Historically, the goal of histologic examination of tumor biopsies was to provide an accurate diagnosis and prognosis of a tumor entity and, in the case of excisional biopsies, to evaluate tumor margins. There is an increasing need for more accurate prognostic and predictive markers in veterinary oncology because of an increasing number of treatment options, the increased financial costs associated with treatment, and the emotional stress experienced by owners in association with the disease and its treatment. Over the last decade the ability of DCPAH to perform these services has been significantly advanced by integrating molecular tools such as immunohistochemistry (IHC) into the biopsy service to more accurately diagnose tumors, to provide an individualized prognosis, and to predict therapeutic responses. In addition, the use of modern technology allows us to deliver images to our clients that significantly enhance the assessment of tumor margins. The purpose of this special edition of Diagnostic News is to familiarize the reader with advances we have made in margin evaluation as well as the current status of the diagnosis, prognosis and therapeutic prediction of some of the most common tumors seen in veterinary medicine: canine cutaneous mast cell tumors (MCTs), canine oral melanomas, and feline intestinal lymphomas.

Margin Assessment
Assessment of surgical margins is a challenging task for the histology technician, pathologist, and submitting veterinarian. It is often difficult to communicate results of a margin evaluation to a submitting veterinarian in a written report, or even by phone. This task is further complicated by the various techniques applied in different laboratories to assess margins. The most commonly used method to evaluate tumor samples is the cross-sectioning method, also known as the radial method or “halves and quarters.” The specimen is bisected along its shortest axis. Then, each half of the tissue is bisected through its longest axis, creating quarter sections that demonstrate the mass in a different plane. While we use this method for routine evaluation of submitted biopsies, this method is not favored for complete margin evaluations since it evaluates a very limited portion of the margin area and makes the erroneous assumption of symmetrical expansile growth of the tumor.

Parallel slicing at regular intervals (complete bread loafing, serial sectioning) increases the percentage of margin area examined (Fig. 1). Since the distance between sections determines the quality of the margin evaluation, the cost of this approach limits its use in veterinary medicine. A modified technique combines radial and parallel techniques (Fig. 2). This allows for evaluation of tissue immediately adjacent to the bulk of the tumor and evaluation of some distant margins of the sample. Tangential sections (shaved edge sections, “orange peel”) provide a complete assessment of surgical margins (Fig. 3). Multiple 2- to 3-mm sections are shaved off the edge of the sample and laid into cassettes with the cut surface down. Any tumor present in the sections is interpreted as incomplete excision. The disadvantage is that the distance of tumor to margins cannot be assessed.

By combining cross-sectioning with tangential margin evaluation, we deliver the most complete margin assessment. While expensive, due to the large number of slides and time required for this method, it should be requested for all mast cell tumors, melanomas, mammary tumors, and other carcinomas to more accurately determine tumor extent. By inking the tumor margins the clinician can guide evaluation of certain regions and insure examination of the surgical margins.

Regardless of the method used to evaluate margins, the clinician needs to know to which margin the tumor extends in order to better direct additional resection or select advanced therapy, e.g. radiation. We therefore provide online photographs of tumors submitted for full margin evaluation that detail the exact position...
The number of AgNORs correlates with the speed of cell proliferation. Research indicates that neoplastic cell proliferation activity cannot be predicted reliably using a single measure, but prognoses developed from this combination of tests are highly correlated with survival and metastasis. ITD mutations in exon 11 of c-Kit have been detected in about 20 to 30 percent of canine cutaneous MCTs. MCTs with such mutations are highly aggressive, but respond well to tyrosine kinase-inhibiting (TKI) therapies, such as Kinaveg or Palladia. ITD mutations in exon 8 of c-Kit are less common and have been detected in 2 to 5 percent of canine cutaneous MCTs. Tumors with this mutation are also expected to respond to TKIs, but no prognostic association has been established. In addition, aberrant KIT expression patterns have been linked with decreased survival. MCT grading, cell proliferation analysis, c-Kit PCR, and KIT IHC results are all linked to MCT-associated survival and metastasis. While each test can be run independently, prognoses developed from interpretation of all tests offer the highest level of certainty, especially for low grade MCTs (Fig. 4). PCR for internal tandem duplication mutations in exon 11 of c-Kit is recommended for all high grade MCTs in order to assist in determining whether or not a TKI drug would be indicated. To download our “Mast Cell Tumor Flowchart” to support therapeutic decision-making based on prognostic parameters, visit our website at animalhealth.msu.edu under Diagnostic Sections > Immunohistochemistry.

