Pathol. Oncol. Res. (2014) 20:719–726
DOI 10.1007/s12253-014-9752-3

RESEARCH

# A 'Waterfall' Transfer-based Workflow for Improved Quality of Tissue Microarray Construction and Processing in Breast Cancer Research

**M. Oberländer · H. Alkemade · S. Bünger · F. Ernst · C. Thorns · T. Braunschweig · J. K. Habermann**

Received: 16 October 2013 / Accepted: 19 February 2014 / Published online: 7 March 2014


**Abstract** A major focus in cancer research is the identification of biomarkers for early diagnosis, therapy prediction and prognosis. Hereby, validation of target proteins on clinical samples is of high importance. Tissue microarrays (TMAs) represent an essential advancement for high-throughput analysis by assembling large numbers of tissue cores with high efficacy and comparability. However, limitations along TMA construction and processing exist. In our presented study, we had to overcome several obstacles in the construction and processing of high-density breast cancer TMAs to ensure good quality sections for further research. Exemplarily, 406 breast tissue cores from formalin-fixed and paraffin embedded samples of 245 patients were placed onto three recipient paraffin blocks. Sectioning was performed using a rotary microtome with a "waterfall" automated transfer system. Sections were stained by immunohistochemistry and immunofluorescence for nine proteins. The number and quality of cores after sectioning and staining was counted manually for each marker. In total, 97.1 % of all cores were available after sectioning, while further 96 % of the remaining cores were evaluable after staining. Thereby, normal tissue cores were more often lost compared to tumor tissue cores. Our workflow provides a robust method for manufacturing high-density breast cancer TMAs for subsequent IHC or IF staining without significant sample loss.

**Keywords** Tissue microarray (TMA) · Biomarkers · High-throughput technique · Immunohistochemistry · Immunofluorescence staining

M. Oberländer, H. Alkemade, S. Bünger and F. Ernst shared authorship.

**Electronic supplementary material** The online version of this article (doi:10.1007/s12253-014-9752-3) contains supplementary material, which is available to authorized users.

M. Oberländer · H. Alkemade · S. Bünger · F. Ernst · J. K. Habermann (✉)
Section for Translational Surgical Oncology and Biobanking, Department of Surgery, University of Lübeck and University Medical Center Schleswig-Holstein, Campus Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany
e-mail: habermann@biobank.uni-luebeck.de

C. Thorns
Institute of Pathology, University Medical Center Schleswig-Holstein, Campus Lübeck, Lübeck, Germany

T. Braunschweig
Institute of Pathology, RWTH Aachen University, Pauwelsstraße 30, 52074 Aachen, Germany

## Introduction

In cancer research, the identification of targetable proteins is essential for the development of new diagnostic and prognostic approaches as well as personalized therapies for higher therapy efficiency [1]. This creates an urgent need for protein-based high-throughput validation methods on clinical material. In general, most archived patient material is collected as formalin-fixed, paraffin-embedded (FFPE) tissue blocks, which preserve protein characteristics to high extend for antibody based staining methods [2, 3]. However, studies on whole sections of tissue samples are time consuming, expensive and are limited for intersection comparability. Therefore, the first steps towards a more economical approach were taken by Battifora and Wan et al. [4, 5] and Kononen et al., who published the first protocol for the construction of a FFPE-based tissue microarray (TMA) [6]. TMAs represent an essential advancement towards a high-throughput technique for in situ analysis of potential biomarker targets by assembling up to thousand human tissue cores in one array being available for several sections. Every single section of the TMA

represents all tissue samples and can be stained with the same amount of reagents and time as needed for one conventional single tissue section. Besides higher efficiency, TMAs yield a much higher comparability between the different samples than whole section staining in separate experiments [7]. The high number of patient samples on each TMA section makes TMAs highly valuable for translational research studies. Thus, much effort is put into optimizing TMA construction and processing [8–13]. However, a common technical limitation is the loss of tissue cores during sectioning and staining procedures. In particular, sectioning strongly depends on the technicians dexterity [14]. The loss of samples during TMA sectioning is more challenging based on mechanical feasibilities. Tempering and leveling of protruding cores in the TMA block may prevent tissue cores from being damaged to a certain extent, and loss of cores could be avoided by using optimized microtome and transfer systems [8]. Therefore, several methods are available to transfer sections from the blade of the microtome to a water bath. On the other hand, cores can be lost during staining for immunohistochemistry (IHC) or—fluorescence (IF) [8]. We here present a workflow using a rotary microtome with a "waterfall" transfer system in combination with specific protocol adaptions providing a robust method for manufacturing high density TMAs for subsequent IHC or IF staining without significant sample loss being expeditious, cost effective and easy to handle.

## Material and Methods

### Study Cohort and Planning of the TMA

The study cohort consisted of 406 breast tissue cores from formalin-fixed, paraffin-embedded (FFPE) tissue samples of 245 patients. FFPE samples were collected between 1989 and 1992 and archived in the Institute of Pathology at the University of Lübeck, Germany. The study was performed in accordance to the approval of the local Ethical Committee (#08-012). Samples were distributed onto three TMA blocks containing 129, 137 and 140 tissue cores with a diameter of 1.5 mm respectively. The cores represented breast cancer tissue, ductal carcinoma in situ (DCIS), benign lesions and normal breast tissue. By choosing 1.5 mm punches, representative cores of ductal hyperplasia (DH) as well as DCIS could be obtained since these small lesions are typically assorted with normal tissue and therefore difficult to obtain with smaller punches [15]. The layout of the TMAs was planned on templates using an Excel® spreadsheet (*Microsoft ® Corporation, Redmond, USA*). Thereby, care has been taken to arrange the tissue cores in a non-symmetrical orientation to distinguish between the different TMA blocks. Furthermore, two to three liver cores per recipient block have been included for orientation in case that tissue cores get lost during sectioning or staining processes.

