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The ultimate objective of our research is to develop an integrated laser-based system for treating burn injury. The first phase of this development effort was funded under the present contract. The objective was to design, construct and optimize a diagnostic system suitable for clinical use in humans that accurately determines burn depth. The diagnostic system utilizes an exogenously administered fluorescent dye to label skin vasculature that is detected by a fluorescence imaging system. During the past year the design criteria for such a system were formulated, the components acquired and the system constructed. A series of experiments have been carried out in animals to test and optimize the system. We have demonstrated that the ratio of fluorescence intensity detected by the system at a burn site to that detected from normal skin, can be used to accurately determine the extent of burn injury. The burn diagnostic system is now ready for clinical deployment to gather further data and refine the system.
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INTRODUCTION

The ultimate objective of our research is to develop an integrated laser-based system for treating burn injury. The system will be capable of assessing the extent of burn injury including an accurate determination of burn depth and will rapidly debride burn eschar under optical feedback control. This complete system will take several years to develop. The first phase of this development effort was funded under DOD Grant # DAMD-17-92-J-2019. The overall objective of that one year proposal was to design, construct and optimize a diagnostic system suitable for clinical use in humans that accurately determines burn depth. The diagnostic system utilizes an exogenously administered fluorescent dye to label skin vasculature that is detected by a fluorescence imaging system. At the completion of the grant term, the unit has been constructed, tested in large and small animals and is ready for clinical deployment. To achieve this goal we:

1. Formulated design criteria for a video fluorescence imaging system based on previous optical measurements in animals and acquired the necessary system components including a high gain charge-coupled device (CCD) camera, optical components, fluorescence excitation light source (flash lamp), image processing electronics and computer hardware.
2. Used the components to construct a user-friendly, reliable system suitable for clinical use. This required extensive optical and mechanical engineering and the development of custom computer software.
3. Tested and optimized the system in large and small animals. Testing included an assessment of accuracy, reproducibility and overall optical performance.
4. Examined speckle as an alternate scheme for improving depth definition.
5. Explored the feasibility of using a semiconductor diode laser as an infrared fluorescence excitation light source.

Approximately 10% of wartime casualties are the direct result of burn injury. In addition, there are more than 100,000 serious civilian burn injuries per year in the United States which result in millions of hospital days and an estimated cost of several billion dollars. Thus, a method to improve the clinical outcome for burn victims would represent an important medical advance. Burn surgery has made tremendous progress by providing aggressive supportive care for burn victims and by performing excision and skin-grafting of deep partial and full-thickness burns in a timely fashion. Two major limitations of this modern approach to burn management are bleeding during burn eschar debridement and an inability to accurately determine depth of burn injury. The ultimate goal of our investigations is to develop an integrated laser-based system that would overcome both of
these limitations. The system would determine extent of burn injury and automatically debride burn eschar in a rapid, hemostatic fashion under feedback control. Furthermore, the system will produce an ablation bed suitable for subsequent skin grafting by conventional skin grafts or cultured autologous keratinocytes. Therefore, when fully implemented this system would represent a major advance in the care of burn victims. In addition, it is likely that the system will contribute to the management of other conditions requiring debridement and skin grafting such as skin tumors, cold (frostbite) injury and skin ulcers.

Prompt burn eschar excision with immediate wound closure decreases mortality and shortens hospital stay and is the standard of care in many institutions (1,2). Ideally excision of burned skin occurs as soon as possible after admission, once the patient is hemodynamically stable (3). In order to conserve surface and subjacent skin during surgery, areas of skin which will heal spontaneously with minimal scarring (superficial-dermal or partial-thickness burns) must be differentiated from areas which will not heal within two weeks and therefore require surgical excision and grafting (deep-dermal or full-thickness burns) (3-5). Clinical criteria to distinguish burn depth including sensitivity to pin-prick, visual appearance and viable cutaneous circulation are often inaccurate in predicting the depth of a burn wound (6) and in guiding surgeons during excisions (7). When conservative tangential excisions are performed in the primary excision and grafting of wounds, the surgeon uses punctate capillary bleeding to delineate underlying viable tissue but this results in significant blood loss (3,8,9). Once within a burn or escharotomy site, clinical criteria can not reliably identify the critical zone of vascular stasis which divides necrotic from viable tissue (7,10-13). It is often easier to excise the entire burned skin and subcutaneous tissue to muscle fascia, which is a distinct, graftable plane (3,14) but this approach may sacrifice significant amounts of viable skin. Thus, the accurate determination of burn depth is a key component of modern burn surgery.

