ISSN: 1220-3173; E-ISSN: 2457-7618

PRE-PROCESSED TISSUE MICROARRAY BIOMIMETIC MATERIAL - APPLICATIONS AND BENEFITS -

MATERIAL BIOMIMETIC DE ȚESUTURI MICROARRAY PRE-PROCESAT – APLICAȚII ȘI BENEFICII –

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ABSTRACT | REZUMAT

Tissue microarrays (TMA) have gained slowly but surely almost universal acceptance as an enabling tool in experimental pathology and histology, particularly when dealing with minute, irreplaceable tissue samples. Recently, sectionable matrices were introduced as an aid in orienting the tissue samples within the TMAs. A sectionable matrix made of biomimetic material was introduced in clinical diagnostic laboratories in 2012 (BxChip™) that allows the multiplexing of core Tru-cut biopsies (6-12, or more) of small diameters (0.8-3mm). Since the current biomimetic material from which these sectional matrices are made requires a liquid storage medium to avoid its drying and deterioration, it was desired to obtain a biomimetic material with superior stability and versatility and determine the potential benefits. The present study aimed to test two types of sectionable matrices (BxFrame™) with different consistency of the biomimetic material at the stage of inserting biopsies (formalin-fixed mouse skin samples up to 17 mm long as well as 3-5 mm thick *in toto* mouse tail segments). In the first group of samples, "wet" matrices pre-fixed with 4% formalin were employed, while in the second group, the matrices were pre-processed and infiltrated with paraffin (complete histological processing protocol: dehydration, clarification, and paraffin infiltration). The resulting composite paraffin blocks were subjected to conventional histological tissue processing and sectioned at 5 microns without major difficulties. Slight distortions were noted for the "wet" matrices following dehydration, as well as some folding of the sections on the flotation bath. The pre-processed matrices demonstrated remarkable properties: they did not deform during biopsy insertion as well as during tissue

Microarray-urile de țesut (TMA) au câștigat lent, dar sigur, acceptarea aproape universală ca instrument de utilizare în patologia și histologia experimentală, în special atunci când se manevrează mostre de țesut minuscule, de neînlocuit. Recent, matricele secționabile au fost introduse ca ajutor în orientarea probelor de țesut în cadrul TMA-urilor. O matrice secționabilă din material biomimetic a fost introdusă în laboratoarele de diagnostic clinic în 2012 (BxChip™) care permite multiplexarea biopsiilor Tru-cut (6-12 sau mai multe) cu diametre mici (0,8-3 mm). Deoarece materialul biomimetic actual din care sunt realizate aceste matrice secționabile necesită un mediu de stocare lichid pentru a evita uscarea și deteriorarea acestuia, s-a dorit să se obțină un material biomimetic cu stabilitate și versatilitate superioare și să se determine potențialele beneficii. Prezentul studiu și-a propus să testeze două tipuri de matrice secționabile (BxFrame™) cu consistență diferită a materialului biomimetic în etapa de inserare a biopsiilor (probe de piele de șoarece fixate cu formol de până la 17 mm lungime, precum și segmente de coadă de șoarece de 3-5 mm grosime. În primul grup de probe au fost utilizate matrice „umede" pre-fixate cu formol 4%, în timp ce în al doilea grup matricele au fost pre-procesate și infiltrate cu parafină (protocol complet de procesare histologică: deshidratare, clarificare și infiltrare cu parafină). Blocurile de parafină compozite rezultate au fost supuse prelucrării histologice convenționale a țesuturilor și secționate la 5 microni fără dificultăți majore. S-au observat ușoare distorsiuni pentru matricele „umede" după deshidratare, precum și unele pliuri ale secțiunilor pe baia de flotație. Matricele pre-procesate au demonstrat proprietăți remarcabile: nu s-au deformat în timpul introducerii biopsiei, precum și în timpul prelucrării țesuturilor, secționarea la microtom a decurs fără probleme, iar secțiunile s-au aplatizat perfect pe baia de flotație. Matricele pre-procesate au stabilitate pe termen lung la temperatura camerei fără utilizarea vreunui mediu de stocare lichid. Matricea secționabilă biomimetică pre-procesată are potențialul de a crește și mai mult valoarea TMA-urilor în aplicațiile

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processing, and microtome sectioning went smoothly while the sections flattened perfectly on the flotation bath. The pre-processed matrices have long-term stability at room temperature without the use of any liquid storage medium. The pre-processed biomimetic sectionable matrix has the potential to further increase the value of TMAs in clinical and research applications in the histology/histopathology laboratory.

