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Modeling Drosophila Stem Cell Development through Ex Vivo Live-Cell Imaging Studies

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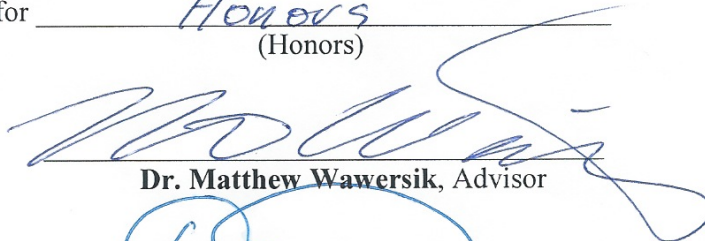
Modeling *Drosophila* Stem Cell Development through *Ex Vivo* Live-Cell Imaging Studies

A thesis submitted to the Department of Biology at
The College of William & Mary

by

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Abstract

Stem cell specification, maintenance, and differentiation are imperative to proper organogenesis and tissue maintenance. Understanding the mechanisms and steps of these developmental processes is invaluable to advancing tissue grafting, cancer treatment, and other organ therapies. The *Drosophila melanogaster* testis is one of the best-characterized systems for studying these processes. Currently, studies in the *Drosophila* testis have thoroughly characterized when stem cells develop and what the resulting adult testis morphology will be. A major question that remains to be answered is how stem cells develop – what controls what cells become functional stem cells and how stem cell fates are dictated. To seek answers to this question, I propose a focus on live-cell imaging studies of the developing *Drosophila* testis. In order to perform live-cell imaging studies in this developmental system, it is necessary to successfully design protocols for: (1) gonad isolation, (2) testis culture, (3) imaging of developing gonads over time in three-dimensions (3D), and (4) tracking cell movement in developing gonads over time. In this thesis, I provide evidence for successful completion of the first three steps towards live-cell imaging, as well as preliminary studies of cell tracking. Testes at different key stages of development, (1) from gonad coalescence through germline stem cell (GSC) niche formation and (2) from GSC niche formation to spermatogonial differentiation, were studied for evidence of successful live-cell imaging. These key developmental events were chosen because they are characterized by major morphological shifts that can be clearly observed via live-cell imaging. These findings provide a promising start to a line of research inquiry that could answer many key questions that have remained elusive to investigators focused on understanding stem cell development (Slack, 2008).

Introduction

Stem cells are characterized by their ability to undergo asymmetric cell division, producing one daughter cell that maintains stem cell character and one that has the capacity to differentiate into at least one other cell type (Slack, 2008). These properties allow adult stem cells to replace dead and dying cells within the adult organ, maintaining healthy cell populations and tissue homeostasis within organ systems. During the development of organs, or organogenesis, stem cells function to give rise to and maintain differentiated tissue types to establish functional organs (Gilbert, 2003).

All stem cells are localized within a niche, a particular microenvironment comprising differing cell populations that assist in the maintenance of stem cells. Stem cell niches vary greatly across organ systems, and dysregulation of stem cells in these microenvironments has been implicated in cancer, senescence, and developmental disorders (Spradling et al., 2001) (Shim et al., 2013). Elucidating the underlying mechanisms of stem cell development and maintenance is imperative to developing stem cell therapies because the long-term benefit of these therapies requires proper engraftment of the appropriate stem cell niche (Kaufman, 2009). However, literature investigating stem cell specification, migration, and fate is limited.

Here, I examine a new line of inquiry to bolster our knowledge of stem cell development using testes from the fruit fly, *Drosophila melanogaster*. Data presented herein strives to examine stem cell development during testis growth and maturation in real-time through live-cell imaging. This introduction will (1) justify the use of the *Drosophila* testis as a model system (2) present an overview of our current understanding of adult stem cell behavior in *Drosophila*, (3) provide additional background on what is known about stem cell development during

organogenesis, and (4) describe how live-cell imaging can be used as a tool to examine remaining questions about stem cell development in the *Drosophila* testis.

Drosophila melanogaster as a model system

Stem cell behavior is currently being studied in a number of model systems including insects, mice, chickens, and humans. *Drosophila melanogaster* (fruit flies) are particularly well suited for laboratory studies. First propagated for research use in 1901, *Drosophila* are one of the earliest and most extensively studied model organisms (Reeve, 2001). Their short generation time (10-12 days at room temperature), high fecundity (laying ~ 100 eggs per day), fully sequenced genome across 4 manipulable chromosomes, and easily isolated adult tissues make this model highly tractable.

In 1918, Miller advanced *Drosophila* research by being the first to employ balancer chromosomes to propagate fly stocks through self-crossing. The multiple inversions found on balancer chromosomes prevent the chromosomal alignment requisite for meiotic recombination. Thus, if crossover were to occur in these flies, the organism would die due to the loss of some chromosomal segments coupled with the acquisition of a second copy of other segments. This ensures that genotype is maintained as fly lines are propagated.

These capacities for gene and tissue manipulability, as well as the relatively easy generation of experimental fly populations, make *Drosophila* an ideal model organism for investigating molecular and developmental pathways.

Drosophila melanogaster testes as a model system to study stem cell development

The *Drosophila* testis is a highly tractable system for studying the regulation of stem cell behavior during spermatogenesis. The adult *Drosophila* testis can be easily dissected from the body, and the cell types that comprise the tissue are well characterized. Furthermore, similarities between the general process of vertebrate and invertebrate primordial germ cell (PGCs) development and spermatogonial differentiation ensure its relevance to other organisms and therapeutic applications (Orkin and Zon, 2008).

The stem cell niche within the *Drosophila* testis is located at the blind end of a single coiled tube; the other end connects to the genital tract (Figure 1). Two populations of stem cells are localized to the testis niche: GSCs and a population of cyst stem cells (CySCs) that regulate GSC maintenance (Jemc, 2011). A group of non-dividing somatic cells, termed the hub, are found at the tip of the testis (nearest the blind end of the coiled tube). Arrayed directly around the hub are about six to eight GSCs. The main cell bodies of the CySCs are slightly displaced from the hub, with only membrane projections that extend in between the proximate population of GSCs directly in contact with the hub. The process of spermatogenesis begins with a GSC undergoing asymmetric division, with one daughter cell remaining at the hub and the other moving away from the hub to differentiate into a gonialblast. The gonialblast divides with incomplete cytokinesis four times, to produce 16-cell spermatogonia. These undergo meiosis, and mature individually into functional sperm. Contemporaneous with each GSC division is a CySC asymmetric division. One daughter cell maintains stem cell identity and hub contact, while the second supports spermatogonial differentiation as a mitotically inactive cyst cell. Cyst cells regulate spermatogonial cell division by growing to encompass and accommodate spermatogonia as differentiation proceeds (de Cuevas, 2011).

Stem cell development during organogenesis

Animal embryos do not contain stem cells in the conventional sense during early embryonic stages (Slack, 2008). Rather, progenitor cells divide symmetrically to generate an adequate pool of progenitor cells to advance development. As development proceeds, cells take on divergent identities, with embryonic progenitors differentiating into distinct germ layers: ectoderm, mesoderm, and endoderm (Gilbert, 2003). Development proceeds hierarchically as cells differentiate into progressively more specific cell types. The specification of a tissue-specific stem cell population is fundamental to organogenesis. In embryonic development, local specification is dictated by a series of developmental events. The presence of certain factors in key combinations leads to the specification of particular cell types (Slack, 2008). The exact origin of a specific stem cell population is incredibly difficult to trace because of changes in the role and salience of particular factors as well as the difficulty in regulating promoter fidelity and strength. Further, more than one pathway can be taken along the steps from original progenitor to final cell type. In other words, some organ systems have stem cell populations that arise from more than one embryonic progenitor (Zaret and Grompe, 2008).

Drosophila testis development begins during embryogenesis, about 10 hours after egg laying (AEL) (Jemc, 2011). The embryonic gonad is initially comprised of primordial germ cells (PGCs) and somatic gonadal precursors (SGPs). During embryonic stages 4-5 (2-3 hrs AEL), PGCs from maternally deposited cytoplasm emerge at the germ plasm, or posterior of the embryo. Next, invagination of the midgut conveys posterior PGCs from the posterior to the interior of the embryo at embryonic stage 7 (~3.5 hrs AEL). Around embryonic stage 9, PGCs migrate through the posterior wall of the midgut to the mesoderm, as required for later PGC development. At embryonic stage 11 (~5-7 hrs AEL), SGPs are specified as bilateral groups in

mesoderm parasegments (PS) 10-13. By stage 12 (~7-10 hrs AEL), PGCs begin to migrate towards and intermingle with these SGPs. Soon after, PGCs and SGPs coalesce tightly such that the SGPs encompass the PGCs by stage (~10 hrs AEL). A subpopulation of SGPs in the anterior of the newly formed testes is specified as hub cells before gonad coalescence around stages 11-12; morphological evidence of their formation is detected around stage 17 (~20 hrs AEL) (Okegbe and DiNardo, 2011). Hub cells then coalesce tightly and function much like adult hub cells with functional GSCs associated with the hub (Sheng et al., 2009). By the end of embryogenesis, hub formation is complete. GSC differentiation then achieve steady state production of gonialblasts by mid- to late- 1st instar larval stage (~30 hrs AEL), implying that the functional GSC niche arises during the embryo to larval transition (Sinden et al., 2012).

The role of live-cell imaging in investigating Drosophila testis development

Our understanding of stem cell development in the *Drosophila testis* is still far from comprehensive. Questions surrounding the mechanisms controlling stem cell population establishment, cell fate, and various steps in between remain to be answered. For instance, what controls which cells become functional stem cells? Which progenitor cells go on to stem cell fates? Alternatively, what determines if a cell *cannot* become a stem cell? The dynamic changes in cell signaling, migration, and morphology that occur during organogenesis confound a comprehensive understanding of the mechanisms surrounding stem cell development. Studying this dynamic system in real-time – by tracking development, specification, migration, and changes in cell fate – may lend important perspective to the study of stem cell development and offer compelling answers to our numerous remaining questions (Slack, 2008).

The current state of literature pertaining to *Drosophila* testis development can tell us *when* stem cells develop and what their final adult morphology appears to be. The question that has yet to be answered is *how* stem cells develop. One means by which we can pursue this line of inquiry is through live-cell imaging studies combined with cell fate tracking. Live-cell imaging has successfully uncovered novel mechanisms of GSC regulation in the adult *Drosophila* testis (Sheng and Matunis, 2011). It would be advantageous to apply this cutting-edge technology to less well-understood systems, such as the developing *Drosophila* testis.

Studying the developing *Drosophila* testis through live-cell imaging allows investigation of real-time stem cell developmental events without killing tissues. However, live-cell imaging of testis development through the fly larval stages is not possible *in vivo* because larvae are mobile, and data collection during development in mobile organisms proves difficult and impractical. Therefore, another challenge for live-cell imaging studies in this system is to sustain and capture development over time in *ex vivo* cultures. Time-lapse capture of developing cells will allow investigators to track the cell lineage of progenitor cells and their progeny, observing stem cell specification and eventual fate.

The process of conducting these live-cell imaging experiments requires successful and consistent protocols for 1) gonad isolation, 2) testis culture, 3) imaging testes in 3D over time, and 4) cell tracking. In 2006, Shigenobu et al. were able to isolate primordial germ cells (PGCs) from embryonic gonads for microarray analysis and gene expression profiling. Sheng and Matunis's work in adult *Drosophila* testis demonstrated the utility of the technique in imaging and tracking cell behavior (2011). For this thesis, I have developed successful protocols for gonad isolation, culture, and 3D imaging in time-lapse (4D) of developing *Drosophila* testes (Figure 2). In the following sections, I will (1) describe the process of protocol evaluation and

optimization, (2) suggest how these techniques may be further modified and refined, and (3) propose future directions for applying live-cell imaging techniques to study stem cell development.

Methods

Fly stocks and gonad isolation

Nos::GMA-GFP ; *His2Av-RFP* flies were used because this line encodes a

Fm7 *Tm Ser*

germ cell-specific promoter that drives green fluorescent protein (GFP) expression at the cell membrane of germ cells, and expresses red fluorescent protein (RFP) fused to a nuclear histone protein; marking both germline and somatic cell nuclei. Fly cages were prepared and embryos collected as specified in Fidler, Boulay & Wawersik, 2013. Flies were collected at ages 13-17 hours and 19-25 hours AEL to examine different key stages of development (hub formation and spermatogonial differentiation, respectively). Once the embryos were aged to the appropriate experimental time point on an apple juice plate, a small but firm paintbrush wetted with Phosphate Buffer Triton X-100 solution with 0.1% Triton X-100 (PBTx) was used to remove the embryos from the plate. Sample was gently wiped with the paintbrush onto the interior ridge of a cell strainer. The cell strainer was placed in a petri dish to catch excess PBTx solution. The paintbrush and cell strainer walls were rinsed using a squirt bottle containing PBTx solution. After all sample was transferred to the cell strainer, the strainer was washed three times in PBTx solution. Solution was discarded in a liquid waster receptacle. Next, the embryos were dechorionated by pouring 50% bleach (NaOCl)/water (ddH₂O) solution into the strainer until the sample rose off the mesh, and kept in the bleach solution for five minutes at room

temperature, with occasional mixing. As soon as five minutes elapsed, the bleach solution was discarded, and the sample in the cell strainer was washed three times with PBTx solution.

Gonad isolation protocol was adapted from Shigenobu, et al., 2006. Specifically, about 50mg of sample was transferred to a glass Potter Elvehjem homogenizer containing 500 μ l of fresh Schneider's Insect medium (Sigma-Aldrich, Catalog No.: S0146). Sample was homogenized with a loose pestle (optimally: seven strokes for samples aged 0-17 hours or nine strokes of loose pestle for sample aged 17 hours or more). After homogenization, 1.5 ml of additional Schneider's Insect medium was then added and the homogenate was then filtered through a 62 μ m-mesh cell strainer into a 50 ml Eppendorf tube and centrifuged at 820g for two minutes at 22°C. Supernatant was discarded, and pellet re-suspended in 2 ml of culture media (85% Schneider's Insect medium, 15% Fetal Bovine Serum (Sigma-Aldrich, Catalog No.: F4135), and 5.5 μ l Pen/Strep (Sigma-Aldrich, Catalog No.: P0781) was added. Filtration, centrifugation, and re-suspension steps were repeated. 40 μ l of re-suspended sample was transferred to a 35 mm glass bottom petri dish (optimally: with a 14 mm Poly-d-lysine coated microwell (MatTek Corporation, Catalog No.: P35GC-1.5-14-C)). Sample was gently spread to an even distribution within the microwell, avoiding bubble formation. An air-permeable YSI Membrane Kit (Yellow Springs Instrument, Catalog No.: 066155) was carefully positioned atop the sample and the membrane-plate junction sealed (to minimize media evaporation) using Halocarbon Oil 27 (Sigma-Aldrich, Catalog No.: H8773) (optimally: 30 μ l). This isolation protocol generally took 1-1.5 hours to complete.

