INTRODUCTION
Back pain is the second most common cause for a visit to a physician, and accounts for $50 billion of annual spending in America. Degenerative disc disease (DDD), the loss of functional properties of the intervertebral discs (IVD), is a leading cause for back pain [1]. Despite a wide range of treatment options available, no current solutions provide a satisfactory solution to DDD. Extracellular matrix (ECM) biomaterials have proven to be suitable biomaterials for promoting the regeneration of many tissues in the body, including regenerate cartilage structures such as IVDs [2]. IVD ECM will be derived through several protocols from the annulous fibrosus (AF) and nucleus pulposus (NP) regions due to site specificity of the ECM. This task proves challenging as the AF and NP regions are distinct in their structure and function. The proposed project will identify an optimized method for the production of AF and NP IVD ECM.

OBJECTIVE
The objective of this particular research is to optimize a decellularization protocol specifically for IVD tissue types.

SUCCESS CRITERIA
In order to optimize a decellularization one hopes to maximize cellular content removal, and minimize native ECM property loss. That is determined through qualitative and quantitative means. The qualitative processes included histochemical staining with H&E, DAPI, Picrosirius red, and Safranin O. Both H&E and DAPI were used to show cell removal. The Picrosirius red was used to show collagen content for the AF, while the Safranin O was used to show glycosaminoglycan (GAG) content for the NP. A quantitative hydroxyproline and glycosaminoglycan assay were also conducted for the AF tissue to determine the collagen and GAG content respectively.

METHODS
Mechanical separation of the IVD into AF and NP regions was required to achieve the needed level of optimization. The fibrous build of the AF requires a harsh physical infusion method which would greatly damage the fragile NP. Decellularization protocols are aimed to maintain the collagenous structure of the AF and the glycosaminoglycan-rich properties of the NP. The study used young rabbit IVDs. Two protocols were used to decellularize the AF. The protocols applied in this study are adjustments to what Xu, H. et al suggested [3]. The AF was placed in a hypotonic Tris-HCl buffer (10 mM, pH 8.0) with 0.1% ethylenediamine tetraacetic acid (EDTA; Sigma) for 24 hours while shaking. The AF was then agitated in Tris-HCl buffer with 3% Triton X-100 and 0.1% EDTA for 72 hours. The washing solutions were changed every 24 hours for both steps. Finally, the decellularized AF was washed with PBS for 24 hours to remove residual reagents. The second protocol uses trypsin to decellularize the tissue. The AF was incubated under continuous shaking in trypsin/EDTA (0.5% trypsin and 0.2% EDTA; both Sigma) in a hypotonic Tris-HCl buffer at 37°C for 72 hours. The solution was then changed every 24 hours. After the 72 hours were completed, the decellularized AF is washed with PBS for 24 hours under shaking in order to remove any residual substances.

PARAMETERS/STATISTICS
The AF used in this study had a large diameter of 1.09 cm ± 0.16, small diameter of 0.57 cm ± 0.05. The NPs of the IVDs had a large diameter of 0.54 cm ± 0.05, and small diameter of 0.33 cm ± 0.08. The IVDs had a thickness of 0.3 cm ± 0.12. The parameter of our protocol design is, as mentioned in previous sections, the detergent species used in the decellularization. Each decellularization protocol was run 3 times with two samples per each cycle. In each assay run, three wells were dedicated for each concentration. A standard was created for each assay run to determine the linear relation between the plate absorption and the concentration. After computing the ECM component of interest concentration, the value was normalized using the mass of dry tissue used to create the digest used in the assay. The average of the three wells was recorded. After which the average of the two decellularized samples were calculated. Finally, From the average of the assays done for each cycle, we obtain a final recording. Thus, each recording is the aggregate of 18 well readings representing 6 IVD decellularizations per detergent.

RESULTS
Many cells are still observed in the Triton X-100 treated tissue as seen in figures 1-A and 1-B. The comparison between the native and decellularized tissue makes it apparent. However, it is also noticeable that the ECM structure wasn’t as disrupted as seen in figures 1-C and 1-D. Trypsin on the other hand displays opposite results. The cellular content was thoroughly removed as observed in figures 2-A and 2-B. But at the same time, the ECM has been greatly disrupted, which is clearly visible when comparing the decellularized and native tissues in figure 2-C and 2-D respectively.
The Hydroxyproline and GAG assays were conducted to determine collagen and glycosaminoglycan retention in AF tissue. Figure 3 provides the glycosaminoglycan quantity within treated AF tissue. An odd distribution is observed due to the normalization of the results. Thus showing the trypsin GAG content superior to the native.

In figure 4, similar observations are made. The triton X-100 shows slight loss of hydroxyproline, an amino acid used as an indicator for collagen. The trypsin shows higher values than the native’s. Again, that is expected due to the normalization process of the concentrations.

**DISCUSSION**

The AF decellularization could be improved with a method to sustain 37°C throughout trypsin treatment. Primary results have showed better cell removal for the trypsin protocol as opposed to the Triton X-100 AF decellularization protocol. However, Triton X-100 shows better GAG retention than trypsin. Triton X-100 also shows better ECM structure preservation than trypsin.

A more improved decellularization for the AF is expected if the EDTA wash was increased back to 48 hours in the Triton X-100 wash as done by Xu, H.et al; that is in order to better cleave the cells from the ECM.

For future considerations, another approach would be to create a mixed wash that utilizes both Triton X-100 and trypsin, hopefully providing the same cellular removal yet with less native ECM property loss. The time factor is also a prime focus for optimization. Since the current protocols take up to a week, a time reduction would definitely accelerate the progress of the research.

**CONCLUSION**

This search is still in its primary levels and still requires more experimentations for applicable results. However, it is showing progress in its initial steps.

**REFERENCES**


**ACKNOWLEDGMENTS**

Funding was provided by the Department of Bioengineering, University of Pittsburgh and the McGowan Institute for Regenerative Medicines, Department of Bioengineering of the University of Pittsburgh.