OPTIMIZATION OF TISSUE PREPARATION FOR THE QUANTIFICATION OF GERMINAL CELLS IN HISTOLOGIC SECTIONS OF FATHEAD MINNOW GONADS

Jeffrey Wolf[1], Tilghman Hall[2], Daniel Dietrich[3], Rosanne Ellis[4], Charles Staples[5], Urs Friederich[6]

[1]Experimental Pathology Laboratories, Sterling, Virginia, U.S.A. [2]Bayer Crop Science, North Carolina, U.S.A. [3]Environmental Toxicology, University of Konstanz, Germany. [4]IPMC-TMC GmbH, Neuwilen, Switzerland. [5]Assessment Technologies Inc., Fairfax, Virginia, U.S.A. [6]Dow Europe, Horgen, Switzerland.

Introduction:

The presented study was performed to determine the optimal combination of fixation, embedding and staining procedures for the histopathological and morphometric analysis of fathead minnow gonads following exposure to 10nM 17 -estradiol (E_2). A further purpose was to provide a basis for the optimal distinction and quantification of cell types in gonads by cell counting techniques.

To date the interpretation of data from histopathological analyses of fish gonadal tissue has been restricted due to:

- Unsatisfactory fixation, embedding, sectioning, and staining techniques not permitting clear identification of and distinction between gonadal cell types.
- Unclear definition of various gonadal cell types resulting in interpretational problems.

Study aims:

- Optimize tissue preparation for gonadal cell type differentiation and quantification using light microscopy.
- Provide a robust and reproducible method to quantify the different cell types.

Methods:

- Sexually mature male and female FHM (*Pimephales promelas*) were exposed to 10 nM 17 -estradiol for 10 days at 25°C ± 1°C.
- Gonad pairs were excised and randomly allocated to one of the fixatives, i.e. to the respective embedding and staining combinations (Table 1).

Optimization of fixing and staining procedures:

- Gonad pairs were effaced to largest longitudinal sectional area. Three step sections (each approx. 3-5 m thickness for paraffin-embedded tissues or 0.5-2 m thickness for glycolmethacrylate (GMA)) were microtomed approximately 5-10 µm apart, and stained with H&E or Trichrome stain.
- The suitability of the fixation/embedding/staining combinations was evaluated by light microscopy.

Experimental Design to Determine Optimal Fixation and Staining

All Animals exposed to 10 nM 17 -estradiol for 10 days	10% NBF / Paraffin		Bouin's / Paraffin		EM Fixative / GMA	
	Male	Female	Male	Female	Male	Female
H&E	1	1	1	1	1	1
Masson's Trichrome	1	1	1	1	1	1

Table 1: Experimental design

Histopathological Examinations:

- **Gonadal Staging:** Gonads were staged using the US Geological Survey's (USGS) Biomonitoring of Environmental Status and Trends (BEST) system developed for carp (1).
- **Manual Type-Tagging of Individual Cell Types:** Image Pro® Plus (IPP) software was used to manually type and tag individual cells on digital images of the gonads.

Type-tagging of ovarian follicles

- 4x objective, two digital photomicrographic images (2.2 x 2.9 m subject area) obtained from left and right ovary sections (i.e. four images total per fish). These four images allowed the detection and type-tagging of >100 follicles (minimum of 100) per fish.
- Ovarian follicles were sequentially type-tagged with one of six different solid-coloured squares (Fig. 5).

Type-tagging of testis cell types

- 40x objective, four digital images (0.22 x 0.29 m subject area) of left and right testis sections (i.e. eight images total per fish).
- A virtual grid consisting of 400 (20 x 20) individual boxes that were separated from one another by 0.015 m horizontally and 0.011 m vertically was applied to each image as an overlay. Each box was tagged with one of six different coloured dots, according to the cell type of interest located beneath the box, i.e. spermatocytes, spermatozoa, spermatids, large intralobular cells (LIC), vacuolated cells (VC), or basophilic body cells (BBC), see Fig. 6.
- From eight images per male, a minimum of 2000 cells were differentially type-tagged.

