RESEARCH REPORT

Sex-specific topological differences in germline cell lineage trees

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ABSTRACT

A conserved phase of gametogenesis is the development of oocytes and sperm within cell clusters (germline cysts) that arise through serial divisions of a founder cell. The resulting cell lineage trees (CLTs) exhibit diverse topologies across animals and can give rise to numerous emergent behaviors. Despite their centrality, sex-specific differences underlying the evolution and patterning of these cell trees are unknown. In *Drosophila melanogaster*, oocytes develop within a highly invariant and maximally branched 16-cell tree whose topology is constrained by the fusome – a branched membranous organelle critical for proper mitosis in females; the same division pattern and topology are widely thought to occur during spermatogenesis. Using highly-resolved three-dimensional reconstructions based on a supervised learning algorithm, we show that cell divisions in male cysts can deviate from the maximally branched pattern, leading to greater topological variability. Furthermore, in contrast to females, fusome fragmentation is common, suggesting germ cell mitoses can occur in its absence. These findings thus add to the repertoire of CLT formation strategies, highlighting the diversity of mechanisms employed during gametogenesis in the animal kingdom.

KEYWORDS: spermatogenesis, network topology, cell lineage trees, fusome, supervised learning

SUMMARY STATEMENT

Diegmiller et al. combine image processing techniques with analysis of cell network structure to identify sex-specific topological differences in germ cell lineage trees generation during *Drosophila* gametogenesis.

INTRODUCTION

Across animals, sperm and egg develop within germline cysts whose cells are connected by arrested and stabilized cytokinetic furrows called intercellular bridges (ICBs) (Fawcett et al. (1959); Fukuda and Hedinger (1975); Fuller (1998); Giardina (1901); Greenbaum et al. (2011); Guo and Zheng (2004); Haglund et al. (2011); Hegner (1914); Hegner and Russell (1916); Moens and Go (1972); Pepling et al. (2015); White-Cooper et al. (2009)). ICBs are conserved from insects to mammals, including humans, where they reveal the history of cell division and with diameters that are several microns large, ICBs are large enough to permit passage of proteins, mRNAs, and organelles (Greenbaum et al. (2011); Haglund et al. (2011); Imran Alsous et al. (2018)). During oogenesis and spermatogenesis, these cytoplasmic channels facilitate germ cell communication and synchronization (de Cuevas et al. (1997); Yamashita (2018); Kaufman et al. (2020); Lu et al. (2017)): during oogenesis, when typically one cell is chosen to become the oocyte, these connections enable symmetry breaking through directional intercellular transport of cell fate determinant (de Cuevas et al. (1997); Yamashita (2018); Nashchekin et al. (2021)), while during spermatogenesis, where all germ cells are equivalent and give rise to spermatids, ICBs enable chromosome dosage compensation and sharing of cellular products among haploid cells (Fig. 1A) (Kaufman et al. (2020); Lu et al. (2017); Yamashita (2018)). An additional feature of developing *Drosophila* germline cysts is the presence of a membranous backbone known as the fusome, which replaces the mid-body-associated microtubules between cell divisions and fuses with the older fusomal material to form one continuous fusome, thus giving rise to a finger-like backbone that extends through the ICBs, connecting all germ cells (de Cuevas et al. (1997); Grieder et al. (2000); Lighthouse et al. (2008)). First described in spermatocytes more than a century ago, the fusome has since been observed in numerous insects, where it is thought to be essential for fertility (de Cuevas et al. (1997); Giardina (1901); Hegner (1914); Hegner and Russell (1916); Lin et al. (1994); Mahajan-Miklos and Cooley (1994); Telfer (1975)).

