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Fluorescence spectroscopy combined with 5-aminolevulinic acid-induced protoporphyrin IX fluorescence in detecting oral premalignancy

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Abstract

Background: Early detection of premalignant/malignant lesions in the oral cavity can certainly improve the patient's prognosis. This study presents fluorescence imaging with the topical application of 5-aminolevulinic as a way to improve detection of various oral tissue pathologies. This procedure depends mainly on comparing the intensity of red and green fluorescence emitted from tissues during examination.
Materials and methods: Seventy-one patients who presented with clinically suspicious oral leukoplakia were recruited for this study. Each of the patients was required to have 5-aminolevulinic acid in the form of mouth rinse prior to fluorescence imaging. Following this a surgical biopsy was acquired from the exact examination site. The results of the fluorescence spectroscopy have been compared with histopathology.
Results: A Student's *t*-test was applied to test the viability of the ratio between red and green fluorescence. The red-to-green ratio was found to increase significantly when the lesion was identified as dysplastic or carcinoma in situ. By applying a threshold line to discriminate between normal and dysplastic lesions; a sensitivity of 83–90% and specificity of 79–89% were obtained.
Conclusion: Fluorescence spectroscopy combined with 5-aminolevulinic acid-induced protoporphyrin IX was found as a valuable tool in the diagnosis of oral premalignancy. This technique offers the potential to be advantageous over other non-optical techniques in terms of providing real-time diagnosis, in situ monitoring, cost effectiveness and more tolerated by patient compared to surgical biopsy.

Keywords: Fluorescence spectroscopy; Non-invasive diagnosis; Oral pre-malignancy; Oral dysplasia; Potentially malignant lesion; 5-Aminolevulinic acid; Fluorescence diagnosis; Protoporphyrin IX; Optical biopsy

1. Introduction

It has become universally known that early detection of premalignant/malignant lesions can substantially reduce the risk or improve the prognosis in oral cancer. Several diagnostic methods have been employed in cancer detection including histopathology, radiology and most recently optical diagnostic methods as well as biological markers.

Clinical differentiation of lesions is usually based on morphological changes in tissues; this technique is quite difficult to implement and clinical experience is usually considered to be a major factor in its success. However it can

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be found to be less sensitive in differentiating between lesions with similar clinical and morphological characteristics, i.e. dysplasia and carcinoma in situ.

A premalignant lesion is always at risk of transforming into a malignant if stimulated by certain exogenous factors or conditions; patients at this stage usually require continuous monitoring which can be difficult sometimes, since this might involve multiple surgical biopsies, which can be uncomfortable, time consuming and costly. Moreover, it is difficult to screen a large population at high risk of developing the disease. Several new alternative methods have been described to increase the visibility of mucosal lesions, such as photography or application of certain dyes, including iodine solution, Lugol's iodine, Toluidine blue and tetracycline; by combining two or more techniques together, this has shown to increase the sensitivity and specificity of detection [1–3].

Recently, there has been increased interest in ''optical biopsy'' systems using tissue spectroscopy to establish pathological diagnoses. The three main techniques currently utilised in the detection of oral dysplasia and malignancy is fluorescence, Raman and elastic scattering spectroscopy.

1.1. Fluorescence spectroscopy (FS)

When cells interact with light they become excited and re-emit light of varying colours (fluorescence) and this can be detected by sensitive spectrometers. All tissues fluoresce due to the presence of fluorescent chromophores (fluorophores) within them. FS can detect these substances and provide characteristic spectra that reflect biochemical changes occurring within the tissue. The resultant spectra not only detect the fluorescence but also are sensitive to the cellular components that absorb light, eg. haemoglobin.

The commonly detected fluorophores include NADH, collagen, elastin and co-factors such as flavins (FAD, FMN) [4]. The fluorescence can either occur as autofluorescence (induced by UV light), or as a laser-induced phenomenon and may also be enhanced by either topical or systemic application of 5-aminolaevulinic acid (5-ALA) and can be used for single-point or imaging measurements. 5-ALA is a precursor of the fluorescent photosensitiser, protoporphyrin IX (PpIX), and can be administered systemically or applied topically to the oral mucosa and facial skin.

