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Quantitative Assessment of Focus Quality in Whole-Slide Imaging of Thyroid Liquid-Based Cytology Using Laplacian Variance

Chan Kwon Jung[1,2,6] · **Chankyung Kim**[3,4] · **Sora Jeon**[2] · **Andrey Bychkov**[5]

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Abstract

Reliable focus is essential for digital cytopathology, yet three-dimensional cell clusters in liquid-based cytology (LBC) slides pose persistent challenges for single-plane whole-slide imaging. We sought to quantitatively compare focus performance among three whole-slide scanners and to evaluate the use of Laplacian variance as an objective focus quality metric. A total of 102,573 image tiles (512 × 512 pixels) were generated from 36 whole-slide images after scanning twelve thyroid LBC slides at 40× magnification using three scanners. Single-plane mode with automatic focusing was applied. Image sharpness for each tile was computed in Python using the variance of the Laplacian, quantified as a focus score and classified into five ordinal categories, ultimately dichotomized as in-focus (variance ≥ 10) versus out-of-focus. Statistical comparisons between scanners were performed. Laplacian variance scores showed strong concordance with visual assessments of focus quality made by pathologists. One scanner consistently achieved the highest median focus scores and the most uniform spatial distribution for SurePath slides, while the other two demonstrated lower median values and greater variability. ThinPrep slides exhibited high focus quality across all scanners (> 95% in-focus), with smaller but still measurable inter-scanner differences. Overall, more than 70% of tiles were in-focus across all scanners, suggesting preserved diagnostic usability. Laplacian variance is a practical and reproducible tool for assessing focus quality in digital cytology. Focus performance varied among scanners, particularly for slides with three-dimensional cell clusters, whereas differences were less pronounced for monolayer ThinPrep preparations. Quantitative focus assessment may support scanner selection, quality assurance, and computational cytopathology workflows.

Keywords Thyroid cytology · Digital cytology · Laplacian variance · Focus · Sharpness · Depth of field · Digital scanner · Whole-slide image

✉ Chan Kwon Jung
ckjung@catholic.ac.kr

Chankyung Kim
chankyung.kim@sa.gov.au

Sora Jeon
thfk38@nate.com

Andrey Bychkov
bychkov.andrey@kameda.jp

[1] Department of Hospital Pathology, College of Medicine, The Catholic University of Korea, Seoul 06591, Republic of Korea

[2] Cancer Research Institute, College of Medicine, The Catholic University of Korea, Seoul 06591, Republic of Korea

[3] Department of Anatomical Pathology, Royal Adelaide Hospital, SA Pathology, Adelaide, SA 5000, Australia

[4] Adelaide Medical School, Faculty of Health and Medical Sciences, University of Adelaide, Adelaide, SA 5000, Australia

[5] Department of Pathology, Kameda Medical Center, Kamogawa City, Chiba 296-8602, Japan

[6] Department of Pathology, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, 222 Banpo-daero, Seocho-gu, Seoul 06591, Republic of Korea

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Introduction

Digital pathology is increasingly gaining recognition as an essential component of modern cytopathology, allowing computational image analysis, artificial intelligence (AI)-assisted large-scale screening, remote consultation, and whole-slide image (WSI) archiving [1–4]. However, optimal digitization of cytology slides remains technically challenging because of the inherently three-dimensional nature of cytologic preparations and the variable thickness of cell clusters [5–7]. In particular, focusing issues are a well-recognized limitation in cytology slide scanning, as a single focal plane may not capture all diagnostically relevant cellular details.

Liquid-based cytology (LBC) has replaced conventional direct smears in some laboratories for thyroid cytology because it offers improved specimen adequacy, cleaner background, better cell preservation, and more reproducible slide preparation [8, 9]. LBC slides are generally considered more compatible with digital scanning than conventional smears [5]. There are two major LBC preparation systems, including ThinPrep (Hologic, Marlborough, MA, USA) and SurePath (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), producing cytology slides with distinct morphologic characteristics due to differences in preparation principles. ThinPrep uses a filtration and transfer method, producing a monolayer of flattened cell clusters, whereas SurePath employs density gradient sedimentation, resulting in larger, three-dimensional cell clusters [10]. Previous studies have suggested that the more complex three-dimensional architecture of SurePath slides may require more frequent focusing during microscopy and can present a greater challenge for automated WSI scanning [11, 12].

In addition to preparation-related factors, scanner hardware and focusing algorithms can influence digital image quality. Systematic comparison of focus performance across multiple scanners using objective quantitative metrics is necessary to guide equipment selection for cytology applications.

In practice, Z-stacking is often recommended to overcome focusing challenges. Z-stacking refers to acquiring a series of images at multiple focal planes along the z-axis and combining them to reconstruct three-dimensional structures or maximize visible detail [3, 6]. Extended depth of field (EDF) techniques further enhance this process by algorithmically selecting the sharpest pixel information from each z-slice and merging them into a single, fully focused image [6]. While these approaches produce diagnostic quality images, their long scanning time and large data size limit their routine use in pathology laboratories [13, 14], where single-plane scanning remains the practical standard. Therefore, technologies that improve focus performance in single-plane imaging are crucial.

