Strain Stabilizes Reconstituted Collagen Fibrillar Network Against Enzymatic Cleavage by Collagenase.

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ABSTRACT

Collagen is the structural molecule of choice in vertebrates and is the most abundant protein on earth. Its primary role is that of bearing and transmitting mechanical (principally tensile) loads. Though collagen degradation is a normal part of collagen homeostasis, excessive collagenolysis has been implicated in a number of human diseases such as arthritis, cancer, and atherosclerosis. Collagen is susceptible to cleavage via bacterial collagenase (Clostridium histolyticum) and members of Matrix Metalloproteinase (MMP) family. *It has been hypothesized that mechanical loads can influence the rate of enzymatic cleavage of collagen molecules in native tissue.* The purpose of the investigation is to study *in vitro* the behavior of reconstituted collagen fibrils in networks supporting mechanical strain and then exposed to enzymatic cleavage by bacterial collagenase and MMP. In a custom designed, environmentally-controlled, reaction micro-chamber, type I collagen solution was self assembled by neutralizing purified collagen (3.0 mg/mL), with PBS buffer for bacterial collagen experiments and with Tris buffer for MMP experiment. Collagen fibrillogenesis (induced by temperature elevation to 37° C) was observed by DIC (using Nikon TE2000-E). Mechanical strain was applied on the fibrillar network using micromanipulators and micropipettes. To determine if the load protected the cleavage of loaded collagen fibril, bacterial collagenase and in the separate experiments MMP-8 was added to the assembled matrix. The results demonstrated that unstrained fibrils were removed quickly while strained fibrils were degraded at a significantly slower rate. Using the edge detection image analysis tool, the degradation curve shows the statistical difference in the start and the end of degradation for loaded and unloaded fibrils. This indicates the strain-stabilization of collagen against enzymatic cleavage. Also, in another result, preliminary data suggest that mechanical signals direct the preferential incorporation of collagen monomers into fibrils. Using the above two conclusions, *in vitro*, we propose a novel method where organized collagenous tissue can be produced merely by application of appropriate strain in the presence of excess of monomers and an appropriate concentration of enzymes.
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Chapter 1: Introduction

[1] Collagen is a structural protein that gives tensile strength to bone, tendons, and various other tissues. Collagen is continually synthesized and degraded in the extracellular matrix. Collagen degradation is a normal part of collagen homeostasis; however excessive collagenolysis has been implicated in a number of human diseases such as arthritis, cancer, and atherosclerosis [2-4]. It is known that there is a significant economic impact because of collagen related diseases. However, little is known about the mechanism that governs collagen degradation and assembly in vivo. The principal role of fibrillar collagen is to bear and transmit mechanical (tensile) loads. It is widely accepted that mechanical loads play a critical role in the development of load bearing collagenous matrices [5].

[6] In the late 19th century, Wolff suggested that bone morphogenesis in the femur head was directed by the mechanical environment. Since the 1950’s, when the spontaneous self-assembly of native-like collagen fibrils from acid-extracted connective tissue was directly observed, much has been discerned regarding the structure, physicochemistry and kinetics of collagen monomer self-assembly into fibrils [7-10]. More recently however, further evidence of the intrinsic “intelligence” of collagen as a structural material has been reported. [11] In 1977, Huang and Yannas reported on the mechanochemical behavior of degrading reconstituted collagen tapes and suggested that mechanical force modulates the rate of degradation, possibly by lowering diffusional access to the collagen. [12] In 1996, Nabeshima et al. suggested that, collagen- bacterial collagenase interaction was modified under uniaxial tensile mechanical load in native tendon. In the separate control experiments, it was indicated that the diffusional access of collagenase into the tendon was not affected by the tension on the specimen. [13] In 2005, Ruberti and Hallab demonstrated that bacterial collagenase preferentially degraded unloaded control fibrils in mechanically-strained, native corneal tissue. This experiment demonstrated that exposure to collagenase and mechanical strain could be used to
“sculpt” or remodel collagenous matrix by culling unloaded fibrils from an existing native tissue.

Taken together, these studies suggest that collagen-enzyme reaction kinetics are likely a function of the state of strain or strain energy density in the system. [5, 14, 15] In the majority of the research performed supported the idea that fibroblasts play the principal role in extracellular matrix morphology control. However the mechanism by which the fibroblasts physically organize the resorption or deposition of each collagen molecule is not well understood. Also it is difficult to explain how the observed fibroblast responses to mechanical signals alone can result in an optimized load adapted remodeling of the matrix. During adaptive matrix remodeling under load, mechanically activated fibroblasts may paradoxically secret both synthetic and degradative extracellular matrix (ECM) molecules [16, 17].

Therefore we hypothesize that there must exist auxiliary mechanochemical mechanisms which operate at the level of the matrix molecules. Such mechanisms should be capable of locally influencing the degradation of collagen based on the state of strain in the ECM. The mechanism by which the collagen appears to be protected or “stabilized” by the applied mechanical load is not yet understood. In another study [18], it has been noted in systems where collagen fibrils under load resist thermal denaturation. In addition, the mere packing of collagen monomers into fibrils has been shown [19] to improve their resistance to both thermal and enzymatic degradation. It has been suggested that the improved stability obtained by packing monomers into fibrils is due to a decrease in the configurational entropy of the collagen molecules by enhancing inter or intra-chain bonding. Similarly, it is possible that mechanical load enhances stability by a similar mechanism. It is also possible that straining the collagen monomer alters the geometry of the enzyme binding site (making binding more difficult) or alters the relative positions of the three alpha-chains (making cleavage more difficult). Regardless of the mechanism by which protection from the enzymatic degradation is affected, if tissue engineers are to take full advantage of collagen fibril strain-stabilization, it is important to observe the kinetics of enzyme mediated degradation of unloaded fibrils in reconstituted, engineered collagen networks via a number of different enzymes including the more
physiologically relevant Matrix Metalloproteinase (MMPs). In this investigation, we have created an environmentally-controlled, reaction microchamber which allows the direct optical imaging of the kinetics and pattern of enzymatic degradation in strained, reconstituted collagen micronetworks. The results obtained from this study will help us to understand the details of collagen/collagenase (Bacterial and MMP) mechanochemistry, and thus have the potential to explain (in part) the etiology of collagenous diseases.
Chapter 2: Background

2.1 Collagen

Collagen is a structural protein found in abundance in extra-cellular matrices of connective tissues. [20] It has more than 27 types and found in many forms like fibrillar, network-forming sheets and filaments. [21] Collagen molecular structure was first demonstrated by Ramachandran and Kartha and is still under investigation. As shown in the Fig 1 [22], it has a unique amino acid composition containing one-third Glycine and rest is primarily proline and/or hydroxyproline or any other amino acid residue. This structure makes the collagen a molecule with high mechanical strength. Hydrogen bonds between glycine of one chain and proline of the other chain and water mediated bonds stabilize the triple helix. The molecular unit of collagen is made up of three polypeptide strands, each of which is a left-handed helix. These three left-handed helices are twisted together into a right-handed

Figure 1: collagen molecule structure
From, Beck, K.etal 1998

Figure 2 Assembly of the triple helix into the fibril, by packing the collagen molecules and illustration of the origin of the banding pattern
coiled coil, called as a triple helix as shown in the Fig 2. The inter-molecular covalent cross links hold them close so that it is suited to the biological function of bearing loads.

