Zaznavanje tehničnih napak in optimizacija izdelave individualnih tkivnih mrež pri presajanju in vlivanju

Detection of Technical Errors and Construction Optimization for Custom Tissue Arrays at Transplanting and Casting

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Izvleček

Namen: *Namen raziskave je prepoznati najpogostejše tipične napake pri izdelavi tkivnih mrež v ključnih fazah, ugotoviti vzroke zanje ter na podlagi ugotovitev optimizirati protokole ročne izdelave tkivnih mrež ob variabilnih vstopnih parametrih in različnih končnih zahtevah. Na podlagi ugotovitev se lahko izognemo zahtevnim popravilom tkivnih mrež, kjer se pri ponovnem premeščanju tkiva pojavijo večje izgube ali celo uničenja vzorca zaradi mehanskih in termičnih vplivov.*

Metode: *V raziskavo je bilo zajetih 720 blokov tkivnih mrež, od katerih so bile nekatere za končnega naročnika, druge pa so bile vključene z namenom eksperimentiranja in izpopolnjevanja protokolov. Uporabljeno je bilo humano in živalsko tkivo. Pod*

Abstract

Purpose: *The purpose of this research is to identify causes for the most common typical defects in the production of tissue arrays at the most crucial stages, and to optimize protocols for custom-made tissue arrays at variable input parameters and different final requirements. In this way, we can avoid difficult repairs of tissue arrays, at which, because of re-allocation, major losses can occur, including destruction of the sample due to mechanical and thermal effects.*

Methods: *Seven hundred and twenty tissue array blocks were included, some for clients and others for experimentation and protocol refinement. Human and animal tissue were used. We tested and evaluated configurations, structural stability, consistency, stability, and practical usability of such*

različnimi laboratorijskimi fizikalnimi pogoji smo testirali in ocenjevali konfiguracije, strukturno stabilnost, konsistenco, obstojnost in praktično uporabnost tako izdelanih tkivnih mrež.

Rezultati: *Prepoznali in analizirali smo 12 napak pri izdelavi tkivnih mrež v histološkem laboratoriju v fazah presajanja in vlivanja. Za vsako preučeno napako smo našli vzroke, podali predloge za prevencijo in navodila za odpravo oz. preprečevanje.*

Zaključek: *Za prikazane probleme obstajajo rešitve, ki so tehnično izvedljive, vendar zahtevajo individualno prilagajanje laboratorijskih postopkov, porabo časa in učenje. To je težje ali celo nemogoče za avtomatizirane laboratorije, saj gre pri opisanem za ročno izdelavo tkivnih mrež. Omenjeni laboratoriji pa morajo tudi takšne izdelke pripraviti maksimalno kakovostno in precizno, kar je možno le s sprotnim odpravljanjem napak. Rezultati predstavljene raziskave so prepoznali najpogostejše napake in doprinesli k optimizaciji izdelave tkivnih mrež.*

designed tissue arrays under different laboratory physical conditions.

Results: *We recognized and analyzed 12 mistakes in the production of tissue arrays in the histology laboratory during the stages of transplantation and casting. At each examined error we found the causes, gave suggestions for prevention, and provided instructions on how to eliminate or prevent those causes.*

Conclusions: *Problems have solutions that are technically feasible but they require individual adjustments of laboratory procedures, time, and learning. This is more difficult or even impossible for automated laboratories, as in the described handmade tissue arrays. However, all laboratories need to produce with maximum quality and precision, which is possible only with addressing problems regularly. The results of this study will contribute to the optimization of custom made tissue array processes.*

INTRODUCTION

The ability to produce tissue arrays is an indispensable tool in modern analytical histology, immunohistochemistry, and genetic analyses in medicine and related biosciences. Histologic tissue slices, just as tissue arrays, allow for simultaneous or serial inspection of multiple smaller, various representative tissue samples, of one or more patients on a single surface or in one tissue slice (1). This shortens the time of review, for it is possible to preform rapid comparisons even with a negative or standardized control. Tissue arrays provide cost savings, since dyeing with expensive antibodies and genetic probes or markers are done on a single sample or a few samples or tissue slices, instead of large batches of tissue samples (1–4). Tissue arrays are created with the transplantation of individual cylindrical tissue parts (cylinders) from one or more different donor tissue blocks onto a receiving empty paraffin block, for which drilled holes have already been prepared for this purpose (Figure 1). While some laboratories within individual institutions have developed and optimized the technique of producing tissue arrays for their own needs, it is still a problem and a technical challenge to make optimum quality tissue arrays for custom requirements. In the preparation of tissue arrays, different widths and depths of donated tissue cylinders are used per the request of the client, and as a consequence, different bore holes, and different formats of sizes and orientations of the tissue array are needed. Additionally, tissue is fixed with different procedures and therefore has different levels of hardness. The embedding medium used is individual per client, and has different physical properties compared with the standards of the receiving laboratory. All of this leads to uncompensated variations in the quality of the final product (tissue slice) and can significantly affect the reactivity and readout values, es-

