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Navigating Tissue Microarray Construction: A Guide for Avoiding Pitfalls and Mastering Key Technical Aspects

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ABSTRACT

Introduction: Tissue Microarray (TMA) is a novel technique that is now integral in pre-clinical and translational research. In resource-limited settings, automated microarrayers and molds are out of reach, and manual TMA construction may be done instead.

Aim: To explore the pitfalls encountered while constructing manual TMAs and to troubleshoot these problems using the available resources.

Materials and Methods: A cross-sectional study was done between September 2019 and March 2021 in the Department of Pathology at a tertiary healthcare center in New Delhi using 60 mastectomy specimens. Five manual TMAs were constructed using simple, cheap, and readily available resources. Problems encountered during the construction were identified and documented. Solutions attempted to troubleshoot the common problems were documented, and their outcomes were evaluated.

Results: Difficulty in core extraction, cracking of TMAs during core insertion, loss of cores, misalignment of cores, bulging of blocks, difficulty in sectioning due to non-uniform cores, and loss of cores during sectioning were major problems identified. Simple measures such as prior warming of both donor and recipient blocks, use of punch biopsy needles with a plunger, maintaining a margin around the cores, using wax cores to align cores, and adequate cooling prior to sectioning helped in overcoming these problems. Other solutions that were attempted but did not yield satisfying results included the use of agarose in paraffin blocks and the use of liquid wax to seal cracks and gaps.

Conclusion: Manual TMA is not only feasible, it is easy to construct once the technique is learned and the problems that commonly arise in its construction are tackled. The modifications suggested in the present study can aid in constructing these microarrays faster and avoid both wastage of time and resources. TMAs can thus be used as an alternative to traditional paraffin-based techniques for research applications in resource-limited centers with high patient loads.

Keywords: Kononen method, Manual Tissue Microarray, Troubleshooting

INTRODUCTION

The TMA is a novel technique that may be used to perform any special test or research technique required on formalin-fixed paraffin-embedded tissue. It was designed as a high-throughput molecular device where tiny amounts of biological samples are organised on a solid support, allowing the analysis of expression of several biomarkers simultaneously on numerous tissue samples [1,2].

TMAs are now integral in preclinical and translational research. They have been predominantly used in the development of biomarker assays for disease characterisation [3]. It is a practical and cost-effective tool for molecular analysis of tissues that further helps in identifying new diagnostic, predictive, and prognostic markers in human malignancies [2]. This technique has several advantages, including simultaneous analysis of a large number of specimens, decreased assay volume, conservation of valuable tissue, its ability to use scarce resources, and time effectively [2].

Some researchers believed that small cores in TMAs might not be representative of the whole tumour. However, a study conducted in the United States of America (USA) found that the microarray core gave the same result for Estrogen Receptor (ER) status as that of the whole section in 96% of cases [4]. Similarly, Camp RL et al., examined the status of ER, progesterone receptor, and Her2/neu in 38 cases of invasive breast carcinoma. They compared the staining of 2 to 10 microarray disks with the whole tissue sections from which they were derived and found that the analysis of two core sections from one case was comparable to the analysis of whole tissue sections in more than 95% of cases [5].

