

Tissue microarrays for high-throughput molecular pathology

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Summary: Modern research technologies, including DNA, protein, and antibody microarrays identify a steadily growing number of clues that are useful in molecular disease classification, drug development, and the prediction of response to treatment. Subsequent validation of the clinical importance of such candidate genes or proteins requires large-scale analysis of human tissues. To date, this analysis constitutes an important bottleneck in the process of discovery because tissue analysis by the conventional slide-by-slide strategy is slow and expensive. To overcome these limitations, tissue microarray (TMA) technology has been developed. TMA allows for the simultaneous analysis of up to 1,000 tissue samples in a single experiment, using all types of in-situ analyses including immunohistochemistry (IHC), fluorescence in situ hybridization (FISH), and RNA in situ hybridization (RNA-ISH). TMA technology has the potential to greatly facilitate the translation of basic research into clinical practice. Potential applications include the establishment of associations between molecular changes and clinical endpoints, testing of potential therapeutic targets using tissue samples from specific cancer patients, standardization of molecular detection of targets, and rapid translation of results from cell lines and animal models to human cancer. Because of its beneficial economic aspects and ability to differentiate ethnic differences in tumor biology, TMA applications may become particularly important in developing countries.

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The sequence of the human genome has recently been unraveled.^{1,2} At the same time, new methods have been introduced that allow for a comprehensive expression analysis of the thousands of genes on the RNA or protein level in a given tissue. As a result, a rapidly increasing number of genes have been identified that may play a role in cancer or other diseases. It is hoped that these findings will eventually lead to clinically useful applications. However, the number of potentially useful candidate genes poses significant problems in the evaluation of their potential utility. Large-scale functional gene analyses and molecular epidemiologic studies are needed for this purpose. Collecting data on molecular epidemiology is often a significant challenge for basic researchers due to limited availability of human tissues with clinical information. Tissue microarray (TMA) technology greatly facilitates large-scale tissue analyses. In this method, up to 1,000 different minute tissue samples can be brought onto one microscope glass slide, and be simultaneously analyzed by all types of in-situ methods.

Tissue microarray methodology

In tissue microarray (TMA) methodology, minute tissue cylinders (typical diameter: 0.6 mm) are taken from various primary tumor blocks (the "donor" blocks) and

subsequently assembled in an array-like format into one empty "recipient" block (Figure 1). Simple and inexpensive arraying instruments can be used for this purpose. Regular microtomes can be used to cut sections from these TMA blocks. Virtually all types of in situ tissue analyses can be done on TMA sections without significant modifications of existing protocols. These include immunohistochemistry (IHC) for protein detection, fluorescence in situ hybridization (FISH) for detection of copy number changes of genes or chromosomes as well as RNA in situ hybridization (RNA-ISH) for quantification of RNA expression levels (Figure 2). Importantly, the small diameter of the specimen taken from each donor block minimizes the damage to patient tissue that might be needed for additional diagnostic procedures. This allows for extensive use of diagnostic tissue samples for research purposes, and at the same time retains sufficient tissue material for additional diagnostic procedures if needed.

TMA's are optimally suited for large-scale expression profiling projects. More than 400 punches can theoretically be obtained from a tissue with a diameter of 25 mm if the entire block can be used for research. All these cores can be placed on 400 different copies of a particular TMA block. As 200 to 300 sections can be taken per TMA block, between 80 000 and 120 000 analyses could be done from a set of TMA's. This means that TMA's would allow the expression

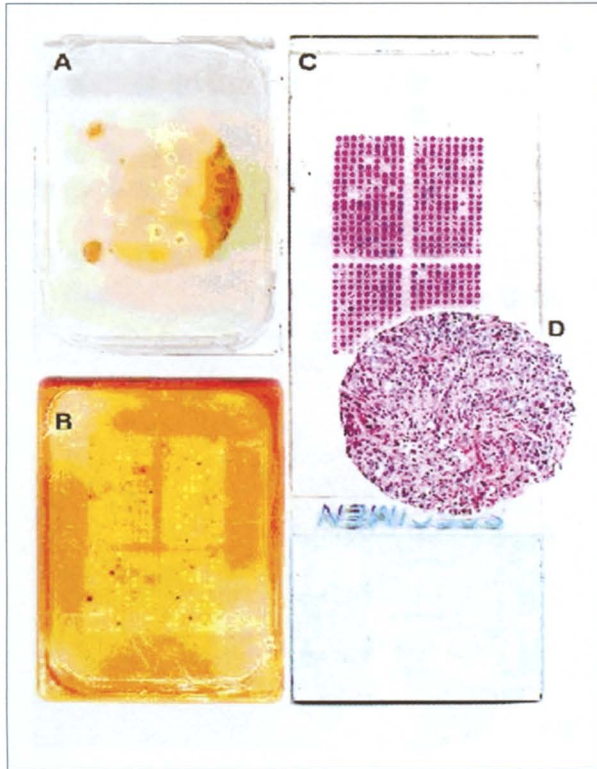


Figure 1. Tissue microarray (TMA) manufacturing. A) Donor block from which several 0.6 mm tissue cores have been removed. Note that the original tissue block remains fully interpretable. B) Recipient block with the completed TMA. C) Hematoxylin & eosin stained tissue section of the TMA. D) Magnification of a tissue spot.

