International Journal of Clinical Images and Medical Reviews

ISSN 2771-6309

Original Research

Selection of FFPE blocks with confocal microscope to reduce routine workload in pathology department

AH Nguyen[1], M Algoe1, M Nap[1,2], FJ van Kemenade[1]

[1]Department of Pathology, Erasmus Medical Center, Rotterdam, The Netherlands

[2]Nap Pathology Consulting, Numansdorp, The Netherlands

Abstract.

Background : Pathology departments face high costs of slide cutting, staining and coverslipping. We employed a confocal microscope (Histolog® Scanner) allowing to scan FFPE blocks to investigate whether this could reduce the number of slides prepared.

Methods: Lung (n=47) and pancreas (n=23) FFPE blocks were imaged with the Histolog Scanner after confirmation that Histolog Dip staining has no impact on routine (immuno)histochemical and molecular tests. Pathologists, residents and one pathologists' assistant diagnosed confocal images. Rate of image rejection prior diagnosis due to insufficient image quality was monitored for costs saving assessments.

Results: Rate of image rejection were 17% for pancreas and 23% for lung tissues reducing the potential cost savings (these blocks would be processed whatever their content in a routine practice). On the non-rejected images, observers were able to recognize tissue features in FFPE block images despite loss of nuclear detail and lack of colour nuances compared to H&E slides. Accuracy to define that the blocks are exclusively composed of normal tissue yielded high scores: overall sensitivity/specificity of 93%/83% for lung (7 pathologists, 4 residents, 1 pathologists' assistant) and 81%/76% for pancreas (4 pathologists, 3 residents, 1 pathologists' assistant). These three observer groups showed similar performances.

Conclusions: Image assessment of FFPE blocks by Histolog® Scanner yields acceptable images compared to H&E slides. Preliminary results showed that good performance of FFPE block screening can potentially be achieved for pancreas and lung tissues with high sensitivity before preparation of histology slides. This allows potential cost reduction in slide preparation and infrastructure costs for slide storage.

Impact Statement: Digital confocal microscopy by Histolog Scanner shows a promising approach to achieve rapid on-site digital imaging of formalin-fixed-paraffin-embedded (FFPE) tissue blocks, in order to allow cost reduction in slide preparation and infrastructure costs for the pathology department. The Histolog Scanner technique can be used as a supportive diagnostic method but this method cannot replace standard H&E-stained slides.

Introduction

In the current setting of a pathology department, the entire workflow from fresh specimen to formalin fixed paraffin embedded (FFPE) tissue blocks and HE stained microscopic slides is resource-intensive due to lack of automation. However, shortcuts in the workflow may be an interesting introduction prior to a more complete automation and double us as a resource saving method in a pathology department. We decided to study such a potential shortcut in workflow by focusing on the step between paraffin embedding and the production of H&E-stained slides. We hypothesized that pathology departments can increase their operational efficiency if they could select blocks of interest beforehand in such a way that it would allow to forego further cutting and staining of slides. This could be an option if it would be likely that such slides would not add information to the final diagnoses. Thus, in case of a high a priori chance of normal microscopy, prescanning blocks would allow the skipping of cutting, staining and cover slipping. In this approach, pathologists or pathologists' assistants would be able to 'prescan' the blocks and assess whether the block content would allow to forego further processing into slides. In particular, if blocks from resection margins of neoplasia specimens are macroscopically entirely unsuspected, then these could be 'prescanned' and assessed without cutting slides. The blocks would then be subsequently stored as blocks lacking any tumor (i.e. showing only normal histology) without producing slides. Such prescans could therefore reduce the numbers of cutting blocks (i.e., slide cutting, staining, cover slipping and analysis by pathologist) and potentially save cost. To be relevant for such a workflow shortcut and subsequent resource reduction by decreasing the number of slides prepared, the prescan process has to be quick, on-site and to produce easy to evaluate information for immediate feedback. In addition, such a 'prescan' step presupposes acceptable quality of the images to safely assess the content of the block.

