A Whole-Mount Approach for Accurate Quantitative and Spatial Assessment of Fetal Oocyte Dynamics in Mice

Safia Malki, Marla E. Tharp, and Alex Bortvin

ABSTRACT

Depletion of oocytes from the embryonic ovary is a key feature of mammalian oogenesis; however, the rational and molecular bases for this phenomenon remain poorly understood. Presently in the field, the most systematic analysis used to understand the effect of a given molecular pathway on fetal oocyte attrition is to count the number of oocytes in ovaries at different stages of development. This analysis is commonly done using a sampling method based on sectioning of the ovary, a technique that includes many laborious steps culminating in an inaccurate estimate of oocyte number contained within that ovary. This inability to generate data that are directly comparable between labs hinders the field and raises questions about the timing and rate of oocyte depletion. Therefore, we set out to implement a robust method that can be easily used by most research laboratories to study the dynamics of oogenesis during fetal mouse ovary development in both normal and experimental conditions. Here we describe an approach to accurately count the total number of oocytes in embryonic ovaries. This method is based on whole-mount immunofluorescence, tissue clearing with sucrose and ScaleA2 reagent, and automatic detection and counting of germ cells in intact ovaries using confocal microscopy and three-dimensional software analyses. We demonstrate the power of the method by assessing variation of fetal oocyte numbers between left and right ovaries and between litters of mice. Finally, we anticipate that the method could be adopted to the analysis of substages of meiotic prophase I and ovarian somatic cells.

INTRODUCTION

Oogenesis in humans and other mammalian species is characterized by the continuous depletion of the ovarian reserve of primordial follicles, which is set at birth, throughout the postnatal life [1, 2]. The quality of the oocyte reserve depends on various developmental processes that take place throughout embryogenesis. One such process, described as a physiologically normal event of fetal oogenesis, is the massive loss of up to 80% of oocytes throughout meiotic prophase I, known as fetal oocyte attrition (FOA) [3–6]. The basis for this oocyte elimination remains unclear and has been studied in the context of many intrinsic pathways and external environmental factors [7], including triggering of meiotic defects [8], persistent retrotransposon activity [9], germ cell cyst breakdown [10, 11], and checkpoint activation [12]. A critical step in assessing roles of a gene or a molecular pathway on oocyte viability is enumeration of the number of oocytes present in the ovary at different developmental stages. However, counting oocytes is not trivial and has been the object of much debate in the field [11, 13–17]. To estimate the number of oocytes contained in embryonic mouse ovaries, a sampling method is most commonly used [13, 18]. This method is based on preparing 6- to 10-μm-thick serial paraffin or cryosections of ovaries followed by histological analyses or immunostaining using a germ cell marker. Oocytes are counted on every 5th or 10th ovary section, and then a correcting factor is applied to extrapolate the number found in this subset to the entire ovary. Unfortunately, this method allows for significant variability in thickness of sections and number of sections analyzed, and, moreover, different correcting factors ranging from 5 to 80 are applied to take into account the total ovary volume by correcting for either thickness of sections and/or volume of ovary analyzed and/or number of sections analyzed [13]. It is also noteworthy that these tissue preparations and calculations imply uniform oocyte distribution within the ovary. This assumption further reduces accuracy of the method because oocytes become confined to the cortex of the ovary and the shape of the ovary changes from an elongated to a more rounder and thicker form throughout embryonic development. As a result, the number of oocytes in embryonic ovaries dramatically varies from one study to another, preventing direct comparison. Alternatively, the number of oocytes could be estimated using a less laborious fluorescence-activated cell sorter (FACS) [19]. However, FACS requires the pooling of several ovaries and does not account for cell loss during ovarian cell suspension preparation and FACS processing, resulting in extremely approximate oocyte counts as well.

In this study, we focused on developing a simple, rapid, robust, scalable, and inexpensive method for whole-mount immunolabeling, imaging, and analysis of mouse ovaries. We first established a whole-mount immunostaining method with the goals of tissue preservation, improvement of antibody penetration, and reduction of fluorescence background. We also established a clearing protocol using sucrose and ScaleA2 reagent [20, 21] that is compatible with our whole-mount immunostaining protocol to produce transparent ovaries and allowing for improved signal detection.