The principle of 5-ALA-induced PpIX is that, in excess, it results in the accumulation of intracellular porphyrins, especially of PpIX which increases tissue fluorescence [5]. Subsequent irradiation of the lesion with visible light matching the main absorption peak of PpIX (405 nm) leads to red fluorescence emission from the PpIX peaking at 635 nm. The difference in fluorescence ratio between normal and premalignant/malignant tissue makes detection and analysis by fluorescence light more applicable in the discrimination between malignant and non-malignant tissues [6]. This intensity is governed by many conditions, such as variations in rinsing procedure, tumour site and type [7,8].

Dysplastic and malignant tissues, as well as having different spectral characteristics, tend to have increased red fluorescence and decreased green fluorescence. Significant increase in the red/green fluorescence ratio is an accurate predictor of dysplasia and malignancy [4].

Several groups have investigated the use of tissue native fluorescence in the early detection of malignant tissues [4,9]. The results were encouraging, but varied greatly between patients and appeared insufficient for others [10]. In addition, there are a high percentage of ''false positive'' and ''false negative'' results which may be attributed to insufficient number of patients.

The aim of this clinical study was to clinically investigate the ability of 5-ALA-induced PpIX fluorescence to distinguish between normal and potentially malignant lesions in the oral cavity.

2. Materials and methods

Seventy-one patients (mean age 59 years, range 37–81 years) with clinically suspicious oral leukoplakia took part in this study in the Maxillofacial Unit, University College Hospital (UCH), London. The trial protocol was approved by the joint UCL/UCLH committees of the ethics for human research.

An information sheet explaining the aim of our study in simple non-scientific terms was given to each patient who was then consented prior to examination. Fluorescence spectroscopy was used then to examine the suspicious area for each of those patients prior to surgical biopsy.

Inclusion criteria were patients over 18 years of age who presented with suspicious oral leukoplakia; while the exclusion criteria were women who were or might have been pregnant, however, there are no known teratogenic effects that FS or 5-ALA might induce, the main reason behind this is the anxiety that might be triggered during the examination.

Three hours prior to examination, topical application to the oral mucosa was performed via a rinsing solution of 0.4% 5-ALA hydrochloride; prepared by dissolving 200 mg 5-ALA in 50 ml of mineral water. Each patient was required to rinse the mouth for 15 min, and maintained at least 5 min of continuous contact to achieve the minimum mucosal saturation. The solution was prepared shortly before (<5 min) each application to avoid unwanted effects reported from the instability of such solutions [11]. Following this, each patient was allowed to rinse with mineral water prior to examination but was warned to avoid teeth brushing. We have considered this relatively short incubation period to be an advantage for clinical routine diagnosis; Leunig et al. [12] found that the absolute fluorescence intensities in tumour tissue decreased 3 h following 5-ALA application; this could lead to the reduction of contrast between premalignant/malignant and healthy tissue.

2.1. Fluorescence spectroscopy system

Fluorescence was excited by a xenon-arc lamp (Medical Light Technologies Ltd., UK) whose output was filtered by a long pass filter (BG12, Schott Glass, Germany) and a bandpass filter centred at 425(±17) nm (Corion Corp., MA, USA). An optical endoscope was used for both illumination and detection of the tissue fluorescence.

The fluorescence image of the tissue was acquired by a high sensitivity single chip charge-coupled device (CCD) colour camera (Sensicam, Personal Computer Optics, Germany) incorporating a red/green/blue (RGB) mosaic filter and coupled to the endoscope to facilitate examination of all oral tissues. The images were captured by a frame-grabber (0.6 s integration time) fitted with an analogue/digital converter (ADC) and analysed and displayed by personal computer loaded with software (Sensicam, Personal Computer Optics, Germany). This allowed fast computation and analysing times. In addition, the RGB system provides wavelength separation allowing only red and green detection for ratio imaging. The output power of the blue light at the endoscope tip was kept to approximately 2 mW/cm; this was found to minimise the photobleaching of the photosensitiser. The imaging technique has been previously described in detail elsewhere [13].

The premalignant/malignant and normal tissue accumulated PpIX was excited via an endoscope with the blue light generated by the filtered xenon-arc lamp which also excited tissue autofluorescence, and both emissions could be imaged using the CCD colour camera. The PpIX fluorescence was imaged by the red channel of the camera and the green channel imaged the autofluorescence.

