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Advances in Digital Pathology: From Artificial Intelligence to Label-Free Imaging

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Abstract
Background: Digital pathology, in its primary meaning, describes the utilization of computer screens to view scanned histology slides. Digitized tissue sections can be easily shared for a second opinion. In addition, it allows tissue image analysis using specialized software to identify and measure events previously observed by a human observer. These tissue-based readouts were highly reproducible and precise. Digital pathology has developed over the years through new technologies. Currently, the most discussed development is the application of artificial intelligence to automatically analyze tissue images. However, even new label-free imaging technologies are being developed to allow imaging of tissues by means of their molecular composition. ***Summary:*** This review provides a summary of the current state-of-the-art and future digital pathologies. Developments in the last few years have been presented and discussed. In particular, the review provides an outlook on interesting new technologies (e.g., infrared imaging), which would allow for deeper understanding and analysis of tissue thin sections beyond conventional histopathology. ***Key Messages:*** In digital pathology, mathematical methods are used to analyze images and draw conclusions about diseases and their progression. New innovative methods and techniques (e.g., label-free infrared imaging) will bring significant changes in the field in the coming years.

Introduction

Digital pathology, in its primary meaning, describes the utilization of computer screens to view scanned histology slides. Digitized glass slide tissue sections can easily be shared for a second opinion, which is even more important under the current restrictions of the COVID-19 pandemic. In addition, it allows tissue image analysis using specialized software tools to identify and measure events previously reported by a human observer. These tissue-based readouts were highly reproducible and precise. The term digital pathology has widened over the years using new technologies. Currently, the most discussed development is the application of artificial intelligence (AI) to automatically analyze tissue images. In addition, even new imaging technologies are being developed to allow label-free imaging of tissues by means of their molecular composition. All these methods are intended to support conventional histopathology to reduce

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workload and to provide more in-depth information for pathological reporting. In this short review, important developments in the last few years will be discussed, in addition to new techniques in digital pathology.

Digital Pathology and Image Analysis

In principle, digital pathology began with the advent of digital cameras and computers on microscopes in the 1980s and 1990s. However, it was not until the commercial availability of digital slide scanners allowing whole-slide imaging at the turn of the millennium that the basis for modern digital pathology was established. Today, digital slide scanners are available for most pathologies, allowing the fast and reliable digitalization of traditional glass histology slides. These images can then be viewed as decentralized on a computer screen or using a handheld device at a resolution similar to that of brightfield or fluorescence microscopy. This procedure places other demands on the workflow used, so the technical requirements (e.g., slide scanner, image storage, and image server), trained staff, and quality control (e.g., in the case of automated scans, control of the consistent color representation, and sharpness) must be ensured [1]. In particular, in light of the current restrictions due to the COVID-19 pandemic, the advantage of digitalization is obvious: cases can be discussed with colleagues in the simplest way without personal contact [2]. Furthermore, the images can be linked to digital patient records for archiving and digital evaluation using specialized software with high reproducibility [3]. In 2017, Philips received Food and Drug Administration (FDA) approval for a digital pathology whole-slide scanning solution (IntelliSite). This was the first global approval of a digital pathology system solution. One year later, the FDA permitted the first medical device using AI to detect diabetic retinopathy in adults (IDx-DR). The entry of these modern technologies into everyday pathology will accelerate in the coming years. All these and the methods presented below are intended to relieve and support the pathologist in his daily work.

Today, besides the digital review of whole-slide images, the most commonly used measures extracted from digitized tissue images are area-based and cell-based and employ object detection. A good introduction and summary of these methods was provided in 2019 in a white paper from the Digital Pathology Association [4]. Thus, a few methods of image segmentation for the identification of cells in histological images are discussed. Many of these algorithms are based on fixed or adaptive thresholds, watershed segmentations, active contour models, or template matching with shape priors. There are many other methods for image analysis, but a description of all of them is beyond the scope of this review, so we will limit ourselves to single examples of today's used approaches to illustrate current developments in digital pathology. One of the most common uses of segmentation is in the field of immunohistochemistry (IHC). For example, with the specific staining of individual proteins, segmentation in IHC helps to count cell nuclei or the ratio between 2 stained protein biomarkers. A frequently used example is the membrane protein human epidermal growth factor receptor 2, which can guide treatment strategies and prognosis in breast cancer [5, 6]. For scoring, the intensities and circumferential patterns of staining were used. The human epidermal growth factor receptor 2 has also been implicated in colorectal cancer (CRC) [7, 8].

