Morphologies of Mitotic Figures (MF)

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Contents

Introduction:

The purpose of Guidelines is to provide standardized methods used to evaluate tumors 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. This Guideline is a “living” document which will be modified as new information becomes available to authors. The Guideline is “generic” and can be used for all animal tumors.

Counting mitotic figures (MF) in histologic preparations is the oldest and most widely used method to estimate cell proliferation in tumors. The mitotic count (MC) is a rapid, inexpensive test which can be performed by any pathologist, is part of many grading schemes and aids in clinical prognostic decisions. The term MC should be used to describe enumeration of MF within a specified area (typically 2.37 mm^2). Variation in MC reproducibility has been demonstrated in multiple human and veterinary studies[1-7] These inconsistencies are attributed to inter-observer variation in MF identification, an inability to efficiently locate hotspots (regions of the highest mitotic activity), an inability to evaluate the complete tumor area or potentially pathologist fatigue. In order to remedy some inter-observer variation with respect to identifying MF, MF morphology has been defined in cytologic[8,9] and histologic[9] preparations (**Figures 1-8** and **Table 1** in the MC Guideline). This Guideline details and provides images to help differentiate MF, atypical mitotic figures (AMF) and mitotic like figures (MLF). A recent manuscript provides numerous images of each of these and provides details of how to correctly identify each.[9]

Mitosis or karyokinesis occurs during the M phase of the cell cycle. Mitosis is the process of karyokinesis while MF are the structures that can be identified with light microscopy. The phases of normal mitosis includes prophase, prometaphase, metaphase, anaphase and telophase.[10,8,11] An important concept to note is that mitosis is a continuum and cells observed in cytologic or histologic preparations may be

arrested in transition between phases. The morphologies of MF and AMF will vary with the phase of mitosis and the transitions between phases such that the nuclear chromatin comprises different shapes and staining characteristics. Identification of each phase is not necessary in order to perform a MC, however, general knowledge of the phases is useful for pathologists to differentiate normal MF and AMF from MLF.

MF are counted from prometaphase through telophase (see **Figures 1-4**). Features to look for that are supportive of MF/AMF include dark purple staining nuclear aggregates with an irregular contour (short rods or spikes of chromosomes, described by van Diest as hairy projections),[11] and lack of a nuclear membrane. Specific features of the normal phases and types of AMF are detailed below and in referenced literature.[9,8]

Histomorphologic Definition of MF, AMF, and MLF[8,9]

Mitotic figures (MF):
Figures 1-4 and Table 1 (MC Guideline 1.0)

1. Dense nuclear aggregates with short rods or projections of nuclear material (chromosomes) and absent nuclear membrane (passed prophase).

2. MF are counted from prometaphase through telophase (Table 1 in MC Guideline 1.0). Prophase cannot be reliably identified in histological preparations but may be seen in cytological preparations.
3. MF have a range of histologic appearances that vary depending upon the phase of mitosis, presence of AMF, fixation, section thickness and staining.

4. The most reliable microscopic feature to identify MF and AMF are the short spikes or blunt projections of chromosomes on the surface of aggregated nuclear material.

    a. Prometaphase: A dark cluster or ring of chromosomes with variably protruding chromosome rods and spikes

b. Metaphase: An equatorial plate (linear or ring-shaped aggregate with short rods or spikes)

c. Anaphase: Two separate aggregates, variable distances apart, linear with projections/spikes

d. Telophase: Further separation of nuclear aggregates (compared to anaphase) at opposite ends of the cell with cleavage furrow between aggregates; cell membranes may be present. Telophase is counted as one MF (see Note C).

e. Anaphase and telophase MF are counted as one, original reports indicated telophase should be counted as two (see below)

AMF:[9,8]

Figures 5-8 and Table 1(MC Guideline 1.0)

1. Chromosome segregation errors during cell division can be observed morphologically as AMF

2. These errors can cause genetic abnormalities in neoplastic cells.

3. There are numerous types of AMF but two broad categories:
   a. Abnormalities of mitotic/polar symmetry
      i. Multipolar (tripolar): The presence of more than two spindle poles during any stage of mitosis
      ii. Asymmetrical bipolar: Unequal sizes of the metaphase axes or anaphase poles
   b. Abnormalities of chromosome segregation
      i. Anaphase bridging
      ii. Lagging chromosomes/lagging chromosome fragments
   c. AMF have been correlated with a poorer prognosis and outcome for some tumors in humans[12-15]
   d. AMF have not been prognostically evaluated in veterinary medicine

