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Thesis

DSDM (Digital Slide Dynamic Morphometry)
Targeting tumor budding in colorectal cancer

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SUMMARY

Background: In histopathology, quantitative assessment of various morphologic features is based on published methods that were conceived on specific areas observed through the microscope and microscopical objective used. Failure to reproduce the same reference field size if using a different microscope may change the score assessed. When visualizing a digital slide on a computer screen, through a dedicated viewer, it is possible to select the adequate objective-magnification (zoom toolbar). However, the field of view is rectangular, different from the circular field area viewed by optical microscopy. In addition, the evaluation of the size of the selected area of the digital slide is not immediately evident and must be estimated with the draw functions of the viewer or the scale/axes grid.

Methods: Using the tools offered by ImageScope, the virtual slide viewer of Aperio Technologies (Vista, USA), a system was conceived to reproduce the various methods published for assessing tumor budding, an important prognostic factor in colorectal cancer. Each method, based on counting tumor budding within a specific area, was reproduced as a colored circle, corresponding to a graphic overlay layer. The various circles were grouped in a target-like shape and then exported and saved as an .xml file. A web page was created giving access to 100 whole-slide digital scans. When each digital slide was opened the file containing the target-like area had to be imported in order to move it onto a tumor budding “hot spot” and to perform the

measurements. Eighteen international experts in gastrointestinal pathology were invited to participate.

Results: Twelve investigators completed the task, the majority of them performing the multiple assessments of each case in less than 12 minutes.

Conclusions: The assessing system proposed appeared to be feasible for pathologists. It allows precise application of methods originally conceived for optical microscopy. The graphical layers that make the tool are freely moved within the digital slide: this system was named DSDM (Digital Slide Dynamic Morphometry). This telepathology extension has various potential applications and might be a useful tool for histopathological assessment of diagnostic parameters that need to be quantified.

INTRODUCTION

Most applications of telepathology have previously been limited to remote frozen intra-operative diagnosis^{1,2} or second opinion consultations.³ More recently, additional applications have included evaluation of interobserver variability of histopathologic diagnosis, using offline telepathology,⁴⁻⁶ and computer-assisted image analysis for morphometric quantification of prognostic factors.^{7,8} In routine histopathology, quantitative assessment of various morphologic features is based on published methods that were based on fields of view specifically obtained with the microscope used by the original descriptors (authors) and expressed as an area (in mm²) or a magnification (high power field: HPF).

This assessing system applies both to inflammatory and neoplastic diseases.

In addition to different microscopes and objectives (magnification) used (thus different field of view areas) the various authors used different cut-offs and different approaches for assessing a specific feature, such as subjective evaluation vs. objective quantification. The assessment is performed throughout the whole lesion (multiple slides) or on the most representative slide, containing the “hot spot”, and/or in multiple additional areas randomly searched. The cut-off values can be arbitrary chosen or based on outcome analysis or on inter/intraobserver analysis.

An example of variation in field areas is given by the results published by different investigators studying inflammatory diseases, e.g the reported mean - SD concentration of mucosal mast cells in the control population of colonic biopsies is 13.3 ± 3.5^9 and 37.3 ± 6.0 per HPF.¹⁰

An example of variation in cut-off values is represented by the number of eosinophils/HPF (based on peak count) used to establish a diagnosis of eosinophilic oesophagitis which has been reported as being 15 in 10 studies, 20 in 8 studies, 24 in 2 studies and 30 in 1 study.¹¹

In neoplastic disease, the mitotic count is an important prognostic factor and is also an essential component of histological grade in breast cancer.

This enumeration is greatly influenced by the variation in HPF when using different microscopes, (the variation being up to 250%) and by the method used for counting mitotic figures and recording results.¹²

In order to routinely apply a given method, one should try to adapt the field size and the cut-off values indicated, with a correction formula considering the particular field of view and the objective magnification of his own microscope. Failure to reproduce the same reference field size will likely change the score assessed.

Although the CAP Invasive Breast Cancer Protocol provides a table to adjust the raw number of mitoses according to the size of the field (either by diameter or area in mm^2) of the microscope used,¹³ mitotic count cutoffs are subject to important sampling errors and prognostic or predictive cutoffs are not well studied.¹⁴

The need for standardizing mitotic count in Gastrointestinal Stromal Tumors, according to the surface area examined (based on size of HPFs) has already been underlined: once more the problem is that there are no agreed-upon definitions in this regard.¹⁵

Tumor budding (TB) is a dedifferentiation process occurring at the invading edge of colorectal cancer (CRC) and of several epithelial malignancies.

It appears as clusters of undifferentiated cells detaching from the surrounding tumor (Figure 1).

Although several studies have shown that TB is independently associated with lymph node and distant metastasis, shorter disease-free survival and overall survival, in stages I to III CRC, there is no consensus in assessing this feature, therefore its clinical impact is still limited.¹⁶

Several original methods have been proposed for assessing TB in CRC. They can be grouped as follows:

1. methods based on a subjective impression on the overall tumor: Hase¹⁷ and Nakamura (Figure 2).^{18,19}
2. methods based on counting with a cut-off in the field with maximum TB advanced by Ueno for early²⁰ and advanced CRC.²¹
3. methods developed with counting in different areas: Morodomi²² and Wang (Figure 3).²³

While some authors report that budding clusters are easily identifiable on hematoxylin & eosin stained sections,^{21,23} others use immunohistochemistry to better assess this feature (Figure 4).²⁴

Such differences in methodology makes it impossible to compare data from different studies.

