counting ovarian follicles

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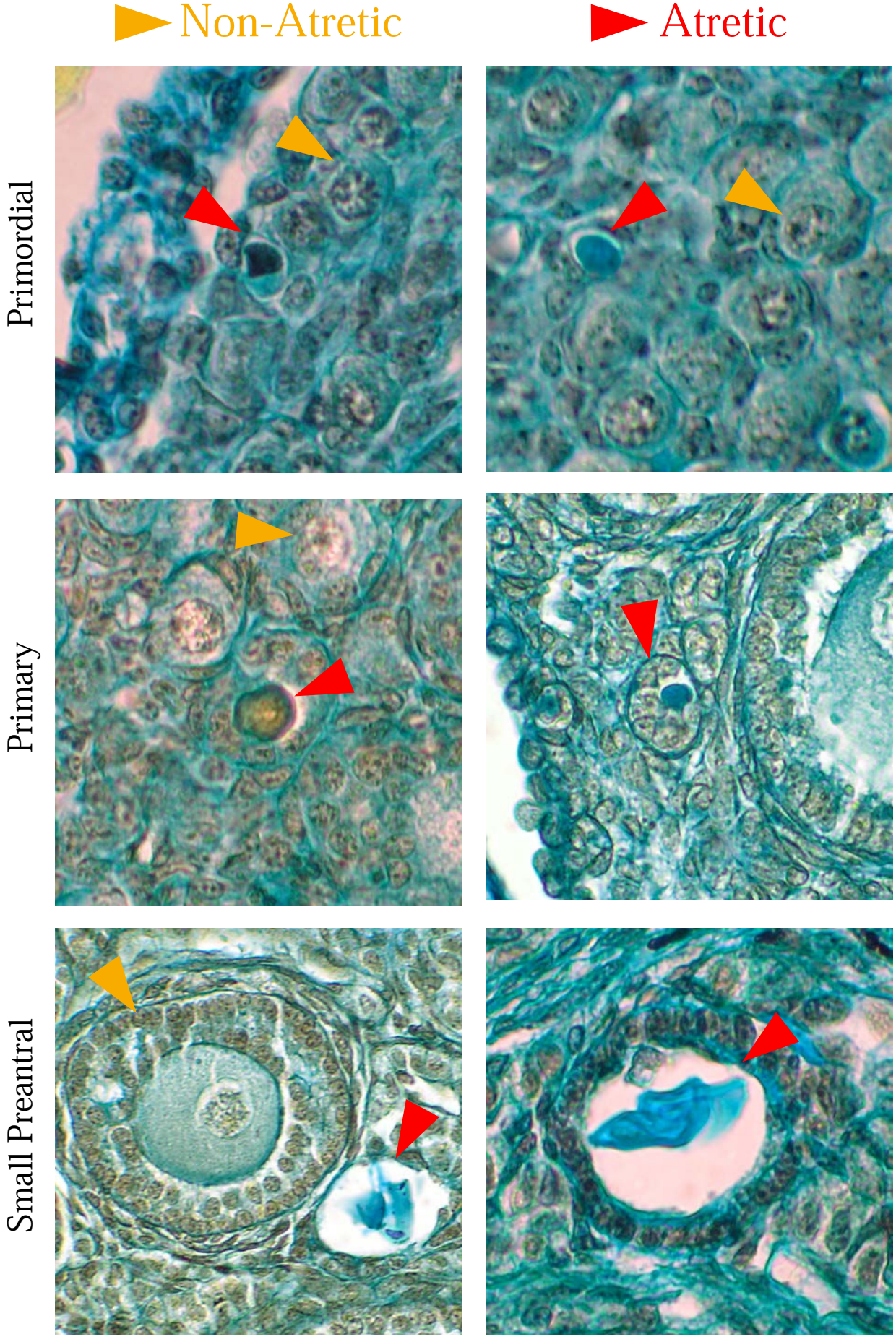
Non-Atretic
Atretic
Primordial
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Non-atretic

