

Digital pathology and image analysis in tissue biomarker research



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ABSTRACT

Digital pathology and the adoption of image analysis have grown rapidly in the last few years. This is largely due to the implementation of whole slide scanning, advances in software and computer processing capacity and the increasing importance of tissue-based research for biomarker discovery and stratified medicine. This review sets out the key application areas for digital pathology and image analysis, with a particular focus on research and biomarker discovery. A variety of image analysis applications are reviewed including nuclear morphometry and tissue architecture analysis, but with emphasis on immunohistochemistry and fluorescence analysis of tissue biomarkers. Digital pathology and image analysis have important roles across the drug/companion diagnostic development pipeline including biobanking, molecular pathology, tissue microarray analysis, molecular profiling of tissue and these important developments are reviewed. Underpinning all of these important developments is the need for high quality tissue samples and the impact of pre-analytical variables on tissue research is discussed. This requirement is combined with practical advice on setting up and running a digital pathology laboratory. Finally, we discuss the need to integrate digital image analysis data with epidemiological, clinical and genomic data in order to fully understand the relationship between genotype and phenotype and to drive discovery and the delivery of personalized medicine.

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1. Introduction

While it is a common misconception that digital pathology and image analysis is new, research on the use of computers and software for analyzing and measuring cells or tissues in pathology date as far back as the 1960's and 70's [1–4]. That's over 40 years ago! Clearly, the hardware and software systems then were limited in their capability by comparison to today – but those studies were the first to demonstrate the value that computer-based imaging, cellular measurement and quantitation could play in pathological diagnosis and discovery.

As computer hardware advanced rapidly in the 1980's and 1990's, there was considerable promise that image analysis would be embraced as part of routine diagnosis in pathology. Some even posited that the technology would ultimately replace human pathologists. There was enormous investment in automated cytology screening based on IA, with the promise that this could be used to reduce cytology workload and improve diagnostic performance across laboratories worldwide. Clearly this did not happen on the scale predicted and even the most state of the art IA systems failed

to significantly change practice in pathology. So the initial enthusiasm for digital IA technology in pathology waivered with the focus shifting to molecular pathology and the promise of diagnostic classification of tissue samples without the need for morphology. Three principle factors changed that: (1) the recognition that molecular pathology still relies heavily on tissue interpretation (2) the drive for targeted therapies based on the presence or absence of tissue-based markers and (3) digital scanning and whole slide imaging (WSI) of entire glass slides in pathology.

The last factor has been hugely instrumental in the recent upsurge in the adoption of image analysis again in both the research and diagnostic sector. Whole slide imaging (WSI), and associated viewing software, allows entire slides to be digitally scanned at high resolution, reviewed by an experienced morphologist, regions selected and image analysis applied to measure specific features. This potentially circumvents the need to use traditional microscopy, manual selection, restricted image capture using a CCD camera, transfer to an image analysis package and subsequent measurement of specific features. WSI can bring these processes together, making image analysis much more practicable and easy to adopt, while facilitating integration into existing workflows in both research and primary diagnostic laboratories.

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Commercial image analysis systems have grown dramatically in recent years. This is likely to continue as the applications in discovery, preclinical and clinical research continue to demand quantitative methods, and as new diagnostic tools are translated into diagnostic practice.

This article aims to provide readers with a rapid overview of the current status of digital pathology and image analysis in biomarker research and diagnostic practice, including practical advice on adopting and developing these technologies.

2. Whole slide scanning and digital slides

2.1. Whole slide scanners

While the digital capture of individual images is still utilized widely in the research and tissue diagnostic community, whole slide scanning (WSI) is by far the most rapidly expanding means of digital image capture in pathology. WSI allows the digital capture of the entire tissue sample at high resolution and with appropriate software allows the viewing of the slide at any position and at any magnification. In this way it replicates what is achievable with standard microscopy, but provides a range of additional advantages – including facilitating image analysis.

Over recent years WSI instrumentation has become more widely accepted and affordable in pathology research laboratories and in primary diagnostic laboratories. However, given the pace of development, there are likely to be further systems available from new providers as the market continues to expand.

Most of the systems rely on two variants of image capture (1) line scanning and (2) tile scanning, both of which generate multiple high resolution images (in the form of lines or tiles) that are subsequently aligned or stitched together to create a complete, composite image of the original whole tissue section. Collecting image data by either method is achieved by passing the slide underneath the objective using a carefully controlled motorized scanning stage or objective assembly. The image data is rapidly recorded as the slide is traversed and image data stitched together in real time.

In most systems the magnification at which the slide is scanned can be adjusted. This is commonly either at 20× or 40× magnification. Other select systems can scan under oil at 63× to provide higher resolution systems. 20× scans are sufficient for most standard H&E remote viewing applications although some institutions prefer to scan at 40× to ensure higher resolution. Fig. 1A shows a whole slide scan of a pancreatic cancer, scanned at 40× magnification where the image can be viewed at any magnification (Fig. 1B) and where multiple slides can be viewed side by side for comparison at any location or any magnification (Fig. 1C). Image analysis can benefit from high resolution scans, particularly for applications that involve nuclear detection and analysis. Applications such as *in situ* hybridization (ISH) can be carried out at 40× with fluorescence but may benefit from higher magnification scans in order to resolve individual spots with chromogenic ISH. Haematology applications may require 63× scanning (restricted to certain models of scanner) in order to better resolve morphology and cell types. There is however a storage premium to pay for high resolution scans.

Accurate focus across large areas of tissue during the scanning process is essential. In most instrumentation, this is achieved by mapping the topography variations that inherently exist across even a very thin tissue section, and rapidly adjusting the focus as the scan is being created [5]. The reliability of this process has improved dramatically over recently years and most systems can automatically scan large batches of slides with no human intervention at all.

Some WSI systems can also generate “multiplane” scans, which capture image data along the z-axis (Fig. 2) as numerous large

images in a stack. With appropriate viewing software, this provides the ability to navigate images in the z-plane, creating a digital focus effect. This is particularly effective for cytology preparations, where the ability to focus is extremely important.

Finally, many scanning systems now offer fluorescence WSI. This makes use of the benefits of fluorescence (see Section 4.6) while providing full slide scans, digitally capturing all relevant data for storage, remote review and image analysis. There are specific challenges associated with fluorescence WSI, not least of which is focus. Fluorescence images tend to contain less contextual background information than brightfield images, and so provide less data to support automated focus over large areas. However most systems provide the ability to select defined regions of interest for scanning, allowing large areas of slides to be successfully scanned under fluorescence.

2.2. Image size and compression

Whole slide digital images are large. Scanning a typical tissue section of 15 × 20 mm in size at 20× viewing magnification (0.24 μm per pixel) can generate images as large as 3.6 GB in size. Scanning at 40× will generate images as large as 14.5 GB. These can be compressed to more manageable sizes (approx. 25:1 compression or more), reducing the file size without impacting on the visual quality of the image. Studies on the compression of images in digital pathology [6] have shown that extensive image compression can be applied without experts being able to visually perceive differences in image quality. Even images with high compression ratios can still be interpretable visually.

An important consideration, however, is whether image compression can affect quantitative image analysis. Commercial systems routinely apply different compression methods and levels as part of their standard configuration and so variation from one instrument to the next could be detrimental. Basic studies have shown that densitometric measurement (which is used routinely for quantitative IHC image analysis) is more sensitive to compression than morphological measurement (e.g., nuclear size). Different compression methods offered by different vendors can have very different effects on image analysis fidelity [7]. Kieran et al. [7] showed that with some methods of compression, 5% of the nuclei were segmented in error, with an error rate that steadily increased as compressed image quality decreased. Care therefore needs to be taken to assess the impact of compression artifacts on image analysis. The impact of compression needs to be validated for each study depending on the features calculated.

2.3. Scanning speeds and automation

Most instruments can now scan slides in 1–3 min, some with the capability of automatically loading multiple slides without user intervention. Some of the larger scanning devices can accommodate in excess of 300 slides, making them ideal for high volume environments, including busy clinical diagnostic laboratories or large scale tissue research facilities where large numbers of slides need to be scanned and archived daily. Smaller scanners are available, which can scan from 1 to 10 slides in a single action. These are ideal for specialist or incidental research requirements, for educational organizations that are scanning relatively small teaching collections, or for diagnostic labs that want to use digital pathology for infrequent second opinion or frozen section review.

2.4. Storage of digital slides

Given the size of digital slides and the numbers that are now being routinely scanned in many research and diagnostic laboratories, storage represents a significant element of the investment

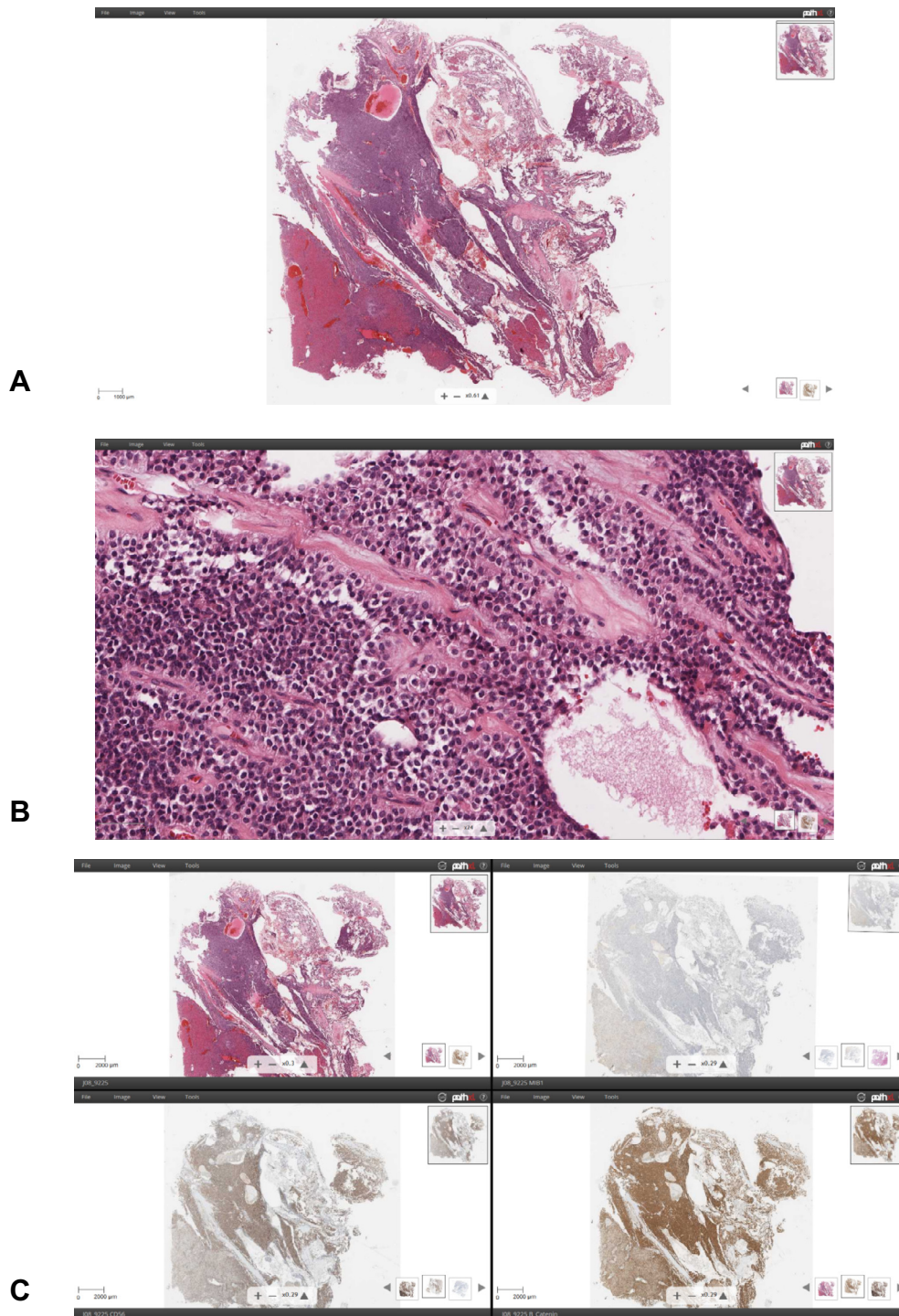


Fig. 1. Example of a digital slide scanned displayed within a digital slide viewer (PathXL Ltd). (A) Full slide overview. (B) High powered view of specific region of slide. (C) Side by side viewing of different immunohistochemical markers on the same case.

required for digital pathology. This investment might actually exceed the cost of the scanner and associated software facilities required for delivering a digital pathology programme! Not only is basic storage essential for these large volume data sets but backup facilities are also normally required, doubling the amount of physical storage needed. Stathonikos et al. [8] (2014), who have undertaken to routinely scan all slides coming into their laboratory in Utrecht, and with approximately 500 slides scanned per day, quote daily storage requirements of 175 GB and the need to store 5 TB of WSI data per month. The storage demands of scanning all

slides are such that this group are currently migrating to a hospital-wide object-based storage solution – a tiered system with scalable capacity to several petabytes – which can archive slides in short-term, long-term and permanent storage environments.

