Tissue microarrays

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The identification of disease-related genes is a major focus of modern biomedical research. Recent techniques, including array-based platforms for molecular profiling of disease tissues such as DNA arrays for expression profiling or matrix comparative genomic hybridization, allow for the comprehensive screening of the whole genome in a single experiment. Consequently, thousands of candidate genes have already been identified that may be linked to disease development and progression, and the process of lead discovery continues unimpeded. The evaluation of the clinical value of such leads is challenging because thousands of well-characterized tissue specimens must be analyzed. Tissue microarray (TMA) technology enables high-throughput tissue analyses to keep pace with the rapid process of lead discovery. With this technique, up to 1000 minute tissue samples are brought into an array format and analyzed simultaneously. The TMA technology is a fast, cost-effective, and statistically powerful method that will substantially facilitate translational research.

INTRODUCTION

During the past decade, enormous efforts were made to uncover the structure and function of the human genome. With the completion of the human genome sequencing project in early 2001, the base sequences of tens of thousands of genes became publicly available, and the emerging cDNA array technology offered a platform for large-scale transcriptome analysis. Meanwhile, hundreds of publications have reported successful applications of this technology. Comparisons of expression patterns between diseased and nondiseased tissues resulted in the identification of thousands of potentially disease-related genes. However, a major disadvantage of the cDNA array technology is that unfixed tissue samples are required. Such samples are usually taken from prospective studies and lack long-term clinical follow-up information. In addition, large-scale cDNA array-based analyses are often cost-prohibitive. Consequently, there is a strong need for validation of promising candidate genes on routinely archived formalin-fixed tissue samples. Such tissue specimens are available in nearly unlimited numbers in pathology institutes, many of them with associated clinical information. However, often thousands of specimens must be analyzed if experiments aim for

a high level of statistical significance. Molecular genetic analysis methods such as multi-tissue Northern or Western blot analyses, protein arrays, or high-throughput real-time PCR facilities have been utilized for this purpose (1–4). Although these techniques are well-suited for high-throughput analyses, they share the disadvantage that tissues must be disintegrated before analysis. This is a considerable drawback because candidate genes can be expressed in multiple different cell types, and it might also be important to distinguish the cellular localization of the gene products. In situ technologies such as immunohistochemistry (IHC), RNA in situ hybridization (RNA-ISH), or fluorescence in situ hybridization (FISH) are therefore optimal for studying molecular epidemiology. However, large-scale in situ tissue analyses are inconvenient and slow when traditional methods of molecular pathology are used. In addition, it is typically not possible to cut more than 200 regular sections from one tissue block. Cutting traditional tissue sections for a high number of in situ analyses would therefore rapidly exhaust valuable tissue resources. To overcome these shortcomings, we recently developed the tissue microarray (TMA) technique (5–7), which is an advancement of the “sausage” block and the “drinking straw” techniques that were previously

recommended for analyzing multiple different tissues on one slide (8,9). With the TMA method, hundreds of different minute tissue samples are placed on one microscope glass slide and subjected to joint analysis by in situ methods.

Tissue Microarray Technology

TMA technology significantly facilitates and accelerates in situ analysis of tissues because it allows for the simultaneous analysis of up to 1000 tissue samples on a single microscope glass slide (5–7). Minute tissue cylinders (typical diameter: 0.6 mm) are removed from hundreds of different primary tumor blocks (the “donor” blocks) and assembled in an array-like format into one empty “recipient” block (Figure 1).

Sections from the TMA blocks can be used for simultaneous in situ analysis of hundreds to thousands of tissue samples. Virtually all types of analysis that can be applied to tissue sections, including IHC, FISH, or RNA-ISH can be used on TMA sections, without significant modifications to existing protocols (Figure 2). The small diameter of the specimen taken out of the donor block maximizes the number of samples that can be taken out of one donor block and minimizes tissue damage. Punched tissue blocks remain fully

interpretable for all morphological and molecular analyses that may subsequently become necessary. Dozens of punches can usually be taken from one tumor without compromising the integrity of the donor blocks.