Gonad culture conditions

The sample plate was incubated in an Okolab Electric CO₂ Microscope Sage Incubator (MSI) with a two-well plate adapter to maintain consistent thermal stability, minimize electromagnetic interference with the microscope devices, and ensure high reading and control accuracy. The in-chamber temperature was set to 25°C, and the lid temperature was set to 32°C. Humidity and CO₂ inputs were not used, though they may prove beneficial in future.

Imaging developing gonads

Sample was imaged using a Nikon A1 confocal microscope with Piezo Z Drive and Resonant scanner. Multiple gonads within a sample plate were selected as 3D-specified points (identified in the X, Y, and Z planes). The following settings were optimized during the course of the project: Perfect focus (PFS) was used to minimize focal drift during the extended time-lapse experiments. During data collection, line averaging was set to 16X, and integration was set to 2X. Pixel size was set to 0.31 μm, scan size was 512 (matching the width and height of 512 used for resonant scanning), Optical resolution was 0.21 μm, Z-step size was 1.30 μm, and Optical sectioning was 3.71 μm. The Z-plane was specified using “Symmetric mode defined by range,” such that the z-center of a gonad was specified and the relative range (top and bottom) were +18.00 and -18.00, respectively (total range: 36.00 μm). Z-plane step size was set to 4.800 μm, minimizing the total number of steps (9 steps in z-plane). Experiment duration was 12 hours with 4 hours intervals.

Object Identification

Slidebook 5.0 software was used to define objects using a “mask”, a two- or three- dimensional set of binary values with the same extent as the image to which it is assigned. Masks are also

referred to as regions of interest (ROIs), and allow for advanced selection of image data as well as image analysis. Masks can be created and edited manually or automatically through intensity thresholding techniques. Each mask was set to detect intensities in the TRITC range such that each cell nucleus (tagged with histone2AV-specific RFP) was identified as a single object.

Results

Efficient isolation of developing gonads

Live-cell imaging of the adult *Drosophila* testis has revealed considerable information about stem cell dynamics and differentiation (Sheng and Matunis, 2011). To assess whether live-cell imaging can be used to study the dynamics of testis development, we first set out to develop a protocol for the isolation of intact testes. Previous studies have been developed for the isolation of primordial germ cells (PGCs) from embryonic gonads for gene expression profiling (Shigenobu et al., 2006). This technique utilized embryo homogenization and centrifugation through a fine mesh, followed by Trypsinization and fluorescence activated cell sorting (FACS) to isolate pure PGC populations. To obtain intact gonads for culture and imaging, I subjected embryos and early first instar larvae (early-L1) to this protocol without inclusion of Trypsinization and FACS sorting. Specifically, homogenization was performed on embryos with newly coalesced gonads prior to formation of the GSC niche (13-17 hrs after egg laying (AEL)) and on older embryos/early-L1 larvae (19-25 hrs AEL) at the time of GSC niche formation. These age ranges were chosen with the intent that successful testis isolation at these times would permit imaging of GSC niche formation and of GSC dynamics and spermatogonial differentiation during testis culture.

As a first step to isolating testis for culture and imaging, I sought to determine the degree of homogenization necessary for efficient isolation of intact gonads. Over-shearing can cause damage to cells or the gonad, removal of associated tissues that promote development, and/or loss of sample due to extensive tissue breakdown, while insufficient homogenization can result in excess tissue that obscures imaging, a loss of sample during filtration (tissue caught in the cell strainer) and a number of other complications due to failure of successful isolation. Therefore, I subjected samples aged 13-17 hrs and 19-25 hrs AEL to 5, 7, or 9 strokes of a loose pestle. Sample was then centrifuged through a nylon mesh, re-suspended in tissue culture media, and the amount of sample obtained immediately after isolation ($t=0$) evaluated via confocal microscopy. Gonads were also examined to determine whether normal gonad morphology was retained after homogenization. For samples aged 13-17 hours, we expect to observe gonad morphology characteristic of gonads prior to GSC niche formation, with spherical GSCs (tagged with cell-surface GFP) intermingled with SGPs (tagged with histone2Av-specific RFP) (Figure 3a). For samples aged 19-25 hours, we looked for GSC niche formation in testes marked by presence of tightly clustered hub cells at the testis anterior (visualized as RFP-tagged somatic nuclei) as well as GSCs with elongated morphology associated with the hub (Figure 3b).

We found that, for embryos aged up to 17 hours, seven moderately brisk strokes of the pestle provide optimal isolation results, while sample aged over 17 hours require nine moderately brisk strokes of pestle for successful isolation. For samples up to 17 hours, seven strokes allow successful isolation of gonads, plated with some associated tissue that may promote development ($n=17$) (Figure 4). Five strokes led to reduced sample size (no or little sample found on plate immediately after isolation, or gonad imaging is obstructed by excess associated tissue), while nine strokes led to fewer gonads plated ($n=7$). In the latter case, the gonads present on the plate

also appeared to be damaged, as GSCs were not tightly coalesced, and some were separated from the main cluster of GSCs.

For gonads aged 19-25 hours AEL, five strokes of pestle led to severely reduced sample size (few or no gonads found on the plate immediately after isolation) and seven strokes of pestle resulted in few gonads on the plate (n=2) and drastic damage to gonads observed on the plate (Figure 4). Nine strokes of pestle permitted gonad isolation, and gonads with a clear niche were observed upon initial isolation (t=0). The results of these shearing experiments demonstrate that we can successfully isolate testes with normal morphology in different stages of development in *Drosophila* embryos.

Testis grow normally in tissue culture after isolation

Successful analysis of testis development *ex vivo* requires that isolated gonads remain viable over time. To test this, testes were imaged immediately after culture (t=0) and then again after 12 hrs. For gonads from 13-17 hr AEL embryos, we looked for evidence of a successful transition from gonad coalescence to formation of the GSC niche (Figure 5a). For samples aged 19-25 hours, we assessed for onset of spermatogonial differentiation in the testes posterior (Figure 5b). GSC niche formation in young, coalesced gonads was assessed via clear evidence of hub formation or presence of elongated GSCs around the hub as previously described. Presence of actively dividing GSCs was also assessed as an additional sign of niche functionality. If GSC niche formation and GSC division was not observed, we looked for the absence of GSC elongation and gonad collapse, both of which are suggestive of cell death. (Lack of GSC elongation may also indicate that the gonad is not a testis, so evidence of gonad collapse is the primary evidence of cell death.) Onset of spermatogonial differentiation was assessed via clear

evidence of gonialblast division away from the hub into 2-, 4-, 8-, or 16-cell groups due to incomplete cytokinesis of spermatogonia. Again, absence of differentiation and evidence of gonad collapse were noted as indicative of cell death.

We find that, in gonads aged 13-17 hr, 21% of testes with normal morphology after initial culture show signs of GSC niche formation. (n=62). Given that 50% of the gonads are likely female, we would ideally see hub formation 50% of the time. The reduced number of gonads showing GSC niche formation indicates that only a subset of isolated testes develop properly under the culture conditions tested. Despite this reduced viability, our data suggest that approximately half of the testes isolated from 13-17 hr embryos are capable of normal development for up to 12 hours in tissue culture.

In gonads aged 19-25 hr, with a fully formed GSC niche upon initial culture, 80% show clear signs of spermatogonial differentiation away from the hub 12 hours later (n = 30). This higher than expected percentage of gonads showing hub formation (50% of the isolated gonads should be female) suggests that developing ovaries from these embryos may show reduced viability in tissue culture. Alternatively, this result may be due to researcher bias for selecting testes that initially show hub morphology characteristic of the male testis niche. Despite these caveats, these data provide strong evidence for testis viability in tissue culture. The successful development of gonads over the 12 hr culture during these morphological shifts illustrates that our *ex vivo* methods and conditions can be successfully employed to assess gonad development during embryonic and early-larval gonad development.

Optimization of testis culture conditions

While developing conditions for successful testis culture, we considered a number of factors that could impact our capacity to successfully image developing testis *ex vivo* including: (1) growth substrate, (2) the potential for media evaporation, and (3) the amount of media used for tissue growth. The substrate on which samples are grown can dramatically influence cell behavior. Furthermore, a substrate that can anchor gonads and minimize movement has potential added benefit during data collection. Therefore, I compared testis development from samples aged 13- 20 h AEL, plated onto two different substrates: 1) a poly-D-lysine coated glass cover slip (n = 87) or 2) an uncoated glass cover slip (n = 63) (Figure 6). Testes were then imaged upon placement in culture and again after 12 hours. As described previously, testes grown on poly-D-lysine coated plates retain the hub, exhibit GSCs with elongated morphology, show evidence of cell division at the testis posterior and eventual spermatogonial differentiation, and retain viability for up to 12 hours. Testes grown on uncoated glass plates initially display a hub and elongated GSCs, but retain viability for a maximum of 8 hours in culture. Signs of cell death after this time include collapse of the hub and fading RFP expression. Thus, poly-D-lysine coated microwells appear to promote greater viability of cultured testes and was used for all subsequent experiments.

To reduce the potential for media evaporation, samples were plated into culture with a nylon membrane placed on top of the sample, and the edges of this membrane sealed with halocarbon 27 oil. However, the use of too little oil could permit media evaporation resulting in loss of culture, while too much oil could impair our capacity to image a sample. To determine the proper amount of halocarbon oil 27 required for successful testis culture, samples were plated under a nylon membrane then sealed with 20-, 30- or 40 μ l of halocarbon oil (Figure 7). Addition of only 20 μ l of halocarbon oil 27 is insufficient for successful culture; leading to media

evaporation and shorter viability of the gonads due to loss of a healthy growth environment (n=9). 30 μ l of halocarbon oil media supports gonad development, as gonads grown under these conditions in *ex vivo* culture show signs of viability – including hub formation, oriented GSC division, and early stages of spermatogonial differentiation – for up to 12 hours (n=100). In gonads sealed with 40 μ l of halocarbon oil 27, even the small excess confounded data collection by seeping into sample media, obscuring gonad clarity, and reducing the number of capture-ready data points. Therefore, we conclude that 30 μ l of halocarbon oil 27 should be used for the purposes of maintaining a healthy culture environment for gonad development for time-lapse experiments that have a duration lasting at least up to 12 hours.

Finally, we assessed the amount of culture media required for successful testis culture. Optimizing culture media volume was necessary to ensure developing gonads had sufficient media while remaining sealed under the nylon membrane. Gonads aged 12-24 hours were isolated and plated under nylon mesh with 40 μ L of halocarbon oil and either 35, 40, 55, or 65 μ l of tissue culture media, then observed over time for signs of viability (Figure 8). Gonads plated in 35 μ l culture media do not remain viable; showing germ cell aggregation and gonad collapse within the first 200 minutes of culture (n=7) (Figure 8a). At 40 μ l (n=72) and 55 μ l (n=4), gonad viability was observed for the full 12 hours (Figure 8b). Fluorescence also remains strong; a further sign of viability. However, using 55 μ l of media, I observed sample drift and rotation that can significantly confound data analysis (Figure 8c). The effects of drift become even more severe when media volume is increased to 65 μ l (n=15), such that the gonad is completely out of focal plane by t=200 minutes (Figure 8d). This makes assessment of the tissue culture impossible, and valuable data from sample is lost due to drift. Therefore, I concluded that 40 μ l of culture media is optimal for purposes of data collection and analysis.

These tests demonstrated that gonad viability in *ex vivo* culture is sensitive to particular culture conditions. Gonads plated on poly-D-lysine substrate, sealed from evaporation with 30 μ l halocarbon oil 27, and immersed in 40 μ l of culture media remained viable for 12 hours, while alternative conditions led to shorter or little viability over time.

Developing gonads can be imaged over time

While we were able to successfully culture testes for time periods sufficient to demonstrate normal development, analysis of cell dynamics during development requires more extensive sampling. Specifically, gonads must be imaged at regular time intervals with multiple image planes in order to track cell dynamics over space and time. As additional sampling can result in photo-bleaching and/or photo-toxicity, we first assessed whether testes cultures remain viable with regular sampling of a single image plane over-time. To do so, gonads cultured at 19-25 h AEL were collected and testes were imaged at 4-h intervals for up to 12 hours (Figure 9). These samples were observed for signs of initial GSC niche formation and cell viability as previously described. I find that cultured testes from embryos aged 19-25 h AEL show clear evidence of development and viability including: GSC division and spermatogonial differentiation in the testes posterior (Figure 9) (n = 96). Thus, imaging of testes cultures at regular intervals appears feasible.

We next sought to image developing testes in three-dimensional space over time (also referred to as 4D capture). Specifically, serial sections were collected from testes aged to 13-17 hrs and 19-25 hrs AEL over time using confocal microscopy, then analyzed for signs of successful development and viability. Multiple z-planes were collected from each sample over 12 hours with 4 hours intervals (Figure 10). I find that GSC niche formation is observed in 21%

of young embryonic testes initially exhibiting coalesced morphology (n=62) (Figure 10c). In older gonads, where the GSC niche morphology is clear at t=0, spermatogonial differentiation is observed during the 4-hr intervals over the total 12 hour duration, confirming viability of these gonads during 4D capture (n=24) (Figure 10d,e). These experiments demonstrate that 4D capture experiments can successfully be employed to evaluate development throughout the gonad during testis development.

Next, I evaluated how rigorous our data collection could be over multiple z-planes to maximize the resolution of data we collect from *ex vivo* gonads in 4D. In particular, we wanted to ascertain how much data in the z-plane we can collect while not diminishing the long-term viability of the gonad. I therefore, collected data under “mild” conditions (9 z-planes with planes 4.8 μm apart) and “harsh” conditions (31 sections with planes 1.3 μm apart). (Figure 11a,b), (Figure 11c,d). Harsher conditions led to tissues loss of viability somewhere between 4 to 8 hours, as indicated by gonad collapse, hub disruption, and cell death during this time period (Figure 11c,d). Larger step size allows tissue to remain viable for a longer period (up to 12 hours) without losing markers of cell growth and development (instances of division, differentiation, migration). Thus, to minimize phototoxic effects, a step size of 4.800 μm (9 steps) was used for my subsequent experiments. It should be noted that future users will need to select a number of z-sections based on whether long-term viability or cell tracking is the research priority. If a study requires maximal viability for extended-time capture, mild conditions should be used. If performing cell tracking, “harsh” conditions may be used, but limited viability (up to ~6-7 hours) should be expected.

The collection of data from gonads over multiple imaging planes will allow us to evaluate, through examination and software cell tracking, the development of full gonads in 4D during testis development.

