Estimation of Fine-Scale Histologic Features at Low Magnification

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• Context.—Whole-slide imaging has ushered in a new era of technology that has fostered the use of computational image analysis for diagnostic support and has begun to transfer the act of analyzing a slide to computer monitors. Due to the overwhelming amount of detail available in whole-slide images, analytic procedures—whether computational or visual—often operate at magnifications lower than the magnification at which the image was acquired. As a result, a corresponding reduction in image resolution occurs. It is unclear how much information is lost when magnification is reduced, and whether the rich color attributes of histologic slides can aid in reconstructing some of that information.

Objective.—To examine the correspondence between the color and spatial properties of whole-slide images to elucidate the impact of resolution reduction on the histologic attributes of the slide.

Design.—We simulated image resolution reduction and modeled its effect on classification of the underlying histologic structure. By harnessing measured histologic features and the intrinsic spatial relationships between histologic structures, we developed a predictive model to estimate the histologic composition of tissue in a manner that exceeds the resolution of the image.

Results.—Reduction in resolution resulted in a significant loss of the ability to accurately characterize histologic components at magnifications less than ×10. By utilizing pixel color, this ability was improved at all magnifications.

Conclusions.—Multiscale analysis of histologic images requires an adequate understanding of the limitations imposed by image resolution. Our findings suggest that some of these limitations may be overcome with computational modeling.

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histologic composition is lost; for instance, diagnostic information associated with nuclear morphology or tissue microstructure becomes difficult to reconstruct because the demarcation between subcellular structures is no longer visually distinguishable. Higher resolutions are therefore needed to resolve these features, which means that pathologists must examine selected regions of the tissue at higher magnification and that CAD algorithms must operate on higher-resolution images.

It has previously been noted that a relationship exists between the histologic identity of pixels and their color properties when H&E-stained tissue is captured at sufficiently high resolution. Since both color and spatial information can provide insight into the microstructural properties of histology images, we hypothesized that increasing spatial resolution is not necessary to accurately characterize the fine-scale features of tissue. We tested whether the color of a pixel at low resolution can be harnessed to infer its underlying histologic composition, effectively providing a super-resolution representation of the image. From these results, we postulate that pathologists synergistically use color and spatial cues to identify suspicious regions at low magnifications, and suggest that this method can be exploited by CAD algorithms to improve the efficiency and performance of multiscale image analysis.

MATERIALS AND METHODS

Case Selection and Data Source

We acquired high-resolution (0.25 μm/pixel) digital images of H&E-stained slides from 88 excised breast specimens at >40 magnification using the Aperio Scanscope XT whole-slide scanner (Leica Microsystems, Wetzlar, Germany). Cases were selected by an honest broker from a database of breast carcinoma cases acquired between 2010 and 2017 that were scanned as part of the clinical activities of the department for the purpose of reporting estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 (Her2) status. All cases were de-identified. The study was approved by the Drexel University’s Institutional Review Board.

Image Region Selection and Preprocessing

For each whole-slide image, we selected 2 regions of interest (ROIs) for analysis, each 800 μm × 800 μm in size, with an effort made to capture epithelium and stroma. The first region of interest was used as a training set to derive a set of color classifiers that described the color attributes of nuclei, cytoplasm, stroma, and the white point of the image, following a previously described method. These classifiers were subsequently used to support color normalization, measures of histologic composition, and nuclear segmentation. The results that we report in this study come strictly from the second ROI, however, which we refer to as the test data set. We discarded 9 of the 88 images that were either too sparse to support 2 ROIs or contained excessive histologic or digital artifacts (eg, blurring, folding). For the purposes of this study, the ROIs from the remaining 79 images were used after being standardized by using a color normalization procedure we previously described.