Most of the work required for TMA relates to logistics. (i) Histologic samples meeting the requirements for the intended array must be identified from databases. (ii) All slides from all these cases must be collected from the slide archive and carefully reviewed by a pathologist. (iii) Representative areas must be marked on the slide to guide the person who will do the punching

work. (iv) The corresponding paraffin blocks must be collected. Depending on the size of the planned TMA, tens of thousands of glass slides and thousands of paraffin blocks may have to be moved. (v) A database must be generated that includes the specimen identifier and all histologic and clinical information. Comprehensive software solutions facilitate the high-throughput analysis and storage of TMA immunostainings (10).

The TMA construction comprises less than 5% of the entire work. Its automation, therefore, cannot substantially accelerate the whole TMA

construction process. The key components of most tissue arraying devices are two needles with a slightly different diameter that can be moved at high precision. With the smaller needle (outer diameter: 0.6 mm), holes are punched into empty recipient paraffin blocks. Subsequently, a slightly larger needle (inner diameter: 0.6 mm) is utilized to transfer tissue cylinders from preexisting donor paraffin blocks into these premade holes at specific coordinates. Regular microtomes can be used to cut TMA sections. To minimize the loss of material during the cutting process, a commercially available adhesive tape system (Paraffin Tape-Transfer System; Instrumedics, Hackensack, NJ, USA) can be utilized.

Paraffin-embedded tissues are mostly used for constructing TMAs because of their availability and easy handling. However, frozen tissue samples can also be utilized (Figure 3) (11,12). Instead of paraffin, Tissue Tek® O.C.T.™ Compound (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands) is utilized as a carrier material. In general, the same arraying devices can be used for both paraffin and frozen TMA manufacturing. However, needles are at a higher risk of bending and breaking than in the case of paraffin-embedded tissues. Frozen arrays are very useful if paraffin-suitable antibodies are not available for the target protein or if different activation states are to be analyzed (e.g., by phosphorylation site-specific antibodies). Frozen TMAs may also be superior for fluorescence-based protein quantification experiments because of their lower autofluorescence compared to formalin-fixed tissues.

Representativity of Tissue Microarrays

Despite the small diameter of the punched tissue cores (usually 0.6 mm), TMAs appear to offer a high degree of representativity compared to whole section analyses of their donor tissues. Obviously, tissue heterogeneity is the most critical issue with TMA analysis. Hodgkin's lymphoma is a paradigm for tissue heterogeneity because it predominantly consists of reactive inflammatory cells with only a few dispersed neoplastic Hodgkin or Reed Sternberg cells. In

Figure 1. Tissue microarray (TMA) manufacturing. (A) Arraying device. (A and B) Two hollow needles can be moved with high precision into predefined positions of the donor block (containing the tissue sample) or the recipient block in which the TMA is built. (B) A 0.6-mm hole is punched in the recipient block using needle B (outer diameter: 0.6 mm). (C) A tissue cylinder is punched from the donor block using needle A (inner diameter: 0.6 mm). (D) The tissue cylinder is released into the hole of the recipient block. (E) Tissue cylinder sitting on the tip of needle A. (F) Donor block showing only little damage even after multiple punches. (G) Completed TMA.

Figure 2. Examples of tissue microarray (TMA) applications. (A) Hematoxylin and eosin (H&E)-stained section of a bladder cancer TMA containing 540 tissue spots. (B) Magnification of an H&E-stained tissue spot measuring 0.6 mm in diameter. (C) RNA in situ hybridization (RNA-ISH) analysis. mRNA expression of the target is detected with a radioactively labeled antisense oligonucleotide probe. Black staining indicates the cells that express the target gene. (D) Immunohistochemistry (IHC) analysis of a tissue spot detecting Her2 protein expression. The inset shows a magnified section of the breast cancer TMA tissue spot with strongly positive (3+) membranous staining. (E) Fluorescence in situ hybridization (FISH) analysis. The tissue spot is counterstained with a blue fluorescing dye to allow for the identification of cell nuclei. The inset shows a magnification of some cell nuclei with specific signals for the *HER2* gene (red signals) and the centromeric region of chromosome 17 (green signals). The high ratio between the red and green signals indicates a *HER2* gene amplification.

such a setting, one might expect that TMA analysis would not be successful. However, three studies have demonstrated that even Hodgkin's lymphoma can be analyzed with the TMA approach (13–15). For example, Hedvat et al. (14) analyzed a TMA containing Hodgkin's lymphomas for CD20, CD30, CD15, Epstein-Barr virus (EBV) latent membrane protein 1 (*LMP-1*), *EBER-1*, and *EBER-2* expression on conventional full sections with the corresponding tumor cores on the TMA. The results from Hodgkin's lymphomas on TMA sections matched exactly with those of the corresponding whole sections.

