

Spiral TMA

Fukuoka et al.

Title:

Spiral Tissue Microarrays as Next Evolutionary Step in the High-density Tissue
Microarray Technology

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Running Title: Spiral TMA

Abstract

Tissue microarray (TMA) is a well-established technique that connects basic research with clinical applications that allow the validation of many pathobiologic events from gene expression dysregulation to genomic aberrations. However, conventional TMAs have several limitations such as limited representation of tissue heterogeneity, destruction of donor tissue blocks due to coring and usage of particular specimens that have limited evaluable material (tissue from thin specimens or needle biopsies). We have developed a novel method, which we termed “Spiral TMA” that generates TMAs that allow for improved representation of the donor tissue while keeping the architectural details of the donor block intact. This technology is ideal for specimens with limited tissue without the need to punch holes into the original block and therefore preserving the tissue integrity. In this report, we describe the methodology of constructing Spiral TMA and demonstrate the validation of tumor representation and tissue heterogeneity by comparing Spiral TMA to conventional TMA using immunohistochemical staining to EGFR and CK7.

Introduction

Since its description by Kononen et al¹, tissue microarrays (TMAs) have emerged as a well-established technique connecting basic research to clinical applications that have become important as a translational research tool. They have proven to be an effective way to provide high-throughput analysis of hundreds of tissue specimens in a single slide while keeping uniform experimental conditions and being cost effective as tissue samples from multiple patients are processed in an identical manner at the same time. TMAs have allowed the identification and validation of the functional and clinical significance of physiologic and pathobiologic events regarding gene expression dysregulation, genomic aberrations, and epigenetic phenomena by exploring the correlations between molecular information and clinico-pathological features²⁻⁶. However, conventional TMAs (CTMAs) have several drawbacks. One pertinent criticism has been that the tissue analyzed on a TMA is not representative of the entire original tissue specimen. This is of special importance in tumors with heterogeneous morphology that have multiple growth patterns such as mixed germ cell tumors with heterogeneous histological component that have various staining patterns, or lung adenocarcinomas with mixed histology⁷.

CTMA blocks are constructed by punching tissue cores in “donor” blocks and transferring them into a “recipient” block. The donor blocks require sufficient amount of tissue, and many surgical pathology specimens especially from biopsies with limited material may not be suitable for CTMA construction allowing for exclusion from investigational studies and further selection bias. This is especially noteworthy because biopsy specimens may represent the only sample that was collected from patients with

advanced cancer prior to neo-adjuvant treatment. Lastly, punching of the paraffin donor blocks inevitably leaves at least one hole in each tissue donor block. This bears considerable significance since interesting or rare tissue samples archived in many institutions may often get exhausted and therefore it is difficult to study cored tissues as part of a multi-center collaboration.

To improve the limitations of CTMAs, we have now developed a novel approach, which we termed “Spiral tissue microarray” (STMA), in which each TMA core consists of a reeled layer of tissue cut as a horizontal section from the donor block and not punched as a vertical cylinder core. With this method, it is possible to construct microarrays from tissue as thin as 20 μm without damaging the donor paraffin block (**Fig. 1**). In addition, since the whole section is represented on the STMA, sampling bias due to tissue heterogeneity is greatly reduced. Here, we describe in detail the construction of STMA and the validation for research use specifically focusing on reducing sampling bias due to tumor heterogeneity.

Results:

Our study cohort consisted of 25 archival paraffin blocks from pulmonary adenocarcinoma patients as described in detail in the Methods section. Morphologic integrity, quality, and tissue representation were verified by hematoxylin and eosin (H&E) stained sections compared to the adjacent H&E stained sections of the original donor blocks. As demonstrated in **Fig. 2**, our array contained 25 cores of reeled tissue (Panel a), each reel representing an entire longitudinal plane of the donor block. All 25 cores contained expected amount of tumor tissue with/without surrounding normal lung tissue where histological findings were identical to H&E staining in the adjacent section of the original blocks (**Fig. 2**, Panels b and c). Although histological architecture is inevitably lost to a certain degree compared to standard sections, the underlying composition of the tumor in regards to tumor cellularity and architectural detail are clearly recognizable (**Fig. 2**, Panel c).

