Mitotic Count (MC)
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Contents

Introduction

The purpose of Guidelines is to provide standardized methods used to evaluate neoplasms in animals and accrue data so that, over time, large data sets with comparable information can be evaluated and studies validated uniformly. Ultimately this will enable meaningful conclusions and accurate prognostic information that will improve patient care. New methods and modifications of present methods are encouraged and should be described in such detail that others can replicate and validate results. Journals should require that new and modified methods be compared to existing standards so others can determine if they are valid, better and what resources are needed. Some Journals have changed their instructions to authors and now require that the materials and methods section of a manuscript describe how the mitotic count (MC) was performed and report the area in mm^2. Additionally, the term "mitotic index (MI)" must be replaced by MC if mitotic figures were enumerated within an area of the neoplasm. Mitotic index is determined by counting mitotic figures (MF) and dividing by the total number of cells counted, which is never done by diagnostic pathologists. Guidelines and protocols are "living" documents which will be modified as better approaches and methodologies are developed or become available.

Counting mitotic figures with an area of a neoplasm is one of the oldest, and most commonly used parameters to help predict a clinical outcome individually or as part of a grading system. However, the method has never been standardized for veterinary oncology and most of our oncology manuscripts defined the area enumerated as 10 high power fields (hpf) which we now know can vary by up to 600% depending on how the microscope is configured. Furthermore 10 hpf is unitless, meaningless for whole slide imaging (WSI) technology which is likely the number one way in which animal tumors are evaluated in the world. The term hpf (and lpf) are outdated and should be replaced with SI units for areas that others can reproduce.This Guideline is intended for all animal tumors, however, future updates will likely provide different methods for different tumors. The MC method presented here is designed to be as

detailed and as standardized as possible given our current knowledge and technologies.However, there are practical issues that prevent full standardization (a few of which are listed e.g what to do if the specimen is <2.37 mm^2). Pathologists and labs should consider offering explanations and or disclaimers for these situations. The area to be used to count and report the MC must be in a Standard International unit for area, mm^2 Most prior animal studies did not define the area (mm^2) in which the MC or other histological features were enumerated beyond 10 hpf, which limits the clinical utility of this data..[1-8] A recent publication detailed how to recognize mitotic figures (MF), atypical MF (AMF)[9] and distinguish these from mitotic like figures (MLF).[10] The MC can be performed manually (conventional- light microscope or digital microscopy) or with software programs that are fully or partially automated (CPATH/AI).[11-15,3,16-20,8,21] **See Guideline 11 Computational Pathology (CPATH).**

The goal of counting mitotic figures in tumors is not to find the average number of MF, or the greatest number of MF/unit area but the number of MF in a selected region of a neoplasm that correlates with one or more outcomes.

Histomorphologic Definition of Mitotic Figures, Atypical Mitotic Figures, and Mitotic-Like Figures

See Guideline 2

Mitotic Count on Digital Whole Slide Images[20,8,21]

The field of view (FOV) varies with the characteristics of the monitor and the magnification at which the image is reviewed. Each pathologist must determine the number of image fields which equate to 2.37 mm^2 in their own workstations (Table 2)

1. At a low magnification, scan the histologic section to identify hot spots of high mitotic activity within viable cellular regions of the neoplasm (note A).

a. Avoid and/or skip (helicopter) areas of the tumor that are cell poor (impacted by hemorrhage, edema, necrosis, cysts, inflammation and/or autolysis).

b. Within identified hot spots, define a contiguous FOV with an area totaling 2.37 mm^2. Calculate the area using one of the three methods in Table 2.

c. Go to magnification appropriate for adequate counting of MF AMF.

