Assessment of mitotic activity in breast cancer: revisited in the digital pathology era

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ABSTRACT
The assessment of cell proliferation is a key morphological feature for diagnosing various pathological lesions and predicting their clinical behaviour. Visual assessment of mitotic figures in routine histological sections remains the gold-standard method to evaluate the proliferative activity and grading of cancer. Despite the apparent simplicity of such a well-established method, visual assessment of mitotic figures in breast cancer (BC) remains a challenging task with low concordance among pathologists which can lead to under or overestimation of tumour grade and hence affects management. Guideline recommendations for counting mitoses in BC have been published to standardise methodology and improve concordance; however, the results remain less satisfactory. Alternative approaches such as the use of the proliferation marker Ki67 have been recommended but these did not show better performance in terms of concordance or prognostic stratification. The advent of whole slide image technology has brought the issue of mitotic counting in BC into the light again with more challenges to develop objective criteria for identifying and scoring mitotic figures in digitalised images. Using reliable and reproducible morphological criteria can provide the highest degree of concordance among pathologists and could even benefit the further application of artificial intelligence (AI) in breast pathology, and this relies mainly on the explicit description of these figures. In this review, we highlight the morphology of mitotic figures and their mimickers, address the current caveats in counting mitoses in breast pathology and describe how to strictly apply the morphological criteria for accurate and reliable histological grade and AI models.

INTRODUCTION
Uncontrolled cellular proliferation is a hallmark of cancer and it is one of the key predictors of tumour behaviour. Cellular proliferation not only influences tumour growth rate and represents tumour aggressiveness, but it also has been playing an increasingly important role in its predictive ability to guide treatment. Several methods have been described to assess the proliferative activity of tumours, the most important are mitotic count (MC), Ki67 labelling index, proliferating cell nuclear antigen (PCNA) and phosphohistone H3 (PHH3). However, these methods still have their limitations (online supplemental table S1) MIB1, which is directed at the Ki-67 antigen targets the whole cell cycle. Therefore, Ki67 immunohistochemical (IHC) staining not only identifies cells committed to cell division but also cells in the interphase stage of the cell cycle. Lack of consensus on scoring methods, cutoffs and reliability of various antibodies also limit Ki67 clinical utility. PCNA targets the S phase of the cell cycle. It is important to remember that the proportion of proliferating cells as measured by Ki67 or PCNA IHC provides incomplete information about tumour growth. PHH3 is a more specific IHC marker for mitotic cells as it stains the phosphorylated nuclear histone protein, formed during late G2 to late anaphase.

However, its widespread routine applicability was limited by the need to use additional IHC techniques and by its still unconfirmed sensitivity in breast cancer (BC). Until now the traditional visual morphological detection of mitotic figures remains the most widely used and accepted method in routine practice, as it is an easy, rapid and simple technique for assessment of growth rate and tumour behaviour.

Assessment of mitotic figures is still used as a key factor in the diagnosis and prognosis of various tumours including BC. In BC, the MC is one of the three parameters used to determine a histological grade (along with tubule formation and nuclear pleomorphism) reflecting both differentiation and proliferation of BC.

In fact, the MC is a strong histomorphological predictor of outcome. Conventionally, MC can be reported as either Mitotic Index (MI) or Mitotic Activity Index (MAI). MI is usually defined as a ratio between mitotic cells and cells that are not undergoing mitosis, regardless of the cell cycle phase, in a given area of a tumour and reported as a percentage, or mitoses per 1000 neoplastic cells. MAI is defined as the numbers of mitotic figures in a given area of tumour and it is reported as an index (the MC/area counted). In BC, the area to be counted in is traditionally considered as 10 high power fields (HPF). Based on the microscope field diameter, the final area of the 10 HPF is estimated and scores of MC is determined. As part of the Nottingham grading system, the absolute number of mitotic figures per 10 HPF is transformed into 3 scores with cut-offs adjusted based on the microscope field diameter, and area size.

Despite all the advantages of this well-standardised counting method, it still suffers low concordance rates. Concordance data from the interpretative National Health Service Breast Screening Programme external quality assurance scheme of breast pathology in UK, indicates a low concordance rate of MC (κ=0.34) compared with other components of grade such as tubule formation (κ=0.46) (particularly for those scored as 3.
(κ=0.62)\textsuperscript{20} and diagnosing invasive lesions (κ=0.91).\textsuperscript{20} Tumours with mitotic score 2 showed the lowest agreement of all components (κ=0.13), whereas tumours with either low or high MC (score 1 or 3, respectively) showed comparable κ values (κ=0.45).\textsuperscript{20} Similar findings were reported by other authors (κ=0.28).\textsuperscript{21} 

Although the basic features of a cell in mitosis are well-documented, these are usually described from biological and genetic perspectives, with less emphasis on the morphological criteria that should be used to identify mitotic figures on H&E-stained slides. The perceived subjectivity and the high discordance rates in routine practice often result from the inconsistent application of such criteria to distinguish mitotic figures from their mimickers.\textsuperscript{17,20,22-24}

Several phases of mitosis are identifiable including normal and atypical forms, some of which can significantly overlap with other structures (mitotic mimickers) such as apoptotic bodies, dark hyperchromatic nuclei and tissue/staining artefacts.