Finally in order to ensure robust, reliable, long term storage that will protect what might be valuable research or clinical data (in the form of images) long term, certain storage standards need to be addressed that meet acceptable criteria for data that may have to be retrieved under law. The DPA white paper on archival and retrieval in digital pathology [9] recommended that disk storage

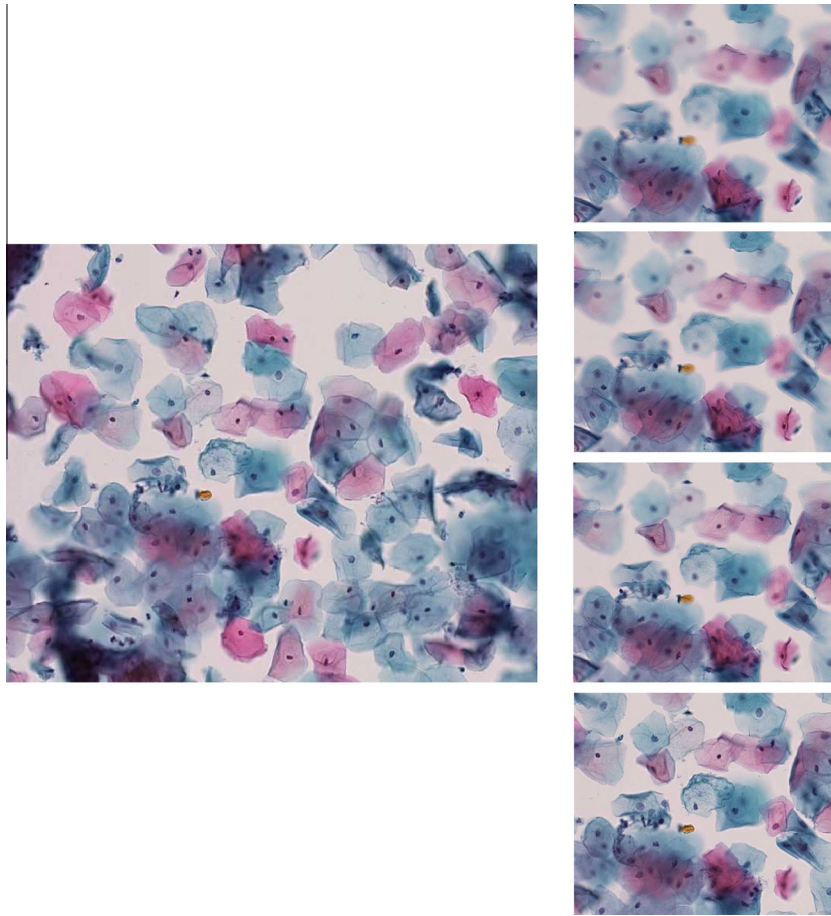


Fig. 2. Multiplane scanning to capture z data and allow digital focusing through the sample.

is configured in RAID 6 with 1 hot spare disk, ensuring that up to three disk failures can occur without affecting data integrity.

Given the storage demands for organizations adopting digital pathology, cloud-based managed storage solutions are also becoming an acceptable model for managing high volume image data. This approach removes the overhead for deployed servers and image management locally. Here, storage is outsourced to third party service providers that manage the backup and security of high volume data on behalf of the organisation. In digital pathology, this often provides better performance in terms of web-based access, viewing speeds and reliability. It also facilitates rapid entry points into the digital pathology market at lower cost for low volume activities. Drawbacks include issues of data transfer speeds to the cloud and additional cost when operating with high volumes of images.

3. The importance of software

While hardware is essential for the scanning of whole slides, software is vital for delivering digital pathology applications. Software underpins all of the functional capability of digital pathology – from digital slide viewing, management and analysis – as well as harnessing the power of workflow applications to support processes across a range of activities.

3.1. Digital slide viewing

As stated previously, digital slide files are large and a single image cannot usually be loaded into local computer memory in its entirety. This therefore requires specialist software to allow digital slides to be viewed without loading or transferring the full image file into memory or across the internet in one go. Most

digital slide images are stored as a tiled, multi-resolution image format where incremental image resolutions are stored as part of a pyramidal file structure (Fig. 3). Here, the lowest resolution representation is at the peak of the pyramid. As one moves down the pyramid, the resolution increases, broadening to the base of the pyramid, which has the highest resolution representation of the image. Each plane in the pyramid is represented by a set of discrete image tiles which can be selectively loaded into view. This means that with appropriate software the user can access a window on the image showing the appropriate resolution (magnification) and set of tiles (x–y position) at any point in the image pyramid, obviating the need to download the entire image. This effectively allows the image to be “streamed” to a viewer, under control of the user, where the image can be navigated, magnification changed and focus adjusted, only loading regions that are being viewed at the time. This is particularly important for web-based viewing of images. Here, dedicated image server software reads the image file requests coming from a remote user via their web-browser, retrieving the appropriate image data from file and serving that across the internet using web services to a remote location.

3.2. Image management: databases, administration and workflow

Most digital pathology applications require the storage, management and recall of many hundreds of digital slides. Appropriate database software is therefore necessary to manage these valuable digital resources, together with unique identifiers and associated metadata so that they can be archived, searched and retrieved for review, analysis and reporting. For example in research, it may be necessary for digital slides to be organized by study type,

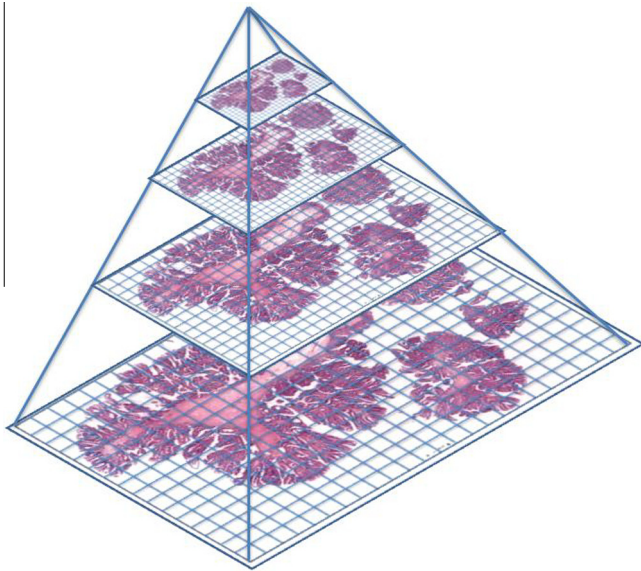


Fig. 3. Digital slide images are represented as a tiled pyramidal structure to allow on demand serving and viewing of images online, by serving image tiles at a specific locations and different resolutions.

label, tissue, trial number, experiment, etc. In addition, comprehensive resource database software in digital pathology permits user management and permissions to be set by an administrator facilitating restricted access to digital resources and functionality based on access rights. This allows for digital slides from numerous research studies or clinical trials to be stored within the database but where access can be selective, allowing certain centres, groups or individuals to view only certain slide sets. Only a small number of vendors provide such comprehensive database solutions for the effective management of digital slides and users, facilitating the integration of digital pathology within very specific workflows that can fast-track and speed up tissue-based research and discovery.

Using digital slides for research or clinical review also requires extensive recording of other metadata that may be associated with digital slides, e.g., unique identifiers for the slide image, bar codes, scan operator, date of scan, tissue type, biomarker name, grade, histological score, IHC score, clinical stage, treatment, survival, response to therapy and potentially molecular data such as expression array data, mutation analysis, next generation sequencing, etc. These data need to be stored in an integrated fashion linked to the image reference in a database so that images and associated data can be retrieved and analyzed together (see Section 8). User interface software is therefore necessary to provide access to the integrated dataset for search, review, analysis and reporting.

An integrated database also allows for the creation of workflow software that can drive certain processes in digital pathology. For example, having the ability to manage users, store slide identifiers, patient information and biomarker scores and present these via a user interface would be essential to streamlining large multicentre clinical trials. Furthermore, managing multiple patient samples in digital tissue microarray (TMA) biomarker experiments requires an underlying database management system and workflow software (see Section 3.3). These demonstrate the importance of having a strong, centralized database-driven management platform to support the wide range of applications in digital pathology.

3.3. Digital slide sharing for multisite collaboration and primary diagnostics

One of the generic benefits of digital pathology is the ability to share microscopic whole slide images between researchers and

pathologists electronically, avoiding the need to physically shift glass between centres. The pathologist no longer needs to be in the same room, building, organization or country as the glass slide. Given the escalating importance of pathology in personalized medicine and biomarker discovery, particularly in solid tumours, digital pathology is now allowing organizations to more efficiently manage their pathology services and support multisite integration and collaboration [10]. This provides organizations with the ability to either de-centralise or centralise their pathology services depending on their specific requirements and needs.

Organisations can now outsource pathology services by scanning slides centrally and distributing these to pathologists anywhere in the world for review, via web-based software. This has distinct advantages for commercial organizations, where pathology outsourcing is made easier and the cost of pathology services can be driven down to the cheapest supplier, since geographic location is no longer an issue. However, regulatory issues and quality remain key considerations. Remote access to digital slides also has advantages for research organizations involved in large multidisciplinary studies where collaboration with distant centres is necessary to access pathology skills.

Similarly, digital pathology can support centralization of pathology services, where scanning can take place across multiple geographic sites with pathologists reviewing these centrally. This can be particularly important in large-scale multinational clinical trials where centralized standardized pathological review is essential. Also important here is the ability to manage multiple scanning devices at each centre and to have software that can support multiple proprietary image formats generated by different scanners. In all of these scenarios, software is essential for digital slide management, the secure distribution of slides to remote pathologists, centralized review and reporting, and for tracking events during complex tissue-based studies.

Tissue microarrays (TMAs) represent an vital means of evaluating and scoring the clinical utility of new biomarkers. While image analysis has a role to play, many studies still rely on manual visual interpretation for candidate biomarkers. Using appropriate digital pathology software, complex TMA experiments can be managed and carried out remotely. This has clear advantages in accessing pathology skills outside an organization and the basic management of TMA experiments benefits from delivering this through a dedicated management interface (Fig. 4). Here, the software can lead the user through the TMA map, presenting each tissue core via a web-based viewer to the pathologists. Each core can be viewed at multiple magnifications and the biomarker score entered via a scoring interface by the user. The software keeps track of progress through the TMA and can relocate to any position on the TMA map. This demonstrates the utility of digital pathology in supporting tissue-based biomarker experiments when software management and workflow underpin the success of a study.

