Early detection and differentiation of venous and arterial occlusion in skin flaps using visible diffuse reflectance spectroscopy and autofluorescence spectroscopy

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Abstract: Our previous preclinical study demonstrated that both visible diffuse reflectance and autofluorescence spectroscopy, each of which yields a different set of physiological information, can predict skin flap viability with high accuracy in a MacFarlane rat dorsal skin flap model. In this report, we further evaluated our technique for the early detection and differentiation of venous occlusion and arterial occlusion in a rat groin flap model. We performed both diffuse reflectance and autofluorescence measurements on the skin flap model and statistically differentiated between flaps with and without occlusions as well as between flaps with venous occlusion and those with arterial occlusion based on these non-invasive optical measurements. Our preliminary results suggested that visible diffuse reflectance and autofluorescence spectroscopy can be potentially used clinically to detect both venous and arterial occlusion and differentiate one from the other accurately at an early time point.

**OCIS codes:** (170.1470) Blood or tissue constituent monitoring; (170.6510) Spectroscopy, tissue diagnostics; (170.4580) Optical diagnostics for medicine; (170.6935) Tissue characterization.

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1. Introduction

Cutaneous flaps are commonly used to provide well vascularized wound coverage in reconstructive surgery. The vascular supply to a flap is crucial to flap success. Any compromise in the vascular supply 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 the periodic examination of capillary refill, flap color, temperature and bleeding patterns; however, this approach relies heavily on the skill and the availability of trained hospital staff. A variety of technologies have been used to augment clinical judgment and decrease the time commitment and skill requirement of clinical staff, which can be broadly divided into two categories: those assessing the peripheral information of flap perfusion such as blood flow and oxygenation, and those assessing cellular metabolism. Methods that rely on the measurement of blood flow are prone to measurement errors and poor reproducibility [1, 4–6]. Inadequate blood flow infers insufficient delivery of oxygen to tissues; however, the direct assessment of cellular utilization of oxygen is not obtained. Near infrared 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 been reported in recent years, which could compensate for the weakness of those methods measuring the peripheral information of flap perfusion. Previously we evaluated both visible diffuse reflectance and auto-fluorescence spectroscopy to identify their values for the early prediction of skin flap viability in a MacFarlane rat dorsal skin flap model [17], our results show that either visible diffuse reflectance spectroscopy or autofluorescence spectroscopy alone can predict skin viability accurately and the combination of the two yields the highest accuracy for early prediction in the first 15 minutes.

Most of the technical studies reviewed above mainly focused on flap ischemic failure without identifying the occlusion type; however, it is vitally important to differentiate the occlusion type since it allows for early intervention and thereby improves the salvage rate in these compromised flaps [18]. Some clinical tissue flap studies focused on arterial occlusion alone [19] despite the fact that venous occlusion are more common and can lead to more severe damage. Some other flap studies focused on partial venous occlusion alone, which may only have the subtle signs of congestion in its early stages before developing into severe thrombosis. Recently, Gioux et al. [20] demonstrated the concept of spatial frequency domain imaging for flap oxygenation monitoring in a human pilot study and later Ponticorvo et al. [21] used the same technique to assess partial vascular occlusions in a swine pedicle flap by quantifying hemoglobin concentration and oxygenation. Their results showed that spatial frequency domain imaging was able to differentiate between venous occlusion and arterial occlusion though only reflectance measurements were involved. In this study, we evaluated visible diffuse reflectance and auto-fluorescence spectroscopy in a rat groin flap model to identify their values in the assessment of flaps with venous occlusion and arterial occlusion. Our preliminary results suggest that both visible diffuse reflectance and auto-fluorescence spectroscopy can be potentially used clinically to detect venous occlusion and arterial occlusion and differentiate one from the other accurately at an early time point. However, diffuse reflectance spectroscopy is more sensitive than auto-fluorescence spectroscopy for the early detection of occlusion in this particular study, which could be attributed to two potential reasons. One is that diffuse reflectance spectroscopy measures blood parameters directly affected by occlusion while auto-fluorescence spectroscopy measures cellular metabolism that is the secondary consequence of blood supply compromise. The other is that the optical signal in diffuse reflectance spectroscopy is much stronger than that in auto-fluorescence spectroscopy.
2. Materials and methods

2.1 Animals and surgical methods

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. Twelve Sprague-Dawley rats at an average age of 16 weeks were acclimated for three days prior to any procedure. All the procedures were performed on animals under 1.2-2% isoflurane inhalational anesthesia. Prior to surgery, the abdomen of the rat was shaved and chemically depilated and the sites at which optical measurements would be made were marked on the exposed skin.