Although published decades ago, the methodology for the assessment of mitotic figures in BC remains subject to interpretation and individual pathologist performance. For instance, standardisation of area for MC was published in the 2019 fifth edition of the WHO classification of breast tumours.\textsuperscript{19}

However, this was based on defining the area of MC when using 10 HPF, rather than defining the best size area to be used for MC and then adjusting the number of HPF based on the microscope field diameter.

This remains to be defined as some authors have demonstrated that MC is best assessed in larger area size (3 mm\textsuperscript{2})\textsuperscript{25} rather than 10 HPF of small field diameter microscopes which can generate an area as small as 1.25 mm\textsuperscript{2} per 10 HPF.\textsuperscript{19} Details for choosing mitotic hotspots and adjustments to account for variation of field diameter of HPF were described in detail in recent publications and are still evolving.\textsuperscript{26,27}

Issues such as the use of 10 HPF vs a defined area size were not addressed. Another major challenge added to identifying mitoses in recent years comes with the use of digitised whole slide images (WSI) and whether the same criteria used on glass slides should be applied as such or be modified to suite WSI visual eyeball counting. Mitosis detection on WSI is affected by the loss of fine-tuning ability, which represents one of the main functionalities of optical microscopes during identification of mitoses. It has been reported that not all mitotic figures seen using microscopes could be identified on WSI.\textsuperscript{28}

Not only the criteria to define mitotic figures should be modified but also the number of mitotic figures per a defined area to be incorporated into histological grade should be defined.\textsuperscript{29} Therefore, a refinement of the methodology for MC in WSI is urgently needed.

In this review, we summarise the morphological definition of mitotic figures and provide an overview of phases of the cell cycle and mitosis, mitosis mimickers, reproducibility of mitotic scoring along with challenges of mitotic figures on WSI; when applied consistently, these should improve the reproducibility of visual assessment in both traditional microscopy and WSI.

**MORPHOLOGICAL CRITERIA**

**Cell cycle phases and mitosis**

The cell cycle is the series of events that takes place in a cell leading to its division in two cells. Mitosis is one of the four main phases of the cell cycle between interphase (G1, S and G2) and the quiescent phase (G0). It represents the step when the newly duplicated DNA is separated, and two new cells are formed.\textsuperscript{30}

![Figure 1](image-url)  

**Figure 1** Schematic illustration and photomicrographic images of different phases of mitosis in routinely stained H&E sections using WSI at ×40x magnification, showing abundant slightly eosinophilic granular cytoplasm, irregular nuclear outline, absent nuclear membrane; the nuclei appear as a condensed clump (prophase), hyperchromatic straight rod (metaphase), two identical sets of chromosomes at the two poles of the cell (anaphase), or two separate identical chromosome sets surrounded by cytoplasm and separated by the newly formed cell membrane (telophase). WSI, whole slide image technologies.

The mitotic phase is a relatively short period of the cell cycle. In a 24 hour cell cycle, the interphase stage generally takes between 18 and 22 hours, whereas mitosis only takes about 2 hours.\textsuperscript{31} In rapidly proliferating cancers, delayed fixation will allow many cells to complete mitosis and progress to the G1 phase, so they will not be detected when the specimen is examined microscopically.\textsuperscript{12,13} Therefore, rapid and adequate fixation of excised pathological specimens, including breast, is considered crucial to accurately assess mitotic activity.

**Morphology of mitosis**

Morphologically, mitosis is defined using a set of features characteristic to its various phases; however, for clinical application, the identification of mitosis regardless of its phases is the target. Description of these phases is only needed in routine practice to recognise mitotic figures and to differentiate them from various morphological mimickers particularly in WSI in which loss of some fine details makes interpretation of these figures more challenging.

