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Accuracy of spectrum estimate in fluorescence spectral microscopy with spectral filters

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Abstract

We establish the accuracy of the spectrum that is estimated with an inexpensive fluorescence spectral microscope utilizing a small set of spectral filters [Soriano et al, Opt. Exp. 10, 1458–1464 (2002)]. The spectrum at an arbitrary image location of the fluorescent sample is estimated as a linear superposition of basis spectra that are derived by singular value decomposition (SVD) or principal component analysis (PCA) from a spectral library of fluorescence spectra. Estimation performance is analyzed as a function of library statistics, filter selection and sequencing, minimum negativity constraint and signal to noise ratio (SNR) of fluorescence image. We consider image SNR degradations that arise from weakening of image intensity, additive Gaussian noise, intensity-dependent Poisson noise and quantization errors. The recovery of specific spectral features like spectral resolution, general similarity and peak alignments, is assessed using Linfoot's criteria of fidelity, structural content, and correlation. We found that estimation with SVD basis spectra is more robust against image noise than that with PCA basis spectra. However for high SNR images, accurate estimation is achieved more quickly with PCA basis spectra and with better response to the application of minimum negativity constraint.

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1. Introduction

The fluorescence spectral microscope (FSM) permits the rapid determination of the emission spectrum of a fluorescent sample at an arbitrary location of its magnified image. It simultaneously functions as a spectrometer and optical microscope for retrieving information about the material composition and morphology of a sample at shorter measurement times and without tedious sample preparation. The FSM is a suitable tool for determining possible relationships between form, substance, and function in microorganisms which is a core objective in biomedical research [1].

The FSM overcomes the inherent limitations of the conventional fluorescence microscope that only reveals the sample morphology, and the spectrometer that outputs only the bulk (average) spectrum of a sample. The bulk spectrum averages out possible local differences arising from inhomogeneities in the spatial distribution of the sample components [2]. Spectrometers are also cumbersome to use with low concentration samples that cannot be cultured or placed in solid matrices [3]. Most are unsuitable for samples that are smaller than 500 μm in diameter [4], and are sensitive to optical misalignments [5].

The FSM has many potential applications in biomedical research where imaging and spectroscopic analyses are still mostly done as two separate time-consuming procedures that often compromise the structural integrity of the sample. For example, from differences in emission signals the FSM can distinguish quickly cell abnormalities (e.g., chromosome arm translocations) [6], differential concentrations of ions within cells [7] cancer cells [8,9], tissue abnormalities [10], and pigment content of samples [11]. Recently, the FSM

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was employed to search quickly for new long-wavelength fluorescent proteins in tropical marine organisms [12].

FSMs are not widely used because those that employ diffraction gratings are often costly and complicated. A grating-based FSM requires special optics for handling emissions from various locations of the sample [10,13–16]. Current research is mostly focused on reducing hardware cost without seriously affecting instrumental performance in terms of resolving power and robustness against image noise.

A promising alternative approach in FSM involves the use of unique narrow-band spectral filters and a spectral library of fluorescence spectra [17]. The unknown spectrum is estimated as a linear superposition of weighted basis spectra that are derived from the library either by principal component analysis (PCA) or singular value decomposition (SVD). The expansion coefficients (weights) are determined from a set of spectrally filtered images of the sample that are recorded with a charge-coupled device (CCD) camera.

Here, we establish the accuracy of the spectrum that is estimated with an inexpensive filter-based FSM that utilizes a library of emission spectra from commercially available fluorescent probes [17]. Estimation accuracy is studied as a function of the library statistics, filter selection and sequencing, minimum negativity constraint and signal to noise ratio (SNR) of the fluorescence image. We consider image SNR degradations that arise from weakening of image intensity, additive Gaussian noise, intensity-dependent Poisson noise and quantization errors. The above factors are important experimental parameters and knowledge of their effects on estimation accuracy is vital in making the FSM into a reliable tool in biomedical analysis.

