Article

Three-Dimensional Printing of Human Skeletal Muscle Cells: An Interdisciplinary Approach for Studying Biological Systems[S]

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Abstract

Interdisciplinary exploration is vital to education in the 21st century. This manuscript outlines an innovative laboratory-based teaching method that combines elements of biochemistry/molecular biology, kinesiology/health science, computer science, and manufacturing engineering to give students the ability to better conceptualize complex biological systems. Here, we utilize technology available at most universities to print three-dimensional (3D) scale models of actual human muscle cells (myofibers) out of bioplastic materials. The same methodological approach could be applied to nearly any cell type or molecular structure. This advancement is significant because historically, two-dimensional (2D) myocellular images have proven insufficient for detailed analysis of organelle organization and morphology. 3D imaging fills this void by providing accurate and quantifiable myofiber structural data. Manipulating tangible 3D models combats 2D limitation and gives students new perspectives and alternative learning experiences that may assist their understanding. This approach also exposes learners to 1) human muscle cell extraction and isolation, 2) targeted fluorescence labeling, 3) confocal microscopy, 4) image processing (via open-source software), and 5) 3D printing bioplastic scale-models (×500 larger than the actual cells). Creating these physical models may further student's interest in the invisible world of molecular and cellular biology. Furthermore, this interdisciplinary laboratory project gives instructors of all biological disciplines a new teaching tool to foster integrative thinking. © 2015 by The International Union of Biochemistry and Molecular Biology, 43:403–407, 2015.

Keywords: *teaching and learning techniques methods and approaches; muscle fiber; cellular biology; additive manufacturing; confocal microscopy*

Introduction

Continually learning new technologies and skills is essential in our rapidly changing world [1], especially in the STEM (science, technology, engineering, and math) fields. This manuscript describes an interdisciplinary laboratory-based approach that combines elements of molecular/cellular biology, kinesiology/health science, computer science, and manufacturing engineering to give learners the ability to better conceptualize complex biological systems. In the science classroom, students are often required to visualize intricate and abstract structures using microscope images or simplified two-dimensional (2D) illustrations. Recent technological achievements allow for viewing and manipulation of virtual images in three-dimensional (3D) space (i.e. in a computer environment). While this advancement provides clear advantages, some students may still have trouble inferring 3D information from these inherently 2D representations.

Additive manufacturing (3D printing) allows for the rapid and inexpensive production of physical models using digital data [2]. This technology has been utilized to produce teaching models at the level of protein structures [3] and gross anatomy [4], but not yet at the cellular level. Therefore, we developed a laboratory activity where students create tangible 3D scale-models of actual human cells using 3D printing. Students

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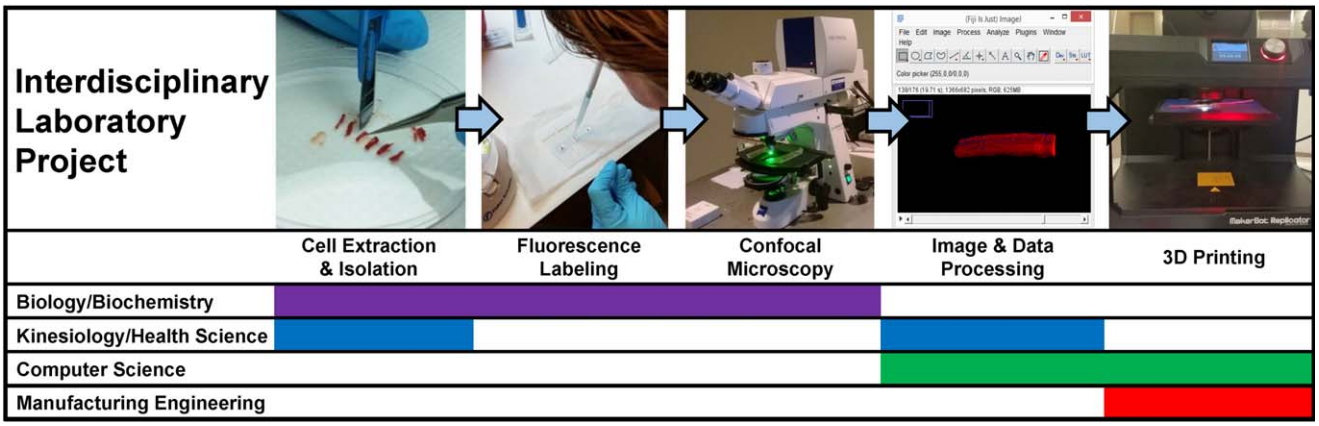

