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Tissue microarrays: bridging the gap between research and the clinic

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Tissue microarrays are a high-throughput method for the investigation of biomarkers in multiple tissue specimens at once. This technique allows for the analysis of up to 500 tissue samples in a single experiment using immunohistochemistry and *in situ* hybridization. Recently, cell lines and xenografts have been reduced to a tissue microarray format and are being applied to preclinical drug development. In clinical research, tissue microarrays are applied at multiple levels: comprehensive analysis of samples in the context of a clinical trial or across a population. Tissue microarrays play a central role in translational research, facilitating the discovery of molecules that have potential roles in the diagnosis, prognosis and prediction of response to therapy.

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Since the invention of tissue microarrays (TMAs), the number of published articles has increased exponentially each year. Initially, TMAs were utilized primarily in basic research, typically in target verification of results from complementary DNA (cDNA) microarrays or other high-throughput approaches [1]. However, with the construction of clinically annotated TMAs, they are becoming an invaluable tool in clinical research. Recently, the methodology has been integrated into the preclinical drug development environment with the construction of arrays from cell lines [2] and xenografts (FIGURE 1) [UNPUBLISHED OBSERVATIONS]. The TMA is becoming firmly entrenched in the arsenal of tools and platforms used in clinical research. Cancer remains the primary focus of research with TMAs as tissue is more readily available. In this review, the application of TMAs throughout the entire spectrum of clinical research will be discussed, with an emphasis on drug development and clinical trials. There are numerous reviews of the impact on TMAs on basic science, demonstrating that with a TMA it is no longer a Herculean challenge to determine the significance of a novel protein [3,4].

A TMA is a platform to interrogate tissue biomarkers in a systematic fashion. These tissue biomarkers can be protein, RNA or DNA

alterations that are correlated with a disease process [5]. In clinical research, there are two areas of concentration in biomarker development. In drug development, the biomarkers that are typically studied are those associated with the molecular mechanism of action. Investigators measure these targets to appreciate the prevalence of a particular target, or to interrogate the functional status of the pathway, and in some instances, determine if the drug is mediating its function through the intended mechanism. On the diagnostic side, biomarkers predict outcome or response to therapy. In some instances, these targets are the same; however, the goal is different. For diagnostics and prognostication, the goal is to reduce the assay to some clinical utility. Developing new biomarkers that improve on current care is challenging, as the biomarker must be faster, cheaper and of greater utility before it can be adopted for clinical use.

TMAs are used throughout the drug development process. With the development of the cell line microarray (CMA), the expression of a protein can now be profiled in a dot-blot-like fashion in 60 or more cell lines at once [2]. Similarly, a xenograft tumor microarray (XMA) can be constructed to explore the expression of a protein in a large panel of

preclinical models [4]. Multitumor TMAs can provide a survey of expression across a broad spectrum of human cancer and normal tissues in as few as four slides [6,7]. This review will address all of these steps, highlighting the benefits and pitfalls of TMAs in clinical research.

Historical perspective on tissue microarrays

The TMA did not come about entirely *de novo*; rather, it is an improvement on previous methodologies. The most commonly cited precursor is the 'sausage roll' of tissue described by Hector Battifora in 1986 [8]. This platform lacked an organized grid of tissue that enabled simple identification of cores to match the

donor tissue, and could not be applied to large numbers of samples with clinical annotation. A further refinement describes a method using squares of tissue ordered in a checker-board pattern, in order to identify every sample better [9]. This method was a significant improvement, but was not optimal for application to archival tissue blocks. In 1998, at the US National Institutes of Health, Juha Kononen developed the current methodology in OP Kallioniemi's laboratory. The basis of this approach was the use of hollow needles on a stationary apparatus (FIGURES 2 & 3) [10]. The initial publication described the measurement of c-myc, c-ErbB2, p53 and estrogen receptor (ER) in breast cancer. More recently, others have described the construction of low-density TMAs with simple spinal needles [11] as well as template-based instruments.

Tissue collection & handling

Regardless of what is being arrayed, ethical issues are paramount. This may be as simple as appropriate material transfer agreement (MTA) for the use of cell lines, but expands rapidly to include the ethical use of animals in research for xenografts or animal model systems. For the use of human tissue, appropriate approval from an institutional ethics review board or other appropriate office/organization is essential [12]. The approval for the use of human tissue varies widely [13,14]. Many TMAs are constructed with anonymous tissue, typically lacking clinical follow-up data. Anonymous tissue is often subject to less oversight and approval is more straightforward. Commercial arrays are available from many different vendors. These arrays tend to be relatively expensive and often lack detailed clinical data. The origin of tissue in these arrays is often poorly documented and the quality of the tissue can be variable. These factors impact on the quality of commercial TMAs and can be an impediment, particularly if comparing different arrays from different vendors. With no clinical data attached, these arrays are of no value in clinical research, but are widely used by basic scientists who do not rely on annotation and are conducting more of a survey approach. However, in the clinical trials arena, the goal is annotated material. To accomplish these goals, the patient's consent must be obtained at the beginning of the trial, typically as a portion of the entire consenting process for the trial. Annotation of tissue requires time and resources; as a result, this