Protocol for **Non-atretic** Follicle Counts:

1. **Primordial Follicle**: Identified as having a compact oocyte surrounded by a single layer of flattened granulosa cells.
2. **Primary Follicle**: (Intermediate-stage) Identified as having a compact or enlarged oocyte with a single layer of mixed flattened and cuboidal or all cuboidal granulosa cells.
3. **Small Preantral Follicle**: Identified as having an enlarged oocyte surrounded by at least a partial or complete second layer of cuboidal granulosa cells but no more than four layers of cuboidal granulosa cells.

Notes: Only those follicles containing an oocyte with a clearly visible nucleus should be scored.

Adjusting the focus (plane of view) may provide a clearer assessment of flat or cuboidal granulosa cells. Use of a 60x objective is recommended when counting follicles.

Atretic

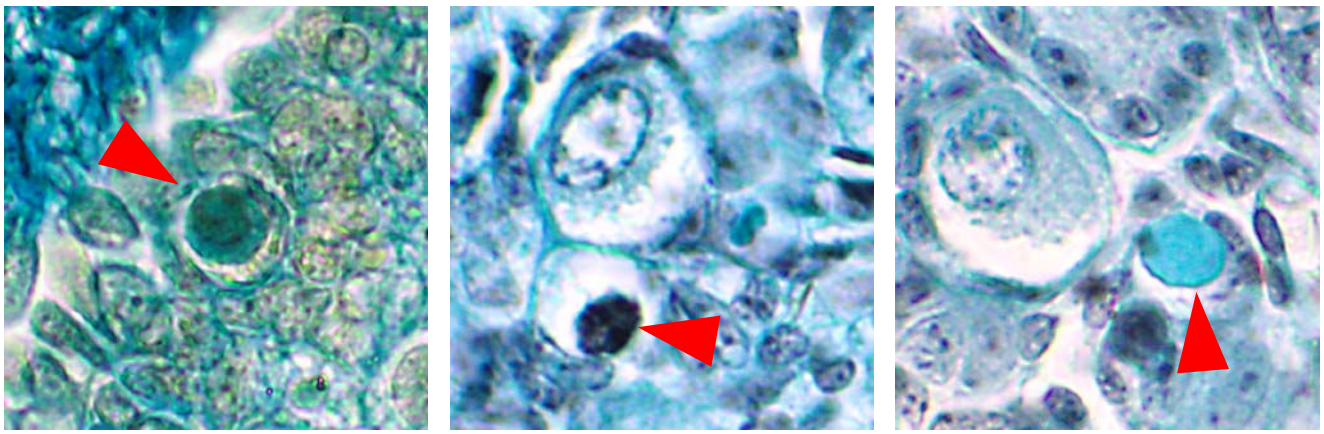
Protocol for **Atretic** Follicle Counts:

1. **Primordial follicle:** A condensed oocyte with a darkened nucleus, with a single layer of flat (squamous) granulosa cells surrounding the oocyte.
2. **Primary and Intermediate-stage follicles:** A condensed oocyte with a darkened nucleus, with a single layer of cuboidal granulosa cells or a mixed population of flat and cuboidal granulosa cells surrounding the oocyte.
3. **Small Preantral follicle:** A degenerating (convoluted, condensed) or fragmented oocyte with discernable nuclear or cytoplasmic material, surrounded by more than one layer of cuboidal granulosa cells.
4. **Grossly atretic follicle ("ZP remnant"):** Zona pellucida remnants from the oocyte lacking discernable nuclear and cytoplasmic material, encased within a basement membrane without or with surrounding granulosa cells.

