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REVIEW

Virtual microscopy as an enabler of automated/quantitative assessment of protein expression in TMAs

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Abstract Tissue Microarrays facilitate high-throughput immuohistochemistry; however, there are key bottlenecks apparent in their analysis, particularly when conducting microscope-based manual reviews. Traditionally Tissue Microarray assessments were performed using a microscope where results were either transcribed or dictated and subsequently entered into flat-file spreadsheets. This process is labour intensive, prone to error and negates the advantages of the high-throughput Tissue Microarray format. In addition, human interpretations of staining intensity parameters are highly subjective and therefore prone to inter- and intra-observer variability. The advent of Virtual Slides has permitted the review of tissue slides across the Internet. In addition, this new technology enables the creation of software solutions to assist in the manual and automated review of Tissue Microarrays, through the use of computer aided image analysis. There are numerous academically developed and commercially available applications which assist in Tissue Microarray reviews; functionality of these systems range in complexity and application domains. The review which follows describes these systems and outlines technical considerations to be assessed when deciding on a Tissue Microarray workflow solution.

Keywords Image analysis · Tissue Microarrays · Immunohistochemistry · Virtual Slides

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Introduction

Immunohistochemistry is a well-established and versatile technique which is routinely used in molecular and surgical pathology (Kononen et al. 1998; Cregger et al. 2006). Immunohistochemistry allows for the identification and localisation of cell-bound antigens and can be performed on numerous cells and tissue preparations (Fejzo and Slamon 2001). The technique is widely used due to its relatively low cost, availability of materials in routine pathology laboratories and relatively rapid turnaround (Conway et al. 2006). The greatest advantage of immunohistochemistry is that it allows the interpretation of histomorphology to discern the complexity of expression patterns which cannot be determined from methods that rely on the extraction of biomolecules (Hewitt 2006). However, recent advances in molecular biology have centred on increases in throughput and quantification of biologic phenomena. No longer is experimental design focused on one gene or one protein, but rather on tens to hundreds of genes, proteins or tissue on analytical platforms (Macbeath 2002). Therefore, the application of immunohistochemical analysis on full-face sections as a means of biomarker validation is increasingly being replaced with Tissue Microarray analysis.

Tissue Microarrays (TMAs) provide high-throughput histomorphologic examination of tissue by means of

arranging multiple tissue samples in a uniform structure on a paraffin wax block. Large amounts of tissue samples are analysed simultaneously based on fluorescence in situ hybridisation (FISH) for genetic rearrangements, RNA in situ hybridisation for genetic expression, or immunohistochemistry for protein overexpression (Kononen et al. 1998; Kallioniaemi et al. 2001; Bubendorf et al. 2001). The technology was developed by Kononen et al. (1998) in order to facilitate gene expression and copy number surveys of large cohorts of tumours. Due to the nature of TMA construction which allows multiple sections to be obtained from a single TMA block, rapid analysis of hundreds of molecular markers on the same cohort of specimens is possible (Moch et al. 2001). TMAs provide substantial value in rapidly translating genomic and proteomic information into clinical applications (Torhorst et al. 2001). When initially created, TMAs were envisioned to make a dramatic impact on basic cancer research and anatomic pathology (Moch et al. 2001); in ten years since TMAs inception this hypothesis has been realised through various studies. TMAs have numerous benefits over full-face analysis including uniform experimental conditions, conservation of scarce tissue and a reduction in the volume of reagents used (Simon and Sauter 2002; Al Kuraya et al. 2004; Milanes-Yearsley et al. 2002; Hoos and Cordon-Cardo 2001).

However, at best, manual immunohistochemical analysis of TMAs is a semi-quantitative technique. In addition, large amounts of tissue and data are associated with TMA reviews, and as a result bottlenecks in microscopic analysis of TMAs have developed (Conway et al. 2006). With the advent of Virtual Slides high-throughput manual analysis of TMAs is possible. In addition image analysis of TMAs provides a high-throughput, reproducible and quantitative means of analysing immunohistochemically stained tissue. Assays for molecular quantification have been in existence for decades. In particular, popular techniques include reverse transcriptase polymerase chain reaction for quantification of nucleic acids or antibody based methods for protein quantification (Camozzi and Razvi 2004). A major drawback to these assays is that they require maceration of tissues and cells to quantitatively assess the amount of particular biomolecules present which leads to loss of critical spatial information (Cregger et al. 2006; Hewitt 2006).

The analysis of immunohistochemical staining patterns usually measures specific single targets rather than the relatively complex and intricate disease patterns, for example those seen on haematoxylin and eosin staining, therefore immunohistochemical studies are inherently amenable to automated image analysis (Joshi et al. 2007). Sources of variability in immunohistochemistry are numerous and include fixation conditions, specimen pre-treatment, reagents, detection methods, and interpretation of results. Although it is not possible to standardise all the potential variables in immunohistochemistry, the interpretation of immunohistochemical results may be standardised through quantitative methods (Cregger et al. 2006).

In theory it is not challenging to quantitate the intensity and area of brown staining using image analysis programs (Braunschweig et al. 2004). However, in comparison with other array platforms TMAs are not easy to analyse automatically. Every slide is stained differently, depending on the laboratory, procedure, stain type and from day-to-day. In addition, every donor block may be fixed differently, which hugely impacts on the quality of staining obtained. All imaging programs need to have the capability to be manually adjusted to facilitate the differences in staining conditions of each slide (Braunschweig et al. 2004). However, despite technical difficulties it has become crucial to automate TMA analysis and provide methods to manage and assess data in order to truly provide high-throughput analysis (Braunschweig et al. 2004).

Automated immunohistochemical protocols in combination with a device that provides quantitative and objective output, can dramatically improve the quality of the data obtained from immunohistochemical studies (Cregger et al. 2006). It has been proposed that computer-based analysis can quantify staining intensity more accurately and with greater reproducibility than manual human-based assessment (Weaver et al. 2003; Johansson et al. 2001). There are numerous commercially available, computer-based systems designed for the quantification of immunohistochemical staining. The aim of this review is to examine the application of automated software solutions for the analysis of protein expression within TMAs. Particular emphasis will be placed on the workflow and infrastructure required to provide a truly high-throughput automated image analysis solution for TMA applications.

TMA technology

Kononen et al. (1998) first illustrated the use of TMAs in 1998. The technique involves the excision of cores of varying diameter (0.6–2.0 mm) from regions of histological importance on donor tissue blocks and the subsequent insertion of these excised cores into precise co-ordinates on a recipient block. This process is repeated until a two-dimensional matrix of cores is inserted into the recipient block. Once the array is complete, sections can be cut from the block, which are then available for any analysis currently performed on full-face tissue sections. The most commonly applied analysis to TMAs is immunohistochemistry, with approximately 80% of all TMAs analysed in this way (Braunschweig et al. 2004; Shergill et al. 2004).

Tissue Microarrays greatly increase throughput of tissue analysis. Analysis of prognostic and predictive markers had traditionally been performed by testing one marker at a time (Torhorst et al. 2001). However, utilising a single TMA block containing 1,000 cores can potentially create 200 slides, and as many as 200,000 individual assays can be performed (Shergill et al. 2004). Therefore, TMAs allow serial selection analysis of multiple markers from the same molecular pathway in a large number of tissue samples, facilitating direct comparison of alterations of multiple molecular targets in virtually identical histologically highly conserved tumour regions (Wang et al. 2002).

Impact of TMA construction and staining on visual interpretation

The numerous challenges associated with immunohistochemistry are often magnified with the use of TMAs due to small sample size of the tissue cores and the diversity of fixation and processing conditions of tissue originating from different sources (Braunschweig et al. 2004). Thus, although immunohistochemistry is no more challenging on TMAs than full-face sections, due to tissue originating from different sources TMA immunohistochemistry is more likely to unmask deficient protocols (Braunschweig et al. 2004).

Nonetheless, there are many advantages associated with the use of TMAs in comparison with full-face sections. TMAs introduce standardisation of protocols into histopathology over and above what is possible with full-face sections (Tzankov et al. 2005), removing the inherent variability in experimental conditions from batch-to-batch analysis. With TMAs all tissue specimens arrayed on the one slide are analysed in an identical fashion. Antigen retrieval, reagent concentrations, incubation times with primary and secondary antibodies, temperatures and wash conditions are identical for each core within a TMA, resulting in an unprecedented level of standardisation which is unattainable utilising full-face techniques (Shergill et al. 2004).