Object identification in developing gonads

Cell tracking during gonad morphogenesis is critical to understanding changes in cell fate and morphology that direct the process of gonad formation. Software with the capacity to track the movement of objects over space and time (4D) is dependent on the how accurately each object can be identified. As a first step to determining whether cell tracking is feasible during gonad development *ex vivo*, we initiated a pilot study to assess whether different cells in the developing gonad could be digitally identified as separate entities. Images of developing gonads with individual cell nuclei marked by expression of the Histone2::RFP fusion protein were imported into the Slidebook 5.0 imaging software and data subjected to image “masking” to convert each marked nucleus into a digital object (see appendix for details). As nuclei in developing gonads are greater than 2 μm in diameter (SGP nuclei $\sim 2 \mu\text{m}$; germ cell nuclei $\sim 5 \mu\text{m}$) and are identifiable in our images by RFP expression above background levels, size and image intensity were used as masking parameters. Specifically, the RFP channel from multiple image planes from a single gonad was subjected to masking at a range of pixel intensities and minimum object size of gated voxels. The number of objects identified digitally after masking was then compared to the number objects counted manually by an experimenter. Through a series of such comparisons, I found that the optimal pixel intensity range is 1400-4080, and the optimal minimal size gate is 10 voxels. Under these conditions, I find that object counts are consistent between investigators visual count and the computed count conducted by the

Slidebook 5.0 software (n=3 gonads examined) (Figure 12). Thus, we have successfully identified relevant objects (RFP-tagged cell nuclei), and are one step closer toward successful object tracking over time.

Image clarity optimization

Imaging developing gonads over time *ex vivo* requires a fine balance of image optimization settings that minimize phototoxic effects and maximize image clarity. The power of successful live-cell imaging lies in the ability of the investigator to analyze images after data collection and to draw representative conclusions. For the purposes of this project, and our goal to eventually employ cell-tracking software to trace cell lineages and map cell fates, clarity of the images is paramount. A number of factors impact the clarity of an image, including (1) confocal laser power, (2) lens objective and digital magnification (zoom), and (3) the use of perfect focus. Overly bright or laser-bleached images may not only cause damage to tissues, but may make it so that data collected from still viable gonads fails to be useful for image analysis due to unrefined image capture. The latter can also be true of images that are too dim, and our ability to successfully analyze the data collected from live-cell imaging is fundamental to its successful utility.

To optimize laser power, gonads were imaged under different laser power settings in gonads until the offset, laser power, and laser intensity allowed for clear image capture for all or most gonads. I tested three settings: (1) FITC: HV=93, Offset= -46, 488 (laser)=21.90; TRITC: HV=71, Offset= -74, 561(laser)=16.65; (2) FITC: HV = 97; Offset = -94; 488 (laser) = 14.14; TRITC: HV = 75; Offset = -74; 561(laser) = 10.50; and (3) FITC: HV=187, Offset= -46, 488(laser)=21.90; TRITC: HV=120, Offset= -74, 561(laser)=26.63. Setting 2 resulted in the

clearest images over time and permitted cell viability for 12 hours were (FITC: HV = 97; Offset = -94; 488 (laser) = 14.14 and for TRITC: HV = 75; Offset = -74; 561(laser) = 10.50), with setting 1 being too dim for clear capture, and setting 3 being too bright and causing photo-bleaching. These settings were recorded and reused for subsequent experiments (Figure 13). Furthermore, data collected from developing gonads under these setting 2 imaging conditions can be analyzed when tracking software tutorials are available.

The resolution of images is affected by the lens objective used to collect imaging data. Oil and water immersion lenses should not be employed during multi-point data acquisition. Therefore, I selected lens objective and digital magnification (zoom) settings by running image experiments with 10X, 20X, and 40X dry lenses (Figure 14). The working distance for both the 10X and 20X objectives are safe for multi-point and multi-plate acquisition without danger of the objective colliding with the stage or sample plate when acquisition mode is engaged. The 10X lens permits clear identification of GFP labeled gonads, but some data on cellular dynamics such as migration, interaction, and division may be lost due to lack of resolution. Under this lens objective, software magnification functionality exacerbates the loss of data due to low resolution. The 20X lens, on the other hand, presents images with greater resolution, and data remains clear (at an effective resolution) with 3X digital magnification. In fact, with 3X zoom, relevant details such as distinct RFP-tagged Histone2Av in nuclei and cell dynamics, such as divisions and differentiation, can be clearly observed. Though we have only tested the 40X images for object tracking at this time, there is realistic potential to use these magnified 20X images for image analysis with Slidebook 5.0 object identification (masking) software. The working distance for the 40X lens is in close proximity to the stage, and may cause mechanical hardware issues for multipoint (or multi-plate) acquisition. Therefore, while the 40X lens may be better for image

resolution, it is not ideal for my general purposes of data collection. Together, these lens objective tests demonstrated that the 20X lens objective with 3X digital magnification is the best option for collecting data during testis development, and would likely be my best option for cell tracking when appropriate tutorials for the Nikon tracking software module become available.

Finally, I tested the need for using the perfect focus (PFS) to prevent microscope drift. PFS finds and measures the distance between the cover slip of the sample petri dish to the software-selected focal plane of the sample. This is meant to minimize focal drift of the microscope during extended time-lapse experiments. However, PFS takes time to calibrate and set before an image is collected from each selected gonad. If the use of PFS is superfluous, more data points can be collected without risk of time loops overlapping and shutting down the software during data collection. With PFS, longer time loops and fewer points must be selected for a successful time-lapse experiment. To determine whether PFS is necessary for data collection, I ran tests runs of gonads over time with PFS on or off. Experiments were conducted either in single plane or, to determine the degree of drift, as 3D capture. Points were selected in the center of each gonad, gonads imaged over a short time intervals and examined for evidence of focal drift. Without PFS, significant focal drift was observed even over short periods and sample was lost from the focal plane (Figure 15a). Using perfect focus, however, each gonad consistently remained in virtually the same focal plane (Figure 15b). Similar results were observed with data collected from 3D capture with PFS on, and therefore there was no need to assess the degree of drift (Figure 15c). Based on these data, it can be concluded that PFS is necessary for successful, consistent imaging of gonads over time.

By optimizing the laser power settings, lens objective and digital magnification, and using perfect focus for our imaging data collection, we are not only able to observe cellular

phenomena over the course of gonad development, but we hope to advance our research in successful cell tracking through the refinement of these imaging parameters.

Discussion

My data demonstrate that live-cell imaging is a feasible means of studying development of the *Drosophila* testis system. Isolation of viable gonads from young embryos to early larvae is possible, and data indicate that successful gonad development occurs through key developmental stages *ex vivo*. These key stages include major morphological shifts, such as that from initial gonad coalescence to GSC niche formation, as well as from full GSC niche formation through spermatogonial differentiation. Indeed, data strongly indicate that germ cells in cultured testes undergo asymmetric GSC division and spermatogonial differentiation.

In order to permit imaging of testes development *ex vivo*, my data also show there are a number of key factors to consider. Indeed, care must be taken to avoid oversampling. Imaging of developing gonads is feasible over time either in a single-plane or in 3D. However, when imaging in 3D, minimizing the number of z-planes collected (for my experiments, nine z-planes per gonad) ensures gonad viability over 12 hours of development when data is collected in 4-hr intervals. However, it may be true that viability is retained with a slight increase in the number of sampling planes. As such, further optimization of multi-plane sampling may prove necessary for future studies. Additionally, optimizing tissue culture and imaging clarity to accommodate development is imperative. Both of these factors are essential to the next step of live-cell imaging analysis: cell tracking. At this time, we have successfully identified objects (cell nuclei) using object masking software in Slidebook 5.0. However, 3D tracking software recently developed by Nikon may provide a more robust platform for cell tracking.

Refinement of testis culture conditions

Optimizing *ex vivo* testis culture to promote cell viability, growth, and development is necessary to producing conditions that will allow proper gonad development. At this time, I have identified conditions that demonstrate viability through two key stages of testis development. However, more work must be done to increase our rate of successful culture and imaging, because only 21% of gonads in the younger age range (developing from coalescence through hub formation) exhibit time-appropriate morphological markers of this key developmental shift. The culture media selected for our purposes included 85% Schneider's Insect Medium, 15% FBS, with the addition of 5.5µl Pen/Strep solution per ml. I found that the poly-D-lysine plate substrate, 30 µl halocarbon oil 27, and 40µl culture media all enhanced culture viability, with alternatives being less successful for tissue survival and/or for data analysis purposes.

Other conditions to consider are temperature, humidity, and CO₂ concentration. Imaging of cultured gonads was conducted in a humidity- and temperature-control chamber. Temperature for the chamber was set to 25°C in-chamber, with a 31°C lid temperature. Interestingly, if the temperature control remained off, gonads could be successfully cultured at ambient room temperature (data not shown). However, the temperature-controlled chamber allows an added degree of thermal consistency. Therefore, I recommend using temperature control at the aforementioned settings. Conversely, humidity control did not produce consistent humidification over time. As such, no extra humidity control is recommended. Finally, we did not alter CO₂ concentration in the imaging chamber. However, many types of tissue culture are grown at 5% atmospheric CO₂ to retain long-term viability. Therefore, future studies may consider manipulation of CO₂ levels in the growth chamber. By refining these, and other growth

conditions, greater culture viability may be achieved, eventually allowing the application cell tracking during gonad development from very early embryonic stages through adult testis formation.

Collecting biologically relevant data

Collecting biologically relevant data for this research entails collecting data from full gonads to capture details of developing tissue dynamics and increasing the number of imaging data points to observe these dynamics in real time throughout development. One way in which we optimized data collection was to vary the number of z-planes we collected to gain greater 3D resolution of viable, developing gonads. As previously discussed, these imaging tests indicated that of the “harsh” and “mild” sampling condition, the mild conditions (nine z-plane sections) both allows us to observe key stages of gonad development and promotes gonad viability over 12 hours. The harsher conditions, on the other hand, increase the 3D resolution of the gonad over time, but lead to reduced cell viability (viability lost somewhere between 4-8 hours). However, it is important to note that 9 z-planes is the minimum possible number of z-planes that can be collected for the z-range chosen (36 μm). Further optimization may demonstrate that a greater number of z-planes also permits tissue development over time; the increased number of z-planes would allow better 3D data resolution, in which case more information can be extracted from each gonad.

Another consideration for collecting biologically relevant data is decreasing the time interval between points of imaging data collection. At present, I have imaged gonad development at 4-hr intervals for a 12-hr duration. As the process of testis formation is highly dynamic, it may be necessary to decrease this time-interval in order to obtain relevant data about changes in cell

location, differentiation and the morphogenic changes that accompany these processes. Further, once we can observe consistent wild-type morphological development in live-cell imaging *ex vivo*, we can compare mutant morphological development to that data.

Optimizing image clarity

Collecting clearer images for the purposes of data analysis will be even more imperative as we move forward in this line of live-cell imaging studies. As imaging analytic tools become more available, the quality and resolution of our images will need to be optimized for the purposes of using newer, different object identification and cell tracking software than was available during the course of my thesis. As such, it is important to consider the software-based imaging processes that provide cleaner image quality at higher resolution. Line-averaging and integration are two examples of such software imaging processes, and both require longer laser exposure to that end. At present, our experiments are conducted with 16X line-averaging and 2X integration. Manipulating these controls to best suit the needs of future researchers and research software, including our new Nikon cell tracking software, will be paramount to the success of live-cell imaging data analysis.

Future Directions

As discussed in previous sections, several important studies must be done to confirm some of my data and to answer questions produced by new data. The experiments focused on culturing and imaging gonads developing from coalescence to GSC niche formation are necessary. Increasing the power of our data through further experimentation will lead to stronger conclusions.

Extending the length of time gonad culture remain viable could also allow for more questions to be answered. Observing real-time development will allow for our greater understanding of stem cell progenitor specification and development, and clearer understanding of the effects of manipulation (i.e. knockout mutations). Studies to investigate the importance of associated tissues *ex vivo* may prove to be especially valuable for extending the duration of viability of gonad cultures. Additionally, insulin has been used to extend viability of adult *Drosophila* ovary cultures while performing live-cell imaging (Prasad et al., 2007). Thus, addition of insulin to developing gonad cultures may also prove fruitful.

At present, we have conducted initial tests of object identification in multiple z-sections of a single gonad. Our next step is to confirm whether we can do so consistently in gonads at various stages of development. This requires a protocol to identify the optimal intensity range to “mask” or identify an object. This process is called “thresholding,” and the “objects” in our experiments with Moesin::GFP; Histone2Av::RFP gonads are the RFP-tagged cell nuclei. The success of thresholding protocols varies from software to software. Therefore, employing software that is compatible with our image file types and has the capacity for both successful object identification and cell tracking over time would be our best next step. The protocol for cell tracking will require training in the software, and optimization of software manipulation for particular experiments in tracking development. Through these experiments, we hope to shed new light on *Drosophila* testis development and other stem cell systems and developmental systems. In particular, we hope to better understand organogenesis, and the specification, establishment, and maintenance of stem cell progenitors and progeny throughout development.

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References

- de Cuevas, M., Matunis, EL (2011). The stem cell niche: Lessons from *Drosophila* testis. *Development* 138, 2861-2869.
- Gilbert, S.F. (2003). *The Saga of the Germline* (Sunderland: Sinauer Associates, Inc).
- Jemc, J.C. (2011). Somatic gonadal cells: the supporting cast for the germline. *Genesis* 49, 753-775.
- Kaufman, D.S. (2009). Toward clinical therapies using hematopoietic cells derived from human pluripotent stem cells. *Blood* 114, 3513-3523.

- Okegbe, T.C., and DiNardo, S. (2011). The endoderm specifies the mesodermal niche for the germline in *Drosophila* via Delta-Notch signaling. *Development* 138, 1259-1267.
- Orkin, S.H., and Zon, L.I. (2008). Hematopoiesis: an evolving paradigm for stem cell biology. *Cell* 132, 631-644.
- Prasad, M., et al. (2007). A protocol for culturing *Drosophila melanogaster* stage 9 egg chambers for live imaging. *Nat Protoc* 2(10), 2467-2473.
- Sheng, X.R., and Matunis, E. (2011). Live imaging of the *Drosophila* spermatogonial stem cell niche reveals novel mechanisms regulating germline stem cell output. *Development*.
- Sheng, X.R., Posenau, T., Gumulak-Smith, J.J., Matunis, E., Van Doren, M., and Wawersik, M. (2009). Jak-STAT regulation of male germline stem cell establishment during *Drosophila* embryogenesis. *Dev Biol* 334, 335-344.
- Shigenobu, S., Arita, K., Kitadate, Y., Noda, C., and Kobayashi, S. (2006). Isolation of germline cells from *Drosophila* embryos by flow cytometry. *Dev Growth Differ* 48, 49-57.
- Shim, J., Gururaja-Rao, S., and Banerjee, U. (2013). Nutritional regulation of stem and progenitor cells in *Drosophila*. *Development* 140, 4647-4656.
- Sinden, D., Badgett, M., Fry, J., Jones, T., Palmen, R., Sheng, X., Simmons, A., Matunis, E., and Wawersik, M. (2012). Jak-STAT regulation of cyst stem cell development in the *Drosophila* testis. *Dev Biol* 372, 5-16.
- Slack, J.M. (2008). Origin of stem cells in organogenesis. *Science* 322, 1498-1501.
- Spradling, A., Drummond-Barbosa, D., and Kai, T. (2001). Stem cells find their niche. *Nature* 414, 98-104.
- Zaret, K.S., and Grompe, M. (2008). Generation and regeneration of cells of the liver and pancreas. *Science* 322, 1490-1494.

