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Use of Imaging Techniques to Assess Skin Wound Healing for Tissue Engineering and Regenerative Medicine

Dale Feldman*

Department of Biomedical Engineering, University of Alabama, USA

Abstract

To accurately assess effectiveness of a skin wound healing treatment, the rate of restoration of structure and function should be measured. The focus of this paper is on the measurements needed to assess effectiveness and ways in which imaging techniques can be used clinically or in animal models to obtain these measures.

The approach was to develop assessments that can show 1) How far away from the "normal" state the tissue is, 2) How it progresses through the restoration of structure and function, and 3) Assess bioprocesses that have the potential to predict 2. In this case 1, would be wound closure and restoration of mechanical properties. The more 2 is done for wounds, the better we would be able to predict the wound trajectory based on 1 and 3.

The specific measures chosen for 1 and 2 were wound size and wound closure rate (healing rate) plus tissue strength and stiffness. This will show how close the wound is to wound closure, plus the percent and rate of restoration of mechanical function. Following these to complete healing is 2. The main predictive bioprocess for 3 is tissue oxygenation, which is related to angiogenesis. Angiogenesis was chosen, since it is the rate limiting step for healing. Also the main bioprocess that influences mechanical properties; collagen production, was also selected with imaging techniques that can assess the amount and orientation of ECM fibers.

The paper showed that imaging techniques have been useful in quantitatively measuring key parameters of skin wound healing: Healing rate, tissue health, and tissue function. As imaging techniques improve they can play an even bigger part as well as provide more accurate data; more closely approximating histological images to allow better clinical assessments without biopsies.

Keywords

Wound healing assessment, Imaging for wound healing, Healing rate determination, Angiogenesis assessment, Mechanical properties of skin wounds

Introduction

To accurately assess effectiveness of a skin wound healing treatment, the rate of restoration of structure and function should be measured. Effectiveness can also be viewed in terms of time (treatment, recovery, return to work, etc.), resource cost (to patient, to healthcare providers, or insurance companies), or human factors (success rate, ease of use, or training required). This study will concentrate on imaging techniques to assess restoration of structure and function. Since imaging techniques are continually evolving, the paper will be organized based on the measurement needed. Then how imagining techniques can be used to obtain these measurements clinically and in animal models. As imaging techniques evolve there will be better methods to obtain these measurements as well as more can be done in the clinical environment. Therefore the measurements needed will not change significantly over time, but the best imaging techniques to use. This paper is not intended to be an exhaustive list of imaging techniques that are cur-

***Corresponding author:** *Dale Feldman, PhD, Department of Biomedical Engineering, University of Alabama, 361 HOEN, AL 35294, Birmingham, USA, Tel: 205-934-8426, Fax: 205-975-4919, E-mail: dfeldman@uab.edu*

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rently being used (clinically or experimentally) to make these measurements; more ones that I have actually used.

Since the effectiveness is in comparison to current treatments, the measurements should be as quantitative as possible to help determine: 1) How far short current treatments are from the desired clinical outcome, 2) How significant a problem this is and 3) How big a difference in restoration rate would actually make a clinical difference.

Current technology has not really produced tissue engineered products that serve as a regenerated functional organ (even in skin), so they need to serve as degradable regenerative scaffolds [1]. The inability to "tissue engineer" functional organs actually follows the *in vivo* model for adult wound healing. Even when tissue or organ grafts can be synthesized *in vitro* the biggest issue is how to incorporate them into the surrounding tissue (e.g. cartilage grafts) and then they still serve more as a degradable regenerative scaffold (like other grafts) [1-3]. The need to be degradable therefore mimics the most common natural scaffold: fibrin [1-4]. In most cases, if the scaffold does not degrade there is no place for the regenerated tissue to go. So the restoration of structure rate is essentially the wound closure (healing) rate. Although this is the important clinical parameter, it should be broken down into regenerative healing vs. repair; which will influence functional recovery [5]. From a design standpoint it is also useful to determine the effect of the treatment on bioprocesses that help determine the healing rate, like angiogenesis. In some cases, a bioprocess may be a rate limiting step (i.e. its rate controls another bioprocess rate) [1]. Typically the measured closure rate for skin is based on epidermal coverage of the wound. Assuming a degradable regenerative scaffold, the epidermal healing rate is dependent on the rate of tissue ingrowth (fibroblasts and the extracellular matrix [ECM] they produce). Fibroblasts need to be close enough to the blood supply to get enough oxygen to proliferate and produce ECM [1,6]. Therefore blood vessel ingrowth limits the rate of fibroblast ingrowth which limits the rate of wound closure. Although wound closure rate is a good clinical outcome measure, restoration of structure does not mean restoration of function. Skin has a number of functions; many of which fully recover only after the wound is closed and the tissue remodels. One clinical outcome measure that imaging has been used for is restoration of mechanical properties. Imaging is also useful in assessing the bioprocess of collagen production, which is a key factor in determining mechanical properties.

These measures (if used clinically) should not only be quantitative, but easy to use, non-invasive, and repeatable [5]. The lack of these clinically used tools poses problems in comparing new treatments or skin substitutes to traditional treatments. Again this paper will discuss the measurement needed to determine clinical effectiveness and then look at imaging techniques to obtain these measurements. In most cases the best imaging technique is histology (microscopy as the imaging technique); which would require a biopsy clinically or use of an animal model. The paper will also cover other imaging techniques that could be used (both clinically and in an animal model). Many of the improvements in imaging techniques are to obtain images with enough resolution to get the measurements with accuracy comparable to histology slides.

Tissue state

Another way to look at assessment of skin wound healing is to measure tissue state as a comparison to the normal state as well as to track the tissue state during treatment. Although there is not a consensus on what is the "normal" tissue state there are certain structural and function properties, which can be monitored over time. An example is mechanical properties (strength and stiffness) assessed by mechanical testing at different points; characterizing the recovery of function. It can be indirectly assessed by imaging; monitoring the amount, type, and orientation of ECM fibers. Other bioprocesses such as collagen deposition and angiogenesis can be assessed over time as well. Healing rate can be considered a bioprocess, but is also a key clinical outcome measure.

For all these measures or bioprocesses a typical trajectory can be plotted (amount or rate at various time points) up until complete restoration of structure and function is achieved. These plots can be used in multiple ways including: 1) Determining how far away a wound is from the "normal" state, 2) Predict the trajectory of the measure based on the "typical trajectory", 3) Measuring how much a given treatment (or pathology) moves closer to (or farther away from) the "normal" state as well as if it changes the trajectory, and 4) Finding other bioprocesses, which are predictive of these trajectories and/or changes in trajectories based on treatment. Using strength as an example, typical increases in properties (trajectories) can be determined as a wound heals and approaches the strength of "normal" skin. Different treatments can be compared on how they change the trajectory of the strength vs. time. Also is the rate of angiogenesis predictive of collagen production and rate of increase in strength.

For skin wounds, such as skin ulcers or burns, these assessments can be put into three groups: Healing rate, tissue health, and tissue function [5]. Healing rate is the speed in which the wound is closed and should be determined independent of wound size, in order to accurately compare treatments [5]. For healing rate, wound volume and surface area are needed to obtain tissue fill rate. Also the wound margin and epithelial margin need to be mea-

sured to determine epithelialization rate and contraction rate. Tissue health would be an assessment of the wound microenvironment in an effort to determine the ability of the wound to heal. An assessment of blood flow or vascularity, which in fact helps determine tissue oxygen level, is a measure of tissue health [5]. Tissue function would be an assessment of the skin's ability to perform its normal functions. Assessments of mechanical properties and epidermal barrier functions can be used to determine tissue function [5].

In general, healing rate and rate of regaining tissue function are the clinical performance design constraints (restoration of structure and function); with tissue health more of an indicator of how the other two will progress in the future. To a degree both of the rate trajectory curves have predictive value for their own tissue state measure as well as the other tissue state trajectory; but the relationships need to be determined. As more and more tissue states are determined, as well as their trajectories with and without treatment or pathology, these predictive models will become more accurate. A chronic wound could be defined as one that does not change its tissue state. Also the benefits of treatments could be compared in terms of predicted changes in tissue state trajectories. Although clinically it is best to select assessment systems that are easy to use, portable, low cost and readily available in all clinical settings; this paper will concentrate on how imaging techniques, in general, can be used.

Healing rate

The assessment of healing rate is an important clinical tool both for comparison of treatments as well as for prognostic value. Clinicians use a variety of methods to assess healing rate and determine clinical endpoints. Traditional measures of healing can generally be divided into methods that determine healing rates over time and those that use clinical endpoints. The former method includes changes in surface area or volume healed, percent epithelialization or closure, and changes in surface area epithelialized or contracted. The latter includes length of hospitalization, time for complete healing or closure, and time for complete epithelialization.

For most of these measures it is difficult to compare wounds of different sizes or even develop a predictable trajectory [7]. For example, a large, shallow wound has an increased wound edge perimeter and can lay down more tissue in a given time than a smaller wound. Similarly, a deep wound has more surface area than a shallow wound and can create more new tissue in a given time in order to fill the wound [5]. This causes a deceptively faster healing in larger or deeper wounds compared to smaller or more shallow wounds (or comparing wounds at the beginning of healing to those near wound closure), even if the two wounds are healing linearly from the wound periphery at the same speed. These limitations cause deceptive, inaccurate results and prevent quantitative comparison of the healing of different wounds or even the same wound at different times. It is therefore important to develop a healing rate that is independent of the area and/or volume of the wound.

Traditionally clinically, wound area and volume measurements have been obtained using contact methods; typically direct measurement or use of molding material (calcium alginate is one type) [5]. Images can be input into image analysis systems to obtain accurate area or volume measures. In animal models, microscope images of tissue slices can also be used to obtain the needed measures.

Tissue health

Since oxygenation, and thus angiogenesis, are rate limiting bioprocesses for wound healing [1,8], methods to assess these bioprocesses would provide insight into the potential of these wounds to heal; or in essence assess tissue health. Imaging can be used to create 2-D maps of the wound to provide spatial (and temporal) comparisons between areas within the wound (or adjacent to it) as well as allow correlations to histological results [5]. Further it would give information on the homogeneity of the tissue health, which is important for determining how representative a given tissue slice is of the whole wound.

Clinically, imaging techniques to assess vascular perfusion and flow include: thermography scanning laser Doppler, and functional MRI [9-13]. Also an Oxymap [14] has been used to get 2-D maps of the cornea with diameter and oxygen saturation of blood vessels shown using microscopy and a light wavelength analyzer. Precision and ease of use should be considered in determination of the best tools to be used for clinical wound assessment. In animal models, vascularity can be accessed from histological images. Although these techniques measure tissue oxygenation indirectly, they still are helpful in assessing tissue health, which will indicate the stage of healing and the likelihood the wound will continue to heal.