We anticipate that this alternative in situ approach for quantification of oocytes will deepen our knowledge of the dynamics of fetal oogenesis. We envision that this approach can be readily used in combination with a variety of meiotic, cytoskeletal, and cell death pathways markers. This method...
should also provide invaluable insights into roles of somatic ovarian cells in establishment of the primordial follicle pool. Finally, we envision that broad implementation of the method will improve reliability of data obtained by different studies or laboratories in the field.

MATERIALS AND METHODS

Animal Husbandry

Swiss wild-type male and female Crl:CD1 (ICR) mice (Charles River) were housed on a 12L:12D regimen with ad libitum feeding and water hydration under specific pathogen-free conditions. We mated wild-type males with wild-type females at 6–8 wk of age. We caged one female with one male and examined her each morning for the presence of a vaginal plug. We recorded the day of plug appearance as Day 0.5 postcoitum (dpc). We monitored cages daily and recorded the number and size of litters. All experimental procedures were performed in compliance with ethical regulations and approved by the Institutional Animal Care and Use Committee of the Carnegie Institution for Science.

Antibodies

We used the following antibodies at indicated dilutions: rat monoclonal anti-TRA98 (1:500; B-Bridge International, Inc.; catalog no. 73-003), Alexa Fluor 488 (Jackson Immunoresearch; catalog no. 712-545-153), Alexa Fluor 568 (Jackson Immunoresearch; catalog no. 712-545-153), and Donkey Anti-Rat IgG (H+L) Alexa Fluor 488 (Jackson Immunoresearch; catalog no. 712-545-153).

Tissue Dissection, Fixation, and Preparation

Pregnant females were dissected at 15.5 or 18.5 dpc. We extracted embryonic ovaries in PBS. We gently removed mesonephros from ovaries from E15.5 embryos and removed E18.5 ovaries from bursa. We immediately fixed dissected embryonic and adult ovaries in freshly prepared 2% PFA (EMS) in PBS on ice or on a nutator at 4°C for 2 h. We separated right from left ovaries in 1.5-ml tubes and pooled them per litter. We washed the fixed samples with 1 ml of PBS 3× 30 min on a nutator at 4°C. Afterward, samples were incubated through a sucrose gradient (10%, 20%, and 30% sucrose in PBS) on a nutator at 4°C. Tissues were then processed directly for whole-mount immunostaining. Embryonic testes were prepared similarly.

Whole-Mount Immunostaining

We treated ovaries for 2 h in a permeabilization solution (PBS, 0.5% Triton X-100), then incubated them in a blocking solution (PBS, 0.1% Triton X-100, 0.15% glycine, 10% normal serum, 3% BSA) on a nutator overnight at 4°C. We applied antibodies at appropriate dilutions in the blocking solution and incubated 48 h at 4°C on a nutator. We washed ovaries the next day for 3× 1 h (PBS, 0.1% Triton X-100). We detected primary antibodies by incubating ovaries for 48 h at 4°C with corresponding secondary antibodies, taking care to protect samples from light. After incubation, we washed samples for 3× 1 h (PBS, 0.1% Triton X-100) and, if needed, counterstained with DAPI (50 ng/ml) for 30 min.

Tissue Clearing

We cleared immunostained ovaries with ScaleA2 reagent (4 M urea, 10% wt/vol) glycerol, 0.1% [wt/vol] Triton X-100, pH 7.7). We first rinsed ovaries 5–10 times in 1 ml of ScaleA2 to wash away any residual salt. We left ovaries in 1 ml of ScaleA2 for several days until tissue became transparent. Solution was refreshed daily.

