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A new technique of performing the cell block using egg whites

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Abstract. The use of the cell block in the cytology laboratory can be a routine procedure, having an important role in establishing the diagnosis and also in performing additional tests without the need for additional sampling of biological material. Numerous attempts have been made to modify and improve the cell block over the years, however, obtaining a cell block from a fluid biological product is a current challenge because lack of cell adhesion to a tissue remains the most common reason for dissatisfaction among cytopathologists. Although various methods for obtaining cell blocks have been proposed and described in the literature, it is noted that each of them has a disadvantage, in addition to advantages. Thus, in this study we propose a new method that eliminates in turn the disadvantages of other methods, obtaining an increase in cell densities, can apply additional testing applications, while minimizing laboratory costs.

***Keywords*:** cell block (CB); technique; egg white (EW); egg or glair/white cell block (ECB); immunohistochemical technique (IHC).

1. Introduction

Cell block (CB) is a routine procedure in the cytopathology laboratory that has become quite important due to the essential role it plays in establishing the diagnosis and performing additional auxiliary studies [1]. CB, first introduced into cytology laboratories more than a century ago [2], by Bahrenburg, and used mainly for ascites, peritoneal and subsequently accepted in practice by 1947 [3].

The main advantage of the cytoblock over the biological product is the maintenance of the cellular architecture, which is quite similar to that observed on the sections of surgical biopsies. Thus, fragments of newly developed tissue may appear as "small biopsies" [4], which are useful for diagnosis, recognition of carcinoma types, as well as for type classification [5] and identification of microscopic features, which might otherwise be quite difficult to appreciate on hypocellular cytological preparations. For those who are less accustomed to the routine examination of cytological smears, the architectural arrangement of cytoblocks can facilitate the diagnosis. In addition, CB as an additional method, supporting classical cytology, increases the efficiency of diagnosis and can be combined with other additional tests [4, 6, 7], thus providing the pathologist with additional samples and even those that have remained unused.

The cytological examination is convenient, with a fairly high sensitivity and sensitivity, with a low potential for errors and allows optimal patient management [8]. Therefore, in recent years, more and more laboratories have introduced in the routine activity of their cytopathology laboratories, the technique of obtaining cytoblock as an important support in the diagnosis of certainty, which provides additional cytomorphological details and also ensures the possibility of immunohistochemical tests and appropriate molecular [9].

The method of obtaining CB refers to the processing of biological products and obtaining or extracting sediments, blood clots or visible solid tissue fragments from the cytological product and including them in paraffin, obtaining blocks that can be sectioned and stained by the same methods, also used in histopathology, bringing additional architectural information about a certain type of tissue. These stains have become indispensable in some pathologies (lung cancer, breast cancer) having both a diagnostic and prognostic role [9, 10].

As early as a century ago, the first changes were introduced to improve the efficiency of the CB method and, although their value is recognized and widely used in all laboratories, CBs are not currently standardized, thus preventing consideration. of the results obtained following the auxiliary tests performed on them [11]. Perhaps they have not been standardized so far, precisely on the grounds that a cell block has not yet been obtained to eliminate all inconveniences. So, obtaining an optimal CB is a challenge, because the inconsistent cellularity, which does not adhere to a fixed tissue, which is variable in terms of quantity and quality, and remains the most common reason for dissatisfaction among cytopathologists. Lately, however, there has been an increase in the demand for CB, imposed somewhat by the growing number of requests for minimally invasive procedures that can be performed in order to obtain biological products that are subjected to examinations in order to establish diagnosis, prognosis and prediction of

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cancer patients, whether or not they are in an advanced stage. The inclusion of routine CB technique significantly increases the workload of the cytopathologist in the laboratory, the costs involved in the procedure and the time allotted.

Peritoneal, pleural and pericardial fluids are among the most common samples used in the cytology laboratory, but they are not the only biological products that can be included in paraffin. It is also possible to examine fine needle aspiration (FNA) samples from breast, lung, pancreatic, solid nodule or bronchial lavage, sputum, pleural fluid, peritoneal fluid, cervix, urine and others [12].

Currently, there is a wide variety of CB processing techniques, but there is no benchmark for this. Most methods are adapted to existing internal techniques, in which several commercially available kits are used, but all have advantages and disadvantages. Among the most commonly used techniques are the plasma / thrombin method, the agar method and the commercially available methods of Histogel- and Cellient CB. In all cases, dissatisfaction is somewhat common [13, 14].

