We thank the editor and all four reviewers for their helpful comments concerning the manuscript we submitted to JoVE. We have incorporated these suggestions into a revised version (please see point-by-point responses to comments below). By doing so, we think that we have significantly improved the quality of the manuscript text and the accompanying figures.

***Editorial comments:***

*1) Please download the most recent version of the Microsoft word document from the "file inventory" to use for any subsequent changes.*

-We followed this instruction.

*2) Please re-write step 2.4 of your protocol section in imperative tense, as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.)*

-We have fixed this step in the revised version.

*3) JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Please remove all commercial sounding language from your manuscript. All commercial products should be sufficiently referenced in the table of materials/reagents. Examples of commercial sounding language in your manuscript are "Kimwipe" in step 3.15 and "ProLong Gold Antifade Reagent" in step 3.17 of the protocol.*

-Commercial product names have been moved from the manuscript to the materials/reagents table in the revised version.

*4) Please revise the manuscript text to minimize the use of any pronouns (i.e. "we", "you", "our" etc.).*

-Personal pronouns have been removed from the revised manuscript.

*5) If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure) and you must cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."*

-In the original Figure 3B, the field of several germline cells included a wild-type round spermatid that had been cropped and presented as a control cell in a previously published manuscript from our lab. To avoid the need to request permission from the previous publisher, we have revised Figure 3B such that a different field of germline cells that does not include the previously published spermatid is now displayed. All of the other figures were generated specifically for inclusion in the current manuscript.

***Reviewers' comments:***

***Reviewer #1:***

*Manuscript Summary:*

*This manuscript is useful and well-done in places and incomplete/inaccurate in others. A fair amount of revision is necessary to make it suitable for publication. Specific comments are listed below, some in the "major concerns" section and some in the "minor concerns" section.*

*Major Concerns:*

*One main concern is that comprehensive methods publications on these techniques have already been published and are not cited. A primary reference is Bonaccorsi, S., M.G. Giansanti, G. Cenci, and M. Gatti. 2000. Cytological Analysis of Spermatocyte Growth and Male Meiosis in Drosophila melanogaster. Chapter 5 in Drosophila Protocols, Eds. W. Sullivan, M. Ashburner, R.S. Hawley. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.*

*Subsequent adaptations of the protocols in the above book chapter have been published in the journal Cold Spring Harber Protocols, with details at the following PubMed entries for protocols relevant to this JoVE submission:*

[*http://www.ncbi.nlm.nih.gov/pubmed/21363941*](http://www.ncbi.nlm.nih.gov/pubmed/21363941) *Live testis preparations*

[*http://www.ncbi.nlm.nih.gov/pubmed/22854561*](http://www.ncbi.nlm.nih.gov/pubmed/22854561) *Formaldehyde fixation*

[*http://www.ncbi.nlm.nih.gov/pubmed/22194254*](http://www.ncbi.nlm.nih.gov/pubmed/22194254) *Paraformaldehyde fixation*

[*http://www.ncbi.nlm.nih.gov/pubmed/21969616*](http://www.ncbi.nlm.nih.gov/pubmed/21969616) *Methanol-acetone fixation*

[*http://www.ncbi.nlm.nih.gov/pubmed/21969617*](http://www.ncbi.nlm.nih.gov/pubmed/21969617) *Immunostaining*

-We include these references in the revised manuscript.

*Only if a truly useful set of videos are produced to accompany this manuscript will this project represent something novel.*

*[* ***Editorial comment:*** *The above comment suggests that the content of your manuscript is not novel, however JoVE is a methods journal and we are not concerned with novelty of results as much as scientific rigor of the protocol. You may disregard the above comment, or you may chose to address it if you feel it will significantly improve your manuscript. However, please attempt to include some of the above references. ]*

- Protocols that describe in words how to carry out imaging of the *Drosophila* testes are available in the scientific literature, and a visual protocol for testes dissection (with no description of further processing of the tissue for microscopic analysis) was previously published in JoVE by another lab. While these protocols are helpful, a visual presentation of methods for preparing *Drosophila* testes for live cell and fixed cell imaging is currently lacking. We therefore believe that our manuscript is ideal for publication in the unique multimedia format offered by JoVE and that we can provide a very useful and unique resource for labs that are inexperienced in working with this model system.

