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TITLE:
Accurate Follicle Enumeration in Adult Mouse Ovaries

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Ovary, oocyte, fertility, primordial follicle, stereology, follicle estimates, direct counts

SUMMARY:
Here, we describe, compare, and contrast two different techniques for accurate follicle counting of fixed mouse ovarian tissues.

ABSTRACT:
Sexually reproducing female mammals are born with their entire lifetime supply of oocytes. Immature, quiescent oocytes are found within primordial follicles, the storage unit of the female germline. They are non-renewable, thus their number at birth and subsequent rate of loss largely dictates the female fertile lifespan. Accurate quantification of primordial follicle numbers in women and animals is essential for determining the impact of medicines and toxicants on the ovarian reserve. It is also necessary for evaluating the need for, and success of, existing and emerging fertility preservation techniques. Currently, no methods exist to accurately measure the number of primordial follicles comprising the ovarian reserve in women. Furthermore, obtaining ovarian tissue from large animals or endangered species for experimentation is often not feasible. Thus, mice have become an essential model for such studies, and the ability to evaluate primordial follicle numbers in whole mouse ovaries is a critical tool. However, reports of absolute follicle numbers in mouse ovaries in the literature are highly variable, making it difficult to compare and/or replicate data. This is due to a number of factors including strain, age, treatment groups, as well as technical differences in the methods of counting employed. In this article, we provide a step by step instructional guide for preparing histological sections and counting primordial follicles in mouse ovaries using two different methods: [1] stereology, which relies on the fractionator/optical dissector technique; and [2] the direct count technique. Some of the key advantages and drawbacks of each method will be highlighted so that reproducibility can be improved in the field and to enable researchers to select the most appropriate method

for their studies.

INTRODUCTION:

The immature, meiotically-arrested oocytes stored within primordial follicles in the ovary are the storage unit for the female germline and comprise an individual's lifetime ovarian reserve. Primordial follicle numbers decline naturally with age[1], or alternatively, can be prematurely depleted following exposure to exogenous chemicals, including some pharmaceuticals and environmental toxicants in air, food and water[2]. Given that the primordial follicle number is finite, the quantity and quality of follicles present within the ovary largely determine female fertility and offspring health. Thus, accurate quantification of primordial follicle number in women is essential for evaluating the off-target impacts of exogenous insults on the ovarian reserve.

In women, analysis of the whole ovary is generally not possible, thus non-invasive surrogate measures of the ovarian reserve must be utilized in a clinical setting. Anti-Müllerian hormone (AMH) is the most widely used surrogate biomarker clinically[3]. Serum AMH levels are often measured in women of advanced maternal age, or before and after cancer treatment, such as chemotherapy. However, AMH is produced by growing follicles and not by primordial follicles, and thus, serum levels do not inform on absolute primordial follicle number.

With the absence of methods to accurately determine primordial follicle number in women in situ, counting ovarian follicles in small animal models, such as rodents, remains an essential research tool to assess the degree by which exogenous insults impact on primordial follicles and thus, fertility. Unfortunately though, reports throughout the literature of primordial follicle numbers in rodent models are highly variable[4]. A major reason for this is widely reported technical differences in the counting method employed. Predominately, there are two different techniques described in the literature for enumerating primordial follicles in mice. These include stereology, which employs the fractionator optical dissector method, and direct follicle counts.

Stereology is widely regarded the gold standard as it uses systematic uniform random sampling[5], making it the most accurate method of quantifying primordial follicle number in whole mouse, or rat ovaries[4,6,7]. Stereology is unbiased, as it accounts for the three-dimensional structure of the object of interest[8]. Using an optical dissector/fractionator method, three levels of sampling are applied to quantify primordial follicles using thick tissue sections (e.g., 20 µm) within a known fraction of the total mouse ovary. Firstly, the sampling interval is chosen (e.g., every 3rd section) at a random start (sampling fraction 1, f_1)[4]. Sections are then sampled in a systematic, uniform manner from this point through the whole ovary. Then, an unbiased counting frame is superimposed over the ovarian section and progressively moved along a defined, randomized counting grid (sampling fraction 2, f_2)[8]. Lastly, a known fraction of the section thickness is optically sampled (e.g., 10 µm) and follicles within this area are counted (sampling fraction 3, f_3)[4]. The raw follicle number is multiplied by the inverse of these sampling fractions to obtain the final value. This method requires expert training and equipment, including a microscope with a motorized stage driven by stereological software. Tissues should be preserved in a specialized Bouin's fixative, and embedded in glycolmethacrylate resin to allow for thick tissue sections to be cut using a glycolmethacrylate resin microtome with a glass knife. This method is designed to account

for tissue shrinkage and deformation, to best preserve the three-dimensional morphological structure of the ovary and follicles[9].

Direct follicle counting is the most frequently used method for counting follicles[10]. More common fixatives (i.e., formalin) can be used, followed by paraffin wax embedding and exhaustive serial sectioning using a standard microtome at a thickness of between 4-6 µm. Follicles are systematically counted in the entire tissue section at a defined interval, and then multiplied by the inverse of the sampling interval to obtain the total follicle estimate. This method is quick, easy, can be performed using archived tissues, and prepared using standard histological techniques. It requires only a light microscope with standard imaging capabilities. However, despite these advantages, direct follicle counting lacks the accuracy and strict counting parameters of stereology, making it more prone to investigator bias. Additionally, tissues may undergo shrinkage and deformation during processing, disrupting the integrity and morphology of the ovary and thus making follicle classification and quantification difficult.

The aim of this article is to describe two commonly-used methods to quantitatively assess primordial follicle number in mouse ovaries: stereology and direct follicle counting. We will provide detailed protocols for these two methods and highlight some of their strengths and weaknesses, in order to enhance reproducibility in our field and allow researchers to make an informed decision of the most appropriate counting method for their studies.

PROTOCOL:

Ovaries were collected from female C57BL6J mice. All animal procedures and experiments were performed in accordance with the NHMRC Australian Code of Practice for the Care and Use of Animals and approved by the Monash Animal Research Platform Animal Ethics Committee.

NOTE: A chemotherapy agent shown to deplete primordial follicle oocytes, as determined using stereology[11] and direct counts[12,13] was used in this report to compare the two counting methods in the same animal. Female, 8-week-old (young adult) mice were weighed prior to a single intraperitoneal injection of 75 mg/kg/body weight of cyclophosphamide, or saline vehicle control (n=5/group). This dose has been shown to cause an approximate 50% depletion of primordial follicles, but not reported to cause morbidity or mortality in mice[14]. Ovaries were harvested 48 hours after treatment. One ovary from each animal was fixed in 10% (v/v) neutral buffered formalin solution for 24 hours, and the other fixed in Bouin's solution for 24 hours. Tissue was then embedded in either glycolmethacrylate resin and serially sectioned at 20 µm, or in paraffin and serially sectioned at 5 µm. All tissues were stained with periodic acid Schiff and haematoxylin.

1. Histological preparation: fixation, processing, embedding and sectioning mouse ovaries

1.1 Dissect mouse ovaries by trimming the oviduct and all surrounding fat, without damaging or cutting the ovary itself. If necessary, use a dissecting microscope and fine blade for this step (**Figure 1A**).