Cell categories:

Ovarian follicles (Fig. 3):

Perinucleolar follicles - generally the smallest follicles, characterized by a thick zone of granular dark basophilic ooplasm that surrounded a sharply-defined core of flocculent pale lavender karyoplasms.

Cortical alveolar follicles - (approx. 100-500 µm diameter), generally larger than perinucleolar follicles, characterized by amphophilic ooplasm that contained numerous, predominately pale pink cytoplasmic bodies (cortical alveoli).

Early vitellogenic follicles - (approx. 200-250 µm diameter), resembled cortical alveolar follicles, except the ooplasm of the former contained a small to moderate amount of bright eosinophilic granular to globular material (yolk granules).

Mid-vitellogenic follicles - eosinophilic yolk granules were larger and more numerous, filling the central area of the follicle. The central yolk area was enveloped by a zone of blue-grey ooplasm that was greater than 50 µm in width.

Late-vitellogenic follicles (approx. 700-1000 µm) large central yolk area, with decreased or in-apparent blue-grey zone.

Testicular cell types (Fig. 4)

Spermatozoa - small (approx. 12 m diameter), dense, circular, essentially acytoplasmic, deep basophilic cells, present in large numbers within the lumina of spermatogenic lobules.

Spermatids - slightly larger (approx. 23 m diameter) cytoplasmic cells, present in small clusters or individually within the superficial germinal epithelium, occasionally free within lobular lumen.

Spermatocytes - primary and secondary (approx. 48 m diameter), well-defined nests of cells within the superficial germinal epithelium. Larger than spermatids, somewhat less densely basophilic (faint) nuclei, and small amounts of very pale cytoplasm with polygonally-shaped borders.

Large intralobular cells (LIC)- small (approx. 5-10 m diameter) clusters or individually within the superficial or deep germinal epithelium. Large, open-faced, pale nuclei, nucleoli prominent, and abundant grainy amphophilic cytoplasm, frequently containing scattered small red granules.

Vacuolated cells (VC) - located within germinal epithelium or as protrusions into the lobular lumen. Large cells with variable degree of cytoplasmic vacuolation. Some vacuolated cells had large clear cytoplasmic vacuoles that did not displace the nucleus (resembled LIC or spermatocyte nuclei). Others had greatly expanded vacuolated cells, pale, foamy cytoplasm and marginated, condensed, sharply angular, hyperchromatic nuclei.

Basophilic body cells (BBC) - vacuolated cells containing one or more variably-sized spherical bodies (basophilic or basophilic with an eosinophilic rim) within one or more large cytoplasmic vacuoles.

Results and Discussion:

Optimal combination for tissue preparation:

