Evaluation of fluorescence measurement techniques for tumour detection in vivo
Saarnak, A.E.
CHAPTER 2

Evaluation of spectral correction techniques for fluorescence measurements on pigmented lesions in vivo

H.J.C.M. Sterenborg, A.E. Saarnak, R. Frank, M. Motamedi

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Abstract

Recently, the use of optical spectroscopy for non invasive diagnosis of malignant melanoma has been suggested. The reliability of such optical measurements can be seriously compromised by spatial variations in optical properties of the tissue that are not related to malignancy. In the present paper we report on a novel approach to fluorescence spectroscopy which allows for elimination of spatial variations in the optical properties of the tissue investigated. To test this concept we performed fluorescence and colour measurements on moles and unpigmented control skin in human volunteers before and after topical application of δ-aminolevulinic acid (ALA). Two types of fluorescence data analysis were investigated; a Single Ratio technique based on the ratio of the red and yellow fluorescence (660nm-750nm/550nm-600nm) at 405 nm excitation and a Double Ratio technique, the red/yellow ratio at 405 nm excitation divided by the red/yellow ratio at 435 nm excitation. The two excitation wavelengths were selected to be located close to the maximum and at some distance from the Soret excitation band of the porphyrins. The Single Ratio showed a significant correlation between fluorescence and colour. The Double Ratio was independent of the colour of the lesion. These findings indicate that the Double Ratio technique is suitable for in vivo detection of local differences in concentration of fluorescent tumour localising drugs in pigmented lesions. This enables in vivo studies of the pharmacokinetics of tumour localising agents in pigmented lesions, and may significantly contribute to the development of a non invasive diagnostic tool for malignant melanoma.
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Introduction

The incidence of melanoma skin cancer has greatly increased in the last decade (Magnus 1991, Muir et al. 1987, Moan 1994). Often, clinical differentiation of melanoma from non-malignant lesions can be difficult, especially during the initial stages of the development of the cancer. Accurate diagnosis requires excision and histopathological evaluation of each lesion. Development of accurate, real-time, non-invasive diagnostic tools to better differentiate benign skin lesions from skin cancer would lead to a reduction in the number of unnecessary diagnostic surgical procedures and might improve the patients' prognosis.

The concept of using fluorescence for in vivo diagnostic purposes was first investigated several decades ago (Policard 1924). During the last decade, the use of fluorescence spectroscopy and imaging have been under investigation in various medical specialities, with an emphasis on applications in cardiovascular disease and cancer (Deckelbaum et al. 1987, Hung et al. 1991, Richards-Kortum et al. 1991, Profio et al. 1983, Silberberg et al. 1994). Although successful of this technique for diagnosis of malignant melanoma in vivo might be of major significance, not much work has been done in this field (Sterenborg et al. 1993).

The first investigations on the use of in vivo fluorescence spectroscopy for diagnosis of malignant melanoma were reported by Lohmann and Paul (1989). These investigators used 365 nm light to excite autofluorescence and found a six fold increase in the fluorescence intensity around 475 nm at the edge of biopsy proven malignant melanomas (N=28) in humans when compared to healthy skin. Such an increase was not observed in benign pigmented lesions (N=54). Recent clinical investigations, conducted by the author of the present study, using a fluorescence imaging device could not confirm these results (Sterenborg et al. 1994). In this study 375 nm excitation was used and fluorescence images were recorded with an image intensified CCD camera detecting in the 418 to 600 nm wavelength range. With this set-up in vivo autofluorescence images were taken in patients (N=17) with either a clinically diagnosed malignant melanoma or a suspected pigmented lesion. We could not distinguish the malignant melanomas (N=8) from benign pigmented lesions (N=9). In an animal study we investigated the excitation-emission maps of the autofluorescence of a K1735P non melanotic melanoma implanted subcutaneously in the ears of CH3/NeH mice. Prominent spectroscopic features appeared to be related to absorption of the excitation light by blood and blood related substances, whereas the differences between tumour and normal tissue were not significant and appeared to be related to oxygenation rather than to specific fluorescent markers of malignancy (Sterenborg et al. 1995). These studies indicate that diagnosis of malignant melanoma on the basis of in vivo autofluorescence spectroscopy is not feasible.

