Chapter 11
Tissue Microarray

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Abstract Tissue microarray (TMA) is a powerful tool for performing population level studies using tissues routinely processed in surgical pathology and cytopathology cell blocks. TMA construction can be done manually or with semiautomated and fully automated equipment on formalin fixed, paraffin embedded (FFPE) tissue produced in routine histology. A variety of other processed materials may also be used, including frozen tissue, cell culture lines, and resin embedded tissue. TMAs are used for a variety of applications including the validation of cDNA array data; validation of the sensitivity and specificity of antibodies; quality assurance in immunohistochemistry; translation of data from cell line, xenograft, and animal models to human cancer; collaborative studies, especially for the aggregation and preservation of rare tumor tissues; molecular profiling of large series of tumors or diseased tissue and correlation with clinical endpoints; and evaluation of diagnostic, prognostic, and therapeutic potential of newly discovered genes and molecules. Most standard histologic and molecular techniques can be applied to TMA sections. Optimal storage conditions of TMA blocks and sections in order to preserve antigenicity continue to be an issue. Image analysis and data management are crucial issues, but many tools are available.

Abbreviations

FFPE	Formalin fixed, paraffin embedded
IHC	Immunohistochemistry
mAB	Monoclonal antibody
TMA	Tissue microarray

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1. What is a tissue microarray (TMA)?

A tissue microarray consists of up to 1000 cores of formalin fixed, paraffin embedded tissue obtained from donor blocks that are placed into a single recipient paraffin block. This recipient paraffin block can subsequently be sectioned, and the sections placed on glass slides. Depending on the length of the cores, as many as 300 slides can be produced from one array [1].

2. What is the scalability of TMAs?

TMAs are population level research tools. Each slide produced from a TMA can be used for a single experiment to probe for a DNA, RNA, or protein target on as many as 1000 tissues at one time [1–4].

3. What are the applications of TMAs?

Validation of cDNA array data: A single cDNA array experiment can generate data about the gene expression patterns of up to 50,000 genes in a single tissue. These genes can be expressed in multiple cell types; however, most molecular methods have a common problem, in that the tissues must be disintegrated prior to testing. TMA experiments can identify the specific cellular type and compartmental localization of the gene products of interest. This is particularly helpful as candidate genes may be expressed in many tissue types other than diseased tissue, so validating protein expression of potential markers using TMA experiments as a screening tool eliminates the time and labor associated with using full tissue sections [1, 3, 5–7].

Validation of the sensitivity and specificity of a newly discovered antibody: Using TMAs containing a variety of tumors and normal tissues is an efficient and cost-effective way to validate the sensitivity and specificity of new antibodies, as well as optimize the appropriate staining protocol.

Utilization of TMAs with cytologic specimens: Biopsies obtained through radiologic procedures in many cases result in a very limited amount of material available for pathologic diagnosis. Often, no surgical resection specimens are taken if the initial cytology specimen is diagnostic. TMAs can be constructed from cell block material, although it may be necessary to add another one or two cores to ensure adequate cellularity on array sections.

Quality assurance in immunohistochemistry (IHC): Performing IHC on TMA sections offers a way to quickly assess the performance characteristics of antibodies, including comparison of different monoclonal antibodies (mABs) that may be directed at different epitopes of a target protein antigen; optimization of staining conditions; interlab comparison of IHC staining results; and standardization of morphologic interpretation. Using TMA sections to test new antibodies is especially useful to quickly optimize staining conditions such as antigen retrieval, reagent concentrations or antibody titers, incubation times with primary and secondary antibodies, temperatures, and wash conditions. This eliminates the variation between batches that use full size tissue sections [1, 6, 8, 9].

Translation of data from cell line, xenograft, and animal models to human cancer: TMAs can be constructed from experimental tissues and cell lines. An array of cell lines makes it possible to screen for amplification of a gene of interest [4, 5, 10].

Collaborative studies: TMAs require little tissue from individual cases and therefore provide a means of making the tissue available for study in a compact form (one slide) that is easily shared. This is a convenient way to share tissue resources in a large collaborative study such as a multicenter clinical trial, while also preserving archived tissues from rare tumors or diseases for additional future studies. This also allows multiple labs to standardize and optimize protocols and use of various probes, as well as validate the data generated from multiple centers [4, 9].

Molecular profiling of large series of tumors or diseased tissue and correlation with clinical endpoints: Clinical data accompanying each core may be crucial for the discovery of significant patterns or alterations in tissue when compared to profiles of nondiseased tissue. This is an important consideration when designing an array and choosing appropriate cases for inclusion. This correlation may result in more refined molecular classifications of tumors and other disease states than was possible in the past, especially if materials are drawn from, and evaluated by, multiple institutions [4, 10].