analysis of all human genes in one set of tissues. TMAs can be made not only from paraffin embedded tissues, but also from frozen tissue samples (Figure 3).^{3,4} Frozen TMAs are well suited for RNA-ISH detection of low-level transcripts, or for IHC if antibodies that work on formalin fixed tissues are unavailable.

General applications

TMAs can be used for many different applications. Most research requiring in-situ tissue analysis can be done in a TMA format. More than 150 publications using the TMA approach have now been published. Most have used TMAs in cancer research to investigate the prevalence of molecular changes and their associations with tumor progression and/or prognosis. However, even simple TMAs containing tissues without any clinical background information can be used for collecting significant new information on the epidemiology of molecular changes in different tumor types.

In one study, a "multitumor" TMA was used to analyze 4788 different samples from more than 130 different tumor categories for cyclin E amplification and overexpression.⁵ More information on the importance of a specific change in one particular tumor type can be collected by using

better-characterized tissues with information on the pathology and/or the clinical outcome. A multitude of studies used TMAs to find associations between molecular alterations and tumor phenotype. For example, associations between cyclin E,⁶ FGFR1, RAF1,⁷ MDM2 or CDK4⁸ amplification or MAGE-A4 expression⁹ and stage and grade were found in urinary bladder cancer. CK7 and CK20 expression were associated with histologic grade in colorectal carcinoma.¹⁰ Several clinico-pathological associations were observed in prostate cancer studies. For example, IGFBP2 expression was related to a hormone-refractory state,¹¹ and EIF3S3 amplification was linked to advanced tumor stage.¹² TMAs were also successfully used to investigate potential heterogeneity between primary tumors and their metastases. Simon et al. previously used a TMA composed of paired primary tumors and metastases from 196 breast carcinomas to demonstrate a high concordance in the HER2 amplification/overexpression between primary tumors and their nodal metastases.¹³ Various molecular features were analyzed for their prognostic significance on TMAs with samples from patients with clinical follow up information, for example in bladder,^{6,8,14} breast,¹⁵⁻²⁰ prostate,²¹⁻²³ brain,²⁴⁻²⁶ liver,²⁷ kidney,²⁸ and colorectal tumors,²⁹⁻³¹ Hodgkin's lymphoma,³² and malignant melanoma.³³ Studies using prognosis TMAs have yielded a considerable amount of novel information. For example, significant associations were found between 17q23 amplifications¹⁸ or COX2 expression¹⁵ and breast cancer prognosis, between TOP2A expression and prognosis in glioblastoma,²⁴ between MYC and AIB1 expression and prognosis in hepatocellular carcinoma,²⁷ and between IGFBP2 and prostate cancer prognosis.¹¹

In all these studies, maximal standardization of the procedure is a major strongpoint of the TMA method. Very often all tissues analyzed are located on one glass section and treated under absolutely identical conditions, including reagent concentrations, temperature, pretreatment, antigen retrieval, slide age, and section thickness. This eliminates the many possible pitfalls of molecular diagnostics and assures maximal comparability of results within one study. We see great applicability of TMA technology not only to basic cancer research but also to anatomical pathology (Table 1).

Tissue microarray applications in developing countries

So far, TMA technology has predominantly been used in European and U.S. laboratories. However, the TMA method may have even greater importance in developing countries. Recently, studies have suggested significant genetic differences between cancers occurring in different ethnic groups. For example, significant differences were found in the pattern of gene amplification between European and Arabian breast cancers (Al Kuraya, unpublished), and between the frequency of HER2 and nm23 expression

between British and Japanese gastric cancers.³⁴ As the management and treatment of cancer patients is increasingly based on molecular tumor characteristics, ethnic variations may be of practical significance.

Currently, our knowledge of the prevalence and clinical significance of molecular tumor features is based on data collected on Western tumors. It now appears possible that not all conclusions drawn from these studies are applicable to patients from other ethnic backgrounds. Large-scale surveys on the molecular epidemiology of clinically important molecular changes are necessary to better define the dimension of ethnic differences in tumor biology. TMAs represent an ideal tool to rapidly determine the epidemiology of important molecular features in different ethnic groups. Once the necessary tissues have been collected and the TMAs manufactured, all these studies could easily be done in a short period of time.