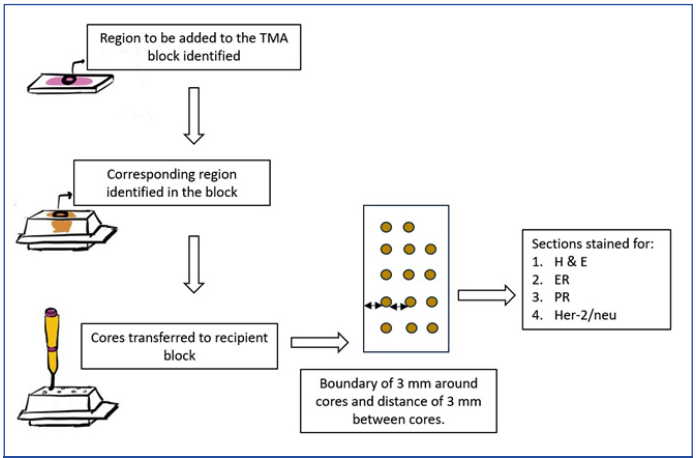
The use of silicone-based TMA molds as marketed by manufacturers such as Ted Pella Inc. and T-Sue™, and automated tissue arrayers, makes TMA construction an efficient and straightforward process. However, in most resource-limited settings, these are out of reach, and hence, manual construction of TMAs remains a mainstay. There are several methods of manual TMA construction such as those described by many previous studies [6-8]. However, few studies have explored the pitfalls, including breakage and cracking of TMA blocks and bulging of cores, encountered while constructing manual TMAs in resource-limited settings or how to troubleshoot the said problems [9,10].

The technique used by Kononen J et al., is one commonly followed method of manual TMA construction [7]. The present study aimed at studying this method of creating manual TMAs with simple, cheap, and readily available resources [7]. It also aimed to identify and troubleshoot the common potential problems that arise while constructing TMAs, which can help in reducing the pitfalls.

MATERIALS AND METHODS

This was a cross-sectional study conducted at a tertiary healthcare center and teaching hospital in Delhi between September 2019 and March 2021 after obtaining Institutional Ethical Clearance (IEC-HR/2019/41/126). Tissues from 60 mastectomy specimens of confirmed breast carcinoma cases were included, while those showing non-availability of representative tumour tissue, poor tissue processing, and those who had received neo-adjuvant chemoradiation therapy were excluded. The authors used the method for TMA construction as described by Kononen J et al., However, instead of using a specially constructed needle, a punch biopsy needle with a plunger of an internal diameter of 3 mm was used, due to its ease of availability, low cost, and less cumbersome usage [7].

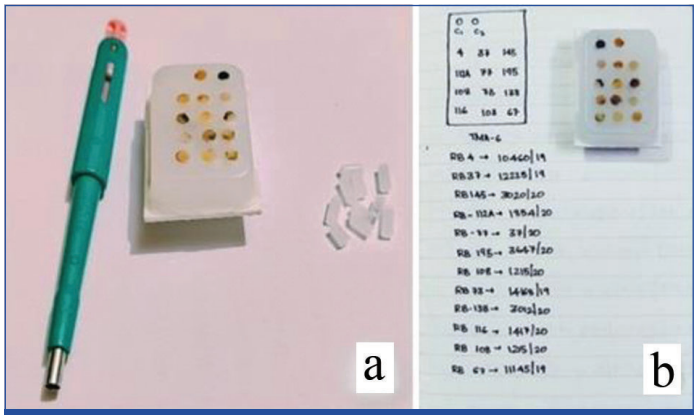
Donor blocks (grossed from the tumour proper of the specimen), punch biopsy needles 3mm (Integra Miltex, ted Pella Inc.); paraffin wax (Merc corporation); micro slides (Pearl Microscope Slides); hot air oven (Tanco Universal oven PLT 125A); stainless steel molds, 6 mm (Yorco);tissue floatation bath (electric tissue float bath, Yorco); microtome blades (Patho Cutter-R, ERMA); semi-automatic rotary microtome (Thermofisher HM 340E);embedding station (Unimeditrek, Kschriom);cooling station (Microm EC 350-2); pen with a rubber grip;incubator (Seco);fine-tipped marker pen;forceps; dissecting Needle (Leica) were used. Specimens reported as invasive ductal carcinomas/invasive carcinomas were re-grossed from tumour areas and embedded into blocks. Hematoxylin and eosin (H&E) stained slides of the tissue were examined to identify the desired tumour area. This area was demarcated on the glass slide using a marker pen. Each demarcated area was assigned a unique identifying code comprising letters and numbers. The Haematoxylin and Eosin (H&E) glass slide was then overlaid on the block, and the corresponding desired area was identified on the block and demarcated using a marker pen. TMA Construction is shown stepwise in [Table/Fig-1].