Digital Pathology and Artificial Intelligence

With ever-improving slide scanners, wealth of data generated, and ever-increasing computational power, digital pathology is also becoming increasingly explored with the use of AI. AI is an umbrella term that covers all computer algorithms exhibiting behavior similar to human intelligence. This can be a simple training of decision trees, for example, to automatically recognize light and dark areas in tissue. However, it can also end up in complex networks that try to emulate human neural pathways and thus learn facts (deep neural networks). All these machine learning methods require training on known and representative data, for example, to recognize a particular tissue type. Here, close monitoring by experienced pathologists is very important to avoid training on wrong features or overtraining. The latter happens when a classifier learns only by heart and knows the training dataset perfectly but fails on majority of other independent samples. In the following section, further interesting aspects of computational pathology in combination with machine learning, especially convolutional neural networks (CNN), are discussed. In recent years, the databases for histological images have been continuously growing, thus allowing for the training of even more complex machine learning algorithms for several issues. Deep neural networks for image analysis, which are already being used for various applications in daily life, such as recognition of traffic signs in cars, have great potential. In pathology, these neural networks can help to make a pre-selection or to learn correlations that are not obvious even to a trained observer, such as a response to therapies [9, 10].

An application is the analysis of the immune context of CRC. This technique was established and designated as the "Immunoscore" [11–13]. The basis of this method is the quantification of lymphocyte populations, in particular CD3- and CD8-positive T cells, both at the tumor center (CT) and at the invasive margin (IM). A scoring sys-