While germline cysts are a conserved feature of oogenesis, across species these cysts have been shown to exhibit diverse forms (Chaigne and Brunet (2022); Diegmiller et al (2022)). Indeed, if one represents cells as nodes and ICBs as edges connecting those nodes, then the germline clusters in the animal kingdom can range from a single supporting cell connected to an oocyte to large, maximally-branched trees (Brubacher and Huebner (2009); Büning and Sohst (1988); Diegmiller et al (2022)). Although there is a wide diversity in cluster size and topology, the factors that govern proper germline cyst development have been most extensively characterized in *Drosophila melanogaster* females, where germline cysts form through four synchronous divisions of a founder cell, giving rise to a highly invariant and maximally-branched cluster of connected cells (Fig. 1B) (de Cuevas et al. (1997); Eikenes et al. (2013); Giansanti et al. (1999); Haglund et al. (2011); Hime et al. (1996); Kaufman et al. (2020); Yamashita (2018)). At each division, one of the two mitotic centrosomes associates tightly with the fusome, a membranous endoplasmic

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Received 18/5/2022; revised 18/5/2022

Development (2022) 00, devxxxxxx. doi:10.1242/dev.xxxxxx

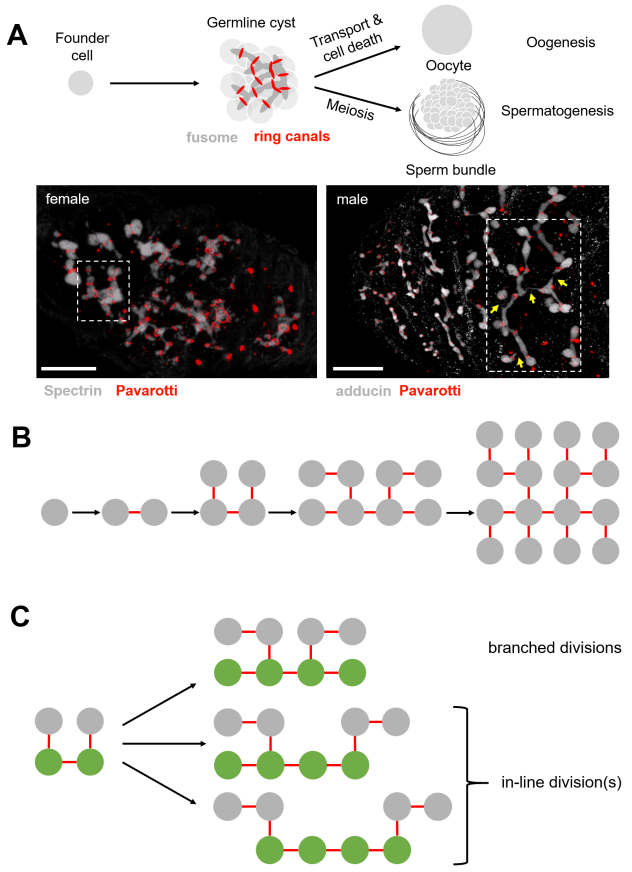

Fig. 1. Visualizing cyst formation in *Drosophila* spermatogenesis. A) Schematic for cyst formation during oogenesis and spermatogenesis. During *Drosophila* oogenesis, one cell to become the oocyte while the remaining cells serve a supporting role, synthesizing necessary biosynthetic components and contributing these to the oocyte for further developmental processes. During *Drosophila* spermatogenesis, one round of meiosis occurs to form clusters of identical spermatids. Despite these differences, both germline cysts contain a membranous organelle known as the fusome, which enables the topology of cell clusters in both instances to be identified across divisions. *Bottom:* Example image of developing cell clusters during *Drosophila* oogenesis (left) spermatogenesis (right) Female fusome is stained for α-Spectrin, while the male fusome is stained for adducin. In both cases, ring canals are marked by Pavarotti. We note the differences in fusome morphologies between male and female cysts (dotted boxes), and the existence of tread-like fusome pieces and fragments in male cysts (yellow arrows, right). All scale bars = 20 μm. B) Series of divisions that is known to form developing female cysts in *Drosophila* and is thought to exist during spermatogenesis. That is, from a single cell, a series of synchronous, sequential divisions generates maximally-branched trees. C) Divisions during gametogenesis can be formed in multiple ways based on the orientation that the internal cells (green) divide. If all internal cells divide to form new branches, the growing cyst will remain maximally-branched. However, these internal cells may also divide without forming new branches, creating in-line divisions that deviate from the maximally-branched structure associated with many developing cysts, particularly that of *Drosophila* oogenesis.

reticulum-derived organelle, orienting the planes of cell division and ensuring the stereotypical branched pattern of the germline cyst (de Cuevas et al. (1997); Storto and King (1989)). The topology of the fusome therefore dictates that of the female germline cyst. A similar sequence of divisions has been thought to govern *Drosophila* spermatogenesis, where the resulting 16-cell cyst gives rise to 64 cells through subsequent meiotic divisions (Insco et al. (2009); Venkei and Yamashita (2015); Yamashita (2018); Rasmussen (1973)) (Fig. 1B). However, the series of divisions governing spermatogenesis in *Drosophila* has not been explicitly characterized.