[Table/Fig-1]: Steps of manual Tissue Microarray (TMA) construction.

A recipient block was constructed using a plain paraffin wax block. Wax was melted at 59°C and poured into a deep mold. A standard tissue cassette was placed on top, and then the block was allowed to cool completely at room temperature. After cooling, the cassette and mold were separated, resulting in the plain recipient block. Using the Miltex punch biopsy needle, the final recipient block was created with a predecided microarray map format of 4×3. The cores were made with the punch, ensuring a perpendicular angle, and the paraffin cores were removed, leaving a hole behind. Two pointer holes were made in one corner for ease of orientation of the TMA [Table/Fig-2].

Previously selected desired tumour tissue areas were taken by punching cores from the donor blocks using a similar punch needle, ensuring that its angle of approach was perpendicular for optimum tissue sampling. After pulling out the biopsy punch, the tissue core was extracted with the help of a plunger and transferred to the holes in the recipient block using forceps, arranged in the previously decided format in the recipient blocks [Table/Fig-2].

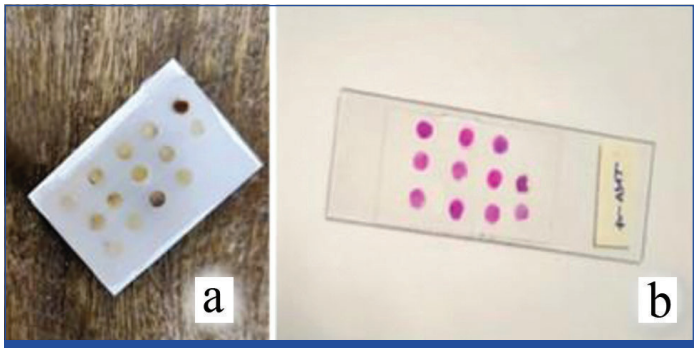


[Table/Fig-2]: A TMA along with Miltex punch biopsy and wax cores taken out while constructing the recipient block (a). An array map with the subsequent TMA that was constructed based on it (b).

On completion of TMA assembly, a clean slide was attached to the face of the TMA block to apply firm yet gentle pressure to press down any protrusion from the surface of the block. The authors designed a TMA yielding 4×3 cores per array with two control pointer cores. Upon completion of construction, the arrays were placed in an oven at 60°C for 10 minutes face down on a clean glass slide to augment the adherence of the cores to the walls of the punches in the recipient block and the surrounding wax.

The slide/block combination was then removed from the oven, and gentle and even pressure was applied, which removed any irregularities in the block surface that may have occurred during core insertion in the recipient block. The TMA blocks were placed on the cooling station (Microm EC 350-2) for complete cooling before cutting.

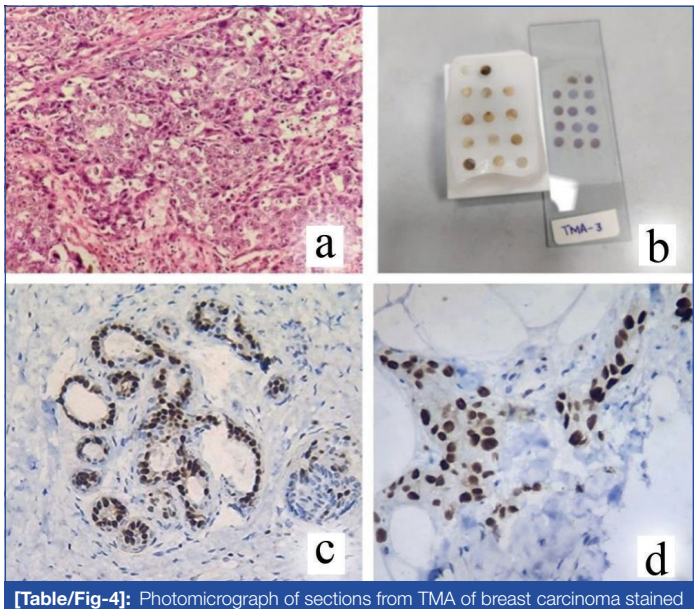
The temperature in the tissue floatation bath was set to 55-60°C. The blocks were sectioned at 3-4 µm on a semi-automatic rotary microtome, both on plain and lysinated slides for H&E staining and IHC, respectively [Table/Fig-3].



