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Wavelength-converted light sources in fluorescence-based methods in medical technology

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Abstract

This contribution proposes phosphors as excitation for fluorescence analysis and evaluates their potential in this application area. Aim of this research is to provide a method which allows to quantify how well a phosphor fits as excitation in a given optical system with spectral filters for fluorescence analysis. The approach consists of a mathematical calculation of crosstalk which is first applied to abstract and subsequentially defined phosphor emission spectra. The resulting crosstalk is used as measure indicating the fit of a phosphor spectrum. Result of this contribution is a detailed description of the applied method as well as an example exercise on a given optical system which gives an impression of possibilities phosphors offer in this application. The presented method is applicable to any (new) phosphor or even LED spectra. Especially evaluations on the example optical system allow conclusions which help to design future optical systems.

Index Terms: phosphor, fluorescence analysis, spectral filter, crosstalk

1 Introduction

Quantitative real-time-PCR (Polymerase Chain Reaction), which we also know as qPCR, is a well-established method in molecular diagnostics. The goal of a qPCR test is to identify one or more DNA-sequences even if they occur in only small concentrations. This high sensitivity is achieved by an amplification of DNA-sequences in several thermal cycles. In a qPCR the concentration of the investigated DNA-sequences is measured after each cycle which is most often performed in a fluorescence analysis. Diagnosing several DNA-sequences in one sample is called multiplex-qPCR and an example is the investigation of Influenza A, Influenza B, SARS-CoV2 and human DNA as reference.

Performing a qPCR analysis in the lab or on a lab-on-chip (LoC) device has many technical facets, but we focus in this contribution on the optical readout of the fluorescence signal.

Purpose of the fluorescence analysis is to determine the concentration of DNA-sequences. All DNA-sequences under investigation are labeled by a fluorescent dye, respectively. Since all dyes absorb radiance in a unique wavelength band and emit again radiance in another unique wavelength band, the concentration of DNA-sequences correlates to the fluorescence of their corresponding dye and different DNA-sequences can be distinguished by the excitation and emission wavelengths of their dyes.

To analyze the dyes, the analyzing unit must provide radiance of different wavelength intervals which fits to the excitation wavelength bands of the dyes as well as detection capabilities in the emission wavelength bands. The excitation unit in low-cost devices usually consists of one or several LED light sources which are optionally paired with spectral filters. State-of-the-art solutions show some drawbacks as high invest in spectral filtering which are discussed in more detail in the section about state-of-the-art light sources. We propose phosphors to overcome the drawbacks of common light sources using the property of a spectrally condensed and stable emission spectrum of certain phosphors which offers the opportunity to reduce filter complexity.

The presented contribution considers an excitation setup with reduced filter complexity, deduces the spectral requirements, and evaluates a large set of phosphors on these requirements. As a result, a set of phosphors is presented which fits best to the considered setup.

The following section introduces crosstalk as measure which helps to evaluate how well an excitation spectrum fits to the considered optical setup. The state-of-the-art section presents common light sources which are used for fluorescence analysis and gives an overview of phosphors. The methods section describes the considered setup in detail, explains how crosstalk is calculated and how spectral requirements as well as a set of suitable phosphors is found. Results on the spectral requirements as well as the selection of phosphors are shown and discussed in the succeeding two sections.

2   Definition of crosstalk

In a multiplex-qPCR analysis the concentration of N different DNA-sequences is investigated. Each type of DNA is labeled with a specific fluorescent dye which allows the measurement of the fluorescence activity of the dye corresponding to the DNA-sequence concentration. Further, the N dyes for N DNA-sequences differ in their absorption and emission spectra which allows to distinguish corresponding DNA-sequences. Typical absorption and emission spectra are shown in Figure 1. To identify the concentration of each DNA-sequence via the fluorescence activity a_j of the corresponding dye, N measurements must be performed. Therefore, the sample with all N dyes is excited N times with different excitation spectra $s_{e,i}$ especially adapted to