Despite the importance of focus quality for diagnostic accuracy, only a few studies have proposed reproducible quantitative methods for assessing scanner focus performance. The variance of the Laplacian is a numerical measure of image sharpness and offers a simple, robust, and computationally efficient approach for quantifying focus quality on a tile-by-tile basis [15–17]. The Laplacian variance metric serves to quantify the degree of local intensity variation within an image, with particular sensitivity to changes occurring along edges. When this metric yields higher values, it indicates the presence of sharp edges and well-defined cellular features within the image, signifying that the image is well-focused. In contrast, lower Laplacian variance values are associated with the presence of blurred contours, suggesting a higher likelihood that the image is out of focus. This direct relationship between Laplacian variance and image sharpness makes it a valuable tool for objectively assessing focus quality in digital cytology slide imaging.

The present study aimed to introduce the use of Laplacian variance as an objective, quantitative measure of image sharpness in cytology slide imaging, compare focus quality between ThinPrep and SurePath thyroid LBC slides, and lastly, evaluate the performance of three commercially available scanners in capturing diagnostically usable WSIs (3).

Methods

Sample Preparation

Twelve thyroid LBC slides diagnosed as papillary thyroid carcinoma were retrospectively selected for this study. To represent a realistic range of specimen quality, cases were intentionally chosen to include slides with both high and low cellularity. Six slides were prepared using the SurePath method and the other six were prepared using the ThinPrep method. All slides were fixed and stained with Papanicolaou stain according to the manufacturers' protocols.

Whole-Slide Image Acquisition

All slides were digitized once, without repeat scanning, as WSIs using three different scanners: LH510 (Vieworks, Anyang, Republic of Korea), GT450 (Leica Biosystems, Deer Park, IL, USA), and S360 (Hamamatsu Photonics, Hamamatsu, Japan) at 40× magnification in bright-field mode. The scanning resolution at 40× was 0.27 µm/pixel for the LH510, 0.26 µm/pixel for the GT450, and 0.23 µm/

pixel for the S360. WSIs were stored in proprietary vendor file formats. All scanners were operated according to the manufacturer's recommended protocols using automatic focusing modes, and no manual focus correction was applied. Single-plane scanning was employed for all slides, and no Z-stacking algorithm was used, thereby allowing a direct comparison of scanner performance under standard single-plane imaging conditions.

Image Tile Generation and Chroma-Based Image Validation

WSIs from thyroid LBC slides were processed in QuPath (version 0.6.0, https://qupath.readthedocs.io) [18]. Regions of interest (ROIs) were manually annotated by a pathologist, and tiles were exported only within annotations using the QuPath TileExporter. Tiles were generated at native resolution with a size of 512 × 512 pixels, without overlap, and saved as TIFF (.tif) files. During tile export, each tile underwent real-time chroma-based background filtering in the YUV color space. For each tile, the U and V components of every pixel were computed, squared, and summed to obtain the mean ($U^2 + V^2$). Instead of applying a single cutoff value, scanner-specific chroma thresholds were empirically adjusted for each so that the number of valid tiles per WSI would be comparable across all three scanners. This method minimized bias arising from device-specific differences in image background characteristics. The surviving tiles were renamed to include spatial metadata facilitating downstream localization within the WSIs.

Focus Quality Assessment Using Laplacian Variance

Tile coordinates were extracted from standardized filenames and converted to grid indices by integer division based on the tile size, allowing spatial reconstruction for visualization. Image sharpness for each tile was quantified using the variance of the Laplacian, computed with a custom Python script, built on standard image-processing libraries. The Laplacian variance was then used to classify the focus quality of each tile into five ordinal levels: tiles with variance below 5 were categorized as very low quality, those with variance between 5 and 10 as low quality, between 10 and 20 as medium quality, between 20 and 50 as good quality, and those with variance equal to or greater than 50 as excellent quality. These thresholds were empirically determined from pilot analyses where pathologists visually evaluated 512 × 512-pixel tiles on a 32-inch medical-grade diagnostic display (LG 32HL512D). The corresponding Laplacian variance values showed consistent alignment with perceived image sharpness, allowing distinct qualitative focus levels. A binary focus status was additionally defined using a variance threshold of 10, such that tiles with variance below 10 were considered out-of-focus, whereas those with variance equal to or above 10 were considered in-focus.

For each WSI, a Comma Separated Values (CSV) file was generated containing the tile filename, coordinates, Laplacian variance, focus status, and quality level. Tiles were also copied into grade-specific folders for manual review. A heatmap visualizing the spatial distribution of Laplacian variance across the WSI was generated using the matplotlib library in Python (version 3.11), with a custom colormap reflecting the predefined focus quality levels. The heatmap provided an overview of spatial heterogeneity of focus quality within the WSI.