In any interconnected cyst, two types of cells exist: internal cells and terminal cells. Upon division, the terminal cells – the cells at the very ends of the cyst, connected to only one other cell – must divide in such a way such that they form a cell connected now to two cells and a cell with only one connection again (Fig. 1C). That is, terminal cell division in germline cysts must yield an internal cell and a terminal cell. On the other hand, internal cells have multiple ways in which they can divide: by forming a new terminal cell, or by yielding two internal cells. When all internal cells divide to form terminal cells, maximally-branched trees are the result (Fig. 1C). However, if in-line divisions occur, maximal branching in the cyst is lost. By tracking the cell-cell connections present in germline cysts, the topology of the network of connections can be derived and analyzed. With these division rules in mind, we sought to catalog the series of connections present within the developing male germline cyst to compare against the known sequence of divisions during *Drosophila* oogenesis.

RESULTS AND DISCUSSION

Supervised learning approach for network topology reconstruction

We performed a systematic and comparative study to reconstruct the three-dimensional structure and topology of germline cysts during spermatogenesis at various stages of cyst formation, using the fusome marker adducin. Specifically, we employed a semi-supervised learning algorithm for pixel classification along with identification of cell-cell adjacencies to reconstruct the network of intercellular connections and fusomal structure in cysts of various sizes (Fig. 2A) (Berg et al. (2019); Diegmiller et al. (2021)). In contrast to the *Drosophila* female germline cyst, where cells are tightly packed and spanned in their entirety by the fusome (Fig. 1A), male germline cysts appeared less compacted, with more elongated fusomes (Fig. 1A). These visualizations also confirmed the presence of thin, thread-like material connecting otherwise disjoint fusome fragments highlighted in previous studies (Eikenes et al. (2013); Kaufman et al. (2020); Miyauchi et al. (2013); Venkei and Yamashita (2015)).

Fusome fragmentation is common during spermatogenesis

Our initial reconstructions demonstrated that fusome fragmentation during male cyst formation is substantial (Fig. 2B). Indeed, we found that in only (50%) of reconstructed samples did the fusome connect all 2^n cells, in contrast to cyst structure in females. Although fusome fragments in developing male germline cysts can encompass fewer than 16 cells, these abnormalities are unlikely to be due to an atypical number of cells. We confirmed this by counting the spermatid tails per sperm tail bundle present across a number of samples, similar to previous studies (Hanna et al. (1982); Liebrich et al. (1982)). We observed an average of 63.15 ± 1.31 ($n = 61$) spermatids per bundle in post-meiotic clusters, consistent with the presence of 16 cells per cluster (Figure S1). These observations suggest that while the number of cells in the developing germline cyst are equal between *Drosophila* males and females, fusome morphology and structure is less stereotypic in spermatogenesis than oogenesis.