[20] Collagen is synthesized intra-cellularly as a precursor procollagen molecule. As shown in the Fig 3, the procollagen molecule by virtue of its globular N-terminal and C-terminal propeptide extensions confers solubility under physiological conditions and prevents aggregation and fibril formation inside the cell. Therefore there has to be controlled cleavage of the propeptides to initiate fibril formation in extracellular matrix. After secretion into the extracellular space these propeptides are then systematically cleaved to trigger fibril formation. [23] In the Smith microfibril native collagen molecular model, five collagen type I molecules are arranged in a “quarter-staggered fashion” to form a micro fibril having a diameter of 4 nm. Due to quarter-staggered arrangement there is a striated look to a collagen sample with periodicity of 64 nm having alternate dark and light bands [8].
The Fig 4 shows the organization of collagen at various hierarchal levels in tendon where collagen triple helices are condensed into fibrils and thicker fibers and finally fiber bundles. Aggregation of collagen micro-fibrils results in the formation of fibrils. The fibrils are cylindrical in shape with a diameter varying from 10 to 500 nm [8].

[24] Mechanical properties have been studied largely at macroscopic levels and indirect studies of X-ray diffractometry predict Young’s modulus as 3-5 GPa. Also, recently nano-manipulation techniques have found that the collagen molecule are much more flexible [25] than believed. This flexibility can possibly be assigned to micro unfolding of the collagen triple helix. The mechanical properties of the connective tissues depend on the composition and organization of collagen molecules. [26] As shown in Fig 5, In tendon, ligament, annulus fibrosus and cornea, collagen is organized such that the fibrils support tensile mechanical loads. Using synchrotron X-ray scattering experiments, it is suggested that for the small amount of strain, there is a straightening of kinks in the collagen structure first at the fibrillar level and then at the molecular level while molecular gliding happens at larger strain, ultimately leading to disruption of fibril structure [27]. Matrix Metalloproteinases (MMP) are capable of degrading an intact triple helical molecule.
2.2 Collagen Degradation

[28, 29] Proteolytic degradation of collagen is important in physiological processes like tissue remodeling, morphogenesis and angiogenesis. It also plays a vital role in induction and perpetuation of pathological states like disuse atrophy, metastasis and cartilage degeneration. Collagen is generally resistant to most proteinases except collagenases [30]. Because of their crucial role in the extracellular matrix degradation, Matrix metalloproteinases (MMP) are implicated in the tissue remodeling processes associated to the growth and development and in several pathological conditions such as osteoarthritis [31]. MMPs are zinc-dependent proteinases which degrade most of the extra-cellular proteins in humans. Fibroblast collagenase (MMP-1), neutrophil collagenase (MMP-8), and collagenase-3 (MMP-13), are classes of MMPs that specifically cleave several types of native triple-helical collagen, and in particular collagen I, at a specific peptide bond between Gly775 and the residue in position 776, leading to the formation of 1/4 and 3/4 fragments [32]. Thus two fragments, a larger N-Terminal three-quarters fragment and a smaller C-terminal one-quarter fragment, are formed. They then get unfolded and are degraded further by gelatinases. [33] One important observation by Gross et al was that these fragments had a denaturation temperature much below than that of native collagen. Therefore it was suggested that collagenase prepares collagen for further degradation by making it thermally unstable at physiological temperatures. The degradation mechanism of collagen by MMPs have been extensively investigated, and preliminary measurements have been carried out on the activation energy of the
catalysis employing porcine collagenases and gelatinases [34]. Previous experiments on the cleavage of native and site-directed mutants of murine collagen by MMP-1, showed the crucial aspects of the recognition mechanism. [35]

Proteolysis of single collagen I molecules has been studied using atomic force microscopy. This shows that simple Michaelis-Menten mechanism is followed in this process. Also the results on bulk solution are compatible with those observed on single collagen molecules. MMP action on collagen proceeds by a ratchet mechanism whereby the enzyme moves along collagen fibrils in steps, alternately binding to and cleaving the available monomers [36], as shown in the Fig 6.

A recent study [37] explains the mechanism of breakdown of collagen by collagenase, typically taken as MMP-1. According to this model, collagenase unwinds triple-helical collagen prior to peptide bond hydrolysis. This can be attributed to the structural dimensions of active site of collagenase and binding site cleft of the substrate, collagen. Thus it was suggested that the conformational state of the substrate significantly influences the activity of collagenase and that unwound collagen is a better
substrate at conditions that decrease the backbone mobility of the triple-helical structure. The strain on the fibrils could alter the conformational state of collagen triple helices and thus makes it difficult or impossible for collagenase to unwind and cleave the specific site. However the exact mechanism and hence the related analytical information of unwinding is not yet understood.

[38] As shown in the table 2, there are 13 types of MMPs based on their domain structures and their preferences for macromolecular substrates. Among the collagenases, [32] interstitial collagenases degrade type I, types II and III collagen into characteristic and fragments; however MMP-8 is more specific to collagen type I and degrades it at faster rate than type II and type III collagen. [38] For identical substrates, MMP-8 has higher $k_{cat}/K_{M}$ values than MMP-1, as shown in Table 1, primarily due to higher $k_{cat}$ values. Thus the specificities of MMP-8 is much higher than MMP-1. Therefore we choose to use MMP-8 for our experiments. However, we suggest that MMP-1 and MMP-13 should also produce the similar results of when tested for strain stabilization.