The spectroscopic differences between normal and abnormal tissue can be enhanced by using optical contrast agents. Currently, fluorescent tumour markers like hematoporphyrin derivative (HPD) are under investigation for localisation of tumours. This concept has shown to be feasible for localisation of cancers of the oral cavity, the bladder and bronchial mucosa (Monnier et al. 1990, Lam et al. 1990, Lin et al. 1983). A recent development in this field is the use of 5-aminolevulinic acid (ALA) for photodynamic therapy of skin tumours (Kennedy and Pottier 1992). Topically applied ALA in aqueous solution passes through the stratum corneum. Thus, ALA can reach both normal and neoplastic cells in the epidermis and its appendages. The mitochondria of these cells...
will then initiate its transfer into the fluorescent compound protoporphyrin IX. Differences in PpIX kinetics between normal and malignant cells may induce temporary fluorescence differences between these cells. Development and evaluation of new tumour localising drugs is currently an important research topic which, if successful, might produce a major breakthrough in cancer diagnosis (van Lier 1989, van Leengoed et al. 1990).

The success of such a technique for the diagnosis of malignant melanoma depends in the first place on the availability of spectroscopic differences between tumour and normal tissue. This problem will not be addressed in the present paper. A second important question is whether it will be possible to measure such differences \textit{in vivo}. A factor complicating optical measurements is presented by the strong spatial variations in the melanin concentration naturally occurring in both benign moles and malignant melanomas (Pehamberger et al. 1987). Successful application of optical contrast agents for detection of malignant melanoma requires a reliable detection technique that is able to compensate for the large spatial variations in the intrinsic optical properties. Two spectroscopic correction techniques, developed to compensate for optical artefacts, will be discussed here.

A technique developed to enable subtraction of an autofluorescence background was first published by Profio et al. (1986). Fluorescence is measured in two wavelength regions, in the red wavelength region where the porphyrin emits, and in a green or yellow wavelength region where the porphyrin has no emission. The red/yellow ratio is taken and the initial ratio, i.e. the ratio measured before presence of any porphyrins, is subtracted. In addition to background subtraction, the advantage of this single ratio technique (SR) over direct measurements is that it corrects for spatial variations in the incident excitation fluence which may occur in endoscopic fluorescence imaging (Profio et al. 1986).

More recently, a correction technique employing two excitation wavelengths and two detection wavelengths has been developed by our group (Sinaasappel and Sterenborg 1993). According to its theoretical basis, this Double Ratio technique corrects not only for spatial variations in the incident excitation fluence rate and other geometrical factors, but also for spatial variations in the optical properties of the tissue. The correction comprises the ratio of the red/yellow ratio measured at the first excitation wavelength and the red/yellow ratio at the second excitation wavelength. When the two excitation wavelengths are chosen to induce a substantially different porphyrin fluorescence intensity, but at the same time have similar absorption and scattering properties, the technique yields a corrected fluorescence signal that only depends on the fluorophore concentration and two calibration constants (Sinaasappel and Sterenborg 1993). These calibration constants are related to the fluorescence properties of the fluorophore used and autofluorescence properties of the tissue investigated, but are independent of geometry and spatial distribution of absorption and scattering coefficients of the tissue. The feasibility of this technique has been demonstrated \textit{in vitro} in an optical phantom. However, the problem of absolute calibration of the two constants \textit{in vivo} has not been solved so far. For the use of this technique for fluorescence imaging, such an absolute calibration of the technique \textit{in vivo} is not necessary (Sterenborg and Motamedi 1993).

In the present paper we report on the \textit{in vivo} evaluation of both the Single Ratio technique and the Double Ratio technique on benign moles.
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Materials and methods

Experiments were performed on 18 moles clinically diagnosed to be benign of 5 healthy male volunteers with ages ranging from 22 to 39 years. All moles were located on the lower arms, back or chest.

ALA, purchased from Jansen Pharmaceuticals (Tilburg, The Netherlands) was dissolved in Instillagel (Farco-Pharma Köln) in a 1:4 weight ratio. Approximately 0.2 cc was applied on each lesion with a 1 cm margin around it. The lesions were covered with a transparent dressing (Tegaderm, 3M) for 4 hours. After removal the remaining gel was wiped off and the skin was cleaned with a moist gauze. Exposure to daylight was avoided for 24 hr.

Fluorescence of the skin was induced with a filtered 100 Watt Mercury lamp using either the 405 nm or the 435 nm emission line. The excitation lines were selected using an automated filterwheel with two interference filters (Oriel # 56541 for 405 nm and Oriel # 56551 for 435 nm) and a position for dark current measurements. Fluorescence was detected with two different detectors, a Hamamatsu R 636-10 photomultiplier with a Schott RG 660 long pass filter to detect the red fluorescence and a Hamamatsu IP 128 photomultiplier with a Schott KV 550 filter for the yellow fluorescence. At equal green sensitivity, the different spectral sensitivities of the two detectors result in a more than 30 times higher sensitivity in the 660 to 750 nm wavelength range for the red detector. Excitation and fluorescence light was transported from set-up to skin and back using three 600 μm core optical fibres. Light from the source was chopped at 330 Hz and the photomultiplier signals were processed using standard lock-in techniques. The intensity of the excitation light was low enough to avoid photobleaching. Each individual measurement, including the change of excitation wavelength lasted less than 30 seconds. The experimental set-up is depicted in Fig. 1.