Microscopy and Image Analysis

For confocal imaging and optical sectioning of whole-mount immunostained ovaries, we used a Leica TCS-SP5 laser scanning confocal microscope (Leica Microsystems). Images were acquired with a 25× NA 0.95 cover glass-corrected water immersion objective (Leica HCX IRAPO L; working distance 2.4 mm). For single-photon fluorescence, Alexa Fluor 488 was excited with a 488-nm laser line, and fluorescence was detected at 500–550 nm. Alexa Fluor 568 was excited with a 561-nm laser line, and fluorescence was detected at 570–625 nm. For two-photon fluorescence, Alexa Fluor 488 was excited at 820 nm with light from a Ti:Sapphire laser (Chameleon Ultra-II; Coherent), and fluorescence was detected at 500–560 nm. For optical sectioning, the distance between planes was 2 μm.

Confocal image stacks were reconstructed and visualized as three-dimensional (3D) volumes with Imaris software (version 7.6.4; Bitplane). The Imaris Spot detection algorithm was used as described by the manufacturer for semiautomatic identification and counting of fluorescently labeled oocytes. Main parameters were absolute thresholding, an object size of 8-μm diameter, and a minimum “quality” score of 100. Errors in the software detection results (both erroneous positive and negative objects) were corrected by manual inspection of the data sets to adjust parameters.

Statistical Analysis

We performed all measurements independently. Random sample populations were analyzed from three different litters. We assessed differences between quantitative variables for normal populations with the two-tailed Student t-test (QuickCalc; http://www.graphpad.com/quickcalc/index.cfm). Throughout the report, statistical significance is indicated as (*) for P < 0.05, (**) for P < 0.01, (***) for P < 0.001, and (ns) for P > 0.05.

RESULTS

Development of an Approach to Image and Identify All Oocytes in Mouse Embryonic Ovaries

In order to better understand numerous facets of the fetal phase of oogenesis, the field requires a method to count oocytes that is efficient, scalable, and accurate. To fulfill this ideal criteria, we turned to an approach that would allow for identification, detection, and quantification of total oocytes in situ, such as in whole-mount embryonic ovaries, without the need for their serial sectioning. Our experimental strategy involves the preparation of embryonic ovarian tissue for whole-mount immunofluorescence staining, optimization of the immunofluorescence protocol, treatment of ovaries with sucrose and ScaleA2 reagent to render tissues transparent, imaging of the entire sample by confocal microscopy, and semiautomatic analysis of the data sets by image processing software (Fig. 1A).

We first aimed to optimize whole-mount immunofluorescence conditions in the embryonic ovary for complete antibody penetration throughout tissues, sharp immunofluorescent labeling of oocytes at different embryonic stages, and maintenance of tissue integrity. We determined that 1-h fixation of freshly dissected embryonic ovaries in 2% PFA in PBS is sufficient to homogenously treat the entire tissue without the need for subsequent antigen retrieval. For the immunostaining, we optimized incubation times and detergent concentrations to modify conditions for whole-mount tissues. We found that 0.5% Triton X-100 in the permeabilization solution improved staining without affecting tissue integrity. Moreover, adding 0.15% glycine pH = 7.4 in blocking solutions prior to applying antibodies strongly decreased autofluorescence. The addition of glycine to these buffers is important when using a glutaraldehyde or paraformaldehyde fixation because it is known to bind free aldehyde groups that would otherwise bind the primary and secondary antibodies and create high autofluorescence. Since the ultimate goal of this proposed method is to precisely detect individual oocytes, we chose to use a germ cell-specific TRA98 antibody that stains nuclei rather than cytoplasm of testicular and ovarian germ cells [22]. For detection of TRA98, we used Alexa-488 or −568 secondary antibodies. Alternatively, use of a secondary antibody in the far red emission spectrum greatly reduces background due to autofluorescence and facilitates detection of positively immunofluorescent cells (Fig. 1B).