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>$k_{cat}/K_{M}$ ($\mu M^{-1} h^{-1}$)</th>
<th>Relative Rate</th>
<th>$k_{cat}/K_{M}$ ($\mu M^{-1} h^{-1}$)</th>
<th>Relative Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly-Pro-Gln-Gly~Ile-Ala-Gly-Gln</td>
<td>0.22</td>
<td>100</td>
<td>4.80</td>
<td>2200</td>
</tr>
<tr>
<td>Gly-Asp-Gln-Gly~Ile-Ala-Gly-Gln</td>
<td>0.038</td>
<td>17</td>
<td>2.20</td>
<td>980</td>
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<tr>
<td>Gly-Ala-Gln-Gly~Ile-Ala-Gly-Gln</td>
<td>0.11</td>
<td>50</td>
<td>1.10</td>
<td>500</td>
</tr>
<tr>
<td>Gly-Leu-Gln-Gly~Ile-Ala-Gly-Gln</td>
<td>0.030</td>
<td>14</td>
<td>1.20</td>
<td>570</td>
</tr>
<tr>
<td>Gly-Pro-Hyp-Gly~Ile-Ala-Gly-Gln</td>
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<td>11</td>
<td>0.73</td>
<td>330</td>
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<tr>
<td>Gly-Pro-Asp-Gly~Ile-Ala-Gly-Gln</td>
<td>0.067</td>
<td>30</td>
<td>2.10</td>
<td>960</td>
</tr>
<tr>
<td>Gly-Pro-Val-Gly~Ile-Ala-Gly-Gln</td>
<td>0.071</td>
<td>32</td>
<td>1.40</td>
<td>660</td>
</tr>
<tr>
<td>Gly-Pro-Leu-Gly~Ile-Ala-Gly-Gln</td>
<td>0.32</td>
<td>150</td>
<td>13.0</td>
<td>5700</td>
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<tr>
<td>Gly-Pro-Arg-Gly~Ile-Ala-Gly-Gln</td>
<td>0.038</td>
<td>17</td>
<td>1.50</td>
<td>700</td>
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<tr>
<td>Gly-Pro-Met-Gly~Ile-Ala-Gly-Gln</td>
<td>0.36</td>
<td>160</td>
<td>7.90</td>
<td>3500</td>
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<tr>
<td>Gly-Pro-Tyr-Gly~Ile-Ala-Gly-Gln</td>
<td>0.44</td>
<td>200</td>
<td>5.10</td>
<td>2400</td>
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<tr>
<td>Gly-Pro-Gln-Met~Ile-Ala-Gly-Gln</td>
<td>0.44</td>
<td>200</td>
<td>6.70</td>
<td>3100</td>
</tr>
</tbody>
</table>

Table 1: Comparison of MMP-1 and MMP-8 Specificity.
Taken from Nagase, Fields (1996)
Bacterial Collagenase:

Since it is expensive to use MMP, for initial demonstration of collagen-collagenase interaction under mechanical load, we used bacterial collagenase. The best characterized bacterial collagenase is that of Clostridium histolyticum. It can be produced under standardized conditions by fermentation. The enzyme is secreted by the bacterium into the culture medium in large quantities. In contrast to mammalian collagenase, the bacterial enzyme cleaves collagen into small peptides.

Although the term collagenase implies that there is a single enzyme produced by Clostridium histolyticum, this is not the case; crude collagenases are mixtures of

---

<table>
<thead>
<tr>
<th>MMP No</th>
<th>Enzyme</th>
<th>Collagen Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>Interstitial collagenase</td>
<td>Collagen I, II, III, VII and X</td>
</tr>
<tr>
<td>MMP-8</td>
<td>Neutrophil collagenase</td>
<td>Collagen I, II and III</td>
</tr>
<tr>
<td>MMP-13</td>
<td>Collagenase 3</td>
<td>Collagen I</td>
</tr>
<tr>
<td></td>
<td><strong>Gelatinases</strong></td>
<td></td>
</tr>
<tr>
<td>MMP-2</td>
<td>Gelatinase A</td>
<td>Collagen I, IV, V, VII and XI</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Gelatinase A</td>
<td>Collagen I, IV, V and XIV</td>
</tr>
<tr>
<td></td>
<td><strong>Stromelysins</strong></td>
<td></td>
</tr>
<tr>
<td>MMP-3</td>
<td>Stromelysin 1</td>
<td>Collagen III, IV, IX and X</td>
</tr>
<tr>
<td>MMP-10</td>
<td>Stromelysin 2</td>
<td>Collagen IV</td>
</tr>
<tr>
<td></td>
<td><strong>Others</strong></td>
<td></td>
</tr>
<tr>
<td>MMP-7</td>
<td>Matrilysin</td>
<td>Collagen IV</td>
</tr>
<tr>
<td>MMP-11</td>
<td>Stromelysin 3</td>
<td>Collagen IV</td>
</tr>
<tr>
<td>MMP-12</td>
<td>Metalloelastase</td>
<td>-</td>
</tr>
<tr>
<td>MMP-14</td>
<td>Membrane type 1 MMP</td>
<td>-</td>
</tr>
<tr>
<td>MMP-15</td>
<td>Membrane type 2 MMP</td>
<td>-</td>
</tr>
<tr>
<td>MMP-16</td>
<td>Membrane type 3 MMP</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2: 13 Categories of MMP family. Ref: Nagase, Fields (1996)
enzymes (mostly proteases) secreted by Clostridium histolyticum. All may contain 10 to 18 components (by electrophoresis), only 8 of which have been identified. The products differ by the amount of the components absolute and relative to each other. [39] The component enzymes in crude are two specific collagenases (measured as FALGA units/mg), clostripain (measured as BAEE after reduction with DTT) and a neutral protease (measured as caseinase). In the crude collagenase, the clostripain is mostly inactive, oxidized. An important feature for use in tissue dissociation is the ratio of collagenase to protease. Effective release of cells from tissue depends on the action of both the two collagenase enzymes and the neutral protease, for either alone is not very effective. Collagenase is activated by 4 gram atom calcium (Ca2+) per mole enzyme. One Collagen Digestion Unit liberates peptides from collagen equivalent in ninhydrin color to 1.0 mmole of leucine in 5 hr at pH 7.4 at 37°C in the presence of calcium ions. Solutions of crude collagenase are stable if frozen quickly in aliquots (at 10 mg/mL) and kept frozen at -20°C. Freeze-thaw cycles will damage the enzyme solution. In aqueous solutions bacterial collagenase loses measurable activity in 3 hr. at 4°C. At pH 7.0 in the presence of 1 mM Ca2+ there is no loss of activity in 1 hr. Molecular weight of crude bacterial collagenase is in the range from 68,000 to 125,000.
Chapter 3: Methodology

3.1 Experimental Setup

Figure 8: (A) Schematic of complete experimental setup to carry out degradation of stretched collagen in the micro chamber and observing the process using Nikon DIC microscopy, with a Nikon perfect focus system. (B) In inset, the schematic of micropipette position and collagen gel in strained and unstrained form inside the micro chamber. (C) Design of micro chamber, made to hold a small volume of collagen, 20µL, also giving access to micropipette through universal capillary holder.

