

Video Article

A Next-generation Tissue Microarray (ngTMA) Protocol for Biomarker Studies

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Abstract

Biomarker research relies on tissue microarrays (TMA). TMAs are produced by repeated transfer of small tissue cores from a 'donor' block into a 'recipient' block and then used for a variety of biomarker applications. The construction of conventional TMAs is labor intensive, imprecise, and time-consuming. Here, a protocol using next-generation Tissue Microarrays (ngTMA) is outlined. ngTMA is based on TMA planning and design, digital pathology, and automated tissue microarraying. The protocol is illustrated using an example of 134 metastatic colorectal cancer patients. Histological, statistical and logistical aspects are considered, such as the tissue type, specific histological regions, and cell types for inclusion in the TMA, the number of tissue spots, sample size, statistical analysis, and number of TMA copies. Histological slides for each patient are scanned and uploaded onto a web-based digital platform. There, they are viewed and annotated (marked) using a 0.6-2.0 mm diameter tool, multiple times using various colors to distinguish tissue areas. Donor blocks and 12 'recipient' blocks are loaded into the instrument. Digital slides are retrieved and matched to donor block images. Repeated arraying of annotated regions is automatically performed resulting in an ngTMA. In this example, six ngTMAs are planned containing six different tissue types/histological zones. Two copies of the ngTMAs are desired. Three to four slides for each patient are scanned; 3 scan runs are necessary and performed overnight. All slides are annotated; different colors are used to represent the different tissues/zones, namely tumor center, invasion front, tumor/stroma, lymph node metastases, liver metastases, and normal tissue. 17 annotations/case are made; time for annotation is 2-3 min/case. 12 ngTMAs are produced containing 4,556 spots. Arraying time is 15-20 hr. Due to its precision, flexibility and speed, ngTMA is a powerful tool to further improve the quality of TMAs used in clinical and translational research.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51893/>

Introduction

Over the last two decades, tissue microarrays (TMAs) have had a remarkable impact on biomarker investigation studies. TMAs are essentially tissue "archives" produced by the repeated transfer of small tissue cores, typically ranging in size from 0.6 to 2.0 mm in diameter, from paraffin embedded 'donor' blocks into a single TMA 'recipient' block (**Figure 1**)¹. Using a small core size, approximately 500 different tissue spots from few or many different patients can be arrayed into one TMA².

The use of TMAs for prognostic or predictive biomarker studies has many advantages. Consider the example where expression of a protein biomarker by immunohistochemistry is to be evaluated on 450 patients. Rather than performing 450 immunohistochemical stains on 450 patient slides sectioned from the same number of blocks, small cores from each sample can be arrayed onto a single TMA block. Even if multiple cores are taken from each individual patient, a minimum number of blocks are produced. This has the considerable effect of drastically diminishing costs and other resources as well as reducing tissue wasting. Additionally this allows appropriately powered studies using a large number of tissues to be evaluated under the same experimental conditions.

TMAs have many different applications. For instance, they can be used to study morphology, protein expression, RNA expression and DNA aberrations following staining with different dyes, or after immunohistochemistry or chromogenic and even fluorescence *in situ* hybridization³⁻⁷. Recent studies have also used TMAs to test intra and interlaboratory variation in staining protocols, establish specificity or sensitivity of antibodies for specific gene mutations, and to determine the interobserver reproducibility of protein expression in international collaborations⁸⁻¹¹.

The construction of traditional TMAs using patient-derived tissues is a long multi step procedure (**Figure 2**). It begins with a search for possible appropriate cases and selection of diagnostic slides from the archives at an institute of pathology, or other institute, from where they are retrieved. The pathologist evaluates each slide per case and selects the most representative slide for the purposes of the study. Next, the region of interest is marked using a pen directly under the microscope. This is often challenging and imprecise and results only in an "estimation" of where tissue punches should be taken from. Next, the paraffin blocks corresponding to these marked slides are retrieved from the archive. A quick comparison between block and slide is made. Using a semi-automated or homemade tissue arrayer, the donor block is punched out in the estimated region of interest and transferred into a recipient TMA block. Construction of TMAs using this arraying technique is labor intensive, time consuming, imprecise, and inflexible. Preparing a TMA of 475 spots in 3 copies is estimated to take about 84 hours of work.

A new approach to the construction of TMAs was recently introduced by the Institute of Pathology, University of Bern that relies on three components: planning and design (or consulting), digital pathology combined with expertise in histology and automated TMA arraying¹². Together, this concept is called next generation Tissue Microarray (ngTMA). Below, a protocol for ngTMA is described based on an example of 134 patients with metastatic colorectal cancer. Here, primary tumors as well as lymph node metastases and liver metastases are to be arrayed into ngTMAs for subsequent biomarker analysis. Additionally, small tissue cores from each patient are desired for future nucleic acid extraction.

