Microarray

A supporting material (as a glass or plastic slide) onto which numerous molecules or fragments usually of DNA or protein are attached in a regular pattern for use in biochemical or genetic analysis.

Merriam-Webster Dictionary of Medical Terms
Chapter 12.1 | Introduction

Recent times have seen the advent of high throughput assays such as array comparative genomic hybridization, cDNA microarray and Next Generation Sequencing techniques, which have led to the rapid discovery of thousands of potential biomarkers. However, these need to be validated in tissue-based studies in large datasets to prove their potential utility. As these datasets are typically present in the form of formalin-fixed, paraffin-processed tissue blocks, immunohistochemical (IHC) methods are ideal for validation. However, performing whole-section IHC on hundreds to thousands of blocks requires a lot of resources in terms of reagents and time. Analysis needs to be batched; batch to batch variability could interfere with the analyses. In addition, an average block will yield less than 300 slides of 5 µm each. The tissue microarray (TMA) technique circumvents some of these problems.

The origin of TMAs can be attributed to Dr Hector Battifora’s humble ‘sausage’ blocks (1), in which a number of tissues, typically from different organs, were thrown together in the same block and the tissue distribution of a particular antigen/protein was assessed. A significant disadvantage of this technique was that when tumors or tissues from the same site were put together it was difficult, if not impossible, to trace them back to the patient. This prevented meaningful analysis of prognostic markers. However, many laboratories, small and large (including our own), adopted this technology to generate multi-tissue or multi-organ tissue blocks. The next step in the development of TMA was described by Wan et al. (2) who used a 16-gauge needle to manually bore cores from tissue blocks and array them in a multi-tissue straw in a recognizable pattern. This method was further modified by Kononen et al. (3) using a 4 mm skin biopsy punch. They used a cast of a small amount of melted paraffin to record the position of each punch specimen. This landmark study lead to the development of a TMA precision microarray instrument with an x-y guide by Beecher Instruments (Sun Prairie, WI). This device enabled real high-throughput analysis, with arraying of up to 1,000 cores in the same block.

Chapter 12.2 | Advantages and Disadvantages of TMAs

Advantages

The major advantage of TMAs is that they allow the performance of tissue-based assays (immunohistochemistry, histochemistry, in situ hybridization, etc.) on a large number of patient samples in an efficient and cost-effective manner. With TMA technology, several hundred representative cores from several hundred patients may be included on a single glass slide for assay. Thus, significantly more tissue can be conserved than if the blocks were to be sectioned serially. TMAs have been generated from all tissue types, including decalcified bone and core biopsies. The latter are usually rotated 90 degrees and embedded vertically to ensure presence of tissue of interest in multiple cuts. In addition, methods for generating TMAs from fresh frozen tissue using blocks made from either optimal cutting temperature compound, or from a mix of gelatin-sucrose, have been described (4). More recently, a technique for developing ‘patch’ TMAs from unstained slides has been described (5).
Disadvantages

The major disadvantage of TMAs is that each core (or set of cores) represents a fraction of the lesion. This was considered a major weakness, particularly in the early days of the TMA. However, multiple studies in different organ systems have now demonstrated that consistent and comparable results can be obtained using TMA cores as with whole sections. In order to obtain comparable results two main strategies have been used. The first is increasing the number of cores from each case. It is typical in breast cancer to use at least two cores from each case when using a 1 mm core; a minimum of three cores from each case for 0.6 mm cores. The number of cores may vary according to the disease site; for example, it is typical to use five cores in colon cancer. Although a 2 mm core might be theoretically considered better than multiple smaller cores, in practice this is usually not true. Smaller cores permit sampling of different tumor areas and are, therefore, more likely to be representative of the entire tumor. In addition, smaller cores tend to inflict a lesser degree of damage on the original tissue blocks. The second strategy consists of increasing the number of tumors included in the study. This method averages out the errors that might result from tumor heterogeneity associated with the use of tissue cores.

When Not to Use TMAs

TMAs are not recommended for certain types of studies. In certain tumors such as glioblastoma, there is such marked heterogeneity within tumors that this feature may not be adequately captured in TMA studies. In addition, TMAs are also not very useful to study rare or focal events, such as number of immune cells in tumors. It is also difficult to study certain facets of tumor biology, such as interactions between the tumor and its stroma, as these stromal components may not be adequately represented in the cores. The use of large cores (2 mm) has been advocated for these types of studies.

Chapter 12.3 | Types of TMAs

The type of TMA to be generated depends on the question being asked within the study. The following are the commonly used types:

Cell Line Arrays

These arrays consist of normal or cancer cell lines that are grown in culture. The major function of these arrays is to survey the presence of proteins that are known to be present in one or more of the cell lines. In addition, cell line arrays can be used to analyze the utility (plus sensitivity and specificity) of an antibody in detecting proteins. The most common example of this type of array is the 3-cell line control that is used with HER2 testing in breast cancer.

