#1 Bestselling Computer Book Series

"After reading your books, I'm considered an expert in computers at the office."
— Kenyon Sayler,

50 MILLION DUMMIES BOOKS IN PRINT

LINEAR UNMIXING FOR DUMMIES®

NEW!

A Reference for the Rest of Us!®

The Fun and Easy Way™

Your First Aid Kit® for Managing and Sharing

The question has arisen several times . . . .

What exactly is LINEAR UNMIXING?

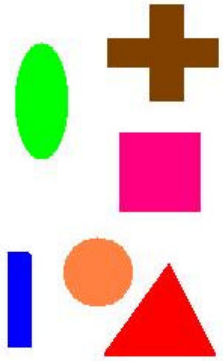
HOW DOES THIS WORK?

Linear Unmixing is a mathematical technique commonly used for the decomposition of chemical elements in an unknown by analyzing the spectral makeup relative to a library of standards. Likewise it is applied to the analysis of lansat images to determine crop or landscape composition.

In the art of confocal microscopy it is now applied to the decomposition of fluorescent components in an unknown.

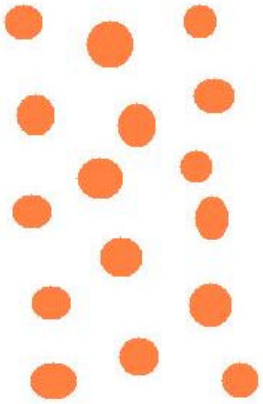
Explanation #1 – conceptual understanding

Consider the small child trying to insert odd shaped pegs into a board of odd shaped holes.



It is quite easy for even a small child to figure out that a round peg will go into a round hole without even understanding what 'round' means. Recognition is so easy because the holes are so different we can quickly determine that the round peg goes into the round hole.

On the other hand consider the task of inserting a round peg into one of the following holes?



Not so Easy!!!!!!!!!!

This requires that you try every possibility to find the <u>best fit</u>!

OR – if you want to be very ANALytical – you take several measurements of each hole and compare those measurements to measurements of your peg. You might measure the radial diameter at say maybe <u>32</u> different angles. This gives you a lot of data to compare, but once you are done you surely will find the best hole to put you peg in.

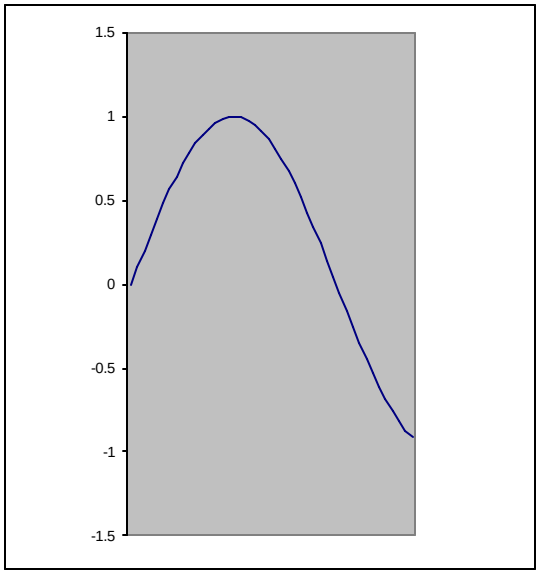
Note: it takes very little deviation to the shape of the hole and the peg to return to a 'no brainer' situation'. Consider . . . . .

Very subtle differences can make our comparison a very easy job again.

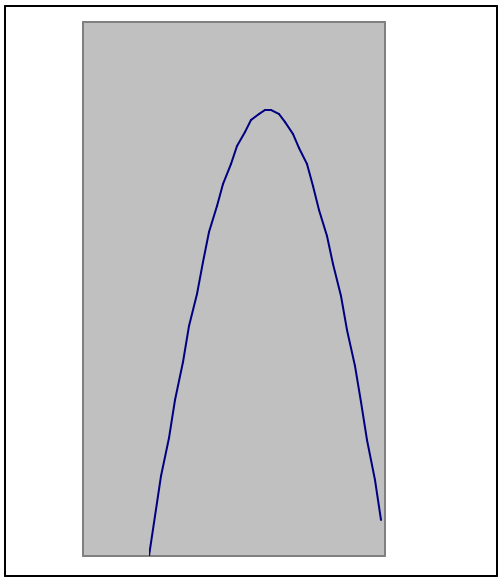
Explanation #2 – conceptual understanding for grown ups

The same idea of pegs and holes can be applied to determining which of 2 reference spectral curves is the best fit for an unknown spectral curve.