However, sub-optimal immunohistochemistry in full-face sections and TMAs can be caused by many factors including poorly fixed/prepared sections; incomplete section drying or dewaxing; use of unclean xylene; insufficient/excess antigen retrieval; inappropriate antibody dilution; and non-specific staining due to endogenous tissue elements. With regard to TMA analysis there are other specific issues that may affect immunohistochemical staining and interpretation. TMAs are susceptible to tissue loss due to wash-off following slide pre-treatments (dewax and antigen retrieval), this can significantly reduce the number of cores available for interpretation. Therefore, it is imperative that replica cores and core sizes are carefully considered when constructing TMAs. For example, four 0.6 mm cores from different regions of a tumour may prove more representative and reproducible than one 2 mm core from the same tumour. In addition, loss of cores or miss-aligned TMAs will also cause difficulties when de-arraying virtual TMAs. De-arraying is the automated process of firstly identifying TMA spots within a virtual array and then subsequently associating each TMA spot with the corresponding information from the TMA map.

Loss of cores can be significantly reduced by utilising adhesive slides which are subjected to baking at appropriate temperatures or alternatively utilising tape transfer techniques which have been found to reduce tissue loss. TMAs containing tissue embedded from several different centres can cause problems at all stages of the TMA process, from construction to IHC staining and interpretation. Disparity can result from variance in fixation and processing protocols and even in the different types of waxes used to embed the tissue. Edge effect and staining artifacts can also lead to misinterpretation of peripheral cores. The occurrence of edge effect can be reduced by using irrelevant tissue cores to form a “moat” around study cores; and by using automated immunohistochemical systems with on-board antigen retrieval and appropriate tissue section coverage (e.g. Leica Microsystems Covertiles or Ventana’s Liquid Coverslip) to prevent reagent evaporation.

The quality of TMAs hugely influences the data obtained from image analysis, even more so than with microscope-based assessments. While it is possible to identify irregularities in the data obtained from image analysis, factors such as edge effect and folding of tissue are problematic for interpretation, and will affect the accuracy of automated image analysis systems. Currently, image analysis systems are not sophisticated enough to decipher edge effect staining from actual staining of interest, unless regions of interest are first annotated and then processed. Therefore, edge effect may be incorrectly interpreted as positive staining. Of a lesser concern is the occurrence of folded tissue, which typically results in the over quantification of protein expression. However, the occurrence of folded tissue can often be identified within the image analysis results by utilising the percentage of tissue present as a classifier for eliminating cores. In our experience, even the presence of tissue dye on the circumference of full-face sections/TMAs hugely affects protein expression quantification, by falsely inflating the level of positive expression observed. Therefore, it is imperative that TMAs are of a high quality; otherwise the investment in image analysis systems is futile. In addition, the presence of positive control tissue across all slides within a single study is imperative where quantitative image analysis will be applied. Positive controls can be utilised to normalise the data, therefore variance in staining protocols and background lighting can be eliminated.

Manual interpretation of TMAs

Histopathology remains the gold standard for most diagnosis and therapeutic decisions in pathology. The interpretation of histologic sections however, is an inherently subjective process based primarily on morphologic features (Cregger et al. 2006). The bulk of cases usually lie between where the research scientists can interpret the data; however, the quality of interpretation would improve with consultation by a pathologist (Hewitt 2006). Traditionally, human analysis has been considered the optimal method for qualifying immunohistochemical staining. Due to the complexity of tissue, the vast majority of TMAs continue to be scored by the human eye. However, the ability to quantify staining intensity by human analysis has produced varied results and is inherently flawed (Conway et al. 2006). In addition, the quantification of immunohistochemical staining is greatly influenced by the complexity of the immunostain under assessment. Human analysis generally quantifies staining intensities into broad categories, rather than assigning exact staining intensity values. At present, alternative methodologies can accurately quantify protein signal when performed in conjunction with computer-assisted analysis, such as densitometry. However, in the majority of instances immunohistochemistry remains the primary technique utilised (Bartlett et al. 2003; Ellis et al. 2000, 2004; Hsi and Tubbs 2004; Hicks and Tubbs 2005; Kay et al. 2004).

It has been proposed that human assessment of immunohistochemistry is considerably easier on TMAs compared to full-face sections, due to the fact that it is possible to compare staining intensities from different specimens on the same TMA. More importantly, interpretation is limited to within a small predefined area. Therefore, the area under investigation is constant for all reviewers, unlike full-face sections where different reviewers will select different areas of importance. In addition, due to the fact that a cohort of samples are typically analysed in a single review seating whereas traditionally this would have involved multiple seating's (Tzankov et al. 2005; Zu et al. 2005). However, observer variability is still evident in the manual assessment of TMAs.

Observer variability can exist in three instances, inter-observer variability, intra-observer variability and inter-laboratory variability. Poor inter-laboratory agreement is usually attributed to variability in tissue fixation, tissue processing, immunohistochemical protocols, antibodies and scoring systems used in different laboratories (Lacroix-Triki et al. 2006). Intra-observer variability has been reported as being less frequent than inter-observer variability. It has been suggested that each pathologist adheres to their own internal standards which in some cases, appear to be consistently reproducible (Kay et al. 1994). Inter-observer variability in relation to microscope-based reviews of immunohistochemically stained tissue has been well-documented in literature.

Inter-observer variability, when performing tumour identification, is hugely dependent on the type of tumour assessed, the antibody under assessment and the standard criteria available to identify the tumour in question (Schnitt et al. 1992; Wei et al. 2004). In addition, inter-observer variability is hugely reduced when well-defined classifiers are in place, for example with the assessment of HER-2 protein expression. The semi-quantitative categories used to classify HER-2 membrane staining are clearly defined and are based on intensity of staining, percentage and completeness of membrane staining. As a result inter-observer agreements when assessing HER-2 expression are greater than in comparison with other membrane antibody assessments for example E-Cadherin protein expression, where universal well defined classifications systems are not in place (publication in draft). Inter-observer variability is the greatest problem associated with human-based microscope assessment. Numerous factors are attributed to influencing human interpretation of immunohistochemically stained tissue, and therefore introducing inter- and intra-observer variability. These factors can be broadly divided into a number of categories, which are briefly described as follows:

Orientation

It is inherently difficult to accurately track the location of individual cores within complex TMAs when performing microscope-based assessments. Reviewers often misplace their orientation and become confused about their location within the slide, which threatens the accuracy of the results obtained. As mentioned previously, misplaced orientation is often exacerbated by poorly created TMAs. For example, TMA cores may be misaligned due to cores moving or washing off during the staining process. The orientation of the array is also crucial when performing automated de-arraying, as the origin of the array has to be known in order to assign the row and column values and associated the TMA spots to the TMA map. Typically control cores are used within TMAs, not only for reference tissue for review, but also for points of reference for orientation within the array. Often distinctive tissue types are housed within the array structure, therefore each row and column are denoted by a different tissue type.