each dye, respectively. The resulting fluorescence is detected in a single detection wavelength band $s_{d,i}$ for each of the N measurements. However, the measured intensity level m_i does not correspond exclusively to the fluorescence activity a_i of the respective dye yet. As shown in Figure 1, both, absorption spectra $d_{a,j}$ and emission spectra $d_{e,j}$ of different dyes, are overlapping each other, which means that it is not possible neither to only excite a single dye nor to measure only the fluorescence of a single dye independent on the chosen excitation and detection bands. As a result, the measured intensity level m_i is composed of the fluorescence of all dyes, excited by the excitation spectrum $s_{e,i}$, in the corresponding detection interval $s_{d,i}$ multiplied with their fluorescent activity a_j. The fluorescence of dye $j = i$ in m_i is denoted as *signal* whereas all other fractions are designated as *crosstalk* from dye $j \neq i$. The described relation can be expressed as matrix-equation

$$\begin{pmatrix} m_1 \\ \vdots \\ m_N \end{pmatrix} = C \cdot \begin{pmatrix} a_1 \\ \vdots \\ a_N \end{pmatrix} \tag{1}$$

where C is the so-called crosstalk-matrix:

$$C = \begin{pmatrix} \int_\lambda (s_{e,1} \cdot d_{a,1}) d\lambda \cdot \int_\lambda (d_{e,1} \cdot s_{d,1}) d\lambda & \cdots & \int_\lambda (s_{e,1} \cdot d_{a,N}) d\lambda \cdot \int_\lambda (d_{e,N} \cdot s_{d,1}) d\lambda \\ \vdots & \ddots & \vdots \\ \int_\lambda (s_{e,N} \cdot d_{a,1}) d\lambda \cdot \int_\lambda (d_{e,1} \cdot s_{d,N}) d\lambda & \cdots & \int_\lambda (s_{e,N} \cdot d_{a,N}) d\lambda \cdot \int_\lambda (d_{e,N} \cdot s_{d,N}) d\lambda \end{pmatrix} \tag{2}$$

Knowing the crosstalk-matrix, the fluorescence activity of the dyes can be calculated multiplying the crosstalk-correction-matrix C^{-1} and the measured intensity values array. However, due to manufacturing tolerances and degradation of chemicals and hardware, the correction with a crosstalk-matrix is never perfect. Hence, it is preferable to keep the crosstalk as small as possible, meaning that C must approach a diagonal matrix.
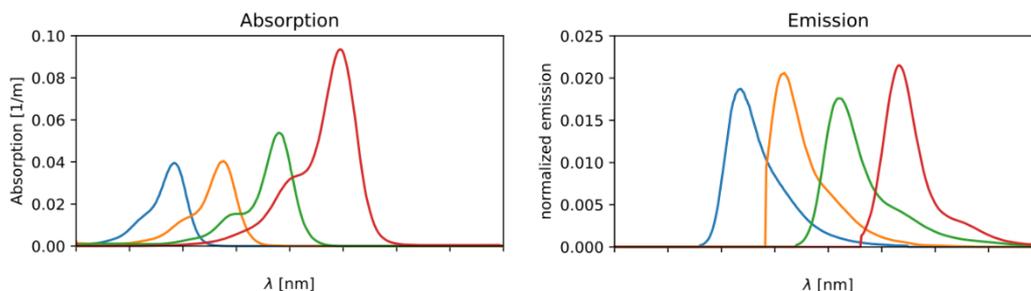

Figure 1: Absorption and emission spectra of four different dyes used for fluorescence labeling. The absorption as well as the emission spectra of the dyes are overlapping each other making it impossible neither to excite nor to measure the fluorescence of only a single dye independent on the chosen excitation and detection wavelength bands.

3   State-of-the-art

This section gives an overview on light sources which are used for fluorescence analyses, with special focus on LoC. The second subsection gives an overview on available phosphors which might be used in the application.

3.1   Light sources for fluorescence-based analyses

In the context of LoC-devices light emitting diodes (LEDs) are used as excitation source. Two different approaches exist. One solution is a combination of a phosphor-converted LED with broadband emission spectrum as known from general lighting or from automotive headlight context combined with interference filters to extract a defined excitation band. These filters are exchangeable to switch between different excitation bands. This solution provides stable radiance but goes along with comparable high invest in expensive interference filters and a mechanical filter changer. Another method is the usage of direct LEDs which are not using a phosphor. They have narrower emission bands than the broadband phosphor-converted LED. Filters are usually required but can be cheaper and be mounted in a fixed setup without moving parts. However, direct LED spectra suffer from manufacturing tolerances as well as temperature drift during operation which induces uncertainties to the measurement if not compensated otherwise. In comparison to the light sources used in lab segment, LEDs provide a low radiance level. For lab-devices higher costs are tolerated which allows to use gas discharge lamps and Lasers [1].

