Video Article

Engineering Fibrin-based Tissue Constructs from Myofibroblasts and Application of Constraints and Strain to Induce Cell and Collagen Reorganization

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Abstract

Collagen content and organization in developing collagenous tissues can be influenced by local tissue strains and tissue constraint. Tissue engineers aim to use these principles to create tissues with predefined collagen architectures. A full understanding of the exact underlying processes of collagen remodeling to control the final tissue architecture, however, is lacking. In particular, little is known about the (re)orientation of collagen fibers in response to changes in tissue mechanical loading conditions. We developed an *in vitro* model system, consisting of biaxially-constrained myofibroblast-seeded fibrin constructs, to further elucidate collagen (re)orientation in response to i) reverting biaxial to uniaxial static loading conditions and ii) cyclic uniaxial loading of the biaxially-constrained constructs before and after a change in loading direction, with use of the Flexcell FX4000T loading device. Time-lapse confocal imaging is used to visualize collagen (re)orientation in a nondestructive manner.

Cell and collagen organization in the constructs can be visualized in real-time, and an internal reference system allows us to relocate cells and collagen structures for time-lapse analysis. Various aspects of the model system can be adjusted, like cell source or use of healthy and diseased cells. Additives can be used to further elucidate mechanisms underlying collagen remodeling, by for example adding MMPs or blocking integrins. Shape and size of the construct can be easily adapted to specific needs, resulting in a highly tunable model system to study cell and collagen (re)organization.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51009/>

Introduction

Cardiovascular tissues have a prominent load-bearing function. In particular content and organization of collagen fibers in the extracellular matrix contribute to the load-bearing properties and dominate overall tissue strength¹. In tissue engineering mechanical conditioning of the construct is used - typically consisting of (cyclic) straining regimens - to enhance tissue organization and mechanical properties^{2,3}. Full understanding of strain-induced collagen organization in complex tissue geometries to create tissues with predefined collagen architecture has not yet been achieved. This is mainly due to our limited knowledge of collagen remodeling in developing tissues. Existing models mainly give information about the final net outcome of collagen remodeling with use of static strain⁴⁻⁶. Here we provide a highly tunable model system that allows the study of collagen (re)organization in a real-time fashion, in 3D, under influence of static or cyclic strain. The tissue constructs are fibrin-based, ensuring that all collagen in the construct is endogenous. Cell and collagen organization in the constructs is visualized, and an internal reference system allows us to relocate cells and collagen structures for time-lapse analysis. In this protocol we will describe the use of the model system for Human Vena Saphena Cells (HVSCs), since these cells are known for their enhanced extra cellular matrix production and ability to remodel the matrix and our established use in engineered cardiovascular tissues⁷, based on the work of de Jonge *et al.*⁸

Protocol

1. Culture of Human Vena Saphena Cells

1. Isolate cells from the vena saphena magna, acquired from a donor in accordance to guidelines for secondary use material, according to the protocol by Schnell *et al.*⁹ and store these in liquid nitrogen. From the part of the vena saphena magna from one donor cut pieces of 2 x 2 mm to culture in a six-well plate. Use 2 pieces per well. Generally enough cells can be obtained to fill about 3 vials with 0.25 x 10⁶ cells in liquid nitrogen. HVSCs are characterized as myofibroblasts, by showing expression of vimentin, no expression of desmin and a subpopulation expressing α smooth muscle actin¹⁰. Next start the protocol to thaw the cells from the liquid nitrogen to increase the number of cells.

2. Place the cells from one vial into a T75 tissue culture flask and add growth medium (GM), consisting of Advanced DMEM, 50 ml fetal bovine serum (FBS), 5 ml penicillin/streptomycin and 5 ml L-glutamax. Change medium every 2-3 days.
3. Cells grow confluent approximately after 14 days. Store 0.5×10^6 cells in one vial in liquid nitrogen, referred to as passage 1.

Note: Freezing of the cells is not necessary when harvesting HVSCs, but is solely used for storage.