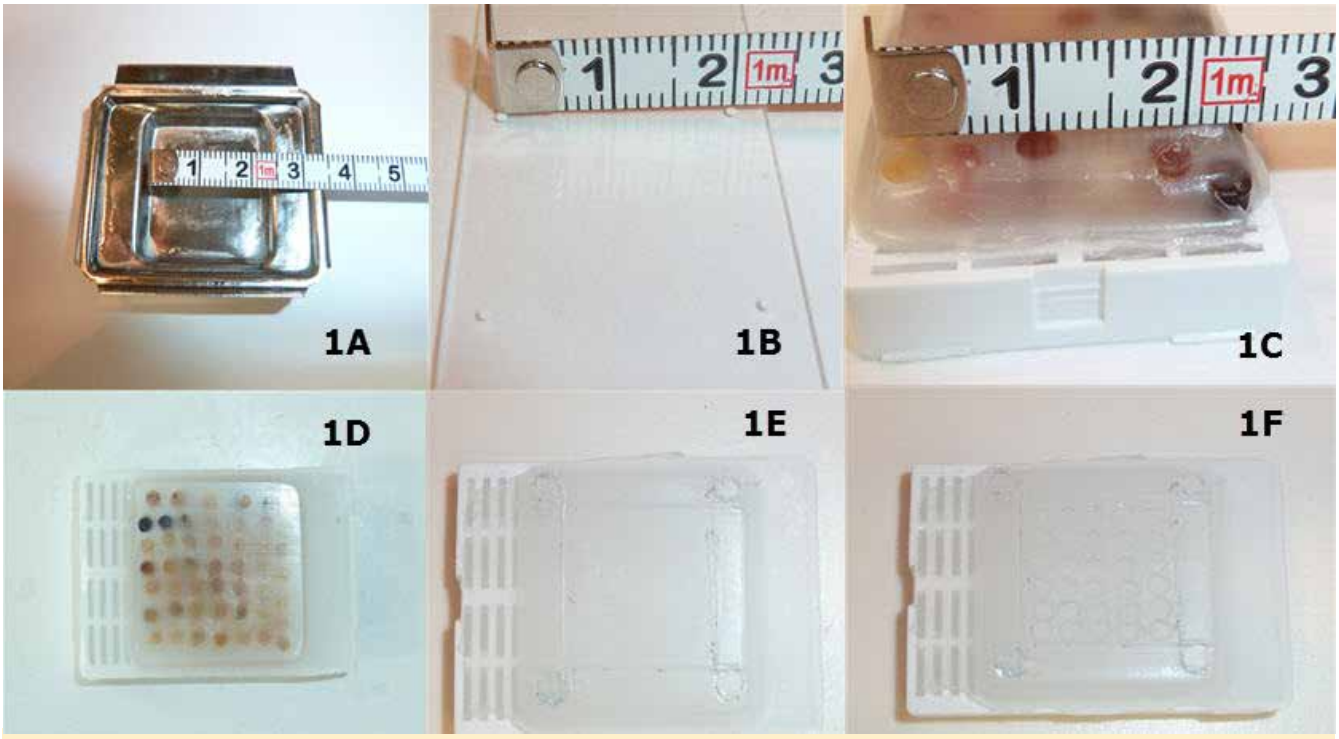

Figure 1. *Construction plan before molding a tissue array. Measuring chosen mold (1A). Maximum 2 x 2 cm array surface for automated staining (1B). An array block that is too large (1C). Crowded array (1D). Distance of one tissue needle diameter on each margin (1E). Written distance of 1 tissue needle diameter between two cylinders or bores (1F).*

pecially in automated systems. Therapy and prognosis are also determined on the basis of this output and the consequences are thus far–reaching (5). Laboratories that have not yet mastered the technology of producing tissue arrays have a difficult time mastering this technology, as there are certain procedures that are "non–standard" and deviate from standard protocols. For example, it is necessary to change the temperature and the speed tissue array blocks casting, the temperature of the working plates in the embedding station, and the time and the temperature of the thermostatic chamber, adding the steps into the order of manufacturing of a particular tissue block. Sometimes it is also necessary to change the embedding medium. All of the foregoing are more difficult to perform in fully automated laboratories, which lack people with fine manual skills (2).

In our laboratory, we have had practice producing tissue arrays for several years and have noticed errors and artefacts in custom–made tissue arrays which are a key influence on further processing and results. Errors in the manufacturing process of tissue arrays are poorly known and described (6, 7). We have described the most common errors and provide technical solutions that we have developed in our laboratory. The described solutions and procedures have been experimentally developed and perfected to avoid future mistakes. The issue varies from case to case and is therefore discussed in more detail below, separately for each analysis.