3.2   Overview of phosphors

Phosphors which are considered in this contribution are materials that absorb radiation at a wavelength and emit radiation at another wavelength in non-thermal equilibrium [2]. This effect is known as fluorescence and occurs in the dyes used to mark DNA-sequences in the sample as well. To avoid misinterpretations between dye and phosphor, this contribution does not consider phosphor excitation and only focuses on the phosphor emission which is used to excite dyes in the sample. The dyes themselves emit radiance at a third wavelength in a second fluorescence process.

Since there are many different dyes in fluorescence analysis, it is important that the phosphor material systems used to excite them are not only supporting the example optical setup, which is considered in section 4.1, but are also suitable to excite other commonly used dyes. Therefore, one or several phosphor material systems must be found, whose emission spectrum peak wavelengths are adjustable in the visual range of the electromagnetic spectrum, which is the relevant spectral range for excitation of fluorescent dyes. Further, a confined emission spectrum is preferred. More precise requirements are evaluated the following sections.

Interesting material systems are Quantum Dots (QDs) and inorganic LED phosphors. QDs are nanoscale semiconductor particles. QD properties differ from that of bulk materials and are strongly dependent on the size of the dots. Using that parameter,

the emission color can be adjusted. Another advantage of QDs is their small emission width as 15 – 40 nm full width at half maximum (FWHM) for CdSe QDs. [3, 4] However, QDs are not considered in subsection 4.4 due to toxicity of the best candidates [4] and nonradiative decay processes limiting their achievable radiance [5].

YAG:Ce^{3+} is one of the most used phosphors for LED and Laser white light generation. It is of type garnet, has a broad emission spectrum and allows for high power densities. [6, 7] Beside garnet type phosphors another relevant material system is given by silicates. Silicates emit a more condensed spectrum than garnets but are not able to withstand power densities as high as garnet-type phosphors [3, 8]. The emission spectrum of silicates ranges from blue over green and yellow (Orthosilicates) to red (Nitridosilicates).

To optimize the achievable power density of a phosphor, the application of the phosphor is relevant. While most vendors sell phosphor as raw powder, a suitable application in a product is the dispersion of that powder in organic or inorganic silicon [9]. A thermally more stable variant is to embed the phosphor into a glass matrix. For laser lighting, phosphor sintered as ceramic [10] or grown as single crystal is preferred. [7, 11]

4   Methods

This section starts with an introduction of the considered optical system and explains details of the applied crosstalk calculation. Further, the procedure to determine spectral requirements for phosphors is explained and the applied method of selecting the best phosphors for the considered optical system is introduced.

4.1   Considered optical system with multi bandpass filter

The considered optical system reduces filter complexity in comparison to N filters. Instead of using one filter for each dye, a single multi bandpass excitation filter is used for all dyes. Therefore, the selection of excitation spectrum is done by changing phosphors instead of changing filters. The number of investigated dyes N equals to four which leads also to four different phosphors as excitation light sources as well as four different excitation and detection wavelength bands. Figure 2 shows a schematic diagram with all components. Independent of the phosphor selection (A) the phosphor emission is filtered by the same multi bandpass excitation filter (B) before the dyes are excited according to their absorptions spectrum (C). The dyes' emission (D) passes one of the four detection filters (E) before it is measured by a spectrally broadband detector (F). For each dye, a certain phosphor and detection filter are provided which results in a coupling of phosphor and detection filter selection.