1.2 Fix tissues immediately by placing into a small labelled tube containing either Bouin's fixative (stereology), or formalin fixative (direct counts) for 24 hours (**Figure 1B**), before transferring tissues into 70% ethanol.

NOTE: Follicle morphology is conserved best within Bouin's fixed ovarian tissue, embedded into glycolmethacrylate resin (**Figure 2**).

1.3 Process whole fixed ovaries and then embed in glycolmethacrylate resin for stereology (**Supplementary File 1**), or paraffin wax for direct counts using a standard histological protocol.

CAUTION: Resin is toxic, so ensure all tissue processing steps are performed in a fume hood and gloves are worn at all times.

1.4 Use a specialized resin methacrylate microtome (**Figure 1C**) fitted with a glass knife (**Figure 1D**) to exhaustively cut thick glycolmethacrylate resin sections (e.g., 20 µm). Collect the sections at a regular interval (e.g., every 3rd section) onto glass microscope slides for stereology.

1.5 Use a standard microtome to cut thin paraffin sections (e.g., 4-6 µm). Collect tissue sections at a regular interval (e.g., every 9th section) onto a glass microscope slide for direct follicle counts.

1.6 Stain the slides with periodic acid Schiff and haematoxylin (**Supplementary File 2**).

1.7 Coverslip with standard DPX for paraffin sections, or thick DPX for glycolmethacrylate resin sections (**Figure 1E**).

CAUTION: Glycolmethacrylate resin DPX is hazardous, so perform this step in to fume hood.

NOTE: Glycolmethacrylate resin DPX is extremely viscous. The glass coverslip must be adhered firmly by pressing it down with a spatula to ensure the DPX is evenly and thinly dispersed, and there are no air bubbles present under the coverslip (**Figure 1F**).

2 Stereological estimation of primordial follicle number using the optical fractionator

2.1 Turn on the computer, the multi-control unit, the camera and the light source within the stereology setup and set the microscope objective to a low magnification (e.g., 10x).

2.2 Open the stereology software.

2.3 Put the first slide securely on the microscope stage.

2.4 Adjust the light exposure by checking **Automatic** under **Exposure** in the **Camera Settings** panel (**Supplementary Figure 1A**). Alternatively, manually adjust the light exposure.

2.5 Use the joystick to locate the first tissue sample and bring the sample into focus.

2.6 Adjust the white balance, either by clicking on **More Settings** (located bottom right of the **Camera Settings** panel), and then click **White Balance** and click on **Automatic** (**Supplementary Figure 1B**). Alternatively, click the **White Balance** button adjacent to the **More Settings** button (or **Select Area** in **More Settings**), to set the white balance manually by selecting a white area on the section.

2.7 Go to the **Probes** drop down menu and click on **Optical Fractionator Workflow**. Then click **Start New Project** and click **OK.**

2.7.1 If an existing sampling configuration has been saved, under **Sampling Parameters** click **Yes** | **…** and select the desired sampling configuration.

2.7.2 If not, click **No** and manually enter the serial section information (**Supplementary Figure 1C**) and define the probe configuration at step 2.13.

2.8 Click on **Next**, set the microscope to **Low Magnification** and choose **10x** magnification from the dropdown menu.

2.9 Click on **Next**, and then trace around the entire ovarian section – start by left clicking around the section and at the end, right click and choose **Close Contour**.

2.10 Click on **Next**, set the microscope to **High Magnification** – choose **100x Oil magnification** from the dropdown menu.

2.11 Place a drop of oil on the tissue section on the slide and move the microscope objective to 100x magnification.

2.12 Adjust the light exposure (as in step 2.4) and click **Next**.

2.13 Set up the **Sampling Parameters** to define the probe configuration. Here, the counting frame was set to 47.5 µm x 47.5 µm (2,256.25 μm^2) and the step length was set to 100 µm x 100 µm (10,000 μm^2) (**Supplementary Figure 2**). Once the sampling parameters are established, save the template and re-open during subsequent analysis sessions at step 2.7.

2.14 Click on **Start Counting** (**Supplementary Figure 1D**). Focus to the top of the sample, click **OK** and begin counting.

2.15 Use the focusing knob to move through the 10 µm sampling depth and count any primordial follicles whose oocyte nucleus comes into focus. Click **Next** to move to the next area.

2.15.1 Classify follicles as a primordial if the oocyte is surrounded by squamous (flattened) granulosa cells, but no cuboidal granulosa cells (**Figure 2A**).

NOTE: Primordial follicles are distinct from intermediate/transitional follicles, which comprise a combination of cuboidal and squamous granulosa cells (**Figure 2B**), and primary follicles, which are surrounded predominantly by cuboidal granulosa cells (**Figure 2C**). These follicle classes should be quantified separately.

2.15.2 Count only follicles in which the oocyte nucleus is visible. The oocyte nucleus must appear within the counting frame or be touching the green inclusion lines of the counting frame (**Supplementary Figure 1E,F**).

2.15.3 If the oocyte nucleus falls outside the counting frame (**Supplementary Figure 1G**) or touches the red exclusion lines of the counting frame, do not count this follicle.

2.15.4 When assessing primordial follicle depletion in response to an exogenous chemical (e.g., chemotherapy), ensure all follicles counted are healthy and thus have normal morphology (**Figure 2**). Count any abnormal or atretic follicles separately. Often, follicle death is induced by insults such as chemotherapy, and quantification of the atretic follicles should be obtained separately in order to distinguish between healthy and atretic follicles, as only healthy follicles comprise the ovarian reserve[15].

2.16 Once counting is complete on that section, do one of the following:

2.16.1 Click **Add New Section**, and then return to step 2.3 to set up the next section for counting.

2.16.2 Click **I've Finished Counting** to end the session. Return the objective to 10x, exit the stereology software and turn off the light source, camera, multi-control unit and computer.

2.17 Obtain the sum raw follicle number (Q) from each tissue section sampled from the entire ovary, then using a spreadsheet, use the equation below to obtain the final value from each replicate animal (N)[4].

$$N = Q \times \left(\frac{1}{f_1}\right) \times \left(\frac{1}{f_2}\right) \times \left(\frac{1}{f_3}\right)$$, where:

N = Total estimated number of follicles within the ovary.

Q = Raw primordial follicle count.

f_1 = Sampling interval. Every 3rd section was sampled thus $f_1 = \frac{1}{3}$ $\left(\therefore \frac{1}{f_1} = \frac{3}{1}\right)$

f_2 = Relationship between the counting frame (sample area) and stepper, calculated as $\frac{sample\ area}{stepper\ area}$. Since the sample area was 2256 µm^2 (47.5 µm x 47.5 µm) and the stepper area was 10000 µm^2 (100 µm x 100 µm), $f_2 = \frac{2256}{10000}$ $\left(\therefore \frac{1}{f_2} = \frac{10000}{2256}\right)$.

f_3 = Fraction of ovarian section sampled. Since 10 µm of the 20 µm section was sampled, $f_3 = \frac{10}{20}$ $\left(\therefore \frac{1}{f_3} = \frac{20}{10}\right)$.