Because clinical assessment of burn depth is difficult, several attempts to develop technical means of predicting burn depth have been made during the last half-century. Unfortunately, none have gained widespread clinical acceptance. Limitations have included inadequate accuracy on selected days post burn, cumbersome and time consuming techniques and toxicity. Passive infrared thermography showed early promise in distinguishing burn depth (15-18) but false positive images were common and thermography has not been able to reproducibly distinguish superficial from deep-dermal burns. High frequency ultrasound was felt capable of delineating the acoustic interface between viable and necrotic skin (19-22) but when tested clinically could not exceed the ability of burn surgeons to gauge burn depth by clinical methods (19). Burn wound depths have also been evaluated by the use of
dioactive isotopes (23) where re-epithelialization and hypercellularity increases isotope uptake in a healing area. This too has not proven to be a clinically viable technique. An optical reflectance technique has been used to evaluate debrided burn injuries (24-25). The ratios of reflected green and red light to infrared light were determined initially with a fiberoptic instrument but more recently a video camera has been used. In clinical testing, the best correlation between reflectance ratio values and burn depth occurred on day three post-burn in areas where the epidermis was removed. The major limitation of this technique is its inaccuracy in the crucially important first three days following burn injury. Blood flow measurements with the Doppler technique have been used to estimate the depth of burn injury in human skin (26-27). Although accurate, the method is slow (measurement at a single point requires 10-15 minutes) and can not provide information over a large area burn.

In our proposal we described a fluorescence technique to assess burn depth. The use of fluorescent vital dyes to detect viable cutaneous circulation and predict burn depth is not new. In 1943, intravenous fluorescein was used to differentiate second from third-degree burns (28). It was hypothesized that the tissue destruction present in a third-degree burn impeded vascular transportation of the drug to the upper layers of burned skin. Studies on medical student volunteers who received injections of fluorescein demonstrated that when examined with a Woods lamp "third-degree" burns appeared black, while "second-degree" burns appeared yellow-green. The observed fluorescence intensity was dependent on two physical factors; a viable cutaneous circulation which transported the dye to the tissue and overlying burn eschar which transmitted, absorbed and scattered the incident exciting Woods lamp (ultraviolet) radiation and subsequent fluorescence emission (560 nm). The shallow (approximately 50 μm) penetration of incident ultraviolet light through skin and eschar (29) limited this method to evaluating intact superficial cutaneous circulation; deeper viable remnants of burned skin covered with eschar could not be detected. It therefore failed to differentiate superficial from deep partial-thickness wounds and did not gain clinical acceptance.

In 1983 another attempt to use intravenous fluorescein for burn depth assessment was made with a fluorometer to quantify fluorescence (30). Quantitative fluorescence measurements during the first 48 hours and between 72 and 144 hours post burn were able to distinguish partial-thickness from full-thickness burns. Partial-thickness burns exhibited fluorescence within 10 minutes after intravenous injection of fluorescein while full-thickness burns showed negligible fluorescence. However, the method failed to distinguish between shallow and deep partial-thickness burns, which in many institutions require different surgical approaches (3).
In our study, indocyanine green (ICG) was used as the fluorescent probe. ICG is a tricarbocyanine dye that has been used for measuring cardiac and hepatic output in humans for thirty years (31-32). It is nontoxic and rapidly excreted by the liver into bile (33). ICG has several advantages over fluorescein for assessing cutaneous blood volume/perfusion and burn depth. Unlike fluorescein which has a single UV absorption band and a mid-visible fluorescence emission, ICG has two major absorption bands, one in the near-UV and one in the near-IR and produces infrared (840 nm) fluorescence. Because near-IR light travels through tissue with little absorption, ICG fluorescence emissions arising deep within tissue can still be detected at the surface; something that is not possible with fluorescein.