The use of digital pathology in clinical trials and for research-only applications does fall under regulatory governance in some areas of practice. In regulated research environments such as in the pharmaceutical industry, in preclinical toxicity testing and in clinical trials, Good Laboratory Practice and Good Clinical Practice guidelines needs to be observed. In the USA and Europe, these tend to concentrate on 21CFR11 or GMP Annex 11 compliancy, which determine best standards for handing access to clinical data.

As digital pathology develops, there has been expanding interest in adopting the technology for routine primary diagnostic tasks. Increased speed of scanning, high volume automated slide loaders and reduced cost of digital storage have made this a realistic possibility. In diagnostic laboratories, digital pathology has a number of clear benefits, including the ability to share images with peers anywhere in the world without shipping glass, being able to archive and retrieve slides conveniently for subsequent digital review, the

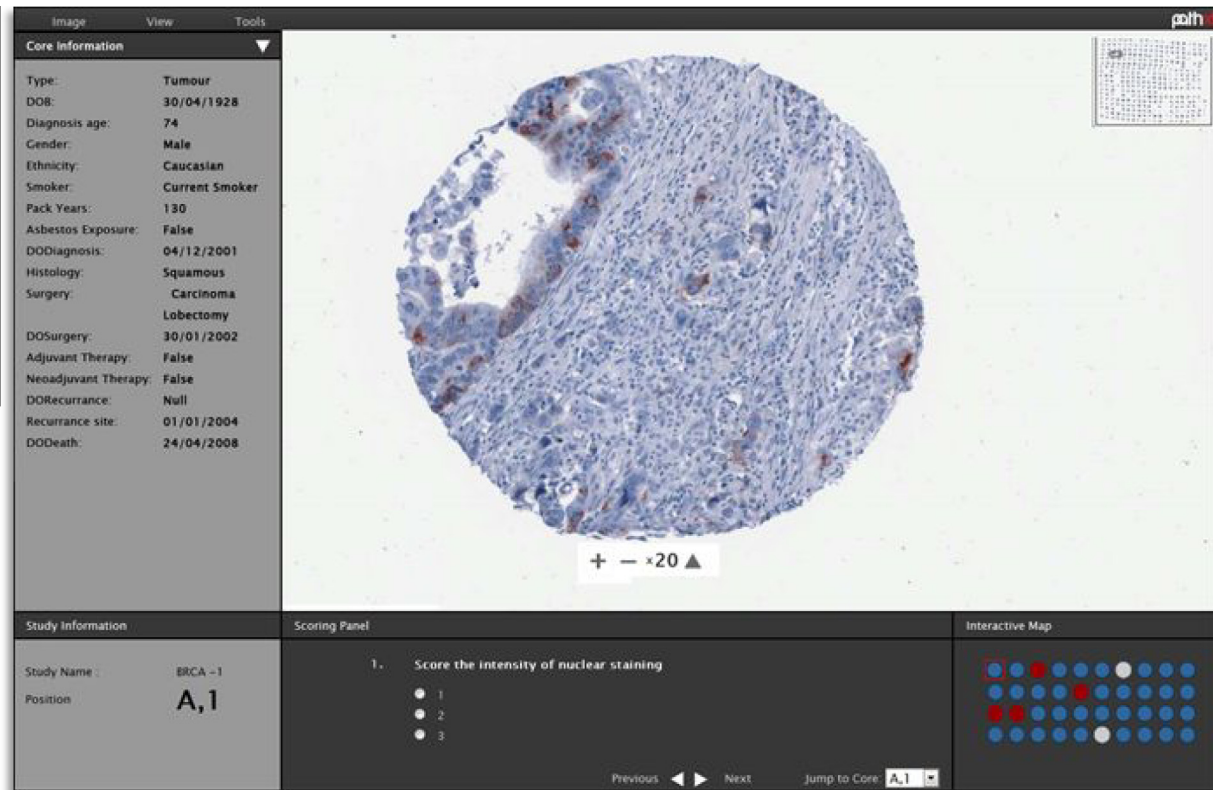


Fig. 4. The interface with PathXL's TMA Toolbox. This software allows web-based management of TMA experiments, scoring of tissue biomarkers and integration of scores and clinic-pathological data.

ability to get rapid pathological diagnostic opinions for intraoperative decision making (e.g., frozen sections), the option to outsource pathology expertise when expertise is not available locally and where the geographical location of the expert pathologist is more or less irrelevant, and the use of decision support tools and image analysis for improved diagnosis. However, concerns still exist over the safety of WSI and the use of digital slides for primary diagnosis. Over the past number of years, several small studies have shown that digital slides do provide a reliable medium for consistent primary diagnosis [11–14], however no large randomized controlled trials have been undertaken. Currently, in the USA, whole slide scanning devices have been categorized as class III devices by the FDA – indicating that safety/efficacy is not proven and further work needs to be done to prove the technology. This prohibits their use for primary diagnostic review, although use for second opinion and frozen section diagnostics is permitted. To date, Europe has been more open in its approach to digital pathology as a surrogate for the microscope in primary diagnosis and several labs are now moving ahead with plans to enable routine digital pathology, at least in some areas of primary diagnostic pathology [8]. In any setting, it is essential that laboratories validate and prove the safety of digital pathology internally before adoption for clinical purposes. Recently, the College of American Pathologists and Laboratory Quality Centre produced comprehensive guidelines on validating whole slide imaging in diagnostic pathology laboratories [15].

4. Image analysis: measuring pathology

It is well recognized that the visual interpretation of tissue structures, IHC and other pathological tissue characteristics using conventional microscopy is subjective. Many studies have shown inconsistency in diagnostic decision-making in pathology, poor reproducibility in grading disease and the variation that exists in

image interpretation [16,17]. Of course this does not at all imply that pathologists are bad at what they do, but rather it highlights the skills necessary to reliably make diagnostic decisions from complex images and identify consistent tissue and cellular patterns that can be used to classify disease in a way that is meaningful to clinical management. But it remains subjective.

Image analysis is a very widely used term to describe the computer-aided quantitative analysis of digital images (small, selected images as well as WSI) in order to extract numerical data on the underlying structures and intensities. This not only allows the measurement of the underlying biological characteristics and processes that would normally be visually assessed, but potentially allows the detection of subvisual characteristics, not discernible to the naked eye. As stated above, image analysis has been used for many decades to better understand the microscopic alterations that occur in disease and better characterize them through measurement. The key advantages offered by image analysis are (i) standardization through tissue measurement (2) automation and (3) improved productivity and efficiency.

Computers now play a major role in extracting quantitative data from digital images for the purpose of interpretation. This covers most applications from simple measurements of length or cell count to more complex approaches to measuring tissue structure using object identification and pattern recognition.

This section of the paper will review some of the key areas where image analysis has had a role to play in pathology and tissue-based research.

4.1. Measuring nuclear morphology, DNA content and augmented visualisation

Many studies have used image analysis to support the quantitative evaluation of nuclei as a key marker of diagnosis and prognosis

in cancer. These have focused on the measurement of (i) morphometric characteristics such as nuclear size and nuclear shape, (ii) densitometric characteristics such as nuclear density and chromatin disorganization and (iii) cell counts such as mitotic indices. Many of these studies originally relied on “semi-automated” methods using computer-aided drawing devices to trace H&E stained nuclei for size and shape measurements or to manually count objects on screen using a mouse and cursor. While still a very valuable approach to image analysis, the trend has been to develop more automated approaches to nuclear and cell identification, with the associated challenges of accuracy and the need for validation. A full range of cellular and cell count measurements can be extracted from pathological images, providing objective data to support a more objective diagnostic taxonomy. While morphometry has been used for years, it is still relevant today for studies which require more quantitative evaluation of tissue and objective identification of disease types.

For example, mitotic cell counts are an important criterion for the grading of several tumour types. In breast cancer such counts have been known for many years to provide prognostic information on clinical outcome, and several authors have advocated the use of a quantitative prognostic index using quantitative mitotic cells counts in combination with other cytometric features [18,19]. Baak et al. [19] proposed a simple mitotic activity index, in combination with tumour size and lymph node status, as a powerful index of prognosis and also as a means of identifying responders and non-responders to chemotherapy [20]. The role for mitotic cell counts continues in tumour pathology as does the drive to develop more automated image analysis-based approaches. In 2013, a standard image library of mitotic counts was used to test a range of mitotic identification algorithms in pathology, and identified that this still posed challenges in the image analysis community due to the difficulty of automated identification of mitotic figures [21].

The use of DNA image cytometry on cell preparations or tissue sections has been extensively explored in cancer. This uses digital imaging to measure the optical density of stained nuclei to identify and measure abnormal DNA content. Minor changes in DNA content can be identified in malignancy and have been advocated as an objective approach to support diagnostic decision making in pathology. DNA image cytometry has certain advantages over DNA flow cytometry, in that tissue organization and context is retained and specific focal lesions can be explored [22]. Work on the use of DNA image cytometry in identifying lesions, such as dysplasia in Barretts Oesophagus, has shown real advantages in predicting clinical outcome [23,24].

Closely allied to DNA content has been the use nuclear densitometric information to evaluate chromatin organization within cell nuclei. Again, subtle changes in chromatin disruption have been shown to be strongly associated with early malignancy in a range of tumour types, tumour progression, as a potential surrogate endpoint marker of clinical outcome [25] and are associated with methylation and acetylation status in cancer [26].

One concept emerging from these studies is that of “augmented visualization”, i.e., the ability to extract quantitative data from marked-up imagery and present these back to the pathologist visually. This can provide the pathologist with images that are much more readily interpretable than standard H&E or IHC alone, providing them with the ability to “see” tissue characteristics that would normally escape the naked eye. Fig. 5 shows an example of this in prostate neoplasia, where image analysis of chromatin disorder can help identify prostate intraepithelial neoplasia.

4.2. Measuring tissue architecture

In histopathological diagnosis, considerable information is contained within the tissue architecture and the disruption that occurs

in diseased tissues. As with cellular analysis, image analysis can be used to measure architectural changes in H&E stained samples in much more precise, reproducible terms. Initially, analysis was confined to manual measurements derived by using computer software to hand-trace tissue structures. However, with advances in image analysis and algorithms to automatically interpret complex patterns, more advanced methods have been proposed. These approaches using “computer vision” have been extensively reported and are based on image understanding methods that model the complex structures seen in tissue images and reconstruct those to identify tissue compartments that can subsequently be measured [27,28]. Some examples are given in Fig. 6 and Fig. 7.

Our ability to develop image understanding solutions in pathology is going to be a significant future challenge in digital pathology and image analysis. It also forms the basis of tumour tissue identification discussed later (Section 4.8). Implementing decision support solutions that can be used within diagnostic practice and augmented visualization in pathology will require increasingly complex imaging tasks that can decipher the content of the image and return valuable new data. While the promise has been there for many years, the technology is getting close to the point where this could be a reality. This will require pathologists, computer scientists and image analysts to combine their skills to derive new innovative technologies that will drive machine vision and image understanding in tissue pathology. While there remains considerable doubt about whether this can be achieved with sufficient accuracy, the potential of this in diagnostic practice could be enormous.

4.3. Quantitative immunohistochemistry (IHC)

Cellular proteins and the use of immunohistochemistry (IHC) are extremely important as tools to identify disease-related single biomarkers which can be used to stratify patients into clinically important groups. These may be potential biomarker candidates under investigation or may have already been shown to be of diagnostic/predictive value and are evaluated in routine diagnostic practice by pathologists. Due to its widespread availability in diagnostic laboratories across the world, IHC represents a very popular target technology for new biomarker development in patient stratification and personalized medicine.