### TMA Construction and Sectioning

To determine an area of interest on the donor tissue samples, 4 μm sections were cut from the FFPE samples in advance and stained with Mayer's Haematoxylin and Eosin (H&E). H&E slides were then evaluated by a senior pathologist (C.T.) marking relevant areas of normal, benign and malignant tissue, respectively. An empty paraffin recipient block was freshly casted 24 h before TMA construction. Therefore, heated paraffin (*Merck KGaA, Darmstadt, Germany, melting point: 56–58 °C, #1.07337*) was casted into a stainless steel mold (*Tissue-Tek*, *Sakura, Netherlands, #4164*). In contrary to our former protocols and as previously describes by others, we did not cool down the block on a cooling plate or ice, but rather let it harden over night at room temperature to avoid the formation of cracks or the trapping of air bubbles under the cassette that might lead to fractions during TMA construction or sectioning [16, 17]. For construction of the TMAs the Tissue Arrayer from Pathology Devices Inc. (*Westminster, Maryland, USA*) was used. Punches were taken from the FFPE donor samples and transferred to an empty paraffin recipient block (Fig. 1). The FFPE recipient block was 5 mm high, 24 mm wide and 29 mm deep. The lateral distance from the cores (each 1.5 mm diameter) to the edge of the block measured 2 mm (Fig. 2). In-between single tissue cores, 0.25 mm were kept as interspaces resulting in a high density TMA-outline (Fig. 2). From each cancer patient, one core of cancerous and normal tissue was punched from each FFPE block and included next to each other on the recipient block. If further available, one core of ductal carcinoma in situ lesions and/or metastatic tissue was included. From patients with benign lesions just one tissue core was included. In total, 406 tissue cores were distributed onto three TMA blocks. To achieve good adherence of the tissue cores to the paraffin after TMA construction, the recipient block was incubated at 37°C for 12 h and, subsequently, overhanging cores were adapted by gently pressing using a glass slide. Hereafter, the TMA block was warmed again at 37 °C for 30 min and finally stored at 4 °C, as described by Hewitt et al. [10]. Cutting 4 μm sections of the TMA blocks was performed with a rotary microtome (*Hyrax M 55, Carl Zeiss, Jena, Germany*) in combination with a cooling clamp (*Cool Cut UKK, Carl Zeiss, Jena, Germany*) and a waterfall section transfer system (*Hyrax STS Section-Transfer-System, Carl Zeiss, Goettingen, Germany*, Fig. 1). Before sectioning, the block was cooled on ice for 30 min. The blocks were faced by cutting 10 μm sections from the TMAs until even sections were obtained. To minimize tissue loss 40 to 46 consecutive sections were cut per TMA block. The blade of the microtome was changed every 20 to 30 sections, meanwhile the TMAs were cooled

