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ORIGINAL PAPER

Spectral analysis of Delayed Luminescence from human skin as a possible non-invasive diagnostic tool

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Abstract In vivo measurements of Delayed Luminescence (DL), the low-level photo-induced emission which lasts for a longer time after switching off the excitation light, have been performed on human skin, with the aim to develop a technique for optical biopsy. Preliminary tests have been performed on healthy volunteers, measuring the time decays of the spectral components (λ_{emiss} = 400–800 nm) starting 10 μs after switching off the excitation (λ_{exc} = 337 nm). Significant differences in the decay trends of DL from different subjects were revealed and quite a good reproducibility for the same subject was observed. The modeling of experimental data has been examined in detail in order to get parameters, characterizing the theoretical fit, whose changes may be correlated with age differences and seasonal variations.

Keywords Laser induced luminescence detection · In vivo skin measurements · Aging effects

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Introduction

Recently several experimental studies (Grasso et al.1992; Van Wijk et al. 1999; Niggli 1996; Cohen and Popp 1997) demonstrated that biological systems, once illuminated, emit a very weak luminescence prolonged in time. This phenomenon has been often called Delayed Luminescence (DL).

Due to its weakness, DL requires very sensitive (single-photon) detection systems to be observed. As a matter of fact instrumentation able to measure the DL from human cell cultures with adequate sensitivity has been built up only recently (Tudisco et al. 2004).

Results obtained with this new set up are quite interesting because they show that UVA-induced DL can provide not only information about the functional state of the cultures but is also able to discriminate between cell cultures of human fibroblasts and human melanoma (Musumeci et al. 2005b). This fact proposed the DL as a possible candidate for the development of an optical biopsy technique which is able to give information on cultures of cells previously obtained from biopsy. Due to the importance of precocious diagnosis in the successful treatment of several pathologies, the extension of these results to a more direct analysis of tissues in vivo should be of great interest.

In this context skin is a suitable choice, because it has many important functions, is affected by several pathologies, and reflects the general health state of the whole organism. Moreover, it is easily accessible to optical analysis techniques.

Indeed, in the recent years, several techniques have been proposed as non-invasive tools for in vivo examination of human skin. Among them two-photon excitation microscopy (Masters et al. 1998), infrared reflectance and fluorescence spectroscopy (Lauridsen et al. 2003), excitation

fluorescence spectroscopy (Tian et al. 2001) can be cited. In some cases multicenter studies of the diagnostic parameters were conducted to determine if optical techniques could be used as a tool for a preliminary selection of patients at increased risk of disease (Chwirot et al. 2001). Anyway, up to now, a completely reliable analysis technique has not been developed yet, so we think that useful information could come by investigating the time resolved DL from skin.

In this paper we report the results of the in vivo DL measurements on human skin, performed with an apparatus obtained with a slight modification of a previously developed one (Tudisco et al. 2004). The obtained data are different for different individuals and seem to be connected with their physiological state, suggesting that DL could become a powerful analysis tool for the status of epidermis and its eventual diseases.

Materials and methods

The properties of photo-induced DL from human skin in vivo have been investigated for two samples of healthy volunteers recruited from the staff in the Department of Physical and Chemical Methodologies in Catania University. They were all Caucasian, their age ranging from 26 to 59 years, and did not present skin diseases or problems connected with the use of plasters. The following standard protocol has been observed for measurements in both groups. In particular, a region of the skin with no *naevi* has been individuated on the lower surface of the left forearm of every volunteer. Then, an adhesive tape stripping procedure (Lauridsen et al. 2003) has been repeated four times on the same skin area before every measure, in order to remove impurities and the outer layer of the *stratum corneum*. Finally, the measurement was performed in a dark room and at room temperature.

The first group (Sample #1), consisting of two men (age 26, 57) and two women (age 29, 49), was chosen with the aim to test for the effective reproducibility of in vivo skin DL measurements, because most of the studies reported in literature involve auto-fluorescence or other different spectroscopic techniques (Masters et al. 1998; Tian et al. 2001; Chwirot et al. 2001; Lauridsen et al. 2003), while DL from human skin has been poorly investigated (Cohen and Popp 1997). During each measure the time trends of four spectral components (460, 567, 645, 763 nm, respectively) of DL emission were registered. The measurement was repeated ten times on every volunteer, and average and standard error have been evaluated. Due to the fact that in literature (Cohen and Popp 1997) it is reported that DL intensities can noticeably vary from one day to another, the measurement protocol has been optimized in such a way that all the repetitions on the same individual were performed inside a maximum time interval of a few hours. Moreover, the whole group of measurements has been performed during three different periods of the year: winter (December 2005), spring (March 2006) and summer (August 2006), in order to check the behavior of DL during different seasonal periods and testing in this way its sensitivity to natural changes in the physiological state of the skin (Van Der Mei et al. 2002).