Confocal microscopy is known for decades as an imaging technique providing histology-like images of biological tissues without the need

***Corresponding Author:** *F.J. van Kemenade, Department of Pathology, Erasmus Medical Center, Rotterdam, The Netherlands

Received: June 06, 2023 **Published:** June 20, 2023

Citation: *A.H. Nguyen .Selection of FFPE blocks with confocal microscope to reduce routine workload in pathology department . Int Clin Img and Med Rew. 2023; 4(2): 1150.

DOI: *10.55920/IJCIMR.2023.04.001150*

of preparing microscopy slides. Confocal laser scanning microscopes (CLSM) have been specifically developed to adapt this technology for the imaging of large tissue surface in a clinical setting (1, 2). The Histolog Scanner is a recent medical device based on CLSM approach allowing fast imaging of surfaces of thick tissue specimens but potentially also of surfaces of FFPE blocks. The image resolution of the Histolog Scanner (HLS) has been shown to provide sufficient morphological information to allow cancer detection in lymph nodes, skin, breast and prostate tissue specimens (1, 3-6).

The objective of this evaluation was to pilot HLS images of two types of resection specimens (i.e. lung and pancreas) where FFPE blocks were selected during the routine workflow to determine if image content would allow identification of block content in order to forgo further block processing. Microscopic assessment by Histolog scans had to be of sufficient quality to allow the pathologists to distinguish normal versus abnormal tissue microstructures and comparative assessment with Whole Slide Imaging.

Material & Methods

Preliminary controls

Innocuity of the Histolog Dip (SamanTree Medical, Switzerland) staining has been assessed on several human tissue specimens to confirm it is not interfering with Standard-of-Care techniques used in our pathology department, such as viewing, special staining, immunohistochemistry and molecular biological tests.

Human sample specimens not needed for further diagnostic purposes (intestine, lung, breast, tonsil, lymph node, skin, prostate, skeleton muscle, kidney, placenta, liver, brain, pancreas) were included in the following assessment. Each tissue specimen is divided into 2 parts: one part was immersed 10 sec in the Histolog Dip solution and then frozen with liquid nitrogen; the other part was directly frozen with liquid nitrogen. 5 microns cryostat slides were cut from the frozen material and stored at -20°C for further processing following laboratory routine diagnosis protocols of histochemistry or immunohistochemistry. Slides were either stained histochemically (H&E, reticulin, trichrome and Giemsa) or stained immunohistochemically for nuclear antigens (MSI proteins, Ki67, ER), common sensitive antigens (PD-L1, Vimentin, Kappa), membrane antigens (CD3, CD8, CD10, CD21, CD30, Beta-catenin), cytoplasmatic antigens (S-100, pancytokeratin, CK8/CK18, Smooth Muscle Antigen, CD1a), neuroendocrine antigens (Chromogranin A) or hormones (glucagon, insulin). Then slides from treated and control parts of the same specimen were compared by two senior pathologists to evaluate if the Histolog staining would have an impact on the diagnosis.

Similarly, the innocuity for molecular testing (CISH, FISH, NGS) has been evaluated on blocks from breast tissue specimens. Each tissue specimen was divided into 2 parts of which one part (test part) was immersed 10 sec in the Histolog Dip solution and then fixed into formalin; the other part (control part) was directly fixed into formalin. After formalin fixation all tissue parts were embedded in paraffin and molecular assessments are performed. Potential effects were assessed by comparing results of the tests between treated and untreated FFPE tissue specimen parts.