Random Tissue/Tumor Arrays

These arrays contain tissues from multiple sites and contain tumor and/or non-tumor tissues. Small arrays of this kind can be used for quality control measures, such as monitoring of existing reagents/antibodies, as well as work-up of novel re-agents. In addition, they can also be used as discovery tools. For example a survey of CD10 in tumors arising in multiple tissue sites lead to the discovery of its utility in diagnosing uterine stromal tumors (6).

Consecutive Case Array

This type of array is constructed using consecutive cases belonging to a single tissue site. These types of arrays are extremely useful for quality control purposes, including identifying shifts and drifts in reagent quality. They are also useful in studying the prevalence of a protein/antigen in a given tumor type, and analyzing the relationships between different biomarkers.

Tumor Characteristic-based Array

This is a special type constructed solely on the basis of a given characteristic, such as patient age or tumor grade. The latter is useful for evaluating the frequency of a marker throughout the spectrum of tumor differentiation. Similarly TMAs can be gen-
erated based on the expression of a biomarker, such as estrogen receptor or HER2/neu positive or triple negative breast cancers. These types of biomarkers are useful in analyzing interrelationships between different cellular pathways.

**Progression Arrays**
These types of arrays are used to analyze the role of protein(s) in cancer progression and consist of normal tissues from patients without cancer, normal tissue from patients with cancer, pre-invasive lesions and tumor (from local and metastatic sites). The addition of normal tissue from close to the tumor and those much further away from the tumor site might enable study of ‘field effect’.

**Outcome-based Arrays**
These special arrays are the most valuable and most difficult to generate, as they involve collation of tissues from patients that have the same disease and have been more or less similarly treated and followed up for a significant period of time. The period of follow-up depends on the type of disease or tumor being studied. These types of arrays are mostly used to evaluate prognostic or predictive biomarkers. The presence of biomarkers in tumor subtypes might then be used to design novel therapeutic strategies.

**Other Special Types**
TMAs can be generated based on specific question being asked, whether it be race (Caucasians versus African Americans), sex (male versus female) or more tissue-oriented questions such as center of the tumor versus invasive edge of the tumor.

The following steps are recommended for breast TMA construction:

**Step 1: Define the question**
As described above TMAs are created to answer specific questions. It is important to define clearly this question at the outset. The question will help define the number of cases and cores that need to be used in the generation of the TMA. For example, a TMA containing 20 cases might be sufficient for routine quality control/assessment, but is not enough for biomarker assessment.

**Step 2: Review the cases to be included in the TMA**
Pull all the cases to be included in the TMA together. If the blocks have been previously cut into for other clinical or research purposes, it is prudent to review a fresh H&E slide to ensure that the slide is representative of the block. Review all the slides and mark areas of interest. It is useful to mark multiple areas from more than one block, as blocks may be depleted or misplaced. Areas to be sampled (tumor, normal, and pre-malignant tissues) should be identified.

**Step 3: TMA core size and number of cores**
Size of the cores: The typical core sizes used for TMA constructs are 0.6 mm, 1.0 mm, 1.5 mm and 2.0 mm. Many workers consider the small 0.6 mm cores as the standard of practice. Use of smaller core diameters, however, allows for a greater number of cores to be extracted from the lesion and a greater number of cores that can fit into the TMA block. In addition, they tend to inflict little damage on the donor and recipient blocks and the cores are easier to remove and replace from these blocks. The larger core sizes have the advantages of being more robust and the cores are more resistant to damage during handling. However, these larger sizes can lead to increased likelihood of difficulty in extracting the
cores from the blocks, as well as greater chance of the blocks being broken or cracked during the TMA generation process.

**Number of Cores**
The optimal number of cores, to be included in the TMA, is marker dependent and can vary depending on the degree of tumor size and heterogeneity. In general, the greater the degree of intratumoral heterogeneity for any given marker, the greater is the number of cores that will be required. When using 0.6 mm sized cores, it is typical to use a minimum of three cores per case. Three 0.6 mm cores are still better than one 1.0 mm core, even though the tissue surface area is essentially identical. For small tumors, three 1.0 mm cores could result in destruction of the donor block; so tumor size would also drive number/size of cores to be taken. Studies that have used 1 mm core punches have tended to use two cores (8).

**Density**
The maximum number of cores that should be placed on a single block will vary depending on core size, block size, and IHC methodology, among other factors. It is best to avoid placing so many cores on a TMA that the surface section of cores becomes larger than the antibody coverage area on the slide programmed by the autostainer (e.g. Dako Autostainer). Similarly, too many cores diminish the amount of paraffin at the edge of the block creating difficulties in sectioning. Cores should start at least 3 mm away from the block edges, to prevent the paraffin from cracking. Maximum number of cores per block should therefore depend on the comfort level of the technician, as well as the pathologist, who is ultimately going to read the slides. For these reason, it is typical for most workers to put somewhere between 100 and 300 of 0.6 mm cores in a TMA block.