Used filter characteristics as well as the dyes' properties are chosen according to an existing and therefore already optimized analyzer system.
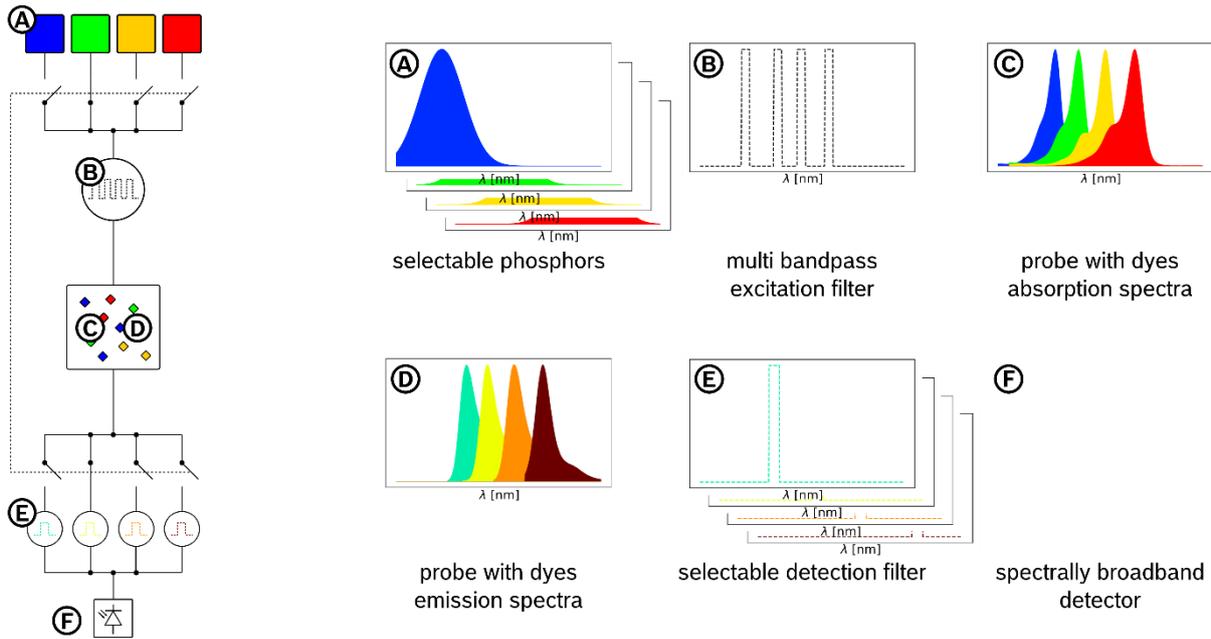
Figure 2: Schematic diagram of the considered optical setup. One of four phosphors is selected (A) whose emission is filtered by a multi bandpass excitation filter (B). Dyes in the sample absorb the excitation light (C) and emit fluorescence (D). One of four detection filters corresponding to the phosphor selection is used (E) before the signal is detected by a spectrally broadband detector (F).

4.2   Crosstalk calculation

The basics of the crosstalk-calculation are given in section 2. The present subsection goes into more detail about the crosstalk-calculation for the given optical setup from subsection 4.1 with four dyes. According to equations (1) and (2) a single measurement value is the sum of the fluorescence of all dyes in the detection wavelength band which is dependent on how well the excitation spectrum fits to the corresponding absorption spectrum of the dye:

$$m_i = \sum_j \left(\int_\lambda (s_{e,i} \cdot d_{a,j}) d\lambda \cdot a_j \cdot \int_\lambda (d_{e,j} \cdot s_{d,i}) d\lambda \right) \tag{3}$$

m_i is the measurement value of channel i. j is the index of the dye. Both, i and j, are in the range of one to four. The first integral in equation (3) represents how well excitation spectrum $s_{e,i}$ fits to the absorption spectrum of dye $d_{a,j}$. The excitation spectrum $s_{e,i}$ is, in case of the considered optical system, given by the selected phosphor emission $p_{e,i}$ filtered by the multi bandpass excitation filter $\tau_{e,mbf}$. Besides the match of excitation spectrum and absorption spectrum, the actual fluorescence a dye emits depends on the fluorescence activity a_j of that dye as well. The fluorescence activity is key parameter to identify in a fluorescence analysis since it correlates with the concentration of the corresponding DNA-sequence. In the crosstalk-calculation the fluorescence activity for all dyes is assumed to be the same and since only relative values are compared in the end, the fluorescence activity can be set to one which allows to omit this variable in further equations. The right integral in equation (3) shows

the emission spectrum of dye j $d_{e,j}$ in the detection wavelength band $s_{d,i}$. The detection wavelength band equals to the selected detection filter $\tau_{d,i}$ in the considered optical system. The spectral sensitivity of the broadband detector is assumed to be constant over the relevant wavelength range. Considering the optical system described in subsection 4.1, equation (3) can hence be re-written as

$$m_i = \sum_j \left(\int_\lambda (p_{e,i} \cdot \tau_{e,mbf} \cdot d_{a,j}) d\lambda \cdot \int_\lambda (d_{e,j} \cdot \tau_{d,i}) d\lambda \right) \qquad (4)$$

