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How to make tissue microarrays

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Abstract

It is now almost 20 years since tissue microarrays (TMAs) were first described by Kononen and colleagues. Today, this high throughput methodology has been widely adopted by investigators who require a cost-effective method to rapidly and simultaneously interrogate large numbers of samples, including those derived from large patient cohorts. Drawing on over 15 years of direct experience of TMA design, construction and analysis, we discuss a variety of TMA applications and provide a detailed description of TMA design, quality control and construction. Advantages and disadvantages are discussed and potential problems and practical solutions are highlighted.

Keywords

Tissue microarray, quality control, design, construction

Introduction

Tissue microarrays (TMAs) were first developed and described by Kononen et al [1] in 1998. Almost 20 years later, TMAs remain a fast and cost-effective solution for multiplexed in situ tissue analysis, and represent a validated method of high throughput simultaneous analysis to investigate a variety of biomarkers.[2]

Currently, TMAs are principally used as a research tool for the investigation of putative prognostic and predictive molecular targets in human cancer tissues. However, TMAs have been utilised for a variety of other applications including for diagnostic staining quality control, inter-laboratory comparisons [3, 4, 5, 6, 7, 8] and infectious disease studies [9, 10].

Collections of tissue organised in a ‘multi-tissue sausage’ were first described by Battifora in 1986 (reviewed by Chan et al [11]). This basic approach was further expanded and developed by Wan et al [12] who produced a library of paraffin embedded cores set within a 'straw' to determine the staining patterns of new monoclonal antibodies. These techniques were refined in 1998 when Kononen and collegues,[1] using the term ‘tissue microarray’ for the first time, described the technology we still use today. In this seminal paper, Kononen eloquently elaborated on the advantages of using TMAs, which allow the processing of up to 1000 tissue specimens in one section, over using a conventional ‘full-face’ tissue section [1].

Types of TMAs

TMAs are generally categorised by their material of origin. If constructed from paraffin embedded material, the term 'tissue microarray' is usually applied, although

this is not universally applicable as tissue samples may also be arrayed into resin as the recipient block medium. The latter is required if very thin sections are needed. However, resin TMA construction is technically challenging, labour intensive and is generally regarded as only being suitable for specialised applications because of constructional complexities when compared to paraffin TMAs.[13] Interestingly, TMAs have also been constructed using frozen tissue samples (cryoarrays) [14, 15, 16] as well as embedding cell lines [17,18, 19, 20] in addition to standard cell blocks [21].

As an alternative to using the material of origin as a basis for classification, TMAs can also be categorised according to their anticipated application. Some examples are listed below:

1. Predictive TMAs, which are used to identify markers that predict response to therapy such as for example HER2 [22]
2. Control tissue TMAs, which are used to establish experimental protocols and also serve as external controls for diagnostic immunohistochemistry (IHC).
3. TMAs for validation of markers discovered by extracted protein, DNA or RNA based studies, [23, 24, 25] (see review by Hewitt SM)[26]
4. Prognostic TMAs for investigation of the relationship between staining results and clinical endpoints [27, 28, 29]
5. Progression TMAs in which cores of a single tissue type derived from different stages of tumour development or different tumour grades. Thus, for example, a progression TMA for breast cancer would include normal breast, ductal carcinoma in situ, invasive tumour and metastatic deposit [30] or for colon

cancer one would array normal colon, adenomas with both, low and high grade dysplasia as well as carcinomas [31].

It is important to note that TMAs are not only used to characterise abnormal tissues, but can also be used to determine the presence and extent of expression of proteins in normal tissues [32, 33].

TMA Design and Construction

1. Tissue sampling

The initial task of TMA design should be regarded as one of the most important stages of TMA construction.

Apriori hypotheses or questions of interest should be defined in advance as this will impact on the sampling strategy used to interrogate the original tissue samples. In particular, if it is intended to compare spatial protein expression patterns between tumour centre and periphery, cores will clearly need to be sampled from the appropriate locations. If, conversely, the task is to characterise overall protein expression for a given marker in a given tissue, then the sampling approach is completely different. Sampling for the first task (comparing the spatial expression of protein between a tumour periphery and centre) could be regarded as a 'targeted' approach whereas 'random' sampling would be the best technique to characterise an overall expression pattern in a tissue [34].