After induction of anesthesia, the animal was placed in the supine position. A 3 cm × 3 cm square cutaneous flap was marked in both groins using a surgical skin marker. The skin was prepared with 1% povidone iodine alcohol solution. Bilateral groin skin flaps were designed and raised according to blood supply from the epigastric vessels on each side. Tegaderm dressing (3M) was placed between the wound bed and the flaps to prevent revascularization from the wound bed. Either the artery, or vein, or neither vessels (control) were ligated and transected according to the study group allocation. The flaps were then secured to their original positions and the wound edges closed with non-absorbable sutures. The animal was then left to recover for 24 hours before occlusion was started. Optical measurements were taken under anesthesia at the desired intervals. The optical measurements taken immediately prior to the occlusion was treated as the baseline. The measurement sites on each flap were selected to evenly cover the entire flap.

2.2 Non-invasive optical measurement

Spectral measurements were taken immediately prior to occlusion creation and further measurements were taken immediately after flap occlusion creation, at a 15-minute interval in the first hour immediately following the occlusion creation, then once every 2 hours in the next 12 hours. The animals were placed under anesthesia only when optical measurement were taken after the occlusion was created. Diffuse reflectance and autofluorescence spectra were measured by a compact spectrometer (USB4000, Ocean Optics, Dunedin, Florida, USA) coupled to a custom built bifurcated fiber optic probe. The entire optical measurement system was illustrated in Fig. 1.

![Fig. 1. (a) Schematic of the optical setup; (b) fiber-optic probe.](image)

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, USA) was used to provide broadband light in diffuse reflectance measurements. A long pass filter with a cut-off wavelength at 420 nm was applied in the collection channel to remove the excitation light in fluorescence measurements. This filter
would be moved away in diffuse reflectance measurements. The fiber-optic probe consisted of one central fiber for illumination and four surrounding fibers for detection. The numerical apertures of illumination fibers and detection fibers were all 0.22. The core diameters of the illumination and detection fibers were 200 µm. The average source-detection separation was around 225 µm. The laser power for fluorescence excitation was maintained to be about 6 mW at all times during measurements. The total time to carry out a full set of diffuse reflectance and autofluorescence measurements at all marked locations in one animal was around 3 minutes.

It should be noticed that the probe-tissue contact pressure could affect tissue optical properties and optical coupling and in turn measured spectra [22–25]. To minimize the variation in probe-tissue contact pressure, we applied a mechanical holder to hold the probe during all optical measurements. Before each measurement we carefully adjusted the distance between the probe and tissue surface to make sure the probe gently touched the tissue surface in order to minimize pressure change across measurements.

With an excitation wavelength at 405 nm, fluorescence was also contributed by porphyrins, which was evident from the fluorescence peak at around 635 nm [26]. However, porphyrin fluorescence is low for wavelengths shorter than 600 nm therefore it should not affect the major peak at around 480 nm that is presumably contributed by NADH and FAD thus indicative of cellular metabolism [27].