MATERIALS AND METHODS

Technical background

When using digital slide viewers it is possible to select the observation magnification (zoom toolbar), because the original high resolution virtual slide is recorded in a pyramidal format, thanks to precomputed subsampled images. However, the field of view delivered by the viewer on the computer screen is rectangular and, as a result, different from the circular field area viewed through an optical microscope. In

addition, the evaluation of the size of the selected area of the digital slide is not immediately evident and must be evaluated using the draw functions of the viewer or the scale/axes grid.

However telepathology seems to be the only way to efficiently and swiftly reproduce the various proposed methods, to compare them and to evaluate inter-observer reproducibility.

This paper details a new strategy, *Digital Slide Dynamic Morphometry*, that allows to precisely reproduce one or multiple assessing methods. This system was tested in the frame of an interobserver study, assessing the reproducibility of a number of tumour budding scoring methods, applied to fifty CRC cases.

Approach

Using telepathology on virtual slides a system was conceived to reproduce the various methods published for assessing TB in CRC.

High-resolution, whole-slide images were recorded from histological slides of 50 CRCs stage I-III of the “G.Fracastoro” City Hospital., using a ScanScope CS microscopical scanner (Aperio Technologies, Vista, CA).

A web page (Figure 5) was built giving access to all the virtual slides (50 hematoxylin & eosin stained histological sections and 50 corresponding AE1-3 cytokeratin immunostained sections, scanned at a magnification of 40x).

The digitized slides were uploaded to the study website http://course.path.mgh.harvard.edu/budding_project/, as tiled TIFF 6 files, compressed in JPEG 2000 at medium quality (70), for online viewing through a digital microscope interface (Aperio ImageScope) that allowed navigation from the desktop computers of the participating pathologists.

It was possible to apply methods based on subjective evaluation on the whole tumor section, just by navigating into the virtual slide.

An original assessing system was conceived for reproducing the methods based on tumour bud enumeration using cut-off values in the field of maximum budding and for also reproducing the methods proposed for counting budding in different tumor areas.

Each method, based on counting TB within a specific area, was reproduced as a colored circle, drawn using the *draw annotations* function of the Aperio ImageScope viewer and corresponding to a graphic overlay layer with all the pertinent information: size, cut-off value, author and year of publication (Figure 6). The various circles were grouped in a target-like shape (Figure 6) and then exported and saved as a .xml file (Figure 7).

When each digital slide was opened the file containing the target-like area was imported in order to perform the assessment (Figures 8-10).

Once the target-like area appeared, it was possible with the *Zoom Toolbar* of ImageSope to magnify or shrink the current field of view until the “target” was fitting

the monitor (Figure 11). The magnification was not important in order to reproduce one method, as the area was fixed.

To move the target around the slide the keyboard keys were used.

It was possible to move all the circles together, holding Shift-Control keys or, after having selected one layer, to move a circle separately from the others holding the Shift key.

So the various layers were not merged together nor burned into the image, allowing a fully dynamic assessment.

To indicate and report the position of the tumor region with maximum TB (the “hot spot”) looking at the *Status Bar* that provides information regarding the coordinates of each pixel indicated by the pointer, as the mouse is moved over the virtual slide (the first is ‘X’ and the second is ‘Y’), after having indicated with the pointer the centre of the target, the coordinates were written down.

A tutorial video was prepared with a screen video capture software (TechSmith Camtasia Studio 6, Okemos, MI, USA) while recording also the audio from a microphone, in order to explain how to perform the assessment. The video was uploaded into the study website as a Windows Media file (.wmv).

An Excel file (Figure 12) to be filled was also provided, containing all the 100 slides (rows) and the 5 methods (columns).

Procedure

Eighteen pathologists and surgeons trained in pathology, experts in gastrointestinal pathology and/or working in a cancer center, were invited to participate.

During the initial contact, the invitees were given basic background information on the aims, methods, and objectives of the study.

The set was reviewed according to the availabilities of each participant between December and February 2009.

The pathologists who agreed to participate received a folder containing:

1. the Excel file to be filled during the TB assessment;
2. the Web Page Address file;
3. a word file containing the detailed description of the project and the various methods for assessing and reporting TB;
4. a .xml file containing three colored circles, each of them representing a method, (the “target”) to be imported and displayed onto each slide;
5. A questionnaire with requests concerning the type of hospital, the specialty, the time expense for filling the Excel table categorized as <10 hours / 10-15 hours / 20-25 hours / >25 hours.

Each participant was asked to evaluate all the 100 slides and to report TB assessment by highlighting Excel cells corresponding to the various TB classifications (such as positive vs. negative; low grade vs. high. grade etc.).

Completed excel files were faxed or e-mailed to the data collector who forwarded them to the study biostatistician for analysis.

RESULTS

Of the 18 invited investigators, 12 completed the task.