1. Nikon TE2000-E microscope and DIC Imaging:
Collagen gelling and degradation were directly observed (magnification 600x) on a Nikon TE2000-E microscope equipped with Differential interference contrast (DIC), an excellent mechanism for rendering contrast in transparent specimens, Fig 8. [40] DIC is a method of obtaining a contrast image from the differences in index of refraction of individual unstained components of a transparent specimen. DIC is appropriately named for “Differential Interference Contrast” because of interference of two different images that result in contrast of the images visualised. As shown in
Fig 9, Typical components in Inverted Nomarski DIC microscopy are a light source, polariser, Upper Nomarski modified Wollaston prism, condenser lens, objective lens, Lower Nomarski modified Wollaston prism, and analyser (lower polariser). Because of the polariser kept in between the light source and condenser, the light is set to one plane polarised light. This light vibrating in one plane passed through the Wollaston prism that separates individual ray of light into ray pairs. These two rays are still in phase but spatially separated and vibrating at 90° apart. The condenser lens helps focusing the light on specimen for good illumination. After passing through the specimen, because of differences in index of refraction, optical path difference is created in ray pairs. As per the specification, the objective lens magnifies the size of image. The ray pair then passes through the lower prism and two perpendicular planes of rays are again combined to one plane. Here the phase difference created because of the specimen is converted to amplitude. Those regions of the specimen where the optical paths increase along a reference direction appear brighter (or darker), while regions where the path differences decrease appears in reverse contrast. As the gradient of optical path difference grows steeper, image contrast is dramatically increased. The analyser after the prism, at the end of optical train helps in rejecting the out of plane light. DIC imaging gives good rejection of out-of-focus interference. Out-of-focus refractive index changes will be blurred and have a shallow spatial gradient in the focal plane, they will therefore not contribute much to the contrast of the image. Nomarski microscopy is generally much more compatible to living specimens than
electron microscopy. However, as Nomarski imaging produces contrast from refractive index gradients, there is very limited scope for developing labeling techniques that can reveal specific components of a specimen.

2. **Nikon perfect focus system:**

As shown in Fig 10, Nikon Eclipse TE2000 PFS system is designed to track the focus automatically so that the point of interest within a specimen is always kept in sharp focus no matter what mechanical or thermal changes take place. PFS uses an LED in the infrared range and an internal linear CCD detector, to detect the focal point, so it does not intrude on wavelengths used for observation. Thus it allows carrying out observation and maintaining focus at the same time, with negligible influence on captured images.

3. **Micromanipulators:**

The microscope is equipped with Eppendorf TransferMan® NK2, Eppendorf, North America which has resolution of 40 nm per micro step for applying strain in collagen gel.

4. **Temperature control system:**

The temperature of the system can be maintained at 37°C using a Bioptechs Delta T4 culture dish controller and objective heater.

5. **Micropipettes:**

The tip of the fire polished Borosilicate (with filament, OD 1.0 mm, ID 0.50mm) pipettes was formed using P-97 Flamming/Brown micropipette puller (Shutter Instrument company, USA), using multi-step program.

Multi-step program was used to achieve the desired shape of a micropipette, as shown in Fig 11. Micropipettes should have enough structural rigidity to resist the forces exerted during collagen gelling also it should not bend while applying strain in
collagen fibril matrix. At the same time, it should not be very thick to avoid interference in DIC optical train. For this purpose, the lower value of pull and lower velocity were used in the beginning to make it thick and short while lower value of pull and higher value of velocity were used at the end to make it thick but long at the tip.

**Program:**

1. Heat=712 Pull=40 Vel=13 Time=200
2. Heat=712 Pull=55 Vel=16 Time=200
3. Heat=712 Pull=65 Vel=12 Time=200
4. Heat=712 Pull=40 Vel=11 Time=200
5. Heat=712 Pull=40 Vel=15 Time=200

![Figure 11: Micropipette design](image)

6. **Surface functionalization:**

Plasma cleaned micropipettes were functionalized to bind collagen using the method of silanization (3 mercaptopropyl – trimethoxysilane) followed by GMBS treatment.
3.2 Experiment Protocol

Following steps were followed to strain the reconstituted collagen network and to induce its degradation by exposure to collagenase:

1. Pipettes were affixed to micromanipulators adjusted into position over the surface of Bioptechs Delta TPG dish. Following guidelines were developed to facilitate in adjusting the micropipettes in micro-chamber:
   a. Make sure that the z knob position is at the bottommost position. Adjust the 60x lens vertical and put a small drop on the lens. Bring 10x lens to position.
   b. Apply little oil on the bioptech stage adapter heating sensor and on the bottom of TPG dish glass in the area of micro chamber. Then fix the dish on the stage adapter.
   c. Insert the micropipettes, made as described in 3.1 #5, in the universal capillary holder and fix the holder at its extreme end on the micromanipulator.
   d. Focus the 10x lens well above (~100 micron) the top surface of the glass. Now slowly move the micropipettes using both, the joystick and the angle adjustment knobs of the micromanipulators. Once the pipettes are visible, take them slowly on the surface of the glass. Keep the micropipettes towards the centre of micro chamber.
   e. By taking the z knob to its bottom most position in every step, move from 10x to 20x and then to 40x and then finally to 60x. In each step take the lens to the glass surface to check if the pipettes are at the centre. Slowly move the z knob upwards till the lens gently touches the glass and tips of micropipettes are in focus. This typically happens at z~3400 micron.

2. As recommended by PureCol, PBS buffer was used for bacterial collagenase assay. However for the suitability of reaction buffer for activation of MMP, buffer was changed from PBS to Tris base [41]. For bacterial collagenase experiments, collagen solution was prepared by mixing: Purified collagen
PureCol™, INAMED, (3.0 mg/mL), buffer (PBS 10X) and NaOH (0.1 M) in 8:1:1. For MMP experiments, collagen solution was prepared by mixing Purified collagen (PureCol™, INAMED, (3.0 mg/mL) with 10x Tris Buffer (Tris-HCl (pH 7.6) containing Tris 0.5M, NaCl 2M and CaCl$_2$ 50mM) in 9:1. By doing this in the final solution, the molarities of Tris, NaCl and CaCl$_2$ were 0.05M, 0.2M and 5mM, which is same as MMP-8 buffer.

3. Neutralized collagen (10 µL) was added to the Delta TPG dish, covered with immersion oil (Cargille, Cedar Grove, NJ, USA, non-drying, Type A, viscosity 150) to prevent evaporation and raised to 37º C in the Delta TPG dish to induce fibrillogenesis both, around and in between the pipettes.