The purpose of this research is to identify the most common mistakes that occur in the manufacturing of tissue arrays during two main stages (transplant of tissue cylinders and casting with embedding media), and on the basis of the findings optimized the protocols for manually created tissue arrays, which have variable inlet parameters and various client requirements. In this way, we avoid difficult repairs of the tissue arrays because of tissue re–allocation, where major losses can occur, including destruction of the sample due to mechanical and thermal effects (8).

MATERIALS AND METHODS

Photographs of tissue array blocks were taken with a Panasonic LUMIX DMC–TZ10 (Matsushita, Panasonic Ltd., Kadoma, Japan) device, using macro features and optical 3D station the Leica M 165C (Leica Microsystems Ltd., Heerbrug, Switzerland. www.leica–microsystems.com), with usage of the zoom function and side lighting. Microscopic clips of tissue slices were captured with the Leica DM2500 device (Leica Microsytems GmbH, Wetzlar and Mannheim, Germany). For tissue mounting and tissue arrays blocks casting we used the paraffin station Leica EG1150 H (Leica Biosystems Nussloch GmbH, Wetzlar and Mannheim, Germany, www.leicabiosytems.com) and McCormic PARAPLAST tissue embedding medium (Leica Biosystems Richmond Inc., USA) The samples were heat conditioned with a precise thermostat Medite TDO 66 (Medite GmbH, Burgdorf, Germany, www.medite.de) and cooling plates Leica EG1150 C (Leica Biosystems Nussloch) and Medite TKF 22 (Medite GmbH) calibrated at –10°C and –20°C, respectively. The measurements of the tissue blocks were made with a very accurate laboratory meter. The statistics were calculated using Excel 2016 (Microsoft Corporation, USA) and SPSS version 25 (IBM Corporation, IBM Business Machines, USA).

A total of 720 tissue array blocks were included in the study, which were for clients, experimentation, and protocol perfection. Human and animal tissue was used. Human tissue underwent routine pathologic testing at different institutes of pathology in Slovenia. Animal tissue was obtained during routine sampling in slaughterhouses and veterinary clinics in Slovenia. The method of fixation and the embedding medium was not known. The size and density of tissue arrays was made according to the client's order or on the needs of our own laboratory, i.e. for research purposes. We tested and evaluated configurations, structural stability, consistency, and practical usability of the tissue arrays under different conditions. During the first stage we checked the quality and usability of each tissue array block individually, on the basis of analysis of optical devices and a general review of mechanical properties. During the second stage, we sliced the paraffin tissue array blocks with the microtome, fixed them on specimen glasses,

Table 1. *Recognized causes for tissue array failures due inappropriate laboratory parameters. Cases (number) of tissue arrays produced under specific laboratory parameters and the ratio of failure (out of 720 total).*

LABORATORY PARAMETER	A reliable cause for failure ?	Number of tissue arrays with this LAB parameter	Number of inadequate output arrays	Number of adequate output arrays	Percent of failures
Too large tissue array area (>2 x 2 cm)	YES	126	126	0	100 %
Too small tissue array area (<2 x 2 cm)	NO	5	0	5	0 %
Cylinder distance less than one tissue needle diameter	YES	202	114	88	56,5 %
Cylinder distance min. or more than one tissue needle diameter	NO	518	0	518	0 %
Too hot tissue needle	YES	24	23	1	95 %
Too cold tissue needle	NO	7	0	7	0 %
Preheated donor/ recipient blocks <40°C	YES	41	37	4	90 %
Preheated donor/ recipient blocks >40°C	YES	5	3	2	60%
Casting paraffin temperature <65°C	YES[1]	10	10	0	100 %[1]
Poor leveling	MOSTLY	7	4	3	57 %
No thermal end-conditioning	MOSTLY	129	69	60	53 %
Tissue array over-cooling (–10°C/–20°C) at the end	YES	70	63	7	90%