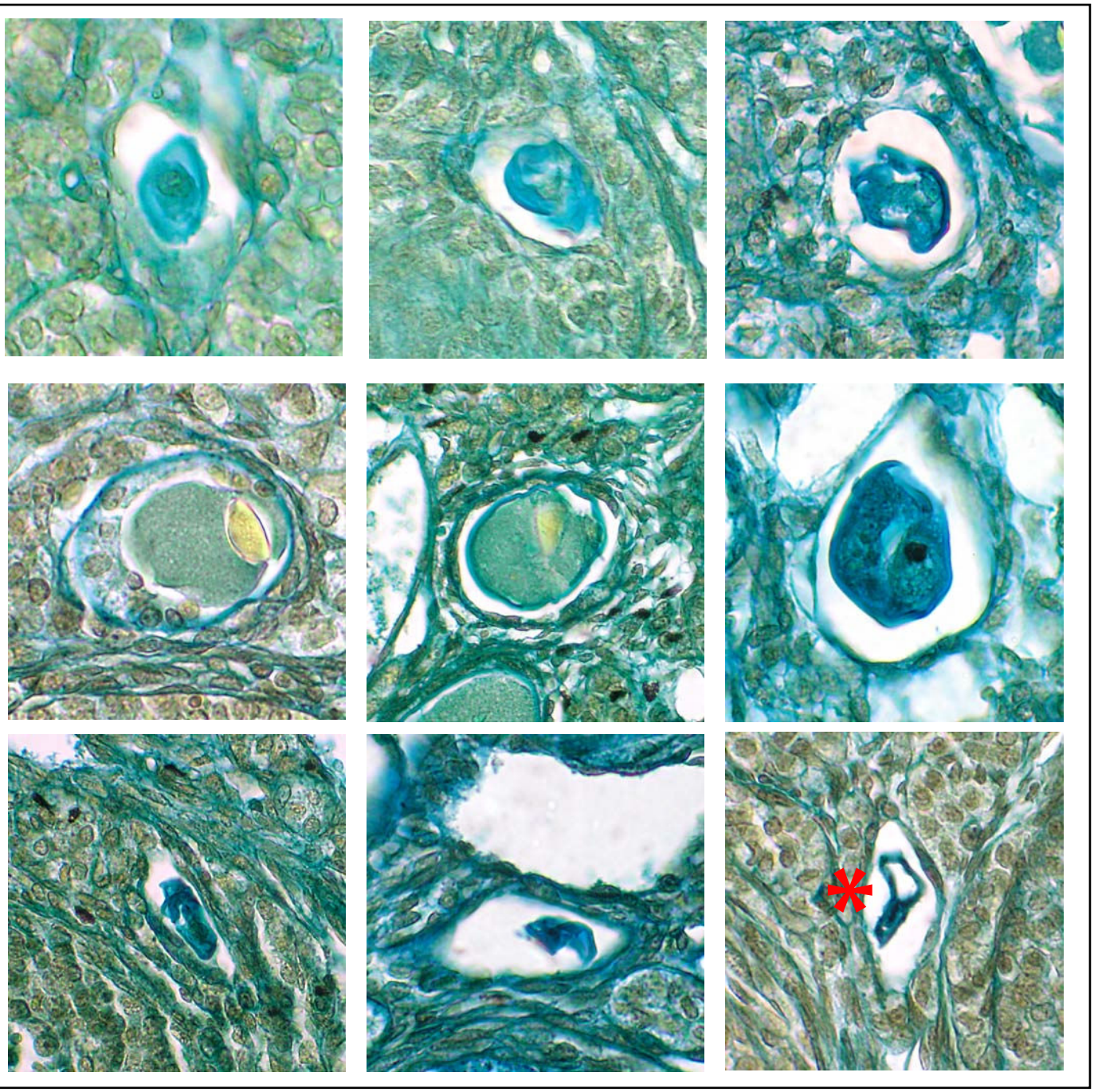
See Page 3, bottom right, for example denoted by *.

