Virtual 3D Microscopy Using Multiplane Whole Slide Images in Diagnostic Pathology

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Abstract

To reproduce focusing in virtual microscopy, it is necessary to construct 3-dimensional (3D) virtual slides composed of whole slide images with different focuses. As focusing is frequently used for the assessment of Helicobacter pylori *colonization in diagnostic pathology, we prepared virtual 3D slides with up to 9 focus planes from 144 gastric biopsy specimens with or without* H pylori *gastritis. The biopsy specimens were diagnosed in a blinded manner by 3 pathologists according to the updated Sydney classification using conventional microscopy, virtual microscopy with a single focus plane, and virtual 3D microscopy with 5 and 9 focus planes enabling virtual focusing. Regarding the classification of* H pylori, *we found a positive correlation between the number of focus planes used in virtual microscopy and the number of correct diagnoses as determined by conventional microscopy. Concerning* H pylori *positivity, the specificity and sensitivity of virtual 3D microscopy using virtual slides with 9 focus planes achieved a minimum of 0.95 each, which was approximately the same as in conventional microscopy. We consider virtual 3D microscopy appropriate for primary diagnosis of* H pylori *gastritis and equivalent to conventional microscopy.*

Virtual microscopy is a new technique that enables users to view digital images of whole microscopic slides in high resolution on a computer display. Because virtual slides can be accessed over the Internet worldwide at any time without a microscope, the advantage of this technique is evident. By now, virtual microscopy has become widely applied in histopathologic teaching[1-7] and proficiency testing.[8,9] We also use Internet-based virtual microscopy in histopathologic teaching (http://patho.med.uni-magdeburg.de). Virtual microscopy was also applied in virtual tumor banking.[10] The extension of virtual microscopy to diagnostic applications was discussed early[11-13] and was recently tested in cervical cytology.[14] However, the use of virtual microscopy in diagnostic pathology has not been widely established. Above all, the reliability of virtual microscopy in diagnostic pathology is not sufficiently verified.

Commonly, today's virtual slides contain a single focus plane, providing the most optimal focus of the scanned area. Although a sharp image of 1 focus plane may be sufficient for diagnostic pathology in many situations, focusing may be necessary for the assessment of special structures. Apart from situations in which sections are too thick or folded, experience shows that focusing is frequently used for the assessment of *Helicobacter pylori* colonization in gastric biopsy specimens. To approach this function in virtual microscopy, more focus planes have to be scanned from microscopic slides to construct 3-dimensional (3D) virtual slides and to enable virtual focusing.

To find out whether virtual 3D microscopy is appropriate for diagnostic pathology and equivalent to conventional microscopy, we digitized slides from 144 cases with gastric biopsies with or without *H pylori* gastritis using up to 9 focus

planes, constituting 144 virtual 3D slides. The cases were diagnosed by 3 consultant pathologists (T.K., M.E., and T.G.) in a blinded manner in 4 rounds using conventional microscopy, virtual microscopy with a single focus plane, and virtual 3D microscopy with 5 and 9 focus planes. On the basis of these results, we discuss the use and the requirements of virtual microscopy in diagnostic pathology.

Materials and Methods

Cases of previously diagnosed gastric biopsy specimens were obtained from the archives of the Department of Pathology, Otto-von-Guericke-University, Magdeburg, Germany. Slides containing the antrum mucosa, stained by a modified Giemsa stain, were obtained for 144 cases. All slides were labeled with a randomized slide number starting from No. 1.

The slides were digitized using a whole slide scanner (NanoZoomer Digital Pathology System, Hamamatsu, Herrsching, Germany). The maximum resolution (0.23 μm/pixel), corresponding to a 40× objective, was used for scanning. Each slide was scanned in 9 focus planes, comprising a middle focus plane (0), 4 upper focus planes (+1, +2, +3, and +4), and 4 lower focus planes (–1, –2, –3, and –4) using NDPScan 1.2 software (Hamamatsu) with a manually set standard interval of 0.5 arbitrary units between the focus planes, resulting in 9 whole slide images. Kakadu software (http://www.kakadusoftware.com) was used for a lossy compression of each image into a JPEG2000 file with a rate of 1.2 bits/pixel, corresponding to a compression ratio of approximately 20:1. The standard W9 × 7 kernel was used with an irreversible transformation, and the bit stream was organized in a resolution-first progression order (RPCL). The compressed images of each case were merged together into a JPEG2000 multidocument file without another wavelet transformation, constituting a virtual 3D slide. An adapted version of the Kakadu viewer, enabling the adjustment of the number of focus planes to be displayed, was used for viewing the virtual slides. An example of views from a virtual 3D slide with 9 focus planes is shown in **Image 1**. Virtual slides with 1, 5, or 9 focus planes as used in this study are available over the Internet (http://patho.med.uni-magdeburg.de/research.shtml).