Statistical Analysis

Quantitative results were summarized with box plots comparing the distribution of focus scores across the three scanners separately. All statistical analyses and graph generation were performed using Rex software (version 3.3.1, RexSoft Inc., Seoul, Republic of Korea). A p-value < 0.05 was considered statistically significant. Statistical graphs were generated in Rex with standardized axis scales and consistent color schemes to allow direct comparison between scanners and preparation methods.

Results

All 36 WSIs from twelve thyroid LBC slides were analyzed and are presented according to preparation method: cases SP1–SP6 were prepared by the SurePath method, and cases TP1–TP6 by the ThinPrep method. Visual inspection of WSIs revealed substantial heterogeneity in focus quality within individual slides. Focus scores based on Laplacian variance closely reflected the focus quality perceived by pathologists during visual review (Fig. 1). A total of 102,573 image tiles were successfully processed (Table 1), and their focus quality quantitatively evaluated using the Laplacian variance metric. This method provided a stable and reproducible measure of image sharpness across all tiles and scanners, enabling objective comparison of scanner performance and visualization of spatial heterogeneity within and across WSIs.

Scanner Performance in SurePath Slides

Quantitative analysis of the six SurePath slides demonstrated that LH510 consistently yielded the highest Laplacian variance values (Fig. 2). The median focus score for