[Table/Fig-3]: A fully constructed TMA block (a) and final section taking from TMA constructed (b) by the method described in this study.

The slides were dried overnight, preferably in a vertical position.

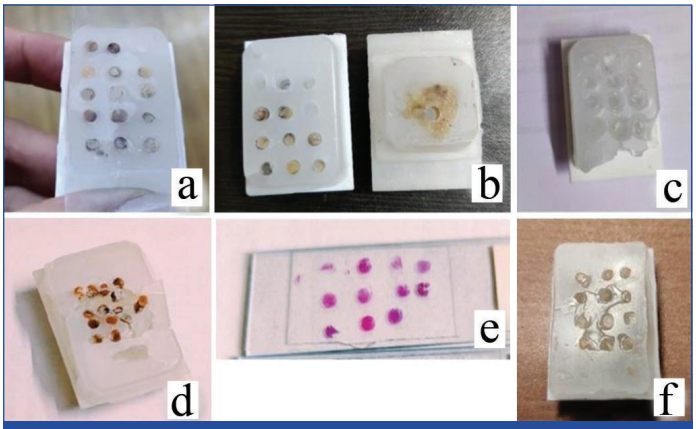
The H&E and routine IHC staining (ER, PR, and HER-2/Neu) for breast carcinoma were done [Table/Fig-4].



[Table/Fig-4]: Photomicrograph of sections from TMA of breast carcinoma stained with H&E stain (a, 400x), TMA block with corresponding section stained with ER (b). Photomicrographs of section from TMA with a core stained with ER (400x, b) and PR (400x, c).

RESULTS

Several problems were encountered while constructing TMA using the Kononen J et al., method, and the authors attempted various solutions to counter these [Table/Fig-5,6] [7].



[Table/Fig-5]: Problems identified in manual TMA construction: TMA block with shorter cores placed deeper than the others (a);TMA block with cracks developed during core insertion (b); Recipient blocks with large sized holes, poor spacing and uneven boundary leading to breakage (c); TMA block constructed by adding agarose to paraffin (d); Loss of cores while sectioning in TMAs with cores of unequal length and also in blocks with uneven surfaces (e); and loss of cores while sectioning in TMAs with cores of unequal length and also in blocks with uneven surfaces (f).

S. No.	Problems identified	Solutions recommended
1.	Difficulty in core extraction	Prior warming of the donor and recipient block
2.	Cracking of TMA during core insertion	Maintaining a margin around the array
3.	Retained wax inside the punch biopsy needle	Removed using tissue dissecting needle
4.	Loss or misplacement of tissue while handling with forceps	Direct insertion of core extracted from donor block into recipient block using plunger
5.	Presence of enlarged holes around the tissue	Add a layer of wax using the embedding station
6.	Presence of longer cores than depth of holes	Use a blade to cut to desired length and re-insert using forceps
7.	Cores shorter than the depth of holes	a. Remove core using a punch biopsy needle of greater size b. Cut and using forceps add a disc of wax of desired length using the wax cores removed c. Re-insert the tissue core d. Add a layer of wax using the embedding station.
8.	Bulging of block when cores placed closely	Ensure a distance of at least 3mm between 2 cores
9.	Difficulty in sectioning due to non-uniform core holes	Add a layer of wax from the embedding station prior to sectioning
10.	Appearance of serrations and loss of cores during sectioning	Adequate prior cooling of blocks
11.	Breakage of blocks during sectioning	a. Maintaining a border of at least 3 mm around the array b. Avoid transferring a fresh TMA block immediately from the oven to the cooling station c. Reducing space between cores increases block stability (3 mm)
12.	Poor availability of hydrophobic pen and ineffectiveness of diamond pencil.	Paraffin pencil constructed using wax used to make paraffin blocks.
13.	Loss of cores during the staining process	Using slides with double coating of lysine

[Table/Fig-6]: Summary of problems encountered and their solutions.