Protocol

NOTE: This study has been approved by the local ethics committee of the Insel Hospital, Bern, Switzerland (07-10-13). Tissues were obtained from the Tumor bank Bern, Institute of Pathology, University of Bern.

1. Planning and Design (Consulting)

1. Think about the research question to be answered. Decide on the tissue types to be included in the project. Determine what histological structures are important to answer the question.
2. Ascertain the most useful core diameter and the number of cores per patient for the project. Decide on the number of copies of the next-generation Tissue Micro Array (ngTMA) based on the amount of biomarkers to be investigated.
3. Consider statistical aspects such as sample size and analysis after the tissue microarray (TMA) is constructed. Determine the number of patients for inclusion in the study and establish control tissues for the array.
4. Retrieve the histological (or diagnostic) Haematoxylin and Eosin (H&E) slides of each patient. Briefly review the slides and identify the ones containing relevant information and histology for the project.

NOTE: Make new sections of the paraffin blocks, if special stain or immunohistochemistry slide is desired for slide scanning and annotation, instead of H&E slides

2. Slide Scanning

1. Turn on PC and slide scanner and open scanning software. Select "automatic mode" for bright field scanning from the preview screen.
2. Click on "scan options" to adjust slide quality parameters. At this time, select whether slides are to be directly scanned to the digital platform accessible by web or to a local drive (or external drive) and set the types of scanning and number of focus points.
3. Click on "Server Parameters" to scan to the "Case center". At this step, add more magazines, label all slides and set the folder in Case Center where the slides should be stored. Each magazine holds up to 25 slides.
4. Check quality/cleanliness of actual histological slides for scanning. If necessary, clean slides with 70% ethanol.
5. Load up to 25 slides per magazine with the labels pointing inward (**Figure 3A**)
6. Place the magazine in the scanner. For more than one magazine, place them on top of each other.
7. Start scanning slide by clicking on the green arrow (**Figure 3B**). When all slides are scanned, unload the magazines and shut off the program, PC and slide scanner.

3. Digital Slide Annotation

1. Enter the browser address for digital slide management center and download the free digital slide viewer software.
2. Open the digital slide viewer software and select the folder containing the digital slides (**Figure 4A**).
3. Add notes, reorder and manage the digital slide folder or assign user rights to colleagues or add attachments to the folder.
4. Click on desired digital slide which appears in the digital slide viewer.
5. Using the magnification tool, evaluate the slide and find the areas of interest for integration into the ngTMA.
6. Using the 'TMA annotation tool', select the size of the desired core and the color of the annotation. Place the annotation on the digital slide by clicking on it (**Figure 4B**).
7. Move the annotation to the desired histological structures for incorporation into the ngTMA (**Figure 4C**).
8. Repeat this process of annotation again by using the annotation tool, selecting a core size and a desired annotation color. Repeat the slide viewing and annotation on all slides in the folder.
9. Alternatively, place tissue cores for PCR into 0.2 ml tubes rather than integration into the TMA. Annotate slides using a different color to identify these spots for further molecular analysis.
10. Create a list of all cases with their corresponding annotations and colors.

4. Tissue Microarray Construction

1. Retrieve the corresponding paraffin tissue blocks for all annotated digital slides, Sort blocks in the desired order for tissue microarraying.
2. Ensure that the tissue blocks have a minimum of 4 mm thickness. If not, reembedding of tissue is necessary.
3. Turn the PC "ON" and automated tissue microarrayer instrument. Open the tissue microarrayer software and provide a name to the project
4. Perform a "Tool Change" and select the required tool diameter for the project (*i.e.*, 0.6, 1.0, 1.5, or 2.0 mm).
5. Load up to 12 'recipient' TMA blocks into the machine. Give an ID to each of the 12 blocks
6. Create a TMA layout for each recipient block. Observe a TMA design with number of rows, columns, and empty lines as well as distance between cores. Create a new layout for each recipient block or use the same layout repeatedly.
7. Place the cursor on the recipient block layout to take the first core.
8. Next, load up to 60 donor blocks into the tissue microarrayer (**Figure 5A**). Place ten blocks in each row A to F. Give each block a donor ID. Observe images of each donor block on the computer screen acquired automatically by the tissue microarrayer.

