Evaluation of fluorescence measurement techniques for tumour detection in vivo
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CHAPTER 6

Influence of tumour depth, blood absorption and autofluorescence on measurements of exogenous fluorophores in tissue


Chapter 6

Abstract
We investigated the influence of tumour depth and differences in blood concentration and autofluorescence between tumour and normal tissue on the fluorescence of a tumour-localising agent. Carotenoporphyrin, CP(Me)$_3$, was injected into rats and nude mice with intradermal tumours. On the tumours an incision was made, uncovering 2 mm$^2$ of the tumour, and fluorescence measurements, including excitation-emission maps and fluorescence ratios, were made on skin, covered and uncovered tumour. The measured fluorescence ratio in the uncovered tumour showed a three- to tenfold increase compared to the covered tumour. We used one-dimensional layered tissue to analyse the data. In conclusion, even with a high tumour-selectivity deeper lying tumours cannot always be detected, particularly if the tumour has a high blood concentration or low autofluorescence intensity.
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Introduction

Fluorescence detection of tumour-localising agents is a promising method for diagnosis of early stage cancer. The method can be applied non-invasively on superficial tumours in skin or organs which can be reached endoscopically such as the lungs, bladder and colon (Sterenborg et al. 1994, Lam et al. 1990, Kriegmair et al. 1996, Schomacker et al. 1992). Several factors determine the process of fluorescence diagnosis. These are the differential uptake of the fluorescing agent, the absorption and scattering optical properties of the tissue and the depth of the tumour. For deeper lying tumours, its depth and the tissue optical properties will determine how much of the excitation light will reach the tumour and how much of the fluorescence will reach the surface.

A new group of tumour-localising agents, the carotenoporphyrins (CP), show promising behaviour for tumour detection. Two types of CPs have covalently linked carotenoid polyenes and either three methyl (Me) or three methoxy groups (OMe). The carotenoid moiety quenches the porphyrin triplet state by vibrational interactions within the molecule preventing phototoxic reactions with oxygen. In this study, CP(Me)$_3$ was used as fluorescent agent because of its tumour-selective properties and because no skin photosensitivity would be induced. In order to compare different animal and tumour models, measurements were performed on rats and nude mice, each animal type injected with a different tumour. Pharmacokinetics and tumour-selectivity of CP(Me)$_3$ and CP(OMe)$_3$ have been reported in a few papers (Reddi et al. 1994, Nilsson et al. 1994, Nilsson et al. 1997). Nilsson et al. (1994) measured fluorescence spectra of intravenously injected CP(Me)$_3$ and CP(OMe)$_3$ in Balb/c mice with MS-2 fibrosarcoma. The CP(Me)$_3$ and CP(OMe)$_3$ both showed two emission bands centred around 654 and 720 nm, and 658 and 722 nm, respectively. After 24 hours CP(Me)$_3$ and CP(OMe)$_3$ fluorescence at 654 nm was 5-6 times higher in the tumours than in surrounding tissue. Higher tumour-normal tissue contrast, on the order of 10:1, was obtained when the difference in autofluorescence between tumour and normal tissue was also taken into account. These measurements were all done following surgical exposure of the tumour. Fluorescence spectroscopy and HPLC has been used to measure distributions of intravenously injected CP(Me)$_3$ and CP(OMe)$_3$ in the same animal and tumour model (Reddi et al. 1994, Nilsson et al. 1997). CP was accumulated in liver, spleen and tumour. Very low concentrations were measured in skin, muscle and cerebral cortex. Reddi at al. (1994) found a tumour/muscle ratio in the order of 10 for CP(Me)$_3$ and 30 for CP(OMe)$_3$ after 24 hours. Photosensitivity studies showed that exposure to light did not cause any necrosis in tumours with CP(Me)$_3$.

The purpose of this paper was to investigate how a layer covering a deeper lying tumour influences fluorescence measurements in vivo. Fluorescence of CP(Me)$_3$ in intradermal tumours was measured on normal skin, covered tumour and uncovered tumour. A one-dimensional mathematical model which described endogenous and exogenous fluorescence in a layered tissue geometry was used to analyse the data. The model was further used to investigate how blood concentration and autofluorescence will influence fluorescence measurements in vivo.
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Materials and methods

Animals and tumour
Two groups of animals were used. Group I consisted of 3 female Fisher 344 rats (Harlan), 170-192 g body-weight. MTF-7 mammalian adenocarcinoma (rat breast cancer cell line) was intradermally injected above the hind legs. One additional rat was used as control.