Mitotic figures can show abnormal features, and these are described as atypical mitoses. The presence of these atypical forms is an expression of pathological division and is considered a sure sign of malignancy. Four main phases of mitosis are recognised, and they are in the following chronological order: prophase, metaphase, anaphase and telophase (figure 1) as below:

**Prophase**

In prophase, the cell prepares to divide with thickening and coiling of the chromosomes. The nucleolus shrinks and disappears. The
end of prophase is marked by the incipient organisation of a group of fibres to form a spindle and by the disintegration of the nuclear membrane. The centriole pair begins to move to each pole of the cell and starts to form spindle fibres. As the centrioles are moving, they become surrounded by clusters of microtubules formed of tubulin, radiating in star-like rays called starburst or Astra. The formation of microtubules and their attachment to the centromeres of duplicated chromosomes then occurs, which induces the appearance of hairy like projections on the nuclear outline.

On H&E sections, mitotic cells in prophase exhibit a central hyperchromatic condensed chromatin mass that retain its rounded nuclear contour despite disappearance of the nuclear membrane. This phase can sometimes be difficult to distinguish from apoptotic bodies and lymphocytes, which typically show rounded condensed nuclear material. Characteristically, mitotic cells in this phase show irregular or ragged outline and hairy-like projections of nuclear material that can be seen on higher magnification views. Fine-tuning the microscope to adjust focus facilitates the identification of the hairy like projections and the irregularity of the nuclear outline, as opposed to other mimickers which typically show smoothly defined nuclear outlines. Prophase mitoses can be considered the most challenging phase of mitosis to be identified, on WSIs due to the reduced ability to identify the hairy like projection of these rounded structures these mitoses typically show irregular ragged outlines, and this together with the size and cytoplasmic features (slightly abundant eosinophilic or basophilic granular cytoplasm) can help to distinguish them from lymphocytes and apoptotic bodies (online supplemental table S2).

Metaphase
Metaphase is a stage where the chromosomes become densely coiled and line up along the midline of the cell (equator) forming a straight line. Histologically, the nuclear membrane disappears, and the chromosomes appear in the cytoplasm of the cell as hyperchromatic straight rods arranged perpendicular to the long axis of the cell. When the cell is caught under perpendicular incidence, spindles can rarely be seen on H&E sections when they appear as thin-stranded structures that radiate outward from the chromosomes to the outer poles of the cell. Although the metaphase mitoses should be easier to distinguish from the nuclei of apoptotic cells and lymphocytes being rod shaped, it can be mistaken for other mimickers especially on WSI, that is, elongated dark hyperchromatic nuclei. The latter are seen in some BC due to compression or crushing of malignant cells. These structures are often seen at the edge of the tumour clusters or compressed between cells and are often multiple, with smooth nuclear outline, with or without cytoplasm. Other mimickers of metaphase include hyperactive elongated stromal cells, which are often seen in regions of poor fixation or at the peripheral surgical margins affected by cauterisation (figure 2A–D). It is worth noting that when the cell is caught in a coronal section, the metaphase chromosomes can be seen as a ring or round layer and is called ring metaphase which may mimic prophase mitoses. Again, the metaphase mitoses can show hairy like projection or irregular outlines which may add to their recognition. The high prevalence of the prophase and the metaphase mitoses makes their recognition on WSI critical.

Anaphase
It is the shortest and most dynamic phase of the cell cycle, when each divided centromere with attached sister chromatids moves towards opposite poles of the spindle. The sister chromatids may take a V shape while they are pulled away. This results in two parallel hyperchromatic rods seen in the same cell. Some authors may divide anaphase into two parts: in early anaphase (anaphase A) the chromosomes appear separated into two groups; in late anaphase (anaphase B), the chromosomes become pulled apart and appear on opposite sides of the cell and a new cell membrane begins to form in the centre between the spindle fibres. In normal mitoses, these have a balanced size and shape, as they contain identical quantities of DNA. Histologically, they appear as two identical sets of chromosomes at the two poles of the cell that appear more oval with an intact cell membrane and an absent nuclear membrane and nucleolus. Anaphase can be considered as the easiest mitotic phase to be identified on WSI due to its unique morphology. Although it can be hard to differentiate anaphase from other atypical mitotic figures like chromosome lagging, polar asymmetry and bridging mitosis, this does not affect their ability to be recognised as mitotic figures regardless of the phase or the typicality.

Telophase
In the last step of mitosis, the chromosomes begin to decondense into chromatin, the spindle breaks down and the nuclear membranes and nucleoli reform. The cytoplasm of the mother cell divides to form two daughter cells, each containing the same number and type of chromosomes. In addition to the microscopic image of its previous phase, the cell membrane can now be seen separating the two dense chromatin masses. It is the least common mitotic phase to be recognised and it can be misinterpreted by some pathologists as two metaphase mitoses however, their close proximity and the overall morphology should be considered, and these should be counted as one mitotic figure. Similar to anaphase, it is typically easy to identify telophase mitoses on WSI due to their characteristic morphology and the lack of mimickers.

