S U M M A R Y

EVALUATION AND STANDARDIZATION OF METHODS USED FOR CREATION OF HISTOLOGICAL PREPARATIONS MULTIPLEX TISSUE MICROARRAY

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The technique of multiplexing several "donor" paraffin blocks in a single "recipient" paraffin block is called "tissue microarray" (TMA). The purpose behind the use of this multiplexing technique was to compare several different tissues on a single histological slide in a cost-effective manner, saving labor and reducing consumables, ensuring the advantages of equally staining conditions for all inserted specimens and the possibility to easily trace the identity of each specimen. The number of specimens that can be multiplexed in a single recipient paraffin block can vary between 2 and 10,000, and their size between 0.1 mm and 5 mm. Specimens that can be multiplexed in a TMA are: cells (from effusions), cell lines and tissues (core biopsies from biopsy needles or resection specimens).

Histological sections resulting from TMA paraffin blocks are used in many categories of analysis (immunohistochemistry, in situ hybridization, histochemistry, etc.) and have proven in multiple tests that these arrays are representative of tissues from donor blocks even if they are small in size. The technique is the foundation for experiments in different fields of research because the obtained results can be transmitted to clinical applications.

Current international methods for obtaining TMA paraffin blocks use exclusively donor paraffin blocks collected from hospitals or research centers, and the areas of interest are often paraffin-embedded cylindrical fragments. The technique of obtaining TMA paraffin blocks is difficult, slow and often the donor paraffin blocks are compromised. To fuse cylindrical fragments with paraffin in the receptor block, the created assembly must be repeatedly heated and cooled, and this process can degrade the molecular targets of interest. Since the donor blocks used to obtain TMA preparations have a very high variability of the pre-analytical methods, the effectiveness of the resulting diagnoses is very low. Therefore, it would be optimal for the tissues of interest to be multiplexed before histological processes.

THE FIRST PART OF THE THESIS is represented by the **BIBLIOGRAPHICAL STUDY**, written in approximately 40 pages and includes the following target topics: generalities and applications of the TMA technique (**CHAPTER I**), techniques for obtaining TMA blocks (**CHAPTER II**) and immunohistochemistry applied to TMA (**CHAPTER III**).

Chapter I describes the conventional method where tissue samples are extracted from archived "donor" paraffin blocks (up to 1000 different blocks) and then inserted into a "recipient" paraffin block. This chapter also describes the matrix model in which the tissue fragments are arranged, which allowed the traceability of clinical data to the individual pieces of tissue, the types of TMA are classified (TMA from normal tissues, TMA from experimental tissues, progressive TMA, etc.), are detailed the qualitative aspects underlying a TMA (the quality of the tissue, the pathological diagnosis, the quality of the experimental procedure, the

quality of the clinical data) as well as the representative nature of the technique (the size of the piece removed from the donor block, the number of tissue fragments needed to construct a TMA, the optimal diameter for a fragment of tissue harvested from a donor block etc).

Chapter II details the most important and different methods for TMA construction in chronological order. In 2014 Ulrich Vogel centralized all these methods, starting with the first description of a TMA in 1965 and ending with the mention of five different techniques for obtaining a TMA in 2013. The TMA technique most often referred to in history of obtaining TMAs is the one used by H. Battifora, in 1986, who obtained a block of multitumor tissues for testing new antibodies for immunohistochemistry. At the end of the chapter, a new technique for obtaining TMAs is described, but for the first time in the history of the technique, it has exclusive applicability in obtaining clinical diagnoses. This technique is based on a sectionable matrix (BxChip™) composed of a biomimetic material with tunable properties specially designed for multiplexing biopsies of small cylindrical diameters (20G, 18G, 16G, 14G, 12G, etc.) preventing their fragmentation or loss during the histological process.

Chapter III exposes the standard methodology of the immunohistochemistry technique and elaborates on the potential factors that can compromise the quality of staining: tissue factors (fixation, processing), factors dependent on solvents and reagents (temperature, buffer solutions and diluents), procedural factors (conditions for preservation of paraffin blocks and histological slides).

THE SECOND PART OF THE THESIS is represented by **PERSONAL RESEARCH** and begins with the general purpose and objectives proposed, followed by four chapters: the characteristics of the sectionable material (**CHAPTER IV**), the BxFrame™ biomimetic matrix (**CHAPTER V**), comparative utility and histochemical staining techniques (**CHAPTER VI**), the BxChip™ biomimetic array (**CHAPTER VII**).