Alternatively orientation spots are positioned outside of the uniform TMA grid structure, in order to identify the actual origin of the array. However, depending on the transfer of the tissue from the microtome to the glass slide and the actual size of the TMA, it is possible for eight different orientations of TMAs to occur, therefore causing confusion when reviewing serial sections from one block. Figure 1

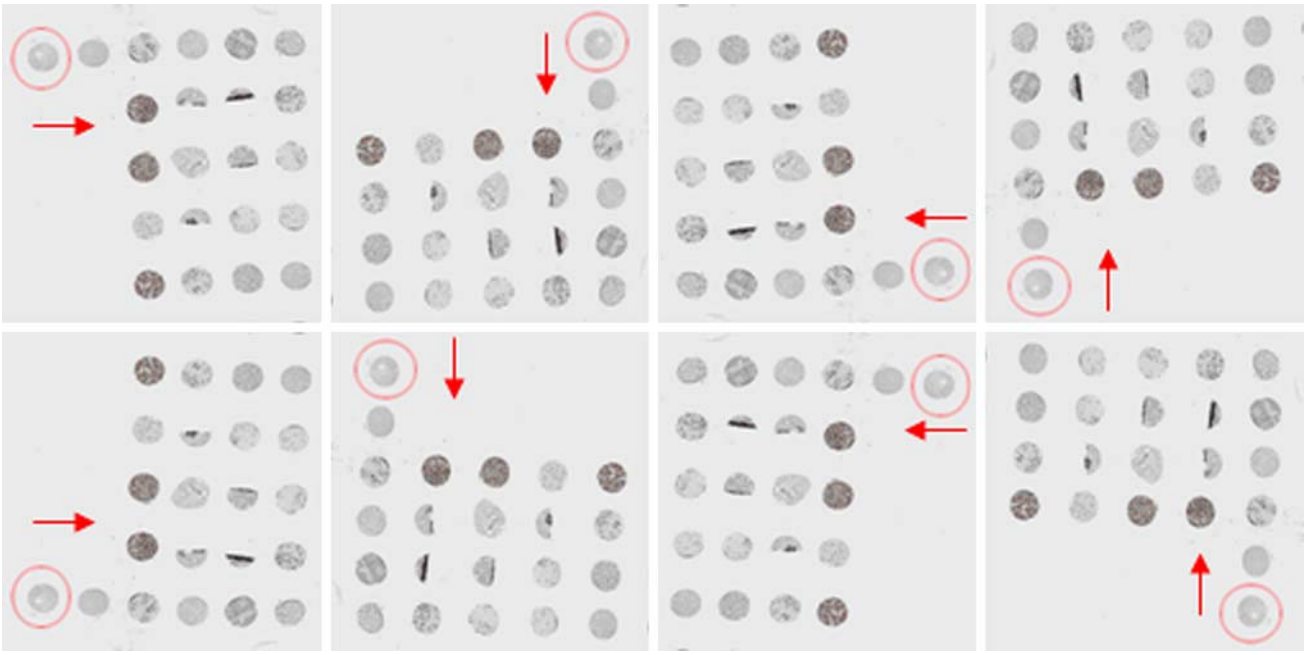

Fig. 1 Illustrates the eight possible orientations of a TMA. Once the tissue is sectioned and transferred to a water bath, there are four possible orientations. However, if the section is inverted when being transferred to the water bath, another four orientations are possible. The *arrow* within the images signifies the direction in which the TMA columns advance

illustrates the eight possible orientations of a TMA slide, and the arrows within the images represent the orientation in which the blocks were constructed.

Sequence of cores reviewed

Typically, the sequence in which the cores are reviewed can also affect reviewer's perception of the tissue. For example, pathologists are extremely knowledgeable when identifying tumour and can clearly recognise cores generated from the same biopsy, especially when the tissue is reviewed in sequence. Perception of staining intensity will also be affected by the sequence in which the cores are reviewed. Reviewers often rely on previously reviewed TMA cores to form their opinion of subsequent cores. For example a moderately stained core could be categorised as weak if the core was reviewed following a series of strongly stained cores, as human assessment is not a true value, rather a form of "comparison" of colours. It can be argue that in order to perform a totally impartial review, TMA cores should be reviewed randomly. Others believe it is of benefit to review all cores from one biopsy in sequence, in order to get an overall understanding of the tumour under review.

Workload and sample size

Pathologists are under increasing pressure to improve productivity and, are therefore generating more data and reviewing more slides. Tackling this workload manually places a constant strain on time, resources, staff, and finances. This burden is magnified when reviewing TMAs due to the volume of samples under analysis. In any field of science dependent on observation, accuracy is essential. However, it is well-documented that, after prolonged visual study, eye and specifically cone-fatigue can significantly affect a person's ability to discern colour changes and identify unusual objects (Habib 2005). The TMA slide format has compounded this effect, and with densities exceeding 500 TMA spots per slide, fatigue quickly becomes an issue.

Management of data

Due to the sheer volume and small sample size of tissue present on TMAs, there are difficulties in performing immunohistochemical reviews using traditional microscope-based assessments. Large amounts of data are associated with TMAs, ranging from information on the tissue (patient information), to their construction, subsequent staining and assessment. As a result of the large amounts of data and the fact that microscope-based assessment typically relies on the manual entry of results first onto a worksheet and then subsequently into a spreadsheet or database system, accurate manual tracking of the TMA core data is challenging, prone to human error and often leads to frustration (Tubbs et al. 2007). Therefore, it is apparent that applications to assist in pathologist's reviews of TMAs are required, ideally online object-orientated databases.

Scoring forms

The general parameters recorded during assessment of immunohistochemically stained tissues using traditional microscopes are intensity, localisation and the proportion of cells of interest that meet the first two criteria (Hewitt 2006). Human assessments can accurately and consistently identify the presence or absence of disease and low or high staining intensity. However, human assessment is not as capable when utilising intermediate categories and huge amount of variation is introduced as a result of over-using the intermediate category available during reviews (Kay et al. 1994).

Manual scoring systems are qualitative or semi-quantitative in nature, either when performing virtual or microscope-based reviews. Quantitative scales are either binary (±) or normative (0, 1, 2, 3). Qualitative scales have limitations in resolution which can be detected by eye, thus many researchers build a simple scale, as 0, 1, 2, according to negative, weak, strong (Braunschweig et al. 2004). Manual review requires interpretative skills of well-trained investigators and frequently the efforts of a specialist primarily pathologist. Staining patterns that are anticipated to be used in clinical practice are usually scorable as positive or negative, whenever possible (Braunschweig et al. 2004). The number and complexity of the categories used to record immunohistochemical staining will affect the levels of inter- and intra-observer agreement.

Illumination

Apart from the quality of the microscope, the next most important item in the reviewing process is the illumination of the slides. Bulbs used in microscopes have a characteristic tint; in general this is yellow or straw coloured. It has been suggested that the bulb tint influences human perception of staining intensity (Conway et al. 2006). If too much or too little light is exposed, information about the intensity of staining is lost. Adjustments in lighting settings from slide-to-slide can introduce huge variability in the reviewing process. Consistency of light between slides and reviews is extremely difficult during microscope-based assessments, as tints and shades can appear to change from one setting or context to another. Figure 2 illustrates an area of tissue which was scanned using different lighting exposure levels. The digital image represents the appearance of tissue under a microscope. Clearly, the perception of membrane staining intensity is affected by background lighting settings.

Human vision limitations

The accuracy of human vision is highly variable from person-to-person and is an extremely complex process; it is also hugely objective. The nature of the human eye is such that every person sees an object slightly differently from the way others see that same object, subjectivity in this regard is therefore innate (Habib 2005). Different observers may report seeing different features on the same object, as may a single observer at different times (Habib 2005). Visual inspection can also be confounded by the inherently subjective nature of human observation, which is affected by context, for example the amount of tumour present, background staining, and stromal staining (Camp and Divito 2005). Numerous facts affect human vision including contrast, borders, and colour, and these affects can be illustrated using a number of optical illusions.

Contrast is the local change in brightness and is defined as the ratio between average brightness of an object and the

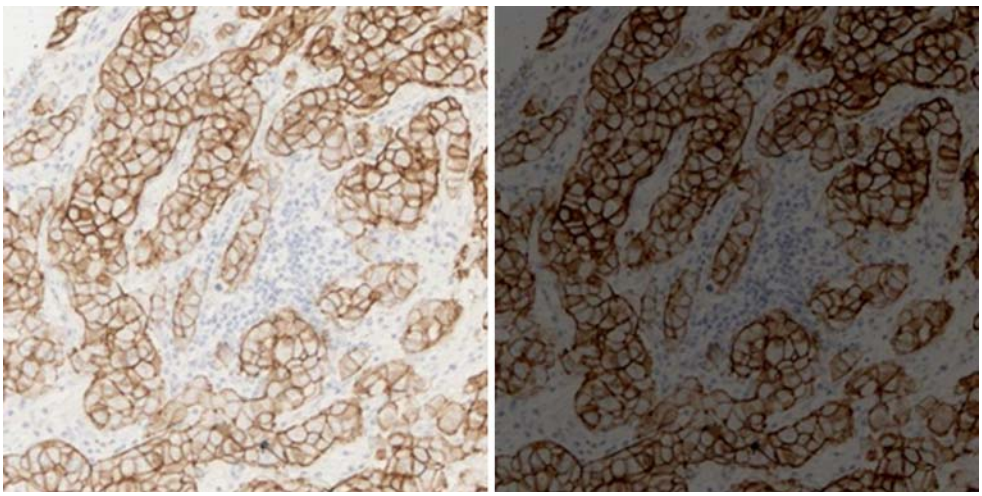

Fig. 2 Represents an area of tissue scanned utilising two different lighting exposure settings. Perception of membrane staining is hugely affected by the lighting exposure at which the slide was scanned. This is also evident and more prevalent with microscope-based analysis