Therefore, $N = Q \times \left(\frac{3}{1}\right) \times \left(\frac{10000}{2256}\right) \times \left(\frac{20}{10}\right)$

NOTE: This protocol describes how to apply these principles of stereological analyses using a

widely cited stereology software (**Supplementary Table 1**); however, other stereological software is available. The principles applied during stereological analyses of ovarian follicles are the same, regardless of the software used to set up the parameters. Stereology is most accurate when 100 or more objects are counted in an adult mouse ovary[4], as this gives a coefficient of error for the estimate below 10%[16]. The sampling parameters outlined here have been optimized to ensure a minimum of approximately 100 objects (i.e., primordial, transitional and primary follicles) can be counted in control adult wild-type C57BL6J ovaries. A pilot study can be conducted including a small sample size to establish the optimal sampling parameters, such as the interval and number of sections to be analyzed and the number of optical dissectors within the sampled sections[17].

3 Estimation of primordial follicle number by direct ovarian follicle counts

3.1 Place the microscope slide securely under a standard light microscope and perform direct counts to obtain raw primordial follicle number.

3.1.1 Classify follicles as a primordial if the oocyte is clearly visible and is surrounded by squamous (flattened) granulosa cells, but no cuboidal granulosa cells (**Figure 2D**).

NOTE: Primordial follicles are distinct from intermediate/transitional follicles, which comprise a combination of cuboidal and squamous granulosa cells (**Figure 2E**), and primary follicles, which are surrounded predominantly by cuboidal granulosa cells (**Figure 2F**). These follicle classes should be quantified separately.

3.1.2 Ensure all follicles counted are healthy and thus have normal morphology (**Figure 2**). Count any abnormal or atretic follicles separately, as only healthy follicles comprise the ovarian reserve.

3.2 Alternatively, take multiple, or stitched high-power (e.g., 20x) photomicrographs of the entire ovarian tissue section to perform counts by opening the image file(s) to perform counts. This can be done manually, or using an automated slide scanner.

3.3 Obtain the sum raw follicle number (Q) from each tissue section sampled from the entire ovary at the predetermined interval. Multiply this number by the inverse of the sampling fraction to obtain the final value for each replicate animal (N), using the equation below.

$$N = Q \times \left(\frac{1}{f_1}\right), \text{ where:}$$

N = Total estimated number of follicles within the ovary.

Q = Raw follicle count (of each individual type, calculated separately).

$\mathbf{f_1}$ = Sampling interval. Every 9th section was sampled thus $f_1 = \frac{1}{9} \left(\therefore \frac{1}{f_1} = \frac{9}{1}\right)$

Therefore, $N = Q \times \left(\frac{9}{1}\right)$

REPRESENTATIVE RESULTS:

A well characterized model of follicle depletion was used, whereby young adult female mice were

administered a single dose of cyclophosphamide chemotherapy, or saline vehicle control (n=5/group) and both ovaries were harvested from each animal after 48 hours. One ovary per animal was prepared as described in Step 1 for each of the two methods: stereology or direct counts. The left and right ovary from each animal was randomly assigned to each group. These data show that when using stereology, a significant depletion of mouse primordial follicles can be detected following chemotherapy treatment (387 ± 11 follicles), versus control (1043 ± 149; p=0.0024) (**Figure 3**). In contrast, using the contralateral ovaries from the same animals, direct follicle counts failed to detect a significant reduction of the ovarian reserve after chemotherapy (490 ± 40), when compared to control (752 ± 139; p=0.1063) (**Figure 3**). Of note, it is clear that primordial follicle number in young adult wild type animals is variable, since the distribution of the saline treated animals is wider, compared to the cyclophosphamide groups, even when counts were performed using stereology (**Figure 3**).

Figure 1. Histological preparation of Bouin's fixed, glycolmethacrylate resin embedded ovaries for stereological analysis. A) An adult mouse ovary (circle, arrow) was closely trimmed of all fat and the oviduct from the mouse ovary using a Feather blade. **B**) Contralateral mouse ovaries (circle, arrow) from the same female adult were dissected, trimmed, then either fixed in Bouin's fixative (left), or formalin for direct counts (right). **C**) A specialized resin methacrylate microtome **D**) fitted with a glass knife (arrow) was used to exhaustively cut glycolmethacrylate resin blocks into 20 µm sections, collected onto glass microscope slides. **E**) Periodic acid Schiff stained ovarian tissue sections on glass microscope slides (arrows) were dipped into fresh histolene (green container) in a fume hood, then a glass coverslip was added on top of drops of GMA DPX. A spatula was used to remove excess DPX and air bubbles. Slides were air dried in the fume hood overnight.

Figure 2. Primordial follicle classification. Representative images of primordial, transitional and primary follicles in glycolmethacrylate resin-embedded (**A-C**) and paraffin-embedded (**D-F**) ovarian sections. **A:** Primordial follicle surrounded by squamous granulosa cells (arrow). Bar = 10 µm. **B:** Transitional follicle surrounded by both squamous (arrow) and cuboidal (arrowhead) granulosa cells. Bar = 10 µm. **C:** Primary follicle surrounded by cuboidal (arrowhead) granulosa cells. Bar = 25 µm. **D:** Primordial follicle surrounded by squamous granulosa cells (arrow). Bar = 10 µm. **E:** Transitional follicle surrounded by both squamous (arrow) and cuboidal (arrowhead) granulosa cells. Bar = 10 µm. **F:** Primary follicle surrounded by cuboidal (arrowhead) granulosa cells. Bar = 25 µm.

Figure 3. Comparison of the optical fractionator and direct counting methods to evaluate mouse primordial follicles. A well-established model of follicle depletion was used as a model, in which 8-week old female C57BL6J mice were treated intraperitoneally with saline, or a 75 mg/kg/body weight dose of the chemotherapeutic, cyclophosphamide (n=6/group). This enabled comparison of the two follicle counting methods to detect changes to the ovarian reserve. In the same cohort of animals, total follicle estimates failed to detect significant follicle depletion, in contrast to stereology which detected a larger and significant depletion of the ovarian reserve.

Table 1. Comparing the strengths and weaknesses of follicle counting methods: stereology vs.

direct counts.

Supplementary Figure 1. Screenshots of the stereology software protocol. A) Adjust the exposure by ticking Automatic (red box) under Exposure in the Camera Settings panel (white box). **B**) Set the white balance by first clicking the More Settings Icon under Other in the Camera Settings panel. Then, select White Balance and click Automatic (red box). **C**) If an existing sampling configuration has not been set, click No (red box) and enter the serial section information (green box). **D**) To begin counting, click click Start Counting (white arrow). **E**) If the nucleus of a primordial follicle is visible within the counting frame, this follicle is counted (white dotted circle). **F**) If the nucleus of a primordial follicle is touching the green inclusion lines of the counting frame, this follicle is also counted (white dotted circle). **G**) If the nucleus of a primordial follicle is not visible within the counting frame or is touching the red exclusion lines of the counting frame, this follicle is not counted (white dotted circle).

