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METHOD FOR PROCESSING LIVER SPHEROIDS USING AN AUTOMATIC TISSUE PROCESSOR

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14. ABSTRACT:
In support of the Ex vivo Console of Human Organoids (ECHO) program, the personnel at the Molecular Toxicology Branch of the U.S. Army Edgewood Chemical Biological Center developed a specialized method for paraffin-embedding liver organoids. Processing the organoids in this manner enables subsequent microtome sectioning to observe the internal structures. Because the tissues that are usually processed by an automatic tissue processor are far larger than organoids, which are typically only 1 mm in diameter, an alternate method from that decribed in the instrument manual was required. The key step that enabled processing of organoids was to first increase their size by embedding them in a plug of agarose gel. This tissue-processing method, which was developed for the preparation of organoids, is described in this report.

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PREFACE

The work described in this report was authorized under project no. CRADA1314C with Wake Forest University Health Sciences. The work was started in July 2014 and completed in July 2015.

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METHOD FOR PROCESSING LIVER SPHEROIDS USING AN AUTOMATIC TISSUE PROCESSOR

1. INTRODUCTION

In support of the ECHO (Ex vivo Console of Human Organoids) program, The Molecular Toxicology Branch of the U.S. Army Edgewood Chemical Biological Center recently acquired a Leica automatic tissue processor (Leica Biosystems, Inc.; Nussloch, Germany). This instrument is used to prepare tissue for histologic study through a process of alcohol dehydration and hot liquid wax infiltration. After the water in the tissue is replaced with wax and cooled, it then becomes possible to cut thin, 5 µm cross sections from the sample without damaging its structural integrity. The processing and cross sectioning of tissue makes it possible to mount samples on glass slides and stain them to enable the tissue to be observed using light microscopy. Tissue cross sections are ideal for light microscopy because they make it possible to observe the microscopic anatomy of the samples. The Molecular Toxicology Branch intends to use the Leica automatic tissue processor to prepare liver spheroids for observation with light microscopy.

2. BACKGROUND

Organoids are structures composed of multiple cell types that are able to self-organize to approximate aspects of the whole organ (Lancaster and Knoblich, 2014). The ECHO liver organoids are composed of stellate, hepatocyte, and kupffer cells, which are the major cells types that make up the human liver. The organoids are approximately 1 mm in diameter, which is significantly smaller than the traditional histology whole-tissue samples. Because of the smaller size of these samples, the procedure varied from that prescribed by the Leica instrument manual. This report describes the procedure that was found to be the most effective for processing and preparing microscopy slides of liver spheroids.

The general process involved formalin fixation, dehydration in a series of alcohol solutions, a final exchange with xylene, and paraffin embedding. The embedded sample was then sectioned to enable subsequent staining procedures, such as hematoxylin and eosin or immunohistochemistry.

3. MATERIALS AND METHODS

3.1 Materials

Laboratory grade chemicals from any supplier are appropriate for this procedure. The following chemicals were used for the procedure:

- Dulbecco’s phosphate-buffered saline (DPBS);
- formalin (37% neutral buffer formaldehyde);
- series of alcohol solutions: 70, 80, 95, and 100% ethanol in water;

- xylene;
- paraffin, low melting temperature;
- hematoxylin;
- blueing reagent; and
- eosin.

The following common laboratory equipment and supplies were used:

- serological pipets;
- centrifuge;
- microcentrifuge;
- microcentrifuge tubes;
- 15 mL conical tubes ;
- vortex mixer;
- KimWipes;
- hot plate;
- beaker;
- thermometer;
- glass microslides;
- waterbath; and
- kitchen twine.

The supplies shown in Table 1 were purchased from Leica Microsystems, Inc.; Buffalo Grove, IL (http://www.leica-microsystems.com):

Table 1. Supplies from Leica Microsystems

Item	Part Number
Automatic tissue processor	TP1020
Paraffin embedding station	EG1150H
Rotary microtome	RM2235
Low profile microtome blades	DB80LX
Surgipath microbiopsy cassettes	Small: 3802731 Large: 38V5P59060-CS
Embedding mold	14038612303

The following was purchased from Bellco Glass, Inc.; Vineland, NJ (http://www.bellcoglass.com):

Table 2. Supplies from Bellco Glass

Item	Part Number
Hot shaker	7746-22110

The following was purchased from Thermo Fisher Scientific, Inc.; Kalamazoo, MI (http://www.thermofisher.com):

Table 3. Supplies from Thermo Fisher Scientific.

