

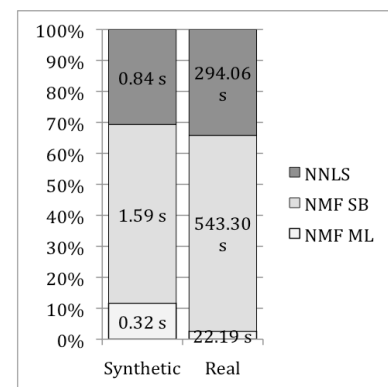
# MULTI-LAYERED NON-NEGATIVE MATRIX FACTORIZATION FOR THE EFFICIENT BLIND SPECTRAL UNMIXING OF FLUORESCENTLY LABELED SAMPLES

Thomas Pengo, Arrate Muñoz-Barrutia, Carlos Ortiz-de-Solórzano

Cancer Imaging Laboratory (LIC)  
Center for Applied Medical Research (CIMA), University of Navarra  
Avenida Pio XII 55, ES-31008 Pamplona, Spain

E-mail: [tpengo@unav.es](mailto:tpengo@unav.es)

In fluorescence microscopy, when multiple fluorescent dyes are used in the same sample, their spectral overlap may cause the emission of a fluorochrome to appear in multiple detection channels, causing spectral confusion and erroneous quantification. The problem of recovering the actual contribution of a given fluorochrome to the detected channel intensity is called spectral unmixing. Spectral unmixing techniques assume that the intensity measured in a given channel is the weighted sum of the emission intensities from all fluorophores of the sample. Techniques to perform spectral unmixing can either measure the weights that must be assigned to each fluorophore to recover the emission intensities (e.g. using Non-negative Least Squares or NNLS), or can calculate the best combination of weights and emission intensities at the same time. In the latter case, the method is said to be blind. Among blind methods, Non-negative Matrix Factorization (NMF) is gaining importance because it naturally enforces the positivity constraint on both the weights and the emission intensities. Recently, a NMF algorithm (NMF SB) has been proposed and applied to biological specimens [2]. We develop on our initial work [1], incorporating the latest evolution in NMF calculus to propose a new algorithm that greatly improves convergence rates (Fig. 1) and still performs better than the latest NMF. Our algorithm (NMF ML) employs two optimizations involving the initialization and a technique called multi-layering, which greatly speeds up its convergence rate. To test the performance of the algorithm, we applied it to two related biological applications. The first is the separation of stained nuclei from autofluorescence. To this end, we use a classifier that exploits the ability of NMF to optimize the linear combination coefficients from different channels to detect the “color” of stained and autofluorescent objects. The second application is the unmixing of strongly overlapping signals in multiply labeled fluorescence in situ hybridization (M-FISH) samples. In both applications our algorithm outperforms the state-of-the-art methods in terms of both residual cross-talk after unmixing and processing speed. We believe the algorithm has the potential to be applied to similar applications in biology and fluorescence microscopy.



**Figure 1 - Processing speed comparison for M-FISH samples unmixing.**

[1] A. Muñoz-Barrutia, J. García-Muñoz, B. Ucar, I. Fernandez-García, C. Ortiz-de-Solórzano, “Blind Spectral Unmixing of M-FISH Images by Non-negative Matrix Factorization”, *Proc. IEEE EMBS*, 23-26 Aug 2007, Lyon, France.

[2] R. A. Neher, Miso Mitkovski, F. Kirchoff, E. Neher, F. J. Theis, A. Zeug, “Blind Source Separation Techniques for the Decomposition of Multiply Labeled Fluorescence Images”, *Biophys. Journal*, **96**, 9 (2009).