

# Analytical Spectral Imaging

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Wavelength dispersive analytical spectral imaging (WDSI) was first developed by the remote Earth sensing community as a tool to characterize pollution, minerals, plant disease etc. over an unlimited field of view (FOV) from either an aircraft or satellite. Because WDSI uses pure spectroscopy it can be used for reflection, transmission and absorption measurements as well as fluorescence and luminescence. Using this capability the absorption characteristics of a fluorophore or chromophore can be determined *in-situ* and *in-vivo*. WDSI is particularly effective because complete high-resolution spectra can be acquired from 400 to 800 nm *simultaneously* with hundreds of wavelength data points (WDP).

In the life sciences, WDSI can be used to quantify, characterize and identify changes in spectral emissions associated with protein-protein interactions (for example FRET) as well as molecular and ionic interactions within living or fixed cells or tissue. In this presentation we will discuss wavelength dispersive and "filter" methods, the impact of spectral resolution, spectral calibration, spectral "unmixing", spectrum delineation, spectral libraries, and examples of how it is possible to create a spectral topographic map of multiple, highly overlapping fluorophores in complex biological materials.

Filter based spectral imaging (FSI) methods take *sequential* wavelength acquisitions through a series of bandpass filters placed in front of a CCD camera. "Filters" can be either dielectric, mounted on a filter wheel, or formed electronically by an AOTF or LCTF device. FSI systems characterize a static FOV to create a "spectrum cube". Each pixel on the CCD chip accumulates a spectrum with the same number of WDP as there are filters. It is usual for researchers to limit the number of WDP acquired because of the extended time it takes to acquire a "full" spectrum.

In a WDSI system the camera forms the detector of a spectrograph and does not image the FOV directly. WDSI systems use either a diffraction grating or prism to disperse entire incident spectra at all wavelengths *simultaneously* across rows of pixels of the CCD. All WDSI systems use a spectrometer with an entrance slit (or an entrance pinhole in spectral confocal systems). An unlimited FOV is translated sequentially across the slit (as would be the case in an aircraft or satellite). By converting, or translating, spectrum data into image data we generate an "image cube" that forms a spectral image of the FOV. Each WDSI spectrum typically contains hundreds of WDP, enough, in fact, to enable exquisite spectral delineation, accuracy and precision. A typical CFP/YFP FRET spectrum would contain over 300 WDP. All of which leads to the subject of "unmixing".

Analytical chemists and the remote Earth sensing community<sup>1</sup> consider that if two or more spectra are almost identical, and highly overlapping, but spatially separate in the FOV, then the task of identifying or delineating one spectrum from another is simply a question of spectral morphology; which is to say pattern recognition. True "unmixing" is required when two or more spectra are truly collocated within a single pixel in the FOV. In this case the issue of spectral identification, delineation, and quantification becomes very complex. There are literally dozens of algorithms for doing this and they all suffer significant advantages and disadvantages. For

example, if a spectrum can shift as a function of binding or pH, or even heterogeneous auto-fluorescence, then most algorithms fail unless the changed spectra can be synthetically simulated or created, so that this information can be included in a "training" session. Linear algorithms, such as such as Principal Component Analysis (PCA) can work in a simplistic non-scattering sample. However, when scattering or other factors occur, PCA can reduce the size of a data set prior to incorporation into the first layer of a neural network<sup>2</sup>. This method is a very powerful means of performing complex non-linear spectral unmixing.

Figure 1, shows a set of highly overlapping spectra emitted by Alexa 555, pseudo colored Yellow; 568 in Green, and 594 in Red. The digital values of the spectra were derived from spectra on the Molecular Probes website. The peak separation was 38 and 14 nm respectively each with a minimum FWHM of 50 nm. Figure 2 shows how these three fluorophores have been delineated in an auto-fluorescing tissue section. (This confidential and proprietary image is reproduced with the permission of Aureon Biosciences Corp.). The fluorophores were identified using the PARISS spectral Imaging system and the data processed with a proprietary variant of spectral waveform cross-correlation analysis (SWCCA). The algorithm enables the use of the logical operator "not" in order to recognize when a spectrum is close to, but "not" identical to an expected spectrum. This is a very effective means of locating spectra that may have shifted as a function of changes in binding, interaction, ionization, or of course FRET.

Figure 3 shows the spectra of CFP, GFP and YFP (numerical data supplied by Clontech) shown on the same scale as the Alexa series in Figure 1. Given the ability to separate these Alexa dyes, it is evident that identifying the locations of each of the individual fluorescent proteins is not a challenge to a WDSI system.

WDSI is a powerful technique for inductive research that enables the researcher to use fluorophores or chromophores as *in-situ* sensors. In addition it is possible to use multiple fluorophores without the constraints and limitations of either filters or traditional imaging methods. This results in the rapid acquisition of accurate, high-content information.

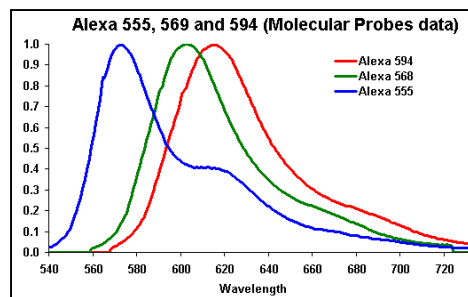


Figure 1: The spectra of Alexa 555, 568 and 594. Note that they are highly overlapping

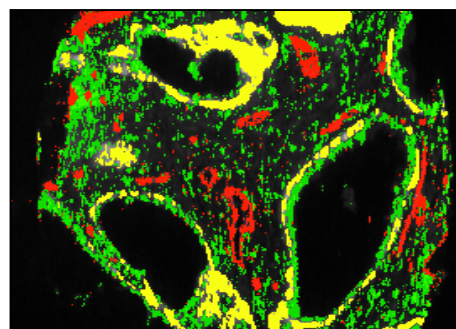


Figure 2: A tissue section with Alex 555, 568 and 594. The spectrum of each dye was acquired *simultaneously*

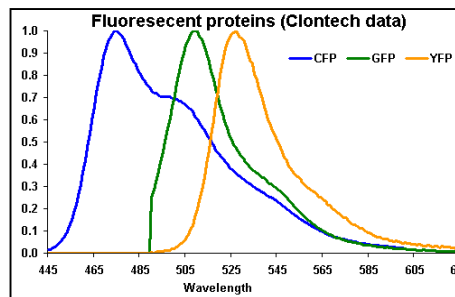


Figure 3: The spectral profiles of CFP, GFP and YFP

1. N. Keshava, "A Survey of Spectral Unmixing Algorithms," Lincoln Laboratory Journal, vol 14, 1, (2003)  
 2. T. Lu, and J.M. Lerner, "Spectroscopy And Hybrid Neural Net Analysis," Proc. IEEE, vol 84, 6 (1996)