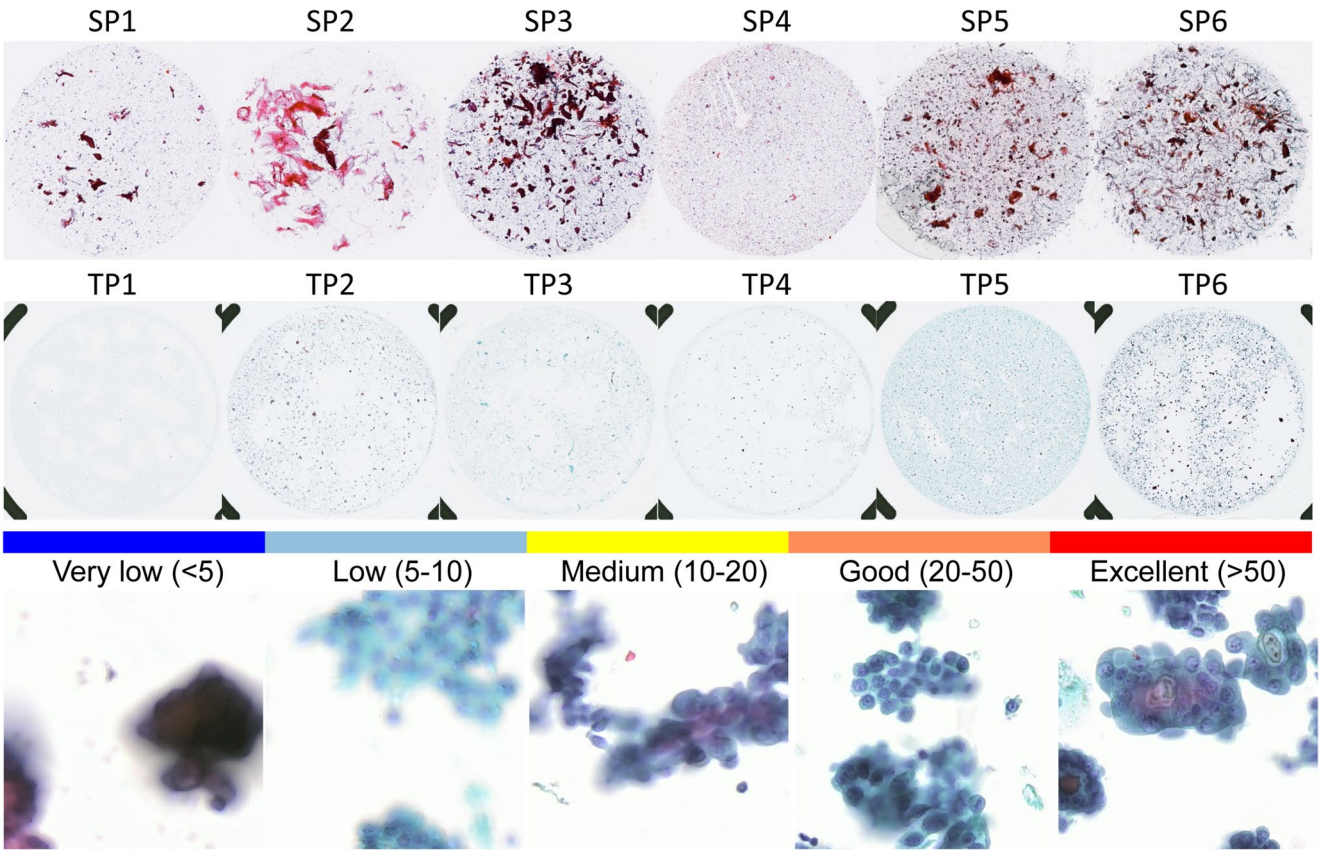

Fig. 1 Representative whole-slide images and high-power views of focus quality samples. Low-magnification overviews of thyroid liquid-based cytology slides show heterogeneous distribution of cellular material. Heatmaps of Laplacian variance are presented using a five-level colormap (very low = dark blue, low = light blue, medium = green, good = orange, excellent = red). The numeric values in brackets indicate the ranges of Laplacian variance. Representative magnified views highlight areas with excellent focus (red) and blurred regions (blue), demonstrating intra-slide variability of image sharpness captured by the Laplacian variance metric

Table 1 Total number of analyzed image tiles and percentage of tiles classified as in-focus for each case and scanner

Case	Liquid-based cytology	GT450		LH510		S360	
		Total *n*	In-focus %	Total *n*	In-focus %	Total *n*	In-focus %
SP1	SurePath	693	95.5%	702	98.1%	700	85.4%
SP2	SurePath	1801	100.0%	1958	98.3%	2191	95.1%
SP3	SurePath	3449	97.9%	3812	94.9%	3598	76.7%
SP4	SurePath	1684	99.9%	1714	100.0%	1490	100.0%
SP5	SurePath	2048	99.2%	2146	95.5%	1947	73.7%
SP6	SurePath	2184	99.9%	2172	98.5%	1952	76.1%
TP1	ThinPrep	2220	100.0%	2829	100.0%	3056	100.0%
TP2	ThinPrep	3845	99.4%	3722	99.7%	4490	100.0%
TP3	ThinPrep	4625	97.5%	5181	97.8%	5405	100.0%
TP4	ThinPrep	1781	90.9%	2075	95.9%	2208	98.2%
TP5	ThinPrep	3840	100.0%	5570	100.0%	5308	100.0%
TP6	ThinPrep	3560	96.7%	3205	99.2%	3412	82.9%

In-focus was defined as Laplacian variance ≥ 10.

LH510 was significantly greater than those of GT450 and S360 in nearly all cases ($p < 0.0001$, Kruskal–Wallis with pairwise comparisons), indicating more precise and uniform focus across tiles. GT450 and S360 produced comparable but lower focus scores, with some cases showing broader interquartile ranges suggestive of greater variability.

Heatmap visualization supported these findings, with LH510 slides displaying widespread orange and red regions