Following examination, the images produced (Fig. 1) were analysed by the computer to identify the area of the highest signal. Then this area was marked with blue ink in a diamond pattern. After that the area was subjected three times to fluorescence spectroscopy in order to reduce the error and ensure the reproducibility at each biopsy site by quick calculation of red-to-green intensity ratio. To prevent any optical interference, care was taken not to capture any fluorescence image after marking. The images obtained were displayed in black and white and the displayed intensity was proportional to the fluorescence intensity. The captured image was intensified and red-to-green ratio was calculated (Fig. 2). For optimal data analysis, images were obtained from the contralateral angle.

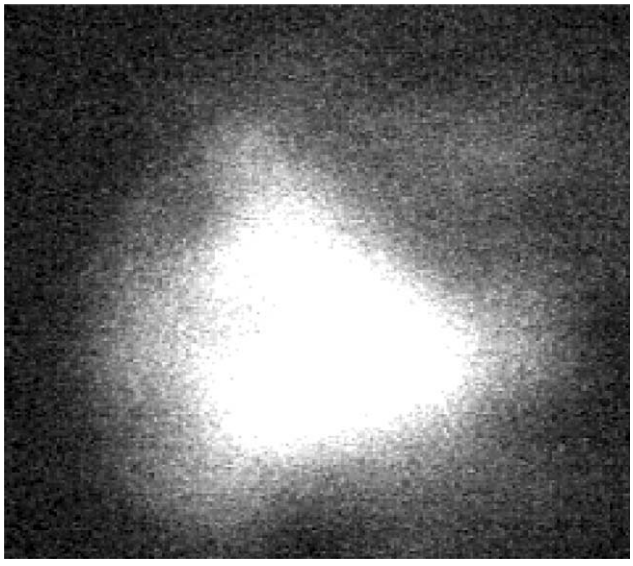

Fig. 1. Representative black and white tissue fluorescence image. Bright area represents the highest fluorescence intensity whereas low intensity is black area.

Within 0.2 s, the data processing stage was complete; this involved digital analysing of the 12-bit red, green and blue fluorescence images. A dimensionless spatial variable (R) was calculated according to the pixel coordination of x, y. The red channel described PpIX fluorescence and red tissue autofluorescence. The green channel recorded the green tissue autofluorescence, while the blue channel showed diffusely back scattered excitation light. To exclude distance dependence of the signals, a red fluorescent spot within normal mucosa was recorded at a different distance and fixed integration time.

The 71 acquired surgical biopsies from the 71 patients have been taken from various oral sites, where the majority originated from the tongue, buccal mucosa and floor of the mouth (Table 1). These biopsies were examined histopathologically and were found to be either normal (normal, inflammatory or hyperkeratotic) or potentially malignant (mild, moderate or severe dysplastic) (Table 2). To allow the correlation of fluorescence images and histology, the red PpIX fluorescence and green tissue autofluorescence

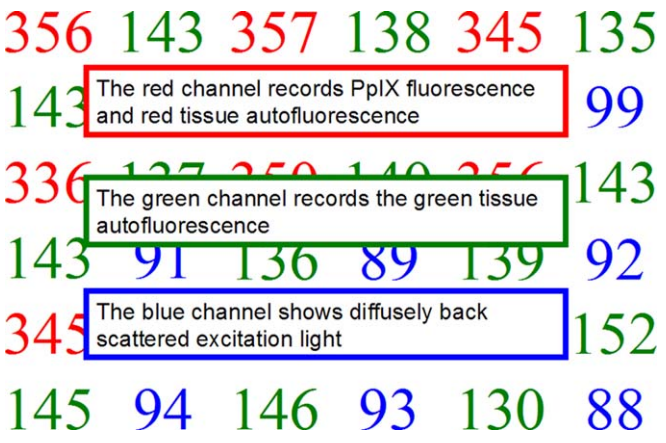

Fig. 2. Magnified pixel colour fluorescence intensity values.