4. Place the HVSCs from one vial into a T175 tissue culture flask and add GM. Change medium every 2-3 days. Cells grow confluent after approximately 7 days. Store 3×10^6 cells in one vial in liquid nitrogen, referred to as passage 2.
5. Place the cells from one vial into a rollerbottle and add GM. Change medium every 2-3 days. Cells grow confluent approximately after 8 days. One rollerbottle contains approximately $20\text{--}30 \times 10^6$ cells. Culture cells up to passage 6. Do not use cells of a passage higher than passage 9, since cell phenotype may change.

2. Engineering of Fibrin-based Tissue Constructs

1. Prepare silicone glue by mixing the elastomer with the curing agent (10:1). Cut 7 x 3 mm rectangular segments of Velcro. Glue the Velcro into a 6 well culture plate, with flexible membranes to form a cross, and to leave a squared space of 3 mm between the Velcro strips.

Notes: Only use the soft side of the Velcro and face this side upwards. When gluing the Velcro, only cover the Velcro with silicone glue, do not spread glue throughout the well. Since the culture plates have silicone membrane bottoms, use something underneath the well plate for reinforcing the flexible membranes, to ensure easy gluing in to the plate.

2. Dry the silicone glue overnight in an oven at 60 °C to ensure hardening of the glue. Sterilize by adding 70% EtOH to the wells and incubate for 30 min. Rinse 3x with PBS and remove all PBS from the wells and the Velcro. Put under UV for 30 min and keep sterile until use.
3. Prepare Tissue Engineering medium (TM), consisting of GM and 130 mg of L-ascorbic acid 2-phosphate.
4. Add 1 mg/ml ϵ -Amino Caproic Acid (ACA) to the TM. ACA is used for the first 7 days of culture to prevent the fibrin from degrading¹¹. Alternatively, aprotinin can be used.
5. To prevent clump formation, allow fibrinogen to reach room temperature before opening. Without clumps fibrinogen will dissolve more easily. Dissolve fibrinogen to a concentration of 10 mg actual protein/ml TM⁷ supplemented with ACA. Sterile filter the fibrinogen solution, multiple filters might be needed. Store on ice until use.

Note: To dissolve fibrinogen mix gently to prevent too much foam formation.

6. Dissolve thrombin to a concentration of 10 IU/ml TM⁷ supplemented with ACA. Store on ice until use.

Note: Storage on ice is needed to prevent early gelation of thrombin and fibrinogen.

7. Trypsinize the cells, resuspend in GM and count.
8. Use 15×10^6 cells/ml for seeding the fibrin-based tissue constructs (concentration based on methods for tissue engineering heart valves⁷). A single gel consists of 100 μ l of gel, and thus of 1.5×10^6 cells. Put 1.5×10^6 cells in one centrifuge tube, resulting in as many centrifuge tubes as the number of gels that will be made.
9. Centrifuge the cells at 350 x g for 7 min and discard the supernatant. Resuspend the cells in 50 μ l thrombin. Add 4 μ l of blue fluorescent polystyrene microspheres to this suspension. Put 50 μ l of fibrinogen in a vial. Use a pipette to take up the 50 μ l thrombin with cells. Increase the volume of the pipette to 100 μ l. Pipette the 50 μ l solution of thrombin with cells into the vial with fibrinogen to mix the thrombin and fibrinogen, and take up the 100 μ l mixture.

Note: The fluorescent polystyrene microspheres are used as internal reference markers for image analysis. When mixing thrombin and fibrinogen, prevent the formation of air bubbles by carefully pipetting the mixture. Air bubbles will result in holes in the fibrin gel.

10. Pipette the gel mixture into and in between the Velcro strips. The typical gelation time for the fibrin gels, once the components are mixed, is of the order of 20 sec.

Notes: Do this as quickly as possible to prevent gelation before the mixture is pipetted in the well plate. Practice before using cells and beads.

11. Incubate suspensions for 30 min at 37 °C in a humidified 95% air/5% CO₂ incubator to allow gelation before adding culture TM with ACA.
12. Replace TM with ACA every 2-3 days for the first 7 days. Gels are stable enough after one week to be cultured without ACA. After the first week replace TM every 2-3 days. Add 6 ml of TM per well. On day 12 cells have produced enough collagen for visualization.