4. Micromanipulators were adjusted to apply a suitable tensile strain.

5. Bacterial collagenase (crude, Sigma-Aldrich C0130, lot 016K1251) made in GIBCO, DMEM media is introduced (10 µL, 0.0125M) in the loaded fibrils and collagen degradation kinetics was observed. In the final solution, there were 12.5 nM of collagenase and 4.39 nM of collagen. Recombinant Human MMP-8 (Chondrex #5001) stored in Tris Buffer (Tris-HCl (pH 7.6) containing Tris 0.05M, NaCl 0.2M and CaCl$_2$ 5mM) was activated by adding APMA [42] dissolved in 0.1N NaOH, final molarity 1mM, in 20:1 ratio and incubating the solution at 37ºC for 1 Hr just before mixing it to collagen. 10 µL of this solution was added to 10µL of collagen gel. The final molarities of collagen and MMP-8 in reaction buffer were 4.39 nM and 0.65 nM respectively.

6. Images of the observed degradation were taken at certain time intervals. The time interval for most of the experiments was taken as 10sec. However, for MMP experiments the time was varied in three phases 20sec, 10sec and 60sec. These time intervals were taken into consideration while analysing the degradation rate. The images were taken by Photometrics® (Pleasanton, CA) CoolSNAPHQ2 Monochrome, fast and high-resolution camera (20 MHz, 1392 x 1040 imaging array). When coupled with 60X magnification, the pixel dimension are 110x110 nm.
The following actions were taken to avoid possible sources of variations:

i) Collagen: control experiment showing that the collagen gelling remains same for the collagen taken from stored neutralize collagen kept at 4°C.

ii) To avoid freeze-thaw cycles, a) for Collagenase: the lot was divided into small volumes and stored at -20°C; b) For MMP: after adding APMA, the vial was divided into small volumes, generally 10 µL, and stored at -80°C. This small volume was used directly thus eliminating variation due to freeze/thaw cycle.

iii) Diffusion: As a control experiment for diffusion variations, degradation of unstrained collagen matrix around and between the pipettes was found to be uniform in all cases.
3.3 Calculations

1) 10x Tris Buffer preparation: PureCol collagen is dissolved in 0.01 N HCl, therefore, the pH is approximately 2. For fibril formation at physiological pH (~7.4), Tris buffer solution (10x) is added to acidic collagen solution. The following method was used to prepare 10x Tris buffer solution:

First, the high pH (~11) of TrisBase was reduced to 7.5 using concentrated HCL (7M). This can be done by titrating 5000 µL of Trizma (1.5M) with 800 µL of concentrated HCL. This will reduce the molarity of Trizma to 1.293M. However the final molarity of Tris should be 500 mM in 10x Tris buffer solution. Following table explains the preparation of individual NaCl and CaCl₂ to obtain their final molarities in 10x Tris buffer solution.

<table>
<thead>
<tr>
<th>Table 3: 10x Tris Buffer molarities</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td>Molecular weight</td>
</tr>
<tr>
<td>In 10 ml, dissolve</td>
</tr>
<tr>
<td>Individual Molarity</td>
</tr>
<tr>
<td>Final Required morality in 10x solution:</td>
</tr>
</tbody>
</table>

* since CaCl₂ used has 2 molecules of H2O

Therefore, for the final molarities of Tris, NaCl and CaCl₂, above 1.293M Tris-HCL, 3432mM NaCl and 1630 mM CaCl₂ are added in 1000:1510:80 µL ratio. Since this 10x solution is added to collagen in 1:9 ratio, in collagen neutralised solution, the molarities of Tris, NaCl and CaCl₂ are brought down to the required 50mM, 200mM and 5mM respectively.

2) Molarities of essential components of substrate and enzyme in reaction buffer:

The dilution buffer should always have Tris-NaCl-CaCl₂ molarities same as MMP buffer. This will keep the best conditions for specified activity of enzyme during incubation. In this case, the molarities in collagen solution were kept same as MMP and we observed the good gelling. Depending on the requirement, collagen
molarities can be adjusted; however it will alter the molarities in reaction buffer which in turn will have a slight adverse effect on MMP activity in reaction buffer particularly in long run of the experiment.

Table 4: Molarities in reaction buffer

<table>
<thead>
<tr>
<th>In the chamber</th>
<th>Molarity of MMP + Collagen Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MMP 8</strong></td>
<td>µL</td>
</tr>
<tr>
<td>Tris Base</td>
<td>NaCl</td>
</tr>
<tr>
<td>0.05</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Dilution Buffer</strong></td>
<td></td>
</tr>
<tr>
<td>Tris Base</td>
<td>NaCl</td>
</tr>
<tr>
<td>0.05</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Collagen</strong></td>
<td></td>
</tr>
<tr>
<td>Tris Base</td>
<td>NaCl</td>
</tr>
<tr>
<td>0.05</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Final Molarity in chamber</strong></td>
<td></td>
</tr>
<tr>
<td>Tris Base</td>
<td>NaCl</td>
</tr>
<tr>
<td>0.05</td>
<td>0.2</td>
</tr>
</tbody>
</table>

3) Activity of MMP and Theoretical time for complete degradation:
1 unit of MMP activity is defined as 1 mL of MMP will degrade 1 ug of collagen in 1 min.

Table 5: MMP Activity and Time for complete degradation

<table>
<thead>
<tr>
<th>Activity and Degradation time calculations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity rate for collagen fibrils</td>
</tr>
<tr>
<td>Collagen degraded in 1 min</td>
</tr>
<tr>
<td>MINUTES required for complete degradation</td>
</tr>
<tr>
<td>Total time for degradation</td>
</tr>
</tbody>
</table>

*The activity for MMP-8 for collagen monomers is specified as 100 units. However, the packing of monomers into fibrils decreases the MMP activity to approximately 10 units [41]. The experimental results show that the activity varies from 10-20 units on collagen fibrils.
4) **Number of collagen and collagenase molecules in reaction buffer:**

Table 6: Collagen and MMP molecules in reaction buffer

<table>
<thead>
<tr>
<th></th>
<th>Collagen</th>
<th>MMP-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration mg/ml</td>
<td>3</td>
<td>0.1</td>
</tr>
<tr>
<td>Volume used in chamber (µL)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mass of collagen in chamber µg</td>
<td>27</td>
<td>1</td>
</tr>
<tr>
<td>Molecular weight (Dalton)</td>
<td>300,000</td>
<td>75,000</td>
</tr>
<tr>
<td>Weight of collagen molecule (gm)</td>
<td>5.01E-19</td>
<td>1.25E-19</td>
</tr>
<tr>
<td>Number of molecules in chamber</td>
<td>5.39E+13</td>
<td>7.98E+12</td>
</tr>
</tbody>
</table>

*1 Dalton = 1.67E-24 gm

Note that the ratio of collagen molecules to MMP molecules is 6.75. This was designed to create preferential choice for MMP to bind and to cleave collagen molecule, anticipating that the MMP molecules will bind onto the unloaded molecules first.
Chapter 4: Results

We here demonstrate directly, using live, dynamic, differential interference contrast imaging, that networks of reconstituted collagen fibrils, exposed to collagenase (Clostridium histolyticum and MMP-8), degrade preferentially based on their state of strain. Specifically, unstrained fibrils were removed quickly while strained fibrils were degraded at a significantly slower rate. The demonstration supports the idea that collagen networks are mechanosensitive such that they are “stabilized” against enzymatic cleavage in the presence of strain. Thus, collagen molecules and their complement enzymes may comprise a truly “smart”, load-adaptive, structural material system which can be utilized by tissue engineers to produce organized connective tissue *de novo*.