Supplementary Figure 2. Setting up the Sampling Parameters. Follow sequential steps in left-hand panel to define the Probe Configuration. **A**) Measure mounted thickness. Ensure 'Refocus to top of section at each grid site' is unticked (red box). Tick 'Manually enter the average mounted thickness' (green box). Enter average mounted thickness as 20.00 µm (blue box). **B**) Define the counting frame size. Under Counting Frame Display, tick 'Force the counting frame display to be square' and 'Centre on live image' (red box). Under Counting Frame Size, enter 47.5 µm for X and Y (green box). **C**) Define SRS grid layout. Manually enter the grid size as 110 for X and Y (red box). **D**) Define dissector options. For top guard zone height, enter 1.00 µm (red box). For optical dissector height, enter 10 µm (green box). Under Focus Method, select 'Manual focus' (blue box). **E**) Save sampling parameters. To save these settings, enter a Name and Comment then click 'Save your Current Settings' (white arrow). **F**) Under Current Sampling Parameter Settings, ensure your settings match those displayed.

DISCUSSION:

This article provides a step-by-step protocol for the gold standard technique for enumerating mouse primordial follicles, stereology, and the more commonly employed method of direct follicle counting. Chemotherapy treatment was used to compare and contrast the results obtained from these two different methods within the left and right ovary from the same animal. Both methods revealed high inter-animal variability in primordial follicle numbers. A significant depletion of the ovarian reserve was recorded using stereology, but counting failed to detect a significant reduction in primordial follicle number after chemotherapy administration versus control.

Notably, it is clear that even in an inbred strain of mice, such as C57BL6J, the ovarian reserve in young adult wild type (control) females is widely distributed, as is the case in humans. Factors that influence this and contribute to the establishment of the ovarian reserve remain under investigation[18]. High variability amongst studies of mouse ovarian function poses numerous challenges for the field to compare, or replicate data and to advance clinical translation of novel therapeutics to protect oocyte number and quality[4]. This variability is likely due to a number of factors including not only the counting methods employed, but additional factors such as

differences between animal strain and age; as well as treatment regimens, such as dose and timing. These factors must all be considered when comparing data from different studies. Regardless, the follicle depletion model employed in this study overall highlights the accurate and sensitive nature of the stereology protocol, whilst also demonstrating that the widely reported direct counts method is unable to detect a known significant primordial follicle depletion.

There are strengths and limitations of each counting method detailed in this article (**Table 1**). Stereology is regarded as the gold standard method due to its accuracy when used to determine cell number in a variety of different organs[5]. However, drawbacks of this technique include the time and cost involved, as well as the requirement for specialized equipment and expertise. Together, this has limited the wide-spread use of this procedure for obtaining the most sensitive data.

In contrast, the direct counts method is quick, easy, can be done using archived tissues, and prepared using standard histological techniques. It requires only a light microscope with standard imaging capabilities. However, it does not account for volume changes induced by histological processing, which may disrupt the three-dimensional structure of the ovary. Consequently, follicle morphology is not always adequately preserved, making identification and counting accuracy challenging, especially for inexperienced investigators. While formalin was the fixative used for direct counts in this study, Bouin's fixed tissues can be embedded into paraffin and this may provide better morphology and help researchers to more easily identify primordial follicles. Furthermore, within the literature, sampling fractions for the direct counts method vary widely, ranging from every 3rd to every 10th 5 µm paraffin section[19], which may contribute to large discrepancies in follicle counts between studies. Given that paraffin wax sections are so thin, counting every 9th section helps to prevent oversampling and needless counting.

Additional methods not outlined in this guide, but commonly reported in the literature, are counting in only one central section from the entire ovary and considering this to be representative of the entire ovary, or follicle density. This first method is highly problematic, since primordial follicles are not evenly distributed in the ovary and can be found in clusters. This means a single, or even a few sections of the ovary are not representative of the entire organ, making systematic sampling an essential component to any counting method. Follicle density involves counting follicles in an ovarian tissue sample, then expressing the counts per area of tissue. Given the limitations of obtaining primary human tissue, follicle density is often used as a surrogate measure for absolute follicle number in human ovary, though reports in mice are also common. However, this quantification method does not account for the uneven distribution of follicles throughout the ovary, or fluctuations in ovarian volume, which occur routinely throughout the ovarian endocrine (luteal) cycle. This is important because an accurate measure of density amongst samples relies on conserved tissue area. Furthermore, this method often only samples a small biopsy or fraction of the ovary when analyzing human tissues, thus is not representative of the entire ovary. Follicle density lacks the accuracy achievable with stereology. Even using the same whole ovary, comparative measures of follicle quantification following stereological analysis, with follicle density, did not correspond in mouse ovaries[20].

Although it is the most commonly used experimental animal, the mouse is only one model species. Obtaining ovaries from larger species, including domestic animals or non-human primates, is possible, thus follicle counts from whole ovarian specimens can be accomplished using a modified stereological protocol[21,22]. Finally, while the ability to analyze follicle counts in whole human ovaries is indeed very difficult, it nonetheless can be done when specimens are available, using a modified stereological protocol[23-25].

Future directions in the field may include the expansion and uptake of new techniques. For example, a new methodology of automated detection and counting for primordial follicle oocytes has been described[26]. This method uses convolutional neural networks driven by labelled datasets and a sliding window algorithm to select test data. However, this algorithm was only tested over two ovaries and widespread uptake remains to be determined. Alternatively, recent advances in optical tissue clearing and light sheet microscopy have permitted comprehensive analysis of intact tissues and some studies have investigated this for follicle enumeration in the mouse ovary[27,28].

In conclusion, whilst direct follicle counting is an easy, quick and cost-effective method for enumerating primordial follicles within the ovary, this technique may lack sensitivity, accuracy and be prone to investigator bias. Therefore, despite its drawbacks, stereology remains the best available technique for accurately and reproducibly defining primordial follicle numbers.

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DISCLOSURES:

The authors have nothing to disclose.