There is one key drawback of IHC for biomarker assessment. The visual interpretation of chromogenic IHC expression is inherently subjective and prone to error, even when carried out by experienced pathologists. Studies on a range of IHC markers including p53 [29] (McShane et al.), ER and PR [30] and Her2 [31] have shown poor reproducibility between pathologists. Recent studies on Ki67 highlight considerable interlaboratory variation in Ki67 evaluation [32,33], a tissue biomarker that is critically important in breast cancer research and clinical assessment of patients. Most tissue-based IHC markers that are approved for clinical use, come with well-defined guidelines on sample preparation, pathological interpretation and scoring. Nevertheless, the evaluations of markers such as Her2 IHC are known to be associated with error rates of up to 20% [34]. Her2 evaluation in other settings such as gastric cancer is also problematic [35].

Computerized image analysis provides the ability to use the quantitative data inherent in a digital image to extract numerical data on the density and distribution of IHC biomarker expression within a tissue sample. Used appropriately, IA can provide important objectivity and repeatability in biomarker studies, allowing the identification of subtle changes in biomarker expression that could have diagnostic, prognostic or predictive value. A variety of image analysis toolboxes and general purpose algorithms are commercially available which allow the quantitative measurement of IHC markers. Few image analysis algorithms work “off the shelf”

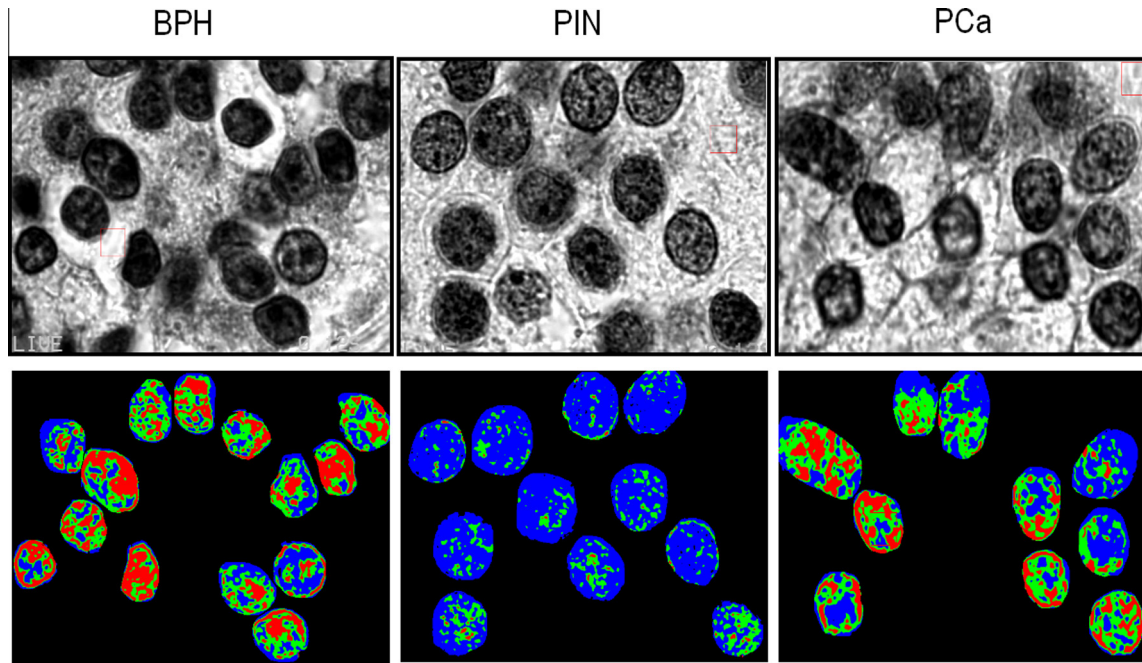


Fig. 5. Augmented visualization in prostate cell imaging, comparing benign prostatic hyperplasia (BPH) prostatic intraepithelial neoplasia (PIN) and prostate cancer (PCa). Measuring the chromatin distribution quantitatively can be used to present colour coded nuclei indicating levels of chromatin disruption in prostate epithelial cells and help distinguish PIN from similar benign and invasive counterparts.

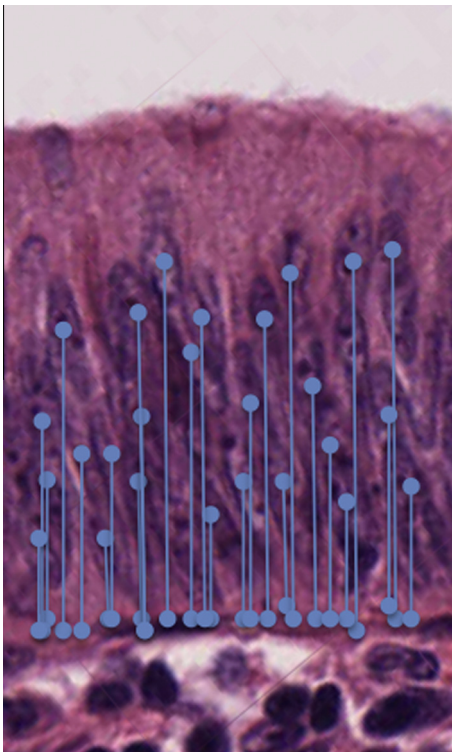


Fig. 6. Shows automated measurement of nuclear stratification in colorectal epithelium using tissue-based machine vision.

and there is invariably the need to configure algorithms for the specific tissue type and biomarker under investigation. In experienced hands, image analysis algorithms can be developed which can reliably segment cellular compartments and extract quantitative data of enormous value. However, in inexperienced hands, image analysis can be very dangerous.

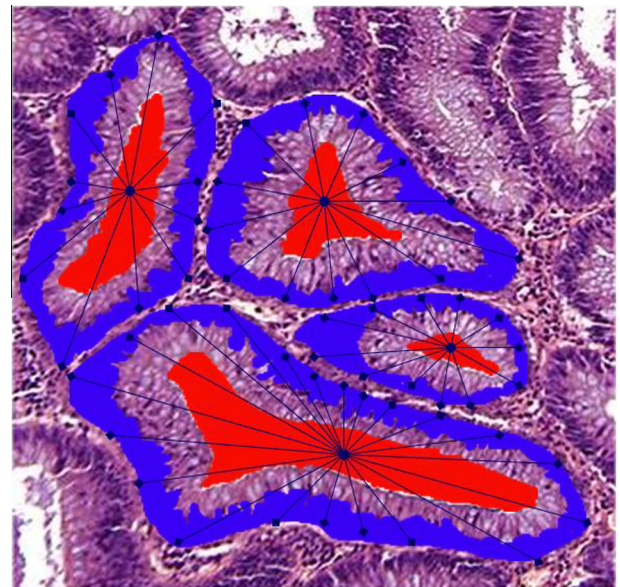


Fig. 7. Using image analysis and computer vision it is possible to measure tissue and cellular organization. This figure shows the measurement of gland proximity, shape and nuclear crowding for determining the grade of colorectal dysplasia.

Generally the research approach is to build a solution in a commercial package by adding together various image processing components that in combination can provide data that works on the biomarker of interest and, following validation studies, provides reliable data. Validation is extremely important and will inevitably involve review by experienced pathologists to ensure appropriate regions of the images are being identified and that density thresholds are set appropriately. When used appropriately, IA algorithms can then be effectively used to identify cellular compartments and measure the protein expression within these compartments (Fig. 8). A range of studies have successfully used image analysis to measure IHC across numerous biomarkers. This has been

empirically shown to improve the reliability and reproducibility of IHC scoring.

Most commercial systems provide general purpose image analysis development platforms that allow algorithms to be constructed for any candidate biomarker and where the development and validation is in the hands of the user. However, there are an emerging number of target-specific algorithms that have been commercially tested and validated for a specific purpose, and under controlled conditions are aimed to operate without any user configuration. For example, several companies (e.g., Aperio/Leica), Ventana Roche) have FDA 510 k clearance for use of specific ER, PR, Her2 and Ki67 algorithms which can be operated by laboratories under defined conditions. These algorithms are generally aligned to specific scanning instruments and must be used exactly as specified by the manufacturer in order to be compliant. They are now used routinely in many centers to measure IHC expression in diagnostic pathology material. A good example of this Her2 IHC, which acts as the companion diagnostic marker for herceptin therapy in breast cancer. While this represents an important tool for patient stratification, it has been widely reported that at least 20% of Her2 evaluations by pathologists could be inaccurate [34]. Bespoke image analysis algorithms that can more precisely measure Her2 IHC in tissue sections and Her2 image analysis has been extensively validated by some laboratories. This has undergone FDA 510 k clearance in USA and also equivalent CE Marking in Europe for clinical use when restricted to certain IHC antibodies and specific scanning platforms. Major organisations such as the American Society of Clinical Oncology and College of American Pathologists have recommended the use of Her2 IHC image analysis, provided it undergoes internal laboratory validation [34]. Similar FDA/MHRA approval has been given for the clinical use of ER, PR and Ki67 image analysis, again as part of breast cancer profiling and selective patient therapy. These and other tissue imaging algorithms are going to be key to the effective development of new markers and the translation of these markers into clinical practice and illustrate the pace at which digital pathology and image analysis is being adopted.

4.4. Tissue microarray analysis

One key area where image analysis can significantly enhance automation and efficiency is in tissue microarray (TMA) analysis. TMAs represent an extremely effective platform for evaluating

the clinical utility of new tissue and cellular biomarkers across many hundreds of patient samples in a single assay. Conventionally each sample needs to be separately scored by eye to determine the expression of a given biomarker. In large studies full TMA datasets which could have many hundreds of tissue samples and that numerous biomarkers need to be evaluated, this soon becomes impractical to do manually. Using IA, TMAs can be analyzed in a completely automated fashion and this is emerging as an extremely important method in biomarker research to fast-track discovery, streamline workflow and produce objective, reproducible biomarker data. Here image analysis automatically identifies the individual tissue cores within the image, registers them to a TMA map so that co-location is retained, analyses each tissue core using a defined algorithm and generates objective data on expression status within that tissue core. This provides quantitative results on each of the hundreds of tissue cores on a slide and maps these to each patient and their clinicopathological data. This is enhanced when combined with high performance computing [36,37]. By parallelising analysis of individual TMAs across multiple computer cores, the complex analysis of multiple biomarkers across large multiplexed TMA experiments [37] is significantly speeded up and can allow for genuine high throughput experimentation in drug/companion diagnostic discovery. For diagnostic companies wishing to validate candidate tissue biomarkers for patient stratification across large cohorts of patients, high throughput image analysis on TMAs is going to become a priority.

