Optimization of Tissue Microarrays from Banked Human Formalin-Fixed Paraffin Embedded Tissues in the Cancer Research Setting

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The tissue microarray (TMA) is a powerful tool for cancer biomarker discovery and validation. Tens to hundreds of samples can be evaluated simultaneously for molecular marker status at the protein or nucleic acid level. Although fully automated or semiautomated technologies for TMA creation provide excellent precision with respect to core transfer, they do not obviate the need for technical expertise to successfully generate high-quality TMA blocks and derivative sections. We have leveraged our expanding bank of formalin-fixed paraffin embedded paired tumor and normal tissues in our academic cancer center to provide a rich source of input material for cancer research TMAs. In this study, we report a stepwise optimization of TMA generation parameters, including paraffin wax selection, tempering protocol, and sectioning conditions, to achieve the best ribbon sectioning.

Keywords: tissue microarrays, TMA, FFPE, optimization

Introduction

HUMAN GENOME SEQUENCING and DNA microarray development have made possible the identification of candidate genes potentially involved in driving tumorigenesis, gaining metastatic capability, and providing prognostic or therapeutic value. In recent years, there has been an emphasis on the identification of tumor biomarkers and/or new therapeutic targets to optimally tailor an individual's cancer therapy. Tissue microarray (TMA) technology is a high-throughput and cost-effective solution to analyzing numerous tissue specimens simultaneously.^{1–6} Instead of the laborious process of cutting each block of interest individually, a TMA can be generated by removing tissue cores from selected regions of interest from each block and arranging the cores into a recipient block. TMAs are highly flexible with respect to design and input tissue to best approach the research question(s). Several different clinical research applications have been described, including predictive, validation, prognostic, progression, and control tissue TMAs.⁵ This technology has gained momentum in the past two decades, contributing to advances in cancer research and biomarker discovery in a range of tumor types.^{3,7–9}

Instruments for automating TMA generation are available; however, these instruments do not completely eliminate the technical expertise needed to construct the TMA block and cut slides for investigative use. Parameters, including design layout, selection of paraffin wax, tempering protocol, and sectioning conditions, are of paramount importance in generating high-quality slides that can be utilized for immunohistochemistry (IHC), *in situ* hybridization, and other applications.^{5,10–12} This study presents the optimization of TMA construction using the TMA Master II (3DHISTECH Ltd., Budapest, Hungary) with minimal or no core loss. We have leveraged the material in our >1975 block formalin-fixed paraffin embedded (FFPE) tissue bank to generate TMAs with various tumor types for research investigation.

Materials and Methods

Donor FFPE tissues

Careful consideration and selection of the donor blocks to be used in construction of the TMA is a time-consuming part of the process. Decisions must be made regarding the appropriate tissue types, disease states, and patient population

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needed to address the research question(s). The anatomic pathology section of the Wake Forest Baptist Comprehensive Cancer Center's Tumor Tissue and Pathology Shared Resource (TTPSR) has been collecting remnant FFPE tumor and normal tissues for ~2.5 years. During that time, >1975 blocks of tumor and matched normal tissues dedicated for research purposes have been collected through an institutional Institutional Review Board (IRB)-approved initiative (BG98-391 and BG04-104). The FFPE tissue bank contains myriad cancer types from a wide range of tissue sites. Once appropriate cases are selected for the TMA, hematoxylin and eosin (H&E) stained slides are reviewed by a board-certified pathologist who marks areas to punch during TMA creation. The marked slides are then captured on a digital camera and downloaded to the TMA Master II for manual overlay during punching. All donor blocks are faced on a HM 325 microtome (MICROM International GmbH, Dreieich, Germany) before punching to ensure a smooth parallel surface across the block. Otherwise, an uneven surface may interfere with accurate measurement of block depth by the TMA Master II touch sensor and result in different core depths in the recipient block.

Paraffin type for recipient TMA blocks

Seven different paraffin wax types (Leica; Thermo Scientific, Runcorn, UK; and 3DHISTECH Ltd.; Table 1) were selected for recipient TMA block generation. Each paraffin wax, excluding wax type #7 that was not available for purchase, was melted in the Thermo Scientific HistoStar embedding station (Thermo Scientific) and manually poured into metal molds supplied from 3DHISTECH Ltd. Blocks were allowed to solidify slowly at room temperature overnight. The next day, molds were briefly cooled on the cold plate of the HistoStar embedding station to allow separation of the metal mold from the cassette. Excess wax was then trimmed from the cassettes. Blocks were stored in a sealed container until use. Facing of the recipient block was done immediately before drilling to ensure a smooth parallel surface.