Table 1 summarises the key morphological features of normal mitotic figures. Mitotic cells have cytoplasm that can be either basophilic or eosinophilic, but occasional figures can show a deep eosinophilic cytoplasm similar to that of apoptotic cells. The size of mitotic cells is approximately the same or larger than the surrounding malignant cells.
Atypical mitoses

All the cell cycle phases are highly regulated by several proteins which control the process of cell division and prevent abnormal mitoses. These checkpoints allow cells to proceed from one phase to another.\(^\text{15}\)

Failure of any of these checkpoints results in aberrant mitotic activity. Morphologically, the presence of atypical mitoses represents deviations from the well-described phases of typical mitosis.\(^\text{39-40}\)

The majority of tumours with atypical mitotic figures show some form of chromosomal instability,\(^\text{41-42}\) and they are frequently seen in highly proliferative and aggressive tumours, and they can also be seen following systemic chemotherapy. In practice, the identification of atypical mitoses is also of great value in distinguishing benign and malignant tumours, along with other criteria.

Recognition of the morphological features of atypical mitoses is critical, as they are of substantial clinical significance and some have different non-mitotic cell mimickers to be differentiated from such as apoptotic and karyorrhectic nuclei. Historically, it has been reported that atypical mitotic figures are most numerous towards the centre of the tumour and are rare at the periphery,\(^\text{43}\) but more recent studies assessing mitotic distribution in the tumour mass are needed. Atypical mitoses result from different mechanisms and therefore can show different morphology which includes the following:

- **Spindle multipolarity:** occurs in metaphase, the cell gains more than the two spindle poles, the chromosomes become distributed along several axes, and instead of having two poles at both ends of the cell, the cell may acquire three poles ‘tripolar’ (figure 3A,a), four poles ‘quadripolar’ (figure 3B,b) and more than four poles ‘multipolar’ (figure 3C,c). Multipolar mitoses are associated with numerical and functional abnormalities of centrosomes,\(^\text{44-45}\) and their unique morphology makes them easily identifiable on WSI as mitotic figures.
- **Chromosome lagging:** lag-type mitosis often exhibits a separated unattached condensed chromatin (figure 3D,d). Histologically, it can appear as chromosomes lagging between two separating masses of chromosomes during anaphase, at one side of the metaphase plate or both sides of metaphase plate.\(^\text{46-47}\)
- **Lagging chromosomes** represent the most common chromosomal segregation defect in cancer cell with chromosomal instability.\(^\text{47}\)
- **Polar asymmetry:** occurs during anaphase, and it appears as asymmetry in the size of the anaphase–telophase poles, occurring when chromosomes are segregated more into one of the two daughter cells.\(^\text{48}\) (figure 3E,e).
- **Anaphase bridge:** is defined as a filamentous connection linking two well-separated and parallel groups of anaphase chromosomes.\(^\text{49-51}\) It can be seen as a string of chromatin extending from one pole of the anaphase to the other (figure 3F,f).

**Table 1** Key morphological features of normal mitotic figures

<table>
<thead>
<tr>
<th>Mitotic phase</th>
<th>Key morphological features</th>
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<tbody>
<tr>
<td>Prophase/prometaphase</td>
<td>Hyperchromatic condensed chromatin, absence of nuclear membrane and hairy extension of nuclear material which appears as thick condensed, clotted or clumped strands of chromosomes</td>
</tr>
<tr>
<td>Metaphase</td>
<td>Perpendicular rod in the centre of the cell; or ring-like in ring metaphase</td>
</tr>
<tr>
<td>Anaphase</td>
<td>Two parallel condensed, clumps in the same cell</td>
</tr>
<tr>
<td>Telophase</td>
<td>Separate identical chromosome sets surrounded by cytoplasm and separated by a newly formed cell membrane</td>
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Detection of an anaphase bridge has been considered a useful method of indirectly evaluating telomere dysfunction.\(^\text{50-53}\)

Ring mitosis: appears as a bizarre figure in which chromosomes are displaced to the periphery of the cell (figure 3G,g). This should be differentiated from the round layer of normal chromosomes in the equatorial plane of the cell during metaphase seen at different plan section (ring metaphase). Ring mitoses can be seen associated with colchicine toxicity.\(^\text{54}\)

Dispersed mitosis: is a type of atypical mitosis where the cell is enlarged and the chromosomes are dispersed, evenly distributed and non-clumped; it shows no specific configuration (figure 3H,h).\(^\text{40-55}\)

This type of mitosis similar to some other forms of atypical mitosis should be differentiated from apoptotic and karyorrhectic nuclei. These cells can show multiple nuclear fragments floating in the cytoplasm with any arrangement and can be mistaken with atypical mitoses on WSI. The cytoplasmic features, size and location of the cells and the chromosome mass outlines can help in the distinction.