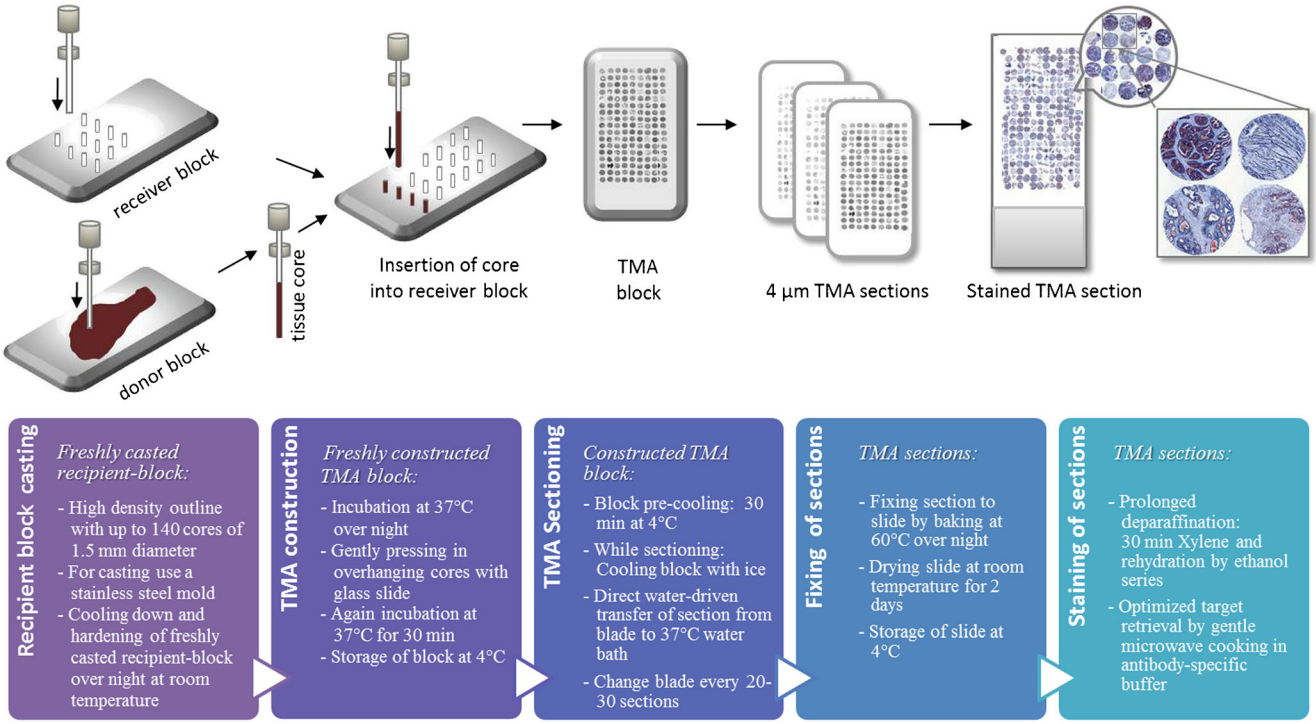


**Fig. 1** Workflow and protocol for improved tissue microarray construction and processing for breast cancer research

with ice without removing the TMAs from the cooling clamp. In order to recover intact sections, the waterfall transfer system was used to convey sections without manual interference from the blade directly into a water bath preheated to 42 °C. Briefly, the water-driven transfer system is comprised of a rail commencing directly beneath the blade of the microtome and leading to the water bath. On this rail a continuous water flow transports the sections directly from the microtome blade to the water bath, where sections stretch out in warm water. The sections do not have to be manipulated manually and the whole process is fully automated. After the sections have strechted out they were immediately mounted centrally on glass slides (*Superfrost Plus, Menzel GmbH, Braunschweig, Germany*) to prevent melting of the lipid rich breast tissue cores. Central positioning of the sections with up to 2 mm distance to the edge of the glass slide is particularly important for subsequent scanning of stained sections e.g. by the Pannoramic Desk Scanner (*3D Histech, Budapest, Hungary*) for digital microscopy evaluation. Thereby, the inclusion of liver tissue cores at well-defined positions helps to orientate the slide image in the right direction for subsequent scoring analysis. To avoid tissue loss during staining procedures, slides were baked at 60˚C over night and dried at room temperature for 2 days. If not processed immediately after

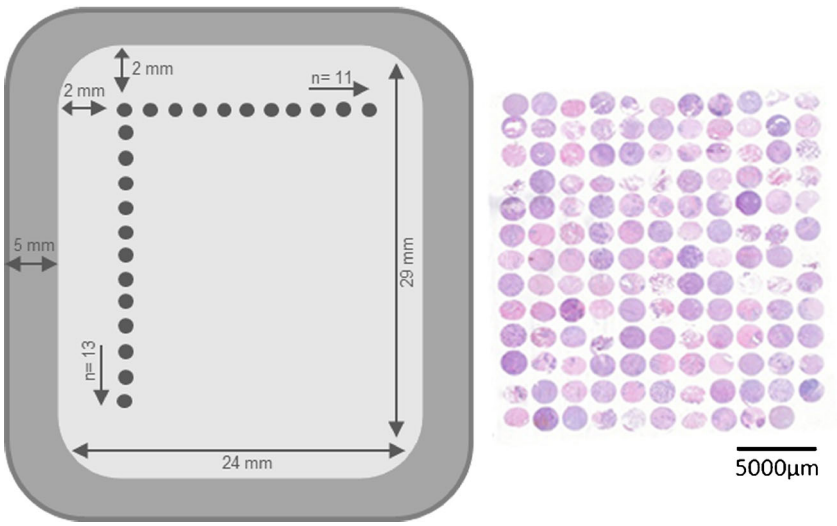


**Fig. 2** Outline and exemplarily image of a high density TMA with 140 cores of breast tissue and two liver reference-cores stained with H&E. The TMA block was 5 mm high, 24 mm wide and 29 mm deep. The distance from the cores (each 1.5 mm diameter) to each border of the block measured 2 mm. In-between single tissue cores 0.25 mm were kept as interspaces resulting in a high density TMA-outline

drying, the slides were stored at 4˚C according to Rimm et al. [7].

### Staining Procedures of TMA Sections

To examine the TMA integrity and the tissue representativeness of each core on the different sections, H&E staining was performed on every 7th section. Additionally, sections were stained with different marker proteins for immunohistochemistry and immunofluorescence. Deparaffination was achieved by incubating slides in Xylene three times for 10 min each followed by rehydration in a graded series of ethanol. For antigen retrieval, sections were cooked in retrieval buffer using a microwave oven according to Shi et al. [18]. Retrieval buffer and other parameters of the protocol were adjusted for the different primary antibodies (Table S1). Antigen retrieval was followed by endogenous peroxidase-blocking with 3 % hydrogen peroxide in methanol (*Merck KGaA, Darmstadt, Germany*). Unspecific background staining was blocked with horse (*Vectastain® Elite ABC Universal Kit, Biologo, Kronshagen, Germany*) or goat serum (*Dako Cytomation A/S, Glostrup, Denmark*) diluted in PBS (*PAA Laboratories GmbH, Pasching, Austria*) (Table S1). The TMA slides were stained immunohistochemically (IHC) for cancer stem cell markers CD326 (EpCAM, *Dako Cytomation A/S, Glostrup, Denmark, M0804),* CD44 (*BD Pharmigen, Erembodegem, Belgium, #550392),* CD166 (ALCAM, a*bcam, Cambridge, UK #ab49496*), CD133 (*Prominin1, Miltenyi, Bergisch Gladbach, Germany, #130-090-422*) mesenchymal markers vimentin (*Sigma-Aldrich, St. Louis, USA, #V6389*), β-tubulin (*Sigma-Aldrich, St. Louis, USA, #T4026*) and the nuclear marker SATB1 (*affibody, kindly provided by R. Kloosters, University of Leiden, Netherlands*). For immunofluorescence (IF), vimentin, ß-tubulin and SATB1 as well as cyclin A2 (*abcam, Cambridge, UK, #ab39*) and cyclin E1 (*Bethyl Laboratories, Montgomery, USA, #IHC-00341*) were stained. For IHC and IF staining of these five markers, 0.1 % Triton X-100 (*Sigma-Aldrich, St. Loius, USA*) in PBS was used as a detergent to improve the intensity of the staining. TMAs were incubated over night at 4˚C with primary antibodies, distinctively diluted in antibody diluent (*Antibody Diluent with Background Reducing Components, Dako Cytomation A/S, Glostrup, Denmark*) (Table S1). Subsequently, slides were incubated with secondary antibodies labeled with biotin (*Vectastain® Elite ABC Universal Kit, Biologo, Kronshagen, Germany*) for IHC and with the fluorescent dyes Atto590 or Atto488 (*Atto-Tec GmbH, Siegen, Germany, #AD590-61, #AD488-61*) for IF. As substrate for IHC, aminoethylcarbazole (AEC) (*Universal staining kit AEC, Biologo, Kronshagen, Germany*) or diaminobenzidine (DAB) (*Liquid DAB+ Substrate Chromogen System, Dako Cytomation A/S, Glostrup, Denmark*) was used in combination with avidin-peroxidase-complex-solution. For IHC, after counterstain with hematoxylin, slides were coverslipped in Aquatex (*Merck KGaA, Darmstadt, Germany*) and analyzed. For IF, slides were counterstained and coverslipped using a DAPI-Mowiol mounting medium (*Sigma-Aldrich, St. Loius, USA*).