The initially thin and elongated fetal ovary gradually transforms to a thicker, rounded form by birth. Consequently, the ovary becomes increasingly opaque even to a confocal...
FIG. 1. Devising an approach to detect and image all oocytes in intact embryonic mouse ovaries. A) Diagram displaying principal steps of an approach to accurately enumerate oocytes in mouse embryonic ovary. B) Lateral view of E18.5 whole-mount ovary immunostained with germ cell-specific TRA98 antibody (white) (top). Inset is a zoom in the center of the ovary on germ cells (bottom). C) Consecutive confocal Z planes of E18.5 ovaries stained with DAPI and imaged by confocal without ScaleA2 treatment (top) after 6 days of ScaleA2 treatment without (middle) or with (bottom) sucrose preincubation. Numbers show depth of tissue in micrometer imaged by confocal microscopy. See also Supplemental Movies S1–S3.
microscope, limiting imaging to cells lying no deeper than 40 μm from the ovarian surface (Fig. 1C and Supplemental Movie S1; Supplemental Data are available online at www.biolreprod.org). Since precisely counting numbers of oocytes within the ovary requires the ability to image through the entire tissue, we had to optically clear ovaries after immunofluorescence staining. A variety of tissue-clearing reagents, such as BABB, dDISCO, CLARITY, methyl salicylate, Mount Clear, Focus Clear, and ScaleA2, have been described to date [23]. We chose the ScaleA2 reagent because of its compatibility with fluorescent proteins, low cost, ease of use, and, importantly, minimal effect on tissue integrity [20]. This urea-based solution has been reported to clear whole brain and thick brain sections of several transgenic mouse lines, thy1-YFP, EGFP, and other GFP-like lines [20]. We initially optimized the application and duration of ScaleA2 incubation on stage E18.5 ovaries when they are thicker and more opaque. We used ovaries that were freshly dissected, fixed, and stained with DAPI alongside ovaries that were incubated in serial sucrose solutions after fixation as a cryoprotectant. Without ScaleA2 incubation, optical sectioning by confocal microscopy was possible through only 40 μm; however, after 6 days or longer of incubation in ScaleA2, this depth increased to 95 μm (Fig. 1C and Supplemental Movie S2). Furthermore, sucrose preincubation of tissues allowed for complete transparency through the entire ~215-μm thickness of ovary (Fig. 1C and Supplemental Movie S3). Hama et al. [20] implicitly mentioned this sucrose step as authors treated thick sections of brain with scale reagents, that is, brain sections that were preincubated in sucrose solution.

In the Hama et al. [20] study, the authors used a transgenic fluorescent mouse line and showed the reversibility of ScaleA2 for retrospective immunostaining, but the direct compatibility of the ScaleA2 reagents with immunoreactions needed to be tested. Therefore, we applied the sucrose preincubation and ScaleA2 clearing steps to immunostained samples using ovaries at both E15.5 and E18.5 stages, when the ovary is thin and thick, respectively. These stages also represent key time points in fetal oogenesis when a large proportion of oocytes are eliminated by FOA [9]. We found that the combination of sucrose preincubation and ScaleA2 reagent sufficiently cleared immunostained ovaries within 3–6 days of incubation without altering immunofluorescence signals. It is important to note that during these long and repeated incubations, care is taken to avoid the accumulation of threads and dust particles that easily stick to the tissue and interfere with subsequent microscopy and analysis. The use of filtered solutions and nonstick 1.5-ml tubes should minimize these complications.

One of our main goals is to develop a robust method to study processes of fetal oogenesis that can be widely used among laboratories so that data can be directly and confidently compared. Therefore, to detect and image positively immunolabeled germ cells throughout entire ovaries, we used single-photon confocal inverted microscopy that is most commonly used among laboratories. Stained ovaries were submerged in ScaleA2 reagent in a glass-bottom dish and imaged using a 25× water immersion objective with a long working distance of 2.4 mm, which greatly exceeds the distance between the objective, the dish, and the top of the ovary (200–400 μm). The refractive index of water is compatible with ScaleA2 reagent. While long working distance objectives compatible with ScaleA2 reagent are commercially available, in our experience, these could be readily substituted by standard objectives. Germ cell nuclei diameter at E15.5 and E18.5 are 8–10 μm. To permit the capture of oocytes through several confocal planes, we performed imaging of the entire ovary every 2-μm confocal plane. This setup allows for limiting exposure of the tissue to the confocal laser and to extract for each oocyte four to five confocal planes. Therefore, the 3D reconstruction of each oocyte within entire ovaries was highly precise. While the signal intensity is obviously stronger in the outer oocytes compared to those positioned deeper in the ovary, we have determined that all oocytes could be sufficiently imaged to be used in postimage analysis of 3D data sets.