9. Starting with the first block, click on "Slide". Observe the annotated digital slide stored in the digital slide management center (or on the external drive) using the digital slide viewer. View the image of the donor block and the digital slide side-by-side.
10. Select reference points on the block image to superimpose the donor block image with the digital slide
11. After clicking "Next", observe the annotations on a larger image of the block. (**Figure 5B**). Click on top of the annotations to confirm and click "Start".
NOTE: This prompts the tissue microarrayer to start drilling a hole of 0.6 mm in diameter in the recipient block at the selected starting point. Then in a second step, using the punching tool, the instrument punches hole in the tissue from the selected donor block at the exact annotated and confirmed region. Cores are then transferred from the donor to the recipient block.
12. After approximately 500 cores, clean the drill and punching tool using a swab of xylene.
13. If tissue cores are desired (rather than punches into an ngTMA), click on the "PCR" tool for the block, confirm up to 4 annotations and hit "Start". This will punch and transfer the cores into a 0.2 ml tube.
14. Repeat point 4.9 to 4.11 with the second donor block, and so on.
15. After using all the donor blocks, enter a second round of donor blocks (61-120) into the tissue microarrayer and continue steps 4.8 to 4.11.
16. Refresh donor and recipient block images while the project progress while drilling and punching is occurring (**Figure 5C**).
17. Perform an "Export" after the project is complete. Locate the .xlsx file with the donor and recipient block IDs, the localization of all punches within the TMA as well as the TMA layout/design, in the exported folder. Save all donor block images with all superimposed annotation as jpg images in the exported folder (**Figure 5D**). The final TMA block is then ready.

Representative Results

Planning and Design

Here the example of 134 patients with metastatic colorectal cancer that are being investigated for a particular series of biomarkers was used. Not only is an ngTMA planned for these patients, but DNA extraction followed by mutational analysis for some specified gene.

During the planning and design phase of ngTMA, the following aspects have been decided. From each patient, 6 different histological regions will be investigated: tumor center, tumor invasion front, areas of densest tumor budding (tumor/stroma interaction), normal tissue, lymph node metastases and liver metastases. Three tumor spots per tumor tissue area and two normal tissue spots are to be included into the project (n=17 spots per patient). Since it is planned that more than 50 biomarkers be investigated on this patient material, two copies of the final ngTMA are desired. Due to the possible small size of lymph node and distant metastases, a small core diameter of 0.6 mm for TMA construction for all tissues is chosen.

Additionally, it is planned that each region be separately arrayed, so that 6 ngTMAs containing different histological areas will be produced: an ngTMA containing tissues from the a) tumor center, b) invasion front, c) tumor budding areas, d) normal tissue, e) lymph node metastases, and f) liver metastases.

For the TMA design using a 0.6 mm tool, a comfortable distance of 0.4 mm between punches will be selected. Because evaluation of tissue spots in long rows and columns is often tiresome, two different designs are selected. For tumor blocks with 3 punches per tissue area, a design containing six parts is created. This leads to a total number of 402 tissue punches. For normal tissue punches, 2 punches per case are to be included. A layout fitting 432 punches in total is chosen.

Moreover, for each case, up to 4 cores at 0.6 mm will additionally be punched out and placed into 0.2 ml tubes. These tissue cores will also be annotated.

To summarize, 6 ngTMAs of different tissues will be produced (5 tumor arrays x 3 punches x 134 patients; 1 normal tissue array x 2 punches x 134 patients) in addition to 4 cores per patient for tubes. Taken together, 2814 annotations will be made.

Next, the corresponding H&E slides from selected patients are retrieved from the slide archive. Each case is briefly reviewed by a pathologist and most representative slides of each tissue type are selected.

Scanning

Digital slides of each histological section are made for future annotation. For this study, 3-4 tissue slides are to be scanned per case, *i.e.*, primary colorectal cancer, with or without adjacent normal tissues, lymph node metastases and liver metastases and possibly an additional slide containing normal mucosa.

Up to 10 different magazines can be fully loaded into the slide scanner; therefore 250 slides can be scanned in a single run. Time for scanning and digital slide size varies with the dimensions of the tissue and the quality factor desired. The quality factor ranges from 0 to 100. Here, a quality factor of 60 is selected, since it produces a smaller scan file size without any noticeable loss of scan quality. Small biopsies can be scanned in under 30 sec while whole tissue sections, like the ones used in this example may take between 2 and 10 min. Digital slide size can also range from 2 MB to 2 GB. Using a quality factor of 60, scans between 600 MB and 1 GB are produced.

Between 402 and 536 slides are required for this project totaling 3 scanning runs. Each run is performed overnight. Approximately 500 GB of storage space is needed. Slides are scanned directly onto a digital platform called Case Center (ngtma.unibe.pathcasecenter). Case Center is accessible from anywhere with web access by entering the designated username and password.

Annotation

For annotation, two aspects should be considered: 1) different tissue areas should be given different annotation colors and 2) the number of annotations should reflect the number of desired copies of the final ngTMA.