Item	Part Number
HistoGel specimen-processing gel	HG-4000-012

3.2 Methods

3.2.1 Harvest and Fix Spheroids

The following steps are used to harvest and fix spheroids:

1. Place a pipette tip near the bottom of the well of a spheroid-containing 96-well plate and aspirate the medium supernatant and spheroids. Be careful to avoid touching the tip to the bottom of the well to prevent crushing the spheroids in the bottom.
2. Pipette the spheroids into a 1.5 mL microcentrifuge tube.
3. Allow gravity to settle the spheroids to the bottom of the tube. Alternately, centrifuge the tube for 10 s at 200 × relative centrifugal force (RCF).
4. Aspirate the supernatant from the tube.
5. Use 500 µL of 4% RCF to fix the spheroids. Spheroids should be fixed at room temperature for at least 1 h or at 4 °C overnight.
6. Remove the fixative.
7. Wash the tissues two times with phosphate-buffered saline. Discard the supernatant.
8. Stain with eoisin, if desired. An eosin stain will make the organoids easier to see and handle during the rest of the procedure.

3.2.2 Create a Plug of Spheroid-Containing HistoGel

After the spheroids have been fixed and stained, follow these steps to create a plug of HistoGel:

1. Heat HistoGel in a 65 °C waterbath to melt the primary ingredient, hydroxyethyl agarose. After the HistoGel has melted, maintain its temperature at 55 °C until it is ready for use.
2. Transfer the liver spheroids from the 96-well plate to a 15 mL conical tube with a 100–1000 µL pipette, and centrifuge the tube for 5 min at 1000 rpm to form a pellet. Remove the supernatant and transfer the pellet to a 1.5 mL microcentrifuge tube. Suspend the pellet in eosin to make the spheroids more visible, centrifuge the tube, and remove excess eosin with a pipette. Wash the spheroids three times in DPBS or until the liquid remains clear. Remove the supernatant and discard it.

3. Cut a short piece of twine (~2–3 in.) and knot it at one end. Place the knotted end into the microcentrifuge tube that contains the spheroids. While holding the twine in place, transfer 1 mL of HistoGel from the tube on the hot plate to the microcentrifuge tube that contains the pellet of spheroids; ensure that the HistoGel surrounds the piece of twine. The knot should be encased in the HistoGel. Close the top on the microcentrifuge tube and place the tube on ice until the HistoGel solidifies.
4. After the HistoGel solidifies, open the microcentrifuge tube, and pull gently on the twine to remove the gel plug that contains the spheroids from tube. Slice the gel below the end of the twine and place the piece that contains the spheroids in a large, labelled histology cassette. Place cassette in 4 °C formalin and allow it to fix overnight. The plug at this stage in the procedure is shown in Figure 1.

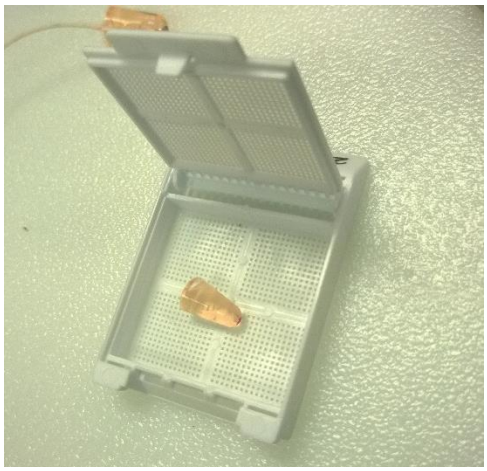

Figure 1. Cassette containing a HistoGel plug before it is processed. The spheroids are visible as a spot of darker pink on the tip of the plug. The plug makes it possible to handle the organoids, which would otherwise be too small to manage.

3.2.3 Process a Plug of Spheroid-Containing HistoGel

Follow these steps to process the HistoGel plug that contains the spheroids:

1. After the overnight fixation, place the cassette with the spheroid-containing HistoGel in the automatic tissue processor basket. Perform the following program steps to use: (a) formalin for 5 min, (b) 70% alcohol for 15 min, (c) 80% alcohol for 15 min, (d) 95% alcohol for 15 min, (e) 100% alcohol for 15 min three times, (f) xylene for 15 min two times, (g) xylene for 20 min, and (h) 55 °C paraffin for 30 min two times. This will dehydrate the sample, and saturate it with paraffin wax to prepare it for embedding and cross sectioning.

2. Remove the cassette from the paraffin immediately after completion of the program, and allow the paraffin to solidify before handling. Remove the processed spheroid-containing plug from the cassette. Figure 2 shows the plug at this stage of the process.

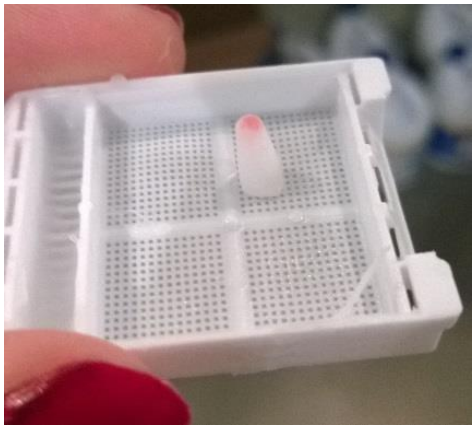

Figure 2. Cassette containing a spheroid-containing plug after it has been processed. The water in the spheroids and HistoGel plug has been replaced with paraffin, making it possible to take cross sections of the sample using the microtome.