Mitotic-Like Figures (MLF)[9,16,17]

1. Structures or processes that appear similar to MF

2. Smooth contours, circular or oval, hyperchromatic bodies (single or multiple)

3. Found in an area where a nucleus should be located
    a. Apoptotic bodies
    b. Hyperchromatic nuclei
    c. Deformed nuclei
    d. Karyorrhexis
    e. Inflammatory cells
    f. Tissue artifacts

4. MLF cytoplasm tends to be eosinophilic, however color is not reliable.
    a. Eosinophilic cytoplasm can occur with denaturation of cytoplasmic proteins, loss of mRNA in degenerating, apoptotic or necrotic cells
    b. Basophilic or amphophilic cytoplasm can occur with increased mRNA (endoplasmic reticulum) and increased protein synthesis. Both normal and neoplastic cells can have active protein synthesis.

5. Pyknotic and karyorrhectic cells have dark, hyperchromatic (darker) condensed nuclei that indicate cell death
    a. Chromatin, a complex of DNA and protein, has two forms:
        i. Heterochromatin (densely compact, staining as dark granules) and
        ii. Euchromatin (active, loosely coiled and light staining).
        iii. Nuclei with greater replicative activity have more euchromatin and open space between granules of heterochromatin, so will be lighter staining.
        iv. Cells in a resting phase have more heterochromatin with a more clumped chromatin pattern and a smaller nucleus.

6. Evaluation of adjacent tissue (context)

    a. A structure within a region of necrosis (multiple cells undergoing nuclear pyknosis, karyolysis, karyorrhexis) is more likely to be MLF

    b. A structure within a region of high mitotic activity is more likely to be a MF.

7. Proceed slowly, taking time for thoughtful evaluation and classification of structures.

Additional Factors that Impact MF, AMF, MLF Identification

Slide preparation

1. Staining technique
2. Thickness of the section

Fixation Delay

1. In tissues which have not been fully fixed, cells remain capable of proliferation[18]

2. Cells can progress through the phases of mitosis until the tissues are fixed or cells exhaust reserves.

3. MF increased up to 3 fold in larger samples with resultant increased tumor grades[18]

4. Also reported in veterinary tissues[19]

5. Other studies: decreased MF or unaltered MF[20-22]

6. At this time, there is no consistent correlation between fixation delay and number of MF.

7. Delayed fixation leads to autolysis and difficulty differentiation MF and MLF

Modality used (light microscopy (LM) vs whole slide images (WSI)

1. WSI: Good agreement between WSI and LM for histologic diagnosis and MC[16,2,23-25]

    a. Possible trend toward underestimating MC with WSI[16,23,24]

    b. Time factor likely negligible with experience

    c. Standardization of monitor size and color calibration may help reduce variability

    d. Scanning resolution does not seem to be a factor[25]

    e. Comparing LM and WSI for individual structures revealed conflicting results[26,27]

        i. Disagreements occur more frequently with smaller objects[27]

AIA (CPATH) recognition of MF, AMF, MLF

(Automated Image Analysis, AIA, see Guideline 11)

1. With sufficient training data, AIA can be used to identify MF or AMF and distinguish from MLF

    a. Benefits of AIA:

        i. The entire slide or several slides can be evaluated quickly

        ii. Algorithms are 100% reproducible

        iii. Accuracy can approach or exceed human abilities (with good datasets and deep learning methods)

    b. Limitations of AIA:

        i. Lack of typical diagnostic variability in training datasets can lead to non-robust algorithms

        ii. No global standards for tissue processing, staining and slide preparation[28]

        iii. MF algorithms developed in one laboratory would need to be adapted and validated in another laboratory

iv. AIA algorithms can be used to identify and classify structures with a provided confidence interval. The pathologist is needed to review these structures and confirm or deny the classification.