In this case, 3 spots per tumor area for a single copy have been selected. For 2 copies, each region must be annotated using 6 spots. Useful are green for normal tissue, blue for the tumor center, yellow for the tumor invasion front, red for areas of tumor budding (tumor/stroma) interaction, black for lymph node metastases, turquoise for distant metastases and finally white for regions to be used for tubes. Annotation of each case requires 2-3 min.

ngTMA Construction

This study requires 6 ngTMA blocks to be constructed in 2 copies, resulting in 12 final blocks. The maximum number of recipient blocks that can be supported by the arrayer is 12. Each tumor block will contain 402 tissue spots and each normal tissue block will contain 268 spots, totaling 4,556 spots across the entire project.

A TMA layout is created for each recipient block and the project is saved (Figures 6A, 6B). 60 tissue blocks can be loaded into the tissue microarrayer in parallel; an image of each donor block is taken. Starting with the first donor block, the corresponding annotated digital slide is retrieved from Case Center. Slide matching is performed, which can take between 1-3 min. Annotations are confirmed and the arrayer is prompted to start punching (Figure 7). Core punching and transfer time is approximately 12 sec. Loss of cores during this procedure is minimal. Total time for arraying this project is approximately 24 hr, including time for slide/block matching and reloading of the instrument. Some examples of ngTMA are illustrated in Figure 8A. Cores for subsequent molecular analysis can also be punched out at this time (Figure 8B).

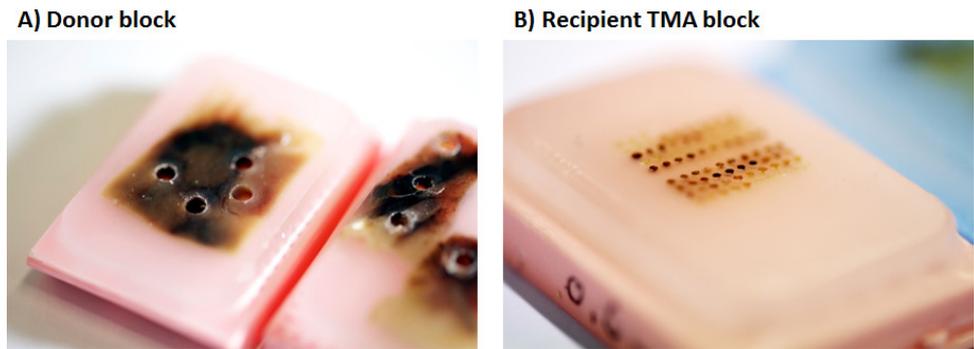


Figure 1. A) A 'donor' block. Cores from the donor block are punched out and transferred into B) a recipient block, or tissue microarray (TMA).

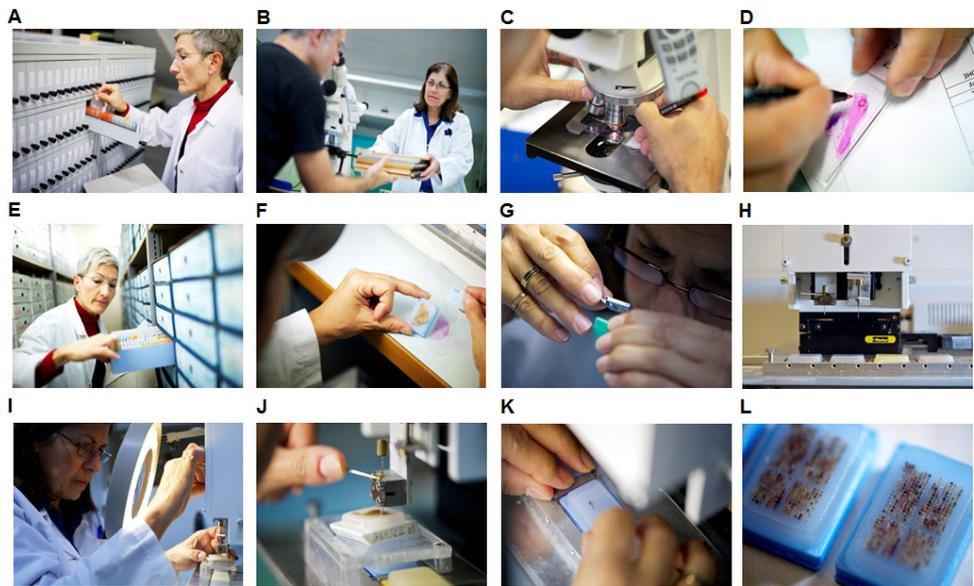


Figure 2. The conventional tissue microarray workflow. A) Histological slides are retrieved from the archive and B) given to the pathologist for review. C-D) Annotations are made on the slides indicating the 'estimated' region for transfer. E) The corresponding tissue blocks are retrieved. F) Block and slide are compared for matching, which can G) sometimes be a challenging task. H-J) Tissue microarraying then takes place. K) Cores are repeatedly transferred and L) a final TMA is constructed.