Group II consisted of 3 male nude mice, 32-35 g body-weight. TU-138 human squamous cell carcinoma (primary source lip with neural invasion) was injected intradermally above two or three legs. One additional mouse was used as control.

Chemicals
Carotenoporphyrins (CP(Me)₃) were synthesised at the Arizona State University according to Gust et al. (1992). A suspension with CP(Me)₃, Cremophor-EL, absolute ethanol and phosphate buffered saline (PBS, pH=7.4) was basically prepared according to Reddi et al. (5). In short, 10 or 20 mg/ml CP(Me)₃ was added to 0.5 ml Cremophor-EL and 0.15 ml absolute ethanol. PBS was added to the final volume 10 ml and the suspension was stirred and sonicated. The suspension with higher concentration of CP(Me)₃ was used for the nude mice.

In both groups 4.2 μmol/kg bodyweight CP(Me)₃ suspension was injected into the animals. The rats were intravenously injected and the mice intraperitoneally injected. As a control, one mouse and one rat with tumours were injected with the same suspension without CP(Me)₃. The animals were anaesthetised with intramuscular injection of 100 mg/kg bodyweight of 1 ml of 10 mg/ml Ace-prominaze added to Ketaset (100mg/ml) to a total volume of 10 ml.

Experimental set-up
The set-up for fluorescence measurements has previously been described by Sterenborg et al. (1995). In short, fluorescence was excited with a nitrogen-dye laser (Laser Science VSL 337 ND nitrogen and DLM 220 dye laser), emitting 3 ns pulses of ~20 μJ. Excitation wavelengths could be chosen between 380 and 500 nm using different dyes. Excitation and emission light was propagated through a 600 μm core fibre. The fibre was in contact with the tissue, defining the spotsize as the area of the fiber. The fibre was coupled to a multifibre bundle which separated the excitation and emission paths. Highpass filters (Schott KV series) were used to avoid influence of reflections of the excitation light. Fluorescence was detected by an optical multichannel analyser with a 0.32 m monochromator (Yobin Ivon, HR 320), and a cooled diode array detector.

Fluorescence measurements
In all animals, except nude mouse nr 1, a piece of the skin covering the tumour was removed, leaving 2-3 mm² of each of the tumours uncovered. Fluorescence was measured on uncovered tumour, covered tumour and on normal skin. In addition to CP(Me)₃ fluorescence, two fluorescence bands centred around 635 nm and 675 nm were sometimes seen. The 635 nm peak only appeared in the rats. The fluorescence peak at 675 nm was reported by Weagle et al. (1996) and was suggested to be caused by pheophorbide-a in the food given to the animals.

Fluorescence ratio measurements
For fluorescence measurements 420 nm was used as excitation wavelength. The fibre was kept in contact with the tissue and three measurements were performed on each site with the fibre repositioned between each measurement. Fluorescence was measured before CP(Me)₃ injection and
at regular time intervals after injection for up to 43 hours. A highpass filter at 470 nm was used. To avoid influence from the 635 nm fluorescence peak and the pheophorbide-a fluorescence, CP(Me)$_3$ fluorescence at 730 nm was used for further analysis. The ratio between 730 and 500 nm fluorescence intensity was calculated to correct the measurements from variations in excitation light intensity and in measurement geometry.

The animals were measured before injection. In order to cover the fluorescence behaviour up to 43 hours each animal was measured at different time intervals after injection.

**Excitation-emission maps**

A nude mouse without the pheophorbide-a fluorescence peak was selected for measurement of excitation-emission maps using excitation wavelengths from 360 to 500 nm with 10 nm intervals. Highpass filters at 398, 470 and 530 nm were used (Schott KV series).

**One-dimensional layered tissue model**

The influence of the upper layer thickness on the fluorescence ratio (730/500 nm) was estimated using a mathematical model of layers with different optical and fluorescent properties. Figure 1 defines the parameters used in the model.

