EVALUATING THE EFFICACY OF TISSUE DECELLULARIZATION

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Tissue and organ failure following disease or trauma are widespread and can require the replacement of the affected tissue. Recently, decellularized tissues or extracellular matrix (ECM) derived from organs/tissues are gaining much interest in various reconstructive surgical applications. Decellularization is the removal of cells and genetic materials while maintaining the structural and biochemical cues of the extracellular matrix (ECM). At iFyber, we apply standard assays for evaluating the efficacy of decellularization processes.

CHALLENGES WITH TRADITIONAL ORGAN FAILURE TREATMENTS

Autografts are the gold-standard treatment for most tissue and organ failures; however, limited donor supply, the possibility of rejection, and the need for immunosuppression have led many researchers to think of alternative methods for reconstructing the tissues. One of the methods used to recapitulate the complexity of the cell microenvironment without causing an immune response is tissue decellularization.

TISSUE DECELLULARIZATION PROCESS OVERVIEW

Decellularization aims to remove the cellular and genetic components of the tissues while maintaining their extracellular matrix ultrastructure, composition, function, and biological activities, and keeping their vascular network. The source and nature of tissues, as well as the manufacturing process, decellularization methodology, and clinical applications of decellularized tissues for repairing various tissues and organs, are all very diverse (Figure 1). To minimize the risk of graft rejection and ensure about retaining an intact ECM, it is imperative to evaluate the efficacy of decellularization.

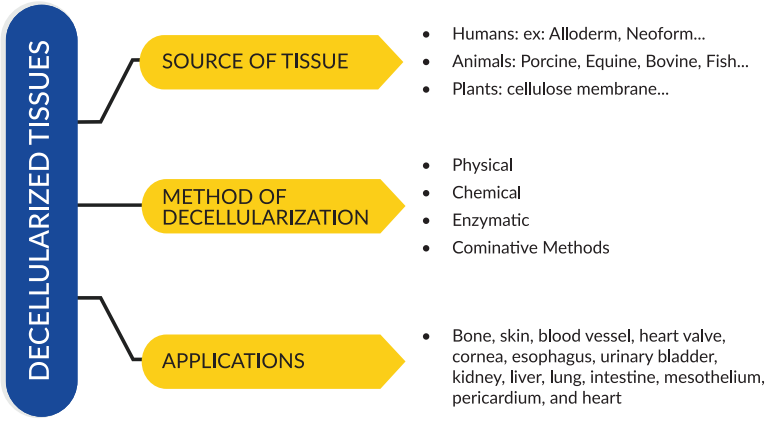

Figure 1: Source and nature of decellularized tissues, manufacturing process and strategies for decellularization, and clinical applications

OVERVIEW OF IFYBER'S RESEARCH CAPABILITIES

iFyber utilizes a variety of methods to quantify the amount of genetic material in tissues; evaluate the efficacy of tissue decellularization; detect the presence of endotoxins in decellularized tissues, assess their biocompatibility, and examine how well the basement membranes of tissues have been preserved following decellularization.

QUANTIFICATION OF GENETIC MATERIAL

DNA is directly correlated to adverse host reactions, and its removal is required to prevent immunorejection of decellularized tissues. While it is impossible to remove 100% of genetic material, to satisfy the intent of decellularization, the amount of double-stranded DNA (dsDNA) per mg tissue/ECM mass should not exceed 50 ng, and the fragment length of this residual DNA should be less than 200 base-pairs (bp).

At iFyber, we utilize gold-standard methods to determine the total amount of DNA in tissue samples before and after decellularization. Different methods to evaluate the presence of DNA in tissues are spectrophotometry, DNA intercalating dyes (e.g., PicoGreen), and qPCR. Figure 2a shows the results of a total DNA quantification that iFyber performed in two decellularized tissues in comparison to the control group using PicoGreen as a highly sensitive fluorometric dye.

Following total DNA quantification, the remnant DNA extract was subjected to agarose gel electrophoresis to reveal the length of DNA fragments in different samples (Figure 2b).