We compare the estimation performance of SVD-derived and PCA-derived basis spectra and investigate the recovery of specific features like spectral resolution, general resemblance and peak alignments between the true and the estimated spectrum using Linfoot's criteria of fidelity, structural content, and correlation [18,19]. Although the SVD and PCA have become standard techniques in many other applications in signal recovery [20], their relative merits in filter-based FSM are not yet fully established.

Filter selection is an important issue in filter-based FSM. At least for high SNR images, estimation quality improves with the number of basis functions that are used to construct the spectrum estimate. However, estimation with many basis functions also involves an equally large number of spectral filters requiring greater alignment stability between the sample and the various FSM components during image sampling. The result is longer FSM image sampling time and difficulty in dealing with motile samples and ultrafast kinetics [21].

Our presentation proceeds with a brief explanation on the use of SVD and PCA methods in FSM (Section 2). The experimental findings are presented in Section 3 and discussed in Section 4.

2. Theory

2.1. Estimation of emission spectrum in filter-based FSM

At the detection plane of the CCD camera in the FSM system, the emission spectrum $C(\lambda; x, y)$ at location (x, y) of the image of a fluorescent sample is given by:

$$C(\lambda;x,y) = E(\lambda;x,y)F(\lambda), \tag{1}$$

where $E(\lambda; x, y)$ is the true fluorescence spectrum of the sample, $F(\lambda)$ is the spectral response of the FSM, and λ is the wavelength. To determine the true spectrum $E(\lambda; x, y)$ one needs to know $C(\lambda; x, y)$ and $F(\lambda)$.

A color image is generated when the light distribution at the detector plane of the CCD camera is recorded. The color image $Q_m(x, y)$ that is obtained from the mth channel output of the CCD camera, is given by:

$$Q_m(x,y) = \int C(\lambda;x,y)S_m(\lambda)\,\mathrm{d}\lambda, \tag{2}$$

where $S_m(\lambda)$ is the spectral sensitivity for the mth camera channel, and index $m = 1,2,\ldots,M =$ number of available camera channels. The integration is taken over the visible wavelength range. The $S_m(\lambda)$ profile may be acquired from the manufacturer or via a straightforward calibration procedure [22].

We assembled a library $\{C_k(\lambda)\}$ from spectra of commercially available fluorescent probes which are widely used to label biological samples [23], where $k = 1,2,\ldots,K$. The $C_k(\lambda)$'s are smooth and their profiles are mostly unimodal with a few that are weakly bimodal (asymmetric unimodal with shoulders). Such spectral characteristics are an accurate description for many biological samples – a majority of the recorded spectra of fluorescent marine organisms or terrestrial plants is unimodal and only a few species have two peaks or secondary shoulders [24].

A set of basis spectra $\{e_i(\lambda)\}$ is derived from $\{C_k(\lambda)\}$ using SVD or PCA. The estimate $C_E(\lambda; x, y)$ of the true spectrum $C(\lambda; x, y)$ is expressed as:

$$C_E(\lambda;x,y) = \sum_{n=1}^{N} a_n(x,y)e_n(\lambda), \tag{3a}$$

$$C_E(\lambda;x,y) = \sum_{n=1}^{N} a_n(x,y)e_n(\lambda) + \langle C(\lambda)\rangle, \tag{3b}$$

where $\langle C(\lambda)\rangle$ is the mean of $\{C_k(\lambda)\}$, a_n is the coefficient for the nth basis spectrum $e_n(\lambda)$, and $N(\leqslant K)$ is the number of basis spectra used to construct $C_E(\lambda; x, y)$. Eqs. (3a) and (3b) correspond to SVD and PCA derivation, respectively.

If we assume that $C_E(\lambda; x, y) = C(\lambda; x, y)$ then Eqs. (2) and (3) can be combined to yield:

$$Q_m(x,y) = \int \sum_{n=1}^{N} a_n(x,y)e_n(\lambda)S_m(\lambda)\,\mathrm{d}\lambda, \tag{4a}$$

$$Q_m(x,y) = \int \sum_{n=1}^{N} a_n(x,y)e_n(\lambda)S_m(\lambda)\,\mathrm{d}\lambda + \int \langle C(\lambda)\rangle S_m(\lambda)\,\mathrm{d}\lambda. \tag{4b}$$