FIG 1 *Interdisciplinary laboratory project schematic showing methods utilized with fields of study represented. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]*

engaging in this interdisciplinary laboratory project will be exposed to 1) extracting/isolating individual cells, 2) targeted fluorescence labeling of organelles/structures of interest, 3) imaging via laser scanning confocal microscopy, 4) processing images using open-source biological imaging software, and 5) 3D printing final scale-models of actual cells made of bioplastic filaments (see Fig. 1). As a "proof of concept" example, this manuscript outlines the methods to investigate organelle organization and morphology on human skeletal muscle fibers (myofibers). The same methodological approach could be applied to nearly any cell type or molecular structure.

Investigating Skeletal Muscle Cells

Myofibers are the largest mammalian cells by volume, and thus must support a substantial cytoplasmic volume. To accomplish this, each muscle cell contains thousands of nuclei (myonuclei) allowing for rapid, simultaneous, and coordinated growth and repair signals to be sent along each cell. Myonuclei are usually located around the cell periphery to maximize contractile component capacity. In healthy muscle, myonuclei occupy non-randomly distributed positions in order to minimize intracellular transport distances [5, 6] as each nucleus can only support a certain cytoplasmic volume for the entire fiber to function properly [7]. Myonuclear quantity and quality can adapt to chronic perturbations (e.g. exercise, nutrition, inactivity, etc.) and studying this system is important when investigating muscle hypertrophy (growth) or atrophy (loss) in response to exercise, aging, and disease. Two-dimensional muscle histology cross-sections prove insufficient for analyzing myonuclear parameters because myonuclei may be misclassified as mononuclear cells located between muscle fibers (e.g. stromal and satellite cells) [8]. In contrast, 3D myofiber imaging can provide accurate and quantifiable structural data distinguishing between intercellular nuclei and myonuclei proper. Creating tangible 3D muscle cell models offers students alternative perspectives regarding the arrangement of skeletal muscle surface structures and organelles (such as myonuclei). Figure 2 shows representative images of a human single muscle fiber 2D projection, 3D rendering, and 3D scale model. Note: The myonuclei and actin contractile filaments can be easily visualized because the basal lamina has not been fluorescently stained.

Goals of This Laboratory Experiment

This manuscript outlines a method to create tangible 3D scale models (×500 larger than actual size) of human muscle fibers using technology available at most university campuses. Primary goals of implementing this experiment are to 1) expose students to multiple technologies (i.e. confocal microcopy and 3D printing), 2) provide tangible models to investigate myocellular and myonuclear organization and morphology, and 3) motivate students to learn more about the invisible molecular world.

Materials and Methods

These methods were piloted with students in the Biochemistry & Molecular Exercise Physiology Laboratory (California State University, Fullerton), which included upper-division undergraduate and graduate students. The entire project consisted of one preparation day (by instructor) and two student-centered laboratory days (this schedule may be altered to fit individual needs). All procedures should be supervised by trained instructors or laboratory assistants. If using human or animal subjects to acquire cells, contact the appropriate review boards and take pertinent safety precautions. Equipment and materials required for this experiment are listed in Table 1.

Muscle tissue used in our application was from a human participant, therefore this study conformed to standards set forth by the Declaration of Helsinki and was approved by the Human Subjects Institutional Review Board (IRB) at California State University, Fullerton. The participant received oral and written information about experimental procedures and potential risks before providing written consent.