### Analysis of IHC and IF Staining

Images of HE and IHC stained TMA slides were archived and documented with the 3D Pannoramic Desk Scanner (*3D Histech, Budapest, Hungary*). Analysis of the IF staining was performed with the fluorescence microscope BZ 9000 (*Keyence, Neu-Isenburg, Germany*).

The number and quality of cores after sectioning and after staining was counted manually for each marker (Fig. 1, Table 1).

### Statistics

Using SPSS Statistics version 19 (IBM Corporation, Somer, NY), the loss/damage of cores was evaluated with respect to the type of tissue being affected. Therefore, univariate tests (oneway anova) were applied to compare mean percentages of loss/damaged cores between control, hyperplastic and cancer tissue. P values of$\leq$0.05 were regarded as statistically significant.

## Results and Discussion

### Evaluation of a Water-Driven Transfer System for Sectioning of a TMA

The outline of our TMA allowed a high density design with up to 140 tissue cores of 1.5 mm diameter on one block (Fig. 2). Sections were cut from the block using a rotary microtome with a cooling clamp combined with a waterfall transfer system. After sectioning, the number and quality of tissue was evaluated.

To minimize tissue loss 44, 46 and 40 sections were cut from the three TMA blocks in one session, respectively. Altogether 130 sections were cut from the three TMAs, yielding a total of 17,578 tissue cores for staining and analysis. During sectioning, 7 % (1,233 cores) were lost from all three TMAs (130 sections) due to misalignment of the sections or deformation of the cores. Thus, 16,345 tissue cores (93 % of all tissue cores) of the three TMAs were available for IHC and IF staining. In more detail, on the sections of the first TMA, 96.1 % (5,456 cores) were intact, on the second TMA 90.3 % (5,690 cores) and 92.8 % (5,199 cores) of the third TMA (Table 1(a)). When compared to sectioning with a sliding microtome, where every third to fourth section of a trial TMA was lost or damaged, sectioning with a rotary

**Table 1** Percentage of lost and damaged tissue cores a) after sectioning and staining, b) during immunohistochemistry (IHC) and c) during immunofluorescence (IF) staining with respect to different grades of malignancy of affected tissues

| a) | | Total (block I-III) | TMA block I | TMA block II | TMA block III |
|---|---|---|---|---|---|
| After sectioning ($n$=130 slides) | Available cores | 16345 (93 %) | 5456 (96.1 %) | 5690 (90.3 %) | 5199 (92.8 %) |
| | Core loss | 1233 (7 %) | 220 (3.9 %) | 612 (9.7 %) | 401 (7.2 %) |
| After IHC/IFstaining ($n$=33 slides) | Available cores | 4161[a] (96 %) | Not applicable | | |
| | Core loss | 174 (4 %) | Not applicable | | |

| b) | | CD44 | CD133 | CD166 | CD326 | Vimentin | Tubulin | SATB1 | MEAN |
|---|---|---|---|---|---|---|---|---|---|
| Evaluable cores after IHC staining ($n$=21 slides) | | 363 (92.6 %) | 362 (92.4 %) | 375 (95.2 %) | 363 (92.4 %) | 384 (97.2 %) | 387 (97.7 %) | 373 (94.7 %) | |
| Core loss during IHC staining | | 29 (7.4 %) | 30 (7.7 %) | 19 (4.8 %) | 30 (7.6 %) | 11 (2.8 %) | 9 (2.3 %) | 21 (5.3 %) | |
| % of lost cores in relation to total number of tissue cores of respective tissue type | normal tissue ($n$=102) | 12.7 % ($n$=13) | 12.7 % ($n$=13) | 7.8 % ($n$=8) | 12.7 % ($n$=13) | 2.9 % ($n$=3) | 5.9 % ($n$=6) | 10.8 % ($n$=11) | 9.3 % |
| | hyperplasia ($n$=47) | 4.3 % ($n$=2) | 6.4 % ($n$=3) | 6.4 % ($n$=3) | 14.9 % ($n$=7) | 8.5 % ($n$=4) | 2.1 % ($n$=1) | 10.6 % ($n$=11) | 7.6 % |
| | tumor ($n$=257) | 5.4 % ($n$=14) | 5.4 % ($n$=14) | 3.1 % ($n$=8) | 3.9 % ($n$=10) | 1.6 % ($n$=4) | 0.8 % ($n$=2) | 1.9 % ($n$=5) | 3.1 % |
| Damage of cores during IHC staining[b] | | 15 (4.1 %) | 45 (12.4 %) | 34 (9.1 %) | 22 (6.1 %) | 66 (17.2 %) | 49 (12.7 %) | 71 (19.0 %) | |
| % of damaged cores in relation to total number of tissue cores of respective tissue type | normal tissue ($n$=102) | 4.9 % ($n$=5) | 18.6 % ($n$=19) | 20.6 % ($n$=21) | 16.7 % ($n$=17) | 30.4 % ($n$=31) | 23.5 % ($n$=24) | 25.5 % ($n$=26) | 20.2 % |
| | hyperplasia ($n$=47) | 4.3 % ($n$=2) | 4.3 % ($n$=2) | 4.3 % ($n$=2) | 4.3 % ($n$=2) | 10.6 % ($n$=5) | 10.6 % ($n$=5) | 10.6 % ($n$=5) | 7.0 % |
| | tumor ($n$=257) | 3.1 % ($n$=8) | 10.5 % ($n$=27) | 4.3 % ($n$=11) | 1.2 % ($n$=3) | 11.7 % ($n$=30) | 7.8 % ($n$=20) | 15.6 % ($n$=40) | 7.7 % |

