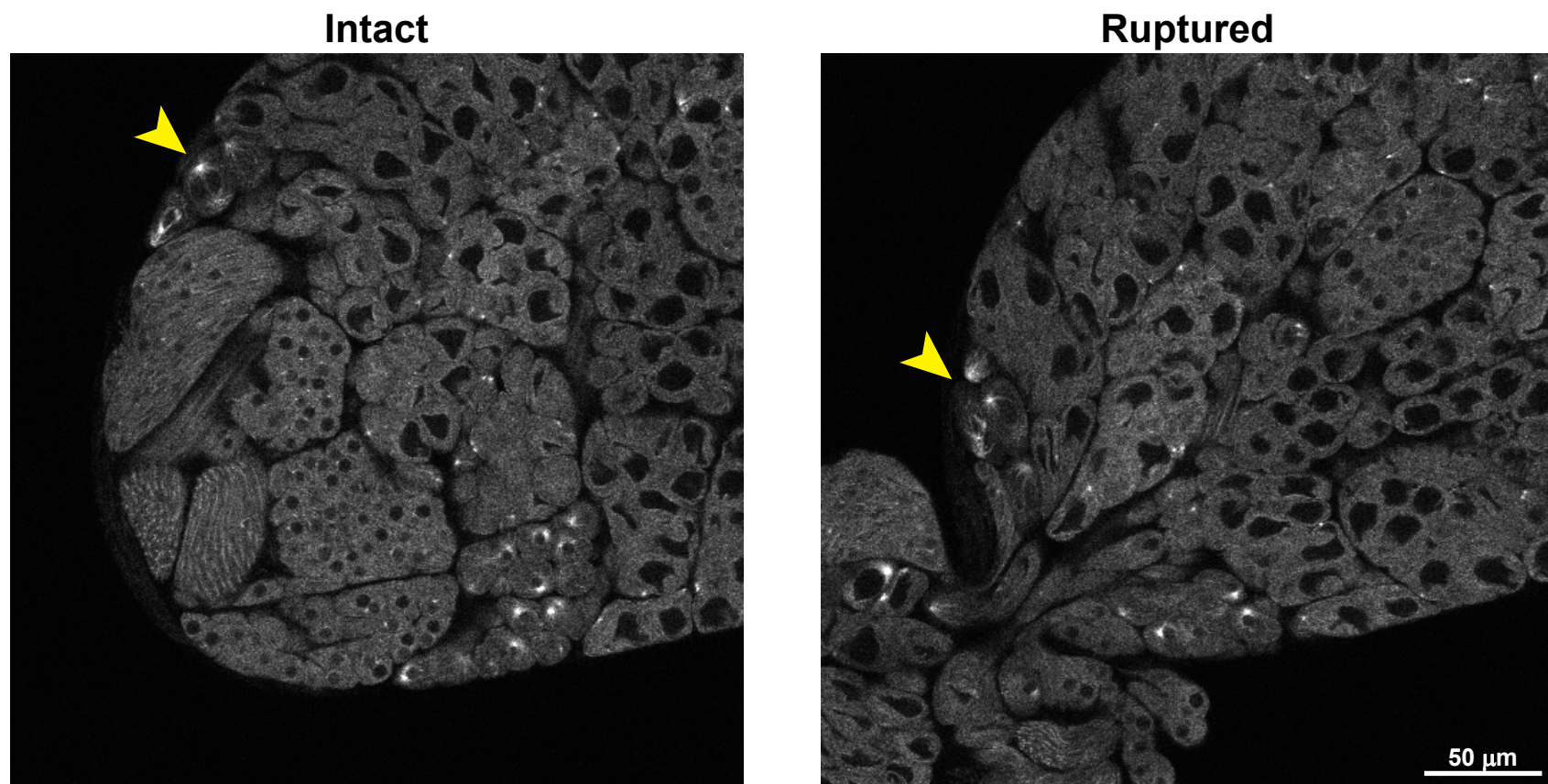
Figure 5

Video 1

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Video or Animated Figure
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Video 2

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Name of Material/ Equipment	Company
5" Dissecting Probe	Fisher
5.75" Glass Pasteur Pipet	Fisher
Bovine Serum Albumin (BSA)	Fisher
Dumont #5 Forceps	Fine Science Tools
Frosted Microscope Slides	Fisher
Halocarbon oil 700	Sigma
Lumox Dish 50	Sarstedt
Microscope Cover Glass	Fisher
Minutien Pins	Fine Science Tools
Nickel Plated Pin Holder	Fine Science Tools
Paraformaldehyde 32% Solution, EM Grade	Electron Microsocopy Sciences
Plan Beveled Edge Microscope Slides	Fisher
PYREX Spot Plates	Fisher
Schneider's Drosophila medium	Fisher
Syringe Filter, 0.22 µm	EMD Millipore
Triton X-100	Fisher
Vectashield	Vector Labs