Keywords: biomimetic materials, dermatology, multiplexing, tissue microarray (TMA), sectionable matrix

clinice și de cercetare în laboratorul de histologie/histopatologie.

Cuvinte cheie: materiale biomimetice, dermatologie, multiplexare, microarray de țesut (TMA), matrice secționabilă

During the evolution of multiplexing methods in histology there have been numerous attempts to obtain materials that can be easily sectioned and can incorporate multiple tissue samples. In most cases, the methods were laborious, required a lot of skill, and also incurred significant risks of deteriorating both the donor and receptor blocks (17).

The main problem in pre-analytical histology processing has always been the lack of standardization such as procedures for harvesting and handling tissue samples, fixation durations and solutions, time, and temperature of the dehydration protocols, etc. In pathology, all these steps must be performed correctly and optimized for the type of tissue to be analysed if accurate diagnoses are to be obtained (4).

In 2011, the first tissue microarray (BxChip™) for clinical diagnosis was described, a sectionable matrix obtained from a biomimetic material that allowed the creation of multiplexed matrices with clinical tissues (10). This medical device is widely used in many countries, in operating rooms or in pathology laboratories, allowing the multiplexing of small cylindrical biopsies (20G, 18G, 16G, 14G, 12G) that have a high risk of fragmentation. The tunable properties of the biomimetic material provide good resistance to handling, dehydration, sectioning, and staining steps, similar to the properties of real tissue samples (11, 7). By using the sectionable matrix for multiplexing core biopsies, the processing time, the duration for reading histological slides as well as the cost of laboratory supplies are significantly reduced. More importantly, by reducing tissue losses during all the stages of the histology flow the sectionable matrices are conducive to a more accurate anatomical pathology diagnostic (9, 2).

To determine the mechanical resistance and stability in decalcification solutions (hydrochloric acid 5%, formic acid 10%, EDTA 10%) of the biomimetic material, in 2020 an experiment was conducted that was focused on two categories of investigations: *i)*: rheology testing, by measuring the deformation of the material through penetration, and *ii)*: chemical resistance in decalcification and dehydration solutions with protocols of different durations. The results showed that the material withstands harsh decalcification protocols as well as dehydration protocols lasting between 4 and 13 hours, protocols routinely used in pathology laboratories (18).

Due to the previously mentioned promising results, a more thorough study on the biomimetic material was planned, a study that proposes testing it in its initial, "raw" formula (biomimetic material pre-fixed with 4% formalin solution) and in a pre-processed, harder formula. The aim is to determine some possible storage and use advantages of the BxFrame™ sectionable matrix in the pre-dehydrated form: stability at room temperature without the need to use storage solutions (formalin 4%, saline, etc.), handling of the matrix without major risk of damage, easy handling during the paraffin embedding stage, obtaining histological sections without folds or splits on the flotation bath and their stainability with routine histochemical dyes (haematoxylin-eosin, picrosirius red, safranin, alcian blue, etc.).

MATERIALS AND METHODS

The present study investigates the utility of Bx Frame™ arrays in three experimental groups:

a) Use of a fixed biomimetic matrix at the time of tissue harvesting and starting of fixation, followed by dehydration and paraffin embedding, further referred to as "raw matrix";

b) Use of a previously dehydrated and paraffin-infiltrated biomimetic matrix further referred to as "pre-processed matrix";

c) Using skin specimens from mouse tails placed independently in tissue cassettes according to the conventional method in histology.

For this experiment Themis Pathology provided the two types of BxFrame™ sectionable matrices (fixed and pre-processed), having the following designs and sizes:

- Raw and pre-dehydrated matrices of overall dimensions 21.3mm x 24.6mm, with 11 straight channels (17mm x 0.5mm);
- Raw and pre-dehydrated matrices of overall

size 30mm x 19mm, with 15 wells, 4.5mm in diameter.

The pre-dehydrated matrices were obtained following a long dehydration protocol (Table 1), with increasing ethanol solutions (Tunic Prod SRL, Romania), clarification being made with two changes of n-butanol (Chimreactiv SRL, Romania) and infiltration with histological paraffin Leica Byosistems, Germany (with a melting point at 55°C).

