Solve your crosstalk and autofluorescence issues while doing Western blot imaging

Introduction

We combine unique spectral imaging and spectral unmixing technologies to provide more accurate detection and measurement in your multi-labeled samples.

Crosstalk and autofluorescence present significant challenges in fluorescence Western blotting techniques. Crosstalk refers to signal bleed-through between fluorophores, where emission from one fluorophore overlaps with the detection range of another (fig 1). This can lead to false-positive signals and inaccurate quantification of target proteins. Autofluorescence, on the other hand, arises from inherent background fluorescence from the PVDF / nitrocellulose blot, which can interfere with the specific signal of interest.

You can optimize the detection range and emission intensity by adjusting the excitation light intensity for each fluorophore, thereby minimizing the extent of crosstalk. However, in cases where the degree of overlap is particularly pronounced, a mathematical restoration of the fluorophores into separate channels may be required. This process, known as spectral unmixing, has emerged as a powerful tool to solve these issues.

Spectral unmixing involves acquiring emission spectra from individual fluorophores and using computational algorithms to deconvolve the overlapping signals, thus enabling accurate quantification and reliable interpretation of Western blot results. This technique has greatly improved the specificity and sensitivity of fluorescence assays, enhancing the overall quality of data obtained in Western blot experiments.

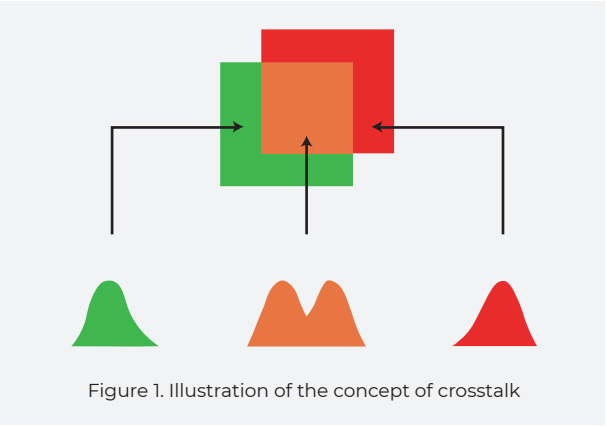

Figure 1. Illustration of the concept of crosstalk

Advantages

Clearly visualize your different signals

Separate the different signals of your fluorophores from each other and distinguish the signal from the autofluorescence with Spectral Unmixing.

The emission spectra of the green dye and the red dye are depicted in Figure 2. In the case of the green dye, approximately 3/4 of the green signal is captured by the green channel, while 1/4 of the signal is detected by the red channel. Conversely, for the red dye, around 4/5 of the red signal is collected by the red channel, while 1/5 of the signal is detected in the green channel.

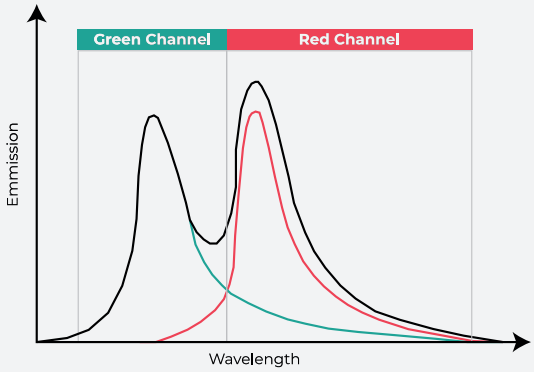

Figure 2. Example of spectral emission overlapping of green and red dyes

When analyzing a sample with dual staining, signals from both dyes will be present. In our example, the green channel will record a combination of 3/4 green dye and 1/5 red dye, while the red channel will capture 1/4 green dye and 4/5 red dye.

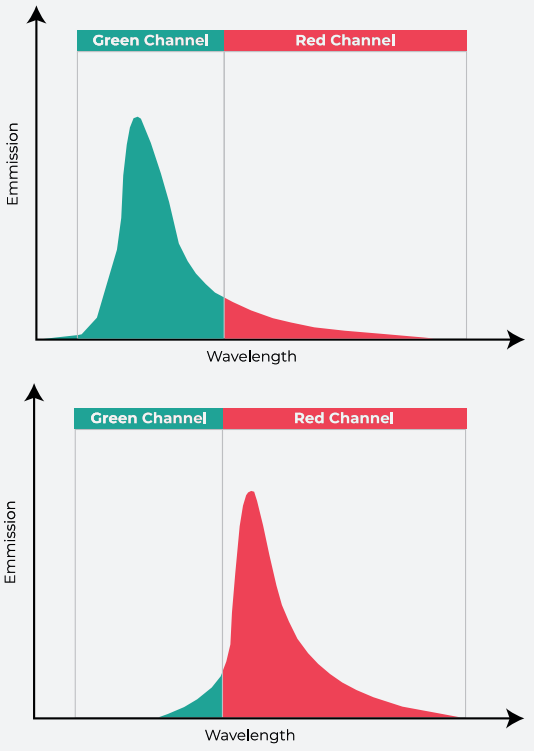

Figure 3. Signal decomposition

Our Spectral Unmixing decomposes a signal from its individual spectral components. In other words, our algorithm understands that there is 1/4 of all green dye emission in the red channel and 1/5 of all red dye emission in the green channel (fig 3). The goal of the mathematical dye separation process is to separate the different signals, ensuring that each channel exclusively contains the signal from a single dye. The result is two images, one with the green signal and one with the red signal only. Spectral unmixing algorithms identify overlapped signals and reassign them to their correct channels, allowing for accurate and precise multiplexing.