Histologic Features and Population Properties

To understand the relative sizes of histologic elements in H&E-stained images, we used cell nuclei as a representative histologic structure and estimated the size of nuclei in our sample. Using the color classifiers from the training set, we classified pixels into their most likely histologic elements, with a measure of classification certainty accompanying each pixel as described in Zarella et al. Cell nuclei were then delineated by using nuclear segmentation. To ensure accurate estimates of nucleus sizes, we discarded overlapping nuclei from this analysis. Since nuclei were largely composed of circular structures, we measured the area, A, of each nucleus and, approximating a perfect circle, computed its effective radius, \( r_0 \) (Equation 1).

\[
 r_0 = 2\sqrt{\frac{A}{\pi}}
\]

Given a distribution of nucleus sizes, we measured the likelihood that a pixel of size \( p \) would be entirely enclosed by a nucleus, and would therefore represent the nucleus without a mixture of other components. We defined this factor according to a geometric model that measured the ratio between randomly placed pixels that overlapped with the nucleus boundary and those that were entirely contained within the nucleus, and termed this the aliasing likelihood \( l \) (Equation 2). We performed this simulation using a total of 85,084 nonoverlapping nuclei, denoted in Equation 2 by \( N \).

\[
l = \frac{1}{N} \sum_{i=1}^{N} 1 - \frac{r_1^2}{r_0^2}
\]

In this formulation, \( r_1 \) represents the effective radius of positions within a circular nucleus that can contain a pixel of size \( p \) without that pixel extending beyond the boundary of the nucleus (Equation 3).

\[
r_1 = r_0 - \sqrt{2p}
\]

Spatial Colocalization of Histologic Elements

To measure the spatial relationship between histologic elements, as defined by the color-based classification paradigm described in the previous sections, we applied a colocalization metric supported by a permutation test. We measured the number of pixels of histologic element \( A \) that neighbored a pixel of histologic element \( B \) within a 40 × 40-pixel neighborhood. To understand how elements \( A \) and \( B \) were colocalized given their underlying spatial structures, we performed the same analysis after shuffling all non-\( A \) pixels in the image. We performed the shuffling procedure 100 times for each comparison and each image. We then compared the mean number of neighboring pixels of class \( B \) without shuffling to the mean number of neighboring pixels of class \( B \) after shuffling to derive a colocalization probability score for each image. We considered probability scores less than 0.05 as significant (ie, fewer than 5 of 100 shuffled images produced higher mean colocalization than the measured value). We counted the number of images in which a spatial relationship between histologic elements was significant, and used this tally to describe the expected frequency of histologic elements being present together in a 100-μm² region (corresponding to the pixel size at ×1 magnification).

We corroborated the results of this analysis by measuring the frequency at which histologic element \( A \) coexisted in the same ×1 pixel as histologic element \( B \). We identified ×1 pixels in which \( A \) was the most frequently observed, and counted the fraction of these in which \( B \) constituted more than 27% of the pixel. We used this as an alternative measure of colocalization.

Histologic Composition Model

Among the central purposes of this study was to estimate the histologic composition of an image at low magnifications. We defined a ground truth for this quantity as the proportion of pixels within a given region that represent nuclei, cytoplasm, stroma, and white space. Region size was defined by the magnification to be analyzed; for instance, estimation of the composition of a 10 μm × 10 μm pixel at ×1 required analysis of a 40 × 40 block of 0.25 μm pixels. Using the color classifiers derived from the training data set, we counted the number of pixels classified as nuclei, cytoplasm, stroma, and white space in the test data set. To confirm the accuracy of this characterization, we measured the concordance between pixel classification by using this method and manual pixel.
classification as described previously. We found that 97.3% of pixels were accurately classified.

To estimate histologic composition from low-magnification images, we developed a model that uses the color of a pixel to surmise its content. By exploiting the spatial relationships between histologic elements, and measuring their individual color properties, we derived axes in hue-saturation-value (HSV) space that can be used to predict the histologic composition of a pixel. We defined nuclear-cytoplasmic (N) and stromal-cytoplasmic (S) axes by the line segments that connected their constituent centroids in HSV space. Centroids were derived after color normalization was applied by measuring the mean color associated with each histologic element. The color normalization step ensured that centroids were approximately the same across images, as demonstrated in Supplementary Figure 1 (see supplemental digital content, containing 3 figures at www.archivesofpathology.org in the November 2018 table of contents). Likewise, we estimated the amount of white space by defining an axis W orthogonal to the line segment that connected the nuclear and stromal centroids and in the direction of the mean white point. For the purposes of visualization, we rotated HSV space to align the page with the plane formed by W and the nuclear-stromal line segment.