*Additional major concerns:*

*p. 6 line 111 (also p.9 line 233): It is not accurate to say that 2-5 day old males are ideal for visulizing spermatids. In fact, very young males (0-2 days old) are ideal for visualizing meiosis and early post-meiotic spermatids of the onion stage and early elongation stages. Only if the final mature sperm are the target is it useful to dissect older males.*

-These two sentences have been modified as follows in the revised version:

“Young males (0-2 days old) are ideal for examining cells throughout the earlier stages of spermatogenesis (e.g. spermatogonia, spermatocytes, and early post-meiotic spermatids), whereas slightly older males (2-5 days old) are ideal for examining cells in the final stages of spermatogenesis (in particular, mature sperm).”

“Older males are preferable if the goal is to visualize mature sperm.”

*It would more complete to add here (in addition to in the discussion) a mention of dissecting larval testes and/or pharate adult testes for the purposes of looking at more cells at extremely early stages.*

-Steps 1.7 and 1.8 (with brief descriptions of methods including references for isolating testes from pharate males and male larvae, respectively) have been added to the protocol section in the revised version. The third paragraph of the discussion section has been modified in the revised version to better fit with the inclusion of methods for isolating pharate and larval testes in the protocol section.

*p.6 line 141: Need much more description with regard to "nicking," as a small nick will cause a failed preparation when cells lyse as they squeeze through a too-small opening.*

-We have used the term “tear open” instead of “nick” in the revised manuscript. The Bonaccorsi protocols describe this step in the same way without additional details: “Tear open the adult testes using either very fine forceps or tungsten needles.” We think that the video demonstration will be very useful for conveying to the viewer how to perform this step.

*p. 7 line 149. Emphasize that all "squashing" is purely from the weight of the cover slip, not from any pressure applied by the researcher.*

-Step 2.3 has been modified in the revised version to make this point:

“2.3) Gently place a glass microscope slide over the cover slip to squash the testes; do not apply pressure manually as the weight of the cover slip alone is sufficient to obtain a properly squashed sample.”

*p.7 line 162: This won't work well unless the slides were treated to make them sticky (e.g. polylysine) and the cover slips treated to make them non-sticky (e.g siliconized). This necessity isn't mentioned here or in the list of reagents.*

-We have found these treatments to be unnecessary, and we do not routinely include them in our protocol. In several of the Bonaccorsi protocols listed above (for example, [*http://www.ncbi.nlm.nih.gov/pubmed/21363941*](http://www.ncbi.nlm.nih.gov/pubmed/21363941) *Live testis preparations;* [*http://www.ncbi.nlm.nih.gov/pubmed/22854561*](http://www.ncbi.nlm.nih.gov/pubmed/22854561) *Formaldehyde fixation;* [*http://www.ncbi.nlm.nih.gov/pubmed/21969616*](http://www.ncbi.nlm.nih.gov/pubmed/21969616) *Methanol-acetone fixation*), nonsiliconized cover slips were actually specified, and no indication was given that the microscope slides were pre-treated in any of the referenced protocols. Nonetheless, in case some researchers find these treatments to be useful for preparing the testes samples for microscopy, we have included them as optional methods in the revised protocol as follows, and product information has been added to the table.

(End of Step 2.1):

“Optional: Use siliconized cover slips to minimize adherence of the tissue to the cover slip later in Step 3.2.”

(End of Step 2.3):

“Optional: Use poly-L-lysine coated microscope slides to promote adherence of the tissue to the slide later in Step 3.2.”

*pages 9-10: Parts of the Representative Results section (particularly the first two paragraphs) seem very redundant with other sections.*

*[* ***Editorial comment:*** *According to the JoVE format, the Representative Results section should include a discussion of the results of all figures, so you may disregard the above comment. ]*

*p. 13 line 369: This paragraph doesn't seem to fit the focus of the article; while it's useful information in some sense, it seems a bit disjointed and out of place.*

-To round out the discussion, we felt it was important to briefly mention that the *Drosophila* testes system can be used for biochemical analysis. A minimal amount of space has been used to make this point because we refer the reader to published literature in which this approach has been successfully taken. In response to a comment from Reviewer #3, we have included an additional reference (Belloni et al., 2012) when providing examples of co-immunoprecipitation experiments using *Drosophila* testes extracts.