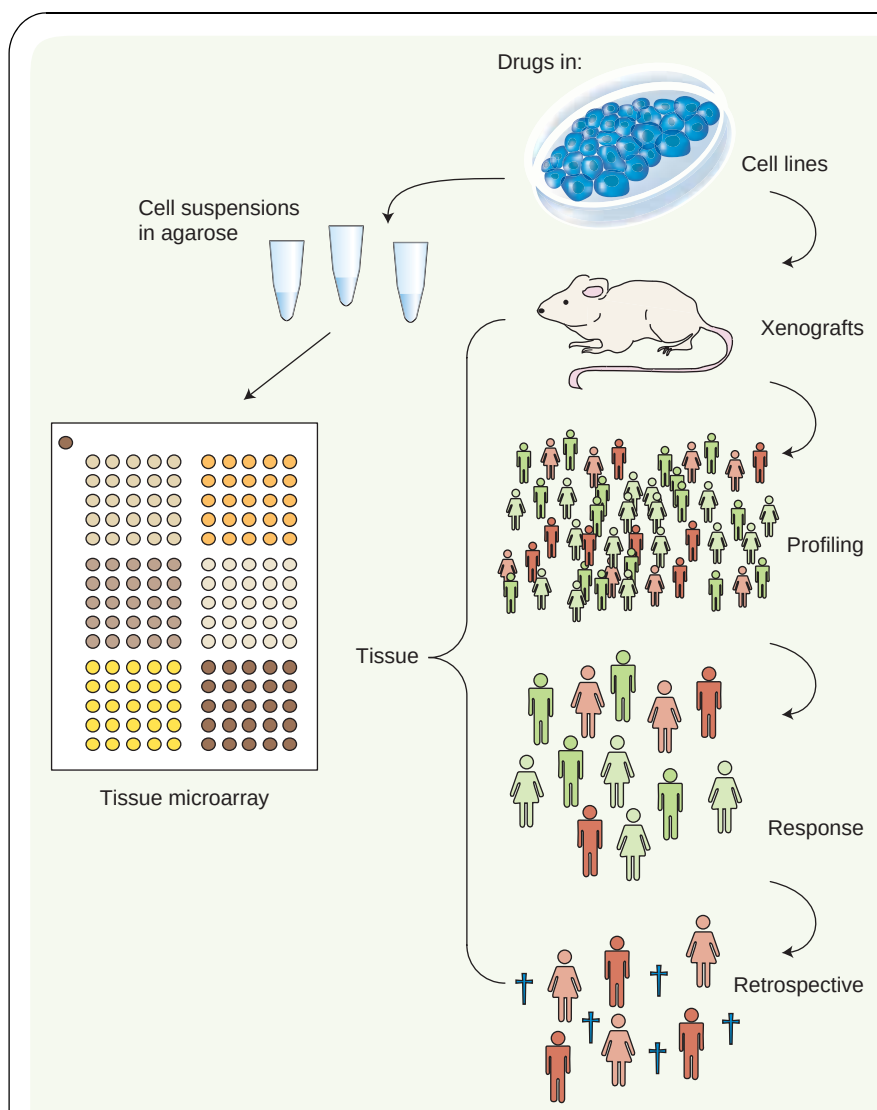


Figure 1. The role of TMAs in drug discovery. TMAs are being used at a number of points in the drug discovery process, including verifying the presence and level of the target proteins or mRNAs in cell lines on CMAs. These findings are then confirmed on xenografts on XMAS. Next, the target is validated on a significant number of unselected tissue samples to characterize its expression in human tumors. In clinical trials, TMAs are used both retrospectively to measure response to therapy and develop prognostic markers. Finally, studies can be performed on a selected subpopulation to examine the survival time or relapse intervals.

CMA: Cell line microarray; TMA: Tissue microarray; XMA: Xenograft tumor microarray.

material tends to be very expensive or only available through collaboration. The collaborators may wish to see extensive preliminary data prior to supplying these annotated arrays.

Method

The vast majority of TMAs are constructed with formalin-fixed, paraffin-embedded tissue. In special applications, 70% ethanol as a fixative may be encountered, more frequently for animal, xenograft and cell line arrays [15,16]. Many antibodies that are routinely used in western blots do not work easily in paraffin-embedded tissue. As a result, Fejzo and coworkers constructed TMAs in optimal cutting temperature (OCT; the generic name for the medium that tissue is imbedded in for pathologic preparation) with frozen tissue [17,18]. These arrays contain fewer but larger cores. Histology is limited, but for particular questions where antibodies that do not work in formalin-fixed tissue or *in situ* hybridization (ISH) is desired, they can be particularly useful. Alternative approaches have included the use of frozen lysate arrays [19]. Regardless of the preservation methods, handling of research tissue is critical to the success of the assays. For cell lines, animal tissue and xenografts, all parameters of handling (time, temperature and process) can be controlled, recorded and standardized. This approach significantly reduces the number of variables that can impact an assay, and provides more reproducible results. However, it is this control that is suddenly lost when confronted with human tissue. Even in the best designed and implemented clinical trials, issues such as warm ischemia time, volume and concentration of fixative and method of dissection are impossible to standardize [20]. As a trial moves from a single institution to multiple institutions, the number of variables escalates to include fixatives and buffers as well as the specifics of processing and paraffins [21,22]. The impact is that any assay that is to be applied must be more robust or hardened to account for these variabilities in order to provide consistent and valid results [5]. These issues pertain to all research tissue; however, they can be magnified on a TMA. For example, one immunohistochemistry (IHC) stain may perform adequately on tissue from one clinical center, but not stain any of the material from a second site, all on the same slide. This result was ultimately traced to different methods of decalcifying the tissue.

With tissue in hand, there are a multitude of parameters the researcher is confronted with to optimize the TMA to the scientific question posed. Frequently, the scientific question is only partially defined, and it will be the discoveries based on the TMA that will determine its final utility. There are many issues when designing an array and the researcher must optimize the use of tissue, appropriate sampling of the tumors being analyzed, as well as construct an array that will answer a scientific question. Often, these are competing issues, and the answer to some questions will remain unknown. If one is using clinical material, it is inappropriate to consume all the tissue entirely on the TMA. Pilot studies may require an over-sampling of the tumor, or there may be a desire to construct multiple replicate arrays. Often, the final design of the array is