Table 1
Number and percentage of surgical biopsies from different anatomical sites

Site of biopsy	Number of lesion	Percentage (%)
Tongue	18	25.4
Buccal mucosa	25	35.2
Floor of mouth	14	19.7
Alveolus	9	12.7
Hard palate	5	7.0

Table 2
Type and percentage of surgical biopsy with different histology

Histology	Number of biopsies	Percentage (%)
Normal	7	9.9
Inflammation	9	12.7
Hyperkeratosis	22	31.0
Mild dysplasia	14	19.7
Moderate dysplasia	9	12.7
Sever dysplasia	8	11.3
Carcinoma in situ	2	2.8

for each site were calculated. The ratio (red/green) was set at 1.2 or 1.3 as the threshold values for demarcation between normal and dysplastic.

2.2. Statistical analysis

The red-to-green values of the suspected areas were calculated and correlated with the histopathology. A Student's *t*-test was used to test the viability of the ratios regarding its ability to discriminate between different disease stages. Sensitivity and specificity determination, histopathological reports (normal, inflammation, hyperkeratosis, mild dysplasia, moderate dysplasia, severe dysplasia and carcinoma in situ) were considered as standard and correlated with red-to-green ratios measured in terms of oral changes in this clinical trial.

3. Results

All obtained images were displayed in the system monitor as black and white and the degree of severity of the lesions was correlated with the brightness of the image. For example, increase in brightness suggests the presence of dysplastic lesion. By using the computer programme, a quick analysis of the bright area reproduced the intensities of RGB fluorescence of that area.

Our data showed that the intensity of red and green fluorescence was directly related to the distance between the endoscope tip and the tissue surface, by increasing the distance the intensity declined; whereas the red-to-green fluorescence ratio remained constant (Fig. 3).

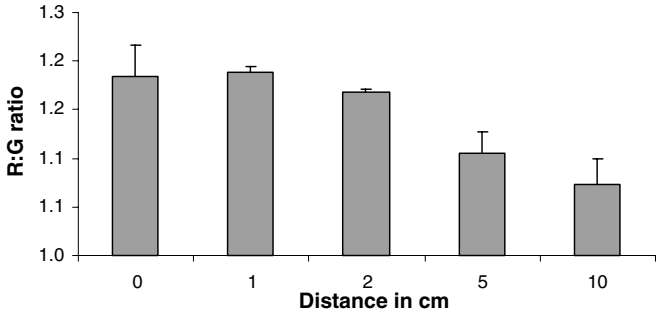

Fig. 3. The distance dependence of the red-to-green (R:G) ratio signals evaluated at various observation distances. Error bars represent the standard deviation of three readings.

When comparing the red PpIX fluorescence to the green tissue fluorescence, we have found a marked increase of the red fluorescence over the green fluorescence in dysplastic lesions and carcinoma in situ (Fig. 4); but the difference was negligible in normal tissue (Fig. 5). The red-to-green ratio was found to increase significantly when the lesions were identified as dysplastic or carcinoma in situ (Fig. 6).

The red-to-green ratio of normal and pathological tissues have been compared (Table 3); *p*-values have been found to be highly significant when comparing the ratio of normal tissue to that of mild dysplasia ($p = 0.002$), moderate dysplasia ($p = 0.039$), sever dysplasia ($p = 0.018$) and carcinoma in situ ($p = 0.027$).

A threshold was set at 1.2 ratio (red-to-green) to discriminate between normal and dysplastic lesions, a sensitivity of 90.3% and specificity of 79% were obtained. A better specificity (89.5%) was obtained when the threshold line

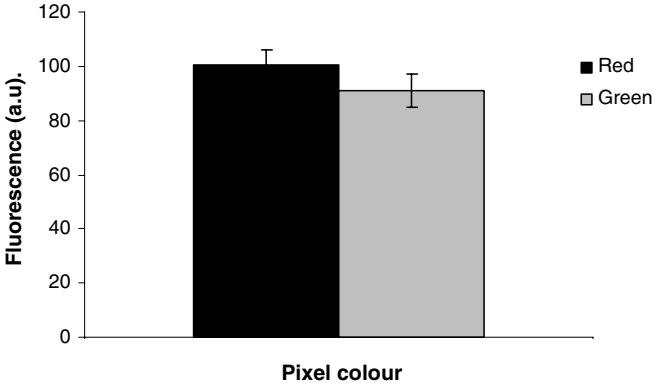

Fig. 4. Fluorescence intensity in red and green in patients with oral mucosal lesions classified histologically as normal. Topical ALA was applied on mucosa for 15 min and fluorescence measurements taken between 1.5 and 2 h. The error bar represents the standard deviation between different anatomical sites in different patients.

