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NOTE

Spectra from 2.5–15 μm of tissue phantom materials, optical clearing agents and *ex vivo* human skin: implications for depth profiling of human skin

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Abstract

Infrared measurements have been used to profile or image biological tissue, including human skin. Usually, analysis of such measurements has assumed that infrared absorption is due to water and collagen. Such an assumption may be reasonable for soft tissue, but introduction of exogenous agents into skin or the measurement of tissue phantoms has raised the question of their infrared absorption spectrum. We used Fourier transform infrared spectroscopy in attenuated total reflection mode to measure the infrared absorption spectra, in the range of 2–15 μm, of water, polyacrylamide, Intralipid, collagen gels, four hyperosmotic clearing agents (glycerol, 1,3-butylene glycol, trimethylolpropane, Topicare^{TM}), and *ex vivo* human stratum corneum and dermis. The absorption spectra of the phantom materials were similar to that of water, although additional structure was noted in the range of 6–10 μm. The absorption spectra of the clearing agents were more complex, with molecular absorption bands dominating between 6 and 12 μm. Dermis was similar to water, with collagen structure evident in the 6–10 μm range. Stratum corneum had a significantly lower absorption than dermis due to a lower content of water. These results suggest that the assumption of water-dominated absorption in the 2.5–6 μm range is valid. At longer wavelengths, clearing agent absorption spectra differ significantly from the water spectrum. This spectral information can be used in pulsed photothermal radiometry or utilized in the interpretation of reconstructions in which a constant μ_{ir} is used. In such cases, overestimating μ_{ir} will underestimate chromophore depth and vice versa, although the effect is dependent on actual chromophore depth.

1. Introduction

Infrared measurements have been used to perform non-invasive probing of biological tissue, including human skin. In pulsed photothermal radiometry (PPTR), radiometric temperature measurements of skin and tissue phantoms after laser irradiation have been used to determine the depth profile of subsurface chromophores (Milner *et al* 1995a, Sathyam *et al* 1996). A potential clinical application of PPTR is determination of port wine stain (PWS) geometry in human skin. Time-resolved radiometric temperature measurements are used to determine the initial temperature distribution immediately after pulsed laser exposure, and hence the chromophore distribution. Computational methods implementing this inverse problem have been investigated (Milner *et al* 1995b, Smithies *et al* 1998). Inversion schemes of the thermal problem rely on knowledge of the thermal and optical properties of the tissue or phantom. One parameter of the inversion algorithm is the infrared absorption coefficient, μ_{ir}, which describes the attenuation of light propagating into the tissue surface. Infrared scattering is much lower than absorption and is neglected in inversion algorithms (Milner *et al* 1995a, 1995b). The value of μ_{ir} is typically assumed to be that of water, or a combination of water and collagen. Similarly, when using phantoms, the infrared absorption spectrum of the phantom material must be known, as departure from the true μ_{ir} in the inversion algorithm may aggravate the instability of its solution.

Glycerol or other hyperosmotic clearing agents may change the μ_{ir} of tissue (Vargas *et al* 1999, 2001). Clearing agents are used to reduce optical scattering of tissue in order to increase the therapeutic laser fluence at a subsurface target. The possible change in μ_{ir} must be considered in any inversion algorithm used in conjunction with this therapy.

Infrared properties of collagen (Yannas 1972, Doyle *et al* 1975), acrylamide (Murugan *et al* 1998, Saraç *et al* 1999, Yavuz *et al* 2000) and glycerol (Schneider *et al* 1998) have been measured. *In vitro* human skin has also been measured in the wavelength range 1000–2200 nm (Troy *et al* 2001). However, a systematic study of phantom materials and clearing agents for the purpose of improving the solution of inverse problems for PPTR in biological tissue has not been performed. In this study, we use Fourier transform infrared (FTIR) spectroscopy to determine the relative absorbance in the 2.5–15 μm range of phantom materials, hyperosmotic clearing agents and *ex vivo* human skin. The phantom materials included collagen gels, polyacrylamide and Intralipid. Clearing agents included glycerol, trimethylolpropane (TMP), 1,3-butylene glycol (1,3 BG) and Topicare$^{\mathrm{TM}}$. The spectra are plotted along with the measured results of water, so that any departure from the water spectrum is emphasized. All data are available from http://omlc.ogi.edu/spectra/.