At least 20 additional studies have compared IHC findings on TMAs and their corresponding traditional “large” sections from solid cancer specimens (13–32). The vast majority of these studies revealed a high level of concordance of results (14–25,27–29,31) and found that two or three samples taken from different regions of each donor provided more representative information than a single sample (19,24,27,29,31). The addition of more than four or five samples did not lead to a massive improvement

in concordance level (19,27).

It is important to realize that all of these studies were based on the assumption that conventional whole sections—the gold standard for molecular tumor tissue analysis—were representative of the entire tumor bulk. However, consid-

ering the tiny volume (0.0018 cm^3) of a typical whole tissue section ($3 \times 2 \text{ cm}$; thickness, $3 \mu\text{m}$) compared to a large tumor bulk (a tumor with a diameter of 1 cm has a volume of 0.52 cm^3), it becomes evident that this assumption is likely to be false. Rather than comparing results obtained on large sections and TMA sections, studies should therefore determine whether or not established associations between molecular features and tumor phenotype or clinical outcome could be found in a TMA setting. In fact, all TMA studies addressing this issue (20–22,33–36) were able to fully reproduce known associations between molecular changes and previously well-established clinicopathological associations, such as between estrogen or progesterone expression (22) or *HER2* alterations (35) and survival in breast cancer patients; vimentin expression and prognosis in kidney cancer (20); as well as between Ki67 labeling index and prognosis in urinary bladder cancer (21), soft tissue sarcoma (33), and Hurthle cell carcinoma (34).

Tissue Microarray Applications in Research

TMAs can be utilized for all kinds of tissue-based research, and their application is not limited to cancer research. However, as of yet, TMA studies have primarily addressed oncologic questions. In principle, three different kinds

Figure 3. Tissue microarrays (TMAs) of frozen tissues. (A) Multitumor TMA made from frozen tissues of different organs. The bigger part of the TMA contains tumor tissues, and the smaller part at the bottom contains normal tissues of the corresponding organs. (B) Frozen TMAs often become more irregular than TMAs from paraffin-embedded tissues. Therefore, a larger spot-to-spot distance was chosen to avoid confusion if particular spots shifted during sectioning. (C) Magnification of a hematoxylin and eosin (H&E)-stained tissue spot (0.6 mm) showing good preservation of histology.

of arrays have been utilized for this purpose; namely, prevalence TMAs, progression TMAs, and prognostic TMAs.

Prevalence TMAs contain tumor samples from one or several tumor entities without further clinicopathological information. They are used to evaluate the prevalence of a particular marker in a tumor population of interest. An ideal prevalence TMA would contain tissue samples from various different tumor entities. It could be used to comprehensively study the epidemiology of molecular features in all types of human neoplasia. Maximal standardization of TMA experiments allows for an effective comparison of frequencies of alteration between different tumor types. In an early study, the known frequencies of amplification for cyclin-D1, c-myc, and *HER2* were confirmed in a multitumor TMA containing 17 different cancer types (36). The largest multitumor TMA manufactured in our laboratory to date contains 4788 different samples from 130 different tumor types (37,38). This TMA has been successfully utilized for the analysis of multiple different markers on the DNA and protein levels. In one study, the frequency of cyclin E amplification and overexpression was analyzed by FISH and IHC, which yielded a comprehensive ranking list of tumor types with cyclin E alterations (Table 1) (38). Of course, prevalence TMAs can also be made from nondiseased normal tissues. Normal tissue TMAs are particularly instrumental for addressing questions related to pharmacology research. For example, if a new drug is developed, then it is crucial to explore the normal tissue distribution of the target protein to estimate potential side effects of the therapy. The placement of small TMAs containing a variety of normal tissues on slides that are used for diagnostic IHC has also been suggested for providing negative and positive control tissues (39).

