

## Consistent fluorescence measurements

Mikkel Brydegaard Sørensen

Applied Molecular Spectroscopy & Remote Sensing  
Atomic Physics, Lund University

Advanced Glycation Endproducts (AGE) is produced during cell division and accumulates during a human lifetime, and thus it is a measure of biological age of the individual, or a count of the total cell division since birth. Eventually during a lifetime crucial part of the DNA will be lost during the copying process, because of the continuous shortening of the DNA sequence. This is why AGE is thought to be highly correlated with a list of deceases such as Cancer, Alzheimer, Sclerosis and Diabetes. Several evidences suggests that estimating cancer risk for screening purpose can be performed using fluorescence spectroscopy, with excitations in the 300-400nm region and emissions in the 400-500nm. This could be explained by AGE fluorescing in that particular region. To measure the AGE fluorescence and thus the biological age in vivo is a challenging task for a list of reasons, and we soon find us self in a general problem in applied spectroscopy, when trying to measure fluorescence in a interrogation volume.

The problem is that reflections, absorption, scattering and fluorescence are all mutual coupled. Thus consistent measures of one property can not be made without taking all other phenomena into account. When determining an optical property in a volume such as refractive index, absorption, scattering or fluorescence, one type of measurement is usually influenced by changes in any of the remaining properties. The problem can be minimized in the laboratory by chemical cleaning and processing, but often spectroscopy is motivated by a possibility to avoid traditional chemical analysis. Further, spectroscopy is often desired for product surveillance where the sample of interest does not meet the requirements for traditional spectrometers. This motivates the elaboration of acquisition systems for simultaneous measurement of multiple optical properties.

Problems with crosstalk and mutual interference occur in almost any measurement of an optical property in a volume, such as a cuvette, a sampled tissue volume or a tube in a process plant. This is especially true when the sample composition can change arbitrary, which often is the case in industrial, medical or environmental liquids.

To illustrate this we will list a few examples which motivate that any optical property cannot be estimated correctly without measuring all other interfering properties, with ever increasing accuracy the more physical phenomena have to be taken into account.

Measurement of refractive index - This category of measurement is often used in process control to estimate salinity or sugar contents of a sample. The measurement is typically based on the Snell law for totally internal reflection or the Fresnel equations, where the reflected intensity  $I(\lambda)$  in a given angle and polarization is compared to the incident intensity  $I_0(\lambda)$ . However, the Fresnel equations are only valid for the interface between two non-absorbing media. Refractive index  $n(\lambda)$  and absorption are coupled via the Kramers-Kronig relations. Precise measurements of both parameters can be performed by ellipsometry which is often a scanning and time-consuming measurement. When involving turbid, scattering media, giving rise to diffuse reflectance or if fluorescence is involved, none of the above mentioned techniques are applicable. Since all sample volumes are finite, the specular reflection will influence light measurements based on light

entering and leaving the sample. For skin measurements it is obvious that the moisture caused by transpiration heavily influences the specular reflectance, causing the well known shiny effect.

Measurement of absorption - The measurement provides information of the chemical composition of the sample, due to the fact that different molecules or atoms might have distinct, absorption spectra within the spectral range covered by the measurement. In classical measurements of absorption, the transmitted intensity,  $I(\lambda)$ , is related to the incident intensity,  $I_0(\lambda)$ , according to the Beer-Lambert law, and determined by the product of the absorption coefficient,  $\mu_{\text{abs}}(\lambda)$ , the concentration,  $C$ , and the path length,  $x$ . Thus the transmittance,  $T(\lambda)$  is obtained as

$$T(\lambda) = I(\lambda)/I_0(\lambda) = e^{-\mu_{\text{abs}}(\lambda) \cdot C \cdot x}$$

The measurement is usually performed using the standard-addition method, by adding known concentrations of the sample in, e.g., a cuvette. This is a time consuming technique, and as we already noticed, the amount of reflection light from the first surface of the cuvette will depend on the absorption. Further, the amount of light from the second reflection, when light leaves the sample will also depend on the absorption. There are of course infinitely many reflections back and forth, with decreasing significance. It is now obvious that a given absorption measurement will change if one were to add, e.g., non-absorbing sugar to the sample volume, which would change the refraction index. This is a classical example of spectroscopic cross talk. Absorption measurements only based on transmittance,  $T$ , yield an underdetermined situation. The problem can be solved by the use of integrating spheres, where both  $T$  and  $R$  are measured. This requires the sample to be measured twice in time, or, alternatively, two integrating sphere of considerable cost can be used.