Table 1

Pre-dehydrated BxFrame™ matrices – dehydration and paraffin embedding protocol

Processor station	Solvent	Time, h	Temp. °C	Pressure/ Vacuum
1	60% Ethanol	24	24	NO
2	70% Ethanol	24		
3	80% Ethanol	24		
4	90% Ethanol	24		
5	95% Ethanol	24		
6	100% Ethanol	24		
7	n-Butanol	24		
8	n-Butanol	24		
9	Paraffin	24	58	YES

For testing the sectionable matrices, skin samples were harvested from the tails of male Albino mice (derived from previous experiments). The samples were divided into seven batches - M, I, II, III, IV, V, VI. Each batch consisted of six samples, the exception being batch M, which had only five.

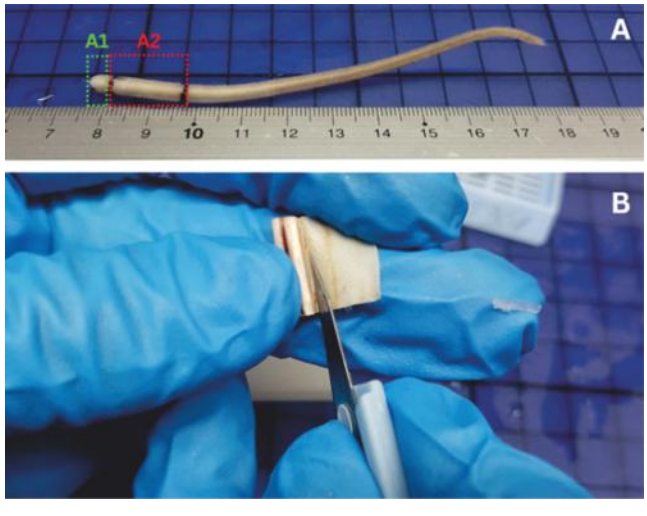

Fig. 1. Tail segment harvesting: (A) Cutting the segment and dividing it into two areas (area A1 and area A2); (B) Removing skin from bone

From each tail, a segment of a maximum of 17 mm was collected from the base of the tail to the tip (see Fig. 1, image A). Each segment was then split into 2 more fragments resulting in area A1 and area A2. Area A1, represented by round pieces of tail with bone, were included directly in "raw" matrices and pre-processed matrices, both with round wells followed by a decalcification protocol with 5% formic acid (2 changes of 48h each). The skin from A2 was cleaved from the bone using a surgical scalpel (Fig.1, image B), then divided into 4 equal pieces and distributed as follows:

– Two pieces were placed in a histological cassette, between 3 reticulated sponges, one below and two others above the skin biopsies, to ensure flattening;

– The third piece was placed into the "raw" TMA matrix;

– The fourth piece was placed in the pre-processed TMA matrix.

After tissue allocation, resulted 6 sectionable matrices with round wells (Table 2), 8 sectionable matrices with long channels (Table 3), and 41 histological cassettes with independent biopsies having the codes Batch M (M1 - M5) T5395 – T5399, batch I (I1 - I6) T5400–T5405, batch II (II1-II6) T5406–T5411, batch III (III1 – III6) T5412 – T5417, batch IV (IV1 – IV6) T5418 – T5423, batch V (V1 – V6) T5424 – T5429, lot VI (VI1 – VI6) T5430 - T5435.

Fine-tip tweezers were used to insert the skin pieces into the raw and pre-processed TMAs, allowing them to be precisely placed due to the distinctive mark in the specially designed channels. After filling the TMAs with biopsies, they were placed in tissue cassettes over which a single reticulated sponge was positioned (Fig. 2).

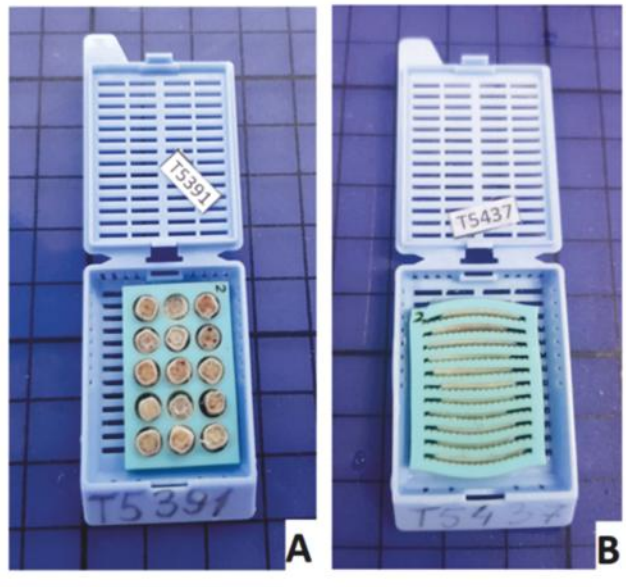

Fig. 2. Sectionable matrices of raw material filled with tissues: (A) Matrices with round wells; (B) Matrices with long channels