Figures

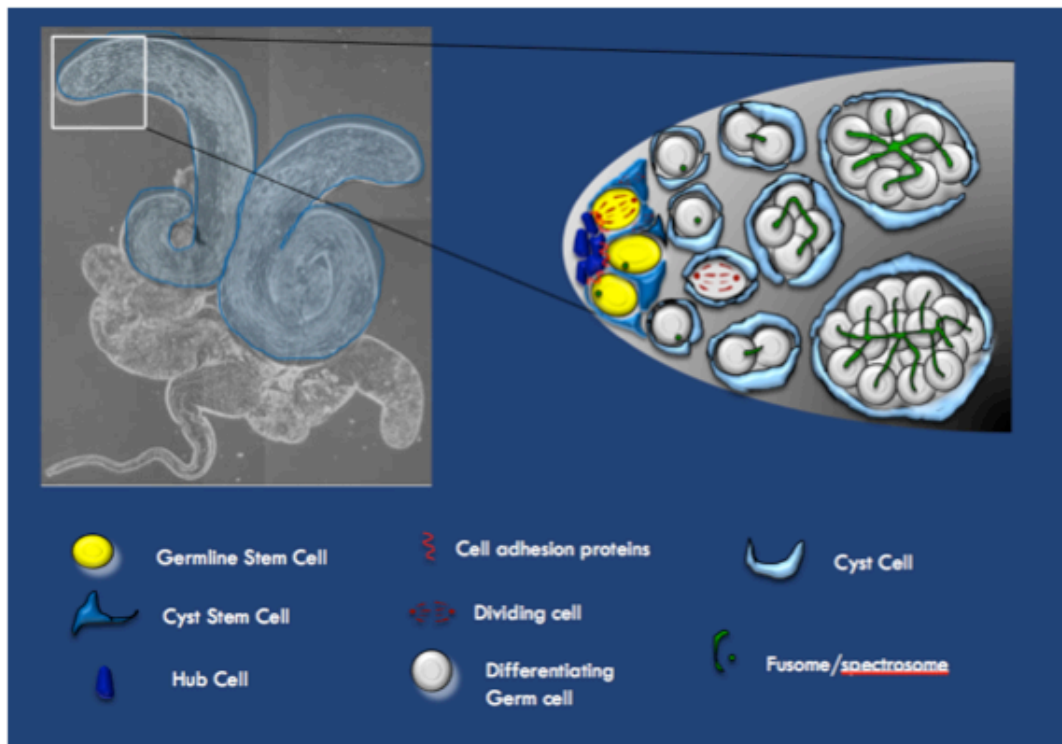


Figure 1 – The adult *Drosophila* testis stem cell niche

In adult *Drosophila* testis, the GSC niche resides at the blind end of the testis coil. 6-8 germline stem cells (GSCs) are arrayed and maintained around a cluster of cells at the testes apex, called the hub. Cyst stem cells (CySCs) inter-digitate the GSCs in order to contact the hub. GSCs undergo asymmetric divisions, self-replicating and differentiating away from the hub. Cyst cells, derived from asymmetric division of CySCs, support germ cells as they divide and differentiate away from the hub; germ cells divide via incomplete cytokinesis into 16-cell spermatogonia clusters enveloped by cyst cells. Spermatogonia then enter meiosis, creating 64 sperm individualized sperm per 16-cell spermatogonial cluster.

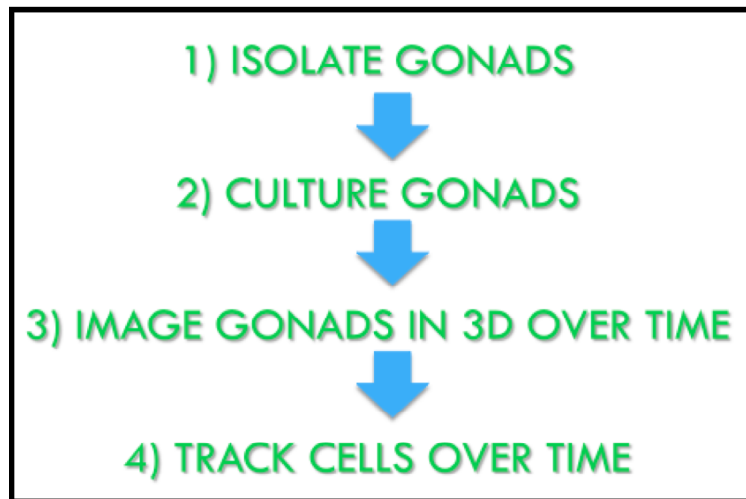


Figure 2 – Thesis objectives for successful live-cell imaging of developing *Drosophila testis*
Live-cell imaging has not been done in the developing *Drosophila testis* before. This schematic illustrates the steps that must be successfully completed and optimized in order for live-cell imaging to be employed to study the developing *Drosophila testis*.

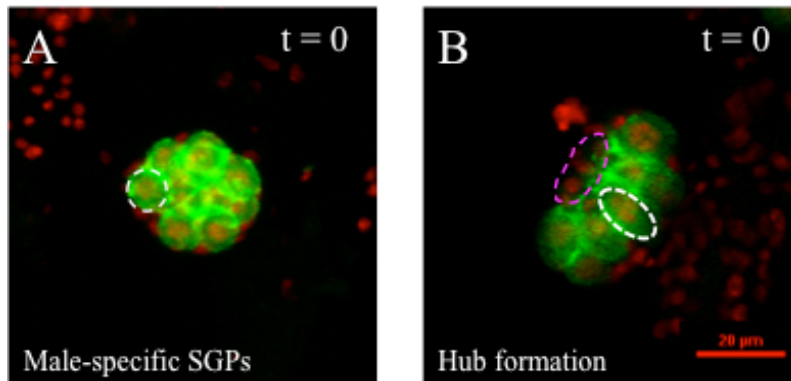


Figure 3 - Gonads retain normal morphology after isolation from different developmental stages: Testes from embryos with germ cells expressing a cell-surface Moesin::GFP fusion protein (green) and all cells expressing a nuclear Histone2Av::RFP fusion protein (red) isolated then immediately imaged. (A) Sample from embryos 11.5-14.5 hrs AEL show a tightly coalesced morphology similar to that observed *in vivo*. (B) Sample from 19-25 hrs AEL embryos show a clear hub (magenta outline, notice nuclei of hub cells in red) as well as presence of elongated GSC around the hub (white outline). Testes anterior oriented left.

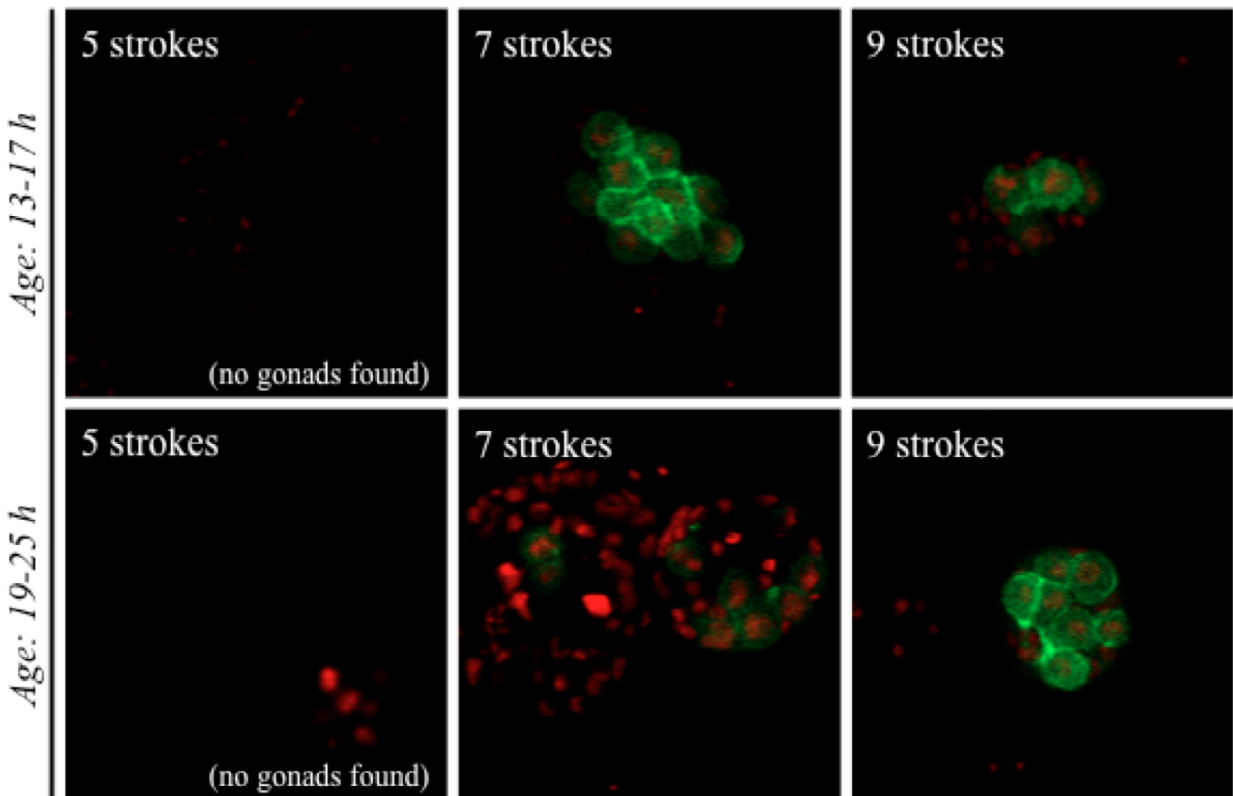
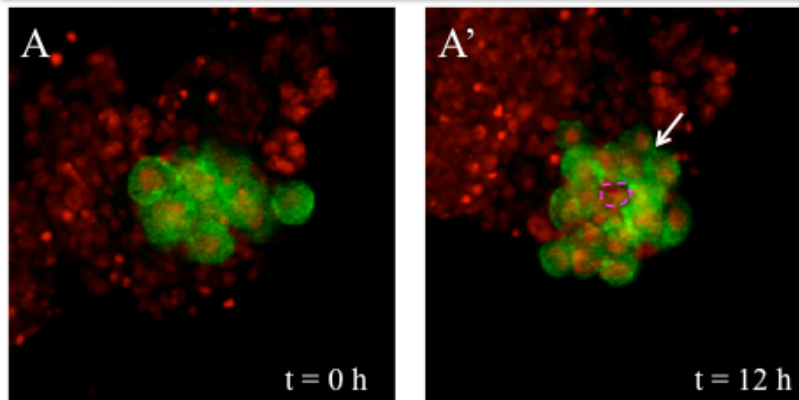


Figure 4 – Number of pestle strokes for isolation differs based on sample age

For gonads aged to 13-17 h AEL before isolation, homogenization via (1) 5 strokes of pestle is insufficient for isolation, and no gonads were observed on the plate, (2) 7 strokes of pestle yields the greatest number of whole gonads, and (3) 9 strokes of pestle yields few to no gonads, and those observed on the plate are damaged (few germ cells or signs of collapse observed). For gonads aged 19-25 h AEL before isolation, homogenization via (1) 5 strokes of pestle was insufficient for isolation, and no gonads were observed on the plate, (2) 7 strokes of pestle yields few to no gonads, those observed on the plate are damaged (few germ cells or signs of collapse observed), and excess tissue obscures imaging, and (3) 9 strokes of pestle yields the greatest number of whole gonads.

Sample age: ~15 h



Sample age: ~23 h

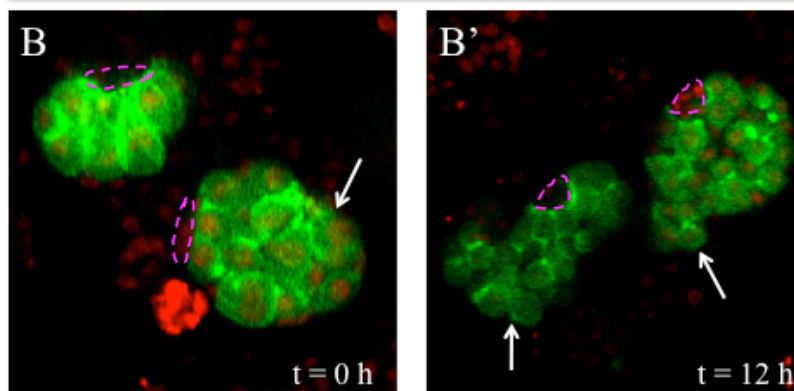


Figure 5 – Cultured testes undergo key developmental events

Cultured testes isolated from *Moesin::GFP; Histone2Av::RFP* gonads aged ~15 hrs (A,A') or ~23 hrs (B,B'). Images show the same testis immediately upon placement into culture (A,B; t=0) or after 12 hours culture (A',B'; t=12). The hub (outlined) and GSCs with elongated morphology (arrow) form in cultured testes isolated from embryos ~15 hr AEL (A,A'). Spermatogonial differentiation (arrow) is observed in cultured testes isolated from ~23 hr embryos (B,B'). Hub morphology (outlined) and elongated GSCs are retained in these later cultures.

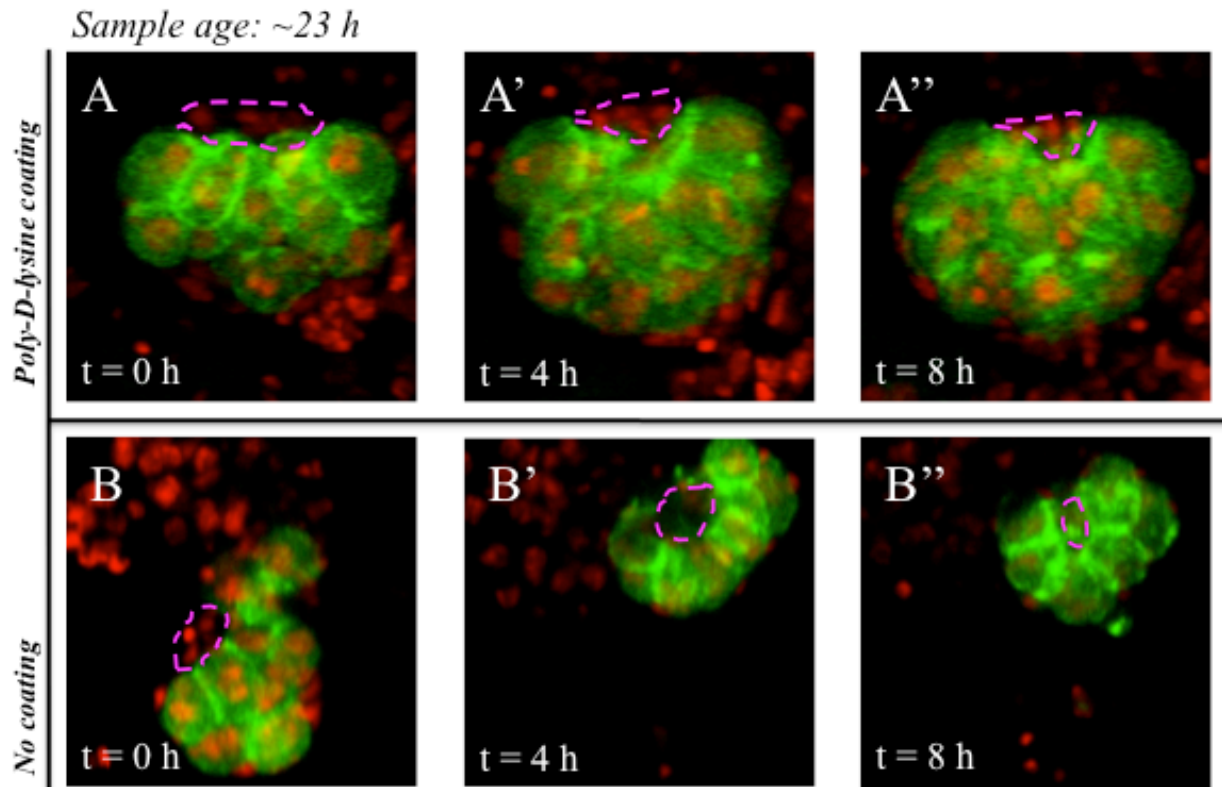


Figure 6 - *Poly-D-lysine extends gonad viability*

Testes from ~23 hr Moesin::GFP; Histone2Av::RFP embryos plated on either (A) poly-D-lysine or (B) an uncoated glass coverslip. Images show the same testis immediately upon placement into culture (A, B; t=0) and after 4 or 8 hours culture (A', B'; t=4 and A'', B'', t=8, respectively). (A-A'') Testes grown on poly-D-lysine coated plates retain the hub (outlined), GSCs with elongated morphology and show evidence of cell division (more cells present) at the testis posterior (arrow). (B-B'') Testes grown on uncoated glass initially display a hub and elongated GSCs, but lose viability after 4-8 hrs culture as observed by faded RFP expression and collapse of the hub (as shown outlined in the three panels).