| c) | | Vimentin | Tubulin | SATB1 | CyclinA2/E1 | MEAN |
|---|---|---|---|---|---|---|
| Evaluable cores after IF staining (Σ) ($n$=12 slides) | | 392 (99.2 %) | 390 (98.5 %) | 383 (97.2 %) | 389 (98.7 %) | |
| Core loss during IF staining (Σ) | | 3 (0.8 %) | 6 (1.5 %) | 11 (2.8 %) | 5 (1.3 %) | |
| % of lost cores in relation to total number of tissue cores of respective tissue type | normal tissue ($n$=102) | 1.0 % ($n$=1) | 2.9 % ($n$=3) | 2.9 % ($n$=3) | 1.0 % ($n$=1) | 1,95 % |
| | hyperplasia ($n$=47) | 2.1 % ($n$=1) | 2.1 % ($n$=1) | 4.3 % ($n$=2) | 4.3 % ($n$=2) | 3,2 % |
| | tumor ($n$=257) | 0.4 % ($n$=1) | 0.8 % ($n$=2) | 2.3 % ($n$=6) | 0.8 % ($n$=2) | 1,07 % |
| Damage of cores during IF staining (Σ)[b] | | 40 (10.2 %) | 50 (12.8 %) | 45 (11.7 %) | 65 (16.7 %) | |
| % of damaged cores in relation to total number of tissue cores of respective tissue type | normal tissue ($n$=102) | 21.6 % ($n$=22) | 23.5 % ($n$=24) | 23.5 % ($n$=24) | 23.5 % ($n$=24) | 23,1 % |
| | hyperplasia ($n$=47) | 19.1 % ($n$=9) | 14.9 % ($n$=7) | 4.3 % ($n$=2) | 6.4 % ($n$=3) | 11,2 % |
| | tumor ($n$=257) | 4.7 % ($n$=12) | 7.4 % ($n$=19) | 7.4 % ($n$=19) | 14.8 % ($n$=38) | 8,6 % |

[a] Only 33 of the 130 manufactured slides were further processed for staining. Remaining slides were stored at 4 °C

[b] Damaged cores were still evaluable

microtome in combination with the waterfall system contributed to an enhanced quality of the TMA sections. Herby, no manual transfer of sections from the microtome blade to the water bath by brush is needed, which could have led to disruption of the sections.

Since the introduction of the TMA technique by Kononen et al., the loss of valuable tissue cores has been one of the main problems of the technique [7, 19–21]. The sectioning of a TMA remains the main bottleneck in the preparation of slides containing all or at least most of the tissue cores, especially of a high density TMA [22]. Instead of manual sectioning, tape-transfer systems try to overcome this problem [23, 10]. One approach introduced by Catchpoole et al. consisted of adhesive tapes which were placed on the block before sectioning; after cutting, the sections stuck to the tape, which was transferred to an adhesive slide, covered with resin and after transfer hardened by UV light [24]. However, the tape-transfer system was found to produce thicker sections which were darker and more densely packed with loss of tissue definition [24, 8, 20]. Furthermore, by producing sticky glued slides, the tape-transfer method shows the disadvantage during IHC of increased background staining and a higher incidence of nonspecific staining, which raised the potential for false positive staining [24, 16]. In our study, tempering of the recipient blocks after embedding the punches and cooling during sectioning already improved the number of tissue cores available on the slides. Furthermore, a substantial improvement to our previous observations using a sliding microtome for TMA sectioning could be obtained by introducing the water-driven transfer system to recover consecutive, high-quality TMA sections from the blade of the microtome. The use of such kind of waterfall system for TMA sectioning is also in use by the Human Protein Atlas Project which deals with global analysis of protein expression patterns of various tissues and therefore strongly relies on high throughput and effective TMA production and sectioning [25]. To our knowledge, up to now there are no data available comparing the recovery of sections with a waterfall section transfer systems to tape-transfer systems or manual microtomy. With the waterfall system described above, 93 % of the tissue cores from all sections were available for further processing, comparable to the amount of cores available using a tape-transfer system [10]. Without specifying a method used for recovery of the sections, Rimm et al. report that 95 % intact cores on a TMA section is difficult to exceed [7]. Furthermore, various groups report tissue losses ranging from 3 % to more than 30 %[26, 20, 27, 28]. In contrast to the here used waterfall system, the tape-transfer system shows the problem that thin sections are frequently uneven in thickness [10] and may present background staining on the slides, especially hindering more delicate analysis such as In situ *hybridization* (ISH) or *Fluorescence* in situ *hybridization* (FISH) [22, 29, 30]. Since our approach does not need any additional solutions or chemicals to be applied on the slides during sectioning, the procedure does not interfere with subsequent staining methods. Moreover, using the rotary microtome in combination with a cooling-clamp and a water-driven transfer system, consecutive and even sections can be cut from the block without the need of removing the TMA block from the microtome and re-cooling it on ice. The sectioning itself and the subsequent retrieval of the sections from the blade are fully automated, fairly easy to operate and expeditious.