[1]in only one transplanting technique

Table 2. *Symptom and solution for each recognized cause of failure.*

FAILURE CAUSE	SYMPTOM(S)	SOLUTION(S)
Too big tissue array area (>2 x 2 cm)	Too big area for many automatic staining machines	Maximum array area is 2 x 2 cm
Cylinder distance less than one tissue needle diameter	Parts on the edge breaking away, parts falling away after microtom slicing	Minimum distance from the edge and in between two cylinders is one tissue needle diameter
Too hot tissue needle	Dirty needle, egg–shaped hole, empty space between cylinder and surrounding, crumpled cylinder	Use only cooled tissue needle for transplanting and boring
Preheated donor/recipient blocks <40°C	Cracking tissue block, tissue needle overforced, crumpled cylinder, parts falling away	Always preheat the donor and recipient block to 40°C
Preheated donor/recipient blocks >40°C	Crumpled cylinders sticking in tissue needle, egg shaped bores	Do not overheat tissue blocks above 40°C
Casting paraffin temperature <65°C	Bad embedding[1]	Set higher casting temperature or use less dense embedding media
Poor leveling	Trouble on slicing, unrepresentative slices	Carefully leveling, side control
No thermal end–conditioning	Empty space between cylinder and sorrounding, trouble on slicing	Should always perform thermal end conditioning and slow cooling
Tissue array over–cooling (−10°C/−20°C) at the end	Cracking tissue blocks, parts breaking away, impossible microtome slicing	Cool only on room temperature

[1]in only one transplanting method

and measured stability and consistency. We checked for missing round surfaces on the transplanted parts of the tissue and whether they merged with the surroundings. For each mistake, we wrote down the number of inadequate samples (blocks of tissue arrays and tissue slices from tissue arrays) with the described error (Table 1). Detailed procedures and results are described for each analysis or issued class specifically (Tables 1,2).

Specific metrics and analysis, identified errors and their solutions

Inadequate size and density of the tissue array, and poor planning.

The most common mistakes are made at the beginning, when the selected mold for casting of the empty paraffin tissue block is too wide or too big (7). All of the molds are defined as "standard" and the 2 × 2 cm ones appear the same at first sight. But, upon precise measurement we see variations up to 0.5 cm (Figure 1A). Some of the molds are narrower at the top compared with the base; therefore errors may occur when calculating the maximum size of the tissue arrays, with poor pouring of the cylinders happening at the base of the mold. It is necessary to precisely measure the mold before use (height, length, width) and to calculate the anticipated size of the tissue arrays only on the basis of its minimum values.

Due to unclear orders by clients, they often receive constructed tissue arrays that have a surface that is too large. This is particularly problematic for automated immunohistochemistry dying and genetic research (e.g. Fluorescence In Situ Hybridization), where automatic devices do not allow or cannot work with tissue slices larger than 2 × 2 cm (Figures 1B, C). Before making each tissue array it is necessary to obtain completely precise information on the maximum permitted gross dimensions of the tissue array and the required diameters of the sampled tissue products. In-

formation about width or diameter of the tissue block is crucial. Depending on the width of the tissue needle we can put differently sized samples on the tissue arrays, setting a maximum number of possible samples in each tissue grid (7). Based on client requirements for the tissue arrays and the manufacturer's attempts to reduce costs, we have seen an exaggeration in the density of the tissue products and distribution in the tissue grid. In this way, there are unstable and arrays that are too dense are made (Figure 1D) that are difficult to slice; edge banding cylinders can fall off or are insufficiently embedded (Figure 1C). Based on experiments, we found that tissue cylinders from the donor tissue block and media in the recipient block have immensely varied expansion coefficients due to the different densities. To prevent separation of the cylinders from the surroundings (and tissue slices falling out), we have introduced a compensation factor for when the tissue arrays are cooled down. This is at least a one–time distance of the width of the used tissue needle between two individual cylinders in the array, equal to the distance from the extreme edge of the tissue array to the first edge of the cylinder in a particular array line. We determined the optimal distribution of cylinders in the tissue array using a fast and practical method that does not require specific jigs. This constructed array allows for the greatest and most stable efficiency of surface.

With the selected size of the tissue needle, we draw a circle in each extreme corner. Then we mark the maximum inner net surface of the tissue array with a ruler (Figure 1E). We then string the drawn circles with the diameter of the chosen tissue needle, so that they do not touch each other (Figure 1F). We start implanting cylinders at the upper left in the first corner of the free circular field of the array.

Improper temperature of the tissue blocks and a tissue needles.

We found that every tissue biopsy needle needs to be heated first in the heating tool grooves at paraffin embedding station, at ~60°C. The optimal frame time for heating is 1–2 min. A longer period of