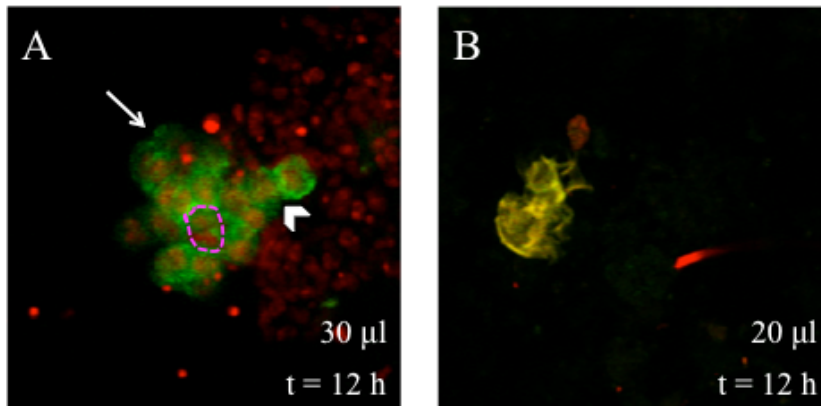
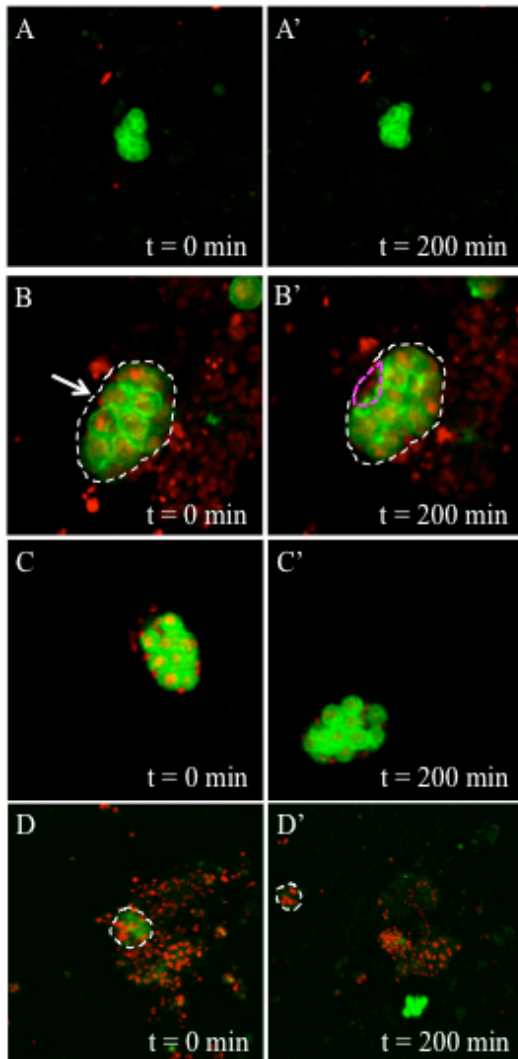


Figure 7 - Halocarbon 27 oil is most effective at volume of 30 μ l

Testes from Moesin::GFP; Histone2Av::RFP embryos plated and sealed with various volumes of halocarbon 27 oil. (A) Testis from ~15 hr embryo sealed with 30 μ L halocarbon oil and cultured 12 hours showing evidence of viability including hub formation (circled), oriented GSC division (arrow head), and early stages of spermatogonial differentiation (arrow). (B) Testis from ~15 hr embryo not visible due to dehydration of sample 12 hours after initial isolation. Gonads sealed with higher volumes of halocarbon oil 27 not pictured above because higher volumes obscure data visibility.



Age range: 12-24 hrs, before isolation

Plating volume	Culture Results
35 µl	Dried up; dead
40 µl	Stable position; viable
55 µl	More sample drift (rotation); viable
65 µl	Sample drift too drastic to observe viability

Figure 8 - Optimal media volume for tissue culture is 40µl

Testes from 12-24 hr Moesin::GFP; Histone2Av::RFP embryos plated with differing volumes of culture media. Images show the same testis immediately upon placement into culture (t=0) and after 200 minutes culture (t=200). (A,A') 35µl plating volume: Initially healthy gonad (A) shows signs of collapse (cell death) 200 min after initial isolation (A'). Minimal sample drift is observed at this volume. (B,B') 40µl plating volume: Testes pre-hub formation (B) shows signs of viability and clear signs of development including hub formation (outlined) and elongated GSCs after 200 min (B'). Virtually no sample drift is observed. (C,C') 55µl plating volume: Cultured testes shows signs of viability including maintenance of shape, fluorescent expression, and cell division (arrow) over time. However, sample drift and rotation was observed. (D,D') 65µl plating volume: Healthy, coalesced gonad centered in focal plane drifts significantly to the left and out of initial plane after 200 min. Evaluation of viability not possible at this high volume. Results summarized in table.

Sample age: ~23 h

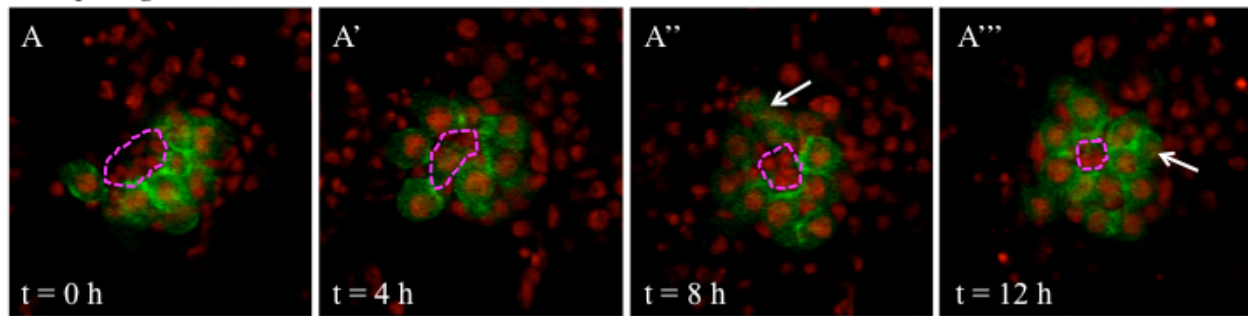
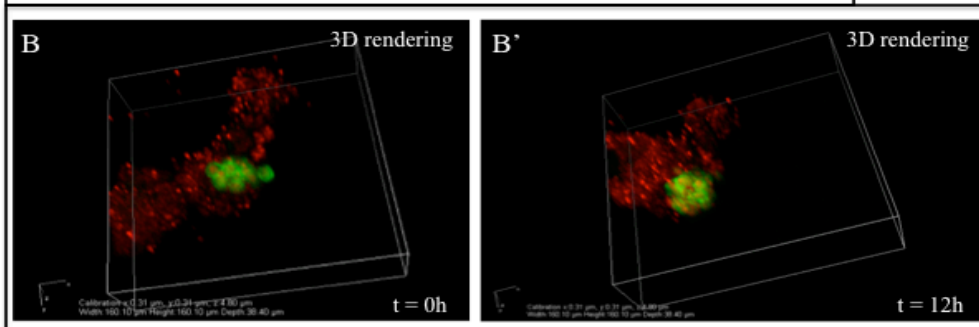
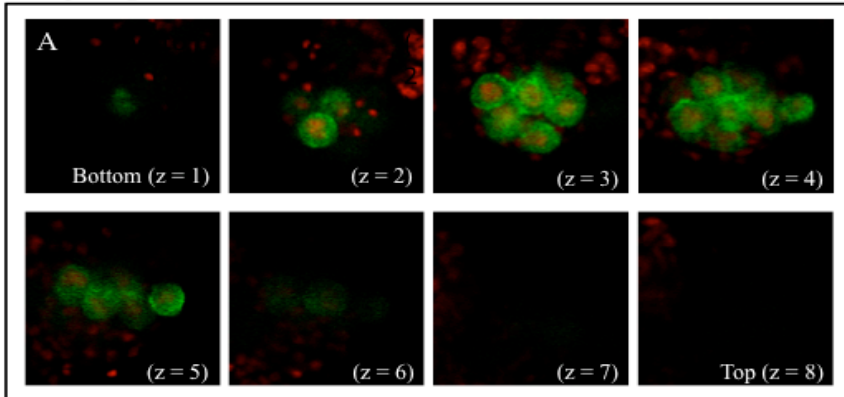


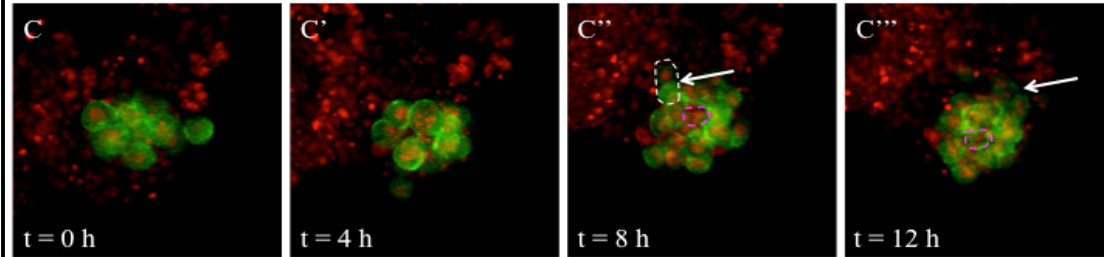
Figure 9 - Cultured testes can be imaged over time in a single plane

Testis from Moesin::GFP; Histone2Av::RFP embryo (aged to ~23 hr AEL) imaged in a single plane at 4 hour intervals for 12 hrs. A coalesced hub (outlined) and elongated GSCs are observed at the start of culture (A; t=0) and there is clear evidence of testis development over time including: cell division and spermatogonial differentiation away from the hub (arrows in A'' and A''', respectively).

Sample age: ~15 h



Sample age: ~15 h



Sample age: ~23 h

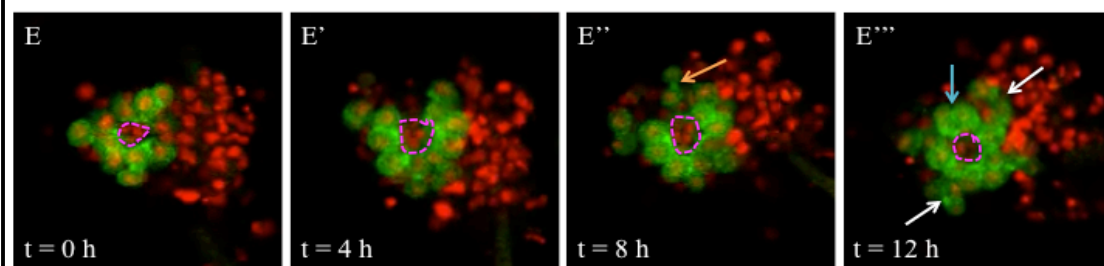
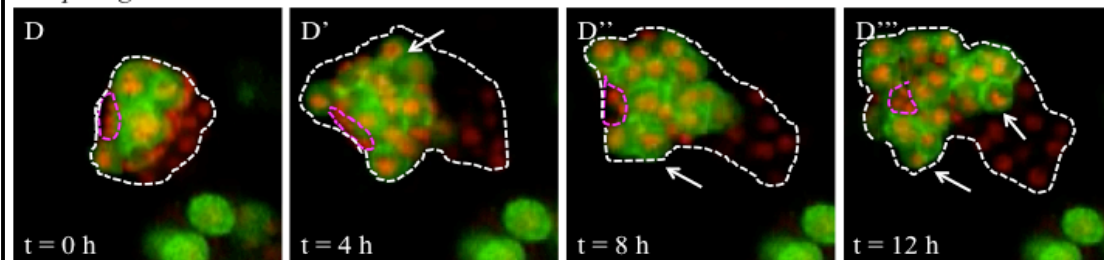


Figure 10 - Gonads cultured *ex vivo* can be imaged in 4D

Cultured testes isolated from Moesin::GFP; Histone2Av::RFP gonads aged ~15 hrs (A,B,C) or ~23 hrs (D,E) can be imaged in 4D (3D over time). (A) Images of individual z-sections at time point immediately after isolation (t=0) from embryos aged ~15 hr. (B) Software manipulation of 4D data produces a “3D rendering” image of the gonad, synthesized from the 9 z-sections collected. Shown are 3D rendering captures of a ~15 h gonad immediately after isolation (t=0) and after 12 hours (t=12). Clear evidence of hub formation by t=12 (outlined, B’). (C-E) Images of testes immediately upon placement into culture (C,D,E: t=0) and after 4, 8, and 12 hours of culture (C’,D’,E’: t=4; C’’,D’’,E’’: t=8; C’’’,D’’’,E’’’: t=12, respectively). ~15 hr gonad imaged in 4D shows morphological development from gonad coalescence to hub formation (outlined in magenta) and early spermatogonial differentiation (arrows) (C-C’’’). ~23 hr gonad and associated tissue (outlined) with a clear hub (outlined in magenta) at t=0 after isolation, exhibits spermatogonial differentiation (white arrows), as well as asymmetrical (orange arrow) and symmetrical (blue arrow) division over 12 hours (t=12) (D-D’’’, E-E’’’).