background brightness. The human eye is logarithmically sensitive to brightness, implying that, for the same perception, higher brightness requires higher contrast (Sonka and Boyle 1993). Apparent brightness depends very much on the brightness of the local background; this effect is called "conditional contrast"(Sonka and Boyle 1993). Figure 3 (a and b) illustrates the fallibility of human perception of contrast (Dodek 2007). Figure 3a illustrates a vertical bar with a single colour throughout. When viewed with the contrast of a white background the vertical bar is clearly a single colour. However, when the vertical bar is superimposed on a background with a changing gradient of colour, our perception of the vertical bar has changed (Fig. 3b). Contrast is extremely applicable in the assessment of membrane-bound immunohistochemical staining. In cases where there is no cytoplasmic staining, membrane staining will appear stronger than in cases where cytoplasmic staining is present.

Object borders carry a lot of information. Boundaries of objects and simple patterns such as circles or lines enable adaptation effects similar to "conditional contrast". The Ebbinghaus illusion illustrates how humans can misinterpret size of particles when displayed in relative comparisons (Plodowski and Jackson 2001). Figure 4a, b displays two circles of the same diameter; however, as they are surrounded by circles of different diameters they appear to have different diameters (Sonka and Boyle 1993).

During the assessment of immunohistochemically stained TMAs the comparison of colour is paramount. As previously mentioned, manual review utilising a microscope is based on comparisons of tissue rather than an independent assessment of the true colour of the tissue under review. The Bezold Effect describes how colours appear differently depending on their relationship to other colours. Figure 5 illustrates that the colour red appears lighter when it is surrounded by a white border, and darker when surrounded by a black border (Lockal 2007).

Contrast is extremely applicable in the assessment of membrane-bound immunohistochemical staining. The contrast between membrane and cytoplasmic staining may be hugely variable and can affect human perception. Figure 6 A1 and B1 illustrates two images of bladder tissue probed with the antibody for E-cadherin. The two images have equivalent membrane staining intensity when quantified by computer-aided image analysis. The areas identified as positive for membrane staining by image analysis are highlighted in green (Fig. 6 A2 and B2). However, the membrane staining intensity appears significantly different within the two images when assessed by human review,

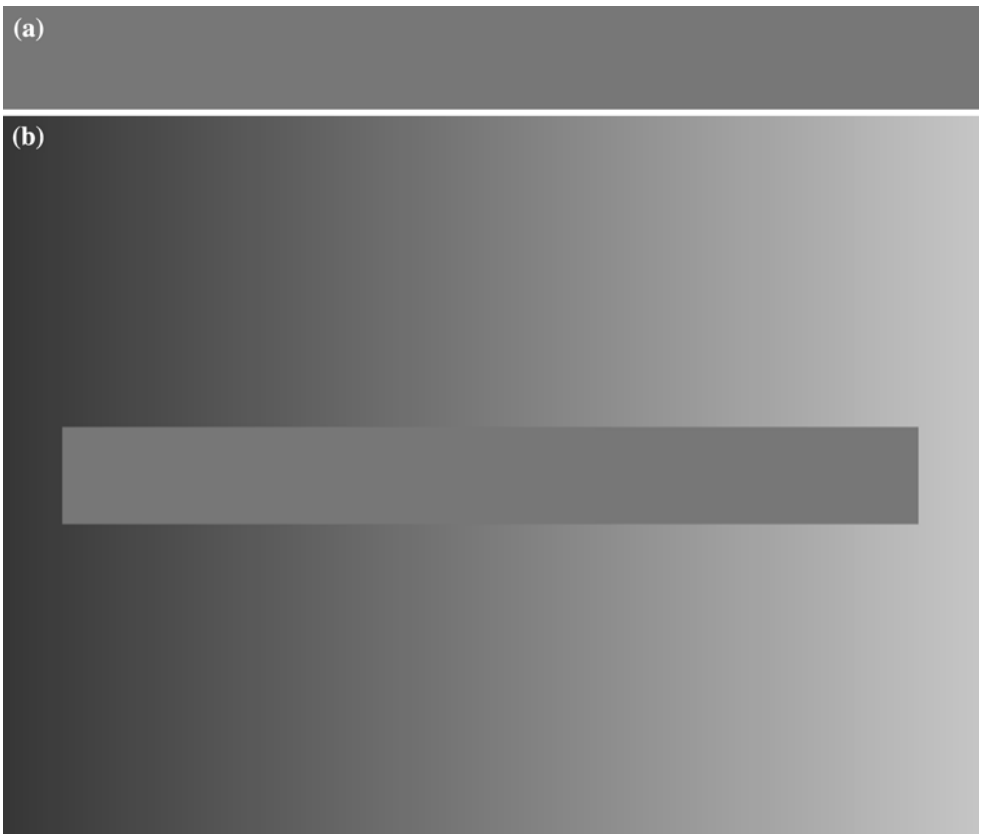

Fig. 3 **a** *Single coloured bar* against *white* background. **b** Illustrates the *identical vertical bar* as in Fig. 3a. However, within this figure the *vertical bar* is surrounded by a background with a changing gradient of colour. As a result, the *vertical bar* no longer appears the same colour throughout

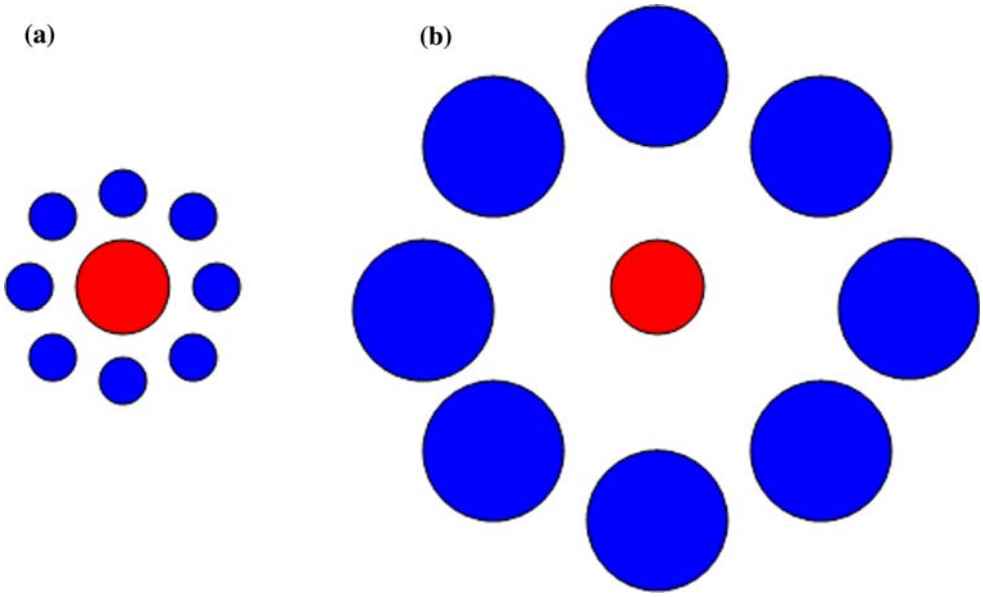

Fig. 4 Ebbinghaus illusion illustrates how the interpretations of the size of objects are relative to their surroundings. The *red circle* within image **a** and **b** are identical; however, perception of the size of the *red circle* is altered by the *blue circles* surrounding them