Equipment

Broached FFPE blocks were imaged with the Histolog Scanner (HLS), a CE-IVD wide field of view confocal laser scanning microscope designed for scanning large biological specimens in a clinical setting. The HLS integrates a computer with a touchscreen display monitor to control the device and review the images (Fig. 1). Images are generated by a laser diode exciting fluorescence at the wavelength of 488 nm. Fluorescence emission is collected in the wavelength above 500 nm. The laser scans multiple tiles and can produce a seamless image mosaic without post-processing. For each image, a 'preview' image of the FFPE block was created, which showed the field of view at lower resolution within 10 seconds, allowing fast overview and possibility for repositioning of the specimen. A higher resolution image could then be acquired within 1 minute to visualize tissue morphology details up to cell nuclei. Purple artificial coloring of the grey values of the fluorescence images is applied to resemble H&E slides. The operation of the device requires simple procedures that lab technician or pathologists' assistant in the lab can execute after a 1-hour training. There are no

Figure 1: *Overview of the Histolog® Scanner device with a broached FFPE block on the scanning surface.*

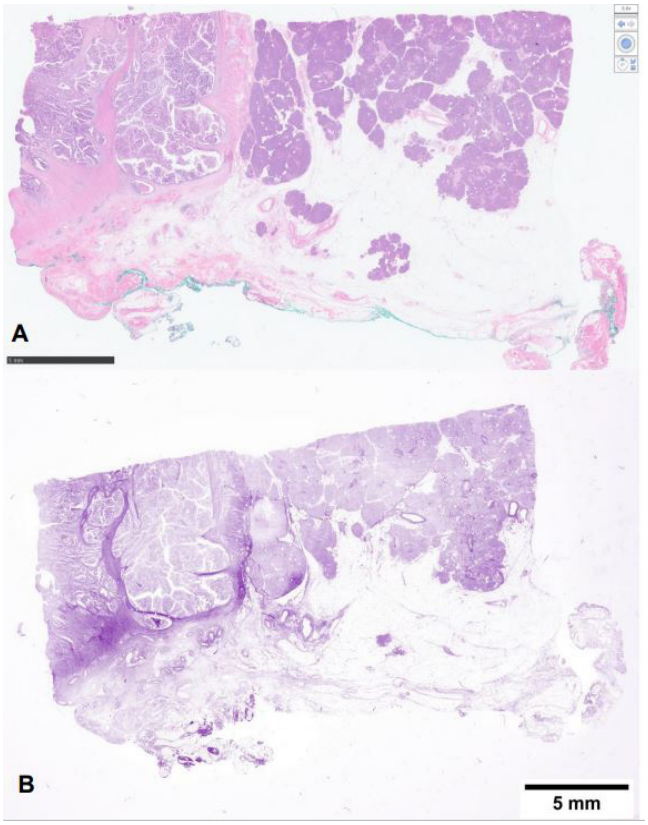

Figure 2: *Representative baseline images of the pilot with an overview stain of pancreas H&E stain (A) and Histolog block scan (B). A neoplasm is seen on the left of the images.*

Table 1: *Techniques and tissue types used to evaluate the Histolog Dip.*

Techniques	Tissue type
Histochemistry on Frozen Sections	
H&E	Intestine, Lung, Breast, Tonsil, Lymph node, Skin, Prostate, Muscle (striated), Kidney, Placenta, Liver, Brain, Pancreas
Gomori	Liver
Jones	Kidney
Masson Trichrome	Kidney, colon
Giemsa	Tonsil
Immuno-histochemistry on Frozen Sections	
MLH1, MSH6	Colon
Ki-67	Tonsil, breast
ER	Breast
PDL-1	Placenta, tonsil
Vimentin	Lymph node
Kappa chain	Tonsil
CD3, CD8, CD10, CD21, CD30	Tonsil, lymph node
Beta-catenin, alpha smooth muscle actin	Liver
S100, CD1a	Skin
Pan-cytokeratin, CK8, CK18	Liver, Colon
Chromogranin	Colon, pancreas
Glucagon, Insulin	Pancreas
Molecular tests on FFPE tissues	
Her2Neu FISH & CISH, NGS	Breast

