Real-time hyperspectral clusterization for two-photon ophthalmic imaging of the mouse retina

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1. Introduction

Hyperspectral multiphoton microscopy is an imaging technique, which enables the study of fluorescence in biological samples. It is powerful, but limited in practical applications due to lack of real-time processing methods allowing for automatic data analysis. In this work, we present a phasor-based [1] processing approach that significantly accelerates the analysis of fluorescence data while maintaining accuracy.

One of the main goals of fluorescence data analysis is clustering, which involves segmenting the image based on the optical spectra recorded in individual pixels. In the case of data represented in the phasor domain, clustering is performed based on the spectral phasor coordinates. One of the more popular clustering methods is the k-means algorithm [2], which iteratively groups data by minimizing the sum of squared distances between points and cluster centers. We propose a new clustering method, spatially-corrected density peak clustering (SC-DPC), which speeds up searching for cluster centers through histogram analysis, and improves clustering accuracy by incorporating intensity image based correction.

2. Results on synthetic data

In SC-DPC, the entire processing flow consisted of four steps. First, hyperspectral data were converted into phasor form. Second, phasor points were filtered using a complex wavelet filter [3], which aimed to remove photon counting error. After removing noise from individual data points, third step was designed to divide all pixels into background and signal based on their intensity using a Poisson background separation [4]. In the last, fourth step clustering was performed.

Next, our method was compared to k-means on synthetic data simulating the presence of two fluorophores in the image. Example spectra of the generated fluorophores are shown in Fig. 1a. The methods were tested for different spectral separations ($\Delta\lambda$) ranging from 20 to 40 nm; below, we present an example for $\Delta\lambda$ = 20 nm. Fig. 1b shows the reference fluorophore spatial distribution pattern, in which the pixel color indicates the fluorophore contributing more than 50% of intensity (orange – fluorophore a, red – fluorophore b). The clustering results obtained using the k-means and SC-DPC methods are shown in Fig. 1c and Fig. 1d, respectively.

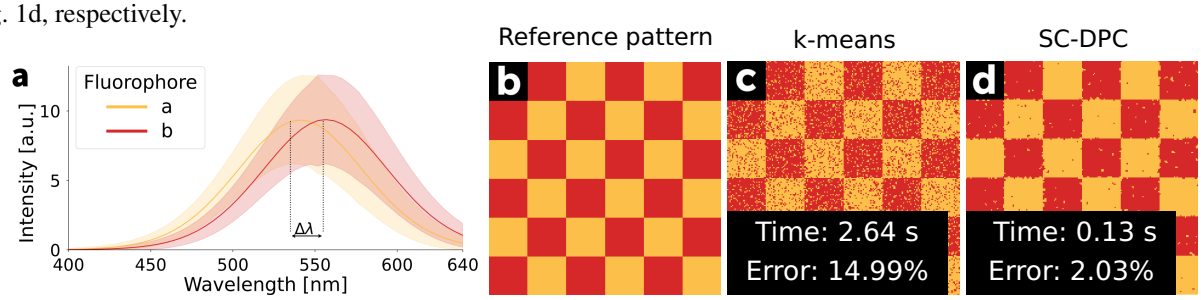

Fig. 1. **Clustering results on synthetic data: (a)** fluorophore spectra generated for $\Delta\lambda = 20nm$, **(b)** reference pattern of fluorophore spatial distribution, **(c)** fluorophore spatial distribution pattern estimated with k-means, **(d)** fluorophore spatial distribution pattern estimated with SC-DPC.

The obtained results showed that the proposed method increases clustering accuracy and reduces processing time. SC-DPC method not only reduces error by almost 13% compared to k-means, but also achieves that in less than 0.2 seconds, yielding more than a 20-fold reduction in computation time. This reduction was made possible by replacing the iterative search for cluster centers with histogram analysis. The application of the method enables the clustering result to be displayed in real time.