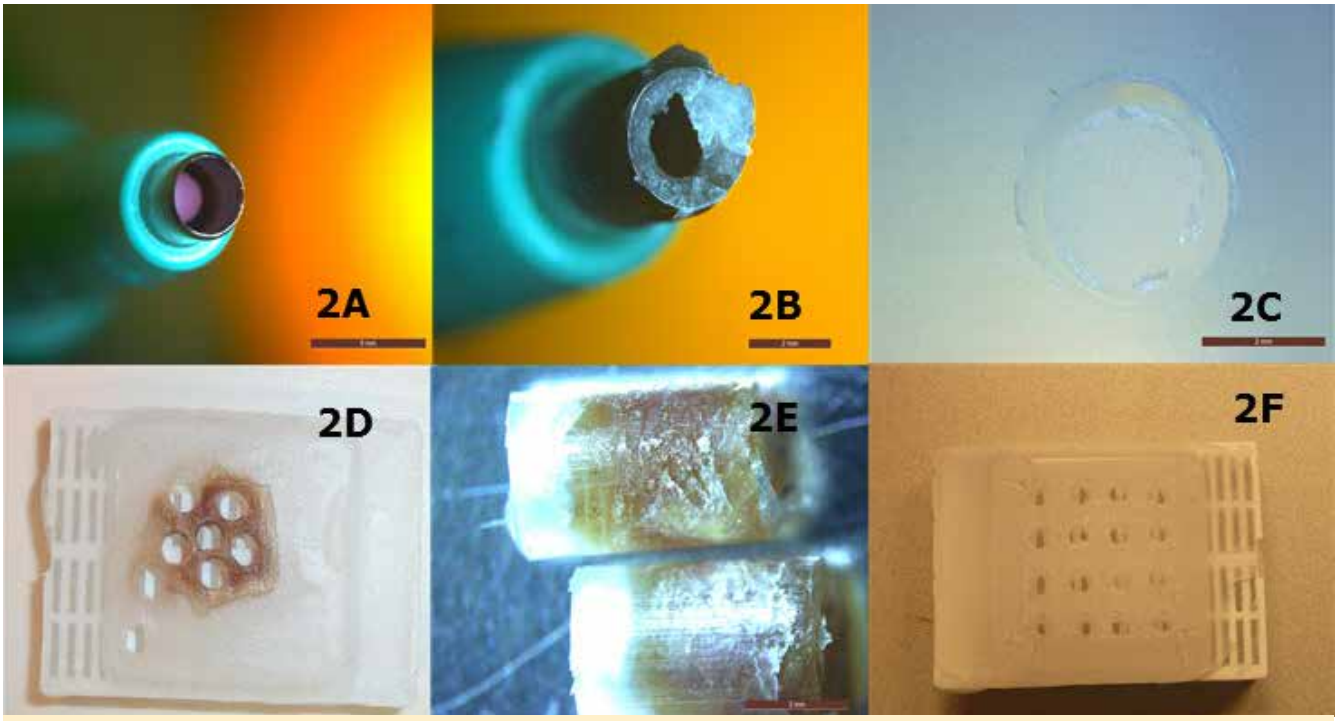

Figure 2. *Optimized tissue needle and tissue block temperatures. A clean needle (2A). Dirty, unusable needle (2B). Cylinder sticking on base surface (2C). Multiple testing bores on animal tissue (2D). Difference between cold tissue block (upper cylinder) and preheated tissue block (bottom cylinder) (2E). Optimal prepared recipient bores (2F).*

time is detrimental because it softens the tip of the needle, which blunts. After heating, every needle must be shaken roughly (like a mercury thermometer) to remove residues of liquid paraffin from the previous transplant. The needle is then placed on the cooling plate for at least 3 min, maintaining the plate temperature at approximately –20°C. At this temperature, the needle can be kept until use. In general, it is no problem when the needle becomes colder. The needle is then completely clean and smooth on all surfaces (Figure 2A). Borings into donor and recipient blocks are always made with a cooled needle; if a hot needle is used, it is contaminated by melted embedding media (Figure 2B) and major anomalies may occur on the recipient block. If the needle clogs with embedding media, it does not stick to the cylinder during removal and leaves wide, dissolved and melted circular stitches (Figure 2C) or egg–shaped cylindrical holes (Figure 2D). If a clogged melted hole is used for transplantation, there is too much empty space around the cylinder which therefore does not blend with the surrounding area and falls off almost 100% (Table 1).

Donor and recipient tissue blocks should be pre–heated before transplantation. The optimal temperature is approximately 40°C for at least 1 h. If the heating time is too short, the entire block is not heated into the depths, which is extremely important for the cylinders to unclasp off the base (plastic grid). A higher temperature of pre–heating is sometimes only meaningful for very hard tissues; otherwise it is not suitable because it softens the tissue and becomes rubbery, pressured, and crumpled while extracted with the needle. A piston in the needle hardly pushes such tissue out; furthermore, a deprived cylinder stutters with the edges during implantation. We always transplant on a plate that is still hot (~60°C) at the embedding station. Donor and recipient blocks should be on the hot panel for at least 2 min (not more than 5 min) before transplantation. Thus, a part of the tissue at the cylinder bottom by the plastic grid will be more heated and easier to separate from the base, especially when using needles wider than 3 mm. It will