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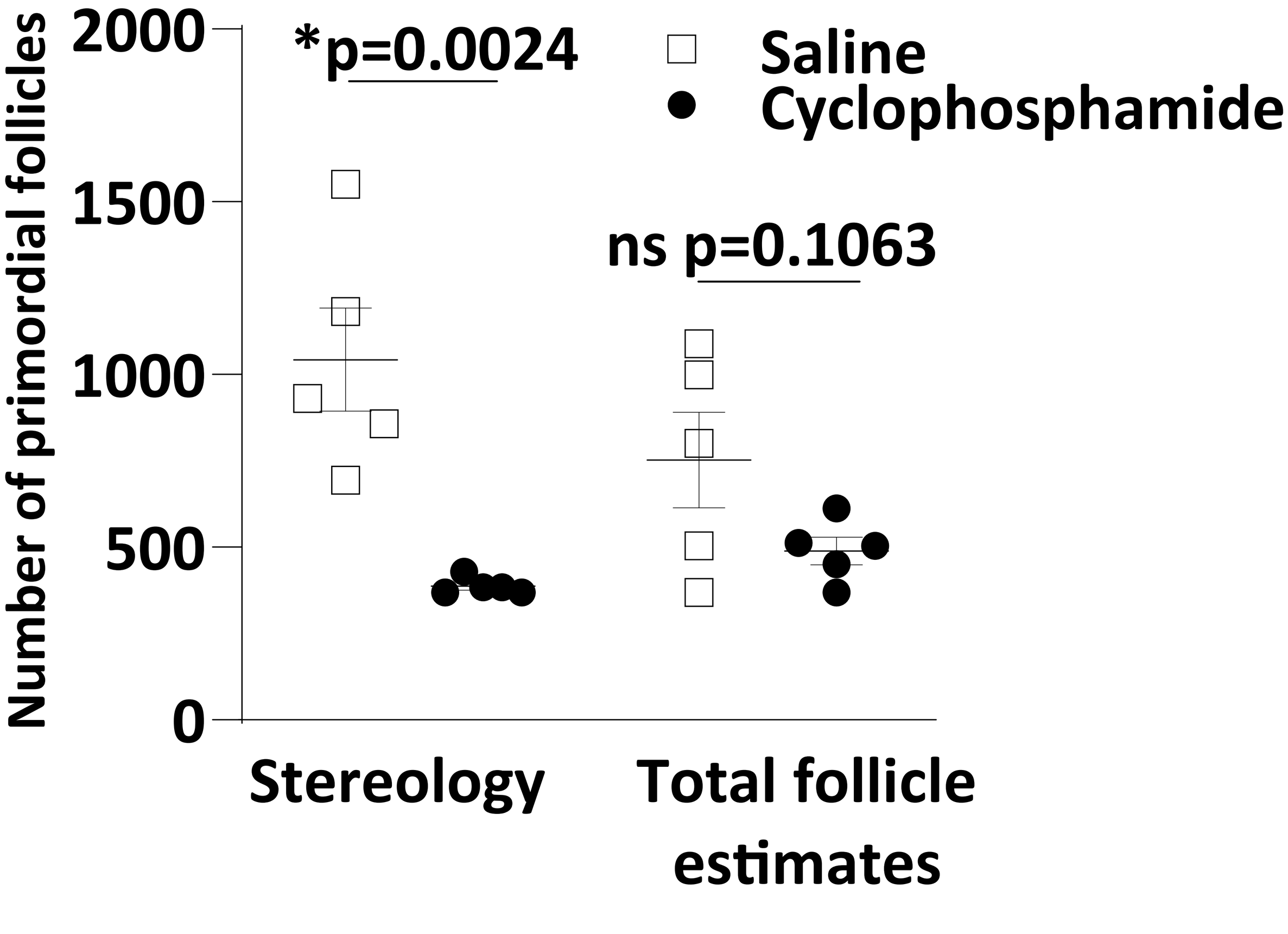

Figure 1

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Figure 1.ai

Figure 2

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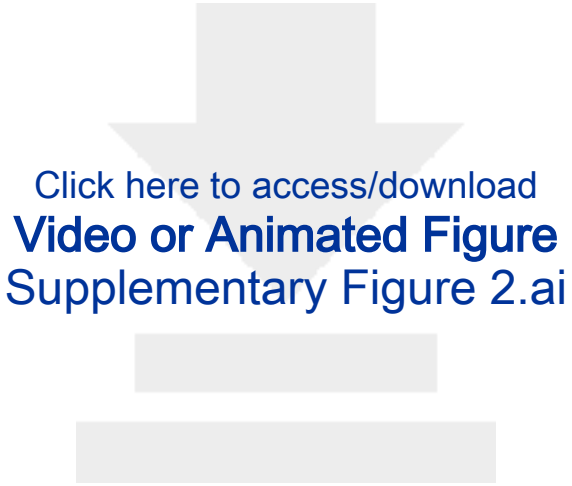
Supplementary Figure 1.ai



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Supplementary Figure 2.ai

Table 1. Comparing the strengths and weaknesses of follicle counting methods: stereology vs. direct counts.

	STRENGTHS	WEAKNESSES
STEREOLOGY	Most accurate way to estimate follicles	Time consuming and costly
	Uses several sampling parameters, making it statistically robust	Requires expert equipment and software
	Highly sensitive, can detect smaller changes in primordial follicle numbers	Samples must be prepared using specific, specialized histological techniques
	Follicle structure is better preserved during tissue processing	Samples must be prepared using specific, specialized histological techniques
	Uniform rules that can be applied by different investigators, thus reducing bias	Samples must be prepared using specific, specialized histological techniques
DIRECT COUNTS	Time and cost effective	Less accurate and sensitive than stereology
	Requires standard laboratory equipment e.g. light microscope	Only one sampling parameter used, making it less effective at detecting small changes in follicle numbers
	Retention of ovarian sections that can be used for immunohistochemical or other analyses	Tissue is more susceptible to changes in volume and follicle structure during processing No uniform set of rules that can be applied, thus more prone to bias from the investigator

Name of Material/ Equipment	Company	Catalog Number
1-Butanol (HPLC)	Fisher Chemical	#A383-1
Acid alcohol	Amber Scientific	#ACDL
Bouin's fixative	Sigma-Aldrich	#HT10132
Cyclophosphamide	Sigma-Aldrich	#C0768-5G
Dibutylphthalate Polystyrene Xylene (DPX)	Sigma-Aldrich	#06522
Ethanol	Amber Scientific	#ETH
Micro Feather opthalmic scalpel with aluminium handle	Designs for Vision	#FEA-745-SR
Formalin fixative	Australian Biostain	#ANBFC
Glass coverslip	Thermo Scientific	#MENCS22501GP
Glycomethacrylate resin RM2165 microtome	Leica Microsystems	#RM2165
Glycolmethacrylate DPX	*made in house	
Histolene	Trajan	#11031
Mayer's haematoxylin	Amber Scientific	#MH
Olympus BX50 microscope	Olympus	#BX50
Olympus immersion oil type-F	Olympus	#IMMOIL-F30CC
Olympus TH4-200 light source	Olympus	#TH4-200
Paraffin wax	Sigma-Aldrich	#03987
Periodic acid	Trajan	#PERI1%
Rotary Microtome CUT 4060	MicroTec	#4060R/F
Schiff's reagent	Trajan	#SCHF
Scott's tap water	Amber Scientific	#SCOT
StereoInvestigator Stereological System	MBF Bioscience	
Superfrost microscope slides	Thermo Scientific	#MENSF41296SP
Technovit 7100 Plastic embedding system	Emgrid Australia	#64709003
Technovit 3040 yellow	Emgrid Australia	#64708805

Comments/Description
Picric acid 0.9% (w/v), formaldehyde 9% (v/v), acetic acid 5% (w/v)
Ethanol 100%
Feather blade for dissections (seen in Figure 1)
22 mm x 50 mm
*Mix 1.5 L Xylene; 800 g polystyrene pellets; 100mL Dibutyl phthalate for 3 weeks
Brightfield microscope fitted with 10x dry & 100x oil immersion objective (numerical aperture 1.3)
Periodic acid 1%
Used to cut paraffin sections
Potassium carbonate, magnesium sulphate, water
Includes StereoInvestigator software, multi-control unit, automatic stage and joystick
1 mm, 72 pcs
500 mL/5 x 1 g/40 mL
100 g/80 mL

Response to reviewer comments JoVE61782
Instructional guide to accurate follicle enumeration in mouse ovaries

Editorial Comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.
   Response: We have thoroughly proofread the manuscript.

Protocol Language

2. Please ensure that ALL text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Some examples NOT in the imperative: 1.1- 1.5, 2.1-2.3.
   Response: We have ensured all text is imperative tense.

Protocol Detail

3. Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
   Response: We have amended the protocol to include more sufficient detail.