*Figure 1 and associated text need to 1) indicate/highlight the fact that cytokinesis is incomplete; 2) describe/indicate what the features of the diagrams are indicated (distinguishing nuclei from mitochondria, for example, and 3) indicate with better accuracy that mature sperm heads are not oval shaped. A full Figure legend is truly needed. The text does not describe all the features.*

-We have revised Figure 1 so that it better depicts the connections between germ cells and the needle-shaped (as opposed to oval-shaped) nuclei of mature sperm heads. We now include a full legend for Figure 1 in the revised manuscript:

“**Figure 1. Schematic of germline cell divisions in *Drosophila* males.** Each stem cell division produces a gonial cell that undergoes four rounds of mitotic divisions with incomplete cytokinesis to produce a 16-cell cyst of primary spermatocytes. Each primary spermatocyte undergoes a prolonged growth phase prior to undergoing the two meiotic divisions, again with incomplete cytokinesis, to form a 32-cell cyst of interconnected secondary spermatocytes followed by a 64-cell cyst of interconnected round spermatids. Round spermatids are characterized by the presence of a phase-light nucleus and a phase-dark mitochondrial aggregate (the Nebenkern) of similar sizes. The round spermatids undergo elongation and individualization to form the mature sperm. Mature sperm heads can be identified by the their needle-shaped nuclei. Nuclei are shown in blue; Nebenkerne (mitochondrial aggregates) in black; cytoplasm in tan.”

*Figure 2: It is confusing to use the letters A and B for the panels as well as for the places to "nick" the testis. Use different designations for the latter.*

-We use numbers rather than letters in the revised version to refer to the various positions at which to tear open the testes.

*Figure 2 (also p. 9 line 239): It is inaccurate to suggest that "Level B" is the place to "nick" the testis to get late stage cells. There will still be mostly primary spermatocytes at that location. The location to open up to get more mature cells is closer to where the curvature starts. This will be a key thing to get right in any video!!­*

-We have modified in the revised version our description of the positions at which to tear open the testes as follows: “To enrich for spermatogonia and spermatocytes, tear open the testis adjacent to its apical tip (level 1, Figure 2B). To enrich for spermatocytes and spermatids, tear open the testis at a position slightly basal to level 1 (level 2, Figure 2B). To enrich for more mature germline cells, tear open the testis closer to where the curvature begins (level 3, Figure 2B).” We agree that the video demonstration will be very useful for conveying to the viewer how to perform this step.

*Figure 2: It is inaccurate to suggest that "nicking" the testes is sufficient. The testes must be truly peeled open. Otherwise cells lyse as they squeeze through a small "nicked" opening. This will be another key thing to get right in a video!!*

-We regret having used the term “nick” in the original manuscript to refer to the process of tearing open the testes. It was not an accurate description. We have expunged the term “nick” from the revised manuscript and have replaced it with “tear open.” Again, we agree that the video demonstration will be very useful for conveying to the viewer how to perform this step.

*Figure 3 legend p. 17 line 496 (also p. 12 lines 321-322): "Occasionally" is inaccurate - it is quite typical to see such fusions since cytokinesis is incomplete to begin with, and the ring canals routinely burst open under the cover slip.*

-We have changed the word “occasionally” to “frequently” in the revised version.

*Need more explanation on incomplete cytokinesis in the text for this all to make sense.*

-We have included a full legend for Figure 1 in the revised manuscript (see note above) that should provide a better explanation of the incomplete cytokinesis that occurs during both the gonial and meiotic divisions. We have also added this sentence to the introduction of the revised manuscript:

“During both the mitotic and meiotic divisions of the male germ cells, cytokinesis occurs incompletely such that the daughter cells remain connected by cytoplasmic bridges known as ring canals (Figure 1).”

*Minor Concerns:*

*p.4 line 61: "a second round of DNA division in meiosis" is not the only event that is needed for the production of 64-cell cysts.*

-In addition to improving our explanation of incomplete cytokinesis in the revised manuscript (see note above), we have modified this sentence as follows:

“Progression through meiosis I and meiosis II results in the formation of 32-cell cysts of secondary spermatocytes and 64-cell cysts of haploid spermatids, respectively.”

*p. 5 line 96: Antibodies and dyes don't actually penetrate the outer layers of the testis (composed of a muscular sheath) very well, and that is one reason we open up the testes to let the cells spill out. Be more precise with language concerning what the antibodies etc are penetrating in these protocols.*

-We have changed this sentence in the revised version to include the following: “antibodies and dyes can easily penetrate cells following their dispersal from the testes.”

*p. 6 line 117: PBS is not the most commonly used dissection buffer; it's worth mentioning that there are others. The Bonaccorsi publication gives recipes for four different buffers.*

-We include the following sentence in the revised manuscript (with reference to the 2000 Bonaccorsi publication):

“Other aqueous solutions have been successfully used for testes dissection.”