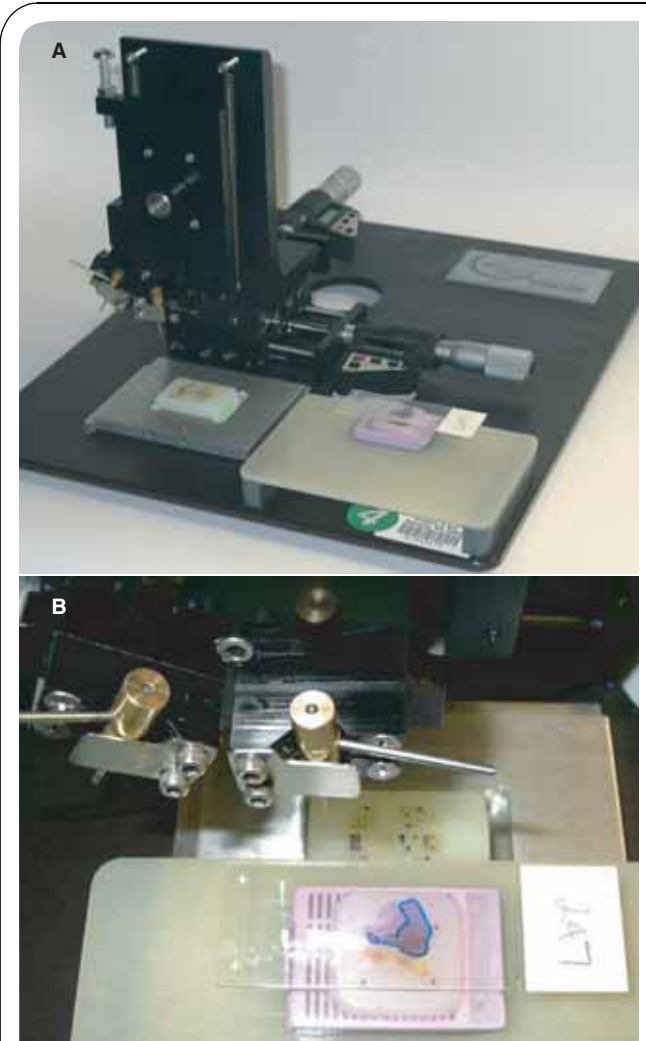


Figure 2. (A) A manual tissue arrayer for the construction of tissue microarrays. This is a Beecher MTA-1 instrument (Beecher Instruments). A pivoting support holds two hollow needles, one to punch out the recipient hole in the recipient block, the other the donor core from the donor block. The support with the needles is moved from recipient block by means of two micrometer screws at right angles. (B) The area of tissue to be punched is determined by overlaying a stained slide that has been marked by a pathologist.

dictated by practical concerns including the amount of tissue available or the number of cores the array can hold. A common failure of some TMAs is a lack of appropriate controls in the form of normal tissue or other tissue types for comparison (FIGURE 4). Many normal tissues are challenging (normal breast) to impossible (normal ovarian surface epithelium) to present on a TMA, but there are appropriate alternatives, either using whole sections or alternative tissues (endometrium is a useful control for both of the above examples).

Assay methods

Since a TMA represents nothing more than a condensed high-throughput platform of pathology, any method that can be applied to a tissue section is applicable to a TMA. IHC is by far

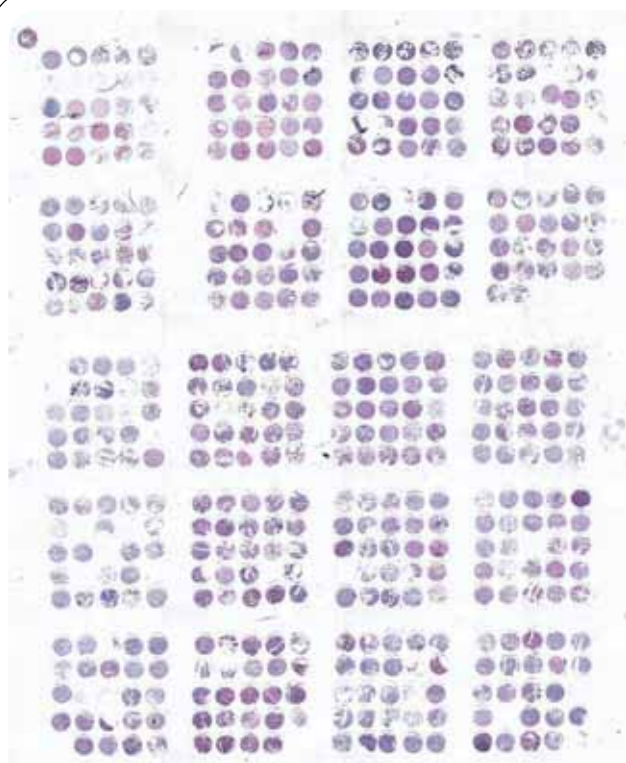


Figure 3. Image of a multitumor TMA from the TARP laboratory of the NCI. This tissue microarray contains 500 cores of tissue: 50 cores of normal tissue representing a variety of tissue types, 25 brain tumors, 25 melanomas, 50 lymphomas, 50 ovarian cancers and 75 samples each of breast, colon, lung and prostate cancer. This TMA is no longer available, but has been superseded by a new family of TMAs that are available from the NCI to researchers on a cost-recovery basis.
NCI: US National Cancer Institute; TARP: Tissue Array Research Program; TMA: Tissue microarray.

the most common application; however, ISH for both DNA and RNA is relatively common. Other applications have been observed, including laser capture microdissection and infrared spectroscopy [23].

Immunohistochemistry

The inherent challenges of IHC on whole sections are likewise found with TMAs. These challenges can be magnified by the small size of the tissue, and diversity of fixation and processing conditions of the tissue on a TMA (FIGURE 4). As a result, a TMA is likely to unmask a deficient protocol, particularly if confronted with tissue from different sources.

New antibodies previously used for western blots are frequently applied to TMAs as immunohistochemical stains [24]. It is estimated that less than 50% of antibodies that perform well in western blots or frozen tissue will work in formalin-fixed, paraffin-embedded tissue. Often, these antibodies are unable to recognize the epitopes after the condensation and crosslinking of proteins by formalin fixation. Validation of staining, by comparison of western blot and IHC results, is important. It is recommended that investigators consult other resources and researchers with experience in this field [25,26].