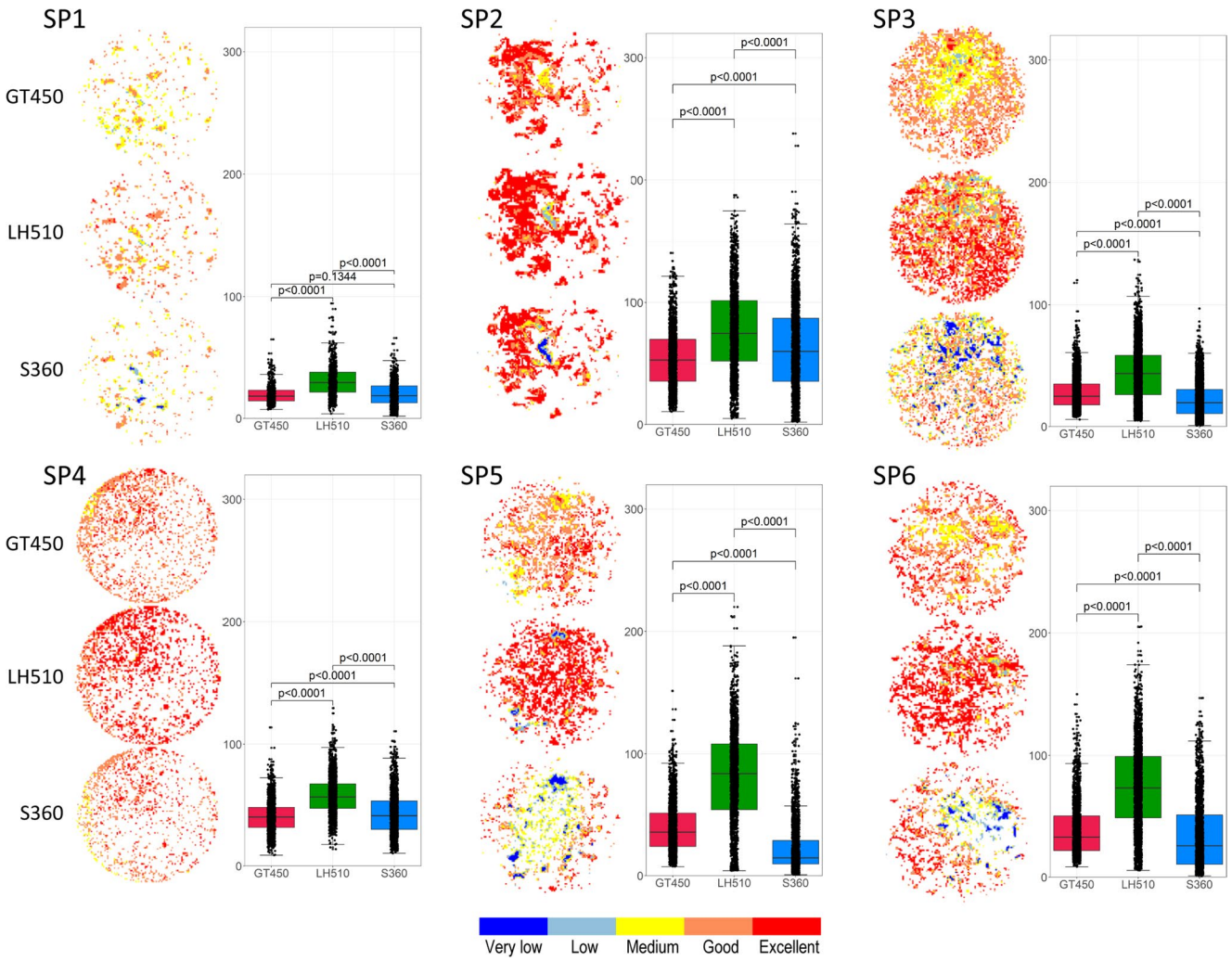

Fig. 2 Focus quality of SurePath thyroid cytology slides scanned by three digital scanners. For cases SP1–SP6, heatmaps display the spatial distribution of tile-level Laplacian variance for GT450, LH510, and S360. The five-level colormap indicates increasing image sharpness from very low (blue) to excellent (red). Corresponding box plots show Laplacian variance distributions per scanner

(high focus quality), whereas GT450 and S360 slides contained larger patches of yellow and blue corresponding to lower focus quality (Fig. 2). These results confirm that LH510 provides superior image sharpness for SurePath preparations.

Scanner Performance in ThinPrep Slides

Focus quality patterns for ThinPrep slides were variable across scanners (Fig. 3). In cases TP2, TP5, and TP6, LH510 exhibited the highest median Laplacian variance values, significantly outperforming both GT450 and S360 ($p<0.0001$). Representative microscopic images from case TP6 further illustrate these differences in focus performance (Fig. 4). In contrast, cases TP1, TP3, and TP4 showed higher focus scores for S360 compared with GT450 and LH510 ($p<0.0001$). The spatial distribution of focus quality was largely homogeneous for LH510 and GT450 slides, whereas S360 images demonstrated more scattered areas of suboptimal focus (Fig. 3).

Binary Focus Status

When tiles were dichotomized into in-focus (variance ≥ 10) and out-of-focus (variance < 10) categories, the proportion of in-focus tiles remained high overall, but meaningful differences emerged between scanners and cases (Table 1).

For SurePath slides (SP1–SP6), the proportion of in-focus tiles exceeded 94% for GT450 and LH510 in all cases, but S360 showed consistently lower values in four of six slides (SP1, SP3, SP5, SP6), with particularly pronounced deficits in SP3 (76.7%) and SP5 (73.7%). LH510 achieved > 98% in-focus tiles in SP1, SP4, and SP6 and reached 100% in SP4. GT450 also performed well, achieving > 97% in-focus tiles for all cases except SP1 (95.5%).