Tumour heterogeneity is a recognised major challenge for TMA users [35]. Taking multiple samples of the tumour seems to be an appropriate method of compensating for potential variability of expression of molecular targets in a given tissue. Although there is no universally agreed standardised tissue sampling method, it is intuitive that the more samples are taken from the donor tissue of interest, the more representative the subsequent TMA staining results. Concerns relating to how representative TMA-derived staining data is when compared to full section staining has led to a large number of validation and feasibility studies in different tissues comparing results from whole sections with those of TMA cores [30, 34, 36 – 56]. How many TMA cores are necessary to achieve a high degree of concordance between results from full sections and TMA cores is a recurring question that is closely scrutinised. Most studies suggest that the results from triplicate TMA cores have up to 98% concordance with the result from full sections [37, 57]. However, a recent study by Goethals et al [58] recommends at least four cores whereas other authors achieved greater than 95% accuracy with only two cores [56].

Beyond heterogeneity, basic technical issues mandate the use of more than one core of tissue per case. Tissue cores can be lost during sectioning and subsequent procedures, or subsequent interpretation is compromised by folding of the tissue core or unacceptably low numbers of tumour cells (assuming tumour cells are the component of interest) per core [59, 60, 61]. Thus by having multiple tissue cores per case, the potential impact of such loss is minimized.

The proportion of 'lost cases' resulting from technical losses has been reported as high as 23% in a TMA study of renal cell carcinomas [62]. We currently construct

TMAs from gastro-oesophageal cancer specimens where we normally sample three 'random' cores from each area of interest. However, in the case of low tumour cell density which in particular is a problem in diffuse type gastric cancer, we often double the number of cores to six per case. Our own unpublished studies in gastric cancer TMAs suggest a mean technical loss rate of 10% of cores.

Interestingly, there is much less debate about the influence of core diameter with respect to tissue sampling. For the commonly used manual arrayer from Beecher Instruments (now manufactured by Estigen Tissue Science), punches with a diameter between 0.6 mm to 2 mm, equivalent to a tissue area of 0.283 mm^2 to 3.141 mm^2, are available. The vast majority of published studies use 0.6mm punches, with cited benefits including a reduction in disruption to the donor block, preservation of more source tissue and incorporation of a larger number of cores in a single recipient block.

Up to 1000 cores of 0.6mm diameter can be placed into a single TMA measuring 25mm x 45mm [1, 27]. However, some authors are more cautious and suggest a maximum of 500 cores per block as a more realistic number ;[57] this reflects our own ongoing practice. From our experience, punches with a diameter greater than 0.6mm are useful in specific applications such as sampling of fatty or connective tissue-rich material as larger cores have better adherence. Also frozen tissue and study of large tissue areas e.g. whole depth of mucosa in the gastrointestinal tract require punches greater than 0.6mm in diameter.

2. Layout of the TMA

Currently there is no general agreement on the optimal layout of a TMA, almost certainly because different studies have different requirements. From our own experience and through correspondence with other laboratories using TMAs, the following components appear to be essential to consider when planning a TMA layout.

As tissue border staining artefacts are a well-recognised problem when performing immunohistochemistry on full tissue sections, we frame all our TMAs with a 'protection wall' (Figure 1A), formed by a row of tissue cores which will not be analysed. Such protection walls are typically formed by any tissue that is available in abundance in the manufacturing laboratory. This protection wall was originally described by Hoos et al [37].