2. Materials and methods

2.1. Apparatus

The apparatus consisted of a Magna-IR 860 FTIR spectrometer (Thermo Nicolet, Madison, WI) operated in multibounce attenuated total reflection (ATR) mode. The beamsplitter in the spectrometer was made from KBr (potassium bromide) and the detector was deuterated triglycine sulfate (DTGS). The wavelength region investigated was 2.5–15 μm and is representative of that studied in PPTR analysis of human skin. The wavenumber resolution was $\Delta k = 4\,\mathrm{cm}^{-1}$ over a total spectrum of $k = 650$ to $4000\,\mathrm{cm}^{-1}$. Measurements of wavenumber ($k = 1/\lambda$) were converted to wavelength. The gels were placed directly onto the ATR cell. For liquids, a drop was placed directly onto the ATR cell and a coverslip was placed on top to prevent dehydration (figure 1). The coverslip was approximately 150 μm

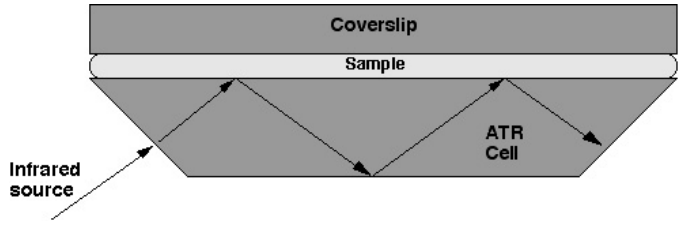

Figure 1. The infrared beam propagates through the ATR cell and reflects off the sample, creating an evanescent wave, which travels through the sample, providing a measure of infrared absorption.

thick and the liquid drop, after placing the coverslip, was 50–100 μm thick. Signal averaging was accomplished with 32 scans taken with each sample. A spectrum of deionized water was taken for comparison with the materials tested.

2.2. Materials

Polyacrylamide (29% by mass) and collagen (29% by mass) gels were prepared at much higher concentrations than normally used in PPTR experiments (17% and 3.5% by mass) to exaggerate the effect of non-aqueous chromophores. For polyacrylamide phantoms, 39.2 g of acrylamide and 0.8 g of bis-acrylamide (Sigma Chemical, St Louis, MO) were dissolved by heating in 100 ml of deionized water. TEMED (tetra-methylethylenediamine) and ammonium persulfate were used to initiate polymerization. For collagen gel, 40 g of collagen powder (Sigma Chemical, St Louis, MO) from porcine skin was dissolved in 100 ml of deionized water. The solution temperature was kept below 45 °C. Gels were set in moulds with dimensions of $10 \times 20 \times 2$ mm^3. The gel samples were placed flat onto the ATR cell and the infrared spectra were measured.

Intralipid is often used as an optical scattering component in tissue phantoms (van Staveren *et al* 1991, Flock *et al* 1992), hence, we also measured μ_{ir} of 20% Intralipid (Abbott Laboratories, Abbott Park, IL).

Four hyperosmotic preparations that are used as skin clearing agents were tested. Glycerol (Merck, Darmstadt, Germany) (chemical formula $CH_2OHCHOHCH_2OH$) was used in its pure state. 1,3-Butylene glycol (1,3-BG) (Celanese, Dallas, TX) (chemical formula $CH_3CHOHCH_2CH_2OH$) was also tested. A 50% mixture of trimethylpropane (TMP) (50% mixture) (Celanese, Dallas, TX) (chemical formula $CH_3CH_2C(CH_2OH)_3$) was prepared by dissolving TMP flakes in heated ethanol. Topicare^{TM}, a proprietary polyolprepolymer-2 of Penederm, Inc. (Foster City, CA), was used as supplied.

The infrared spectra of cryo-preserved, *ex vivo* human skin (Community Blood Center, Portland, OR) were measured. The skin was thawed in a 32 °C water bath and cut into samples approximately 2×2 cm^2 in area and 1–2 mm thick. The skin samples were carefully dried afterwards to remove excess water. The skin was spread flat onto the ATR cell. Two skin samples from different human sources were tested. Measurements were made from the epidermal and dermal sides.