Evaluation of diagnostic, prognostic, and therapeutic potential of newly discovered genes and molecules: TMAs provide an easy and quick means to evaluate new markers and alterations or responsiveness of tissues to new candidate drugs in a large number of tissues simultaneously [4, 10].

4. What are the common types of TMAs designed?

Multitumor TMAs: These TMAs contain samples of several different tumor types in order to screen for the prevalence of a molecular alteration across many different tumor types or to test the specificity of a new mAB. A sample size of 30–60 per tumor type is recommended [4, 11].

Progression TMAs: These TMAs contain samples of one tumor type at different stages in order to study changes or similarities in gene amplification or protein expression at different stages in a tumor type. At least 50 samples per stage or category are recommended in order to ensure an adequate sample size for statistical analysis [4, 5, 11].

Prognosis TMAs: These TMAs contain samples of tumors with associated long-term follow-up clinical data in order to identify and evaluate prognostic markers and alterations for the prediction of clinical response to therapeutic interventions such as chemotherapeutic drugs or radiation therapy [4, 5].

Normal tissue TMAs: These TMAs contain samples of normal tissues in order to evaluate the expression of candidate genes in multiple tissues for their potential as therapeutic targets. Normal tissue arrays are also useful in the validation of new antibodies by confirming the localization of antigens [6, 12].

Rare tumors or unusual diseases: In order to preserve as much tissue as possible for future studies of rare malignancies or diseases, TMAs provide the means to utilize a small sample that can be arrayed with tissue cores obtained from multiple institutions, preserving very limited resources.

5. What types of assays can be applied to TMAs and are there special considerations?

Immunohistochemistry: IHC is the technique most commonly applied to TMA sections. It is a multiparameter assay involving many variables that need to be optimized. The most important step is choosing a primary antibody that is specific for the protein of interest. Ideally, the antibody should usually only produce one detectable band on a Western blot, but often there are multiple bands. When applied to tissue, it needs to produce a staining pattern in the appropriate cellular compartment of the expected cell types. IHC in TMAs faces the same difficulties as full-size tissue sections; however, TMA sections are particularly susceptible to tissue loss during deparaffinization and antigen retrieval steps. They are susceptible to edge staining effect. These can be avoided by using replicate cores placed in different locations within the array. Some labs also place a rim of cores around the edge of the array of other tissue types, finding that more fibrous tissue is helpful. There are two categories of proteins that are challenging for IHC, transcription factors because there are very few copies per cell that may not be visibly detectable and cytokines which may diffuse throughout tissue and produce nonspecific staining [8, 13].

Chromogenic in situ *hybridization (CISH)*: CISH requires intact cells to probe for the nucleic acid sequence of interest. Formalin fixation and tissue processing not only preserve tissue morphology but also cause conformational changes that affect probe attachment. The chromogenic signal is usually localized to the nucleus and cytoplasm, and interpreted similarly to IHC [13, 14].

Fluorescent in situ *hybridization (FISH)*: FISH is a method used to detect translocations, gene deletions, and gene amplifications in tumors. Formalin fixation preserves the structure of tissue by forming crosslinking methylene bridges, but this has the disadvantage of reducing the accessibility of the hybridization target. Pretreatment steps, such as use of sodium thiocyanate and pepsin digestion to unmask the target DNA, need to be optimized for each tissue type. Since multitumor TMA sections contain many different tissue types that may require different pretreatment conditions, this fact needs to be considered when designing the appropriate array for an experiment. For more information on problems encountered in FISH assays in TMAs and possible solutions, see Ref. [15].

Quantum dots (QDs): Quantum dots are nanoscale semiconductor crystals with several properties that offer advantages over conventional organic and fluorescent dyes. Their broad excitation spectra and narrow emission spectra allow for simultaneous excitation and quantifiable observation of up to 10 QDs in the same sample. QDs are highly resistant to photobleaching, unlike both organic and fluorescent dyes. They have been used in imaging tumor vasculature, studying apoptosis, and performing gene expression analysis. They have been applied to TMA sections as multiplex assays to distinguish renal carcinoma from normal tissue by detecting MDM-2 and B-actin protein targets, as well as examining simultaneous antigen expression of EGFR, E-cadherin, and cytokeratins in xenograft lung cancer specimens [16, 17].

Multiplex immunoblotting: Proteins can be transferred from a single TMA section to a stack of up to ten replicate membranes. These membranes can be probed using conventional immunoblotting techniques, increasing the number of antibodies that can be utilized in an experiment. The advantage is that this method works with phosphor-specific antibodies, as well as many antibodies that do not work well in FFPE tissue samples [18–20].