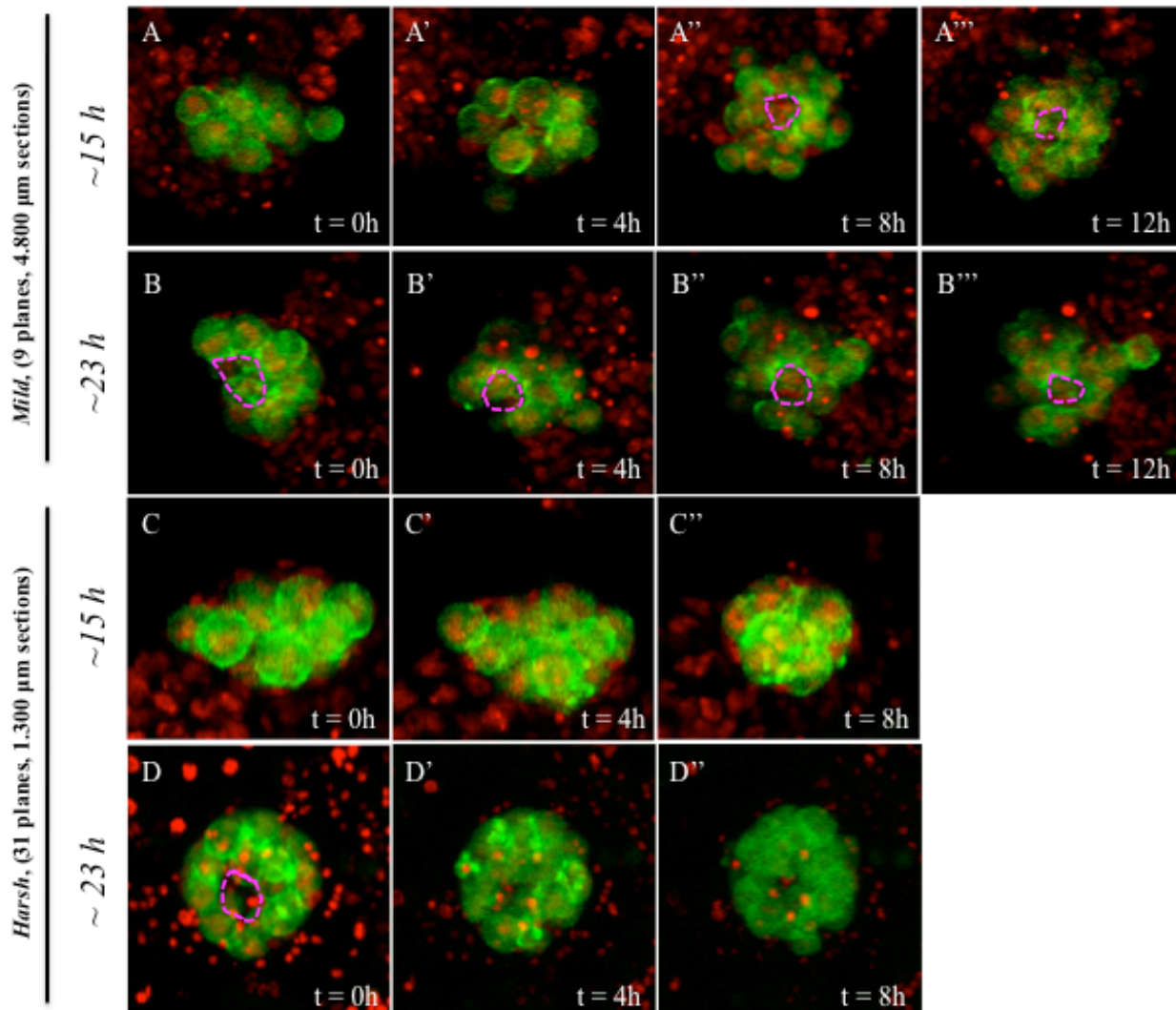


Figure 11 *The number of z-sections recorded affects gonad viability over time*

Testes from Moesin::GFP; Histone2Av::RFP embryos imaged under either “mild” (nine 4.800 μm z-sections) (A-B), or “harsh” conditions (thirty-one 1.300 μm z-sections) (C-D). Images show the same testis immediately upon placement into culture (A,B,C,D: t=0) and after 4, 8, and 12 hours after initial culture (A',B',C',D': t=4; A'',B'',C'',D'': t=8; A''',B''',C''',D''': t=12, respectively). Under mild conditions, testes from ~15 hr or ~23 hr embryos show signs of viability. In the younger testis (A-A'') morphology shifts from gonad coalescence (A) to hub formation (outlined) and spermatogonial differentiation (arrow)(A''-A''') are observed. In the older testis (~23 h), a hub is clearly formed at t=0 (outlined, B), and spermatogonial differentiation occurs over time (arrows, B'-B'''). Under harsh conditions, testes from ~15 hr or ~23 hr embryos show signs of tissue death over 12 hours. (C-C'') Testis from ~15 hr embryo, shows hub formation and presence of elongated GSCs after 4 hrs in culture (C'), but gonad collapse and cell death occur by 8 hours (C''). (D-D'') Testis from ~23 hr embryo showing a normal GSC niche upon initial culture (D), but loss of viability marked by reduced fluorescence and gonad collapse after 4-8 hours if culture (D',D'').

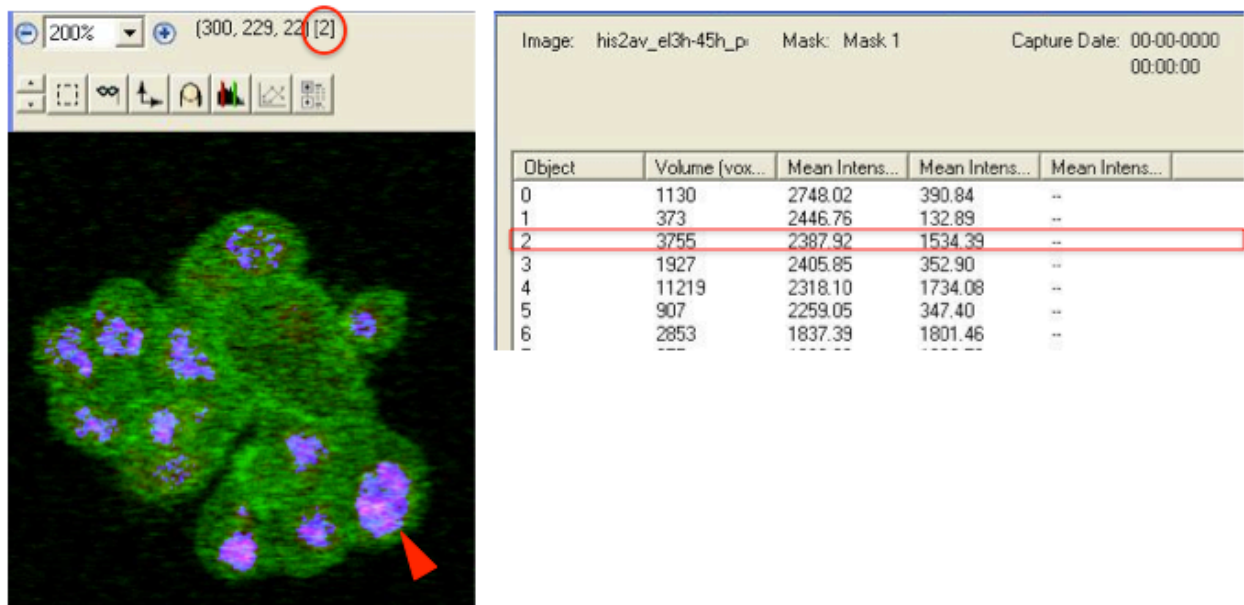


Figure 12 – Object Identification in Slidebook 5.0

Objects are identified through masking in the TRITC range, with the optimal intensity range to 1400-4080. Each object (i.e. nucleus indicated by the red arrowhead) is identified by the software and assigned a number (red circle), and associated with its X, Y, and Z coordinates (numbers to the left of red circle). Statistics on each object can be calculated by the software calculation and displayed in a statistics window (statistics for Object 2 outlined to the right in the red rectangle). Software counts match investigator counts.

Sample Ages: 13-46 h

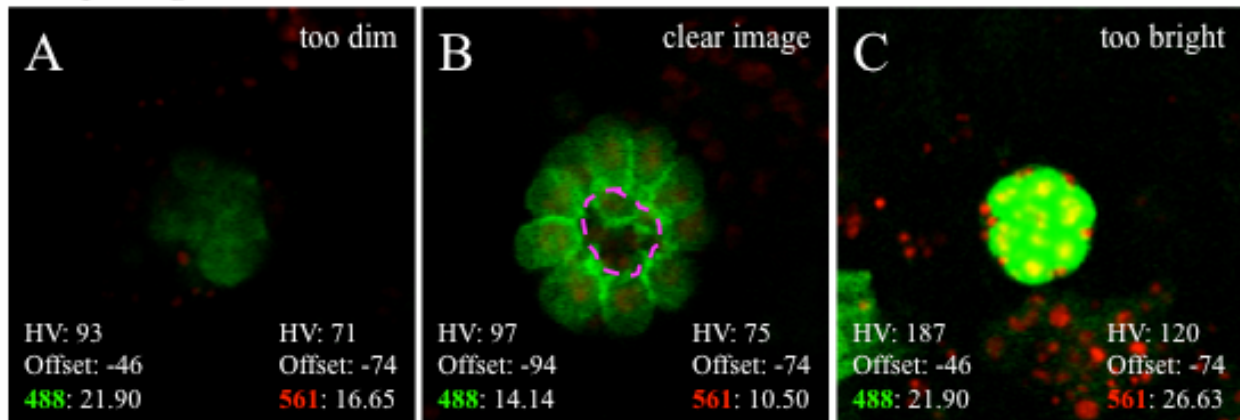


Figure 13 - Laser power settings must be optimized for effective data collection and analysis
Tests from Moesin::GFP; Histone2Av::RFP embryos under different laser power settings. (A) Gonad is too dim to properly observe cell dynamics or use software tools to manipulate the image and/or identify objects. (B) Gonad from 13-46 hrs with hub (outlined in magenta) shows clear morphology. Good example of image to be manipulated and/or used for object identification and eventual cell tracking. (C) Gonad morphology washed out due to laser power settings being too strong. Cannot use software manipulations to effectively analyze data.

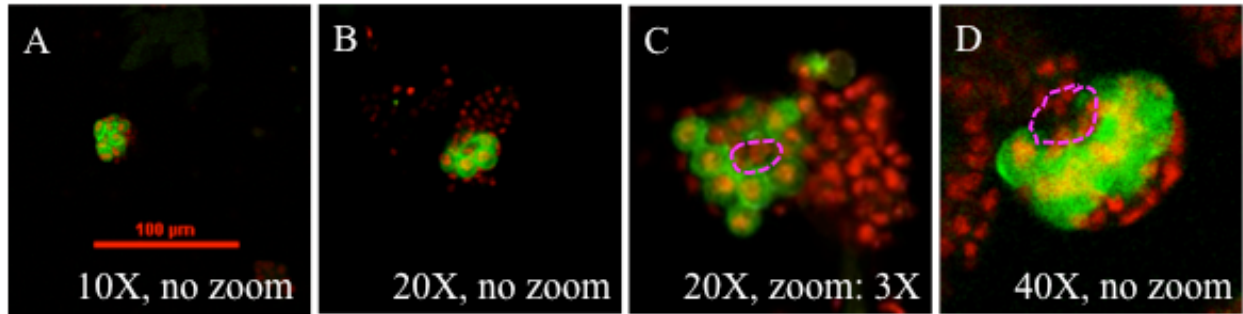


Figure 14 - 20X lens objective with 3X digital magnification is optimal for data collection

Tests from Moesin::GFP; Histone2Av::RFP embryos under different lens objective settings and varying degrees of digital magnification (zoom). (A) Gonad imaged under 10X objective is too small to properly observe cell dynamics or use software tools to manipulate the image and/or identify objects. (B) Gonad imaged under 20X without digital magnification shows clear morphology (hub outlined), but may still be difficult to identify relevant objects for future cell tracking. (C) Gonad imaged under 20X with 3X digital magnification shows clear hub morphology (outlined) with distinct cells that can be easily distinguished from fluorescent “noise”. Good example of image to be manipulated and/or used for object identification and eventual cell tracking. (D) Gonad morphology is clear and distinct (hub outlined). However, use of this setting is risky for capturing data from multiple gonads in a single experiment because it could cause significant hardware damage when using multiple tissue wells due to proximity of the lens to the sample stage. Furthermore, viability over time is diminished at this high-resolution setting (not shown).

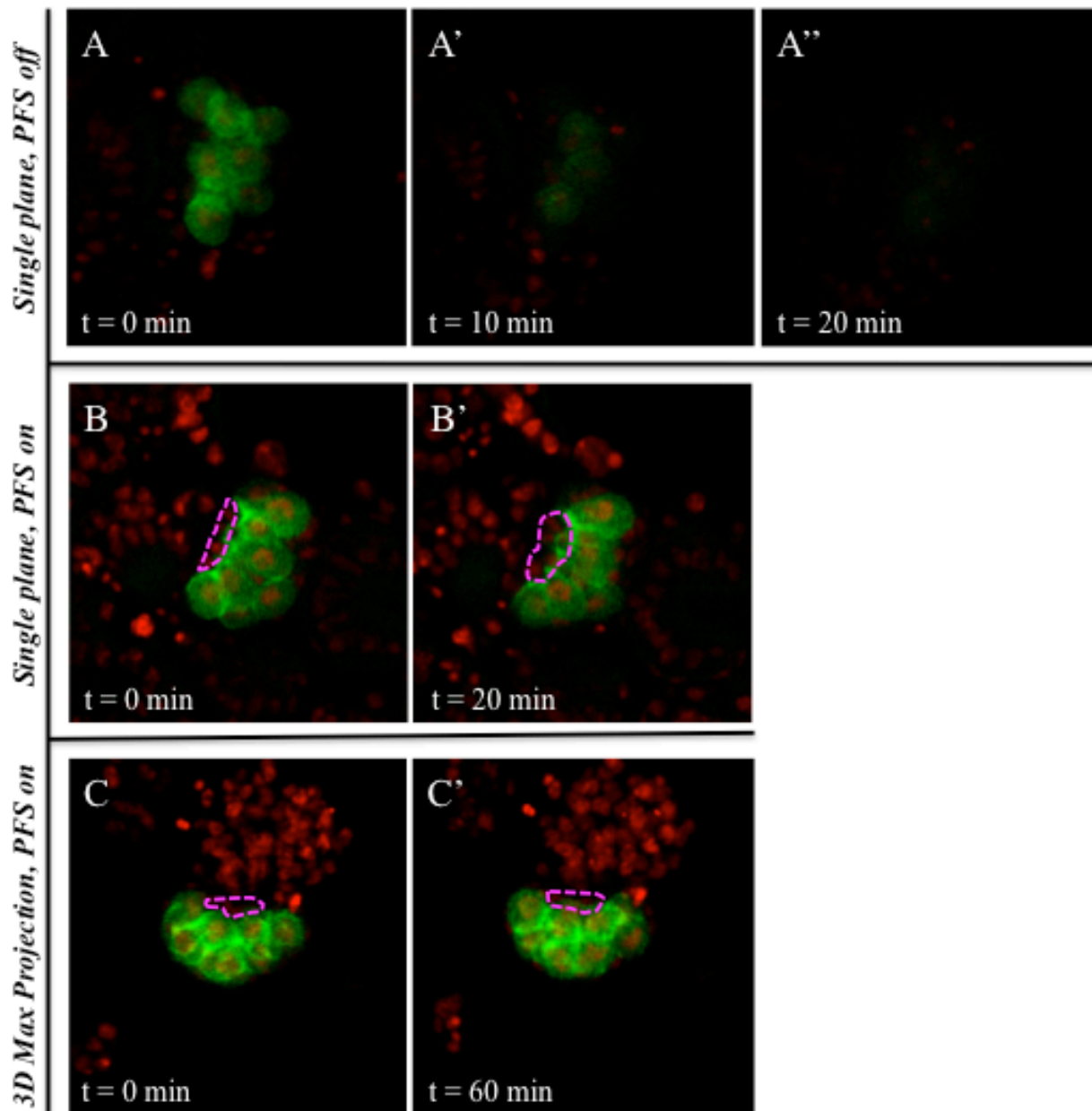


Figure 15 Perfect focus (PFS) necessary to collect clear images over time

Tests from Moesin::GFP; Histone2Av::RFP embryos with and without PFS. Images show the same testis immediately upon placement into culture (A,B,C; t=0). A' and A'' show images from the same testis in A at 10 min and 20 min after t=0 (A': t=10, A'': t=20). B' shows the same testis in B at 20 min after initial capture (B': t=20). C' shows the same testis in C at 60 minutes after initial capture (C': t=60). (A) PFS OFF, single-plane capture: Gonad moves out of focal plane as quickly as 10 minutes after initial capture, and moves further out of plane by 20 min (A', A''). (B) PFS ON, single-plane capture: Gonad remains consistently in focal plane for duration of 20 min (B'). (C) PFS ON, 3D capture: Gonad remains consistently in-plane over 60 min (C').

