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Title: Accurate Follicle Enumeration in Adult Mouse Ovaries

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VO Talent: please record interview statements

Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

3. Interview statements: Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

☒ Interview Statements are read by JoVE's voiceover talent.

4. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **Y**

Protocol Length
Number of Shots: **y**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **JoVE Voiceover Talent**: We hope these in-depth protocols will help to enhance reproducibility in our field, while also allowing researchers to make an informed decision when selecting a counting method for their studies **[1]**.

1.1.1. Use 2.2.1. Talent opening software

REQUIRED:

1.2. **JoVE Voiceover Talent**: As it uses several sampling parameters, stereology is highly sensitive and accurate. However, direct counts are quicker and easier and can be performed on samples prepared with standard histological techniques **[1]**.

1.2.1. Use 2.4.1. Settings being selected

OPTIONAL:

1.3. **JoVE Voiceover Talent**: Stereology is used in studies of ovarian biology for evaluating the impact of toxicants on ovarian reserves. It can also be applied to life sciences for study of the kidney and brain **[1]**.

1.3.1. LAB MEDIA: Figure 3 *Video Editor: can emphasize black data points or no animation*

OPTIONAL:

1.4. **JoVE Voiceover Talent**: Familiarizing yourself with the morphology of the follicles requires experience. Using previous studies to define the morphology, along with establishing standardized criteria of classification, can help with consistency **[1]**.

1.4.1. Use 2.14.1.

Introduction of Demonstrator on Camera

1.5. **JoVE Voiceover Talent**: Demonstrating the procedure will be Lauren R. Alesi (ah-LESS-ee) and Urooza C. Sarma, PhD students from Karla Hutt's laboratory **[1]**.

1.5.1. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera *Videographer: Please film*

Protocol

2. Stereological Primordial Follicle Number Estimation *Videographer: Please collect some B-roll of computer mouse being clicked or Talent working at computer if possible to be used to break up screen captures during editing*

2.1. To estimate the primordial follicle number by stereology using an optical fractionator, turn on the computer, multi-control unit, camera, and light source within the stereology setup **[1]** and set the microscope objective to a low magnification **[2]**.

2.1.1. WIDE: Talent turning on instrument(s)

2.1.2. Talent selecting objective

2.2. Open the stereology software **[1]** and place the first slide securely onto the microscope stage **[2]**.

2.2.1. Talent opening software, with monitor visible in frame
2.2.2. Talent placing slide onto stage

2.3. Use the joystick to locate the first tissue sample **[1]** and bring the sample into focus **[2]**.

2.3.1.1. ADDED SHOT: Talent using joystick to locate tissue sample
2.3.1.2. ADDED SHOT: Talent bringing sample into focus

2.4. In the **Camera Settings** panel under **Exposure**, click and drag the sliding scale to adjust the light exposure **[1]**.

2.4.1. SCREEN: Screenshot1: 00:05-00:10

2.5. In the **Camera Settings** panel, click the **White Balance** button and click a blank area of the section to adjust the white balance **[1]**.

2.5.1. SCREEN: Screenshot1: 00:11-00:18

2.6. Open the **Probes** menu to select the **Optical Fractionator Workflow [1]**. Then click **Start New Project** and **OK [2]**.
2.6.1. SCREEN: Screenshot1: 00:19-00:24
2.6.2. SCREEN: Screenshot1: 00:25-00:31

2.7. If an existing sampling configuration has been saved, click **Yes** under **Sampling Parameters** and select the desired sampling configuration **[1-TXT]**.

2.7.1. SCREEN: Screenshot1: 00:32-00:44

2.8. Click **Next**, set the microscope to **Low Magnification** and select the **10x** magnification **[1]**.

2.8.1. SCREEN: Screenshot1: 00:45-00:56

2.9. Click **Next** and left click to trace the entire ovarian section **[1]**. Right click to end the selection and select **Close Contour [2]**.