Extraction of Tissue Core

The first problem encountered in this study was the difficulty in extracting tissue cores from the donor block and wax from recipient blocks. This was especially encountered during the winter months when the low temperature made the wax hard. The insertion of the punch biopsy needle needed considerable force, which displaced the needle from the intended area of interest and hence posed a significant problem. This was easily resolved by warming the blocks slightly at 40°C for five minutes, which made the wax softer without melting it and considerably eased the procedure.

Blockage of Punch Bore

The punch biopsy needle, when used continuously, sometimes retained wax within its punch bore. This created a problem by modifying the core size and preventing smooth extraction of tissue wax from the block. Moving the plunger once or twice usually addressed the problem, but at times the plunger also got stuck. This problem was tackled using a sharp needle (e.g., dissecting needle), with which the wax was extracted from the bore while ensuring that the edge is not harmed and the plunger can be pushed up manually to reset the needle.

Cracks in Recipient Block

While constructing the recipient block, several holes were punched into a plain paraffin block, which considerably weakened the block's strength. This was particularly evident when cracks appeared in the block during core insertion or subsequent heating in the oven [Table/Fig-5]. Several solutions were attempted to address this issue. At first, an attempt was made to seal these cracks by heating the blocks at 60°C in an oven for 15 minutes and applying gentle and even pressure while the wax was still soft. However, this only held temporarily, and the cracks re-appeared on sectioning, sometimes leading to complete breakage of the TMA block. Following this, an attempt was made to add a layer of liquid wax using the nozzle of the embedding station. However, the wax added here solidified separately and fell off upon cooling. Finally, an attempt was made to seal these cracks by putting the block face-first into the liquid wax present in the paraffin tray of the embedding machine for 30 seconds. Then, it was removed, flipped over immediately, and a glass slide was placed on it. Gentle but firm pressure was applied to mold the wax into place, followed by putting it on the cooling tray. Though this solved the issue, it was discovered that the best way is to avoid cracks altogether by maintaining a margin of 3 mm around the entire array to increase its stability.

Transfer of Cores to Recipient Block

The transfer of cores to the recipient block using forceps was cumbersome, as it was disastrous when the cores fell and were lost. The best way to avoid this was to retain the extracted core in the punch biopsy needle and transfer it directly to the intended hole using the plunger present in the needle itself. Gentle force was used to push out the tissue so that the core directly entered the hole without the edge of the needle touching the block. Slight pressure using a glass slide was applied to gently push down any cores that remained protruding.

Size of Recipient Hole

Sometimes, while creating recipient holes or inserting tissue cores, the holes' size increased due to trauma from the needle edge or forceps tip. Although most of these holes could be sealed using gentle pressure after heating in the oven, the cores present in the center were sometimes resistant to this. Initially, an attempt was made to add tiny pellets of wax to the holes before putting the TMA into the oven. However, this only sealed the surface and did not traverse the entire core length, creating problems during sectioning. Ultimately, the technique used above of putting the blocks for 30 seconds in the paraffin tray and the subsequent steps explained above proved useful for this problem as well.

Size of Core

At times, the cores extracted were too long for the hole created in the recipient block. Attempts to accommodate these by trying to re-punch the hole deeper mostly disfigured the hole further. Trying to push the tissue gently using a glass slide was also ineffective. Ultimately, it was decided to cut down the size of the longer donor core. The discrepancy in length was noted using a ruler. Using forceps or merely covering the tissue with the punch biopsy needle and pulling it out with gentle pressure without using the plunger or the plunge mechanism (to avoid tissue damage), the core was ejected and placed on a clean flat surface. A clean, sharp microtome blade was used to cut it to the desired length. It was then replaced into the hole using forceps or a punch biopsy needle.