**Figure 1**

![Figure 1](image)

*Figure 1. Illustration of the model. a) Two-layered model where the upper layer 1 has thickness $z_1$ and layer 2 is semi-infinite. Irradiance $I$ is incident on layer 1 at $z=0$. b) and c) are semi-infinite media with the properties of layer 1 and layer 2, respectively.*

Three different cases were considered. First (Fig. 1a), a two-layer model where the upper layer 1 had thickness $z_1$, and layer 2 was semi-infinite. The other two cases (Figs. 1b and c) were both a semi-infinite medium with the properties of layers 1 and 2 in Fig. 1a, respectively. The properties of layers 1 and 2 were assumed to be those of skin and tumour, respectively. The two-layer model (Fig. 1a) was supposed to simulate the covered tumour. The calculations, which were strictly one-dimensional, are described in detail in Appendix A.

The fluorescence ratios in the skin, covered and uncovered tumours were described with the model using one excitation wavelength $n$ (420 nm if chosen according to the measurements) and two fluorescence wavelengths $i$ and $j$ (730 and 500 nm, respectively). The calculated fluorescence ratios were denoted $R_1$, $R_2$ and $R_{1+2}$ for skin, uncovered tumour and covered tumour, respectively. The fluorescence ratio on the covered tumour, $R_{1+2}$, was calculated using the fluorescence ratios on uncovered tumour and skin, measured at the same time on the same animal. The model included
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macromolecular fluorescence yields of autofluorophores, $A_i$ and $A_j$, and an exogenous fluorophore, $C_i$ and $C_j$. The ratio between $A_{2i}$ and $A_{1i}$, the autofluorescence yield in the tumour and the skin at wavelength $i$, was dependent on the autofluorescence intensity at wavelength $i$ and on the effective attenuation coefficients (mm$^{-1}$) at wavelengths $i$ and $n$. The effective attenuation coefficient (mm$^{-1}$) at excitation wavelength $n$, $\mu_n$, was assumed to be equal in layer 1 (skin) and layer 2 (tumour). The same assumption was made for the effective attenuation coefficients at the two fluorescence wavelengths $i$ and $j$, $\mu_i$ and $\mu_j$. With these assumptions, the ratio $A_{2i}/A_{1i}$ could be estimated from the measurements (Eq. 21 in Appendix A). The skin thickness $z_1$, representing the tumour depth in the skin, was assumed to be equal for all tumours. The effective attenuation coefficients used were calculated from measured absorption and reduced scattering coefficients in rat skin found in literature (Vijverberg et al. 1993). Table 1 shows the values of $\mu_n$, $\mu_i$, and the effective attenuation coefficients $\mu_{eff}$.

Table 1. The optical properties found in literature (Vijverberg et al. 1993) and the calculated effective attenuation coefficients. In the text $\mu_{eff}$ is denoted as $\mu_n$ and $\mu_i$ at 420 and 730 nm, respectively.

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>$\mu_n$ (mm$^{-1}$)</th>
<th>$\mu_i$ (mm$^{-1}$)</th>
<th>$\mu_{eff}$ (mm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>420</td>
<td>0.3</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>730</td>
<td>0.05</td>
<td>1.2</td>
<td>0.43</td>
</tr>
</tbody>
</table>

The model was further used to calculate the fluorescence ratio in the covered tumour divided by the fluorescence ratio in the skin. A tumour/normal (T/N) ratio was calculated expressing the calculated fluorescence ratio in the two-layered geometry divided by the calculated fluorescence ratio in the medium with the properties of layer 1. The influence of depth $z_1$, and of differences in blood content in tumour and skin on the T/N ratio was investigated. In the calculations $A_{1n}$, the autofluorescence yield factor in the skin, was assumed to be equal to 1 and the other factors ($A_{2i}$, $C_{1i}$, $C_{2i}$) were chosen relative to this. First, equal autofluorescence and effective attenuation coefficients in skin and tumour were considered, i.e. $A_{1i}=A_{2i}=1$, and the exogenous fluorophore concentration was varied in tumour and skin by choosing different $C_{1i}$ and $C_{2i}$. The tumour-selectivity ($C_{2i}/C_{1i}$) was chosen to equal 1, 5, 10 and 20. Thereafter $C_{2i}/C_{1i}$ was kept equal to 10 and the blood concentration in layer 2 (tumour) was varied. The absorption coefficient at wavelength $n$ of oxyhaemoglobin, $\mu_a=300$ mm$^{-1}$ (Welch et al. 1995), was used to calculate $\mu_{2n}$. A blood concentration of 0.1, 0.3, 1 and 3% blood in the tumour was used. The autofluorescence intensities in tumour and skin were chosen to be equal ($F_{skin}=F_{uncov}=1$ in Eq. 19 in Appendix A).