2. AIA's potential role in a diagnostic workflow

a. AIA can identify the region of highest mitotic activity faster than pathologists[29]

b. Candidate MF/AMF/MLF can be identified by computer assisted systems and verified by pathologists

c. Vigorous testing and validation would be required before usage by veterinary pathologists

d. Correlation with outcome assessments may reveal new prognostic trends

e. correlation with outcome assessments can be used to compare pathologist (manual) vs AIA (machine) prognostic capabilities

Discussion:

The goal of counting MF for the purposes of obtaining the MC is to accurately reflect the mitotic activity within a tumor. Structures that are identified as definitive MF or AMF should be counted, regardless of the exact phase of mitosis of type of AMF; the specific phases of MF nor the type of AMF need not be identified. The key is differentiation of MF and AMF from MLF so that the same MCs are obtained with microscopes, WSI or AIA. If the morphologic criteria described here are adhered to for identification of these structures, highly reproducible MF identification and MC results are possible. This, combined with other methods of standardization will improve inter-pathologist variation for obtaining MCs. The most important determination is simple: should the candidate structure be counted as a MF? Since some structures are difficult to classify and inter-pathologist variation is inevitable, the most straightforward way to handle these and other confounds is to simply count definitive MF and AMF.

Notes:

A. The most problematic phases (in terms of definitive identification and pathologist agreement) are prophase, prometaphase and telophase. Prophase is characterized by

uniformly, lightly condensed chromosomes[5,8] without obvious spikes and cannot be reliably identified in histologic sections, therefore, should be excluded from MC.[5] This has been previously recommended because there were many discrepancies between pathologists when interpreting prophase for the purposes of grading human breast cancer biopsies.[5] MF previously characterized as prophase are likely to be more accurately identified as prometaphase or MLF. Prometaphase is characterized by circular clusters or a ring of dark purple chromatin with sometimes inconspicuous rods or spikes projecting from the cluster (Table 1 in MC Guideline). It is likely that prophase can be recognized in cytological preparations.

B. The ring form of prometaphase and metaphase is a normal MF. This occurs when the plate of chromosomes is viewed “head-on” or parallel to the spindle apparatus, in which the dark cluster of chromosomes has a circular ring form. This is referred to as a “starburst” or “rosette”.[8,9] This form is not an AMF and should not be counted or annotated as such.

C. Telophase should be counted as 1 MF (Figure 4, Table 1 in MC Guideline). The original recommendation to count the two separated chromosome clusters as two MF was based upon speculation regarding future automated systems. It is now clear that AIA can be trained to recognize a telophase figure as a single MF. Since histologic sections of MF represent a two-dimensional image obtained from a three-dimensional structure, slender attachments between cells that appear to have undergone cytokinesis may not be visible. Therefore, it is proposed a minimal distance of at least one tumor cell width apart in order to count 2 MF. If MF arise from one cell, they should be counted as one. Therefore, cells that are less than one tumor cell width apart should be counted as 1 MF, even if a clear connection between cells cannot be appreciated.[9]

D. Inter-pathologist variation is recognized when identifying MF, for both human and veterinary tumors. Causes and contributing factors include: 1) Difficulty differentiating

MF from MLF (human error), 2) An inability to efficiently locate the region of highest mitotic density and 3) Impaired reproducibility of MF identification or classification while performing repetitive tasks.[29,1,2,30,3,31-33,12,4,5,34,6,35] Deep learning methods with robust algorithmic training can be integrated into the pathologists diagnostic workflow in order to alleviate some of these factors. Combined human and machine intelligence will likely increased pathologist efficiency with automation or semi-automation of repetitive tasks.[36]

Future Considerations:

1. Evaluate prognostic utility of restricting MF identification to those most easily identified (eg metaphase, anaphase and telophase) for the purposes of obtaining the MC and correlating with outcome data.