Block integrity was crucial, and any blocks that had cracks or had formed air pockets were discarded. Waxes showed variable susceptibility to these issues, with fewer issues seen using waxes with higher polymer content (e.g., Type H and EM-400). Potential opportunities for unintentional air introduction included adding molten wax too quickly to the mold and air trapping under the embedding

cassette when placed on top of the mold. We found that slow manual addition of the wax to the metal mold yielded blocks with the best integrity. Tapping the cassette with forceps after placing on the mold to release air bubbles was also helpful.

TMA layout and construction

Recipient TMA blocks were constructed with the TMA Master II (3DHISTECH Ltd.) following the user guide instructions. Different insertion starting positions, gap width between cores, number of cores per block, block sizes, and drill/punch sizes were tested, and the final layout design was determined from these results. All cores in the recipient block were drilled before any donor tissue insertion. For the current studies, a variable number of cores from each donor FFPE block were used in TMA creation.

Tempering the block

After all cores were punched and inserted into the recipient block, the newly constructed TMA block was removed and tempered. Four conditions were tested:

Protocol 1 (64°C for 10 minutes): The TMA block was placed with cores facing upward for 10 minutes in a 64°C oven and then cooled to room temperature for 10 minutes. This cycle of 10-minute incubations at 64°C and room temperature was repeated three times.

Protocol 2 (50°C for 1 hour): The TMA block was placed with cores facing upward for 1 hour in a 50°C oven, and then cooled to room temperature.¹⁰

Protocol 3 (60°C for 10 minutes): The TMA block was placed on a clean glass slide with cores facing downward (Fig. 1) for 10 minutes in a 60°C oven and then cooled at room temperature.¹³

Protocol 4 (37°C overnight): The TMA block was placed on a clean glass slide with cores facing downward (Fig. 1) in a 37°C oven overnight. After overnight incubation, the slide was pressed gently against the block to level the cores and remove any air bubbles. The block was then cycled through a 1-hour incubation at 4°C followed by a 1-hour incubation at 37°C for a total of four cycles.

The wax temperature during the tempering process must be warm enough to anneal the donor cores to the surrounding paraffin wax in the recipient block, but high temperatures

TABLE 1. PARAFFIN WAX FOR RECIPIENT TISSUE MICROARRAY BLOCKS

Wax no.	Paraffin wax	Vendor	Melting point (°C)	Additives
1	Paraplast X-tra	Leica	52	Low molecular weight polymers and highly purified paraffins
2	Paraplast	Leica	56	Highly purified paraffin with plastic polymers
3	EM-400	Leica	56–57	Purified paraffin with synthetic polymers
4	Type H	Thermo Scientific	55–57	High polymer and refined paraffin
5	Histo LP	Thermo Scientific	50–54	Low melting point wax made of paraffin and plastic polymers
6	Type 6	Thermo Scientific	55–57	Highly refined paraffin, no polymers, but blended with microcrystalline wax
7	3DHISTECH Ltd. premade block	3DHISTECH Ltd.	Not available	Custom wax from 3DHISTECH Ltd., no information on components

FIG. 1. The TMA block is oriented with the cores facing downward on a clean glass slide in a temperature-controlled oven for tempering. TMA, tissue microarray.

can cause the cores to shift in the block.¹⁴ Early evaluation of tempering protocols with a limited number of blocks demonstrated the clear inferiority of Protocol 1 due to block deformation at high temperature, and this condition was not pursued. The remaining protocols were tested with 4–11 blocks each depending on performance. Before microtomy from each tempering condition, the block was cooled in an ice water bath for 30 minutes before sectioning.