Being able to unambiguously identify individual cores within the TMA section is crucial as any confusion or doubt about the origin of a core will make the assessment of the staining impossible. We recommend use of two separate features to ensure unambiguous orientation within the TMA section as well as unambiguous identification of the TMA block itself. Most authors add 'orientation cores' in specific positions usually outside the overall geometric margin of the array (Figure 1B). However, we were always concerned regarding loss of these crucial orientation cores and therefore incorporate orientation 'gaps' into the TMA design. Using a combination of intentionally left empty core positions, it is possible to unambiguously macroscopically identify the TMA block as well as to orientate the cut TMA section (Figure 1A). In addition, we include control tissue cores in every TMA

and place them asymmetrically into the grid further aiding orientation within the section. Thus, control tissue cores serve as internal 'orientation cores' as well as both positive and negative internal experimental controls. We usually establish the staining pattern of the marker under investigation on sections from a control tissue TMA which contains the cores from the same control tissues as included in the final TMA.

The arrangement of TMA cores for a given design will depend on the type of study and on how many cores are sampled from each donor block. Ideally, cores from the same donor block should not be placed adjacent to each other as only a random distribution of cores from the same donor block within a given TMA would ensure results from individual cores are recognised as 'independent results' from a statistician's perspective.

However, from a practical perspective, randomly distributed TMA cores derived from the same donor block significantly increases the manufacturing workload and is therefore rarely done when using a manual TMA arrayer. Random distribution of cores is less of an issue if using an automatic TMA arrayer. We typically cluster cores from the same tumour and the matched normal tissue next to each other (Figure 1A) along the horizontal axis (from left to right). However, we recognise that other investigators or commercial suppliers of TMA sections use a completely different design (for example see Figure 1B). We would like to emphasise that our design presented in this paper is only one of many options for TMA design and individual investigators need to identify which methodology best suits their purpose.

3. Technical procedure

After appropriate cases of interest have been identified and tissue blocks retrieved from archive, a fresh full face 5µm H&E section should be cut and reviewed using a conventional microscope or corresponding scanned image. The area of interest is identified and marked on the glass slide (or annotated on the virtual slide) so that the corresponding area on the tissue block can be sampled (Figure 2).

3.1 TMA instruments

Several instruments are commercially available. Examples include a manual and automated tissue arrayer produced by Beecher Instruments (manufactured by Estigen Tissue Science; https://www.estigen.com), a semi-automated tissue arrayer produced by Veridiam (www.veridiamtissuearrayer. com), the Quick Ray manual and automated tissue arrayer from Unitma (www.unitma.com), and the TMA Master II automated arrayer by 3DHistech (http://www.3dhistech.com). In addition, several 'home made' array methods have been reported [63, 64, 65, 66].

All devices utilise a hollow needle/punch to extract tissue cores from 'donor' blocks in a process similar to that of a cutaneous punch biopsy. These donor cores are subsequently reassembled in new paraffin 'recipient' block. Once cut, sections from the recipient TMA block show a series of circular samples of tissue from multiple or reduplicated sources organised in a grid like formation.

In this review, we describe the procedure of how to construct TMAs from paraffin embedded tissue using the manual Beecher tissue arrayer (now produced by

Estigen Tissue Science, https://www.estigen.com/). Users of other TMA machines should consult the appropriate documentation relating to their device.

3.2 The recipient block

Recipient blocks are manufactured by forming blank paraffin blocks using existing metal moulds of variable size. We have previously identified that air bubbles can be generated within the paraffin block during the cooling procedure when using conventional metal moulds. These air bubbles are not readily apparent until after TMA construction and subsequent sectioning of the TMA block when they can lead to the collapse or severe distortion of the constructed TMA (Figure 3). As a quality control measure, we therefore now x-ray all recipient blocks prior to use with an x-ray device used for x-raying breast specimens in a routine histopathology laboratory. Air bubbles can easily be seen within the block on the x-ray and unsuitable recipient blocks can be melted for reuse (Figure 4). We, and others, have found that the formation of air bubbles is related to the use of metal moulds and can therefore be minimised by using moulds made from paper or plastic material.