**Distance**
The distance between cores should NOT exceed the core diameter. It is easier for the microscopist to follow the rows and columns if he/she can “lead” from one core to another. If the distance between cores is large, it difficult to follow the chain of cores and may result in skipping of lanes and false recording of data when performing manual interpretation.

**Step 4: Identify control tissues to be included in the block**
Controls should be placed on each TMA block, for quality control and to address tumor heterogeneity. Three types of control tissues may be used:

- **Tissue-specific controls:** Normal tissues and cell lines from the organ site can help in comparative analysis of the marker expression status, in addition to helping ensure standardization.
- **Biology-associated controls:** It is useful to insert ‘pathway associated’ controls to ensure that the reagents are working well; thus functioning as good internal controls within the TMA block. Common examples include endometrium for hormone receptor, testes or lymph node or tonsils, for proliferation.
- **Organ system controls:** examples include adrenal gland, brain, breast, colon, kidney, liver, lung, pancreas, placenta, prostate, testes, salivary gland, uterine myometrium (smooth muscle). These controls are particularly useful when the TMA is being used to analyze novel markers, as one or more of these tissues can serve as internal controls. Normal tissue TMAs, at a minimum, should contain: liver, kidney, endometrium, lymph node, colon, and testis.

![Figure 12.3 TMA map and block design](image-url)

TMA layout should be asymmetric and irregular so that it is relatively easy to orient the TMA block. This irregularity should be obvious to the histotechnician who is cutting the block, so that all the cuts from the block are taken on the slides in an identical manner. In addition, locating the controls in an asymmetric manner is also helpful when reading the slides. For example, the following features may be included: 1) Blank rows and columns that do not run down the center lines of the TMA blocks, but to one side, so that the block is cut into two-third and one-third grids. 2) A blank corner for orientation or a tail coming out from close to one of the corners. 3) Asymmetric distribution of control cell lines and tissue controls. Placing stained cores of control tissues at the edge of the grid can be useful to mark orientation.
**Step 5: Make a TMA map depicting the layout**

The TMA map may consist of a simple Excel sheet, or may be a more sophisticated datasheet made using one of the TMA generation programs. This map also serves as a guide in order to arrange blocks in the sequence in which they need to be arrayed. Thus, the TMA map will contain the exact location of each case, including the duplicate samples, and where controls are located. Mini-arrays (“City Blocks”) of the cores (3x5, 4x5, 5x5, 6x5) can be spaced for easy orientation, with control tissue in the rows between the mini-arrays.

**Issues Related to Layout**

- TMA layout should be asymmetric and irregular to assist orientation (see Figure 12.3).
- If multiple TMA blocks are being made for the same project, one consideration is to carry a small proportion of cases onto other blocks (e.g., 10%).
- Cores from the same case: Ideally, if same-patient cores are to be placed on the same block, they should be dispersed on the block. This will decrease the risk of interpretation bias. However, some researchers prefer this arrangement since it permits immediate ‘normalization’ or ‘confirmation’ of the results of the different cores from the same patient. If same patient cores are to be dispersed across multiple blocks, it is better to place them in different regions of the array (outer and inner); (outer in one block and inner in the other), with random placement, rather than placing them in the same location in each block. This is done to prevent similar artifacts affecting all cores.

**Step 6: Creating the TMA itself**

**Instrumentation**

The need for specialized instrumentation for creating TMAs is entirely based on the number of cores and value of the tissue being inserted in these TMAs. For TMAs being constructed for quality control/quality assessment or work-up of new reagents, the number of cores inserted is relatively low. This enables use of larger cores and diminishes the need for specialized instrumentation. However, for TMAs to be made from valuable cases with scant materials, it is necessary to use these instruments. The simplest of these devices consists of hand-held punches and is generally not very useful for a serious TMA project, where it is necessary to use at least an intermediate grade device. These intermediate grade devices consist of a stand, in addition to a positioning apparatus, and ensure vertical punching of the blocks and proper placement within the grid. Fully automated devices additionally have integrated computers that can be programmed to select the donor sites from different blocks and transfer them in the recipient block.

**Donor Block**

The block from which a core will be taken is referred to as the donor block. The area of the donor block to be cored for TMA should be selected by a pathologist. Although it is intuitive, it must be stated that the donor blocks should be optimally processed and should not contain any poorly processed areas. Similarly, cores should be obtained from the block before the block gets depleted. The thicker the donor blocks the greater the number of useful sections that can obtained from the TMA. Core punches should be pushed gently into the TMA block, and not too deeply as this can damage the needle as well as the block. When using semi-automated devices it is easier to mark the depth of the punch to the level of the plastic of the cassette. It has been suggested that heating the tissue core for 10 minutes, before inserting it into the recipient block, allows better fusion of the paraffin within the core and that surrounding the core. Fusion avoids loss and folding of tissue cores during sectioning of the TMA.