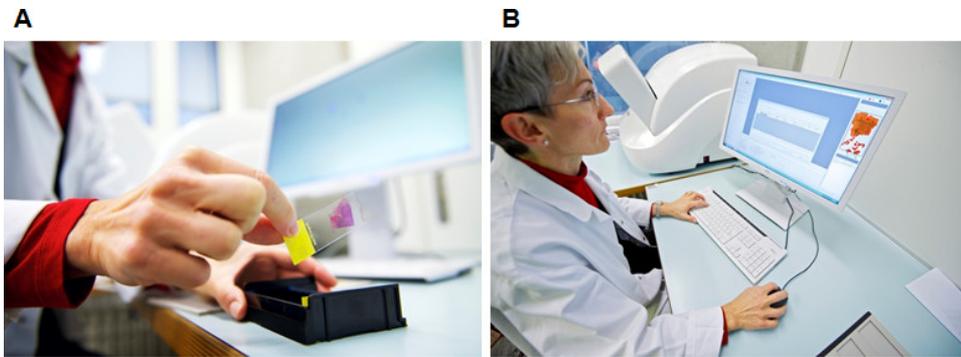


Figure 3. A) Up to 25 slides can be placed in each magazine for scanning. B) Adjustments to the quality factor and size can be made. Scanning progress can be monitored.

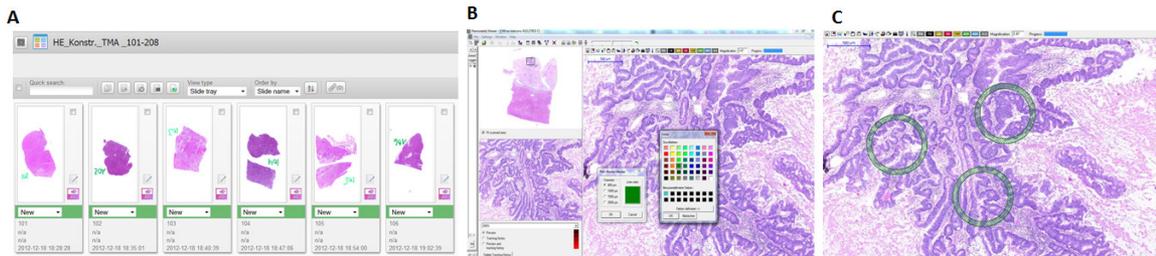


Figure 4. A) Scanned slides can be uploaded onto the web-based platform, Case Center. B) Digital slides can be viewed. The TMA annotation tool allows the user to select the core size and color required for annotation. C) Annotations can be placed directly onto the digital slide. [Please click here to view a larger version of this figure.](#)