3.3 Step-wise TMA construction

The manual Beecher Instruments tissue arrayer from Estigen, as per most other arrayers, has two needles (also known as punches) with slightly different diameters. The smaller one is used to extract the paraffin core from the recipient block in order to create the space for the core from the donor block and the larger one to extract the tissue core from the donor block. Both needles can be positioned with high accuracy along the x and y axis using two manually-adjustable micrometers. A magnet holds the recipient block holder in place and the 'bridge' slides over the

recipient block to accommodate the donor block. An adjustable depth stop enables a constant depth of hole to be created in the recipient block (Figures 5A and 5B). One of the first steps is to position the recipient “blank” TMA block into the block holder or mount; align the needles, so that both needles are exactly on top of each other when the needle holder is moved to the opposite side and move the needles to the starting point of the array, usually the upper left corner.

Next, the hole in the recipient block is created, the donor core extracted and inserted into the recipient block with the help of the steel stylet as show in (Figures 5A, 6A and 6B). Some authors advocate stacking several cores on top of each other if the donor tissue core is very short [67]. After the recipient hole is filled, the needles are moved along the x axis to the right using the micrometer. We use a centred distance of 1mm between the cores when using 0.6mm punches.

The entire procedure is repeated according to the design plan. It is mandatory that both, the block of origin and the TMA position of each individual core are documented with great care throughout the procedure. Figure 7 shows a completed recipient block (TMA). This block will be placed upside down onto a glass slide and placed into an oven at 40^{o}C overnight to facilitate bonding of the donor cores with the paraffin wax of the recipient block. The following morning, the glass slide attached to the TMA block will be used to level the TMA block surface by gently pushing the cores into the block if necessary. After cooling, the TMA block is ready for sectioning.

The TMA block can be sectioned on a microtome as any other paraffin block. However, we recommend that only experienced technicians should cut TMA blocks as the TMA section needs to be cut and picked up from the hot water bath with great care to avoid distortion prior to aligning it in parallel with the edge of the glass slide. Our own experience using a tape transfer methodology for this is not as favourable as reported by others. [59, 68, 69] In practice we have very good experience with cutting TMA sections at 5 µm thickness and subsequent mounting of sections onto 4% APES coated Superfrost Plus glass slides. However, it is critical to avoid hot plating of the cut TMA section as this could lead to disintegration of the arranged TMA grid (Figure 8). See Figure 9 for an example of a Haematoxylin/Eosin stained TMA section at low magnification.

Advantages and Disadvantages of TMA technology

1. Advantages

TMAs allow fast, simultaneous (and thus high-throughput) study of immunohistochemical expression patterns, gene copy number patterns (using in situ hybridisation) or other features in a large number of cases (usually 500+). TMAs significantly reduce the experimental handling time compared to using individual full sections. Furthermore, simultaneous processing ensures identical experimental conditions for all tissues assembled within the TMA block. The necessary reagent volume can be reduced by up to 80 times making it a very cost-effective method [54]. Using TMAs preserves precious and finite tissue resources and maximises the number of experiments that can be performed with the material present in one paraffin block (confusingly known as 'tissue amplification') [70]. From a normal paraffin block, approximately 200 to 300 5µm sections can be cut. Assuming that a full

section block is completely cut through and each section used to look at a different molecular target, then the number of markers investigated would represent roughly 0.75% of the estimated 40,000 genes in the human genome [27]. However, assuming the tissue in this block measures 10mm by 10mm and a TMA needle with a diameter of 0.6mm is used; then approximately 256 cores can be extracted from the tissue and placed into different recipient blocks. This 'amplification' of tissue would enable the investigation of 76,800 markers instead of 300 when full sections are used.

2. Disadvantages

The costs of a manual tissue arrayer or even more expensive automated models mandate that the equipment is in constant use. Therefore, TMA methodology is not cost effective for laboratories who only need to array small patient series once every five years or investigate only very few markers on the same tissue.

The ease of use of TMAs leads to many experiments being performed in a short period of time and the generation of a large amount of data to be analysed. The knowledge and experience required to analyse such complex datasets is much greater than for conventional small scale immunohistochemical studies in particular if results from TMA studies need to be integrated with studies from other high throughput methods such as gene expression arrays or array CGH.