Results

Excitation-emission maps

Excitation-emission maps measured on skin, covered and uncovered tumour in a nude mouse are shown in Figs. 2a-c. The iso-intensity lines in the maps are logarithmically plotted with a factor 0.5 between each line and the grey colour increases with increasing fluorescence intensity. The area in the upper left corner was not within the measurement range, since fluorescence was measured at wavelengths longer than the excitation wavelength.
Figure 2. Excitation-emission maps measured on a) skin, b) covered and c) uncovered tumour in a nude mouse. The contour lines in the maps are logarithmically plotted with a factor 0.5 between each line. The area in the upper left corner was not within the measurement range.

In the lower left area of the map of the skin (Fig. 2a) the autofluorescence can be seen as a broad band with highest fluorescence intensity around 450 nm and decreasing towards longer emission wavelengths. Horizontal valleys at 370 and 415 nm represent increased absorption of the excitation light. The absorption peak at 415 nm is probably caused by oxyhaemoglobin. The 370 nm absorption peak might be a haemoglobin breakdown product (Sterenborg et al 1995). The most efficient excitation wavelength for the autofluorescence is 360 nm, and with longer excitation wavelengths the autofluorescence decreases. In the middle of the map a slightly increased fluorescence area is seen centred around the CP(Me)$_3$ fluorescence peaks at 652 and 730 nm. On the covered tumour (Fig. 2b) this fluorescence area has increased, showing highest fluorescence after excitation with 420 nm. On the uncovered tumour (Fig. 2c) the CP(Me)$_3$ fluorescence is more pronounced in the middle of the map and the autofluorescence on the left hand side is weaker compared to skin.
**Fluorescence measurements**

In Figs. 3a and b the fluorescence ratio measured on skin, covered and uncovered tumour on the 3 rats (a) and the 3 nude mice (b) is shown.

**Figure 3a**

- **skin**
- **covered tumour**
- **uncovered tumour**

**Figure 3b**

The mean and standard error of all tumours on each animal has been calculated, averaging measurements during 2-12 hour time intervals (n=6-42 for skin and n=12-90 for tumours). At t=0 hours the mean and standard error of all rats or mice are shown.

The mean and standard error of all tumours on each animal has been calculated, averaging measurements during 2-12 hour time intervals (6-42 measurements for skin and 12-90 for tumours). During these intervals fluorescence was measured 2-10 times. At t=0 hours, the measurement before CP(Me)$_3$ injection, the mean and standard error of all rats or mice are shown. The other columns in
Fig. 3 are calculated per animal since the measurement time interval after injection was different for each animal.

The covered as well as the uncovered tumours show an increase in fluorescence ratio compared to skin. The maximum difference in the fluorescence ratio between tumours and skin is seen at 21-24 hours after injection in the rats and 24-35 hours in the mice. At this time the fluorescence ratio in the covered tumours of the rats are 3.4 times higher than in the skin and the uncovered tumours 14.6 times higher than in skin. For the mice the fluorescence ratio in the covered tumours is 1.7 times higher than in skin and the uncovered tumours 9.8 times higher. Comparison of the fluorescence ratios in uncovered and covered tumours show that the uncovered tumours in the rats have a fluorescence ratio 4-7 times higher than in the covered tumours up to 43 hours after injection. In the nude mice fluorescence ratio in the uncovered tumours were 1.3-5.6 times higher than in the covered tumours.

Figure 4 shows the fluorescence ratio measured on the control animals that received the same suspension without CP(Me)$_3$. Using the unpaired t-test ($\alpha=0.05$), the rat shows a slight but statistically significant increased fluorescence ratio 20-24 hours after injection. The spectra showed humps at 670, 710 and 730 nm, which might have been due to pheophorbide-a, giving support to the observed increase in the fluorescence ratio.