We projected pixels to the N, S, and W axes by calculating the dot product between a vector formed by the HSV value of a pixel p and a unit vector \( \mathbf{u} \) aligned with each axis (Equation 4, where \( x \) denotes 1 of the 3 axes).

\[
x_x = p \cdot \mathbf{u}_x
\]  

(4)

This calculation produced 3 projections \( x \) per pixel, each representing a scalar value along the N, S, and W axes. We restricted these projections to fall within the range of 0 to 1. From these values, we computed the histologic composition \( C \) of the pixel, an estimate of the relative proportion of each histologic element within the space confined by the pixel, by using the relationships in Equation 5.

\[
\begin{align*}
C_W &= x_W \\
C_N &= (1 - x_W) x_N \\
C_S &= (1 - x_W) x_S \\
N &= (1 - x_N) (1 - x_S)
\end{align*}
\]  

(5)

\( C_W, C_N, C_S \), and \( N \) represent the relative proportions of white space, nuclei, stroma, and cytoplasm in a given pixel, respectively.

**Histologic Composition Maps**

To visually represent the measured quantities, we generated pseudocolor maps by modulating the hue and saturation of the map while keeping the value equal to 1. Hue varied according to the projection of a pixel to the N or S axis, generating green for pure nuclei, blue for pure cytoplasm, and red for pure stroma (corresponding to hues of 2\pi/3, 4\pi/3, and 2\pi, respectively). Saturation was defined as 1 minus the projection to W. Although these values were arbitrarily chosen, by remapping the color of the pixel we exaggerated the mixture of histologic elements to aid in visualization, while the contribution of the white component emulated its actual impact on pixel saturation. Pixels with high proportions of white appeared “washed out.”

We measured the concordance between the measured histologic composition maps \( x \) and the predicted composition maps \( y \) by calculating the normalized mutual information (NMI) between the maps (Equation 6).

\[
\text{NMI}_K = \frac{I(x, y)}{I(x, x)}
\]  

(6)

The mutual information \( I \) represents the amount of information common to both maps and is expressed in units of bits. By dividing this quantity by the mutual information between the ground truth and itself, we forced the range of this metric between 0 and 1 with an upper limit defined as the ability to perfectly reproduce the ground truth from the predicted map. Although a value of 0 represents the minimum NMI value allowed, we used a procedure that randomly shuffled pixels in the image to establish a baseline NMI value corresponding to the comparison of dissimilar images. To ensure that the NMI could be compared across the varied magnifications and resolutions found in this study, we computed mutual information by using a random sample of 6400 pixels from each map. For the analysis at \( x_1 \), this meant using all pixels in the 80 \( \times \) 80-pixel maps. However, for analyses at higher magnifications, only a subset of map pixels was used.

**RESULTS**

We analyzed 79 images acquired at \( \times 40 \) magnification using whole-slide imaging. Images were stored in a proprietary format that enabled direct access to the image at lower resolutions, thereby reducing bandwidth and facilitating rapid loading for viewing and analysis. At full magnification, each pixel represented 0.25 \( \mu \)m of the slide. The native resolutions stored in the image files corresponded to \( \times 40, \times 10, \times 2.5, \) and \( \times 1.25 \) magnification, as well as a “thumbnail” image with a variable resolution that typically corresponded to \( \times 0.25 \) to \( \times 1 \) magnification.