Tissue function

The skin is the largest organ in the body, and its normal functions are essential to survival. Skin provides coverage and protection against water loss, thermal loss, and chemical, infectious, and other environmental agents. It provides mechanical integrity and protects against external forces. The skin is a multilayer composite structure with varying thicknesses over the body. Anatomically, the skin is divided into two structurally distinct layers, the epidermis and the dermis, which as a unit provide

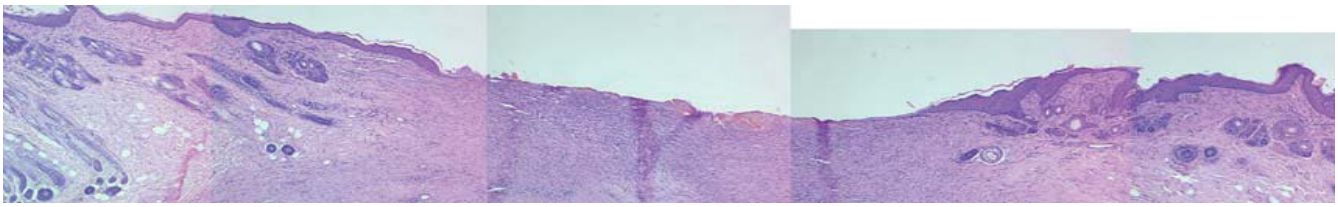

Figure 1: Healing skin wound with normal skin on each side. The epidermis is the thin dark layer on the top, with the bottom layer the dermis. Many of the structures, particularly hair follicles, help show the original wound edge.

the functions of the skin (Figure 1). The epidermis is the cellular outermost layer that is primarily responsible for barrier functions. The dermis is a thicker, mostly acellular layer that gives the skin its mechanical properties as well as provides nutrients to the avascular epidermis.

Healing of skin injuries proceeds through a combination of epithelialization, new dermal tissue formation, and in the contraction to achieve wound closure. The figure shows new epithelialization partially growing over the new dermal tissue in the center area. After wound closure (complete reepithelialization), the tissue remodels to restore function (mostly mechanical). The thickness of the injury is an important factor in determining the extent of each component in the healing process. Injuries to the epidermis and superficial dermis primarily heal by epithelialization from the wound perimeter as well as from epithelial islands around hair follicles and sweat glands. Injuries to the deeper dermis primarily heal by contraction. These deeper wounds, in some cases, exhibit excessive healing and the formation of hypertrophic scar tissue. Contracture, the pathologic shortening of scar tissue, can also occur and is both deforming and debilitating. In both cases, this results in an unacceptable clinical result due to the increased stiffness, or reduced elasticity, of the skin and poor cosmetic appearance [15-18].

Although the goal of skin wound healing is a complete regeneration of the original structure and function, it is difficult if not impossible to achieve this currently clinically. In reality, the best result is probably a functional repair that closely approximates the original tissue and provides virtually all the functions. Skin barrier functions are important in the early stages of healing, to prevent desiccation and infection. The use of skin grafts for burns and specific wound dressing, however, can provide adequate coverage and protection until the epidermis can regenerate. The restoration of acceptable stiffness or pliability long term is still a primary challenge to clinicians. E.g. after a meshed graft heals, in a burn wound, the rehabilitation process to help regain skin flexibility can take a year or longer [1,18].

Researchers and clinicians have devised a number of techniques and instruments to measure the various functions of the skin. Techniques to measure the barrier function have been used by several researchers, especially techniques to measure transepidermal water loss (TEWL) [19,20]. The regenerative ability of the epidermis, and the availability of short-term coverings makes measurements of barrier function less important than assessments of ultimate skin functionality.

Techniques

Again this paper will be organized based on the measurement needed for the particular tissue state. Then how imagining techniques can be used to obtain these measurements clinically and in animal models. This paper is not an exhaustive list of imaging techniques that are currently being used (clinically or experimentally) to make these measurements, but is intended to show the importance of imaging techniques in obtaining these measurements. Further, even though the imaging techniques will continue to improve and evolve the measurements needed will probably not change significantly over time.

Healing rate

Skin wound healing rate in a shallow wound and outward growth rate in cell culture are similar. It has been shown that the epidermal migration rate is relatively constant at approximately 1-2 mm/week [8]. So the radial change in a shallow wound or outgrowth from a bolus of keratinocytes on a cell culture dish per unit time can be relatively constant and should be close to the epidermal migration rate [5]. The amount of new tissue (epidermis in this case) that can be laid down is proportional to the perimeter, so the change in area is not a constant, per unit time, for either a shrinking wound or a growing group of cells [5,7]. It gets even more complicated for deeper wounds where there should not only be epithelialization and contraction rates, but a tissue fill rate (which can also be a radial change per unit time) [5].

Again the other measures that use change in area like percent of wound healed or comparing the percent healed over time curves; relationships that give average radius, or the radial change divided by the average radius [1,20-23], do not give the actual healing rate. Any measure tied to change in area will not be independent of wound size and not be the actual healing rate and at

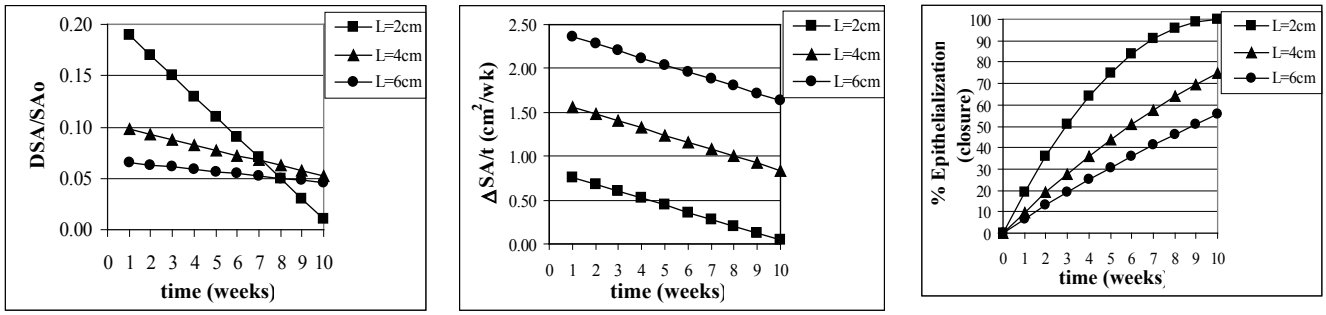

Figure 2: Model Justification (2D). Mathematical model of wound healing shows that traditional measurements of healing change over time. Models assume square shaped wound (side of L) with constant linear inward healing of 1 mm/week. The traditional measurements shown here are as follows: A) Change in surface area of wound (ΔSA) divided by original wound area (SAo); B) Change in wound surface area over time and C) Percent epithelialization (or closure) over time.

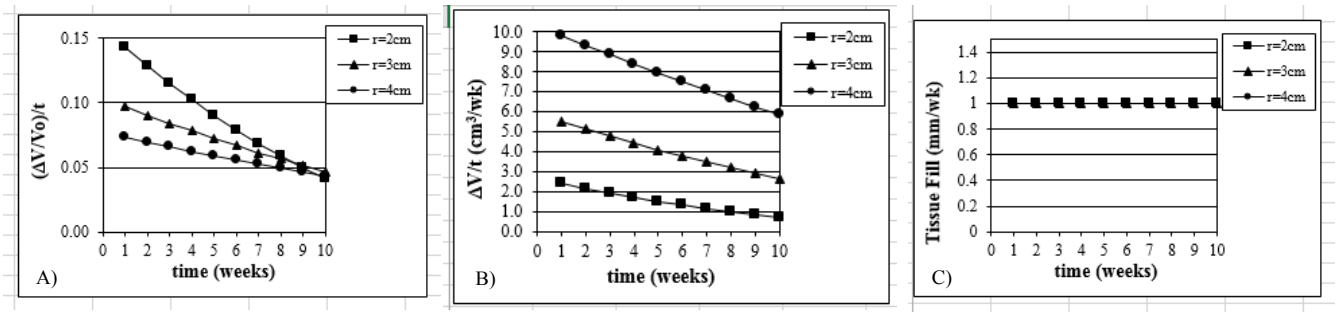

Figure 3: Model Justification (3D). Mathematical model of wound healing shows that traditional measurements of three-dimensional healing change over time. The model assumes a hemispherical wound (radius R) with constant linear inward healing of 1 mm/week. The measurements shown here are as follows: A) Change in volume of wound (ΔV) divided by original volume (V_o); B) Change in volume over time and C) Tissue fill over time.

best will be "an indicator of healing"; providing a trajectory over time that makes it difficult to compare wounds of different sizes or quantifies the effect of a given treatment. Figure 2 shows how, assuming a constant healing rate, the other measures give values that change over time (trajectories); with Figure 2A, Figure 2B and Figure 2C showing measurements typically used.

Although deep wounds do have the tissue fill component, clinically the time of complete epithelialization is an important clinical endpoint (although all the function has not returned yet); so epithelialization and contraction rates are the most critical. It is still important to give a true healing rate. Measures related to average size would have to be validated to prove they relate to time to heal--a healing rate does not because it already is the outcome measure. Again (Figure 3) shows how, assuming a constant TF (tissue fill rate) (Figure 3C), the other measure typically used (Figure 3A and Figure 3B) give values that change over time.

Because of this confusion, currently the FDA uses time to heal as an outcome measure. Wounds of different sizes, even if they heal at the same rate heal at different times making time to heal a noisy parameter (meaning a large sample size is needed to get statistical significance) [1-5]. What we should shoot for is a healing rate to complete healing. This is an important factor in the delay in seeking FDA approval for treatments for wounds that heal, like pressure ulcers, in which the goal is to speed healing. This has forced most investigators to pursue approval for wounds that typically don't heal unless we use the proposed intervention--such as diabetic foot ulcers.

Justification: Traditional models often combine all types of healing into a single measurement and don't normally determine an actual rate. Healing rates of full-thickness or deeper wounds are, however, a combination of three types: epithelialization, contraction, and tissue fill [5,7,24]. The amount of healing via each type depends on the type of wound, depth of wound, and many other variables [24]. A better measurement would isolate these three types and determine the individual rates. In particular, contraction rate, associated with scarring (and increased stiffness) and thus undesirable, should be determined separately. Clinical trials that use endpoints such as total closure can often take months or years to show efficacy, especially if dealing with chronic wounds like diabetic ulcers. The proper model should be

able to give clinically relevant results at all points during the course of healing. Finally, the model should be able to accurately compare the healing of different wounds by being independent of size and shape.