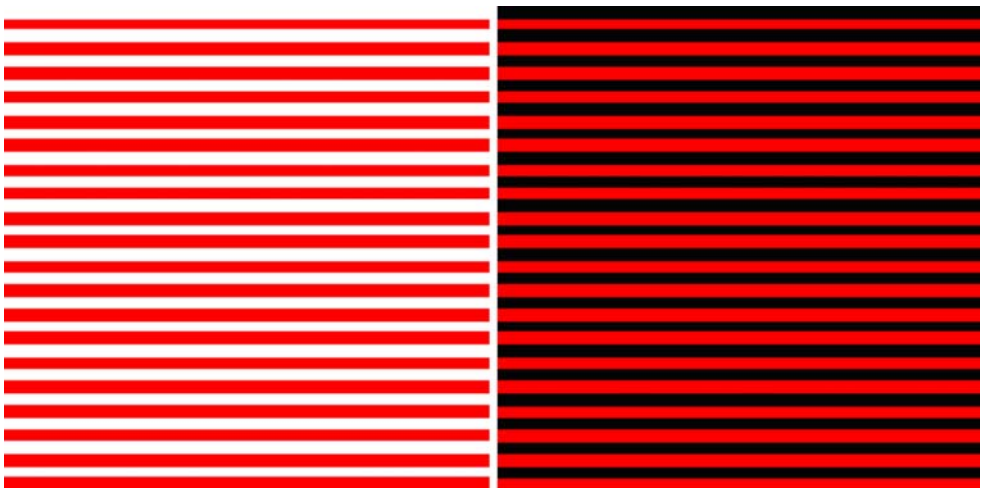

Fig. 5 Bezold Effect illustrates how the appearance of colour is altered by the colours that surround them. In this case, the colour *red* appears lighter when surrounded by *white*, and darker when surrounded by *black* Watanabe (2007)

firstly due to the differential tumour morphology, and secondly due to the presence of cytoplasmic staining within image 6A1. Figure 6 illustrates how both size and contrast affects human perception of staining intensity.

Virtual microscopy

Virtual Slides is a term used to describe the digitisation of traditional glass slides. Virtual Slides overcome problems attributable to sampling bias and interpretation resulting from limited field selection, allowing telepathologists to navigate to any field of view, at magnifications comparable to that of a conventional microscope, using images of sufficient resolution to render a correct diagnosis (Costello et al. 2003). In this technique, a conventionally prepared glass slide is placed on a microscope with a motorised stage and an automatic focusing facility or alternatively a specialised scanning device. The slide is scanned using a 10×, 20× or 40× objective lens and these images are integrated to produce a single large image file. This file can then be viewed on any computer with a virtual microscope interface where a user can press keys to change magnification from an overall low-power view up to the resolution at which it was scanned (Cross et al. 2002). Virtual Slides provide users with similar functionality of a microscope, but with numerous additional benefits, including concurrent access for multiple users, tracking of review movements and image annotation.

Advances in new technologies for complete slide digitisation in pathology have allowed the development of a wide spectrum of solutions for full-face slide scanning (Rojo et al. 2006 Vicente). Typically, acquisition devices can be broadly categorised based on their modes of action, of which three currently exist. Firstly, field of view devices which digitise slides based on capture of many small

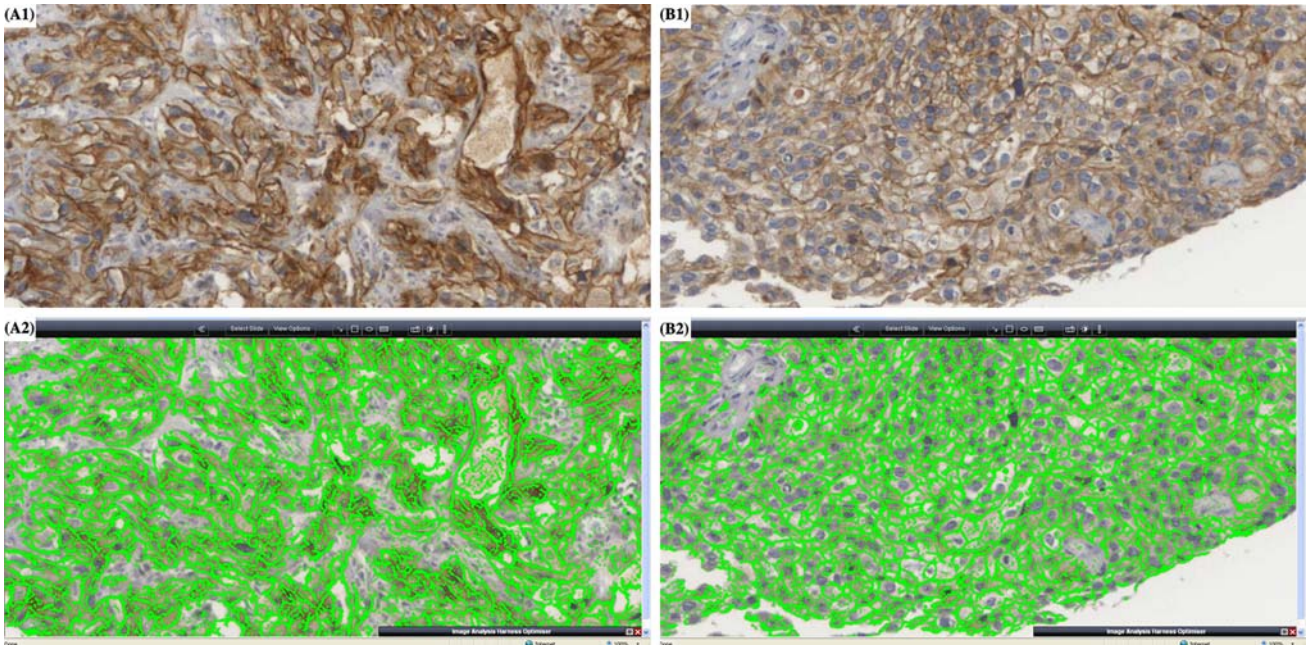

Fig. 6 Both images A1 and B1 have equivalent membrane staining intensity when assessed by image analysis; however the intensity appears different when assessed by eye. Within images A2 and B2 the *green* colouring represents the positive membrane staining assessed by image analysis

regions of the slide via a microscope with a traditional charged coupled device (CCD) mounted camera. The numerous images are then stitched together to create one large digital image. Numerous providers utilise field of view technology within their instruments, for example, Olympus dotSlide (Olympus UK Ltd), 3DHistech Ltd (Hungary) and Genetix (formally Applied Imaging, UK). Secondly, linear array devices which capture a small number of contiguous overlapping image stripes (Aperio-Technologies 2008). Linear array devices continuously move the microscope slides during image acquisition, therefore, facilitating rapid slide digitisation and seamless images. The key providers which use linear array devices are Aperio (Aperio Technologies, Inc., USA) and Hamamatsu (TDI-CCD technology, Hamamatsu Photonics, UK). Finally, area array scanners utilise many objectives rather than one and therefore, can digitise large areas faster than when using traditional field of view devices. At present Dmetrix Inc, (USA) are apparently the only vendor utilising this technology.

A number of new technologies are developing in the field of virtual microscopy. Hybrid scanners which provide numerous additional functionalities in addition to bright-field scanning are beginning to emerge. Extended depth-of-field and multi-focal scanners are broadening the domain of virtual microscopy to cytology applications for example cytology. Currently, Hamamatsu provides a scanner that is capable of multi-focal plane scanning, whereas 3DHistech provide a scanner that can facilitate extended depth-of-field scanning. In addition, the development of optical projection tomography (OPI) microscopy which facilitates the 3D imaging of biological specimens facilitates the mapping of multiple proteins distributions within the same tissue (Sharpe 2008). Currently, Bioptonics (MRC Technology, UK) claim to provide a scanner that can generate OPI images under 30 min.

The memory requirements for storing a digitised full-face slide/TMA is dependent on the area of tissue being scanned, the optical resolution (magnification) it is scanned at and the image file format/compression algorithm used for its storage. Typically, full-face scanning of a single standard paraffin-embedded slide (18 × 22 mm) at an optical resolution equivalent to 40× requires up to 1–1.2 GB of storage. Storage of up to 250–300 MB is required while scanning at an optical resolution equivalent to 20×. In order to calculate the storage requirements for a project for 1 year estimate the approximate number of slides to be scanned at a particular resolution. For example 1,000 slides at $40\times = (1{,}000 \times 1.2\ \text{GB}) \sim 1.2\ \text{TB}$. As a baseline for most projects it is recommended that sufficient storage be provided for at least 3 years. It is also important to consider redundancy/backup requirements for image data.