The biopsy specimens were diagnosed in a blinded manner by 3 consultant pathologists according to the updated Sydney classification.[15] Four parameters, including chronic inflammation, neutrophil activity, *H pylori* density, and intestinal metaplasia, were graded as absent (0), mild (1), moderate (2), or marked (3), and the results were recorded for each case. Four test rounds, including all 144 cases, were made by every pathologist using conventional microscopy, virtual microscopy with 1 focus plane (0), virtual 3D microscopy with 5 focus planes (–2, –1, 0, +1, and +2), or virtual 3D microscopy with 9 focus planes (–4, –3, –2, –1, 0, +1, +2, +3, and +4). All pathologists used the same Windows XP workstation connected to a 20-in monitor (Dell 2001 FP; Dell, Frankfurt, Germany) for virtual microscopy.

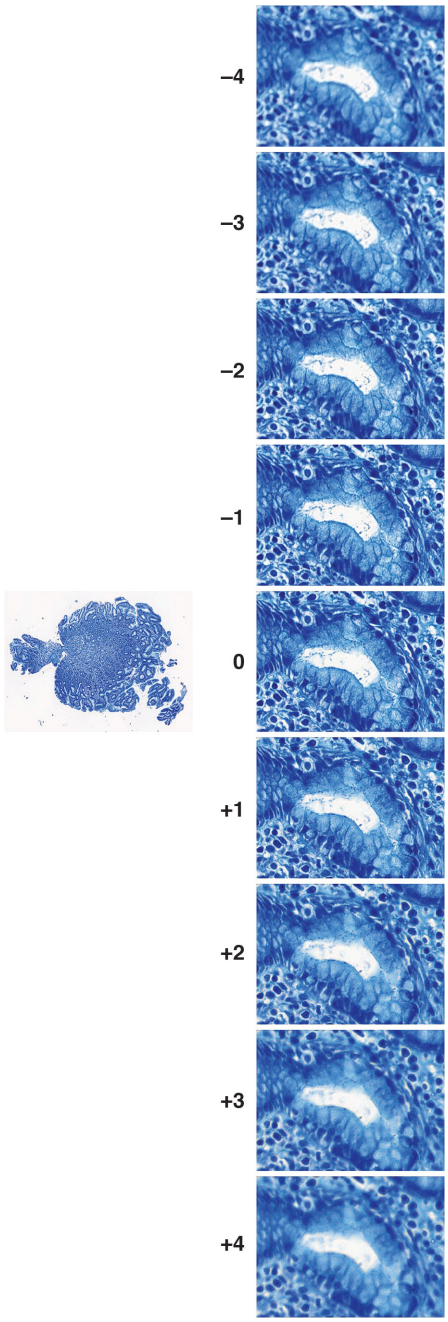

Image 1 Views of a virtual 3-dimensional slide with 9 focus planes representing a gastric biopsy specimen with active *Helicobacter pylori* gastritis. The overview of the slide is shown on the left. Views in high magnification from all 9 focus planes (–4, –3, –2, –1, 0, +1, +2, +3, and +4) with clearly identifiable *H pylori* colonization are shown on the right.

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SPSS software (SPSS, Chicago, IL) was used to perform all statistical analyses. A *P* value of less than .05 was considered statistically significant.