Given the fact that finding abnormal mitotic figures is of great clinical value, the lowest level of agreement was found on assessing atypical mitoses.

A study by Barry et al.,\(^\text{56}\) revealed poor agreement between pathologists (kappa 0.19) on the recognition of atypical mitoses, and in an unpublished study by our group, the interobserver reliability in counting atypical mitotic figures was limited (interclass correlation coefficient = 0.49). However, the assessment of atypical mitoses for BC grading can be considered from a different perspective.
mitoses, karyorrhectic nuclei/apoptotic cells often have dense ‘pink’ refractile cytoplasm and homogeneous chromatin.\textsuperscript{61} Identification of apoptotic figures is important as high levels of apoptosis, have been linked to a poor prognosis and have been reported as a poor prognostic factor in BC.\textsuperscript{52–64}

**Artefacts, pigments and crushed cells**

Formalin pigment artefacts (figure 4F) (black to brown finely granular birefringent deposit), delayed fixation (cause shrinking and cytoplasmic clustering), sipping dark inks, tattoo pigments (generally refractile), crushed artefacts and haemosiderin deposition are just a few of the artefacts that can mimic the hyperchromasia of a mitotic figure. The refractile nature of these mimickers or their presence across the entire mounting medium (even beyond the histological section) should distinguish them from true mitoses. Particularly, crushed artefacts or poor fixation are seen within a range of areas, so they should be recognised with care.

**Dark hyperchromatic nuclei**

Sometimes the hyperchromasia of malignant cells can be mistaken for the condensed chromatin seen in mitotic figures, especially in prophase (figure 4G–I). The decision to classify as mitosis is based on the absence of a nuclear membrane, seen as hairy extensions or irregular outlines. When using light microscopy these can be easily distinguished; however, on WSI we lack the ability to fine-tune between adjacent focal planes or to test for birefringence/refractile ability.

**Lymphocytes and other inflammatory cells**

Lymphocytes, granulocytes (figure 4J,K) and other inflammatory cells can have small hyperchromatic nuclei (figure 4O). Recognising these cells is often easy using the microscope; however, on suboptimally prepared slides it is often difficult to differentiate these cells from prophase. The presence of cytoplasm, hairy-like projections or at least a slightly irregular nuclear outline favour a mitotic figure in prophase. Furthermore, the distribution of chromatin within the nucleus is more even and appears denser in lymphocytes. Plasma cells often have abundant basophilic cytoplasm and an eccentric nucleus with a well-defined nuclear membrane and a characteristic cartwheel distribution of chromatin. They also contain a pale zone of the Golgi apparatus. The segmentation of the nuclei in neutrophils can help in the distinction from mitotic figures.

**Mitotic intratumour heterogeneity**

In many tumours, a substantial variation in mitotic rates is noticed in different areas of the same tumour as a result of intratumoural heterogeneity.\textsuperscript{63} As MC should be based on the highest count rather than the average, the choice of hotspots can be a major cause of low reproducibility among observers.\textsuperscript{65} For this reason, it is recommended that mitoses should ideally be counted on larger tissue sections to account for heterogeneity and to prevent sampling errors.\textsuperscript{65} There were many attempts to standardise the area for counting mitoses. Gal et al.\textsuperscript{66} proposed drawing a 2 mm\textsuperscript{2} rectangle on a coverslip in the most mitotically active area of the tumour. The range of mitoses counted by different observers using this method was found to be narrow, indicating that variability is greatly reduced when establishing a specific area to count in. However, this seems a time-consuming method, and this technique is not routinely used in practice. In addition, it does not help when the MC is assessed by one observer as is the case in routine practice. Identifying areas of highest MC does not only rely on the experience and skills of the reporting pathologist, but also on the speed of reporting in order

**Mitotic mimickers**

Many cellular morphological features can be mistaken for mitotic figures in histological sections. These include apoptotic bodies (online supplemental table S3), lymphocytes, darkly stained nuclei, dying or crushed cells and tissue artefacts. Although mitotic figures in H&E-stained sections have typical characteristic features as described above, distinguishing them from these mimickers is often challenging and requires careful assessment of these structures.

**Apoptotic bodies**

Apoptosis, also called ‘programmed’ cell death, is a distinctive form of cellular death that involves the genetically determined elimination of cells.