Protocol Numbering:

4. Please add a one-line space after each protocol step.
   Response: We have amended the text spacing.

Protocol Highlight:

5. Ensure that the highlighting is under 2.75 pages.
   Response: Highlighting is under 2.75 pages.

6. The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
   Response: A more cohesive narrative has been written.

7. Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
   Response: Complete sentences and headings are highlighted.

8. Notes cannot be filmed and should be excluded from highlighting.
   Response: Notes are no longer highlighted.

Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused.

9. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

    Response: The discussion has been amended to cover these topics.

References:

10. Please spell out journal names.

    Response: References have been amended.

Tables:

11. Please remove the embedded Table from the manuscript. All tables should be uploaded to the Editorial Manager site in the form of Excel files. A description of the table should be included with the Figure legends.

    Response: The table is now in an Excel file.

Commercial Language:

12. JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Stereo Investigator,
    1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

    Response: All commercial language has been removed from the manuscript text.

13. If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

    Response: The table and figure are original.

Reviewer #1:

Nothing to address.

Reviewer #2:

14. Page 1: the authors should consider including a paragraph on the issue of counting one representative slide vs. counting longitudinal sections within an entire ovary. As they well know, the majority of publications on follicle counts as endpoints in the literature are based on taking sections from the central portion of the ovary as a representative sample. Since this is clearly entrenched in current practice, some mention of its inaccuracy is warranted here.

    Response: This is a good suggestion and we have added this to the Discussion. Please see page 7.

15. Page 1: While the ability to analyze follicle counts in whole human ovaries is indeed very difficult, it nonetheless can be done when specimens are available. Likewise, obtaining ovaries from domestic animals or other non-rodent species is less problematic, thus follicle counts from whole ovarian specimens can also be accomplished. This reviewer realizes this manuscript is focused on the mouse ovary, but a brief paragraph on how accurate follicle counts would also be useful for many aspects of ovarian biology in many species would be a nice addition to the Introduction as well as mentioned in the Discussion.

    Response: We have now acknowledged the mouse is only one model species, but a commonly used one. We have added references to other animal species in the Discussion section.

16. Page 1: There are a number of publications for unbiased determination of primordial follicle numbers in primate (macaque and human) ovaries based on combining random sampling, use of a physical dissector and fractionator techniques that allows for estimation of the variance of the overall estimate. This system has been used as the basis for primordial follicle counts according to various stages leading to menopause in women. This reviewer wonders if the authors would or have considered using this method to compare to the data in the present manuscript. To be clear, the present manuscript does not need these data to be complete, just something to think about in the future because the reality of most researchers is that they will most likely never use the GMA-stereology method.

    See the following publications, some of these should be included as references when discussion human ovaries: Miller PB Biol Reprod 56:909-915, 1997; Miller PB Biol Reprod 61:553-556, 1999; Charleston JS, Hum Reprod 22:2103-2110, 2007; Hansen KR Hum Reprod 23:699-708, 2008; Hansen KR Menopause 19:164-171, 2012

    Response: Thank you for this comment. We have amended the Discussion section to include these seminal contributions to the ovarian biology field, using gold standard stereological techniques. We have not previously used a physical dissector in our studies, but agree that a comparative methods study for follicle enumeration would be interesting and useful in the future.

17. Page 7, lines 332-334: as mentioned above, most researchers are not going to embed their precious ovarian samples in GMA for stereology because the sections in between those counted for follicle numbers cannot be used for immunohistochemical or other analyses. So, while stereology may be the most accurate, how much accuracy is needed at the cost of getting so much more information out of ovarian tissue? Perhaps a compromise could be the combined physical dissector/fractionator method which could be mentioned here, and that comparisons could be done to see whether this has similar accuracy to stereology as described within this manuscript.

    Response: While beyond the scope of this instructional guide, we agree with this reviewer on this important point and are currently working on a comparative methods study.

18. Page 10, Table 1: Please add in the Strengths for Direct Counts "retention of ovarian sections that can be used for immunohistochemical or other analyses."

    Response: This has been added to Table 1.

19. Page 2, lines 107-108: How was the well-established depletion of primordial follicles determined as referenced in this sentence since the standard image method in this manuscript shows no differences in follicle number? By the stereology method?

    Response: Yes, the well-established depletion model has been determined using stereology and we have clarified this in the text.

20. Page 3, lines 162-163: this reviewer suggests deleting "should either not be counted" and leave "counted separately." If the authors' message is accurate counting, then both classes of primordial follicles should be included in counts so as not to bias results because counts of just healthy follicles do not represent total number of follicles. Or, make some distinction here about the accuracy of total vs. healthy vs. atretic follicles.

    Response: A line about the distinction between healthy and atretic follicles has been added to this section.

21. Do the authors have any data on counting primordial follicles in young vs. old mice comparing the two methods?

    Response: This is an interesting question but we have not done this analysis.

Reviewer #3:

22. The authors state the importance of stereology for cell counting but the manuscript does not explain how the optical or physical dissector methods work. These are crucial aspects of unbiased sampling in the stereological method.

    Response: This is a good suggestion and more detail about the optical dissector/fractionator method has been added to the introduction. We have not added further information about physical dissectors, as we did not use this type of probe in this instructional guide.

23. Lines 236, 237 and 449 refer to sampling 10 µm of the 20 µm section. In supplementary fig 2 the optical dissector is set to 18 µm.

    Response: This error has been corrected.

24. There is no instruction given for accounting for the oversampling of the optical sections. The diameter of the primordial follicle oocyte nucleus shown in Figure 2a is 11 µm. The optical section width used by the authors appears to be 10 µm. Therefore, if it is possible to see an oocyte nucleus in which say, the bottom 1 µm just intersects with the top of the optical section (and this thus counted), then 10 µm-worth of nuclear volume extends above the optical section. The same principle applies to oocyte nuclei that intersect with the bottom of the optical section. In this example the nuclei being counted can extend up to 10 µm above and below the optical section and thus the objects being counted theoretically extend over a 30 µm section depth, not a 10 µm depth. This will lead to oversampling and overestimation of primordial follicle numbers. Potential solutions to this problem include not counting objects that are in focus at the top of the optical section (but still counting objects that intersect at the bottom of the optical slice) or applying an Abercrombie correction to account for the oversampling.

    Response: Every 3rd 20 µm section is sampled, consecutive sections are never counted and only the middle 10 µm of that 20 µm section is evaluated. Thus, the same primordial follicle could not be counted twice. However, this reviewer is correct that if 1µm of the nucleus is detected in the optical section, then it should be counted, as described by the protocol. In practice, this is likely to be a rare event and it is difficult to confidently identify a structure as a primordial follicle when only 1 µm of the nucleus in seen (even if a little bit more is visible above this). Thus, overall, the risk of significant oversampling is probably very low.

25. The "direct follicle counts" section does not employ the physical dissector stereology method (see Myers et al. 2004 in the manuscript reference list). Failing to use this leads to oversampling caused by biased counting in the z-axis of the sectioning plane and does not meet the high technical standards required by the journal. The appropriate method should be described and the advantages/disadvantages section updated accordingly.