4.5. Tumour heterogeneity

Interestingly, in addition to average biomarker expression in tissues, tumour heterogeneity is emerging as an important contributor to pathological misinterpretation and as a clinical indicator of response to treatment. For example, Her2 heterogeneity in breast cancer may account for the 30% of patients that fail to respond to, or acquire resistance to herceptin therapy. However, visually assessing the degree of heterogeneity in tissue samples is visually challenging and compounds the difficulties of objectively scoring the mean intensity of Her2 IHC protein expression by eye. Image analysis provides the perfect tool to better measure spatial heterogeneity of biomarker expression within a tissue sample. By measuring IHC expression on a cellular level across the slide or by gridding the tumour into distinct compartments, spatial heterogeneity can be visualized by color coding and mapping IHC image

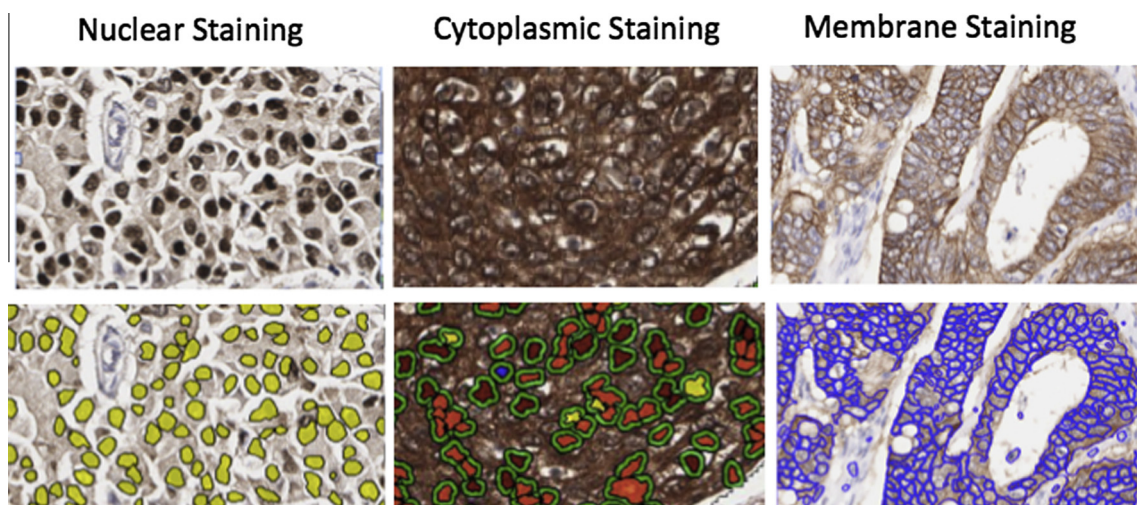


Fig. 8. Image analysis for the quantitative measurement of IHC positivity in the nucleus, cytoplasm and cell membrane. The top panel shows the original images. The lower panels show the image analysis detection and markup of positive regions (Definiens Tissuestudio).

analysis data across the tissue image or by measuring the variation in IHC expression across the defined tissue compartments. Some have developed algorithms, previously used for measuring ecological heterogeneity, to assess IHC expression heterogeneity in breast cancer. It may be that measuring heterogeneity of Her2 expression in breast cancer using IA could act as a more efficient means of stratifying and selecting patients for herceptin therapy [38].

4.6. Fluorescence imaging in digital pathology

4.6.1. Advantages of fluorescence over chromogenic dyes

Chromogenic stains gained popularity for immunodiagnostics when the primary interest has been in assessing the presence or absence of the stain, alongside visualization of tissue structure [39]. However as attention shifts to the quantification of expression as an aid to predicting therapeutic response, it has proven to be extremely difficult to visually distinguish between even a small number of discrete levels of staining intensity – such as low, medium and high; an issue that is further complicated by variations among laboratories, slide scanners and computer monitors. While objective measurement of pixel intensities by automated image analysis as described above offers a partial solution, it should not be considered a panacea; for example, widely-used DAB-based stains are known to have differing spectral properties at high and low intensities [40], which reduces the ability of computational techniques such as colour deconvolution to recover accurate stain intensities from red, green and blue channel data in a general sense [41], while further uncertainty arises from the fact that the dynamic ranges of such stains remains poor relative to the range of protein concentrations found *in vivo* [40].

One approach to overcome these issues is to use fluorescence imaging. Fluorescence images are formed from the detection of photons emitted from fluorochromes/fluorescent molecules within a specimen, whereby there is expected to be a linear relationship between pixel values and the number of photons that struck the detector. By matching filters to the emission spectra of the fluorophores being used, it is largely possible to ensure that the information contained within individual channels in the image already corresponds to the markers of interest, without a need for stain separation. In cases where this has not been possible and there is a significant bleedthrough between channels ('crosstalk'), spectral deconvolution or linear unmixing may be applied to correct for these errors [42]. Fluorescence imaging is also more suited to 'multiplexing', i.e., the assessment of multiple markers in a single image [39,43].

4.6.2. Considerations for acquisition and interpretation

While more quantitative than chromogenic dyes, when it comes to interpreting fluorescence data it should be kept in mind that pixel values themselves are in arbitrary units, dependent not only on the number of photons emitted by the sample (a function of fluorophore concentration, illumination intensity and exposure time), but also on many other factors, such as gain settings and conversion factors inherent to the acquisition hardware [44]. Consequently, drawing valid conclusions based on absolute pixel values depends on a detailed understanding not only of the physical and spectral properties of the specific fluorochromes/fluorescent molecules under consideration, but also of the imaging process and settings [44,45]. In many cases in which intensity measurements are deemed important, a more practicable approach is to reduce variation as far as possible by standardizing the experimental setup and acquiring images with identical settings, and then comparing only differences in relative intensities [45]. However even here, care must be taken during acquisition and image storage to ensure that the linearity between detected photons and pixel values is not compromised. One major danger is data

'clipping', which occurs when the 'true' pixel value derived from the detected photons exceeds the range permitted by the image bit-depth (0–255 for an 8-bit image, 0–4095 for a 12-bit image), so that the image contains only the closest valid value. It may not be possible to discern visually that clipping has occurred from looking at the image alone, but it is evident in the form of peaks at either extreme when viewing the image histogram. Solutions to overcome clipping include reacquiring the data with an increased image bit-depth (at a cost of larger file sizes), or having reduced the exposure time or microscope gain setting. The second main pitfall is the use of a lossy compression method (e.g., JPEG) when saving the acquired image, which will also modify pixel values and thereby influence intensities, in addition to introducing artifacts that may prohibit the detection of small structures [46]. Because fluorescence signals fade over time, digital archiving – rather than storage of the physical slide only for later re-scanning – is needed if the data might be re-evaluated at a later date [47], but storing the raw, uncompressed data can be problematic for whole slide scanning if this produces prohibitively large file sizes. Nevertheless, some reduction can be possible by discarding unused channels, using lossless compression (e.g., LZW, lossless JPEG 2000), or storing only cropped regions of the entire dataset.

4.6.3. Fluorescence *in situ* hybridisation

Currently, the primary use of fluorescence in pathology is in fluorescence *in situ* hybridization (FISH), which provides a view of gene copy number rather than protein expression as normally measured by IHC [48]. Although considered more time consuming and costly, FISH has been shown to be a better predictor of trastuzumab response than traditional IHC scoring in retrospective reports looking at breast cancer, and has therefore been recommended for the evaluation of HER2 status in all cases when IHC scoring has been deemed equivocal [48,49]. A rapid screening approach to FISH analysis using tissue microarrays has been proposed to improve throughput and reduce cost [50], while analysis time may also be reduced by the integration of automated nucleus identification and spot counting [51,52].

4.6.4. Fluorescence algorithms generally

As fluorescence slide scanners become more widely available and file storage costs decrease, the advantages of fluorescence data in terms of quantitation and multiplexing make it likely to expand more into areas traditionally served by brightfield imaging. This raises new challenges, in that specially-adapted image analysis algorithms are required to deal with the differences in information content within the images, in particular the unavailability of some of the textural or contextual features familiar from IHC or H&E stains. Recent studies have begun to address this by looking at the identification of tumour regions based only on features and relationships calculated from the cell nuclei detected in the DAPI channel of fluorescence images [53,54], or registering images of tissue sections imaged first with fluorescent markers and subsequently with H&E dyes [55].

Exploiting the additional possibilities of fluorescence in these applications has perhaps been demonstrated most fully by the AQUA (Automated Quantitative Analysis) set of algorithms [56] [18]. The primary distinguishing feature of this approach is the use of fluorescence data to produce an 'AQUA score', which is a measure of co-localization calculated by dividing the signal intensity in the channel corresponding to the marker of interest by the compartment area (e.g., nucleus, cytoplasm or plasma membrane) [57] [58]. Because this provides a continuous measurement of a biomarker that makes use of intensity information relating to expression, it becomes possible to identify potential subpopulations amidst the data that would not be captured by a coarse-grained classification of 'low' or 'high' staining, and provide a

new viewpoint on biomarkers previously analyzed by standard immunohistochemical evaluation [57–60].

4.7. Quantitative biomarker discovery and stratified medicine

Stratified medicine is central to the delivery of personalized therapies in cancer and other diseases. “Stratification” is the process of (i) identifying and separating different variants of a disease into new, previously unrecognized, sub-types, (ii) developing new therapies for these new patient subtypes and (iii) showing improved clinical outcomes for patients identified and treated in this more personalized format. Most of the drive towards personalized medicine has been underpinned by our better understanding of disease at a molecular level. By identifying mutations and their impact on molecular pathways and disease development, it is possible to develop new antibodies or small molecule inhibitors that can target these anomalies. However, the development of new drugs for this purpose requires the concurrent development of “companion biomarkers” that can identify which patients will benefit from the new drug. The drug discovery/development pipeline must therefore be mirrored by an associated biomarker discovery/development pipeline (Fig. 9) and this is presenting enormous challenges to the pharmaceutical and diagnostics industries.

Digital pathology has very significant roles to play that map across this drug/biomarker pipeline, particularly in developing new drug/biomarker combinations for solid tumours. These are illustrated in Fig. 9. This includes the role that digital pathology is playing in biobanks (Section 4.9), analysis of biomarkers in TMAs (Section 4.4) and the development of companion image analysis algorithms. Companion image analysis algorithm development represents a significant area of investigation and is used widely in the identification and validation of new tissue biomarkers. Here image analysis allow researchers to more precisely determine the clinical utility of protein expression or ISH markers. This is essential in driving down costs associated with drug/diagnostic development, in that candidate markers can be ruled in/or ruled out more efficiently. Once developed for use in research, the potential then exists to extend the application of imaging for the routine evaluation of clinically valuable IHC or ISH markers. Again, a good example of where this is happening is in HER2 IHC, where commercially available FDA/MHRA approved algorithms can be used for routine decision support and allowing for more accurate selection of patients for herceptin therapy. We expect to see many more companion algorithms being developed for use in cancer and in other diseases which provide more precise data on patient phenotype/genotype and their selection for targeted therapy.

4.8. Automated tumour detection and molecular pathology

Molecular pathology relies on identifying gene-specific anomalies using PCR or sequence-based analysis of DNA or RNA extracted from tissues. This is becoming extremely important in solid tumours where molecular stratification of patients can be used to determine effective treatment and improve patient outcomes. Examples include EGFR mutations and ALK fusions in lung cancer, KRAS and NRAS mutations in advanced colorectal cancer, and BRAF mutations in malignant melanoma.

While these biomarkers are “molecular”, they still strongly rely on pathological examination of solid tumour tissue samples, annotation for possible tumour cell enrichment and the estimation of % tumour to ensure sample adequacy. Two aspects are important here: (i) the percentage of tumour tissue as a fraction of the entire sample and (ii) the percentage of actual tumour cells within the diseased tissue, which invariably consists of a mixture of cell types. Since the latter is the ultimate determinant of the tumour DNA yield, evaluating the fraction of tumour cellularity is important in

ensuring the accuracy of molecular test results. However, assessing this visually can be highly subjective and poorly reproducible. For example, Smits et al. [61] have shown in H&E stained lung tumour samples that there is gross variation in how pathologists report tumour cell percentage in the same samples. Only 14% of the observations were considered correct, with tumour cell percentage being overestimated in 45% of cases. Differences between pathologists were statistically significant and could be as high as 40 points on the % scale for the same sample. Over a third of pathological reviews deviated from the actual tumour cell count by as much as 20 points on the % scale. Most importantly, of the samples that actually fell below the crucial 20% threshold for direct sequencing, over one third were overestimated by pathologists, raising the likelihood of false negative EGFR test results in these cases and subsequent serious consequences for patient treatment. Viray et al. [62] have demonstrated similar findings in a multi-institutional diagnostic trial on colorectal cancer and KRAS testing. These studies emphasize the difficulty of reliably estimating tumour cell percentage by eye and the complexities involved in subjective morphological interpretation³. These issues are not just restricted to routine molecular diagnostics, where misdiagnosis may result in treatment failures and litigation. They also highlight the potential errors that may arise in the use of nucleic acid-based diagnostic test from tissue samples in research and clinical trials. Improvements are necessary in the quality, standardization and automation of tumour cell measurement and macrodissection. Without this, discovery, validation and clinical trials of new companion diagnostics may be flawed.