2.3 Data analysis

The qualitative analysis on diffuse reflectance spectra was restricted to a wavelength range of 470-700 nm, while the analysis on autofluorescence spectra was restricted to an emission wavelength range of 450-700 nm. Each measured spectrum was smoothed using a digital median filter with a bandwidth of 10 nm and then calibrated following a standard procedure [28, 29]. To calibrate diffuse reflectance, each smoothed diffuse reflectance spectrum was divided by the diffuse reflectance spectrum measured from a reference standard (SRS-99-010, Labsphere Inc., New Hampshire, USA) wavelength by wavelength. In contrast, the smoothed fluorescence spectrum was multiplied by the ratio of the reference spectrum of a standard lamp source (RS-15-200, Gamma Scientific, San Diego, USA) to the lamp spectrum measured using the emission channel of the probe and the spectrometer wavelength by wavelength for calibration. The shape of averaged spectra measured from control flaps, flaps with venous occlusion and flaps with arterial occlusion were compared and analyzed at different time points. In addition to the qualitative analysis on the spectra shape, a classification study was also performed as follows. The calibrated and smoothed reflectance spectra and fluorescence spectra were normalized by dividing each data point by the maximum peak intensity in the spectrum first, then the partial least squares (PLS) analysis [30] was conducted on the normalized diffuse reflectance or autofluorescence spectra to find PLS components (PCs) that represented the measured spectra. The first 15 PCs of each spectrum that accounted for over 99% variance in the original spectra were retained for the following classification analysis. Two types of classification on the spectral data have been performed. In the first type of classification, the differentiation between control flaps and occluded flaps, and the differentiation between flaps with venous occlusion and flaps with arterial occlusion were conducted independently. In the second type of classification, the flaps were classified as the occluded group or control group in the first step; then the flaps assigned to the occluded group will be further classified as flaps with venous occlusion or flaps with arterial occlusion. The first type of classification enables to quantify the capability of the combination of diffuse reflectance and autofluorescence spectroscopy techniques just in the differentiation between flaps with venous occlusion and those with arterial occlusion. The second type of classification mimics the clinical situation in which an unknown flap, which can be venously occluded, arterially occluded or un-occluded, needs to be diagnosed. In both types of classification, a Wilcoxon rank-sum test [31] was applied first to identify a subset of the PC scores that show statistically significant differences (p<0.05) between the two groups at each time point. This subset of PC scores up to a range of early time points were then fed
into a linear discriminant analysis [32] (LDA) classifier to predict skin status. A leave-one-out cross validation method [33] was used in the analysis to obtain an unbiased estimate of the prediction accuracy. The overall accuracy, sensitivity and specificity of prediction using diffuse reflectance spectra alone, autofluorescence spectra alone and their combination were compared.

It should be noted that there are two multivariate statistical analysis techniques, i.e. principal component analysis (PCA) [34], and partial least squares regression (PLS) [35], commonly employed for spectral data reduction. Both methods project the set of spectra onto a subspace expanded by multiple components to represent the spectral data with a few variables, which account for most of the variance in the original spectral data set. In this study we only adopted PLS for our data analysis similar to a previous comparison study [28], in which it was found that PLS had better performance than PCA in their breast cancer diagnosis study using spectral data.

3. Results

3.1 Flap grouping

Using the chosen flap model, the flaps with venous occlusion and arterial occlusion likely became necrotic in 6 hours. Figure 2 shows the typical flaps with venous occlusion, with arterial occlusion, and the control without any occlusion at different time points respectively. The area of necrosis can be identified by the black eschar. As expected, the appearance of the control flap did not change with time, while the occluded flaps changed dramatically in color and shape with time after occlusion. However, it was hard to differentiate between flaps with venous occlusion and those with arterial occlusion based on clinical observation at early time points. At 6 hours, the flaps with venous occlusion were much darker than the flaps with arterial occlusion. It implied that these flaps were likely dead by then. Totally 24 flaps each with a size of 3 cm × 3 cm, one on each groin of 12 Sprague-Dawley rats, were created. Four or five sites were measured at each flap as highlighted by yellow circles in Fig. 2 in the study. A total of 102 flap measurement sites of interest were assessed for 12 hours. These included 83 occluded sites and 19 control sites. Among 83 occluded sites, 47 sites were located on flaps with venous occlusion while the other 36 sites were located on flaps with arterial occlusion. It should be noted the fluorescence signals measured from flaps with venous occlusion became too weak to detect in 3 hours after the creation of the venous occlusion, thus only the spectral data obtained in the first 3 hours were analyzed.
3.2 Quantitative analysis of diffuse reflectance spectra

The average diffuse reflectance spectra measured in the groups of control flaps, flaps with venous occlusion and flaps with arterial occlusion in the first 3 hours after the creation of occlusion are shown in Fig. 3. The overall diffuse reflectance intensity in flaps with occlusions monotonically decreased with time while that in control flaps varied randomly with time. It can be found that the diffuse reflectance intensity values from 500 nm to 600 nm dropped more significantly in flaps with arterial occlusion compared to flaps with venous occlusion.