Microtomy

Sectioning the newly constructed TMA blocks was performed on a HM 325 microtome (MICROM International GmbH) with a clearance angle of 10° and a thickness of 4 µm along the width of the block (Fig. 2). Before facing the block, proper alignment on the *x*-axis and *y*-axis was checked to prevent excessive tissue loss until a parallel surface was achieved for smooth continual ribbon sectioning. Five different high-profile microtome blades were compared for ease of sectioning (Table 2): Surgipath DB80 HS (14035843490), High-Profile 818 (14035838926), and the High-Profile thin

FIG. 2. The TMA block is oriented along the width of the block on the microtome for sectioning to generate consistent sections with minimal or no core loss.

blade (3802123) from Leica (Leica Microsystems, Inc., Chicago, IL); and the HP35N Ultra noncoated (3151735) and HP35 Ultra from Thermo Scientific (3153735). At least 50–100 sections were cut per block to assess ease of cutting and section quality.

H&E staining and IHC

Sections were cut 40–50 levels at a time with intermittent cooling of the block. Slides were dried vertically and stored in a plastic slide box placed inside a closed plastic bag at –80°C. Slides were removed from the –80°C freezer and allowed to dry at room temperature for 1 hour before staining. Sections were stained with H&E on the Thermo Scientific Gemini AS using the user guide protocol for Hematoxylin-7211 and Eosin-Y 7111 (Thermo Scientific). All other reagents were from Cardinal Health (Waukegan, IL). IHC for CK7 (Leica, NCL-L-CK7-OVTL) was performed on a Leica Bond-III, Bond Polymer Refine Detection system at a dilution of 1:200 for 20 minutes.

Results

FFPE tumor and normal tissues banked in the TTPSR provided excellent quality donor material for TMA generation from a range of tissue types. After testing variables in layout and design, the following parameters proved effective for reproducible high-quality easily interpretable sections: a 37 or 30 mm block with a core depth of 7 mm, insertion starting position of –300 µm, a gap width (space between the cores) of 800 µm, with a 6×9 layout, a 3–4 mm clearance from the block edge to prevent cracking during drilling, and a drill/punch size of 1.5 mm. For orientation, one undrilled core was placed at the top left corner as the starting point, and three to four undrilled cores were placed in the mid-section of the block as a second point of reference. These orientation spots were chosen in an asymmetrical pattern in the same location for all TMA blocks. With this template, we designed TMAs with matching tumor and normal tissues using FFPE tissue blocks available in our TTPSR bank.

Our initial efforts to prepare recipient blocks using the deep metal molds supplied by 3DHISTECH Ltd. were problematic. We quickly discovered that the blocks had to solidify at room temperature overnight, since cooler temperatures caused the blocks to crack regardless of wax type. After overnight room temperature incubation, the molds were cooled briefly on the embedding station cold plate and then removed.

The selection of paraffin wax is paramount in TMA generation. The paraffin wax that performed most favorably through mold preparation, tempering conditions, drilling, and sectioning was Type H (Thermo Scientific). This paraffin wax provided consistent durable blocks able to withstand temperature changes throughout the process with preserved integrity. EM-400, a blend of synthetic polymer and paraffin, and Paraplast X-tra, a low molecular weight polymer (Leica Microsystems, Inc.), were both easy to section as well, but the blocks intermittently formed tiny cracks during tempering, which can lead to distortion of the array when sectioning. The blocks supplied from 3DHISTECH Ltd. are an acceptable choice as well; the wax is softer, but it drills and sections easily. However, the 3D HISTECH Ltd. wax is not available for purchase so the premade recipient blocks must be purchased from the company.

TABLE 2. MICROTOME BLADE COMPARISON

Vendor	Blade	Catalog no.	Length (mm)	Width (mm)	Angle (°)	Height (mm)
Leica	Surgipath DB 80 HS	14035843490	80	14	35	0.25
Leica	High-Profile 818	14035838926	80	14	35	0.35
Leica	High-Profile Thin	3802123	77	14	35	0.30
Thermo Scientific	HP35N Ultra High-Profile, noncoated	3151735	75	14	34	0.31
Thermo Scientific	HP35 Ultra High-Profile, with PTFE coating	3153735	75	14	34	0.31

A variable that is equally as critical as paraffin wax selection in the successful TMA generation is the procedure for tempering the TMA block after donor core insertion. This process facilitates integration of the donor core wax and recipient block wax for secure donor core adherence within the TMA block. Early testing of Protocol 1 revealed that the high temperature (64°C) resulted in block distortion and cores were dislodged from the block during sectioning. For Protocols 2 and 3, cores inconsistently annealed to the surrounding wax, particularly those with lower polymer content, and cores had a tendency to roll during sectioning. The tempering protocol most successful in our laboratory was Protocol 4 described in the Materials and Methods section, in which the TMA block is initially incubated at 37°C overnight with cores facing down on a glass slide. The following day, with the slide still attached to the block, the block cycles between 1-hour incubations at 4°C and 37°C for a total of four cycles.