Four investigators preferred to perform the assessment as groups of two, therefore the total number of submitted files was ten.

The reasons for the failure of the remaining 6 investigators to participate included: technical problems viewing the tutorial video (one); a technical problem opening the digital slides (one); and a missed deadline (four).

Two assessment were performed by Japanese surgeons with a vast experience of CRC histopathology, being also authors of the methods for assessing TB; the other participants were pathologists.

The assesment took less than 10 hours for both hematoxylin & eosin stained slides and corresponding cytokeratin immunostains in 6 of 10 performances; for two investigators the assessment of hematoxylin & eosin stained slides lasted longer (15-20 vs. 10-15 and <10); for one investigator it was the opposite (15-20 hours vs. 20-25). The remaining assessment was performed for both stainings in more than 25 hours.

Therefore the majority of the assessments took less than 10 hours for each 50 case set, (i.e. less than 12 minutes per case, keeping in mind that for each case, 5 different assessment methods were tested).

CONCLUSIONS

The assessing system proposed appeared to be feasible and time economical for pathologists considering the multiple different methods assessed.

Viewing the tutorial video was essential to understanding how to perform the morphometric analysis.

The assessing system allows simultaneous application of different methods originally conceived for optical microscopy, by using customized tools prepared to precisely reproduce those methods. The graphic overlay layers that make the tool can be freely moved within the digital slide and one can keep them separate or grouped. For this reason, the system was named DSDM (Digital Slide Dynamic Morphometry). This telepathology extension has various potential applications and might be a useful diagnostic tool for histopathological assessment of parameters that need to be quantified. This approach might be also a tool for quality control of grading protocols, whose results **influence prognosis and therapy decisions**.

FIGURES

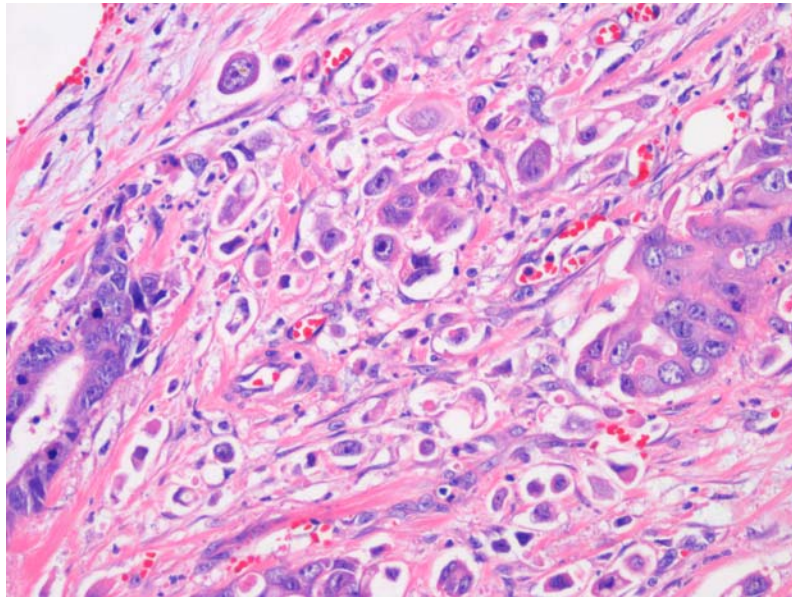


Figure 1. The tumor budding (TB) appears as clusters and single undifferentiated cells detaching from the surrounding tumor. Hematoxylin & eosin.

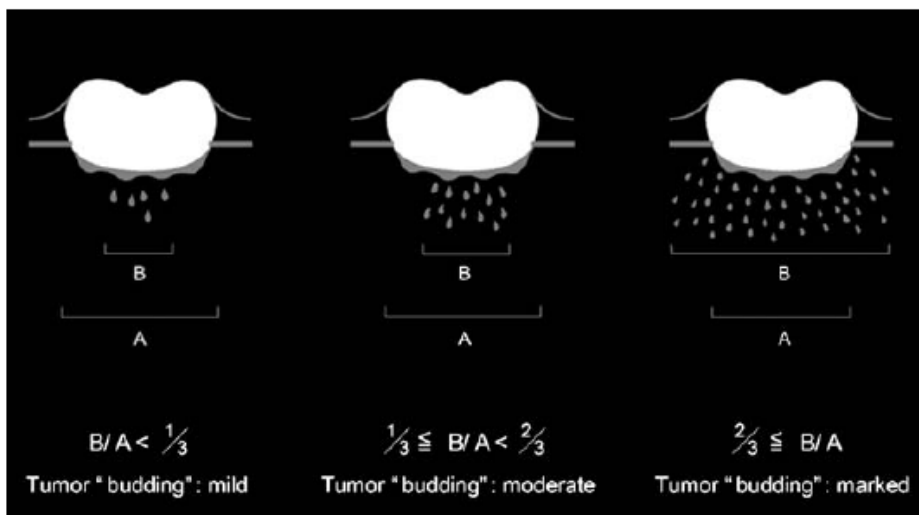


Figure 2. The Nakamura method for assessing TB, based on the subjective impression on the overall tumor. Extracted from reference n°18.