3.3 Quantitative analysis of autofluorescence spectra

The averaged autofluorescence spectra measured from control flaps, flaps with venous occlusion, and flaps with arterial occlusion in the first 3 hours after the creation of occlusion are shown in Fig. 4. It can be observed in Fig. 4 that the overall autofluorescence intensity measured from flaps with occlusion was lower than that from control flaps. The overall autofluorescence intensity around 480 nm and 635 nm in flaps with arterial occlusions decreased nearly monotonically with time while those in control flaps and flaps with venous occlusion varied randomly with time.
3.4 PC scores showing significant differences between different groups

The PC scores, which were extracted from diffuse reflectance and autofluorescence spectra, showing statistically significant differences (p<0.05) between the control group and flaps with occlusions as well as between flaps with venous occlusion and flaps with arterial occlusion at each time point were analyzed. It should be noted that the number of significantly different PC scores could be more than one at each time point, while the general trends in different PC scores with time are similar thus only the first PC score that was significantly different between two groups under comparison was shown. At each time point, the first PC score with a p-value smaller than 0.05 for the two group under comparison were averaged and plotted in Fig. 5. The error bar stands for plus/minus one standard deviation.

Obviously, the differences in PC scores between each pair of different groups as shown in Fig. 5 are more significant compared with the intensity value at any wavelength as shown in Fig. 3 and Fig. 4. Statistical analyses show that these differences are all significant with p-values smaller than 0.05, which is the basis for high accuracy of classification based on PC scores after PLS even the differences in measured optical spectra, especially in measured autofluorescence spectra, between each respective pair of groups are subtle. The differences in diffuse reflectance PC scores are generally more significant than those in autofluorescence PC scores especially within the first hour. The differences in PC scores between the control group and the occlusion group are more significant than those between the venous occlusion group and the arterial occlusion group.

3.5 PLS component spectra showing major features used for classification

To confirm the major spectral features used in the classification, the first two PC spectra at the timing point of 3 hours that generated significantly different PC scores among different
groups are shown in Fig. 6. Based on the PC spectra shown in Fig. 6(a) and 6(c), it can be seen that the major spectral features in diffuse reflectance spectra, i.e. the PC spectra that are useful for classification, match those of hemoglobin absorption spectra. In particular, the trend of dramatic reflectance reduction at around 600 nm in PC 1 spectra agrees with the shape of hemoglobin absorption spectrum after filtering out two peaks. In contrast, the two peaks in PC 2 spectra overlap with those in the oxygenated hemoglobin absorption spectrum. Similarly, the major features in autofluorescence spectra useful for classification are PC spectra with peaks close to 480 nm and 635 nm as shown in Fig. 6(b) and Fig. 6(d). This suggested that NADH and FAD fluorescence as well as porphyrin fluorescence all contain diagnostic information for differentiation between venous occlusion and arterial occlusion.

3.6 Classification accuracy using LDA followed by PLS regression

Table 1 shows the accuracy values calculated from LDA classification between the control groups and occluded 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 occluded group was treated as positive while the control group was treated as negative in the calculation of sensitivity and specificity. The overall classification accuracy when using diffuse reflectance spectra alone was always higher than 92%. The overall accuracy when using autofluorescence spectra alone was slightly lower but it reached 96% in around 3 hours.