Development (2022) 00, devxxxxxx. doi:10.1242/dev.xxxxxx

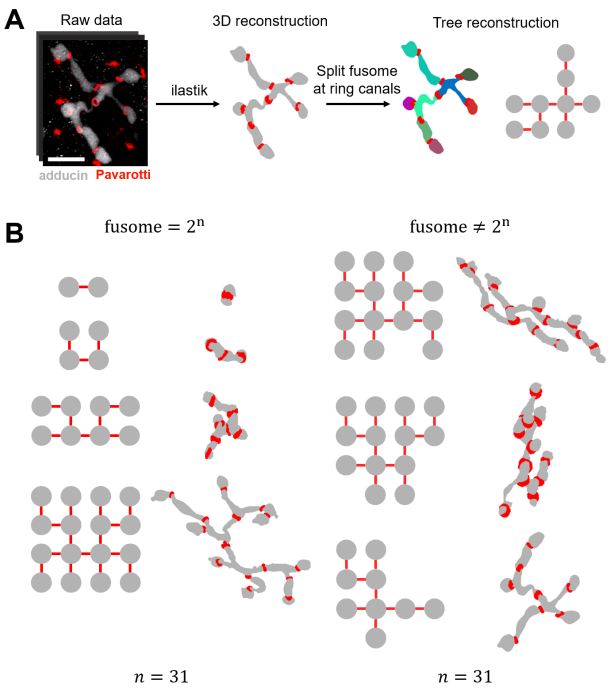

Fig. 2. Distribution of cyst sizes in male germline clusters. A) Pipeline for reconstructing male germline cyst connections. For each stack of images, a supervised learning algorithm allows for the identification of fusome (gray, adducin) and ring canals (red, Pavarotti), which can then be split into component parts and used to generate cell adjacencies for graph reconstructions. B) From $n = 62$ isolated cyst samples, a range of sizes can be found by identifying the largest connected portions of fusome within each sample. These fusome portions, and their corresponding ring canals, can be used to identify how many cells are connected by each fusome piece. While many of these cysts did contain a power of 2 number of cells (n=31), there were just as many that did not. We note that some fusome pieces within cysts appear disconnected, and it is unlikely that cysts truly contain cell numbers that cannot be represented as a power of 2. However, fusome fragments seem to appear during spermatogenesis with a much higher frequency than oogenesis.

Atypical cyst topology can arise at the 16-cell stage

To explore the effects of fusome and fragmentation on male germline cyst topology, we analyzed the various topological patterns present that could be recapitulated through fusomal connections in males. Starting from a single cell, there is only one path towards forming a four-cell cyst via synchronous divisions: a linear fusome fragment with three ICBs. Upon dividing synchronously again, multiple eight-cell cysts are possible, depending on whether the divisions occur in a maximally-branched fashion; that is, whether or not each division forms a new terminal fusome fragment budded off from the parent (Fig. 1C). For cells at the terminal ends of the cyst, the fusome can only elongate in one manner; however, for the inner cells, the fusome either adds a branch or continues growth in a linear manner. By symmetry, one is left with three possible configurations for the cyst; of these, only one configuration is maximally-branched, whereby each division creates a new branch (Fig. 1C). Importantly, of all fusome reconstructions encompassing 5-8 cells (n = 21), we found none were inconsistent with the maximally-branched connections of an 8 cell cyst; maximal branching was not found to be lost at the 8-cell stage during spermatogenesis.

Given that only the maximally-branched 8-cell cyst was observed as a precursor, we found 36 possible configurations that could be obtained through another division to a 16-cell cyst, with only one resulting in a maximally-branched network. We analyzed fusome reconstructions connecting 9-16 cells and, while 16-cell maximally-branched cysts were observed, we uncovered trees with underlying connections that could not be embedded into the connections of a maximally-branched 16-cell tree (Fig. 3A). Even in cysts whose fusomes did not connect 16 cells, the topology of the largest fragment was sufficient in some instances to demonstrate that divisions had not occurred in a maximally branched manner. The presence of non-maximally branched cysts suggests that the stereotyped pattern of connections ubiquitous in oogenesis is not preserved during spermatogenesis.

Evidence of in-line divisions during 8-to-16 cell transition

To rationalize how these ‘atypical’ germ cell cluster topologies can arise, we analyzed the orientation of mitotic spindles in these clusters throughout divisions, focusing on the fourth set of divisions where the cluster increases in cell number from 8 to 16. In female germline cysts, mitotic spindles align such that newly-formed cells divide to create a maximally-branched tree as opposed to ’in-line’ (Fig 1C); the fusome follows cyst growth, leading to its branching throughout the cyst (de Cuevas et al. (1997)). This division pattern was observed in all dividing 2- and 4- male cell cysts, but not in 8-cell samples, where we found mitotic spindles that were parallel to two disjoint fusomal fragments (Fig. 3B) – consistent with cell division elongating the fusome rather than giving rise to a new branch. These findings suggest that during spermatogenesis, deviations from maximally-branched structures can occur at the fourth division during cyst formation, in contrast to the highly invariant pattern observed during oogenesis.