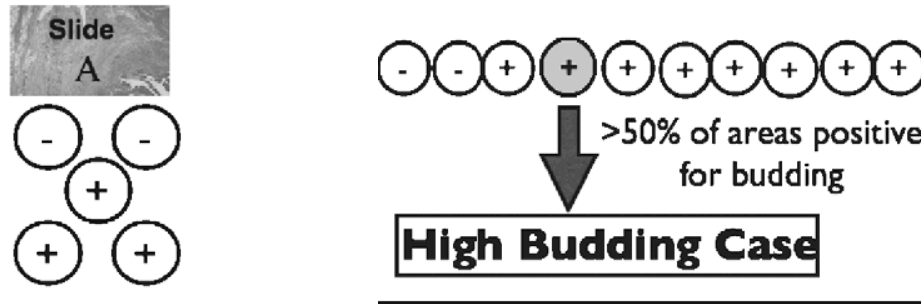


Figure 3. The Wang method developed with counting in different areas. Extracted from reference n° 23.

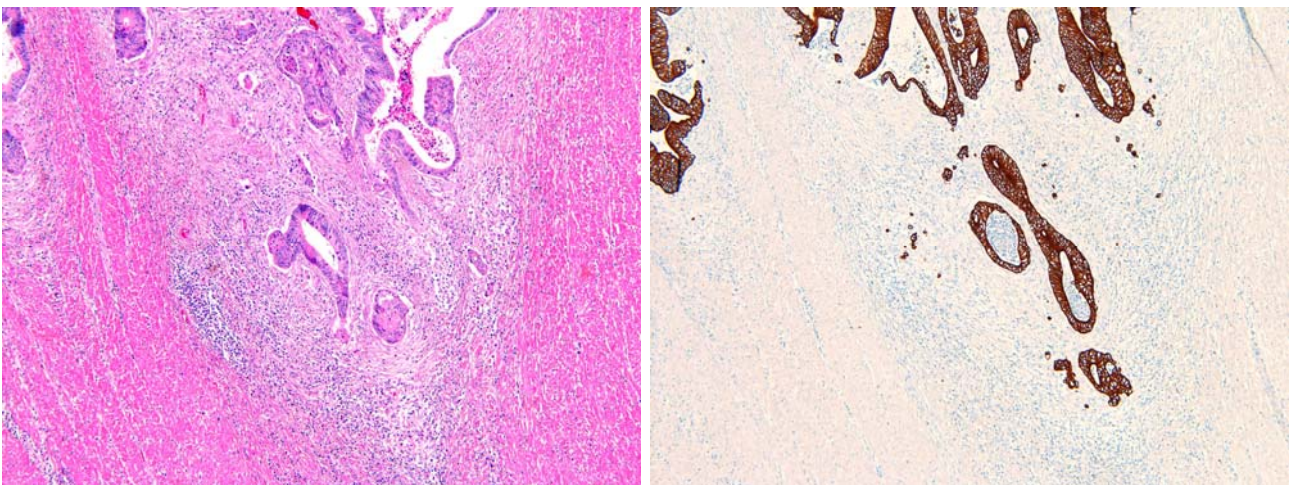


Figure 4. The advancing edge of a CRC showing TB, stained with hematoxylin & eosin (left) and with immunohistochemistry (right, AE1-3 cytokeratin).