Table 1. Overall classification accuracy, sensitivity and specificity achieved with linear discriminant analysis (LDA) between control flaps and occluded flaps using selected PC scores.

| Time  | 5 min | 15 min | 30 min | 45 min | 1 h  | 3 h  |
|-------|-------|--------|--------|--------|------|------|
| Overall accuracy (%) |
| R     | 92.2  | 98.0   | 97.1   | 98.0   | 97.1 | 97.1 |
| F     | 88.2  | 87.3   | 86.3   | 86.3   | 94.1 | 96.1 |
| R + F | 93.1  | 98.0   | 97.1   | 97.1   | 96.1 | 94.1 |
| Sensitivity (%) |
| R     | 89.5  | 100    | 94.7   | 100    | 100  | 94.7 |
| F     | 68.4  | 68.4   | 63.2   | 73.7   | 100  | 97.6 |
| R + F | 84.2  | 100    | 94.7   | 94.7   | 94.7 | 89.5 |
| Specificity (%) |
| R     | 92.8  | 97.6   | 97.6   | 97.6   | 96.4 | 97.6 |
| F     | 92.8  | 91.6   | 91.6   | 89.2   | 92.8 | 96.4 |
| R + F | 95.2  | 97.6   | 97.6   | 97.6   | 96.4 | 95.2 |

Legend: “Time” = time point after flap elevation; “R” = diffuse reflectance data alone; “F” = autofluorescence data alone; “R+F” = both diffuse reflectance and autofluorescence data.
Table 2 shows the accuracy values calculated from LDA classification between the group with venous occlusions and that with arterial occlusions. Similarly, the PC scores obtained by PLS regression and Wilcoxon rank-sum test were used to select those PC scores showing significantly differences between the two groups for classification. It is interesting to see again that diffuse reflectance spectroscopy yielded much higher accuracy than autofluorescence spectroscopy. The overall accuracy obtained from diffuse reflectance data was always higher than 94% while the accuracy obtained from fluorescence data was less than 82% in the first hour but it reached 88% around 3 hours. It can be seen that the accuracy for the differentiation between flaps with venous occlusion and flaps with arterial occlusion as shown in the Table 2 is lower than that for the differentiation between control flap and occluded flap as shown in Table 1.

| Time | 5 min | 15 min | 30 min | 45 min | 1 h | 3 h |
|------|-------|--------|--------|--------|-----|-----|
| Overall accuracy (%) | | | | | | |
| R | 94.0 | 94.0 | 97.6 | 97.6 | 97.6 | 96.4 |
| F | 73.5 | 78.3 | 81.9 | 77.1 | 80.7 | 88.0 |
| R + F | 91.6 | 91.6 | 92.8 | 95.2 | 89.2 | 90.4 |
| Sensitivity (%) | | | | | | |
| R | 91.7 | 91.7 | 100 | 100 | 100 | 94.4 |
| F | 66.7 | 61.1 | 72.2 | 69.4 | 75.0 | 80.6 |
| R + F | 91.7 | 83.3 | 88.9 | 94.4 | 86.1 | 91.7 |
| Specificity (%) | | | | | | |
| R | 95.7 | 95.7 | 95.7 | 95.7 | 95.7 | 97.9 |
| F | 78.7 | 91.5 | 89.4 | 83.0 | 85.1 | 93.6 |
| R + F | 91.5 | 97.9 | 95.7 | 95.7 | 91.5 | 89.4 |

Legend: “Time” = time point after flap elevation; “R” = diffuse reflectance data alone; “F” = autofluorescence data alone; “R+F” = both diffuse reflectance and autofluorescence data.

Table 3 shows the accuracy values calculated by the second type of classification, i.e. two-step LDA classification, described previously. Totally three groups, i.e. the control group, the group with venous occlusion and the group with arterial occlusion, were classified. The visible diffuse reflectance spectroscopy always yielded accuracy higher than 92% except the first 5 minutes. In contrast, autofluorescence spectroscopy provided relative lower accuracy. The overall accuracy obtained by fluorescence spectroscopy increased with time in general and reached 84% around 3 hours after the creation of occlusion.

| Time | 5 min | 15 min | 30 min | 45 min | 1 h | 3 h |
|------|-------|--------|--------|--------|-----|-----|
| Overall accuracy (%) | | | | | | |
| R | 87.3 | 92.2 | 95.1 | 95.1 | 95.1 | 95.1 |
| F | 69.6 | 71.6 | 71.6 | 68.6 | 82.4 | 84.3 |
| R + F | 86.3 | 91.2 | 91.2 | 93.1 | 89.2 | 79.4 |

Legend: “Time” = time point after flap elevation; “R” = diffuse reflectance data alone; “F” = autofluorescence data alone; “R+F” = both diffuse reflectance and autofluorescence data.