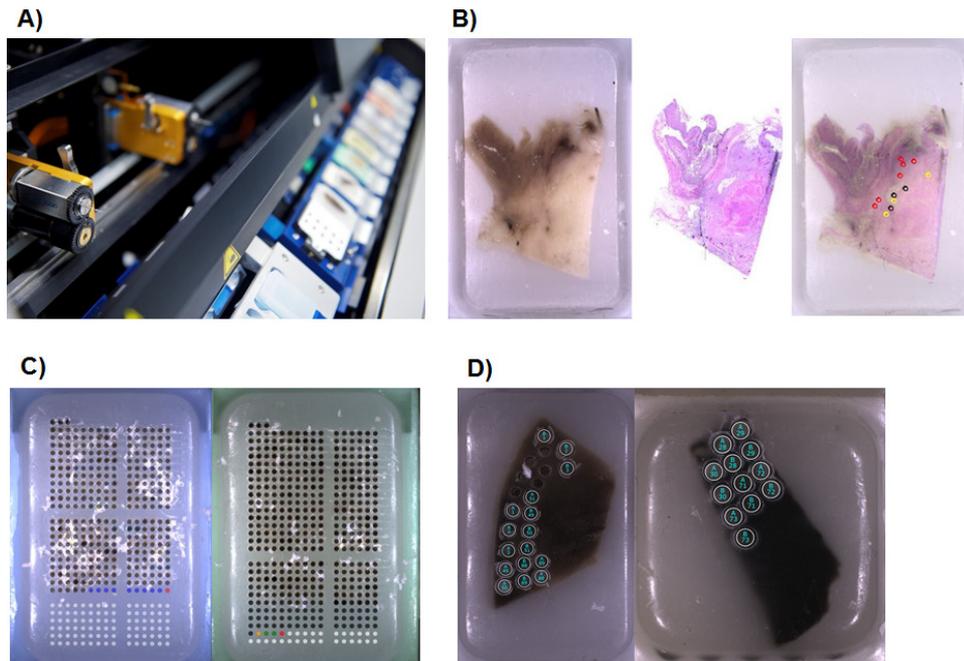


Figure 5. A) Up to 60 blocks can be loaded into the tissue microarrayer, namely 10 donor blocks per row and 12 recipient blocks as well. B) An image of each donor block is taken, the digital slide is retrieved. Matching of block and slide is done and annotations are used for block punching. C) The progress of the arraying can be monitored while the instrument is punching. D) After punching is performed, an export of the location of each spot in the layout but also an image of each donor block with corresponding annotations is made.

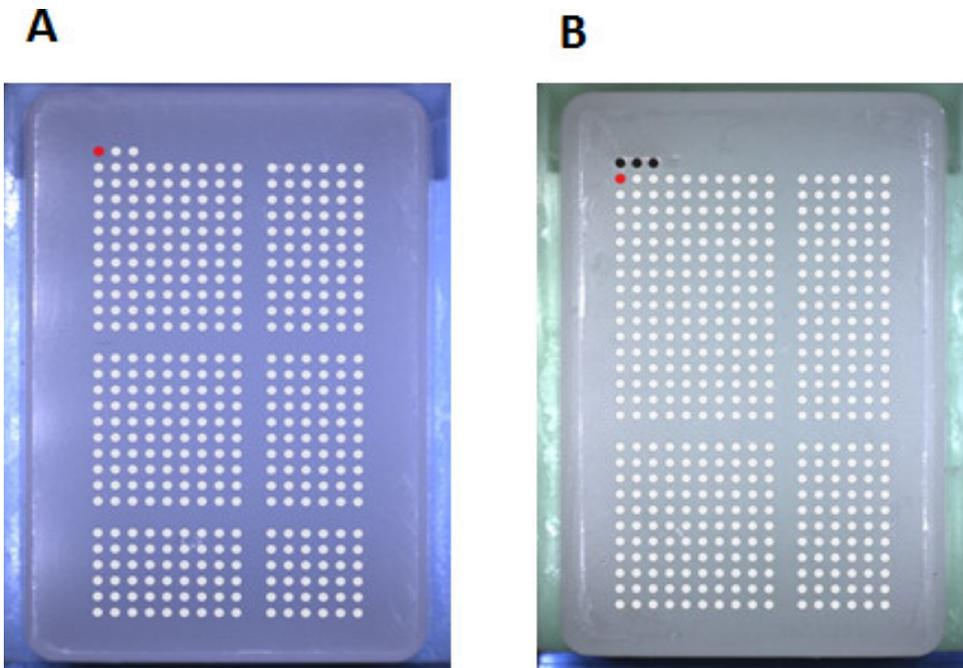


Figure 6. These TMA layouts were used for arraying the **A)** tumor blocks and **B)** normal tissues.

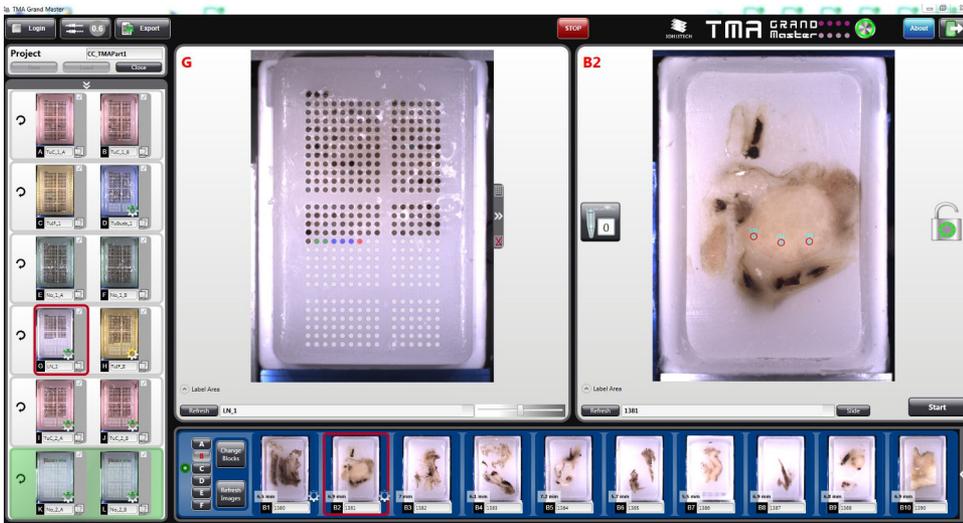


Figure 7. Screenshot of the tissue microarrayer software. On the left, 12 recipient blocks can be placed in the instrument. On the bottom, images of each donor block are taken. In the center, the TMA layout, the progress of the punching as well as enlarged images of each donor block with annotations is found.