## Adjustments of the Pretreatment of TMAs to Improve Stability of Tissue Cores During Staining Procedures

Eleven representative sections of each TMA were used for IHC staining of the cancer stem cell markers CD44, CD166, CD133 and CD326 as well as the mesenchymal markers vimentin and ß-tubulin and the nuclear marker SATB1. For IF staining we chose vimentin, ß-tubulin, SATB1 and the cyclins A2 and E1. Altogether, the 33 sections contained 4.335 cores. Due to the harsh pretreatment using a microwave oven for IHC and to a lesser extend for IF, 5.4 % (149 cores)

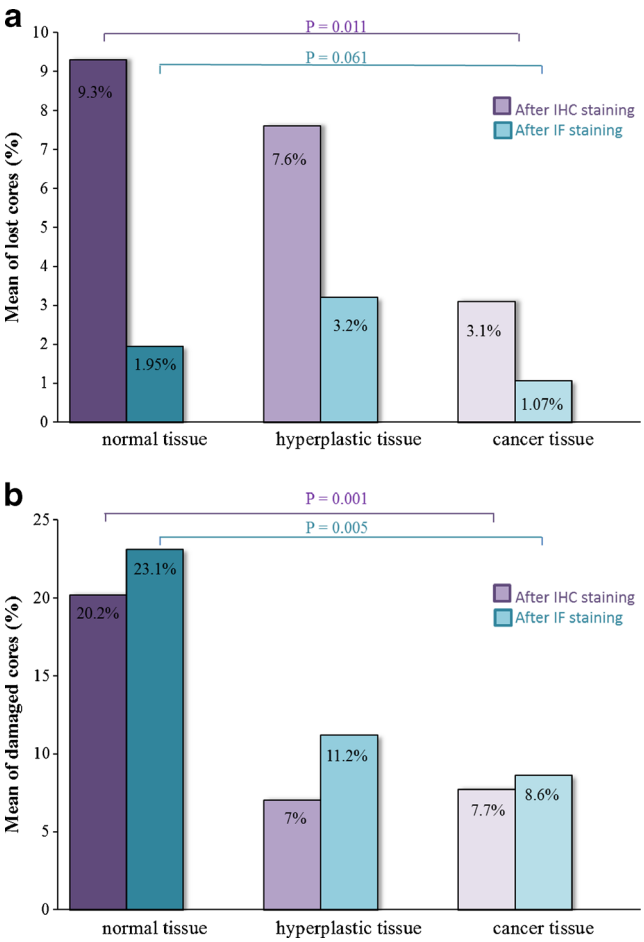


**Fig. 3** Comparison of mean percentages of a) lost cores or b) damaged cores with respect to type of tissue (control, hyperplastic and cancer tissue) being affected after IHC and IF

for IHC and 1.6 % (25 cores) for IF were lost during the staining of the sections (Table 1(a)). Breast tissue is intermingled with a lot of adipose tissue and therefore does not adhere well to glass slides. Cooking of the slides with high energy over a long time, as described above, leads to the detachment of the tissue from the slide and tissue cores are washed away. On the other hand this treatment is necessary due to formalin fixation in clinical material during routine workflow. During the establishment of the staining protocol for the breast cancer tissue we experienced extensive tissue loss during antigen retrieval. To prevent this sample loss, we baked the TMA slides at 60˚C for 12 h after sectioning and subsequently dried them at RT for 2 days. This procedure improved adherence of the tissue to the slides. When we finally used the TMA slides for staining, 96 % of all cores on the 33 sections (4,161 cores) were available for analysis on the slides after the staining of all three TMAs (Table 1(a)). The range of cores suitable for further analysis of the three different TMA slides stained with one antibody reached from 92.4 % for IHC of CD133 to 99.2 % for IF of vimentin (Table 1(b) and (c)).

Thereby, we detected differences of core loss and core damage with respect to the tissue type being affected: After IHC, significantly more normal tissue cores (9.3 %) were lost compared to hyperplastic (7.6 %) and cancer tissue cores (3.1 %, $P$=0.011, Fig. 3a) in relation to the total number of tissue cores of the corresponding tissue type. A similar observation could be made for fractions of damaged cores after IHC, with 20.2 % for normal tissue, 7 % for hyperplastic and 7.7 % for cancer tissue cores ($P$=0.001, Fig. 3b). This trend could also be observed for damaged cores with 23.1 % of normal tissue, 11.2 % for hyperplastic and 8.6 % for cancer tissue cores ($P$= 0.005, Fig. 3b) after IF. Interestingly, more tissue cores got lost during IHC than IF staining possibly caused by antigen retrieval and counterstaining processes. Therefore, the use of more normal tissue cores should be taken into account for TMA setups for breast cancer research. Thus, while planning TMA setups, one should account for tissue loss of individual sample groups of up to 25 % which could be substantial especially for smaller cohorts of distinct tissue types.