2. Count all MF and AMF within the defined 2.37 mm^2 FOV area. Annotate MF and AMF with a counting tool if it is available in the image software to prevent duplicate counting of MF and AMF.
3. The total sum of MF and AMF counted is the MC.
   a. MC is reported as a number within a specified area (2.37 mm^2)

Mitotic Count - Glass slides Microscope

1. The total number of fields to be counted to equate to 2.37 mm^2 will vary between microscopes (FN of ocular and objective magnification)[14,22] and each pathologist needs to determine the number of image fields that equate to 2.37 mm^2 (Table 3).
2. At a low magnification, scan the histologic section to identify hot spots of mitotic activity within viable cellular regions of the neoplasm (note A).[13,19,20,8,21]
   a. Avoid and/or skip (helicopter) areas of the tumor that are cell poor (impacted by hemorrhage, edema, necrosis, cysts, inflammation and/or autolysis).
3. Select an area of hot spot and begin counting MF AMF in a contiguous, non overlapping fashion in a total area of 2.37 mm^2. Recommended magnification is 400X (Table 3).
4. Count all MF and AMF within 2.37 mm^2 area: 400 X FN 22 = 10 hpf
5. The total sum of MF and AMF counted is the MC.[10]

a. MC is reported as a number within a specified SI unit of area mm^2 (2.37 mm^2) See Table 3 to determine FOV in individual microscopes.

Practical Issues Encountered When Performing MC: Recommendations

MC Result Close to Clinically Established Cut-Offs

1. If MC is close to a clinically significant cut-off (as determined by the type of neoplasm being assessed), repeat the MC procedure in different hot spots; recommend an additional 2-5 MC if sufficient neoplastic tissue is present.[23]
2. Report the highest MC and each MC (recommendation)
3. Reports have advocated performing the MC multiple times and some advocate averaging the numbers obtained but there is no comparable data for animal tumors.[18,6] Pathologists and labs will develop their own policies and explanations of what was done in these situations; example: Multiple MC were performed because the first value obtained was close to a reported cutoff for this tumor. Consider evaluating other clinical and histological parameters when the MC is close to a reported cutoff.

Specimen size smaller than 2.37 mm^2

1. Determine and report the area available that is adequate for performing the MC.
2. Count all MF and AMF within that area
3. Report total sum MF and AMF/ area mm^2 counted
4. Consider:
    a. Offer to cut more sections and perform MC until an area of 2.37 mm^2 is attained - recommended
    b. Extrapolate the sum of MF and AMF counted to approximate a 2.37 mm^2 area. Example:
        i. Tumor only has a 1.185 mm^2 area available for adequate MF and AMF counting.

ii. Within the 1.185 mm^2 area, a total of 10 MF and AMF are counted.
iii. Multiply the total MF and AMF count by 2 to approximate the result (20) to a 2.37 mm^2 area. Report the calculated result and the actual count (3. above). A problem with this approach is it assumes a uniform distribution of MF AMF, however there is not uniform distribution of MF in tumors.

5. Consider adding an explanation and disclaimer of what was done due to small sample size and that a larger sample should be submitted. Example: Quantity of tissue not sufficient for MC, consider submitting additional tissue.

Large Specimen with multiple sections

1. If multiple sections of tumor are available, screen several slides for mitotic density (hot spots) and perform MC on the most appropriate tumor section. There is not (yet) a standard procedure of how many sections should be evaluated to determine MC. The recommendation is to only perform one MC. However, if multiple counts are performed the highest MC and each MC should be reported rather than the average of all counts.

Specimen has numerous spaces – vascular, ducts, acini, desmoplasia, tumor matrix, necrosis

See Note A

1. There is not a standard way to approach these situations. Skipping over spaces and non-neoplastic tissues and resume contiguous field counting when tumor is present in FOV is recommended. However, when a tumor has large areas that should not be counted it is difficult to estimate how many new areas should be counted to compensate for the skipped areas. Another approach is to perform the MC in the areas which have the most cellular density with the fewest spaces due to ducts, blood vessels etc. In tumor biology there will be unique events that make 100% rules impossible.