3.2.4 Embed the Processed Spheroid-Containing Plug for Sectioning

Follow these steps to embed the spheroid-containing plug in wax for sectioning:

1. Remove the spheroid-containing plug from the processing cassette.
2. Using the paraffin-embedding station, fill the depressed portion of a metal mold with liquid paraffin and, with the spheroids facing the bottom of the depressed portion of the mold, place the spheroid-containing plug in the paraffin wax. Separate the lid from the base of the cassette and adhere the base of the cassette to the metal mold. The cassette base enables attachment ot the spheroid-containing wax block to the microtome. Allow the wax in the cassette to solidify on the cold plate, and then place it in ice for 10 min until the wax is fully solidified.
3. Remove the cassette from the ice and separate the plastic cassette containing the wax and spheroids from the metal mold.

3.2.5 Section the Embedded Spheroid-Containing Plug and Create a Slide

After the spheroid-containing plug has been embedded in wax, follow these steps to section the plug and create a slide:

1. Secure the cassette in the rotary microtome, align it with a new blade, and slice the paraffin at 5–6 µm by turning the wheel in a clockwise direction.
2. Collect a ribbon of paraffin that contains the spheroid cross sections and suspend the ribbon in a 42 °C water bath.

3. Hold a glass microscopy slide beneath the floating ribbon and lift the slide to remove the ribbon from the water bath.
4. Allow the slide to air dry.

3.2.6 Rehydrate and Stain the Spheroid Cross Section Slides for Observation Under Microscope

Follow these steps to prepare the slide for microscopic examination:

1. Remove the paraffin from the slide by washing it in xylene for 5 min and repeating this step three times.
2. Rehydrate the sample by washing the slide multiple times using: (a) 100% alcohol for 3 min and repeating this three times, (b) 95% alcohol for 3 min and repeating this twice, (c) 80% alcohol for 3 min once, and (d) distilled water for 3 min once.
3. Stain the spheroids by (a) placing the slide in hemotoxylin for 5 min then rinsing gently with distilled water; (b) placing the slide in bluing reagent for 15 s then rinsing gently with distilled water; and (c) placing the slide in eosin for 3 min then rinsing gently with distilled water.
4. Observe the spheroid cross sections under a light microscope. An example image of liver spheroids is shown in Figure 3.

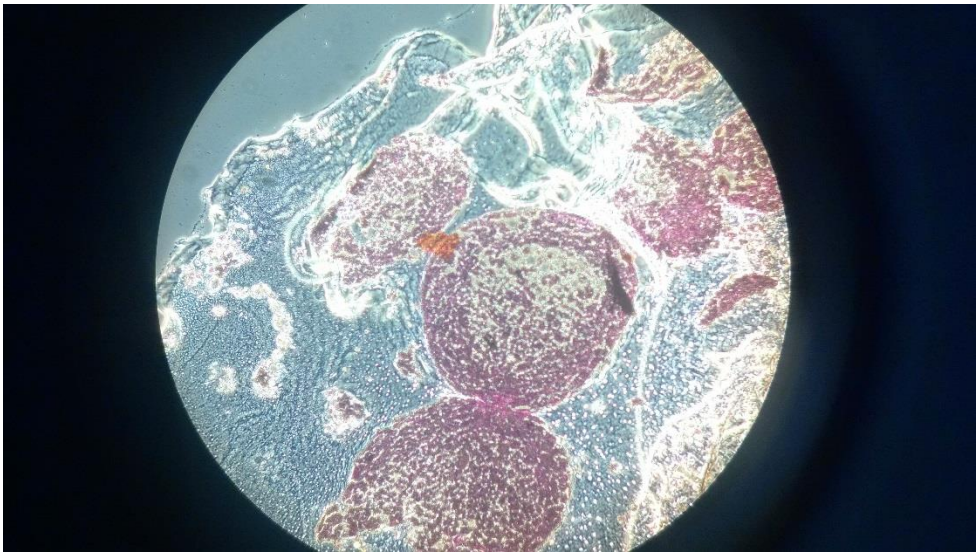

Figure 3. Cross sections of liver spheroids that were prepared with this method. These spheroids were stained with eosin, cross sectioned, and viewed with a light microscope. Histological processing makes it possible to create two-dimensional images of this section of the spheroids and observe their internal structures.

4. CONCLUSIONS

Three-dimensional organ models are becoming increasingly important as they provide a middle ground between the study of cells and tissues. Their emergence requires a process with which to study the anatomical structure of organoids while using a size scale comparable to that of cells. As organ models, the structure of organoids and the impact that toxicity testing may have on that structure are important to understand the risks to full-sized tissues and organs, but the small size of organoids makes it challenging to use traditional histology methods. However, the procedure developed by the Molecular Toxicology Branch for the histological processing, embedding, cross sectioning, and staining of liver spheroids makes it possible to overcome that challenge. These procedures may be further developed for processing other three-dimensional organ models such as heart, lung, and minibrain organoids.

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