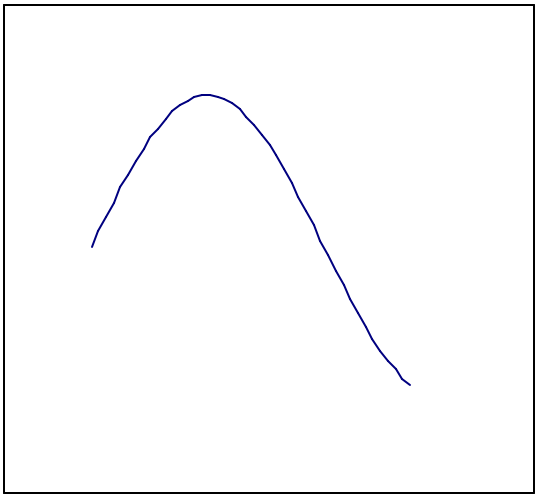
Ref #1



Ref #2



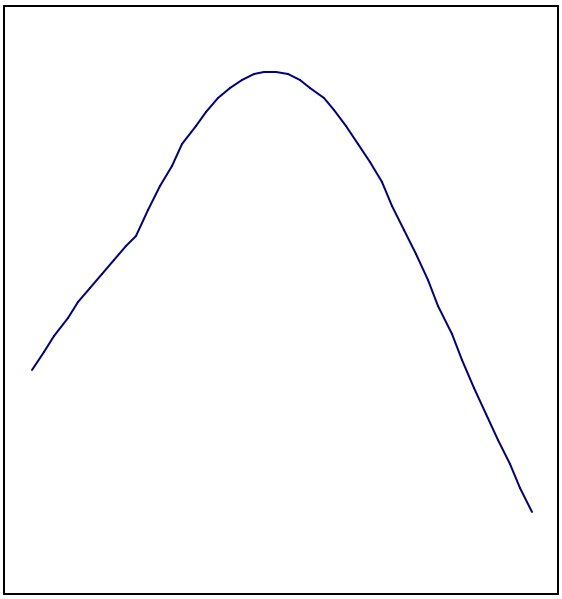
Unknown



If you compare or fit the Unknown to the reference spectra you quickly determine that the best fit is Ref #1. It just looks right.

What you are doing is a fast mathematical calculation of best fit.

This becomes much more difficult when the Unknown can be a combination of BOTH of the reference spectra. Consider this unknown . . . .



You stand little chance of determining how much of Ref #1 and how much of Ref #2 is in this unknown spectrum without the aid of a computer.

We just happen to have a computer, and FYI this unknown is a perfect combination of 50% Ref #1 and 50% Ref #2.

And just like the peg and hole analogy, a subtle difference in the characteristics of the curves make the identification much easier. So even if the reference curves look very similar it take only the slightest difference to trigger an easy match. But, subtle difference or not, a computer can unmix these mathematical mixtures very fast and very easy.

Explanation #3 – Mathematical understanding

Consider the measured spectrum of a single pixel in a sample known to contain only CFP and YFP, we can represent the measured fluorescence to be a combination of the 2 components:

$$S(?) = A_1 CFP(?) + A_2 YFP(?)$$

S(?) = the measured spectra at a given point = an array of up to 32 elements to represent the 32 wavelengths ranges from the META detector.

C,Y = our previously measured reference spectra with the same 32 elements

A1, A2 are the constants we need to solve for – that is - the % of each component at the given pixel

The way we do the comparison between the unknown and the reference spectra is to calculate simultaneously all the difference equations of the unknown minus the 2 reference curves for all values of A1 and A2. We solve for the minimum value of these simultaneous equations – that is what values for A1 and A2 make this result a minimum.

$$S(?) - A_1 CFP(?) - A_2 YFP(?) = \min.$$

To find the best fit (the smallest difference) we do a least squares fit. That is we create a function that is the square of this equation and then solve for the minima.

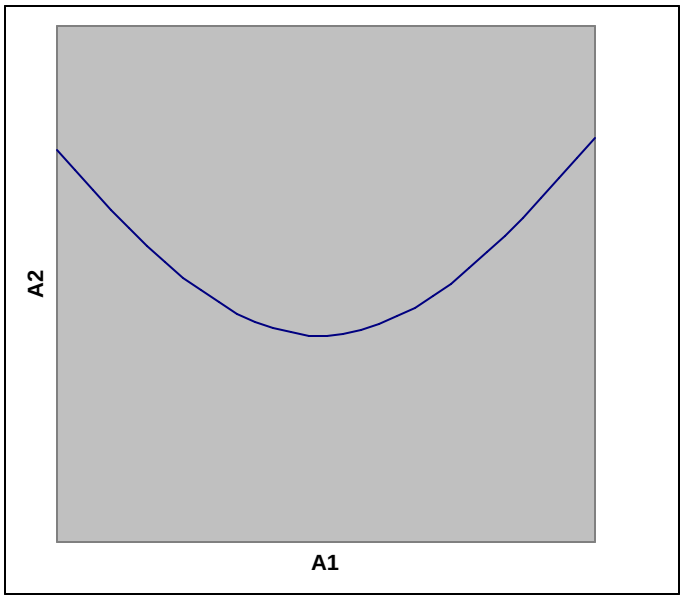
$$S_i \left[S(?i) - [A_1 CFP(?i) + A_2 YFP(?i)] \right]^2$$

[It is important to note that before any of these calculations are made the reference and measured curves are normalized so as to remove any influence from the intensity of each point. We look only at the relative shapes of the curves!]