Atretic

Primordial



Small Preantral

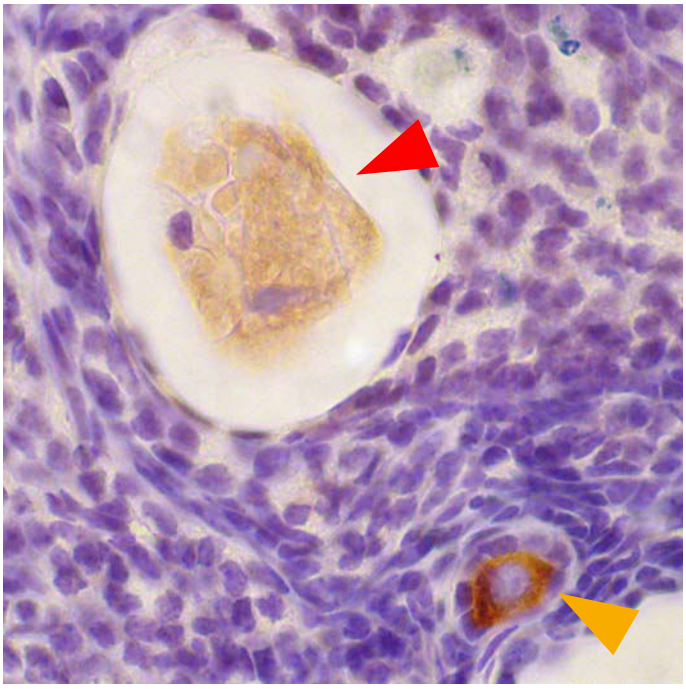


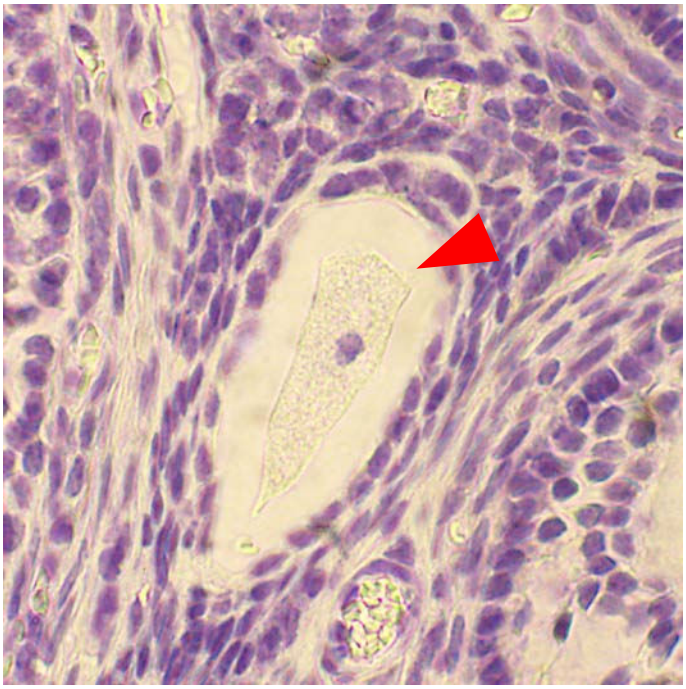
Non-atretic

Atretic

Immunohistochemical detection of MVH: maintenance of signal in fragmenting oocyte

Control lacking first antibody





Routine immunostaining of adult mouse ovaries with anti-Mouse Vasa Homologue (MVH) antibody reveals that fragmented (atretic) oocytes retain MVH protein (brown), especially when DNA is still visible via hematoxylin counterstaining (violet) (left panel). This finding supports our categorization and counting of atretic follicles separately from those scored as "grossly atretic," which may be composed solely of zona pellucida (ZP) remnants. A control where the anti-MVH antibody was omitted is shown (right panel).

General Information for Preparation Prior to Counting:

Remove ovaries, rinse in PBS, and fix in Dietrich's fixative. Ovaries may be transferred into 70% Ethanol after overnight fixation or stored long-term in Dietrich's.