Healing rate model: A quantitative model for calculating healing rate proposed by Gilman [25] and later illustrated by Gorin, et al. [26] fits the necessary requirements of independence of wound size and shape. The method allows for the calculation of a linear healing rate perpendicular to [24-27] the wound edge as a function of wound area and perimeter: Healing rate = $\Delta SA/(P_{avg} \times \Delta t)$, where ΔSA is the change in surface area over the time period and P_{avg} is the average wound perimeter between initial and final time points (Δt). This model provides an accurate, quantitative way to assess healing rates or closure rates at various times during the course of healing. When possible, however, it is better to separate out epithelialization rate from contraction [28-31]; although this is easier in an animal model. Researchers have used this model of a linear healing rate to assess the healing of various skin wounds such as diabetic ulcers, pressure ulcers and skin grafts [5,24.27,32-35].

For shallow wounds like skin grafts or partial thickness wounds, this model is adequate for calculating healing rates. For deeper wounds such as a Stage 3 or 4 pressure ulcer, the 3-dimensional healing rate of the wound should be determined along with the 2-D measures of epithelialization and contraction rates. This 3-D healing rate, or tissue fill rate, can also be determined independent of wound volume [5,7,24]: Tissue Fill (TF) = $\Delta V/(SA_{avg} \times \Delta t)$, where ΔV is the change in volume over the time period, and SA_{avg} is the average surface area of the wound (in 3-D) between the two time points (time). The equation parallels the 2-D closure rate with a change in dimension of the variables. These two calculations work together to quantitatively assess the healing of a wound independent of size, depth, or shape. The TF rate, however, loses some accuracy if there is significant undermining of tissue as found in some pressure ulcers.

Using these calculations, healing rate (HR) can be separated into its three basic components: HR = ER + CR in 2-D and HR = CR + TF in 3-D, where ER is the Epithelialization Rate, CR is the Contraction Rate, and TF is Tissue Fill. Epithelialization rate is defined as the growth of new epithelium either solely from the wound periphery, as in deep wounds like third degree burns, or also radially from epithelial islands present around hair follicles and sweat glands, as in partial-thickness wounds:

$$ER = |\Delta SA_{epithelium}|/(P_{avg} \times \Delta t).$$

Contraction rate represents the change in wound size due to centripetal contraction:

$$CR = \Delta SA/(P_{avg} \times \Delta t).$$

Tissue fill represents the change in wound volume centripetally 'inward' from the 3-D volumetric surface area of the wound. The equation has been described earlier:

$$TF = \Delta V/(SA_{avg} \times \Delta t).$$

Combining these equations allows for comparisons of wounds whether deep or shallow [5].

An even more accurate measure can be obtained by adjusting ER and TF. Because significant contraction can give artificially high ER and TF values, a better value is obtained by adjusting ER and TF to the values that would have occurred with no contraction. Clinically, even in deep wounds, the wound closure rate can be just ER + CR, since it is the easiest to measure. If TF is not measured it is OK, especially if similar wounds are compared. Both the ER and CR, however, are influenced by the TF (in fact each one affects the other two). The epidermis needs a healthy well vascularized dermis to migrate over, since it does not have its own blood supply [8]. Although CR is different in different species, it seems to increase at lower TF. For example, in meshed skin grafts (Figure 4) the faster the mesh fills in the less scarring and contraction is seen [5].

In addition to the independence of size and volume considerations, meshed types of wounds (Figure 4) heal differently and one component of healing may be more important in a particular application than others (meshed skin grafts are typically used in burn wounds to allow a larger area of coverage especially when donor sites are limited). By using the previous equations, the contribution of each healing mechanism can be investigated. The ability to isolate healing rate into components allows for unique analysis of different types of wounds. For example, while it may be perceived that skin grafted

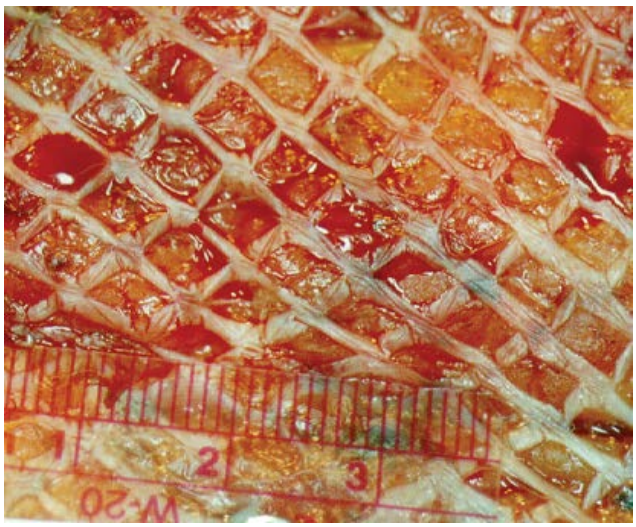

Figure 4: A meshed skin graft. To heal in, the underlying blood vessels have to grow into the graft. Each rectangle acts like a separate wound.

regions in humans heal primarily by epithelialization, in reality there is a combination of epithelialization and contraction involved in graft healing. Pressure ulcers, on the other hand, heal mainly through contraction and tissue fill. In each case, the individual mechanisms may be either isolated or combined for specific types of analyses. With this analytical model of healing rate, wounds of different shapes and sizes can be accurately and consistently compared to assess the effectiveness of different treatments on wound healing. In addition, the effectiveness of the treatment from week to week can also be compared.

Model justification: The importance of developing measures independent of wound size is illustrated for shallow wounds in Figure 2 and full-thickness wounds in Figure 3. Only the measure of HR (HR = $\Delta SA/(P_{avg} \times \Delta t)$) as described above demonstrates a measure of healing rate independent of wound size. In a clinical situation wounds of many different sizes and many different healing rates are encountered. This independence of wound size and shape is critical for accurate quantitative comparison of healing [5].

The derivation for $\Delta V/(SA_{avg} \times \Delta t)$ is similar to the one for 2D [5,7]. For a hemispherical wound, the change in radius in a 2D histology slide would be the same as the 2-D measure $\Delta A/(P_{avg} \times \Delta t)$, which is equivalent to $\Delta V/(SA_{avg} \times \Delta t)$; based on stereological principals [5,36].

Specifically for a hemispheric wound that changes radius from r_0 to r_1 (ΔR) in a given time:

$$\Delta V / SA_{avg} = \left[\frac{4/3\pi r_1^3 - 4/3\pi r_0^3}{2}\right] / \left[\frac{4\pi r_1^2 + 4\pi r_0^2}{2}\right] / 2$$

$= 4/6\ [r_1^3 - r_0^3] / [r_1^2 + r_0^2]$

$= 2/3\ [(r_0 - r_1)(r_0^2 + r_0 r_1 + r_1^2) / [r_1^2 + r_0^2]$

$= 2/3\ \Delta R\ [(\Delta R + r_1)^2 + (\Delta R + r_1) r_1 + r_1^2] / [r_1^2 + (\Delta R + r_1)^2]$ since $\Delta R = r_0 - r_1$

$= 2/3\ \Delta R\ [\Delta R^2 + 3\Delta R r_1 + 3r_1^2] / [\Delta R^2 + 2\Delta R r_1 + 2r_1^2]$

$= 2/3\ \Delta R\ [3r_1(\Delta R + r_1)] / [3r_1(\Delta R + r_1)]$ since $\Delta R^2 << r_1^2$ $\Delta R^2 \rightarrow 0$

$= 2/3\Delta R$

In deep open wounds, any epithelialization will occur at the wound edge. In shallow wounds, epithelial growth may also occur from epithelial islands within the wound. In these wounds, epithelialization rate is a sum of epithelial growth from the wound edge and from the islands, or $ER = ER_{edge} + ER_{islands}$. Growth from the wound edge is inward, while epithelial growth from islands is outward.

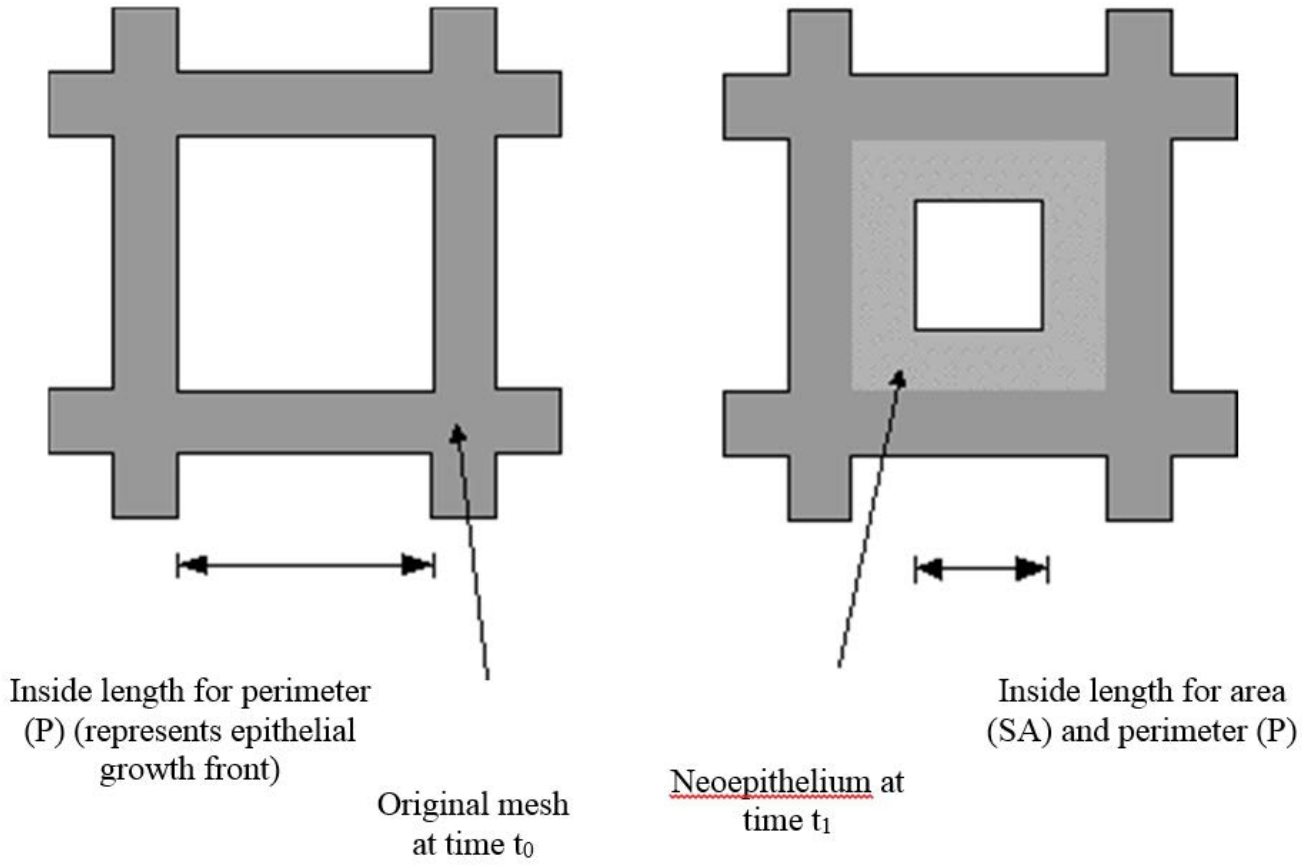

Figure 5: An individual rectangle of a meshed graft works just like a single wound. For the calculation, the following formula is still used: $ER = (SA_0 - SA_1)/[(P_0 + P_1)/2] \times (t_1 - t_0)$.