Catalog Number	Comments/Description
08-965-A	
13-678-20A	
BP9703-100	
11252-20	Straight forcepts with fine tips
12-544-2	Slides for mounting fixed tissue, with frosted writing surface
H8898-100 mL	
SAR946077410	Gas-permeable tissue culture dish
12-541-B	22x22 mm, #1.5 glass coverslip
26002-15	Insect pins used to make scalpel tool
26018-17	
15714	
12-549-5	Slides used for dissections
13-748B	9-well glass dissecting dish
21720-024	
SLGS033SB	
BP151-500	
H-1000	Microscopy mounting medium

JoVE60961

"Preparation of *Drosophila* larval and pupal testes for analysis of cell division in live, intact tissue"

Darya Karabasheva and Jeremy T. Smyth

Rebuttal to Editorial Comments

1. We have proofread the manuscript and fixed spelling and grammar issues.

2. A copy of the webpage that describes the editorial policy that allows re-prints for Nature Publishing Group has been included. This pertains to Figure 4, which contains data reproduced from our earlier manuscript published in *Scientific Reports*.

3. All text is 12 pt and single-spaced.

4. Numbering of the Protocol has been changed to follow JoVE instructions.

5. Protocol steps are separated by a one-line space.

6. Steps longer than 4 lines were separated into two steps where possible.

7. Appropriate Greek characters have been used for SI units.

8. Commercial language ("Vectashield") in lines 276-280 has been changed to "microscopy mounting medium". The Table of Materials has been updated to indicate that Vectashield refers to the microscopy mounting medium.

9. All journal titles have been spelled out fully in the References.

Rebuttal to Peer Review Comments

Reviewer #1

We appreciate that the reviewer found our protocol to be thorough and adequately detailed. The reviewer's suggestions for improvements were very helpful to us, and we have made the following changes in response to these suggestions:

1. The authors repeatedly claim that intact testes can be maintained ex vivo for upwards of 24 hours but do not demonstrate this themselves nor provide citations for previous literature. I would like to see the authors demonstrate this with their own culture conditions, especially since

*they state "our methods are optimized for long-term culture of testes on the microscope stage".
To substantiate this claim the authors should provide data on the following:*

1) Percent survival of testes after extended culture
*2) Whether there is an increase in cell cycle abnormalities (cell division time increases, number
of lagging chromosomes, activation of division checkpoints, etc) after extended culture*

We agree that it is important to determine whether long-term culture of testes results in
increased cell cycle abnormalities, especially because our statement of "upwards of 24 hours"
suggests that testes can be maintained for several days. To address this, we have first changed
"upwards of 24 hours" to "up to about 24 hours" (line 79), which more accurately reflects
culture durations that we have done in our lab. We have also added a new paragraph to the
Discussion (lines 397-414) explaining the utility of long-term culture and explaining that in our
hands, testes maintained overnight remain healthy and viable based on the routine presence of
cells actively undergoing cell division. Unfortunately, we do not currently have data directly
comparing cell cycle parameters for freshly dissected versus long-term cultured samples,
though we agree with the reviewer that including such data would strengthen the protocol. We
now address this in the new Discussion paragraph by advising the reader that precautions
should be taken to determine whether, for their respective assays and conditions, long-term
culture adversely affects the physiology of the testes and cells within. This includes, as
suggested by the reviewer, assaying for increased incidence of meiotic abnormalities.

*2. The authors should take care when describing their system to use in vivo versus ex vivo
appropriately.*

We have changed "in vivo" to "in intact tissue" or "ex vivo" in several places in several places in
the manuscript (e.g., lines 102 and 132).

*3. The authors should consider adding a troubleshooting section where they discuss common
technical pitfalls in their protocol and how to recognize them.*

We have included all troubleshooting steps and pointers in the Discussion section, as prescribed
in the *JoVE* manuscript preparation guidelines. This includes sections on avoiding damaging
testes during dissection, preventing bursting of the testes during mounting, and considerations
regarding fixation methods and proper preservation of cell and tissue architecture.

4. It was unclear why highlighting was used for portions of the text.

Highlighting was used to indicate steps that should be included in the video, as prescribed in
the *JoVE* manuscript preparation guidelines.

Reviewer #2

We thank the reviewer for their favorable comments. In response to the suggesting to "include other panels to describe some important steps", we feel that the video will provide adequate visualization of the important steps of our protocol.

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