After the bone decalcification protocol was completed, all the raw and pre-processed TMAs were rinsed extensively under running water for 10 minutes,

Table 2

Round well sectionable matrices centralizer – matrices coding and tissue placement

Sectionable matrices with round well											
Raw material						Pre-processed material					
T5390	M1	M2	M3	M4	M5	T5393	M1	M2	M3	M4	M5
	I1	I2	I3	I4	I5		I1	I2	I3	I4	I5
	I6	II1	II2	II3	II4		I6	II1	II2	II3	II4
T5391	II5	II6	III1	III2	III3	T5394	II5	II6	III1	III2	III3
	III4	III5	III6	IV1	IV2		III4	III5	III6	IV1	IV2
	IV3	IV4	IV5	IV6	V1		IV3	IV4	IV5	IV6	V1
T5392	V2	V3	V4	V5	V6	T5395	V2	V3	V4	V5	V6
	VI1	VI2	VI3	VI4	VI5		VI1	VI2	VI3	VI4	VI5
	VI6						VI6				

Table 3

Long channel sectionable matrices centralizer – matrices coding and tissue placement

Sectionable matrices with long channel							
Raw material				Pre-processed material			
T5436	T5437	T5438	T5439	T5440	T5441	T5442	T5443
M1	II1	III6	V5	M1	II1	III6	V5
M2	II2	IV1	V6	M2	II2	IV1	V6
M3	II3	IV2	VI1	M3	II3	IV2	VI1
M4	II4	IV3	VI2	M4	II4	IV3	VI2
M5	II5	IV4	VI3	M5	II5	IV4	VI3
I1	II6	IV5	VI4	I1	II6	IV5	VI4
I2	III1	IV6	VI5	I2	III1	IV6	VI5
I3	III2	V1	VI6	I3	III2	V1	VI6
I4	III3	V2		I4	III3	V2	
I5	III4	V3		I5	III4	V3	
I6	III5	V4		I6	III5	V4	

pooled in one single batch, and subjected to a 13-hour dehydration protocol (Table 4) performed on the Tissue Tek Sakura VIP 2000 processor.

After completing the dehydration and paraffin embedding protocols, the resulting paraffin blocks (Fig. 3) were sectioned at 5 µm with a Leica RM2235 rotary microtome (Leica Microsystems, Heidelberg, Germany). Slides were stained with 3 different histochemical stains: Haematoxylin-Eosin, Picrosirius Red, and Safranin-Fast Green (6).

After completion of histochemical stains, the slides were mounted with Cytoseal XYL (Richard-Allan Scientific™, Kalamazoo, MI, USA) and cover slippers 24 x 40 mm (Heinz Herenz Hamburg Gmb, Germany), then examined with a MOTIC BA310 microscope (Motic Europe, S.L.U., Barcelona, Spain).

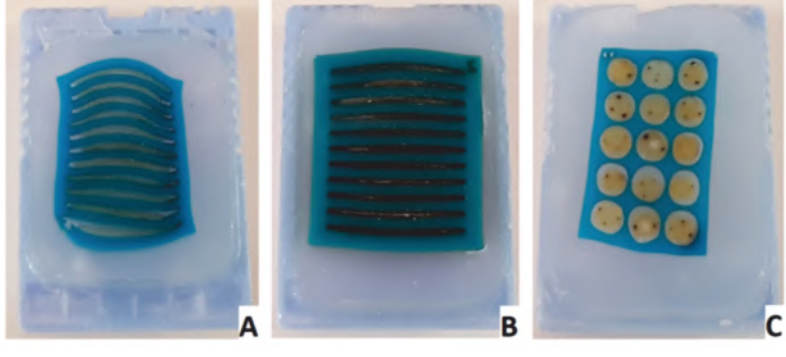

Fig. 3. TMA paraffin blocks: (A) Sectionable matrices of raw material; (B) Sectionable matrices of pre-processed material; (C) Sectionable matrices of raw material with round wells