Automatic Detection of Positively Immunolabeled Germ Cells to Accurately Estimate the Total Number of Oocytes in Whole, Intact Mouse Embryonic Ovaries

Several 3D reconstruction software packages, such as Volocity, Metamorph, or Imaris Bitplane, can be used for visualization, detection, and counting of TRA98-immunolabeled germ cells. Furthermore, additional information, such as spatial organization of oocytes or statistical analysis of the distribution of oocytes within ovaries at different stages, can be obtained from these images. We used Imaris Bitplane to reconstruct 3D images from confocal planes of E15.5 and E18.5 ovaries (Fig. 2A and Supplemental Movies S4 and S5). As suggested by germ cell staining on ovarian sections, we observed widespread dispersal of oocytes throughout the entire E15.5 ovary, while oocyte distribution in E18.5 ovaries is biased toward the cortex of the organ (Fig. 2B and Supplemental Movie S6).

To detect and extract numbers of TRA98-positive cells, we used the Imaris Bitplane Spot detection algorithm. For this analysis, principal parameters are set to define the size of the detected object (oocytes), the quality of object of interest, and the intensity of signal. Prior to whole ovary analysis, we identified optimal detection parameters by examining a representative ~100-μm³ volume of the ovary and subsequently applied these parameters to the entire ovary. A first critical parameter is the diameter of the object of interest: the oocyte nucleus. Based on our measurements of the size of TRA98-positive nuclei of female germ cells in the representative volume of interest, we established the optimal diameter parameter to be 8 μm. Next, to exclude objects that do not satisfy the diameter parameter, we set a minimum quality score of 100 as suggested by the Imaris software. Finally, to account for experimental variability of TRA98 signal among germ cells, we determined the signal mean intensity in the TRA98-labeled cell (200–4100 a.u., a median of ~600 a.u.). The lowest signal intensity value (200 a.u.) was used to set up the Imaris Intensity parameter to detect TRA98-positive cells. Importantly, the mean intensity background signal in somatic cells did not exceed 100 a.u. and, therefore, none of these cells will be scored. Based on our experience, we recommend setting these parameters for every experimental replicate. We then applied our optimized parameters to the entire ovary and checked for fidelity of assignment of spots to visualize positively labeled cells, as shown for E15.5 and E18.5 stages (Fig. 2C and Supplemental Movies S7–S9). Detailed observation showed imperfect matches that led us to establish the percentage error of automatic detection of positively labeled TRA98 cells by the Spot detection algorithm. To facilitate proofreading of detection of TRA98-positive cells by Imaris software, we virtually extracted a longitudinal representative section from the center of ovary and applied the same parameters used for the entire ovary. We then manually removed the spots that were erroneously assigned to nonspecific fluorescent signals from debris, and we added spots that were missed in areas of high cell density where multiple nuclei

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FIG. 2. Automatic detection of positively labeled germ cells. A) Top, middle, and bottom confocal planes of E15.5 and E18.5 ovaries. Whole-mount ovaries are immunostained with germ cell-specific TRA98 antibody (white) and cleared with sucrose and ScaleA2 reagent. See also Supplemental Movies S4 and S5. B) Front, side, and back views of volume occupied by germ cells in E18.5 mouse ovary. See also Supplemental Movie S6. C) Colocalization of TRA98-positive cells (white) and spot-detected TRA98-positive cells in E18.5 ovary by Imaris built-in algorithm (magenta). Number indicates TRA98-positive cells detected. See also Supplemental Movies S7–S9. D) Steps to estimate percentage error when counting germ cells using Imaris built-in algorithm (top). Example of
were indistinguishable (Fig. 2D). This human proofreading step permits calculating the percentage of error made by the software on E15.5 and E18.5 ovaries ranging from 9% to 14% error. Therefore, the number of cells detected on whole-mount immunostained ovaries represents the absolute number of all germ cells contained in each ovary with a 9%–14% margin of error. It is worth noting that the use of multiphoton confocal microscopy will greatly improve the imaging of positively labeled TRA98 cells (Supplemental Movies S4 and S10). We showed this improvement using Imaris Bitplane software to generate a heat map that displayed the color-coded intensity of signal, from blue to red coding, from weaker to stronger signal intensity along X, Y, and Z positions, and comparing these heat maps between immunostained ovaries imaged with single- or two-photon confocal microscopy (Fig. 2E). The signal intensity is more homogeneous throughout an entire ovary imaged by two-photon compared to single-photon microscopy, therefore improving intensity parameters of the spot tools and consequently decreasing the percentage of error.