Recent technology is addressing this by using image analysis for automated tumour markup and % cell calculations, automating this process and providing more objective, reliable measurements for tumour cell content (Fig. 10). The digital annotation can then be used by the laboratory technician to macrodissect tissue samples. By developing bespoke algorithms for each individual tumour type and building this within a digital slide management workflow this approach can now be used to support busy molecular pathology laboratories and research organizations undertaking large clinical biomarker trials that require tumour % thresholds or macrodissection for tumour cell enrichment.

4.9. Digital pathology and image analysis in biobanking

Biobanks aim to collect high quality biological samples linked to well defined data sets and as a resource provide the critical bridge to support translational research across a range of diseases. Modern biobanks provide the necessary tissue samples to develop new companion diagnostic markers and are therefore essential in translational medicine and the development of personalized therapies [63] (Hewitt 2011).

In tissue centric biobanks, digital pathology has an emerging role in sample archiving and sample retrieval. This ability to (i) archive digital slides associated with physically stored tissue samples, (ii) retrieve these for rapid digital review ahead of sample retrieval and (iii) to enrich the information associated with stored samples through tissue and biomarker analysis is becoming central to modern biobanking. Biobanks are also increasingly the guardians of TMA constructs, which can be generated from the millions of retrospective samples available in pathology laboratories across the world. The integration of digital pathology imagery with physical samples and their linked clinical and pathological data represents the perfect example of how data integration is essential to discovery [63].

As an example, the Northern Ireland Biobank routinely scans and digitally stores all H&E and biomarker slides stored within the biobank programme. Focusing on the prospective collection samples across six cancer types, this currently equates to

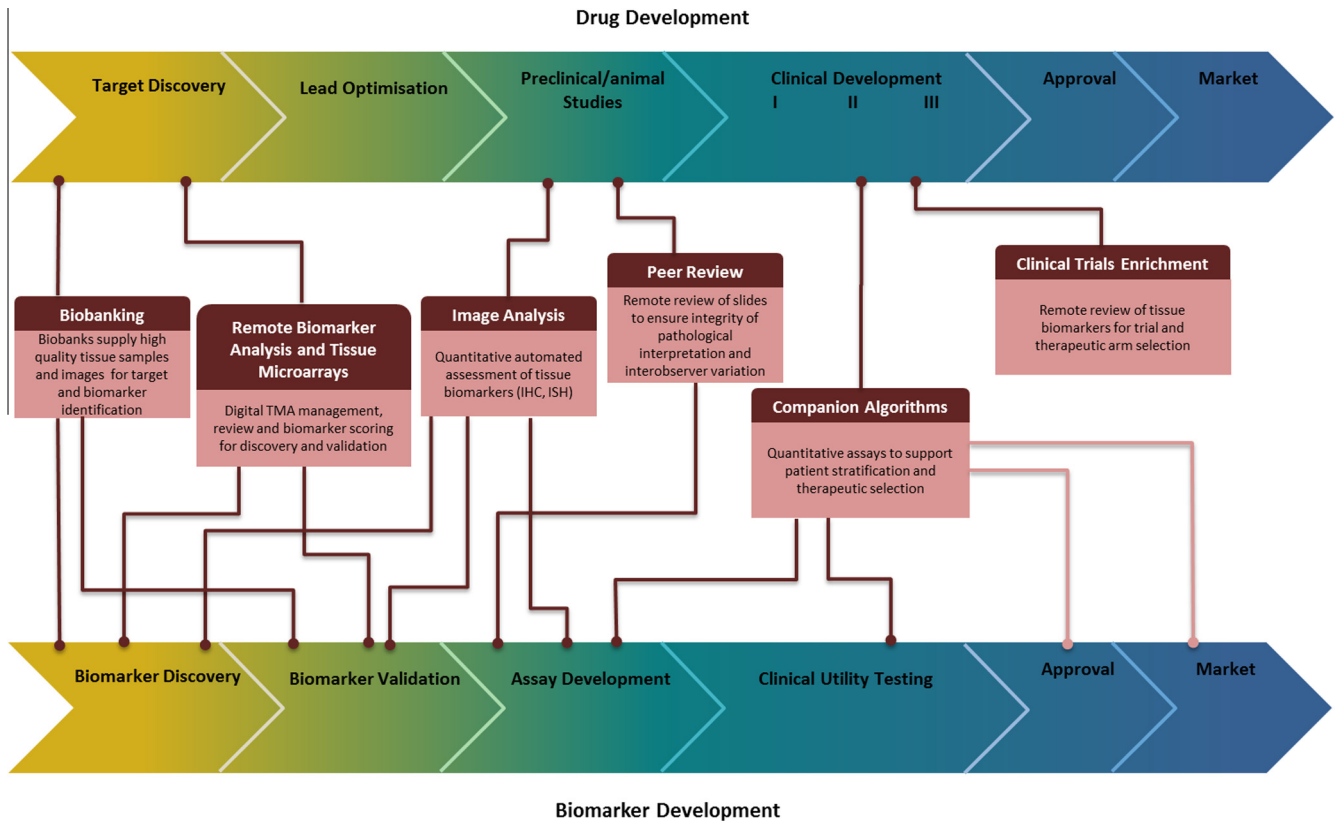


Fig. 9. The role of digital pathology in drug development and companion biomarker discovery and validation.

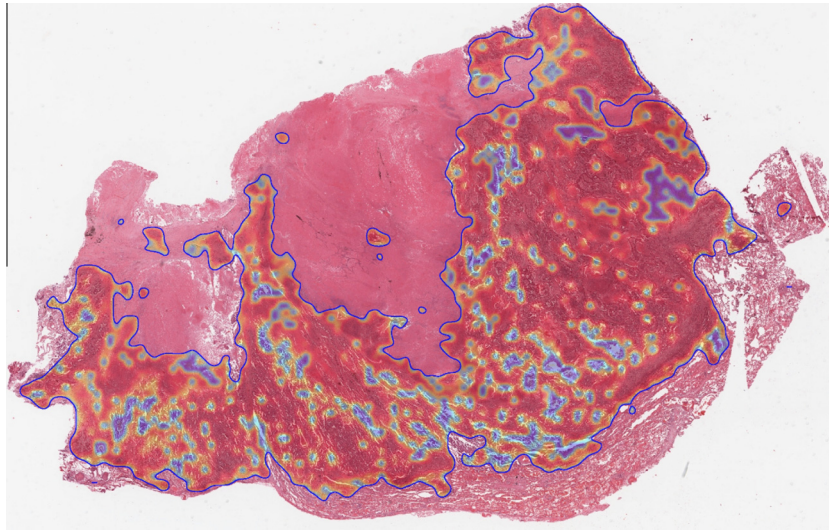


Fig. 10. Automated annotation of tumour tissue in lung cancer using PathXL TissueMark software. This uses pattern recognition image analysis and here shows the tumour boundary and “heat-map” of tumour rich areas. This approach enables rapid and reliable calculation of tumour area in real units, but also as a proportion of the entire tissue section. It also quantitatively calculates the % tumour within the annotated region.

approximately 3000 scanned slides and 2 Tb storage per annum. Hosted on an secure imageserver (PathXL Ltd), this allows researchers to review tissue histology and biomarker expression ahead of sample retrieval and provides researchers with a pre-existing rich source of image related data for their subsequent work. In addition, the automated image analysis of biomarkers within biobanking further enriches the data available to researchers. In the future, biobanks will increasingly host analytical data derived from the tissue specimens including single biomarker assays such as IHC and multivariate signatures derived from next

generation sequencing. Digital pathology and pathology informatics software will be the enabling technology that supports these developments and will put biobanks at the centre of developments in personalized medicine.

5. Pre-analytical variables and controlling variation

While tissue measurement provides objective data on underlying morphology, IHC expression or FISH signals, the reliability of these data is in turn reliant on a range of processes that the tissue

sample is subjected to prior to analysis. For example, it has been shown that delay in tissue fixation can have a significant impact on the IHC expression of certain proteins. Reduction in progesterone receptor (PR) and oestrogen receptor (ER) expression by IHC and HER2 FISH can be seen with delays in fixation >1 h [64,65], with delays of >12 h having a major impact on expression status (and therapeutic decision making) [64]. Current guidelines for ER and PR IHC from American Society of Clinical Oncology/College of American Pathologists recommend fixation within 1 h [34] – but this is not always achieved in routine labs. The use of archival tissue samples by research labs, therefore potentially introduces a background variation in expression which is not due to the underlying biology of the tissue, but which is due to how it was handled prior to analysis.

Delay in fixation is just one factor that can potentially influence biomarker expression. These sample processing steps are called **pre-analytical variables** and Table 1 shows a list of candidate variables, many of which are understudied and where the real impact on analysis is largely unknown.

Controlling these factors within strict limits in a busy diagnostic laboratory which is receiving samples from the operating room or theatre without well-defined retrieval, delivery and handling protocols will be difficult. Biobanks on the other hand are established with the sole aim of controlling many of these variables through the prospective collection of tissue samples under strict standard operating and quality procedures. The role of digital pathology in biobanking has been discussed already in Section 4.9. But reducing the noise associated with pre-analytical variables will also improve the ability of image analysis to detect subtle, subvisual variations in quantitative IHC or immunofluorescence – variations which may be biologically or clinically important, and which would otherwise be missed. The importance of this in TMA research has been highlighted by Ilyas et al. [66].

An additional benefit of image analysis is that it can also be used effectively to measure the variation introduced by pre-analytical variables. As an example, Bai et al. [67] demonstrated a significant impact of cold ischaemic time on antigenicity of a variety of biomarkers by comparing matched biopsies (fast) and resection (slow) specimens and using the AQUA fluorescence imaging technology. Subsequently, Neumeister et al. [68] showed how image analysis of fluorescently labelled breast cancer panel on specifically prepared TMAs, could measure marker antigenicity as a function of time to fixation. While the core set of breast cancer markers (i.e., ER, PR, HER2 and Ki67) were relatively robust over short selective delays in fixation, others were more susceptible. Further work by this group has attempted to define a tissue quality index (TQI), based on the use of image analysis to precisely measure the expression of a chosen series of baseline biomarkers in breast cancer [69]. By quantitatively measuring a select set of variables known to be positively and negatively associated with time, new samples can be quality assessed by the ratio of these

measurements. This represents a potential step forward in the use of image analysis for quality assessment in biobanking and retrospective sample cohorts which rely on tissue quality for scientific investigation.

6. Setting up a digital pathology core facility

Digital Pathology equipment and software are expensive, and most organizations cannot afford to replicate resources across multiple groups. For this reason, most digital pathology facilities, in both private and public organizations, are centralized into “core facilities” that are available to a wide range of research programmes.

There are a number of considerations necessary when setting up core facilities. These are listed in Table 2 and discussed below.

