Novel Acridine Orange Staining Protocol and Microscopy with UV Surface Excitation Allows for Rapid Histological Assessment of Canine Cutaneous Mast Cell Tumors

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Introduction
- Mast cell tumors (MCTs) represent up to 27% of all malignant cutaneous tumors in dogs. Diagnosis is easily made with cytophogy, however the grade of the tumor determines prognosis and required margins.
- Grade is not typically known until at least 48 hours after surgery, pre-surgical incisional biopsies are imperative due to high metastatic risk to the patient.
- Up to 30% of all mid to high grade mast cell tumors recur when incompletely excised. Recurrence is correlated with progression to malignancy in 55% of cases.
- Low grade tumors are unlikely to recur regardless of margins chosen. When removed with wide margins, the patient endures unnecessary short term morbidity and long term complications.
- MCTs commonly occur in areas such as the face, perineum, and distal limbs where removal may not be realistic.
- Very recent attempts to solve this marginal assessment problem have been unsuccessful (improvised staining, 2017) as they are possibly dangerous for the patient (in vivo fluorescent probe detection, 2016).

Hypothesis
A novel Acridine Orange (AO) tissue staining protocol for MUSE, once optimized, accurately highlights mast cells and the most critical histological features of mast cell tumors in fresh, thick tissue slices.

Materials & Methods
- Fixed tissue from 7 MCTs (Kings™ grade 2, high grade, 5, low grade) and fresh tissue from one low grade MCT were obtained from the UC Davis Veterinary Pathology service. Slides from the above tissues as well as from various other 'look-a-like' round cell tumors stained and analyzed.
- The new protocol (Fig. 1) is a significant modification of the Zahrach (1967) method for fluorescence microscopy. Images were analyzed with Zyla, open-source software QMatPha™ and ImageJ. Statistical analyses were performed in R. Data analysis was mostly restricted to slides and tissues from the MCT removed on July 27th as it carries a high signal significantly improved image performance.
- Correlation between thick slice and slide staining (Figs. 6, 7) will be measured by comparing overall to one stained of large regional and with a comparison of average difference in hue between MC nuclei and cytoplasm. An RGB spectral phantom (www.opsin.com) will be used to remove the background signal.
- All stained slides were first imaged with the MUSE microscope (Fig. 7), most completely stained with 400x magnification and 10x glacial acid acid; then re-stained with 0.5% Toluidine Blue (TB) at pH 2.20 and 0.01% Eosin counterstain. Each count was scanned with a Geica digital scanner and compared on a (Figs. 6) and in ROI with the same protocol (Figs. 9).
- A 14 AO Thin MUSE and corresponding TB ROI tiles containing a mixture of tumor and non-tumor cells were selected semi-randomly, assigned a random identifier, and flipped or rotated to provide blind analysis during these ROI's included 25 common areas of interest.
- Color thresholds with fixed ranges (generated with an independent consultant) measured the positively stained (purple or orange) pixels (Figs. 7b, 8b), the combined area of which was assessed as a percentage of the TB. Percent area positively stained area was assessed with Breath-Alcohol 97.5% limits of agreement and a 95% CI (Williams Signed-Rank test) around the mean difference in percentage area stained.
- Stain agreement regarding the number of mast cells in a given ROI was assessed with a Williams Signed-Rank test for the blinded cells counted by the primary authors across the 14 paired ROIs as well as the blinded median cells counted provided by 7 additional people not affiliated with the project.
- Detectionality of the AO stained mast cells in the thick tissue by a machine learning algorithm will be quantified in fresh thick tissue ROIs containing tumor and nontumor areas. First, a random classifier has only required an average of about 85 training objects-to-identify mast cells appropriately.
- 1268 low grade mast cell and 1194 high grade mast cells were measured for nuclear complexity and activity and compared in an attempt to assess metabolic pheomorphology, however, hypothesis testing failed to find any significant differences between high and low grade cells.
- To quantify the correlation between AO fluorescence signal intensity and the granularity of a given cell, saturation intensity (mean gray value, HSB slice 2) of the cytoplasm were compared as a percentage of the saturation intensity of a common baseline, heavily stained cell for both slices. These findings will be interpreted along with staining code (4) of staining intensity of the individual cells from the surveyed outside evaluators.

Results
- AO and TB strongly agreed regarding the percentage of area stained (thickened) across 14 paired ROIs. The median difference between the percentage area stained was just 0.94% and a Williams Signed-Rank test found a 95% CI of 2.42 to 1.74, 2.71. The Breath-Alcohol 97.5% limits of agreement was 5.62% (Fig. 10).
- Cells across the 14 paired ROIs matched fairly well but not perfectly. The author’s counts and the median counts of the slice outside evaluators had median difference (and Williams 95% CI) across the paired ROIs of 8 cells (3.93, 13.9) and 7.5 cells (17.5, 48.5) respectively. 86.97% limits of agreement were 22.7 cells and 16 cells, respectively. Count differences were most likely due to confusion over processing artifacts.
- Metachromasia of Acridine Orange is the result of ionized dye aggregates inside the low pH cytoplasmic vacuoles of mast cells which fluoresce at 630 nm (orange). This is consistently apparent with 280 x excitation and allows clear distinction between MC nuclei, MC cytoplasm, and background.
- Negative control slides from 6 different denufural cell tumors did not feature the bright orange cytoplasmic staining of MCs but some did fluoresce with a (potentially useful) lighter orange or peach color (Fig. 11).
- Two high grade MCTs had distinctive morphological features seen in thick tissue. One was extremely cellular and the other was highly infiltrative of healthy tissue with clusters of MCs (Fig. 12).
- Higher magnification and better resolution are needed to reliably detect mitotic figures or visualize chromatin detail.

Conclusions & Future Aims
- The simple and fast Acridine Orange staining method developed in this study reliably highlights mast cells and distinguishes them from other inflammatory cells.
- The stain is highly agreeable with Toluidine Blue. Ongoing statistical evaluation is attempting to quantify the correlation between the AO staining of the thick tissue and slide-mounted specimens as an additional assessment for fluorescence intensity and granularity at the level of an individual cell.
- The stain appears to perform significantly better in fresh tissue than in fixed. This is highly encouraging for the intended purpose but made it difficult to draw definitive conclusions as only one known mast cell tumor has been analyzed so far.
- This method could also be used by pathologists to provide a more complete margin assessment than is typically assessed with radial section sampling.
- A much larger sample size of fresh tumors is needed to determine if this method and associated digital analyses can predict the grade. Grading is a challenging task but this study has identified multiple relevant, measurable parameters that may eventually provide clues and thus warrant investigation.
- Future staining modifications and image processing techniques will also attempt to find a solution to the pervasive problem created by the inability to distinguish mast cells near the tumor margins from healthy mast cells being called in via inflammatory cytoines.

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References
[1] Cho, all participants who provided data for the visual stain assessment.
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