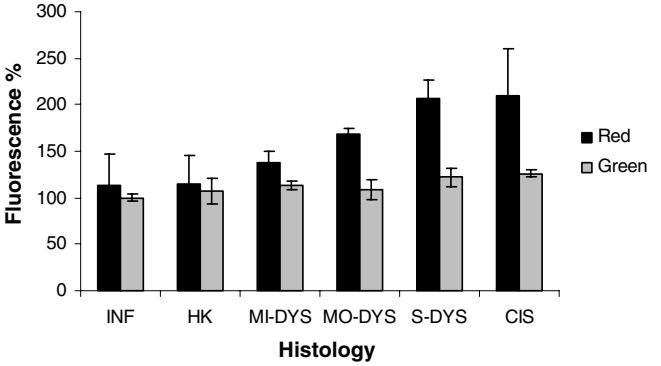

Fig. 5. Fluorescence intensity versus histopathological stages of oral lesions. Topical ALA was applied on mucosa for 15 min and fluorescence measurements were taken between 1.5 and 2 h. The data represents the % of fluorescence intensity in red and green compared to lesions diagnosed histologically as normal. The error bar represents the standard deviation between different anatomical sites in different patients: INF, inflammatory; HK, hyperkeratosis; MI-DYS, mild dysplasia; MO-DYS, moderate dysplasia; S-DYS, sever dysplasia; CIS, carcinoma in situ. Error bars represent the standard deviation between patients.

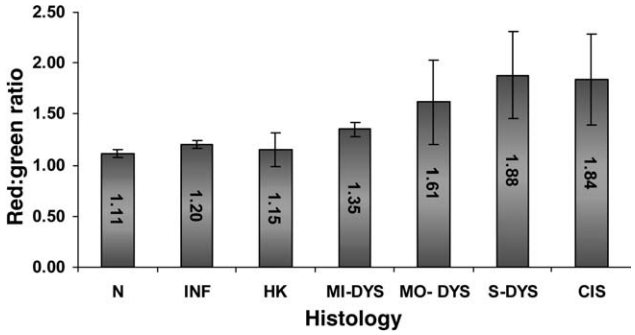

Fig. 6. Red-to-green ratio versus histopathological stages of oral lesions: N, normal; HK, hyper dysplasia; INF, inflammation; MI-DYS, mild dysplasia; MO-DYS, moderate dysplasia; S-DYS, sever dysplasia; CIS, carcinoma in situ. Error bars represent the standard deviation between patients.

Table 3
p-Values (*t*-test) of correlation of red-to-green ratio with histopathology of oral lesions

	N	INF	HK	MI-DYS	MO-DYS	S-DYS
N	–					
INF	0.0207					
HK	NS	NS				
MI-DYS	0.002	0.020	0.041			
MO-DYS	0.039	NS	0.004	NS		
S-DYS	0.018	0.051	0.000	0.053	NS	
CIS	0.027	0.074	0.001	0.073	NS	NS

NS, non significant; N, normal tissue; INF, inflammation; HK, hyperkeratosis; MI-DYS, mild dysplasia; MO-DYS, moderate dysplasia; S-DYS, sever dysplasia; CIS, carcinoma in situ.

Table 4
Classification of oral tissue red-to-green fluorescence ratios

	Red-to-green ratio	
	1.2	1.3
Number of biopsies showing dysplasia histologically	31	31
Number of dysplastic sites diagnosed by optical fluorescence ratio as dysplasia (true positive)	28	26
Number of dysplastic sites diagnosed by optical fluorescence ratio as normal (false negative)	3	5
Sensitivity (%)	90.3	83.9
Number of biopsies found to be normal histologically	38	39
Number of normal fluorescence ratios found to be normal (true negative)	30	34
Number of normal fluorescence ratios found to be dysplastic (false negative)	8	4
Specificity (%)	79	89.5

was shifted to 1.3; but this reduced sensitivity by about 6% (Table 4).