Unlike dogs, most cutaneous MCTs in cats are benign and cured by complete surgical excision. Occasionally, more aggressive feline cutaneous MCTs and systemic mastocytosis (generally affecting the spleen and/or liver) are seen. For these more aggressive forms, we also offer PCR to detect mutations in exon 8 of c-Kit. Again, tumors with a c-Kit mutation are expected to respond to TKI therapy.

**Figure 4: Flowchart to support therapeutic decision-making based on prognostic parameters**

DCPAH is a full-service, fully accredited veterinary diagnostic laboratory offering more than 800 tests in 11 service sections.
Melanomas
Melanoma is another challenging tumor. Definitive diagnosis of amelanotic melanocytic tumors is often difficult based on microscopic examination alone; these tumors can closely mimic poorly differentiated malignant neoplasms such as carcinomas, soft tissue sarcomas, and round cell tumors. It is, however, of the utmost importance to accurately diagnose melanocytic tumors, as prognosis and therapy vary greatly between differentials. IHC stains are often required to confirm the diagnosis. IHC staining is most commonly identified within nests of intra-epithelial neoplastic cells that are located adjacent to the underlying mass. Thus, the likelihood of correct diagnosis increases dramatically when samples contain ample non-ulcerated, overlying and adjacent epithelium. We offer a diagnostic melanocytic tumor panel that includes IHC staining using a cocktail of antibodies against Melan-A, PNL-2, TRP-1, and TRP-2 (Fig. 5). This IHC cocktail is highly sensitive and 100% specific in detecting amelanotic melanocytic tumors and is more efficient and cost-effective compared to a panel of individual antibodies.

Figure 5: Junctional activity (the presence of neoplastic cells within the basal layer of the epithelium) is an important diagnostic feature of oral melanomas.

Figure 6a: Low malignancy canine oral melanoma. Few neoplastic cells have nuclear Ki-67 immunolabeling.

Figure 6b: High malignancy canine oral melanoma. A high proportion of the neoplastic cells contain Ki-67 immunostaining.

Accurately determining the prognosis of canine melanocytic tumors, especially those of the oral cavity, is also difficult. Recent evidence suggests that a subset of canine oral melanocytic tumors may have a more favorable prognosis than historically thought. Nuclear atypia is a predictive histological feature for oral and cutaneous melanocytic tumors; however, it is subject to inter-observer variation and difficult to assess in some tumors. Mitotic index is also helpful in predicting prognosis but is not as predictive as nuclear atypia or Ki67 index. Assessment of Ki67 IHC labeling has been shown to be highly predictive of prognosis, based on survival times, for both oral and cutaneous melanocytic tumors (Figs. 6a and 6b). A prognostic melanocytic panel includes immunolabeling with Ki67 as well as assessment of nuclear atypia, mitotic index, and degree of pigmentation for prognostic information.

Feline Intestinal Lymphomas
A geriatric cat presents with vomiting and diarrhea. Is it primary intestinal lymphoma, inflammatory bowel disease (IBD), or hypersensitivity? While microscopic analysis can’t differentiate small lymphocyte infiltrates into neoplastic versus inflammatory, particularly when practitioners submit endoscopic samples as opposed to full-thickness biopsies, a specialized panel of molecular tests can distinguish between these differentials with a high degree of certainty. This panel uses an experimentally-developed diagnostic algorithm to improve diagnostic certainty, allowing practitioners to more confidently choose treatment options for the animals under their care.