One-dimensional layered tissue model

Figure 5a and b show the measured normalised fluorescence ratio on the covered tumour plotted against the normalised calculated fluorescence ratio $R_{1+\lambda}/R_{1+\lambda,0}$ (Eq. 20 in Appendix A) using the fluorescence ratio of uncovered tumour and skin on mice and rats, respectively. $R_{1+\lambda,0}$ denotes the fluorescence ratio without an exogenous fluorophore present. All measurements after administration of CP(Me)$_3$ are plotted. The straight line shows the ideal case where measured and calculated fluorescence ratios are exactly equal. An average depth $z_1$ was found by using constant effective
attenuation coefficients in the exponent $-z_1(\mu_a+\mu_t)$ in Eq. 20 (Appendix A). Using the least squares method with $z_1$ as variable the calculated data in Figs. 5a and b were fitted around the ideal line. The data showed correlation coefficients $r^2=0.69$ in Fig. 5a and $r^2=0.11$ in Fig. 5b. Depths $z_1$ found in this way were 0.45 mm for the mice and 0.55 mm for the rats.

**Figure 5a**

![Graph showing measured vs. calculated fluorescence ratio for mice](image)

**Figure 5b**

![Graph showing measured vs. calculated fluorescence ratio for rats](image)

*Figure 5. The measured fluorescence ratio on the covered tumour in a) the mice and b) the rats plotted against $R_{1+2}$, the calculated fluorescence ratio. All measurements on the tumours have been plotted and $R_{1+2}$ has been calculated using the measurements on the uncovered tumour and the skin. The straight line shows the ideal case where the measured and calculated fluorescence ratios are equal.*

The T/N ratio (Eq. 22 in Appendix A), normalised at the T/N when no exogenous fluorophore is present, is plotted against $z_1$ in Fig. 6a. The parameters used in the calculations are indicated in the figure. The autofluorescence has been put equal in tumour and skin. $C_{2i}/C_{1i}$, the ratio between the macroscopic fluorescence yields at wavelength $i$ in layer 2 (tumour) and layer 1 (skin), respectively, has been used as a measure for tumour-selectivity. With an increasing $z_1$ the T/N ratio decreases. At a depth of 0.2 mm the curves have decreased by 50% and at 0.65 mm the T/N ratio has decreased by
90%. In Fig. 6b the blood percentage in the tumour is denoted in the upper right corner. With increasing blood concentration the slope of the T/N ratio decreases faster. With 1% blood in the tumour the T/N ratio has decreased by 90% at a tumour depth of 0.09 mm, compared to 0.1% blood where a 90% decrease is seen at a tumour depth of 0.33 mm.

Figure 6a

Figure 6. The T/N ratio as a function of the depth $z_i$. a) The numbers in the upper right corner denote $C_{2i}/C_{1i}$, the ratio between the exogenous fluorescence yields in layer 2 and 1. The effective attenuation coefficients at 420 nm ($\mu_a$) and 730 nm ($\mu$), used in the calculations, are given. b) The blood concentration in layer 2 is varied according to the number in the upper right corner.

Discussion

Figures 5a and b show that the model did not provide a perfect correspondence between calculations and measurements. We attributed this to the assumptions that effective attenuation coefficients and depths $z_1$ did not vary between the measurements. The optical properties of the tissue, tumour depth
and fluorescence yield factors $A_{ij}$ and $C_{ij}$ depend on the animal and tumour model used. However, since the in vivo optical properties are not available we chose a simple approach and used effective attenuation coefficients found in literature on albino rat skin (Vijverberg et al. 1993). In the calculations of $R_{i+2}/R_{i+2,0}$ (Figs. 5a and b) an average depth was used for all tumours since the actual depth of the tumour at the spot where the fibre was placed during the measurement was difficult to determine. The location where the fibre was held varied slightly between different measurements and it was histologically impossible to determine the actual depth at every measurement. The tumour depth could vary between the tumours, or one covering skin layer could have different local thicknesses.