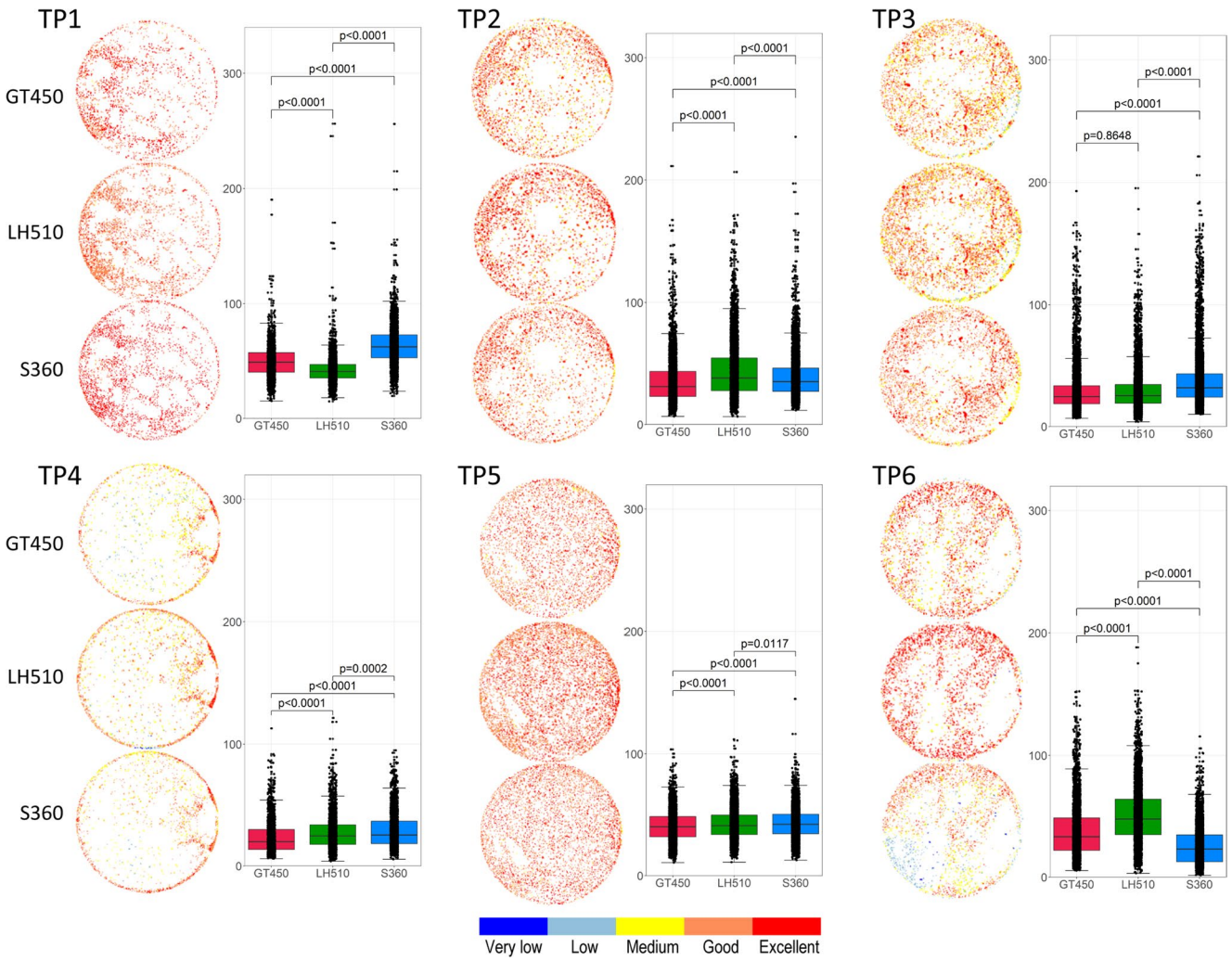

Fig. 3 Focus quality of ThinPrep thyroid cytology slides scanned by three digital scanners. For cases TP1–TP6, heatmaps and box plots are shown as in Fig. 2. ThinPrep slides generally exhibit high focus quality across all scanners, with smaller inter-scanner differences than SurePath slides. The five-level colormap indicates increasing image sharpness from very low (blue) to excellent (red). Corresponding box plots show Laplacian variance distributions per scanner

For ThinPrep slides (TP1–TP6), the overall in-focus proportion was high across all scanners, exceeding 95% in most cases. All three scanners achieved 100% in-focus in TP1, TP5, and near-perfect performance in TP2 and TP3. However, differences emerged in TP4 and TP6: S360 maintained the highest in-focus rate in TP4 (98.2%), while GT450 achieved only 90.9% in TP4, but the proportion dropped markedly in TP6 (82.9%) for S360, whereas LH510 and GT450 achieved 99.2% and 96.7% in TP6 respectively, overall indicating relatively superior consistency of LH510 and GT450 with challenging slides.

Spatial Patterns of Focus Quality

Across both preparation types, regions of low focus quality were frequently located at slide peripheries and in areas containing thick cellular material. Central slide regions were generally well focused. When accounting for both SurePath and ThinPrep preparations, based on qualitative inspection of the spatial heatmaps, the LH510 appeared to maintain more uniform focus across the slide, with relatively fewer visually appreciable peripheral regions of low Laplacian variance.

Discussion

This study provides a systematic, quantitative evaluation of focus quality in thyroid LBC slides scanned using three different whole-slide scanners under standardized single-plane conditions. Using the variance of the Laplacian as an objective sharpness metric, we demonstrated measurable