6. What instruments are available to construct TMAs?

Manufacturers offer both semiautomated and automated (see Fig. 11.1) tissue arrayers and a list is included in the Resources section at the end of this chapter. Silicone molds and punch needle tools are also available for manual production. All arrayers work similarly and employ hollow needles with slightly different diameters—a larger bore needle to remove a core of tissue from a recipient block and a slightly smaller bore needle to remove the tissue from a donor block for insertion into the hollow cylinder created in the recipient block. Since most of the costs of constructing a TMA are associated with the time spent in the selection of tissues to be arrayed, there is little advantage to using the more expensive automated arrayer, unless the lab is a core facility that will be making arrays daily. The punching needles on the automated arrayers wear out more quickly. Semiautomated and manual production of an array is tedious, but when compared to an automated arrayer, it is easier to get all cores level, it is simpler to repair, and the cores can be punched out more precisely within a localized area. However, advances in the software available with automated arrayers are helping to overcome limitations associated with precision punching and make scanning, viewing, and scoring array spots easier. It is not recommended to use the automated arrayer on calcified tissue or bone [5, 21].

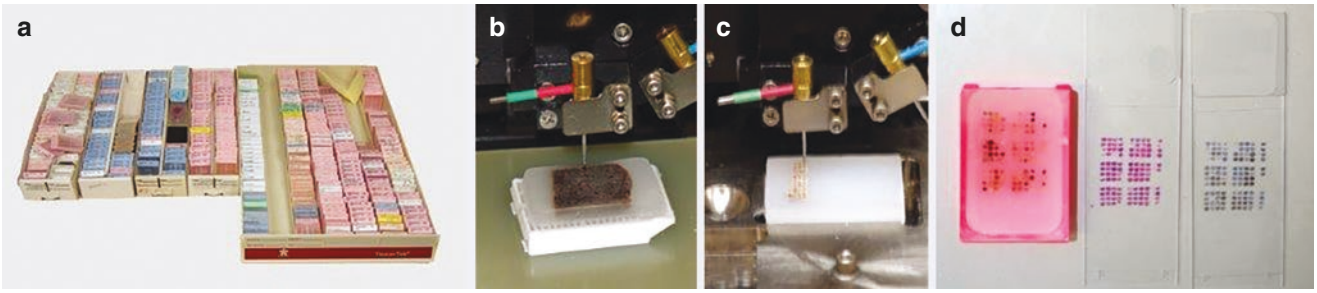

Fig. 11.1 Workflow for constructing TMAs. (**a**) The most time-consuming step in building a TMA is selecting appropriate cases and the most representative FFPE blocks containing adequate tissue from those cases for inclusion in the array. (**b**) The areas to be cored from a donor block are usually marked on an H&E-stained slide that can be overlaid on the donor block, then the core of tissue in the selected area can be removed from the donor block. (**c**) The donor core is then placed into a predetermined hollow cylinder that was previously removed from the recipient block with a hollow needle that is slightly larger in diameter than the donor core. (**d**) The completed TMA should be tempered to let the donor cores anneal to the recipient block paraffin, then the TMA can be sectioned, and the resulting slides stained similarly to routine histology practices

7. How is a TMA designed?

Selecting tissue: The most important consideration is the availability of clinical data that can be compiled and associated with each sample in an array. Even though a TMA may be initially designed for a specific set of experiments, the remaining sections may be used for future experiments and the value of those sections may depend on the accompanying annotated data. Donor blocks of FFPE tissues should be selected for thickness. Ideally, the tissue should be 3–4 mm thick; however, cylindrical spaces in the recipient block can be filled in by stacking multiple donor cores in the recipient space from thin tissue sections and from core needle biopsy blocks, as they will anneal during tempering of the completed recipient block. The tissue should not be necrotic or contain heavily calcified or bony areas that have not been decalcified. In situ lesions or thin epithelial cancers can be difficult to obtain representative cores. It is also difficult to array certain microanatomical structures, such as an entire hepatic lobule or the full thickness of an epithelium [1, 5, 22–24]. A technique has been described for using cores that have been previously arrayed. This is useful, particularly in rare tumors or when many other cores in the block have been exhausted in sectioning [25].

Use of cell blocks: Tissue cores may be taken from a cell block preparation. It is recommended that an H&E slide demonstrate at least six tumor cells per high power field (HPF). Three cores, each 2 mm in diameter, should be included in the array. Each core should potentially contain 100 tumor cells (40× objective with 0.5-mm field diameter; 2-mm core = 16 HPFs), so using three cell block cores will be the equivalent of a tissue core [26].

