Chemometrics and Intelligent Laboratory Systems 142 (2015) 95–106

Contents lists available at ScienceDirect

Chemometrics and Intelligent Laboratory Systems

journal homepage: www.elsevier.com/locate/chemolab

# Complete parameterization of temporally and spectrally resolved laser induced fluorescence data with applications in bio-photonics

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ARTICLE INFO

*Article history:*
Received 30 July 2014
Received in revised form 19 January 2015
Accepted 23 January 2015
Available online 31 January 2015

*Keywords:*
Biophotonics
Fluorescence lifetime
Optical spectroscopy
Dynamic models
Population dynamics
Laser diagnostics

ABSTRACT

We present a set of spectrally and temporally resolved clinical fluorescence data—with two separate excitation wavelengths—that was recorded in vivo. We demonstrate that data in the spectral and temporal domains are in certain ways coupled and provide a method for integrated and effective parameterization of spectrally and temporally resolved fluorescence (i.e., time-resolved emission spectra). This parameterization is based on linear algebra, matrix formulation and system identification. We demonstrate how to empirically extract single exponentially decaying components and provide rectified emission spectra without prior knowledge. We investigate the potential for improved cancer diagnostics according to the reduced parameters along the various domains. In this case, in terms of cancer diagnostics, we were unable to identify any benefits of simultaneously measuring both the temporal and spectral properties of the observed fluorescence. However, we note that this may be explained by an important experimental bias present in many studies of optical cancer diagnostics, namely, that, in general, suspected lesions always differ visually from the neighboring healthy tissue.


## 1. Introduction

### 1.1. Outline

In this article, we first review the historical background of fluorescence spectroscopy applied to biomedicine, including the different approaches for dealing with emission quenching leading to time-resolved fluorescence spectroscopy. We then briefly review the parallel development of linear algebra and its application to population dynamics in ecology and nuclear physics before presenting an instrument capable of acquiring spectrally and temporally resolved fluorescence data as well as two *in vivo* clinical data sets recorded using this system. We note that the time-resolved fluorescence spectrometer and the clinical data sets have both been reported previously. Using these clinical data sets as an example, we evaluate the degree to which the temporal and spectral domains provide complementary information and discuss how to systematically parameterize both domains with an integrated model. This dynamic model can be further used to obtain a unique rectification of the pure fluorescence emission (decay and spectrum) from each individual transition. We demonstrate how to turn the arbitrary spectral components—typically obtained using singular value decomposition (SVD), similar to principal component analysis (PCA)—into unique, pure emissions from each transition. This could increase the inter-comparability of fluorescence data recorded in different studies or using different instruments. Our approach is entirely empirical, requires no *a priori* knowledge and offers a way to obtain the pure fluorescence components from fluorophores in their natural environment in complex biological matrices. Finally, we present the reduced parameters with respect to clinical oncological measurements.

### 1.2. Historical background

In the 1980s and 1990s, physicists started to explore new opportunities for electro-optical diagnostics in biomedicine, with the aim of enabling non-intrusive measurements and immediate evaluation. The new trends were permitted by emerging technologies including widely available personal computers for data interpretation, compact lasers at new wavelengths and charged coupled device (CCD) cameras. Varieties of both optical fiber probes (for single point measurements) and imaging systems were developed [1,2]. Several successful applications based on measurements of the dominating chromophores (such as oxy- and de-oxy-hemoglobin) were presented [3,4] and, related to these techniques, a discipline studying light transport in highly scattering media

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http://dx.doi.org/10.1016/j.chemolab.2015.01.014
0169-7439/

was established [5–12]. Biomedical fluorescence was also widely studied, including both tissue autofluorescence [1,13–18] and fluorescent sensitizers such as protoporphyrin nine, PpIX [19,20]. Since fluorescence is a secondary effect of absorption, the measured intensity of inelastic (fluorescence) photons is weaker by orders of magnitude than that of elastic (scattered) photons. As such, acquired fluorescence data are heavily perturbed by the properties of absorption and elastic scattering at both the wavelength of excitation and the wavelength of emission. For this reason, methods for deriving the intrinsic fluorescence were developed. Many such techniques required measurements of both the elastic and inelastic properties of a sample in order to then compensate for the elastic perturbations in the fluorescence signal using the theory of light transport in scattering media [21–27]. Due to its complexity, practical applications of this technique were not widespread (besides the use of the ratio of the total escape of elastic and inelastic light as an approximation of the intrinsic fluorescence [28,29]). Instead, the instrumentation was extended to include multiple fiber probes [30] or to provide spatially resolved reflectance and/or fluorescence measurements [31,32]. Alternative approaches were based on temporally resolving the fluorescence decays, as the fluorescence lifetime should be insensitive to the elastic properties of the sample. The instruments used were based on the measurement of a transient fluorescence decay following pulsed laser excitation [27,33,34], on the demodulation and phase delay observed in frequency domain methods [35–38], or on single photon counting using mode-locked lasers [39,40]. Whereas steady-state measurements of fluorescence emission were discretized in a large number of spectral bands, fluorescence decays were usually collected over a broad emission band determined simply by the sensitivity of the detector (typically a photo multiplying tube (PMT), an avalanche photodiode (APD) [34] or an intensified camera (ICCD)) [33,41]. It was quickly realized that the decay of the autofluorescence from biological tissue could not be explained using a mono-exponential model but required a sum of several exponential functions. With a good signal-to-noise ratio (SNR), it is possible to fit up to three exponential decays, each with a different amplitude and lifetime (e.g., [42]). However, using this approach, the fitted coefficients are not necessarily unique and the fitting procedure can be incorrectly biased by a number of factors including the initial fitting conditions and erroneously estimated background levels (both of which are typically chosen manually). The fact that data from single photon counting instruments gives Poisson distributed intensities further complicated fitting with, for example, least squares approaches [43]. In addition, a sum of multiple exponentials is sometimes necessary to adequately describe the measured decays even in purely synthetic substances (such as elastin or collagen) [42].