The feline intestinal lymphoma panel includes microscopic evaluation, IHC phenotyping, and confirmatory PCR for lymphocyte clonality. Microscopic evaluation of the routine formalin-fixed biopsy sample (endoscopic or full thickness) is used to screen for morphologic hallmarks of lymphoma, including marked lymphocytic infiltration of the tunica muscularis (full thickness samples only) and epitheliotropism (nests or plaques of lymphocytes accumulating within the epithelial layer). IHC is then used to differentiate between B- and T-cells, and to visualize the location of T-lymphocytes in the epithelial layer and identify heterogeneous inflammatory cell populations versus homogeneous neoplastic cell infiltrates. The diagnosis is confirmed based on the combined interpretation of morphology and IHC results, or if the diagnosis is inconclusive, PCR tests for T- and/or B-lymphocyte clonality are run to differentiate neoplastic from inflammatory lymphocytes. Lymphoid neoplasms are monoclonal expansions of malignant lymphoid cells, whereas lymphoid cells in an inflammatory reaction are usually polyclonal. This test is often referred to as PCR for Antigen Receptor Rearrangements (PARR). We are the only diagnostic lab that currently performs duplicate or quadruplicate amplification, heteroduplex analysis, and capillary electrophoresis, which allows us to achieve the highest possible sensitivity as well as avoid pseudoclonals, which are false positive results. Thus, it is very important to inquire how a specific laboratory performs these tests. Histology, IHC, and PCR results interpreted in context dramatically increase diagnostic certainty in differentiating feline intestinal lymphoma from inflammation (IBD).

In addition, IHC and PARR testing are useful in both cats and dogs to differentiate follicular lymphomas such as T-zone lymphoma or marginal zone lymphoma from hyperplastic reactions in the spleen and lymph nodes and can also be used to characterize lymphohistiocytic proliferations in the skin or other organs. PARR results should never be interpreted independent of morphology. We therefore only offer PARR in combination with a biopsy or a second opinion and immunophenotyping.

What Samples Should Be Submitted?
All of the described tests for MCTs, melanomas, and lymphomas can be performed on routine formalin-fixed biopsy material as well as previously submitted biopsy samples. Alternatively, clients can submit the paraffin block of the lesion in question or at least 10 unstained positively charged slides of this block. For more information, please contact the Anatomic Pathology lab at 517.353.1683, or visit our website at animalhealth.msu.edu.
Bone Radiograph Panel

Bone biopsies are essential to differentiate between neoplasia and infectious etiologies (bacterial or fungal). Obtaining diagnostic bone core biopsy samples can be difficult, especially when there is significant periosteal reaction. Oftentimes, the core biopsies we receive are not taken deep enough in the bone and are non-diagnostic (Fig. 7). In these cases, we recommend additional and/or larger bone biopsies from multiple sites and varying depths and/or consultation with a veterinary radiologist. The most common instrument used for bone biopsy is the Jamshidi needle biopsy. Two radiographs, lateral and cranio-caudal, are taken and reviewed. The center of the lesion is located using palpable anatomical landmarks. It is important to biopsy the center of the lesion, as a biopsy of the periphery may result in sampling reactive bone surrounding the tumor. The center of the lesion is localized using the anatomical landmarks. A 2mm stab incision is made in the skin using a number 11 blade. The Jamshidi bone needle, with the stylette in place, is pushed through the soft tissue until it contacts the bone cortex. The stylette is removed and the cannula is advanced through the near bone cortex into the medullary cavity using a twisting motion. Advancement is stopped before penetrating the far cortex to prevent contamination of uninvolved tissue planes. The bone needle is rocked to allow the sample to break off within the cannula. The instrument is withdrawn and the specimen is pushed out of the base of the cannula by inserting the probe into the cannula tip. Four or five samples are obtained by redirecting the needle through the same stab incision. Material for culture is taken from the samples prior to fixation in formalin.

Interpretation of bone biopsy samples ideally should always be performed in conjunction with radiographic interpretation. It is also extremely helpful if the location of the bone biopsy is indicated on the radiograph. We now offer a panel that includes decalcification of the bone biopsy, microscopic evaluation of the bone specimen by a board certified pathologist, and radiographic interpretation of the bone lesion by a board certified radiologist. The radiologist and pathologist consult with one another and report their interpretations in a single combined report. Either plain films or digital images are accepted. Entire amputated limbs can also be submitted for this panel but additional processing charges apply.

Figure 7: A superficial bone biopsy core specimen (left, shown in red) only contains periosteal reactive bone formation and would be considered non-diagnostic. A biopsy core taken from the center of the lesion (right, shown in green) contains neoplastic osteoblasts producing osteoid consistent with a diagnosis of osteosarcoma.