Females

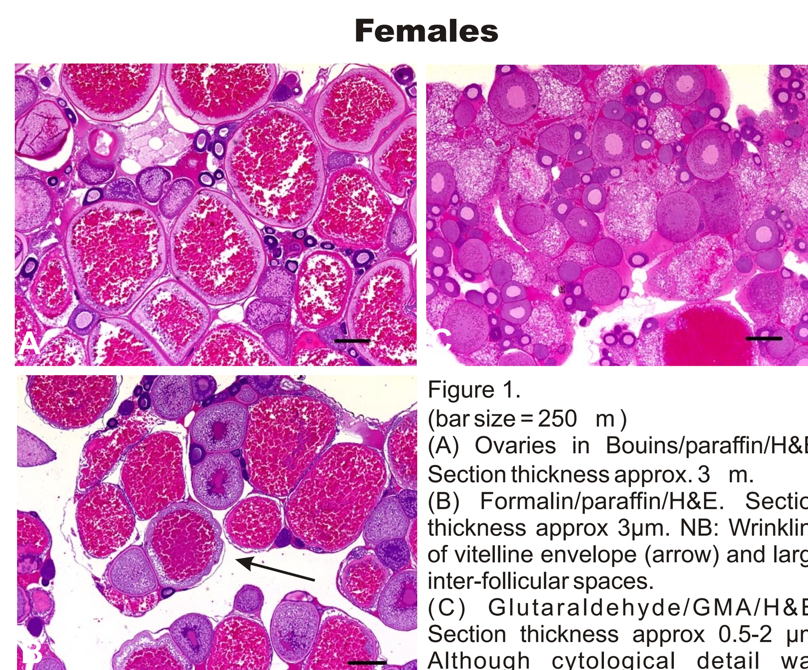

Figure 1. (bar size = 250 m) (A) Ovaries in Bouins/paraffin/H&E. Section thickness approx. 3 m. (B) Formalin/paraffin/H&E. Section thickness approx 3µm. NB: Wrinkling of vitelline envelope (arrow) and large inter-follicular spaces. (C) Glutaraldehyde/GMA/H&E. Section thickness approx 0.5-2 µm. Although cytological detail was superior in these sections, this increased level of detail did not facilitate the differentiation of follicle types.

Males

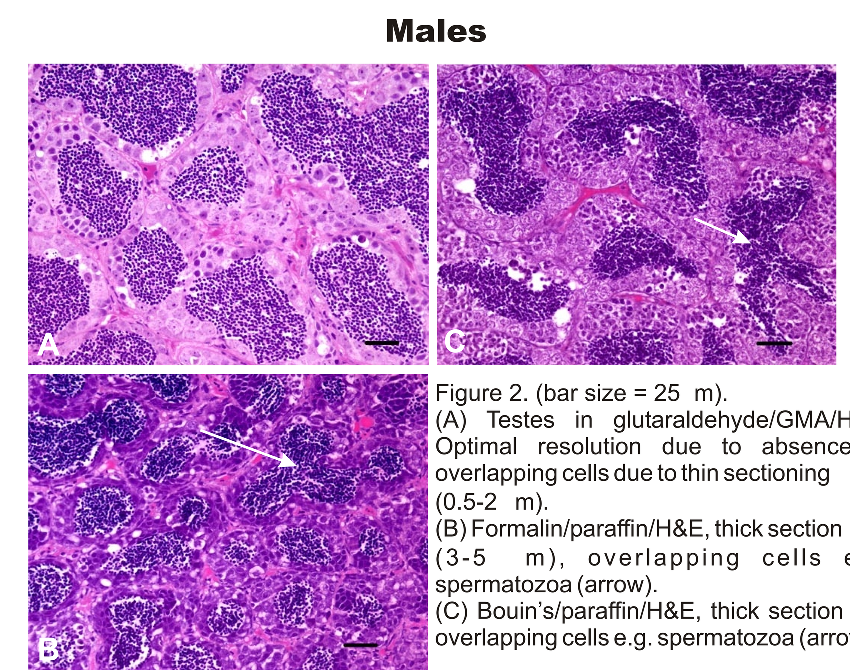

Figure 2. (bar size = 25 m). (A) Testes in glutaraldehyde/GMA/H&E. Optimal resolution due to absence of overlapping cells due to thin sectioning (0.5-2 m). (B) Formalin/paraffin/H&E, thick section (3-5 m), overlapping cells e.g. spermatozoa (arrow). (C) Bouin's/paraffin/H&E, thick section and overlapping cells e.g. spermatozoa (arrow).

Recommended:
Combination of Bouin's Solution (fixation), paraffin (embedding) and H&E (staining) is recommended for optimal quality (Fig. 1A).

Not recommended:
Staining with Masson's trichrome did not provide sufficient cytological detail and differential staining. Example figure not presented.

Recommended:
Glutaraldehyde fixation, GMA (embedding) and H&E (staining) is recommended for optimal quality (Fig. 2A), providing one single cell layer (0.5-1 m) and excellent resolution.

Not recommended:
Formalin and Bouin's (fixation) and paraffin embedding was deemed unacceptable (Figs. 2B & 2C) due to too many overlapping cell layers for optimal cell type recognition.

Testes stained with Masson's trichrome did not provide enough cytological detail and differential staining. Example figure not presented.