The discrepancies in core size can also be avoided by ensuring that the block surface is smooth and that all the arrays are made in the same plane. This can be done by trimming the plain paraffin wax before the construction of the microarray.

However, when the cores were shorter, they tended to be placed deeper [Table/Fig-5], and hence, did not reach the surface. It was assumed that flipping the TMA on a glass slide and allowing gravity to pull the core down to the surface would tackle this problem. However, this method did not work well, mostly because of the adhesive nature of wax. Heating the flipped TMA on a glass slide and applying pressure for the realignment of the core was also found to be ineffective. Ultimately, a larger-sized punch biopsy needle was used to remove the tissue after measuring the discrepancy using a measuring tape. The wax cores removed while constructing the recipient block were used to cut out small wax disks of the requisite size. These disks were then placed in the recipient hole using forceps, followed by the tissue core. The block was then placed in the oven, as mentioned above. The remaining hole created by the larger-sized biopsy was filled using the embedding station, as described above.

Bulging of Recipient Block

When cores were placed close together, the block began to bulge. Increasing the space between the cores (at least 3 mm) reduced the bulging.

Difficulty in Sectioning

During sectioning, the uneven surface of the TMA created problems [Table/Fig-5]. Despite ensuring that all recipient blocks were trimmed before construction, this problem persisted due to the traumatic nature of punch biopsy insertion and removal. To address this, adding a layer of wax from the embedding station prior to sectioning proved to be beneficial.

Appearance of serrations and loss of tissue cores on sections were observed [Table/Fig-5]. This issue was easily rectified by ensuring that the blocks were sufficiently cooled before sectioning. It was observed that a minimum of three hours was required for easy sectioning. The use of fresh microtome blades for each block also helped.

Breakage of blocks during sectioning was another problem encountered [Table/Fig-5]. This was addressed by utilising the techniques previously mentioned for countering the cracking of TMAs. At the same time, it was found that keeping a minimum distance between the cores, i.e., 3 mm, helped increase the stability of blocks. Also, the practice of immediately transferring the block from the oven to a cooling station further increased the possibility of breakage. An attempt was made to add agarose to our paraffin recipient blocks, as described by Catchpoole et al., [11]. However, these attempts were unsuccessful and led to breakage of the block at the core insertion stage itself [Table/Fig-5]. Here, instead, the TMA block with the attached glass slide was removed from the oven and transferred to an incubator set at 37°C, where it remained overnight. It was observed that allowing the blocks to cool down and return to room temperature before removing the glass slide from its surface and then placing the block on the cooling station was an effective solution to the breakage problem.

Difficulties Encountered During Staining

During the IHC procedure, loss of cores mainly occurred during the bring-to-water and buffer washing steps. This problem was tackled by using slides with a double coating of lysine. Also, due to resource constraints and the ineffectiveness of a border made using a diamond pencil, it was difficult to appropriately block our antibodies, super-enhancer, and poly-HRP on the large area of the slide covered by the TMA cores. Hence, the present study experimented with other hydrophobic materials such as wax crayons, parafilm, and paraffin. It was found that a paraffin pencil constructed using a rubber pen gripper, steel molds, and the paraffin nozzle of the embedding station was an efficient solution [Table/Fig-7]. Unlike the other options, it did not wash off easily, so there was no need to reapply it at each step, and it was both cost-effective and easy to obtain.

[Table/Fig-7]: Picture of a wax in-house pencil made to mark the region where the stain was put.

Considering the learning curve in this method of TMA construction, it is also recommended that dummy or practice blocks be first constructed to identify and troubleshoot problems that may arise during the process.

