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Caigang Zhu: He developed the optical system and performed optical measurements. He also processed the data and wrote this manuscript.

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Christopher Hoe-Kong Chui: He performed the flap surgery in all animals and he was also involved in manuscript preparation.

Bien-Keem Tan: He designed and participated in the flap surgery and he was also involved in the discussion of this manuscript.

Quan Liu: He designed this project and critically revised this manuscript.

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Abstract

Accurate and early prediction of skin flap viability is vitally important in reconstructive surgery. This is the first pilot study to evaluate the simultaneous use of both visible diffuse reflectance and autofluorescence spectroscopy on a reverse MacFarlane rat dorsal skin flap model in the early prediction of skin viability, to our best knowledge. A total of 62 flap measurement sites from eleven Sprague Dawley rats were monitored for 72 hours. Both statistical analysis using measured spectra and quantification of physiologically relevant tissue parameters using empirical methods were performed. The statistical analysis results suggest that either visible diffuse reflectance spectroscopy or autofluorescence spectroscopy alone can predict the skin viability accurately; however, autofluorescence spectroscopy is more sensitive to tissue changes in the first two hours after induction of ischemia. The pilot study shows that it is feasible to predict flap failures in the first two hours when using autofluorescence spectroscopy alone; moreover, it is possible to predict flap failures even in the first 15 minutes with high accuracy when using diffuse reflectance and autofluorescence spectroscopy simultaneously. Meanwhile, several physiologically

relevant parameters including hemoglobin oxygenation, total hemoglobin concentration and redox ratio indicators estimated from diffuse reflectance and autofluorescence spectra show distinctively different trends over time for non-viable and viable skin. These findings will be helpful to clinicians for making precise judgment on flap viability. Furthermore, our results highlight the advantage of using autofluorescence spectroscopy in the early prediction of skin flap viability relative to diffuse reflectance spectroscopy.

Key words: skin viability, early prediction, plastic surgery, diffuse reflectance spectroscopy, autofluorescence spectroscopy

1. Introduction

Cutaneous flaps are frequently used to provide well vascularised wound coverage in reconstructive surgery. The vascular supply to a flap is crucial, and if compromised may lead to flap failure [1, 2]. Most flap losses occur within 72 hours of surgery [3], thus it is critical to closely monitor flap perfusion during this period and detect any impairment as early as possible to maximize the chance of flap salvage. Flaps are traditionally assessed clinically with periodic examination of capillary refill, flap color, temperature and bleeding patterns; however this relies heavily on the skill and the availability of trained hospital staff.

A variety of technologies have been used to augment clinical judgement, these can be broadly divided into two categories: those that assess blood flow and those that assess cellular metabolism. Methods that rely on measurement of blood flow are prone to measurement errors and poor reproducibility [1, 4-6]. Inadequate blood flow infers inadequate delivery of oxygen to tissues; however, direct assessment of cellular

utilisation of oxygen is not obtained. Near infrared (NIR) diffuse reflectance spectroscopy has been investigated for assessing tissue viability by measuring hemoglobin oxygenation [7-9], hemoglobin concentration [8], and tissue hydration [9]. Visible light spectroscopy has also been used to characterize tissue viability by measuring tissue oxygenation and total hemoglobin concentration [10, 11]. Fluorescence spectroscopy utilizing exogenous [12] and endogenous fluorophores [13-16] to measure the metabolic rate of tissue has also been reported in recent years. Diffuse reflectance spectroscopy and autofluorescence spectroscopy provide complementary information about tissue status and have shown excellent potential for assessing tissue viability separately, however their simultaneous use to characterize the skin flap metabolism and their respective accuracies in flap viability assessment have not been studied to our best knowledge. In this study, for the first time, we performed both visible diffuse reflectance and autofluorescence measurements simultaneously on a reverse MacFarlane rat dorsal skin flap model to evaluate their value in the early assessment of tissue viability.

2. Materials and Methods

Animals

All animal experiments were conducted in compliance with the SingHealth Institutional Animal Care and Use Committee (IACUC) animal welfare committee's requirement for the care and use of laboratory animals in research. The animals were housed separately. Eleven Sprague-Dawley rats at an average age of 16 weeks were acclimated three days prior to any procedures. All the procedures were done under 1.2-2% isoflurane inhalational anesthesia. Prior to surgery, the dorsum of the rat was

shaved and chemically depilated and the sites at which optical measurements would be made were marked on the exposed skin.