Meshed skin grafts would work similarly (Figure 5) [5].

Two-dimensional healing rate techniques

Clinically: The critical measures for use in the model are area of the wound and perimeter of the wound as well as the change from a previous time point. If possible the original wound edge could also be identified. Specialized imaging techniques (thermography, scanning laser Doppler imaging, ultrasound, etc.) have been proposed to identify the amount of new epithelium laid down. It typically, is relatively easy to differentiate the unhealed part, from the newly epithelialized part, laid the normal skin (Figure 6A). This is only important, if the contraction rate is to be separated out from the epithelialization rate.

Normally wound images are analyzed with image analysis software. It is crucial however, that a reference distance marker, such as a transparent ruler, is included in the image and the image is taken as perpendicular to the surface as possible (note rulers in two directions can help determine, if the image is angled"). From the image, the software can be used to calculate surface area and perimeter of the wound. The identification of neo-epithelium allows for the calculation of ER and the identification of the wound periphery allows for the calculation of CR. For skin grafts, ER can be calculated from the average neo-epithelialization from a group of individual units of the mesh graft as seen in Figure 4 and Figure 5 [5,27]. The contraction rate can be calculated by examining the change in area of a given number of individual units of the mesh as seen in Figure 7 [5,27]. The true ER (ER_t), can then be determined by normalizing for the CR. Thus for shallow wounds such as skin grafts, the ER and CR independent of wound size can be isolated for accurate analysis of healing.

In an animal model: In this case, tissue sections are used (Figure 1). To get this micrograph, it is important to have the tissue slices come from the center of the wound and perpendicular to the wound. Normally the wound is cut in half and embedded and sectioned from the middle outward. It is also helpful to attach the excised wound to a rigid structure (stapling around the wound to cardboard is one method) prior to fixation and embedding to limit shrinkage during processing. It is also helpful to have square wounds to give more leeway than a spherical wound where sectioning through the actual diameter is not always possible.

From a tissue section the measures are width of the wound and length of new epithelium from each side. In an animal model with hair, the wound margins are easily detectable from where the hair follicles start. The calculation of epithelialization rate, contraction rate, and healing rate are then more straight forward in an animal model, since the wounds are the same size initially. The rates desired are still the change in radius per unit time so the change in width of the wound per unit time is divided by two to determine contraction rate and the epithelialization rate is the average length of new epithelium (from each side) per unit time. The rates can be determined from the initial wound or change from a different previous time point; depending on whether the rate of healing over time is desired or just the cumulative healing rate.

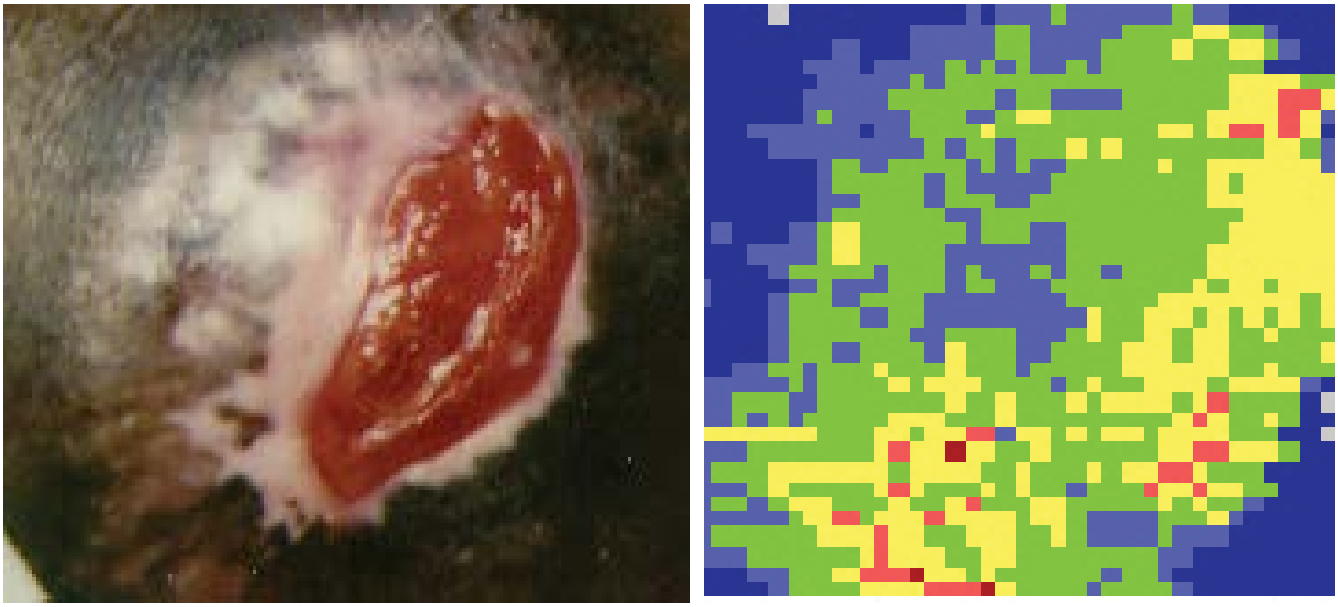

Figure 6: A) Pressure ulcer and B) Corresponding LDI (Laser Doppler Image).

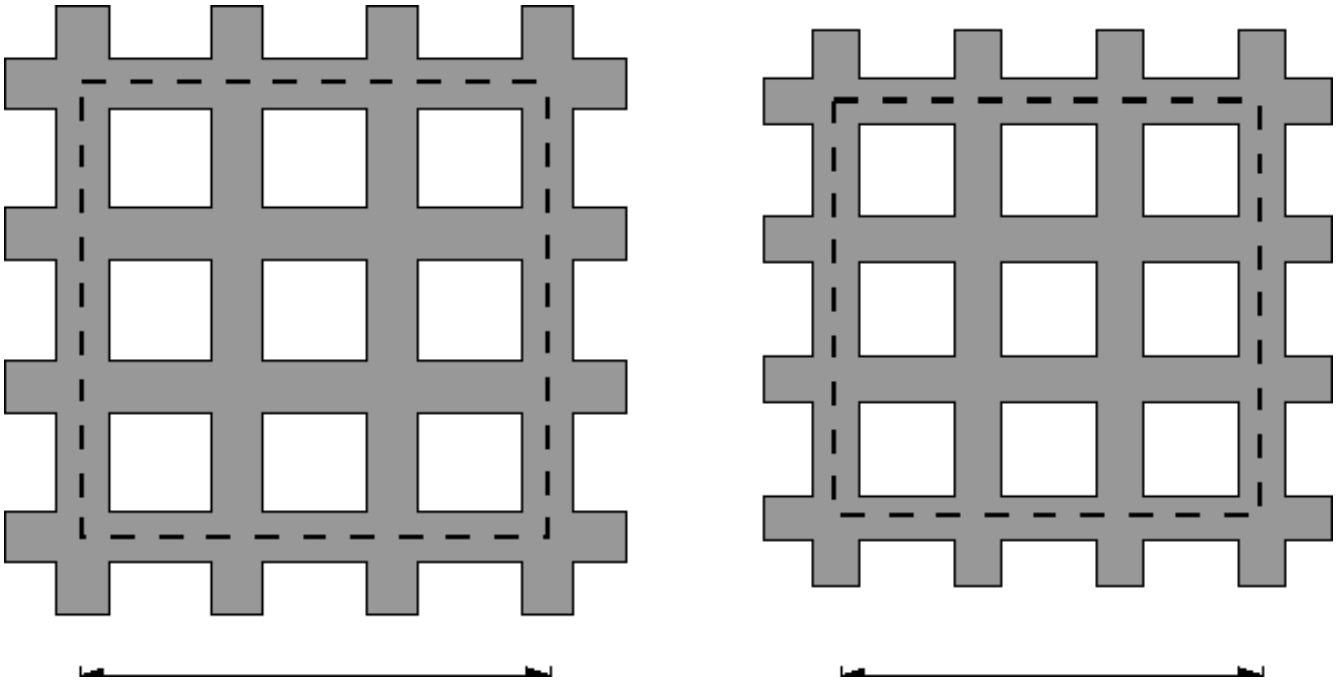

Figure 7: Calculation of Contraction Rate (CR) from a 'box' of meshes of a skin graft. For the calculation, the following formula is used: $CR = (SA_0 - SA_1)/[(P_0 + P_1)/2] \times Time$.

Clinical relevance: In numerous studies, the ER and CR were determined in an animal model [37-41]. In many of these studies, the ratio of ER/CR was used as an indication of the amount of healing that was regenerative vs. scarring. This was very useful for comparing treatments. From a statistical perspective a paired t-test could be used in comparing the treatments to the controls at each time point. Also week to week comparison was useful for some treatments that had a time lag like systemic injection of stem cells [38,41].

Clinically, the ability to measure a true healing rate and do it week to week was critical in determining the effect of the treatment and the time frame it was most useful (Figure 8). In a clinical study, using electrical stimulation on pressure ulcers; statistically significant results were shown with just four patients [42]. In addition, the figure shows that the treatment worked best for three-weeks at a time, so the clinical protocol was changed to a week of no treatment between each three-week period. Although it is not known for sure, it has been suggested that the cells exhibit a phenomenon similar to electroporation. Even though it is a much lower voltage it is over a three-week period and the voltage X time is approximately equivalent.

Three-dimensional healing rate techniques: Traditionally, a number of methods have been employed to measure wound volume. While these methods are relatively inexpensive to perform, most require wound contact, leading to possible wound disruption, contamination, and increased chance of infection. Imaging techniques can be used with some of these methods as well as can be used to obtain these measurements clinically, without direct wound contact. The key measures are wound volume and surface area of the unhealed surface. The surface area and volume of the original wound edge is important to get contraction rate. It can, however be approximated by determining the 2D contraction rate as an estimate of the 3D contraction rate, particularly when there are no histological slices to help determine the original wound edges.