Staging/ Cell categories:

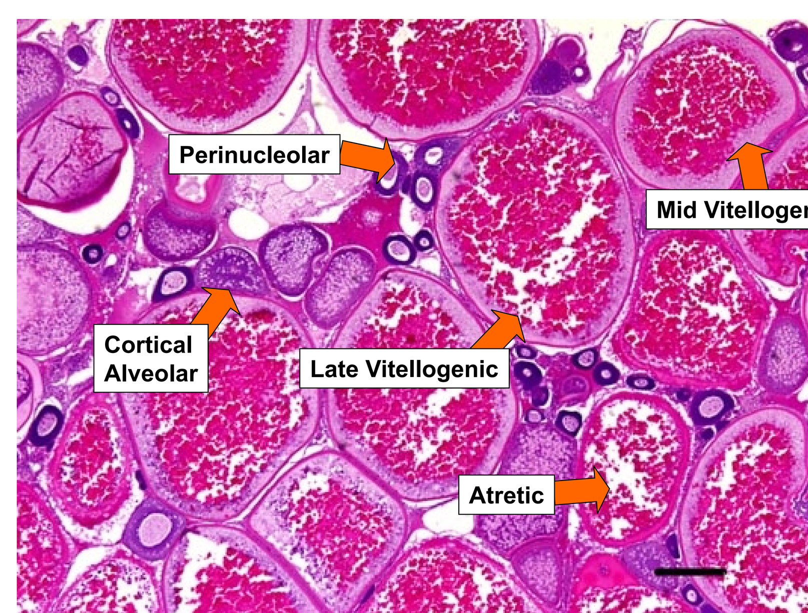

Figure 3. Various stages in the developmental differentiation progression of ovarian follicles (bar size = 250 m).

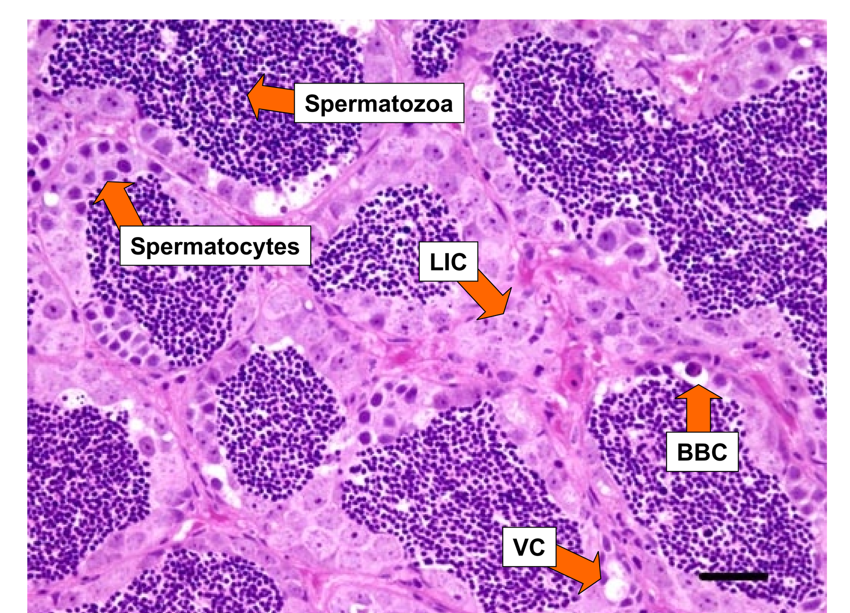

Figure 4. Individual cell types recognized in glutaraldehyde/GMA/H&E sections (bar size = 25 m).

Typical testes cell types e.g. spermatocytes, spermatids and spermatozoa were easily recognizable in 17 -estradiol exposed males, however LICs, VCs and BBCs were present.
LICs, VCs and BBCs are likely to be of spermatogonial or Sertoli cell origin, however, an unequivocal association as to which cell origin could not be determined, which is why descriptive terms were used.

Gonadal Staging and Histopathology:

- Stages 0 through 3
- Follicular atresia
- Follicles in late stages of vitellogenesis prior to their degeneration.
- Similar effects were observed in earlier studies with fathead minnows and other small fish species exposed to17 -estradiol (2;3) and other compounds (4).