2. Determine the prognostic significance of AMF for veterinary tumors

    a. Easily recognizable AMF (tripolar)

    b. Include all categories of AMF

    c. Use AIA to identify AMF

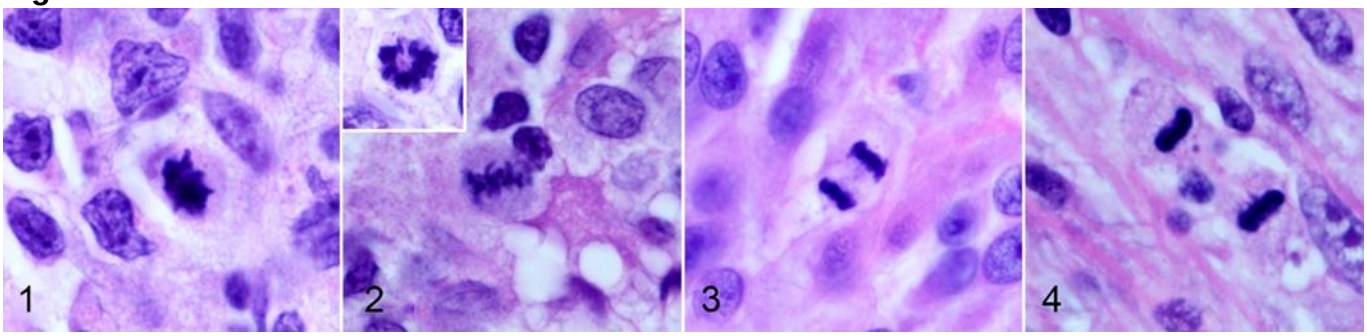
3. Use immunohistochemistry (PHH3) as a “gold standard” for MF/AMF identification vs MLF

    a. Compare this marker with MC obtained with H&E

    b. Investigate whether identification with IHC has prognostic significance

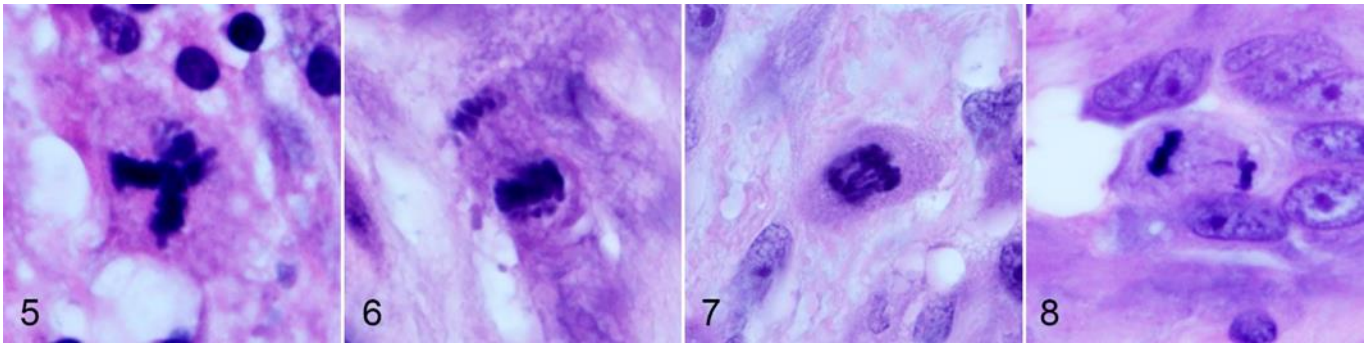
    c. Determine whether identification with IHC will facilitate AIA determined region of highest mitotic activity (eg hotspot)

Figures:



Figures 1-4:

MF are characterized by dark aggregates of nuclear material with short rods and projections. **Figure 1**: Prometaphase/metaphase (dense nuclear cluster with short protruding rods). **Figure 2**: Metaphase with linear equatorial plate of darkly staining nuclear material and short protruding rods and spikes. Inset: Ring form of metaphase with end-on (nonperpendicular) view of the equatorial plate. **Figure 3**: Anaphase MF with two separate nuclear aggregates with irregular contours and short protruding spikes. **Figure 4**: Telophase MF with aggregates at opposite ends of the cell and formation of a cleavage furrow.[9]



Figures 5-8:

Atypical MF (AMF). **Figure 5**: Tripolar AMF (more than two spindle poles during any stage of mitosis). **Figure 6**: Asymmetric AMF (unequal sizes of the metaphase axes or anaphase poles). **Figure 7**: AMF with anaphase bridging (chromosomes stretching from one pole to the other). **Figure 8**: Lagging chromosomes left behind during anaphase (small dark purple streak in center of cell).[9]

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