Finally, each term in the sum is a scalar, representing a relative intensity due to dye j in detection interval i excited by phosphor i. Corresponding to the nomenclature in section 2 the term j = i is denoted as signal, whereas all terms j ≠ i are considered as crosstalk of dye j. The sum of all terms j ≠ i results in the overall crosstalk. The crosstalk is usually compared to the signal as quotient of crosstalk divided by signal and most often expressed in percent.

Figure 3 selects the measurement interval i = 2 and shows corresponding spectra in the different steps of the calculation. Underneath each spectrum illustration the part of equation (4) which is used to calculate the respective spectrum is shown. Figure 4 has a closer look onto the calculated spectrum at the broadband detector before the broadband detector integrates over all wavelength to return a single value m_i. In the example the signal is given by dye j = i = 2 in yellow while crosstalk portions mainly come from the dyes j = 1 and j = 3.

4.3 Spectral requirements for phosphors using Gaussian-approximated spectra

To minimize the range of possible phosphors fitting to the considered optical setup, requirements on the width of a phosphor emission spectrum are estimated based on the method described in this subsection. All phosphors are modelled as Gaussian-approximated spectra which are defined by a peak-wavelength and an FWHM. The peak wavelengths of the four phosphors are chosen to exactly match the central intervals of the multi bandpass excitation filter. An adjustable FWHM in the range of 5 nm to 215 nm in 5 nm steps allows to estimate the crosstalk for different spectral widths. Section 5.1 shows four different graphs showing the crosstalk normed to the signal as result of this calculation.

4.4 Phosphor-screening

To represent real phosphors, their emission spectrum must be modelled in more detail. Since not all phosphors have only one peak in their emission spectrum a more general approach models the spectrum as a sum of Gaussian-approximated spectra at different peak-wavelengths and with different FWHMs as well as different intensities. Phosphors are not available for all peak-wavelengths. This unavoidably requires collecting concrete phosphor emission spectra in a database. Selected phosphors from

[12, 13, 14, 15] are included. For each entry in the database, it is possible to calculate the crosstalk as described in subsection 4.2. Having the crosstalk of each database entry the spectrally, best fitting phosphor for a specific dye equals to the database entry with the lowest overall crosstalk for the respective channel. This leads to four spectrally best fitting phosphors for the considered optical system.
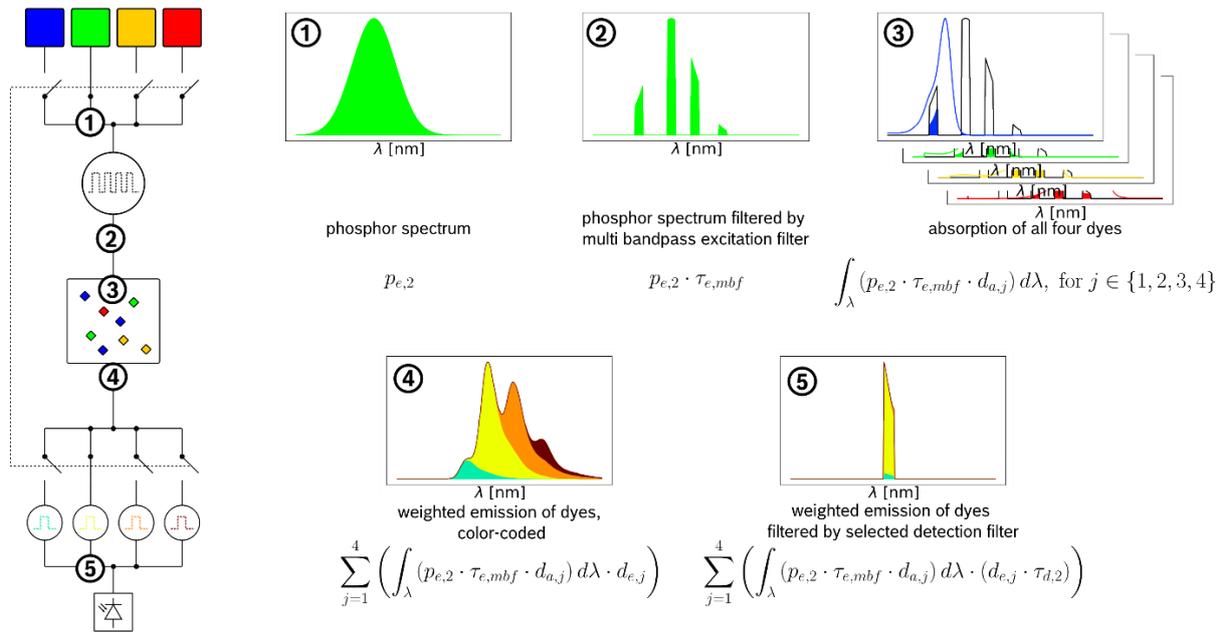