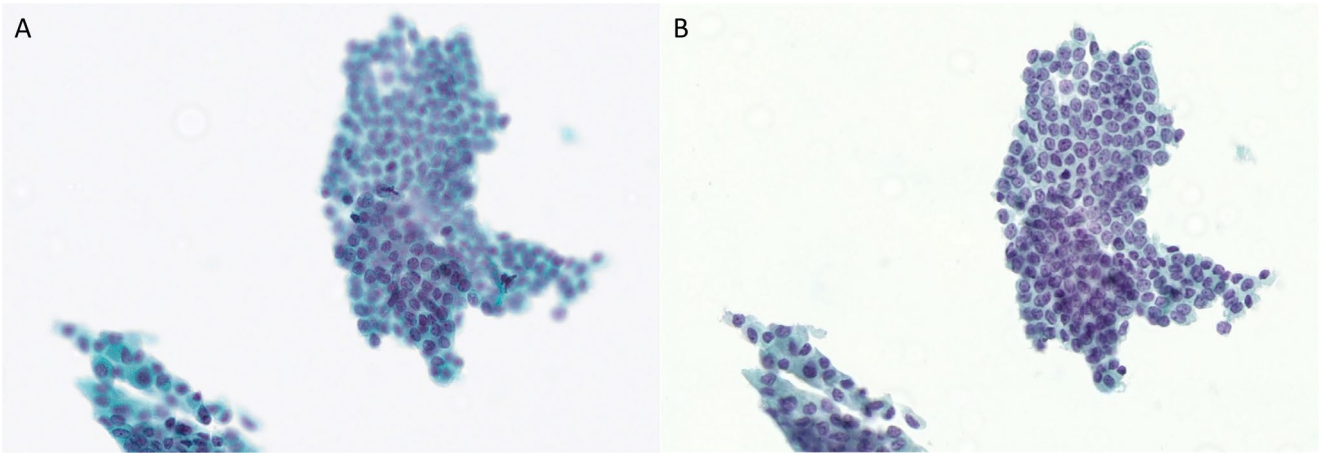

Fig. 4 Representative microscopic comparison of focus quality in case TP6. Panel A shows an area scanned with suboptimal focus, exhibiting blurred nuclear contours and loss of chromatin detail. Panel B, captured from the same cellular cluster, demonstrates well-defined nuclei and sharp cytoplasmic borders, corresponding to higher Laplacian variance and improved image sharpness. Both images are from the same thyroid ThinPrep slide scanned by different scanners at 40× magnification

and statistically significant differences in focus performance among whole-slide imaging systems. One scanner consistently achieved higher median focus values and a greater proportion of in-focus tiles, particularly in SurePath preparations. These findings confirm that measurable differences in focus quality exist between scanners and underscore the importance of objective image quality assessment in digital cytopathology.

Prior studies have reported that SurePath preparations, due to their three-dimensional cell clusters and greater cellular thickness, present more frequent focusing challenges during both microscopy and whole-slide scanning, whereas ThinPrep slides generally produce monolayered, evenly distributed cells that are easier to capture in a single focal plane [11, 12]. Our results corroborate these observations: SurePath slides exhibited greater spatial heterogeneity in focus quality and more pronounced performance gaps among scanners. Two scanners maintained > 98% in-focus rates in most SurePath slides, whereas one scanner showed substantial drops in half of the cases (down to 73.7%), confirming that scanner hardware and focusing algorithms can critically influence digital image quality in three-dimensional preparations.

In ThinPrep slides, differences between scanners were less pronounced. One scanner yielded the highest focus scores in three of six cases (TP2, TP5, TP6), but another performed comparably or better in TP1, TP3, and TP4. The consistently high in-focus rates (> 95%) across all scanners for most ThinPrep slides suggest that ThinPrep preparations are less sensitive to autofocus limitations, likely due to their planar morphology.

The superior performance of one scanner observed in this study, particularly for SurePath preparations, is likely attributable to the multi-focal strategy used in its whole-slide imaging system. This approach employs simultaneous acquisition of information from three distinct focal planes in a single scan pass and algorithmically merges the sharpest pixels from each layer. Functionally, it approximates the benefits of Z-stack or extended-depth-of-field imaging while maintaining the efficiency and data footprint of standard single-plane acquisition. For cytology, where three-dimensional cell clusters are common and large slide volumes must be digitized efficiently, this design may represent a practical compromise between focus quality, scanning speed, and storage requirements.

These findings have important implications for laboratories transitioning to digital cytopathology. Scanner selection should consider the predominant preparation method in use [6]. Institutions processing primarily SurePath samples may particularly benefit from scanners with multi-plane acquisition or focus-enhancement capabilities to ensure effective capture of diagnostically relevant details without compromising storage efficiency.

Laplacian variance has been described as a measure to assess for focus quality of WSIs in histopathology [19], but to our knowledge, no studies have explored the use of Laplacian variance in LBC. Dealing with blurred WSIs is a particularly common problem for cytology and is one of the main reasons why many laboratories would hesitate to adopt digital cytopathology for their routine practice [14]. By classifying WSIs based on their focus quality, using Laplacian variance, laboratories will be able to identify out-of-focus slides using a quantitative measure for quality assurance. Furthermore, this approach may enable the implementation of an AI-based autofocusing system as a quality control step prior to allocation or applying cytology AI software in the future, resulting in improved efficiency [20]. Our study demonstrated that objective metrics such as

Laplacian variance can serve as reproducible tools for image quality benchmarking and quality assurance, supplementing traditional visual inspection in LBC [21]. Furthermore, this study could enable real-world data collection from multiple laboratories for independent, comparable evaluation and feedback to manufacturers, to improve their devices.