Surgical methods

After induction of anaesthesia, the animal was positioned in a prone position and 2 x 8 cm reverse McFarlane flaps without panniculus carnosus were raised as described previously[17]. The wound was closed underneath the flaps to prevent revascularisation from the wound bed and the flap was secured to the underlying skin with non-absorbable sutures. Final skin viability was determined clinically 72 hours after flap elevation, prior to euthanasia of the animals.

Non-invasive optical measurements

Visible diffuse reflectance and autofluorescence spectra measurements were taken immediately prior to surgery and further measurements were taken immediately after flap elevation, at hourly intervals in the 4 hours immediately following the surgery, then 4 hourly for the next 68 hours. Diffuse reflectance and autofluorescence spectra were measured by a compact spectrometer (USB4000, Ocean Optics, Dunedin, Florida, US) coupled to a custom bifurcated fiber optic probe. The scheme of the entire optical measurement system and custom-built bifurcated fiber-optic probe was shown in Fig.1.

Fig.1. (a) Schematic of optical spectroscopy measurement system; (b) custom-built bifurcated fiber-optic probe.

A diode laser at 405 nm (PhoxX®405-120, Omicron, Germany) was utilized to excite autofluorescence, while a compact white light source (HL-2000-FHSA, Ocean Optics, Dunedin, Florida, US) was used to provide broadband light in diffuse reflectance measurements. The fiber-optic probe as shown in Fig. 1(b) consisted of one central fiber for illumination and four surrounding fibers for detection. The core diameters of the illumination and detection fibers were 200 μm . The average source-detection separation was around 490 μm . The total time to carry out a full set of diffuse reflectance and autofluorescence measurements at all marked locations in one animal was around 15 minutes. It should be noted that although the flaps were monitored for 72 hours, autofluorescence measurements at these sites would not be reliable once the measured skin region became necrotic. These autofluorescence spectra were much noisier than those measured earlier at the same location, which was likely due to physical changes such as formation of eschar following ischemia. Thus only the data obtained in the first 12 hours were analysed.

Data analysis

The qualitative analysis on diffuse reflectance spectra was restricted to a wavelength range of 450-700 nm that covers the major portion of the visible spectrum, while the analysis on autofluorescence spectra was restricted to an emission wavelength range of 420-700 nm that covers the emission peaks of most tissue fluorophores. Each measured spectrum was calibrated following a standard procedure [18, 19] and smoothed using a digital median filter first, and then normalized by dividing each data point by the maximum peak intensity in the spectrum. Next, the partial least square (PLS) analysis [20] was conducted on the normalized diffuse reflectance or autofluorescence spectra to find principal components (PCs) to represent the measured spectra. The first 15 PCs of each spectrum were retained, which accounted for over 99% variance in the original spectra data. A Wilcoxon rank-sum test [21] was applied to identify a subset of the PC scores that show statistically significant differences ($p < 0.05$) between the non-viable and viable groups, which would be diagnostically important. This subset of PC scores up to a range of early time points were then fed into a linear discriminant analysis [22] (LDA) classifier to predict skin viability at the end of 72 hours. A leave-one-out cross validation method [23] was used in the analysis to obtain an unbiased estimate of the prediction accuracy. The reason for using PC scores instead of raw spectra in prediction is that not every spectral data point contains equally useful diagnostic information. The PLS and Wilcoxon rank-sum test sorted out the data in a transformed form that contain the most meaningful diagnostic information to yield higher accuracy than raw spectra. The non-viable group was treated as positive case while the viable group was treated as negative case in the calculation of sensitivity and specificity. The overall accuracy, sensitivity and specificity of prediction using diffuse reflectance spectra alone, autofluorescence spectra alone and their combination were compared.