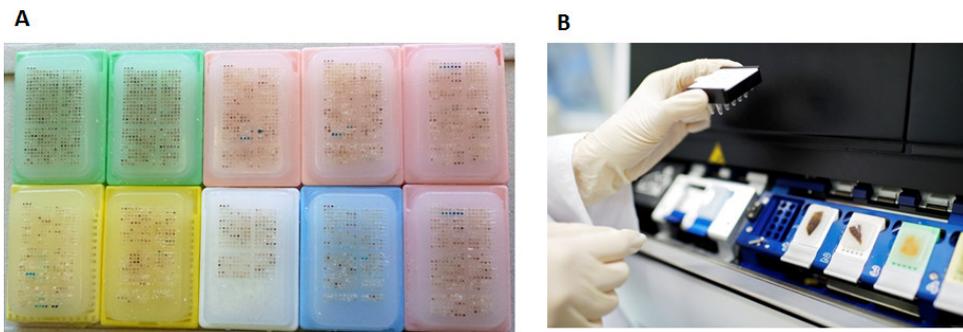


Figure 8. A) Some examples of newly created ngTMA blocks. **B)** Additional tissue punches can be taken for subsequent molecular analysis. These cores are punched out and placed in 0.2 ml tubes.

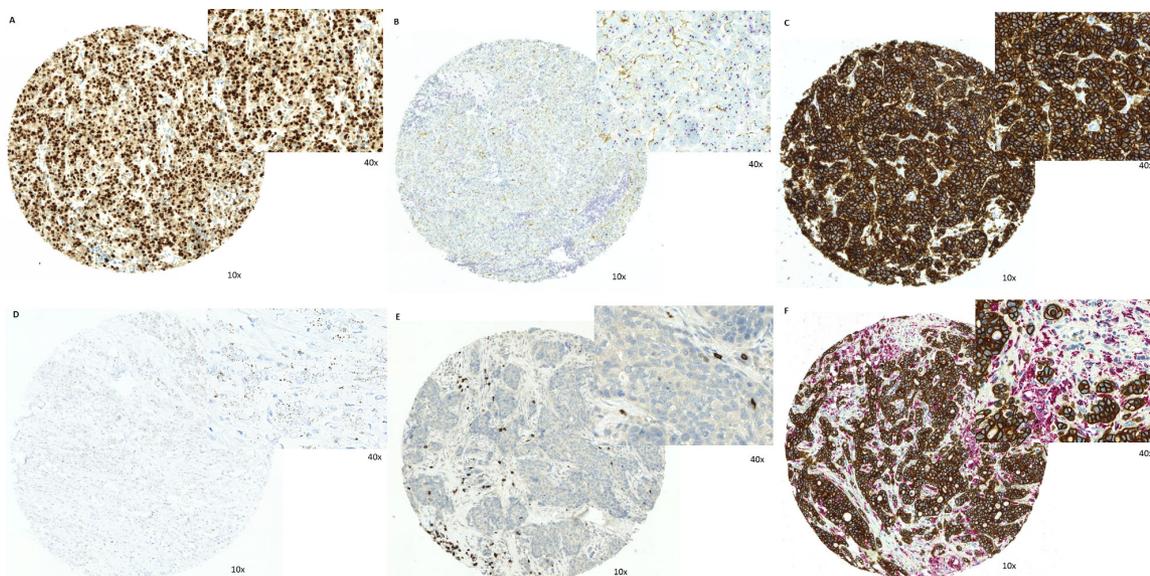


Figure 9. Examples of ngTMA biomarker applications. **A)** Immunohistochemistry staining of Ki67 in colorectal cancer. **B)** Dual HER2 immunohistochemistry/*in situ* hybridization assay showing low expression of protein (brown) and no amplification of the Her2 gene (black) relative to the centromere (red); **C)** high expression of the protein (brown) and Her2 gene amplification (black) relative to centromere (red). **D)** Chromogenic *in situ* hybridization of Her2 showing single mRNA transcripts in breast cancer ngTMA. **E)** Immunohistochemistry for CD8 in colorectal cancer. **F)** Double immunohistochemistry stain for pan-cytokeratin marker AE1/AE3 (brown; tumor cells) and CD68 (red; macrophages) highlighting tumor microenvironment in colorectal cancer. [Please click here to view a larger version of this figure.](#)

Discussion

In this paper, a protocol for ngTMA is outlined. ngTMA is a newly established concept for tissue microarraying involving planning and design, digital pathology and slide annotation as well as automated tissue microarraying¹².