3. Results

3.1. Phantom materials

Figure 2 shows infrared absorption spectra of collagen gel, Intralipid and polyacrylamide in comparison to the measured water spectrum. A close up of the data in the 6–10 μm range is shown in figure 3.

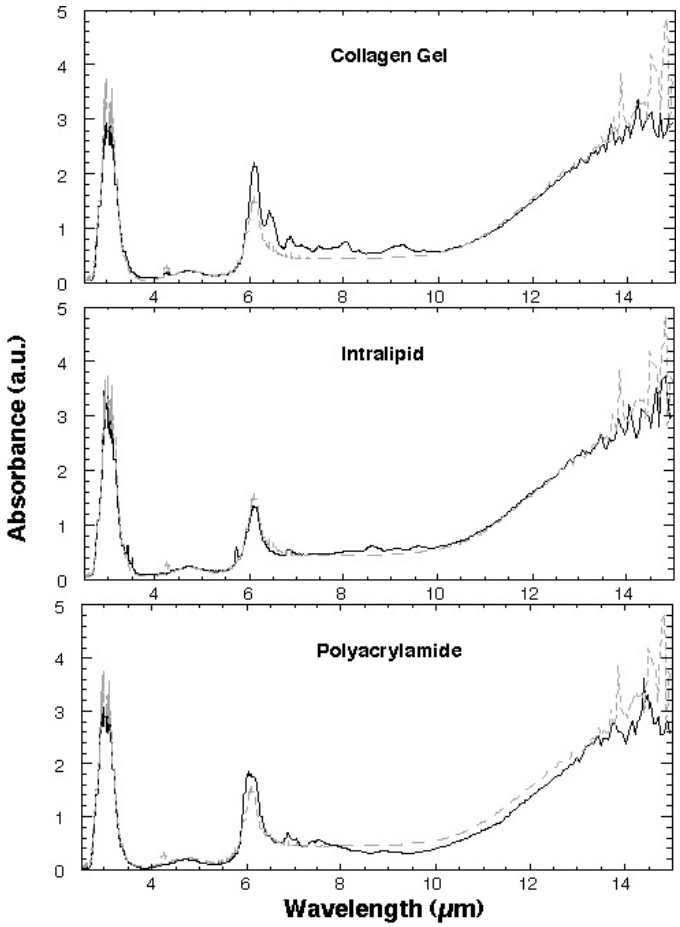

Figure 2. Absorption spectra of collagen gel, Intralipid and polyacrylamide from 2.5 to 15 μm are shown alongside the water spectrum (dashed line). In the collagen gel spectrum, additional absorption peaks are present in the 6–10 μm range.

3.2. Clearing agents

Infrared spectra of the clearing agents are shown with the water spectrum in figure 4 indicating molecular absorption bands not present in water.

3.3. Ex vivo skin

The spectra of the stratum corneum and dermis are shown in figure 5. ATR measurements were taken on the epidermal and dermal sides. The water spectrum (dashed line) is included with the dermal spectrum. The spectrum of the stratum corneum is shown without the water spectrum as the scale was lower by a factor of 4.

4. Discussion and conclusions

FTIR spectra were taken to determine the difference between μ_{ir} of the samples and that of water. The water spectrum was measured under identical conditions as the other