In addition, we compared the staining of immunohistochemical markers EGFR and CK7 between CTMA and STMA. We used pulmonary adenocarcinomas because of their vast tissue heterogeneity⁸. It is well known that more than 90% of lung adenocarcinomas represent a mixture of various architectural subtypes. In addition, we noticed intra-tumoral differences in immunostaining intensity as a reflection of staining heterogeneity. For each tumor we computed an immunostaining intensity score (IS) difference as the largest variation in staining intensity. Among the 25 cases analyzed in CTMA, EGFR expression was diffuse and homogeneous in 22 cores and CK 7 expression in 18 cores, respectively. Among the 25 cases analyzed in STMA, EGFR expression was diffuse and homogeneous in 14 reels and CK 7 in 6 reels, respectively. In CTMA IS differences of 1 were detected for EGFR in 3 cores and for CK7 in 7 cores.

In STMA IS differences of 1 were detected for EGFR in 8 cores and for CK7 in 10 cores. There was no core in CTMA showing IS differences of 2 for either EGFR or CK7, whereas 3 and 9 reels were found to have IS difference of 2 for EGFR and CK7, respectively in STMA. The range of the staining intensity, expressed by subtraction of minimum IS from maximum IS, was significantly increased in STMA than CTMA in both EGFR and CK7 ($P < .01$ for both) (**Fig. 3**, Panel a-c). Of note, in the STMA, the mean size of each tissue fragment is 1.44mm^2 (range 1.0 to 2.04mm^2), while in a CTMA is 3.14mm^2 .

Discussion:

Our results show that we can achieve an improved representation of multiple areas in a tumor while still maintaining the advantages of the array technology such as high-throughput, experimental uniformity, and cost-effectiveness^{1,9}. This eliminates the selection bias as well. Since STMA uses only one planar tissue section, blocks with little remaining tissue that cannot be used for CTMAs can be included in studies. Furthermore, many core needle biopsy samples, such as transrectal prostate biopsy specimens or breast biopsy which cannot be routinely used for TMA construction with conventional approaches are amenable to the STMA technique. As the tissue is too thin to be sampled vertically, it has insufficient depth to be sampled for a TMA. These biopsy specimens are however ideal to be arrayed with STMA technology as the entire biopsy can be represented in a reel on the array (see Supplementary Fig.1). The clinical implication for the evaluation of diagnostic and prognostic biomarkers on such needle biopsy specimens can be substantially improved.

Recently, there are a few reports describing the clinical application of CTMA to evaluate biomarkers for breast cancer^{10,11}. In these reports, high agreement of expression of HER2 and estrogen and progesterone receptors between cored breast cancer TMA and standard tissue section using immunohistochemical analysis are demonstrated and it is suggested that the routine use of TMA technique for biomarker evaluation may be applicable. Our method allows for maintaining the donor tissue block integrity as well as facilitating the collection of tissue samples, which will promote inter-institutional collaborations and ideally clinical applications.

We believe that validating prospective diagnostic or prognostic markers on STMA in combination with CTMAs may facilitate and accelerate biomarker discovery.

Another advantage of our approach is that sections that will be arrayed on a STMA are identical to the adjacent hematoxylin-eosin sections taken from the paraffin blocks. Therefore, the tissue that will be represented on the array can be validated. In CTMA on the contrary, there is increased variability since the original HE section only corresponds to the first few sections of the cores but not the deeper ones¹². We observed a greater range of immunostaining in STMA tissue fragments compared to CTMA fragments which indicates a greater representation of tissue heterogeneity in STMAs than in CTMAs. One potential limitation of STMAs may be the limited representation of tissue architecture compared to CTMA due to smaller tissue fragment size. However, we were able to show in this report that 100 µm-thick sections are representative of the entire tumor morphology. Increasing section thickness may enhance the tumor architectural detail if necessary. Increased tumor representation on STMA leads to increased space demands on the array block and therefore fewer samples can be arrayed. From our current experience, we estimate that an STMA can accommodate as many as 50 cores in one paraffin block which is less than in a CTMA block. However, increased representation of tumor heterogeneity may alleviate the necessity to sample multiple cores of one donor block as is routinely done in CTMAs where about 600-800 cores are assembled from 200-300 patients.

LeBaron et al also suggested a method to create TMA without coring the blocks¹³. By their method, the spot for each case is a tiny square for which advantages of STMA described above cannot be applied.

Other experimental and clinical applications commonly used for CTMA and better applicable for STMA are for tissue banking and for positive/negative controls of various antibodies used in the immunohistochemistry laboratory¹⁴. Since no special

device is required for the sectioning part, keeping a few additional thick sections for tissue banking and/or control array is an easy application in the routine clinical practice.