Economical factors are another strong reason for using TMAs. Studies have confirmed that TMAs help to save reagents, manpower and money because one can investigate up to 1000 tumors in one glass slide section with the same amount of reagent that was previously necessary to analyze one tumor (in the large section format). So, in laboratories with limited financial resources one of the major advantages of TMA is the small amount of reagents needed for scientific studies. For example, more than 10 000 tumors have been analyzed in the University of Basel for amplification. If regular sections were used for this purpose, the reagent costs would have exceeded US\$200,000 per 10,000 tumors. In the TMA setting, we stained 30 slides, containing 10 000 tumor sections for about \$600 (Table 2).

We hope that strong collaborative projects can be initiated in the future that involve research laboratories in different developing countries. Such collaborations are highly important to clarify whether patients from different ethnic backgrounds are equally prone to benefit from modern targeted cancer therapies. TMAs will be optimally suited to highlight such potentially important ethnic differences in a cost effective manner.

Is representativity a limitation of tissue microarray technology?

The most significant and obvious limitation to the TMA technique is the size of the arrayed tissues, which measure only 0.6 mm in diameter. Initially, the method was criticized because it was questioned whether such small tumor samples could be representative and give meaningful information for an entire tumor. To address this question, at least 20 studies have compared IHC findings on TMAs with their corresponding traditional "large" sections—the current gold standard for molecular tumor tissue analysis.^{16,20,26,28,32,35-49}

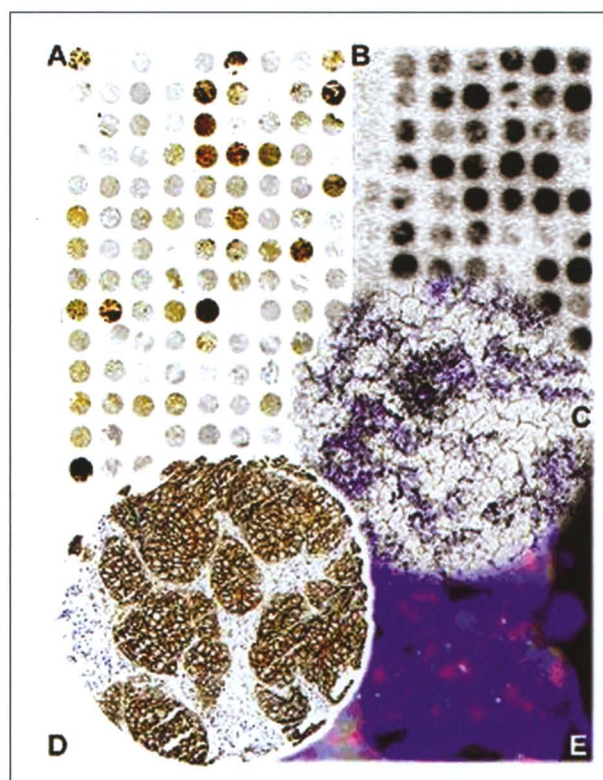


Figure 2. Tissue microarray applications. Overview of TMA sections investigated by A) immunohistochemistry and B) RNA-ISH. C) Tissue spot showing focal expression (black granular staining) of BTG2 mRNA as investigated by RNA-ISH. The TMA section was coated with a photographic emulsion for detection of a radioactively labeled antisense-RNA probe. D) Tissue spot from a breast cancer array showing strong (3+) membranous staining of the HER2 protein by immunohistochemistry. E) Sector from the same tissue spot as in D) analyzed by FISH, using probes detecting HER2 gene amplification (clusters of red signals) and centromere 17 copy numbers (green signals).

Table 1. Potential applications of tissue microarray technology.

- Establishment of associations between molecular changes and clinical endpoints
- Testing of potential therapeutic targets using tumor samples from specific cancer patients
- Standardized molecular detection of targets
- Rapid translation of results from cell lines and animal models to human cancer

Table 2. Cost analysis of 10,000 tumors.