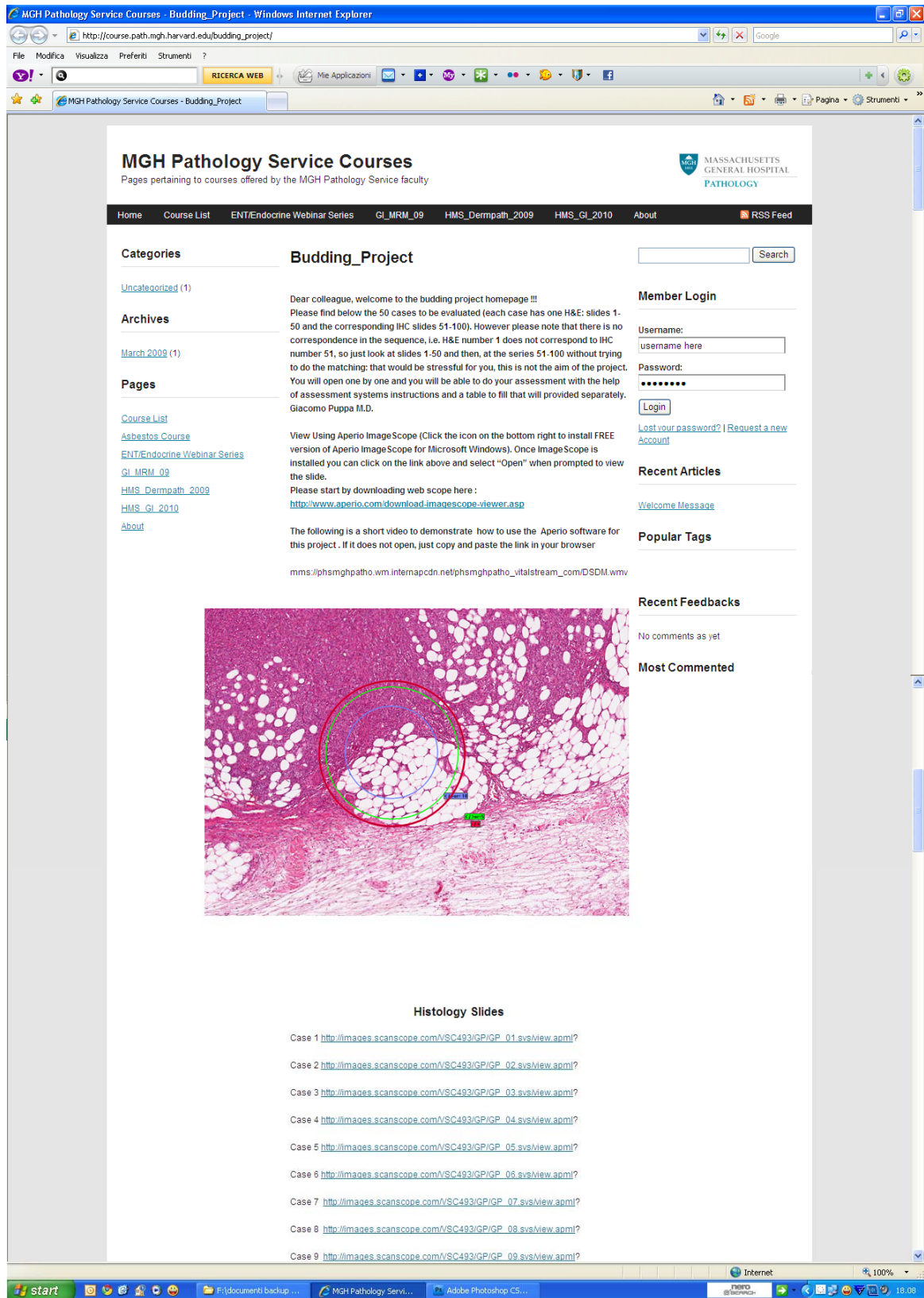


Figure 5. The web page giving access to the 100 virtual slides.

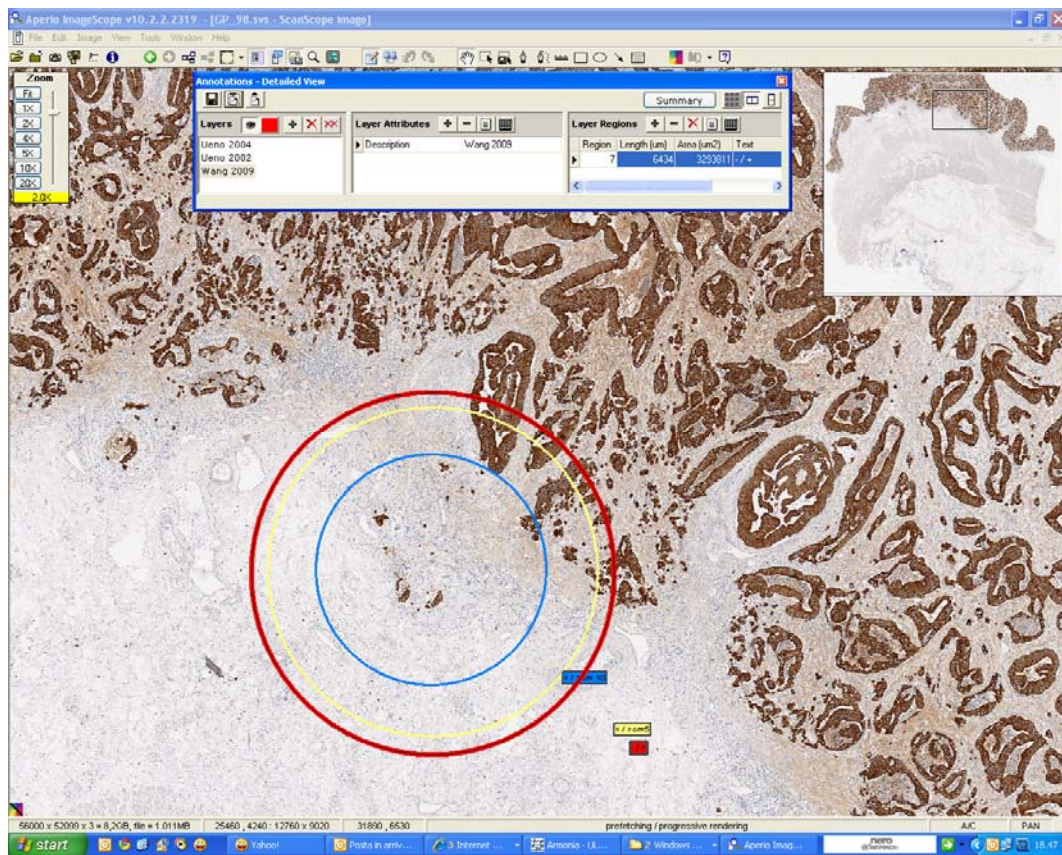


Figure 6 The advancing edge of a CRC showing TB, stained with immunohistochemistry (AE1-3). From the ImageScope viewing window, the following elements are selected: Zoom Toolbar indicating the magnification; Thumbnail Window showing what part of the entire image is under evaluation; Status Bar showing the target coordinates ('X' and 'Y'). The annotation panel showing the multiple (three) annotation layers, saved in different colours, and organized with descriptions (Author, year of publication; size area, and cut-off values used by such author; the same cut-off value is also reminded, highlighted with the corresponding colour, in the lower right of the circles).