In addition to the common chromogenic detection methods, fluorochromes can also be utilized [27]. This requires the use of fluorescent microscopy, which offers some advantages: greater dynamic range and the simultaneous use of different fluorophores [28]. Fluorescence does not solve all problems as there are a number of inherent challenges: transient nature of the stains, requirement for high magnification for quantification and localization, and issues concerning interpretation and pattern recognition without a counter stain. However, for some questions, fluorescence is the best approach, particularly when precise quantification is desired. For precise localization, confocal microscopy can be applied to TMAs. In applying confocal microscopy, the goal is typically describing the relationship between multiple proteins in a cell, or the location of a protein in the cells, and is best applied to a CMA; however, XMAS and routine TMAs can also be used [2]. Some researchers have tried to use immunofluorescence or chemiluminescence quantification, scanning the slides with different types of scanner instrumentation [29]. The limited resolution of this approach results in a bleeding effect and frequently does not provide sufficient histomorphology to analyze heterogeneous samples in many human tissues; however, CMAs can be analyzed using this method.

No discussion of TMAs and staining methodologies is complete without confronting image analysis. Before the introduction of TMAs, image analysis was applied to whole sections or preselected regions of whole sections. The intersection of TMAs, high-quality digital cameras and faster computers has accelerated the image analysis industry. Now, with the capacity to capture high-resolution images rapidly, the new challenge is the algorithms for analysis. By examination and scoring by eye, it is easy to abstract the location and apply a qualified score of the staining. In contrast, for image analysis, algorithms must be programmed and tested and adjusted to every individual stain. One challenge is the variability of expression pattern, such as nuclear, membranous or in the cytoplasm. The authors' experience using different instruments and software packages is that interpretation by a pathologist correlates very well with image analysis [30]. This approach is particularly strong with XMAS and CMAs (FIGURE 5), where samples are rather homogenous, but the reproducibility and continuous data that image analysis provides can quickly outstrip the impact of tissue heterogeneity that a pathologist can interpret and exclude. These advanced techniques are expensive, challenging to implement and do not diminish the role of the pathologist. Rather, the work of the pathologist is to program and oversee the application of image analysis.

In situ hybridization

ISH for RNA expression is an invaluable tool to characterize spatial and temporal response of specific gene targets in tissue sections [31]. However, the complexity and expense of the approach has severely restricted its application. Due to the high-throughput nature of TMAs, there has been a resurgence in ISH on paraffin-embedded tissue. Some investigators continue to use probes labeled with radioactive nucleotides, which remains the most sensitive approach [7]. Recent advances have led to an

increased use of chromagenic (C)ISH using digoxigenin-labeled riboprobes. These methods have been used for detection of amplified RNA and DNA viral sequences for some time, and new protocols incorporating tyramide amplification have recently come to the market [31–33]. Interphase fluorescent (F)ISH for the detection of DNA targets is a popular application with TMAs [10,34]. The greatest challenge with FISH is microscopy, which require high magnification, and the counting of nuclei within each core to detect the specific targets.

Applications

Applications of tissue microarrays to model systems

Increasingly, TMAs are being applied to alternative tissue sources rather than archival human tissue. Most of these platforms are focused on drug development with a goal of increased throughput and development of assays that have application in human tissue.

Cell line microarrays

The construction of a CMA has been dubbed a 'western on a slide' approach. Reducing a series of cell lines to a CMA has a number of benefits, as well as some trade-offs (FIGURES 4 & 5) [25,35]. The platform is a low-cost, high-throughput means of exploring protein expression in cell lines. It offers the capacity to multiplex the assay, interrogating different proteins and or

activated forms of a protein on individual slides at an economy of scale that cannot be accomplished with western blot approaches. In this fashion, a CMA becomes a functional protein array. Currently, the 60 cell lines that the US National Cancer Institute (NCI) uses in drug screening have been formulated into a CMA platform (FIGURE 5) [101]. It is essential that the antibody be validated prior to application to a platform such as this. Others have used this platform as a high-throughput means to explore cellular localization of proteins [2]. CMAs can be constructed with less than half a million cells and some investigators use this approach as a protein profiling methodology when they wish to profile a large number of conditions or agents for changes at the proteome level [25]. An additional utility of CMA-like platforms is the inclusion of cell lines as staining controls for clinical interpretation [36]. Although not a purely quantitative approach to protein levels by IHC, this method is anticipated to reduce interobserver variability in scoring IHC.

Xenograft tumor microarrays

With molecularly targeted therapy and the necessity to investigate the mechanism of action of these agents, xenografts are frequently utilized as a preclinical model system (FIGURE 1). As assays are developed that measure either the susceptibility or response to