Clinically: A common method of measuring wound volume is that of casting, or creating a mold of, the wound with a foreign material. Filling the wound with a foreign material, however, increases the chance of wound contamination, irritation or an allergic reaction [43]. A number of methods have been employed to obtain volume from the casting. Imaging techniques have been used to digitize the shape and allow calculations based on those images. Hayward and associates used MRI for digitization, while others have used CT (computerized tomography) [44-46].

Similar to obtaining 3-D information from molds; these techniques can also measure volume and surface area directly. In many cases these techniques produce slices through the structure in one or more orientations. There is readily available software to turn these 2-D

slices into 3D images; some are built into these imaging systems. MRI and ultrasound are two of the better techniques to image skin wounds. Ultrasound requires a special probe to look at skin vs. the probes that look at deeper organs and tissue; plus a membrane is needed over the wound surface [47].

Also two types of laser scanners have been employed in the measurement of wound volume. These systems have been used to digitize facial contours for burn masks, design other burn dressings or pressure garments, plan plastic surgery and to calculate volume of foot ulcers.

The first system consists of a laser beam projected onto a wound, positioned horizontally on an x-y table. As the table moves, distance to the wound surface is recorded and used to reconstruct the wound surface. The primary disadvantages of this system are the expense and the lack of portability. Also, the patient must be lying with the wound horizontal. Systems also tend to be less accurate with larger wounds; with values being up to 25% off for wounds up to 60 mL [48,49].

A second, larger system employs a projected laser line, and a camera that is rotated around the study surface (e.g., a face or wound), taking images every few degrees. Images are based on the concept that a straight line projected onto a curved surface will appear as a distorted line when viewed at an angle. Coordinates are identified by measuring the distance from the axis of rotation to a line representing the study surface. Custom software is used to construct a model of the surfaces scanned, and to make calculations. Systems like this used for wound volume and surface area measurement are made by both Cyberware [50,51] and Cencit [52]. The main advantages of these laser scanning systems include direct data acquisition and processing, the ability to record color, and very fast scanning time. The main disadvantages are the high cost, the lack of portability, and the necessity of positioning wounds vertically, which may be difficult with some patients.

Like large 3-D laser scanning systems, structured light is based on the concept that a vertical line projected onto a curved surface appears curved when viewed at an angle. Unlike 3-D laser scanning, however, a structured light system does not necessarily require costly equipment. Structured light systems can consist of just a light projector system, a camera, a method of positioning the components appropriately, and software to help calculate wound and surface area from the obtained image. Costs can be further reduced by making the necessary calculations directly off the structured light photograph, instead of using software. For structured light used with wounds, stripes or dots have been projected directly onto the area to be examined [52-58]. By spacing the lines or dots apart a known distance, depth on the image can be easily calibrated to obtain volume and area values.

Plassman and Jones have developed a structured light system for wound measurement that employs color-coded lines [57]. Using the system, they take two pictures of each wound: one with a homogeneous beam of light shone on the wound, to identify the wound boundary and to map reflectance; and a second picture, with 60 color-coded lines shone on the surface, to obtain the structured light image for analysis. The structured light images are then analyzed using custom software, which includes algorithms to adjust for reflectance, camera non-linearity, and lens error. With this system, they obtained depth measure accuracy of ± 0.4 mm in real wounds, and ± 0.02 mm in an idealized situation. Advantages to their particular system include portability and easy use due to a handheld device and direct transmission of the images to a computer.

Stereophotogrammetry has been used for two decades to measure contours on the body. The most common application in the medical field has been for digitization and reconstruction of facial contours. More recently, stereophotogrammetry has been employed to measure wound volume of pressure ulcers and deep burn wounds [5,27].

Stereophotogrammetry is based on the principle that two 2-D images taken from two different angles can be combined to create a 3-D image. Three-dimensional images of wounds have been constructed by manually selecting points with a metrograph, or using a stereocomparator [45,59]. A camera setup involving two cameras, placed on both sides of the surface, captures images to be used for image reconstruction. Specialized software is used to combine these images to calculate wound volume, area and perimeter.

Bulstrode, et al. found high precision and accuracy of area and volume measures of leg ulcers using Stereophotogrammetry [59]. They also observed high precision and accuracy associated with detection of the epithelialization edge in full-thickness excisional wounds in pigs [59]. Primary advantages of steroephotogrammetry are the high degree of accuracy and precision, and the acquisition of a permanent, digitized image. The main disadvantages include high equipment cost, the time needed to collect the data, low portability and the elaborate training required [5,27].

In an animal model: This can be done using MRI or ultrasound (as discussed for clinical applications) or using histological slides. Multiple slices can be done through a wound using histology to mimic the MRI images. Again, there is readily available software to turn these 2-D slices into 3-D images; some are built into ultrasound and MRI systems. For light or electron microscope micrographs

histomorphometry (stereology) can be used to obtain a surface area measure per unit volume, which can help approximate the needed measures for 3-D healing [36]. Also an open wound cross-x can be considered half a 2-D wound and the previously calculated radial healing rate can approximate the 3-D tissue fill rate. Each technique is better for certain types of tissue and may require addition of fluids, contrast medium, or stains to help visualize the wound. In most cases currently, histology is needed to get all the measurements accurately enough to calculate useful healing rates. Again, imaging techniques are improving all the time and will eventually reduce or eliminate the need for histological images to obtain these measurements.

Tissue health

Tissue health may be defined as the potential, of a selected tissue, to undergo the healing process. The assessment of tissue health should not only help predict the trajectory of wound healing, but also act as an indicator of where the wound is in the healing process (how far the wound is from "normal").

The level of oxygenation at various parts of the wound is able to do this. Research has shown that hypoxia reduces fibroblast proliferation, collagen synthesis, angiogenesis, and epithelialization [60-63]. Oxygen is also vital in the prevention of infection [64]. This is important, since infected wounds do not heal. Investigators have determined that a tissue pO_2 of at least 30 mmHg is necessary for efficient bacterial killing [62]. In addition, it has been shown that wounds with decreased oxygen display characteristics of delayed granulation and epithelialization [65]. This tissue health can be determined by directly measuring oxygen tension or indirectly by skin temperature or blood flow.

Clinically

Oxygen tension: Oxygen tension, a measure for oxygenation, may be determined by a number of non-imaging techniques. Imaging techniques, however, have the advantage of providing the spatial comparisons needed without multiple sequential measurements.

An Oxyscope is one word determines the tissue oxygen concentration via a light excitation and emission. Porpherine dye is injected into the tissue of interest. A detection device measures the phosphorescence of the dye as it is being quenched by the oxygen within the tissue. Two devices presently on the market include the OXYSPOT (Medical Systems Corp., Greenvale, NY), which measures a single point, and the OXYMAP (Medical Systems Corp., Greenvale, NY), which is the imaging technique able to image an entire area. Both techniques provide a quick assessment of the local tissue oxygen levels [5].

Vascularity: Although it is the vascularity or angiogenic response, which determines the rate of wound healing; it is mostly due to the oxygen level. The key is the distance between fibroblasts and the nearest blood vessel, since it is a diffusion process. Histomorphometry can give average distance between blood vessels, but clinically blood flow is the measure most commonly used, which is an indirect measure of vascularity.

There are a number of non-imaging techniques that can assess vascularity including: ^{133}Xe-washout and Laser Doppler Flowmetry. Again imaging techniques, however, have the advantage of providing the spatial comparisons needed [5].

Thermography: Thermography is the measurement of tissue heat, and in the case of skin wounds this would correspond to skin temperature. Although there are many variables that influence skin temperature, such as ambient temperature, radiation, evaporation, etc., if skin temperature is measured with relatively constant environmental factors, local changes in skin temperature can be determined [66]. These changes are a mainly a result of heat conduction followed by local heat production. Heat conduction is a result of blood and lymph flow, while local heat production is due to energy producing processes [5]. Therefore, thermography acts as an indirect imaging measure of blood flow and thus perfusion and oxygenation. This technique is a non-contact method that is able to measure and model an entire wound with surrounding tissue, assessing both the spatial and temporal dynamics of a wound [67,68]. This technique, however, is limited in that, at best, it is only an indirect measure of blood flow and difficulty in maintaining constant environmental factors hampers the ability to accurately assess changes in the wound over time.

Laser Doppler and Imaging: Laser Doppler Imaging (LDI) was introduced in the late eighties in an effort to overcome the limitations of Laser Doppler Flowmetry (LDF) [69-72]. LDI is based on similar principles, yet, is able to measure the Doppler shift, and thus blood flow, over a large area, within a short time, and without any tissue contact. This measurement is done with the use of a laser head positioned 7 to 30 cm from the tissue. The laser head consists of a raster, or scanning laser, and a detection device, which is able to pick up the Doppler shifted light emitted from the tissue (Figure 9). A data acquisition board is used to input this data to an analysis program, which then produces a map of the wound perfusion. These characteristics allow the LDI to provide a portable, non-contact blood perfusion assessment of the spatial variability within the entire wound [60,73,74].

LDI has been used in numerous applications both in the clinic and for research to measure blood perfusion in tissues such as skin, brain, muscle, and liver [60-

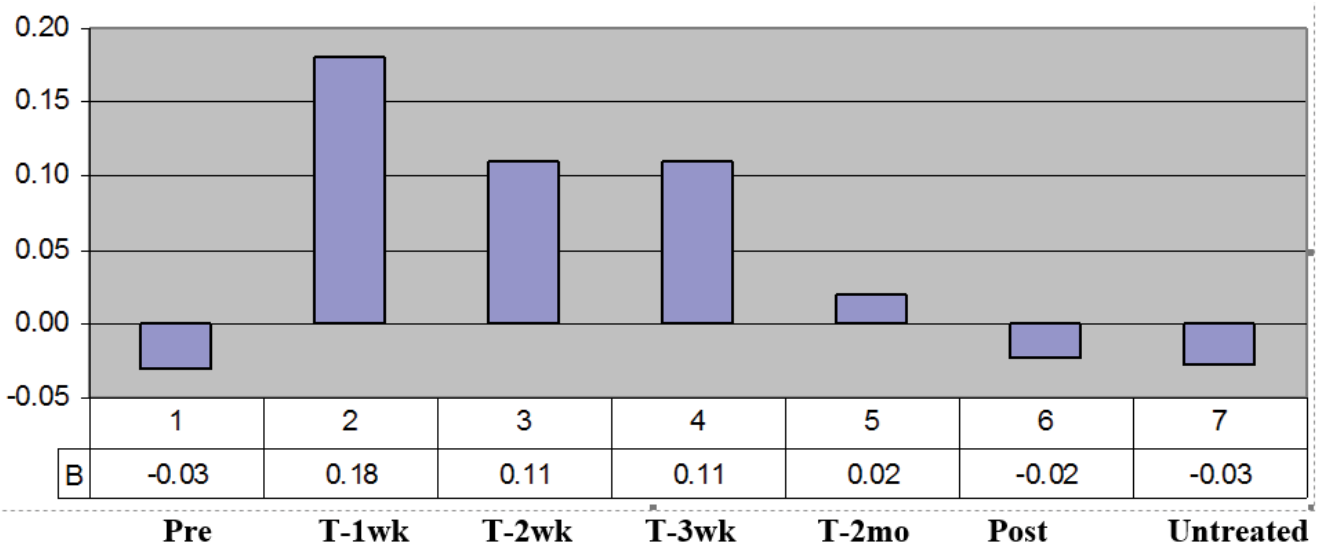

Figure 8: The average overall healing rates for five spinal cord individuals with pressure ulcers. The chart shows the healing rates (cm/week) in the untreated period (Pre) for each of the first three week time periods, for the 2-month treatment period, for the 2-month follow-up period (Post), and for all the untreated (Untreated) time periods.