The automated ClientTM cell locking system has been proposed, which is a new automatic method of obtaining cell blocks, a system which, however, has a main disadvantage, that it is based on the methanol fixation of the obtained "part", in most cases it is based on formaldehyde, commonly used in fixing small biopsies. This can lead to false positive or false negative reactions in the immunohistochemical examination [15], respectively to errors of interpretation and diagnosis; there are also other automated techniques for rapid processing of the cytoblock, which allow to obtain good processing times of about 3 hours but are also quite expensive [16].

In many laboratories, cytoblocks can be easily integrated into the existing work circuit to obtain a definite diagnosis, representing an auxiliary test. The expert consensus opinion recommends obtaining CB in addition to cytological smears, for EGFR mutation and evaluation of ALK rearrangement in all cases of lung cancer [17]. However, an update on this topic is expected to review the current literature and evaluate the role of cytology specimens in testing for lung cancer mutation [18]. Many laboratories, in agreement with commercial companies, tend to draw up and use specific panels for each type of cancer directly on cytology samples, respectively on cytoblocks [19].

Extracting from all that known techniques of obtaining the cell block performed so far, the following positive aspects have been obtained so far: maintaining the cellular architecture so as to resemble as much as possible the one observed on the section obtained from the surgical biopsy only in cases in which is noted macroscopically the presence of "micro-biopsies", and which are necessary for the recognition of tumor types, identification of microscopic characteristic features, sub- classification and diagnosis of tumors). A suitable CB from which numerous sections can be obtained (useful in the evaluation of all existing cells and for performing complementary tests, such as: special and immunological staining, ultra-structural analysis or molecular and immunocytochemical tests) [20]. The above mentioned auxiliary tests were applied on bioptic parts included in paraffin which were fixed in formalin, so for CB processed in this way no further validation is required.

The problematic aspects that have not been eliminated until now are: the impossibility to guarantee the formation of a "clot" (precipitate) after the centrifugation of the biological product; irregular distribution of cells per section; some plasma / thrombin-based agents may also contain rare epithelial cells that may interfere not only with the diagnosis but also with the interpretation of subsequent studies; the hypocellularity offered by biological fluids, compared to that existing in the biopsy; CB may not contain enough diagnostic material; irregular distribution of cells per section, in the palette, poor cell preservation and cell architecture, intensity and presence of background staining (interfering with the background) [21].

Fetch *et al*. evaluated the immunoreactivity on three CB obtained differently, by techniques already used in laboratories: air-dried cytospins fixed in ethanol, CB FFPE prepared by the thrombin clot method and thrombo-protein. Following this study, they reported that cytospins and thrombo-protein reacted similarly. It was noted that in 66% of cases, the CB included in the study tended to have a fairly large background staining, especially in CBs that contained three-dimensional cell groups. There were also discontinuous membrane stains that led to misinterpretations, false positive results. In only 17% of cases, the CB of the smear was clear and the results were close to those reported in the surgical pathology literature [13]. Starting from this information and also to minimize the time and costs of the procedure, in the current study we tried to replace the use of these thrombin-based compounds, commercially available, with a natural product, often used in various experiments egg white, a clear liquid of moderate viscosity, whose biochemical composition includes proteins (albumin, mucoproteins and globulins, etc.) [22], the main constituent of the formation of a clot. Knowing the high protein content of albumin (approximately 56% of egg proteins) [23] and also knowing the important role of proteins in clot formation, we used this "product" in order to obtain an optimal "clot" to be able to be incorporated in paraffin. Starting from the known fact that unprocessed peritoneal fluids can be stained with toluidine blue without altering cellular integrity [24], in this study we used toluidine blue in order to mark and highlight the sediment to be included in paraffin.

2. Experimental

2.1. Materials

The cytological samples used in this study were obtained by aspirating the liquid, under ultrasound guidance and local anesthesia (thoracentesis, pericardiocentesis, paracentesis or lavage) and exfoliative cytology, depending on the sample location.