6.1. Equipment

Hardware: As indicated previously, there are a variety of scanning systems currently available on the market. Depending on workload and available staff, a decision needs to be made on the sophistication of the scanner required. Lower throughput laboratories will be able to manage with cheaper low volume scanners, while larger laboratories will require high throughput systems. The latter also inevitably comes with staffing requirements. While high throughput systems are generally described as automated, there remains the need to stack the slide trays, manage issues that may arise during the scanning process, coordinate the large numbers of digital images that are being generated on such systems and quality control the results. Some systems can provide integrated functions within a single system, such as multilevel scanning and fluorescence. Other manufacturers separate functions such as brightfield and fluorescence into different dedicated systems meaning that if both functions are required it is necessary to purchase two separate systems. Other functions such as oil scanning are very specialist and only offered by a small number of vendors. Many fully equipped DP laboratories have several scanning systems to cope with workload and varied applications, often from different vendors.

Software: As discussed in Section 3, selection of appropriate software is extremely important in running a digital pathology laboratory. Two considerations are important when choosing software. The first relates to the specific management and workflow requirements of the laboratory. It is essential that the software supports the workflow that laboratories operate – whether that is for simple archiving, sharing or image analysis – and ensures the secure, reliable and trackable management of digital slides. If multiple scanners are in operation or where collaboration with other centres is required, having software that can natively read multiple image formats is important. Also, if possible the software should be 21CFR11 (FDA) or GMP Annex 11 (EU) compliant. These regulatory guidelines define the standards for commercial software in regulated environments including security, validation, data integrity, electronic signatures and audit, all of which are important in healthcare-related research.

Storage: Given the size of scanned digital images, it is important to consider at the outset the storage requirements for the laboratory. This can be expensive. Purchasing hardware for a lab requires a scalable solution that can be added to over time. Small research laboratories or biobanks might only scan 10 slides daily, equating to approx. 3.5 Gb per day, 910 Gb per annum. Other large diagnostic labs (discussed earlier) may generate 400 slides per day, generating 175 GB per day and over 5 TB annually. It also inevitably requires internal IT manpower to manage the hardware, support software and coordinate backup. This can prove to be expensive

Table 1

Selection of pre-analytical variables that potentially impact on the measurement of tissue morphology and molecular markers (modified from [65]).

Pre-analytical variables
Warm ischaemia time
Time to fixation (warm and cold ischaemia times)
Fixation concentrations
Fixation pH
Fixation times
Size of sample
Dehydration and cleaning times
Section thickness
Temperature and duration of slide drying
Temperature and duration of paraffin block storage

Table 2
Checklist for establishing a digital pathology core facility.

Equipment
Scanning hardware selection
Digital pathology image management software
Image analysis software
Image storage including backup
Servers and network
Service contracts
People
Dedicated trained technical staff
Ethics, anonymisation and legal requirements
SOPS
Evidence of ethical approval
Slide tracking paperwork/software
Slide storage

over time. Alternative solutions include cloud based services which allow all of the storage, management and backup to be outsourced to a specialist provider.

Servers and Network: Most laboratories require the need to move digital data between the scanner, internal servers or cloud-based storage systems. This requires networks that have sufficient bandwidth to manage large data transfers without enormous delays. Most universities can manage such data transfer rates, but on occasions specialist provisions need to be made such as dedicated, protected high bandwidth lines to the service provider backbone. Many health care providers have much more limited bandwidth provisions and this can pose significant problems when serving images into and out of the institution.

6.2. People

Proper training of staff is essential in running a DP core facility. The processes involved in scanning are relatively straightforward, but troubleshooting, ensuring proper functioning of the instruments and digital image quality is important. More challenging applications, such as fluorescence or oil scanning, demand a higher level of skill and experience. In our view, having a dedicated digital pathology technician to look after instrumentation, slide handling, digital file management, SOPs and paperwork has been essential.

The integration of image analysis within a digital pathology program brings its own considerable requirements. This tends to require individuals with specific skills and interests to develop and deliver image analysis solutions, and to manage the data generated from such studies. If more ambitious goals are being set, in which bespoke algorithms need to be developed for specific problems, it is often necessary to recruit computational scientists who can write software and develop more complex solutions independently or by using existing image analysis development platforms.

6.3. Ethics, anonymisation and legal requirements

A key consideration in offering a digital pathology service is to ensure that all appropriate SOPs and protocols are in place to manage human tissue samples and that regional/national laws are adhered to. In the UK, the Human Tissue Authority (HTA) has strict regulations on handling, tracking and carrying out research on human tissue samples. In the USA, Department of Health and Human Services, HIPAA, CLIA and CAP oversee activities research and clinical activities on human tissues. Ethical approval and appropriate anonymisation of patients data is essential and should be observed for core facilities undertaking to scan slides. Important to this is removing any “identifiable” labels that might allow identification of patients. Most scanners automatically capture an image of the slide label, which is stored with the slide. Care needs to be taken that for the purposes of research, patients cannot be

identified from these labels by the end user. In clinical applications of digital pathology it is essential that the pathologist can read the slide labels clearly. Most DP scanners can switch off the slide label function so that that information is not captured and this is recommended in cases where the patient name is included in the barcode label.

It is important that slides and associated digital slide images can be appropriately tracked within a slide scanning service operation – and this is often a legal requirement (e.g., CLIA, HIPAA, CPA, HTA). For small operations, this can usually be handled by using paper-based methods or basic workflow software. For more substantial laboratories where numerous slides need to be processed, scanned, catalogued, stored and returned. This can be facilitated by bar code systems with automatic reading of bar codes by digital pathology scanners and image file creation and archiving.

7. Integromics and image analysis

Digital pathology and the data derived from image analysis only represents one facet of sample/patient characterization. In reality, biomarker image analysis is valueless on its own without having associated high quality clinical and pathological data against which their diagnostic, prognostic or predictive power can be evaluated. Similarly, the ability to integrate IA data with other diverse analytical data such as genomic and other tissue sample derived “omics” datasets will be essential for discovery, translational medicine and targeted therapies. Unfortunately in most centres, these data exist in different silos and the integration necessary to make full use of these diverse datasets is challenging.

Converging imaging and molecular data allows for the combined analysis of the relationship between phenotype and genotype. Data reduction on the basis of the genetic profiling alone will allow for a clear interpretation of immunohistochemistry markers in tissue-based digital pathology research allowing insight into aberrant expression at the protein level. This, coupled to DNA methylation and mRNA expression patterns, will proffer a more reliable identification of robust biomarkers for potential therapeutic targeting. Big-data approaches allow for a holistic interpretation

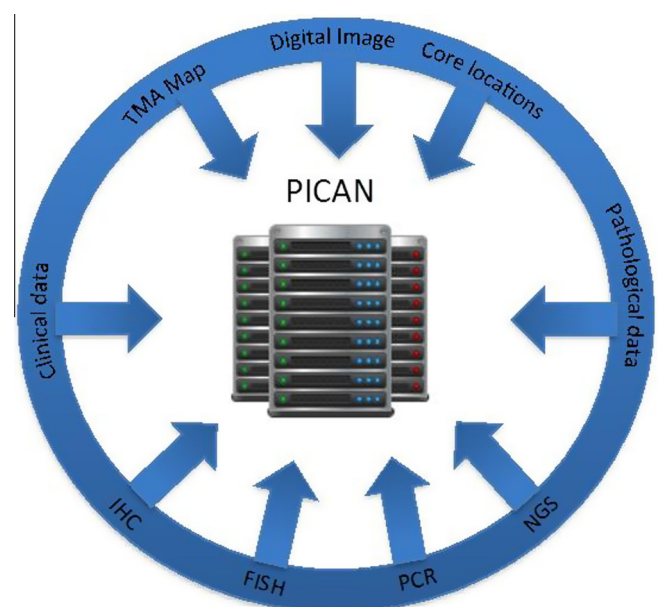


Fig. 11. Demonstrates a model for data integration and analysis of tissue-based imaging and genomics. This is based on the PICAN (Pathology Integromics in Cancer) approach adopted by the authors.

of the genetic landscape and reveal the level of molecular complexity in modalities such as tumour heterogeneity [70,71]. Statistical data mining will be greatly enhanced across patients, cancers and markers [72]. Understanding the relationship between phenotype and genotype in quantitative terms will allow us to provide a more precise taxonomy of disease, in terms of drug targets, companion biomarkers, patient stratification and personalized medicine.

It could be that biobanks act as the centralized repository for these data-sets, matched to high quality clinical samples, aimed at supporting future tissue-based discovery. Similarly, molecular pathology laboratories will also be driven to focus on combining conventional histopathological review, quantitative image analysis of tissue and cell phenotype and genomic analysis including DNA sequencing and other methodologies to support diagnostic and therapeutic decision making. Regardless of who drives this “integratics” initiative (Fig. 11), once an integrated approach is adopted the benefits will be extraordinary.