![Diagram of measurement set-up](image)

Figure 1. Layout of the measurement set-up. The filter wheel selects either the 405 or the 435 nm emission line from the mercury arc. Red fluorescence is detected with a red sensitive photomultiplier (R 928) combined with a 660 nm long-pass filter; yellow fluorescence is detected with a photomultiplier with a decreased red sensitivity (1P28) with a 550 nm long-pass filter.

Two series of three individual measurements were performed for each mole, one on the mole and one on the adjacent unpigmented skin that served as control. On both the mole and the control skin ALA had been applied. Between each individual measurement the fibre probe was removed from the skin and repositioned. For each series the three measurements were averaged. Measurements were performed before ALA application, after four hours of ALA application and two hours later.
Before ALA application, the colour of the mole and the adjacent control skin was measured with a Minolta CR300 colour measurement system equipped with a home made 3mm diameter aperture. Colour values were expressed in the X-Y-Z tristimulus system, yielding values which are directly related to the reflection coefficients in the red, green and blue spectral areas, respectively (Wyszecki and Styles 1982). This standard colour measurement system measures the tristimulus values based on the colour matching functions of the CIE 2° standard observer. Again, two series of three individual measurements were performed for each mole, one on the mole and one on the adjacent unpigmented control skin. Between each individual measurement the colour meter was removed from the skin and repositioned. For each series the three measurements were averaged.

The Single Ratio; as well as the Double Ratio were calculated, where the Single Ratio is defined as the ratio of the red and the yellow fluorescence excited with 405 nm and the Double Ratio as the ratio of the Single Ratios measured at 405 nm excitation and at 435 nm excitation. The performance of the two correction techniques was evaluated, first by investigating the correlation between fluorescence and colour and second, by a paired comparison between the values on the mole and the value from the corresponding control skin, the latter having the advantage of being independent of local and individual variations in ALA application and porphyrin metabolism (Sokal and Rohlf 1981).

As we were not able to biopsy our volunteers no information on actual PpIX concentrations could be obtained. However, knowing that the PpIX concentration increases in time we tested the sensitivity of the technique to detect differences in the PpIX concentration by comparing the fluorescence values at different times for each measured area (18 moles + 18 control areas).

Results

The red (660 - 750 nm) fluorescence excited with 405 nm as a function of the colour (X value; blue, low value is dark, high value is light skin) of the mole or control skin is depicted in Fig. 2. Before as well as after ALA application the fluorescence tends to increase with increasing colour value. The correlation coefficients, listed in table 1 are all significantly different from 0, indicating that there is a significant correlation between fluorescence and colour (t-test, p<0.05, double sided (Sokal and Rohlf 1981)).

![Figure 2. Fluorescence intensity (660 nm - 750 nm, in arbitrary units) measured on the moles and the control skins as a function of the skin colour, before and 4 and 6 hours after ALA application. The value of the colour parameter is a measure of the reflection in the blue region. The lines are drawn to guide the eye and represent a linear regression fit. The correlation coefficients listed in Table 1 indicate a significant correlation between fluorescence and colour.](image-url)
The Single and the Double Ratio as functions of colour (X value) are shown in Figs. 3a and 3b. The correlation coefficients, listed in table 1 show that for the Single Ratio there is still a significant correlation with colour, for all colours and before as well as after ALA application (t-test, p<0.05, double sided). In all cases the Single Ratio decreases with increasing colour value. For the Double Ratio there is no significant correlation with colour (t-test, p<0.05, double sided) although a slight decrease with colour value appears to be present before ALA application and a slight increase with colour value after ALA application.