Selecting controls: The inclusion of normal tissue on all arrays is essential. If a biotinylated detection system will be used, either liver or kidney should be included to demonstrate false background staining due to endogenous biotin in the tissue [23].

8. How is a paraffin-based TMA constructed?

(see Fig. 11.1)

1. Search databases for appropriate cases.
2. Examine H&E-stained slides to select appropriate blocks and mark areas to be sampled. Use different colors of markers on the slide to indicate different areas to be cored and arrayed, such as black for tumor, green for normal benign tissue, blue for inflammatory changes, etc.
3. Acquire paraffin blocks that correspond to the slides selected and marked. Examine to check for block integrity (our laboratory had a fungus colonize several years' worth of paraffin blocks in storage) and to see if adequate tissue remains for sampling.
4. Design the TMA block in a diagram. We designed three templates in MS Excel that show a grid with the coordinates of each core based on the core diameter to be used. The case number, paraffin block, and color of the sample area on the H&E slide for each core are indicated in individual cells on the spreadsheet. This map is crucial for the person constructing the array and for data collection after the TMA is sectioned. This also lets the array designer know how many spaces are available to be filled.
5. Prepare recipient blocks. There are several options for the production of recipient blocks.

   (a) *Semiautomated arrayers*: Semiautomated arrayers may allow up to four replicate blocks to be produced simultaneously. The recipient blocks should be formed from low melting point paraffin with a high polymer content such as Type H available from ThermoFisher Scientific. This paraffin appears to be the most durable and withstands all steps of construction through sectioning with fewer problems such as cracking [27]. Standard metal pans used for tissue blocking in histology can be used, although many labs prefer to make blocks of double thickness so that

longer cores can be arrayed. A tissue cassette should be placed on one side of the block, so that it can be cut in a fashion like routine histology blocks. Set coordinates on the arrayer and remove the first core of paraffin from the recipient block. Remove a core from the donor block by aligning the marked slide over the block face to find the region of interest. Place the donor core into the hollow cylinder in the recipient block. If the tissue in the donor block is thin, multiple cores can be removed, the excess paraffin trimmed from the cores, and the shorter tissue cores can be stacked inside the hollow cylinder. Repeat these steps until the array is completed. This process will cause the paraffin to buckle, so occasional rows should be left empty in order to maintain alignment. Designing and building the array in sectors with empty spaces also makes locating specific cores easier when manually scoring staining patterns [5, 6, 21, 23, 28].

(b) *Manual array molds*: Array recipient blocks may be produced using silicone molds that eliminate the need to drill out cores in the recipient block. These molds can be filled with paraffin to make a donor block that will receive FFPE cores; alternatively, the same molds can be filled with OCT, resulting in a recipient block that can receive cores of frozen tissue. The preformed core holes can be filled with tissue cores that are extracted from donor blocks using a punch needle tool. These methods are described in Bingle et al. [29].

(c) *Automated tissue microarrayer*: Automated tissue microarrayers use a software program to detect premarked areas on glass slides and align these with the corresponding areas on donor blocks. The instrument then extracts cores and places them into premade recipient blocks. See Fig. 11.2.

Fig. 11.2 Example of an automated tissue arrayer system (3DHistech; Budapest, Hungary)

9. Can TMAs be constructed from materials other than paraffin embedded tissue?

Frozen tissues in gel: Recipient blocks can be formed from a gel made of formalin, gelatin, sucrose, agarose, and PBS. Arrays of frozen tissue can be arrayed in this block at room temperature or at −5 to −10 °C. This helps preserve RNA and protein quality [30, 31].

Frozen TMAs using cryo-embedding compounds: Recipient blocks can be formed by freezing tissues in a cryo-embedding compound such as OCT (optimal cutting temperature; Tissue-Tek® OCT Compound is available from Sakura Finetek USA, Inc.; Torrance, CA). The advantage is preservation of DNA, RNA, and proteins; however, morphology may be distorted, as compared to paraffin or gel arrays. Lipids will also be preserved, another advantage over FFPE tissues. Fewer cores of tissue can be arrayed due to the brittle nature of frozen OCT and the completed TMA must be stored in the frozen state. Antibodies that do not work well on formalin fixed tissue may work better on frozen tissues. In situ hybridization (ISH) assays also work well on frozen tissues [13, 14, 32–34].

Resin TMAs: Howat and Wilson have described a method that combines cold acetone fixation with array construction using glycol methyl acrylate resin, combining the advantages of better antigenicity in frozen tissue with the thinner sectioning and better optical clarity of resin embedment. Acetone fixed cores are embedded in agarose to hold them in place for embedment in resin. The resin should be of a hydrophilic type, allowing most stains and immunohistochemistry applications to be performed on TMA sections [35].