DISCUSSION

The TMA is an efficient and time-effective technique that plays a significant role in centers catering to a large patient population. However, the high cost of automated TMAers and silicone molds limits their use in resource-limited countries like India. Manual TMAs, therefore, are good cost-effective alternatives [3,12].

The total cost of building the TMAs by the method described in the present study was 500 rupees ($6.86), including the cost of the punch biopsy needle and other stationary items, which is comparable to other Indian studies using manual TMAs [3,12,13]. The TMAs constructed were both cost and resource-effective and did not show any core loss or breakage. These were constructed using the method explained by Kononen J et al., however, suitable modifications were made upon identifying the problems in this method of manual TMA and hence troubleshoot them [7].

Singh A et al., compared the construction of manual TMAs using two techniques, Kononen J et al., and Chen N et al., methods [3,6,7]; While using the Kononen J et al., method, they also encountered a few problems similar to the present study. They recommended warming the donor blocks before core extraction

to prevent the development of cracks. The present study also found that warming the donor blocks eased the extraction process, but the authors would also recommend warming the recipient block beforehand for the same reasons. They also encountered bulging of the block when cores were placed close together, which they resolved by increasing the space between cores. However, they did not specify the recommended distance between cores. They also recommended facing off the recipient block before construction to facilitate the even placement of cores to address the uneven surface of TMAs. Additionally, the authors recommend adding a layer of wax from the paraffin tray on the TMA surface (using the method we have described above) to ensure an even surface.

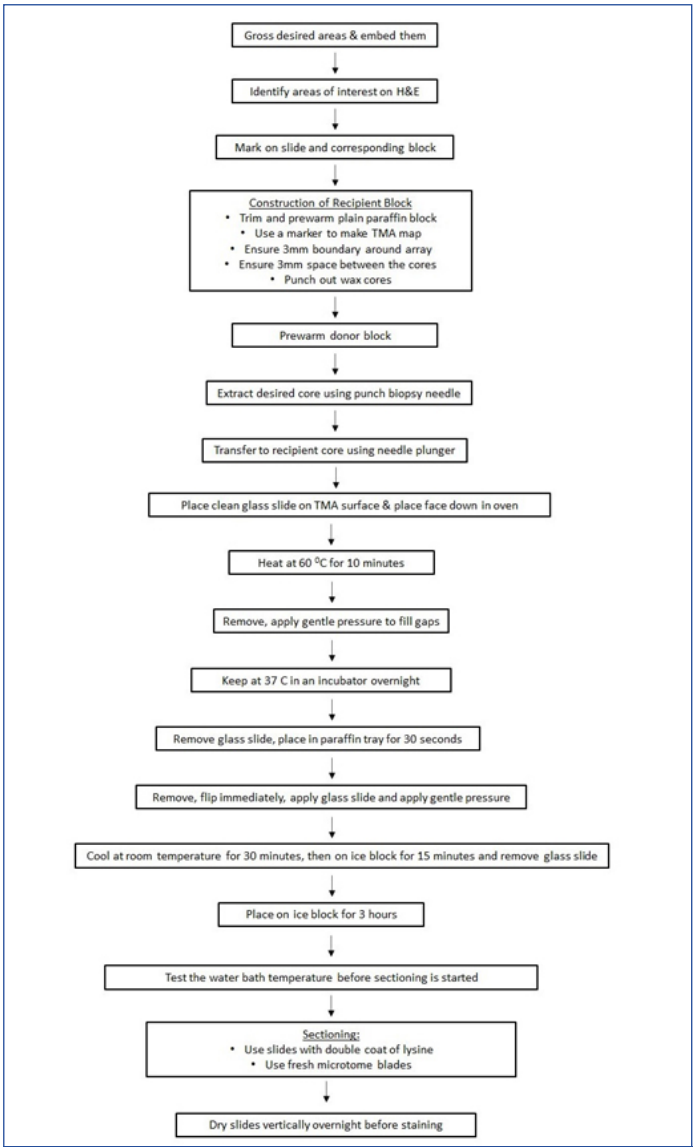
Singh et al., further recommended a margin of 2.5 mm throughout the array to prevent breakage. The authors found that a margin of 3 mm was better at increasing the stability of the block. They addressed the problem of too-long cores, similar to our method, by cutting off the extra length. However, their solution to too-short cores was different. They recommended removing these cores using a needle of smaller size, leaving behind a rim of tissue in the block, and replacing it with another appropriately sized core. This not only leads to wastage of tissue core but also increases the chance of errors. In contrast, the authors found that the method of removing the core with a rim of wax using a larger needle, adding appropriately sized wax disks, replacing the core, and filling any gaps created using liquid paraffin removed the chances of core wastage and errors while keeping the block itself stable. Additionally, they also recommended using practice blocks to test the temperature of the water bath and suggested that a magnifying glass may be used during array construction. Though the researchers here found that a practice block was indeed useful at all steps, they did not find the need to use a magnifying glass during construction.