In Fig 12(A), collagen fibrils in the matrix are clearly visible. Mechanical strain was applied to the observable network using micromanipulators and micropipettes. In Fig 12(B), Bacterial collagenase has begun degrading the matrix (principally the “unstrained” matrix surrounding the pipettes). Fig 12(C) demonstrates that the fibrils under load were “protected” and that the surrounding unloaded matrix was preferentially removed or “culled” from the system. The .tif images obtained from the Nikon NIS were subjected to edge detection as described in the Image analysis section. The edge detected black and white images were obtained using an ImageJ software plugin which uses Deriche-Canny edge detection algorithm [43]. The images shown are full length however the edge detection was done based on the region of interest (ROI) taken in between the pipettes for strained collagen fibrils and another away from the pipettes for unstrained collagen fibrils, as shown in the Fig 16.
4.1 Preferential Degradation of Collagen by Collagenase

Figure 12: Time sequence (A,B,C) of collagen degradation by bacterial collagenase: [left side: DIC images; right side: Edge detected images]

#1 - ROI corresponds to unloaded fibrils.
#2 – ROI corresponds to loaded fibrils.
#3 – Note that the bunch of fibrils degraded as fast as unloaded no-bunched fibrils, indicating that diffusion of enzyme is not limited.
The graph below shows the degradation of collagen fibrils by bacterial collagenase as detected by edge detection algorithm as described in the Image Analysis section 4.3. In graph A, the number on the Y-axis represents the number of white pixels (and hence the presence of fibrils) in the edge detected ROI. Note that since the collagen gel is highly disorganized, the number of fibrils in the same area of ROI could be different. Therefore as shown in the graph B, we have normalized the graph A for proper comparison. Because of the pipette vibrations, the fibrils go in and out of the focal plane. This causes fluctuation in the graph.

Figure 13: Plots of collagen fibrils degradation by bacterial collagenase.
4.2 Preferential Degradation of Collagen by MMP-8

Following are image sequence from the experiment where the collagen fibrils under strain in between the pipettes and the unloaded fibrils away from the pipettes were subjected to degradation by MMP-8. It can be noticed that the fibrils under load could resist the enzymatic attack and they stayed longer than fibrils under no load.

Figure 14: Time sequence of collagen fibril degradation by MMP-8, indicating the preferential degradation of unloaded fibrils by MMP-8.
The graph below shows the degradation of collagen fibrils by MMP-8 as detected by the edge detection algorithm as described in 4.3.

Figure 15: Plots of collagen fibril degradation by MMP-8

Fig 15 (A) : ROI data  Fig 15(B): Normalized data
The collagen- bacterial collagenase experiment (n=12) which showed the preferential degradation when under loaded were subjected to two tailed unequal variance Student’s T test and it shows P=0.03136 for Figure 16 (C), indicating a significant difference between the respective times observed. Only 3/21 experiments didn’t show the significant preferential degradation when under load. The table shows the initial time for the start of degradation, degradation time for the unloaded fibrils and for the loaded fibrils.

Figure 16: Degradation times for loaded and unloaded collagen fibrils in bacterial collagenase experiments.

T11, T22 - Average time for the start and end of the unloaded collagen fibrils
T33, T44 - Average time for the start and end of the loaded collagen fibrils.
T12 = T22-T11 ; T34 = T44 –T33 ; T14 = T44-T11.

P value for Fig 16 (C) P=0.03136 ; for Fig 16 (D) P=0.00636
For each of the MMP experiments (n=4), there was a significant difference in the
time to degrade the unloaded and loaded fibrils. (Fig 17) Thus, MMPs are more
sensitive to strain induced molecular distortion given their specificity for one
cleavage location and the requirement that the collagen monomer is reoriented
during cleavage.

![Graph showing degradation times](image)

Figure 17: The degradation time of collagen by MMP-8
4.3 Image Analysis

The following steps were followed to detect the onset of degradation and the rate of degradation from the image sequence obtained from DIC microscopy. The image sequence present in Nikon NIS Element software is present in .nd file. The images from .nd files were extracted in .tif format. The LUT was adjusted for 100% pixel under the curve and gamma line (gamma=1) varying from 0 to 256.

1. The sequence of images was imported in ImageJ software [43]. It is suitable to convert the images to 8 bit format while importing. Also, it is convenient to specify the initials of files names and sort them numerically to get the correct sequence in the stacked files.

2. The region of interest (ROI) was selected using rectangular box, one in between the pipettes in strained region and other away from the pipettes in unstrained region. These ROIs can be stored by “save as → selection” for future reference. Individual ROI was cropped and saved using the name “strained_original_” and “unstretched_original_” respectively.

3. The ROIs were treated with brightness and contrast adjustment. The width of gamma line was reduced from 0-256 to minimum and maximum value in the histogram of first image of either strained or unstrained image, whichever is higher. This reference width remains constant for all the images in both the ROIs. Reducing the width enhances the brightness and contrast in the image which is helpful for edge detection and the same time keeping it same for all images assures that the contrast in both the ROIs is same and hence can be compared. These files are stored by the function by suitable names.
4. The contrast adjusted files were then opened in ImageJ. All files in the stack were blurred using a small Gaussian blur (r=1). This will reduce the noise particularly from the images with less fibrils. The files were then subjected to edge detection using ImageJ plugin “Deriche Canny” edge detector. The parameter “alpha” was adjusted to 0.3 (this can be set to 0.5 depending on the image clarity). Of the two image sequences generated, “Canny-Deriche suppr” was taken for further analysis. These images were stored by converting them to 8bit format by the name “strained_edge_” and “unstretched_edge_” respectively for strained and unstrained ROIs.

5. These ROI edge detected images were subjected to MATLAB code (1), to obtain the degradation rate results in strained and unstrained region.

6. In case, if the experiment contains floating debris or a small number of bacteria, those were removed using the following procedure:

*Figure 19: Image contrast adjustment*

**Fig A:** The histogram distribution as taken from NIS software.

**Fig B:** Stretching the lower value of histogram to 0 and higher value of histogram to 256, thus introducing more contrast in the FIRST image.

