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Review

A review of attenuation correction techniques for tissue fluorescence

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Fluorescence intensity measurements have the potential to facilitate the diagnoses of many pathological conditions. However, accurate interpretation of the measurements is complicated by the distorting effects of tissue scattering and absorption. Consequently, different techniques have been developed to attempt to compensate for these effects. This paper reviews currently available correction techniques with emphasis on clinical application and consideration given to the intrinsic accuracy and limitations of each technique.

Keywords: tissue fluorescence; correction techniques; absorption; scattering

1. INTRODUCTION

The measurement of fluorescence intensity from tissues has the potential to facilitate the diagnoses of pathological conditions such as cancer and ischaemia. Tissues contain many native fluorophores including nicotinamide adenine dinucleotide hydrate (NADH), flavin adenine dinucleotide (FAD), collagen, elastin, tryptophan and porphyrins (Richards-Kortum & Sevick-Muraca 1996; Andersson-Engels *et al.* 1997), and quantitative data about their concentrations and change over time would yield important information about tissue state.

NADH and FAD for example are molecules involved in metabolic processes within cells, and changes in their fluorescence are directly related to changes in the reduction–oxidation state of cells (Horvath *et al.* 1994). During periods of low perfusion of oxygenated blood to tissues (ischaemia) or during organ storage, reduced activity of complex I of the mitochondrial respiratory chain leads to an increase in the concentration of NADH relative to its non-fluorescent oxidized form NAD^+, with a consequent increase in NADH fluorescence. Prolonged ischaemia leads to irreversible tissue damage and full oxidation of NADH with a corresponding loss of NADH fluorescence (Thorniley *et al.* 1995). Measurement of NADH fluorescence would therefore enable tissue damage assessment to be made during ischaemia (Coremans *et al.* 2000). Information of this nature would be of vital importance during tissue transplantation and other surgical procedures.

Changes in the fluorescence of collagen and elastin are associated with changes in tissue structure since they are both proteins that provide tissues with rigidity (Ramanujam 2000). Elastin fluorescence, for instance, has been found to be lower from atherosclerotic aortic tissue than from normal aortic tissue (Richards-Kortum & Sevick-Muraca 1996) and collagen fluorescence has been shown to decrease during laser-induced thermal damage of tissues (Tang *et al.* 2000). Furthermore, changes in collagen, elastin, NADH and tryptophan fluorescence have all been associated with the onset of cancer (Richards-Kortum & Sevick-Muraca 1996; Georgakoudi *et al.* 2002; Sokolov *et al.* 2002).

In other work, the use of exogenous fluorophores has been studied to aid medical diagnoses. In particular, measuring the fluorescence of photosensitizing agents used in photodynamic therapy has improved the detection and demarcation of tumours (Ramanujam 2000).

However, the interpretation of fluorescence intensity measurements is problematic since fluorescence from tissues is significantly affected by absorption and scattering at both the excitation and emission wavelengths. The fluorescence power per unit area escaping the tissue (F) can be written as a function of H^{in} and H^{out}, where H^{in} is related to the distribution of the excitation radiation within the tissue and H^{out} is the fluorescence escape function, which relates F to the fluorescence power emitted at position $\boldsymbol{r}$ within the tissue (Richards-Kortum 1995; Gardner *et al.* 1996*a*),

$$
\begin{aligned}
F_{\mathrm{e,m}}(\boldsymbol{s}) = \int\limits_{\substack{\text{irradiated}\\\text{surface}}} \mathrm{d}\boldsymbol{s}'^2 E_0(\boldsymbol{s}') \int\limits_{\substack{\text{tissue}\\\text{volume}}} \mathrm{d}\boldsymbol{r}^3 H_{\mathrm{e}}^{\mathrm{in}}(\boldsymbol{s}',\boldsymbol{r}) \\
\times \left[\sum_k \ln(10)\varepsilon_{\mathrm{e}}^k C^k(\boldsymbol{r})\Phi_{\mathrm{m}}^k \right] H_{\mathrm{m}}^{\mathrm{out}}(\boldsymbol{r},\boldsymbol{s})
\end{aligned}
\tag{1.1}
$$

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Table 1. Nomenclature. (Subscripts 'e' and 'm' denote values at the excitation (e) and emission (m) wavelengths.)

intensity	the radiant flux density at a point as given by the time-averaged magnitude of the Poynting vector (W m^{-2})
r	position within the tissue (m)
s	position on the tissue surface (m)
ε^i	extinction coefficient of ith fluorophore (m^{-1} M^{-1})
Φ^i	quantum yield of ith fluorophore
C^i	concentration of the ith fluorophore (M)
μ_a	absorption coefficient (m^{-1})
μ_s	scattering coefficient (m^{-1})
g	scattering anisotropy
μ_t	total attenuation coefficient $= \mu_a + \mu_s$ (m^{-1})
μ_s'	reduced scattering coefficient $= (1-g)\mu_s$ (m^{-1})
E_0	irradiance of the excitation light on the tissue surface (W m^{-2})
F	fluorescence power escaping the tissue per unit area (W m^{-2})
F^{det}	output of the detector measuring F (arbitrary units)
f^{det}	corrected value of F^{det} (arbitrary units)
R	diffuse reflectance power escaping the tissue per unit area (W m^{-2})
R^{det}	output of the detector measuring R (arbitrary units)
$H^{\text{in}}(s,r)$	the excitation fluence rate at r due to radiant power incident on the tissue surface at s per unit incident power (m^{-2}). The total excitation fluence rate is given by: $\phi(r) = \int_{\text{tissue surface}} E_0(s) H^{\text{in}}(s,r) \mathrm{d}s^2$
$H^{\text{out}}(r,s)$	the radiant excitance at s due to an isotropic fluorescence source at r per unit power of the source (m^{-2}). The total radiant excitance is given by: $F(s) = \int_{\text{tissue volume}} \left[\sum_k \ln(10)\varepsilon_e^k C^k(r) \Phi_m^k\right] \phi(r) H^{\text{out}}(r,s) \mathrm{d}r^3$

H^{in} and H^{out} are functions of the optical properties at the excitation and emission wavelengths respectively, and these properties generally vary through the tissue (table 1). The intrinsic fluorescence is defined as the observed fluorescence when there is no scattering or absorption, and can be obtained by measuring the fluorescence intensity from an optically thin tissue sample. A transfer function can be used to relate the intrinsic fluorescence to the measured tissue fluorescence.

