

Original Article

## Spectral Separation of Quantum Dots within Tissue Equivalent Phantom Using Linear Unmixing Methods in Multispectral Fluorescence Reflectance Imaging

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### Abstract

#### Introduction

Non-invasive Fluorescent Reflectance Imaging (FRI) is used for accessing physiological and molecular processes in biological media. The aim of this article is to separate the overlapping emission spectra of quantum dots within tissue-equivalent phantom using SVD, Jacobi SVD, and NMF methods in the FRI mode.

#### Materials and Methods

In this article, a tissue-like phantom and an optical setup in reflectance mode were developed. The algorithm of multispectral imaging method was then written in Matlab environment. The setup included the diode-pumped solid-state lasers at 479 nm, 533 nm, and 798 nm, achromatic telescopic, mirror, high pass and low pass filters, and EMCCD camera. The FRI images were acquired by a CCD camera using band pass filter centered at 600 nm and high pass max at 615 nm for the first region and high pass filter max at 810 nm for the second region. The SVD and Jacobi SVD algorithms were written in Matlab environment and compared with a Non-negative Matrix Factorization (NMF) and applied to the obtained images.

#### Results

PSNR, SNR, CNR of SVD, and NMF methods were obtained as 39 dB, 30.1 dB, and 0.7 dB, respectively. The results showed that the difference of Jacobi SVD PSNR with PSNR of NMF and modified NMF algorithm was significant ( $p < 0.0001$ ). The statistical results showed that the Jacobi SVD was more accurate than modified NMF.

#### Conclusion

In this study, the Jacobi SVD was introduced as a powerful method for obtaining the unmixed FRI images. An experimental evaluation of the algorithm will be done in the near future.

**Keywords:** Fluorescent Reflectance Imaging, Jacobi SVD, Non-Negative Matrix Factorization, Multispectral Imaging, SVD

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## 1. Introduction

Fluorescent Molecular Tomography (FMT) is a widely used modality in biomedical fields such as dermatology [1, 2] which can detect co-localized fluorophores with highly overlapping emission spectra. The overlapped emission spectra are difficult to separate using traditional, barrier-type optical filters due to the close spectral overlap [3]. The overlapped spectra are resolved by the linear decomposition algorithms, also termed spectral unmixing. The spectral unmixing methods are based on the assumption that the measured signal at each pixel is linearly proportional to concentration of individual fluorophores in the sample [4]. There are three different unmixed algorithms including Principal Components Analysis (PCA), Supervised Classification Analysis (SCA), and linear unmixing. PCA is a powerful method but its main set back in biomedical applications is its lack of sparseness [5-8]. In this sense, linear unmixing is the preferred method since the results are comparable to other methods for co-localizing fluorophores [9]. In this method, the spectrum of any pixel is a linear sum of the emitted spectra which is weighted by some factors that depend on the local concentration of each probe [10]. The weighing factors are then obtained using the QR decomposition method or singular value decomposition method (SVD) [11]. The SVD is used because the spectral matrices are not square [12]. However, SVD requires prior knowledge of the reference spectra for a given dye and needs constraints such as non-negativity of components on the fit coefficients. Recently, a blind source separation algorithm called Non-negative Matrix Factorization (NMF) was proposed for FMT using non-negativity constraints [13]. To the best of our knowledge, the SVD and NMF methods have not been compared in the same condition using FMI method. Therefore, the aim of this article is to separate the overlapping emission spectra of quantum dots within tissue-equivalent phantom using SVD, Jacobi SVD, and NMF methods in the fluorescent reflectance imaging method.

## 2. Materials and Methods

In this article, a tissue-equivalent phantom and an optical setup in reflectance mode was developed and implemented. The algorithm of multispectral imaging method was then written in Matlab environment.

The phantom was made of 6 g agarose (BioGene, Kimbolton, UK), 24 ml Intralipid 20% (Fresenius SE, Bad Homburg, Germany) and 18  $\mu$ l Indian ink (Pelikan Holding, Schindellegi, Switzerland) [14] dissolved in 600 ml of water. The optical properties of the phantom (with a radius of 10 cm) were measured with a frequency-domain tissue oximeter (oxiplexTS, ISS, Champaign, USA). The reduced scattering and absorption coefficients, at 692 nm, were then found to be  $\mu_s=8 \text{ cm}^{-1}$  and a  $\mu_a=0.11 \text{ cm}^{-1}$ , respectively. The same mixture was poured into four Plexiglas molds of 6x6 cm and thickness ranging from 2 to 4 mm. The width of the mold was more than 10 penetration depths (0.6 cm) which fulfilled the requirement for a semi-infinite boundary medium. After solidification, the agarose layers were removed from the molds and positioned by a fixed holder on the top of a solid spherical source. The sources with radius 2-6 mm contained 5 mg/l quantum dots (Plasma Chem., Germany) with emission wavelength at 610 and 710 nm [15].