Apoptotic cells are smaller in size, with a dense, eosinophilic cytoplasm.\textsuperscript{59} The nuclei shrink and become condensed (pyknosis) and then fragment (karyorrhexis) and the cellular membrane detaches.\textsuperscript{60} Then the nuclear fragments dissolve (karyolysis). On histological examination, apoptotic bodies can resemble prophase mitoses (figure 4A–E). Conversely, the nuclear shrinking, smooth nuclear outlines, small size and the dense eosinophilic cytoplasm are in favour of apoptosis. Unlike the delicate or clumped appearance of chromatin that is associated with slightly eosinophilic cytoplasm and the absence of nuclear membranes seen in true...
to comply with clinical time requirements. This may explain at least partially the interobserver discordance. Evidence-based data on the best approach for selecting hotspots in BC is still needed. In addition, the method to identify hotspots remains less defined. Although it is recommended that these should be selected from the periphery of the tumours, this periphery can include many areas to be assessed and some tumours can be more cellular towards the centre. Further definition of hotspots is the subject of active research by our group.

**Tissue processing and practice-related factors**

**Sectioning and staining quality**

Adequate gross sectioning of the tumour as to be representative and to account for intratumoural mitotic heterogeneity is essential. Another point to raise is that sections thicker than 5 µm can lead to a high tumour cell volume with a potential bias towards a higher MC. The staining quality can also affect the visibility of the delicate chromatin structures needed to differentiate mitosis from other structures.

**Fixation time**

Delayed formalin fixation, known as ‘cold ischaemia’, has a detrimental effect on BC morphological features particularly mitosis. In addition to allowing many cells to progress to the G1 phase and not being able to detect them during microscopic examination, it can cause stromal retraction or even autolysis that can further affect the identification of these figures. Although it may not affect concordance rates, it will result in underscoring cases and under grading tumours with subsequent management implications on individual patients.

**Using conventional microscopes with different sized HPF**

It is a common practice to count mitotic figures in 10 HPF at ×40 magnification in several fields of pathology, including breast; but in certain tumours where MC can be highly variable, more HPF are required to account for this variability. For instance, in adrenal gland and gastrointestinal stromal tumours, mitotic figures are counted in 50 HPF or in 5 mm² areas.

Although a gradual firm move has been made towards standardising the area of measurement, HPF is still widely used without score adjustments throughout different fields of pathology apart from BC and few other tumours. Adjustment of mitotic scores based on the field diameter of the microscopes only provides standardisation of counting and may improve the concordance of scores when using the same microscopes, but does not address the clinical impact of the MC identified or the concordance when using different microscopes with variable field diameters. Theoretically and even in practice, MC in 10 HPF, which is usually equal to 2–2.5 mm² in most modern used microscopes are not reliable.

Even the original Nottingham grade authors have mentioned the need to count more than 10 HPF, but it has not been sufficiently highlighted, and only by increasing the sample size, this problem can be addressed.

Emerging evidence indicates that an area larger than 2 mm² is needed to produce an actual representation of MC in BC, a move towards standardising the area size with adjustment of the number of HPF examined should be considered for better concordance and representation of the actual proliferative activity of the tumours. Current evidence indicates that in BC, despite standardisation to account for the field diameter of each microscope, the reproducibility of mitotic scores remains low, especially when close to cut-off points. These cases can benefit from using larger areas for counting, which indirectly means more HPF rather than just adjusting the cut-offs. Using WSI in scoring may make these issues easier to address.

**Digital pathology and computer-assisted mitosis detection**

Digital pathology (DP) is a relatively new diagnostic technique, that entails converting diagnostic glass slides into digital images in the clinical settings using whole slide scanners with the full capacity to navigate these slides, meanwhile allowing detailed image analysis. Since the introduction of WSI scanners, several researchers have tried to improve and incorporate this new technology into diagnostic processes, and with further advancements in this field, DP eventually made its way from the research field to clinical practice. The labour-intensive nature of counting mitotic figures during grading, besides, the low concordance across pathologists, made it an attractive target for computer-assisted detection algorithms. These detection models are continuously and rapidly improving, but, before diving into artificial intelligence (AI)-assisted diagnostics, challenges of using WSIs have to be addressed first, together with the adoption of evidence-based clinically approved protocols.