Compared to conventional tissue microarraying, ngTMA offers many advantages. In a first step, the planning and design phase is critical. The focus is on answering a targeted research question. This should take into consideration the histological issues (e.g., which regions and how many spots do I want to include?), the statistical planning (e.g., sample size? How do I analyze my samples later?) and logistical considerations (e.g., how many biomarkers and therefore how many ngTMA copies?). A specific ngTMA is made for specific needs, whether it is for biomarker screening and high-throughput or intended to study specific histological aspects on only a few well selected cases.

One, if not the, most important disadvantage of conventional tissue microarraying is the low accuracy with which markings on the histological slides are made. The study of specific histological structures, cells or regions is made nearly impossible. ngTMA allows for high accuracy because annotations are placed directly on the digital slides. This allows the researcher to precisely select the regions to be punched, including specific cells. In this example, various areas within the same tissue block are punched out for arraying, such as the tumor center, the invasion front and areas of tumor/stroma interaction highlighted by the presence of small tumor cell clusters or even single cells. This accuracy can only be achieved using ngTMA. Thirdly, because slides are scanned onto a web-based digital platform, slide viewing and annotating can be made through the computer rather than with the microscope. The tissue arraying software provides a user friendly interface with a high degree of flexibility, therefore different layouts and ngTMA design can be achieved. Since TMA construction is performed automatically by drilling, there is little need for hands-on maneuvering and the amount of time for construction is significantly reduced. The time for TMA construction in this example here is between 24 hr. Using a conventional TMA approach and estimating 15 punches per hr, this project would take approximately 304 hr.

ngTMA can be applied to study protein expression, mRNA or DNA as well as combinations of these. **Figure 9** illustrates several of these applications to potential biomarkers. Standard immunohistochemistry can be applied to determine the proliferation index of cancers using Ki-67. A combined approach to investigate protein expression and DNA amplification for genes such as HER2 can be used. Tissues can be gathered together on a single ngTMA to reduce costs, tissue usage and other resources. In addition, chromogenic mRNA *in situ* hybridization for HER2 and other genes can be performed to identify single mRNA transcripts in a large number of cases using a minimal number of tissue slides. Immunohistochemistry of immune markers such as CD8 can be visualized in the context of the tumor microenvironment. Double immunohistochemistry can also be used to highlight particular regions of interest such as interactions between immune cells (labelled in red) and tumor cells (labelled in brown) at the invasion front of cancers. Such a region of interest could not have been captured using conventional tissue microarraying.

Nonetheless, this protocol contains some limitations. The most important challenge is the overlap between donor block and digital slide. Several factors can influence this step. Firstly, the latest section from the block should be used for slide scanning. In many cases, the H&E is not the last section of the donor block, rather it is an immunohistochemical or other stain. In this case, also a stained slide can be used as long as the last stain is scanned and annotated or a new H&E should be made. Care should also be taken when tissue sections are being made as tissues may expand in the water bath which leads to challenging matching of slide and block. Secondly, at the moment projects are limited to 12 recipient TMA blocks processed at a single time. Larger projects exceeding 12 TMAs must be assigned to a second project name. Third, the donor blocks

must be made using standard molds and cassettes as the instrument cannot adjust itself to various sizes. Finally, donor blocks must exceed the minimal height (4 mm) to achieve optimal drilling. In some cases, this requires reembedding of tissues.

Hundreds of publications over the last few years highlight the TMA as an invaluable tool for biomarker research. TMAs have been used to study lung¹³, colorectal⁷, breast¹⁴, prostate¹⁵, pancreas¹⁶, bladder¹⁷, and gastric¹⁸ cancers, to name a few. An increasing number of authors have combined the use of TMAs with image analysis and significant strides are being made in this direction¹⁹⁻²¹. However, beside a handful of research groups that have published innovative TMA ideas²²⁻²⁴, little attention has been given to optimize the TMA technique itself. Automated tissue microarrays, such as the ATA-27 by Estigen/Beecher do provide layout design and expedient and automated tissue punching. This represents however only 1 aspect of the ngTMA concept.

ngTMA is a substantial improvement over conventional tissue microarraying techniques. It incorporates expertise in histology and TMA design with the flexibility of digital pathology and the precision of digital annotations with the speed and reliability of automated TMA construction. The combination of ngTMA and image analysis for evaluation of protein and molecular biomarkers will be a powerful tool to further improve the quality of clinical and translational research in the future.

Disclosures

The authors have no disclosures

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