65,75,76]. The device is capable of penetrating various depths into the tissue, depending on the wavelength and tissue density [77]. For skin a He-Ne laser is capable of penetrating up to 200-300 μm, which is into the dermis but above the underlying muscle and away from the major vessels. This allows assessment of the tissue health of skin from a vascular standpoint. These devices have been used in previous studies to help determine healing and predict healing rate in a number of different wounds [11,12].

For accurate quantification of the clinical assessment, it is important; however, to know the limitations of the technique as well as quantify the physical variables that affect the perfusion values. The effect on perfusion level of physical variables, such as ambient light, distance from the tissue and presence of wound dressings should be known and quantified in order to develop standardized clinical assessment protocols. In addition, it has been shown that the amount of skin pigmentation has a direct effect on the perfusion values obtained. Several researchers are attempting to solve this limitation by the use of lasers with differing wavelengths [78]. Another concern has been the ability to match perfusion scans with wound landmarks. This is critical in evaluating temporal changes of many types of wounds.

Clinical relevance: These measures of tissue health are important for developing relationships between bioprocesses and clinical outcomes. Although it is known that increased angiogenesis helps the healing process, the amount necessary to have a clinical impact is not. The model that has to be developed is the relationship of oxygen level and gradient to ER, CR, and TF. These measures quantify the relationship between angiogenesis and healing, but it would be helpful to know how these angiogenic measures relate to tissue oxygen level and gradients. Ultimately you want to select treatments that stimulate the desired angiogenic response to produce the desired oxygen gradients to speed the overall healing (the desired amount) while limiting the scarring and contraction compared to regenerative healing (also at the desired level).

Usage in two clinical wounds, burns and pressure ulcers, will serve as examples of how this can be used as a tissue health measure.

For meshed grafted burn wounds, the tissue health was evaluated over a three-month period (Figure 10) [79]. Both the average perfusion and the spatial variability are determined for each site. To account for patient to patient variability and measurement errors, the perfusion values used in the statistical analysis are the differences between the treatment sites and their respective internal controls. This allows more accurate comparison of temporal changes in blood perfusion. The perfusion values (Figure 10) show that the two treatments (fibrin with and without FGF-1) kept the perfusion level higher than controls during the wound closure phase (first three weeks) as well as the remodeling phase [5,79]. Ultimately these wounds closed sooner (about one week) and had significantly less scarring; leading to shorter rehabilitation time [5,79].

Although the quantitative relationship between perfusion level and healing is not known, at this point it can serve as an explanation for the change in healing trajectory. Future work can help make this measure more of a predictor of changes in trajectory.

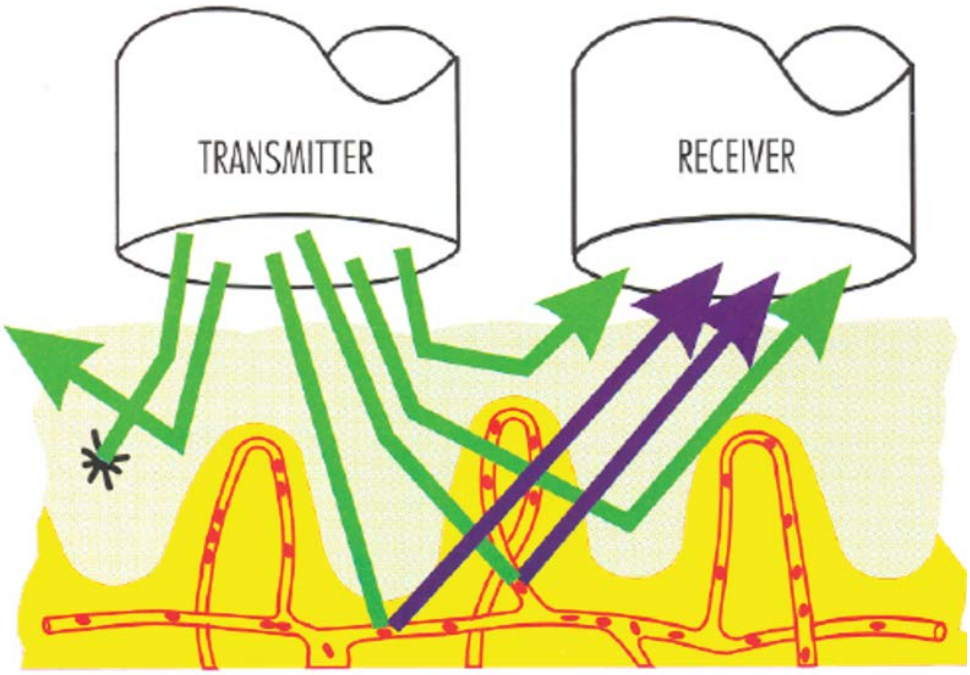

Figure 9: Diagram of the principle behind LDI.

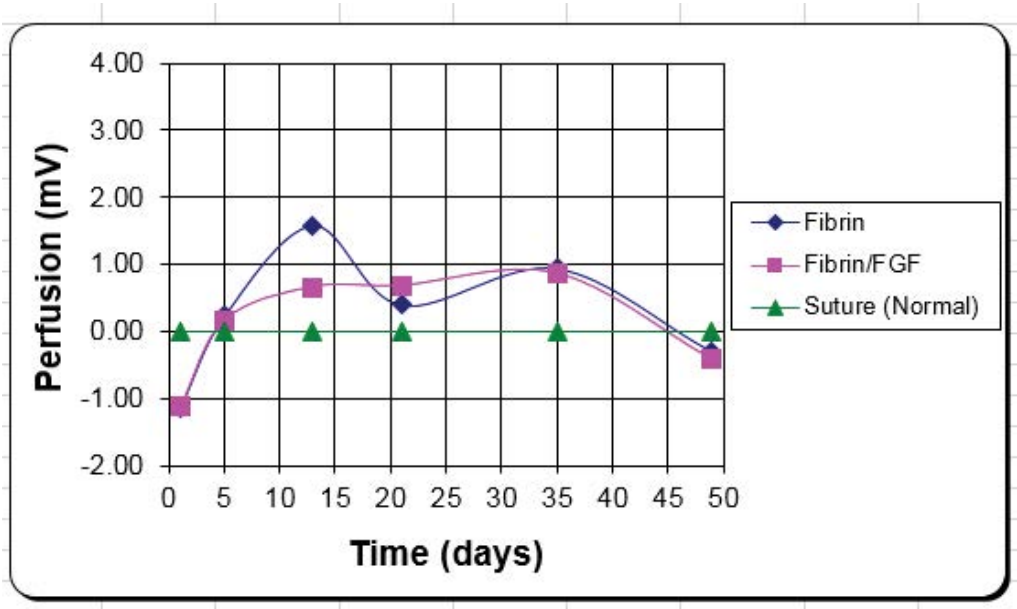

Figure 10: Laser Doppler Imaging (LDI) results for a burn patient. Mean perfusion of alternate treatment types minus the mean perfusion of the standard treatment type. LDI shows the increase in perfusion for each of the two treatment types over time.

In another clinical study [80], the tissue health of Stage II-IV pressure ulcers were evaluated over a two-month period for outpatient Spinal Cord Injured (SCI) patients (Figure 6). Figure 6 shows both the wound image (Figure 6A) and the corresponding LDI image (Figure 6B). Scans were taken at the time of treatment and at each monthly visit thereafter. From the scans, the averaging blood flow values, above the control, of both the healed and unhealed portions of the pressure ulcer could be determined. Three metallic markers were placed at fixed distances around the wound to give landmarks that show up in the scan and in photographs taken. This allows better determination of the wound regions in the scan and distances within the scan. As seen in Figure 6 this technique not only shows how good the perfusion is, but also, if there are differences in levels within the wound that should be looked at further. Again this technique proved to serve as an explanation for why certain wounds or parts of wounds healed faster than others.

Others have suggested that change in perfusion level

due to a provocation (temperature, pressure, occlusion) can be used to get a more accurate assessment than the absolute levels [60,80]. This in essence is similar to a clinical assessment used for pressure ulcers (non-blanchable erythema--the color stays red even after pressure is applied). This measure seems to be a better indicator of tissue health and may be useful to help in determining how much sitting can be done without re-injury to better control the sitting protocols after pressure ulcers are healed [80].

In animal models: All the techniques used clinically can be used in animal models. In addition the angiogenic response can be determined using stereology principles (off of micrographs) similar to those used for ER. Again equations have been developed to obtain 3-D measures from 2-D images such as microscope slides. This means it is easier to do this in an animal model vs. get a skin biopsy in a healing wound. An important assumption in stereology, however, is that the structure is relatively homogeneous. For a skin wound it is best to divide the wound into two or four parts and assess multiple regions in each region. There should at least be the inside and the outside of the wound (i.e. covered by new epithelium). If the wound is deep it should be also broken down into a top and bottom layer. Some of the other imaging techniques can also give an indication of the number of areas a wound should be divided into.

Stereology allows a number of different 3-D measures; but what is important for angiogenesis assessment is the ability to provide oxygen and nutrient exchange via blood vessels. As stated before, the key is providing enough oxygen for the fibroblasts to proliferate and lay down collagen. For blood vessels, the oxygen diffuses out from the red blood cells; so two useful measures are the volume fraction of blood vessels and the average distance between vessels (since that is easier to measure than the average distance between fibroblasts and the nearest vessel). It may be helpful to use special stains to pick up endothelial cells, but missing capillaries is not going to have a significant effect, since it is the comparison that is important. In healthy tissue, cells need to be within 100 μm of a blood vessel to survive and be active (150 μm in bone) [5,81]. So the average distance between blood vessels can be up to two times these values to assure that the cells are within the needed distance.