We simulated the effect of reducing magnification by using spatial averaging to downsample \( \times 40 \) images. This enabled us to mimic the influence of aliasing and its corresponding loss of spatial information while retaining the ability to directly compare the downsampled image to the original image. Figure 1, B, depicts an example of aliasing due to a 40-fold reduction in the resolution of the image in Figure 1, A. Although individual nuclei and stromal detail are readily visible in the \( \times 40 \) image, at \( \times 1 \) this detail is lost. We measured the frequency of occurrence of aliasing by developing a model that used cell nuclei as a representative histologic element. We measured the sizes of nuclei across our image sample and compared these values to the pixel sizes (Figure 1, C) associated with different magnifications. We estimated the likelihood that a single pixel represented multiple histologic elements, and found that nuclei were sensitive to aliasing effects at all magnifications, an effect that was especially prominent at magnifications below \( \times 20 \) (Figure 1, D). At \( \times 8 \), for instance, nearly 60% of pixels that contained nuclei were likely to contain other histologic elements as well, such as cytoplasm. These results demonstrate that cell nuclei were generally smaller than the pixels at low and intermediate magnifications, establishing the widespread incidence of aliasing in histology images and the potential loss of information associated with it.

To understand the consequence of aliasing on histology images, we compared the color attributes of images at \( \times 40 \) and \( \times 1 \) magnification. We plotted the hue, saturation, and value of pixels in a representative \( \times 40 \) image (Figure 2, A; black points) by rotating HSV space to optimally reveal the variance of pixels in H&E-stained images. We performed the equivalent measurement at \( \times 1 \) (Figure 2, B) and found that nearly all pixels mapped to a region of HSV space between the mean nucleus, stroma, and cytoplasm colors (each indicated by red points), implying that \( \times 1 \) pixels generally reflect a mixture of colors associated with their underlying histologic composition. At \( \times 1 \), nearly every pixel is lighter than the mean color of a nucleus determined at \( \times 40 \). The effect is less prominent for stroma, though many pixels that contain stroma shift to a mixture between the mean color of stroma and some mixture of white or cytoplasm. Color normalization allowed us to align the color means across images with different staining attributes.

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to confirm this observation over the population. The heat map shown in Figure 2, C, represents the relative density of
pixel colors at \(3^1\) across all 79 samples. Overall, a compression of the color dynamics was observed for all 79
images as a result of aliasing (the standard deviation of the
points about the mean was 0.134 for \(3^{40}\) images and 0.087
for \(3^1\) images). These findings are consistent with the
expectation that the color of a pixel at a low resolution is the
composite of the colors of the constituent pixels.

We hypothesized that the histologic composition of \(3^{40}\)
pixels can be reconstructed from an \(3^1\) image based on
color. We built a model that estimates the relative proportions of nuclei, cytoplasm, stroma, and white space
from the color of a pixel at \(3^1\), given 2 assumptions: (1) the
constituent pixels have well-defined colors that correspond
to their histologic identity; (2) only certain histologic
elements are colocalized within the same \(3^1\) pixel. Although
the first assumption is unlikely to be true for most images,
color normalization is a powerful preprocessing tool that
can artificially make pixels with the same histologic identity
conform to a single color with very little interimage
variability.\(^{11,14}\) We tested whether the second assumption
naturally occurs in histology images, and if so, to what
extent this could be harnessed to enable a super-resolution
prediction of histologic composition in low-resolution
images. We measured the colocalization of nuclei, cyto-
plasm, stroma, and white pixels within 40 \(3^{40}\) blocks of
pixels (corresponding to the 40-fold increase in pixel size at
\(3^1\)). We compared this value to that expected by chance by
applying a permutation test that destroyed the spatial
structure of the histologic elements by randomly shuffling
pixel position (eg, Supplementary Figure 2). This analysis
effectively provided a likelihood measure of the co-
ocurrence of 2 histologic elements within the same 10
\(\mu m \times 10 \mu m\) pixel at \(3^1\). As expected, we found a strong
association between nuclei and cytoplasm (Table 1), where