Figures 6a and b show the influence of depth $z_i$ on the T/N ratio, and the influence of differences in blood absorption between the layers. The influence of blood absorption and autofluorescence are hard to separate from each other since a lower autofluorescence intensity in tumour compared to skin would give the same result as in Fig. 6b (see Eq. 19). In our case, as seen in the excitation-emission maps (Figs. 2a-c), the autofluorescence in the tumour decreased in intensity compared to the autofluorescence in the skin. Comparison of the fluorescence ratio between the two different tissues includes the change of CP(Me)$_3$ fluorescence as well as changes in autofluorescence. This was also seen by Nilsson et al. who reported that CP fluorescence in tumour compared to normal tissue was twice as high when autofluorescence was taken into account compared to measuring red fluorescence only (Nilsson et al. 1994). In our study, the autofluorescence in skin was found to be about three to 25 times higher than in the tumour, based on the measurements before CP(Me)$_3$ was administered and Eq. 21 in Appendix A. However, we cannot be certain that the tumours and the skin contained the same concentration of blood. The measurements on the uncovered tumour might have been influenced by blood from the incision, even if the incision was rinsed with a saline solution before each measurement.

As described above, the calculations of the T/N ratio were dependent on some assumptions and estimations. These estimations are dependent on the intrinsic fluorescent and optical properties of the tissue and of the tumour-selectivity of the exogenous fluorophore. However, Figs. 6a and b gives an indication of how the layer of skin on a tumour as well as blood absorption and autofluorescence can influence the measured fluorescence ratio. Using a longer wavelength for excitation where the absorption of blood is lower and the penetration depth in tissue deeper might be a solution to avoid these effects. In these calculations, the penetration depth of the excitation light ($1/\mu_b$) was 0.33 mm. With a low tumour-selectivity of the exogenous fluorophore, or high blood absorption in the tumour, the tumour might not be detectable even if it is situated within the penetration depth of the excitation light.

**Conclusions**

A layer of tissue covering the tumour strongly influences fluorescence ratio measurements. Consequently, even with a high tumour-selectivity deeper lying tumours cannot always be detected, particularly if the tumour has a high blood concentration or low autofluorescence intensity.
Appendix A

In the two semi-infinite media (Fig. 1), the fluence rate $\Phi_n$ at depth $z$ was estimated with an exponential approximation using the one-dimensional transport theory (van Gemert et al. 1997, Gardner et al. 1996):

$$\Phi_n(z) = I \exp(-z\mu_n), \quad [W/mm^2]$$

(1)

where $I$ is the incident irradiance [W/mm$^2$] at wavelength $n$ and $\mu$ the effective attenuation coefficient:

$$\mu = \sqrt{3\mu_a(\mu_a + \mu_s')}, \quad [mm^{-1}]$$

(2)

The fluorescence escape function $E_i$ of fluorescence at wavelength $i$ generated at depth $z$ was estimated as (Gardner et al. 1996)

$$E_i(z) = \frac{1}{2} \exp(-z\mu_i). \quad [-]$$

(3)

The factor 1/2 is due to the isotropic distribution of fluorescence from the fluorophore, which in one dimension gives 1/2 in each $z$-direction (+$z$ and -$z$).

In our model we assumed that two different kinds of fluorophores were present, an exogenous fluorophore and autofluorophores. The fluorophores were assumed to be homogeneously distributed in each layer but with different concentrations in the layers. The factors $Q_i$ and $A_i [mm^{-1}]$ represented the macroscopic fluorescence yield for the exogenous fluorophore and the autofluorophores, respectively. The macroscopic fluorescence yield factor is dependent on the concentration [mm$^{-1}$], absorption per concentration [-] and fluorescence quantum yield per concentration [-] of a fluorophore emitting fluorescence at wavelength $i$.

The total fluorescence $F_i$ at wavelength $i$, excited with wavelength $n$, and measured at $z=0$ was a function of the fluence rate, the factors $A_i$ and $C_i$ and the escape function:

$$F_i(z = 0) = \int_{z=0}^{\infty} \Phi_n(z)(A_i + C_i)E_i(z)dz. \quad [W/mm^2]$$

(4)

The ratio $R$ between two fluorescence wavelengths $i$ and $j$ was calculated as

$$R = \frac{F_i(z = 0)}{F_j(z = 0)} = \frac{\int_{z=0}^{\infty} \Phi_n(z)(A_i + C_i)E_i(z)dz}{\int_{z=0}^{\infty} \Phi_n(z)A_jE_j(z)dz}$$