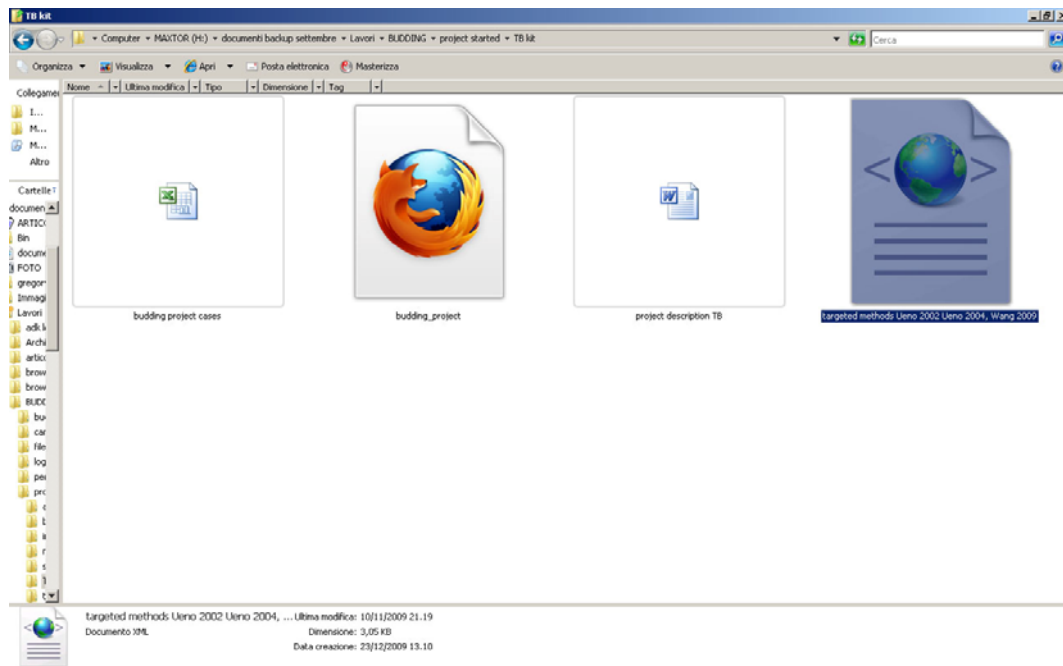


Figure 7. The TB kit that each participating pathologist received as folder containing from the left to the right: the Excel file to be filled during the TB assessment; the Web Page Address file; the word file containing the detailed description of the project and the various methods for assessing and reporting TB; the .xml file (highlighted) containing the “target” to be imported.

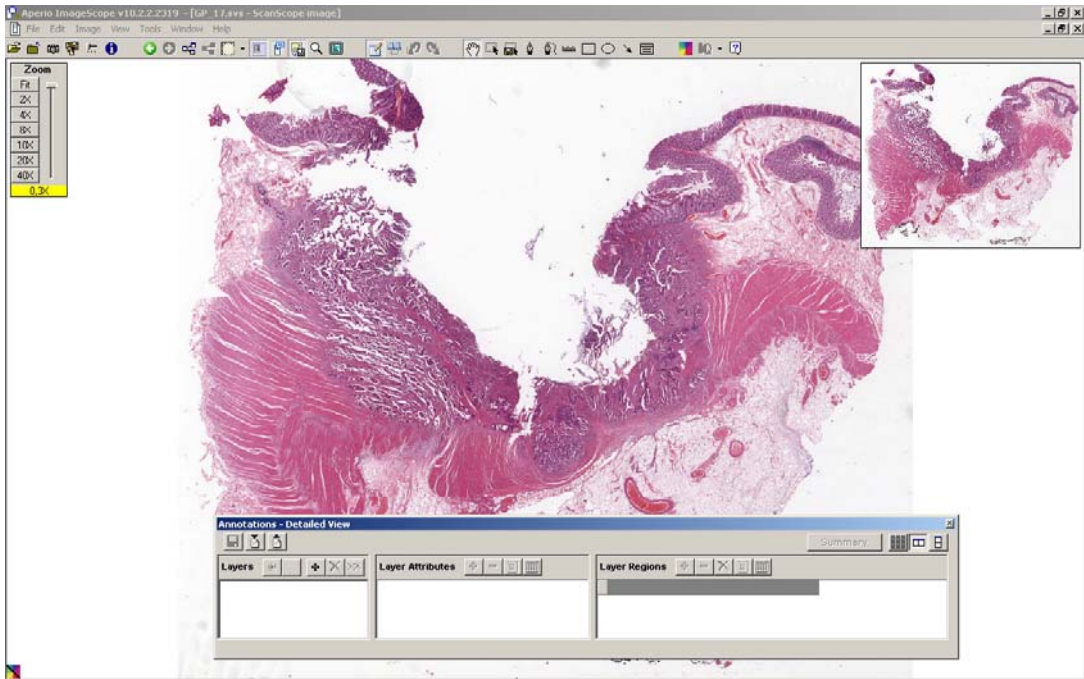


Figure 8. The annotation panel is selected.