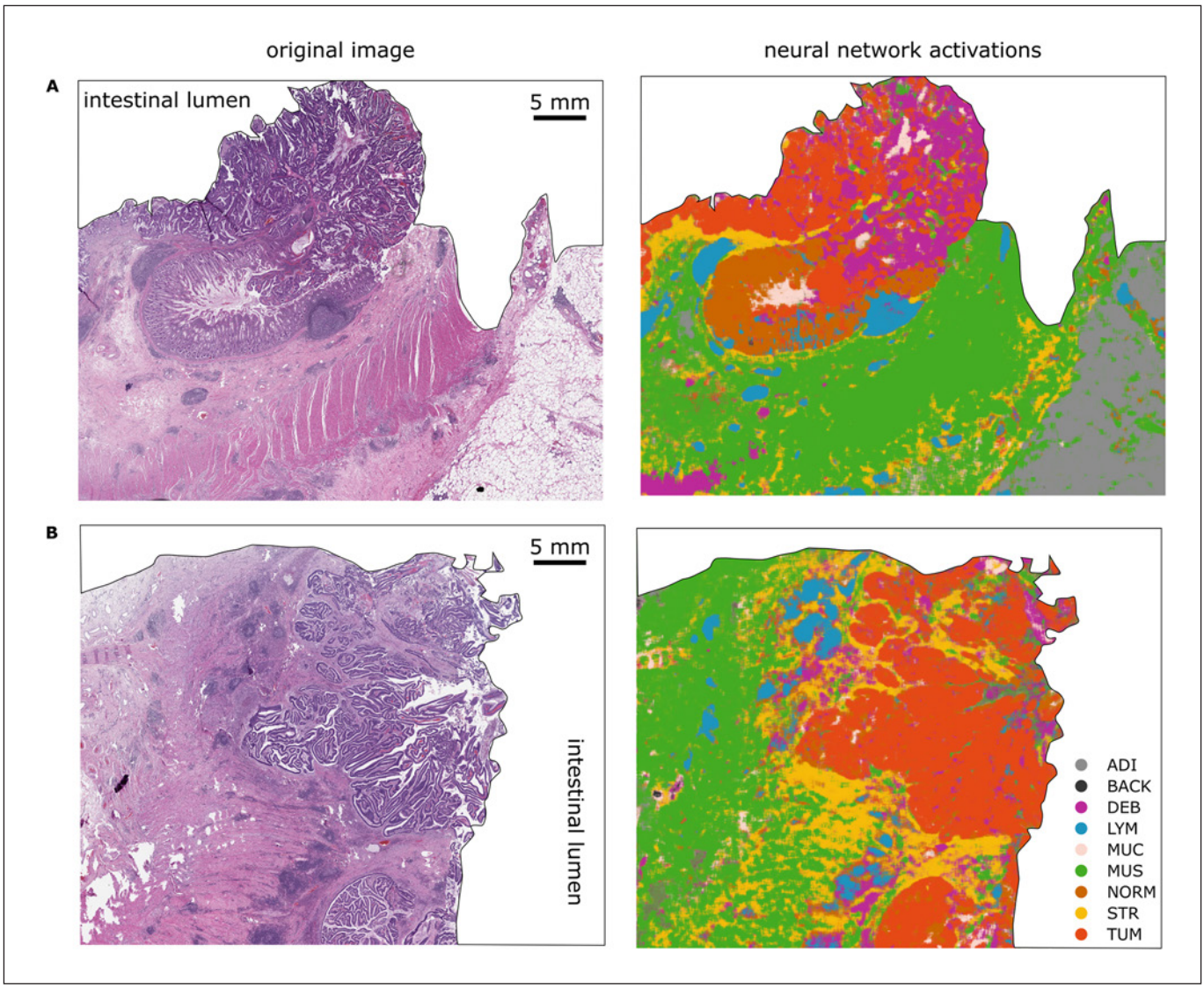

Fig. 1. A CNN classifier was used to classify real-world images from the DACHS cohort. **A**, **B** Two representative example images. Left: original HE image; right: classification map. Even fine structures are recognized by the neural network even in regions of suboptimal tissue quality. Only the tissue is shown in this example, and because the tissue does not occupy a rectangular area on the pathology slide, the whole-slide image was manually segmented by an observer trained in pathology to show only the tissue without background for better clarity (background is white). This figure was previously published in a study by Kather et al. [28] published in *PLoS Medicine*. ADI, adipose tissue; BACK, background; CNN, convolutional neural network; DACHS, Darmkrebs: Chancen der Verhütung durch Screening; DEB, debris; HE, hematoxylin-eosin; LYM, lymphocyte aggregates; MUC, mucus; MUS, muscle; NORM, normal mucosa; STR, stroma; TUM, tumor epithelium.

tem was used, ranging from low immune cell densities found at both the CT and the IM (Immunoscore 0–I0) to high densities classified as Immunoscore 4 (I4), with increasing scores correlating with longer patient survival [14, 15]. In brief, CD3- and CD8-immunostained tissue thin sections were scanned, and the 2 corresponding digital images were validated by the operator. The images were then analyzed using a dedicated software (Immunoscore Analyzer; HalioDx). In the first step, the software automatically detects the tissue histologic structure using a trained CNN. Second, an operator defines the tumor (adenocarcinoma), healthy tissue (submucosa, muscularis propria, and serosa), and epithelium (mucosa). In this step, all areas of necrosis, abscesses, and artifacts (bubbles, folds, torn areas, and background) were marked for exclusion to avoid false positives. Thereafter, the software calculates the Immunoscore by counting the IHC-stained cells based on a proprietary computer vision algorithm. The entire procedure, including all materials, instrumentation, and software, has been validated and approved for use in clinical practice [14, 15]. Even with this already highly developed and validated method, its usability in a

routine manner must still be evaluated. This includes points such as feasibility, simplicity, cost, robustness, and reproducibility. While cost and reproducibility are self-explanatory, the feasibility and simplicity of the methodology include whether it is feasible in any pathology work setting without much effort. For example, independence from errors caused by misalignment of the microscope used and incorrect operation of the software must be ensured. This is also linked to the robustness of the methodology, which describes the reliability in the face of such influences. The Immunoscore has the potential to fulfill these key aspects, but it is still a way to prove all these points in clinical studies. Thus, studies on whether the results are reproducible in everyday clinical practice and at independent clinical centers should be continuously done [15]. This example, which is already very close to application, shows that the interaction between the user and automated evaluation is necessary to ensure successful diagnostics. After all, a classifier cannot recognize anything that it has not previously learned. Unfortunately, in contrast to humans, classifiers usually do not ask questions but simply make a decision. Therefore, a pathologist is indispensable for making the final decision.