Baking the slides at 60˚C for 12 h with subsequent drying for 2 days allowed to maintain almost 99 % of the stained cores for evaluation. Without this preparation IHC staining may be accompanied by higher loss of tissue cores [31, 13]. To our knowledge there is no detailed description available in the literature that evaluates the outcome of TMA sections after staining with IHC or IF using a tape-transfer system. Some publications describe only qualitatively better adhesion of tissue cores or the overall percentage of interpretable cores after immunohistochemistry by using a tape-transfer system [23, 32]. Regarding the minimal tissue loss observed with our pretreatment and the problems that may evolve by using a tape-transfer system as described above, we refrained from using such a system.

## Conclusion

In this report we describe the use of a waterfall section transfer system and a protocol for the sectioning and staining of high density TMAs. The aim of this study was to improve the yield of tissue cores on TMA sections and their adhesion to glass slides during IHC and IF staining. For this purpose we used three high density TMAs with up to 140 human breast tissue cores and stained them after sectioning with antibodies against cancer stem cell markers, mesenchymal and nuclear markers.

For TMA sectioning we used a rotary microtome together with a cooling clamp and a waterfall section transfer system, for optimal, fast and consecutive cutting. A protocol routinely used in our research lab for sectioning following deparaffination and antigen retrieval of whole sections was adjusted for this purpose. A major drawback in the sectioning of TMAs is the loss of tissue cores due to misalignment, tearing of the cores or disengaging of complete cores. Moreover, tissue cores are lost during harsh pretreatment procedures e.g. heat-induced antigen retrieval for IHC and IF.

The water-driven transfer system seems to be as protective for the TMA sections during cutting as the tape-transfer system while bearing fewer obstacles for further handling and staining of the sections. The waterfall section transfer system is effective, automated, easy to operate and minimizes the risk of uneven tissue sections which might vary in thickness. Baking and drying of the sections before staining preserves the tissue cores during antigen retrieval for different staining methods. Furthermore, more normal breast tissue—at least two tissue cores per case—should be included in the TMA setup to account for the more frequent loss of this more fragile tissue. In conclusion our protocol and the use of a rotary microtome with a waterfall section transfer system provide a robust method to manufacture outright sections of high-density TMAs and to stain them subsequently with IHC or IF protocols without significant sample loss.

**Acknowledgments** This study was performed on behalf of the Interdisciplinary Center for Biobanking—Lübeck (ICB-L) and in connection to the Surgical Center for Translational Oncology—Lübeck (SCTO-L), the North German Tumorbank of Colorectal Cancer (ColoNet), the latter being generously supported by the German Cancer Aid Foundation (DKH e.V. # 108446) and the European Union 7th Framework Programme (FLUODIAMON #201837).

**Author Disclosure Statement** The authors declare that they have no competing interests.

## References

1. Martini M, Vecchione L, Siena S, Tejpar S, Bardelli A (2012) Targeted therapies: how personal should we go? Nat Rev Clin Oncol 9(2):87–97. doi:10.1038/nrclinonc.2011.164