Figure 1. A representative hematoxylin-eosin (H&E) image depicted at \(\times 40\) (A) and a simulation of this image at \(\times 1\) (B). The simulated image was
computed by averaging RGB values in 40 \(\times 40\)-pixel blocks. C, The size of a pixel at a given magnification was calculated as the base pixel size (0.25
\(\mu m\) in length) acquired at \(\times 40\) magnification as a fraction of zoom level. D, The percentage of pixels in simulation that overlap with the boundary of a
nucleus, termed the aliasing likelihood, is shown as a function of magnification. Overlap with a nucleus boundary implies that other histologic
elements, or background, are therefore present in the pixel, and that the mean color of the pixel no longer strictly represents the nucleus. Every
detected nonoverlapping nucleus from all test images was considered, and an aliasing likelihood was computed separately for each nucleus. Error
bars represent the standard deviation of aliasing likelihood values across all tested nuclei.
statistically significant colocalization was observed in all images ($P < .05$). Likewise, stroma and white space were often colocalized (significant in $77\%$ of images), likely due to the striated pattern typically observed in stromal areas. Cytoplasm generally exhibited an association with all other histologic elements; the frequent interaction between stroma and epithelium is an especially prominent source of the colocalization observed between cytoplasm, stroma, and white pixels, although it did not reach statistical significance in all images. Importantly, nucleus pixels rarely colocalized with stroma pixels. This finding suggests that nucleus-stroma color mixture does not often occur in $x\times1$ pixels. We confirmed this observation by measuring the frequency at which stroma points were present in nucleus-dominant $x\times1$ pixels, and vice versa. Fewer than $2\%$ of pixels exhibited a strong interaction between nucleus and stroma, compared to a nucleus-cytoplasm interaction rate of $64\%$ and stroma-cytoplasm rate of $18\%$.

We exploited this observation to build a histologic composition model that considered stroma-cytoplasm, nucleus-cytoplasm, and tissue-white spatial colocalization. We defined axes that connected the points in HSV space corresponding to nuclei, cytoplasm, stroma, and white pixel color, shown schematically in Figure 3, A. To estimate the histologic composition of an $x\times1$ pixel, we projected its HSV value onto these axes. The interpretation of these projections defined the relative ratios of histologic elements present in the $x\times1$ pixel, providing super-resolution insight into low-resolution images. For instance, a pixel that projected one-third the length of the white axis and to the middle of the cytoplasm-stroma axis was estimated to have equal proportions of cytoplasm, stroma, and white pixels. Conversely, a pixel that projected to a value of $0.8$ along the cytoplasm-nucleus axis and $0.1$ along the white axis was estimated to be composed of $72\%$ nuclei, $18\%$ cytoplasm, and $10\%$ white. As demonstrated by the distribution of pixels falling on the nucleus-cytoplasm and cytoplasm-stroma axes (Figure 3, B), $x\times1$ pixels were rarely composed of purely nucleus pixels, consistent with our observation that nuclei tended to be smaller than the size of an $x\times1$ pixel. Stroma, with a much more variable spatial structure, predictably had a broader distribution of mixtures that often reached (or exceeded) a value of $1$ on the cytoplasm-stroma axis. When the composition of white pixels was also considered, it became apparent that a modest mixture of white generally accompanied most $x\times1$ pixels (Figure 3, C), with a notable peak near $1$ occurring for blank regions of the slide (arrow). Further analysis of these projections shows that pixels that were estimated to contain a nucleus majority (Figure 3, C; purple line) tended to contain much smaller proportions of white, whereas pixels with higher stromal composition (Figure 3, C; yellow line) often contained a much greater proportion of white pixels. These estimates are consistent with measured proportions of histologic composition in $x\times1$ pixels, where the relative proportions could be directly computed from the identity of the pixels at $x\times40$. In those measurements, the mean proportion of white is $14\%$ in stroma-dominant regions and $8\%$ in nucleus-dominant regions, suggesting concordance between predicted and measured histologic composition.