The quality of the used hardware as well as the software can significantly and synergistically affect the computer-assisted mitosis detection. The hardware, such as scanner digital camera and resolution, sensors, scanner lens magnification and scanning operations, as well as the display monitor characteristics have an impact on the quality of the digital images and subsequently on the ability to detect mitotic figures and to differentiate them from their mimickers. The latter relies on the recognition of some fine details that can be lost in poor-quality images. The quality of the digital images also relies on other variables including the software used such as the image viewing software and the mitotic-cell identification algorithms in addition to the slide preparation quality. All these variable must be addressed carefully because errors in either hardware, software or in the slide preparation and scanning stages can lead to unsatisfactory results, and the sensitivity of computer assisted mitosis detection can undoubtedly be affected.

**Mitosis counting in the DP era**

There are additional challenges when using WSIs in counting mitoses.

In BC grading studies using WSIs, the interobserver and intraobserver agreement on mitotic scores was higher on glass slides, indicating that there is a slight tendency to underestimate the number of mitoses with a reduced ability to accurately define mitotic figures on WSIs.

As stated above, it is recognised that loss of fine-tuning capability when using WSI and lacking a refined list of objective criteria for mitosis identification can impede detection of true mitotic figures on WSI and hamper differentiation from mimickers. While scanning glass slides on multiple focal planes may provide a z-axis to WSI, however, this can increase scanning time, also, the storage requirements will be impractical for routine pathology work. Another point to note is that counting mitoses on WSIs has proved to be more time-consuming than counting mitoses with conventional microscopes, this extra time is usually spent on annotating the area for counting, marking each mitotic figure and counting the total number of annotations in the end.

In a recent unpublished study, we reviewed the mitotic figures on WSI, and corresponding glass slides using microscope and found that mitotic figures on WSI showed more hyperchromasia, which may be not recognised due to similarity with other mimickers. Moreover, when matching mitotic figures between the same regions on glass
and methodologies. Indeed, when a standardised methodology is not used, quite variable results may be obtained on the same material by different observers. By following a strict protocol, excellent reproducibility results could be gained. We have summarised a few key issues relating to MC with a particular emphasis on morphology and practicality. The use of digital images will bring new challenges in assessing mitoses and the further application of AI can only begin from clearly defined datasets.

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Review


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**Supplementary Table (S1)**- Common current markers of proliferation (advantages and Limitations).
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<thead>
<tr>
<th>Marker</th>
<th>Advantages</th>
<th>Limitations</th>
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<tbody>
<tr>
<td><strong>Immunohistochemistry</strong></td>
<td></td>
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</tr>
<tr>
<td><strong>Ki67</strong></td>
<td>Prognostically significant in invasive BC.</td>
<td>Field selection and stain intensities disagreement. Suboptimal reproducibility. Assesses all cells entering the cell cycle not only mitoses.</td>
</tr>
<tr>
<td><strong>PHH3</strong></td>
<td>High specificity to mitotic cells.</td>
<td>Clinical utility is still under investigation.</td>
</tr>
<tr>
<td><strong>Ki-S1</strong></td>
<td>Identifies a cell cycle associated antigen.</td>
<td>No prognostic results.</td>
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<tr>
<td><strong>Topoisomerase IIα</strong></td>
<td>Marker of cells ongoing division and similar to Ki67.</td>
<td>Similar to Ki67</td>
</tr>
<tr>
<td><strong>Cycline A</strong></td>
<td>Expressed in the late cell cycle phases S, G2, and M. Has prognostic importance in BC.</td>
<td>Similar to Ki67</td>
</tr>
<tr>
<td><strong>Nuclear antigen proliferating cells (PCNA)</strong></td>
<td>Targets the S phase of the cell cycle.</td>
<td>No prognostic results.</td>
</tr>
<tr>
<td><strong>Geminin</strong></td>
<td>Correlated with cell proliferation.</td>
<td>No prognostic results.</td>
</tr>
<tr>
<td><strong>Minichromosomemaintenance (McM)</strong></td>
<td>Involved in the control of DNA replication</td>
<td>No prognostic results.</td>
</tr>
<tr>
<td><strong>Stains</strong></td>
<td>Crystal violet, Toluidine blue, Giemsa stain and fluorescent microscopy.</td>
<td></td>
</tr>
<tr>
<td><strong>Cytometric phase S fraction</strong></td>
<td>Prognostically significant.</td>
<td>Use fresh / frozen material</td>
</tr>
<tr>
<td><strong>Argyrophilic nuclear organiser regions in Ki67 positive cells</strong></td>
<td>Prognostically significant</td>
<td>Difficult to evaluate</td>
</tr>
<tr>
<td><strong>Thymidine labelling index</strong></td>
<td>Prognostically significant.</td>
<td>Prognostic significance similar to Ki67 and MAI.</td>
</tr>
<tr>
<td><strong>Bromodeoxyuridine (BrdU) and tritiated thymidine labelling index</strong></td>
<td>Prognostically significant.</td>
<td>Use fresh material, Intravenous treatment and/or includes radioactivity</td>
</tr>
</tbody>
</table>
# Supplementary Table (S2)- Morphological features of mitoses.