Appendix – Detailed Protocols

I) *Preparation of Fly Collection Cage*

- 1) Use CO₂ to anesthetize fruit flies to be collected.
(Genotype: Nos::GMA-GFP ; His2Av-RFP)
Fm7 Tm Ser

Note: The phenotype for this fly line is such that a germ cell-specific promoter expresses green fluorescent protein (GFP, which glows green) and all nuclei (specifically, nuclear histones) express red fluorescent protein (RFP, which glows red).

- 2) Deliver anesthetized fruit flies into collection cage.
- 3) Put a dime-sized amount of yeast paste in the center of a ready-prepared apple juice agar plate. Place the plate on the open side of the fly collection cage. Secure the plate onto the cage with tape.
- 4) Seal the gap between the plate and cage circumference with parafilm.
- 5) Place the cage in a secondary container so as to ensure its stability. Position the cage so that the apple juice agar plate is at the base. Allow the fruit flies to reproduce for an appropriate period of time at a defined temperature, as warranted by the experiment.
- 6) When the time period for collection is complete, tap the cage on the lab bench so that the fruit flies fall away from the apple juice agar plate. (Anesthesia with CO₂ is not necessary for this step). Quickly replace the used plate with a new one. (Remember that the plate should contain a drop of yeast paste.) Secure the plate onto the cage with tape and parafilm as before, and store the cage once more with the apple juice agar plate at the base.
- 7) From the used apple juice agar plate, carefully remove the drop of yeast paste with a metal spatula. Place a plate lid on the used plate, and allow laid embryos to age as needed for the experiment.

II) *Isolation of Embryonic/Larval Gonads*

- 1) Once the laid embryos have aged to the appropriate experimental time point on the apple juice agar plate, use a small but firm paintbrush wetted with Phosphate Buffer Triton X-100 (PBTx) solution to remove the embryos from the plate.

Note: If working with older, mobile larvae, check the inside of the plate lid for sample. These larvae may similarly be collected and removed via paintbrush.

- 2) Gently wipe the sample from the paintbrush onto the interior ridge of a cell strainer. Place the cell strainer on a petri dish cover. Rinse the paintbrush as well as the walls of the cell strainer using a squirt bottle containing PBTx solution. (The petri dish cover will capture the PBTx solution.)
- 3) After all sample has been transferred to the cell strainer, pour PBTx solution into the strainer until the sample rises off the mesh. Then, lift the cell strainer so that the solution drain through the mesh, and pour the contents of the petri dish into a liquid waste receptacle.
- 4) Repeat step 3 three times to remove yeast and fruit fly waste that may have been transferred with the sample.
- 5) Dechoriation: Pour 50% bleach (NaOCl)/water (ddH₂O) solution into the strainer until the sample rises off the mesh. Allow the sample to remain in solution for five minutes at room temperature, mixing occasionally. Then lift the strainer and pour out the content of the petri dish into a liquid waste receptacle.

Note: This step is required for samples aged 0-22 hours to remove the chorionic membrane. For older samples, the application of bleach can be omitted, though its inclusion is not detrimental (Fidler, et al, 2013). If opting to for omission, replace the bleach in this step with PBTx. 50% bleach solution should be used within 24 hours of preparing the diluted solution.

- 6) Wash newly dechorionated sample with PBTx. Pour PBTx into strainer until the sample rises of the mesh. Allow the sample to sit in solution for three minutes. After three minutes have elapsed, lift the strainer and pour the contents of the petri dish into a liquid waster receptacle.
- 7) Repeat Step 6 three times.
- 8) After washing the sample three times, transfer ~50mg of sample to a glass Potter Elvehjem homogenizer containing 500 μ l of fresh Schneider's Insect medium (Sigma-Aldrich).
- 9) Homogenize sample with seven strokes of a loose pestle.

Note: Use seven strokes of a loose pestle for samples aged 0-24 hours. Use nine strokes of loose pestle for sample aged 24 hours or more.

- 10) Add 1.5 ml of Schneider's Insect medium to the homogenizer. Add the media such that sample that may have aggregated on the walls of the homogenizer is washed from the walls and sinks to the bottom of the homogenizer.
- 11) Filter the 2 ml homogenate through the center of a 62 μ m-mesh cell strainer into a 50 ml Eppendorf tube. Keep flow-through.

- 12) Prepare dissecting media by adding 2.550 ml of Scheider's Insect medium, 450 μ l Fetal Bovine Serum (FBS), and 16.5 μ l of Pen/Strep to a 15 ml Eppendorf tube.
- 13) Use permanent marker to mark one side of the sample-containing Eppendorf tube. Place the tube in a centrifuge with the marked side facing the outer edge of the centrifuge. Make sure to balance the centrifuge by placing an empty 50 ml Eppendorf tube opposite the tube containing flow-through. Pellet the flow-through via centrifugation at 820g for two minutes at 22°C.

Note: g = rcf, which is approximately equivalent to 2500 rpm on Eppendorf 5702R centrifuge with swinging bucket 15 ml tube holding rotor (Shigenbou et al, 2006).

- 14) Discard supernatant by pipetting from the **unmarked** side of the Eppendorf tube.

Note: This technique will minimize sample loss because the pellet will be concentrated on the marked side of the tube.

- 15) Re-suspend pellet in 2 ml of dissecting media (prepared in step 12).
- 16) Filter the 2 ml re-suspension through the center of a fresh 62 μ m-mesh cell strainer into a fresh 50 ml Eppendorf tube. Keep flow-through.
- 17) Repeat steps 13 and 14.
- 18) Add 40 μ l of fresh dissecting medium to resuspend pellet.
- 19) Gently pipette 40 μ l of resuspended sample into the center of a 35 mm glass bottom petri dish with 14 mm Poly-d-lysine coated microwell. Use the tip of the pipette to gently distribute the sample evenly within the microwell. Avoid any bubble formation.
- 20) Use forceps to carefully cover the sample with a pre-prepared air-permeable membrane. (This membrane has been cut to the size of the microwell.)
- 21) Seal the junction between the air-permeable membrane and microwell by pipetting 30 μ l halocarbon oil 27 along the edge of membrane. Ensure that no bubbles form as the gap between the membrane and plate is sealed.
- 22) Close the petri dish by replacing its lid.

III) *Live-Imaging: Setting Up the NIS Elements System and Software*

- 1) Switch on the Main Power Switch (labeled #1) on the surge protector. The :Hg lamp, XYZ, and scope are now switched on.
- 2) Turn on Switch #2, which turns on the Intensilight, and fluorescence light.
- 3) Turn on Switch #3, which controls the tower of three units: the Spectral Detector, Detection, and Control.
- 4) Turn Main Laser Key (labeled #4) to horizontal position. This will turn on all four lasers.
- 5) Record your name, the date/time, and fluorescence bulb hours in the log book.
- 6) Turn on the computer and log in to lab folder.
- 7) Switch on the temperature and humidity control unit (to the left of the scope, labeled #6).
- 8) Open NIS Elements software from the desktop icon. This fully automated confocal imaging system will begin calibrating and booting up.
- 9) Go to the **A1 Compact GUI** window and click **Resonant** in the top right corner. Go down to the **Line Average** icon, and select **4x** from the dropdown. Set **Integration** at **2x**. The **Pinhole** should be set to 1.8 AU (34.5 μm).
- 10) Select the checkboxes next to **FITC** (488) and **TRITC** (561). Set their parameters as follows:

FITC:

HV: 96

Offset: -94

Laser (488): 14.14

TRITC:

HV: 73

Offset: -74

Laser (561): 10.50

- 11) From the NIS toolbar, go to **A1 Scan Area**. Change the **Zoom** setting to 4, **Pixel size** to 0.31 μm , **Scan size** to 512 (matching the width and height of 512 used for resonant scanning.) **Optical resolution** is 0.21 μm , **Z-step size** is 1.30 μm , and **Optical sectioning** is 3.71 μm .
- 12) Next, go to the top of the NIS Elements toolbar and click **ND Acquisition** icon. A window will appear with tabs labeled **Time**, **XY**, and **Z**.
- 13) Click on tab **Z**. Select the second mode icon, **Symmetric mode defined by range**. Press the **Relative** button, and set the **Range** to 36.00 μm . Set **Step** to 4.800 μm , so that the total number of steps is minimized (9). Ensure that the Z Device selected from the dropdown is the **Nikon A1 Piezo Z Drive**.

IV) *Live-Imaging: Culturing Drosophila melanogaster gonads ex-vivo in Dissecting Media*

The following steps occur in a dark room, with the door partially open for some increased visibility.

- 1) Place the petri dish containing newly isolated gonads and dissecting media in the two-plate temperature and humidity control stage chamber of the confocal microscope. The petri dish should be positioned such that the microwell with sample is at the base, and the lid is on top. To seal the petri dish in the temperature and humidity control stage chamber, place the glass chamber cover squarely down on the recessed chamber, and tighten the screw at the top left until it stops turning, then unscrew it a half turn. The lid should be firmly and snugly in place, with no gap for air to enter the chamber.
- 2) Ensure that the scope objective is set to 10X or 20X (preferred) so that nothing will be damaged during initial set up. These lens objectives will remain at a safe operating distance during calibration.
- 3) Follow Steps 1-8 from Section III: *Live-Imaging: Setting Up the NIS Elements System and Software*.
- 4) After the system is ready for use and NIS Elements is open, use the stage control joystick to center the sample over the 20X objective.
- 5) Use course focus to get the sample down to a z-plane of about 3000 μm . Then, go to the **A1 Compact GUI** window in the NIS Elements software. Click the **Eye Port** button. While looking through the eye-piece, carefully use course focus to hone in on the sample. When sample appears nearly in focus, push the **PFS** (perfect focus) button on the microscope base.

Note: **Perfect focus (PFS)** will allow the scope to measure distance from the cover slip of the petri dish, minimizing the loss of data during collection due to the tendency

of sample to drift out of focus over time. This will allow better data collection over several hours.

- 6) Use the **PFS Offset Controller** to adjust the fine focus.

Note: The singular, square blue button on the base of the PFS Offset Controller indicates course and fine focus. When the blue button is depressed, the PFS is operating fine focus. Otherwise, the PFS is operating course focus.

- 7) Once the sample is clearly in focus, use the Nikon TI-S-EJOY joystick to find a gonad of interest. Center and focus on one gonad in the plane visible through the Eye Port.
- 8) In the NIS Elements software window, find the **A1 Compact GUI** display. Click **Scan**. A new window will appear, displaying the region being scanned.
- 9) Click the **Mouse XY** icon. Then, use the cursor to click and drag the scanned area so that the gonad is centered in the window.
- 10) Next, use the focus wheel of the **PFS Offset Controller** to find the middle of the gonad in the z-plane. To do this, turn the focus wheel clockwise until the top of the gonad is reached. Note the z-plane value. Then, turn the focus wheel counter-clockwise until the bottom is reached. Note the z-plane value. Find the approximate average of the top and bottom z-plane values, and turn the focus wheel until it reaches that point in the z-plane.
- 11) Go to the **ND Acquisition** window in NIS Elements and select the **XY** tab. Double click the space with default Point Name “#1”. The X, Y, Z coordinates and PFS value for this point should then be displayed in the row corresponding to point #1.
- 12) Repeat steps 7-10 for each gonad of interest. Then, go to **A1 Compact GUI > Line Average** and select **16x** from the dropdown menu.
- 14) Go to the **ND Acquisition** window. Click on the **Time** tab. Set **Interval** to the desired time period between data collection. Set **Duration** to the desired time period for the entire experiment. **Loop** number will update automatically.


Note: Alternatively, setting the Loop number after specifying a given Interval will automatically update the Duration time.

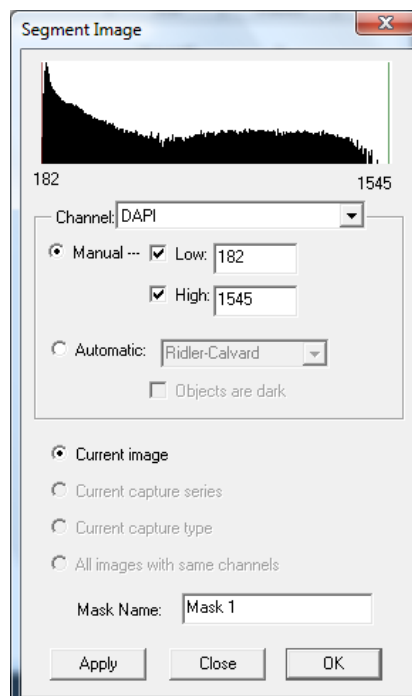
- 13) To save the experiment that will be run, go to **ND Acquisition** window, and find **Experiment: ND Acquisition**. Check the box that says Save to File. A line specifying **Path** will appear. Click **Browse** to the right of this line, and navigate to the desired folder. Select the appropriate folder and click **OK**. Then, choose an appropriate **Filename** for the experiment.

- 14) Press **Run now** in the bottom right of the **ND Acquisition** window. Watch a single loop to ensure that the experiment is running smoothly. Then, shut off the monitor and close the door to the room to minimize noise during data collection.