The variation of tissue optical properties with wavelength gives rise to distortion of fluorescence excitation and emission spectra and as a result it is difficult to identify the fluorophores responsible for the fluorescence and their relative contributions to it. Furthermore, changes in fluorescence intensity due to variations in fluorophore concentrations cannot be easily distinguished from those arising from variations in absorption and scattering. It is worth noting however that the scattering and absorption themselves may yield significant information. For example, changes in absorption can reflect changes in chromophore concentrations or their oxygenation state, and changes in scattering are indicative of changes in tissue structure (Sokolov *et al.* 2002).

Clearly, quantitative information about fluorophore concentrations can only be determined if apposite correction techniques can be used to compensate for the effects of scattering and absorption. Currently available correction techniques fall into three broad categories, namely empirical techniques, measurement-method based techniques and theory based techniques. Empirical techniques generally involve forming a combination of measurements related to the intrinsic fluorescence but independent of the tissue optical properties. Measurement-based techniques essentially entail selectively recording the least attenuated proportion of the fluorescence. Theory-based techniques generally necessitate calculating the transfer function relating intrinsic to measured fluorescence. This paper reviews the correction techniques available in terms of their accuracy, limitations and suitability for clinical application. In the final section, the application of Monte Carlo simulations is discussed.

2. EMPIRICAL TECHNIQUES

2.1. Utilizing reflectance

Many investigators have employed measurements of the reflected illumination intensity in order reduce the effects of tissue optical properties on fluorescence. The most common techniques are based on a ratio of fluorescence to reflectance. More recently, techniques utilizing the spatial variations of fluorescence and reflectance have been developed.

2.1.1. Subtraction technique. This technique was developed during the pioneering studies of *in vivo* tissue fluorescence to take account of the effects of absorption by blood on NADH fluorescence (Jöbsis *et al.* 1971; Franke *et al.* 1976; Harbig *et al.* 1976; Mayevsky & Chance 1982). Changes in fluorescence due to blood content changes were compensated for by subtracting a proportion of the corresponding change in the reflected excitation light intensity from the fluorescence intensity (excitation at 366 nm, emission centred on 450 nm). The theoretical basis for this method was put forward by Jöbsis *et al.* (1971), who postulated that blood vessels would almost totally absorb both excitation and emission light. Changes in fluorescence were therefore expected to be in direct proportion to changes in reflectance. This relationship was confirmed experimentally over a limited range of blood pressures and haemocrits. However, the constant of proportionality was found to vary considerably between different tissue preparations and fields of view. The technique was

extended by Harbig *et al.* (1976) to take into account haemoglobin oxygenation changes by measuring the fluorescence at an isosbestic wavelength for haemoglobin absorption, although the oximetric effects were not completely eliminated since the excitation wavelength was not isosbestic.

Kramer & Pearlstein (1979) suggested, from experience, that the subtraction technique did not provide accurate correction. The technique was superseded by ratio techniques.

2.1.2. Ratio techniques. In 1979, Kramer & Pearlstein (1979) argued that it would be more appropriate, for changes in tissue absorption, to utilize a ratio of fluorescence intensity to backscattered excitation light intensity rather than an algebraic subtraction. However, early use of a fluorescence–reflectance ratio yielded inconsistent results (see Coremans *et al.* (1996) and references within). It was argued by Coremans *et al.* (1996) that unpredictable contributions from specularly reflected incident light might be responsible for the inconsistency and that it was essential to employ cross-polarization methods to reject the specular component. Coremans *et al.* (1997) considered the efficacy of the method when measuring fluorescence in a narrow wavelength range (10 nm bandwidth at half maximum). Theoretical considerations, which combined the theories of front-faced fluorimetry and Kulbelka–Munk, suggested that the fluorescence–reflectance ratio should be approximately independent of absorption at high values of absorption, provided that the amount of scattering is constant. At high absorption, both reflectance and fluorescence vary linearly with μ_{ae}, whereas the variation of fluorescence with the other optical properties is an order of magnitude less (Beuthan *et al.* 1996). Experiments involving the imaging of tissue phantoms, which contained increasing amounts of haemoglobin, confirmed that the fluorescence–reflectance ratio reaches a constant value at high absorption (haemoglobin concentrations greater than 85 μM). However, at lower haemoglobin concentrations, the ratio was found to decrease as concentration increased. This accords with the theoretical considerations of Wu *et al.* (2003). The efficacy of the technique was evaluated *in vivo* using NADH fluorescence from perfused rat hearts. The effects of blood absorption were investigated by interrupting the supply of oxygenated blood with flushes of oxygenated Tyrode solution. It was found that the ratio remained constant for all practical purposes.

A limitation is that the correction factor fails to take account of the optical properties at the emission wavelength. This limitation becomes apparent when measuring either fluorescence spectra or the total fluorescence emission over a large wavelength range. When imaging fluorescence in the emission range 470–650 nm from tissue phantoms containing various amounts of blood, Qu & Hua (2001) found that while the ratio only varied by up to approximately 7% for low blood content, the ratio increased significantly with higher blood contents.

Recently, a number of correction factors incorporating the diffuse reflectance at the emission wavelength have been proposed in an attempt to overcome this limitation. Chen *et al.* (2002) and Stamatas *et al.* (2002) explored the possibility of correcting spectra by dividing by the diffuse reflectance at the emission wavelength. However, Biswal *et al.* (2003) found that this technique did not successfully recover spectral lineshape or intensity information of tissue phantoms with μ_a/μ_s' in the range 0.32–1.8. Theoretical work, using the photon migration model of light transport, suggests that this method is only accurate when absorption is weak (Müller *et al.* 2001). This limitation arises because the optical properties at the excitation wavelength and the isotropic nature of the fluorescence emission are not taken into account.

Finlay *et al.* (2001) incorporated the diffuse reflectance at both the excitation and emission wavelengths into an experimentally determined correction factor, specifically for use with their fibre-optic probe. Correction was achieved by dividing by $(R_e^{det})^{0.8} R_m^{det}$. This approach has been generalised by Hull *et al.* (2004), who proposed that correction for local variations in optical properties can be achieved by dividing by the factor $(R_e^{det})^{k_e}(R_m^{det})^{k_m}$. Collagen fluorescence from human skin was measured *in vivo* and the exponents k_e and k_m quantified by determining the values which minimised the variance of the corrected fluorescence for repeated measurements on the same subject. It was assumed that the intrinsic fluorescence remained constant for all repeat measurements. No detailed quantitative information is currently available with regard to the accuracy of this method.