The second group (Sample #2) consisted of eight volunteers, five men (age: 26, 36, 37, 37, 58 years, respectively) and three women (age: 35, 41, 49 years, respectively), and measurements were performed with the aim to give complementary information about a possible sex-related dependence of DL on age, as reported for other skin spectroscopic analysis techniques (Lauridsen et al. 2003). In order to save time, measurements for this group were performed in summer, only for two spectral components (460 and 645 nm) and repeated only two times.

All measurements have been performed with an improved version of the ARETUSA set-up, a sensitive instrument able to detect the photo-induced DL from cells in suspension, developed at Laboratori Nazionali del Sud in Catania (Tudisco et al. 2003). The old set-up has been slightly modified in order to be able to perform DL measurements of skin tissue directly in vivo, by means of an optical fiber probe. Indeed, according to the scheme depicted in Fig. 1, the light from a high-intensity pulsed nitrogen laser (Laserphotonic LN203C, λ = 337 nm, pulse width = 5 ns, maximum energy = 100 μJ/pulse) was sent to the skin area of interest through one branch of a bifurcated fiber bundle. The head of the probe was firmly mounted on a suitable holder, at 45° incidence angle, in such a way to maintain the same geometric configuration

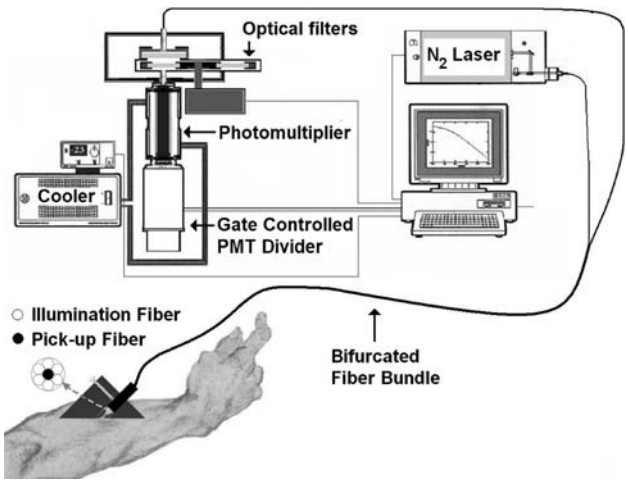

Fig. 1 Schematic view of the set-up used to measure Delayed Luminescence (DL) of skin in vivo

during different measures, with an excited area of about 25 mm^2. The photo-induced emission, coming out from the same skin area, was collected by the inner part of the same bundle and carried up through a second branch to a single photon counting photomultiplier (Hamamatsu R-7206-1, multialkali, spectral response 300–850 nm) cooled down to –30°C. A special feature of the DL set-up was the electronic control of the photomultiplier gate, which, acting as a fast shutter, delayed the start of the acquisition process with respect to the end of excitation. Thus the DL emission has been observed in a dynamic window ranging from about 10 μs to 100 ms. The whole data acquisition process was performed and controlled by a personal computer through a multi-channel scaler (Ortec MCS PCI) plug-in card. The low level of the emitted intensity did not allow detecting signals with a high spectral resolution. So the spectral analysis has been performed by a set of broadband (80 nm FWHM) Thermo-Oriel interference filters.

Raw data have been processed taking into account the sensitivity factor of the apparatus, that is the product between the optical transmittance of the filtering system and the quantum efficiency of the photomultiplier (Musumeci et al. 2005a). A smoothing procedure has been used (Scordino et al. 1996) in order to reduce random noise: experimental points have been sampled in such a way that it results $\Delta t_i/t_i = constant$.

Results and discussion

Figure 2 reports the typical spectral DL decays $I(t)$ from skin in the time interval 10 μs–10 ms. Data refers to one set of data (Sample#1, Age = 26, Month = December), that is the average values of the ten repetitions of the same measurement on the same individual in the same day. Similar pictures have been obtained for the other sets of data. The reproducibility of the measurement performed on the same volunteer for each seasonal period has been tested: percentage error on the DL intensity values $I(t)$ (average of the ten repetitions as above specified) was less than 5% at shorter time and became larger, but less than 20%, at time $t > 1$ ms.