Results

The gastric biopsy specimens were classified by 3 pathologists (A, B, and C) using conventional microscopy, virtual microscopy with 1 focus plane, and virtual 3D microscopy with 5 and 9 focus planes according to the updated Sydney classification. The results showed slight differences among the pathologists and the methods used for classification **Table 1**. The results of the 3 tested variants of virtual microscopy were compared with the results of conventional microscopy for each pathologist. Significantly different results (Mann-Whitney *U* test) were observed in the *H pylori* density category for 2 pathologists using virtual microscopy with 1 focus plane ($P < .05$). Significantly different results in this category were confirmed by the Wilcoxon test ($P < .05$). No significant differences were found in other categories using virtual 3D microscopy with 5 or 9 focus planes, except in the chronic inflammation category, in which 1 pathologist categorized biopsy specimens with a significantly higher grade using virtual 3D microscopy with 9 focus planes as compared with conventional microscopy.

Next, we analyzed the number of cases with identical classification in each category using conventional or virtual microscopy **Table 2**. The greatest variation in the number of these cases was observed in the *H pylori* density category, in which we found a positive correlation between the number of matching cases and the number of focus planes used in virtual microscopy. The use of virtual microscopy with 5 focus planes instead of 1 focus plane increased the number of matching diagnoses by 8 to 10 cases (5.6% to 6.9%). The use of virtual 3D microscopy with 9 focus planes instead of 5 focus planes raised this number by another 2 to 10 cases (1.4% to 6.9%).

Because a therapy is entirely dependent on whether *H pylori* is present, we dichotomized the *H pylori* density score into *H pylori*– (no *H pylori* colonization) or *H pylori*+ (including grades 1, 2, and 3) **Table 3**. Regarding these results, we found a greater number of matching cases using conventional or virtual microscopy, with a positive correlation between the number of matching cases and the number of focus planes used in virtual microscopy. The highest agreement in this category was up to 100% using virtual 3D microscopy with 9 focus planes.

Table 1
Results of Histopathologic Classification of 144 Gastric Biopsy Specimens Using Conventional Microscopy and Virtual Microscopy by Three Pathologists*

Classification/ Grade	Pathologist/Microscopy Method											
	A				B				C			
	M	VM-1	VM-5	VM-9	M	VM-1	VM-5	VM-9	M	VM-1	VM-5	VM-9
Chronic inflammation												
0	8 (5.6)	14 (9.7)	11 (7.6)	12 (8.3)	0 (0)	0 (0)	0 (0)	0† (0)	14 (9.7)	12 (8.3)	14 (9.7)	14 (9.7)
1	73 (50.7)	69 (47.9)	72 (50.0)	71 (49.3)	48 (33.3)	36 (25.0)	35 (24.3)	21† (14.6)	65 (45.1)	68 (47.2)	67 (46.5)	63 (43.8)
2	59 (41.0)	60 (41.7)	60 (41.7)	60 (41.7)	90 (62.5)	105 (72.9)	107 (74.3)	119† (82.6)	63 (43.8)	63 (43.8)	63 (43.8)	64 (44.4)
3	4 (2.8)	1 (0.7)	1 (0.7)	1 (0.7)	6 (4.2)	3 (2.1)	2 (1.4)	4† (2.8)	2 (1.4)	1 (0.7)	0 (0.0)	3 (2.1)
Neutrophil activity												
0	72 (50.0)	74 (51.4)	74 (51.4)	74 (51.4)	75 (52.1)	70 (48.6)	68 (47.2)	61 (42.4)	74 (51.4)	75 (52.1)	75 (52.1)	74 (51.4)
1	45 (31.3)	49 (34.0)	48 (33.3)	47 (32.6)	26 (18.0)	50 (34.7)	43 (29.9)	45 (31.3)	42 (29.2)	41 (28.5)	40 (27.8)	41 (28.5)
2	26 (18.1)	21 (14.6)	22 (15.3)	23 (16.0)	43 (29.9)	24 (16.7)	33 (22.9)	38 (26.4)	27 (18.8)	28 (19.4)	29 (20.1)	28 (19.4)
3	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.7)	0 (0.0)	0 (0.0)	1 (0.7)
Helicobacter pylori density												
0	82 (56.9)	93 (64.6)	85 (59.0)	83 (57.6)	84 (58.3)	99† (68.8)	90 (62.5)	82 (56.9)	84 (58.3)	100† (69.4)	92 (63.9)	84 (58.3)
1	30 (20.8)	29 (20.1)	33 (22.9)	34 (23.6)	19 (13.2)	27† (18.8)	28 (19.4)	29 (20.1)	23 (16.0)	19† (13.2)	18 (12.5)	21 (14.6)
2	25 (17.4)	15 (10.4)	19 (13.2)	20 (13.9)	36 (25.0)	16† (11.1)	24 (16.7)	30 (20.8)	34 (23.6)	23† (16.0)	30 (20.8)	35 (24.3)
3	7 (4.9)	7 (4.9)	7 (4.9)	7 (4.9)	5 (3.5)	2† (1.4)	2 (1.4)	3 (2.1)	3 (2.1)	2† (1.4)	4 (2.8)	4 (2.8)
Intestinal metaplasia												
0	114 (79.2)	114 (79.2)	113 (78.5)	114 (79.2)	108 (75.0)	113 (78.5)	110 (76.4)	112 (77.8)	113 (78.5)	113 (78.5)	113 (78.5)	113 (78.5)
1	21 (14.6)	22 (15.3)	23 (15.9)	22 (15.3)	26 (18.1)	21 (14.6)	23 (16.0)	22 (15.3)	23 (16.0)	24 (16.7)	23 (16.0)	23 (16.0)
2	8 (5.6)	7 (4.9)	6 (4.2)	7 (4.9)	8 (5.6)	10 (6.9)	11 (7.6)	10 (6.9)	7 (4.9)	6 (4.2)	7 (4.9)	7 (4.9)
3	1 (0.7)	1 (0.7)	2 (1.4)	1 (0.7)	2 (1.4)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.7)	1 (0.7)	1 (0.7)	1 (0.7)