In Figure 4, we show an example H&E image (Figure 4, A), its corresponding histologic composition at $x\times1$ measured by analyzing the identity of $x\times40$ pixels (Figure 4, B), and its estimated histologic composition using the histologic composition model (Figure 4, C). Red corresponds to stroma-dominant pixels, green corresponds to nucleus-dominant pixels, and dark blue corresponds to cytoplasm-dominant pixels. Mixtures of these elements are denoted by a change in the hue of the pixel; purple, for instance, implies a mixture of stroma and epithelium, while aqua implies a mixture of nuclei and cytoplasm. The contribution of white pixels modulates the saturation of the color in these maps so that pixels with high white content appear washed out. Consistent with the distribution in Figure 3, B, there are very few green pixels in this map. The green pixels generally correspond to large or overlapping nuclei whose sizes approach the pixel size. The dense epithelial region in the lower right hand side of the image shows a relatively strong nuclear concentration, present in both the estimated and

| Table 1. Histologic Structure Colocalization |
|--------------------------------------------|
| White | Nuclei | Cytoplasm | Stroma |
|-------|--------|-----------|--------|
| White | —      | 0.16      | 0.16   | 0.63   |
| Nuclei| 0.16   | —         | 0.95   | 0.10   |
| Cytoplasm| 0.53 | 1.00 | —     | 0.68   |
| Stroma| 0.77   | 0.09      | 0.30   | —      |

Figure 2. A, Pixel colors, in hue-saturation-value (HSV) coordinates, of a sample image are plotted relative to the mean color of stroma, cytoplasm, nuclei, and white space (red points). The plane represents a rotation in HSV space formed by the line segment that connects the stroma and nucleus red points and the orthogonal line segment that connects the white space point. The pixel colors were determined after color normalization was applied. B, Pixels from the same image are shown after reduction to $x\times1$ magnification. C, Pixels from all test images were aligned on this plane after color normalization. The heat map represents the relative density of points computed by using the kernel density estimate with $\sigma = 8$ pixels. The red points in (C) correspond to those in (A) and (B).
Figure 3.  A, A schematic representing histologic composition estimation. Pixels are projected to axes in hue-saturation-value (HSV) space formed by the line segments connecting stroma (Str) and cytoplasm (Cyt) means, nucleus (Nuc) and cytoplasm means, and an orthogonal axis toward the direction of white space (Wht). Axes drawn for clarity. B, The distribution of values formed by the projection of simulated ×1 pixels to the N (nuclear-cytoplasmic) and S (stromal-cytoplasmic) axes is shown. Pixels were pooled across all images. Values that projected close to Cyt were given a score of 0, while values that projected close to Nuc or Str were given a score of 1 on their respective axes. To demonstrate the continuous nature of this quantity when transitioning from the N to the S axis, the 2 axes are represented on the same plot; the intersection of these distributions is denoted by a circle. The sharp peak near 0.6 coincides with the high concentration of white pixels observed on the right side of (A) and denoted in (C) by the arrow. C, The distributions of values formed by the projection of simulated ×1 pixels to the orthogonal (white) axis is shown. As in (B), pixels were derived from all test images. They were categorized according to their positions on the N and S axes and separately plotted by each category. Pixels that were greater than 0.5 on the N axis are identified as the “nuclei” group; pixels greater than 0.5 on the S axis are identified as the “stroma” group; the remaining pixels are identified as the “cytoplasm” group. These quantities were normalized by group to aid in their comparison. The arrow denotes the peak in the “stroma” group that corresponds to pixels that represented entirely white spaces.

Figure 4. The relative proportions of pixels classified as nuclei, cytoplasm, stroma, and white space were calculated in blocks of 40 × 40 pixels from the sample hematoxylin-eosin (H&E)–stained image (×40) shown in (A). B, The representation of this proportion is shown in a map whereby nuclei are represented by green, cytoplasm is represented by dark blue, stroma is represented by red, and white space is represented by white. Mixtures of these quantities are represented by modulating the hue and saturation. The spatial scale of this map is equivalent to an ×1 image. C, A corresponding map is shown for the image in (A) in which the proportions are derived from estimates computed directly from the pixel colors at ×1. The normalized mutual information between these 2 maps is 0.67.
measured maps and consistent with a manual count of nuclei in this region (467 nuclei in the lower right versus 320 nuclei in a region of comparable size on the lower left). These results support the notion that the color properties of pixels at ×1 magnification can predict the fine-scale histologic composition of the tissue.