(5)
where wavelength j was chosen so that the exogenous fluorophore did not fluoresce. In the two-layer model the fluence rate and the escape function were described as

\[
\Phi_n(z) = I \exp(-z \mu_{1n}), \quad \text{[W/mm}^2\text{]} \quad z \leq z_1.
\]

\[
\Phi_n(z) = I \exp(-z \mu_{1n}) \exp(-(z - z_1) \mu_{2n}), \quad \text{[W/mm}^2\text{]} \quad z > z_1.
\]

\[
E_i(z) = \frac{1}{2} \exp(-z \mu_{ii}), \quad [-] \quad z \leq z_1.
\]

\[
E_i(z) = \frac{1}{2} \exp(-(z - z_1) \mu_{2i}) \exp(-z \mu_{ii}), \quad [-] \quad z > z_1.
\]

The total fluorescence at \( z=0 \) was

\[
F_i(z = 0) = \int_{z=0}^{z_1} \Phi_{1n}(z)(A_{1i} + C_{ii})E_{ii}(z)dz + \int_{z_1}^{\infty} \Phi_{2n}(z)(A_{2i} + C_{2i})E_{2i}(z)dz, \quad \text{[W/mm}^2\text{]} \quad (10)
\]

and the ratio \( R \)

\[
R = \frac{\int_{z=0}^{z_1} \Phi_{1n}(z)(A_{1i} + C_{ii})E_{ii}(z)dz + \int_{z_1}^{\infty} \Phi_{2n}(z)(A_{2i} + C_{2i})E_{2i}(z)dz}{\int_{z=0}^{z_1} \Phi_{1n}(z)A_{1j}E_{1j}(z)dz + \int_{z_1}^{\infty} \Phi_{2n}(z)A_{2j}E_{2j}(z)dz}. \quad (11)
\]

Since the fluorophores were assumed to be homogeneously distributed in the layers, the factors \( A \) and \( C \) could be taken outside the integrals. Solving the integrals in Eqs. 5 and 11 gave three different fluorescence ratios \( R \) for the three geometrical cases:

\[
R_1 = \left( \frac{A_{1i} + C_{ii}}{A_{1j}} \right) \left( \frac{\mu_{1n} + \mu_{1j}}{\mu_{1n} + \mu_{ii}} \right), \quad (12)
\]

\[
R_2 = \left( \frac{A_{2i} + C_{2i}}{A_{2j}} \right) \left( \frac{\mu_{2n} + \mu_{2j}}{\mu_{2n} + \mu_{2i}} \right), \quad (13)
\]

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\[ R_{1+2} = \frac{A_{1i} + C_{1i} \left( 1 - e^{-z_1 (\mu_{ln} + \mu_{li})} \right) + A_{2i} + C_{2i} e^{-z_1 (\mu_{ln} + \mu_{2i})}}{\mu_{ln} + \mu_{li} + \mu_{2n} + \mu_{2i}} e^{-z_1 (\mu_{ln} - \mu_{2n})} e^{-z_1 (\mu_{ln} - \mu_{2n})} \]  

\[ R_{1+2} = \frac{A_{1j} \left( 1 - e^{-z_1 (\mu_{ln} + \mu_{li})} \right) + A_{2j} e^{-z_1 (\mu_{ln} + \mu_{2j})}}{\mu_{ln} + \mu_{li} + \mu_{2n} + \mu_{2j}} e^{-z_1 (\mu_{ln} - \mu_{2n})} e^{-z_1 (\mu_{ln} - \mu_{2n})} \]  

where \( R_1 \) and \( R_2 \) were the fluorescence ratios in the semi-infinite media with properties of layer 1 and 2, respectively, and \( R_{1+2} \) the fluorescence ratio in the two-layered medium. If the excitation wavelength \( n \) and the emission wavelengths \( i \) and \( j \) were chosen according to the measurements, \( R_1 \), \( R_2 \) and \( R_{1+2} \) described the fluorescence measurements on skin, uncovered tumour and covered tumour, respectively.