2.2. Utilizing spatially resolved reflectance

These techniques utilize the variation of the fluorescence and reflected excitation light with distance away from an excitation optical fibre. Measurements made at a particular distance selectively record photons which have travelled, on average, a particular path length through the tissue. Canpolat & Mourant (2000) proposed that the concentration of an exogenous fluorophore could be recovered by determining the fluorescence–reflectance ratio at a specific distance. According to Beer's law, the ratio should vary proportionally with fluorophore concentration provided the product of fluorophore absorption coefficient and path length is less than 1, and the background absorption remains constant. The proportionality was confirmed experimentally for distances of less than 1 mm.

Weersink *et al.* (2001) measured the escaping fluorescence and reflectance at two different distances from an excitation fibre. Experimental investigations and Monte Carlo simulations were carried out and it was established that there was an optimum combination of the distances at which the effects of changes in scattering and absorption on the fluorescence–reflectance ratio were minimised. Fluorophore concentrations were retrieved with a root mean square (r.m.s.) error of 14.6% over a wide range of optical properties. However, no simple rationale for the

optimum distances has been suggested; the distances have to be obtained experimentally.

Like the subtraction technique, these techniques do not take into account tissue optical properties at the emission wavelength and application is restricted to measurements made a at single emission wavelength.

2.3. Utilizing fluorescence

In 1979, Kramer & Pearlstein (1979) suggested that a ratio of fluorescence intensities at two emission wavelengths might be used to correct for changes in blood volume and oxygenation when measuring cerebral cortical NADH fluorescence *in vivo*. An exogenous fluorophore (rhodamine B) was used as an internal standard by which changes in NADH redox state could be distinguished from haemodynamic and oximetric effects. Rhodamine B was chosen because its emission spectrum does not overlap with that of NADH for excitation at 366 nm. The ratio of NADH fluorescence to rhodamine fluorescence at emission wavelengths of 448 and 549 nm, respectively, was then determined, the wavelengths being chosen to coincide with the isobestic wavelengths of haemoglobin absorption. Qualitatively, it was found that the ratio remained unaffected by changes in blood volume or oxidation. Similarly, Koretsky *et al.* (1987) used an exogenous fluorophore to evaluate the NADH fluorescence from perfused rat hearts. More recently, Saarnak (Aalders *et al.* 2000) applied the technique to evaluate exogenous fluorophore concentrations, and devised the normalised fluorescence ratio. The normalised fluorescence ratio is obtained by dividing the fluorescence ratio by its value when exogenous fluorophores are absent. This ratio can be used as a measure of exogenous fluorophore concentration provided that the autofluorescence remains constant.

Avrillier *et al.* (1997) used a similar technique to Kramer & Pearlstein (1979), but relied entirely on endogenous fluorescence. Fluorescence intensity was measured at two emission wavelengths where the haemoglobin absorption coefficients are similar in value. The scattering coefficient remains relatively constant over the ultraviolet and visible regions, so any changes in optical properties were expected to similarly affect both intensities.

The accuracy of these fluorescence ratio techniques was evaluated by Sterenborg *et al.* (1996) when measuring the fluorescence from an exogenous fluorophore applied to human skin. It was discovered that there was a significant correlation between the ratio and skin colour, indicating that the ratio was not an effective correction technique. However, it is worth noting that the intensities were measured over a relatively large wavelength range and the emission wavelengths were not specifically chosen for the absorption coefficients to be comparable.

2.4. Utilizing correlation techniques

Other work has concentrated on developing correction equations by experimentally determining correlations between fluorescence and reflectance. Na *et al.* (2001)

and Sandby-Møller *et al.* (2003) investigated the relationship between autofluorescence and skin pigmentation (a measure of the melanin content) and redness (a measure of the haemoglobin content) using multiple regression analysis.

The possibility of utilizing the partial least squares method to extract the concentration of fluorophores from intensity measurements has been explored by Durkin & Richards-Kortum (1996). In essence, a matrix of calibration constants, relating measurement data to underlying physical parameters, is determined using a set of 'training' data. The method was found to be effective for tissue phantoms with optical properties typical of human tissues. Application of the method to tissue fluorescence, however, is constrained by the characteristics of the training data, which must include accurate values of tissue fluorophore concentrations and optical properties.

3. MEASUREMENT-METHOD BASED TECHNIQUES

A number of techniques have been proposed to selectively record fluorescence photons that have only travelled a relatively short distance through tissue. The intensity will be less affected by distortion since the photons will have experienced relatively few interactions.

A confocal detection technique was utilized by Pogue & Hasan (1996), in which the excitation light is focused on to a small area of tissue and the resultant emitted fluorescence light is detected through a pinhole sized aperture. The diameter of the pinhole controls the size of the region from which the emitted fluorescence is measured and, if the region is smaller in extent than the average mean free path for scattering, the detected intensity should not be significantly affected by tissue optical properties. Experimental work coupled with Monte Carlo modelling confirmed that the confocal technique was effective in reducing distortion. However, the technique has the intrinsic disadvantages of a small measurement region. Small regions may not be representative of the entire tissue due to local variations in fluorophore concentrations and tissue optical properties, due for example to the presence of blood vessels. It may be necessary, therefore, to carry out measurements in a number of different areas to gain a more comprehensive assessment of tissue state.

Pogue & Burke (1998) investigated optimizing fibre-optic design and suggested that if a single fibre-optic was used for the excitation and capture of fluorescence, then the smaller the fibre diameter the shorter the path length the captured light will have travelled on average through the tissue. Monte Carlo simulations indicated that for a fibre radius of less than 100 μm, the average number of elastic scattering events undergone by the detected fluorescence photons was less than one for most tissues. Experimental work on tissue phantoms confirmed that absorbance effects were significantly reduced when using a test probe comprising 30×100 μm radii fibres compared to a standard bundle. The use of multiple optical-fibres ensured a good signal to noise ratio was obtained. Diamond *et al.* (2003b) extended

the work by establishing a calibration curve to allow fluorophore concentrations to be quantified. For wavelengths in the red to near infrared region, fluorophore concentrations were recovered from tissue phantoms with an r.m.s. error of approximately 10%. A disadvantage of calibration however is the requirement of *a priori* knowledge of the range of optical properties likely to be encountered. Errors may also be incurred if a tissue phantom is used for calibration, because of the difference in the phase function of the phantom and that of the tissue.