An increasing number of morphologically driven CNN-based models have been successfully used for tumor detection, classification, gland segmentation, and grading, especially for bladder [16, 17], brain [18], breast [19], gastric [20], lung [21], and prostate [22, 23] cancers. To date, some of the largest studies using CNNs have been conducted for CRC. Several approaches have been published in the pathologic image analysis of CRC. However, until today, the studies have many limitations, such as the use of annotated data with limitations, a relatively small number of training and validation datasets for generalization, and impaired study design for an appropriate level of evidence. A recent review has discussed several recent studies [24]. Therefore, only a few of the latest studies are presented here. Significant work in the last 2 years has been done by a German group who has presented various deep learning models that detect/predict CRC tumors, microsatellite instability (MSI), mutation patterns, and survival [25–28]. The CNN segmentation is shown in Figure 1. In their most recent studies, they further tested their models for MSI detection [26] and presented pan-tumor mutation detection [27]. Both were detected in tumor samples on routine histological slides (H&E stained). For MSI, 8,836 colorectal tumors (of all stages) included in the MSI-DETECT consortium study were analyzed. The CNN was trained to identify samples with MSI. Performance was assessed by cross-validation (N = 6,406 specimens) in an external cohort (n = 771 specimens), resulting in an AUROC curve of 0.92 (lower bound, 0.91; upper bound, 0.93) and an AUPRC of 0.63 (range, 0.59–0.65), or 67% specificity and 95% sensitivity, in the cross-validation development cohort. In the validation cohort, an AUROC of 0.95 (range, 0.92–0.96) without image preprocessing and an AUROC of 0.96 (range, 0.93–0.98) after color normalization were reached. As an independent validation dataset, the authors showed the performance of their MSI detector on endoscopic biopsies, which are quite different samples than the resectates used for training, and achieved a much lower AUROC of 0.78. This shows that the dataset with thousands of samples does not precisely cover the special features or technical artifacts of very small samples well, and that the neural network could not learn to recognize them perfectly on the used training dataset [26]. Barriers in AI development are technical artifacts including fragmented tissue, small tissue areas, and cauterization, as well as biological artifacts (sampled from the luminal portions of the tumor only) [24]. As the authors of the presented study state, the clinical application of this technology requires high performance and multisite validation, which have not yet been performed. All these aspects have not yet been addressed and significantly limit all of these systems, as they will always rely on human assistance.

Digital Pathology, Artificial Intelligence, and Label-Free Imaging

Most approaches discussed in digital pathology relate to the analysis of stained tissue sections, as in the previous examples. This results in several difficulties; for example, it has high requirements for consistent staining quality. In addition, many different stains with an increasing number of biomarkers to be analyzed per patient sample are needed in order to achieve a fully comprehensive analysis of the tissue. AI now opens up a new field where staining of thin tissue sections is no longer necessary, wherein the physical properties of molecules are used to extract spatially resolved specific information about the tissue. To do so, label-free vibrational spectroscopic methods (infrared [IR] and Raman spectroscopy) are used to analyze thin tissue sections. Here, we will focus on IR imaging for automated classification of cancer tissues [29]. In combination with AI, IR imaging allows morphological and molecular analyses of a single tissue thin section within only a few minutes. This involves examining the thin tissue sections in an IR microscope, which is very similar to a brightfield microscope, except that the specimen is irradiated with infrared rather than visible light. The IR interacts with the molecules in the sample and incites molecular vibrations [30]. This results in a specific intensity being lost as it passes through the sample, called absorption. Infrared cameras can thus be used to record the spatially resolved IR spectra. For individual molecules, specific information about the molecule can be ob-

tained from the IR spectra measured in this manner. For complex systems such as tissues, the spatially resolved IR spectra represent an integral "fingerprint" of the entire proteome, genome, transcriptome, lipidome, and metabolome. This fingerprint can then be used by AI to analyze tissues, similar to the methods presented previously. In contrast to staining-based methods, the IR fingerprints of a single section can be used to consider not only morphology but also molecular composition. Furthermore, the tissue remains unmodified, undamaged, and can therefore be used for further analysis (e.g., omics).