The reagents were used as received, without further purification (staining kits for the Papanicolaou and Giemsa, absolute ethanol, formaldehyde, toluidine blue paraffin and xylene for deparaffining, were purchased

from Diapath, and monoclonal antibodies with hematoxylin stain, from Tunic BioLab).

For centrifugation we used the Rotofix 32 centrifuge and, for mixing liquids in test tubes, Stirrer / shaker (both purchased from the HETTICH Company).

2.2. Methods

In the first stage of the study, we identified the types of biological specimens that, by inclusion in paraffin blocks, will provide additional information for the cytological diagnosis and which one bring problems in the process of inclusion in the paraffin block.

We chose 80 cytology samples (malignant and benign cases) and we prepared 80 CB using egg – cell block method (ECBK). From all these samples used, 12 were bronchial aspirates (obtained by bronchial lavage), 16 were sputum samples, 12 peritoneal fluids, 12 ascitic fluids, 12 pleural fluids, 8 cervico-vaginal secretions and 8 urinary samples.

Only bronchial aspirate samples were collected and fixed immediately in 96% alcoholic solution or formalin, the rest were processed unfixed. After we obtain two cytological smears (used for routine cytological evaluation, which were stained with Papanicolaou and Giemsa kit stainer), the remaining sediment is fixed with 5 ml absolute ethyl alcohol (if it has not already been fixed); 5 minutes centrifugation (Rotofix 32 A) at 1000 rpm, the supernatant is removed, and after that we added 1-2 drops of toluidine blue and mix 1 minute.

The next step was represented by adding EW (egg white) (1:1, the equal quantity with the sediment), which precipitated in the presence of absolute alcohol when you mixed for 30 seconds.

After this step, the mixture obtained is centrifuged by the Thin Prep method, glued to the flat bottom of the glass tube and rotated at 2000 rpm for 10 minutes. The tubes are then gently and vertically removed from the centrifuge, taking care not to disturb the thin layer set with sample cells at the bottom.

The supernatant is removed, and the "piece" obtained is inserted in the cassette block and then in 10% formaldehyde (between 3-6 hours, depending on the thickness of the clot we obtained) (Fig. 1), and after that is included in paraffin, according to the tissue processing protocol.

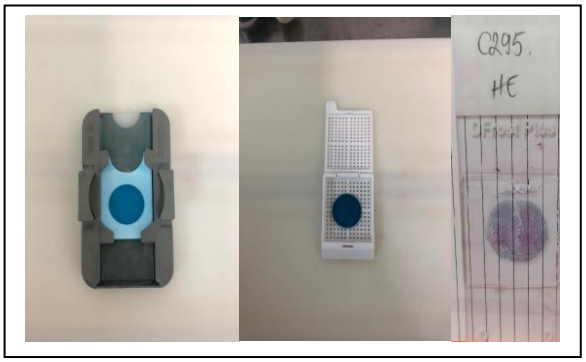

Figure 1. The whole cell material is arranged in a single plane (top - in centrifuge cyto-funel / cyto-chamber, bottom - in cassette block prepared for paraffin inclusion).

After inclusion in paraffin, the ECBK is sectioned. Were obtained several smears, some were stained H&E for cyto-morphological evaluation, the rest were used for immunohistochemical tests (IHC).

Immunohistochemical technique was performed according to the protocol from MasterDiagnostica, using monoclonal antibodies from Tunic BioLab. The antibody panel selected to differentiate a carcinoma from a mesothelioma included: p63, AE1/3, Calretinin, TTF1, CK7, CK20, CDX2, PAX8, anti-G15, GT3 (Fig. 6, 7, 8, 9).

3. Results and discussion

The current study aims to describe a new challenging method for obtaining the CB, based on the use of unfertilized chickens egg glair (also called egg white, albumen or the glair/glaire).

In the first stage of the study, we conducted a broad literature search on different CB preparation techniques, using internet search engines using the following keywords: cell block, cytoblock, biological products included in paraffin block, cytopathology, CB preparation methods, fixatives used in cytology, immunocytochemical tests performed on CB.

Since the limits of the methods currently used in laboratories are somewhat common, we aimed to optimize all the disadvantages that appeared in other techniques used so far to obtain the cellular CB: the impossibility to control the clot / precipitate formation, low cellularity per section, cellular distribution in pallet, poor cell preservation and architecture, intensity and presence of background coloring (possible background reactions) and dependence on certain reagents which involves additional costs.