Measured diffuse reflectance spectra were used to estimate the indicators of total hemoglobin concentration (THB) and hemoglobin oxygen saturation (StO₂) as previously described [24]. THB was derived from autofluorescence spectra using a ratio-metric method [15]. In addition, the indicator of redox ratio was also estimated from autofluorescence spectra. The reduction-oxidation (redox) ratio is an important metabolic biomarker that has been widely used in tissue characterization. It is indicative of the redox state and reflects cellular metabolism and oxygen consumption of the tissue. The redox ratio can be calculated using the autofluorescence intensities of two major fluorophores in tissues, i.e. reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD). It is commonly defined by the following equation [16],

$$redox \text{ ratio} = \frac{[FAD]}{[FAD] + [NADH]}$$

where the [FAD] and [NADH] represent the concentrations of FAD and NADH respectively. In this report, the measured autofluorescence intensity around 530 nm and 470 nm were used to represent the FAD and NAD concentrations since these two wavelengths corresponded to the emission peaks of FAD and NADH, respectively.

The trends of these four derived parameters over time were shown in table 2 to table 5 and Fig. 3 to Fig. 6 respectively. Every data point shown in the tables or figures has been divided by the baseline value in the same set that was measured shortly prior to the surgery. Although the four parameters were reported as relative to the baseline value instead of absolute concentrations, it is sufficient to demonstrate the trend of each individual parameter over time. The p-values shown in the tables were obtained by conducting a t-test on the data for comparison between the non-viable and viable groups.

3. Results

Flap viability

Using this model, an area approximately half the length of the flap (furthest from the pedicle) always became necrotic by 72 hours. Fig. 2 shows a typical skin flap created in this study at different time points. The area of necrosis is identified by the black eschar. A total of 62 flap measurement points from eleven Sprague-Dawley rats were assessed for 72 hours. Among these points, 31 were deemed non-viable and 31 deemed viable after 72 hours.

Fig.2. A typical flap shown at 15 minutes, 4 hours, 12 hours and 72 hours post flap elevation. Spectroscopic measurement sites are indicated by blue circles on the flap. The non-viable skin at the cranial half of the flap becomes black and dark after 72 hours.

Classification accuracy obtained by PLS regression

Table 1 shows the accuracies calculated from LDA classification between non-viable and viable groups using those PC scores obtained by PLS regression and Wilcoxon

rank-sum test that demonstrated statistically significant differences with a p-value of 0.05. The overall classification accuracy when using diffuse reflectance spectra alone increased with time and it was always higher than 90%. The accuracy when using autofluorescence spectra alone was always higher than 92%. It is worth highlighting that the accuracy when using autofluorescence spectra alone reached a maximum of 97.6% in just 2 hours after flap elevation. In terms of sensitivity, autofluorescence spectroscopy always provided slightly better or equal accuracy in the first 12 hours. In terms of specificity, autofluorescence spectroscopy provided significantly better accuracy in first 3 hours and equal accuracy from 4 hours to 12 hours.

Table 1. The overall classification accuracy, sensitivity and specificity achieved with linear discriminant analysis (LDA).

Time (h)	0.25	1	2	3	4	8	12
Overall accuracy (%)							
R	90.3	90.2	90.2	92.7	93.5	95.2	98.4
F	95.2	92.7	97.6	97.6	95.2	95.2	96.8
R+F	98.4	92.7	97.6	95.1	95.2	95.2	90.2
Sensitivity (%)							
R	93.5	95.7	91.3	95.7	93.5	96.8	96.8
F	100	95.7	95.7	95.7	96.8	96.8	96.8
R+F	100	95.7	95.7	91.3	96.8	93.5	90.3
Specificity (%)							
R	87.1	83.3	88.9	88.9	93.5	93.5	100
F	90.3	88.9	100	100	93.5	93.5	96.8
R+F	96.8	88.9	100	100	93.5	96.8	90.3

Legend: "Time" = number of hours after flap elevation; "R" = diffuse reflectance data alone; "F" = autofluorescence data alone; "R+F" = both diffuse reflectance and autofluorescence data.

Oxygen saturation indicator

The mean oxygen saturation indicator as derived from reflectance spectra for the non-viable group decreased to less than 32.3% of the baseline value within the first hour of ischemia and remained low. In contrast, the mean oxygen saturation for the viable

points decreased to no less than 61.2% of the baseline value within the first hour and then stabilized as shown in Table 2 and Fig. 3.