Methods

Step 1 : Spectral data acquisition

Capture optical spectrum at every pixel of an image.

Each fluorophore emitting within the detected spectral range can be individually characterized and analyzed, even in the presence of spectral overlap between different fluorophores. A series of images (typically 6) is acquired at different wavelengths and a spectral data “cube” is generated, with x, y, and wavelength as its three dimensions. Each pixel within this cube corresponds to a spectrum.

This spectral data provides valuable insights to solve our 2 main issues. By examining the complete emission spectra, it becomes possible to differentiate specific signals from the fluorophores of interest and unwanted signals from autofluorescence. The entire process enables precise quantification and analysis of Western blot data, improving the accuracy and reliability of the experimental outcomes.

Step 2: Unmixing

Obtain unmixed signals thanks to our powerful N.M.F. (non-negative matrix factorization) algorithm.

SPECTRAL UNMIXING

The algorithm that we use operates on the assumption that the total emission signal S of each λ channel can be expressed as a linear combination of the contributing dyes FluoX. The amount of contribution by a specific fluorophore is represented by the coefficient Ax. To achieve this, the algorithm employs spectral signatures, which serve as references in the form of emission spectra. Even in cases where the emission signals are combined and mixed in multi-fluorescence images, the algorithm effectively separates them into the individual dyes that contribute to the overall signal. In other words, the system calculates the distribution coefficients of all the dyes in the different channels:

$$S(\lambda) = A1 \times Fluo1(\lambda) + A2 \times Fluo2(\lambda) + A3 \times Fluo3(\lambda)...$$

The linear combination of fluorescent light emissions allows for the mathematical “unmixing” of crosstalk. As long as the distribution coefficients are measured, the sample can be analyzed. The output is a series of images containing the pure light for each dye present in the image.

Output

Revolutionary non-negative matrix factorization

Non-negative matrix factorization (NMF) is considered the optimal procedure for spectral unmixing due to several reasons. Firstly, NMF is specifically designed to handle spectral data with linearly combined emission spectra components. It assumes that each emission spectrum can be expressed as a positive linear combination of known reference spectra. This enables accurate modeling and separation of the individual spectra. Furthermore, NMF enforces non-negative constraints on the generated factors, which is optimal for fluorescence intensities in images. This constraint ensures that the results remain meaningful, preventing negative values that would be uninterpretable in the context of protein detection.

Another advantage of NMF is its ability to perform dimensionality reduction. A comprehensive reference spectrum is provided, which allows for a more concise representation of complex spectral data, facilitating interpretation and visualization of the results.

After the unmixing option has been selected, the spectral unmixing calculations are performed completely internally by the program.

Acquiring spectral data from each channel is essential for ensuring consistent quantitative results. When the spectra of the desired signal(s) and autofluorescence are known or can be inferred from the data, images that accurately represent the abundance of each protein isolated from the others can be generated. Since autofluorescence is essentially another spectral signal, it can be effectively separated and removed from exogenous signals during the unmixing process.

Get your results easily

Use our technology with ease and visualize the concentration of each fluorophore in each pixel.

- Specify the spectrum files (recommended)
- Click on “Go unmixing”

(See Figure 4)

Summary

In summary, non-negative matrix factorization is a preferred procedure for spectral unmixing due to its ability to model emission spectra, its non-negativity constraint that aligns with fluorescence intensities, its dimensionality reduction capabilities, and its ease of use. These qualities make it a powerful and robust method for spectral data analysis and solving spectral mixing problems.

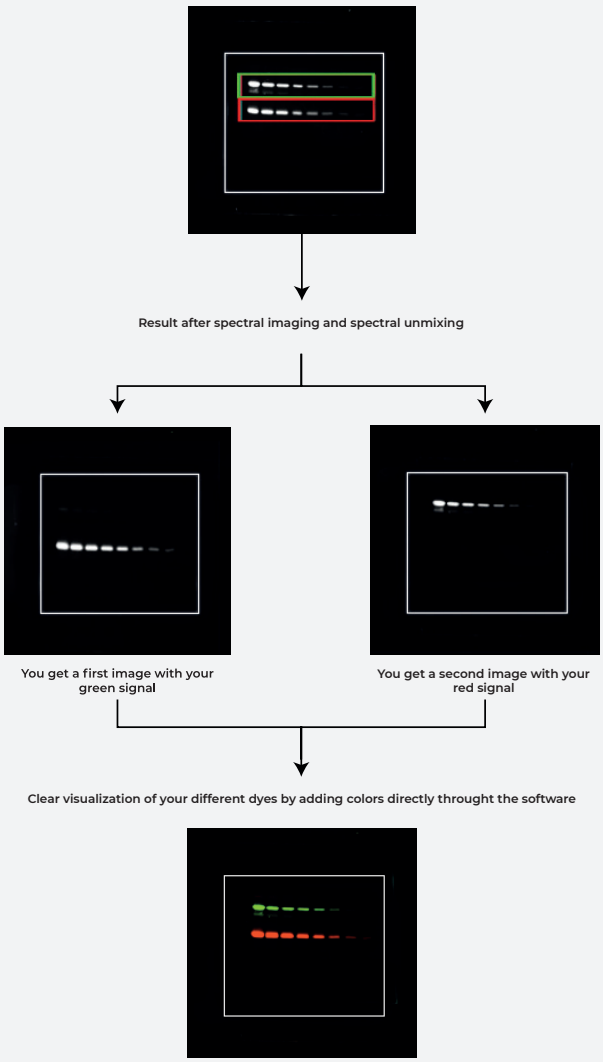

Figure 4. Example of a Western blot with overlapped dyes