Due to the small size of the tissue core, TMA cores can be vulnerable to tissue loss during processing when compared to full sections, especially those containing abundant connective tissue, keratin or bone [54] (see also above). If TMA sections are read manually, one core after another, using a conventional light microscope, keeping track of the position of each core can become very difficult when there are hundreds of spots on one slide. The observer may 'get lost' in the grid and

consequently assign a result to the wrong core or case. As mentioned above we use orientation gaps and asymmetrically located control tissue cores to facilitate and ensure proper orientation (Figure 1A). Sauter [35] favours the use of smaller subarrays in an attempt to prevent orientation problems when reading the glass slide (for example see Figure 1B). This potential problem can be solved by using software to construct the array and / or subsequent digital scanning and image analysis for the evaluation of results [71]. Open source software for this purpose is available from Stanford University (http://genome-www.stanford.edu/TMA/), Johns Hopkins University (http://tmaj.pathology.jhmi.edu/) and the University of Leeds (http://www.virtualpathology.leeds.ac.uk/research/systems). Scanning of TMA sections and making images available and analysable via the internet has greatly facilitated international collaborative studies in our laboratory.

By constructing TMAs from hundreds of different tissue blocks, one potentially combines tissues which may be differently fixed, have different storage time in paraffin, or have been embedded in different paraffin mixtures. Therefore, the established staining protocol may only be optimal for a subset of the tissues included within the TMA, whereas others may be over stained or give false negative results. The inclusion of multiple control tissue cores not only representing different tissues but also representing different fixation and embedding protocols may be one way of tackling this potential problem. A second option is to quality control the TMA regarding immunoreactivity by immunohistochemical staining for vimentin as suggested by Battifora [72].

Strict and comprehensive quality control is necessary for TMA design, construction and use (see below); however this has clear limitations in very small tissue areas such as TMA cores. This is in particularly true when making the decision whether a

particular core contains tissue with dysplasia or invasive carcinoma, a distinction that sometimes cannot be made reliably and reproducibly on TMA cores. [73] Such errors can potentially lead to the exclusion of cases from the final analyses, even after careful quality control.

Quality control

Strict quality control (QC) is necessary at many different steps of the TMA manufacturing and analytical process. The initial review of the H&E section in order to select the area of interest should be performed by an experienced histopathologist in close collaboration with the end-user as appropriate selection of material is crucial for the success of the whole experiment.

We have demonstrated [73] that the quality control of the H&E stained TMA section in order to confirm (or not) whether the expected tissue (e.g. tumour) is present at the pre-defined position in the TMA is most reliably performed by an experienced histopathologist as very junior pathologists have greater difficulty in providing a correct diagnosis in a given tissue core. However, the relative regular organisation of our TMAs (see FIGURE 1A) makes the quality control process challenging as the blinded observer can more or less predetermine what type of tissue to expect in a given core at a certain location and may therefore be biased in his/her independent assessment.

Apart from confirming the qualitative content of the core (e.g. that the core represents tumour tissue), some studies may require a more quantitative assessment of the number of tumour cells per core. Unfortunately, there are currently no guidelines regarding the minimum number of tumour cells in a core to be of acceptable quality.

As for full section staining, every staining procedure needs to include appropriate negative and positive controls. We include all tissues that were used to establish experimental protocols within all our TMAs. This enables us to assess whether the staining has worked as expected in a particular TMA section.

In addition, the evaluation of the staining ('scoring') needs to be quality controlled which can be done by a) double scoring all slides or a subset of the slides and b) by producing 'reference slides' with agreed staining scores which are used by all observers. Both of these methods are in use in our laboratory.

The issue of x-raying the recipient block in order to exclude air bubble containing blocks and the issue of performing vimentin immunohistochemistry in order to assess antigenicity have already been discussed above.