With the aggregate parameters outlined earlier, we successfully generated TMA blocks for cancer research from our banked FFPE tissues. The final step yielded high-quality

sections with all cores represented. We found that cutting the TMA block along the width of the block with a Surgipath DB80 HS high-profile blade (Leica Microsystems, Inc.) produced consistent sections with minimal to no core loss. Using the aggregate parameters, a TMA for prostate cancer research was constructed, with prostatic adenocarcinoma Gleason scores 6 through 9 represented (Fig. 3). H&E stained sections of the prostate TMAs demonstrated excellent morphology. In addition, a kidney cancer TMA was created and sections were used for CK7 IHC with excellent results (Fig. 4). IHC is a technique widely applied to TMAs for biomarker investigation.

Discussion

TMAs have emerged as a powerful tool for biomarker evaluation in human cancer research. Depending on the core size and TMA design, numerous tissues from tens to hundreds of samples can be interrogated simultaneously on a single slide. This approach conserves tissue as well as decreases the reagent cost of assessing a single biomarker.

FIG. 3. (A) Color-coded layout of prostate cancer TMA showing tumor and relative normal tissue as well as indicating the Gleason score of prostate cancer tissues. (B) Prostate cancer TMA block generated using Type H paraffin wax. Accompanying H&E stained slide of prostate cancer TMA. (C) Representative 1.5 mm prostate cancer cores stained with H&E. H&E, hematoxylin and eosin.

FIG. 4. (A) Kidney cancer TMA with block generated using Type H paraffin wax and accompanying H&E stained slide and CK7 immunostain. (B) Representative H&E stained 1.5 mm cores of normal kidney (left) and kidney cancer (right). (C) CK7 IHC of 1.5 mm cores of normal kidney (left) and kidney cancer (right).

TMAAs have been used in a range of human cancer investigations, including prostate cancer,^{15,16} breast cancer,⁷ and colon cancer.^{8,17,18}

The tumor bank within our Comprehensive Cancer Center has been actively collecting FFPE tumor and normal tissues from patients preoperatively consented to IRB-approved tissue procurement protocols to build an FFPE tissue bank to complement our long-standing frozen tissue bank. We have leveraged this resource to generate TMAAs for cancer research. Using these tissues dedicated to research provides an appealing alternative to compromising the clinical FFPE blocks used in diagnostic evaluation.

Automated TMA technology has provided consistency in the process of drilling the recipient block and insertion of donor cores; however, it has not eliminated the need for technical expertise and optimization of TMA generation. In this study, we have outlined the evaluation of key variables in TMA preparation, including paraffin wax selection, tempering protocol, and TMA block sectioning. The paraffin selected as the highest performing wax in our laboratory was Type H from Thermo Scientific. It has a high polymer content, which is good for drilling, but it also maintains its shape during mold preparation without forming air pockets. Type H wax was also identified as a high performing wax by Chang et al.¹⁰ No agarose was added to the paraffin in this study, as has been proposed by some groups to aid in core integration into the recipient block.^{12,14} Of note, the paraffin wax used in our banked FFPE blocks is Leica EM-400, which contains plastics similar to Type H. The plastics may provide the necessary support for TMA integrity, while maintaining the softness of the wax for ease of cutting.

Temperature was a key variable in multiple steps during TMA generation. During initial generation, allowing recipient blocks to solidify at room temperature overnight proved important to minimize cracking. After the completion of donor core insertion, the tempering process is critical to promote core integration into the recipient block. Numerous different tempering protocols with various temperatures and incubation times are reported in the literature. After testing

four sets of conditions, we determined that the optimal protocol involved repetitive cycles of 1-hour incubations at 37°C and 4°C. Although this is a time-consuming process, the importance of this step in preparing the block for successful sectioning cannot be overemphasized.