References

- [1] P.H. Bartels, L.G. Koss, J.J. Sychra, G.L. Wied, *Acta Cytol.* 22 (1978) 387–391.
- [2] A.I. Spriggs, *J. Clin. Pathol. Suppl. Coll. Pathol.* 3 (1969) 1–7.
- [3] O.A. Husain, J.H. Tucker, B.A. Roberts, *Biomed. Eng.* 11 (1976) 161–166.
- [4] P.H. Bartels, G.L. Wied, *J. Histochem. Cytochem.* 22 (1974) 660–662.
- [5] M.C. Montalto, R.R. McKay, R.J. Filkins, *J. Pathol. Inform.* 2 (2011) 44.
- [6] D. McCleary, J. Diamond, D. Crookes, H. Grabsch, P.W. Hamilton, *J. Pathol.* 217 (2009) s13.
- [7] D. Kieran, Y. Wang, D. Fennell, J. Quinn-O'Brien, Crookes D. WeiQi, P.W. Hamilton, Poster session presented at 11th European Congress on Telepathology and 5th International Congress on Virtual Microscopy, Venice, Italy, 2012.
- [8] Nikolas Stathonikis, Mitko Veta, André Huisman, Paul J. van Diest, in: *J. Pathol. Inform.* 4 (2013) 15.
- [9] (DPA) Digital Pathology Association. White Paper: Digital Pathology Archival and retrieval Systems. <http://digitalpathologyassociation.org/_data/files/Archival_and_Retrieval_in_Digital_Pathology_Systems.pdf>.
- [10] S.J. Potts, *Drug Discovery Today* (2009).
- [11] S. Al-Janabi, A. Huisman, S.M. Willems, P.J. Van Diest, *Hum. Pathol.* 43 (12) (2012) 2318–2325.
- [12] S. Al-Janabi, A. Huisman, A. Vink, R.J. Leguit, G.J.A. Offerhaus, F.J.W. ten Kate, P.J. van Diest, *J. Clin. Pathol.* 65 (2) (2012) 152–158.
- [13] S. Al-Janabi, A. Huisman, A. Vink, R.J. Leguit, G.J.A. Offerhaus, F.J.W. ten Kate, P.J. van Diest, *Hum. Pathol.* 43 (5) (2012) 702–707.
- [14] P.S. Nielsen, J. Lindebjerg, J. Rasmussen, H. Starklint, M. Waldstrom, B. Nielsen, *Hum. Pathol.* 41 (12) (2010) 1770–1776.
- [15] L. Pantanowitz et al., *Arch. Pathol. Lab. Med.* (2013), <http://dx.doi.org/10.5858/arpa.2013-0093-CP>.
- [16] P.W. Hamilton, P.J. van Diest, R. Williams, A.G. Gallagher, *J. Pathol.* 218 (3) (2009) 285–291.
- [17] P.I. Dalla Palma, P. Giorgi Rossi, G. Collina, A.M. Buccoliero, B. Ghiringello, E. Gilioli, G.L. Onnis, D. Aldovini, G. Galanti, G. Casadei, M. Aldi, V.V. Gomes, P. Giubilate, G. Ronco, NTCC Pathology Group, *Am. J. Clin. Pathol.* 132 (2009) 125–132.
- [18] Stenkivist et al., *Cytometry* 1 (1981) 287–291.
- [19] Baak et al., *Cancer* 56 (1985) 374–382.
- [20] P.J. Van Diest, J.P.A. Baak, *Hum. Pathol.* 22 (1991) 326–330.
- [21] Roux Ludovic, Racoceanu Daniel, Loménie Nicolas, Kulikova Maria, Irshad Humayun, Klossa Jacques, Capron Frédérique, Genestie Catherine, Naour Gilles Le, Metin N. Gurcan, *J. Pathol. Inform.* 2013 (4) (2012) 8.
- [22] Qin Huang, Yu Chenggong, Xiaoli Zhang, *BMC Clin. Pathol.* 8 (2008) 5.
- [23] J.M.I. Dunn, G.D. Mackenzie, D. Oukrif, C.A. Mosse, M.R. Banks, S. Thorpe, P. Sasieni, S.G. Bown, M.R. Novelli, P.S. Rabinovitch, L.B. Lovat, *Br. J. Cancer* 102 (2010) 1608–1617.
- [24] Ming Fang, Edward Lew, Michael Klein, Thomas Sebo, Yingyao Su, Raj Goyal, *Am. J. Gastroenterol.* 99 (2004) 1887–1894.
- [25] Nor F. Rajab, Declan J. McKenna, Jim Diamond, Kate Williamson, Peter W. Hamilton, Valerie J. McKelvey-Martin, *Cytometry Part A* 69 (2006) 1077–1085.
- [26] J.A. Orr, P.W. Hamilton, *Anal. Quant. Cytol. Histol.* 29 (2007) 17–31.
- [27] Stephen J. Keenan, James Diamond, W. Glenn McCluggage, Hoshang Bharucha, Deborah Thompson, Peter H. Bartels, Peter W. Hamilton, *J. Pathol.* 192 (2000) 351–362.
- [28] Peter W. Hamilton, Peter H. Bartels, Rodolfo Montironi, Neil H. Anderson, Deborah Thompson, James Diamond, Sidney Trewin, Hoshang Bharucha, *Anal. Quant. Cytol. Histol.* 20 (1998) 443.
- [29] L.M. McShane, R. Aamodt, C. Cordon-Cardo, R. Cote, D. Faraggi, Y. Fradet, H.B. Grossman, A. Peng, S.E. Taube, F.M. Waldman, *Clin. Cancer Res.* 6 (5) (2000 May) 1854–1864.
- [30] Anthony Rhodes, Bharat Jasani, Andre J. Balaton, *Am. J. Clin. Pathol.* 115 (2001) 44–58.
- [31] David Gancberg, Tero Järvinen, Angelo di Leo, Ghizlane Rouas, Fatima Cardoso, Marianne Paesmans, Alain Verhest, Martine J. Piccart, Jorma Isola, Denis Larsimont, *Breast Cancer Res. Treat.* 74 (2002) 113–120.
- [32] M.Y. Polley, S.C. Leung, L.M. McShane, D. Gao, J.C. Hugh, M.G. Mastropasqua, G. Viale, L.A. Zabaglio, F. Penault-Llorca, J.M. Bartlett, A.M. Gown, W.F. Symmans, T. Piper, E. Mehl, R.A. Enos, D.F. Hayes, M. Dowsett, *J. Natl Cancer Inst.* 105 (2013) 1897–1906.
- [33] Mitch Dowsett, Torsten O. Nielsen, Roger A'Hern, John Bartlett, *J. Natl Cancer Inst.* 103 (22) (2011) 1656–1664.
- [34] A.C. Wolff, M.E. Hammond, J.N. Schwartz, et al., *Arch. Pathol. Lab. Med.* 131 (2007) 18–43.
- [35] M. Salto-Tellez, E.X. Yau, B. Yan, S.B. Foz, *Arch. Pathol. Lab. Med.* 135 (2011) 693–695.
- [36] Yin Hai Wang, David McCleary, Ching-Wei Wang, Paul Kelly, Jackie James, Dean A. Fennell, Peter Hamilton, *Cell. Oncol.* (2010).
- [37] Yin Hai Wang, Kiernan Savage, Claire Grills, Andrena McCavigan, Jacqueline A. James, Dean A. Fennell, Peter W. Hamilton, *PLoS One* (2011), <http://dx.doi.org/10.1371/journal.pone.0026007>.
- [38] S.J. Potts, J.S. Krueger, N.D. Landis, D.A. Eberhard, G.D. Young, S.C. Schmechel, H. Lange, *Lab. Invest.* 92 (2012) 1342–1357.
- [39] D.L. Rimm, *Nat. Biotechnol.* 24 (8) (Aug. 2006) 914–916.
- [40] C.M. van der Loos, *J. Histochem. Cytochem.* 56 (4) (Apr. 2008) 313–328.
- [41] A.C. Ruifrok, D.A. Johnston, *Anal. Quant. Cytol. Histol.* 23 (4) (Aug. 2001) 291–299.
- [42] A.R. Hibbs, G. Macdonald, K. Garsha, in: J.B. Pawley (Ed.), *Handbook of Biological Confocal Microscopy*, 3rd ed., Springer, 2006, pp. 650–671.
- [43] W. Schubert, B. Bonnekoh, A.J. Pommer, L. Philipsen, R. Böckelmann, Y. Malykh, H. Gollnick, M. Friedenberger, M. Bode, A.W.M. Dress, *Nat. Biotechnol.* 24 (10) (Oct. 2006) 1270–1278.
- [44] J. Pawley, *Biotechniques* 28 (5) (2000) 884–886.
- [45] A.J. North, *J. Cell Biol.* 172 (1) (Jan. 2006) 9–18.
- [46] J.C. Waters, *J. Cell Biol.* 185 (7) (Jun. 2009) 1135–1148.
- [47] M.B.K. Lambros, R. Natrajan, J.S. Reis-Filho, *Hum. Pathol.* 38 (8) (Aug. 2007) 1105–1122.
- [48] A. Kovács, G. Stenman, *Pathol. Res. Pract.* 206 (1) (Jan. 2010) 39–42.
- [49] D.G. Hicks, R.R. Tubbs, *Hum. Pathol.* 36 (3) (Mar. 2005) 250–261.
- [50] D. Faratian, A. Graham, F. Rae, J. Thomas, *Histopathology* 54 (4) (Mar. 2009) 428–432.
- [51] L.A. Brown, D. Huntsman, *J. Mol. Histol.* 38 (2) (2007) 151–157.
- [52] Z. Theodosiou, I.N. Kasampalidis, G. Livanos, M. Zervakis, I. Pitas, K. Lyrudouia, 450 (2007) 439–450.
- [53] B. Lahrmann, N. Halama, H.-P. Sinn, P. Schirmacher, D. Jaeger, N. Grabe, *PLoS One* 6 (12) (Jan. 2011) e28048.
- [54] D. Padfield, B. Chen, H. Roysam, C. Cline, in: *Proceedings of 1st Workshop on Microscopic Image Analysis with Applications in Biology*, 2006, pp. 86–92.
- [55] A. Can, M. Bello, H. Cline, X. Tao, *Biomed. Imaging* 668 (2008) 288–291.
- [56] R. Camp, G. Chung, D. Rimm, *Nat. Med.* (2002) 1323–1328.
- [57] A. McCabe, M. Dolled-Filhart, R.L. Camp, D.L. Rimm, *J. Natl Cancer Inst.* 97 (24) (Dec. 2005) 1808–1815.
- [58] G.G. Chung, M.P. Zerkowski, S. Ghosh, R.L. Camp, D.L. Rimm, *Lab. Invest.* 87 (7) (Jul. 2007) 662–669.
- [59] S. Pozner-Moulis, M. Cregger, R.L. Camp, D.L. Rimm, *Lab. Invest.* 87 (3) (Mar. 2007) 251–260.
- [60] M.A. Rubin, M.P. Zerkowski, R.L. Camp, R. Kuefer, M.D. Hofer, A.M. Chinnaiyan, D.L. Rimm, *Am. J. Pathol.* 164 (3) (Mar. 2004) 831–840.
- [61] Alexander J.J. Smits, J. Alain Kummer, Peter C. de Bruin, Mijke Bol, Jan G. van den Tweel, Kees A. Seldenrijk, Stefan M. Willems, G. Johan A. Offerhaus, Roel A. de Weger, Paul J. van Diest, Aryan Vink, *Mod. Pathol.* (2013).
- [62] H. Viray, K. Li, T.A. Long, P. Vasalos, J.A. Bridge, L.J. Jennings, K.C. Halling, M. Hameed, D.L. Rimm, *Arch. Pathol. Lab. Med.* 137 (11) (2013) 1545–1549.
- [63] R.E. Hewitt, *Curr. Opin. Oncol.* 23 (2011) 112–119.
- [64] T. Khoury, S. Sait, H. Hwang, R. Chandrasekhar, G. Wilding, D. Tan, S. Kulkarni, *Mod. Pathol.* 22 (2009) 1457–1467.
- [65] K.B. Engel, H.M. Moore, *Arch. Pathol. Lab. Med.* 135 (2011) 537–543.
- [66] M. Ilyas, H. Grabsch, I.O. Ellis, C. Womack, R. Brown, D. Berney, D. Fennell, M. Salto-Tellez, M. Jenkins, G. Landberg, R. Byers, D. Treanor, D. Harrison, A.R. Green, G. Ball, P. Hamilton, *Histopathology* 62 (2013) 827–839.
- [67] Yalai Bai, Juliana Tolles, Huan Cheng, Summar Siddiqui, Arun Gopinath, Eirini Pectasides, *Lab. Invest.* 91 (2011) 1253–1261.
- [68] V.M. Neumeister, V. Anagnostou, S. Siddiqui, A.M. England, E.R. Zarrella, et al., *J. Natl Cancer Inst.* 104 (2012) (2012) 1815–1824.
- [69] Veronique M. Neumeister, Fabio Parisi, Allison M. England, Summar Siddiqui, Valsamo Anagnostou, Elizabeth Zarrella, Maria Vassilakopoulou, Yalai Bai, Sasha Saylor, Anna Sapino, Yuval Kluger, David G. Hicks, Gianni Bussolati, Stephanie Kwei, David L. Rimm, *Lab. Invest.* (2014), <http://dx.doi.org/10.1038/labinvest.2014.7>.
- [70] D. Gonzalez de Castro, P.A. Clarke, B. Al-Lazikani, P. Workman, in: *Clin. Pharmacol. Ther.* 93 (3) (2013) 252–259, <http://dx.doi.org/10.1038/clpt.2012.237>.
- [71] M. Gerlinger, A.J. Rowan, S. Horswell, J. Larkin, D. Endesfelder, E. Gronroos, P. Martinez, N. Matthews, A. Stewart, P. Tarpey, I. Varela, B. Phillimore, S. Begum, N.Q. McDonald, A. Butler, D. Jones, K. Raine, C. Latimer, C.R. Santos, M. Nohadani, A.C. Eklund, B. Spencer-Dene, G. Clark, L. Pickering, G. Stamp, M. Gore, Z. Szallasi, J. Downward, P.A. Futreal, C. Swanton, *N. Engl. J. Med.* 366 (10) (2012 Mar 8) 883–892.
- [72] P.R. Quinlan, A. Ashfield, L. Jordan, C. Purdie, A.M. Thompson, *Breast Cancer Res.* 12 (2010) P27.