Overall, these studies reveal that, contrary to previous assumptions, *Drosophila* male germline cysts do not strictly observe the stereotypical division pattern observed in females; instead, male CLTs exhibit greater topological variability. To our knowledge, the reconstructions of the fusome-ring system within the male germline cyst presented here are novel; however, we are not the first to demonstrate that *Drosophila* spermatogenesis may exhibit ’atypical’ division patterns: a manual reconstruction by Rasmussen fifty years ago acknowledged that the structure of cell-cell connections did not align with the known pattern found in the female germline (Rasmussen (1973)) (Fig. 3A). Unfortunately, the reconstruction and observations made my Rasmussen have been largely disregarded by the field, with some authors later stating that "the distribution of ring canals among the cells of the cyst appears to be the same as described by King for oogenesis...; the recent report to the contrary by Rasmussen probably represents an exceptional cyst." (Lindsley and Tokuyasu (1980)).

The analysis by Rasmussen concluded that this atypical male cyst must have formed as the result of five mitotic divisions (Rasmussen (1973)). Our results, however, yield an alternative mechanism through which cysts without a maximally-branched structure can arise: four synchronous mitotic divisions, where the final one may occur without branching. Male germline cysts can thus be generated in a way such that the number of ring canals does not necessarily reflect the history of divisions within the cyst,