3. Applying Strain and Inducing Changes in Strain and Constraints

1. Static strain is applied by the cells directly, due to cell traction and compaction¹². To induce collagen reorganization, cut the tissue construct loose from two Velcro strips in one direction. This results in unidirectional constraints (**Figure 1A**). Perform this on day 12, since the construct is then stable enough and collagen has been deposited.
2. Apply cyclic strain by applying a vacuum to the bottom of the culture plates with flexible membranes, with the use of the Flexcell FX4000T system. Place the plates on a baseplate, on top of loading posts (which are a part of the cyclic loading device). The pump applies a vacuum onto the membrane and thereby pulls the membrane over the rectangular post lying underneath. Due to the cross shaped attachments of the tissue constructs to the membrane (via the Velcro) and the rectangular post the applied cyclic strain is uniaxial (**Figure 1B**).
3. Initially, use static culture for 5 days to achieve initial mechanical integrity, before application of cyclic strain starting on day 6. Program a cyclic strain protocol into the controller for the vacuum pump. For example use a previously established intermittent cyclic strain protocol, with uniaxial direction¹³. This consists of an intermittent strain of a sine wave with 1 Hz, straining from 0 to 5% strain, for periods of 3 hr, alternated with 3 hr resting periods. Perform this cyclic strain protocol for 7 days, inducing an aligned collagen organization.

- Typically after 12 days HVSCs have produced an aligned collagen organization. After an aligned collagen organization is reached, change the uniaxial cyclic strain direction, to be perpendicular to the original strain direction. Do this by turning the rectangular posts by 90°.

4. Visualizing Cells and Collagen

- To visualize active, real-time collagen remodeling, label samples with probes that do not interfere with cell viability or collagen formation. Use probes to fluorescently stain the cell cytoplasm and collagen.
- Alternatively second harmonic generation using confocal laser scanning microscopy can be used to visualize collagen structures using autofluorescence¹⁴, with excitation at 780 nm and detection between 500-550 nm.
- Remove the culture plates from the setup and the incubator, for the cyclically strained samples during the 3 hr rest period, and transport them to a confocal laser scanning microscope to visualize cells and collagen.

Representative Results

This model system allows for culturing myofibroblast-seeded fibrin gels. **Figure 1A** shows a tissue cultured first under static biaxial constraints. Tissue constraints are released by cutting the fibrin gel from two constraints, to create uniaxial static constraints, and tissue compacts and remodels afterwards (**Figure 1A**). For cyclic strain, the tissue is cultured under static biaxial constraints as well. After 5 days cyclic uniaxial strain can be applied (**Figure 1B**). To induce collagen reorientation, uniaxial strain direction can be changed to the perpendicular direction (**Figure 1B**). The microspheres seeded with the gel mixture, display a random pattern, which is used to relocate predefined locations for time-lapse imaging (**Figure 2**). **Figures 2A** and **2C** show an image of cells, collagen and beads. These same images are scanned 3 days (**Figure 2B**) and 2 days (**Figure 2D**) later, respectively. Cells and collagen patterns have changed, but bead patterns are used to relocate the cells and collagen. Culturing samples for 12 days results in endogenous collagen production in quantities sufficient to be visualized with confocal microscopy (**Figures 3** and **4**). Using either static or cyclic culture results in distinctly different collagen organization, where biaxial static culture gives rise to a random collagen organization (**Figure 3A**) and uniaxial cyclic strain applied to a biaxially constrained tissue results in an aligned collagen structure (**Figures 4A** and **B**). This collagen organization can be studied over time, while changing the static constraints or changing the cyclic strain direction. When static constraints are changed from biaxial to uniaxial, collagen orientation changes from a random orientation (**Figure 3A**) to an aligned orientation (**Figure 3B**). Cyclic strain induces a change of orientation at the surface of the tissue (**Figures 4A** and **C**), but after 3 days, no change in the core of the tissue is observed (**Figures 4B** and **D**).