Figure 3: Different spectra which are calculated in the system and the corresponding formula for an example phosphor spectrum in interval i = 2. (1) Shows the phosphor spectrum which is filtered by a multi bandpass filter (2). In (3) the integral of the excitation spectrum from (2) with the absorption of the dye in the sample is calculated, respectively. (4) shows the combined color-coded (j = 1: green, j = 2: yellow, j = 3: orange, j = 4: brown) fluorescence of the dyes according to the chosen excitation. The result of (4) is filtered by the selected detection filter leading to the spectrum at the broadband detector (5).
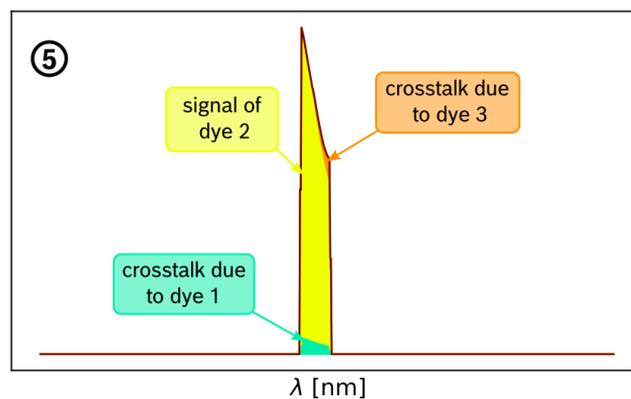

Figure 4: Detailed look on spectrum (5) from figure 3. The yellow part shows the signal from dye j = i = 2 whereas the green and orange part is crosstalk from dye j = 1 and j = 3.

5 Results

The result section first shows results of the requirement analysis which is described in subsection 4.3 and presents the selection of four spectrally best fitting phosphors determined according to subsection 4.4.