Table 4

Dehydration protocol

Processor station	Time h	Temp. °C	Pressure/ Vacuum
NBF	1	37	YES
70% Ethanol	1		
80% Ethanol	1		
95% Ethanol	1		
95% Ethanol	1		
100% Ethanol	1		
100% Ethanol	1		
Xylene	1		
Xylene	1		
Paraffin	1	60	YES
Paraffin	1		
Paraffin	1		
Paraffin	1		

RESULTS AND DISCUSSIONS

In the first category analysed in this experiment, BxFrame™ raw material matrices, a slight tendency to curl was observed in the long channel matrices due to the skin tissues having unremoved hair on their surface. The dividing walls of the raw material matrices curved and remained so throughout the dehydration protocol. Biopsies remained firmly fixed in the cavities of the sectionable matrices during the transfer from the tissue cassettes to the paraffin mould and during block casting. No cracks or deformations were observed in the sectionable matrix material. Microtome sectioning was unproblematic for BxFrame™ raw material. To obtain a good quality paraffin ribbon and complete histological sections, the histotechnologist recorded times between 20 and 60 seconds. The histological sections from the raw material occasionally presented small folds on the flotation bath (Fig. 4), and the tissues moved in the middle of the channels during the dehydration protocol, therefore a slight fragmentation of the specimens is observed (images A, C, E – red arrows).

For the second category of matrices in the experiment, pre-processed BxFrame™ matrices, the loading of tissue specimens was rapid, and the channels no longer curved due to the harder material walls, forcing the skin and hair to remain perfectly straight throughout the entire histological process. Slight stiffness was noted during microtome sectioning, and the histotechnologist was able to obtain a paraffin ribbon in less than 30 seconds. In the case of matrices made of pre-processed material during the dehydration protocol, the tissues remained in place and adhered to the left wall of the channels (Fig. 4, B, D, F - yellow arrows). No significant differences were observed between Bx Frame™ array designs (round wells vs.long channels).

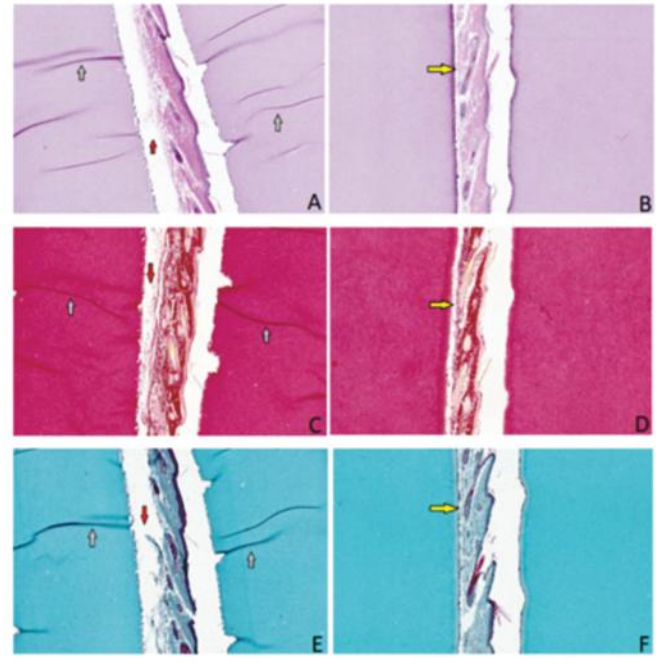

Fig. 4. Differences in the structure after processing of sectionable matrices, 4x objective – (A, C, E) Histological section of a matrix with raw material, folds highlighted by gray arrows and detachment from the channels of biopsies, highlighted by red arrows (Haematoxylin-Eosin staining, Picrosirius Red, Safranin-Fast Green); (B, D, F) Histological section of a pre-processed sectionable matrix, remaining tissue in the channel, highlighted by yellow arrows (Haematoxylin-Eosin, Picrosirius Red, Safranin-Fast Green stain)

In the case of the histological sections from paraffin blocks that had tissues in independent cassettes, it is observed that the structures of the biopsies have frayed, either because of skin detaching from the underlying bone, or because of the compression generated by the sponges used to maintain flatness. This layer separation can be seen in Fig. 5 (image C), where fragmentation of the hypodermis is highlighted, an aspect absents in the biopsies inserted in the sectionable matrices (images A and B). Panel D shows the cross-section of one tail, with all anatomical layers intact.