Histomorphometry (stereology in tissue) is based on using a grid placed over the tissue. The key principle is that $V_f = A_f = P_f$ (volume fraction equals area fraction equals point count fraction); therefore the percentage of grid points landing on a blood vessel (just the lumen) is equivalent to the area fraction, which is equivalent to the volume fraction [36]. Surface area is the number of intersections with grid lines divided by the total length of the grid lines (or fraction of the grid lines that pass through the endothelial lining of a blood vessel) divided by 2, since a line will intersect two sides of circle [36]. To be more accurate (but it isn't much more accurate), the endothelial layer of each blood vessel is circled. Then the grid is essentially whatever the pixel density is (e.g. 1080 × 480). The mean free path is 4 times the area fraction outside of the blood vessels (1- area fraction of the blood vessels) divided by the surface area (fraction of grid lines crossing the endothelium) [36]. Note a number of investigators count the number of blood vessels. Based on stereology this approximates the length of blood vessels *in vivo*.

This has been used in a number of studies to determine the angiogenic response to different materials and treatments [37,38, 82-86]. In one case [38], this was measured with wounds with fibrin glue (with and without an angiogenic agent [FGF-1]) as well as oxygen treatment. Histomorphometry measurement of the blood supply for the various treatments showed the differences in the angiogenic response particularly how deep into the tissue (edge vs. center of the wound) it was seen. Although the clinically important parameter is healing rate, angiogenesis is an important bioprocess to help explain the differences in healing seen. In this study, overall the oxygen treatment elicited the best angiogenic response both at the edge and the center of the wound. The FGF-1 was also high on the outside and only with the fibrin was it high in the wound center. As expected, the oxygen and FGF-1 in the fibrin had the highest epithelialization rates, but also the highest contraction rates.

Again, imaging techniques are improving all the time and will eventually reduce or eliminate the need for histological images to obtain these measurements.

Tissue function

Restoration of mechanical properties is also an important function that can be measured using imaging techniques. Predominantly, this is stiffness and load carrying ability. Load carrying ability is important to prevent recurrence of the wound and the need to protect the wound from mechanical loading. E.g. A sitting protocol is normally used for spinal cord injured individuals with pressure ulcers; limiting the time they can sit on the healing area until it is fully healed [5]. In many other cases the strength of the wound places limits on the types of rehabilitation exercises that can be done. Again, there is a typical trajectory of restoration of load carrying ability until wound closure, which normally continues during the subsequent remodeling phase.

Restoration of stiffness usually parallels the trajectory of restoration of load carrying ability, since they both are related to cross-sectional area of newly healed tissue.

Stiffness, however can surpass normal levels due to scarring and is one of main causes of impaired tissue function in wound healing. Again it is the reason for the long rehabilitation period for meshed skin grafts, even after they heal as well as part of the reason passive movement is introduced as early as possible during rehabilitation; before any strengthening exercises. So a measure of stiffness increase over time relative to native tissue would not only monitor healing rate, but also be an indicator of the length of time for rehabilitation (as well as monitor progress on reducing tissue stiffness). In addition, indicators of mechanical properties are measurements of collagen density and orientation. Imaging techniques are useful in obtaining this data both clinically and in animal models.

Imaging techniques are currently limited to monitoring deformation under a load (for stiffness measurements) or assessing collagen density and orientation. Clinically techniques such as ultrasound and MRI can give some area measures of connective tissue as well as density comparisons [87]. There have also been studies to use specialized MRI (T2 mapping) to get collagen orientation [88]. There are also some systems that use imaging to measure deformation of the skin subjected to a specific load (usually a vacuum) to obtain a stiffness measure.

For animal models, additional assessments can be done. Mechanical loading can be done on open wounds to failure allowing both stiffness and load to failure measurements throughout both the healing and remodeling phases *in situ*. In addition, the wounds can be removed to allow other types of loading conditions. Histology can also be done for animal models (although limited use of biopsies can be done clinically) to obtain collagen density (as well as type) and orientation. There are a number of techniques to better identify collagen fibers including: polarized light (since collagen is birefringent), special stains, and TEM (the banded structure is relatively easy to identify at this magnification).

Clinical assessment

Mechanical properties: For mechanical properties it is not possible to get load to failure as a wound heals. Although it is possible to determine loads that can be withstood below failure levels, typically stiffness is what is measured clinically. Imaging, however, is just used for the deformation part of stiffness. It can also be used to indirectly determining mechanical properties by assessing collagen amount and collagen orientation.

The assessment of scar characteristics is an important clinical tool to determine tissue function and there have been several attempts at developing clinical rating scales to better quantify scar healing [89-91]. These rating scales are often based primarily on visual assessment of scar color and thickness, rather than stiffness, and are rather subjective and inconsistent.

There have been a number of attempts to quantify stiffness clinically. One type that typically uses imaging are the vacuum techniques. Systems typically consist of a vacuum chamber (for application of load) and a video camera (for measurement of tissue deformation). The system calculates deformation with the corresponding pressure value to generate a pressure-deformation, or stress-strain curve.

From the resultant pressure-displacement curves, an elastic modulus and total energy under the curve can be calculated. Both measures may be representative of distinct mechanical characteristics of a healing wound. In addition, the area under the 'toe' region of the stress-strain curve and the slope of this early region may reveal specific elastic contributions.

For example, a two-month post-graft site and a contralateral control area were each tested; with imaging used to determine the deformation [5]. Figure 11A

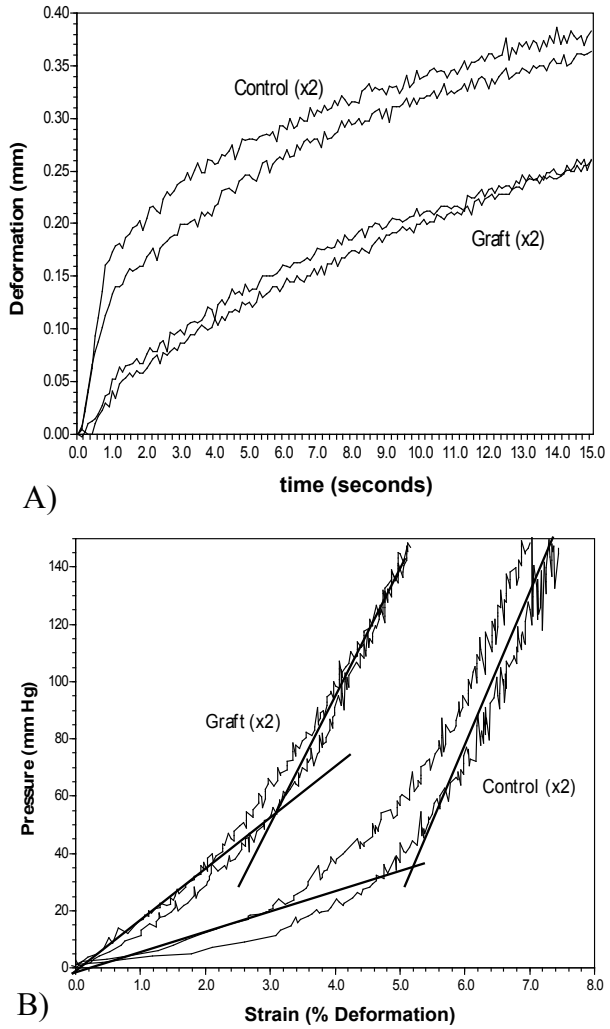

Figure 11: Results from a typical vacuum deformation test of a grafted site and a contralateral control. A) Deformation vs. time; B) Pressure vs. strain.

shows the deformation versus time curves for the two regions; based on imaging data. The control site had an approximately 40 percent increased total deformation. Figure 11B shows the pressure versus strain (percent deformation) curves for the regions. The total area under the curve for the control site was 50 percent higher than that of the graft site. As illustrated by the regression lines, there was a significant difference between the slopes (elastic modulus) of the two curves subjected to small pressures, at higher applied loads, up to the final 150 mmHg, the modulus of the two sites were similar. Results similar to this have been reported for *in vitro* mechanical testing of skin as well [91].

ECM fibers: In many cases, assessment of the anisotropic properties (non-uniform properties) of the newly healed tissue are desired. The collagen in dermis is not randomly oriented and tends to line up along Langer's lines [5]. Skin tension is lower parallel to the lines vs. perpendicular and is used to make incisions with less tension on them [5]. Similarly strength and stiffness are highest parallel to the Langer's lines [5]. Using a vacuum system this information could be obtained by using more than two non-collinear markers; to assess mechanical properties in along more than one axis. It also can be obtained by assessing the orientation of the fibers in the ECM (predominantly collagen).

The options clinically are somewhat limited, but imaging techniques such as ultrasound and MRI can be used as well as for skin biopsies. Skin biopsies will be described in the *in vitro* section, since the use of biopsies in healing wounds is limited. As imaging techniques improve they will be more comparable to histology. Goal is more to show what need to know vs. how to get it.

Again the goal will be to show the information needed and describe some imaging ways to obtain this information.

The two critical parameters are flexibility (stiffness) and load carrying ability in a specific direction. Predicting these properties in a fiber reinforced composite (in this case collagen fibers in mostly a don't capitalize glycosamino (GAG) matrix) usually requires making some simplifying assumptions, which do not always work well in a viscoelastic material such as skin [5]; i.e. small deformation, one-directional loading, and either homogeneous or unidirectional fiber orientation. Assuming the stiffness and load to failure for collagen fibers and the GAG are known in tension these parameters can be approximated by knowing the volume fraction of the fibers, the direction of loading compared to the fiber orientation and the thickness of the skin. It helps to look at the properties relative to loading along the fibers. In this case the amount of deformation for a given load (stiffness) and load to failure are related to the area fraction of fibers in a cross-sectional slice. The stiffness is the stiffness of the fiber material times its area fraction plus the stiffness of the matrix times its area fraction. The load to failure is determined by the ultimate tensile strength of the material (which is the load to failure normalized to be independent of cross-sectional area). "Therefore the higher the area fraction of the fibers the higher the load to failure can be without exceeding the ultimate tensile strength. If the load is not parallel to the fibers both the stiffness and load to failure decrease related to the square of the angle between fiber orientation and loading. Specifically, it is the square of the sin or cos of the angle. Since $\sin^2$ plus $\cos^2$ equals one it is either the $\cos^2$ fraction of the original or reduced by the $\sin^2$ fraction of the original. Therefore the area fraction and actual cross-sectional area of collagen are needed as well as the angle between the loading and the collagen fiber orientation.

High frequency ultrasound scanners can give tissue slices, for wound healing measures, as well as collagen assessment. Judgements can be made on amount and orientation of collagen in a wound [92,93].