To quantify this observation across our sample, we measured the correspondence between the estimated and measured histologic composition maps by using a normalized version of the mutual information metric. An NMI of 1 implies perfect agreement, whereas an NMI of approximately 0.3 occurs when 2 maps lack a spatial relationship (shuffled NMI = 0.298 ± 0.045). We found that the mean NMI of the 79 images decreased with magnification, reaching a minimum of 0.66 at ×1. For context, this value is equivalent to the NMI obtained by artificially manipulating histologic composition by randomly shuffling a small subset of an image’s pixels, which produces only a modest distortion of the image (Supplementary Figure 3). These results confirm that an NMI of 0.66 represents substantial agreement between maps, and suggest that the estimation of histologic composition using color remains accurate even at low magnifications.

To demonstrate the increase in information content by estimating histologic composition, we compared the NMI by using the estimation method to the NMI by using color classification alone (Figure 5). Naturally, we expected that classification of an ×1 pixel as a singular histologic element, based on classical methods, would result in a loss of detail about the presence of other elements within that pixel. For both methods, NMI decreased as magnification was reduced, though at a much lower rate when histologic composition was estimated than when it was ignored (Wilcoxon signed rank test, P < .001). The performance of estimation at ×1 was similar to the performance of color classification at ×20, demonstrating a vast increase in information obtained by estimating histologic composition from pixel color. These results reveal that histologic composition can be accurately estimated at low magnifications and contains information not captured by conventional methods of pixel classification.

For each case, the entire processing pipeline was self-contained. That is, for a given case, color normalization was performed by transforming the image’s colors to a predefined and somewhat arbitrary target, color classification was performed by using a classifier derived from the training image from that particular case, and the projection of points to the axes in the histologic composition model was independent of other images. However, we also tested whether the classifiers derived from other normalized images can be used instead of deriving them from each case de novo. We performed k-folds cross-validation to derive a color classifier from a set of images that did not include the image under test, and recomputed the NMI values presented in Figure 5. We found that applying a generalized color classifier to normalized images had a negligible impact on NMI (Table 2), suggesting that the algorithm we describe can be applied in practice without the need for recomputing the color classifier each time. This result underscores the importance of an accurate color normalization procedure on the success of the algorithm.

### DISCUSSION

The magnifications that pathologists use to examine a slide inherently result in a tradeoff between image detail and field of view and impact the overall efficiency of the process. At low magnifications, pathologists often look for coarse-scale features that may guide further examination at higher magnifications. The classical view suggests that the intrinsic limitations of image resolution, whether due to optical factors when viewing through a microscope or the capabilities of the digitization system and computer monitor, impose a hard limit on what is visible to the pathologist. In this article, we report that the color properties of pixels intrinsically contain information that enable observers to extrapolate histologic content that is smaller than the size of the pixels. We developed a model to demonstrate this effect and to measure the accuracy at which it may occur. We hypothesize that the human brain naturally performs a similar computation, and we suggest that this may depend on a number of factors including the experience of the pathologist. Analysis of gaze fixation times during low-magnification scanning reveals that experienced observers spend less time and commit fewer diagnostic errors than less experienced trainees. Therefore, pathology training not only includes learning the complexities of the microscopic features characteristic of the hallmarks of disease, but may also include honing the basic visual mechanisms that support the efficient examination of a slide. Although the results that we describe demonstrate one way in which this may occur, further research is needed to explicitly test this hypothesis.