Due to the size of the images, specialist viewer software must be used in order to view Virtual Slides. Numerous software applications are available which facilitate distribution of images, locally and via the Internet. Image viewers typically facilitate the viewing and panning of Virtual Slides, however more complex functionality, for example

annotations and analysis is outside the scope of typical image viewers. The majority of commercially available image viewers are provided by the scanner vendors in conjunction with the hardware. For example, Aperio provide ImageScope, and Hamamatsu provide NDI Viewer. However, each of these viewers are vendor specific and therefore do not support alternative vendors file formats. As a result, collaboration with institutes utilising alternative scanners would require additional software. There are some image viewers that have non-priority formats, for example Zoomify Inc. (USA). Zoomify Droplet is a Macromedia Flash application which uses the original scanned image as an input and converts it into a set of JPEG image tiles. This tileset, once uploaded to a webserver, can be displayed via the Internet using the Zoomify embedded object within a conventional web page (Conway et al. 2006). Typically, image viewers are only used to verify the quality of the scan.

Utilising Virtual Slides it is possible to overcome some of the problems experienced when performing microscope-based TMA reviews. Uniform lighting conditions can be achieved across many TMAs when scanning slides. This eliminates the possibility of variance of interpretation due to background lighting. Integrating Virtual Slides within TMA workflow software facilitates the integration of TMA review data with the digital image of the TMA slide. The sequence in which cores are reviewed can be customised, which in return reduces sample bias. Finally, by using automated image analysis systems which are quantitative and produce continuous data sets, the elimination of categorised assessments in what is continuous data can be eliminated. High-throughput automated image analysis systems also reduce workloads, compensate for limitations in human vision, and as a result reduce inter- and intra-observer variability. Figure 7 illustrates the limitations of microscope-based assessments, and the solutions that virtual microscopy provides.

Software workflow solutions for TMAs

The development of TMAs has significantly increased the throughput of tissue analysed using immunohistochemistry, compared to more traditional full-face methods. Initial TMA studies were uni-dimensional, one stain, hundreds of samples. Management of the data could be easily recorded with a simple spreadsheet. In some instances this remains true, such as when a TMA is used to confirm a "hit" from a microarray experiment (Braunschweig et al. 2004). However, studies are now applying multiple stains to a single TMA or series of TMAs, generating large and complex datasets. Datasets with clinical outcome and epidemiologic information paired with immunohistochemical data can be in excess of 50,000 elements. As a result object-orientated databases are essential to manage the data. Many investigators who began with simple spreadsheets have had to abandon them as their datasets have grown, and have migrated to more robust enterprise level server based platforms (Conway et al. 2006). These issues are especially problematic for users who wish to maintain images of the individual TMA cores within the database (Braunschweig et al. 2004). Therefore, TMAs require specific management tools (Rojo et al. 2006).

The ability to create association between TMA spot images and data is fundamental for successful Virtual

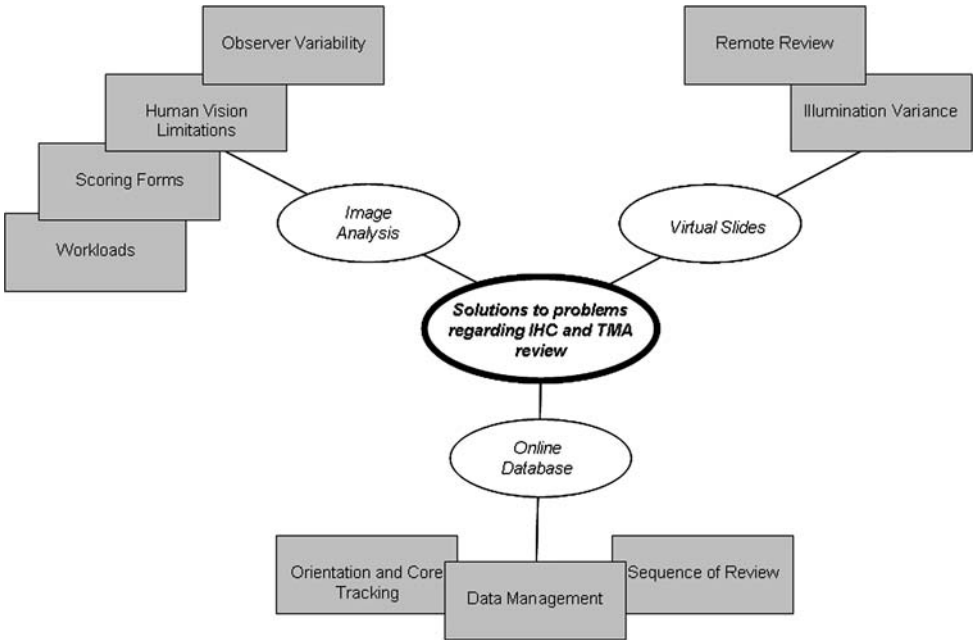

Fig. 7 Summaries the general limitations of microscope-based manual review of TMAs, and the solutions that Virtual Slides and automated image analysis systems can provide

Microscopy. Even with the development of automated analysis it remains necessary to manually inspect and verify the images and data at some point (Hewitt 2006). Therefore, software which can facilitate the manual analysis and storage of large images and associated data is imperative, especially with regard to the additional complexities associated with TMA reviews.

There are numerous software applications that facilitate review and data storage of TMAs. The technology varies from academic offerings to highly sophisticated commercial applications (Conway et al. 2006; Manley et al. 2001; Liu et al. 2002). Although Microsoft Excel™ spreadsheets are traditionally used by scientists to store data, there is always a significant risk of human error, as large amounts of data entry are required and the object-oriented nature of the data does not lead to optimal data storage in spreadsheets, also data is vulnerable to file corruption. In addition, the ability for numerous users to edit spreadsheets introduces potential opportunity for human errors without any protocol for tracking the authorships of files. However, it is imperative that TMA data is in a format that is easily available for distribution. The importance of distributing TMA data is evident from the creation of a TMA data exchange specification, which is a community-based open source tool for sharing TMA data. In 2001, the Association of Pathology Informatics hosted the first in a series of four workshops co-sponsored by the National Cancer Institute to develop the open community supported TMA exchange specification, which allows researchers to submit their data to journals and to public domain repositories and to share and merge data from different laboratories (Berman et al. 2003).

Academic software which facilitates the storage of TMA data and images have the advantage of being low cost and freely assessable to other low volume researchers. However, software created in an academic setting are typically hardcoded and therefore do not facilitate on-the-fly modifications. In addition, customer support is limited and access is restricted to researchers only. One of the most successful academic offerings is TMAJ, which is reported to consist of a database and set of open source software tools to manage TMA data and images. TMAJ is presently implemented at The Johns Hopkins TMA Laboratory, USA and is freely available as an open-source software tool for academic use only. TMAJ contains data from over 13,500 specimens, 7,000 blocks and 235 TMA's containing greater than 35,000 tissue cores (De Marzo 2003).

There are numerous commercial offerings which provide a complete TMA workflow, these systems range in complexity and functionality. The leaders in the field of TMA specific software include; SlidePath's (OpTMA), Aperio's (TMALab II) and Alphelys (Tisalys®). The systems functionality varies; however, all the above systems provide the utility to perform manual and automated image analysis reviews and store the review and epidemiological data in an associated database. Key features of any TMA workflow solution should incorporate the ability to upload and de-array TMAs with automatic identification and association of cores with case information. The system should provide a rapid review interface to facilitate manual reviews, with the instant embedding of scoring data into case information files. The ability to perform high-throughput consolidation across numerous reviewers' data for multiple cores per biopsy or patient cases should also be possible. Ideally, the ability to view virtual arrays of all cores pertaining to a biopsy or patient that have been immunohistochemically stained with numerous biomarkers would be possible. Finally the system should be fully searchable to provide rapid retrieval of the review and associated data. Software solutions that support some or all of the above features have relieved the bottlenecks in TMA review and data management. However, to truly realise the full potential of TMA technology, high-throughput automated image analysis should be considered.