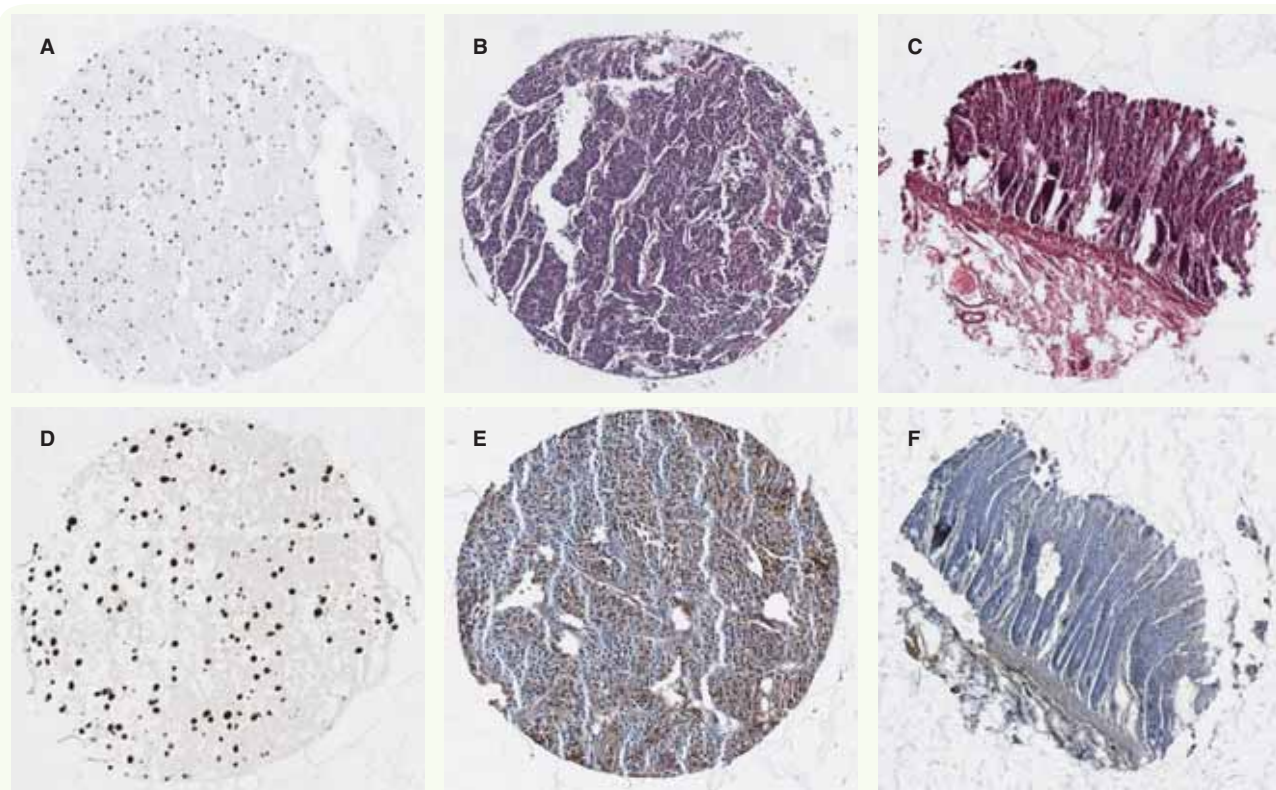


Figure 4. Examples of cores of a CMA (A,D), XMA (B,E) and TMA (C,F). In the upper row are hematoxylin and eosin stains of a lymphoma cell line (A), a neuroblastoma xenograft (B) and a tissue core of normal colon epithelium (C). In the second row are representative immunohistochemistry of the above cores from the same CMA (D), XMA (E) and TMA (F).

CMA: Cell line microarray; TMA: Tissue microarray; XMA: Xenograft tumor microarray.

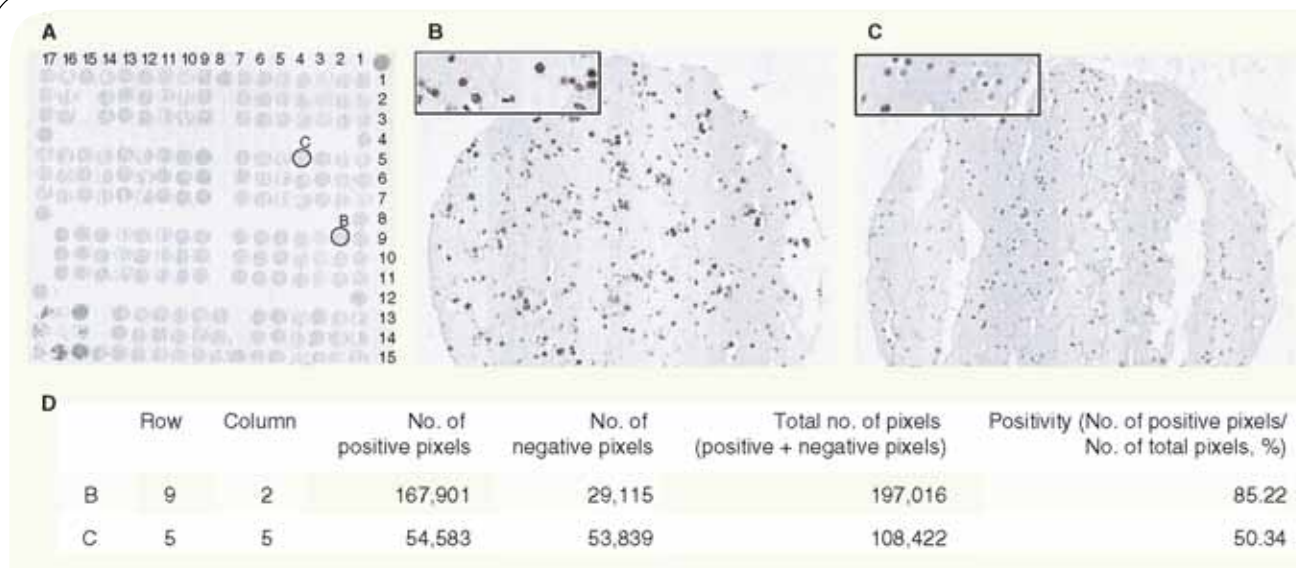


Figure 5. Example of immunohistochemistry of a CMA (folate receptor on the NCI60 CMA). The slide was scanned using the Scanscope T2 (Aperio Technologies), as in an overview of the whole section shown in (A). Then it was analyzed using TMA Lab, from Aperio, showing a reduced table of results in (D). Control tissue cores are placed in the CMA as well. Panel (B) shows strong stain in a human renal carcinoma cell line with a high number of positive cells and more intense staining. In panel (C), a human colon epithelial cell line with fewer positive cells and lower expression. CMA: Cell line microarray; NCI60: US National Cancer Institute, 60 cell lines.

a drug, there is increased demand that these assays be translated to clinical medicine. Toward this end, assays that had often been western blots are now being reformulated as IHC assays. To support these endeavors, the authors have constructed a number of xenograft arrays for preclinical models (FIGURE 4). Some of these platforms are aimed at a specific tumor system and response to therapy, while others are broader, consisting of a panel of tumors in which investigators can interrogate similarities and differences in protein expression between different tumor types [102].

Animal tissue-based tissue microarrays

Animal tissue and tumors are extensively used in the construction of TMAs. For researchers not focused on cancer, animal tissue is the most common material used in TMAs. Researchers have used TMAs to study the cellular response to toxic agents, while others have used TMAs to study the expression of cytokines in the liver of mice after infection with eggs of *Schistosoma mansoni* [37]. Another example is the examination of MeCP2 protein expression in the neurons of mice as it relates to the Rett syndrome [38]. TMAs of animal tumors as models of human cancer in clinical trials have been used with great success. In 2002, Khanna and coworkers evaluated the codrug octreotide pamoate in carboplatin treatment in osteosarcoma in dogs using a TMA [39]. Although unsuccessful as an agent, the TMA has contributed significantly to exploring the role of ezrin in metastasis [40].