Gonadal Staging and Histopathology:

- Stages 2 and 3a
- Infrequently diagnosed, sperm necrosis, granulomas and mineralisation, LIC hyperplasia, multinucleated cells.
- Numerous LICs, VCs, and BBCs observed instead of characteristic cell types (Sertoli cells, spermatogonia, spermatocytes, spermatids and spermatozoa) typical of normal testes.

Quantification/ Cell-type tagging:

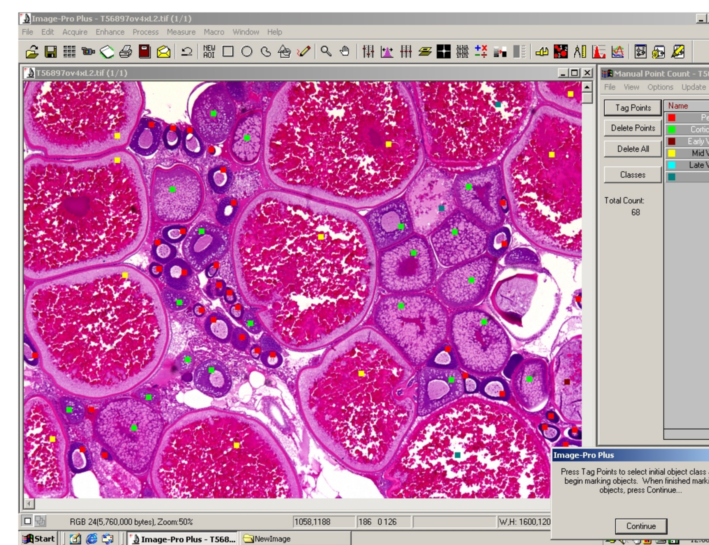

Figure 5. Ovary manual tagging using the Image Pro processing system under low magnification.

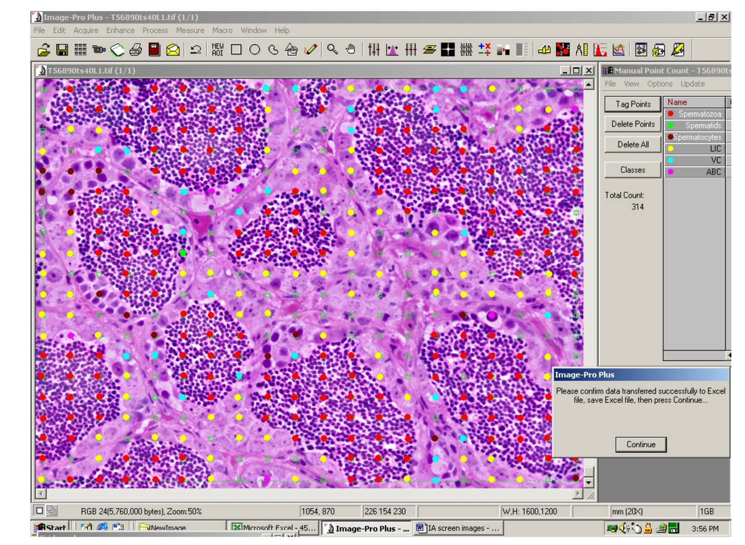

Figure 6. Testis manual tagging using the Image Pro system under low magnification.

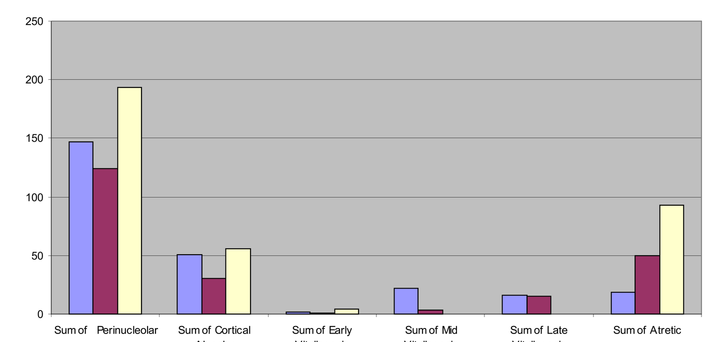

Figure 7. Relative frequency of follicular cell types in left and right ovaries of 3 females (PS-012, PS-014, PS-016) following exposure to 17 -estradiol.