Biswal *et al.* (2003) proposed an alternative technique of using polarized excitation light and measuring the fraction of the fluorescence and reflectance that retains the polarization. Experimental data using tissue phantoms revealed that the polarization-retaining fraction of the fluorescence was less distorted than the unpolarized fraction. Polarization is lost during collisions, so the polarized fraction will on average have undergone less scattering events than the unpolarized fraction and will therefore have travelled less distance on average through the medium. It was observed that changing the absorption and scattering coefficients had remarkably similar effects on the spectra of both the polarized fluorescence and the polarized backscattered illumination light. Consequently, correction was achieved by dividing the polarized fluorescence spectrum by the polarized reflectance spectrum. For a constant fluorophore concentration, it was found that the corrected fluorescence spectra were within 10% of each other for a range of optical parameters (μ_s'/μ_a in the range 0.32–1.2). The results were less encouraging for higher values of μ_s'/μ_a.

A disadvantage of these types of techniques is that the measured fluorescence is on average from shallower depths in comparison with standard measurement techniques. The application of these techniques may be restricted therefore to ascertaining the fluorescence from upper tissue layers only.

4. THEORY BASED TECHNIQUES

The propagation of light in turbid media can be described by the radiative transfer equation, if the wave nature of light is disregarded. There is, however, no general analytical solution to this equation for propagation of fluorescence with anisotropic scattering (Patterson & Pogue 1994). Instead, approximations to the equation, namely the modified Beer–Lambert law, the Kubelka–Munk theory and diffusion theory, have been employed as a basis for proposing a number of correction techniques. The modified Beer–Lambert law provides a reasonably good approximation for distances of up to 50 mean free paths (Welch *et al.* 1995) and can be used when absorption dominates scattering (Welch *et al.* 1987). The Kubelka–Munk and diffusion theories are suitable for use when scattering dominates absorption ($\mu_s' \gg \mu_a$) and light can be considered to be effectively isotropically scattered (Cheong *et al.* 1990; Hoffmann *et al.* 1998; Richards-Kortum & Sevick-Muraca 1996). The Kubelka–Munk theory is not applicable however when the incident radiation is collimated. Diffusion theory can be used in this situation provided

that measurements are not made close to the source (Star *et al.* 1988). More recently, an analytical model of photon migration has been developed (Wu *et al.* 1993*b*). All of these theory-based techniques assume that the optical properties of the medium are uniform. Other approximations, which may be employed, include considering the tissue to be of infinite width and depth, and/or to have a refractive index equal to that of the surrounding medium.

4.1. Modified-Beer–Lambert law

Richards-Kortum *et al.* (1989) used a one dimensional exponential attenuation model of light transport to derive the following equation relating tissue fluorescence to underlying tissue optical properties, assuming an infinitely broad, uniform beam of light illuminates the tissue:

$$F_{e,m}^{det} = kE_0 \frac{\frac{1}{2} \sum_{i=1}^{N_f} \mu_{ae}^i \Phi_m^i}{\sum_{j=1}^{N_a} \mu_{te}^j + \mu_{tm}^j}, \qquad (4.1)$$

where the sum over '*i*' is for fluorophores (N_f is the total number of fluorophores), the sum over '*j*' is for all chromophores (N_a is total number of chromophores), and k is a constant taking into account the measurement scheme and the refractive-index change at the tissue surface. Fluorophore concentrations can be determined by fitting the above equation to the measured fluorescence spectrum, with the concentration of the fluorophores and chromophores as variable parameters. It should be noted that the quantum yields and attenuation coefficients of each species present need to be known. Durkin & Richards-Kortum (1996) investigated the efficacy of the method using tissue phantoms and found that, whilst the least-squares fitting method could be used to accurately reproduce the measured spectra, the best-fit concentrations were far from accurate, being generally significantly larger than the measured concentrations and subject to a significant amount of random error. Some of the discrepancy may be attributed to problems in accurately determining fluorophore quantum yields *in vitro*. However, the method has been used successfully to develop schemes for identifying pathological conditions (Richards-Kortum *et al.* 1989; Ramanujam *et al.* 1994).

In other work, Lin *et al.* (2001) developed a technique to correct for the absorption affects associated with superficial blood on tissues, which accrues during surgical procedures and can have a significant influence on optical spectra. In essence, the technique comprises calculating the ratio of the fluorescence intensity to the *h*th power of the diffuse reflectance, where h is related to the blood absorption coefficients at the excitation and emission wavelengths. Beer's law can be used to show that this combination is independent of the quantity of blood. The application of Beer's law, however, is only valid provided the blood layer is thin ($< 100\ \mu m$) and this may only be the case when a contact fibre-optic probe is used. Nonetheless, data from experimental work together with Monte

Carlo simulations have indicated that this technique can be successfully used to compensate for the absorption effects of superficial blood.

4.2. Kubelka–Munk theory

Durkin *et al.* (1994) developed a correction technique based on a two-flux Kubelka–Munk model of light transport. The model assumes that the illumination light is diffuse and that the tissue refractive index is the same as that of the surrounding medium. Correction is attained by calculating the transfer function by using values of the Kubelka–Munk absorption and scattering coefficients. In practice, these coefficients can be obtained from diffuse reflectance and transmission spectra. Experimental work, using aortic tissue soaked in haemolysed blood, showed that intrinsic fluorescence spectra could be recovered with reasonable success, except at wavelengths where haemoglobin absorption is maximal. The method was used in conjunction with a fitting procedure to extract the fluorophore concentrations from tissue phantoms (Durkin & Richards-Kortum 1996). It was found that the calculated intrinsic spectra could be accurately reproduced, but the best-fit concentrations were significantly larger than the measured concentrations by, on average, a factor of 5.5. This factor was incorporated into the fitting procedure to improve the accuracy and, consequently, the concentrations could be recovered with an average relative error of less than 20%.

A major disadvantage associated with this technique is the need to determine transmission and reflectance data as part of the experimental procedure and this technique may therefore only be feasible *in vitro*.