Now, by adding the light scattering process to the problem, we realize that attenuation is also changed by scattering processes which change the propagation of the light, partly preventing it from reaching the detector. Photon migration can somewhat be approximated by the diffusion equation or, better, by Monte Carlo simulations. We again find the previously mentioned methods to be underdetermined. We now need to add an additional measurement to separate data taking for collimated and non-collimated transmittance. The problem can somewhat be solved by time-of-flight spectroscopy, where the exact sample path lengths is known for each photons received. This technique involves pulsed lasers and time-resolving detector electronics. The instrumentation cost of time- or frequency-resolved spectroscopy is typically extreme and also many techniques also require considerable measurement time.

Finally, when adding fluorescence phenomena to the possible events occurring during an absorption measurements, we understand that absorbed light can now be reemitted at longer wavelengths. If neglecting this, it can cause  $T$  and  $R$  to pass 100% or result in negative concentrations according to the Beer-Lambert law. This occurs if when ordinary paper is measured with an unfortunate light source. None of the above mentioned available techniques treats this problem, since both incident and resulting light have to be dispersed in wavelength. In other words, only data on the diagonal of the emission excitation matrix (EEM; explanation) of the sample should contribute to the absorption measurement. Most techniques sum along rows or columns, neglecting fluorescence peaks not falling on the diagonal. Furthermore, photon delays in time-of-flight spectroscopy are now not only governed by path length but even by fluorescent lifetimes.

Measurement of scattering - The scattering probabilities  $\mu_{\text{sca}}(\lambda)$ , the anisotropic scattering,  $g$ , and the polarization dependence provide information of the microstructure of the sample. Typically, information on particle size, concentration and refractive index of particles is derived e.g. in diary

products. Scattering processes in the optical regime are described by Rayleigh and Mie theory. As already discussed, scattering is heavily correlated with the absorption and refractive indices discussed above. Often assumptions of constant refractive index over wavelength are applied and poorly conditioned iterative algorithms are needed to extract scattering from the measured properties. Also assumptions of the spectral dependence of the scattering are done, even though interference might cause spectral features.

Measurement of fluorescence - Acquisition of one or many fluorescence spectra in the EEM provide powerful and detailed information on the chemical composition of the sample. Often, in cases where absorption proves incapable of distinguishing substance, fluorescence is the natural next step. Even in cases where distinction cannot be performed in the EEM matrices, one might find differences in the fluorescence lifetime decay of each EEM element. EEMs are traditionally measured by two scanning monochromators on a cuvette in 90° observation configuration. Such measurements are very time consuming. Lifetimes are measured with pulsed lasers in combination with streak cameras or by frequency-domain methods. Thus only a few columns in the EEM can be measured. In general, there are never any units on a EEM measurement; this reflects the fact the measurement is influenced by all the above listed optical properties, which are not measured in a traditional EEM setup. An EEM measurement relies on excitation light at  $\lambda_1$  being transported through the sample to a fluorophore, absorbed, reemitted at  $\lambda_2$  and transported through the sample to the detector. The transport is governed by  $n(\lambda)$ ,  $\mu_{\text{abs}}(\lambda)$ ,  $\mu_{\text{sca}}(\lambda)$ ,  $g$ , and polarization effect in scattering and fluorescence for both  $\lambda_1$  and  $\lambda_2$ . Apart from difficulties in modeling we will find that we again have an underdetermined equation for estimating absolute fluorescence yields in the EEM. Besides being influenced by photon transport, EEM measurements might also depend on excitation exposure inducing bleaching and photokinetics, and, further, EEM measurements typically also depend on sample temperature,  $T_{\text{sample}}$ .

In vivo tissue fluorescence involves photon migration of the exciting light from the light source to the fluorophores and once again from the fluorophores to the detector. In skin samples the interrogation volumes can be expected to be highly heterogeneous and varying largely between individuals. Several factors such as additional fluorophores, quenchers, absorbing skin pigments, reflecting skin moisture, hairs, different interrogation volumes, skin heterogeneity, underlying scattering fat layers, blood circulation, bleaching, repeated cellular interference, mechanical deformation by the instrument, background radiation and varying temperatures of both light sources, sample and detector together ensembles a scenario way too complex for any straight forward modeling. Still a list of methods in electronics, optics, and mathematics enables both consistent measurements over longer time in changing conditions and meaningful interpretation. In the summer school several portable spectroscopic systems and geometries for simultaneous acquisition of multiple coupled optical properties will be presented. Systems are based on broad band sources such as Light Emitting Diodes (LEDs) and Xenon flashes. Geometries involve double dispersive instruments for instantaneous emission excitation matrix (EEM) spectroscopy with capability for simultaneous absorption and scattering properties by green-function analysis. A compact LED EEM and reflectance sensor and a liquid spectrometer using a novel combinatorial light paths method will be presented. Local temperatures in LED sources are accessed via electrical characteristics for long time stabilization of emissive yield, band gap and band width. Singular Value Decomposition (SVD) is used both for calibration and data compression. Multi variate modeling is used to train spectral algorithms for interpretation and estimation of biological age.