Table 1. The coefficients of correlation, r, between the fluorescence and colour, for the red fluorescence, the Single Ratio and the Double Ratio. The t values result from a t-test of the hypothesis r = 0; i.e. no correlation. The underlined t-values indicate where there is no significant correlation (p<0.05) between fluorescence and colour.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>X (blue)</th>
<th>Y (green)</th>
<th>Z (red)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red fluorescence</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r 0</td>
<td>0.3571</td>
<td>0.3503</td>
<td>0.3379</td>
</tr>
<tr>
<td>t</td>
<td>3.175</td>
<td>3.107</td>
<td>2.983</td>
</tr>
<tr>
<td>r 4</td>
<td>0.6512</td>
<td>0.6554</td>
<td>0.6345</td>
</tr>
<tr>
<td>t</td>
<td>4.929</td>
<td>4.985</td>
<td>4.716</td>
</tr>
<tr>
<td>r 6</td>
<td>0.7531</td>
<td>0.7521</td>
<td>0.7832</td>
</tr>
<tr>
<td>t</td>
<td>6.576</td>
<td>6.557</td>
<td>7.237</td>
</tr>
<tr>
<td>Single Ratio</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r 0</td>
<td>-0.4499</td>
<td>-0.4645</td>
<td>-0.3946</td>
</tr>
<tr>
<td>t</td>
<td>-4.185</td>
<td>-4.357</td>
<td>-3.567</td>
</tr>
<tr>
<td>r 4</td>
<td>-0.4765</td>
<td>-0.4675</td>
<td>-0.4157</td>
</tr>
<tr>
<td>t</td>
<td>-3.114</td>
<td>-3.038</td>
<td>-2.626</td>
</tr>
<tr>
<td>r 6</td>
<td>-0.4675</td>
<td>-0.4437</td>
<td>-0.4452</td>
</tr>
<tr>
<td>t</td>
<td>-3.038</td>
<td>-2.844</td>
<td>-2.856</td>
</tr>
<tr>
<td>Double Ratio</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r 0</td>
<td>-0.2045</td>
<td>-0.2163</td>
<td>-0.2069</td>
</tr>
<tr>
<td>t</td>
<td>-1.748</td>
<td>-1.854</td>
<td>-1.769</td>
</tr>
<tr>
<td>r 4</td>
<td>0.2572</td>
<td>0.2605</td>
<td>0.3015</td>
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<tr>
<td>t</td>
<td>1.529</td>
<td>1.550</td>
<td>1.816</td>
</tr>
<tr>
<td>r 6</td>
<td>0.1764</td>
<td>0.2102</td>
<td>0.2033</td>
</tr>
<tr>
<td>t</td>
<td>1.029</td>
<td>1.235</td>
<td>1.193</td>
</tr>
</tbody>
</table>
A paired comparison of the measurements on each mole and on their corresponding control skin was made in Figs. 4a and 4b. Here we plotted the Single and the Double ratio measured on each mole versus the value on the corresponding control skin. For each point in these graphs we calculated the relative difference between the measurement on the control skin and the mole; i.e.

\[
\Delta SR = \frac{(SR_{control} - SR_{mole})}{(SR_{control} + SR_{mole}) / 2}
\]
where SR and DR stand for the Single and Double Ratios, respectively and ΔSR and ΔDR for the relative differences. The average values listed in Table 2 indicate that the differences between mole and control are much smaller than for the Double Ratio. The Single Ratio on the moles is 32.1% larger than on the control skin, while the Double Ratio is 8.6% smaller. Both are significantly different from 0 (t-test, p<0.001, single sided).

Figure 4a

![Figure 4a](image)

**Figure 4a** Single Ratio (a) and Double Ratio (b) values measured on the mole versus the values measured on the corresponding control skin. The lines are drawn to guide the eye and represent a linear regression fit.
Table 2. Relative differences in Single and Double Ratios between control skin and mole, calculated with Eqs. 1 and 2 for each mole and then averaged. Both show a significant difference between control and mole (t-test, p<0.001, single sided), although for the Double Ratio this difference is much smaller.

<table>
<thead>
<tr>
<th></th>
<th>ΔSR</th>
<th>ΔDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>-0.321</td>
<td>0.086</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.358</td>
<td>0.088</td>
</tr>
<tr>
<td>N</td>
<td>54</td>
<td>54</td>
</tr>
<tr>
<td>t</td>
<td>6.34</td>
<td>7.03</td>
</tr>
</tbody>
</table>

For each measurement location, the 18 moles and the 18 control sites, the fluorescence values (average and standard deviation of the series of 3 individual measurements) at different times were calculated and compared with a t-test. Table 3 lists the number of locations that showed a significantly larger fluorescence at the later time (p=0.05, single sided). The average values and standard deviations are shown in Fig. 5.

Table 3. At each measurement location the fluorescence values were compared at different times using a double sided t-test. Listed are the number of locations, N, out of the 36 on which a significant difference (p<0.05) between two consecutive times was detected. The parameter t stands for the average of the resulting t-values.