4.3. Diffusion theory

4.3.1. Utilizing reflectance. Zhadin & Alfano (1998) have developed a correction method based on a one-dimensional diffusion model with isotropic scattering. Illumination is assumed to be by an infinitely wide beam, incident at a given angle to the tissue surface. Fluorescence is expressed as a function of the reflectance at the excitation wavelength and the 'darknesses' at the emission and excitation wavelengths (the darkness being given by μ_a/μ_s'). Correction of fluorescence emission or excitation spectra is achieved by dividing by a wavelength dependent correction factor, calculated from the diffuse reflectance spectrum. However, this correction factor is also a function of a parameter related to the mean free path for scattering at either the excitation wavelength (for emission spectra) or emission wavelength (for excitation spectra). This parameter cannot be readily determined in practice, so the technique may be limited to recovery of relative, rather than absolute, intensities. Overall, for darkness values in the range 0.004–0.9, the normalised corrected fluorescence emission and excitation spectra of tissue phantoms were in good agreement with the spectra obtained in the absence of the scatterers and absorbers. However, the authors stress that further testing is required for full evaluation.

A correction technique to determine the fluorescence quantum yield of an exogenous fluorophore *in vivo* has been developed by Du *et al.* (2001). The work of Del Nido *et al.* (1998) was extended to derive a quantity that is independent of fluorophore concentration and tissue optical properties. This quantity is the ratio of fluorescence intensity to $A_{e,m}R_e$, where $A_{e,m}$ is given by

$$A_{e,m} = \log\left(\frac{R_e}{R_m}\right). \qquad (4.2)$$

In the derivation, it was assumed that the scattering properties are the same at both wavelengths, which is reasonable provided the difference between the wavelengths is less than 100 nm for the visible and near infrared regions. In addition, the wavelengths must be selected such that the respective reflectances are similar in value. The technique was applied to determine the effects of intracellular calcium concentration changes on the quantum yield of Rhod-2 when loaded into mouse hearts.

4.3.2. Utilizing spatial variations of fluorescence and reflectance. A considerable amount of work has been carried out to develop relationships between the variations of fluorescence and reflected illumination light with distances away from an excitation fibre-optic. These relationships have been incorporated into correction techniques that attempt to match theoretical predictions to experimental data. Diamond *et al.* (2003*a*) derived two equations based on the theoretical considerations of Li *et al.* (1996). It was established from the second, more complex but more theoretically accurate equation, that fluorophore concentrations could be recovered with an overall r.m.s. accuracy of approximately 11% for values of μ_s' in the range 0.6–2.4 mm^{-1} and μ_{ae} in the range 0.001–0.1 mm^{-1}. Hyde *et al.* (2001) extended the technique to ascertain the variation of fluorophore concentration with depth. A numerical algorithm was developed to calculate fluorescence and reflectance for arbitrary variations of the optical properties and fluorophore concentration with depth. Excitation by a thin beam is assumed but an allowance is made for a refractive index change across the tissue–air boundary. Diamond *et al.* (2003*a*) applied the algorithm to homogenous tissue phantoms and found, for the range of optical properties given above, fluorophore concentrations were recovered with an overall r.m.s. accuracy of 8%. Stasic *et al.* (2003) utilized the algorithm for tissues phantoms where the fluorophore resided in a top or a bottom layer. Fluorophore concentrations and upper layer thicknesses were generally recovered to within 20% provided that the upper layer was shallow. The accuracy of the algorithm is limited by the ability to distinguish between the spatial distributions obtained for different sets of optical parameters.

4.4. Combined Beer–Lambert law and diffusion theory

Most theory-based techniques suffer from the assumption that the tissue is uniform in terms of its optical

properties. In general, however, tissues comprise distinct layers, each layer having different optical properties. A given light transport theory may therefore not be applicable to all layers under consideration. In recognition of this, Chang *et al.* (2004) combined the Beer–Lambert law with diffusion theory to describe the fluorescence induced in the epithelial and stromal tissue layers. The Beer–Lambert law was applied to the epithelium since this layer has a relatively low albedo and is in close proximity to the excitation source, while diffusion theory was employed for the highly scattering stroma. The model incorporates light reflection at the interface between the two layers and assumes that measurements are made with a fibre-optic probe. Using optical properties typical of cervical tissue, predictions of fluorescence were generally found to be in good agreement with Monte Carlo simulations. It was proposed that the recovery of optical parameters could be achieved by finding the set of optical parameters that provide the optimum agreement between predictions and measurements.

4.5. Photon migration theory

In 1993, Wu *et al.* (1993*a*) applied the photon migration model of light propagation in tissue to fluorescence. The model depends on ascertaining the probability of an excitation photon being absorbed by a fluorophore after 'i' scattering events and the resultant emission photon escaping the tissue after $n-(i+1)$ scattering events (ρ_{ni}; Müller *et al.* 2001). The fluorescence can be written as follows:

$$F_{e,m} = \sum_{n=1}^{\infty} \sum_{i=0}^{n-1} \omega_{ni} \rho_{ni}, \qquad (4.3)$$

where ω_{ni} gives the contributions to the fluorescence intensity from those photons. Monte Carlo simulations have shown that ρ_{ni} can be represented by an exponential function. Müller *et al.* (2001) combined expressions for reflectance and fluorescence to derive the following equation, relating measured to intrinsic fluorescence

$$f_{e,m}^{det} = \frac{F_{e,m}^{det}}{\dfrac{1}{\mu_{se}L}\left(\dfrac{R_{0e}^{det} R_{0m}^{det}}{\alpha_e \alpha_m}\right)^{1/2} \dfrac{R_e^{det}}{R_{0e}^{det}}\left(\dfrac{R_m^{det}}{R_{0m}^{det}} - \alpha_m\right)}, \quad (4.4)$$

where α is $e^\beta - 1$ and β is $S(1-g)$. S and L are constants for the given illumination-collection scheme, and subscript 0 denotes a quantity when no absorbers are present.

Although R_{0e}^{det}, R_{0m}^{det}, μ_{se} and g are unknowns, the analytical expression for the diffuse reflectance derived by Zonios *et al.* (1999) can be used to obtain values for these quantities (Zhang *et al.* 2000; Müller *et al.* 2001). The parameters S and L were determined by calibration using a tissue phantom of known optical properties. Experiments on tissue phantoms ($\mu_s \leq 100 \text{ cm}^{-1}$ and $\mu_a \leq 40 \text{ cm}^{-1}$) and minced and bulk oral cavity tissue confirmed that the intrinsic fluorescence spectra could be recovered with good accuracy (Müller *et al.* 2001).