The assessment of burn depth can be viewed as a determination of viable cutaneous circulation at different levels in the skin. Because UV light penetrates skin and eschar superficially compared with near-IR light (29) superficial and deep intravascular ICG can be differentiated by comparing fluorescence following UV and near-IR excitation, respectively. This will potentially be useful in guiding the laser debridement of burn eschar. In addition, ICG is strongly bound to serum proteins and when compared to fluorescein (which is less than 50% protein bound), ICG is much less likely to leak from the vasculature into surrounding tissue.

**BODY**

Specific Goal *1*

Formulated design criteria for a video fluorescence imaging system based on previous optical measurements in animals and acquired the necessary system components including a high gain charge-coupled device (CCD) camera, optical components, fluorescence excitation light source (flash lamp), image processing electronics and computer hardware.

Through a series of iterative experiments, the following design criteria for the fluorescence imaging system were established.

Source:

A xenon flash lamp is used as the excitation source. This source's wide spectral band width provides a great deal of flexibility (see spectrum in Fig. 1). As mentioned earlier, ICG can be excited at more than one wavelength (absorption spectrum of ICG is shown in Fig. 2). Hence, with a broad band source, the wavelength of choice can be selected by using an appropriate bandpass filter. This allows this device to be used in the debridement phase of the project where the UV excitation wavelength will be used in addition to its use as the diagnostic system where the IR excitation wavelength is used.
We desired an extremely intense excitation source to allow maximum flexibility in the design criteria of other components. Thus the choice of a pulsed source which can produce high intensity while minimizing heat build up. We acquired a Speedotron (Model 202VF/2405) 2400 Joule flash lamp.

Fig. 1: Spectrum of a xenon lamp

Fig. 2: Absorption spectrum of ICG in water
Filters:

Filters at the selected excitation and emission wavelengths of ICG were acquired. These wavelengths were determined after performing in vivo measurements of ICG fluorescence. Burns were created by applying a brass block heated to 100 C to the flank of a hairless, fuzzy rat for 10 s. The rat was then intravenously injected with ICG (2 mg/kg). A fiber optic probe coupled to a SPEX fluorometer was used to take excitation and emission scans from the burn site. The excitation peak was found to be fairly broad, with a peak at 800 nm. The emission peak was found to occur at 825 nm. (See Figs. 3 and 4). Using this information, custom manufactured bandpass filters were obtained. Because it is difficult to design filters that can discriminate two closely spaced wavelengths with a high degree of rejection, we chose to use an excitation wavelength of 780 nm. Fortunately, the absorption peak of ICG is broad, and this results in only minimal loss of efficiency. The emission filters used in our system are centered at 825 nm. Spectral performance of the filters is shown in Fig. 5.

The fluorescence signal is very weak relative to the excitation flash. To isolate this weak signal, the light outside the band pass of the filters must be well attenuated. Measurements indicated that a rejection factor of 10^12 was needed, which we attained by using 2 filters of OD 6.

![Fig. 3: Excitation scan at burn site](image-url)
Fig. 4: Emission scan at burn site

Fig. 5: Spectra of 780 and 825 nm filters
Detector:

Our system uses a high gain CCD camera to acquire fluorescence signals in a two dimensional image. CCD arrays were selected because they have high sensitivity in the near IR (the region of sensitivity required by this system). After examining the performance of numerous CCD array cameras, we selected a Dage (model C72) camera. This camera has digitally adjustable gain and possesses adequate sensitivity for our application. The adjustable gain allows us to vary the gain of the camera in an accurate and reproducible fashion. We selected a fixed focal length lens (D. O. Navitar 37.5 mm) with a large aperture (f/1.1) to maximize light collection. Color images of the burn site are acquired with an Elmo (model Mw401E) color camera. This camera has an extremely small head and can be positioned very close to the Dage allowing both cameras to acquire images from the same vantage point.