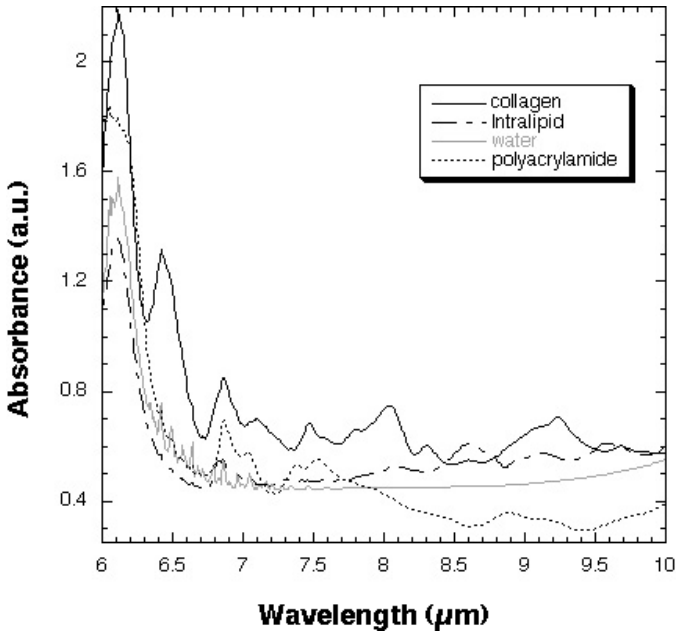

Figure 3. Spectra of the phantom materials at 6–10 μm, revealing the presence of molecular absorption bands. The bands at 6.1 μm and 6.45 μm correspond, respectively, to hydrated Amide I and Amide II. The 6.8 μm and 7.4 μm bands correspond to the C–H bend, 8.1 μm corresponds to the C–N stretch and 8.6 μm to the $(CH_3)CH$ stretch.

samples. Since all samples were measured in ATR mode, the absorbances represent relative measurements that may be compared directly to the measured water spectrum. The small peak in water at 4.2 μm shown in figure 5 is probably an artefact due to CO_2.

4.1. Phantom materials

The spectra of the phantom materials did not differ substantially from that of water, although there were additional molecular absorption bands (Ellis *et al* 1999) in the 6–10 μm range, most notably Amide I and II, N–H, and C–N in collagen, and the $(CH_3)CH$ stretch in Intralipid (figure 3). These slight differences should not substantially affect the solution of the inverse algorithms, especially noting that the present collagen and polyacrylamide concentrations (29%) were higher than those normally used in phantoms prepared for PPTR measurements (3.5% and 17%, respectively). Thus, using the infrared absorption of water in the inversion algorithms should be adequate for proper reconstruction from the PPTR signal.

4.2. Clearing agents

The clearing agents showed molecular band structure in the range of 6–12 μm (figure 4). The data indicate that inversion algorithms should account for additional infrared absorption in this range. The spectra were taken of pure or highly concentrated clearing agents; however, in a PPTR measurement of tissue, clearing agents would only constitute a small component of the tissue and would be present in a dilute state within the tissue and thus, discrepancies between the absorption spectra of the agents and water would be reduced. There have been no measurements of clearing agent concentration in skin, thus no approximation is given.

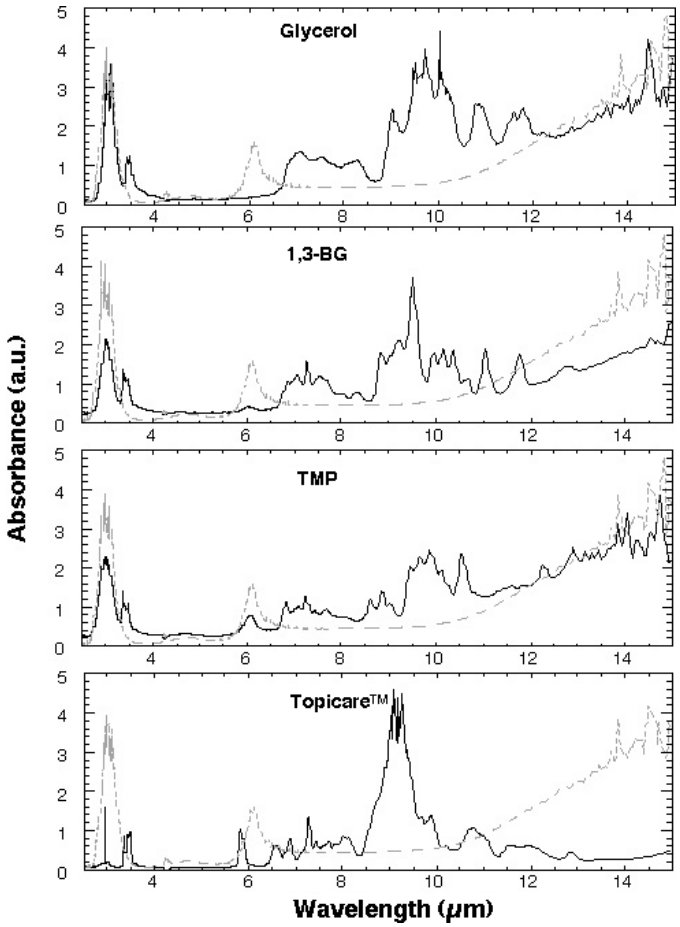

Figure 4. Spectra of the clearing agents along with water spectra (dashed line).