In summary, we have developed a novel approach for tissue microarray construction, Spiral TMAs, which has several advantages to the conventional construction of TMAs while maintaining the advantages of high-throughput array technology. Specifically, 1) the increased representation of tissue heterogeneity, 2) the possibility to include small biopsy specimens and 3) the fact that donor tissue is sectioned and not destroyed by punching holes allows for the inclusion of surgical pathology specimens which has been previously not amenable to research due to limited material. Due to the new technique we are describing in the current study, STMA may expand the investigation of entirely new tissue repositories. We believe that STMA is another step in the evolution of high-density tissue microarrays and will further facilitate translational research that will enrich our knowledge and expand the information regarding molecular abnormalities in cancer.

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Author Contributions:

J.F. developed the hypothesis, designed the experiment, performed the bulk of the experiments, analyzed all data and wrote the manuscript

M.D.H. contributed to analyzing the data and wrote the manuscript

T.H. contributed to the construction of STMA and conducted data analysis

T.T. contributed to preparing the case cohort and wrote the manuscript

S.T. and R.O. contributed to the scoring of immunostaining data and to the statistical analysis.

L.R.C. contributed partly to the hypothesis and wrote the manuscript.

Figures Legends:**Figure 1. A schematic overview of Spiral Tissue Microarray (STMA) construction.**

Regions of interest are marked with sharpie pen directly on the block. 20 - 100 μm thick slices of the sample block are cut and rolled up into cylindrical reels. This cylindrical reel is divided into 2-3mm tall including marked regions of interest and transferred vertically into the recipient block/cassette with arranged 3.3 mm diameter holes. A STMA block can be then sectioned in the usual histopathologic fashion.

Figure 2. Histological appearance of Spiral Tissue Microarray (STMA)

(a-c) STMA holding 25 rolls of pulmonary adenocarcinoma. Low-power magnification of STMA (H&E stain) (a). Low power view of one representative reel in the STMA (b). Higher magnification indicates preserved morphology of the adenocarcinoma in the left side, and normal lung area in the right side (c).

Scale bars in image a, b and c indicate 2.0mm, 0.5mm and 0.1mm, respectively.

Figure 3. Comparison of tissue heterogeneity seen in conventional and Spiral TMAs.

a-b) CK7 immunohistochemical staining in the conventional TMA (CTMA) (a) and Spiral TMA (STMA) (b). Both core and reel are taken from the same donor block. Note intensity score (IS) 3 (arrow) and IS 1 (arrow head) can be identified in the same reel in STMA while diffuse IS 3 staining is seen in the CTMA. Scale bars in both a) and b) indicate 0.5 mm.

c) The difference of IS in the same core/reel, calculated by subtraction of minimum IS

from maximum IS, in CTMA and STMA. The shaded bars are for EGFR expression while gray and black bars are for CK7 expression. No differences of IS of EGFR and CK7 were found in the majority of cases (22 and 18 cases, respectively) in CTMA, whereas more cases show increased ranges of staining intensities in STMA compared to CTMA.

Supplementary Figure 1.

(a-c) A reel of Spiral TMA created from the core needle biopsy of breast cancer patient. a) Lower magnification of the reel (H&E staining). Note three thin biopsied fragments are lined together in a roll shape. b) Higher magnification of the same reel. c) Hercep test performed on the same reel. Scale bars indicate 1.0, 0.2 and 0.2mm, respectively.

Materials and Methods**Construction of Spiral TMAs.**

STMA block holding 25 reeled cores of pulmonary adenocarcinoma cases.

We selected 25 paraffin blocks holding pulmonary adenocarcinoma tissue larger than 1cm square from the pathology archive of Toyama University Hospital, Toyama, Japan. The blocks were heated around 40C on the surface, and 100 µm-thick sections were cut with a standard microtome. The tissue sections were manually rolled up into tissue reels. Each tissue reel was then cut into 3 mm-tall subcylindrical reel. Each subcylindrical reel was vertically embedded into plastic tissue-holding-cassette with 26 holes of 3.3 mm diameter (Azumaya Co. Ltd., Tokyo). Melted paraffin was pored into the cassette to re-embed the reeled tissue followed by cooling down. Lastly, the STMA block was sectioned at 4 µm.

CTMA holding cores from same 25 blocks was also constructed to compare the degree of tissue heterogeneity coverage. Cores with two mm diameter were transferred to the same plastic cassette using manual tissue microarrayer (JF-2, Pathology Institute, Toyama). The cassette was re-embedded using same method as described previously.