The measurements at \( t=0 \), i.e. with no exogenous fluorophore present, were described as Eqs. 12-14 with \( C_{1i} = C_{2i} = 0 \):

\[ R_{1,0} = \frac{A_{1i} \mu_{ln} + \mu_{li}}{A_{1j} \mu_{ln} + \mu_{li}} \]  

\[ R_{2,0} = \frac{A_{2i} \mu_{2n} + \mu_{2j}}{A_{2j} \mu_{2n} + \mu_{2j}} \]  

\[ R_{1+2,0} = \frac{A_{1i} \left( 1 - e^{-z_1 (\mu_{ln} + \mu_{li})} \right) + A_{2i} e^{-z_1 (\mu_{ln} + \mu_{li})}}{\mu_{ln} + \mu_{li} + \mu_{2n} + \mu_{2i}} e^{-z_1 (\mu_{ln} - \mu_{2n})} e^{-z_1 (\mu_{ln} - \mu_{2n})} \]  

Using Eqs. 12-17, \( R_{1+2}/R_{1+2,0} \) could be expressed as:

\[ \frac{R_{1+2}}{R_{1+2,0}} = \frac{R_{1,0} \mu_{ln} + \mu_{li}}{A_{1i} \mu_{ln} + \mu_{li}} \frac{R_{2,0} \mu_{2n} + \mu_{2i}}{A_{2j} \mu_{2n} + \mu_{2j}} e^{-z_1 (\mu_{ln} + \mu_{li})} \]  

The ratio \( A_{2i}/A_{1i} \) can be found using the fluorescence intensity at 730 nm in the uncovered tumour and the skin with no exogenous fluorophore:

\[ \frac{F_{\text{uncov}}}{F_{\text{skin}}} = \frac{\int \Phi_{2n}(z) A_{2i} E_{2i}(z) \, dz}{\int \Phi_{ln}(z) A_{li} E_{li}(z) \, dz} = \frac{A_{2i} \mu_{2n} + \mu_{2i}}{A_{1i} \mu_{ln} + \mu_{li}} \]
Chapter 6

Assuming that $\mu_i = \mu_2$, $\mu_j = \mu_2$ and $\mu_{in} = \mu_{2n}$, Eq. 18 could be written as

$$R_{1+2} = \frac{R_{1,0}}{R_{1+2,0}} \left( 1 - e^{-z_i (\mu_{in} + \mu_{ii})} \right) + \frac{A_{2i}}{A_{li}} \frac{R_2}{R_{2,0}} \cdot e^{-z_i (\mu_{1n} + \mu_{ii})}$$

where in this case

$$\frac{A_{2i}}{A_{li}} = \frac{F_{in, cov}}{F_{skin}}$$

Here, $A_{2i}/A_{li}$ can be calculated according to Eq. 19.

R1+2, R1 and R2 are parameters which have been measured at different time intervals. $R_{1+2}/R_{1+2,0}$ is the fluorescence ratio on the covered tumour normalised at the measurements at $t=0$. $R_1/R_{1,0}$ and $R_2/R_{2,0}$ are the normalised fluorescence ratios on the skin and the uncovered tumour, respectively.

To solve Eq. 18, $R_{1+2}$, $R_1$ and $R_2$ have to be measured at the same time after administration. For each tumour $A_{2i}/A_{li}$ was estimated as the ratio between the fluorescence intensity at 730 nm in the uncovered tumour and in the skin at $t=0$, the measurement before CP(Me)$_3$ was administered.

The calculated fluorescence ratio in the two-layered geometry, $R_{1+2}/R_{1+2,0}$, divided by the fluorescence ratio in the medium with the properties of layer 1, $R_1/R_{1,0}$, would represent a tumour/normal ratio (T/N ratio) of fluorescence ratios. Using Eqs. 12-17 the T/N ratio was described as

$$T = \frac{R_{1,0} \cdot \frac{1 - e^{-z_i (\mu_{1n} + \mu_{ii})}}{\mu_{1n} + \mu_{ii}} + \frac{A_{2i}}{A_{li}} \frac{R_2}{R_{2,0}} \cdot e^{-z_i (\mu_{1n} + \mu_{ii})}}{1 - e^{-z_i (\mu_{2n} + \mu_{2i})} + \frac{A_{2i}}{A_{li}} \cdot e^{-z_i (\mu_{1n} + \mu_{ii})}}$$

Here, $A_{2i}/A_{li}$ can be calculated according to Eq. 19.

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