Cell line microarrays: Drug development studies often require the use of cell lines rather than FFPE archival tissues. Methods have been developed for cell culture lines to be fixed, paraffin embedded, and arrayed [23, 32, 36–38]. We use tumor cell line TMAs as control material for immunohistochemistry stains.

Xenograft tumor arrays: TMAs can be constructed using tissues from xenograft models or from transgenic or knockout mice. These TMAs provide a means for rapid assessment of gene expression and drug responsiveness [32, 39].

Cutting edge matrix assembly (CEMA): Tissues can be shaved from paraffin blocks, trimmed into rectangular primary plates, then stacked and bonded either by heat that anneals the paraffin together or with cyanoacrylate glue that will dissolve during processing. Stacks of bonded primary plates are then transversely cut and bonded edge-to-edge, forming high-density arrays lacking an intervening matrix between the tissue samples. This has several advantages over TMAs, in that up to 12,000 pieces of tissue can be arrayed in a block, and those pieces of tissue are much longer than the cores used in TMAs, so more slides can be produced from a CEMA. Because the tissue samples are bonded together and are of the same length, less tissue is lost when the array is sectioned and floated on a water bath for placement on a

glass slide. CEMA solves the problem of creating arrays of thin-walled structures such as blood vessels or ducts, full-thickness representation of layered epithelia, or larger micro-anatomical structures such as hepatic lobules [32, 39, 40].

Tissue immunoblotting: TMA sections on a glass slide can be treated with enzymes and the proteins subsequently transferred to a stack of nitrocellulose membranes, producing up to ten replicate protein arrayed membranes. These protein arrays can then be probed with antibodies for quantitative protein analysis [18, 32].

Patch TMA: Construction of a so-called patch TMA from an existing glass slide is possible using a mounting medium for tissue transfer followed by a tape transfer system. This will allow for production of a limited number of slides containing a few cores. The patch TMA technique may be useful when immunohistochemistry studies need to be performed but the paraffin blocks are unavailable [41].

10. How are sections cut from an array block?

The array block should be tempered prior to cutting. Place the block in a 37 °C incubator for 10 minutes, then gently press the surface with a glass slide to flatten the cores. Place the block back in the incubator for 1 hour, then cool on an ice block or in a −4 °C freezer for 15 minutes and repeat incubation and cooling two more times. This helps the paraffin in the recipient block adhere to the cores, so that the cores do not pop out and tissue spots are not lost during sectioning. The tissue spots on the slides will have fewer defects if this is done correctly. This is why recipient blocks should be molded from low melting point (58–65 °C) paraffin [23]. Sexton et al. provide a comparison of four tempering protocols [27], and they determined that one similar to that which we have described is optimal.

Sections may be cut from the block on a microtome and floated on a water bath for placement onto a positively charged slide. Some tissue spots may be lost in this process and to prevent this, many laboratories employ a tape sectioning aid (Instrumedics Inc., Hackensack, New Jersey, USA). This system includes adhesive-coated slides, adhesive tape for section transfer, and a UV lamp. In our experience, a competent histotechnologist does not require such an aid. It is difficult to clear all the adhesive residue from the slide and this residue may interfere with ISH, FISH, and phosphorylated antibodies in IHC, but the use of slide coating and adhesives does not affect the epitope stability. This residue can also make automated image analysis more difficult. It has also been reported that IHC using the capillary gap method will not work with the tape transfer system. In addition, the oil coverslip of some automated stainers seems to interfere with the staining process on the adhesive-coated slides [5, 7, 14, 36, 42, 43].

We recommend performing an H&E stain on the first section and every 50 sections thereafter for quality assurance. If an array has not been sectioned and stained for some time, it may also be beneficial to perform stains to check on the viability of antigens. Examples would be vimentin or pancytokeratin for IHC, or a probe for housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase, beta-actin, or histone H3 for *in situ* hybridization methods [23].