Chapter IV develops the experiments performed on the biomimetic material following the resistance of 15 sectionable materials of different consistencies by immersing them in different decalcification solutions routinely used in pathology laboratories (formic acid 10%, hydrochloric acid 5% and EDTA 10%) and subjecting them to rheology tests. After completing the immersion periods in the decalcification solutions, the materials are histologically processed with a 9-hour dehydration protocol, being compared within the same protocol with other tissues: spleen, liver, skin, brain, kidney, heart and lung. The results are delimited by the application of qualifications (Very good, Good, Sufficient and Insufficient). Also in this chapter, the structural preservation of the 15 biomimetic materials is followed by subjecting them to dehydration protocols with different durations (Protocol 1 – 4h30min, Protocol 2 – 5h50min, Protocol 3 – 7h, Protocol 4 – 9h, Protocol 5 – 13h). Shrinkage of materials is observed by measuring them in length, width and height before and after carrying out the dehydration processes.

Chapter V aims to develop a new sectionable matrix, BxFrame™, for multiplexing irregularly shaped tissues of different sizes. In the first sub-chapter, 7 models of rectangular matrices with a thickness of up to 3 mm are tested, having pre-set wells of different shapes (square, round, hexagonal wells) all numbered to improve tissue traceability. These matrices are tested with pig tissues (kidney, liver, heart and skin). Due to this experiment with promising results, a single model is established, the BxFrame™ GRID, mesh-type sectionable matrix accompanied by a perforated base of the same material to support the loading of biopsies. In the second sub-chapter the efficiency brought by BxFrame™ in terms of time and costs of consumables by the multiplexing method compared to a usual histological process is proven. Testing is performed on a limited number of tissues: tail, intestine, brain and heart of C57BL/6 mice, domestic pig skin. The new multiplexing method reduces the required working time for the same number of biopsies by three times compared to the conventional method, and costs can be reduced by up to 50%.

Chapter VI presents the testing and use of BxFrame™ matrices in two different states during the histological process: raw, 4% formalin-fixed, and pre-dehydrated (using a slow dehydration protocol). The purpose of this experiment is to determine possible advantages of using arrays depending on the stage of the histological process of tissue multiplexing. The experiment is performed on Albino mouse tails and described in the first subsection. The testing of three histochemical stains (hematoxylin-eosin, safranin and Fast Green,

pricrosirius red) on matrix sections are presented in the second subsection. In subchapter three, the role of sectionable matrices in the educational field is highlighted, a project conducted with the help of the Department of Pathological Anatomy of the Faculty of Veterinary Medicine. This project was made with kidney donor paraffin blocks to create educational TMA arrays.

The last chapter in the thesis, **chapter VII**, is represented by the BxChip™ sectional matrix and is composed of two subchapters. In the first sub-chapter, the increase in the diagnostic yield is proven due to the use of the previously mentioned matrix, through a study conducted in the clinical hospital "Prof. Dr. Theodor Burghele" from Bucharest. For 24 patients, biopsies were collected through the TRUS (Transrectal Ultrasound) procedure, of which the usual histological process was performed for the first half of the patients, in which the biopsies were folded in thin histological paper and subjected to the process of dehydration and infiltration with paraffin, and for the second half biopsies were placed in BxChip™ arrays following the same dehydration and paraffin infiltration protocol. In both categories of patients, the harvested biopsies were measured before and after the dehydration process (raw biopsies, then biopsies on histological slides). The study is completed by mentioning the multiple advantages brought by the BxChip™ sectionable matrix and underlines that because its use the diagnostic yields increase by at least 10% compared to the conventional method. The second subchapter offers a new perspective within the history of TMA, namely that multiplexing is and will be used with great interest in digital pathology. This subsection describes the BxChip™ array together with the BxLink™ histological slide viewer software. Due to the compact form of this matrix, laboratory technicians can apply three BxChip™ paraffin sections (representing three levels of depth in the paraffin block) to histological slides. The resulting slides are stained with standard histochemical stains and then scanned for input as images into computer systems. Once in the BxLink™ storage system, they can be easily observed using the size scale similar to microscope objectives and accessed anywhere and anytime by medical specialists and pathologists to determine diagnoses.