T2 MRI mapping has also been used to characterize tissue collagen [94]. In addition, a number of other imaging techniques are being used to non-invasively analyze the ECM in skin including multiphoton excited fluorescence imaging [95-97], dermoscopy (epiluminescence microscopy) [98,99], and reflectance confocal microscopy [100].

In animal models: Although ultimately the clinical recovery of mechanical properties is desired, there are limits on what a vacuum system can do (e.g. cannot determine the strength of the wound). Part of this is the importance of separating out the regenerative healing from scarring (as was done for healing rate). Use of an animal model and mechanical testing can allow determining the recovery of both strength and stiffness over time as well as compare treatments to the control. Again there can be an evaluation of the ECM as a way to explain some of the differences seen.

Mechanical properties: Removing tissue from the body alters its mechanical properties (lose support from surrounding tissue) as well as storage time and type can affect the mechanical properties. It is helpful to do this as more of a comparison to the control (i.e. treated the same way) to minimize the problems of trying to get an exact value. Also the results can be as a percent of uninjured tissue, to get the recovery trajectory; a functional healing rate.

As an example, data from a study to assess the recovery of incisional wound strength will be presented [40,101]. In this case, there was a control sutured wound and treatments that included stem cells or an adenovirus that would transfect cells to overproduce fibromodulin a moderator of TGF-β. Imaging was used to determine the

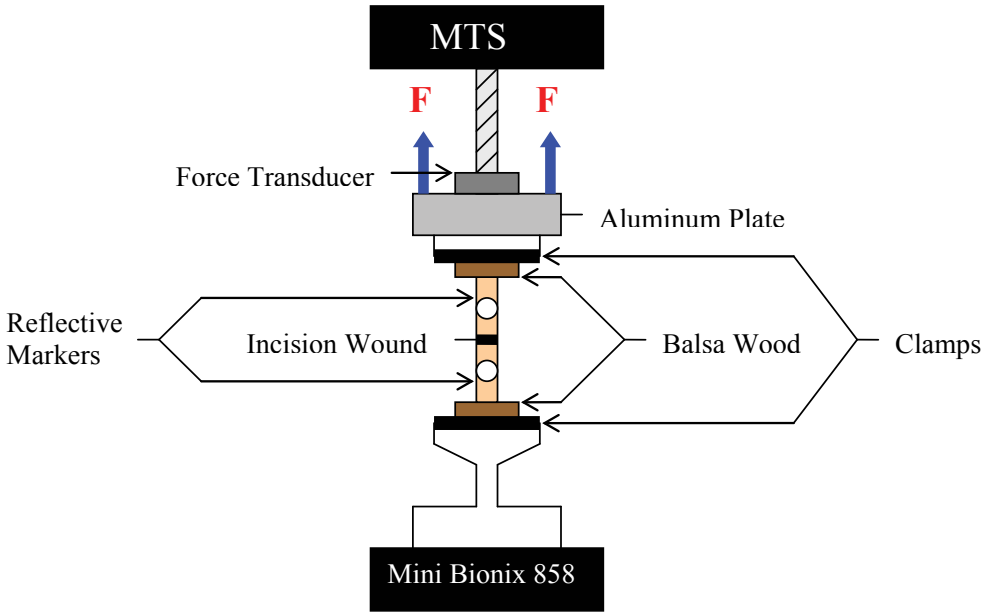

Figure 12: Axial tensile test diagram. Balsa wood was placed on the front and back of the specimen and clamped into the material testing system. Reflective markers were placed above and below the wound site (to aid in imaging the deformation). Axial tensile testing occurred at 1 mm/s until failure with a force perpendicular to the wound site.

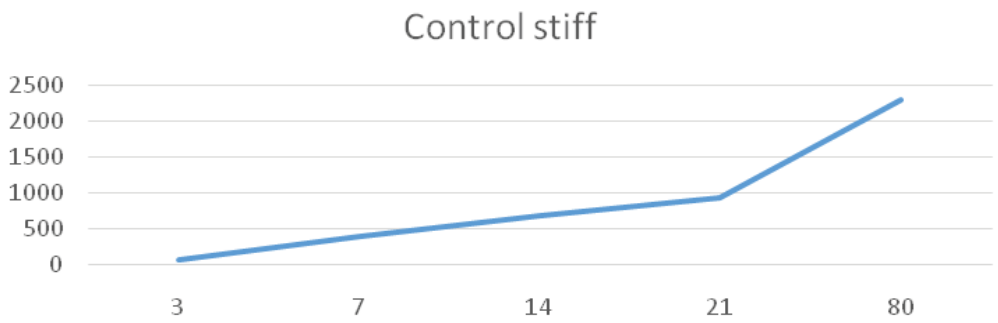

Figure 13: Trajectory of a stiffness and strength over time as percent of native skin.

deformation (Figure 12). The trajectory curves show the recovery rate over time for both stiffness and strength of treatments (Figure 13); with imaging used for the stiffness measure. The fact that the control exceeded the stiffness of native tissue showed that some of the healing was due to scar formation. Another way to look at this is to assume that recovery rate of strength and stiffness should parallel each other, since they are both proportional to cross-sectional area. So if stiffness is increasing faster than strength recovery, then that is due to scarring.

Although the rate of recovery of mechanical properties is similar to healing rate, since both stiffness and load carrying ability are proportional to amount of new tissue formation, healing rate should be measured as a change in radius of new tissue vs. a volume or cross-sectional area change. It is further complicated by the remodeling aspect of wound healing; with type III being laid down first, before becoming the stronger type I. Also the square or cube root (depending on the wound shape) of the mechanical recovery rate does not translate well to clinical practice. For an incisional wound the change in new tissue is closer to the 2-D cross-sectional area versus the volume of new tissue. If the change in properties is proportional to the cross-sectional area (the change in cross-sectional area as a percent of the area when completely healed) as well as the radial healing rate is constant, the trajectory curve will be the same independent of size of the wound (the square of the radial percent recovery, i.e. it takes 70% of the time to reach half of the area vs. 50% of the time for a linear function). So recovery rate of mechanical properties is slower than healing rate (initially), but increases over time to allow most of the recovery of mechanical properties at the time of wound closure.

ECM fibers: Similarly to *in vivo*, the recovery of mechanical properties can be linked to the amount and orientation of new ECM fibers. The type of ECM can help explain the influence of remodeling on recovery rate of mechanical

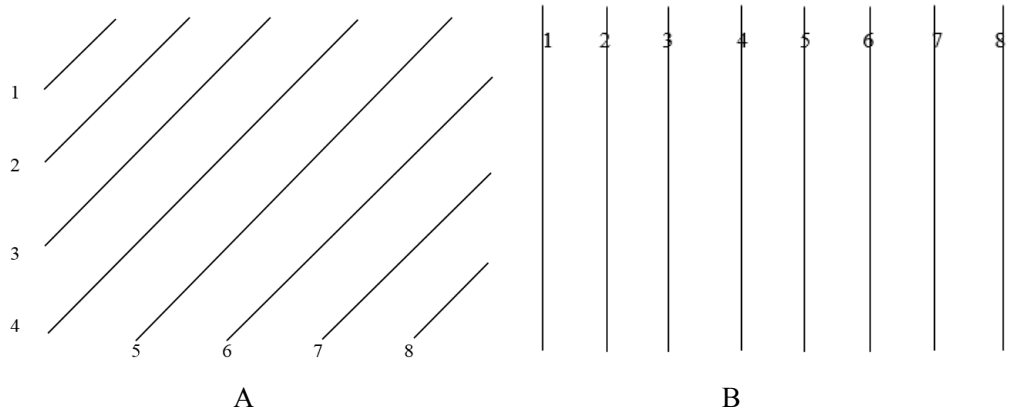

Figure 14: Determining fiber orientation. The image in Figure 4 of a meshed skin graft would be an example that would show anisotropy. A, B are examples of grid lines which can be placed over the image to determine the number of intercepts per unit length of test line.

properties. This information can be determined using the imaging techniques described for clinical testing as well as histomorphometry from micrographs (which can also be done using biopsies, but on a smaller area) [36].

In this case the volume fraction can be done similar to previously described for blood vessels. Orientation can be done a number of different ways. Histomorphometry can be used to determine if there is alignment (isotropy) in one or more directions [36]. The more directions done the more accurately the directionality can be done (to a point), if lines are used in four different directions a general idea of the isotropic nature of the structure can be determined. Plotting the number of intercepts vs. direction (x, y plot), is called the rose-of-the-number of intersections and can show directions of anisotropy [36].

For illustration, we can use the meshed skin graft in Figure 4 attached with fibrin glue, used in the burn study discussed earlier. This, however, needs to be done with collagen fibers (possibly using special stains or at the EM level). For this case, the isotropic nature can be measured by making intercept counts in four different directions: Vertical direction (moving from left to right across the image), horizontal direction (moving from top to bottom), and the two diagonal directions (from top left to the bottom right of image and from bottom left to top right). Figure 14 shows examples of grid lines that can be placed over the image. The higher the number of intercepts in a specific direction, per unit length, the more the fibers are aligned perpendicular to that direction.

Conclusions

Imaging has been useful in quantitatively measuring key parameters of skin wound healing: healing rate, tissue health, and tissue function. As imaging techniques improve they can play an even bigger part as well as provide more accurate data; more closely approximating histological images to allow better clinical assessments without biopsies.

The focus of this paper therefore was more on what measurements are needed and how imaging techniques can currently get these measures. Although most uses of the imaging techniques allowed direct measurement of the key parameter, in some cases imaging was used to get one part of the measure (typically deformation for mechanical testing). There will continue to be new imaging techniques or modifications of current ones that will be able to more accurately get these measurements as well as allow better non-invasive clinical assessments.

Specifically the goal was to talk about the critical measures in quantifying wound healing (particularly in tissue engineering/regenerative medicine) and how to use them. For *in vitro* and *in vivo* assessment microscopy currently is one of the best imaging techniques, but is hard to do clinically; with many imaging techniques getting closer and closer to obtaining similar information. Although currently imaging for mechanical properties tends to be to get distance measures more accurately (for deformation); a number of techniques can get fiber volume fraction and orientation; which can be used to approximate mechanical properties.

Although the emphasis was on what is needed to make these quantitative measures and how imaging techniques can be used, imaging techniques can also get information on some of the bioprocesses that control or help determine these measures. Although angiogenesis is one of these bioprocesses that helps control healing rate, it was singled out as part of Tissue Health. There are still many other bioprocesses that control the key wound healing measures selected: wound healing rate, angiogenesis, tissue stiffness, and tissue strength.

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Conflict of Interest Disclosure Statement

The author is CEO of Surface Integrity, a company that received STTR funds to develop a Mg alloy for internal fixation particularly in orthopedics.

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