In this work particular attention has been devoted to the curve-fitting analysis of the experimental data, from which useful information can be gathered (Musumeci et al. 2005a, b). For the sake of brevity we report the results of such analysis for the data of Fig. 2 corresponding to the emission wavelength λ_{em} = 645 nm and redrawn as markers in Fig. 3a.

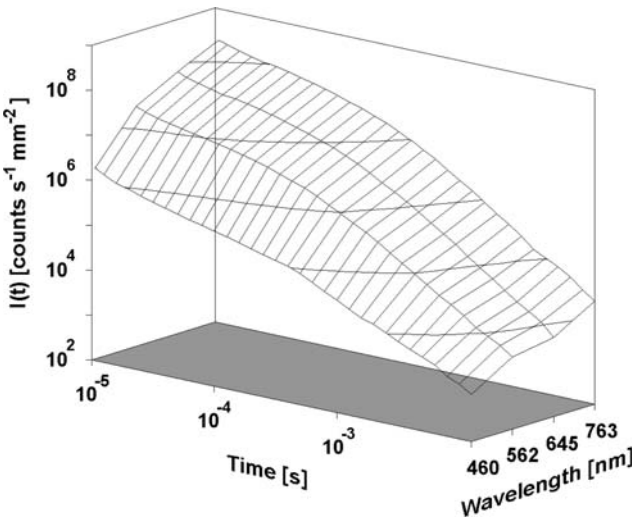

Fig. 2 Typical temporal trend of DL from skin following UVA-laser induction at 337 nm, as a function of the emission wavelength (Sample#1, Age = 26, Month = December)

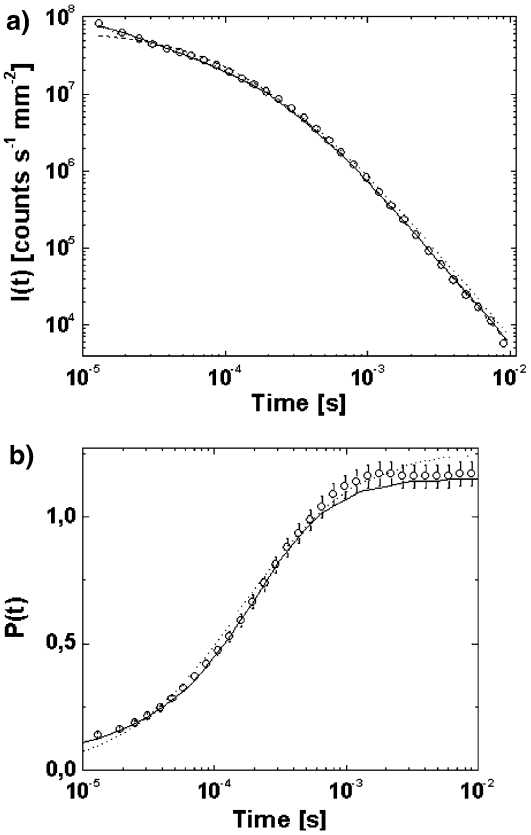

Fig. 3 Comparison between experimental data and theoretical predictions. **a** (*open circle*) DL intensity $I(t)$ for the spectral component at emission wavelength λ_{em} = 645 nm (Sample#1, Age = 26, Month = December); (*dashed line*) hyperbolic fit according to Eq. (1) (reduced $\chi^2 = 0.82$); (*dotted line*) bi-hyperbolic fit according to Eq. (2) (reduced $\chi^2 = 0.86$); (*solid line*) fit according to Eq. (4) (reduced $\chi^2 = 0.33$). **b** (*open circle*) Experimental probability of decay $P(t)$ values corresponding to DL time trend shown in Fig. 3a: (*dotted line*) theoretical $P(t)$ curve corresponding to hyperbolic fit; (*solid line*) theoretical $P(t)$ curve corresponding to Eq. (4) fit

For a great variety of biological systems (Scordino et al. 1996; Triglia et al.1998; Musumeci et al. 2005a) DL temporal trend can be modeled by a power law (hyperbolic) function as

$$I(t) = I_0/(1 + t/t_0)^m \quad (1)$$

Eq. (1) has been used to describe relaxation of complex systems (Frauenfelder et al.1999; Popp and Yan 2002).