5.1 Spectral requirements for phosphors using Gaussian-approximated spectra

Evaluating Gaussian-approximated phosphor spectra leads four graphs from figure 5. Each of the graphs represents a single measurement channel i and plots the crosstalk over different spectral widths of phosphor emission spectrum i. The overall crosstalk is separated into segments whose color represents the origin of the crosstalk: A green color corresponds to dye 1, yellow to dye 2, orange to dye 3 and brown to dye 4. To compare the crosstalk between all channels, the y-axis scale is chosen to be same for all graphs.
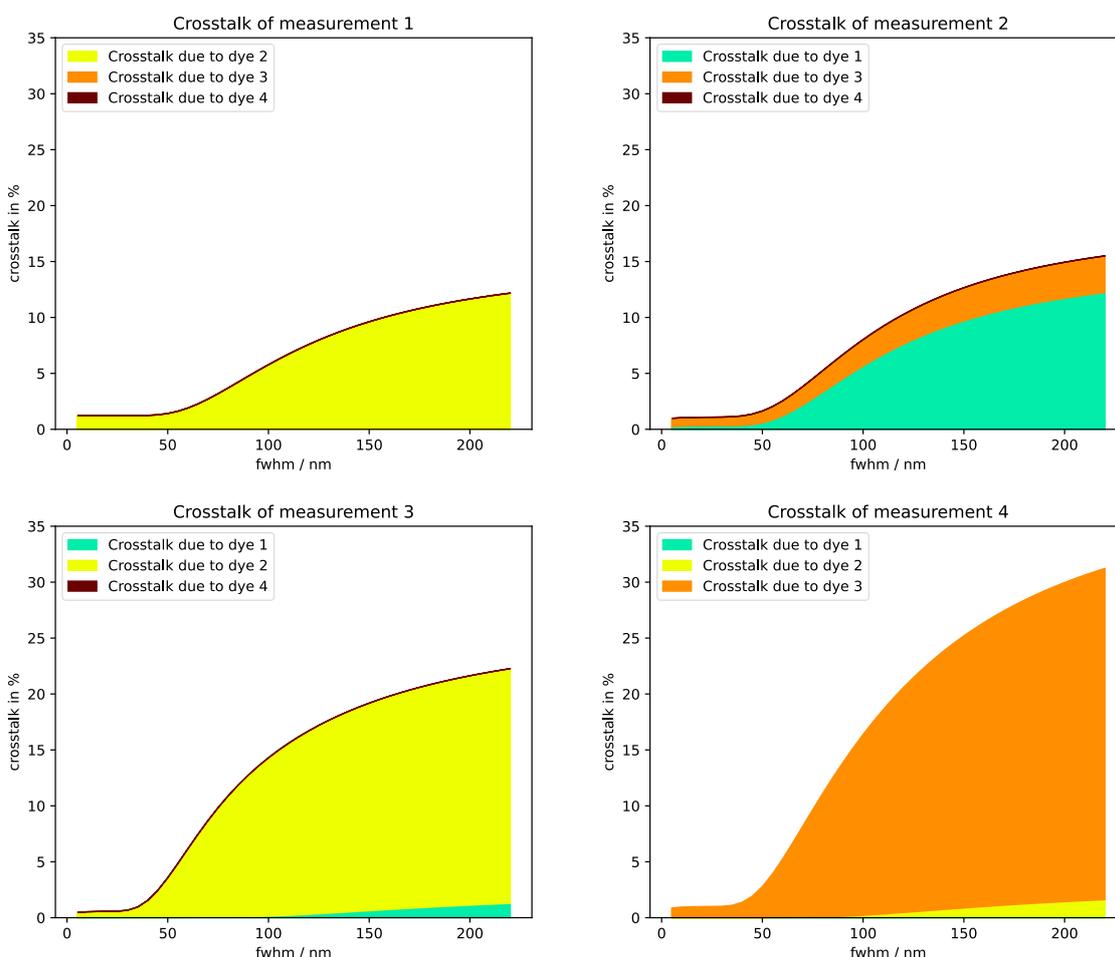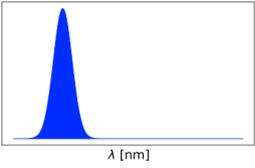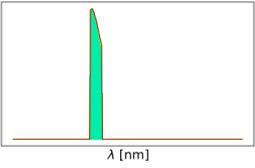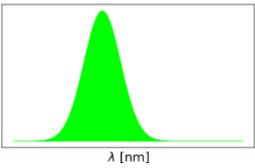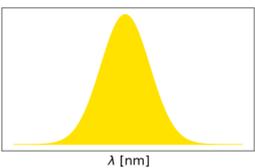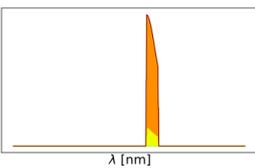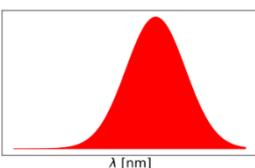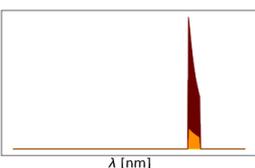

Figure 5: Results of the crosstalk calculation with Gaussian-approximated phosphor spectra. Four graphs are given, one for each measurement channel. In each graph the summed crosstalk, normed to the signal level, is displayed. The overall crosstalk is separated into distinct parts which are color-coded to visualize the dye which is the reason for a certain fraction of the overall crosstalk (j = 1: green, j = 2: yellow, j = 3: orange, j = 4: brown). All graphs have the same x- and y-scale to allow for comparability.

5.2 Phosphor-screening

Table 1 shows the spectrally best fitting phosphor to excite each dye, the corresponding emission spectrum, the spectrum at the detector and the resulting overall crosstalk, respectively.