The optical setup includes diode-pumped solid-state laser [DPSS] (B&W Tek, Germany) at 479 nm, 533 nm, and 798 nm, achromatic, telescopic and objective lenses (Thorlabs, USA), mirror, high pass and low pass filters (Thorlabs, USA), and EMCCD camera (Andor company, UK) (Figure 1).

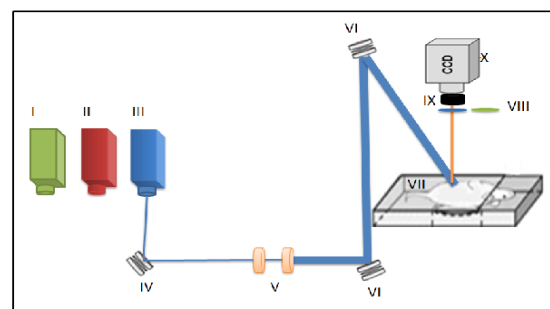


Figure 1. Schematic view of the multispectral imaging in reflection mode.

The 1 mm diameter laser beam exiting from the DPSS lasers (I, II, and III) is focused on the mirror (IV). The reflected beam was transmitted through telescopic lenses (V) and expanded to a 10 mm diameter. The expanded laser beam was reflected from a mirror (VI) to excite fluorophores in the tissue-like phantom at the selected depth. The fluorescent signal then passed through band pass filters (VIII) to reach the EMCCD camera and provide fluorescence reflectance image (FRI).

The FRI images were acquired by the CCD camera using band pass filter centered at 600 nm and a 615 nm high pass filter for first region and a 810 nm high pass filter for the second region. The Region of Interest (ROI) in the FRI images was selected and denoised using a median filter algorithm. Additionally, reference multispectral images were obtained from the Institute of Electronic Structure and Laser (Foundation of Research and Technology Hellas) for evaluating the proposed algorithm. The experimental and reference images were unmixed by singular value decomposition (SVD). The SVD was used to decompose the image matrix (W) into the product of three matrices [16]:

$$W = U\Sigma V^T \quad (1)$$

Where W, U, V, and  $\Sigma$  are  $m \times n$  matrix and a diagonal unitary matrix with non-negative elements in descending order, respectively. In order to find the SVD, the unitary matrix  $\Sigma$  is first reduced to a bidiagonal matrix by Householder transformation and the SVD of the bidiagonal matrix was then computed. Finally, the inverse of W matrix was obtained [16]:

$$W^{-1} = V\Sigma^+U^T \quad (2)$$

The inversed matrix was multiplied to the main image matrix for obtaining the unmixed images.

Additionally, we used Jacobi SVD for providing the unmixed images. The Jacobi SVD algorithm is an iterative method for the calculation of the Eigen values and Eigen vectors of a real symmetric matrix.

The SVD and Jacobi SVD algorithms were compared with a Non-negative Matrix Factorization (NMF). It factorizes a non-negative multivariable matrix V into two non-negative matrices W ( $n \times r$ ) and H ( $r \times m$ ) [16]:

$$V = WH \quad (3)$$

Where r is smaller than n and m. The factorization uses an iterative method starting with random initial values for W and H. The iteration continued until cost function is minimized. However, NMF is an iterative algorithm which may provide a local optimum solution and unmixed even when no prior information of spectra of the fluorophores was available [17].

The SVD, Jacobi SVD, and NMF algorithms were written in MATLAB environment. The algorithms were applied to the same ROI in the obtained images. The performance of the algorithms was compared by calculating the average of Peak Signal to Noise Ratio (PSNR), Root Mean Square (RMS), Signal to Noise Ratio (SNR), and Contrast to Noise Ratio (CNR).

### 3. Results

After implementation of the optical setup, the point spread function and the resolution of the imaging system was retrieved as 1.8 mm and less than 400  $\mu\text{m}$ , respectively. The experimental setup was used for taking the multispectral images in the spectral ranges of 550 to 780 nm and more than 780 nm (Figure 2). The reference images were also used for confirming the accuracy of the proposed algorithm (Figure 3).