In Fig. 3a the fit curve according to Eq. (1) is reported: it appears that this function failed to describe our data, especially at shorter times (where experimental uncertainty is lower). Neither a better accord is obtained if one considers a bimodal behavior (Librizzi et al. 2002) according to the equation

$$I(t) = I_1/(1 + t/t_1)^{m_1} + I_2/(1 + t/t_2)^{m_2} \quad (2)$$

In order to determine a more appropriate mathematical model, we consider the dimensionless function $P(t) = (-\mathrm{d}n/n)/(\mathrm{d}t/t)$ where $n(t)$ represents the total number of excited levels at time t. In our experimental condition (see Materials and methods) $P(t)$ is related to the experimental probability that Δn_i levels of the n_i excited ones decay radiatively (Musumeci et al. 2005b) and permits to evaluate how the decay probability changes in time along with the number of excited levels. Since the intensity $I(t)$ of luminescence is related to the number of excited levels $n(t)$ by the expression $I(t) = -\mathrm{d}n(t)/\mathrm{d}t$, starting from the experimental data of $I(t)$, the $P(t)$ function can be evaluated as

$$P(t) = \frac{I(t) \cdot t}{\int_t^\infty I(t)\mathrm{d}t} \quad (3)$$

In Fig. 3b the $P(t)$ curve relative to the data of Fig. 3a is reported, along with the expected theoretical trend according to Eq. (1). It appears that the trend $P_{\mathrm{th}}(t) = (m - 1)\, t/(t + t_0)$, obtained by inserting Eq. (1) into Eq. (3), is different from $P_{\mathrm{exp}}(t)$, obtained from the experimental points, which follows a slightly sharper transition and does not converge so rapidly to zero when time decreases, exhibiting a sigmoid-like trend. So, in order to accomplish this behavior, another trial model function has been considered according to the following equation:

$$I(t) = \frac{I_0}{(t/t_0)^m} \frac{t^n}{t^n + t_0^n} \quad (4)$$

in such a way that the $I(t)$ trend reduces to a simple power law not only for times $t >> t_0$ (as the hyperbolic decay does) but also for times $t << t_0$ (where a simple hyperbolic function goes toward a constant value). In fact, for $t >> t_0$ it results $I(t) \rightarrow I_0/(t/t_0)^m$, while for $t << t_0$ it is $I(t) \rightarrow I_0/(t/t_0)^{m-n}$, which accounts for a lower but non-zero decay probability of the excited states. The solid line in Fig. 3a, b shows the theoretical trend according to Eq. (4). The lowering of reduced χ^2 values, along with the decreased number of free parameters to be determined, with respect to the case of Eq. (2), encourages the use of a model curve Eq. (4). It is evident that for temporal intervals exceeding the present experimental one, the theoretical trend of $I(t)$ could be extrapolated without any physical significance.

Figure 4a shows the trend, as a function of the subject age, of the parameter m in Eq. (4) for the different spectral components. Data refers to the measurements performed in the wintertime. It appears that m values for the different components of DL emission from the same individual are different according to the different trends shown in Fig. 2, but for every component the m parameter always decreases with the age, assuming in any case values greater than 1. The parameter n shows a less evident dependence on the

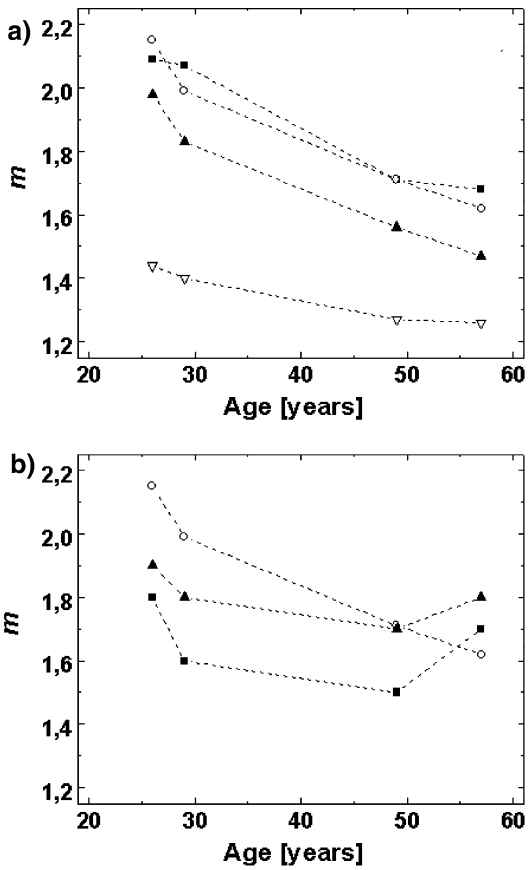

Fig. 4 The parameter m Eq. (4) as a function of the subject age. **a** Data refer to DL measurements performed in December at different emission wavelengths: (*filled square*) λ_{em} = 763 nm, (*open circle*) λ_{em} = 645 nm, (*filled triangle*) λ_{em} = 562 nm, (*open inverted triangle*) λ_{em} = 460 nm. **b** Data refer to DL measurements performed at λ_{em} = 645 nm in different period of the year: (*open circle*) December, (*filled square*) March, (*filled triangle*) August