Notes

A. The current standard area of a neoplasm to perform the MC is in a region(s) of viable tumor with high mitotic activity (hot spots) which is located by scanning the tumor, finding an area of high cell density and proceeding to higher magnifications to confirm MF.[11-13,24,18-20,8,21] If no MF are found during scanning, select a cellular area of the tumor with good tissue preservation and start the count. Early reports stated selecting a "random" region to start counting MF, however that has been replaced with starting in a region with high mitotic activity. Counting at the periphery is recommend for some human tumors.[11-13,24,18,19,25,20,8,21] If a necrotic area is encountered while performing a MC, this region is "skipped" (not counted; "helicopter") and a viable area of equivalent size to the region skipped must be included in the final tally of MF. The tumor cells adjacent to the area of necrosis are counted if the pathologist deems them viable. How viable (healthy) they are cannot be determined histologically. No matter how carefully a method is defined there will be an element of subjectivity and judgment. MC can only be accurately performed in areas of the tumor that are viable, proceeding slowly and using well defined criteria to recognize MF, AMF and MLF

B. Determination of the area of highest mitotic activity (hot spots) by routine light microscopy is subjective and a source of interobserver variation.[13,26,19,21] A study of canine cutaneous mast cell tumors showed variability of MCs in different tumor areas and interobserver variations in MC, particularly in tumors with borderline/intermediate number of MF.[13] This study and another showed that pathologists were not as capable of finding the hotspots as compared to computer assisted locations of hot spots.[27] However, outcomes were not known, therefore the clinical significance of this is not known. It has also been recommended to count MF at the periphery of tumors, the "invasive front" and tumor is better preserved.[11,12,19] This is the recommendation for many human

tumors. The periphery has been defined as the outer 1-2 mm,[15] 2-4 mm,[26] 2-5mm,[12] or as a percentage of tumor area;[13] others have said count in a region of invasion. A study of human breast carcinoma reported that the periphery contained more hot spots (using Ki67) than other regions and percentages of Ki67 positive nuclei obtained at the periphery changed the prognosis.[15] Other studies in humans reported that using Ki67 in hot spots which were not just at the periphery of breast carcinoma contributed the most prognostic information as compared to other methods.[26] Additionally, a study of canine cutaneous mast cell tumors found that the regions of highest mitotic activity were not always at the periphery.[13] Selecting the area of a tumor that is *predictive* of outcome(s) or treatments needs to be found, for each tumor type. Until those results are established, select the region of highest mitotic activity (standard). If a region of the tumor other than hot spots was used to count MF AMF that should be indicated in the report e.g. periphery, center. Studies need to be validated and MC from different regions correlated with known patient outcomes.

C. MC is reported as a number within a specified area (2.37 mm^2).[13,10,17] To avoid confusion to clinicians consider stating that this is comparable, but more accurate and replaces a number per 10 hpf. Prior reports of animal tumors did not specify the area in which MC were performed beyond 10 hpf 400X, however, this area can vary up to 200% depending on how microscopes are equipped.[14,22] These studies need to be repeated with standardized methods. Today the majority of MC are likely to be performed on WSI, for which 10 hpf is nebulous, regardless, hpf or 10 hpf are not standard units of area.[12,14]

D. Most pathologists prefer 400X magnification for performing a MC. High magnification objectives with high numerical apertures (NA) will produce sharp FOV making the correct identification of MF, AMF and MLF easier. The NA number is engraved on the side of high-quality objectives and higher numbers

mean greater resolution, but this does not change how the area in the FOV is calculated. All objectives have an FN and it is engraved on some objectives; it indicates the maximum FOV diameter that can be achieved using this objective, however it is the ocular FN which limits the size of FOV in a standard microscope, not the objective FN (See Appendix 1.0 Definitions).

Future Considerations:

MC is an important parameter in tumor assessment but the present methods to determine the MC can be improved. We encourage others to investigate new methods and modify existing methods for the MC and all the parameters used to evaluate tumors. Changes in our methods need to be data driven on robust numbers of patients and validated in subsequent studies.

1. Our foundation oncology studies, and more recent ones, that did not define an area (mm^2) in which structures were enumerated need to be repeated with standardized methods correlated to accurate patient outcomes. MC performed with H&E stained slides should be compared to other means such as IHC for Ki67, MIB-1, PHH3 or molecular PAM50; even slides stained with toluidine blue that will enhance nuclear chromatin should be considered.