The IR imaging technique, which is still very new in pathology, has been developed and tested in basic research over the last few decades. Two to 3 years ago, the most commonly used form was Fourier-transform infrared (FTIR) imaging. This has been applied by a number of research groups worldwide to first identify spectral differences between different (normal) tissue types, between normal and diseased tissues [31–35], between tissues with different disease types [36], and as a method to elucidate prognostic information [37, 38]. Currently, IR imaging has been applied for the automated, label-free classification of tumorous tissue [39], including colorectal [40, 41], lung [42, 43], prostate [44], bladder [45, 46], and skin [47] cancer and many more. These studies demonstrated sensitivities and specificities of the technique >90%, as compared to histopathology and IHC as the ground truth.

For CRC, different tissue structures that occur in the colon wall were found to have distinctly different spectrochemical signatures. The structures identified spectrally, namely, the lamina propria mucosae, the lamina muscularis mucosae, the crypts, and the lumen filled with mucus, connective tissue, and cancer were classified by an AI algorithm called random forest. The classifier was trained on a spectral dataset representing different tissue types. The classification of CRC using FTIR imaging has high accuracy, sensitivity, and specificity (96% accuracy, 94% sensitivity, and 100% specificity) [41]. Later, this approach was extended by differential cancer diagnosis, including grading [48]. All these studies demonstrated that FTIR imaging has the power to classify tissue morphology without staining based on spectral fingerprints. The results are highly comparable to the annotation by a trained expert, but staining and observer independence are done in an automated manner. Now, one may ask why this innovative method has not yet found its way into clinical practice, to which one can attribute to the slow speed and high sensitivity to laboratory conditions of FTIR imaging (e.g., temperature changes and the need for liquid nitrogen). For slowness, a CRC sample of, for example, 1 × 3 cm in size can take several days for analysis. Therefore, its use in clinical everyday business is not yet feasible.

In recent years, new IR imaging systems have been developed. These are no longer using FTIR spectrometers.