Immunohistochemical staining:

Anti EGFR and CK7 immunohistochemical staining was performed for 4 µm thick specimens of each block as described previously ⁶. Briefly, after sectioning and deparaffinization of sections, the specimens were heated up to 95C using a water-bath for 40 minutes in Tris/EDTA buffer at pH 9 (Target Retrieval Solution; DAKO). A DAKO autostainer was used for all immunohistochemical process (DAKO, Kyoto,

Japan). Clones and titrations of each antibody were as follows: EGFR (clone DAK-H1-WT, IgG1, titration 1:50, DAKO) and CK7 (clone OV-TL 12/30, DAKO, 1:100). Envision+ system (DAKO) was used for the signal enhancement. The reaction products were visualized with DAB+ (DAKO). The nuclei were lightly counterstained with Mayer's hematoxylin solution. All procedures were carried out at room temperature.

Scoring of the staining results and statistical analysis

Staining intensity (IS) was scored as 0 (no signal), 1 (weak signal), 2 (moderate signal) or 3 (marked signal) as previously reported^{5,6}. To compare the heterogeneity covered by both TMA techniques, we scored maximum and minimum IS in the same core/reel. The range of the staining intensity, expressed by subtraction of minimum IS from maximum IS, was considered as the degree of covered heterogeneity. The range was scored in both STMA and CTMA using same 25 pulmonary adenocarcinoma cases. Mann-Whitney's U test was used for the statistical analysis to compare the differences of the two groups. *P* value less than 0.05 was considered significant.

This study was approved by the Institutional Review Board of Toyama University Hospital (No.19-12, Toyama, Japan).

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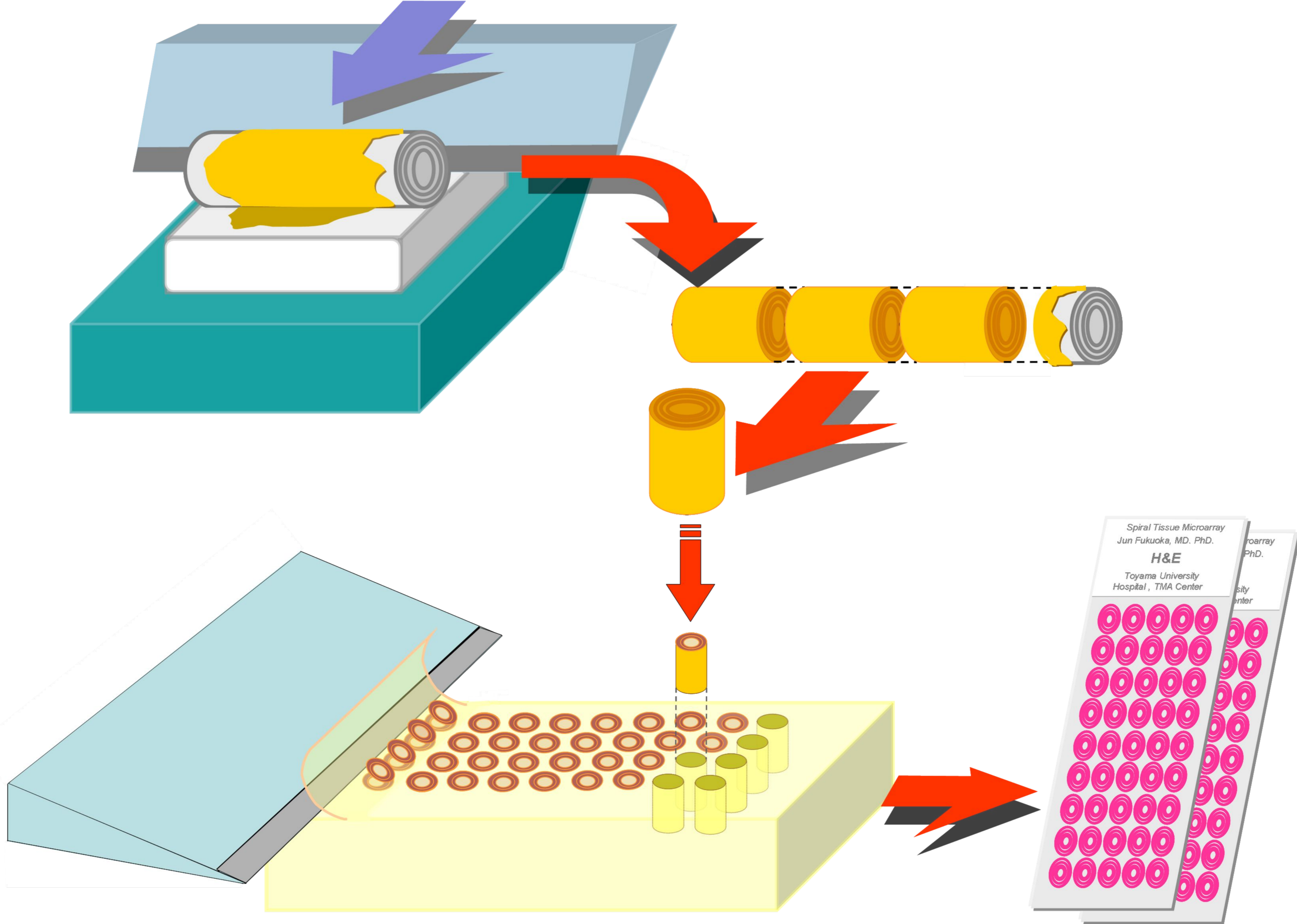


Figure 2.