M, conventional microscopy; VM-1, virtual microscopy with 1 focus plane; VM-5 and VM-9, virtual 3-dimensional microscopy with 5 and 9 focus planes, respectively.
* Data are given as number (percentage). Percentages may not add to 100% due to rounding.
† Significantly different results ($P < .05$) for this category as compared with results of conventional microscopy for the same pathologist; Mann-Whitney *U* test, as confirmed by the Wilcoxon test.

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Table 2
Number (Percentage) of Cases With Identical Classification by Each Pathologist Using Conventional or Virtual Microscopy

	Pathologist								
	A			B			C		
Category	M/VM-1	M/VM-5	M/VM-9	M/VM-1	M/VM-5	M/VM-9	M/VM-1	M/VM-5	M/VM-9
Chronic inflammation	131 (91.0)	134 (93.1)	133 (92.4)	117 (81.3)	117 (81.3)	105 (72.9)	140 (97.2)	140 (97.2)	141 (97.9)
Neutrophil activity	131 (91.0)	134 (93.1)	135 (93.8)	103 (71.5)	101 (70.1)	106 (73.6)	134 (93.1)	135 (93.8)	137 (95.1)
Helicobacter pylori density	120 (83.3)	130 (90.3)	132 (91.7)	96 (66.7)	104 (72.2)	109 (75.7)	119 (82.6)	129 (89.6)	139 (96.5)
Intestinal metaplasia	133 (92.4)	133 (92.4)	132 (91.7)	122 (84.7)	126 (87.5)	125 (86.8)	143 (99.3)	144 (100.0)	144 (100.0)

M/VM-1, conventional microscopy compared with virtual microscopy with 1 focus plane; M/VM-5 and M/VM-9, conventional microscopy compared with virtual 3-dimensional microscopy with 5 and 9 focus planes, respectively.

Table 3
Number (Percentage) of Cases With Identical Classification as +[*] or –[†] by Each Pathologist Using Conventional or Virtual Microscopy

	Pathologist								
	A			B			C		
Category	M/VM-1	M/VM-5	M/VM-9	M/VM-1	M/VM-5	M/VM-9	M/VM-1	M/VM-5	M/VM-9
H pylori positivity	133 (92.4)	141 (97.9)	143 (99.3)	115 (79.9)	124 (86.1)	124 (86.1)	128 (88.9)	136 (94.4)	144 (100.0)

M/VM-1, conventional microscopy compared with virtual microscopy with 1 focus plane; M/VM-5 and M/VM-9, conventional microscopy compared with virtual 3-D microscopy with 5 and 9 focus planes, respectively.
[*] Including grades 1, 2, and 3.
[†] No *Helicobacter* colonization.