The mechanism of clearing is not understood either. It has been hypothesized that clearing agents reduce scattering by drawing water out of tissue; thus it is possible that there is no clearing agent within the tissue itself. Such measurements are the subject of further research.

4.3. Ex vivo skin

The dermal spectrum was nearly identical to that of water (figure 5), although the shoulder at 6.45 μm may be evidence for the presence of Amide II. The dermal spectrum is vertically offset by about 0.5 absorbance units to prevent obscuring the water signal. As mentioned above, the small peak in water at 4.2 μm is probably due to CO_2. Measurements from the epidermal side (stratum corneum) showed the absorbance to be approximately 25% of water. The absorption structure clearly shows Amide I and II peaks, typical of collagen. The lack of water is probably due to the intact stratum corneum, which comprised the bulk of skin interrogated by ATR measurement on the epidermal side. Walsh *et al* (1988) indicated that the water content of the stratum corneum was 30%. One conclusion of our analysis of figure 5 is that PPTR measurements from intact skin will have to account for infrared absorption due to these peaks, as they are different from the water spectrum. Prior to PPTR measurements,

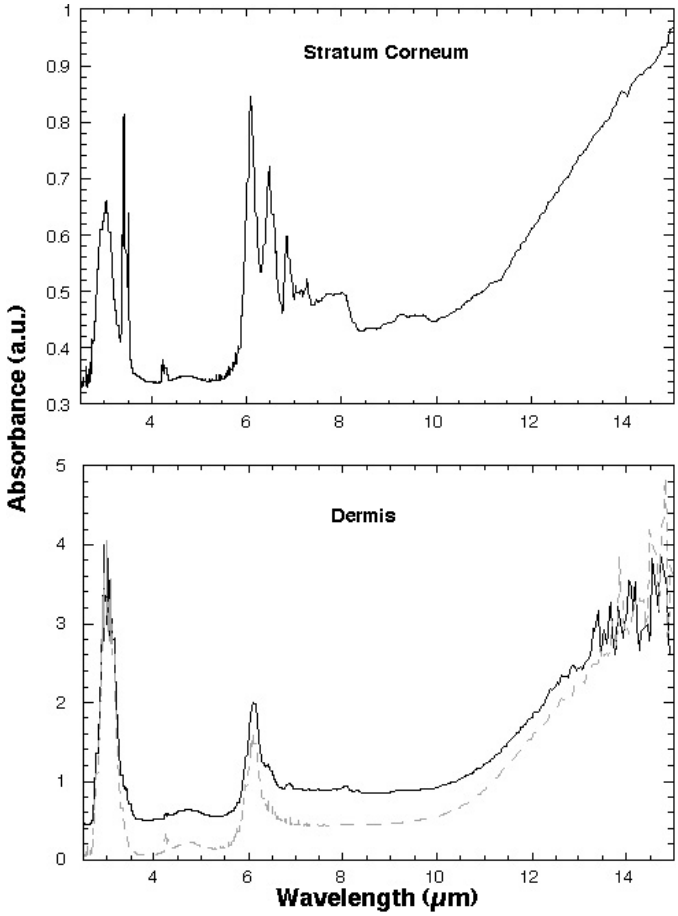

Figure 5. Spectra of human skin *ex vivo*. Reflectance measurements were taken on the epidermal and dermal sides. The water spectrum (dashed line) is included with the dermal spectrum. The spectrum of the stratum corneum is shown without the water spectrum as the scale was lower by a factor of 4.

the stratum corneum can be removed, arguably enhancing the contribution of water to infrared absorption. Further experiments are planned to investigate the effect of removing the stratum corneum on the infrared absorbance spectrum of skin.