11. How many cores of tissue are considered representative of a tumor?

Many studies comparing IHC findings in core samples from TMA blocks to large section histology have been conducted in several tumor types for a variety of markers. The concern is whether tumor heterogeneity can be accounted for in such small samples and what size and number of cores are optimal. The consensus is that three 0.6-mm diameter cores provide adequate representation and most researchers are assuming that one of the three tissue spots will be lost during transfer to a glass slide or during staining, so that two cores will be available for scoring. Similarly, two 1.0- or 1.5-mm cores are also considered representative. There does not appear to be a consensus on which core diameter is optimal, but at least one study has shown that a 0.6-mm core is equivalent to a 1.0-mm or 1.5-mm core for estimation of protein concentration [44]. A 1.0-mm core may be better than a 0.6-mm core to evaluate epithelia such as urothelium where staining may vary in different layers [45]. Use of 2.0-mm cores may allow one to demonstrate larger structures, such as a hepatic lobule, but they are more difficult to array in the recipient blocks due to fractures created in the paraffin when removing cores. Use of larger cores may also be necessary in samples of tissue that may have low cellularity such as thyroid follicles with abundant colloid.

Taking the cores from different tissue blocks may help account for tissue heterogeneity, especially in diseases like lymphoma or when looking at markers of hypoxia. Adding more than four or five core samples does not appear to improve concordance with large sections. These studies have included prognostic markers such as estrogen and progesterone receptors and Her-2/neu, as well as proliferation markers such as Ki-67 and all have shown excellent concordance (most were between 94% and 100%) with large tissue sections. TMA cores may be more representative than larger sections in necrotic tumors because the best areas are sampled and much of the background staining associated with necrosis is eliminated. Some studies have also shown that fixation differences between central and peripheral regions of tumors do not impact the concordance between TMA cores and large sections. One important fact to remember is these studies assume that standard tissue sampling practice is the gold standard and representative of an entire tumor. Consider that a tumor measuring 3 cm × 2 cm × 3 cm has a volume of about 18 cm^3, one large tissue section represents about 1/7500 of the tumor volume. A 0.6-mm core represents

about 1/1600 of the volume of a standard large tissue section, so the greater problem is not correlation between TMA cores and large tissue sections, but between large tissue sections and large volume tumors [1, 3, 5, 7, 10, 22, 42, 46–54].

12. How do TMA immunohistochemistry (IHC) stain results compare to large tissue slide results in various tumors?

TMA IHC results have been reported in many cancers including (but not limited to) non-small cell lung carcinoma, breast carcinoma (both invasive and *in situ*), Hodgkin lymphoma, non-Hodgkin lymphoma, acute myelogenous leukemia in bone marrow trephine biopsies, endometrial carcinoma, ovarian carcinoma, kidney carcinoma, urinary bladder carcinoma, sarcomas, thyroid carcinoma, and cell blocks from effusions. All studies looked at multiple markers and the concordance of phenotypic expression patterns ranged from 80% to 100%. All studies were able to reproduce known associations between phenotypic expression patterns or molecular changes and clinical endpoints. Advantages of TMA studies compared to those of large tissue section studies are the degree of consistency and standardization of staining on a single TMA slide versus the corresponding large tissue sections. Scoring of large tissue sections is also more subjective as one chooses what is most representative, whereas a TMA tissue spot is usually scored in its entirety [3, 22, 46–51, 55–60].

13. How long do formalin fixed, paraffin embedded (FFPE) tissues and unstained slides retain their antigenicity?

Previous studies have suggested that tissue oxidation starts when a block is sectioned and plays a role in the loss of protein antigenicity. Other studies have shown that some proteins will retain their antigenicity for greater than 60 years if they were originally fixed in neutral buffered formalin. Antigens in a cytoplasmic or nuclear distribution may be less susceptible to long-term storage degradation than those with a membranous distribution [43, 48, 61, 62]. Similarly, unstained TMA slides demonstrate a decrease in immunohistochemical staining intensity as they age, but they may still correlate well with molecular and pathologic findings, making them valuable for research [63].

More recent studies suggest that tissue oxidation may not be the cause of loss of antigenicity, and that using current techniques avoids some of the problems detected in the earlier studies. A recent study employing formalin fixed tissues placed in a TMA compared storage temperatures, slides stored with and without paraffin coating, and different lengths of storage time up to 1-year duration. Using modern antigen retrieval and signal detection methods, they found no significant loss of antigen expression by IHC or problems with DNA or RNA detection by *in situ* hybridization techniques under the various storage conditions. Compartmental localization of antigens was not a factor [64]. Another important study suggests that insufficient dehydration during tissue processing, as well as high ambient humidity may actually be the culprit for loss of antigenicity rather than tissue oxidation. The solutions to prevent loss of antigenicity may include longer tissue processing to ensure optimal dehydration of tissue, as well as storage of paraffin block and unstained slides at lower temperatures and more importantly, in a low humidity environment [65].