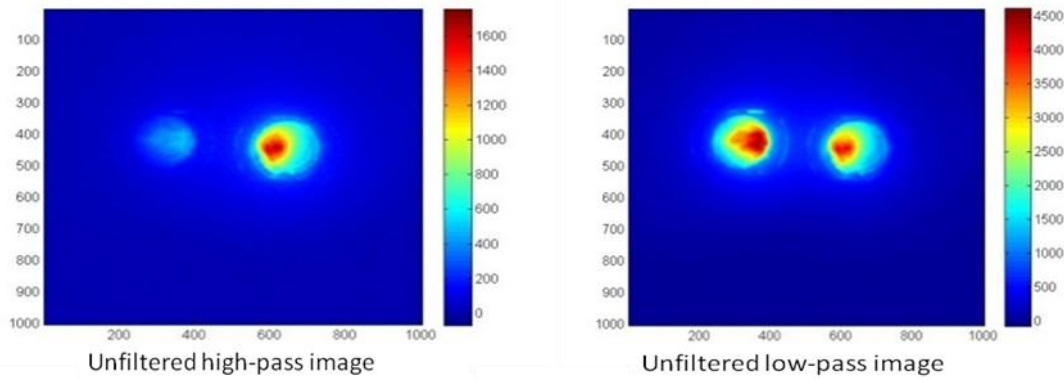


Figure 2. Images of two point-sources containing QD 660 & 710 in different filtering ranges.

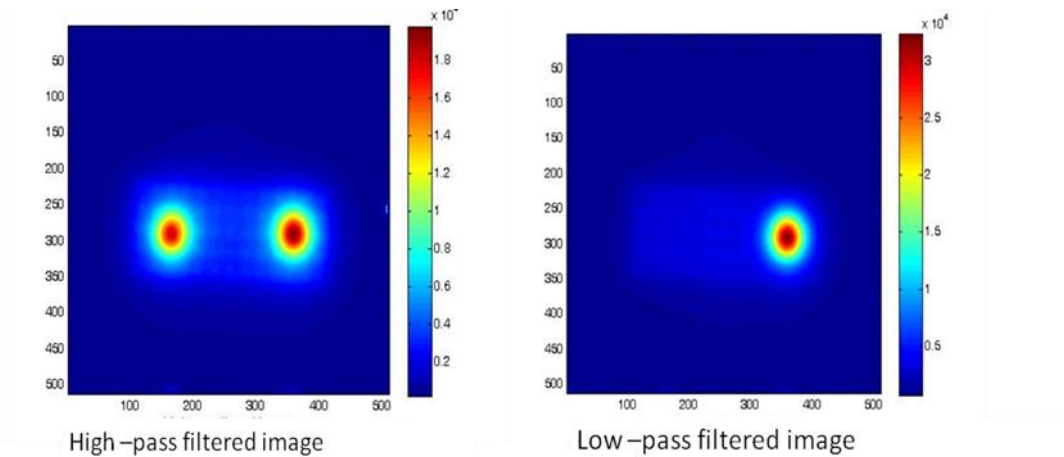


Figure 3. Reference images were used to verify the proposed algorithm.

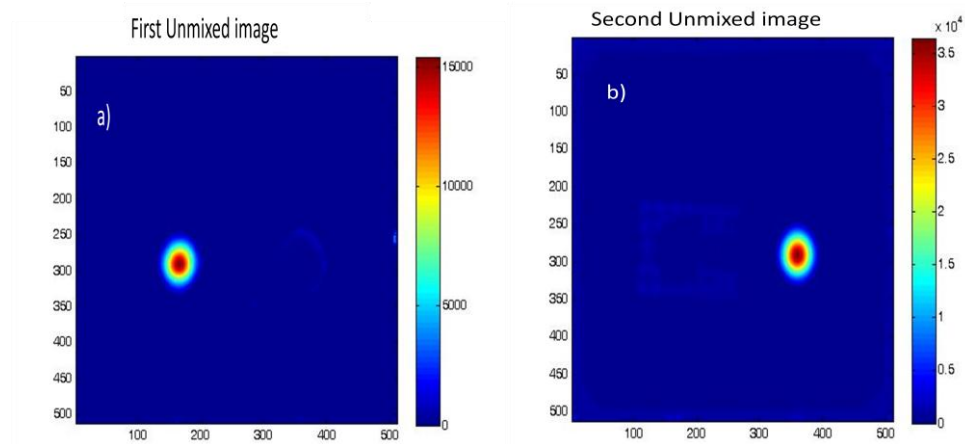


Figure 4. Multispectral images were obtained using linear unmixing algorithm based on Jacobi SVD. a) the first source, b) the second source.