    Response: The purpose of this instructional guide is to provide step by step instructions for how to perform primordial follicle counts using stereology and the most commonly used method in the literature, which is by direct counts. We are not advocating that direct counts is an ideal method and agree that it does have sampling flaws. We do urge that caution must be applied when using direct counts and indicate that stereology is preferred. Whilst we use optical dissector/fractionator stereology ourselves, we have found that direct counts can provide acceptable results in some contexts and are currently working on a study to address this issue in detail.

26. The paraffin-embedded tissue in figure 2 appears damaged and the histology quality is poor. The same authors recently published an article with high quality thin-section ovary histology (https://academic.oup.com/humrep/article/doi/10.1093/humrep/deaa128/5865163). The experiments used for this manuscript should be of the same quality. The low quality of the histology raises concerns about the accuracy of the cell counts.

    Response: This manuscript is an instructional guide for stereology. One of the issues with the commonly used paraffin-embedded, direct counting technique is that formalin fixed, paraffin embedded tissue can vary during the histological processes. The figure here shows that tissue can be variable, and how to recognise the structure of different follicles in this.

27. Were the primordial follicle counts significantly different between the optical dissector counts and direct follicle counts in the control animals? The study is sufficiently powered to detect a difference of this size but the main concern is whether the sample size is large enough to rule out the possibility of type 1 error. The mean of the optical dissector control group is influenced by one large value and the mean of the direct follicle counts are influenced by two points at the lower end. Is it possible that the differences in means arises from random variation? Are the rank orders for each animal the same in the optical dissector counts and direct follicle counts or do they fluctuate?

    Response: There was no significant difference in the counts for control animals using optical dissector counts and direct follicle counts. We have not assessed the rank orders as there were no significant differences.

28. Line 436 - Why does the figure legend and supplementary figure refer to the caudate putamen, a structure in the brain, instead of the ovary?

    Response: This error has been corrected and this term removed from the figure and figure legend.

29. The terminology used in the x-axis in figure 3 is not consistent with the text.

    Response: This has been fixed.

30. What is the numerical aperture of the 100x objective lens?

    Response: The numerical aperture is 1.3. We have clarified this in the table of equipment and reagents.

31. Please define what is meant by "stepper area".

    Response: The stepper area refers to the area encompassing each step (i.e. 100 µm^2). This has now been more clearly outlined within the stereology equation (lines 280-289).

Reviewer #4:

32. Protocol 1.5: I am concerned that an adult ovary counted at only every 9th section is missing a lot of primordial follicles (reflected by your control group in Fig 3). If this manuscript is to put forward a standardised method can you provide firmer definitions around sectioning intervals for staining?

    Response: The 20 µm glycolmethacrylate resin sections are counted at every 3rd section, with 10 µm of each section sampled, whereas every 9th 5 µm paraffin section is counted. This interval was chosen to maintain consistency between the sampling fraction applied between the two techniques, and has been clarified within the protocol. Additionally, within the literature, sampling fractions commonly used range from every 3rd to every 10th 5 µm paraffin section. Given these sections are so thin, counting every 9th section helps to prevent oversampling and needless counting. We have commented on this in the manuscript.

33. Protocol 3.16: F1 value is poorly explained. Although you are taking 20uM sections, the size of your entire ovary may not fit within size range (i.e. divisible by 20). How do you account for the start and end sections?

Response: The sampling designs were all performed in a systematic, uniform manner from a random start. This means that regardless of how the ovary is organised within its embedding material, the sections were chosen as every 3rd one from the first cut of the ovary and continued through the entire structure. A line has been added to the description in the manuscript to explain this.

34. Protocol 3.16 F3: How do you determine what fraction of the whole section has been analysed? This is not clear from the previous steps. Clear explanation of these numerical details is critical in ensuring a standard approach.

    Response: The F3 value of 20/10 is obtained since 10 µm of the 20 µm section is sampled. This has now been clarified within the introduction.

35. A limitation that is noted by the authors is that the "Stereo Investigator software" is one of many additional stereology software packages. Hence the detailed protocol can only be applied to this software. Is there further justification for selecting this software package over others or an option to provide additional generalised methods for stereology follicle assessment?

    Response: Stereo Investigator software is widely cited for a variety of organs/tissues and is a user-friendly software. We are unable to mention 'commercial sounding language' or registered trademarks in the manuscript, so any mention of Stereo Investigator software has been removed and referred to in Supplementary Table 1. For this reason, we are unable to provide justification within the manuscript, however a line was added to the protocol to clarify that other softwares are available and that the principles applied during stereological analysis of ovarian follicles remain the same regardless of the software used to set up the parameters.

36. Results Figure 3 are in opposition to your direct count method. A conserved method of depletion should demonstrate a significant depletion in follicle number. The current result suggests that this method is inaccurate and unsuitable. You have successfully captured the depletion of your treatment groups. However, as pointed out, the control ovary counts are highly variable and trending lower in you direct counts (do you have stats for variation across methods?). The justifications in your discussion for variability (strain age etc) have been controlled for in this study. I am concerned that the variation is a result of insufficient sampling (see point 1 above).

    Response: This is a how-to guide comparing the gold standard technique for quantifying the ovarian reserve in mice (stereology), with the most widely used, but less accurate technique (direct counts). The follicle depletion model employed in this study overall highlights the accurate and sensitive nature of the stereology protocol, whilst also demonstrating that the widely reported direct counts method is unable to detect a known significant primordial follicle depletion. Though not published, we have in the past, increased section frequency in our lab, but found no benefit (the results for counting every 6th section were no different to when every 9th were counted). Nonetheless, as now described in our discussion, researchers have the option of increasing section frequency.

37. Figure 3: How reflective are your total counts to those previous published for this mouse strain/model?

Response: Variability in primordial follicle counts amongst the literature is likely due to a number of factors including not only the counting methods employed, but additional factors such as differences between animal strain and age; as well as treatment regimens, such as dose and timing. These factors must all be considered when comparing data from different studies.

Using the direct counts method, previous data from out lab in the same animal strain has shown a significant depletion of the ovarian reserve 28 days after single dose cyclophosphamide treatment at the same dose of 75 mg/kg/body weight (Saline 731 ± 101 (SEM) versus cyclophosphamide 365 ± 73, p=0.0193).[1] However, the data presented for direct counts in Figure 3 of this study in mouse ovaries collected 48h after a single dose of 75mg/kg cyclophosphamide, or saline vehicle control (Saline 752 ± 139 versus cyclophosphamide 490 ± 40, p=0.1063). Reports from other research groups using the direct counts method have been performed, but not in the same mouse strain. Female 8-9 week-old Balb/c mice were treated with a single dose of 75 mg/kg/body weight cyclophosphamide (Saline 673 ± 286 (SD) versus cyclophosphamide 307 ± 85, p<0.004).[2]

Stereological counts were performed in Figure 3 of this study in mouse ovaries collected 48h after a single dose of 75mg/kg cyclophosphamide, or saline (Saline 1043 ± 149 versus cyclophosphamide 387 ± 11 follicles, p=0.0024). In comparison, stereological counts have only been performed on C57BL6 mouse ovaries following a much higher dose of cyclophosphamide (300mg/kg/body weight) and collected either 5 d after treatment (saline 6852 ± 510 versus cyclophosphamide 720 ± 141, p<0.0001)[3], or 24 h, with 33% of primordial follicles remaining (p<0.01).[4]

Based on the many discrepancies in follicle counting technique, animal strain, age and time of tissue collection in the available literature, the comparison of two different counting methods using both ovaries from the same animal, as performed in this study is the most accurate means of comparison.