Embed ovaries in paraffin, cut 8 micron serial sections, and place sections onto glass slides.

1. Place slides on warmer for 30-60 min. at about 62 degrees.
2. Take slides from warmer and put in 1st, 2nd, 3rd xylenes for 5 min. each. (Shake slides)
3. Place in 100% ethyl alcohol (2 times, 1st quick rinse then for 3 min.)
4. 95% Ethanol for 3 min.
5. 80% Ethanol for 3 min.
6. 70% Ethanol for 3 min.
7. Distilled cold water 1 change. (Shake of excess water.)
8. Place slides in Weigert's Iron Hematoxylin for 10 min. (Critical Step.) Shake slides.
9. Wash slides in running tap water for 2 min. until a bluish purple color.
10. Counter stain in Picric Acid Methyl Blue for 6 min.
11. Wash excess stain off in 95% Ethanol for 3-5 min. 2-3 changes. (1st wash dispose of as necessary, save second, third wash for next staining.)
12. Dehydrate slides: 95% ETOH 2 min.
    100% ETOH for 4 min.
    100% ETOH for 2 min.
    Shake slides out well.
13. Dehydrate with 4th, 5th, 6th xylenes for 2 min. each.
14. Clean slides of xylenes with ETOH and cover slip with 1-2 drops of mounting medium, clean off excess from slides. Avoid small bubbles if possible. Let dry 24/48 hrs.

Solutions and Equipment:
Dietrich's fixative: 30% Ethanol (v/v), 10% Formalin (v/v—using aqueous 37% Formaldehyde solution), 2% Glacial Acetic Acid (v/v); filter prior to use.

Slides: Fisher Superfrost/Plus Microscope slides-Precleaned (Cat#12-550-15) size – 25x70x1.0mm
Cover slips: Fisher Scientific Premium Cover Glass (Cat# 12-548-5p) size- 24x60-1

Weigert's Iron Hematoxylin: 1:1 mixture of Solution A and Solution B

Solution A: Weigert's Iron Hematoxylin 1g in 95% ethanol 100ml

Solution B: Ferric chloride, 29% aqueous 4ml, Distilled water 95ml, HCl 1ml

Picric Acid, Methyl Blue solution: Picric acid, saturated aqueous 100ml Methyl Blue 40mg

Fisher Scientific Laboratory Counter
Grids to place within microscope oculars: Klarmann Rulings Inc, 10mm x 10mm Grids 16 squares total, Cat # KR-404 (27mm).

Mounting Medium used for slide preparation: Richard-Allan Scientific Cytoseal-60 Low Viscosity (Cat# 8310-16).

OOCYTE COUNTING SHEET **Non-atretic __ Atretic__**

/ / **/ /**

section #	**primordial**	**primary**	**preantral**	**Grossly**	**section #**	**primordial**	**primary**	**preantral**	**Grossly**
5					**5**				
10					**10**				
15					**15**				
20					**20**				
25					**25**				
30					**30**				
35					**35**				
40					**40**				
45					**45**				
50					**50**				
55					**55**				
60					**60**				
65					**65**				
70					**70**				
75					**75**				
80					**80**				
85					**85**				
90					**90**				
95					**95**				
100					**100**				
105					**105**				
110					**110**				
115					**115**				
120					**120**				
125					**125**				
130					**130**				
135					**135**				
140					**140**				
145					**145**				
150					**150**				
155					**155**				
160					**160**				
165					**165**				
170					**170**				
175					**175**				
180					**180**				
185					**185**				
190					**190**				
195					**195**				
200					**200**				
205					**205**				
210					**210**				
215					**215**				
220					**220**				

TOTALS:
x 5 :

*** Multiplication factor of 5 for every 5th section counted per ovary.**
The precision of measurements when follicles are independently counted should be within 10%.