age and in any case it results $n < m$ (data not shown). In the other periods of the year (spring and summer), when the skin pigmentation can become greatly influenced from climatic conditions, the monotonic trend appears less evident as can be observed in Fig. 4b where there are reported data at the emission wavelength 645 nm. However, it must be noted that the monotonic trend remains if we consider the data separated according to gender (see later).

In order to take into account for the complex processes underlying luminescent phenomena, it is widely accepted to describe time-resolved luminescence decays as continuous distributions of decay times or rate constants by introducing a probability density function $H(\gamma)$ such that:

$$I(t) = A\int_0^\infty H(\gamma)\ \exp(-\gamma t)\mathrm{d}\gamma \tag{5}$$

where γ denotes the rate constant and A is a normalising factor having the same units as $I(t)$.

According to Eq. (5) the distribution function $H(\gamma)$ can be determined by the inverse Laplace transform of $I(t)$. Such inversion can be easily performed if the observed data can be fitted by a function $I(t)$ that can be analytically Laplace inverted, as it occurs in the case of a pure hyperbolic decay trend (Musumeci et al. 2005a) where $H(\gamma)$ is represented by a Gamma distribution. In our case the experimental data are well accorded by Eq. (4) whose analytical Laplace inversion is not known. However it must be noted that, if we operate changes in the variables t and γ and introduce new ''scaled'' variables $\tau = t^n$ and $g = \gamma^n$, the theoretical trend $I(t)$ Eq. (4) becomes:

$$I(\tau) \ = \ I_0\frac{\tau_0^{(m/n-1)}}{\tau^{(m/n-1)}}\frac{\tau_0}{\tau+\tau_0} \tag{6}$$

that can expressed as the product of two functions whose analytical Laplace inverse transform in g-domain is known (see Appendix). So the idea was to evaluate

$$I(\tau) = I_0\int_0^\infty F(g)\exp(-g\tau)\ \mathrm{d}g \tag{7}$$

or alternatively

$$I(t) = I_0\int_0^\alpha f(\gamma)\exp[-(\gamma t)^n]\mathrm{d}\gamma \tag{8}$$

that is to consider the temporal trend Eq. (4) as a continuous distribution of stretched exponential decays. In literature the stretched exponential function has been proposed as an alternative model (Benny Lee et al. 2001; Berberan-Santos et al. 2005) to fit luminescence decay of complex systems, in particular biological samples, characterized by decay rate that, instead to be constant as it occurs in the exponential relaxation, changes in time during the decay, that is our case.

After determining $F(g)$ (see Appendix), the probability distribution function $f(\gamma)$ can be evaluated being:

$$f(\gamma)\mathrm{d}\gamma = F(g)\mathrm{d}g. \tag{9}$$

Figure 5 shows the distribution function $f(\gamma)$ for data obtained from measurements on Sample#1 in December at λ_{em} = 645 nm. It appears that the value γ_{max} corresponding to the maximum value for the $f(\gamma)$ function shifts towards slower decay rates for younger volunteers, especially if one distinguishes between women and men.

The necessity to separate the two classes of women and men before extrapolating any correlation with age appears evident in the data of the more populated Sample#2. As a matter of fact Fig. 6 shows the t_0 parameter values obtained by the fit of experimental data according to Eq. (4). Measurements have been performed in the summer period on the eight persons constituting Sample#2 at λ_{em} = 645 nm. Both for men and women this parameter shows a monotonic decrease as a function of age. Moreover the experimental points related to the men-set can be well accorded to an exponential trend (reduced χ^2 = 0.5) with a time-constant of 13 years, while the accord to an exponential trend for the data of the women-set is less significant (reduced χ^2 = 16.5).