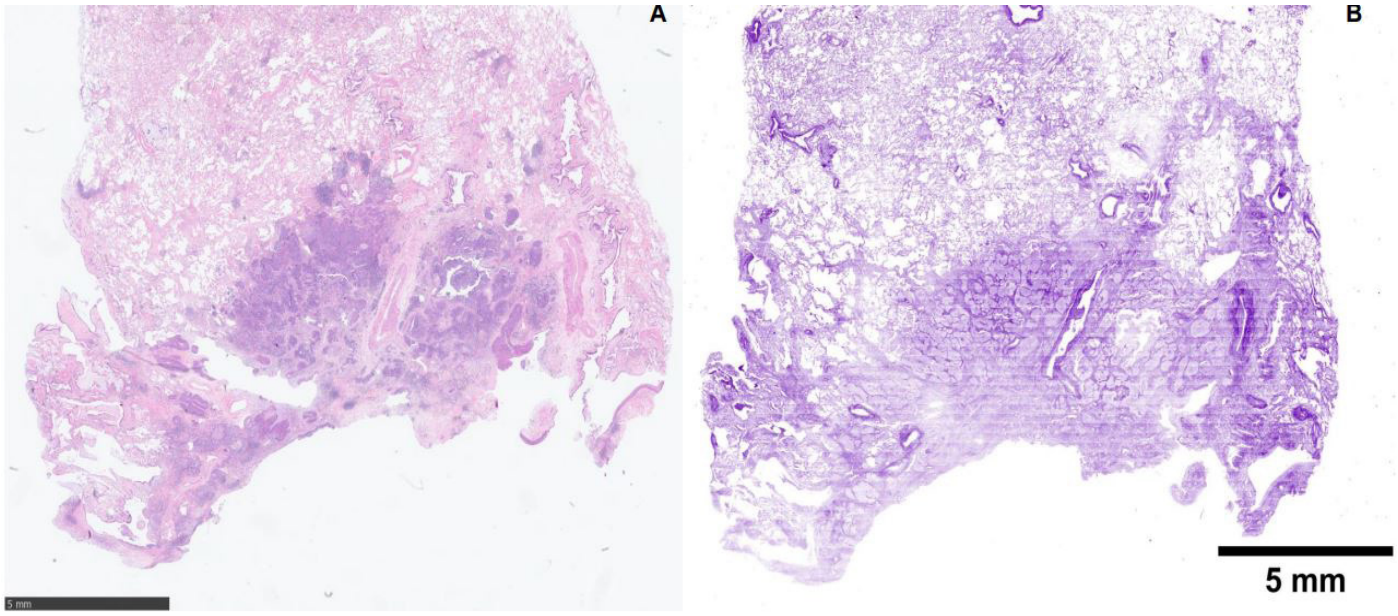

Figure 3: *Representative baseline images of the pilot with an overview stain of lung H&E stain (A) and Histolog block scan (B). A neoplasm is seen in the center lower part of the image.*

Table 2: *Abnormalities performance detection achieved by pathology staff in HLS images of selected lung and pancreas tissue blocks.*

	Lung Tissue	Pancreas Tissue
Number of Assessed blocks	47	40
Number of Assessors (overall)	n=12	n=8
Mean Sensitivity (overall)	93,3%	81,1%
Mean Specificity (overall)	83,3%	76,4%
Number of Assessors (Residents)	n=4	n=3
Mean Sensitivity (Residents)	95,7%	84,4%
Mean Specificity (Residents)	81,3%	77,8%
Number of Assessors (Pathologists)	n=7	n=4
Mean Sensitivity (Pathologists)	91,3%	76,1%
Mean Specificity (Pathologists)	94,5%	76,4%
Number of Assessors (Technician)	n=1	n=1
Sensitivity (Technician)	97,1%	90,9%
Specificity (Technician)	83,3%	72,2%

Table 3: *Percentage of potential block saving with routine resections.*

	Average number of blocks	Average number of blocks for resection margins	Average number of normal tissue blocks	Blocks saved per requisition (%)
Lung	9	1,6	3,6	17,8-40%
Pancreas	34,8	4,8	5,4	13,8-15,5%
Average				15,8-27,6%

preliminary calibration or parameter to set up prior imaging promoting standardization of the procedure.