Adequate sectioning of the TMA block provides interpretable cores spanning the entire block. Orienting the block on the microtome to cut along the width of the block, as recommended by Dennis et al.,¹¹ led to significantly less core loss as opposed to cutting along the length of the block. The use of an adhesive tape system to transfer sections onto the slide was recommended early on in the development of TMAAs.¹ However, this can lead to undesirable staining artifacts and, like other groups,^{5,10} we were successful when using the traditional water bath approach. To facilitate consistent placement of the section on the slide, we applied the same TMA template with orientation “gaps”⁵ across TMA blocks. This approach is also helpful to the pathologist reading the slides to follow the same pattern every time.

In summary, the parameter optimization presented in this study led to successful TMA generation for cancer research at our institution. Banked FFPE matched tumor and normal tissues provide invaluable resources for TMA creation. In other active cancer research settings, developing and maintaining an FFPE bank may be helpful to provide an alternative to potentially compromising diagnostic clinical blocks by punching cores for TMAAs. Applications for TMAAs continue to expand with the increasing emphasis on molecular biomarkers of cancer.

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Author Disclosure Statement

The authors declare no conflicts of interest, except for Dr. Gregory L. Kucera who is a scientific advisor to Spherix, Inc.

References

1. Kononen J, Bubendorf L, Kallioniemi A, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 1998;4:844–847.
2. Hewitt SM. Tissue microarrays as a tool in the discovery and validation of predictive biomarkers. *Methods Mol Biol* 2012;823:201–214.
3. Camp RL, Neumeister V, Rimm DL. A decade of tissue microarrays: Progress in the discovery and validation of cancer biomarkers. *J Clin Oncol* 2008;26:5630–5637.
4. Vogel U. Overview on techniques to construct tissue arrays with special emphasis on tissue microarrays. *Microarrays (Basel)* 2014;3:103–136.
5. Parsons M, Grabsch H. How to make tissue microarrays. *Diagn Histopathol* 2009;15:142–150.
6. Radhakrishnan R, Solomon M, Satyamoorthy K, et al. Tissue microarray—a high-throughput molecular analysis in head and neck cancer. *J Oral Pathol Med* 2008;37:166–176.
7. Rossing HH, Talman ML, Laenkholm AV, et al. Implementation of TMA and digitalization in routine diagnostics of breast pathology. *APMIS* 2012;120:341–347.
8. Goos JA, Coupe VM, Diosdado B, et al. Aurora kinase A (AURKA) expression in colorectal cancer liver metastasis is associated with poor prognosis. *Br J Cancer* 2013;109:2445–2452.
9. Ponten F, Schwenk JM, Asplund A, et al. The Human Protein Atlas as a proteomic resource for biomarker discovery. *J Intern Med* 2011;270:428–446.
10. Chang H, Peluso D, Hussain S, et al. Optimizing tissue microarray construction procedure to improve quality of sections. *J Histotechnol* 2014;37:95–100.
11. Dennis J, Westra J, Bell A, et al. The type and quality of paraffin wax is important when constructing tissue microarrays. *Mol Pathol* 2003;56:306.
12. Kim KH, Choi SJ, Choi YI, et al. In-house manual construction of high-density and high-quality tissue microarrays by using homemade recipient agarose-paraffin blocks. *Korean J Pathol* 2013;47:238–244.
13. Yan P, Seelentag W, Bachmann A, et al. An agarose matrix facilitates sectioning of tissue microarray blocks. *J Histochem Cytochem* 2007;55:21–24.
14. Choi CH, Kim KH, Song, JY, et al. Construction of high-density tissue microarrays at low cost by using self-made manual microarray kits and recipient paraffin blocks. *Korean J Pathol* 2012;46:562–568.
15. Ouellet V, Aprikian A, Bergeron A, et al. The Terry Fox Research Institute Canadian Prostate Cancer Biomarker Network: An analysis of a pan-Canadian multi-center cohort for biomarker validation. *BMC Urol* 2018;18:78.
16. Hennigs JK, Muller J, Adam M, et al. Loss of somatostatin receptor subtype 2 in prostate cancer is linked to an aggressive cancer phenotype, high tumor cell proliferation and predicts early metastatic and biochemical relapse. *PLoS One* 2014;9:e100469.
17. van den Broek E, den Uil SH, Coupe VMH, et al. MACROD2 expression predicts response to 5-FU-based chemotherapy in stage III colon cancer. *Oncotarget* 2018;9:29445–29452.
18. Zhang L, Zhao Y, Dai Y, et al. Immune landscape of colorectal cancer tumor microenvironment from different primary tumor location. *Front Immunol* 2018;9:1578.

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