The advantages of the method presented were highlighted by comparing it with the simplest method known today, the thrombin-plastin method. The comparison was made by carrying out the following examinations: morphologic examination and IHC technique on ECBK.

By including the remaining biological material, we ensure that all cells will be examined, either on classical smears either on sections of the CB.

Due to the current conditions of the SARS-COV2 endemic, the laboratories have adapted and provided additional protection measures to block the spread of the virus, previously fixing the lung biological products.

For a better view of the floor on which the cells are concentrated (especially in hypo-cellular specimens where cells are not macroscopically visible at all), we used toluidine blue (for "marking" the cells in the sediment). The cellular area of interest could be viewed in this way, with the dark light being exposed during sectioning. This possibility to monitor the area of interest would prevent the sectioning of the level in which the cells are not concentrated and also the too superficial sectioning, as well as the risk of obtaining cellular smears.

In particular, in the case of bronchial aspirates and urinary sediment, their inclusion in the CB is often difficult, due to the presence of mucus and the low amount of protein that prevents the formation of a clot

through the precipitation process. The precipitate must include the dispersed cells in order to obtain an "artificial tissue", which can be subsequently included in the paraffin, without the cells dispersing. By adding the egg white, precipitation was obtained in all cases included in the study.

Compared to other approaches, the method proposed by us also aimed to solve the critical feature of the classical protocol used for the preparation of cell blocks from relatively hypo-cellular specimens, with few free cells scattered separately. Thus, the entire area of interest, in which the cells are concentrated, can be visualized and exposed during sectioning.

The purpose of this centrifugation step is to concentrate the cells in a single layer, as close as possible to the sectioning surface of the final block.

Cytological examination provided informations on the cellularity, cell architecture and cell morphology, as well as on the uniform /or non-uniform distribution in the pallet of the cells (Fig. 2, 3, 4, 5).

In Fig. 2, we notice, in addition to the groups of tumor cells, there is a background covered with cellular detritus, which can cover or obscure the visibility of the cells of interest. On the smear obtained by sectioning the ECBK it can be observed that the inflammatory infiltrate is concentrated, focally, far away from the cells of interest (Fig. 3, 4, 5). The amorphous background is the result of the precipitation of the protein from the egg into the alcohol.

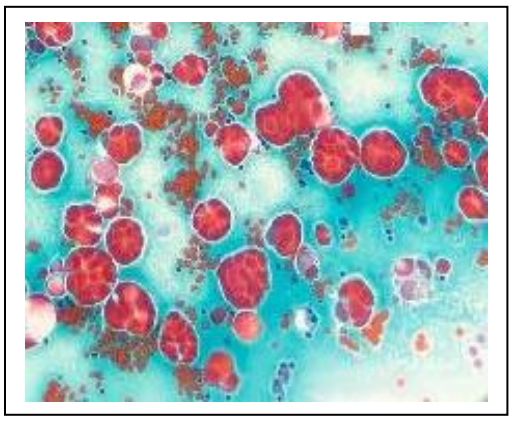

Figure 2. Cytological smear from a pleural effusion from a 43 years old/female, known with breast carcinoma; objective (ob.) 20x, Papanicolaou stain (PAP)

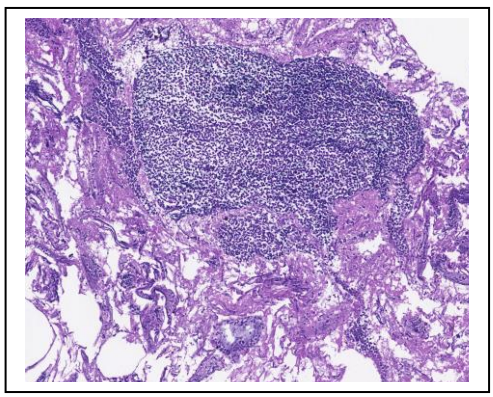

Figure 3. Overview of cell distribution and inflammatory infiltrate - ECBK. Pleural effusion from a 66 years old/male; ob.10x, col. Giemsa.