14. How should TMA blocks and slides be stored?

There is no consensus on the best method of storage. Some labs section the entire TMA block all at once, whereas others cut 20 sections at a time and then coat the surface of the block with a thin layer of paraffin. Cold storage at 4 °C appears to slow antigen degradation but does not stop it entirely. Other labs report that overall immunoreactivity appears to be best preserved with storage at −20 °C, without paraffin coating or vacuum sealing. Some stores cut slides in a refrigerator inside a sealed container with a desiccant. Other labs coat freshly cut slides in paraffin and may store them at room temperature, in a refrigerator (some in a nitrogen desiccator), or in a freezer; however, adequate deparaffinization prior to staining may be problematic and a freezer storage may introduce an ice crystal artifact. Paraffin coating seems to have the most beneficial effect on slides stored at room temperature. Vacuum sealing seems to preserve some epitopes, but it is detrimental to others. Slides stored in a desiccator at ambient or cold temperatures do not appear to lose immunoreactivity, but this is impractical. Cold storage at −20 °C appears to be the best option [14, 43, 48, 66–69].

15. Do standards exist for TMA data exchange?

An open-source TMA data exchange specification was developed by the Association for Pathology Informatics to facilitate data sharing between laboratories that employ a variety of information systems containing source data for tissue, experimental protocols, imaging modalities, data capture, and data storage. It consists of an XML document that defines 80 common data elements and 6 semantic rules. The data exchange specification has been evaluated and validated with files from AIDS and Cancer Specimen Resource TMA data [70, 71].

16. What features should TMA data management software offer?

1. Registration of patients and specimens
2. Means to catalog and manage paraffin block and frozen tissue archives
3. Management of common data element sets for general and organ-specific clinical information

4. Management of common data element sets for TMA construction and studies
5. De-identification of data for HIPAA compliance
6. Data mining tools
7. Web accessibility
8. Experimental results scoring, both quantitative and qualitative
9. Data exporting functions to other database and spreadsheet programs
10. Data importing functions from other database and spreadsheet programs
11. Security features including audit trails
12. Access for collaborators

17. What software is currently available for TMA data management?

Open access software: Stanford University currently offers TMAD for designing, viewing, scoring, and analyzing TMAs [72]. Another set of open-source software tools for managing TMA data and images is TMAJ [8]. QuPath is an open-source digital image analysis software that may be used to score IHC staining in TMA spots. The software also has learning algorithms, so that it can be trained to distinguish between tumor and non-tumor cells on whole slide images. A link is provided in the Resources section at the end of this chapter [73].

Commercial software: There are numerous commercial software packages available with a wide range of features. Many are associated with whole slide imaging equipment. For a partial list, please see Resources at the end of this chapter.

18. What statistical methods have been utilized to handle the large data sets associated with TMAs?

The statistics utilized depend on the study design and questions to be answered. The following is a brief summary of a few of the more complex mathematical models that have been employed. For open access software related to these models, please refer to the Resources listed at the end of this chapter.

Hierarchical clustering analysis: This technique has been applied previously to gene expression microarray studies to detect patterns of expression and can be similarly applied to TMA data. In this model, relatedness is independent of clinical or histological parameters and is therefore considered unsupervised. IHC scoring has a narrower range than that of cDNA array results and the clustering of data may therefore be less defined in IHC. The cluster analysis process groups similar IHC profiles together into columns in a clustergram. The relationships between cases and IHC profiles can then be depicted as a dendrogram [13, 74–76].

Hierarchical naïve Bayes model: This is a population level model that takes sample heterogeneity into account where replicate measurements are available for the same sample [77].

Tissue microarray object model: This is a data model that attempts to manage different sets of clinical and histopathologic information, as well as integrate those data with other biological data such as gene expression or proteomics data [71].

Random forest clustering: Unlike other clustering models, this one does not depend on dissimilarity measures between tumor samples. It is not dependent on other covariates but does look at relatedness between covariates. The model will also accommodate missing values [78].

19. What are the advantages and limitations of manual analysis of TMA IHC slides?

A pathologist can manually score approximately 1000 TMA tissue spots over a 1–2 hour period using a standard microscope if a single antibody marker is used, normal cells are easily distinguished from neoplastic cells, and artifacts are easily identified.

Sources of variability in TMA IHC manual analysis include:

Orientation: The greatest difficulty is keeping track of which tissue spot you are examining and scoring when the slide contains several hundred spots, making it easy to lose orientation. This is further complicated when arrays have misaligned cores, or when tissue spots are lost during transfer from the microtome to a water bath to a glass slide. It helps to have distinctly different control tissues scattered throughout the array.

Classification protocol: A well-defined system of classifiers will help reduce interobserver variability. It should address attributes of both the tumor and the antibody used such as expected staining patterns and grading of staining intensity.