Table 1: Results of the phosphor screening. The table shows the spectrally best fitting phosphor, corresponding emission spectrum, resulting spectrum at the detector as well as the resulting crosstalk for all dyes, respectively.

Dye	Phosphor	Phosphor emission spectrum	Spectrum at the detector	crosstalk
1	Orthosilicate A			1.23 %
2	Orthosilicate B			4.27 %
3	Orthosilicate C			15.3 %
4	Nitridosilicate			23.4 %
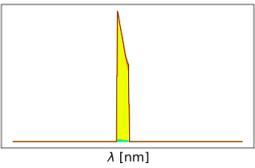
6 Discussion

As the result section, the discussion section is separated into a subsection discussing spectral requirements for phosphor emission spectra and a subsection focusing on best fitting phosphors for the considered optical system.

6.1 Spectral requirements for phosphors using Gaussian-approximated spectra

With the plots in figure 5 it is possible to estimate the requirements on spectral confinement of a phosphor emission spectrum in the proposed application. Usually, a device allows a certain crosstalk level, which is used to determine the maximum allowed FWHM of the phosphor emission spectrum. For an example overall crosstalk

threshold of 5 %, the maximum allowed FWHM of the phosphor spectra for all measurement channels i is given in figure 5 by 90 nm, 75 nm, 60 nm, and 60 nm for the measurement channels in ascending order.

The presented analysis with Gaussian-approximated spectra delivers the spectral requirements a phosphor emission spectrum must provide to be under a certain threshold value. However, it is important to be aware of the limitations this evaluation entails. Real phosphor emission spectra differ from simple Gaussian-approximated spectra. Further, it is assumed that the peak wavelength of the phosphor emission spectrum is perfectly matching the peak wavelength of the band in the excitation spectrum. To assess real phosphors, the evaluation in subsection 4.4 models phosphor emission spectra in more detail.

6.2 Phosphor-screening and outlook

For measurements in band i = 3 and i = 4, the crosstalk is above the example crosstalk-threshold of 5 %. Hence, phosphors in the current database cannot be used in the considered optical system if the crosstalk should be below the example crosstalk level.

So far, we have not identified a phosphor combination suitable for a four-channel setup with one multi bandpass excitation filter yet. In the following, some examples are presented, which adapt the optical system or parameters of the analysis to allow the use of phosphors. One possibility to decrease crosstalk is to reduce the number of channels to three or two. Comparing the plots for measurement channels 1 and 3 in figure 5, both, dye 3 and 1, are not resulting in much crosstalk for the mentioned measurement channels, respectively. Another idea is to use dyes which are covering a broader spectral range which allows to spectrally separate the bands in the multi bandpass excitation filter from each other. Also possible is the usage of two different multi bandpass filters which are both only supporting two bands, one filter band 1 and 3, the other band 2 and 4. Finally, the acceptable crosstalk level can be increased by investing more effort in calibration which allows a more detailed crosstalk correction.

The selected phosphors are silicates which are typical LED phosphors. It is possible to adapt the central emission peak of silicates which allows to find suitable phosphors also for excitation bands at other wavelengths.

A limitation in this kind of evaluation is the quality of database entries. Since phosphor emission spectra are usually only available as plots in datasheets rather than as list of spectral values, a transformation of a plot into a computer-readable format must be applied. To fit a phosphor in the database, the spectrum is approximated as described in subsection 4.4 as a manual step which is error prone. The following evaluation itself can only be as good as the database. For control, the real spectra of the best candidates, either from the supplier or measured, should be evaluated in the optical system.

7   Conclusion

Summing up, the presented contribution describes a method to calculate crosstalk depending on a phosphor emission spectrum for a given optical setup. This method is used in combination with Gaussian-approximated spectra to estimate requirements for phosphor emission in the investigated optical system. The same method is used to find a set of spectrally best fitting phosphors in a database. For the considered optical system of four phosphors with one mutual four-bandpass filter, phosphors resulting in a crosstalk level between 23 % and 1% are found. The scope of application of the presented evaluation comprises arbitrary optical systems and is not limited to only phosphor emission spectra. Hence, future decisions may benefit from the here presented method.

8   Acknowledgement

9   References

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