Table 2. Normalized oxygen saturation indicators derived from reflectance spectra

Time (h)	0	0.25	1	2	3	4	8	12
Mean $\pm$ Standard Deviation of normalized oxygen saturation indicator								
Non-viable	1.0 $\pm$ 0.0	0.45 $\pm$ 0.1	0.32 $\pm$ 0.1	0.29 $\pm$ 0.1	0.23 $\pm$ 0.1	0.29 $\pm$ 0.1	0.22 $\pm$ 0.1	0.24 $\pm$ 0.2
Viable	1.0 $\pm$ 0.0	0.75 $\pm$ 0.2	0.75 $\pm$ 0.3	0.69 $\pm$ 0.3	0.61 $\pm$ 0.3	0.61 $\pm$ 0.3	0.67 $\pm$ 0.3	0.69 $\pm$ 0.2
p-value	NA	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Legend: "NA" = "not available"

Fig. 3. StO₂ indicator derived from diffuse reflectance spectra over time from flap elevation.

Total hemoglobin concentration indicator

Mean THB from reflectance spectra for the non-viable group increased gradually after flap elevation. In contrast, mean THB for the viable skin group plateaued at around 1.5 as shown in Table 3 and Fig. 4.

Table 3. Normalized THB indicators derived from reflectance spectra

Time (h)	0	0.25	1	2	3	4	8	12
Mean $\pm$ Standard Deviation of normalized THB indicator								
Non-viable	1.0 $\pm$ 0.0	1.89 $\pm$ 0.6	1.93 $\pm$ 0.8	2.23 $\pm$ 1.0	2.39 $\pm$ 1.1	2.82 $\pm$ 1.4	4.46 $\pm$ 2.9	5.61 $\pm$ 3.8
Viable	1.0 $\pm$ 0.0	1.45 $\pm$ 0.5	1.62 $\pm$ 0.8	1.45 $\pm$ 0.5	1.50 $\pm$ 0.5	1.44 $\pm$ 0.6	1.47 $\pm$ 0.8	1.46 $\pm$ 0.7
p-value	NA	<0.01	>0.05	<0.01	<0.01	<0.01	<0.01	<0.01

Legend: "NA" = "not available"

Fig.4. THB indicator derived from diffuse reflectance spectra over time from flap elevation.

The results obtained from autofluorescence spectra were consistent with those obtained from reflectance spectra; see Table 4 and Fig. 5.

Table 4. Normalized THB indicators derived from autofluorescence spectra

Time (h)	0	0.25	1	2	3	4	8	12
Mean $\pm$ Standard Deviation of normalized THB indicator								
Non-viable	1.0 $\pm$ 0.0	1.34 $\pm$ 0.1	1.43 $\pm$ 0.2	1.46 $\pm$ 0.1	1.48 $\pm$ 0.2	1.42 $\pm$ 0.3	1.60 $\pm$ 0.4	1.64 $\pm$ 0.5
Viable	1.0 $\pm$ 0.0	1.23 $\pm$ 0.2	1.24 $\pm$ 0.2	1.23 $\pm$ 0.2	1.21 $\pm$ 0.1	1.19 $\pm$ 0.1	1.16 $\pm$ 0.2	1.16 $\pm$ 0.2
p-value	NA	<0.01	<0.01	<0.01	<0.05	<0.01	<0.01	<0.01

Legend: "NA" = "not available"

Fig.5. THB indicator derived from autofluorescence spectra over time from flap elevation.

Redox ratio indicator

The mean redox ratio value of the non-viable group decreased to 76.7% in the first hour and kept decreasing. In contrast, the mean redox ratio value for the viable group decreased to 88.0% in the first hour but then normalized to the baseline level by 12 hours. See Table 5 and Fig.6.

Table 5. Normalized redox ratio indicators derived from autofluorescence spectra

Time (h)	0	0.25	1	2	3	4	8	12
Mean $\pm$ Standard Deviation of normalized redox ratio indicator								
Non-viable	1.0 $\pm$ 0.0	0.81 $\pm$ 0.1	0.77 $\pm$ 0.1	0.75 $\pm$ 0.1	0.75 $\pm$ 0.2	0.72 $\pm$ 0.1	0.70 $\pm$ 0.2	0.64 $\pm$ 0.2
Viable	1.0 $\pm$ 0.0	0.92 $\pm$ 0.2	0.88 $\pm$ 0.1	0.90 $\pm$ 0.1	0.90 $\pm$ 0.1	0.91 $\pm$ 0.1	0.97 $\pm$ 0.2	1.00 $\pm$ 0.2
p-value	NA	<0.01	<0.05	<0.01	<0.01	<0.01	<0.01	<0.01

Legend: "NA" = "not available"

Fig.6. Redox ratio indicator derived from autofluorescence spectra over time from flap elevation.