Sequence of review: Most pathologists can easily recognize cores taken from the same tumor. Scattering cores from the same tumor into different sectors in the same array may help to ensure that each core is scored independently, especially regarding staining intensity.

Workload: Prolonged visual study results in eye fatigue and it becomes more difficult to reliably distinguish color changes.

Quantitative versus qualitative scoring: The number and complexity of categories used for scoring affects inter- and intraobserver agreement. Qualitative scoring (present vs. absent, or + vs. −) is simple but does not indicate intensity. Quantitative scoring is usually a numeric scale (e.g., 0, 1, 2, 3, 4) that accounts for staining intensity, but the intermediate

categories tend to be overused because the human eye cannot always reliably discriminate between subtle differences in staining color or intensity. When scoring tissue samples, we recommend the following parameters be used for consistency and ease of comparison between studies or institutions:

Indicate a semiquantitative scale for staining:

- Negative = no staining
- 1+ = <25% of appropriate cells stain
- 2+ = 26–50% of appropriate cells stain
- 3+ = 51–75% of appropriate cells stain
- 4+ = >75% of appropriate cells stain

Indicate intensity of staining:

- Strong, intermediate, or weak

Indicate compartmental localization:

- Nuclear, cytoplasmic, membranous, or combinations

Illumination: The typical light bulbs used in microscopes usually have a yellow tint that may influence the perception of staining intensity. The contrast introduced by filters and condenser settings will also influence the amount of light transmitted and can change the observer's perceptions.

Human vision: Vision is highly variable and every person views objects and colors slightly differently. Vision is very subjective and IHC observations will be affected by the amount of tumor present, background staining, and stromal staining. Contrast, or the average brightness of the tissue versus the background brightness, is especially important when assessing membranous staining patterns. If no cytoplasmic staining is present, membranous staining is perceived as more intense than if cytoplasmic staining were present. This effect is called conditional contrast [5, 8, 79, 80].

20. What are the advantages and limitations of automated analysis (virtual microscopy) of TMA IHC slides?

Virtual microscopy via a slide scanner helps to overcome some of the limitations of human vision as subtle differences in staining intensity and color can be detected and scored on a continuous scale with highly reproducible results. Slide scanners are not subject to eye fatigue and illumination is standardized. However, virtual microscopes and their associated software programs have difficulty accounting for daily variability in slide staining, uneven color patterns, and other artifacts such as edge effect in IHC, air bubbles, tissue folds, and cells that appear merged, all of which pathologists recognize easily.

Several types of scanners are available. There are three basic types: field of view devices that capture a digital image of a field and use software to stitch the images together; linear array devices that scan a slide in strips that are stitched together by software; and area array scanners that have several fiber-optic cameras take an image of a slide simultaneously and the software stitches the images together. The area array scanners are the fastest (about 1 minute per slide) but they are much more expensive. Important features to look for in a scanner include scanning time, image resolution, z-stacking capability of the software (equivalent to focusing up and down on a microscope), file formats (many are proprietary) and types of compression for images, and usability of software for slide viewing. Commercially available scanners offer brightfield microscopy, and many offer fluorescence capabilities. An excellent overview of automated image analysis by Foran et al. describes the current state of computer-assisted diagnostics, including applications and issues associated with TMAs [81]. For a list of vendors, please see the Resources section at the end of this chapter.

Another limitation of automated systems is image storage requirements. The image size depends on the scanning resolution and area scanned in two dimensions. Images for research will probably need to be maintained for several years and should have a backup system. Indexing of images for easy retrieval is essential [8].

Resources for TMA

Tissue arrayer instrument vendors:
- https://www.3dhistech.com
- http://www.alphelys.com/alph01/prod/us/minicore/minicore.php
- https://estigen.com
- https://www.everbiotech.com/
- http://www.micaarray.com
- http://www.pathologydevices.com
- http://unitma.com

TMA supplies (manual punches and molds):
- https://www.alphametrix.de
- https://www.antigen-retriever.com
- http://www.ihcworld.com/tissuearray.htm
- https://www.tedpella.com/histo_html/tissue-microarray-kits.htm
- http://www.thermoscientific.com

Open-source software for TMA analysis:
- http://genome-www.stanford.edu/TMA/
- https://github.com/qupath/qupath
- https://qupath.github.io
- http://tmalab.jhmi.edu/software.html

Commercial software TMA tools:
- http://www.premierbiosoft.com/tissue-microarray
- http://www.alphelys.com/alph01/prod/us/spotbrowser3/spotbrowser3.php

Slide scanners with TMA management software:
- https://www.3dhistech.com
- https://www.leicabiosystems.com
- https://www.usa.philips.com

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