The derived intrinsic fluorescence spectra for bulk tissue samples were found to be sufficiently similar to those of minced tissue to suggest that layer structure does not adversely affect the efficacy of the technique. Examples of the application of the technique to *in vivo* measurements are shown in figure 1. The measured spectra of human oesophageal tissue vary significantly in line-shape and magnitude between different sites and patients, the variation being attributed to differences in the haemoglobin content of the tissue. The correction technique significantly reduces this variation, and produces corrected spectra having essentially the same line-shape and similar magnitudes.

The technique, however, is expected to run into difficulty at extremely high absorption levels ($\mu_a > 3\mu_s'$), when the assumptions that fluorescence and reflectance photons take similar paths through the tissue and that the scattering phase function can be treated independently of scattering and absorption break down. Nonetheless, the method should be applicable over the wavelength range 337–700 nm. The main disadvantage of the technique is that prior knowledge of a number of optical parameters is requisite. If the analytical expression of Zonios *et al.* (1999) is employed to this end, the reflectance spectrum is needed and must be measured via a fibre-optic probe. A further disadvantage is that calibration is necessary before the technique can be applied to a given illumination-collection geometry.

4.6. The double ratio correction technique

This technique was developed by Sinaasappel & Sterenborg (1993) to evaluate the concentration of an exogenous fluorophore, and involves the measurement of fluorescence intensity at two emission wavelengths for two different excitation wavelengths. The double ratio is defined by the following equation:

$$\text{DR}(\boldsymbol{s}) = \frac{F_{e_1,m_1}^{det}(\boldsymbol{s}) F_{e_2,m_2}^{det}(\boldsymbol{s})}{F_{e_1,m_2}^{det}(\boldsymbol{s}) F_{e_2,m_1}^{det}(\boldsymbol{s})}. \qquad (4.5)$$

From general theoretical considerations, the ratio can be shown to be independent of the tissue optical properties providing that the following assumptions are valid.

(i) The fluence rate of the excitation light varies only with depth in tissue.
(ii) The penetration depth of the excitation light is much smaller than that of the fluorescence light, and therefore only fluorophores located superficially contribute.
(iii) Exogenous and endogenous fluorophores concentrations are uniform over the excitation volume.

Originally, the theory was based on illumination and detection by a fibre-optic probe. However, the theory is applicable to measurements made using an imaging system providing that the lateral spread of the excitation light is negligible and that the spatial variations in the fluorophore concentrations occur on a scale larger than the resolution of the imaging system. The double ratio can be written in terms of the
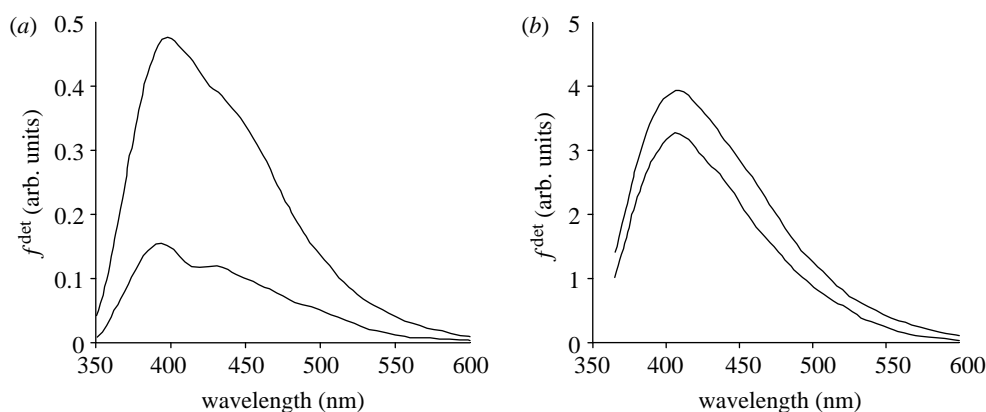
Figure 1. Two examples of the *in vivo* fluorescence spectra of human esophageal tissue, before (*a*) and after (*b*) correction using the technique of Müller *et al.* (2001). (Adapted from Müller *et al.* (2001) with permission from the Optical Society of America).

exogenous fluorophore concentration (C^{t}) as

$$\mathrm{DR}(\boldsymbol{s}) = k\frac{1 + aC^{\mathrm{t}}(\boldsymbol{s})}{1 + bC^{\mathrm{t}}(\boldsymbol{s})}, \qquad (4.6)$$

where k, a and b only depend on the optical properties of the exogenous and endogenous fluorophores present. For constant autofluorescence, the double ratio depends solely on the extrinsic fluorophore concentration. A calibration curve may be constructed for *in vitro* measurements to evaluate the absolute target fluorophore concentration. However, *in vivo* application is currently limited to determining relative concentrations. Furthermore, if the autofluorescence properties of the tissue were to vary significantly with position, accurate comparison of the double ratio from different positions would be precluded.

Bogaards *et al.* (2001) established that the double ratio is in good agreement with equation (4.6) over a wide range of optical properties (μ_{s} in the range 4–69 cm^{-1} and μ_{a} in the range 1.8–4.2 cm^{-1}) when measured using an imaging system. Sterenborg *et al.* (1996) investigated the effectiveness of the technique *in vivo*. The effect of skin pigmentation on the double ratio fluorescence of human skin was studied after the topical application of 5-aminolevulinic acid (AlA). No correlation was found between the two, indicating that the ratio is not affected by changes in tissue absorption.

A limitation of the technique is that sensitivity reduces with increasing fluorophore concentration, the double ratio tending towards an upper asymptotic value at high concentrations. Aalders *et al.* (2000) found this to be problematic when determining the distribution of a fluorescent marker for malignancies (AlA-induced protoporhyrin IX) in rat abdominal cavities. The double ratio could only be successfully used at low drug concentrations ($<5\ \mathrm{mg\ kg}^{-1}$). Furthermore, Sterenborg *et al.* (1996) surmised that the fluorophore concentration from a layered tissue would be underestimated if the optical penetration depth exceeded the thickness of the layer containing the exogenous fluorophore.

5. MONTE CARLO SIMULATION BASED TECHNIQUES

Monte Carlo simulations are becoming increasingly popular as a means of gaining an understanding of

fluorescence spectra in terms of the contributions made by a number of different fluorophores, and the effects of tissue scattering and absorption (Welch *et al.* 1997; Drezek *et al.* 2001; Liu *et al.* 2003; Swartling *et al.* 2003; Zhu *et al.* 2003; Chang *et al.* 2004). A major attraction of the simulations is that any particular tissue structure and range of optical properties can be modelled together with any illumination-collection geometry. The only downside is the long time taken to run simulations, particularly for multiple emission or excitation wavelengths. In practice, Monte Carlo simulations have been successfully used to accurately reconstruct the fluorescence spectra of tissues from measured microscopic optical properties (Zeng *et al.* 1997; Churmakov *et al.* 2003). Recently, attenuation correction techniques have been proposed which utilize information obtained either directly or indirectly from such simulations.