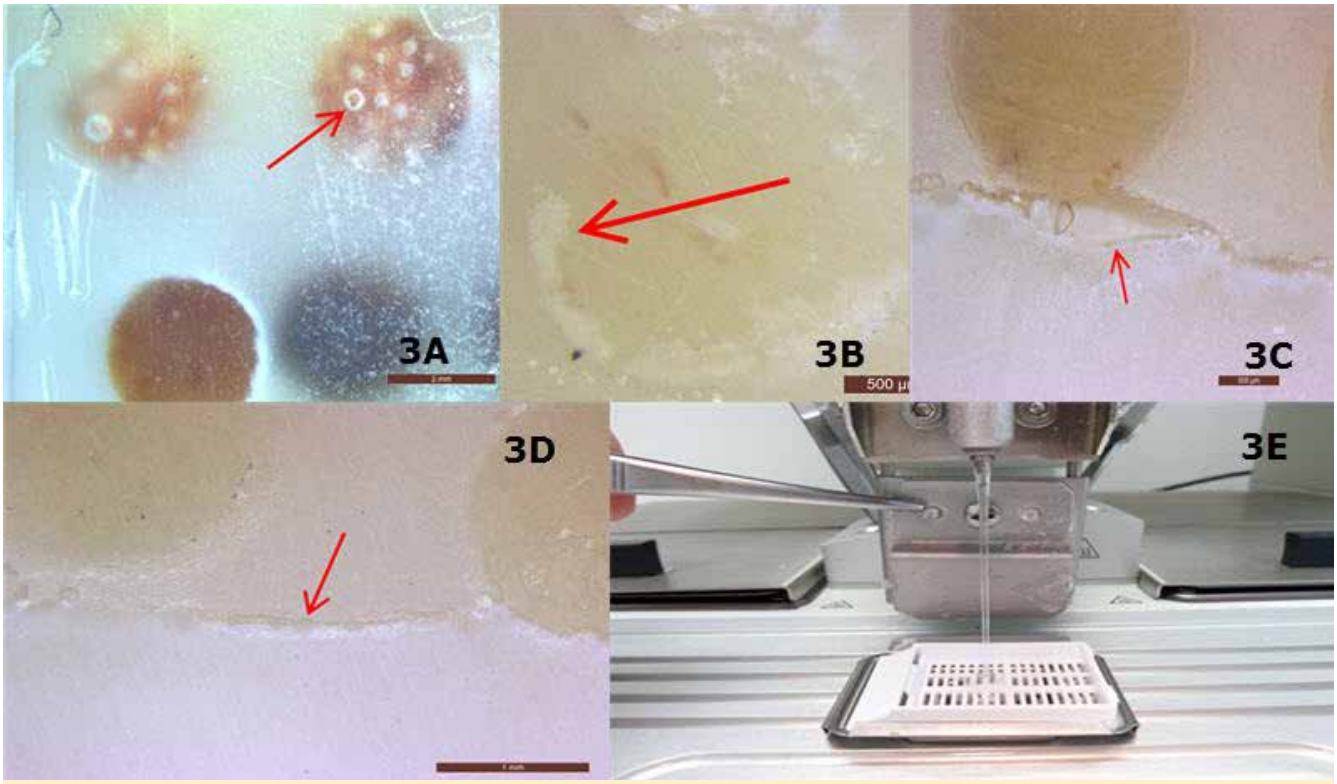

Figure 3. *Air inclusions and cracking at improper casting (3A–3D). Optimal casting temperature, quick distribution of embedding media (3E).*

be easier for the implanted cylinder to clog with the plastic grid in the recipient block. We must also not forget that the tissue needle must be positioned perfectly vertical during the transplantation process; most people overlook this point. If it is not vertical, irregular egg–shaped drillings and empty spaces around the transplanted cylinder may occur. The consequences of appropriate needle and tissue block temperatures are depicted in Figure 2E. The macro photo of the upper tissue cylinder (lateral projection) is from the cold donor tissue block. The cylinder lateral surface is edgy, rough, and hardly slips into the receptor bore. The bottom depicted cylinder is coated with smooth paraffin, which is a consequence of a cold needle contacting a hot cylinder in the donor block. Such a cylinder will not stick in the needle and will smoothly slip into the recipient bore. A well–made recipient block is shown in Figure 2F, where you can see straight, round bores throughout its depth, to the plastic grid.

Wrong temperature and physical parameters of the embedding medium.

Before casting or pouring of tissue arrays, it is necessary to heat the paraffin to maximum liquidity. Only then will it have the lowest viscosity, being smooth and quickly filling all the empty spaces in the casting tissue array block. The thick liquid paraffin is unable to do so, resulting in a porous tissue array with many air inclusions (Figures 3A,B). Empty spaces or separating of cylinders from its surrounding medium (Figures 3C,D) can also occur, especially when using the method of pouring grids (the China study) or ex–post corrections in filling or surface levelling. Standard settings for paraffin temperatures are from 55°C to 60°C, as used by histological laboratories; this is satisfactory for making tissue blocks but it is too low for casting of tissue arrays. The most suitable temperature of paraffin in our case was 65°C, where it was highly transparent and flowing, quickly flowing through the grid of the tissue blocks without accumulation (Figure 3E), and beautifully wrapping itself around the cylinders without inclusions.