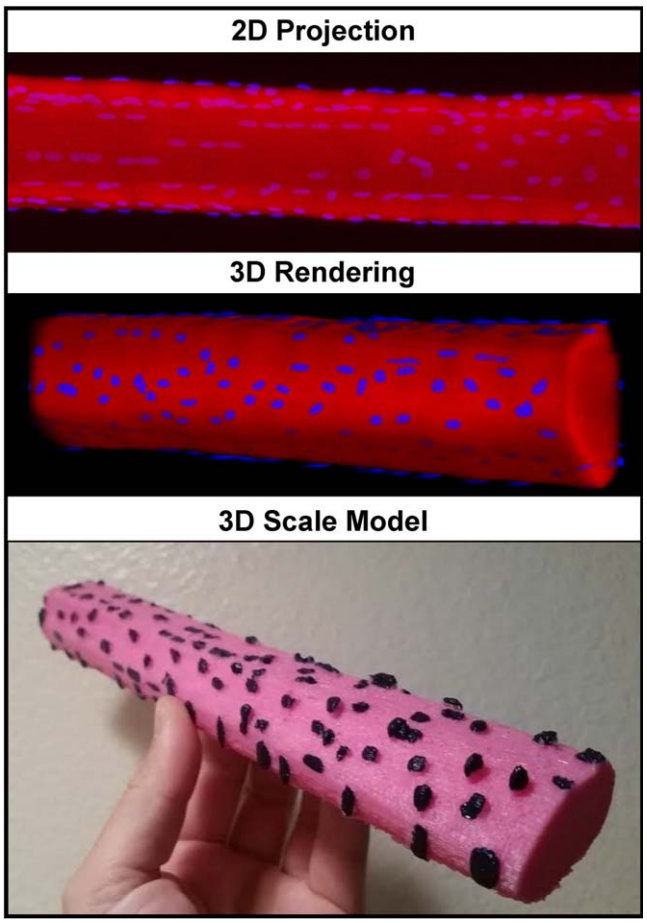

FIG 2 *Representative images showing a two-dimensional (2D) projection, three-dimensional (3D) rendering, and 3D bioplastic scale model of a human muscle cell (500× larger). Blue: myonuclei, Red/Pink: cell body (delineated by actin filaments). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]*

Day 1: Laboratory Preparation by the Instructor

Solutions

Stock solutions should be prepared by instructors or laboratory assistants prior to initiating the experiment. Skinning solution [(in mM): 125 K propionate, 2.0 EGTA, 4.0 ATP, 1.0 $MgCl_2$, 20.0 imidazole (pH 7.0), and 50% (vol/vol) glycerol] may be stored at −20°C for up to 1 year. Relaxing solution [(in mM): 7.0 EGTA, 20.0 imidazole, 14.5 creatine phosphate, 1.0 free Mg^{2+}, 4.0 free MgATP, KCl, and KOH] may be stored at 4°C for up to 6 months and has an ionic strength of 180 mM and a pH of 7.0 [free Ca^{2+} concentration of *p*Ca 9.0 (where $pCa = -\log Ca^{2+}$ concentration)].

The following solutions should be prepared fresh immediately before each laboratory. Permeabilizing solution (vol/vol): 0.1% Triton X-100 (Thermo Fisher Scientific, Waltham, MA) diluted with relaxing solution. Actin Label: Phalloidin conjugated with AlexaFluor 568 (Molecular Probes, Eugene, OR) diluted with relaxing solution (1:40). Note: Protect the actin label solution from light to minimize photo bleaching.

Muscle Tissue Extraction

One healthy male volunteer (age = 29 yr) underwent a *vastus lateralis* muscle biopsy conducted by trained researchers (at the Center for Sport Performance, California State University, Fullerton) as described previously [9]. Longitudinal muscle sections (∼20 mg) were placed directly into 1.5 mL cold skinning solution (in a cryogenic vial) and stored at −20°C for later analysis (at least one week to allow for smooth muscle fiber isolation). This tissue sample contained thousands of muscle fibers that may be stored for several months until use.