<table>
<thead>
<tr>
<th>Phases of mitosis</th>
<th>Nuclear membrane</th>
<th>Hairy projections</th>
<th>Shape</th>
<th>Colour</th>
<th>Size</th>
<th>Chromatin homogeneity</th>
<th>Cytoplasm present or not seen</th>
<th>Cytoplasmic colour</th>
<th>Cell membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prophase</td>
<td>Intact</td>
<td>Absent</td>
<td>Round to oval</td>
<td>Intensely basophilic</td>
<td>No increase</td>
<td>Chromatin condenses into chromosomes that are coarse clumps of similar size</td>
<td>Present</td>
<td>Abundant granular slightly eosinophilic or basophilic</td>
<td>Intact cell membrane</td>
</tr>
<tr>
<td></td>
<td>nuclear membrane</td>
<td></td>
<td>Absent nucleolus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prometaphase</td>
<td>Absent</td>
<td>Present</td>
<td>Round to oval</td>
<td>Intensely basophilic</td>
<td>No increase</td>
<td>Condensed chromatin</td>
<td>Present</td>
<td>Abundant granular slightly eosinophilic or basophilic</td>
<td>Intact cell membrane</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Absent nucleolus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metaphase</td>
<td>Absent</td>
<td>Present</td>
<td>The metaphase plate may be:</td>
<td>Intensely basophilic</td>
<td>No increase</td>
<td>Condensed chromatin</td>
<td>Present</td>
<td>Abundant granular slightly eosinophilic or basophilic</td>
<td>Intact cell membrane</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- linear and perpendicular to the long axis of the spindle apparatus</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- parallel to the axis with chromosomes in a ring or round layer</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- “ring metaphase” forms can be seen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Absent nucleolus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaphase</td>
<td>Absent</td>
<td>Present</td>
<td>2 uniform groups of dark chromatins are being pulled away from each other</td>
<td>Intensely basophilic</td>
<td>No increase</td>
<td>Condensed chromatin</td>
<td>Present</td>
<td>Abundant granular slightly eosinophilic or basophilic</td>
<td>Intact cell membrane</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Absent nucleolus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Telophase</td>
<td>Absent</td>
<td>Present</td>
<td>Nuclear envelope forms around 2 uniformly sized separate clusters of chromosomes Nucleolus reforms</td>
<td>Intensely basophilic</td>
<td>No increase</td>
<td>Chromatin de-condensation</td>
<td>Present</td>
<td>Abundant granular slightly eosinophilic or basophilic</td>
<td>Cell membrane separates the 2 new cells</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Supplementary Table (S3)** - Comparison between mitotic and apoptotic figures in routinely stained haematoxylin and eosin slides.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Mitosis</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell size</strong></td>
<td>A slight increase in size.</td>
<td>Reduced cell size.</td>
</tr>
<tr>
<td><strong>Chromatin</strong></td>
<td>Condensed chromatin</td>
<td>Pyknosis (small condensed nuclei) – karyorrhexis (nuclear fragmentation) – karyolysis (dissolved nuclear material) and formation of apoptotic bodies</td>
</tr>
<tr>
<td><strong>Nuclear membrane</strong></td>
<td>Absent nuclear membrane.</td>
<td>Intact nuclear membrane.</td>
</tr>
<tr>
<td></td>
<td>Hairy projections of the nuclear material.</td>
<td>No hairy projection of nuclear material.</td>
</tr>
<tr>
<td><strong>Cytoplasm</strong></td>
<td>Abundant, slightly eosinophilic granular cytoplasm.</td>
<td>Densely eosinophilic cytoplasm.</td>
</tr>
<tr>
<td><strong>Cell membrane</strong></td>
<td>Intact; separates only during telophase resulting into two daughter cells</td>
<td>Intact; altered structure. Loss of cell to cell contact; blebbing, cytoplasmic buds, and formation of apoptotic bodies at late stages.</td>
</tr>
</tbody>
</table>
**Supplementary Figure (S4)**-Photomicrograph showing different mitotic figures and mimickers (H&E) using W5Is at 40x magnification.

- **(a-e):** Mitotic Figures (H&E) showing mitotic figures in prophase and metaphase.
- **(f-j):** Mimickers (H&E).
- **(f):** Apoptotic figure, **(g) Pigments, (h) Karyolitic cell, (i) Compressed elongated hyperchromatic cells, (j) Hyperchromatic cell.**