Block imaging

After fixation and dissection of specimens, tissue for blocks were selected with and without abnormalities and subsequently embedded in paraffin blocks following standard procedures. Freshly cut FFPE blocks (broached) were then stained with Histolog Dip fluorescent solution (Saman Tree Medical, Switzerland) for 30 seconds and rinsed in 0,9% NaCl solution to remove excess dye. The Histolog Dip is a proprietary formulation of Acridine Orange, and preliminary controls confirmed that the staining with this solution did not affect subsequent staining with H&E nor other techniques routinely used in our laboratory. After staining, FFPE blocks were placed on the Histolog Dish over the imaging window of the Histolog® Scanner (Saman Tree Medical SA, Switzerland) (Figure 1). A max of five hundred grams of pressure was applied on the FFPE blocks during imaging to ensure optimal contact between the imaging window area and the FFPE block (not shown). Time to produce HLS images is monitored. Average time to generate an HLS image was 9 minutes.

Histolog images assessment

JPEG versions of the images were exported from the HLS with an external USB device. Then, these files were manually uploaded in Midview software (also used for Hamamatsu nano zoomed scans in our lab) to allow routine assessment by clinical staff. Prior to this assessment quality control was performed i.e., block images of insufficient image quality were excluded by one resident. Rate of this image rejection was monitored. Without preliminary training on HLS images, pathologists, residents and one senior pathologists' assistant were asked to assess the selected Histolog images dichotomously. i.e. they were asked to indicate whether the scans contained normal or abnormal tissue. Lung (n=47, assessors: 12) and pancreas (n=40, assessors: 8) FFPE blocks were evaluated to test the ability of the observers to distinguish normal from abnormal blocks. FFPE block content determined using H&E images was used as reference for this assessment. These tissue types have been chosen because Erasmus Medical Center is a center of reference for pancreas surgery and lung diseases. Time to evaluate the content of HLS images was monitored. Average time needed for evaluation was 2 minutes.

Results

Assessing potential effects of the Histolog Dip

The imaging with the HLS includes prior staining of the specimen with the Histolog Dip fluorescent solution. We wanted to exclude any interfere of the Dip with the routine techniques performed in our laboratory. First, frozen sections of thirteen types of human tissues stained with the Histolog Dip solution have been prepared and either histochemically or immunohistochemically stained (see Table 1). Two senior pathologists have compared these slides with the control slides coming from the same tissue specimen but not stained with Histolog Dip and prepared following the same procedures. This comparison showed no noticeable difference that could impeded the diagnosis. Then, four breast tissue specimens stained with Histolog dip and after paraffin-embedding together with the corresponding untreated parts, were submitted for molecular testing to confirm that such a staining has no impact on genomic related tests (Table 1). Comparison of the values obtained with the treated specimen parts and the control were performed by a certified senior pathologists' assistant and no difference were found between the two parts of a same specimen.

Preliminary block Assessment with the Histolog Scanner

The Histolog Scanner was easy to handle. The generated images appeared in a purple color after digital staining, resembling a conventional H&E stained slides. Automatic contrast adjustments could be done. The estimated time to generate and evaluate a Histolog image was 9 minutes. Preliminary quality control of HLS block images was performed with the following criteria: images had to be scanned completely with minimal scanning artefacts (minimal contact loss resulting in empty spots or mal alignment shifts producing ridges in the images). These comparisons concluded that 68,1% and 64,5% of lung and pancreas block HLS images, respectively, were of suitable quality for image content assessment by pathology staff. Rejected scans of FFPE blocks due to limited image quality, obviously would need to be processed and thus reduce potential savings by our approach.

Comparison of Histolog blocks Scans with routine H&E slides.