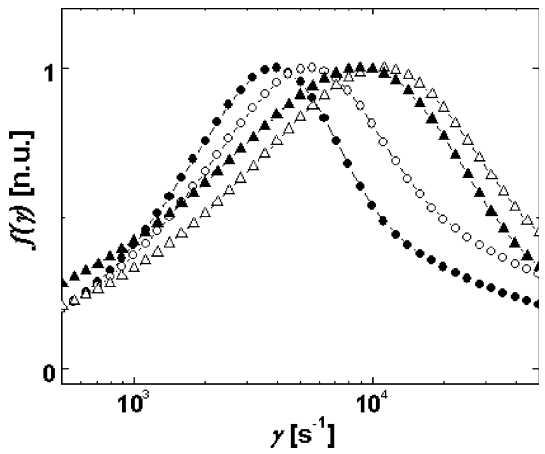

Fig. 5 Decay probability distribution function $f(\gamma)$, normalized to the maximum values, for the 645 nm emission spectral component. Data refer to DL measurements performed in December on different subjects. Age = 26 (man) (*filled circle*); age = 29 (woman) (*open circle*); age = 49 (woman) (*open triangle*); Age = 57 (man) (*filled triangle*)

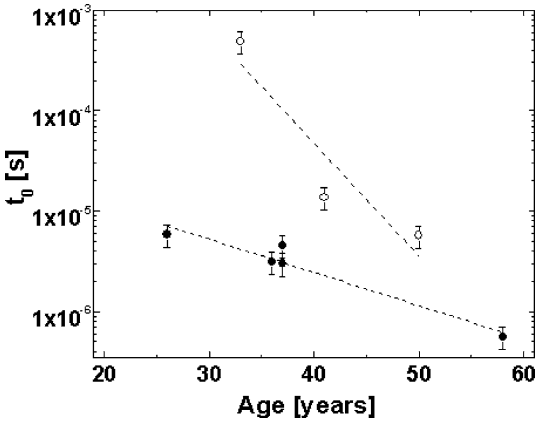

Fig. 6 Experimental values of parameter t_0 in Eq. (4) for different sex and age. *Filled circle* men, *open circle* women, *dashed line* theoretical fit to an exponential trend (reduced $\chi^2 = 0.5$ and 16.5, respectively)

Conclusions

Results reported in this paper constitute a preliminary test which permits us to assess that DL from human skin can be measured with good sensibility and reproducibility. Moreover experimental data can be modeled to a theoretical trend which represents a slight modification of the usual hyperbolic trend, and fitting parameters can be correlated to different characteristics of the sample (age of the subject, period of the year), showing in such a way the sensitivity of the technique to the physiological status of healthy skin.

Finally the significant results obtained for longer emission wavelengths open to the possibility of using the red spectral region also for the excitation, letting to penetrate deeper than ultraviolet excitation in highly scattering tissues as in vivo human skin, and to reduce damaging effects of phototoxicity and photobleaching.

Measurements on specified phototypes have been planned before extending the study to subjects who are healthy and affected by some skin pathologies in order to test the potentiality of the technique as a completely non-invasive diagnostic tool.

Appendix

Given the function

$$I(\tau) = I_0 \frac{\tau_0^{(m/n-1)}}{\tau^{(m/n-1)}} \frac{\tau_0}{\tau+\tau_0} = I_0 L_1(\tau) L_2(\tau) \tag{10}$$

in order to evaluate the inverse Laplace transform $F(g)$ such that:

$$I(\tau) = I_0 \int_0^\infty F(g)\ \exp(-g\tau)\ \mathrm{d}g \tag{11}$$

we consider

$$L_1(\tau) = \frac{\tau_0^{(m/n-1)}}{\tau^{(m/n-1)}} = \int_0^\infty F_1(g) \exp(-g\tau)\mathrm{d}g \tag{12}$$

and

$$L_2(\tau) = \frac{\tau_0}{\tau+\tau_0} = \int_0^\infty F_2(g) \exp(-g\tau)\mathrm{d}g. \tag{13}$$

Analytical Laplace transform inversion of L_1 and L_2 functions gives, respectively, (Abramowitz and Stegun 1972):

$$F_1(g) = \frac{\tau_0^{(m/n-1)}}{\Gamma\left(m/n-1\right)} g^{(m/n-2)} \quad \text{and} \quad F_2(g) = \tau_0 \exp(-\tau_0 g)$$

so that

$$\begin{aligned} F(g) &= \int_0^g F_1(x)F_2(g-x)\mathrm{d}x \\ &= \frac{\tau_0^{(m/n)}}{\Gamma\left(m/n-1\right)} \exp(-\tau_0 g) \int_0^g x^{(m/n-2)} \exp(\tau_0 x)\mathrm{d}x \end{aligned} \tag{14}$$

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