38. Title: This manuscript describes primordial follicle enumeration in adult mouse ovaries only.
    Response: We have modified the title.

39. Introduction: "Tissues should be preserved in a specialized Bouin's fixative," Can you explain why Bouin's fixative is necessary for stereology preparation?
    Response: Fixing in Bouin's fixative and embedding in glycolmethacrylate resin allows for the best preservation of the three-dimensional morphological structure of the ovary and follicles. A line has been added to the introduction to clarify this.

40. This manuscript self-cites previously published literature. It may be of value in developing the necessity of these methods to incorporate a more expansive cohort of supporting literature.
    Response: We have referenced a broader range literature.

41. Protocol 3.9 - what dictates the "region of interest"?

Response: The "region of interest" is a term used within the Stereo Investigator software and in this case was intended to refer to the ovarian section. This phrase has been removed and replaced with "trace around the entire ovarian section" within the protocol to avoid ambiguity.

42. Protocol 3.13 - how and why were those "sampling parameters" selected? For a standard protocol more information should be defined here.
    Response: These sampling parameters were selected to allow for a minimum of approximately 100 primordial follicles to be counted per ovary in control animals (i.e. adult wild-type C57BL6J mice) and were determined previously by conducting a pilot study. The requirement for 100 objects to be counted in intrinsic to the stereological method and applies to any object being counted (i.e. is not particular to the ovary or follicles). A note has been added to the protocol to clarify this.

43. It would be helpful to advise on average how many primordial follicles can be counted in a single section using both approaches. I appreciate this is variable but perhaps you could include the raw data for your control sections.
    Response: As this is primarily a how to guide, we have only included minimal data. The range for the number of primordial follicles counted using stereology (raw numbers) is between 34-105. The range for the number of primordial follicles counted using direct counts (raw numbers) is between 41-121.

44. In the PDF manuscript provided to reviewers, supp Figures 1 and 2 are blurred and cannot be read.
    Response: All figures have now been saved as vector files to rectify this.

45. Were L and R ovaries randomly or consistently assigned to assessment groups?
    Response: Left and right ovaries were randomly assigned to assessment groups. A line has been added to the Representative Results section to clarify this.

46. Discussion: "volume changes induced by histological processing" can you explain why this is problematic is obtaining accurate primordial follicle counts.
    Response: Volume changes induced by histological processing, namely by fixing in formalin and embedding in paraffin wax, are known to impact the three-dimensional structure of the ovary. This can lead to follicle morphology being disrupted and making identification and counting of follicles difficult. A line has been added to the introduction and discussion to clarify this.

47. Discussion: "follicle density" could this technique be elucidated or referenced?
    Response: We have expanded this paragraph in the discussion to provide more detail about follicle density, please see page 8.

References

1 Winship, A. L. *et al.* The PARP inhibitor, olaparib, depletes the ovarian reserve in mice: implications for fertility preservation. *Hum Reprod.* doi:10.1093/humrep/deaa128, (2020).

2 Meirow, D., Assad, G., Dor, J. & Rabinovici, J. The GnRH antagonist cetrorelix reduces cyclophosphamide-induced ovarian follicular destruction in mice. *Hum Reprod.* **19** (6), 1294-1299, doi:10.1093/humrep/deh257, (2004).

3 Nguyen, Q. N. *et al.* Loss of PUMA protects the ovarian reserve during DNA-damaging chemotherapy and preserves fertility. *Cell Death Dis.* **9** (6), 618, doi:10.1038/s41419-018-0633-7, (2018).

4 Nguyen, Q. N. *et al.* Cisplatin- and cyclophosphamide-induced primordial follicle depletion is caused by direct damage to oocytes. *Mol Hum Reprod.* **25** (8), 433-444, doi:10.1093/molehr/gaz020, (2019).

Supplementary File 1. Tissue processing protocol for Bouin's fixed, glycolmethacrylate resin embedded ovaries for stereological analysis.

S1.1 Rinse Bouin's fixed ovaries after 24 hours, using 70% ethanol and leave for another 24 hours.

S1.2 Dehydrate the tissues as follows, whilst constantly agitating, for example, using an automated shaker, or roller:
70% ethanol 1 x 1 hour
100% ethanol 3 x 1 hour
100% butanol 1 x 1 hour, then 1 x 3 hours (or overnight).

S1.3 Infiltrate tissue in infiltration solution overnight, whilst agitating.

NOTE: Infiltration solution: Using Technovit 7100 kit – dissolve 1 gram of Hardener I in 100 mL of Technovit 7100 solution. Stir for approximately 30 minutes with a magnetic stirrer until dissolved. (This solution can be stored at 4 °C for 1 month in a glass jar with silica gel).

S1.4 Place ovary tissue in plastic embedding moulds and fill the mould with embedding solution and allow to set at room temperature overnight.

NOTE: Embedding solution: Using 15 mL of the above infiltration solution, add 1 mL of Hardener II and mix thoroughly with a wooden stick. Make this solution up in batches of 30 mL or less, as it can set quickly.

S1.5 Adhere backing block to methacrylate block using Technovit 3040 resin, using a ratio of 2:1 powder to liquid. Stir quickly with a wooden stick and put a couple of drops onto the methacrylate block, then quickly place backing block on top and allow to set.

NOTE: The resin sets very quickly, so do not make up more than 6 g powder and 3 mL liquid at one time. The resin is also used to make the backing blocks in the embedding moulds.

CAUTION: Glycolmethacrylate resin is hazardous, so perform these steps in to fume hood, until resin is set. Hardener II should be added to all infiltration solution and allowed to harden before it can be disposed of. Butanol is hazardous and should be used in the fume hood and deposed of through appropriate chemical waste disposal protocols.

Supplementary File 2. Periodic-acid Schiff tissue staining protocol

S2.1 Warm both periodic acid and Schiff's Solution to room temperature prior to use.

S2.2 Clear sections in histolene for 5 minutes and repeat once in fresh histolene. Rehydrate tissue sections using a graded series of ethanol, including 2 x 2 minutes in 100% ethanol, followed by 2 x 2 minutes in 70% ethanol. Wash tissues in distilled water for 2 minutes.

S2.3 Transfer slides to Periodic Acid for 5 minutes, followed by a 5-minute wash in distilled water.

S2.4 Transfer slides to Schiff's Reagent for 10 minutes and then follow by washing slides under running tap water, or changing water until the color turns pink.

S2.5 Counterstain tissues in Mayer's Haematoxylin for 5 minutes, then rinse using distilled water.

S2.6 Dip the slides in Acid Alcohol (containing 70% ethanol and HCl), then remove immediately and transfer to distilled water.

S2.7 Transfer the slides to Scott's tap water for 1 minute, then rinse in distilled water for 5 minutes.

S2.8 Move the slides through fresh solutions of 70% ethanol (2 x 2 minutes), then 100% ethanol (2 x 2 minutes), and finally histolene (2 x 3 minutes), before securing a glass coverslip and DPX.