3. Application to multiphoton imaging of the mouse retina

Our method was evaluated using data from in vivo two-photon fluorescence imaging of the mouse retina. The images were obtained with 730 nm excitation from the eye of an albino mouse ($Abca4^{PV/PV}Rdh8^{-/-}$ genotype) without any staining and show retinal pigment epithelium (RPE) cells (Fig. 2a). Fluorescence spectra were acquired with a Leica TCS SP8 microscope equipped with an integrated spectral detection system. For each image pixel, the fluorescence emission spectrum was recorded, separated into 24 wavelength channels spanning 400–640 nm. The average fluorescence spectrum of the image is shown in Fig. 2b, indicating the presence of two distinct fluorophores. However, after direct phasor transformation, the signals from both fluorophores appeared to be strongly mixed (Fig. 2c).

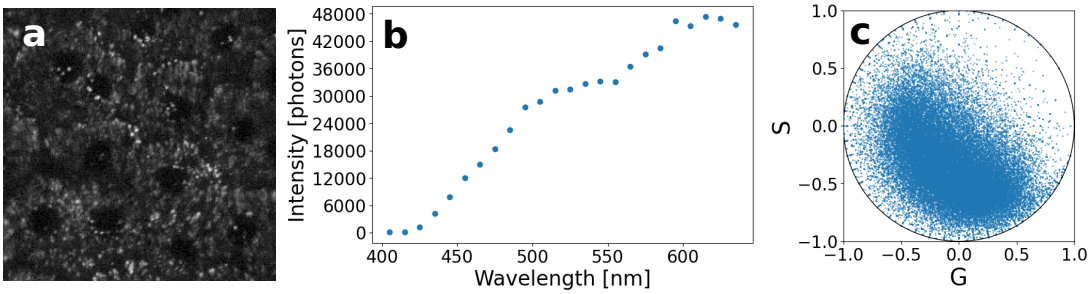

Fig. 2. **Multiphoton imaging of the mouse retina: (a)** intensity image (gamma correction was applied for better structure visibility); data previously published in Ref. [6] and reused here, **(b)** average fluorescence spectrum of the image, and **(c)** unfltered phasor data.

Spectral phasor data were clustered using both k-means clustering and the SC-DPC algorithm into two groups: k-means clustering produces sharply delineated clusters in the phasor plot, whereas SC-DPC yields more gradual, spatially coherent transitions between clusters, reflecting its incorporation of spatial context. The results demonstrate the advantage of the spatial awareness incorporated in SC-DPC, which can correct intensity contributions from pixels located between cluster centers (Fig. 3a,b). This correction yields a smoother spatial distribution of fluorophores (as seen in Fig. 3c,d). The results of clustering demonstrated that the fluorophore with an emission peak near 520 nm (likely retinyl esters) is predominantly localized in granules near the cell borders, while the fluorophore with a peak around 630 nm is more uniformly distributed throughout the retinal pigment epithelium (RPE) cells, which we hypothesize to be A2E. The results are consistent with those presented in [5].

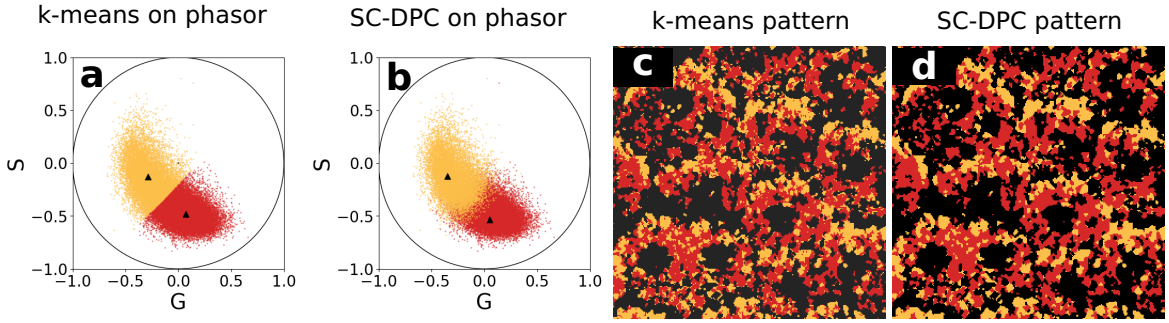

Fig. 3. **Clustering results for mouse retina image: (a)** k-means results presented on filtered phasor plot, **(b)** SC-DPC results presented on filtered phasor plot (black triengles represent estimated centers of the clusters), **(c)** fluorophore spatial distribution estimated with k-means, **(d)** fluorophore spatial distribution estimated with SC-DPC.

4. Acknowledgment

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5. References

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