While continuous Laplacian variance values provide subtle gradations in image sharpness, the binary classification into in-focus vs. out-of-focus tiles is also clinically relevant. Although one scanner demonstrated lower performance in several SurePath cases, all scanners produced more than 70% in-focus tiles in every slide, indicating that overall diagnostic usability was preserved. Performance in focus quality was especially consistent for ThinPrep preparations, with only one slide showing a noticeable decrease in the proportion of in-focus titles (82.9%). However, lower in-focus proportions may have implications for downstream tasks such as automated cell detection or quantitative image analysis, which are sensitive to defocus artifacts.

Strengths of this study include the use of harmonized ROI selection, chroma-based tile filtering, and standardized single-plane scanning conditions to ensure fair comparison across scanners. Furthermore, the focus-assessment workflow was implemented using transparent, script-based procedures in QuPath (for reproducible tile extraction and chroma validation) and Python (for Laplacian-based focus quantification and tile-level data aggregation). By relying on openly available software tools and explicitly defined processing steps, this study provides a reproducible and extensible computational approach that can be readily adopted, verified, and modified by other laboratories seeking to evaluate or optimize focus performance in digital cytology. Because the computation of Laplacian variance itself is computationally lightweight and scales efficiently with tile-based processing, this approach could, in principle, be incorporated into routine quality-control workflows for cytology WSIs. However, practical implementation would require vendor-level integration, harmonization of threshold settings, and validation across diverse cytology specimen types, particularly because total processing time can vary substantially depending on hardware performance and tiling configurations.

Limitations include the relatively small sample size and focus on thyroid cytology slides only. Different specimen types (e.g., effusion cytology, cervical cytology, aspirates from various organs) and different staining methods may present different focusing characteristics. An additional methodological limitation is that all scanners were operated strictly under the manufacturer's default automatic focusing modes without any manual focus refinement. Autofocus algorithms in certain devices are primarily optimized for histologic sections and may intentionally reduce the number of focus points to accelerate scanning, potentially disadvantaging cytology slides with greater three-dimensional complexity. Therefore, the observed differences in focus quality partly reflect each scanner's default autofocus behavior under routine single-plane conditions.

Although our analysis demonstrated measurable differences in focus quality across scanner systems under controlled single-plane conditions, these findings should not be interpreted as a definitive ranking of scanner superiority. Performance may vary depending on specimen type, cellularity, staining characteristics, scanning protocols, and software settings. In addition, scanning speed is an important but multifactorial parameter that warrants a dedicated, methodologically optimized study, ideally incorporating standardized queue loads, repeated measurements, and controlled slide characteristics. Therefore, comprehensive validation across a broader range of cytology preparations, stains, and clinical practices will be essential to determine the generalizability of these observations. Additionally, we did not compare single-plane scanning with Z-stack or extended depth-of-field modes directly. Future studies should investigate whether manually optimized focus settings, tri-focal layer merge or other multi-plane approaches improve diagnostic accuracy and computational pathology performance across use cases.

Conclusions

This study demonstrates that objective quantification of focus quality in WSIs of the thyroid LBC is feasible using Laplacian variance, which showed strong concordance with expert visual assessment. Under standardized single-plane scanning conditions, measurable differences in focus performance were observed across whole-slide scanning platforms, particularly for SurePath preparations, where three-dimensional cellular architecture posed greater autofocus challenges. These findings underscore the importance of scanner-specific evaluation when implementing digital cytology workflows and highlight the value of reproducible sharpness metrics for validation and quality assurance. Incorporating objective focus assessment into routine practice may support more informed scanner selection, enhance digital image fidelity, and facilitate the robust deployment of computational pathology tools.

Author Contributions Conceptualization: CKJ; Data curation: CKJ; Formal analysis and investigation: CKJ, CK, SJ, AB; Methodology: CKJ, SJ; Visualization: CKJ, CK, SJ, AB; Funding acquisition: CKJ; Resources: CKJ; Supervision: CKJ; Writing - original draft preparation: CKJ; Writing - review and editing: CKJ, CK, SJ, AB.

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Data Availability All data analyzed during this study are included in this published article.

Code Availability The custom scripts used for image processing and focus quality analysis (QuPath Groovy and Python code) are available from the corresponding author upon reasonable request.

Declarations

Ethics Approval This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of Seoul St. Mary's Hospital (KC24ENSI0589), The Catholic University of Korea.

Consent To Participate Informed consent was waived by the institutional review board because of the retrospective nature of this study, which used anonymous clinical data.

Consent for Publication All authors approved for the publication of this paper.

Competing interests The authors declare no competing interests.

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