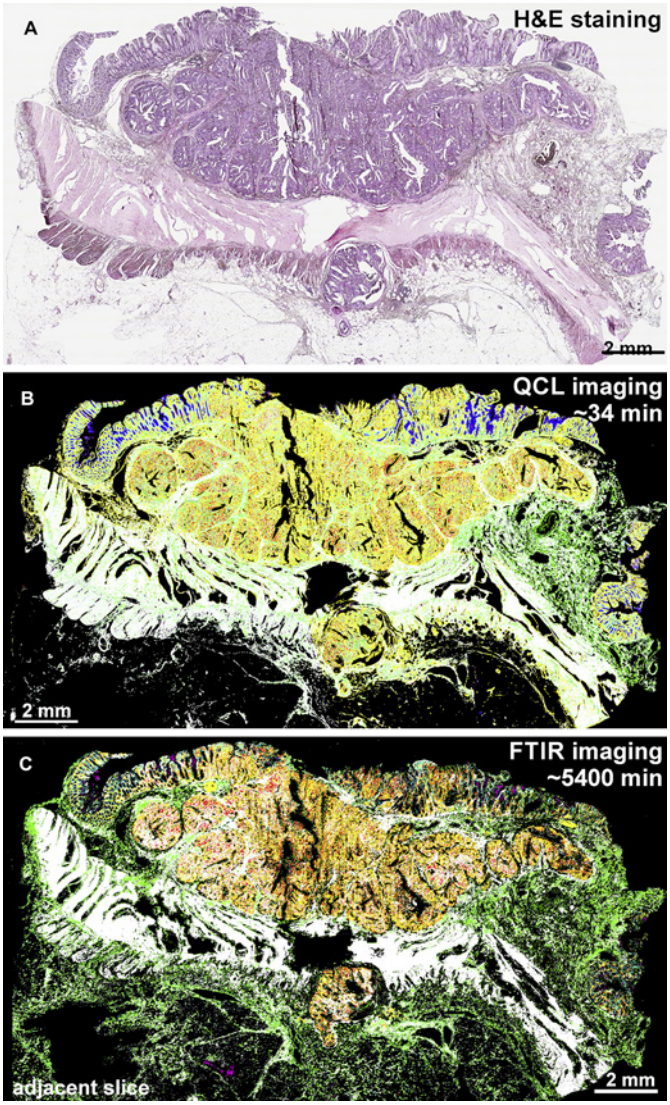

Fig. 2. Colorectal cancer tissue analysis using H&E staining as the gold standard (**A**), the Spero QT system (**B**), and an FTIR-based imaging system (**C**). The listed times illustrate the duration of the measurements. Red, pathological region comprising tumorous regions and infiltrating inflammatory cells; white, muscles; green, connective tissue; cyan, crypts; and blue, lumen. The comparison of the images convincingly demonstrated that the QCL IR imaging results are in good agreement with those obtained using the FTIR imaging. The observed deviations seem to be caused mainly by the use of an adjacent slice and the training of the FTIR classifier on samples of another study which could slightly differ in sample handling and processing. Furthermore, the previous FTIR classifier was performing the classification in one-step which is less accurate by means of tumor detection. However, the improved classifier for the QCL imaging recognizes infiltrating inflammatory cells in the first-level RF and cancerous regions in the second-level RF which allows a much more accurate classification. This figure was previously published in the supplement of a study by Kuepper et al. [54]. RF, random forest.

Instead, quantum cascade lasers (QCLs) are used as high-power light sources. The groups of Bhargava and Petrich made novel discoveries in the field of chemical imaging with homemade QCL-based microscopes [49–51]. This

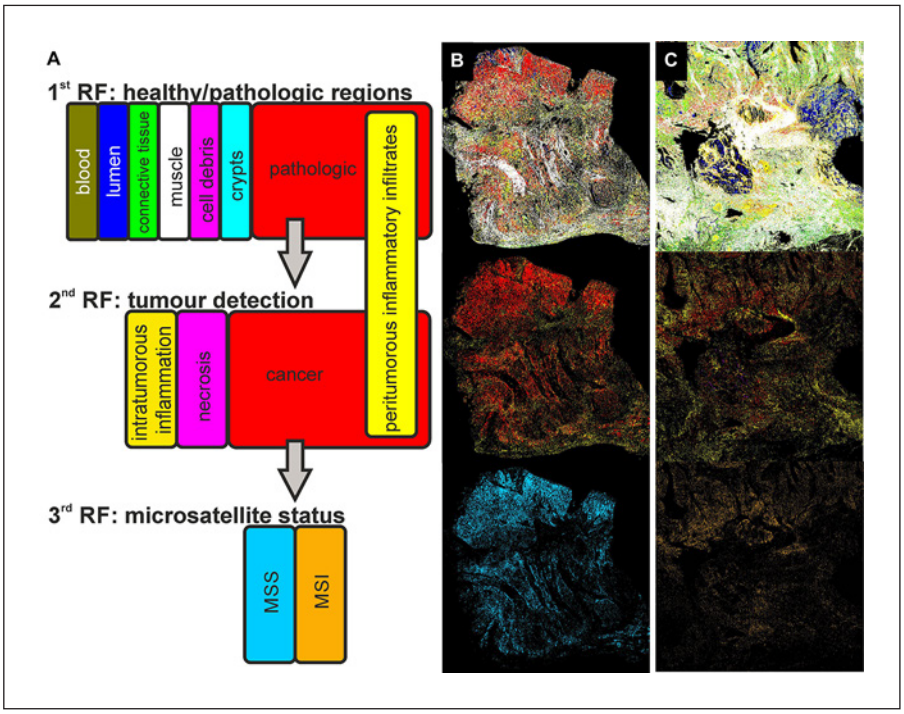

Fig. 3. A The RF classifier structure and the corresponding color code are shown schematically. The first RF (first row) determines healthy and pathologic regions, the second RF (second row) further classifies the pathologic regions to identify cancer regions, and the third RF (third row) determines microsatellite status of cancer regions. **B**, **C** The resulting IR index color images are shown for a MSS CRC (**B**) and MSI-H CRC (**C**). This figure was previously published in a study by Kallenbach-Thieltges et al. [55]. RF, random forest; IR, infrared; MSS, microsatellite stable; MSI-H, high microsatellite instable.