**Fig C:** However the above width is kept constant about the mean value of histogram, in the subsequent images to assure that no additional contrast is introduced with respect to first image.
a. The ROI which has bacteria was imported to ImageJ software. Using the plugin “Manual Tracking”, X-Y data for each bacterium was obtained by simply clicking by mouse on its centre, in each of the entire image sequence. This data is stored in .xls file named as “TrackingData”. Using Matlab code(3), the ROI images were subjected to Gaussian filter in a small circle around the bacteria present in the XY coordinate as per TrackingData.xls.

b. These files were then saved by the program using the names generated by another code, as explained in the Matlab code (2), run previous to running the step (6a).
4.4 Collagen Gelling and Polymerization under Strain

It is shown in the experiment, see figure 20 and also in the literature that in the absence of load, collagen self assembles into a network fibrils which is highly disorganized. However it has been hypothesized that the self assembly of collagen could be preferential along the direction of load. Following procedure was followed to observe the above. Micropipettes of fire polished Borosilicate (with filament, OD 1.0 mm, ID 0.50mm) were made using the P-97 Flamming/Brown micropipette puller. The pipette has an opening of around 5-7 µm. These pipettes were fixed to universal capillary holder (Eppendorf) and manipulated by Micromanipulator (Eppendorf). The capillary holders were attached to manual piston pumps (Eppendorf CellTram Air and CellTram Oil). This is to provide control over suction of collagen during the initial phase of the experiment. The above two micropipettes were adjusted above the glass surface of small chamber in a delta dish observed through 60x objective of Nikon DIC microscope. Tips of the two pipettes were kept almost touching each other. Collagen type 1 solution, neutralized by Tris-HCl buffer (containing NaCl and CaCl₂, as described above), was added to the chamber (approximately 15µL). Low viscosity immersion oil was added as a top layer on collagen solution to avoid evaporation during long the run of the experiment.

To initiate the self assembly of collagen, the temperature of collagen solution inside the chamber was raised to 37°C using Bioptechs delta dish controller. The gelling between the pipettes was initialized by a small suction. Once few fibrils were formed between the pipettes and were put under tensile load, the suction was stopped and

![Figure 20: Disorganized collagen self assembly](image)
the distance between the pipettes was increased using micromanipulator. The rate of increase of this distance was maintained as low as a micron per second. It was observed that the higher strain rate can break the collagen fibrils. However the range of sustainable strain rates and its effect on polymerization should be carried out as a future work. The final distance between the pipettes was around 30 µm. It was observed that during this extension from 0 µm to 30 µm, the collagen between the pipettes remain stretched and the width of bunch of collagen fibrils between the pipettes remained constant. This can be explained if the soluble monomers around the stretched fibrils preferentially self assembled on the stretched fibrils. It was interesting to notice that less fibrils were formed in the area where there was no load as compared to where the fibrils were under load.

![Figure 21: Time sequence of collagen polymerization under strain](image)

Figure 21: Time sequence of collagen polymerization under strain
Chapter 5: Discussion

5.1 Collagen Degradation by Collagenase

The results, in the above section, provide significant evidence that strain stabilizes collagen against enzymatic degradation by collagenase (bacterial and MMP-8). In past studies [11-13], the experiments were performed at tissue level using only bacterial collagenase; however in this study we directly observed (using DIC) the phenomenon of strain stabilization of collagen at fibril level. Also, the demonstration using physiologically more relevant recombinant human MMP-8 was the first of its kind. In the series of experiments performed, the degradation time data was collected. A novel method of finding the degradation times and hence the rate of degradation using series of images was developed primarily through edge detection algorithm. As explained in the Image analysis section, DIC images taken from Nikon NIS software were converted to edge detected files using ImageJ Canny-Deriche plugin. These edge detected files were then converted to black and white images. The white color in this image represents the presence of fibril and black color is background. Thus from the graph it was easy to detect the start of degradation and loss of fibrils (and hence fibril edges) with time and the complete degradation of fibrils. It was found that the time for degradation varies between the experiments with the best possible identical setup. Despite of the control measures as described in the experiment protocol section 3.2, the time for the start of degradation shows significant variation in different experiments. However for an experiment under the
consideration, the fibrils in loaded and unloaded matrix were exposed to the same state of variable parameters. Thus state of strain was only the parameter different for the fibrils in between the pipettes and away from pipettes. For a future work, an innovative method for determining the strain in collagen fibrils is required. This can provide us a relationship of strain and degradation rate. Also for sustainable higher values of strain, the cross linking of collagen fibrils [44, 45] is required.

5.2 Polymerization of Collagen under Strain

Since early 1960s, the phenomenon, mechanism and kinetics of collagen self assembly in vitro has been the interest of study [8-10]. Mechanical properties of collagen fibrils, both self assembled and native, have been studied [45, 46]. Also, there are studies on effect of temperature, buffer chemicals, and pH on collagen self assembly [47]. For the polymerization experiments, we are interested in how applied mechanical strain changes the rate of polymerization of collagen fibrils and the organization of the resulting fibrils. The results obtained during the polymerization of collagen under tension suggest that collagen monomers tend to incorporate into fibrillar structures which are under strain. The fibrils in the direction of load appear to be thicker than the unloaded fibrils. However no quantitative measurement is done to assess the rate of incorporation or its relation with the amount of strain. Also, since the micropipettes used in this experiment were hollow and were subjected to initial suction, therefore it is possible that some fibrils formed inside the pipettes were
drawn out. However the suction was stopped immediately after the formation of first few fibrils in between the pipettes. So the “draw out” effect can be neglected.

As discussed before, some of the studies suggest that epigenetic adaptation of connective tissue ECM is largely provided by resident fibroblasts which transduce mechanical loads. However one of the most attractive implications of preferential polymerization of collagen under load will be that it relieves fibroblast of the burden of knowing which fibrils to retain and which to degrade. Thus collagenous matrix remodeling should be directed by most relevant control signal: mechanical strain at the matrix level.

For future work, we propose a novel method using FITC labeled collagen monomers. In this experimental setup, collagen fibrils should be loaded using micropipettes as described in the experiment protocol section above. Once the fibrils are under load, fresh FITC labeled collagen monomers should be introduced near the loaded fibrils. Fibrillogenesis of this FITC collagen monomers should be observed using Fluorescence microscopy. These results will provide us the exact kinetics of incorporation of monomers onto the loaded fibrils.
Chapter 6 Conclusion

In our experimental setup, some of the randomly reconstituted collagen fibrils were put under mechanical load using micropipettes. Both using bacterial collagenase and recombinant human MMP-8, we have demonstrated that the enzymatic degradation of loaded fibrils proceeds more slowly than it does for unloaded fibrils. Thus we conclude that strain stabilizes collagen against enzymatic degradation. Also, in another result, preliminary data suggest that mechanical signals direct the preferential incorporation of collagen monomers into fibrils. Taken together, it suggests that in vivo the load bearing collagen with its complementary enzymes constitutes a smart mechanochemical system capable of self optimizing in extracellular matrix primarily using the signal of mechanical loads. This idea significantly simplifies the complex process of collagen formation and degradation based on fibroblast directed adaptation.

