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Research Article

Preparing and Processing Complex Tissue Constructs Composed of Cellular Spheroids for Histological Examination

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Abstract

Using cellular spheroids to build tissues and organs is a rapidly emerging technology that will have great impact on the biomedical industry. As tissues are fabricated *in vitro*, it will be critical to histologically examine tissue composition, function and organization for comparison with native tissues. Conventional automated histologically methods are not recommended for fixation and processing of tissues composed of spheroids due to their delicate nature and the need to preserve tissue morphology. Given this, there is a need to develop a manual method to prepare and process tissues composed of cellular spheroids for histological sectioning and examination. Here, we report a manual processing technique developed for preparing larger tissues composed of spheroids for histological examination based on our lab's work using magnetic forces to pattern and assemble tissues composed of Janus Magnetic Cellular Spheroids for tissue engineering applications. The results presented here contribute to understanding the organization of cells, studying extracellular matrix content, analyzing phenotypic expression and localizing iron oxide magnetic nanoparticles in tissues composed of Janus Magnetic Cellular Spheroids.

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Introduction

Tissue engineering uses knowledge of biology, material science, biochemistry, engineering and physiological systems to replace or repair damaged, diseased or lost tissues in the human body with functional tissue that mimics native tissue. One of the biggest challenges in the field of tissue engineering is the *in vitro* fabrication of functional tissue engineered constructs with high cell density, high cell viability and an Extracellular Matrix (ECM) network that mimicsnative tissue. Three dimensional cell cultures, or spheroids, are being investigated as building blocks for scaffold-free tissue engineering applications due to their ability to mimic the native cellular and ECM environment of natural tissues [1]. Spheroids can be fabricated with one cell type, or co-cultured with other cell types, which is desirable for fabricating tissue types with varying complexities and functionalities [2,3]. Tissue assembly and fabrication methods include 3D printing, cell sheet techniques, and patterned molds [4-9]. These methods assemble the spheroids or cells into a desired position through passive contact, but do not encourage active contact mediated by forces to promote fusion of the tissue over time. This can cause issues with tissue homogeneity, can alter tissue geometry and can lead to increases in fabrication time. Our lab has developed the Janus Magnetic Cellular Spheroid (JMCS) structure to safely incorporate iron oxide Magnetic Nanoparticles (MNPs) into cellular spheroids [10-13]. This method allows for spatial control over MNPs to form two distinct domains within the spheroid: (1) cells and ECM and (2) extracellular MNPs. Safely incorporating MNPs allows for utilizing magnetic forces at a distance to pattern JMCSs into desired geometries for building larger, more complex tissue constructs such as rings, spheroid sheets and tubes for various tissue engineering applications [12].

Further, it is critical to understand and visualize the fusion and maturation of tissues composed of JMCSs over time. To understand these mechanisms, studies must include analyzing phenotypic expression, visualizing the spatial orientation and viability of cells, studying ECM organization and localizing iron oxide MNPs throughout the developing tissues over time. Tissue stains, such as the Hemotoxylin and Eosin (H&E) stain, the Masson's Trichrome stain, the Verhoeff-Van Gieson stain, Turnbull's Blue Reaction stain, and immunohistochemical stains have been developed for these purposes. While histological examination allows for analysis of the tissues composed of spheroids, preparing and processing these tissues for staining and analysis is challenging. It is not recommended to use conventional histological processors or standard processing methods, due to the delicacy of the samples, which makes it necessary to use manual processing to increase the survival of the samples and preserve tissue morphology. Through our work, we encountered the need for extensive histological analysis of assembled tissue constructs. Here, we report the techniques developed in our lab for use in the processing of larger, complex tissues for histological examination.

Materials and Methods

Cell culture

Primary rat aortic Smooth Muscle Cells (SMCs) were used for all studies. All cells were cultured in monolayer cultures at 37°C and 5% of CO_2 until spheroid assembly. SMCs were cultured using Dulbeco's Modified Eagle Medium: F-12 (ATCC, Virginia, USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Georgia, USA) and 1% penicillin-streptomycin-amphotericin (MediaTech, Inc., Virginia, USA).