Human tissue microarrays & the application to clinical research

TMAs are used in a wide variety of clinically related research areas, from molecular epidemiology to analysis of molecular end points in clinical trials. Some of the more common applications are summarized, with a discussion of strengths and weaknesses.

Diagnostic markers & classification of tumors

Naturally, any method of profiling a large number of pathology specimens at one time will be extensively used to develop new diagnostic markers for surgical pathology. The first large-scale demonstration of this was using expression microarrays on B-cell lymphoma [41]. In the same fashion, TMAs have been used for classification of tumors. In some instances, the TMAs have been used for validation of the results from expression microarray platforms [1], and in other instances, the TMA has been the primary platform, with targets identified from the literature or other approaches. Nishizuka and coworkers screened a panel of cell lines and identified genes that were differentially regulated between colon and ovarian cancers [1]. They identified *villin* and *moesin* as genes that can be used to differentiate colon and ovarian adenocarcinomas. These findings were validated on a variety of platforms, and ultimately confirmed on a large multitumor TMA. Starting with expression microarrays and then moving to TMAs, Hans and coworkers used a variety of markers to distinguish subgroups in diffuse large B-cell lymphoma (DLBCL), the most common non-Hodgkin lymphoma [42]. A subset of three out of seven markers was identified to discriminate germinal center B-cell-like (GCB) and non-GCB forms of DLBCL.

Prognostic oncology

Interestingly, TMAs have had less of an impact on new markers for diagnosis, and rather a greater impact on development of prognostic markers. The most common approach to identify markers for prognosis is to build a TMA of tissues from patients with different survival or relapse-free times and to show a correlation between the expression of a marker and a specific outcome, such as survival. These data are typically plotted

in a Kaplan–Meier plot. There are numerous examples of IHC for different proteins as predictive of good or poor prognosis. Initial work with ezrin on dog osteosarcoma was successful enough to expand the work to human osteosarcoma, and currently, the impact of ezrin is being explored in a diversity of tumor types [40].

Issues concerning validation of prognostic markers

Despite the large number of publications touting new prognostic markers discovered and validated on TMAs, few of these have moved beyond a research setting at this time. In most studies, the IHC was scored by eye by a pathologist, using either a qualitative scoring system (positive/negative or 0, 1, 2, 3 ordinal approaches). However, IHC as a proteomic method is not very robust and without a strong correlation to outcome, prognostic markers have proven difficult to implement in a clinical environment. Studies that rely on a low versus intermediate or strong stain to differentiate prognostic groups are particularly problematic. The data may be very powerful, but implementing this type of assay in a clinical environment is challenging at best and is too often unreliable for clinical use. There is no single stage in the process that results in this problem; rather, there are multiple stages that together contribute to the challenge. These challenges pertain to all IHC, but with the advent of the TMA, they have received far greater attention than before. Many IHC assays fail to meet the definition of a hardened assay, which performs the same in the investigator's laboratory as it does in a collaborator's, regardless of the source of specimens [5]. Clinical laboratories that perform IHC as a diagnostic test go to great lengths to validate their IHC conditions, run routine quality assurance against known positive and negative controls, and must meet certain standards put forth by regulatory bodies. It is documented that nearly every stage of tissue handling and staining can impact the outcome of a stain. One particular challenge is interpretation, which has extensive inter- as well as intraobserver variability. Automated image analysis is only just now being applied, although it promises improved reproducibility (FIGURE 5) [43–45]. Automated approaches may not be truly quantitative; however, they do produce a reproducible and continuous variable type data, allowing different analytic approaches. The TMA has become a driving force in image analysis as the volume of samples, albeit small cores, has become overburdening for a pathologist to review, and the analytical methods that can be applied to qualitative data are limiting.

Screening of pathways & targets of molecular targeted therapy

Researchers have defined many different pathways, related or independent receptors, surface molecules and cell–cell signaling molecules that play a role in the development and progression of cancer, as well as other diseases. Alterations that have been described include upregulation of members of a cell proliferation pathway (e.g., ER [46], c-Kit [47], epidermal growth factor receptor [EGFR] [48]), downregulation of members of apoptotic pathways (e.g., p53 [49,50]) or inappropriate

expression of surface molecules (e.g., HER-2 [51], CD antigens [53,54]) or enzymes (e.g., cyclooxygenase [COX]-2 [54]). These pathways are typically elucidated *in vitro* in cell lines, and may not function in the same fashion in all cell types. Numerous studies are underway, targeting proteins that played roles in the development and progression of cancer such as EGFR, mammalian target of rapamycin (mTOR), Ras, Raf-1, mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), Bcl-2, Akt and nuclear factor (NF)-κB [55,56]. This strategy is well demonstrated, although it is not without risk. Inhibitors of EGFR have not been as clinically successful as anticipated by the initial *in vitro* data. As a result, there is increased interest in profiling these cellular targets before moving into clinical trials. The preclinical data are often based on animal models and *in vitro* screens, both of which are inadequate to provide a broad picture of expression across all normal tissues, or a spectrum of tumors. Pathway activation and propagation can vary between tumor types. When a pre-treatment diagnostic is desired to determine expression of the target protein, TMAs are used to baseline the expression across different tumors, as well as develop the parameters of a clinical assay that will be ultimately used to select patients for these therapies. For most of these targets, IHC is the approach that has been utilized to measure cellular proteins, although in some instances FISH/CISH may be an appropriate assay for transcription or gene amplification. Increasingly, it is no longer sufficient to assay only the targeted protein, rather, investigators are forced to look downstream of the target, and determine the functional status of the remainder of the pathway in the tumors of interest [30]. IHC remains the preferred method as the expression pattern of these proteins can be appreciated in their histomorphologic context. Currently, the authors' laboratory has been profiling the Akt pathway in an effort to better understand its significance in human cancer and define optimal targets of therapy as well as appreciate the changes that occur after treatment with different targeted drugs.