2. Address the practical considerations outlined in this Guideline.

3. Avoid creating MC cutoffs (thresholds) that are based on a single number (above or below). Develop scoring systems, confidence intervals, ranges of predictability for different tumors.
   Example: a score of 1 represents 0–5 MC/2.37mm^2; 2 represents 6–10 MC/2.37mm^2 and a score of 3 for > 10 MC/2.37mm^2

After creating a scoring system statistical analyses may indicate a single digit threshold is discriminating. Single digit thresholds are statistically sound and are easy to use but do they make biological sense? A single count on either side of the threshold should not change the prognosis alone.

4. Investigate different methods to perform MC

    a. should the area counted be changed for different tumor types? compare different size areas (e.g. 1 mm^2 5 mm^2 10 mm^2 etc.). Compare different tiered cutoffs.

    b. where should counting MF AMF be performed? (hot spots; random; at periphery or an invasive front?). Might there be a different clone of tumor cells at the invasive portion and this clone is more predictive of outcomes.

    c. perhaps the proportion of a tumor that is *hot* or *cold* is more predictive than one hot spot e.g. most MCT are indolent, perhaps knowing the proportion of a MCT that is *cold* vs *hot* will be predictive of behavior

    d. determine if counting only AMF is predictive; compare utility of counting only metaphase MF vs counting all phases of mitosis;
    employ IHC

    d. determine the best number of sections needed for MC

    e. determine if an *index* is more predictive than MC e.g., evaluating a standardized number of cells would provide an index per 5000 cells. The denominator should be manipulated during investigations.

    f. determine if delayed fixation influences MC and or its predictability.[16]

5. Use CPATH/AI to lead investigations for each of the above. CPATH will permit rapid enumeration and reiterations of MC in various regions of tumors while avoiding

interpathologist subjectivities. Compare results obtained with CPATH to manual (pathologist) for MC, hot spots etc.

6. Develop methods to enumerate MF/AMF with respect to tumor cell density, such as true mitotic index or volume-corrected MC and compare to standardized MC.

7. Develop grading systems based on scores of 4-5 histological features, then combine different features and evaluate statistical significance (e.g. = how do tumors behave if they have one, 2,3,4 or all of the features?)

8. Discover and compare new methods to existing standards. For MC, compare new methods to those that can be performed with H&E stained histological sections to determine if the new method is an improvement, and if so at what investment of resources and expertise.

Tables and Figures:

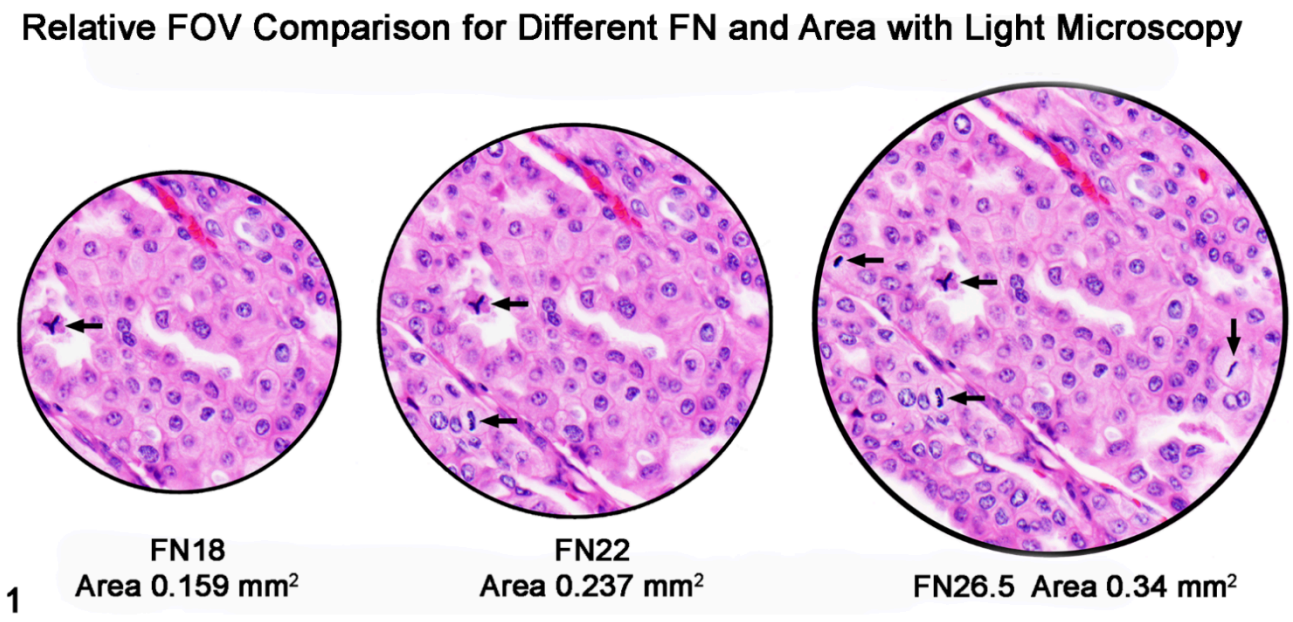


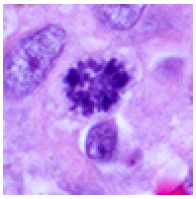
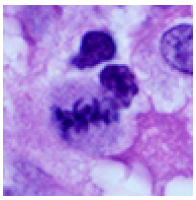
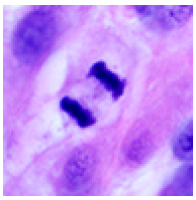
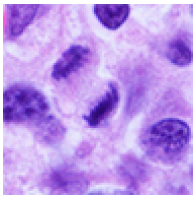
Figure 1:

Schematic representation comparing field of view (FOV) at 400X magnification. The differences in diameter, area, and the number of cells and MF seen are proportional to the differences seen with different oculars: Field number (FN) 18mm narrow FOV, FN 22mm and FN 26.5mm wide FOV. The FOV with ocular FN 26.5mm has 4 MF, and the area is approximately 100% larger (2X) than the FOV with ocular FN 18mm which has 1 MF. Older microscopes were commonly equipped with ocular FN 18 (smaller FOV) in the 1980s compared to current microscopes which commonly have oculars of FN 22 or greater. Mitotic counts (MC) reported in terms of high-power fields (hpf) without specific SI units of measurement (mm^2) cannot be compared to other MC as the area of a hpf can vary 100-600% depending on the configuration of the microscopes. To clarify study results and enable comparison between studies, reports of histologic parameters which are enumerated (MC, nuclear pleomorphism, microvascular density, etc) must be reported in precise units of area (ie, mm^2) not in terms of hpf. Alternatively, a

standardized number of cells that were evaluated e.g. an index in 5000 cells. Urothelial carcinoma, dog.

Table 1.

Criteria for Defining Mitotic Figures (MF), Atypical Mitotic Figures (AMF) and Mitotic-like Figures (MLF)[10]

Photomicrograph Example	Structure	Characteristics	Helpful Features
	Prometaphase MF	Dark aggregate (cluster or ring shape) with spikes/projections	May be difficult to differentiate from MLF; look for spikes
	Metaphase MF	Dark aggregate (linear or ring shape) with spikes/projections	Common; ring form represents end-on (parallel) view of chromosomes in relation to spindle; normal MF
	Anaphase MF	Two separated aggregates variable distances apart; linear with spikes/projections	Common, can have morphologic overlap with telophase
	Telophase MF	Two separated aggregates at opposite ends of the cell; cleavage furrow	Uncommon, may be very thin cytoplasm connection between 2 daughter cells; cell borders are less than 1 cell width apart