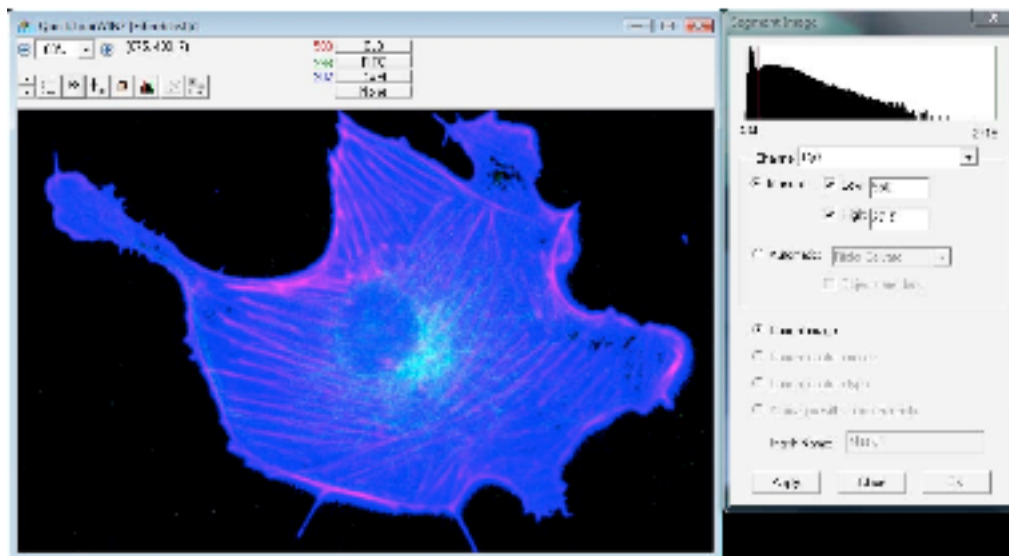
V) Object Identification: Creating a Mask with Thresholding Techniques

Note: In Slidebook 5.0, a “mask” is defined as a two- or three- dimensional set of binary values with the same extent as the image to which it is assigned. Masks are also referred to as regions of interest (ROIs), and allow for advanced selection of image data as well as image analysis. Masks can be created and edited manually or automatically through thresholding techniques. To cover these techniques, Slidebook 5.0 provides example slides. The following steps can be applied to experimental slide images as well.

- 1) Open Slidebook 5.0 application. In the top left of the application screen, select **File > Open Slide** and find *QuickTourWIN2.sld* in the *Documentation* folder within the *SlideBook* directory. A slide window will open that contains two images. (In this step, you can navigate to the experimental slide (.sld) to be masked. A slide window will open with the experimental image(s).)
- 2) Double-click the “Fibroblast” thumbnail (or an experimental image thumbnail) to open a Main View of the first image.
- 3) To create a threshold mask, click on the mask menu icon  and select **Create Segment Mask**. An alternative to reach this step is to go to the toolbar menu and select **Mask > Segment**. The following window will appear:




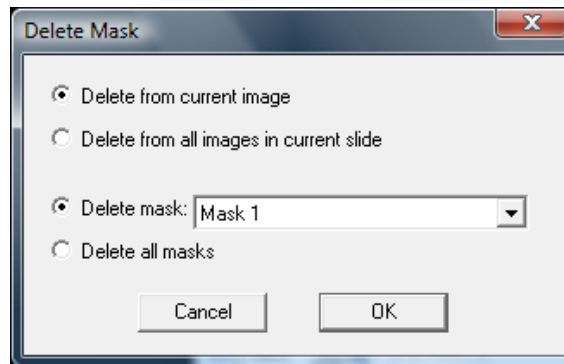
- 4) The **Channel** dropdown allows you select a channel for which to set a threshold. If the **Manual** radio-button is selected, then the range (bracketed by a low and high intensity) of intensities can be manually set. **Mask Name** allows you to specify a referent title for this mask.
- 5) Once the desired intensity range is set, select **Apply**. The Generating Mask window will appear, detailing the segmentation progress by plane. Upon segmentation completion, the entire image will be overlaid in blue. Slidebook has selected and masked those pixels with intensity values between the red (low) and green (high) bars in each of the three channels.
- 6) Select **Cy3** from the dropdown menu.
- 7) By moving the red bar to the right (mouse: left-click, drag, and release), the image segmentation can be updated with new intensity range parameters and the mask will be updated.
- 8) Set the low threshold to “550” and click **Apply**.



Now Slidebook is masking any pixel with a Cy3 channel intensity value from 550 to 2715.

- 9) Use the scroll wheel (or up and down arrows in the info/tool bar) to scroll through the image. The mask remains through the entire image (every plane).
- 10) Close the Segment Image dialog box by pressing **OK**.
- 11) Change the red channel from Cy3 to **None**. Observe the mask displayed.

- 12) To remove the mask from the display, click the mask menu button  in the toolbar and select **None**.
- 13) To replace the mask, select the desired mask title from the mask menu. (In the case illustrated above, select **Mask 1** from the mask menu. The mask is now displayed.)
- 14) To delete a mask, select **Mask > Delete**. A dialog box will appear:



- 15) Click **OK** to delete the mask from the image.

VI) *Object Identification: Defining Multiple Objects*

In the experiments for my project, it has been necessary to mask several distinct objects (cells) with similar intensities. Thus, it is necessary to create a mask that can be divided into objects.

- 1) Open a Main View of the desired experimental image.
- 2) Select **Create Segment Mask** from the mask menu to create a new thresholding mask. The window similar to the one below will appear:



- 3) Set the threshold values for your image (in TRITC my experimental values were about 770 to 1200, masking the RFP-tagged nuclei in the image).

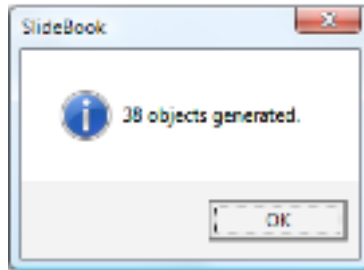
Note: Alternatively, the red and green bars on the histogram can be clicked, dragged, and released to set the low and high intensity range. As a bar is dragged, take note of the masked areas on the image. Note, too, that the data being masked is displayed in white on the histogram. Pixels with intensities in the gray area of the histogram will not be masked.

- 4) Select **Apply** to set the threshold values.
- 5) Select **OK** to create the mask.
- 6) Next, select **Masks > Define Objects in Mask**. The window below will appear:

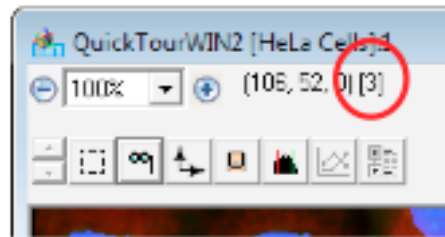


Here you may choose to exclude objects based on minimum and maximum size gates. For my experiments, the minimum size chosen was 100.

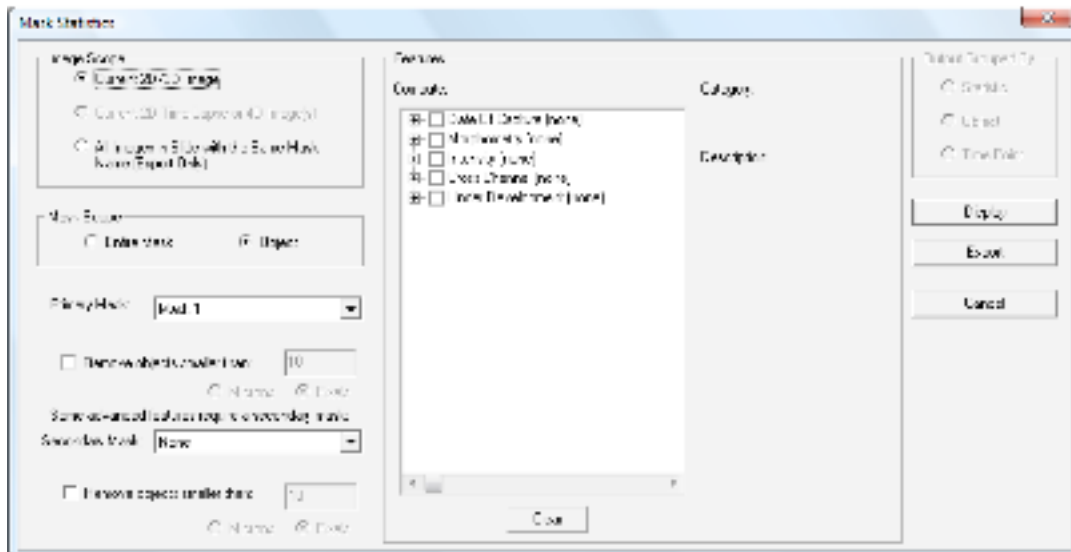
- 7) Click **OK** in the *Define Objects* window. A dialog box with the status of object generation will appear. At completion of object generation, a dialog box similar to the one below will appear, reporting the number of objects generated.



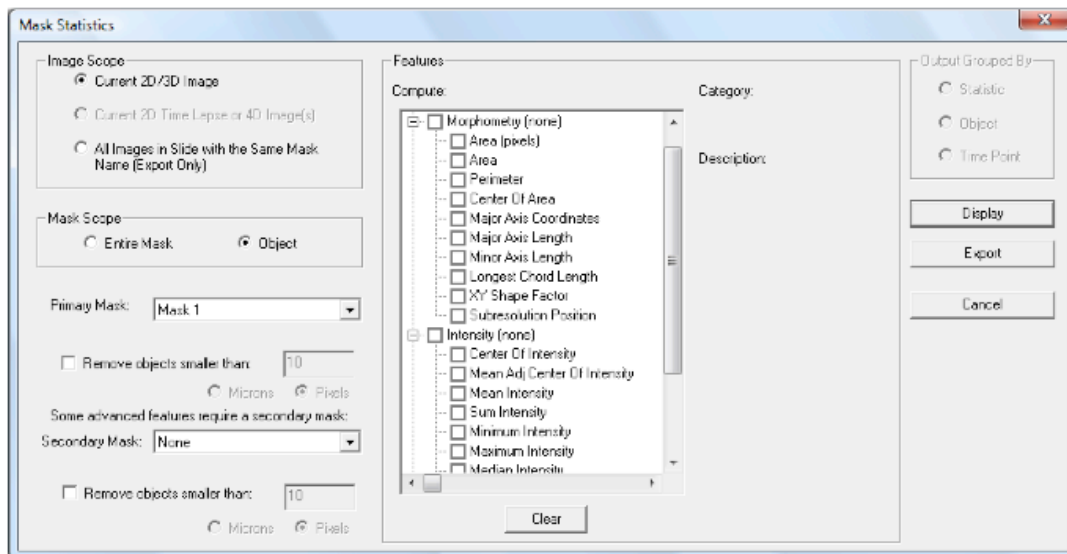
- 8) Click **OK**. If you move the cursor over one of the objects in the Main View, the object number will appear to the right of the cursor location coordinates in the info/tool bar.



- 9) Slidebook can generate individual statistics for each object. Go to the Slidebook toolbar and select **Statistics > Mask Statistics**. The following window will appear:



- 10) Click the [+] to the left of the checkboxes to expand Morphometry and Intensity statistics selections.



- 11) Check the box next to **Area (pixels)** and **Mean Intensity**, then select **Display**. The statistics will be generated, and a table similar to the one below will appear.

QuickTourWIN2 [HeLa Cells]

Image: HeLa Cells Mask: Mask 1 Capture Date: 10-20-2001 17:52:44

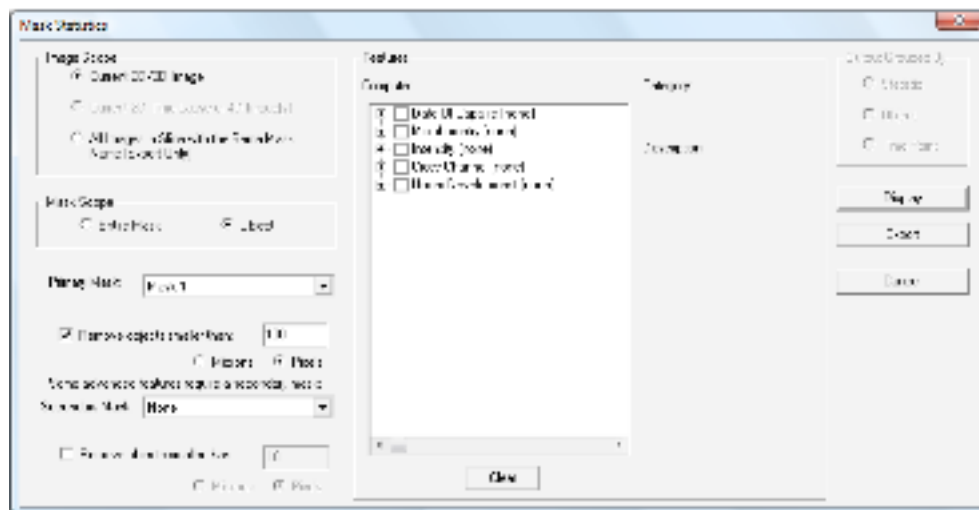
Object	Area (microns ²)	Mean Intensity (ADU), ch1:FITC LP	Mean Intensity (ADU), ch1:CY3
0	132.81	792.94	531.53
1	74.31	720.95	310.68
2	146.69	714.09	332.74
3	294.70	801.26	343.21
4	103.71	736.57	373.06
5	141.99	704.45	357.33
6	0.51	587.60	281.40
7	2.55	587.56	285.64
8	2.04	601.80	587.20
9	168.33	732.54	423.63
10	204.88	879.43	407.58
11	182.11	842.91	407.78
12	217.12	776.80	369.23
13	157.92	737.40	389.16
14	189.97	826.57	345.61
15	73.60	698.46	338.21
16	0.71	599.43	647.57
17	1.74	595.76	673.76
18	160.06	816.09	428.59

Export Close

- 12) Expand column headers by clicking and dragging the edge of the header. To highlight an object in the image (appears pink), click any object number in the table.

Note: Some of the objects may be very small in size. To exclude smaller objects, go to **Mask > Update Object Definitions** (This differs from the earlier “Define Objects” menu item because the objects already exist for the image.) Check the box next to **Gate Objects by Size** and enter the appropriate minimum size gate. This will exclude objects smaller than 100 voxels from being masked. To update object definitions, select **OK**. This should generate fewer objects than if there were a lower (or no) size gate specified. Updated statistics can be generated on the new objects as outlined in step 9-12.

Alternatively, size can be gated in the Statistics View. Select **Statistics > Mask Statistics**. The following window will appear:



Click the **Remove objects smaller than:** checkbox (under the **Primary Mask** dropdown menu) and enter a minimum size gate (100 pixels, for instance) in the active edit box. Now, to update the statistics, select those statistics desired for computation (as outlined in step 11) and click **Display**. SlideBook will remove objects smaller than the minimum size gate while statistics are computed.

- 13) The statistics table may be exported to other programs by clicking **Export**. A window appears that will allow the user to choose the file destination. The file will be saved as a text file (*.txt), and can be opened by various applications, including Microsoft Excel.
- 14) Close the slide by clicking the in the upper right corner of the slide view. Alternatively, go to the Slidebook main toolbar and select **File > Close Slide**.