Large section method	Tissue microarray method
1 tumor per slide @ \$20 per slide	334 tumors per slide @ \$20 per slide
10 000 tumors = \$200 000	10 000 tumors = \$600

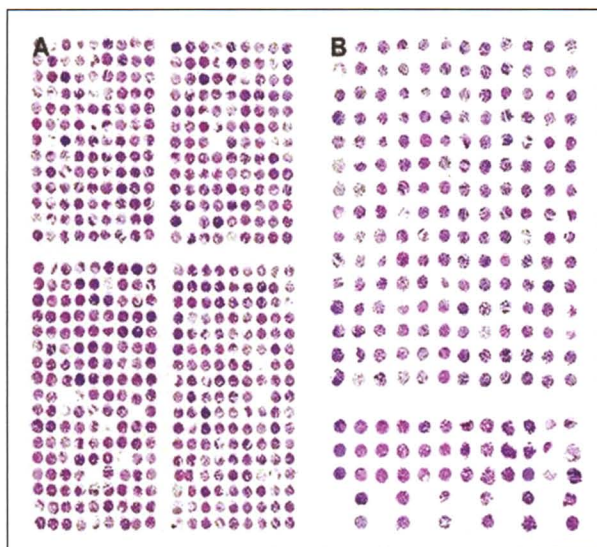


Figure 3. Tissue microarrays from paraffin (A) and frozen tissues (B). TMAs from frozen tissues become more irregular and distorted than TMAs from formalin fixed material because the commercially available arrayers have not been designed for frozen array making. Therefore a larger space between samples is recommended (e.g., 1 mm).

A high level of concordance has been found in most of these studies.^{16,20,26,28,35-42,44,45,47,48} Some results suggest that two or three samples may provide more representative information than a single sample,^{38,41,44,45,48} and that adding more than four or five samples does not improve the concordance level to any important extent.^{38,44} However, it is necessary to understand that the general assumption of classical large sections, that they are representative of an entire tumor, may be wrong. In the optimal case, a “large” section will contain about 0.0018 cm³ of tumor tissue (3 cm x 2 cm x 3 μm). This is only about 1/7500 of 14 cm³, which represents the approximate volume of a tumor measuring 3 centimeter in diameter. Considering these numbers, the representativity problem is obviously much greater between the entire tumor and a traditional “large” section than between a TMA sample and a “large” section.

Rather than comparing results obtained on large sections with TMA sections, studies validating the TMA technique should determine whether associations between molecular features and tumor phenotype or clinical outcome can be found in a TMA setting. In fact, numerous studies have confirmed previously well-established associations between molecular findings and clinical outcome.^{18,28,39,50-53} For example, significant associations were found between estrogen or progesterone expression⁵² and HER-2 alterations¹⁸ and survival in breast cancer patients, between vimentin expression and prognosis in kidney cancer²⁸ as well as between Ki67 labeling index and prognosis in urinary bladder cancer,³⁹ soft tissue sarcoma,⁵⁰ and Hurthle cell carcinoma.⁵¹ Studies

that successfully used tissues from Hodgkin's lymphoma for analyses on TMAs provide further evidence that highly heterogeneous tumors can be analyzed in a TMA format.^{32,37,47}

Future developments

Currently, the availability of TMA sections is limited. TMAs that are available from commercial sources or from non-profit organizations are often small and have little clinical data attached. Academic sources that have large TMA repositories are overloaded with requests for collaboration and cannot meet all demands. However, the number of institutions with TMA facilities is increasing. Virtually all institutions dealing with tissue analyses will be using TMAs in the future. Also the quality of TMAs will improve as more are made from particularly precious tissue resources like rare tumor types at referral centers or tissues from patients included in clinical trials. In fact, multiple clinical trial groups are now implementing TMAs as a part of their trial protocols.

Automation of TMA analysis is another major need since manual TMA reading is cumbersome, slow and subjective. TMAs are optimally suited for automated IHC analysis because the most critical step for automation—the selection of the area to be analyzed—has already been accomplished. In a “low tech” approach, the automated analysis would more or less be limited to measurement of the total signal intensity per tissue spot. Although this approach cannot distinguish neoplastic from non-neoplastic epithelial cells or from stroma cells, our home-made system revealed the expected associations with outcome information in all cases. More sophisticated analyses could include multicolor fluorescent detection systems for IHC or intelligent pattern recognition software solutions. In contrast to TMA analysis, automation of tissue arraying devices has little importance because the availability of tissues and their pathological characterization, and not the arraying process itself, is the rate-limiting step in TMA manufacturing.

Large databases that include the results of in situ analyses obtained on large and well-documented TMA resources constitute another expected development. Such databases may not only contain the staining results of all tissues and related clinico-pathological information, but potentially images of each individually stained spot. The total size of such databases will be enormous, as they will include millions of images. Implementation is largely dependent on the development of suitable data storage solutions.

TMAs will significantly contribute to an accelerated transition of laboratory observations to clinical applications. Once a gene product is measurable by an in situ test, it is possible, in one examination, to analyze all normal tissues, and all different tumor types, as well as a variety of tissues from non-neoplastic diseases to immediately determine where the gene of interest might have clinical utility.

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