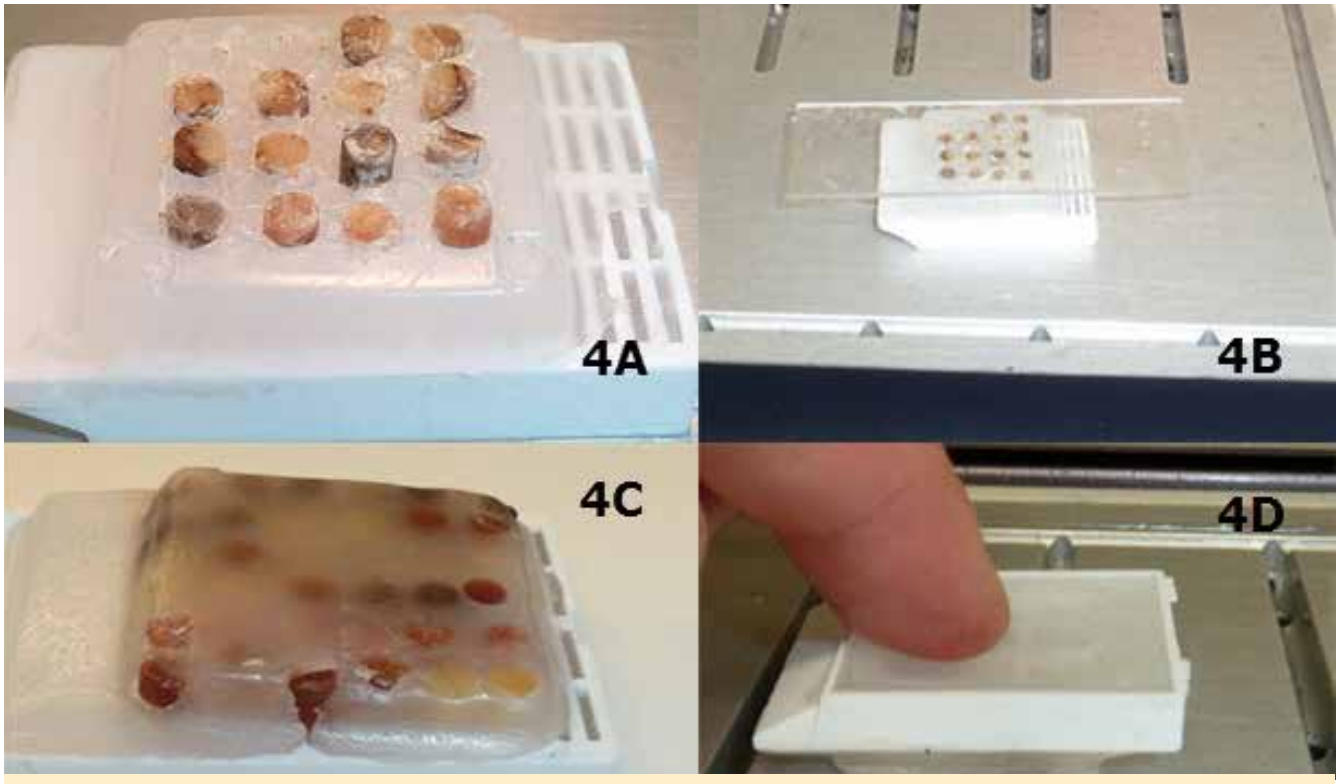

Figure 4. *Leveling of tissue cylinders. Unleveled cylinders (4A). Leveling with cold glass (4B). Too much pressure on one side at leveling, bad casting (4C). Skating the surface after leveling (4D).*

Poor processing and levelling, after the tissue transplantation.

Immediately after cylinder implantation, in depth planing is necessary so that they are on the surface of the tissue block all in one plane. It is necessary to construct the correct depth of the receiving block (Point 1) first. We must level the heated tissue grids (Figure 4A) in the hot tissue block (40°C–50°C) with cold glass cooled to approximately –20°C (Figure 4B). The cooled glass will shock the contacted surfaces of the cylinders, which will slightly harden and clog to the glass, so that they will not slide to the side when pushed or be extended on the contact surface due to pressure. Because they are hot, the cylinders will slightly expand in the lower area, while entering the receptor bore, causing friction with the surrounding media and better incorporation with the surroundings. Some people level cylinders with hot glass, which melts and shortens them on the surface, leading to the described problems. In aligning the glass, one must be as cautious as possible to hold it steady. Many errors in levelling (Table 1) occur due to uneven pressure, when the tissue array blocks become unilaterally crumpled (Figure 4C) and unfit for latter cutting; also the tissue in such grids is compressed and morphologically changed. A changed density of the tissue also gives a denser reaction for further investigations . We cannot compare and evaluate the reaction of the entire surface of one tissue slice. When the tissue array block is aligned, it is recommended to make a surface "skating" on a hot panel of the embedding station (Figure 4D), heated to at least 55°C.This action heats the tissue block from the upper side (during transplantation it is heated from the bottom side) and enables all the structures within the tissue block to be evenly divided.