2. Braunschweig T, Chung JY, Hewitt SM (2005) Tissue microarrays: bridging the gap between research and the clinic. Expert Rev Proteomics 2(3):325–336. doi:10.1586/14789450.2.3.325
3. Hewitt SM, Takikita M, Abedi-Ardekani B, Kris Y, Bexfield K, Braunschweig T, Chung JY (2008) Validation of proteomic-based discovery with tissue microarrays. Proteomics Clin Appl 2(10–11): 1460–1466. doi:10.1002/prca.200800003
4. Battifora H (1986) The multitumor (sausage) tissue block: novel method for immunohistochemical antibody testing. Lab Invest 55(2):244–248
5. Wan WH, Fortuna MB, Furmanski P (1987) A rapid and efficient method for testing immunohistochemical reactivity of monoclonal antibodies against multiple tissue samples simultaneously. J Immunol Methods 103(1):121–129
6. Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi O-P (1998) Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 4(7):844–847
7. Rimm DL, Nielsen TO, Jewell SD, Rohrer DC, Broadwater G, Waldman F, Mitchell KA, Singh B, Tsongalis GJ, Frankel WL, Magliocco AM, Lara JF, Hsi ED, Bleiweiss IJ, Badve SS, Chen B, Ravdin PM, Schilsky RL, Thor A, Berry DA (2011) Cancer and leukemia group B Pathology Committee guidelines for tissue microarray construction representing multicenter prospective clinical trial tissues. J Clin Oncol 29(16):2282–2290. doi:10.1200/JCO.2010.33.2023
8. Gately K, Kerr K, O'Byrne K (2011) Design, construction, and analysis of cell line arrays and tissue microarrays for gene expression analysis. Methods Mol Biol 784:139–153. doi:10.1007/978-1-61779-289-2_10
9. Gillett CE, Springall RJ, Barnes DM, Hanby AM (2000) Multiple tissue core arrays in histopathology research: a validation study. J Pathol 192(4):549–553. doi:10.1002/1096-9896(2000)
10. Hewitt SM (2004) Design, construction, and use of tissue microarrays. Methods Mol Biol 264:61–72. doi:10.1385/1-59259-759-9:061
11. Onozato ML, Hammond S, Merren M, Yagi Y (2013) Evaluation of a completely automated tissue-sectioning machine for paraffin blocks. J Clin Pathol 66(2):151–154. doi:10.1136/jclinpath-2011-200205
12. Torhorst J, Bucher C, Kononen J, Haas P, Zuber M, Kochli OR, Mross F, Dieterich H, Moch H, Mihatsch M, Kallioniemi OP, Sauter G (2001) Tissue microarrays for rapid linking of molecular changes to clinical endpoints. Am J Pathol 159(6):2249–2256. doi:10.1016/S0002-9440(10)63075-1
13. Zhang D, Salto-Tellez M, Putti TC, Do E, Koay ES (2003) Reliability of tissue microarrays in detecting protein expression and gene amplification in breast cancer. Mod Pathol 16(1):79–84. doi:10.1097/01.MP.0000047307.96344.93
14. Buesa RJ (2010) Productivity standards for histology laboratories. Ann Diagn Pathol 14(2):107–124. doi:10.1016/j.anndiagpath.2009.12.005
15. Yang XR, Charette LA, Garcia-Closas M, Lissowska J, Paal E, Sidawy M, Hewitt SM, Rimm DL, Sherman ME (2006) Construction and validation of tissue microarrays of ductal carcinoma in situ and terminal duct lobular units associated with invasive breast carcinoma. Diagn Mol Pathol 15(3):157–161. doi:10.1097/01.pdm.0000213453.45398.e0
16. Dancau AM, Simon R, Mirlacher M, Sauter G (2010) Tissue microarrays. Methods Mol Biol 576:49–60. doi:10.1007/978-1-59745-545-9_4
17. Fedor HL, De Marzo AM (2005) Practical methods for tissue microarray construction. Methods Mol Med 103:89–101
18. Shi SR, Key ME, Kalra KL (1991) Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue Sections. J Histochem Cytochem 39(6):741–748
19. Tsao SC, Wu CC, Wen CH, Chai CY, Chen YT (2013) Improved technique for manually constructing tissue microarrays for large-core arrays. Appl Immunohistochem Mol Morphol/Off Publ Soc ApplI mmunohistochem 21(1):85–89. doi:10.1097/PAI.0b013e3182553527
20. Hoos A, Cordon-Cardo C (2001) Tissue microarray profiling of cancer specimens and cell lines: opportunities and limitations. Lab Invest 81(10):1331–1338
21. Shebl AM, Zalata KR, Amin MM, El-Hawary AK (2011) An inexpensive method of small paraffin tissue microarrays using mechanical pencil tips. Diagn Pathol 6:117. doi:10.1186/1746-1596-6-117
22. Kajdacsy-Balla A, Geynisman JM, Macias V, Setty S, Nanaji NM, Berman JJ, Dobbin K, Melamed J, Kong X, Bosland M, Orenstein J, Bayerl J, Becich MJ, Dhir R, Datta MW (2007) Practical aspects of planning, building, and interpreting tissue microarrays: the Cooperative Prostate Cancer Tissue Resource experience. J Mol Histol 38(2):113–121. doi:10.1007/s10735-006-9054-5
23. Camp RL, Neumeister V, Rimm DL (2008) A decade of tissue microarrays: progress in the discovery and validation of cancer biomarkers. J Clin Oncol 26(34):5630–5637. doi:10.1200/JCO.2008.17.3567
24. Catchpoole D, Mackie N, McIver S, Chetcuti A, Henwood A, Graf N, Arbuckle S (2011) Tape transfer sectioning of tissue microarrays introduces nonspecific immunohistochemical staining artifacts. Biotech Histochem : Off Publ Biol Stain Comm 86(6):421–428. doi:10.3109/10520295.2010.527859
25. Kampf C, Olsson I, Ryberg U, Sjostedt E, Ponten F (2012) Production of tissue microarrays, immunohistochemistry staining and digitalization within the human protein atlas. J Vis Exp: (63). doi:10.3791/3620
26. Fergenbaum JH, Garcia-Closas M, Hewitt SM, Lissowska J, Sakoda LC, Sherman ME (2004) Loss of antigenicity in stored sections of breast cancer tissue microarrays. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 13 (4):667–672
27. Hoos A, Urist MJ, Stojadinovic A, Mastorides S, Dudas ME, Leung DH, Kuo D, Brennan MF, Lewis JJ, Cordon-Cardo C (2001) Validation of tissue microarrays for immunohistochemical profiling of cancer specimens using the example of human fibroblastic tumors. Am J Pathol 158(4):1245–1251. doi:10.1016/S0002-9440(10)64075-8
28. Mucci NR, Akdas G, Manely S, Rubin MA (2000) Neuroendocrine expression in metastatic prostate cancer: evaluation of high throughput tissue microarrays to detect heterogeneous protein expression. Human Pathol 31(4):406–414. doi:10.1053/hp.2000.7295
29. Watanabe A, Cornelison R, Hostetter G (2005) Tissue microarrays: applications in genomic research. Expert Rev Mol Diagn 5(2):171–181. doi:10.1586/14737159.5.2.171
30. Packeisen J, Korsching E, Herbst H, Boecker W, Buerger H (2003) Demystified…tissue microarray technology. Mol Pathol 56(4):198–204
31. Henriksen KL, Rasmussen BB, Lykkesfeldt AE, Moller S, Ejlertsen B, Mouridsen HT (2007) Semi-quantitative scoring of potentially predictive markers for endocrine treatment of breast cancer: a comparison between whole sections and tissue microarrays. J Clin Pathol 60(4):397–404. doi:10.1136/jcp.2005.034447
32. Divito KA, Berger AJ, Camp RL, Dolled-Filhart M, Rimm DL, Kluger HM (2004) Automated quantitative analysis of tissue microarrays reveals an association between high Bcl-2 expression and improved outcome in melanoma. Cancer Res 64(23):8773–8777. doi:10.1158/0008-5472.CAN-04-1387