Immunohistochemical examination (IHC) provided information on the suitable monoclonal antibody and also on the eventual interfering with the section background (positive or negative reaction). For this, multiple tests were performed with different biomarkers, in order to obtain predictable positive / negative reactions. The IHC technique was applied in all cases using different panels of monoclonal antibodies, depending on the type of carcinoma, so that in particular positive reactions are obtained, while also using control blocks (Fig. 6, 7, 8, 9, 10).

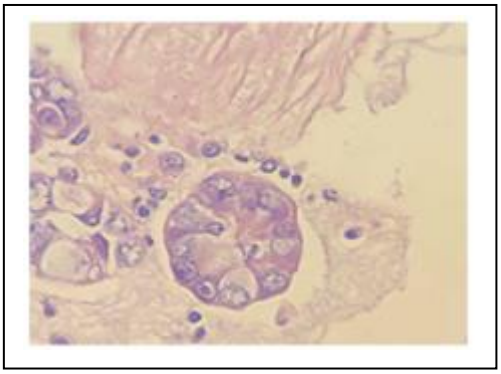

Figure 4. Pleural effusion from a 43 years old/f, breast carcinoma; ECBK, ob.40, col. H&E. The architecture of cell groups is intact and unaltered.

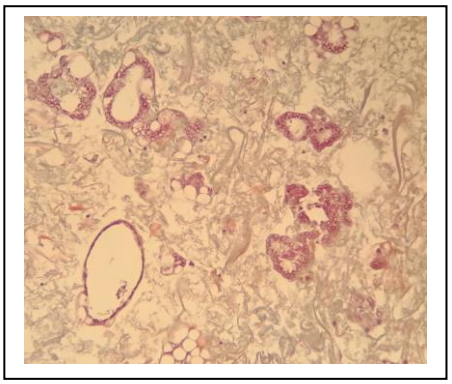

Figure 5. Ascitic fluid from a 58 years old/f, mucinous ovarian adenocarcinoma; ECBK, ob.20, col. PAP. The architecture of cell groups is intact and unaltered.

Samples collected in alcoholic media were not affected, and individual antibodies were reported for optimal signaling. After immunohistochemical staining with the target antigen, the smear is counter-stained with hematoxylin and eosin, to provide the necessary contrast, which helps to notice the primary staining in intense brown.

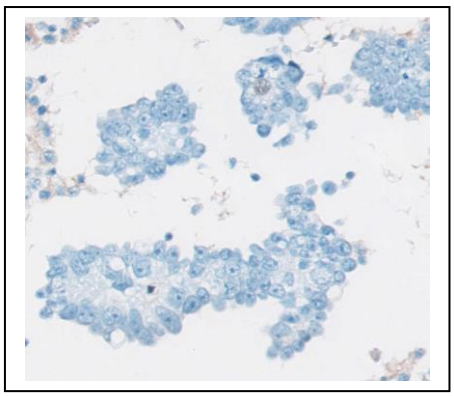

Figure 6. Section from ECBK. Peritoneal effusion from a 58 years old/female; GT3 negative reaction – clean and clear background; ob.40x, IHC

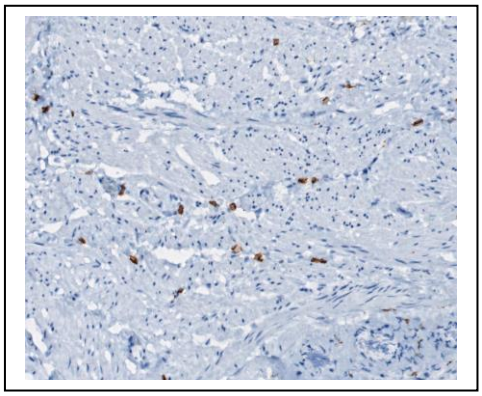

Figure 7. IHC performed on thin sections of ECBK. Positive reaction with Pax 8 (specific nuclear reactivity); the background of the smear stained with hematoxylin provide the contrast, ob. 10x

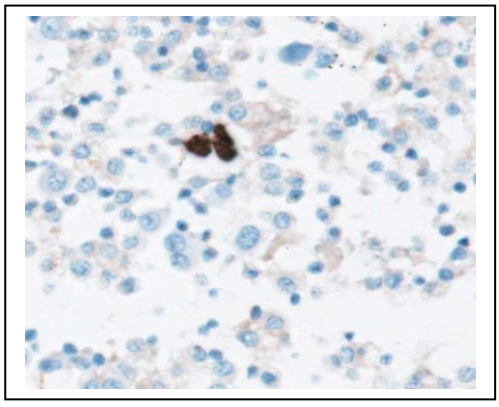

Figure 8. Hight-power IHC on EGBK, p63 specific nuclear reactivity; ob. 40x.