<table>
<thead>
<tr>
<th></th>
<th>6 vs 0 hr</th>
<th>4 vs 0 hr</th>
<th>6 vs 4 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single ratio</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>31</td>
<td>25</td>
<td>21</td>
</tr>
<tr>
<td>t</td>
<td>7.23</td>
<td>6.09</td>
<td>2.28</td>
</tr>
<tr>
<td>Double ratio</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>34</td>
<td>32</td>
<td>7</td>
</tr>
<tr>
<td>t</td>
<td>15.25</td>
<td>10.43</td>
<td>0.316</td>
</tr>
</tbody>
</table>

Figure 5. Fluorescence values for the two ratio techniques at different times after ALA application, averaged over all measured sites (18 moles and 18 control areas). The error bars represent the standard deviation.
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Discussion

Correlation with colour

The Double Ratio presented a small (8.6%) but statistically significant difference between mole and control skin (Table 2), whereas the correlation with colour (Table 1) was not significant. This apparent contradiction may be due to variations in the procedure. The topical application of ALA on the skin is performed manually and is not well controlled. Moreover, different anatomical locations and different individuals are involved, which may introduce variations in PpIX production due to differences in ALA penetration and PpIX metabolism. This induces additional experimental variations. In the paired comparison we looked at relative differences between the moles and the corresponding control sites which are not influenced by these experimental variations. Thus, the paired comparison is more sensitive in detecting systematic differences between mole and control.

Sensitivity

The sensitivity of the double ratio technique in detecting differences in concentration of the fluorophore is known to be better at small concentrations (Sinaasappel and Sterenborg 1993). The Double Ratio, DR was derived to equal:

$$ DR = \frac{1 + aC}{1 + bC} $$

where C represents the concentration of the fluorophore and a and b are calibration constants related to the autofluorescence properties of the tissue and the fluorescence properties of the tumour localising dye. The ratio DR equals 1 at zero fluorophore concentration, increases with concentration with initial slope equal to a and gradually flattens off. It is obvious that the upper limit of the Double Ratio is determined by the ratio \( \frac{a}{b} \). From the derivation of Eq. 1 given by Sinaasappel and Sterenborg (1993) it can be derived that the ratio \( \frac{a}{b} \) is nearly equal to the ratio of the excitation strength of PpIX at the two excitation wavelengths. In the current geometry using 405 and 435 nm the ratio \( \frac{a}{b} \), and thus the upper limit of the double ratio, lies around a value of 2.5. The values measured at 4 and 6 hours after ALA application are in the range from 1.5 to 2, already reaching the flatter part of the curve. This may explain why the Double Ratio technique is not able to see significant differences in fluorescence between 4 and 6 hours, whereas the differences with 0 hrs of application appear to be larger (table 2). This suggests that experiments investigating differences in kinetics using the Double Ratio technique should be performed at lower PpIX concentrations. In practice this could be realised by using a lower ALA concentration or by performing the measurements earlier after ALA application.

The theory of the Double Ratio assumes a semi-infinite medium. In the case of topically applied ALA, however, it is realistic to assume that the porphyrin concentration varies with depth. The precise effect of this on the double ratio is hard to estimate as it depends on the precise depth distribution of porphyrins and excitation light. In case the ALA penetration depth would be less than the penetration depth of the excitation light, then the Double Ratio measured would be an average over a superficial area with porphyrins and a deeper area without porphyrins. The measured Double
Ratio would then be less than the Double Ratio expected on the basis of the superficial porphyrin concentration.

The technique described enables accurate measurement of the pharmacokinetics of tumour localising drugs in pigmented lesions like malignant melanoma and opens a new field of research that may be of major impact on the early detection of this form of cancer.

**Conclusions**

The results of this experiment show that the Double Ratio is not influenced by the colour of the skin, whereas the Single Ratio, as well as the raw data show a significant correlation with skin colour.

The sensitivity for detecting the difference in fluorescence between t = 0 hr and t = 4 hr and between t = 0 hr and t = 6 hr (or a low PpIX and a high PpIX concentration) was very good and roughly the same for both techniques, although for the Single Ratio technique the observed differences were less significant. For t=4 hr and t=6 hr (the difference between two high PpIX concentrations) the techniques were less sensitive, whereas the Single Ratio technique performed best.

These findings indicate that the Double Ratio technique is suitable for in vivo detection of spatial or temporal differences in concentration of fluorescent tumour localising drugs in pigmented lesions, independently of the optical properties.

**Acknowledgements**

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The technique described enables accurate quantification of the fluorescence signal. However, it can be improved by localising drugs in epidermal lesions. This will assist in the treatment of cutaneous malignancies and offers a new tool for the early detection of skin cancer.

Conclusions

The results obtained from this study support the use of the Double Ratio technique in the quantification of fluorescence. The Double Ratio technique is more accurate and reliable compared to the Single Ratio technique.

Acknowledgements

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References