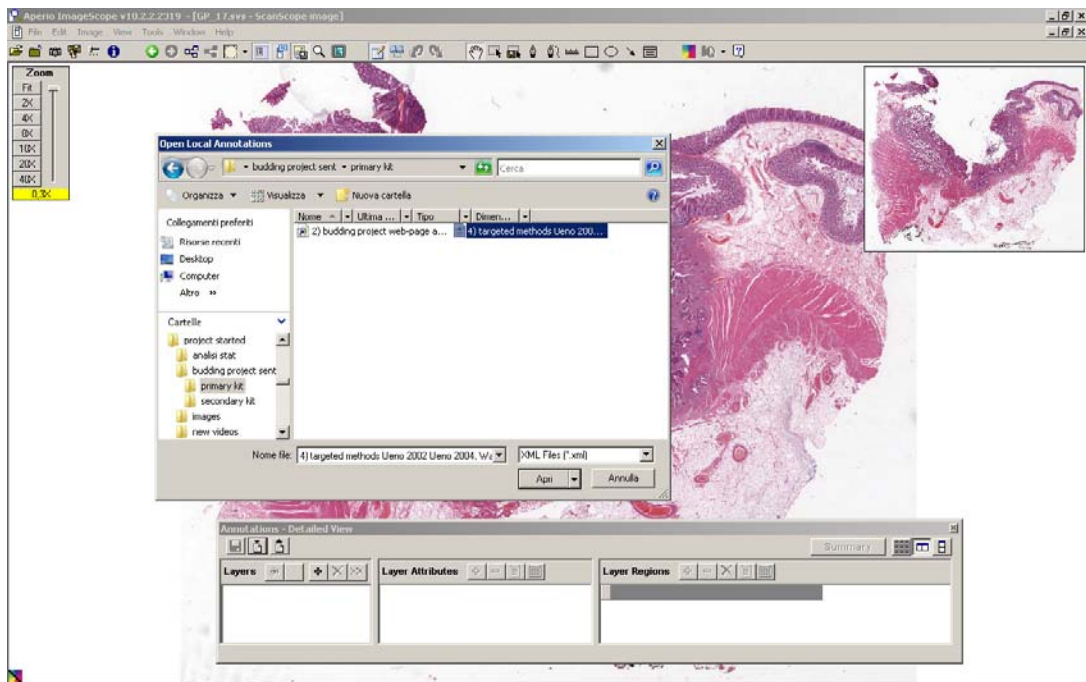


Figure 9. Within the annotation panel the import annotation from file is selected and the panel containing the file to be imported appears.

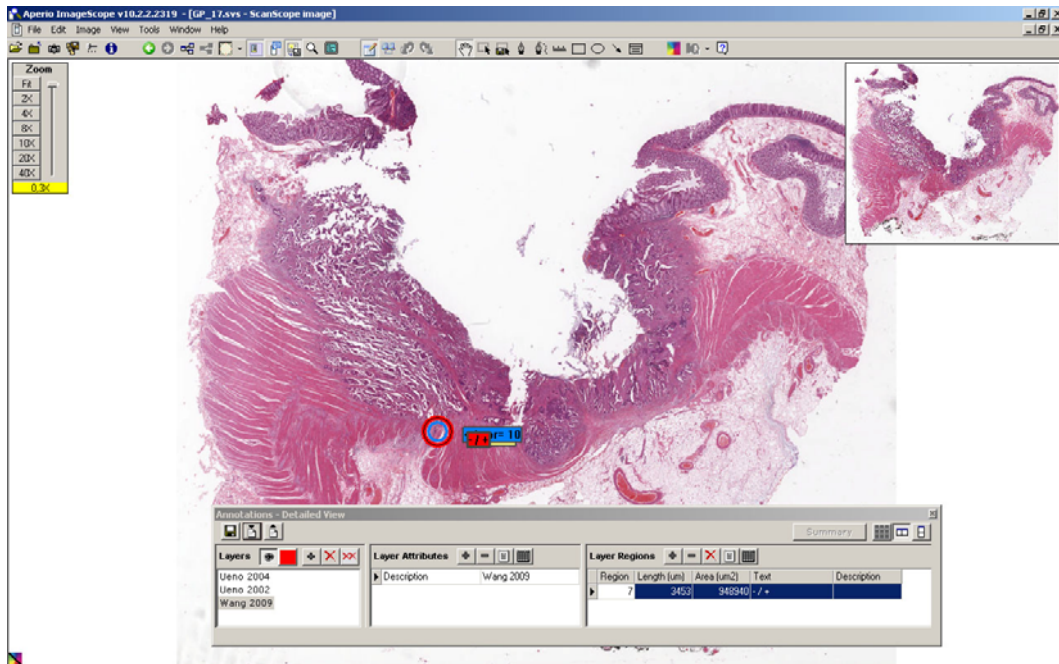


Figure 10. The file selected is imported and appears with all the layers.