**Recipient Block**

The block into which the cores are placed is referred to as the recipient block. It is best to place the cores towards the center of this block in order to prevent cracking of the block. After the cores are inserted, place the TMA at 37 °C degree overnight, and then on the cold plate of the tissue embedding station, with subsequent two to three 1-hour cycles of hot/cold to temper the array. Multiple sections from the block should be cut at the same time to prevent wastage of tissue. Incomplete sections should not be discarded; these can be used for standardization of staining technique (see below).

**Staining TMAs**

When performing staining of the TMA, the step is to ensure that the staining procedure actually works in the laboratory and the procedure has been standardized. If the TMA has been obtained from an outside institution, it is important, if possible, to get other tissue processed in that laboratory, or alternatively...
poor-quality sections (incomplete or discards) from the TMA, for practice and standardization; following standardization good quality TMA sections should be used for analysis. As TMA sections are usually larger, they require special care to ensure that the entire section (the whole array) is covered with reagents, otherwise uneven staining will be observed.

One of the limitations of the TMA is that the tissues in the tumor cores have been processed at different times and often with different protocols. This will lead to optimal staining of some tumors, but also sub-optimal staining (over or under-staining) of quite a few tumor cores. However, the large number of cases included in the TMA can to some extent compensate for this limitation.

**Step 7: Validation and quality assurance**

Measures for the TMA should include the following:

**Validation**

The use of TMAs enables analysis of large datasets, however this ability does not by any means suggest that the dataset is not skewed (10). This skewing may be the result of the institution’s location (population distributions with regards to race, ethnicity, and access to health care), or type of practice (community hospital versus referral center). These biases collectively might influence the tumor size, grade and subtype composition of the cases in the dataset. Such abnormalities of the dataset need to be recognized and allowed for in interpretation of findings; the involvement of a biostatistician from the start (i.e. at case selection) helps to prevent the creation of biased TMAs. It is useful to perform common biomarker analysis on sections from the created TMA to confirm the “normal” distribution of known parameters. Comparison of this data with prior clinical data (e.g. ER analysis) obtained from whole section analysis is particularly useful to validate utility of the TMA. Alternatively the incidence of expression of a number of biomarkers in the TMA should be compared to that in published literature (using whole sections).

**Quality Assurance Measures**

It is critical to perform and analyze H&E sections from the TMA to confirm the presence of tissue of interest (usually tumor) in the TMA sections. In addition, H&E should be performed at regular interval (e.g. on every 25th slide) from the TMA blocks. The above tests should be reviewed by a pathologist familiar with the study.

**Chapter 12.5 | TMA Analysis**

The analysis of the TMA has two components. The first involves analysis of the slides and recording of the data. The second involves data analysis.

**Slide Analysis**

The TMA slides can be analyzed manually; alternatively automated image analysis programs that can assist with the analysis are also available. The need for these programs is based...
on the work volume as well as density of the TMAs. Some programs generate a virtual slide (Whole Slide Image – WS) of the TMA and further analysis can be done using a computer screen. Use of a WSI has the advantage of avoiding burn to the slides and all the cores can be analyzed at the same “optical and illumination” conditions. It additionally permits electronic storage of the fresh images and later re-analysis if required. This capability is particularly beneficial for FISH sections, which fade with viewing and time.

Data Analysis

**Step 1: Data cleaning**

Given the large number of samples in a typical TMA study, analysis of the data can become quite a challenge. One needs to exclude the cases that are not informative; it is not unusual to lose up to 10% of cases due to insufficient representation of tissue of interest. On the informative cases, strategies for conversion of multiple values (one per core) for each case into a single data point have to be devised. The commonly used strategies include using the highest value or a numerical mean of the values obtained (for review see (11)). Each method used for normalization has its own advantages, as well as limitations.

**Step 2: Statistical analysis**

The tests used to determine the $p$ value will be dependent on the type of data (i.e. nominal or categorical), as well as the degree of variance within the data. For simple analyses of relationships, contingency tables, and chi-squared tests are used. For demonstration of survival distributions, most researchers use the Kaplan-Meir plot and then apply Log-rank analysis to test survival differences between groups. The most frequently used analytical strategy is to subdivide patient material into high- and low-risk groups, based on the expression of novel biomarkers. Some commercially available computer programs, such as X-tile program (11), may assist the selection of the best cut-off point. This cut-off point needs to be confirmed in a separate series of cases to validate its utility. The NCI – EORTC group has developed the REMARK (Reporting recommendations for tumor marker prognostic studies) (12) guidelines which should be followed whenever possible.

References