Kononen J et al., suggested using a larger-sized donor core and smaller-sized recipient holes to ensure better adhesion and prevent folding [7]. However, this was not effective in all cases, and it was difficult to squeeze cores into smaller-sized holes [14]. Kononen J et al., and Vogel U used specially constructed arraying tools, which are both expensive and inaccessible to resource-poor settings [7,14]. To address the cracking of TMA blocks during sectioning, the use of an adhesive paraffin tape transfer system has been suggested [7]. However, apart from being expensive ($3466), it also increases tissue damage, loss of cores, and causes non-specific staining [11,14,15].

Other researchers such as Shebl et al and Foda et al., have attempted to improve upon the Kononen technique of constructing TMAs using mechanical pencil tips [9,16]. However, in the present study, it was found that the pencil tips were poorer at producing even holes in donor and recipient blocks compared to punch biopsy needles. This produced the problems with variable core length and hole depth. Additionally, the pencil tips were flimsy and often broke during the process, leading to damage to the block [9,16].

Palo S further suggests the use of a blunt needle or ballpoint pen refill to extract the cores from the biopsy needle hub. They also suggested arranging the cores in an array and then pouring molten wax on it to construct a donor block [10]. However, during the present study, it was found that transferring the cores as such from the donor block to a pre-constructed recipient hole prevented core loss and avoided any damage to the core, as caused while using a ballpoint refill or a blunt needle, even when used gently. Furthermore, pouring the wax after arranging the cores led to problems such as cores falling down and displacement due to the molten wax. These issues were bypassed by using the modifications suggested in this study to the standard Kononen method [7].

[Table/Fig-8] describes these steps of manual TMA construction using the Kononen J et al., method, modified to avoid the problems commonly encountered and suggest solutions compatible with resource-poor settings [7].



[Table/Fig-8]: Flow diagram of steps of TMA construction with suitable recommendations.

Limitation(s)

Though the authors tried to identify all the major problems arising in this manual construction method in the present setting, this study was limited to a small sample size. The researchers constructed only five TMAs for 60 cases in this study. Construction of more TMAs using this methodology might reveal further problems that we have not encountered. Also, during the learning curve, the construction of each TMA took 36-48 hours, which came down to 24 hours with experience. Given its long construction time, this technique might not be beneficial for developed countries with the facilities of automated arrayers. However, it can be of immense utility in developing countries with limited resources.

CONCLUSION(S)

Manual TMA construction is a cost-effective method of constructing TMAs. The authors have addressed the common problems encountered during manual TMA construction and have tried to come up with economical solutions. Thus, manual TMA construction is not only feasible but also easy to construct once the technique is learned. It serves as an alternative to traditional paraffin-based techniques for research applications, especially in resource-limited centers with high patient loads.

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- Manual Googling: Jan 18, 2024
- iThenticate Software: Feb 24, 2024 (6%)

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