**Analysis of Fetal Oocyte Attrition Using a Whole-Mount Immunofluorescence Approach**

A great decrease in the numbers of oocytes between E15.5 and E18.5 followed by a lesser loss of oocytes between E18.5 and few days after birth has been previously reported [9, 14]. The analysis of 19 E15.5 ovaries from three litters of CD1 mice by the whole-mount method described in this article showed that the total number of oocytes ranges from 3970 to 9954 with an average of 6260 ± 1601 (±SD) oocytes per ovary. The analysis of 33 E18.5 ovaries from three litters of CD1 mice by this same method showed that the total number of oocytes range from 3023 to 6991 with an average of 4534 ± 900 (±SD) oocytes per ovary (Fig. 3A). When comparing the number of oocytes per ovary in mice of the same age and same genetic background (CD1), carefully estimated by the widely used sampling method by sections, we observed numbers more than twofold lower than numbers detected by the whole-mount method (∼18,000 and ∼10,000 at E15.5 and E18.5, respectively) [14]. As previously reported, the use of this method to accurately quantitate number of oocytes per ovary showed a loss of oocytes between E15.5 and E18.5. We observed a loss of ∼27% of oocytes in CD1 background, while the previous sampling method shows a loss of ∼44%.

In both studies, the standard deviation appeared to be greater for E15.5 stages compared to E18.5. The analysis of large numbers of ovaries showed a greater range of the total number of oocytes per ovary at E15.5 compared to E18.5. The scalability and precise logistic of the whole-mount method allowed us to obtain more robust data and perform statistical analyses to address biological effects on the number of oocytes. We first wanted to address a long-standing question in the field by placing one male with one female during their dark cycle for 12 h. By convention, the development is counted as 0.5 dpc the next morning, as if the mating occurred around midnight. All dissections were performed at the same time of day within a 30-min difference between litters. The statistical analysis of number of oocytes per ovary from different litters showed great variation between E15.5 litters, while numbers of oocytes per ovary do not significantly differ between E18.5 litters (Fig. 3C). The setting up of mating offers an explanation regarding uncertainty of exact timing of mating and fertilization, which could explain differences in developmental progression between litters, notably if the rate of development is different between E15.5 and E18.5. We therefore reanalyzed our images and closely looked at size of ovaries. Indeed, we noted variation of ovary size at E15.5. For example, we observed E15.5 ovaries that were about 970 μm in length and other smaller ovaries of about 730 μm in length compared to an E18.5 ovary of about 700 μm in length (Fig. 3D). While one can better control for timing of mating by controlling the time a female and male are exposed to each other, it is impossible to control the exact moment of fertilization in vivo. Therefore, using a scalable method, such as the method we present in this article, offers the advantage of robust statistical analysis and acknowledgment of physiological variation.

To further analyze the dynamics of embryonic oogenesis, we took advantage of additional information provided by the Imaris Bitplane software. For example, we determined the ovarian region within which oocytes are distributed at E15.5 and E18.5 stages by extracting the volume of these regions using the Imaris software. We observed a wide variability for both stages and a decrease in volume occupied by oocytes between E15.5 and E18.5 stages, from 6.39 to 12.9 μm³ with an average of 9.36 ± 1.86 μm³ and from 5.04 to 10 μm³ with an average of 7.15 ± 1.23 μm³, respectively (Fig. 4A). We plotted the number of germ cells per ovary related to the volume occupied by oocytes and observed a strong correlation for E15.5 stages, while the correlation for the E18.5 stage was weaker (Fig. 4B). This observation is in accordance with variability observed in terms of number of oocyte per ovary in E15.5 and E18.5 stages.