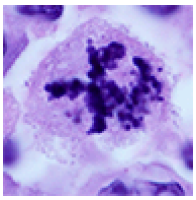
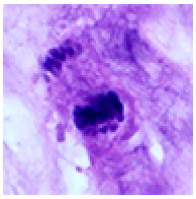
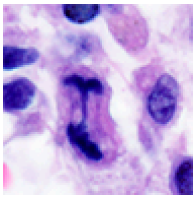
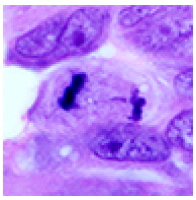
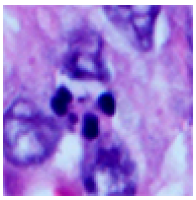
	Tripolar AMF	More than 2 chromosome clusters (spindle poles) during any phase, appears as 3 or more linear plates	Common, AMF has been correlated with poorer prognosis in some human cancers
	Asymmetric AMF	Unequal sizes of two chromosome clusters in anaphase/ unequal sizes of metaphase plate	Less common form of AMF; unequal clusters may be influenced by sectioning artifacts
	Chromosome bridging AMF	Chromosomes stretching from one anaphase pole to the other (touching both), occurs in anaphase or telophase	Morphologic overlap with chromosome lagging
	Chromosome lagging AMF	Chromosomes not in contact with larger aggregate/linear cord; occurs in anaphase and telophase	Morphologic overlap with chromosome bridging
	MLF/Hard Negative	One or more hyperchromatic bodies, smooth surfaces, no spikes/projections; may have eosinophilic cytoplasm or nuclear membrane	Morphologic overlap with some MF (prometaphase); 3 helpful features: surface contour, context, cytoplasm color

Table 2:

Methods to determine standard area for Counting MF using WSI

Calculate the standard area using one of the following three methods:

1. Create a rectangle encompassing one FOV* at magnification appropriate for identification of MF AMF on your monitor. Computer software will calculate the area of the FOV annotated. Divide 2.37 mm^2 by the area of one FOV = number of fields to be counted.

2. Calculate the area in one FOV at the desired magnification by measuring field image length and width with the measuring tool; length X width = one FOV. Divide 2.37 mm^2 by the area of one FOV = number of fields to be counted.

3. Use an annotation tool to create a box = 2.37 mm^2. The box will only be completely visible at low magnification and can be dragged to the appropriate area.

*FOV- field of view

Table 3.

Standardized Area for Mitotic Counts Using a Light Microscope (Replacing 10 High-Power Fields with a Consistent SI unit of area mm^2).

There are two ways to determine the area in the FOV for microscopes. Look at the FN engraved on the ocular eyepiece and calculate the area as described below or measure the diameter in one FOV at 400 X using a high precision stage micrometer..
Standard area in one FOV: 40x objective and 10x ocular with FN 22 = 0.237 mm^2

Calculation: FN/objective magnification= diameter of one FOV

FN 22/40x objective= 0.55 diameter; area circle= πr^2 = 0.237 mm^2 x 10 fields = 2.37 mm^2

As the FN on the ocular increases, the area in the field of view increases. Objectives also have an FN but it may not be engraved on the objective and even if it is ignore the FN on the objective when calculating the FOV area - see Abbreviations and Definitions for details and explanations of this.

If MC is performed with an ocular other than FN 22, consider these adjustments:

FN 18/40x objective = 0.45 mm diameter; 0.159 mm^2; 33% smaller, count 15 fields
FN 20/40x objective = 0.5 mm diameter; 0.196 mm^2; 17% smaller, count 12 fields
FN 22/40x objective = 0.55 mm diameter; 0.237 mm^2 count 10 fields

FN 24/40x objective = 0.6 mm diameter; 0.283 mm^2; 19.4% larger, count 8 fields
FN 26.5/40x objective = 0.66 diameter; 0.34 mm^2; 30% larger, count 7 fields

FN = field number on ocular in mm.

A second way to determine the area in the FOV is to measure the diameter of one FOV with a stage micrometer.

The fine divisions at one end of a high-quality stage micrometer can be used to determine if the microscope system, objectives and oculars yield a FOV diameter at specimen level that is expected for the FN of the oculars used e.g. 40X objective, 10 X ocular FN 22 the FOV diameter is 0.55 mm. Numerical aperture (NA) and the FN engraved on an objective is not used to determine the area in FOV see Abbreviations and Definitions for details and explanations of this. When the FOV diameter is known use the calculation for the area of a circle (above) to determine the area in mm^2. Microscope companies have stated that the precision used to manufacture high quality microscopes used by pathologists is such that calculating the FOV area by using the FN on ocular along with objective magnification is more accurate than the human eye and a stage micrometer.

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