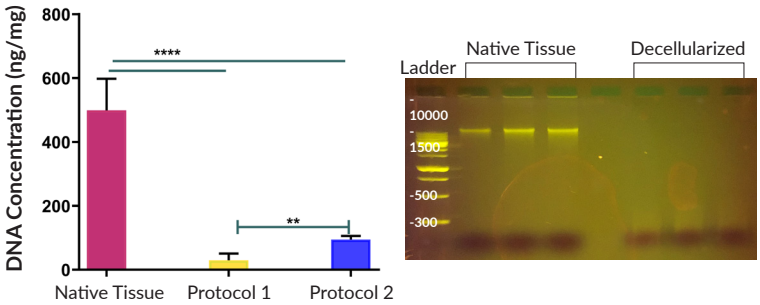

Figure 2: Amount of total DNA in tissue and decellularized tissues prepared by two protocols (a) DNA fragment length revealed by agarose gel electrophoresis (b).

VISIBLE SIGNS OF CELLS

Residual cellular material within the ECM of processed tissue may contribute to cytocompatibility issues. iFyber's performance of histological staining with 4',6-diamidino-2-phenylindole (DAPI) or

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hematoxylin and eosin (H&E) serves as a qualitative method for verification of decellularization. Hematoxylin stains nuclei blue, and eosin binds to the cytoplasm protein, giving the collagen a pink color, while DAPI gives a blue color to the nucleus. Successful decellularization is defined as the absence of nuclei from H&E staining as well as DAPI staining, and preservation of the dermis structure (Figure 3).

DETECTION OF ENDOTOXINS

Endotoxin, a complex lipopolysaccharide (LPS) in the gram-negative bacteria cell wall, may contaminate biologically derived materials and stimulate acute inflammatory responses. The U.S. Food and Drug Administration (FDA) regulates the limits for endotoxins in all medical devices, including materials made from decellularized tissues. The standard for a biologic scaffold eluate is 0.5 EU/mL for most tissues and 0.06 EU/mL for cerebrospinal devices. Because endotoxin determinations are required for ECM-derived materials, iFyber uses the well-established Limulus Amebocyte Lysate (LAL) gel-clot test as a highly sensitive and accurate assay for this purpose. LAL will form a firm gel in the presence of bacterial endotoxin (Figure 4).

BIOCOMPATIBILITY

Cytocompatibility and safety of decellularized tissues depend on the successful removal of cellular-derived DNA, endotoxins, and decellularization agents. Many decellularization processes use chemical treatments, and residuals may be cytotoxic. A reduction of cell viability by more than 30 % is considered a cytotoxic effect. iFyber utilizes various types of indirect and direct assays using ISO 10993 standards for biomaterial and medical device testing to determine the cytotoxicity of tissues (Figure 5). The indirect assay (using extract of tissues) is a very common method to detect toxins leached from exposed surfaces of materials and devices used in the body. On the other hand, the direct assay yields direct contact of the sample with seeded cells, which is more correlated to in vivo situations. iFyber selects the best assay for evaluating the product's biocompatibility based on our knowledge of the applications of each cell culture assay and the properties of each company's product.

The idea of biocompatibility doesn't only mean a lack of cytotoxicity; a biocompatible product that is not cytotoxic should also be able to perform the expected function in the body. Decellularized tissues are expected to provide an appropriate environment for cell infiltration after implantation and should slowly be replaced with host cell-derived ECM. Using the direct assay, we can evaluate the recellularization potential of the tissue (Figure 6).

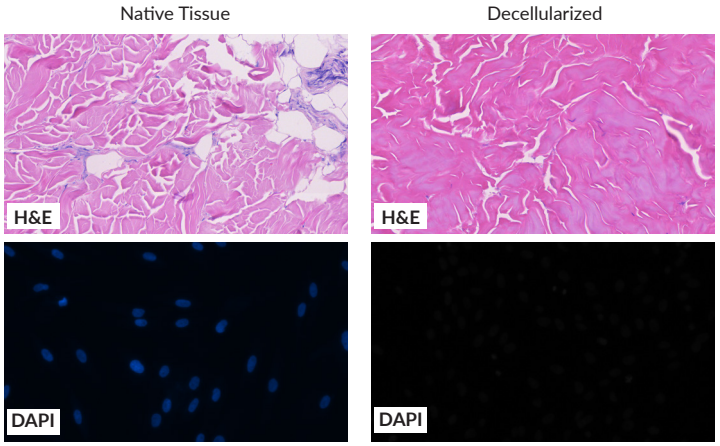


Figure 3. Histological sections of cellular and decellularized tissues stained with H&E and DAPI.