On the selected block images, observers were able to recognize tissue features in HLS block images despite lower nuclear details and colour contrast compared to routine H&E slides (Figure 2 and Figure 3). Accuracy for recognizing presence of abnormalities in the blocks, yielded similar scores for both tissue types (Table 2). Mean sensitivity/specificity of 93%/83% was obtained for lung tissue blocks when the performance of all assessors was combined (7 pathologists, 4 residents, 1 senior pathologists' assistant). Mean sensitivity/specificity of 81%/76% was obtained for pancreas tissue blocks when the performance of all assessors was combined (4 pathologists, 3 residents, 1 senior pathologists' assistant). Interestingly, similar performances between the three observer populations were found for the detection of abnormalities in HLS images of lung and pancreas FFPE blocks (Table 2).

Assessment of resource savings

If the absence of abnormalities could be determined directly with the confocal imaging by the HLS on FFPE blocks, preparation of the corresponding microscopy slides could be avoided. Obviously, the routine embedding of normal margins in oncological resection specimens are likely candidate to test the ability of direct Hisology scans. We computed percentage of potential blocks saved that could be stored without cutting, staining, coverslipping in an academic pathology laboratory. This would estimate potential savings in terms of slide production by technical staff.

However, there are some limitations to our assumptions in this scheme. Firstly, quality assessment of the scans prior to examination by microscopists, decreases potential cost savings. Secondly, not all FFPE blocks are suitable for HLS images due to e.g., inherent architectural difficulties. Lastly, the suboptimal specificity renders false positives, leading to unnecessary slides while suboptimal sensitivity would misjudge scans implying incorrectly assessing margins as without abnormalities.

Lung and pancreas specimen have an average of 1,6 and 4,8 blocks for margin assessment per specimen and an average of 3,6 and 5,4 blocks with normal tissue (including margins), respectively. Only these blocks, potentially, would be suitable for HLS imaging. Average blocks per specimen are 9,0 and 34,8, respectively. Table 3 shows that an average of 15,8-27,6% blocks can be saved per routine for lung and pancreas resections.

Since 68,1% and 64,5% of lung and pancreas block HLS images, respectively, are of suitable quality for image content assessment, the number of tissue type of FFPE blocks needed to scan for HLS images should be increased to explore cost savings.

Discussion

We tested the ability of a confocal laser scanning microscope (Histolog) to reduce routine workflow in routine academic pathology. We conducted a pilot comparing direct confocal microscope (the Histolog® Scanner) generated images with routine microscopy as control within the diagnostic infrastructure by having pathologists compared the scans dichotomically (either 'normal' or 'not normal') in the normal diagnostic surroundings. We conclude that, thus employed, the Histolog scanning could in principle reduce workflow of cutting those blocks, that have a high a priori chance of containing normal histology. We estimate around 10 to 25% of all cut blocks in the labs to contain normal tissue, depending on the specimen. Since this pilot is a demonstration of the principle, there are some caveats for implementing in routine workflows.

First, technical staff should be trained prior using the device to achieve a rough understanding of image content and increase the proportion of readable images. This will reduce rejects caused by artefacts. Our results were obtained with clinical staff that had no prior experience with confocal images. A training presenting the tissue content in Histolog images with the support of H&E slides is expected to improve the detection performance as previously seen in a recent study (1). Secondly, close approximation of block- and scan surface are needed to both reduce scanning time and artefact. As a result, blocks must be broached. The use of tools to increase approximation with the HLS imaging window could reduce the presence of these artefacts without increasing length of reading time (4). Thirdly, our results were obtained with clinical staff that had no prior experience with confocal images. Finally, image review by clinical staff was performed on jpg version of Histolog images for practical reasons. Working with the proprietary format of the Histolog images is thought to bring higher detection performance with deeper image dynamics. This will be made possible soon with a new version of the Histolog Scanner compatible with standard imaging formal such as DICOM and a CE-IVD Histolog Viewer software.

For potential implementation, there are some issues that need to be addressed.

Although a high level of concordance between pathologist, residents and one pathologists' assistant demonstrated that normal tissue can be accurately distinguished from abnormal tissue using the HLS, accuracy needs to be monitored. Our measured sensitivity in our pilot was acceptable, but we suspect that this could be increased by training and experience of pathologists. Interestingly, missing nuclear details did not prevent observers to qualify specimens as 'normal' or 'not normal'. Results were similar between the three observer populations. Specificity can be similar limiting for application.