4.4. Implication for inverse problems

As stated in the introduction, knowledge of μ_{ir} is required for solving the inverse problem of determining the initial temperature distribution in tissue after pulsed laser exposure. The theory is elegantly explained in Milner *et al* (1995b) and is summarized here. The inverse problem is based on the Green's function formulation relating the radiometric temperature measurement, alternatively called the PPTR signal, $\Delta\mathbf{S}$, the Green's function of the tissue, $\mathbf{K}$ and the initial temperature distribution, $\Delta\mathbf{T}$:

$$\Delta\mathbf{S} = \mathbf{K}\Delta\mathbf{T}. \tag{1}$$

This equation can be inverted to give

$$\mathbf{K}^{-1}\Delta\mathbf{S} = \Delta\mathbf{T}. \tag{2}$$

The inverse problem uses equation (2), although iterative schemes use equation (1). The PPTR signal at time t, $\Delta\underline{\mathbf{S}}(t)$, can be thought of as a smearing of the temperature at all depths at time, t, attenuated by μ_{ir}:

$$\Delta\underline{\mathrm{S}}(t) = C_{\mathrm{d}}\mu_{\mathrm{ir}}\int_{z=0}^{z=\infty}\Delta\mathrm{T}(z,t)\exp(-\mu_{\mathrm{ir}}\,z)\,\mathrm{d}z. \tag{3}$$

C_{d} is a proportionality constant of the infrared detection system. The integral is taken from $z = 0$ to ∞ as the problem is posed in an infinite half space, modelling a planar tissue geometry. Evaluation of the integral in equation (3) results in the Green's function used in the inverse problem:

$$\mathrm{K}(z',t) = \exp(-z^2/4Dt)\left\{\operatorname{erfc}x(u_-) + \operatorname{erfc}x(u_+) - \frac{2h}{h-\mu_{\mathrm{ir}}}[\operatorname{erfc}x(u_+) + \operatorname{erfc}x(u_1)]\right\} \tag{4}$$

where D is the thermal diffusivity, $\operatorname{erfc}x(u) = \exp(u^2)\operatorname{erfc}(u)$ is the exponential complementary error function, h is the boundary loss coefficient and u is the relationship between depth, time, D and h (Milner *et al* 1995a):

$$u_\pm = \mu_a\sqrt{Dt} \pm z/2\sqrt{Dt} \qquad u_1 = h\sqrt{Dt} + z/\sqrt{Dt} \tag{5}$$

The evaluation of equations (1) and (2) depends on μ_{ir}, which occurs in the Green's function (equation (4)).

Previously, the value for μ_{ir} has been based on the infrared absorbance spectrum of water. As we have shown, the spectra of phantom materials, clearing agents and skin depart from the spectrum of water. If the PPTR signal from such materials is collected at wavelengths where the spectrum is close or identical to that of water, such as polyacrylamide at 3–5 μm, then the μ_{ir} of water is satisfactory for use in the inversion algorithm. In the case that μ_{ir} for water differs from the phantom materials, etc, then the difference must be addressed, either before inversion, or afterward in the interpretation of the results.

Majaron *et al* (2002) showed that including the spectral variation in the reconstruction algorithm gave improved results, although the computational burden was increased. Limiting the infrared detection to a band in which μ_{ir} is constant or nearly so, such as 4.5–5 μm, gave accurate reconstruction results. However, in the case where detection is in a range where μ_{ir} varies greatly from that of water (e.g., 8–11 μm), the data given in this paper would be necessary for determining the depth of chromophores.

As shown in figures 3 and 4, when the infrared absorption of the phantom or clearing agent materials differ from that of water, it is usually an increase in absorption. For example, in the 6–12 μm range, the clearing agents show several molecular absorption bands rising above the water spectrum. Although the magnitude of the increase is diminished by the dilution of clearing agents in tissue, the increased infrared absorption will attenuate the radiometric signal within the tissue. The effect would be a decrease in the PPTR signal, resulting in an initial temperature reconstruction in which the subsurface chromophores are deeper than if the algorithm had used the actual μ_{ir}. Thus, an underestimate of μ_{ir} overestimates the chromophore depth.

Conversely, a decrease in infrared absorption would result in a larger PPTR signal, causing the reconstruction to underestimate chromophore depth. The exact effect depends on the difference between the measured infrared absorption of the tissue and the value used in the inversion algorithm. The data supplied herein allow use of the actual μ_{ir} in the inversion