Figure 4. Example of test readout. Left: negative result – no gel formation. Right: positive result – gel has formed that does not collapse when the vial is inverted.

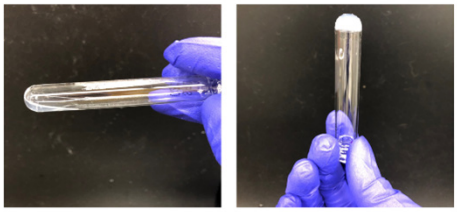
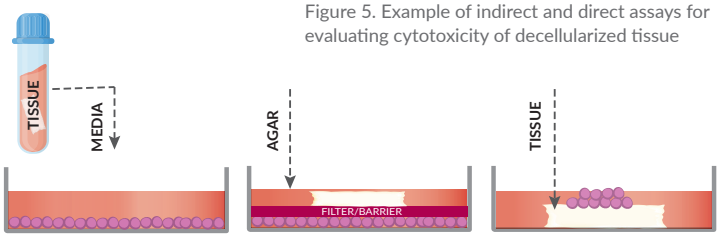


Figure 5. Example of indirect and direct assays for evaluating cytotoxicity of decellularized tissue



EXTRACT ASSAY

Groups:
- Negative control group: cells in regular medium
- Native Tissue's Extract
- Decellularized Tissue Extract

AGAR DIFFUSION ASSAY

The use of the agar diffusion assay for the assessment of cytotoxicity shall be justified

DIRECT ASSAY

Groups:
- Native Tissue
- Decellularized Tissue

Pros: direct assay mimics physiological conditions
Cons: It cannot provide a quantitative comparison with negative control

PRESERVATION OF BASEMENT MEMBRANE

Finally, while decellularization aims to remove cellular and genetic materials, it should not have a detrimental effect on the remaining ECM components and native architectures of the decellularized tissues. Structural proteins like collagen, fibronectin, laminin, glycosaminoglycans (GAGs), and growth factors are essential for

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ECM's proper function and features. Using commercially available kits, iFyber can quantify the amount of the ECM proteins like elastin, collagen, and sulfated glycosaminoglycans (GAGs) in decellularized tissue to ensure that the tissue has retained the desired ECM components following decellularization. Alcian blue and Sirius Red staining, immunohistochemistry and immunofluorescence, as well as western blotting, are also options iFyber uses as qualitative methods to check the native architectures.

In summary, iFyber's decellularization assessment methods can help manufacturers evaluate the quality and consistency of their tissue decellularization protocols when reporting new techniques and compare their decellularized technologies with commercial products.

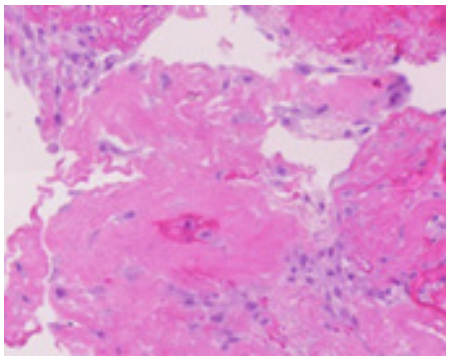

Figure 6. Evaluating recellularization by H&E staining after direct seeding of cells on the surface of decellularized tissue.

References:
1. Crapo PM, Gilbert TW, Badylak SF. An overview of tissue and whole organ decellularization processes. Biomaterials. 2011 Apr;32(12):3233-43. doi: 10.1016/j.biomaterials.2011.01.057.