Spheroid assembly

Spheroids were prepared using a method previously described and developed in our lab [10-12]. Our lab has developed a Janus Structure of Magnetic Cellular Spheroids (JMCS) and this provides spatial control of Magnetic Nanoparticles (MNPs) to create two discrete domains: extracellular MNPs and cells [12]. Uniform volumes of solutions comprised of suspended iron oxide MNPs (Fe_3O_4, 20-30 nm, Sky Spring Nanomaterials Inc., Texas, USA), collagen (Bovine, Type I, Life Technologies, California, USA), and cells in cell culture media were mixed and dispersed onto Petri dishes using the hanging drop technique into 15 µL droplets. For these studies, spheroids were fabricated with 20,000 cells each. For tissue rings and tissue sheets, the collagen concentrations were 0.017 mg/mL and 0.24 mg/mL, respectively. For all spheroids, the iron oxide concentration used was 0.3 mg/mL. Primary rat aortic smooth muscle cells were used for the tissue samples presented in this work. Spheroids were left to form for three days prior to use. Collagen was prepared according to the recommendations of the manufacturer and kept on ice before use in all samples.

Fabrication of tissue rings

Rings composed of JMCSs were assembled using ring magnets (2 mm outer diameter, 1 mm inner diameter, 1 mm thick, vendor calculated pull force = 0.16 lbs, Super Magnet Man Inc., Alabama, USA) (Figure 1A). The magnets were attached to the bottom of glass chamber slides containing cover glass bottoms. The wells were filled with cell culture media and 25 individual JMCSs of the same formulation (same cell number, ECM concentration and iron oxide MNP concentration) were carefully patterned in a monolayer around the magnetic ring using pipette tips. The rings were incubated at 37°C and 5% CO_2 for 48 hours to allow fusion to occur, after which the magnets were removed. This was found to be the minimum time required to manipulate the rings, while keeping them intact.

Fabrication of tissue sheets

Sheets composed of JMCSs were assembled using a strip of magnets (2.5 mm outer diameter, 5 mm length, K&J Magnetics, Inc., Pennsylvania, USA) (Figure 1B). Five magnets were attached to the underside of a 6 well plate (Greiner Bio-One, North Carolina, USA) and a glass cover slip (micro cover glass 22x30 mm, VWR, Pennsylvania, USA) was positioned directly over the magnets within the well plate to prevent spheroid adherence to the well plate. The wells were filled with cell culture media and 1,000 JMCSs were carefully aligned on the cover slip using a pipette tip and incubated at 37°C and 5% CO_2 for 3 days to allow fusion to occur. Three days was found to be the minimum time required to manipulate the tissue sheets while preserving morphology. To remove the tissue sheets, a sterile pipette tip was used to gently scrape the sample off the surface of the glass so that it could be collected for processing.

Fabrication of tissue tubes

After 3 days, fused tissue sheets composed of JMCSs on glass cover slips were removed from the 6 well plate. Next, fused tissue sheets were wrapped around a silicone tube (5 mm outer diameter, 2.5 mm inner diameter, Cole-Parmer, Illinois, USA) containing a magnet (2.5 mm diameter, 5 mm length, pull force = 1.8 lbs, Super Magnet Man Inc., Alabama, USA) within its lumen to effectively wrap the sheets around the tube (Figure 1C). To avoid oxidation of the magnet by culture media, the ends of the silicone tube were plugged with silicone rubber. Samples were incubated in culture medium at 37°C and 5% CO_2 for 7 additional days. This was found to be the minimum time required to manipulate the tissue tubes without them breaking apart upon removal of the sample from the silicone tube.