Figure 1. Constructs cultured under biaxial constraints, used to reorganize collagen by (A) releasing static constraints in one direction, or by (B) changing cyclic strain direction. Loading posts are indicated by dotted black lines. Scale bars indicate 3 mm.

Figure 2. Typical example of the use of bead patterns to relocate predefined locations in 3D, in this case in statically cultured samples. Cells are shown in red, collagen in green and beads in blue. **A** and **C** show an image of cells, collagen and beads. These images are scanned 3 days (**B**) and 2 days (**D**) later, respectively. Bead patterns are used to relocate cells and collagen. Scale bar indicates 50 μm .

Figure 3. Representative images of 12 day static strain with biaxial constraints (A) and reorientation due to uniaxial constraints within 7 days (B). Scale bar indicates 50 μm .

Figure 4. Representative images after 12 days (5 days static, followed by 7 days cyclic strain) for cyclic strain at the surface (A) and ~60 µm into the tissue (B). After a change in cyclic strain direction (after 12 days), at the surface cells and collagen reorient (C) but no changes are seen in the core of the tissue (D) after 3 days.

Discussion

The described model system of cell-populated fibrin constructs has great potential for the study of cell and collagen (re)organization (de Jonge *et al.*¹⁵), e.g. to be used for tissue engineering purposes. By using fibrin as the initial cell carrier, after fibrin degradation, a tissue is created with cells and endogenous matrix only. In this way, cells are stimulated to react to strain, either static or cyclic in nature, by applying contractile forces^{16,17}, sensing boundary stiffness¹², or displaying strain avoidance.

Significance: Fibrin constructs have been used before to study collagen (re)organization¹⁸, but not with the ability of applying cyclic strain and/or studying the constructs in a time-lapse manner, both of which are possible in this presented method. The Velcro strips that provide the constraints in this model system have been used before¹⁹, but combining this technique with culture plates with flexible membranes allows to apply cyclic strain for prolonged periods of time. Culturing in these plates allows for easy addition and removal of medium and visualization of the constructs while still attached and sterile. This enables studying relevant remodeling processes time-lapsed or even real-time.

Modifications and future applications: Future applications of the system can be found in manipulating the 3D remodeling process, for instance by adding metalloproteinases or agents that interfere with integrin assembly or signaling. Next to the application of additives, the components of the model system can be easily modified when studying cell and collagen (re)organization. Different cell sources, healthy and diseased cells, the maturity of the collagen matrix (e.g. by varying culture time), and the shape and size of the construct can be adapted to specific needs. The system is well suited for other cell sources, instead of the currently used HVSCs. We have already used this system to culture endothelial colony forming cells (ECFCs; PlosOne, accepted for publication), where we observe that ECFCs orient their produced collagen differently upon cyclic strain than HVSCs.

Limitations of the technique: A limitation of the current model system is the relatively large size of the tissues, requiring 1.5×10^6 cells per construct when using a cell density of 15×10^6 cells/ml. This may pose a problem in cases where less available cell sources will be used.

Another limitation is the current thickness of the constructs (approximately 1 mm), which is due to the (thick) Velcro attachments. This limits the confocal scanning to only a portion of the entire sample. It is possible to reach a depth of max 200 μ m in the tissue, which is enough to visualize cellular responses within the core of the current tissues, but does not result in a full thickness overview.

Critical steps and troubleshooting: As the model system is based on the formation of tissue between the Velcro attachment points, this also encompasses the critical steps in the protocol. Correct cutting and gluing is essential and special attention should be paid to the careful pipetting of the gel mixture into the Velcro strips for proper attachment of the tissue, which is crucial for applying cyclic strain. Further determinative steps can be found in the choice of cell source and medium additives. Optimization will be needed when culturing with a different cell source. Currently, constructs are cultured with ACA for 7 days, to stop the fibrin from degrading. A different cell source may also result in a different time frame with regard to both collagen formation and fibrin degradation.

Disclosures

The authors declare that they have no competing financial interests.

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