Gardner *et al.* (1996*b*) used Monte Carlo simulations to develop equations to model the variation of both excitation fluence rate and fluorescence escape function with depth over a wide range of optical properties. The equations are functions of the effective penetration depth and a number of free parameters correlated with diffuse reflectance. Experimental results suggest the equations are accurate providing:

(i) the tissue optical properties are uniform over the volume sampled;
(ii) the tissue sample size is large enough to ensure that no boundaries (other than the upper surface) affect the measurements (tissue dimensions should be greater than 12 penetration lengths);
(iii) the collimated incident light beam diameter is greater than four times the penetration depth.

An attenuation correction technique has been subsequently presented (Gardner *et al.* 1996*a*) which utilizes the equations to calculate the fluorescence transfer function. The technique is based on measurements of diffuse reflectance at excitation and emission wavelengths, a prerequisite being that the effective penetration depths at all wavelengths either need to be known or estimated (since they may prove difficult to measure in practice). The intrinsic fluorescence can then be recovered from predominantly absorbing to

predominantly scattering media. Experimental work with tissue phantoms has established that fluorophore concentrations can be recovered with an average standard deviation of 15% from the measured value. Determination of absolute intrinsic fluorescence, however, first requires calibration to be carried out to allow for the relative position of the detector.

Beuthan *et al.* (1996) have developed a rescaling procedure to compensate for the effects of statistical variations in optical properties between people/samples. An equation, derived from theoretical considerations, is used to establish a calibration curve of fluorescence intensity versus fluorophore concentration for a set of tissue samples of the same type. The resultant curve corresponds to that of a single tissue having optical properties and background fluorescence given by the mean of the tissue samples. Prior to rescaling, Monte Carlo simulations need to be carried out to determine the variations of fluorescence and excitation reflectance with μ_{ae}. For a given tissue sample, the fluorescence intensity is then corrected in proportion to the deviation of μ_{ae} from its average value, as determined by the deviation of the reflectance from its average value. The calibration curve can then be used to determine the corresponding fluorophore concentration. For liver tissue samples it was found that fluorescence intensities differed by less than 10% from the calibration curve after taking into account deviations in μ_{ae}. The procedure can be used with any range of optical properties and/or measurement system, and may be extended to take variations in the other optical properties into account (Minet *et al.* 2002). The need to establish a calibration curve may, however, limit the use of the procedure in practice.

Avrillier *et al.* (1998) and Tinet *et al.* (2001) applied Monte Carlo simulations directly in order to correct fluorescence spectra, employing scaling relations and fast-Fourier-transform convolutions to reduce simulation times. However, both techniques depend on knowing the optical properties of the tissue at the particular excitation and emission wavelengths. This information may prove difficult to obtain in the clinical environment, and using *in vitro* values may give rise to significant errors e.g. due to unpredictable absorption by blood. This limitation has been recently overcome by Finlay & Foster (2005), who employed a fitting procedure to extract either the optical properties from the diffuse reflectance spectrum or both the optical properties and the fluorophore concentrations directly from the fluorescence spectrum. The technique involves using a Monte Carlo simulation to construct reference tables of the absorbed energy and fluorescence escape function at various positions within the tissue and at different distances travelled by the light through the tissue. The simulations were carried out assuming a uniform medium, for a range of scattering coefficients but a fixed value of the scattering anisotropy. Linear interpolation was employed to accommodate any particular value of the scattering coefficient, while a scaling relation was used to accommodate different absorption coefficients. Application of the technique requires a model of the tissue to be developed in terms of the different scatterers and absorbers present. In addition, knowledge of the dominant fluorophores is required when fitting the fluorescence spectrum directly. It was found that the intrinsic fluorescence spectra of tissue phantoms could be accurately recovered at nearly all wavelengths with the exception, when using the reflectance spectrum, of those wavelengths where the absorbance was close to maximum (due predominantly to haemoglobin). The direct fitting of fluorescence spectra yielded better results since the measured spectrum influences the best-fit absorption and scattering coefficients as well as the best-fit fluorophore concentrations. figure 2 shows a typical example of a tissue phantom's fluorescence spectrum before and after correction using the direct fitting method. As can be seen, the corrected line-shape is in good agreement with that of the intrinsic spectrum. Overall, the relative contributions to the fluorescence from two fluorophores present and the haemoglobin concentration and oxygenation were all recovered with good accuracy.

The results, however, were less promising when the technique was applied to the fluorescence of EMT6 tumours in mice. Only a simplified tissue model was used in this particular case, however, and more accurate results might be expected if all the dominant scatterers, absorbers and fluorophores are incorporated into the model.

It is worth noting that the choice of scattering phase function may be a potential source of inaccuracy in Monte Carlo models. Kienle *et al.* (2001) and Baranoski *et al.* (2004) found that different scattering phase function could precipitate significantly different results. Care must be taken therefore to ensure that the function selected is as close an approximation as possible to that of the tissue under consideration.