led to the launch of the first commercially available QCL-based IR microscope, Spero (Daylight Solutions, San Diego, CA, USA). Studies using this instrument have reported promising results [39, 52, 53]. However, limitations of coherence effects, low laser stability, and offset edges in mosaic datasets must be overcome. In the second generation (since 2017) of this instrument, these effects were minimized, thus allowing a combination with a newly developed classification model for CRC. In the first study, it was shown that the results of this new instrument are comparable to those of previously used FTIR spectrometers [54]. It is now possible to analyze thin tissue sections within a few minutes, matching the same time range used for an immediate section. The QCL-based microscopes were 160 times faster as shown in a study with 100 samples (UICC stage II and III CRC tissue) as well as 20 tumor-free tissue samples from 110 randomly chosen patients older than 18 years. The automated tissue classification reached a sensitivity of 96% and a specificity of 100% comparable to the slow FTIR imaging (Fig. 2) [54]. The speed-up of the methodology was the most important step toward possible translation of label-free digital pathology into clinics because it allows for larger studies, which further enables label-free digital pathology to follow the same path as stain-based digital pathology for validation studies and subsequent translation into clinics. For example, by using QCL-based IR imaging, it became possible to detect MSI in unstained CRC tissue samples. For this purpose, the tissues from 100 patients were analyzed. Forty patients were used to train the classifier and 60 for verification. In validation, this method achieved 100% sensitivity and 93% specificity (Fig. 3) [55]. This study showed, for the first time, that molecular changes can be represented. Since IR fingerprints reflect all molecular changes, future extension to the mutational level is very likely. However, similar to the use of machine learning on stained samples, these numbers should always be viewed critically until external validation is performed.

Similar to the developments in the image analysis of stained tissues, the application of deep learning to vibrational spectroscopic images was performed. It was the sole introduction of fast IR microscopes that made this possible, as the availability of large datasets is crucial for deep learning. These approaches will allow for correlation of spatially resolved molecular information by IR imaging with morphological information directly from the spectral data cube. It was demonstrated that CNNs with architectures designed to process both spectral and spatial information could significantly improve classifier performance over per-pixel spectral classification for both Raman and IR imaging [56–58]. For better visualization, some groups try to digitally stain the IR images, which allow the transfer of spectral information to well-known visualization for clinical experts [59]. Thus, by using deep learning, any morphological staining, as well as a molecular analysis of the tissue sample, can be generated from a single IR imaging analysis. Considering the nonexistent variance due to staining, this represents a unique advantage. Larger studies will follow, further bringing this innovative new label-free technology closer to clinical application.

Digital Pathology and Molecular Analysis

For both the stain-based and label-free methods, there is yet another application besides classical tissue analysis. For example, spatially resolved classifications can be used to excise tissue using laser capture microdissection (LCM) and subsequently analyze it using omics methods. On the one hand, this enables automated tissue collection for established tests. On the other hand, however, a deeper molecular understanding of the disease can be gained if, for example, the classifier recognizes other tissue regions as significant as the classical tool would. The label-free methods described are particularly superior for biomarker research, since tissues are not altered and can be collected in as natural a state as possible. By integrating FTIR imaging with LCM for subsequent proteomic analysis, a new protein biomarker was identified. The protein biomarker helps to differentiate severe cystitis from carcinoma in situ, which is a high-grade carcinoma [46]. Especially in large consortia for biomarker discovery, such automated methods may be of interest to further minimize bias between participating partners in tissue selection.

Conclusion

This brief review is intended to provide an overview of current developments and go beyond the scope of the much-discussed approaches in digital pathology. Thus, methods of classical image analysis have long since found their place in everyday pathology, such as for counting cells. These approaches are now very advanced, but still require experienced users to recognize and avoid errors. However, the ongoing standardization of procedures, starting with the cutting and staining of thin tissue sections and ending with microscopic image acquisition, is making great strides. Today, classical image analysis is supplemented by AI, which allows systems to be trained for specific tasks for automated analyses. The studies so far are promising, but they still have some weaknesses that prevent their daily use. For example, it has hardly been shown how AI approaches react to staining errors or artifacts in samples, such as cauterization or freezing artifacts. If this is tested in clinical trials in the next few years, and their robustness is proven, AI will be an indispensable support tool in everyday pathology, as it promises significant time savings. Unfortunately, AI methods are highly dependent on the number of samples available for training. This can be a problem especially for complicated or rare questions. The label-free IR imaging described above holds great promise. It allows not only morphological insights but also resolves detailed molecular changes in the genome and proteome. This makes it possible to work with significantly smaller sample quantities in AI which leads to equally good automated detections. It remains to be seen to what extent all these methods will revolutionize classical pathology, but it is certain that they are all methods that will support their operators, rather than replace them.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

F.G. wrote the manuscript. H.J., K.G., and A.T. critically revised the manuscript.

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