Computer:

We chose a Macintosh platform (Quadra 900) for this system to allow for maximum user-friendliness. A Perceptics frame grabber board was installed to acquire and digitize the fluorescence images. This board is supplied with a library of Lab View routines for software control. A RasterOps video board acquires the color images. For documentation purposes, photographic quality hard copies of the images can be obtained on a GCC dye sublimation color printer.

Specific Goal #2

Used the components to construct a user-friendly, reliable system suitable for clinical use. This required extensive optical and mechanical engineering and the development of custom computer software.

The components were assembled into a portable burn diagnostic system (BDS). The flash lamp and cameras were mounted into a movable head. The excitation wavelength is isolated with 2 filters centered at 780 nm. The diffuse light produced by the lamp-filter combination is collected and focused onto a plane 24 inches from the head using a fresnel lens. The Dage CCD camera views the area illuminated by the flash and detects the fluorescence generated. Two emission filters centered at 825 nm isolate the fluorescence signal from the excitation flash and other contaminating wavelengths. The Elmo color camera provides a visual image of the same area. The head is mounted on an arm, which in turn is in turn mounted on a pneumatic lift. This permits the head to be positioned over the area of interest at the appropriate working distance. The working
distance is identified by 2 cross-pointing diode lasers whose beams overlap in the appropriate plane. A picture and a schematic of the system are shown in Figs. 6 and 7, respectively. The system has been engineered for use in a standard hospital ward or operating room. It meets the standards for shielding of electrical noise as defined by MGH.

Fig. 6: A picture of the Burn Diagnostic System
The entire image acquisition process is controlled by a custom software package titled "Burn Imaging System" (BIS). This program is based on the Lab View program. Concept VI’s provided with the Perceptics image acquisition board are used to communicate with the frame grabber board. The top level program is written in C and creates a user-friendly, menu driven graphical interface (see Fig. 8). In operation, the user adjusts the head to visualize the desired area with the help of a live color image displayed in one window. Once the area of interest is set, fluorescence image acquisition is initiated with a “capture” button. A custom built electronics circuit controls the timing so that the flash is triggered at the appropriate time with respect to the camera frame sequence. The frame corresponding to the flash is then acquired and displayed on the computer screen adjacent to the color image. This image pair can then be saved or discarded at the discretion of the user.
Specific Goal #3

Tested and optimized the system in large and small animals. Testing included an assessment of accuracy, reproducibility and overall optical performance.

The BDS has been tested and optimized in animals. A series of experiments was undertaken during the construction phase of the project to iteratively establish design criteria and then to verify the design choices. The reproducibility of the flash and uniformity of the illumination were tested. The flash reproducibility was measured with a photodiode to be within 2% once it has stabilized for half an hour. The area visualized by the camera is 5 x 5 inches. Over this area, the flash uniformity was found to be 5%. This was measured in two ways. We imaged the fluorescence from a flat dish containing an ICG solution and imaged the reflectance of the flash from a reflectance standard (gray color card). We then measured the uniformity of the detected grey levels in the images. The working distance of 24 inches was determined based on optimizing this uniformity. The fluence of the lamp at this working distance is 2 mJ/cm². This fluence was found to
be sufficient to adequately detect a fluorescence signal from a very weak concentration of ICG. We were able to detect a fluorescence signal in the rat animal model described earlier with a dose as low as 0.02 mg/kg.

The accuracy of the system was tested in an experiment using a pig animal model. The goal of the experiment was to test the ability of the system to perform the desired task of burn wound differentiation. Burns that would heal in 21 days and hence, should be left alone need to be differentiated from burn that will not heal in 21 days and hence, need to be grafted. In particular, the system's performance with variations in burn location (and thus in skin thickness) and time following burn injury was studied. Both these factors are variable in the “real world” and so the system should either be independent of them or should be able to correct for them.