**DISCUSSION**

During embryonic life, major events, including epigenetic reprogramming, meiotic prophase I, and germ cell cyst breakdown, shape the ovarian reserve of primordial follicles. Much of our current knowledge of how these processes impact the establishment of the ovarian reserve comes from histological techniques, immunofluorescence labeling of ovarian sections, or nuclear meiotic spreads. While providing much insight into the oogenesis, none of these methods allows analysis of oocytes as a cell population in their 3D environment of the ovary. By comparing the widely used sectioning method with the whole-mount immunofluorescence method described in this study, we have come to the conclusion that our new method could provide reliable quantitative and qualitative data on the dynamic and spatial aspects of fetal oogenesis.

The whole-mount method described in this work offers the following advantages over the classical sectioning approach

**Figure 2**. A, B) Heat map of fluorescence intensity signal on whole-mount, immunolabeled E15.5 ovaries imaged by single (top) or multiple (bottom) photons. Color-coded scale is shown. See also Supplemental Movies S4 and S10.

**Figure 3**. A) Heat map of fluorescence intensity signal on whole-mount, immunolabeled E15.5 ovaries imaged by single (top) or multiple (bottom) photons. Color-coded scale is shown. See also Supplemental Movies S4 and S10.
FIG. 3. Quantification of oocytes detected in E15.5 and E18.5 ovaries. A) Box-and-whisker plot of oocyte number per E15.5 and E18.5 ovary. Table displays descriptive statistics. B) Box-and-whisker plot comparing oocyte number in E15.5 and E18.5 left and right ovaries. Table displays descriptive statistics. C) Box-and-whisker plot comparing oocyte number in E15.5 and E18.5 ovaries per litter. Table displays descriptive statistics. **$P < 0.01$ and ns for $P > 0.05$. D) Lateral view of whole-mount TRA98-labeled E15.5 (left and middle panels) and E18.5 (right panel) oocytes. Length in micrometer of each ovary is indicated.
First, it preserves tissue integrity using whole-mount ovaries rather than ovary sections or dissociation. This provides the ability to obtain important information regarding the spatial dynamics of oocytes during embryonic development. Second, it is less laborious, and even though incubation times are longer overall, the hands-on time is reduced fivefold. Third, it is scalable, and the increased number of tissue samples that can be processed simultaneously should lead to statistically sound results and conclusions. We suggest that this new approach provides a more systematic analysis of the dynamics of oocytes during embryonic development.

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<td>Tissue integrity</td>
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FIG. 4. Variation in ovarian size. A) Box-and-whisker plot comparing volume occupied by oocytes in E15.5 and E18.5 ovaries. Table displays descriptive statistics. **P < 0.01. B) Correlation between number of oocytes per ovary and volume occupied by oocytes.

FIG. 5. Comparison of sectioning and whole-mount methods. Comparative table for major concerns when counting oocytes in embryonic ovaries using sectioning versus whole-mount methods.
ACCURATE ENUMERATION OF MOUSE FETAL OOCYTES


Oogenesis and will improve inaccuracies in oocyte quantification that currently prevent comparison of data between laboratories. Although the whole-mount method does not provide an exact absolute number of germ cells, the actual percentage of error can be calculated. Fourth, this method can be used in combination with different markers for meiotic factors, DNA repair, or cell death pathways to redefine oocyte subpopulations of interest at different stages of embryonic development. The preservation of tissue integrity is also advantageous to study oocyte organization in clones and cysts using lineage tracing and markers of intercellular bridges. In addition, the clearing of adult ovaries with sucrose and ScaleA2 reagents and confocal microscopy could help detect and identify the entire population of oocytes developing through folliculogenesis based either on specific immunostaining, on cell organization using a nuclear dye, or on nuclear volume of oocyte [25]. Finally, this method can also be applied to embryonic testsis and provides the opportunity for the quantitative analysis of this critical period in the male germ cells and the gonad (Fig. 6).

ACKNOWLEDGMENT

We thank Eugenia Dikovsky for animal care and Mahmud Siddiqi for microscopy assistance.

REFERENCES