Tissue microarrays & routine clinical care

Currently, TMAs are not used as a platform for diagnostic pathology. There has been speculation that IHC could be performed in a batch mode on a weekly basis as a cost-containment strategy. Currently, concerns of tissue/tumor representation on TMAs have hindered this approach. There are many studies that demonstrate that TMAs accurately present patient samples for IHC analysis [57–61]. However, these studies are based on the correlation of multiple cases and not the analysis of the individual sample. Without a documented improvement in clinical care, it is unlikely that constructing TMAs of patient material will be adopted as a part of clinical care.

Although patient samples are unlikely to be constructed into TMAs for routine clinical care, TMAs do have a role in routine diagnostic pathology. In breast cancer therapy, testing for ER and c-ErbB expression and/or HER-2/neu amplification is standard [62,63]. Recently, staining for expression of c-Kit (CD117) has become routine for chronic myelogenous leukemia

and gastrointestinal stromal tumours. There are efforts underway to construct mini-TMAs that would be present on the same slide, or stained on a separate slide at the same time, to function as references for scoring. TMAs are routinely used to validate IHC and provide inter- and intralaboratory equalization [64–66].

Tissue microarrays in clinical trials

Clinical trials are becoming increasingly expensive and complex. Trials no longer only examine pharmacokinetics and survival rates, but may be based on molecular end points as determined by IHC or other measurements. Clinical trials are being co-opted for biomarker development, where investigators develop assays that predict response to therapy [5]. Tissue and serum are frequently collected for additional laboratory analysis of molecular end points. With the added emphasis on tissue in clinical tissue, it is not surprising to see TMAs increasingly applied.

Profiling of tumors before therapy

Although TMAs are not used in routine clinical care, they are used to expression profile patients at the protein level in clinical trials. TMAs offer a standardized platform, reducing concerns about stain quality, as well as reducing assay cost. TMAs are currently being constructed of patients' tumors with the tissue collected prior to initiation on the trial. These TMAs are often constructed from the original surgical specimen, which may have been removed significantly prior to enrolment on the trial. Alternatively, a new specimen may be obtained from the patient prior to the initiation of the investigational therapy. The objective of these TMAs is to define the functional status of the tumor prior to the therapy. The results of assays on the TMA are not used in clinical decision making. The goal is to develop biomarkers that predict response or failure to the therapy, as well as document expression changes in the tumor. In many ways, this is much the same as using TMAs to profile pathways; however, the focus is on a smaller number of patients, and frequently on more focused sets of targets related to the agent in the clinical trial.

Tissue microarrays & response to therapy

During therapy, patient follow-up is often performed using noninvasive methods such as x-ray or computerized axial tomography scans or magnetic resonance imaging and tissue is rarely available. However, there is increasing effort to obtain tumor (and in some instances normal) tissue during or after treatment in clinical trial. This tissue is often limited to biopsies, and there is great interest in utilizing it for TMAs to study the response to therapy [30]. Recent results of this approach include examining heat shock protein (HSP)27 expression as a marker for drug resistance in prostate cancer [67]. A different study demonstrated increased staining for HSP27 in hormone refractory prostate cancer, but with positive correlation to the expression of insulin-like growth factor-binding protein (IGFBP)2, as an indicator of switching from one proliferation pathway to another [68]. Another example is in renal cell carcinoma, where overexpression of carbonic anhydrase IX correlated with response in interleukin-2 treatment [69].

End point analysis & tissue microarrays

A large number of TMA-based studies consist of retrospective analyses of expression patterns in tumor samples in respect to survival time, local relapses, metastases, tumor progression and aggressiveness. The goal is to develop markers that predict patient outcome or methods of selecting patients for different therapeutic regimes. There are too many examples to review in their entirety, and only the examples in breast cancer are highlighted to provide an overview of their use and utility. TMAs constructed of breast tumors have been developed based on survival time, local or metastatic relapse. Many studies are paired with cDNA microarray data, often with a smaller sample size of outcome-selected patients. The cDNA expression microarray platform is used as a discovery mechanism, and the TMA a validation step for the results. Typically, the new markers have not been previously applied in IHC, such as colony-stimulating factor (CSF)-1 [70] or 14-3-3 σ , a myoepithelial marker [71]. Other studies have examined the expression levels of known markers, such as telomerase [72], that have not been evaluated systematically in large cohorts. Nevalainen and coworkers demonstrated that loss of Stat5a staining in breast cancer is associated with metastasis and poor outcome [73]. Unfortunately, a negative marker is not very applicable, as a well-stained counterpart is essential to validate the assay in individual samples. Jirstroem and coworkers analyzed six different antibodies and demonstrated a correlation between strong expression of cyclin D1 and a longer relapse-free period in breast cancer [74]. TMAs can be used in progression models, constructed of different disease stages, such as normal tissue, hyperplastic lesions, *in situ* lesions, invasive tumors, and lymph node and visceral metastases [75].

Tissue microarrays in molecular epidemiology

The majority of publications about TMAs in clinical settings describe screening for known or putative new markers or targets. These papers focus less on determining if a tumor type will respond to a specific therapeutic approach, and are more descriptive. There are various approaches: examining expression levels in a selected entity [76], or on a TMA containing multiple tumor histologies [6]. There are several studies describing screens for established markers as c-ErbB2 or c-Kit in different entities or on multitumor platforms [47,78]. Others have focused on classifying different malignancies based on other parameters including velocity of growth or expression of proteins associated with invasion and metastasis [78,79]. One approach is to screen a panel of antibodies against proteins in different pathways and/or with different cell functions in a shotgun fashion. Alonso and coworkers used 39 antibodies and evaluated a pattern for radial and vertical growing melanoma using multivariate analysis [80].

Unfortunately, many of these TMA surveys do not have strict epidemiology underpinnings that provide a population-based analysis of tumors. The tissue is based on what is available at a particular hospital. The analysis is of tumors examined and lacks a perspective of evaluating different stages of disease or other potentially confounding influences. Other large arraying projects of epidemiology-based tumor collections, including

those from the NCI-sponsored Surveillance, Epidemiology and End Results (SEER) repositories, are now underway. It is hoped they will provide a platform to evaluate diagnostic and prognostic biomarkers in a more population-based approach, and avoid the pitfalls that are commonly encountered when new markers are developed, such as small sample size and ascertainment bias. Other epidemiology studies are incorporating TMAs into their design. These studies extend for years and encompass large cohorts numbering from hundreds to thousands of patients.