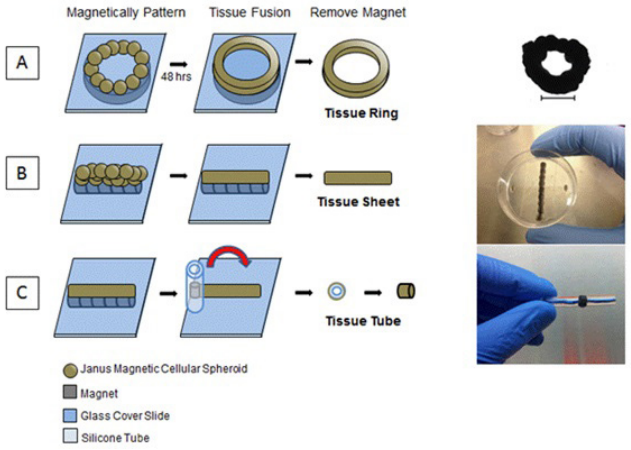

Figure 1: Magnetic force assembly of JMCSs to fabricate tissue rings, sheets and tubes.

To study the fusion of JMCSs, spheroids were magnetically patterned into various geometries to represent various tissue engineering applications. A schematic for the magnetic patterning of tissue rings (Figure 1A), tissue sheets (Figure 1B) and tissue tubes (Figure 1C) is shown with pictures of each respective tissue after fabrication. (Scale = 500 µm).

Tissue fixation, processing, embedding and sectioning

Ring tissues composed of JMCSs were left in their well plates and the cell culture media was removed and replaced with Z-Fix for overnight fixation (buffered zinc formalin, Anatech Ltd., Missouri, USA) for overnight fixation. Tissue sheets were left in their well plates and the cell culture media was removed and replaced with Z-Fix for overnight fixation. The tissues were left in their respective well plates and the magnets were left in place for overnight fixation to prevent any issues that could arise from handling the tissues and to preserve tissue morphology. Tube tissues were left on the silicone mandrel and placed into a glass vial to be submerged in Z-Fix for overnight fixation. The tissue was left wrapped around the silicone mandrel and the magnet left in the lumen for overnight fixation in order to preserve tissue morphology.

After overnight fixation, the Z-Fix was removed and the tissue samples were dehydrated using a series of ethanol and xylene incubations (Table 1) [14]. Immediately prior to the first ethanol incubation, the tissue tube was removed from the silicone mandrel. Given that our lab has done extensive histological examination of spheroids, a protocol was developed for preparing and processing these individual spheroids. However, since the tissue rings, tissue sheets and tissue tubes are larger, more complex and composed of

Step	Reagents	Time
1	Z-fix	Overnight
2	70% Ethanol	30 minutes
3	80% Ethanol	30 minutes
4	95% Ethanol	30 minutes
5	95% Ethanol	30 minutes
6	100% Ethanol	30 minutes
7	100% Ethanol	30 minutes
8	Xylene	15 minutes
9	Moltene Paraffin	Overnight

Table 1: Protocol for Fixing and Processing Tissue Composed of Janus Magnetic Cellular Spheroids.

many spheroids, the times for the ethanol incubations were doubled to ensure proper infiltration. For ethanol solutions, deionized and distilled water were used for dilutions. The times selected for incubation were chosen in accordance with literature values with automated and manual processing [15]. The times used for incubation are also comparable to what automated tissue processing programs regularly use (SAKURA Tissue TEK VIP) for standard tissue processing. After incubation in xylene, the solution was removed and replaced with molten paraffin (Surgipath EM-400, 56˚C, Leica, Illinois, USA) using a Tissue-Tek TEC Tissue Embedding Console System (Sakura, California, USA). Samples submerged in molten paraffin were placed in one of the thermal chambers of the embedding console for overnight infiltration of paraffin at 60˚C.

After overnight incubation in molten paraffin, tissue samples were transferred to appropriately sizedem bedding molds filled with molten paraffin. Here, care was taken to properly orient the tissues in the center of the mold for ease of sectioning. The embedding molds were each covered with a numbered embedding block holder and placed on a cold plate at -4˚C for at least 1 hour. Following, the molds were removed and embedded tissue blocks were placed in an ice water bath for 10 minutes in order to rehydrate the tissue samples for ease of sectioning. A microtome was used to cut 5 µm thin sections from the tissue samples. These sections were suspended on the surface of a heated deionized water bath (44˚C) and placed on a silanized glass histology slides (Adhesive Coated Slides, New Silane White Frosted, Newcomer Supply, Wisconsin, USA), due to the delicacy of the tissue samples [16]. Each slide was visually examined under a microscope to see if the sample was observed and to check for tears, folds or irregularities of the sample. The slides containing samples were incubated overnight in an oven (65°C) to remove excess paraffin.