4. Discussion

Optical diagnostics have proved to be a reliable source that can be used to give an instant diagnosis of soft and, more recently, hard tissues. In the field of head and neck malignancy, most of the experimental spectroscopy work has been performed using fluorescence, Raman and elastic scattering spectroscopy. Currently, there is a continuous development, not only in spectroscopy technology and data acquisition, but also in the analysis methods used. The ultimate aim of this development is to be able to provide diagnostic algorithms that may be used instantaneously in the clinical setting.

One of the earliest studies that described the use of autofluorescence spectroscopy to differentiate between normal and malignant tissues were published by Tata et al. in 1986 [14]; the group used rat kidney tissue. Since then several authors have registered their findings with this technique [15–17]. The technique has also recently been used to map out the individual characteristics of healthy oral mucosa at several anatomical sites within the oral cavity which can be used as a baseline for further studies [18]. Onizawa et al. [3] examined the usefulness of fluorescence spectroscopy for oral cancer diagnosis. 130 oral lesions from 130 patients were subjected to fluorescence spectroscopy; 72/79 (91.1%) and 6/7 (85.7%) of carcinoma and epithelial dysplasia were identified, respectively.

Current routine methods applied for the detection of oral neoplasms is limited to the appearance of the suspected tumour area and it appears difficult for even experienced clinicians to differentiate between different disease stages. Therefore, the development of an enhanced fluorescence imaging system for non-invasive demarcation between normal and altered tissues can establish a revolution in early clinical diagnosis. The imaging technique applied in this study was initially developed by our previous workers and was clinically tested on only a few patients [19].

Here, more detailed clinical digital fluorescence images were applied with a correlation to the conventional histological results for different oral anatomical sites following the topical application of an ALA solution. Two channels, red and green, and their ratio diagnostic algorithm were used to quantify the fluorescence intensities. The approach was similar to previous work but with differences in ratio imaging techniques, despite its capability of classifying the different histological stages of oral lesions.

Our clinical data demonstrated that dysplastic lesions have significantly higher red fluorescence than benign oral lesions without changes in green autofluorescence. This is in agreement with previous reported studies [6,20]. In clinical trials, a combination of autofluorescence and ALA-induced PpIX fluorescence caused an enhanced demarcation between malignant and healthy tissues [21]. Many mechanisms have been proposed in order to explain the enhanced intensity of PPIX in the abnormal tissues, such as the role of plasma lipoproteins, low pH in tumour tissues and tumour vasculature [22–24].

The application of a red-to-green ratio algorithm set to 1.2 cut threshold between normal and abnormal tissues resulted in 90.3% sensitivity and 79% specificity in discrim-

inating between dysplastic lesions, including mild, moderate and severe dysplasia, and benign lesions, with inflammatory and hyperplastic tissue changes. The possible explanation of low specificity could be due to the porphyrin synthesised by oral microorganisms present in plaque and superficial necrotic tissues [20,21]. The 10% reduction in sensitivity could be related to the limited penetration depth (less than 1 mm) [25], which may lead to poor registration of some cytological and biochemical changes occurring in dysplastic tissues. This supports the data from previous studies, such as Zheng et al. [6] who investigated ALA-induced PpIX fluorescence on 28 patients with suspicious malignant oral lesions combining both red-to-green and red to blue ratios. This group reported a high sensitivity and specificity of 95% and 97%, respectively. Similarly, others found high specificity by applying a red to blue ratio to discriminate between malignant and pre-malignant bladder lesions, although the red-to-green ratio was less applicable [9].

In order to enhance specificity, a 1.3 cut threshold between dysplastic and benign tissues was applied; this however gives us more ''false negatives'', which could be due to insufficient tissue fluorescence following the improper application of ALA. It is difficult to compare quantitatively the ratios imaging with other fluorescence systems due to inter-system variation, such as spectral width, background noise and sensitivity.

5. Conclusions

With standardisation of instrumentation and proper diagnostic algorithms, fluorescence spectroscopy has great potential both to non-invasively identify dysplastic changes at an early stage and to be used as a screening tool for detection of subsequent primary lesions. It characteristically produces real time, high quality image maps with clear demarcation. Applying ratio imaging may avoid the misclassification results from the comparison of the obtained fluorescence intensity of the suspected area with the control of normal adjacent tissues in the same individual.

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