The proposed algorithm based on SVD was initially applied to the reference images and the results are shown in Figure 4.

The image of fluorescent point source which has the emission spectrum in the range of 550 to 780 nm is shown in Figure 4a. On the other hand, Figure 4b shows the unmixed image of

the second probe. The unmixed images confirm the accuracy of the application of the proposed algorithm. Finally, the SVD and NMF methods were applied to the images which were taken by the optical experimental setup (Figure 5).

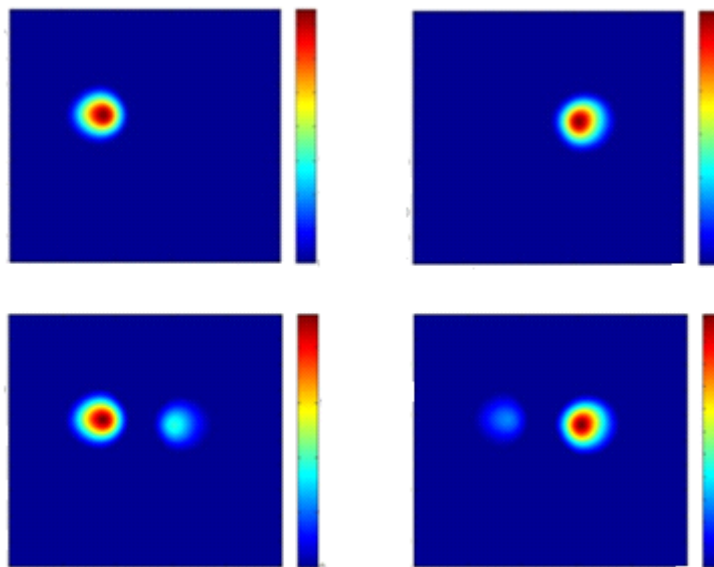


Figure 5. Images obtained using SVD (top) and NMF (bottom).

#### 4. Discussion

In fluorescent molecular imaging modality, several fluorescent dyes can be simultaneously accumulated in a lesion and irradiated with a laser light. The excited fluorophores have the overlapped emission spectra which are monitored by a cooled CCD camera. The overlapped emission spectra can be separated from each other using unmixing approaches which have recently been introduced. The goal of this article is spectral separation of quantum dots within tissue-equivalent phantom using linear unmixing methods in multispectral fluorescent reflectance imaging. Therefore, we proposed a new multispectral imaging algorithm based on a linear unmixing method. The first step of the algorithm was applying a denoising method to the obtained images.

The background subtraction method was introduced for reducing noise in multispectral images [18, 19]. Analysis of the images by SVD showed that the algorithm can be used to find matrices that are numerically very close to singular. For computing the accurate singular values, the Jacobi SVD methods were then applied to the FRI images. The algorithm was quantitatively evaluated in the specified ROI by calculating PSNR, RMSE, SNR, and CNR. The SNR of the proposed method was 30.1 dB [20] obtained by unmixed microscopic imaging using SVD method. The results showed that SNR was

27.8 dB. The 8.3% SNR increment in our method was due to the application of a median filter.

Additionally, we wrote a NMF algorithm based on the Lee and Seung method [17, 21] and applied to the FRI images. The disadvantage of the NMF method was using a cost function which converged to a not well-defined point. Therefore, the modified NMF algorithm was written in MATLAB environment with an improved cost function. The complete description of the modified NMF algorithm can be found elsewhere [21] and was used to unmix the multispectral images. The result showed that the CNR of the images was 0.3\_db. Montcuquet et al. [14] applied the NMF method to in vivo optical fluorescent images. Validation studies in mouse xenograft models showed that the CNR was 0.67 dB. Reduction of CNR in our study was due to using random initial values instead of the real numbers.

The Jacobi SVD, NMF, and modified NMF were compared by the one-way ANOVA and post-hoc test (Tukey-Kramer). The result showed that the difference of Jacobi SVD PSNR with PSNR of NMF and modified NMF algorithm was significant ( $p < 0.0001$ ). The statistical results showed that the Jacobi SVD method was more accurate than modified NMF. This is due to using previous knowledge in the Jacobi SVD instead of random initialization of the NMF.

## 5. Conclusion

In conclusion, the Jacobi SVD is a powerful method for obtaining the unmixed FRI images. The results showed that SNR, CNR, and PSNR of the unmixed images were 39, 0.706 and 30.1 dB, respectively in the phantom

study. In future studies, the algorithms will be evaluated in the *in vivo* imaging modalities.

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