Hematoxylin and eosin stain

The cell nuclei of tissue constructs composed of spheroids (rings, sheets and tubes) were visualized using an H&E stain. The following reagents were used: Hematoxylin I (Richard-Allan, #7221, Thermo Scientific, Massachusetts, USA), Eosin-Y (Richard-Allan, #7411, Thermo Scientific, Massachusetts, USA), Bluing Reagent (Richard-Allan, #7301, Thermo Scientific, Massachusetts, USA), and Clarifier I (Richard-Allan, #7401, Thermo Scientific, Massachusetts, USA). Care was taken during water wash and staining steps to prevent tissue samples from being washed from histology slides, which can occur with tissue samples composed of spheroids [16].

Masson's trichrome stain

The collagenous ECM of spheroid tissue samples was visualized using Masson's Trichrome stain and was performed in accordance with the manufacturer's protocol (Masson's Trichrome Method for Connective Tissue, Poly Scientific R&D Corporation, New York, USA). It is recommended by the protocol to incubate samples in Analine Blue reagent for 5 minutes. However, our lab has found that 20 minutes is required to adequately visualize the ECM in tissues composed of JMCSs. During the staining procedure, slides containing the tissues were placed on trays and stains applied with transfer pipettes. This method prevents tissue specimens from potentially being washed off the slides during the staining process.

Tissue sheet viability

After three days of fusion, tissue sheets were fixed overnight using Z-Fix and their viability was qualitatively studied using a LIVE/DEAD cell viability assay (Life Technologies, CA, USA) with confocal microscopy. The staining was performed according to the manufacturer's protocol and imaged using a Nikon Eclipse Ti microscope.

Immunohistochemistry for functional protein markers

Immunofluorescence microscopy was used to qualitatively confirm SMC phenotypic expression within tissue tube samples that had fused for 7 days. First, OCT embedded histology sections (5 µm thick) were collected and cleared via two washes (5 min) in PBS and 1 wash in water. Samples were circled with a diamond tip pen. Samples were treated with a 0.1% Triton X-100 to permeabilize samples (10 min). Slides were washed 3x with water (5 min), followed by a 30 minute incubation with Background Buster (Innovex Biosciences, CA, USA). Next, samples were washed 2x water and 1x PBS. Once samples were prepped, they were incubated with primary antibodies overnight at 4˚C in a humid chamber. Samples were washed 3x PBS, followed by incubation with secondary antibodies for one hour at room temperature. As a control, samples were incubated with just the secondary antibody to ensure controlled binding was occurring. Finally, samples were washed 3x PBS followed by incubation with Hoechst 33342 to stain for nuclei (10 min, room temperature). Samples were washed 3x PBS and cover slipped, and fluorescently imaged for Smooth Muscle Myosin Heavy Chain (SMHC) and Smooth Muscle 22 (SM22) (FITC, Ex/Em 492/520), Smooth Muscle Alpha Actin (SMAA) (Cy3, Ex/Em 552/570), and nuclei (Hoescht 33342, Ex/Em 343/483) using a Nikon Eclipse Ti microscope. SMHC, SM22 and SMAA are known phenotypic markers of smooth muscle cells. Three tissue samples of were analyzed to confirm that results were consistent.

Imaging

A Nikon (Nikon Instruments, Florida, USA) AZ100 multizoom microscope was used to image stained tissue sections. For fluorescent immunohistochemistry staining, a Nikon Eclipse Ti microscope was used. The NIS-Elements software package from Nikon was used to process the images.