6. DISCUSSION

All the correction techniques developed to date have their own advantages and disadvantages and so the choice of technique will depend on the specific application. The selection of an appropriate technique will be governed firstly by the desired information, which will generally be either absolute or relative fluorophore concentrations. The determination of absolute fluorophore concentrations is important, for example, during photodynamic therapy treatment of cancer where the effectiveness of the treatment depends on the photosensitizer concentration. Relative fluorophore concentrations encompass the relative concentrations of different fluorophores, the relative concentration of a given fluorophore in different tissue areas/organs, and the relative changes in a fluorophore concentration over time. Such information would facilitate, for example, the assessment of organ viability before and after transplantation (using NADH and FAD fluorescence) and the demarcation of cancerous regions. It is worth noting that the determination of the absolute or relative concentrations of different fluorophores is the most difficult to achieve since the correction technique must take into account the variation of the tissue optical properties with wavelength.
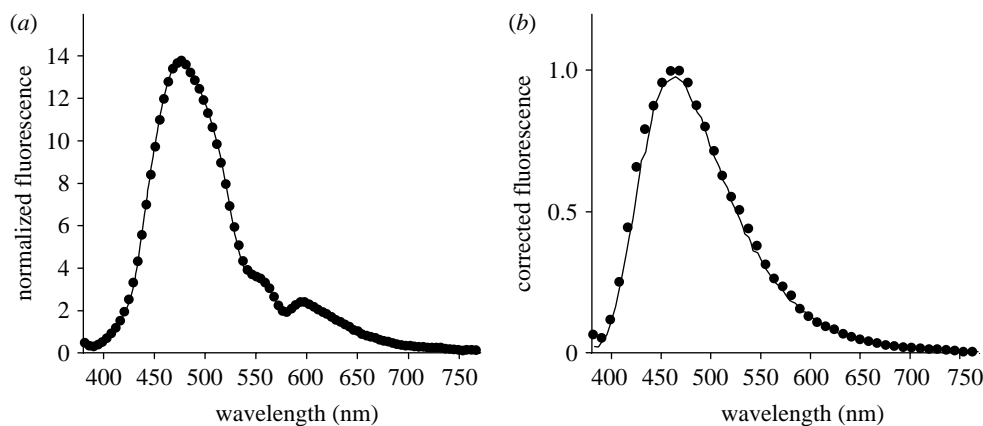
Figure 2. A typical fluorescence spectrum obtained from a tissue phantom before (*a*) and after (*b*) correction using the technique of Finlay & Foster (2005) when directly fitting the fluorescence spectrum. In (*a*), the spectrum is normalized to 1 at 650 nm and the solid line gives the best-fit spectrum of the model. In (*b*), the corrected spectrum is normalized to 1 at the peak and the solid line is the phantom's intrinsic spectrum. (Adapted from Finlay & Foster (2005) with permission from the Optical Society of America.)

Secondary factors that will influence the selection include the accuracy of the technique over the expected range of tissue optical properties and the measurement system itself. The choice of measurement system may, however, ultimately depend on the correction technique chosen. For example, techniques that are reliant upon the measurement of the spatial variation of the fluorescence necessitate systems capable of illuminating and detecting fluorescence from small areas of tissue (with a dimension typically in the range 10 μm–1 mm). It is worth noting that imaging systems (which might incorporate an endoscope for internal examination) have the distinct advantage over fibre-optic probe based systems in that the information can be simultaneously acquired over larger areas of tissue, facilitating assessment of whole organs or the detection of abnormal regions. However, a significant proportion of the correction techniques currently available are applicable to fibre-optic based measuring systems only.

Taking all of the above factors into consideration, empirical techniques are generally only suited to the measurement of relative fluorophore concentrations and, other than those employing correlation methods, they involve combining measurements in such way that the outcome is independent of changes in tissue optical properties. Most of these techniques fail to take tissue optical properties at both the excitation and emission wavelengths into account, making them unsuitable for determining fluorescence spectra or total fluorescence emission intensities. Consequently, they are generally only suited to the measurement of relative fluorophore concentrations in different tissue sites/organs or concentration changes with time. Furthermore, all these techniques fail to take tissue heterogeneity into account and caution needs to be exercised in circumstances where more than one tissue layer is responsible for the fluorescence. Notwithstanding their limitations however, reliable results are achievable over a broad range of optical properties, although the actual range cannot be determined readily without a theoretical underpinning. They also have the advantage of being simple to implement and, depending on the circumstances, this may weigh heavily in their favour.

Measurement-based techniques rely on a specific illumination–detection scheme to selectively record a relatively un-attenuated fraction of the fluorescence, and are principally suitable for determination of relative fluorophore concentrations. Although calibration would enable absolute fluorophore concentrations to be recovered, a given calibration may only be appropriate for a limited range of optical properties and hence for certain tissue types. The main disadvantage of these techniques is that the specific measurement scheme may not be readily transferred to the clinical environment and problems may be experienced with low signal to noise ratios. For the measurement of relative concentrations, the technique described by Biswal *et al.* (2003), which is reasonably accurate over a wide range of optical properties, should be given serious consideration.

Theory based techniques may be used to recover absolute as well as relative concentrations, although as a consequence they are often complicated in application. Of necessity, the theories are all based on a number of approximations, and care needs to be taken in the interpretation of results from *in vivo* applications. Moreover, the techniques are frequently limited to a specific range of optical properties and may only be suitable for a particular illumination–detection arrangement. A further difficulty is that, with the exception of the technique developed by Chang *et al.* (2004), the techniques fail to take tissue heterogeneity into account and this can clearly be problematic when more than one tissue layer is responsible for the fluorescence. Nevertheless, for absolute fluorophore concentration recovery, the techniques described by Müller *et al.* (2001) and Diamond *et al.* (2003*a*) are particularly worthy of consideration, and the double ratio technique of Sinaasappel & Sterenborg (1993) is particularly suited to the determination of changes in exogenous fluorophore concentrations. It is worth noting that whilst the technique of Müller *et al.* (2001) requires calibration for the recovery

of absolute concentrations, the calibration determines constants relating to the measurement-system alone (in contrast to the calibration necessary for measurement-based techniques), and needs to be carried out only once using, for example, a single tissue phantom.

Monte Carlo simulation based techniques have considerable potential and versatility in that they can be used to recover absolute and relative fluorophore concentrations and can accommodate any particular tissue structure and optical properties, as well as any illumination–detection geometry. Even though the techniques currently suffer from many of the same limitations as empirical and theory-based techniques, the on going development of faster techniques (e.g. Finlay & Foster 2005), to incorporate the matching of simulation to measured data would overcome most of the limitations. At the present time, the techniques are only generally applicable in situations where time is not of the essence.

In summary, even though research has been conducted into correction techniques for over thirty years, the development of a successful and practical correction technique remains a considerable challenge. An ideal technique would encompass high accuracy over a wide range of optical properties, be readily adaptable to different measurement systems, recover absolute and relative fluorophore concentrations, operate in real time and be simple to implement. Currently, progress towards this end is most likely to be made through further development of Monte Carlo simulation based techniques, since they offer the greatest prospective accuracy, constrained only by the quality of tissue model employed. Although these techniques are relatively complex and time consuming in their implementation, greater computing power should enable them to be applied in near real time in the not too distant future. In the meantime, however, it would prove profitable to direct effort towards the further development of procedures aimed at reducing simulation run times (e.g. the perturbation method of Hyaykawa *et al.* (2001) and the reverse Monte Carlo techniques of Lu & Hsu (2003)), and to their incorporation into the Monte Carlo based correction techniques.

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