4. Discussion

In this pilot study, it is found that that the diffuse reflectance spectroscopy is able to differentiate venous occlusion, arterial occlusion and no occlusion in the first 30 minutes with an accuracy of 95% as shown in Table 3; in contrast, fluorescence spectroscopy is able to differentiate these flaps in the first 3 hours with an accuracy of 84%. This suggests the great potential of using these two techniques for the identification of venous occlusion and arterial occlusion from no occlusion and from each other in the clinical setting.
Tables 1 to 3 all showed that diffuse reflectance spectroscopy yields better performance compared to autofluorescence spectroscopy at the early timing points, which can be attributed to larger differences in diffuse reflectance spectra between each pair of groups compared to autofluorescence spectra shown in Fig. 3 and Fig. 4. One possible reason for the lower performance in fluorescence spectroscopy compared to diffuse reflectance spectroscopy in the differentiation between control flaps and occluded flaps, which is different from our earlier flap viability study, is that the occlusion of arterial or venous blood vessels can rapidly cause considerable changes in hemoglobin oxygenation and total hemoglobin concentration but may not lead to changes as quickly and significant in cellular metabolism therefore no quick or dramatic changes in autofluorescence measurements. While arterial occlusion, which blocks the supply of oxygenated blood, reduces hemoglobin oxygenation quickly and decrease total hemoglobin concentration, venous occlusion, which blocks the draining of deoxygenated blood, may decrease hemoglobin oxygenation at a slower rate but accumulate hemoglobin rapidly. Cellular metabolism, which is measured by autofluorescence spectroscopy, will be compromised similarly in either occlusion but at a slower rate because this is the consequence of insufficient oxygen supply. This can be seen from the observation in Table 1 that diffuse reflectance spectroscopy can reach 98% accuracy in 15 min but autofluorescence spectroscopy reach 96% in 3 hours. The same reason can also explain the finding in Table 2 that autofluorescence spectroscopy always yields considerably lower accuracy in the differentiation between arterial and venous occlusion than diffuse reflectance spectroscopy. This implies that diffuse reflectance spectroscopy could be more effective than autofluorescence spectroscopy in detecting progressive blood vessel occlusion and differentiating arterial and venous occlusions before considerable changes in cellular metabolism occur. Autofluorescence spectroscopy appears more effective in detecting serious compromise in blood supply that lead to detectable cellular metabolism variation based on our earlier flap viability study. To detect both types of compromises in flaps, it would be desirable to use the two techniques jointly when monitoring flap conditions.

In our experience, the majority of flap failures are caused by the occlusion of major feeding vessels. Micro-clotting can cause partial flap necrosis; however, this study did not look at this phenomenon. Since micro-clotting would induce generalized ischemic changes, the proposed technique is expected to work in detecting micro-clotting as well. However, a further study is needed to develop our technique to identify areas of general ischemia caused by micro-clotting.

In this pilot study, we employed a point-measurement spectroscopy system, which was portable and cheap but can only measure a small region at a time. It should be highlighted that, a spectral diffuse reflectance and autofluorescence imaging system that can measure a much larger region with high spatial resolution could offer similar sensitivity. This work is currently ongoing in our group.

5. Conclusion

Our study demonstrated that both visible diffuse reflectance and autofluorescence spectroscopy can detect and differentiate between venous occlusion and arterial occlusion in a rat model. However, the results showed that diffuse reflectance spectroscopy could be more sensitive than autofluorescence spectroscopy in detecting progressive blood vessel occlusion and differentiating arterial from venous occlusions before considerable changes in cellular metabolism occur.

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