*p.7 line 154: Could add here the application of visualizing fluorescence of endogenous proteins (e.g. a GFP-tagged protein encoded by a transgene in the dissected fly), as mentioned later in the discussion. In the discussion (e.g. p.12 line 343), it should be emphasized that this visualization can be done in live preparations.*

-We have made these changes in the revised version as follows:

(From protocol section)

“2.4) Use preparation immediately (ideally within 15 min of preparation) to observe live cells by phase-contrast microscopy; for transgenic flies with expression of fluorescently tagged proteins in the testes, live cells can be examined by fluorescence microscopy at this step. Alternatively, proceed with fixation and antibody staining (Step 3).”

(From discussion section)

“If antibodies against a protein of interest are unavailable, transgenic *Drosophila* lines expressing a fluorescently tagged (e.g. mCherry or GFP) version of the protein can be generated as an alternative approach. The subcellular localization of the protein can then be determined by using a microscope to view the GFP fluorescence in live or fixed preparations of tissue; alternatively, anti-GFP antibodies can be used to detect the fusion protein in fixed samples. In Figure 4, microtubules in a fixed testes preparation were visualized via the intrinsic fluorescence of GFP-tagged beta1-tubulin (expressed from a transgene under control of a globally expressed ubiquitin promoter).”

*Figure 2: The dark field images are hard to make out. Would other optical conditions be more suitable?*

-We have taken the highest-quality images that we can with our microscope. We have brightened the image in the revised figure in an effort to make it easier for the reader to discern the dissected specimens against the dark background.

*Figure 4 legend p. 17 line 508: What evidence is there that that is a primary and not a secondary spermatocyte undergoing division?*

-We can usually tell the difference with some degree of confidence because primary spermatocytes are much larger than secondary spermatocytes. When both primary and secondary spermatocytes are present within the field of view, it is easier to distinguish between them based on their relative sizes. Because Reviewer #1 has raised this concern, however, combined with the fact that only one spermatocyte is present in the field of view in Figure 4D, we have changed “dividing primary spermatocyte” to “dividing spermatocyte in the revised version.

*p.22 in list of reagents: "forceps" is misspelled.*

-We have fixed this error in the revised version.

*Additional Comments to Authors:*

*Other than the issues noted, the manuscript is generally clear and accurate, though not comprehensive in terms of possible variations.*

***Reviewer #2:***

*This manuscript presents the method for isolating and preparing Drosophila testes samples for imaging by phase-contrast and fluorescence microscopy. This is well desigend and well written manuscript. There are some quick fix and staining methods are provided, however, most of the methods presented here already known, except few. Some of these methods are not cited such as:*

*Cold Spring Harb Protoc. 2012 Aug 1;2012(8).*

*Anal Biochem. 2013 May 1;436(1):55-64*

*Methods Mol Biol. 2008;450:45-59.*

*Cold Spring Harb Protoc. 2012 Jan 1;2012(1):105-6*

*Cold Spring Harb Protoc. 2011 Oct 1;2011(10):1273-5*

-We include these references in the revised manuscript.

*Blocking the tissue before primary antibodies incubation is not given in method.*

-We do not routinely include a blocking step when performing our immunostaining experiments, but it may be very important for some antibodies. The revised manuscript includes a description of an optional blocking step (new Step 3.8) and modification of the primary antibody incubation and wash conditions if blocking was performed (Step 3.10):

“3.8) Blocking step (optional): Immerse slides in PBS plus 1% BSA for 45 min at room temperature.”

“3.10) Add 30-40 μl of primary antibody (diluted in PBS-T, 1:400 to 1:50, depending on antibody) to tissue within the circle. If blocking was performed, dilute primary antibody in PBS-T plus 1% BSA.”

“3.11) Wash slides in PBS for 5 min at room temperature three times. If blocking was performed, wash twice in PBS-T and once in PBS (5 min at room temperature each)”

*Details of source of the antibodies for primary (GFP, tubulins) and secondary antibodies are not provided.*

- Information about the anti-gamma tubulin antibodies and Cy3-conjugated secondary antibodies used in Figure 4 has been added to the reagents/equipment table in the revised version. We did not use anti-GFP antibodies; instead, we visualized the fluorescence of GFP-tagged beta-tubulin expressed from a transgene.

***Reviewer #3:***

*This manuscripts describes protocols for the visualization of cells in Drosophila testes. In addition it provides useful tips on how to enrich preparations for specific stages of spermatogenesis*

*Although protocols for live analysis and and immunostaining of Drosophila testes have been already published, they are focused on male meiotic divisions.*

*Major Concerns:My major concern the absence of any description of how to dissect larvae. A large number of mutants die at the third instar larval stage.*

*Thus adult testes cannot be performed. A small section describing how to dissect larvae can be important.*

-Please see response (above) to similar point raised by Reviewer #1.