Automated image analysis of immunohistochemically stained TMAs

It is possible to create image analysis algorithms which quantify protein expression within TMAs utilising generic programming applications for example, MatLab® (The MathWorks, Inc., USA) or ImageJ (National Institute of Health, USA) (Carmona et al. 2007; Francisco et al. 2004). Alternatively, it is possible to utilise commercially available image analysis applications which allow general researchers to write and record application specific macros in order to facilitate automated quantification of protein expression, for example Image-Pro Plus® (Media Cybernetics, Inc., USA). Utilising Image-Pro Plus, it is possible to extract features with spatial tools that isolate an area of interest from the rest of the image, or with segmentation tools that extract features by colour or intensity value. The greatest advantage of Image-Pro Plus is that non-programmers can create an effective algorithm. However, Image-Pro Plus is only of benefit for field of view analysis, as the manual segmentation of large images into tiles of areas of interest is highly labour intensive. While Image-Pro Plus is accurate, reproducible and quantitative the software alone will not increase throughput of analysis.

There are numerous commercial systems available that are specifically designed for the quantification of immunohistochemical staining including; IHCscore (Bacus Laboratories, Inc, USA); iVision (BioGenex Laboratories, Inc., USA), TissueMap (Definiens, Germany), VIAS (TriPath Imaging Inc, USA); PATHIAM (BioImagene Inc, USA);

ACIS-Automated cellular imaging systems (DakoCytomation, USA); AQUA-automated quantitative analysis (HistoRx Inc, USA) and TMAx (Beecher Instrument's, USA). Cregger et al. (2006); comprehensively reviewed the functionality of the image capture devices and image analysis capabilities of numerous vendors. In addition, Rojo et al. (2006); performed a comparative review of 31 digital slide systems in pathology, describing hardware and software functionalities. However, there are number of software solutions specifically designed for the TMA workflow analysis. These applications include Aperio (TMALab II), Alphelys (Spot Browser®), Genetix (Ariol-SL-50) and SlidePath (OpTMA). However, the fact that the majority of image analysis systems only perform field-of-view analysis is a major limitation with regard to high-throughput analysis. There are only a limited number of vendors that provide full-face and TMA high-throughput analysis, for example, SlidePath and Aperio. A brief description of the TMA specific vendors functionality follows.

Aperio's device, the ScanScope is designed for image acquisition. Aperio currently has five generations of the ScanScope; (T3, T2, CS, GL and XT) (Aperio Technologies, Inc, CA, USA) (Cregger et al. 2006). The ScanScope is capable of high-speed digital slide creation, management, and analysis. Aperio also provide software, namely TMALab II which facilitates the storage, manual/automated analysis of TMAs and storage of associated data and images with web-based software (Rojo et al. 2006). Using TMALab II it is possible to view, score and annotate TMAs, and in addition images and data can be exported from the database. Utilising TMALab II, it is possible to analyse entire immunohistochemically stained TMAs, spots or regions of interest using the following algorithms; nuclear, membrane, colour deconvolution and co-localisation. In addition, TMALab II also supports third party algorithms, for example those written using Image-Pro Plus or MatLab®. However, the software will only support Aperio's own image format SVS, therefore collaboration between other institutes using alternative scanners can not be supported. Aperio's software is extremely popular within the USA; however, in Europe where Zeiss, Hamamatsu and Olympus scanners are widespread the limitation of the software's proprietary file format restricts the application of TMALab II (Aperio 2008).

Alphelys provide Tisalys®, a database for archiving, reviewing and processing images and data generated during TMA analysis. Alphelys also provide Spot Browser®, an image analysis workstation integrated with microscope, visualising and capturing images through colour CCD camera and using a motorised stage. It allows rapid scanning of TMAs to build the TMA map, assignment of defined coordinates to tissue spots to track and provide a user's interface for pathologist's visual inspection and TMA browsing. Spot Browser® facilitates the analysis of TMAs either through visual inspection on the oculars or on the high resolution screen, or through automated detection of specific events for example, nuclei counting, signal quantitation, surface determination, morphometry or both methods simultaneously. All data collected can be exported to Excel for further data processing (Alphelys 2008).

Genetix (formally Applied Imaging) provide the Ariol *SL-50*, a TMA analysis application which combines an automated scanner and high-throughput automated image analysis application for the quantification of biomarkers on microscope slides in both brightfield and fluorescent imaging. Ariol has been FDA approved for in vitro diagnostic use of HER-2/neu, ER and PR Immunohistochemistry. The Ariol *SL-50* system quantifies nuclear, cytoplasmic and membrane immunohistochemistry protein expression utilising both nominal and quantitative scales. Both images and data are archived in case file. Utilising industry standard SQL and XML facilitate export of data and images from the Ariol *SL-50* system to third party databases. However, Ariol SL-50 operates purely on field of view analysis (Genetix 2008).

SlidePath provides a software product called OpTMA, which is a secure web-enabled information management system that facilitates integration of project information, digital slides (full-face and TMAs) and multimedia files (for example, PDF's, Microsoft Word) into a fully searchable, hierarchical database. OpTMA enables easy curation of digital slide archives and rapid retrieval of slides based on associated data attributes. OpTMA also allows users to create customised databases in order to store TMA images and clinical pathological data. In addition, the software fully automates the dearraying process of TMAs, and then automatically associates tissue spots with data. OpTMA also facilitates online reviews of virtual TMAs whilst storing the generated data within the database. The functionality to consolidate review data generated from multiple cores from a single biopsy/patient is also available. Users are presented with a virtual array of cores and associated review data, a consolidation form is then used to record the overall observation of the multiple cores, and data is returned to the database. The software facilitates high-throughput automated image analysis, utilising nuclear, membrane, cytoplasmic and positive pixel algorithms. Results can be presented as either nominal or quantitative data. SlidePath created an image analysis grid computing system which distributes images across multiple processing nodes, therefore facilitating truly high-throughput automated analysis across entire full-face sections and TMAs. In addition, third party algorithms, for example those created using ImageJ or MatLab®, can be integrated into the image analysis harness. However, the greatest advantage of SlidePath's products is the software is vendor neutral. Currently, SlidePaths software supports Zeiss (Mirax), Aperio (SVS), Bacus (BLISS), Nikon (VSL), Olympus (WebImage) and Hamamatsu (VMS and

NDPi) image file formats, in addition the software also supports non-propriety file formats for example JPEG, TIFF and Bitmap (SlidePath 2008).

With respect to image analysis, it is important to note that human analysis is still the gold standard when it comes to feature recognition and object classification. Human reviewers can easily identify and classify tumour from non-tumour and differentiate cell types from each other. Image analysis, on the other hand, is extremely accurate at quantification of staining extent and intensity. Image analysis in conjunction with TMAs (which are punched by qualified human observers from appropriate regions of tissue) is a combination that helps eliminate the obvious deficits that this technology experiences and allows developed algorithms to focus on quantification over object recognition.

Systems performance

Automated image analysis systems need to be reproducible and at least as accurate as traditional methods of analysis. Typically, the accuracy of these systems are validated by comparing protein expression levels when quantified by automated means with manual review data, traditional laboratory tests (FISH and ELISA), and prognostic outcome. A number of commercially available imaging systems have received FDA premarket approval to quantify biomarker expression as an aid in diagnosis. In order to obtain FDA approval the level of concordance between manual and automated image analysis is assessed. Table 1 illustrates the total number of automated imaging systems that have received FDA approval, and the levels of concordance between automated and manual reviews. The table illustrates there is a high level of correlation between manual and automated analysis. However, as previously described human analysis is inherently flawed. Therefore, correlation of biomarker expression with prognosis is a more robust evaluation of an image analysis system.

Table 2 lists the numerous publications that have utilised image analysis systems as a means of quantifying protein expression. The table illustrates the level of correlation between automated imaging and manual review, laboratory tests and prognostic data. The majority of publications have utilised ACIS and AQUA systems, which as the results illustrate are highly accurate when quantifying protein expression. However, currently the systems do not have a specific TMA workflow in place. Also the majority of antibodies that have been assessed are membrane specific for example HER-2 protein expression, or nuclear specific.

Factors to consider when deciding on image analysis applications

As with all experiments the quality of the results obtained are dependent on the procedure and raw materials utilised.