Using the above two conclusions, in vitro, we propose a novel method where organized collagenous tissue can be produced merely by application of appropriate strain in the presence of excess of monomers and an appropriate concentration of enzymes. This has a straightforward application in growing tissue engineering constructs.
References


Sigma-Aldrich, COLLAGENASE, CRUDE, General Use from Clostridium histolyticum (Sigma Prod. No. C0130).

Appendix: 1 Collagen degradation by bacterial collagenase

1) 15May2007
(2) 11 Jun 2007
(3) 10May2007
(8) 18Jun2007
Appendix: 2 Collagen degradation by MMP-8

(1) 17 April 2008
(2) 01August2008
(3) Control experiment

![Image](image.png)

![Graph](graph.png)
Appendix: 3 MATLAB Codes for edge detection

1. For generating degradation graphs in strain and unstrained ROIs

% First asking for the previous results, if any.
clc;
reply = input('Do you want to use the previous Results? Y/N [Y]: ','s');
if reply == 'Y';
    % Reading from .MAT file generated in the previous run of this program
    load('degradationDATA','datax', 'dataROI1', 'dataROI2');
    figure
    plot(datax,datayS,'r');
    hold on
    plot(datax,datayNS,'b');
elseif isempty(reply)
    reply = 'Y';
else
    [datax, dataROI1]= getthedata('strained_edge_*.tif');
    figure
    plot(datax,dataROI1,'r');

    [datax, dataROI2]= getthedata('unstreched_edge_*.tif');
    hold on
    plot(datax,dataROI2,'b');

    % Save the result data in .MAT file for future quick reference.
    save('degradationDATA','datax','dataROI1', 'dataROI2');
    xlsdata=[datax;dataROI1;dataROI2];
    % Generating an excel file with Strained and Unstrained data
    xlswrite('AutoLUT_matlabIMGADJUST_Blur_edge_true',xlsdata');
    clear;

2) Details of “getthedata” function to calculate the number of white pixels in the ROI.

```matlab
function [datax datay] = getthedata(filenamelogic)

dirOutput = dir(filenamelogic);
fileNames = {dirOutput.name}';
numFrames = numel(fileNames);

datax=zeros(1,numFrames);
datay=zeros(1,numFrames);

time=0;
for frame=1:numFrames
    if frame<362
        datax(frame)= time;
        time=time+20;
    elseif frame<1443
        datax(frame)= time;
        time=time+10;
    else
        datax(frame)= time;
        time=time+60;
    end
    I = imread(fileNames{frame});
    J=im2bw(I,0.15);
    [countS, s]= imhist(J);
    datay(frame)= countS(2);
end
```

```matlab
% [countS, s]= imhist(I); %     sum_intensity=0;
% for i=1:256
%     sum_intensity = sum_intensity + countS(i)*s(i);
% end
% datay(frame)=sum_intensity;
end
```
3) Generating file names for the images without bacteria

dirOutput = dir('degra*.tif');
fileNames = dirOutput.name';
numFrames = numel(fileNames);

for frame=1:numFrames
    if frame<=9
        numbertostring=int2str(frame);
        strnumberframe=strcat('000',numbertostring);
    elseif frame<=99
        numbertostring=int2str(frame);
        strnumberframe=strcat('00',numbertostring);
    elseif frame<=999
        numbertostring=int2str(frame);
        strnumberframe=strcat('0',numbertostring);
    else
        strnumberframe=int2str(frame);
    end

    newfilenameframe=strcat('NoBacteria_',strnumberframe);
end

save('JUSTfilenames','newfilename');
4) Removing bacteria from ROI files using Gaussian filter:

dirOutput = dir('NoBacteria*.tif');
fileNames = dirOutput.name';
numFrames = numel(fileNames);
rc=17; % Specifying the radius of bacteria in the file in term of pixels
Trackingdata=xlsread('TrackingData');
load('JUSTfilenames','newfilename');
[length, width]= size(Trackingdata);
numberofbacterias=length/numFrames;
h=fspecial('gaussian',25,5);
for frame=1:numFrames
    I = imread(fileNames(frame);
    for nthbacteria =1:numberofbacterias
        xc=Trackingdata((frame+(numFrames*(nthbacteria-1))),1);
        yc=Trackingdata((frame+(numFrames*(nthbacteria-1))),2);
        [c r]= getcircle(xc, yc, rc);
        BW = roipoly(I, c, r);
        I = roifilt2(h,I,BW);
    end
    imwrite(I,newfilename(frame),'jpg'); %files are stored in 8bit format
end

function “getcircle” to generate a circle around the XY center of bacteria

function [c, r] = getcircle(xc,yc,rc)
c=zeros(1,360);
r=zeros(1,360);
for i=1:1:360
    c(1,i)=xc+rc*cosd(i);
    r(1,i)=yc+rc*sind(i);
end
5) To adjust the intensity of images keeping the constant width of histogram:

```matlab
I=imread('unstreched_original_0001.tif');
imhistI=imhist(I);
Imin=double(min(I(:)));
Imax=double(max(I(:)));
Imean=double(mean2(I));
vmax=255;

Imin_percentage = Imin/vmax;
Imax_percentage = Imax/vmax;
Imean_percentage = Imean/vmax;

both_side_chop_off = 0.02;

Left_adjustment = Imean_percentage -
    (Imin_percentage+both_side_chop_off);
Right_adjustment = Imax_percentage -
    (Imean_percentage+both_side_chop_off);

dirOutput = dir('strained_original_*.tif');
fileNames = {dirOutput.name}';
umFrames = numel(fileNames);

datax=zeros(1,numFrames);
datay=zeros(1,numFrames);

for frame=1:numFrames
    I = imread(fileNames{frame});
    mean_percentage=mean2(I)/vmax;
    left=mean_percentage-Left_adjustment;
    right=mean_percentage+Right_adjustment;
    J=imadjust(I,[left,right]);
    %J=imadjust(I);
    if frame<=9
        numbertostring=int2str(frame);
        strnumber{frame}=strcat('000',numbertostring);
    elseif frame<=99
        numbertostring=int2str(frame);
        strnumber{frame}=strcat('00',numbertostring);
    elseif frame<=999
        numbertostring=int2str(frame);
        strnumber{frame}=strcat('0',numbertostring);
    else
        strnumber{frame}=int2str(frame);
    end
    newfilename{frame}=strcat('Strained_Adjusted_',strnumber{frame});
    imwrite(J,newfilename{frame},'jpg');
end
clear;
```