Table 4 shows the number of cases classified as *H pylori*– or *H pylori*+ using conventional microscopy compared with the original findings and virtual microscopy. We found a negative correlation between the number of false-negative diagnoses and the number of focus planes used in virtual microscopy. No false-positive diagnoses were produced by 2 pathologists, whereas 1 pathologist diagnosed up to 11 false-positive cases using virtual microscopy.

Based on the data in Table 4, we calculated the specificity, sensitivity, and κ statistics for conventional microscopy and virtual microscopy **Table 5**. As expected, we found a positive correlation between the sensitivity and the number of

Table 4
Number of Cases Classified as *Helicobacter pylori*+ or *H pylori*– by Each Pathologist Using Conventional Microscopy Compared With Original Findings[*] and With Results of VM-1, VM-5, and VM-9

	Conventional Microscopy/Pathologist							
	A (n = 144)		B (n = 144)		C (n = 144)		All 3 (n = 432)	
	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive
Original[*]								
Negative	81	1	81	3	83	1	245	5
Positive	4	58	4	56	2	58	10	172
VM-1								
Negative	82	11	77	22	84	16	243	49
Positive	0	51	7	38	0	44	7	133
VM-5								
Negative	82	3	77	13	84	8	243	24
Positive	0	59	7	47	0	52	7	158
VM-9								
Negative	82	1	73	9	84	0	239	10
Positive	0	61	11	51	0	60	11	172

VM-1, virtual microscopy with 1 focus plane; VM-5 and VM-9, virtual 3-dimensional microscopy with 5 and 9 focus planes, respectively.
[*] Obtained from the pathologist's reports by different pathologists.

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Table 5
Specificity, Sensitivity, and κ Statistics of Conventional and Virtual Microscopy*

	Pathologist											
	A			B			C			All 3		
	Specificity	Sensitivity	κ	Specificity	Sensitivity	κ	Specificity	Sensitivity	κ	Specificity	Sensitivity	κ
Original†	0.95	0.98	0.93	0.95	0.95	0.9	0.98	0.98	0.96	0.96	0.97	0.93
VM-1	1.0	0.82	0.84	0.92	0.63	0.57	1.0	0.73	0.76	0.97	0.73	0.73
VM-5	1.0	0.95	0.96	0.93	0.78	0.71	1.0	0.87	0.88	0.97	0.87	0.85
VM-9	1.0	0.98	0.99	0.87	0.85	0.72	1.0	1.0	1.0	0.96	0.95	0.9

VM-1, virtual microscopy with 1 focus plane; VM-5 and VM-9, virtual 3-dimensional microscopy with 5 and 9 focus planes, respectively.
* Specificity, sensitivity, and κ statistics were calculated based on the classification of the gastric biopsy specimens as *Helicobacter pylori*+ or *H pylori*– as shown in Table 4.
† Obtained from the pathologist's reports by different pathologists.

focus planes used in virtual microscopy. The highest sensitivity, with an average of 0.95 for all pathologists, was achieved using virtual 3D microscopy with 9 focus planes. Two pathologists achieved a specificity of 1.0. The average specificity was 0.96 to 0.97 for all pathologists. The specificity and sensitivity of virtual 3D microscopy using 9 focus planes were approximately similar to the specificity and sensitivity of conventional microscopy (0.96 and 0.97, respectively) for all pathologists. A κ score of 0.9 was achieved for all pathologists combined using virtual 3D microscopy with 9 focus planes.

Discussion

The use of virtual microscopy in diagnostic pathology has not been established. Several requirements have to be met for everyday use. Generally, the basis of virtual microscopy is powerful technical equipment, including at least one slide scanner, a speedy network, and servers with high capacity. Moreover, digital data management is necessary to process the huge amount of data, including the registration, distribution, and archiving of the digitized slides. The integration of virtual microscopy in an existing information system (IS) including a picture archiving and communication system (PACS) is a prerequisite for digital pathology.[16] We recently reported on digital workflow management using IS[17] and on a DICOM-conform model for the integration of virtual microscopy into IS/PACS using JPEG2000/JPIP-based streaming of virtual slides.[18]

Apart from the technical requirements, the application of virtual microscopy in diagnostic pathology has to be tested and evaluated in detail. Special diagnostic questions may require certain qualities of virtual slides. Comparative investigations are needed to determine whether virtual microscopy is appropriate for diagnostic pathology and equivalent to conventional microscopy.