Table 1 FDA 510k Approved Automated Image Analysis Systems and their performance

Manufacturer	System	Approved use	Assay	Sample size	Automated vs manual score % concordance
Genetix	Ariol	HER-2	DAKO HercepTest	124	[a]
		HER-2 (FISH)	Abbott Vysis PathVysion DNA Probe kit	82	98
		ER	Kisight nuclear IHC	75	93.2–98.6[b]
		PR	Kisight nuclear IHC	75	84.4–96.1[b]
TriPath Imaging	VIAS	HER-2	Ventana PATHWAY anti-HER-2/neu (clone cb11)	201	77
		HER-2	PATHWAY (4B5)	206	86
		PR	Ventana anti-ER	210	88.2–94.1[b]
		ER	Ventana anti-PR	210	94.6–98.5[b]
		P53	Ventana CONFIRM anti-p53	204	86–98[b]
		Ki67	Ventana anti-Ki-67	207	88.4–97[b]
Chromavision	ACIS	HER-2	DAKO HercepTest	90	75
		ER&PR	No data	No data	No data
Cell analysis	QCA	ER	DAKO Cytomation (1D5)	192	85.15
BioImagene	PATHIAM	HER-2	DAKO HercepTest	176	80.4
Aperio	ScanScope XT System	HER-2	DAKO HercepTest	180	86.5

[a] In general, the likelihood of the image analysis systems to produce a consistent score on a given slide is as likely as the pathologists are to agree with each other

[b] Depending on cut-off thresholds of pos ≥1, 5 or 10% positive stained tumour cells

Table 2 Publications which describe image analysis as a means of quantifying protein expression, the table also illustrates the level of correlation observed between the automated systems and manual scoring/ELISA/ FISH analysis

Author	System	Biomarker	Sample size	Automated vs manual score	Correlation with ELISA	Correlation with FISH	Marker utility
Gokhale et al. 2007	ACIS, Ariol	ER	64	93% Concordance			
Leys et al. 2007	Ariol	ERp57	749				ERp57 was found to be prognostic marker for patients with gastric cancer with protein expression significantly lower in cancer and metastases in comparison to normal gastric mucosa ($P = 0.001$)
							Low ERp57 expression was associated with a statistically significant post-operative survival advantage ($P = 0.009$) compared to those patients with high expression
Turbin et al. 2007	Ariol	ER	3,484	No significant difference between methods, $P = 0.64$			
Zhang and Wang 2006	ACIS	SKP2	58				SKP2 was found to be a statistically valid predictive marker for grade of follicular lymphoma with the ability to distinguish between G1 and G3 ($P = 0.002$) and G2 and G3 ($P = 0.0002$)
Faith et al. 2004	ACIS	TFF3	635	Good correlation $R^2 = 0.84$			
Tawfik et al. 2006	ACIS	Her-2	247			94% Concordance	
Messersmith et al. 2005	ACIS	EGFR	18		Good correlation $R^2 = 0.69$		
Wang et al. 2001	ACIS	Her-2	189			91% Concordance	
Camp et al. 2002	AQUA	ER	340	Good correlation $R^2 = 0.88$			
Camp et al. 2003	AQUA	Her-2	300	Good correlation $R^2 = 0.70$			
Stromberg et al. 2007	TMAx		200	Excellent correlation $R^2 = 0.94$			
Divito et al. 2004	AQUA	Bcl-2	402				Bcl2 was found to be a valid prognostic marker for melanoma with increased expression associated with good prognostic outcome $P = 0.004$
Harigopal et al. 2005	AQUA	E-Cadherin	341				Strong E-cadherin expression was found to be strongly associated with improved survival rates ($P = 0.007$) in patients with breast cancer
Zerkowski et al. 2007	AQUA	Cox-2	669				Cox-2 expression was found to be associated with overall survival rate ($P = 0.0055$) in patients with breast cancer
Dolled-Filhart et al. 2006	AQUA	B-Catenin	600		Good correlation $R^2 = 0.85$		
Perner et al. 2007	ACIS	PSMA	450				PSMA was found to be a statistically valid predictive marker for prostate specific antigen with the ability to distinguish between benign prostate tissue, localised prostate cancer and lymph node metastases ($P < 0.001$). PSMA was strongly associated with increased risk of PSA recurrence ($P = 0.004$)
Brennan et al. 2008	In-house	Survivin	102				Different prognostic information is supplied by nuclear and cytoplasmic survivin in breast cancer. Nuclear survivin is a poor prognostic marker in breast cancer. Cytoplasmic to nuclear ratio of survivin, as determined by image analysis, is an independent prognostic factor

In the case of image analysis applications this translates to image and stain quality (Hewitt 2006). Image analysis performed on poorly scanned Virtual Slides or tissue with staining artefacts will result in all likelihood in inaccurate protein expression quantification.

There are numerous factors to consider when selecting image analysis software. Firstly, the system has to be user friendly. There are numerous publications listing the merits of image analysis as a means to quantify protein expression on TMAs. However, the influx of commercially available image analysis applications utilised to quantitate protein expression maybe extremely complex and difficult to use. Researchers have to decide upon creating their own algorithms using software which facilitates macro development for example, Image-Pro Plus or to purchase complete TMA software solutions for example OpTMA (SlidePath). However, if algorithms are created using applications like Image-Pro Plus extensive validation of the quality of the results obtained is required. Typically, it is not possible to purchase off-the-shelf systems that require no knowledge or understanding of image analysis. The intended users should comprehend the basic principles of image analysis, and also be able to interpret the large amounts of data that are generated from image analysis reviews. This skill set is not innate in scientists, and therefore it is often more viable to out-source image analysis requirements.

Secondly, there is a perception that image analysis and virtual applications are prohibitively expensive. Researchers tend to focus on the most expensive component within telepathology, which is the purchase of the scanning device. Currently, the cost of purchasing high-throughput scanners usually run at between 60,000 and 180,000 Euros (Rojo et al. 2006). As a result, commercial systems are not always viable in research or small laboratories (Camp and Divito 2005; Camp et al. 2002, 2003), especially, as researchers typically only produce a small volume of TMA slides per year. However, collaborations can be created between institutions, where a scanner is purchased by a consortium of institutes, and slides are posted for digitisation. By using a web-based information management system, slides are then available for manual and automated image analysis. The costing of software solutions that provide high-throughput automated analysis is relatively low in relation to other "materials" that are purchased in wet laboratories. The costing of these systems has to be off-set against the return on investment. Automated systems are proven to increase accuracy of results and are more reproducibility than manual assessments. However, most importantly automated analysis systems increase throughput and therefore save pathologists' precious time. In addition, numerous vendors provide managed services whereby the digitisation and image analysis requirements can be out-sourced, if the scale of project does not merit the purchase of software or a scanner (for example SlidePath).

Thirdly, a major consideration when utilising image analysis systems is how best to interpret the data. Typically, quantitative computer aided image analysis results in continuous variable data rather than an ordinal parameter. Therefore, the users must decide how best to classify the biomarker expression data, if at all. If the objective is to identify a prognostically significant biomarker it is possible to evaluate the data as a continuous variable, by using the Cox proportional-hazard regression. However, if users wish to persist with ordinal or nominal classification (i.e. use of Kaplan–Meier analysis) the dataset must first be segmented into categories, by implementing arbitrary cut-points. Unbiased assignment of cut-points can be achieved by creating two categories above or below mean or modal continuous variable value or by assessing top vs bottom quartile of a continuous variable range. However, assignment of cut-points based on minimisation of *P* values is a flawed strategy. Users of this approach will have to divide their datasets into training and test sets, validating the significance of these cut-points in a separate cohort of patients. This in turn creates a requirement for the provision of greater numbers of patients to increase statistical power. X-tile (Camp et al. 2004) is a particularly useful utility to help identify the optimal cut points in continuous data based on *P* value minimisation strategies.

Conclusions

Tissue Microarrays facilitate high-throughput biomarker validation, by arranging hundreds of tissue samples in a uniform structure on the surface of a glass slide. However, due to the sheer volume of tissue present within TMAs, there are bottlenecks when performing microscope-based reviews. In addition, human interpretation of staining intensity is inherently flawed. Utilising Virtual Microscopy, it is possible to overcome the bottlenecks associated with traditional microscope-based TMA reviews. Numerous software solutions exist which provide an end-to-end solution for TMA-based analysis, facilitating both manual and automated reviews. Currently, Virtual Microscopy is preferable to traditional microscope-based reviews. In addition, image analysis has proven to be more accurate when quantifing biomarker expression. However, human interpretation of feature recognition is still superior to any image analysis system currently available.

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