Progression TMAs contain samples of different stages of one particular tumor type (5,40). For example, an ideal breast cancer progression TMA would contain samples of normal breast from patients with and without breast cancer history, several different non-neoplastic breast diseases, ductal and lobular carcinoma in situ, invasive cancers of all

stages, grades and histologic subtypes, as well as metastases and recurrences after initially successful treatment. Progression TMAs have been successfully utilized to detect associations between tumor phenotype and genotype in a wide variety of cancer types. For example, relevant findings include associations between cyclin E (41), *FGFR1*, *RAF1* (42), *MDM2*, or *CDK4* (43) amplification, or *MAGE-A4* expression (44) and stage and grade in bladder cancer; *CK7* and *CK20* expression and grade in colorectal carcinoma (45); *IGFBP2* expression and hormone-refractory state (46); *EIF3S3* amplification and stage (47); E-cadherin expression and tumor size (48) in prostate cancer; or *SHP1* expression and tumor development in lymphomas (49). Progression TMAs are also suited to study potential heterogeneity between primary tumors and their metastases. In one such study, we used a TMA to demonstrate a high concordance in the *HER2* amplification/overexpression between 192 primary breast cancers and their corresponding lymph node metastases (50).

Prognosis TMAs contain samples from tumors with available clinical follow-up data. They are optimally suited to evaluate suspected associations between genetic alterations and clinical outcome. A multitude of studies has used prognosis arrays to detect or reevaluate associations between molecular alterations and patients' prognoses, such as in bladder (41,43,51), breast (18,22,35,52–54), prostate (40,55,56), brain (28,57,58), liver (59), kidney (20), colorectal tumors (60–62), Hodgkin's lymphoma (13), and malignant melanoma (63). For example, significant associations were established between 17q23 amplifications (35) or *COX2* expression (52) and breast cancer prognosis; *TOP2A* expression and prognosis in glioblastoma (57) and in bladder cancer (64); *MYC* or *AIB1* expression and prognosis in hepatocellular carcinoma (59); and between *IGFBP2* and prostate cancer prognosis (46).

TMAs have also been constructed from a variety of experimental tissues including cell lines (43,65) or xenografts (46). For example, cell line TMAs can be easily made from formalin-fixed paraffin-embedded cell chunks and are

Table 1. Results of a Multitumor Tissue Microarray Analysis of Cyclin E Alterations

Tumor Type	<i>n</i> ^a	Cyclin E Alteration		
		Amplification Only (%)	Expression Only (%)	Amplification and Expression (%)
Urinary bladder, small cell carcinoma	3	66.7	33.3	0.0
Malignant fibrous histiocytoma	3	66.7	0.0	33.3
Urinary bladder, adenocarcinoma	2	50.0	0.0	50.0
Gall bladder, adenocarcinoma	5	20.0	0.0	20.0
Nonserous ovarian cancer	11	18.2	18.2	0.0
Urinary bladder, invasive carcinoma	23	17.4	0.0	4.3
Serous ovarian cancer	40	15.0	0.0	7.5
Endometrial carcinoma	88	11.4	1.1	0.0
Uterine cervix squamous cell carcinoma	9	11.1	11.1	0.0
Stomach adenocarcinoma	29	3.4	0.0	17.2
Medullary breast cancer	4	0.0	0.0	25.0
Small intestine adenocarcinoma	3	0.0	0.0	33.3
Rhabdomyosarcoma	1	0.0	0.0	100.0

In a single experiment, 13 of 128 tumor types were identified that may be affected by cyclin E amplification and/or overexpression.
^aTotal number of samples showing any cyclin E alteration.

especially suited to quickly identify cell lines expressing a particular gene for subsequent functional analyses.

Future Developments

The TMA technology provides the platform to keep pace with high-throughput lead identification methods such as cDNA or protein arrays. Whereas TMAs were limited to a few academic institutes in the early phase after development, they have now become broadly available through nonprofit organizations such as the National Human Genome Research Institute (National Institutes of Health, Bethesda, MD, USA) (<http://research.nhgri.nih.gov/tma/>), universities, or from commercial sources. The TMA technology has successfully passed the evaluation phase and is an established standard tool. It can be expected that a large fraction of tissue-based research will be carried out on TMAs in the future and that the TMA reading process will become a major new bottleneck that limits the rate of research throughput. Automated TMA analysis using sophisticated hardware and software tools is an emerging field that will significantly contribute to the resolution of this problem.