An obvious conclusion of this study is that matrices from pre-processed material show great promise in multiplexing tissue samples for both research and clinical diagnostics. Pre-processed matrices have a more

stable structure that allows the insertion of fixed as well as paraffin-embedded tissues. The property of the material to support any geometric pattern encourages future experiments where paraffinized tissues of different shapes and sizes should be tested. It would be very interesting to test the applicability of this approach for surface biopsies in dermatology ("shave" or "punch" biopsies). The epidermal tissue removed in a shave biopsy is flat and thin, no more than 1 mm thick (14), and for these types of biopsies, the sectionable long-channel matrices described in the article could be used. Biopsies from this procedure are the most frequently performed due to technical simplicity, wounds heal fast, are low cost, and reveal the epidermis and upper dermis (19,13,8,3). Since biopsy orientation during paraffin block casting is very important, by using BxFrame™ matrices with the long channel pattern, biopsies of this type can maintain their orientation throughout the histological process and they can be inserted by the physician performing the excision.

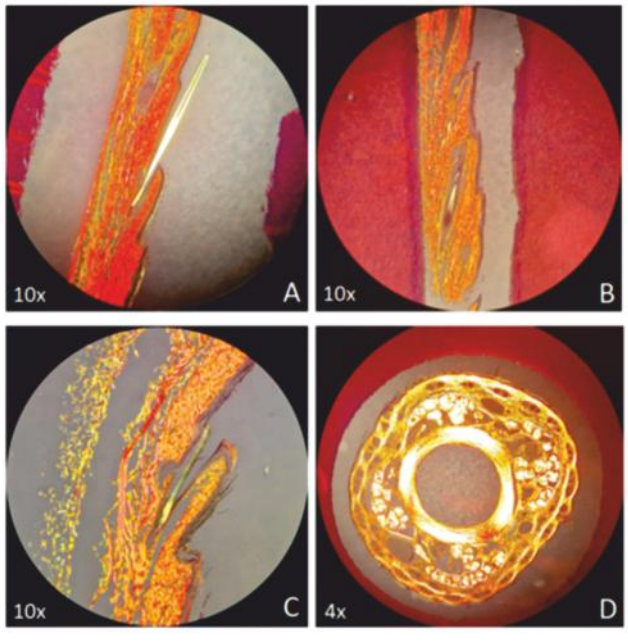

Fig. 5. Sectionable matrices and tissues, Picrosirius Red staining in polarized light – (A) Skin biopsy with hair in a raw material matrix, 10x objective; (B) Skin biopsy with hair follicles in matrix with pre-dehydrated material, 10x objective; (C) Hairy skin biopsy without sectioning matrix, 10x objective; (D) Transverse histological section of a mouse tail segment, 4x objective

For punch biopsies, collected primarily for diagnostic subcutaneous tissue (2 to 8 mm diameters), BxFrame™ round-well sectionable matrices could be used. Because these types of biopsies are cylindrical in shape with a bigger depth (16), up to 4 mm thick, lesions suspicious for melanoma (5, 15) are generally excised only after a more thorough history of the patients and the fulfilment of possible criteria such as asymmetry, edges, colour, diameter, and evolution (1, 12). When collecting multiple punch biopsies from the same patient, the sectionable arrays facilitate the traceability of suspicious areas for melanoma and provide the pathologist depth information on the harvested tissues necessary to obtain an accurate diagnosis.

Through this experiment, it was observed that the matrices can be used in both types with various designs, either having raw material that requires a storage solution (formalin 4%, saline, etc.) or having pre-processed material, where the storage can be long term at room temperature in low humidity conditions.

CONCLUSIONS

BxFrame™ sectionable arrays are very useful and versatile adjuncts for tissue multiplexing, but also for traceability of histologically analysed specimens by maintaining their placement and orientation. Regardless of the type of sectionable matrices, with raw or pre-dehydrated material, they can be subjected to decalcification treatments without being affected structurally. At the dehydration stage, raw material matrices tend to deform if the dividing walls are thin and flexible, while pre-processed matrices, regardless of the thickness of the walls or their arrangement maintain their configuration and do not fold on the flotation bath. Both categories of matrices subjected to histochemical staining were easily distinguishable under the microscope. Even though the utility of BxFrame™ sectionable matrices may vary (use in diagnostic laboratories before tissue processing,at the paraffin embedding stage, or in certain scientific research projects) they can undoubtedly be used with multiple benefits.

ACKNOWLEDGMENTS

The first and second authors contributed equally to the achievement of this paper.

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