Another limitation is the need for a good quality control and familiarity with the scan and its inherent lower quality compared to slides. This starting point needs to be accepted by a staff of pathologists, inherently keen on highest quality, but have to accept the scans that only allow normal versus not normal. Nevertheless, a high level of concordance between several pathologist, residents and a pathologists' assistant demonstrated that normal tissue can be accurately distinguished from abnormal tissue using the HLS. Therefore, it is possible to engage grossing room technicians to perform the actual histology, but also to engage them to actually screen the Histolog images. If the scans could be assessed by technical staff of the pathology department, it would more fully exploit the workflow improvement by Histolog scanning. It would simultaneously reduce the pathologists' workload.

In our department we considered that the load of tissue blocks scanned, needed to be 5-10% in order to incur sufficient savings to

consider an implementation in the routine practice. Our pilot was performed on lung and pancreas tissue blocks but in order to be viable in the routine process of a pathology department, further tissue types need to be tested to support a business model with Histology to reduce block cutting workload in a laboratory. We aimed for a reduction of at least 5% of the FFPE blocks produced into microscopy slides.

This is the first time that confocal microscopy, such as this one, can be used to image full FFPE tissue blocks. It is providing promising applications for the tissue assessment in the medical field. In addition to the resources saving purpose discussed above, it provided sufficient level of information for a meaningful documentation of its content for further assessments when the case needs to be re-evaluated later on or for bio-banking purposes without further consumption of the tissue block.

Interference by the necessary pre scan proprietary Histolog Dip (an acridine orange solution) used with the Histolog Scanner was excluded. Histochemical stainings, immunohistochemical stainings and molecular tests were evaluated on a large variety of human tissues. In comparison with an unstained control, none of them has shown any negative effect that could be linked to the staining processes. To the best of our knowledge, it is the first time that is it shown on such a large panel of techniques and tissues. In addition, it is in agreement with the findings of the previous studies performed with the Histolog Scanner (1, 4-6) and with other articles using acridine orange solution in the context of confocal microscopy (2, 7).

Conclusion

This report shows a promising approach to achieve on-site imaging of FFPE, using the Histolog Scanner to image FFPE blocks in order to forgo cutting, staining and cover slipping. The present study shows that HLS technique can be used as a supportive diagnostic method but this method cannot replace standard H&E-stained slides. Results showed that good performance of FFPE block screening can potentially be achieved for lung and pancreas tissues with high sensitivity and specificity before the preparation of histology slides. This allows potential cost reduction in slide preparation and infrastructure costs for slide storage to be assessed in the next step of our project.

Acknowledgements: We thank Frédéric Schmitt, Myriam Ballo, Hans de Bruin, Johannes van Brakel and Jenny Giang for their input and technical assistance.

Availability of data and materials : The datasets used and analysed during this study are available from the corresponding author on reasonable request.

Compliance with Ethical Standards: The Board of the Medical Ethics Committee Erasmus MC of Rotterdam, The Netherlands, has decided that the rules laid down in the Medical Research Involving Human Subjects Act (also known by its Dutch abbreviation WMO), do not apply to this pilot study. A waiver was granted (MEC-2021-0010), to do this study without informed consent and in an anonymized manner. The study did not interfere nor perturb the regular workflow in the pathology lab. It did not incur any risk for patients.

Conflict of Interest and Funding

The Histolog Scanner® and its utensils were provided during this pilot study by SamanTree Medical SA, Lausanne, Switzerland. No funding was received to assist with the preparation of this manuscript. No funding was received for conducting this study. AH Nguyen, M Algoe and FJ van Kemenade have nothing to disclose. M Nap reports the following role: medical advisor for SamanTree Medical SA, Lausanne, Switzerland.

Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by AH Nguyen, M Algoe, M Nap and FJ van Kemenade. The first draft of the manuscript was written by AH Nguyen and all authors commented on previous versions of the manuscript. All authors approved the final manuscript.

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