Results

Histological examination of the tissue sheets and tubes shows that the manual processing method yielded intact tissue structures with preserved morphology. Tissue sheet sections were stained using an H&E stain and demonstrate the presence of cell nuclei throughout the

fused tissues after 3 days (Figure 2A). The Masson's Trichrome stain demonstrates the presence of collagenous ECM throughout the fused tissue (Figure 2B). A LIVE/DEAD stain of the fused tissue sheet qualitatively confirms the presence of viable cells throughout the tissue, as seen by the large population of viable (green) cells with only a small portion of dead (red) cells (Figure 2C). Tissue tube sections were also stained using H&E stain and demonstrate the presence of cell nuclei throughout the fused tissue after 7 days (Figure 3A). The Masson's Trichrome stain demonstrates that collagenous ECM is present throughout the tissue tube (Figure 3B). Tissue tubes were fluorescently stained for known phenotypic smooth muscle cell markers: Smooth Muscle Myosin Heavy Chain (SMHC) (Figure 3C), Smooth Muscle 22 (SM22) (Figure 3D) and Smooth Muscle Alpha Actin (SMAA) (Figure 3E). Results demonstrate positive expression of each of these key phenotypic markers in the tissue tubes (red for SMHC and SM22, green for SMAA), as well as the presence of nuclei throughout the tissue using a Hoechst stain for nuclei (blue).

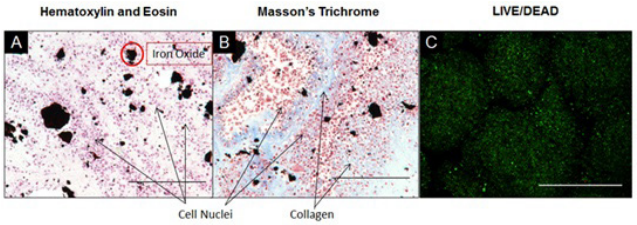

Figure 2: Histological Examination of Tissue Sheets Composed of JMCSs.

Tissue sheets composed of magnetically patterned JMCSs were left to fuse for 3 days. After three days, tissue sheets were fixed, processed, sectioned and then stained using an H&E stain (Figure 2A), a Masson's Trichrome stain (Figure 2B) and a LIVE/DEAD viability stain (Figure 2C) (Scale Bars = 500 μm). The H&E stain demonstrates the presence of cell nuclei throughout the fused tissue sheet. Nuclei are visualized as blue or dark purple and cytoplasmic tissue elements are visualized as various shades of pink. The Masson's Trichrome stain demonstrates the presence of collagenous ECM throughout the fused tissue sheet. Nuclei are visualized as dark red, collagen is visualized as blue, and cytoplasmic tissue elements are visualized as various shades of red or pink. The iron oxide magnetic nanoparticles are black in the images. The LIVE/DEAD stain demonstrates that the tissue contains a large population of viable cells (green), with a small population of dead cells (red) after 3 days of fusion.

Discussion

Cellular spheroids are attractive for use as the building blocks for larger and more complex tissues because they mimic the 3D native orientation of tissues and produce their own ECM over time [7,17]. The bottom up, scaffold free tissue engineering approach with cellular spheroids is promising for the fabrication of larger tissues because industrial systems are being developed to robotically scale tissue and organ fabrication using automated spheroid bioprinters [17]. To compare tissues fabricated in vitro from spheroids to native tissues, it is critical to develop an automated method to rapidly process these tissues for histological examination of tissue structure, cellular orientation and ECM organization. Tissues composed of spheroids are subject to poor mechanical properties, due to their fluidic nature and lack of maturation [18]. Thus, care must be taken with these delicate samples to preserve tissue integrity and tissue morphology during the processing steps.