Conclusion

Analytical platforms utilised by biomedical researchers are becoming increasingly complex. Despite such advancement, TMA technology remains a major tool in the field of biomarker discovery and in validation of genomic, transcriptomic and proteomic data. Innumerable studies have demonstrated that TMAs are both time and cost effective as well as allowing the maximum amount of information to be gained from precious tissue resources. With growing expertise in the automated scoring of stained TMAs, particularly in the field of image analysis and deep learning, even larger experiments can be performed as the manual scoring of TMA sections is undoubtedly time consuming and arguably, inaccurate. Despite the disadvantages and challenges, we remain convinced that the use of TMA technology in well characterised clinical material will enable us to bridge the gap between the

laboratory-based science and the routine application of predictive and prognostic biomarkers in a clinical setting.

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Figure legends

Figure 1: Tissue microarray design

1A: TMA 'Leeds Version 1': Components considered essential are (i) the 'protection wall' which frames the array and can be produced from any tissue type (blue spot); (ii) asymmetric location of control tissues (green spot) for experimental positive and negative controls and orientation within the array; (iii) gaps (black circles) for orientation and macroscopic identification of the TMA block. Tumour tissue (red spot) and matched normal tissue (black spot) are arrayed in triplicate from left to right; six cores are taken in cases with low tumour density.

1B: TMA 'Subarray Design': The whole array is subdivided into regular blocks of smaller subarrays which are separated by asymmetric rows of gaps (black circles). Control tissues (green spot) are arrayed in subarrays, asymmetrically located at one side of the array but are not distributed in between the rest of the cores (see 1A for comparison). Two additional orientation cores (pink spots) at one side of the array.

Figure 2: Marked Haematoxylin/Eosin stained full section

HE stained tissue section from a colon cancer where an area with tumour tissue (Tu) and an area with normal mucosa (N) have been marked with a black pen by a pathologist. This slide will be used to identify the areas to punch in the donor block.

Figure 3: HE stained section of TMA with air bubble containing paraffin block

Low magnification picture of an HE stained TMA section that was cut from a TMA block which contained a large air bubble. The air bubble was only detected upon sectioning into the block (star indicates position of air bubble). Although the hole was

filled with paraffin to rescue the TMA block, the distortion caused by the air bubble is clearly visible.

Figure 4: X ray of recipient paraffin blocks

Arrows indicate where air bubbles are visible in the paraffin block. Please note that these air bubbles are not visible from outside.

Figure 5: Overview of the Beecher Instruments Tissue Arrayer

5A: The usual position of the hands in front of the arrayer when extraction a core from the recipient block where the right hand is pushing the movable holder in front of the machine downwards into the block. Note that the adjustable depth stop (see arrow) can only be used to control the depth of the hole in the recipient block. Also visible the micrometer for the movement along the Y axis at the back of the instrument.

5B: Close up view of the front of the machine with recipient block in block holder which is attached to the rest of the machine via a magnet. Note the micrometer for the positioning along the X axis. The two punches are set up for a right handed person with the punch for the extraction of the core from the recipient block on the left side (A) and the one for the donor block on the right hand side (B).

Figure 6: Extraction of the donor core and insertion into recipient block

6A: A bridge slides over the recipient block and accommodates the donor block. After identifying the area to be punched, the larger needle (B) will be pushed into the donor block. As soon as tissue enters the hollow needle, the stylet moves upward.

6B: Bridge and donor block have been moved away from the recipient block. The index finger of the left hand is used to push the donor core into the recipient hole. Note that the thumb of the right hand underneath the front holder of the machine is the counterforce that prevents the donor needle (B) from sliding into the recipient block.

Figure 7: TMA paraffin block with cores

0.6 mm cores have been inserted with a centred distance of 1mm. The picture shows the surface of the block before warming overnight at 40 Degree Celsius. Note that a space is left between the edge of the paraffin block and the most outer row of cores in order to allow pick up of the cut section from the microtome with forceps.

Figure 8: TMA section after hot plating

HE stained TMA section showing the disintegration of the cores of the TMA when hot plating the section after cutting.

Figure 9: HE stained TMA section (overview)

Overview of an HE stained TMA section demonstrating the protection wall (blue overlaid spots) and the intentionally empty cores for identification and orientation of the block (grey coloured areas). Note that although some of the cores are truly lost (yellow coloured areas), orientation is still possible.