Incorrect final thermal treatment.

After alignment of the tissue array block, it must be thermally conditioned in a thermostatic heater. This process takes 2 h at temperatures of 40°C–45°C. Consequences of incorrect temperatures or non–implementation of this step are shown in Table 2. We then take the hot block and if needed, even out the edges and surface with cold glass (–20°C) in order to thicken the structure. While performing this step, we should not apply too much pressure, so as to not deform the tissue array block. We allow the block to passively and slowly cool to room temperature. We must keep in mind that there are greater stretching coefficient variations in a tissue arrays block than in a standard tissue block, which contains only one piece of tissue. Therefore, tissue arrays are much more sensitive to rapid and deep cooling on a cooling plate (–10°C to –20 °C) and can crack after a few minutes of cooling (Figure 5). The same caution is used when cutting tissue arrays with a microtome.

RESULTS

In this study, we discovered and recognized five of the most common errors in the production procedures of tissue arrays. Of these, there are seven major and some less common causes. Of the 720 samples of tissue arrays, 518 were free of errors and

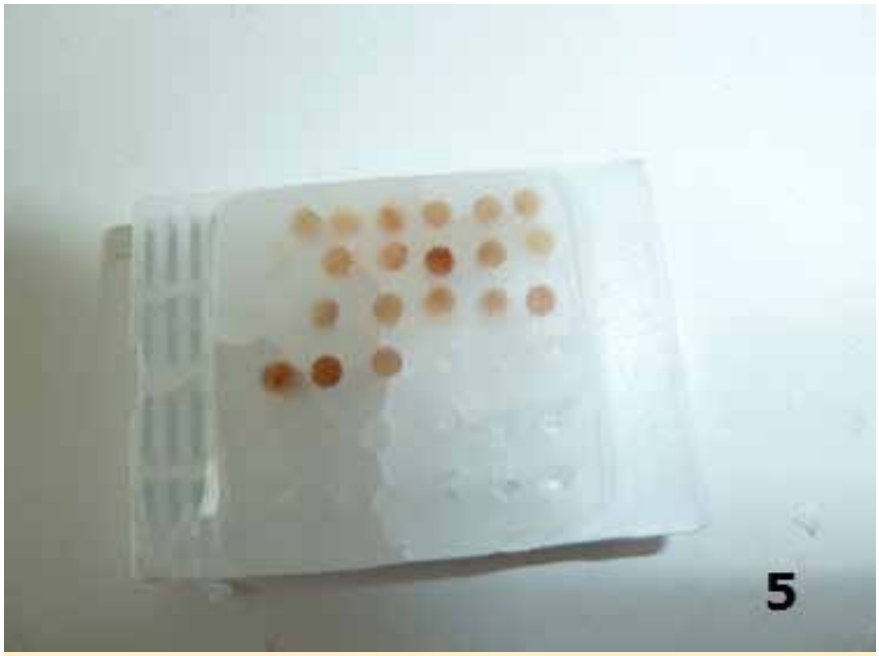

Figure 5. *Cracked tissue array block after extensive and quick cooling.*

performed according to protocol, or within laboratory values already described and recommended in the Methods. We assessed these tissue arrays as adequate. At almost all of the remaining manufactured tissue arrays (n=147), which were not prepared according to recommended laboratory protocols, had one or more errors. These tissue arrays were assessed as inadequate. In total, we found 12 types of solitary recognizable errors. All errors can be resolved with appropriate alteration of the laboratory protocol, better planning, and cautious work. In the Methods section we provided a solution that we experimentally tested and confirmed for each individual error (Table 2). We did not notice any difference between the results of human and animal tissue samples. A summary of the most common and interesting results are shown in table 1.

CONCLUSIONS

There are solutions to optimize custom ordered and institutionally designed tissue arrays. These solutions are technically feasible but require adjustment of laboratory procedures, time, and learning. This is difficult or even impossible for automated laboratories, as we have described problems arising in handmade tissue arrays. Such tissue arrays are not marketable and are intended for scientific purposes, so are made only by specialized laboratories. These products also require maximum quality and precision, possible only by continual correction of mistakes, finding solutions, and adapting protocols. From the results of this research, we have contributed to the optimization of tissue arrays production which we believe will benefit laboratories that are beginning to implement these techniques.

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