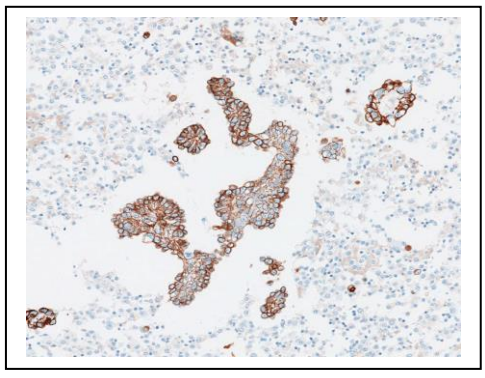

Figure 9. Positive reactivity of Ck7 (cytoplasmic staining) on ECBK; ob.10x.

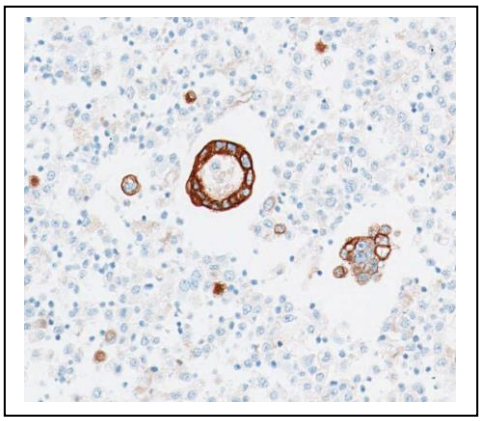

Figure 10. Hight-power IHC on ECBK; cytoplasmic reactivity; ob.20x

The biological product can be stored for later use at room temperature. The egg white can be stored in the refrigerator at temperatures of 3-5 ºC, not more than two weeks. ECBK method is not much more elaborate than other methods, instead, it tries to cover all the drawbacks of the methods used so far.

In 49 of 80 cases (61, 25% adequacy rate), malignant and benign, cellular density on the section area was superior with ECBK relative to all others methods, that use simple centrifugation, even if the morphology is comparable across methods.

In 39 of 40 confirmed malignancies (97.5% adequacy rate) and 10 of 40 confirmed benign cases (25%), cellularity in ECBK was higher than that seen on classical cytological smears and had a suitable material, well preserved, without cell lysis, optimal to confirm the morphological characters or to perform IHC tests.

By serial sectioning of the paraffin shell, at least 30 sections of 3.5 μm thickness were obtained from the entire ECBK, which has optimal cellularity. The increased cellularity per section as well as the uniform distribution of cells/section, is provided with the help of the centrifuge used to obtain preparations in liquid medium.

IHC was excellent in all cases and the addition of formaldehyde or absolute alcohol during the EGBK process, did not influence the IHC staining quality. Samples collected in alcoholic medium were not affected, and individual antibodies were reported for optimal signaling.

Also, it should be noted the cost of the material used, which, in the case of the egg, is very cheap, compared to any other products used in other methods. This new technique, based on egg-glair, is simple, cost-effective, ready adaptable in routine hospital laboratories and can be successfully used on all types of preparations.

The advantage of a block of paraffin over a sample collected in cellular hairspray consisting of storage time. The sample collected on cellular hairspray is valid for a maximum of one month, while the cell block can be stored for up to 10 years.

4. Conclusions

This method proposed by us can be a reliable method of CB processing that can also be easily used in the routine practice of laboratories. The advantages of this method

include, in addition to those mentioned above, the evaluation of the entire amount of biological product and thus the reduction of sampling error, the possibility of unlimited storage and additional testing even after a few years, advantages identical to those of histological samples. This ECBK can be used both to diagnose non-gynecological specimens and those in the gynecological sphere.

It is important, however, for the laboratory to opt for the processing method that best suits the existing sample. Having a wide variety of CB processing protocols at its disposal, the laboratory must adapt to the specific characteristics of each specimen, so as to obtain the best cell block variant.

Conflict of interest

The authors declare no conflict of interest.

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