Day 2: Laboratory Bench Work by Students

Muscle Cell Isolation

After at least one week incubation in skinning solution, a small bundle of muscle fibers was placed in a 35-mm dish containing ∼2 mL of relaxing solution. Using fine tipped tweezers under a stereo dissecting microscope, muscle fiber segments (∼2.5–3.0 mm) were mechanically isolated by carefully holding one corner of the bundle and pulling individual fibers.

Targeted Fluorescence Labeling

Immediately following isolation, individual muscle cells were placed on glass microscope slides (∼5–10 per slide) surrounded by a hydrophobic barrier (Note: the small area surrounding each fiber was used for the following incubations to save reagents). Once all fibers were placed on the slide, they were incubated in permeabilizing solution for 15 min, then rinsed in relaxing solution (3 × 5 min). Myofibers were then incubated in the actin label for 30 min to stain F-actin filaments. Subsequently, single fibers were rinsed with relaxing solution (3 × 5 min) and labeled with 4,6-diamino-2-phenylindole (DAPI) in mounting medium (ProLong Gold Antifade with DAPI, Molecular Probes, Eugene, OR) to directly label myonuclei (Note: ensure that the mounting medium does not contain any bubbles when placed on fibers). A Parafilm window was cut and placed around pooled fibers on the microscope slides to prevent cover slips from compressing the myofibers. Glass coverslips (0.5 mm) were carefully placed over grouped fibers to keep cells as close together as possible for efficient imaging, then sealed with clear nail polish. Slides were stored in the dark at 4°C until imaging to minimize photo bleaching (Note: Imaging can be done immediately, but this is a logical time to break and image during the next lab meeting or have students image on their own time, depending on the confocal microscope availability).

Day 3: Imaging and 3D Printing by Students

Laser Scanning Confocal Microscopy

Muscle fibers (∼1–2 mm) were imaged using a Leica TCS SP2 Inverted Scanning Confocal Microscope (Nano- & Micro-Visualization Laboratory, California State University, Fullerton) equipped with a Plan-Apochromatic ×20/0.75 numerical aperture (NA) objective. An undamaged region of interest was

TABLE 1 *Interdisciplinary laboratory project equipment and materials*

Muscle cell extraction and isolation	
Muscle tissue sample	Human skeletal muscle biopsy from the *vastus lateralis*, stored in skinning solution[a] at −20°C
Dissecting microscope w/light source	Olympus SZ60 (Olympus, Tokyo, Japan) or equivalent stereoscope with 20× zoom capabilities
Culture dish w/relaxing solution	Corning 35 mm culture dish (Corning Inc., Corning, NY) filled with relaxing solution[a] (∼1.5 mL)
Fluorescence labeling	
Microscope slides	Fisherbrand Superfrost Plus (25 mm × 75 mm) (Thermo Fisher Scientific, Waltham, MA)
Hydrophobic pen	ImmEdge Pen (Vector Labs, Burlingame, CA)
0.1% Triton x-100	Triton x-100 (Thermo Fisher Scientific, Waltham, MA) diluted in relaxing solution[a]
Actin label (Phalloidin)	Phalloidin conjugated with AlexaFluor 568 (1:40; Molecular Probes, Eugene, OR)
Myonuclei label (DAPI)/mounting medium	ProLong Gold Antifade with 4,6-diamino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR)
Cover slips	0.5 mm micro cover glasses (Electron Microscopy Sciences, Hatfield, PA)
Confocal microscope imaging	
Laser scanning confocal microscope	Leica TCS SP2 Confocal Microscope (Leica Microsystems GmbH, Wetzlar, Germany) or equivalent
3D image processing	
Open-source software	ImageJ, Fiji Distribution
Plugin	3D Object Viewer
3D printing final scale models	
3D printer	MakerBot Replicator 2 Desktop 3D Printer (MakerBot Industries, New York, NY) or equivalent
Acrylic paint	Acrylic based paint (i.e. nail polish) to distinguish surface organelles and structures