Development (2022) 00, devxxxxxx. doi:10.1242/dev.xxxxxx

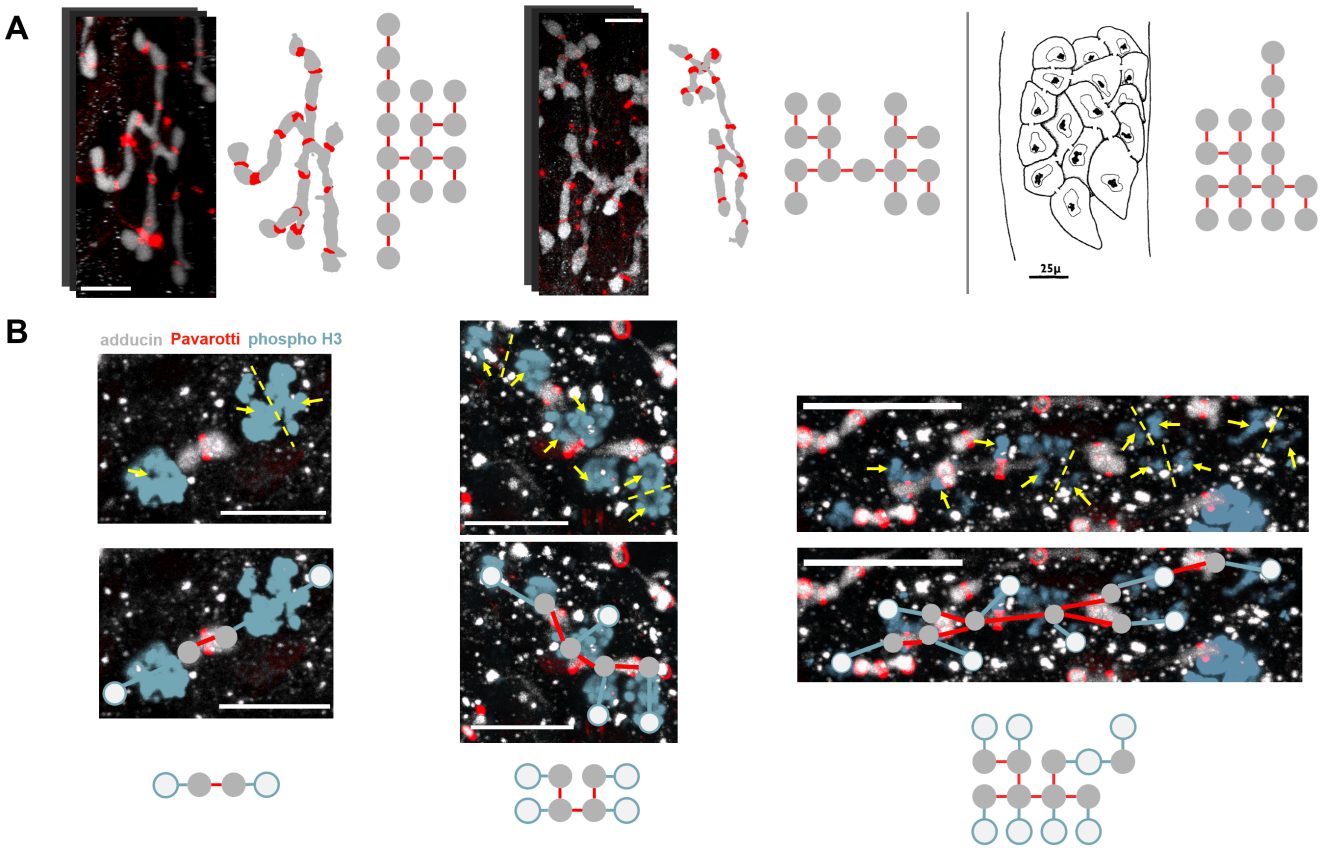

Fig. 3. Example network topologies of male germline clusters. A) *Left:* Two examples of cysts (16 and 15 cells, respectively) where maximal branching could not have occurred during cyst generation. In this case, at least one division must not have generated a new fusome branch, in contrast to the well-documented and highly invariant divisions that occur during *Drosophila* oogenesis. *Right:* Hand-drawn reconstruction of the cells and intercellular connections of a 16-cell sperm cluster from Figure 1 of the 1973 work by Rasmussen (Rasmussen (1973), reproduced with permission). Here, although the pattern of connections do not align with the conventional maximally-branched pattern (as shown in gray), this work was dismissed as representing an atypical example of spermatogenesis. Our work aims to correct this view and restore proper credit to this observation B) Here, we show examples of a 2-cell (left), 4-cell (middle), and 8-cell cyst (right) actively dividing. It is clear from the fragmentation of the fusome, as well as the orientation of the mitotic division that in at least location in the 8-cell cyst that cells are dividing without forming a new branch, a deviation from the cell network shape observed in oogenesis and further evidence of differences in gametogenesis in *Drosophila*. However, this behavior was not observed for any dividing 2- or 4- cell cysts. For all examples, yellow dotted lines denote the likely plane of division for actively dividing cells, while yellow arrows depict clear bundles of aggregated or migrating chromosomes. In addition, nodes representing the locations of existing cells, as well as the likely positions of newly-formed cell based on the plane of division are shown. In all images, fusome is shown in gray (adducin), ring canals are shown in red (Pavarotti), and mitotic chromosomes are shown in cyan (phospho Histone 3). Scale bars = 10 μm, unless otherwise indicated.

in contrast to female germline cysts, where ring canal number informs when a cell was formed.

Investigating the role of the fusome during gametogenesis

These findings can be interpreted in the context of the divergent outcomes of gametogenesis in both sexes. Specifically, while the outcome of spermatogenesis is a cluster of equivalent spermatids, that of oogenesis is a single cell or small number of cells that become the oocyte at the expense of supporting cells. As such, there may be less of a need for a highly stereotyped pattern of connections in the male, as directed and large-scale cytoplasmic transport is not as essential for the coordination and distribution of vital developmental components in the male as in the female. This conclusion is supported by the presence of fragmented or elongated fusome pieces present in the male germline. Moreover, we propose that the denser, more tightly-packed fusome present in the female germline enables control of division orientation such that cells form as new branches with each mitotic division (de Cuevas et al. (1997)). Our results also suggest this control can be lost in the male germline, particularly where the fusome has become thread-like or fragmented.

Recent work has demonstrated that cell network topology can affect cell cycle synchrony and coupled oscillations (Shao et al. (2021)). It is unknown, however, how intercellular connections can evolve to form different cell-cell connections and still coordinate proper signaling and transport during gametogenesis. It is also unknown how cyst divisions are terminated during oogenesis at four divisions. Although the protein Bag of marbles (Bam) has been implicated in regulating the number of divisions, its mechanism of action and how this could differ between males and females with different cell-cell connections has not been characterized (Insco et al. (2009); McKearin and Spradling (1990)).