In this study, we investigated the use of virtual microscopy in diagnostic pathology of gastric biopsy specimens. Because focusing is an essential and frequently used function in the microscopic assessment of *H pylori* colonization, we included virtual 3D microscopy in our investigation. In contrast with today's common virtual slides, which contain a single focus plane, virtual 3D slides contain multiple focus planes and enable digital focusing. Although the number of focus planes in virtual 3D slides is unlimited, we confined ourselves to a maximum number of 9 focus planes covering the useful focus range. Even though we used identical spacing between the focus planes, individual 3D slides had a slightly different quality, as the middle focus plane, which was determined by autofocus, did not correspond exactly to the center of the useful focus range in single cases. Therefore, some marginal focus planes in virtual 3D slides, such as –4, –3 or +3, +4, turned out to be superfluous because they were out of the useful focus range. Anyway, this problem can hardly be solved, as even slight irregularities in the sections may cause it, at least in limited areas of the slide. However, it indicates the need for virtual focusing.

Because multiple focus planes multiply the data amount of virtual slides and extend the time of digitizing, today's use of virtual 3D microscopy seems to be limited. Therefore, further investigations are necessary to define diagnoses for which virtual 3D microscopy is essentially required. Regarding gastric biopsy specimens, we tested 4 diagnostic categories, including chronic inflammation, neutrophil activity, *H pylori* density, and intestinal metaplasia. By using virtual microscopy with a single focus plane, the identification of *H pylori* was unsuccessful in several cases, leading to significantly different results in the corresponding category as compared with conventional microscopy. Apart from minor individual differences in the other categories, we did not find any significant differences using conventional microscopy or virtual microscopy with 1 focus plane. Therefore, we conclude that cases can be sufficiently diagnosed in these categories without using virtual focusing.

Regarding the number of focus planes in virtual 3D microscopy, we found a positive correlation with the number of matching diagnoses determined by conventional

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microscopy in the *H pylori* density category. By using virtual slides with 9 focus planes, we observed the highest agreement. The number of false-negative diagnoses in the assessment of *H pylori* colonization decreased with a higher number of focus planes, indicating the advantage of virtual focusing. By using virtual microscopy, only 1 pathologist diagnosed false-positive results, which may be attributed to inexperience with the interpretation of the digital images because the technical equipment was identical for all pathologists. Because therapy depends on the precise diagnosis of *H pylori* colonization, we conclude that the use of virtual 3D slides with at least 9 focus planes can be accepted generally as a standard in diagnostic virtual microscopy of *H pylori* gastritis. Concerning *H pylori* positivity, the specificity and sensitivity of virtual microscopy using virtual 3D slides with 9 focus planes or conventional microscopy showed no marked difference; the specificity and sensitivity of both methods achieved a minimal value of 0.95 in each case and both methods had an average κ of 0.9 for all pathologists. Therefore, we consider virtual 3D microscopy with 9 focus planes appropriate for the primary diagnosis of *H pylori* gastritis and equivalent to conventional microscopy.

Although further studies on the use of virtual 3D microscopy are needed, we are convinced that virtual 3D slides will succeed in diagnostic virtual pathology. As demonstrated, virtual focusing overcomes previous functional limitations of virtual microscopy. Next, standardization of the spacing between the focus planes will be necessary to achieve comparable results. The capability of virtual 3D microscopy can be extended by the inclusion of additional whole slide images, eg, scans in polarized light, to further approach the functions of conventional microscopy. Certainly the most essential investigation in the field of diagnostic virtual microscopy will be the evaluation of the maximum tolerable compression rates of virtual 3D slides. In this study, we used a lossy compression of approximately 20:1, which was found to be below a threshold at which recognizable compression artifacts may occur.[18] However, compression rates other than 20:1 need to be evaluated in diagnostic virtual microscopy and compared with conventional microscopy. We will discuss this topic in a forthcoming report.

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