In parallel and independent of the optical community, research in control theory also experienced vast progress during the 1980s and 1990s. While the discipline was previously based on analog electronics, microprocessors and computers could now be used for sophisticated control—in particular through linear algebra—allowing real-time applications. So-called state space models [44] were developed to describe the dynamics and interaction of populations of kinetic and potential energy in robot joints. Advanced methods for creating such dynamic models from measured data were established in the discipline of system identification [45–49], and these methods spread to other communities, for example, multispecies population ecology [50–53]. Here the models could effectively describe the size of populations of interacting species in an ecosystem over time. Such models were also previously applied to describe populations of nuclear isotopes, their decay, and their interaction in terms of mother and daughter activity [54–58].

In optical spectroscopy, only a few groups have started to adopt system identification approaches, e.g., in bleaching studies [59,60]. Here the simple linear models are capable of describing complicated phenomena. For example, one chromophore bleaching out of the interrogation volume and ceasing to obscure underlying chromophores [61], or the phenomenon of a fluorophore bleaching into another fluorescent rest product with a different spectral signature [62–64]. Furthermore, the production of fluorophores or inflow of fluorophores into the interrogation volume [65,66] can also be explained by adding a simple bias to the model. To our knowledge, the progress in system identification and linear algebra based dynamic models has not yet been fully exploited in fluorescence lifetime spectroscopy. Considering absorption as electrons migrating from a ground state population to an excited state population, and fluorescence as electrons migrating from the excited state to lower lying states, it is clear that several routes would produce fluorescence where a sum of exponential decays would not accurately reflect the actual electron population sizes. In the case of one excited state having two radiative decay routes with different spectral signatures, the depletion of the excited state would be governed by the total transition rate; this could even occur in a complex biological matrix where one fluorophore pumps another fluorophore [67]. All such mechanisms can be described by a simple linear dynamic model.

In this paper, we present an effective and systematic way of completely parameterizing both spectrally and temporally resolved fluorescence, obtained using an instrument presented previously [68,69]. Our approach is based on empirical observations of the information down to the given noise limits of the system. It does not rely upon theoretical assumptions that might not be valid in a given practical situation or might be underdetermined for the given signal-to-noise conditions. Using this method, we evaluate a clinical data set (also presented previously [ref 70]) and assess the extent to which the temporal and spectral domains provide independent information. Finally, we discuss how this dynamic model can be used to obtain the pure spectral signature of each decaying transition.

## 2. Materials and methods

### 2.1. Sample set

A cohort of 25 patients participated in a study in the dermatology clinic at Lund University Hospital. In total, they had 27 body locations where lesions were suspected. At each location, both the suspected lesion and the surrounding tissue were subject to several measurements. 120 measurements of spectrally and temporally resolved fluorescence were made. For cross-validation, 45 measurements of steady-state fluorescence and reflectance spectra were also performed on the same locations using a second instrument. Each suspected lesion was visually evaluated by the dermatologist, and a subset of the lesions was subject to subsequent biopsies and histological evaluation. According to the final diagnosis, 10 locations were classified as basal cell carcinoma, 6 as squamous cell carcinoma, 4 as melanoma types, 3 as actinic keratosis, 3 as benign lesions, 1 as Kaposi's sarcoma, and 1 as pyogenic granuloma. Participating patients spanned the age 41–94 years, with the median age being 74 years. 76% were men, 88% were blue eyed Caucasians, 32% had smoked during their lifetime, 20% mentioned particular exposure to sunlight, and 20% additionally suffered from diabetes. The clinical context of the measurements, the patient distribution (as described above), and the data recorded are reported in an earlier, more clinically directed paper [69].

### 2.2. Instrument for spectrally and temporally resolved fluorescence

The apparatus for measuring spectrally and temporally resolved tissue autofluorescence *in vivo* was developed in the Photonics group of Imperial College, London. Details regarding the instrument can be found in two previous papers [68,69]. In summary, the instrument excites the tissue using either a frequency tripled Yb:Glass fiber laser at 355 nm or a diode laser at 445 nm, emitting ~10 ps and ~100 ps pulses, respectively, with 37 MHz repetition rates. Excitation light is delivered by a 200 μm non-contact optical fiber and the resulting