Thus, the sex-specific differences observed in these studies during *Drosophila* cyst generation may also be observable in other systems. The algorithm developed for this work is applicable to any developmental system containing a fusome-like structure surrounded at cell-cell connections by an ICB and is readily extended to systems containing only rings and cell membranes (Diegmiller et al. (2021)). Our work thus contributes a valuable computational tool that can be used to recapitulate germline cyst topology and morphology, and uncover the factors that govern cyst formation, thus allowing for comparative studies of gametogenesis within and across species. Is division orientation less strictly defined in animals without fusomes? How do division patterns affect oogenesis and spermatogenesis in these animals? An exciting future avenue for research would investigate how the diverse cyst topologies across animals are generated during oogenesis and spermatogenesis through synchronous and asynchronous divisions, and commonly in the absence of a fusome.

MATERIALS AND METHODS

Fly stocks and dissection

Ubi-Pavarotti-GFP flies were obtained from the Glover lab and dissected according to standard protocols (Minestrini et al. (2002)). Immunofluorescence staining was performed as described previously (Cheng et al. (2008)). Briefly, tissues were dissected in PBS, transferred to 4% formaldehyde in PBS and fixed for 30 minutes. Tissues were then washed in PBS-T (PBS containing 0.1% Triton X-100) for at least three 10 minute washes, followed by incubation with primary antibody in 3% bovine serum albumin (BSA) in PBS-T at 4°C overnight. Samples were then washed for three 20 minutes washes in PBS-T, incubated with secondary antibody in 3% BSA in PBS-T at 4°C overnight, washed as above, and mounted in Vectashield with DAPI (Vector Labs). The following primary antibodies were used: rabbit anti-Phospho H3 (Ser10) (1:200, Thermo Fisher) and mouse anti-Hts (1:20, 1B1, Developmental Studies Hybridoma Bank). Fluorescent images were acquired using a Leica TCS SP5 confocal microscope with a 63x/1.3 NA oil objective.

Image acquisition and processing

Confocal stacks were processed in FIJI by first isolating individual cysts for further analysis and to reduce data input within the machine learning software. Samples were uploaded into ilastik and trained to assist in assigning probabilities for voxels forming parts of an ICB, fusome, or background of the image (Berg et al. (2019); Diegmiller et al. (2021)). The probabilities for ICBs and fusome were then inserted into MATLAB for further analysis.

Fusome segmentation was performed similarly to previous work, using skeletonization to isolate ICBs and the ICBs themselves to iteratively divide the fusome into its constituent parts. When the fusome was fragmented, network reconstruction was performed manually throughout the sample to ensure accurate connections were identified. The collection of fragmented fusomal structures found across samples motivated counting spermatid bundles from acquired post-meiotic samples, which were performed manually in FIJI.

Samples stained with Phosphohistone 3 were additionally trained within ilastik and inserted into MATLAB for further analysis for positioning. 3D reconstructions allowed for the identification of cells dividing without the formation of new fusome branches.

Acknowledgements

The authors would like to thank Tomer Stern for his coding expertise, and Eric Wieschaus, Trudi Schüpbach, Hayden Nunley, and Matt Smart for helpful discussions.

Competing interests

The authors declare no competing or financial interests.

Contribution

S.Y.S. and Y.M.Y. designed the project; D.L. conducted experiments; R.D. acquired images and classified training algorithms; R.D. developed codes; R.D. and J.I.A. analyzed data; R.D., J.I.A., D.L., Y.M.Y., and S.Y.S. wrote and edited the manuscript.

Funding

This work was supported by the National Institutes of Health [F31HD098835 to R.D., R01GM134204 to S.Y.S.].

Data availability

All MATLAB codes used in this study have been made available at `https://github.com/Shvartsman-Lab/MaleReconstruction`. Sample input image stacks, ilastik training sets and output files for these codes will be made available upon request.

Supplementary

Supplementary information available online at:

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