Expert commentary: challenges

Although TMAs are being used daily in translation research, this is not to suggest they cannot be improved. As previously noted, two of the greatest challenges in utilizing TMAs are the annotation available on the tissue and the quality of the tissue being utilized. There are broad efforts underway to improve both of these issues, but only time will tell their impact. Currently, the majority of TMAs are constructed manually (FIGURE 2). Automated instruments do exist; however, paraffin-embedded tissue is a challenge to work with, and improvement to the instrumentation is essential.

The one area in which dramatic improvements in technology will impact TMAs is image analysis. Image analysis has been hampered with low numbers of specimens examined and, as a result, unable to demonstrate broad utility. The TMA readily solved this. Recent advances in instrumentation (digital cameras and automated stages) and computers (both speed and storage capacity) now allow researchers to image and store an entire slide at the limits of optical resolution in 20 min or less. Current software is providing reasonable measurement and quantification of IHC. Individual groups are using image analysis and publishing results [44,57]. Improvements are still required, and there are no widely agreed approaches. The capacity for other groups to replicate these experiments as well as the reduction to widespread clinical utility is not at hand. With improvements in data collection, new analytical approaches will be necessary. Many investigators attempt to analyze TMAs as if they were expression microarrays. This approach is often flawed, as the choice of antibodies applied as IHC is directed and, as a result, inappropriate for unsupervised analytical approaches.

Five-year view: tissue microarrays in the future

There remains continued debate on the role of the TMA in clinical care. Some pathologists believe the TMA will be utilized in routine clinical care to reduce cost and improve assay reproducibility. This has been proposed for IHC; however, it is more likely for *in situ*-based protocols, or theoretically for *in situ* single nucleotide polymorphism analysis. Numerous medicolegal issues need to be confronted before the TMA is utilized in this fashion. However, it is anticipated that TMAs will be increasingly utilized in immunopathology as a tool for proficiency testing of staining, and providing reference standards for scoring of samples.

Initially, TMAs were anticipated to drive the development of a host of new diagnostic markers of diagnostic pathology; however, the progress has been slower than expected. This may reflect that there have been more pressing uses of TMAs or the complexity of developing useful IHC markers for diagnosis. As TMAs are used to define profiles of expression levels of proteins, and not to demonstrate the expression of a single protein, the development of useful diagnostic profiles based on multiple markers should be anticipated. Multivariate analysis will be required, and may be reduced to simple algorithms in which results are entered and a diagnosis or prognosis along with confidence intervals is rendered.

Over the next 5 years, as new clinical trials are designed, the TMA will support an increased interest in molecular pathology end points of therapeutic trials. Trials will no longer be measured by only clinical or radiologic response, but will include measures of change in protein expression of targeted therapies. It has been proposed that TMAs will be utilized as a means of identifying patients for enrolment in clinical trials. Hospitals would construct TMAs of all patients' tumors, which would subsequently be assayed for a variety of markers, and based on the profile obtained for a patient, they could be invited to join a clinical trial focused on their disease and the profile of the tumor they possess. It is unlikely that this approach would be taken in the USA. However, in Europe or other parts of the world, this approach would be aimed at increasing participation in clinical trials, and centralizing the task of patient recruitment and enrollment.

In the preclinical environment, TMAs and their variants CMAs and XMA will provide a useful high-throughput tool to measure protein expression that cannot be obtained with western blots. Rather than just large panels of cell lines or xenografts representing a single time point or condition of growth, more complex arrays including multiple samples of the same cell line or xenograft grown under different conditions will be common.

Although TMAs have been used with virtually every assay that involves tissue on a slide, the basic methodology of TMAs will expand to support new approaches to molecular pathology. Instead of obtaining tissue for analysis by PCR from a slide, a core may be used as the source. The authors have developed a method of transforming the TMA to a protein array by transferring the proteins to replicate membranes, which can then be probed as if it were a dot-blot [UNPUBLISHED OBSERVATIONS]. New imaging platforms, including high-throughput confocal microscopy [2], infrared and Raman spectroscopy [23] are being applied to dissect tissue in new ways that were impossible without the high-throughput platform of the TMA.

The TMA is a platform that is here to stay in translational research. Although already a well-defined and robust approach to study the expression of proteins and nucleic acids in tissue, improvements in analysis of TMAs will increase their utility even further. TMAs will expand in their role in drug development, driven by the demands of molecularly targeted therapy. As robust, clinically annotated TMAs become more common, TMAs will contribute new biomarkers for diagnosis and prognosis.

Information resources

- Tissue Array Research Program at the NCI
http://ccr.cancer.gov/tech_initiatives/tarp
(Viewed May 2005)
- Beecher Instruments – tissue array instrument manufacturer
www.BeecherInstruments.com
(Viewed May 2005)
- TMA database exchange
www.biomedcentral.com/1471-2105/5/19
(Viewed May 2005)
- Pediatric Tumor Xenograft Panel
<http://ctep.cancer.gov/highlights/POPPTAPsummary.html>
(Viewed May 2005)

Key issues

- Tissue microarrays (TMAs) are a cost-effective approach for studying protein or nucleic acid expression in tissue.
- TMAs conserve patient material, requiring less tissue per assay than traditional whole-section analysis.
- With hundreds of samples on one slide, analysis of molecular pathways with multiple members or branches can be undertaken, using tools such as multivariate and correlation analysis.
- TMAs can be made from cultured cells (cell line microarrays), xenografts (xenograft tumor microarrays) and animal tissue as well as archival human tissue.
- Cell line microarrays and xenograft tumor microarrays are being used as powerful preclinical screening tools.
- The TMA platform enables image analysis and has become a driving force in the development of image analysis tools.
- Clinical annotation of the tissue on a TMA greatly adds to the value of the platform and enables high-throughput pathology and analysis of outcomes.
- In the future, TMAs will be used as a screening method to integrate patients in clinical trials.

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