4x4 cm brass blocks were heated to 100 °C. Three (20 kg) farm pigs were shaved and sterilely prepped. Burns were induced on the skin by applying the heated brass blocks to the skin for varying durations of time. ICG (0.2 mg/kg) was injected after a certain time delay. Burns were then imaged with the BDS after fifteen minutes. The location of the burn, duration of the burn, and time of imaging were set according to the following algorithm:

for each of 2 time durations (24, 48 hours)
  for each of three locations (back, flank, abdomen)
    apply three (4x4 cm) burns to location – 4, 6 and 8 s burns
    wait time duration
    inject ICG (0.2 mg/kg)
    wait 15 minutes
    image fluorescence
    dress wounds
    observe healing for 21 days
  end
end

Dressings were changed every three days. Antiseptic Silvadene cream was applied along with sterile gauze pads to the wounds to prevent infection at every dressing change. The binary healing endpoint at Day 21 as determined by an experienced burn surgeon (healed/did not heal) was then compared to the fluorescence ratio measured from the acquired images. The fluorescence ratio is defined as:
Post-inject fluorescence at burn site - Pre-inject fluorescence at burn site
Post-inject fluorescence at normal skin - Pre-inject fluorescence at normal skin

The results are as follows:

<table>
<thead>
<tr>
<th>Burn Location</th>
<th>Burn duration</th>
<th>Time imaged</th>
<th>Flu. ratio</th>
<th>Healing result</th>
</tr>
</thead>
<tbody>
<tr>
<td>abdomen</td>
<td>4 s</td>
<td>24 hours</td>
<td>0.68</td>
<td>healed</td>
</tr>
<tr>
<td>abdomen</td>
<td>8 s</td>
<td>&quot;</td>
<td>0.63</td>
<td>did not heal</td>
</tr>
<tr>
<td>flank</td>
<td>4 s</td>
<td>&quot;</td>
<td>1.0</td>
<td>healed</td>
</tr>
<tr>
<td>flank</td>
<td>6 s</td>
<td>&quot;</td>
<td>0.97</td>
<td>healed</td>
</tr>
<tr>
<td>flank</td>
<td>8 s</td>
<td>&quot;</td>
<td>0.65</td>
<td>did not heal</td>
</tr>
<tr>
<td>back</td>
<td>4 s</td>
<td>&quot;</td>
<td>0.79</td>
<td>healed</td>
</tr>
<tr>
<td>back</td>
<td>6 s</td>
<td>&quot;</td>
<td>0.81</td>
<td>healed</td>
</tr>
<tr>
<td>back</td>
<td>8 s</td>
<td>&quot;</td>
<td>0.82</td>
<td>healed</td>
</tr>
<tr>
<td>abdomen</td>
<td>4 s</td>
<td>48 hours</td>
<td>0.63</td>
<td>healed</td>
</tr>
<tr>
<td>abdomen</td>
<td>8 s</td>
<td>&quot;</td>
<td>0.46</td>
<td>did not heal</td>
</tr>
<tr>
<td>flank</td>
<td>4 s</td>
<td>&quot;</td>
<td>0.88</td>
<td>healed</td>
</tr>
<tr>
<td>flank</td>
<td>6 s</td>
<td>&quot;</td>
<td>0.82</td>
<td>healed</td>
</tr>
<tr>
<td>flank</td>
<td>8 s</td>
<td>&quot;</td>
<td>0.41</td>
<td>did not heal</td>
</tr>
<tr>
<td>back</td>
<td>4 s</td>
<td>&quot;</td>
<td>0.79</td>
<td>healed</td>
</tr>
<tr>
<td>back</td>
<td>6 s</td>
<td>&quot;</td>
<td>0.76</td>
<td>healed</td>
</tr>
<tr>
<td>back</td>
<td>8 s</td>
<td>&quot;</td>
<td>0.57</td>
<td>did not heal</td>
</tr>
</tbody>
</table>

Examining the results (see Fig. 9), it is evident that there is some overlap in the fluorescence ratios of the two outcomes. However, a ratio of 0.68 will differentiate the two classes, erring slightly on the side of safety. Further testing will, of course, have to be carried out for statistical significance. The system is, however, ready to be used on human as well as animal patients to gather data and further refine the segmentation algorithm.