2.9.1. SCREEN: Screenshot1: 00:57-01:59 *Video Editor: please speed up*
2.9.2. SCREEN: Screenshot1: 02:00-02:03

2.10. Click **Next**, set the microscope to **High Magnification**, and select the **100x oil magnification [1]**.

2.10.1. SCREEN: screenshot_1: 02:04-02:19 *Video Editor: can speed up*
2.9.1.1. ADDED SHOT: (Noted as 2.9.1.1 on shoot day) Talent changing objective from 10x to 100x and placing a drop of oil over the tissue section on the slide Either shot is fine to use for this. Maybe live shot to break up severl screens in row?

2.11. After placing a drop of oil on the tissue section on the slide, adjust the light exposure as demonstrated **[1]**, refocus the sample and click **Next [2]**.

2.11.1. SCREEN: Screenshot1: 02:30-02:42 *Video Editor: can speed up*
2.11.2. SCREEN: Screenshot1: 02:43-02:55 *Video Editor: can speed up*

~~2.12. If an existing sampling configuration has not been saved, set the **Sampling Parameters** to define the probe configuration and save the template **[1]**.~~

~~2.12.1. SCREEN: To be provided by Authors: Sampling Parameters being set, then template being saved~~

2.13. Next, click **Start Counting [1]**, focus at the top of the sample, click **OK**, and begin counting **[2]**.

2.13.1. SCREEN: Screenshot2: 00:03-00:09 *Video Editor: can speed up*
2.13.2. SCREEN: Screenshot2: 00:10-00:29 *Video Editor: please speed up*

2.14. Use the focusing knob to move through the 10-micron sampling depth and look within the counting frame for any follicles in which the oocyte nucleus comes into focus, then click next to move to the next site **[1]**.

2.14.1. SCREEN: Screenshot3_Take3: 00:02-00:23 Can use Take 1 or Take 2 if preferred

2.15. Classify a follicle as primordial if the oocyte is surrounded by flat squamous granulosa cells but no cuboidal granulosa cells **[1]**.

2.15.1. LAB MEDIA: Figure 2A *Video Editor: please emphasize white arrow*

2.16. Count only follicles in which the oocyte nucleus is visible **[1]**. The nucleus must appear within the counting frame or be touching the green inclusion lines of the counting frame **[2]**.

2.16.1. LAB MEDIA: Supplementary Figure 1E *Video Editor: please emphasize purple circle within dashed white line*
2.16.2. LAB MEDIA: Supplementary Figure 1F: *Video Editor: please emphasize green inclusion line and/or purple circle touching green line*

2.17. If the oocyte nucleus falls outside the counting frame or touches the red exclusion lines of the counting frame, do not count this follicle **[1]**.

2.17.1. LAB MEDIA: Supplementary Figure 1G *Video Editor: please emphasize purple circle in dashed white circle outside of red box*

2.18. When assessing primordial follicle depletion in response to an exogenous chemical, ensure that all of the counted follicles are healthy with a normal morphology **[1]**. Count any abnormal or atretic follicles separately **[2]**.

2.18.1. LAB MEDIA: 2.18.2_Normal
2.18.2. LAB MEDIA: 2.18.2_Atretic

2.19. When all of the sites in a section have been counted, click **Add New Section** to set up and count the follicles in the next section **[1]** or click **I've Finished Counting** to end the session and exit the software **[2]**.

2.19.1. SCREEN: Screenshot4: 00:03-00:06
2.19.2. SCREEN: Screenshot4: 00:07-00:10

2.20. Then obtain the sum raw follicle number from each tissue section sampled from the entire ovary and use the formula as indicated to calculate the final value for each replicate animal **[1]**.

2.20.1. SCREEN: Screenshot5: 00:02-00:35

3. Direct Ovarian Follicle Count Primordial Follicle Number Estimation

3.1. To estimate primordial follicle numbers by direct follicle counting, obtain multiple stitched high power photomicrographs of the entire ovarian tissue section **[1]**. To analyze the images, open them in ImageJ Software **[2]**.