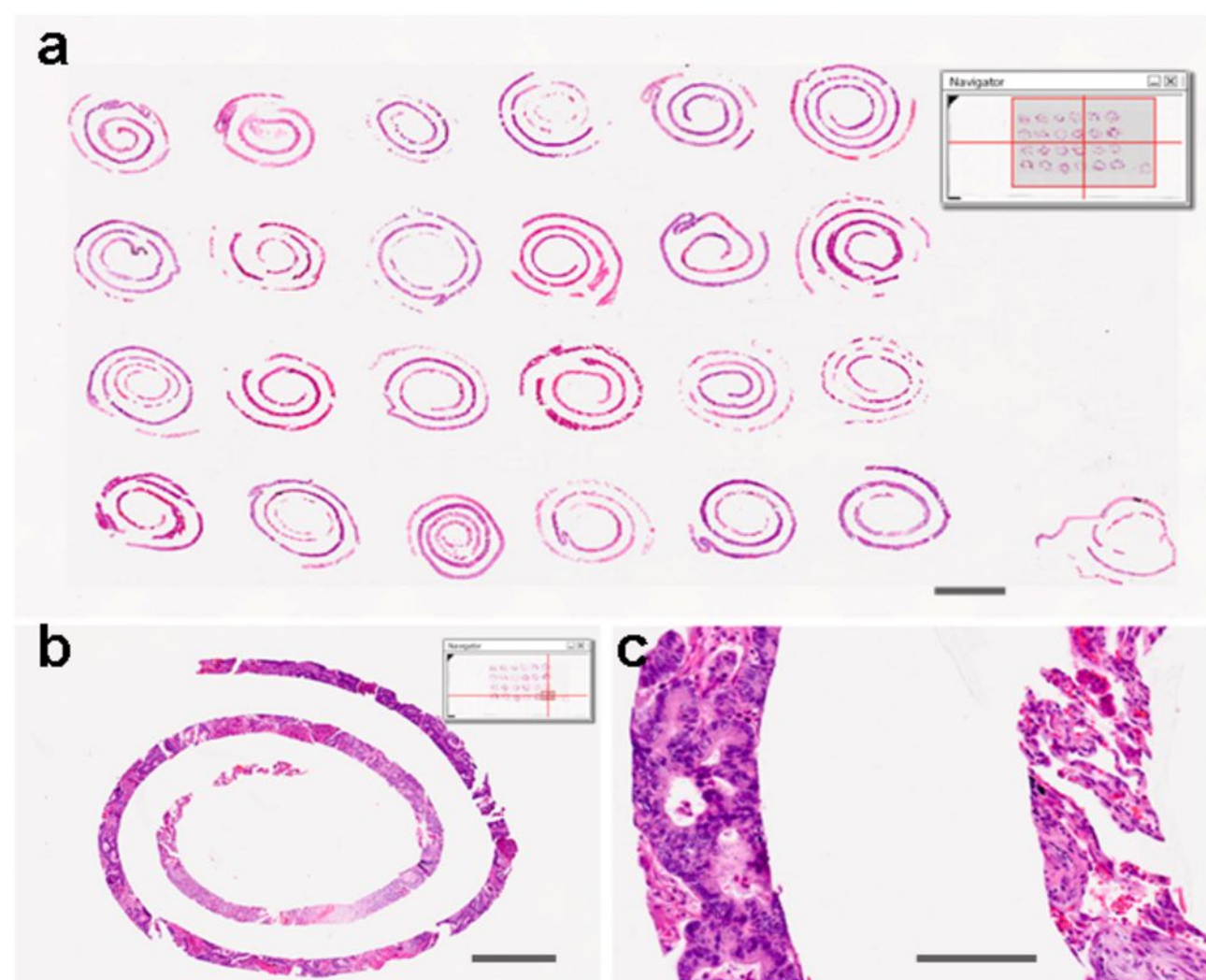


Figure 3.