In this work, a manual histological processing technique for tissues composed of spheroids was presented. This technique uses overnight fixation and also overnight infiltration in paraffin. The chosen manual method of processing has been compared to automatic processing with a SAKURA Tissue TEK VIP. When using the

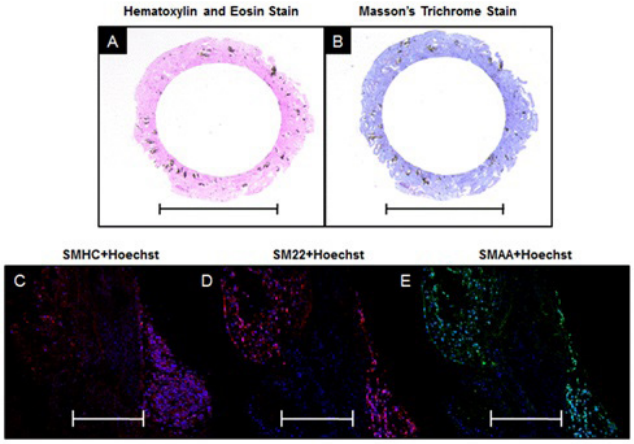

Figure 3: Histological Examination of Tissue Tubes Composed of JMCSs.

Tissue tubes composed of fused JMCS tissue sheets were left to fuse for 7 days. After 7 days, tissue tubes were fixed, processed, sectioned and then stained using an H&E stain (Figure 3A), Masson's Trichrome stain (Figure 3B), or fluorescent IHC markers for smooth muscle cell phenotype analysis (Figures 3C, 3D and 3E) (Scale Bars = 5 mm for Figures 3A and 3B; Scale Bars = 500 μm for Figures 3C, 3D and 3E). The H&E stain demonstrates the presence of cell nuclei throughout the fused tissue tube, as well as the gross structure of the tissue tube. Nuclei are visualized as blue or dark purple and cytoplasmic tissue elements are visualized as various shades of pink. The Masson's Trichrome stain demonstrates the presence of collagenous ECM throughout the fused tissue tube. The iron oxide magnetic nanoparticles are black in the images. Results of staining tissue tube sections for smooth muscle cell phenotypic markers demonstrated positive expression of SMHC (red) (Figure 3C), SM22 (red) (Figure 3D) and SMAA (green) (Figure 3E). The presence of nuclei throughout the tissue was demonstrated using a Hoechst stain for nuclei (blue).

automatic processor, spheroid samples have been lost and tissues composed of spheroids have had their morphologies compromised. This is especially critical for tissue tubes composed of spheroids, where it is important to maintain the morphology of the lumen for further analysis of cells, markers, and ECM distribution. In general, tissue samples composed of spheroids are delicate and the manual processing allows for proper care to be taken. Manual processing allows for careful solution exchanges and prevents samples from being lost because the sample can always been visualized. This research offers a reliable method for preparing tissues to study the localization and viability of cells, the organization of extracellular matrix and phenotypic expression of tissues composed of cellular spheroids. This understanding is critical when developing tissue engineered constructs that mimic native tissue. Though the method is sufficient for small scale histological analyses, an automated histological processer must be developed in the future to meet the need at the industrial scale. Morales et al., developed a method to rapidly fix, process and embed tissue specimens in 1 hour by using a continuous flow of the working solutions [19]. Another strategy for making the process more high-throughput could be to embed multiple spheroid samples throughout the same paraffin block, which reduces the amount of blocks to be made and sectioned [20].

Using the manual technique presented here, tissues of various shape, size and composition were able to be processed and prepared for histological examination. Sectioned specimens were stained using H&E, Masson's Trichrome, LIVE/DEAD and various IHC reagents. The H&E stain allowed for visualization of cell nuclei, the Masson's Trichrome stain highlighted collagen throughout the tissue, the LIVE/DEAD stain demonstrated high cell viability in tissue sheets and IHC staining demonstrated phenotypic expression expected

of smooth muscle cells. These results qualify the presented manual method of tissue processing for preparing tissues for standard stains, like H&E, as well as specialized stains, like fluorescent IHC staining. The presented method allowed for visualization of cells, characterization of collagen, localization of iron oxide MNPs and analysis of phenotypic expression throughout tissues composed of cellular spheroids. Having a full understanding of these tissue molecules and components is critical for developing a tissue engineered construct that mimics native tissue.

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