It should be noted that burn durations of 4, 6 and 8 s were used because they were found to represent the zone of crossover from healing to non-healing burns. That is, 2s burns were found to heal well within 21 days, while 10+ s burns did not heal.
Specific Goal #4

Examined alternate schemes for improving depth definition.

We have proposed to determine burn eschar thickness by measuring the spatial modulation of near field laser speckle in tissue. Near field laser speckle is a phenomena that arises when coherent photons cross paths at the surface of a scattering medium. These photons interfere coherently and produce a grainy pattern on the surface of the medium.

A two layered model is used to simulate the anatomy of burn tissue. This model consists of a "dead" layer (eschar) overlying a "live", vascular layer (viable tissue). The scatterers in the dead layer are static, for it contains no blood flow. Thus the photons that propagate through the static layer give rise to a near field speckle pattern that remains stationary over time. The viable layer, however, contains moving scatterers in the vasculature. Scattered photons that reach the vascular layer form a near field speckle pattern that is modulated at the surface (Fig. 10).
Diffusion theory and Monte Carlo simulations have shown that photons propagating deeper into a turbid media have a higher probability of leaving the media further away from the source entry point. Thus, the measurement of the diameter of the stationary speckle pattern should enable the determination of the static layer thickness.

The method consists of irradiating the tissue with a coherent point source and acquiring a frame-averaged near field speckle profile. The modulated speckle is blurred during the frame averaging period, so only the static speckle remains visible. Once the speckle profile is acquired, the diameter of the stationary speckle is measured. The optical properties of the point in question are then determined by diffuse reflectance spectrophotometry. Finally, isofluence contours computed by Monte Carlo simulations are used to determine the eschar thickness as a function of the static speckle diameter.

A feasibility study has been performed using gelatin phantoms to simulate the static layer and a scattering liquid to represent the moving layer. The results of this initial study show that the diameter of the static speckle profile directly correlates with the thickness of the static layer (Fig. 11).

In addition, in vivo near field speckle profiles of animals burned using the standard burn model discussed earlier have been imaged. The static speckle diameter can be seen to increase with the time of application of a heated brass block (Fig. 12). These preliminary results demonstrate the viability of this method for humans burn patients in vivo. Future studies using both phantoms and live animal subjects are planned that will further investigate the accuracy of burn eschar thickness measurement by analysis of near field speckle modulation.
Experimental Data

Monte Carlo Results

Fig. 11: Static speckle diameter vs. gelatin phantom thickness

Fig. 12: Near field speckle profiles as a function of duration of burn
Specific Goal #5

Explored the feasibility of using a semiconductor diode laser as an infrared fluorescence excitation light source.

Semiconductor diode lasers are an attractive choice as an infrared excitation source due to their compact size, high efficiency and monochromacy. We studied a diode laser made by Star Medical Technologies and found it to be a viable candidate for use in a dedicated Burn Diagnostic System. Because it is monochromatic, contamination due to cross talk of band pass filters (as our current system is implemented) is completely eliminated. Thus the signal to noise ratio (and hence, the sensitivity) of such a scheme is far superior. The laser we tested is powerful enough with the beam spread out to uniformly illuminate a 5x5 inch area to perform at least as well as the BDS.

CONCLUSIONS

In summary, we have successfully developed a video fluorescence imaging system that detects fluorescence from the dye indocyanine green. Extensive testing in small and large animal models of burn injury suggests that the technique can accurately distinguish burns that will heal spontaneously within 21 days from those that will not. The diagnostic accuracy appears to be present as early as one hour after burn injury and is superior to any previously reported method for assessing burn depth. In future experiments we plan to further refine the image analysis algorithms and extend the study to human subjects.

The burn diagnostic system will be a valuable diagnostic tool to burn surgeons. The improved accuracy provided by this device will assist surgeons by allowing them to analyze burn wounds early in the course of a patient’s care. The system is also the first major step towards achieving our ultimate goal of developing a laser-based system for the semi-automatic debridement of burn injury. In future studies the use of optical techniques to guide laser ablation as well as optimization of the laser ablation process itself will be addressed.
REFERENCES