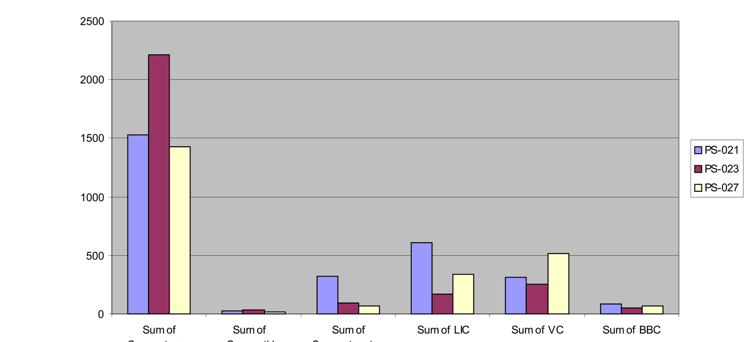

Figure 8. Relative frequency of testicular cell types in left and right testes of 3 males (PS-021, PS-023, PS-027) following exposure to 17 -estradiol.

Manual Tagging:

- Manual tagging provided a precise and straightforward basis for cell type identification, tagging and quantification, therefore accurate evaluation of changes in gonadal cell proportions. Such changes in cell proportions have been reported to occur in fish following exposure to xenoestrogenic compounds (5-7; 8).
- Manual tagging of cells in digital images is recommended for quantitative differentiation of gonadal cell types, because: 1) ability to enlarge digital images as needed allows for more detailed morphological inspection of gonadal cell types; 2) the virtual grid used for testis cell selection facilitates robust, repeatable, and precise tagging of different cell types; 3) it is a rapid technique compared to direct evaluation of glass slides; 4) it affords a permanent and reviewable record of each cell counting analysis, including outlines of the tagged cells.

Conclusions:

This study has identified optimal techniques for 1) preparation, preservation and processing of fathead minnow gonadal tissues for light microscopic examination, and 2) manual tagging of cells in digital images for identification and quantification of gonadal cell types.

References:

1. McDonald, K.K., Gross, T.S., Denslow, N.D., and Blazer, V.S., Reproductive Indicators In: Biomonitoring of Environmental Status and Trends (BEST) Program: Selected Methods for Monitoring Chemical Contaminants and their Effects in Aquatic Ecosystems. Washington D.C., U.S. Department of the Interior, U.S. Geological Survey, Biological Resources Division., 2000, 37. **2.** van Aerle, R., Pounds, N., Hutchinson, T.H., Maddix, S., and Tyler, C.R., *Ecotoxicology*, submitted, 2002. **3.** Gimeno, S., Ed., *OECD Technical Workshop on Gonadal Histology of Small Laboratory Fish*, OECD, Bilthoven, Netherlands, 2002. **4.** Arcand-Hoy, L.D. and Benson, W.H., T Schlenk, D. and Benson, W.H., Eds., New York, Taylor & Francis, 2001, 175p. **5.** Miles-Richardson, S.R., Kramer, V.J., Fitzgerald, S.D., Render, J.A., Yamini, B., Barbee, S.J., and Giesy, J.P., *Aquatic Toxicology*, 47, 1999, 129p. **6.** van der Ven, L., Wester, P.W., and Vos, J.G., *Environmental Toxicology and Chemistry*, in press, 2002. **7.** Sohoni, P., Tyler, C.R., Hurd, K., Caunter, J., Hetheridge, M., Williams, T., Woods, C., Evans, M., Toy, R., Gargas, M. and Sumpter, J.P., *Environmental Sciences and Technology*, 35 (14), 2001, 2917p. **8.** Kinnberg, K., Korsgaard, B., Bjerregaard, P. And Jespersen, A., *The Journal of Experimental Biology*, 203, 2000, 171p.