*Minor Concerns: I found a bit confusing the protocol describing fixation and antibody staining between point 3.8 and 3.9. After using a hydrofobic barrier PAP pen to draw a circle around the preparation it is not clear whether the preparations are left to dry or should be maintained wet when adding the antibody. In my experience the preparations should be kept wet.*

-We agree that the preparations should be kept wet. To make this point more clearly, we have modified Step 3.8 in the revised version as follows:

“3.8) Use a hydrophobic barrier PAP pen to draw a circle on the slide around the squashed tissue (easily visible by eye) in order to confine the antibody solutions (added in steps 3.9 and 3.11). The tissue should be kept moist at all times while performing immunostaining.”

*In the list of reagents the authors should also give the recipe for PBS. There are several PBS buffers available.*

- We include a description of our “homemade” PBS (130 mM NaCl, 7 mM Na2HPO4, and 3 mM NaH2PO4) in Step 1.4 of the Protocol section in the revised manuscript.

*IN the same section please change Foreceps to Forceps.*

-We have fixed this error in the revised version.

*The paper of Belloni et al., 2012: Mutations in Cog7 affect Golgi structure, meiotic cytokinesis and sperm development during Drosophila spermatogenesis.*

*J Cell Sci. 2012 Nov 15;125(Pt 22):5441-52. doi: 10.1242/jcs.108878. Epub 2012 Sep 3. should be also cited when referring to Co-IP using Drosophila testes.*

-We have cited this reference in the revised version when referring to co-immunoprecipitation experiments using *Drosophila* testes.

*Additional Comments to Authors:*

*N/A*

***Reviewer #4:***

*Manuscript Summary:*

*N/A*

*Major Concerns:*

*None*

*Minor Concerns:*

*line 57: The term "hub" should be reserved for the niche cells that support the stem cells.*

-We have changed “hub of germline stem cells” to “population of germline stem cells” in the revised version.

*line 60: Please describe the length of time that spermatocytes remain in G2 and the increase in cell volume.*

-We have added information to this sentence in the revised version as follows:

“After premeiotic S phase, primary spermatocytes enter G2, a prolonged growth period of ~90 hours during which cellular volume increases ~25-fold [1](#_ENREF_1).”

*line 77: Please descrive the "specialized organs" as spermathecae.*

-We have made this change in the revised version.

*line 96: The testis is not always easily penetrated by antibodies and can require additional permeabilization (e.g. increased detergent)*

-Reviewer #1 also noticed a problem with our wording in this sentence (see response above). We have made it clearer in the revised version that we are referring in our methods to the staining of cells dispersed from the testes as opposed to staining of the intact testes.

*line 130: Most transgenic strains have transparent testes as the mini-white gene found in P-elements does not result in pigment accumulation in testes.*

We have added the following sentence to the first paragraph of the Discussion section: “Most transgenic strains, which are typically in a *white* background, also have white testes because the mini-*white* gene found in *P*-elements does not promote pigment accumulation in the testes.”

*Section 2.2:The authors state that germline stem cells can egress from the testis - Do GSCs really detach from the hub?*

-We were incorrect to suggest that germline stem cells can egress from the testis. To fix this error, we have removed “germline stem cells” from this sentence in the revised version.

*Should a "2.5" be added - along the lines of "Gently wick liquid from under the coverslip using a Kimwipe to allow flattening of the preparation until the germ cells are clearly in focus"?*

-We have added the following Step 2.5 to the revised manuscript:

“2.5) Gently wick any excess liquid from under the coverslip using a cleaning wipe to allow flattening of the preparation until the germ cells are clearly in focus.”

*line 228: I would not descrine testes as "thick" but at their maximal diameter.*

-We have changed “thick” to “at their maximal diameter” in the revised version.

*Figure 1: it would be useful to show the connections between germ cells.*

-We have revised Figure 1 so that it depicts the connections between germ cells.

*Figure 2: It is not optimal to use red arrows on different panels to refer to different cell types.*

-We have revised Figure 2 so that the color scheme used for the arrows and arrowheads will be less confusing for the reader.

*Reagent list: Forceps is not spelled correctly.*

-We have fixed this error in the revised version.

*Additional Comments to Authors:*

*Did the authors not find any need to siliconize coverslips or treat slides to allow tissue to attach to the slide and not the coverslip*

-Please see response (above) to similar point raised by Reviewer #1.