In principle, TMAs are well suited to automated analysis. However, there are two major issues associated with automation. First, the area for auto-

mated tissue analysis has to be carefully selected. This has already been resolved during TMA manufacturing. Second, it is important to distinguish between tumor cells and nonmalignant tissue components including stroma, inflammation, or non-neoplastic epithelium that may obscure the outcome

of analysis. Some investigators have significantly contributed to technical solutions that will help to resolve this issue. Rao et al. (66) adapted their quantitative fluorescence image analysis (QIFA) system for TMA analysis. Compared to classic IHC, QIFA analysis allows for the detection of target proteins with strongly enhanced sensitivity. Camp et al. (67) developed the automated quantitative analysis (AQUA) system using different fluorescence tags to separate tumors from stroma and to define subcellular compartments (67). Haedicke et al. (68) recently applied simultaneous double-direct immunofluorescence detection of one test and one reference antigen to normalize for the cellular content of detectable protein in each TMA spot. Custom-built software was utilized for these studies; however, there are also commercially available solutions suitable for TMA analysis, such as the iCsy™ (CompuCyte, Cambridge, MA, USA) or ARIOL system (Applied Imaging, Newcastle Upon Tyne, UK). Although the superior sensitivity and multicolor properties of fluorescence-based IHC TMA analyses are trendsetting, they also have important limitations compared to classic IHC.

Figure 4. Tissue microarray (TMA) analysis: manual versus automated. (A) Immunohistochemistry showing progesterone receptor (PR) expression (brown nuclear staining) in a breast cancer tissue spot. (B) Same image as in panel A, with green color indicating automated thresholding for positive PR staining. Survival plots from both (D) manual and (E) automated analyses indicated the known shortened survival time for patients with the loss of PR expression.

Complex staining protocols have to be developed and evaluated, which is a time-consuming and expensive process. In addition, manual reevaluation of fluorescence stainings is difficult if questionable stainings need morphological clarification; even the most sensitive label does not resolve the inherent problems of IHC, primarily caused by inhomogeneous tissue processing.

Therefore, several companies concentrate on automated analysis of conventionally immunostained TMA sections. Most systems are based on an automated microscope with a charge-coupled device (CCD) camera attached to it. An overview image is generated from several tiled images at low magnification, which is used to identify the localization of each tissue spot in the TMA for subsequent high-resolution scanning. For example, the BLISS system (Bacus Laboratories, Lombard, IL, USA) or the Virtual Slice Module (MicroBrightField, Williston,

VT, USA) allow the user to create a virtual slide that supports navigation and different magnifications similar to a real microscopic slide. ACIS[®] (ChromaVision, San Juan Capistrano, CA, USA) is specifically designed for routine colorimetric analysis of immunostainings and has been adapted to TMA technology. *i*VISION[™] (Bio-Genex, San Ramon, CA, USA) and TMA-Discovery (Molecular Devices, Sunnyvale, CA, USA) are similar systems for research purposes. Aperio Technologies (Vista, CA, USA) and Trestle (Newport Beach, CA, USA) have developed systems that use a scanner instead of a CCD camera, allowing for image acquisition of a typical 20 × 30 mm TMA area in less than 20 min. For existing automated hardware, software solutions such as Array^{f(x)}[™] from TissueInformatics (Pittsburgh, PA, USA) are available for automated analyses.

Compared to the fluorescence-based

approach, these “low tech” solutions that only quantify the total signal intensity per TMA spot are easy to establish. For example, the analysis of multiple markers with our custom-made system revealed the expected associations with outcome information in all cases. For example, the results obtained for manual and automated progesteron receptor staining analysis in breast cancer are shown in Figure 4 (M. Ramseier, S. Hänggi, J. Wirth; personal communication).

The management of data from TMA studies is another challenge. Simple spreadsheet software can be used to collect raw data, however, the integration of spot images or statistical analysis tools will require more sophisticated software. For example, Bova et al. (69) presented a Web-based application for storing and viewing TMA images, and a fully relational database for TMA data handling has been build by Manley et al. (70). A collection of TMA software tools is also

available in the public domain (e.g., from the Stanford TMA Consortium at http://microarray-pubs.stanford.edu/tma_portal/index.shtml).

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