4. Discussion

Successful reconstructive surgery requires vigilant postoperative monitoring that accurately detects early disturbances in tissue perfusion. An ideal monitor would allow continuous monitoring of tissue metabolism, allow rapid and accurate detection of irregularities in perfusion, and be minimally invasive.

In this pilot study, it is found feasible to predict flap failures in the first two hours when using autofluorescence spectroscopy alone; moreover, it is possible to

predict flap failures even in the first 15 minutes with high accuracy when using diffuse reflectance and autofluorescence spectroscopy simultaneously. This observation suggests that this technology has great potential for clinical use in flap monitoring or intra-operatively to determine tissue viability. Although flap failures usually occur within the first 72 hours after surgery, currently it can be only guessed whether and exactly when they will occur. In order to salvage a failing flap, it needs to be identified in a timely manner so that the disturbance in perfusion can be corrected with as little ischaemic injury to the flap as possible. This technology has the potential to provide continuous monitoring of skin flap status and alert the surgeon within 15 mins of a threat to flap viability.

From Fig.3 to Fig.6, clear trends in the indicators of StO₂, THB and redox ratio with time were observed after flap elevation. The trend of StO₂ was consistent with the previous findings [7, 9, 10]. Increases in THB were most likely due to venous congestion. When the venous outflow was inadequate it could lead to eventual skin necrosis [10]. Measuring the redox ratio using autofluorescence spectroscopy to predict skin viability has not been reported previously, but it seemingly holds great potential according to this study. In addition to its accuracy in prediction, the measurement of redox ratio using autofluorescence spectroscopy can non-invasively provide an indicator of in vivo cellular metabolism without requiring administration of exogenous fluorophores and measurements can be repeated frequently with no morbidity to patients. Inadequate oxygen supply, which is a common consequence in ischemia, would cause a slower oxidation and in turn a decrease in redox ratio in autofluorescence spectroscopy [25, 26]. Our findings in the trends of redox ratio for flaps shown in Fig. 6 are consistent with several published reports [13, 27].

Diffuse reflectance spectroscopy typically is more cost effective in system setup compared to autofluorescence spectroscopy; however, autofluorescence spectroscopy offers the advantage of earlier detection of tissue ischemia, so it would be preferred for early diagnosis in a non-contact setup [28]. The preference of one or the other technique depends on the tradeoff between the cost and performance. Diffuse reflectance spectroscopy alone at a low cost provides decent overall accuracy for early assessment of skin viability in the first 2 to 3 hours and an excellent overall accuracy within 12 hours. Autofluorescence spectroscopy alone, at a relatively high cost, offers excellent overall accuracy in the first 2 hours. The combination of both techniques yields excellent overall accuracy even in the first 15 minutes.

At this time, there is no single commercially available technology that has widespread use in flap monitoring thus clinical monitoring is still considered the gold standard. Laser Doppler flowmetry is one non-invasive technique that has shown promise[1]. In an animal study involving islanded flaps in pigs, Jia et al [29]found that laser Doppler scanning could predict skin viability with an accuracy of 91.3%. Improved accuracy has been achieved when it was combined with tissue spectroscopy to measure oxygen saturation [4]. However, laser Doppler flowmetry requires careful interpretation [30] even though it can only measure the blood flow, which is inadequate for flap assessment. Moreover, it cannot distinguish venous and arterial occlusion [1], which is very important in flap monitoring. In contrast, our proposed technique could overcome most limitations associated with laser Doppler flowmetry and offer considerably higher accuracy.

For the point measurement system used in this report, the area measured each time is around 0.8 mm^2 as shown in Fig.1. We are currently developing the technique in an imaging setup that would offer a much larger field of view and better spatial

resolution, which should facilitate the potential application of the technique in skin flap surgery.

5. Conclusion

Our study demonstrated that both visible diffuse reflectance and autofluorescence spectroscopy can accurately predict skin flap viability in a rat model, while autofluorescence spectroscopy is more suitable for early prediction of skin viability when imaging at high spatial resolution is desired.

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