[a]*Solution recipes are shown in* Materials and Methods*. Other equipment not listed above includes: PC computer, flash drive (for transferring data), pipettes, medical grade tweezers, Kimwipes, Parafilm, cryogenic vials (for tissue storage), and proper personal protective equipment (i.e. gloves, goggles, lab coat).*

identified on each cell (∼500 μm long) and myofibers were imaged at resting sarcomere length (∼2 μm), which corresponds to normal sarcomere spacing in humans [10]. Z stacks were acquired by collecting consecutive images from the top to the bottom of each myofiber. Nuclei labeled with DAPI were detected at a laser line of 405 nm using a band pass filter (BP) 420–480 nm. Actin filaments labeled with AlexaFluor 568 phalloidin were excited with the laser line at 543 nm and collected with a long pass filter (LP) 560 nm. Pixel time was set at 2.17 μs and data were collected in 12-bit for both channels. All images were sampled at Nyquist frequency in *xy*-axis (0.98 × 0.98 μm^2) and 2× Nyquist sampling for the *z*-axis.

3D Image Processing

3D image stacks were processed and analyzed using ImageJ v1.48i (Fiji distribution), an open-source biological

image-analysis program [11]. ImageJ is a Java-based image processing tool developed at the National Institutes of Health (Note: this program is extremely customizable and the following commands may be "pre-programmed" or set with "hot keys" for future use). For each myofiber image stack, an undamaged region of interest was selected on the *xy*-plane (500 μm per fiber) away from outer edges. This region of interest was cropped in both the actin and DAPI channels and duplicated for 3D rendering (Note: image stacks should be saved as ".tif" files). Each stack was then converted to binary with standard settings (Process → Binary → Make Binary). Subsequently, each fiber underwent 3D rendering to globally view structures using the 3D Viewer plugin in Fiji [12]. Briefly, the actin and DAPI image stacks were opened in the 3D Viewer (Plugins → 3D Viewer tab) and displayed as "Surface" with standard settings. Then, when both surfaces were open they were converted to an ".stl" file (File → Export Surfaces → STL (binary)).

3D Printing Final Scale Model

The ".stl" file created using Fiji was saved onto a flash drive and transferred to the MakerBot Replicator 2 3D printer (Division of Information Technology, California State University, Fullerton). The file was prepared for printing by following MakerBot software command prompts (Note: the image can be scaled, moved, or rotated as necessary). In our case, the original muscle fiber was 500 μm long was scaled up to ×500 original (25 cm). Myonuclei were visible on the 3D printed bioplastic cell, but blue acrylic paint was used to color them to highlight their organization and morphology. The final model is shown in Fig. 2 (Note: The ".stl" file of this muscle fiber has been uploaded as Supporting Information and can be opened with ImageJ).

Conclusion

University science curriculum often incorporates laboratory projects using advanced biological methods, microscopy, and computer imaging to independently investigate various systems. We built upon these methods to design this interdisciplinary laboratory project, which includes additive manufacturing (3D printing). Applications of 3D printing are limitless, and the project presented here only begins to highlight the potential for integrating this relatively new technology into laboratory curriculum. While we investigated human skeletal muscle cells, 3D modeling could be adapted to study many cells or structures, giving teachers of all biological disciplines a new tool to engage students in interdisciplinary research. Bioplastic scale models, created by students, may help promote their interest in the invisible world of molecular and cellular biology.

Initial implementation of this project was conducted with upper-division undergraduate and graduate students in the Biochemistry & Molecular Exercise Physiology Laboratory (California State University, Fullerton). Future research will investigate 1) the efficacy of such projects at various educational levels (i.e. upper-division undergraduate and graduate), 2) potential increases in student's understating of myofiber structure over using 2D and 3D computer renderings, and 3) the feasibility of studying other biological systems using bioplastic scale models.

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