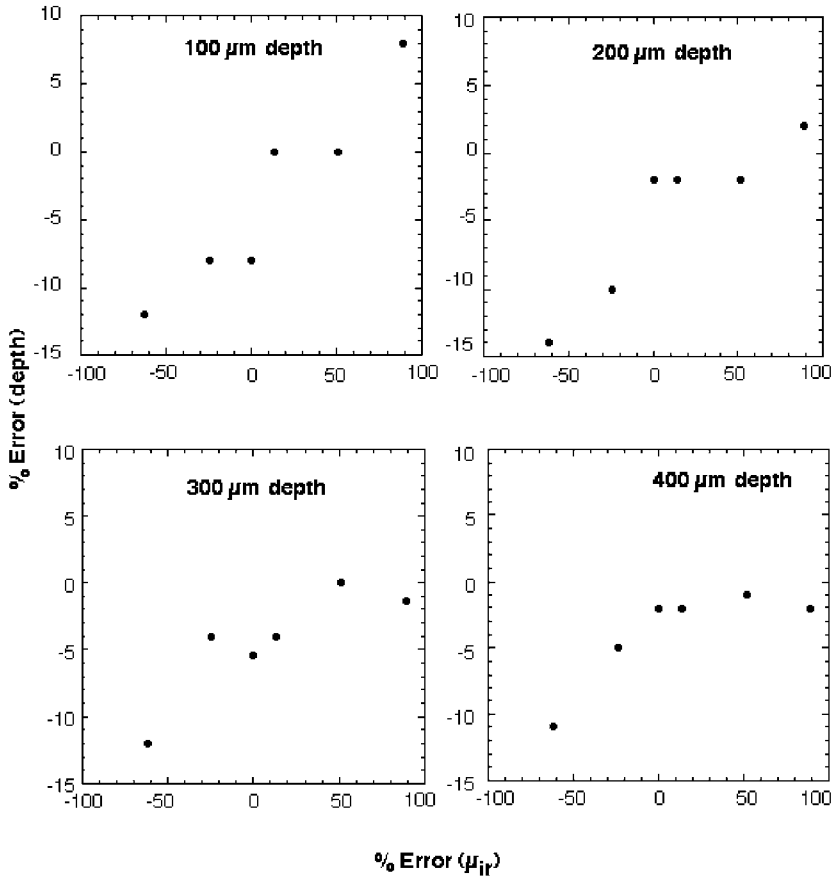

Figure 6. Error created by under- and overestimating μ_{ir} in the reconstruction algorithm. Underestimating μ_{ir} overestimates chromophore depth and vice versa, though the results are depth dependent. As all reconstructions were performed with μ_{ir} set to 265 cm^{-1}, underestimation corresponds to a positive error in μ_{ir}.

algorithm for improved reconstruction. If μ_{ir} for water is still used, knowledge of the actual infrared absorption will allow proper interpretation of the results.

We performed four simulations to demonstrate the above using a 4 μm thick chromophore buried in tissue at 100, 200, 300 and 400 μm depths. Resultant PPTR signals (1 s duration, 700 time points) were generated. The thermal diffusivity was 1.1×10^{-7} m^2 s^{-1}, similar to that of skin (Duck 1990). The infrared absorption used to compute the signals was set to 100, 200, 265, 300, 400 and 500 cm^{-1}. Reconstructions were performed using the positive-constrained, conjugate gradient scheme described in Milner *et al* (1995a, 1995b) and Smithies *et al* (1998). The infrared absorption of skin is approximately 265 cm^{-1} (Majaron *et al* 2002), hence that value was used in all reconstructions. Additionally, the reconstruction was evaluated as a 1 mm thick sample with 125 spatial points and 20 iterations of the conjugate gradient algorithm.

The results are consistent with the claim above regarding under- and overestimates of μ_{ir}, although the absolute deviations are depth dependent. Figure 6 shows the four graphs corresponding to four depths of the embedded chromophore. As μ_{ir} of the tissue is greater than 265 cm^{-1}, corresponding to a positive % error, the chromophore depth is overestimated,

as shown by a positive % error in chromophore depth. This effect changes with depth, so that with deeper chromophores underestimates of μ_{ir} give ever smaller overestimates of depth. Overestimates of μ_{ir}, however, give consistent underestimates of chromophore depth. The depth dependence of the reconstruction results indicates a complexity that may be difficult to interpret. A signal to noise ratio of 1000:1 was included in the simulations to give a more realistic interpretation. Even this low level of noise contributed to an imperfect reconstruction, as shown in figure 6. In conclusion, using the actual μ_{ir} in reconstruction gives better results in the inverse solution, albeit with greater computational burden.

Acknowledgments

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