3.1.1. WIDE: Shot showing Talent's face Not sure whether this shot is needed. Maybe just use 3.1.2. for 3.1.1. and 3.1.2.1. for 3.1.2.
3.1.2. Talent opening image, with monitor visible in frame
3.1.2.1 ADDED SHOT: Talent setting up grid.

3.2. To classify the follicles as primordial, oocytes must be clearly visible **[1]** and surrounded by squamous granulosa cells but not cuboidal granulosa cells **[2]**.

3.2.1. LAB MEDIA: Figure 2D *Video Editor: please emphasize oocyte in center of image*
3.2.2. LAB MEDIA: Figure 2D *Video Editor: please emphasize white arrow/squamous granulosa cells indicated by white arrow*

~~3.3. Ensure that all of the follicles are healthy and have a normal morphology **[1]**. Count any abnormal or atretic follicles separately, as only the healthy follicles comprise the ovarian reserve **[2]**.~~

~~3.3.1. LAB MEDIA: To be provided by authors: Shot of normal follicle~~
~~3.3.2. LAB MEDIA: To be provided by authors: Shot of abnormal and/or atretic follicle~~

3.4. Use the counting tool **[1]** to obtain a raw follicle number of the primordial follicles with each section sampled from the tissue **[2]**.

3.4.1 Talent setting up counting frame
3.4.2. Talent at computer, counting follicles, with monitor visible in frame

3.5. Once the sum raw follicle number from each tissue section sampled has been obtained, multiply this number by the inverse of the sampling fraction to obtain the final value for each animal as demonstrated **[1]**.

3.5.1. SCREEN: screenshot_6: 00:01-00:12

Results

4. Results: Representative Optical Fractionator and Direct Counting Method Comparison for Mouse Primordial Follicle Evaluation

4.1. When using stereology **[1]**, a significant depletion of the mouse primordial follicles can be detected following chemotherapy treatment **[2]** compared to that detected in control animals **[3]**.

4.1.1. LAB MEDIA: Figure 3
4.1.2. LAB MEDIA: Figure 3 *Video Editor: please emphasize black Stereology data points*
4.1.3. LAB MEDIA: Figure 3 *Video Editor: please emphasize white Stereology data points*

4.2. In contrast, using the contralateral ovaries from the same animals **[1]**, direct follicle counts failed to detect a significant reduction of the ovarian reserve after chemotherapy **[2]** compared to controls **[3]**.

4.2.1. LAB MEDIA: Figure 3
4.2.2. LAB MEDIA: Figure 3 *Video Editor: please emphasize black Total follicle estimates data points*
4.2.3. LAB MEDIA: Figure 3 *Video Editor: please emphasize white Total follicle estimates data points*

4.3. Notably, it is clear that the primordial follicle number in young adult wild type animals is variable **[1]**, as the distribution of the saline treated animals is wider compared to the cyclophosphamide groups, even when the counts are performed by stereology **[2]**.

4.3.1. LAB MEDIA: Figure 3
4.3.2. LAB MEDIA: Figure 3 *Video Editor: please emphasize standard deviation brackets for both sets of white data points*

Conclusion

5. Conclusion Interview Statements

5.1. **JoVE Voiceover Talent**: It is important to be able to accurately classify primordial follicles, to differentiate between healthy and atretic follicles, and to appropriately include or exclude individual follicles from the overall count **[1]**.

5.1.1. Use 2.14.-2.17.

5.2. **JoVE Voiceover Talent**: These growing follicle and corpora lutea quantification techniques can be used in conjunction with immunofluorescence and cell viability marker staining to quantify the number of follicles expressing specific markers of interest **[2]**.

5.2.1. Use 3.5.1. Talent at computer, counting follicles

5.3. **JoVE Voiceover Talent**: Stereology and direct counting are used to evaluate how disease and exogenous insults can alter the ovarian reserve, leading to more comprehensive evaluations of toxicants and their impacts on female fertility **[1]**.

5.3.1. Use 2.19.1. Counting results being calculated