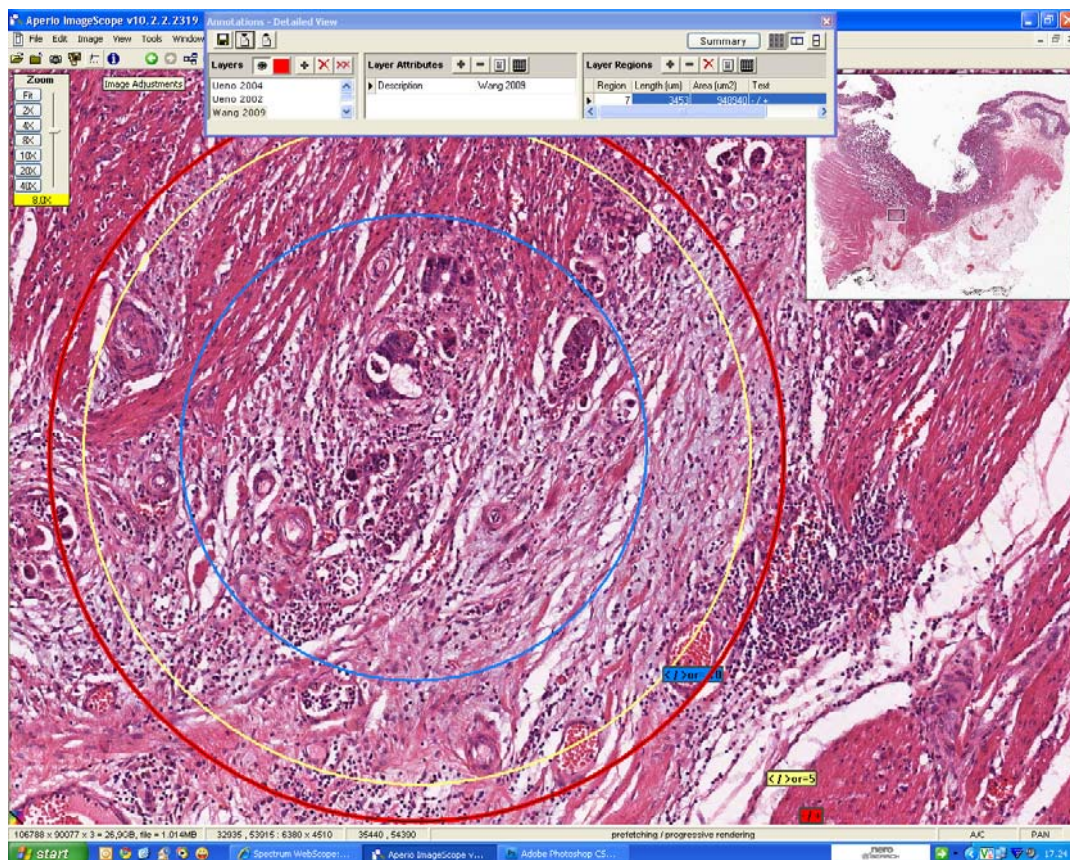


Figure 11. With the zoom toolbar it is possible to adjust the magnification, until fitting the monitor, for a precise assessment.

1	A	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y
2		1		2				4		3				5									
3		Hasse predominant pattern		Nakamura along the entire invasive margin				Ueno area 0,385 mm ²		Ueno area 0,785 mm ²			target centre coordinates	Wang 0,949 mm ²									
4		case n°	none or minimal moderate or severe	none	mild < 1/3	moderate 1/3 - 2/3	severe > 2/3	low <10	high ≥10	negative <5	positive ≥ 5	"x" coordinate	"y" coordinate	1 ° area	2 ° area	3 ° area	4 ° area	5 ° area					
4	1	1	2					1	2	1	2	387	532										
5	2	1	2	0	1	2	3	1	2	1	2	282	432	0	1	0	1	0	1	0	1	0	1
6	3	1	2					1	2	1	2	407	201										
7	4	1	2					1	2	1	2	856	224										
8	5	1	2	0	1	2	3	1	2	1	2	180	354	0	1	0	1	0	1	0	1	0	1
9	6	1	2	0	1	2	3	1	2	1	2	276	401	0	1	0	1	0	1	0	1	0	1
10	7	1	2					1	2	1	2	390	433										
11	8	1	2					1	2	1	2	236	727										
12	9	1	2	0	1	2	3	1	2	1	2	937	648	0	1	0	1	0	1	0	1	0	1
13	10	1	2					1	2	1	2	113	274										
14	11	1	2	0	1	2	3	1	2	1	2	705	526	0	1	0	1	0	1	0	1	0	1
15	12	1	2					1	2	1	2	185	356										
16	13	1	2	0	1	2	3	1	2	1	2	177	587	0	1	0	1	0	1	0	1	0	1

Figure 12. The Excel file to be filled by highlighting the cells, containing all the 100 slides (rows) and the 5 methods (columns).

Additional data files.

The video file (.wmv) shows how to navigate into a digital slide, how to import the annotations that is the “target” and the movement of the circles that make the target grouped or dissociated for a fully dynamic assessment and multiple methods application.

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