The results of this study have broad applicability not only to understanding the visual processes used to examine glass slides and whole-slide images, but also to provide a novel...
processing algorithm to support CAD. For example, the histologic composition model can be applied as a pre-processing step to identify regions of interest by detecting fine-scale features from low-resolution images. The prognostic features associated with stromal characteristics,\textsuperscript{16,17} cellular density,\textsuperscript{18} lymphocyte concentration,\textsuperscript{19–21} and nuclear-cytoplasmic ratio\textsuperscript{22,23} can potentially be revealed at lower magnifications than previously possible. As shown in Figure 5, conventional classification achieves an NMI at ×20 that is similar to the NMI achieved by the histologic composition model at ×1; this 20-fold difference in magnification therefore has the potential to improve computational efficiency by a factor of up to 400.

The histologic composition model that we describe relies on an expectation that the histologic structures of interest contain stereotypic color properties—an expectation that is not always observed in practice. There is often significant variability in the H&E staining characteristics of tissues, even when comparing across the same type of tissue from the same laboratory.\textsuperscript{10} However, we used color normalization to force images to adhere to a rigid set of color attributes. We used a separate training set to train the color classifiers necessary for this step to demonstrate that an initial normalization procedure can be applied in practice by using a small region of the image to initially train the classifiers that can then be applied to the image in its entirety. Although we used a color normalization algorithm based on structure classification and color transformation, we have no reason to believe that the histologic composition model relies on this particular method. Other forms of color normalization, such as those based on color deconvolution\textsuperscript{24} or unsupervised stain separation,\textsuperscript{25} are likely to be sufficient, although we did not explicitly test this assertion. Notably, color normalization is not available when looking at images through the microscope. However, human observers may rely on visual mechanisms such as color constancy\textsuperscript{26} to accomplish a similar effect. Improvements in diagnostic performance\textsuperscript{27} and image quality\textsuperscript{28} have been shown by using color correction, further supporting the notion that color plays a role in diagnosis.

The projection of pixels to a color mixture space represented by 3 axes was based on the observation that nuclei and stroma did not usually coexist in the same ×1 pixel. We used 2 methods to establish this finding: a colocalization model and direct measurements of pixel content in 10 \( \mu \text{m} \times 10 \mu \text{m} \) regions, corresponding to the size of an ×1 pixel. We found a very low, but non-zero, co-occurrence of stroma and nuclei, likely due to the proximity of fibroblasts and lymphocytes to stromal tissue. Analysis of the 7 images that exhibited statistically significant colocalization of stroma and nuclei revealed that these images were particularly rich in fibroblasts and lymphocytes. Regional analysis of the discordance between the estimated histologic composition and ground truth in these images shows that its effect on NMI is in fact strongest in these areas. We caution, therefore, that estimates of histologic composition in stromal regions with high fibroblast or lymphocyte concentration may overstate the presence of cytoplasm.

We used a single whole-slide scanning system to capture histology images for the purpose of this study. However, we believe that the results not only extend to other whole-slide imaging systems but also to routine microscopic analysis. For clarity, we expressed the results in terms of equivalent magnification, but it is important to note that resolution is impacted by other factors.\textsuperscript{29} In digital imaging, resolution is generally determined by pixel size, with an upper limit usually dictated by the image sensor or computer monitor. In light microscopy, the effective resolution is determined by the optical resolution of the microscope, illumination, or the visual acuity of the observer. However, a relationship between resolution and magnification can be estimated for any given system, as we did in Figure 1, C, and the results of our analysis can be extended to other systems by a corresponding change in the analysis parameters.

One of the principal findings in this article is that low-resolution viewing results in significant aliasing of the content of the image, using cell nuclei as a representative example of the microstructural elements commonly found in histology images. Indeed, some analyses may focus on higher-order structures that do not necessarily require the precise identification of nuclei, and therefore exhibit a larger space constant. We argue, however, that our findings are applicable across a broad array of magnifications and space constants. In Figure 5, we show that the difference between the estimate of histologic composition and conventional pixelwise classification is significant even at ×20 magnification, where the aliasing likelihood for nuclei is still quite small. Therefore, even larger histologic structures that exhibit a small aliasing likelihood at ×1 may benefit from understanding the role of color in H&E interpretation and analysis.

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