To solve this equation we use Linear Algebra since S, C, and Y are arrays with up to 32 elements.

Linear Algebra* is designed to solve simultaneously multiple equations with multiple variables - in our case we try to solve as many as 32 equations in 8 unknowns. (FYI - CFP and YFP with 12 PMT channels would be 12 equations with 2 unknowns)

The least squares function above will have a shape something like this



All we need to do is find the point where this function reaches its minimum – <u>that is the difference between the measured spectra and the 2 reference curves is at its minimum</u> – and you have found the best fit.

*While linear algebra is a rather simple and <u>very</u> fast tool, it does require some mathematical background knowledge (basic algebra, calculus, abstract algebra, vectors, etc. .) to understand how the linear algebraic tools are derived and applied. We won't discuss that here – you will have to refer to 'Linear Algebra for dummies' for that understanding.

Once you have calculated the best values for A1 and A2 you have determined the concentration or % of each component (CFP and YFP), which exists in the measured pixel.

Multiply A1 times the total intensity of the original pixel and put it in output image C and multiply A2 times the total intensity of the pixel and put it in output image Y. Repeat this over the entire image and you have successfully used linear unmixing to separate out the components of the entire image.

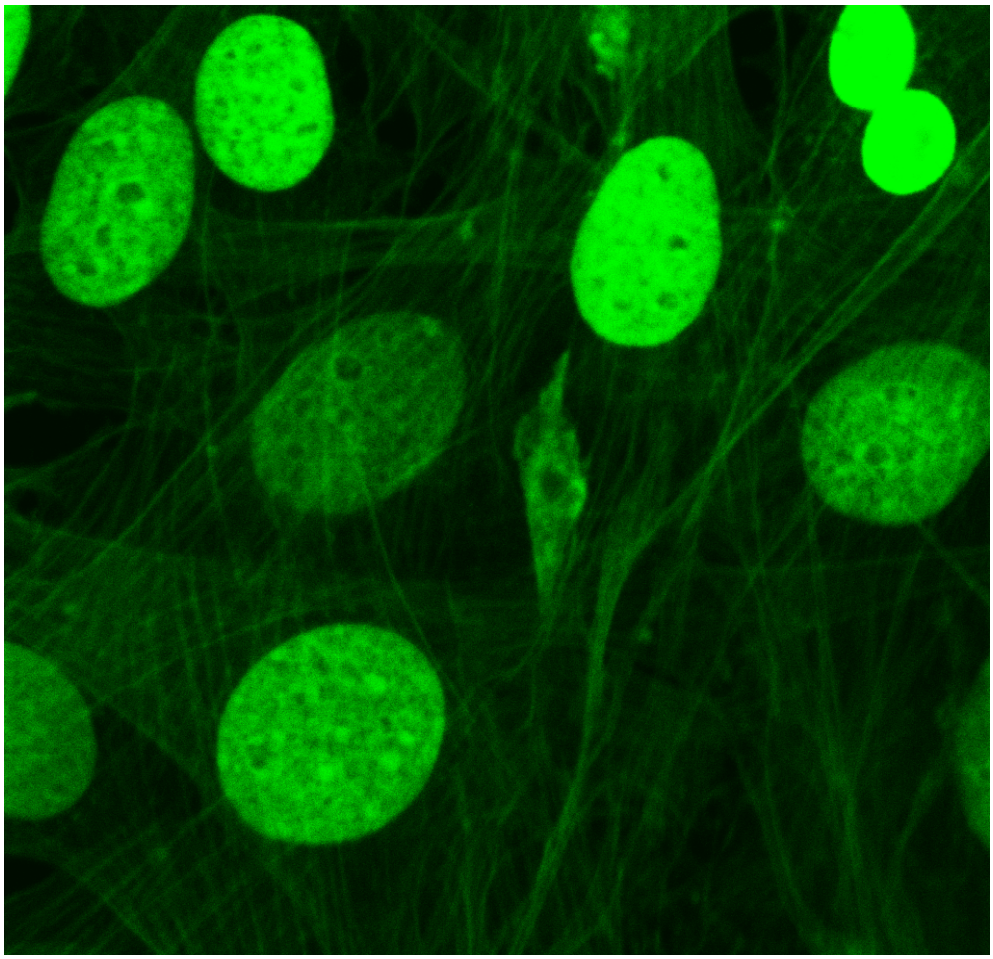
SUMMARY (easiest verbal explanation): We calculate the minimum difference between the unknown spectra and the reference spectra for all possible mixtures of the reference spectra. Since we only solve for the minimum value of this difference equation the calculation is straightforward and FAST.

Explanation #4 – visual understanding – seeing is believing

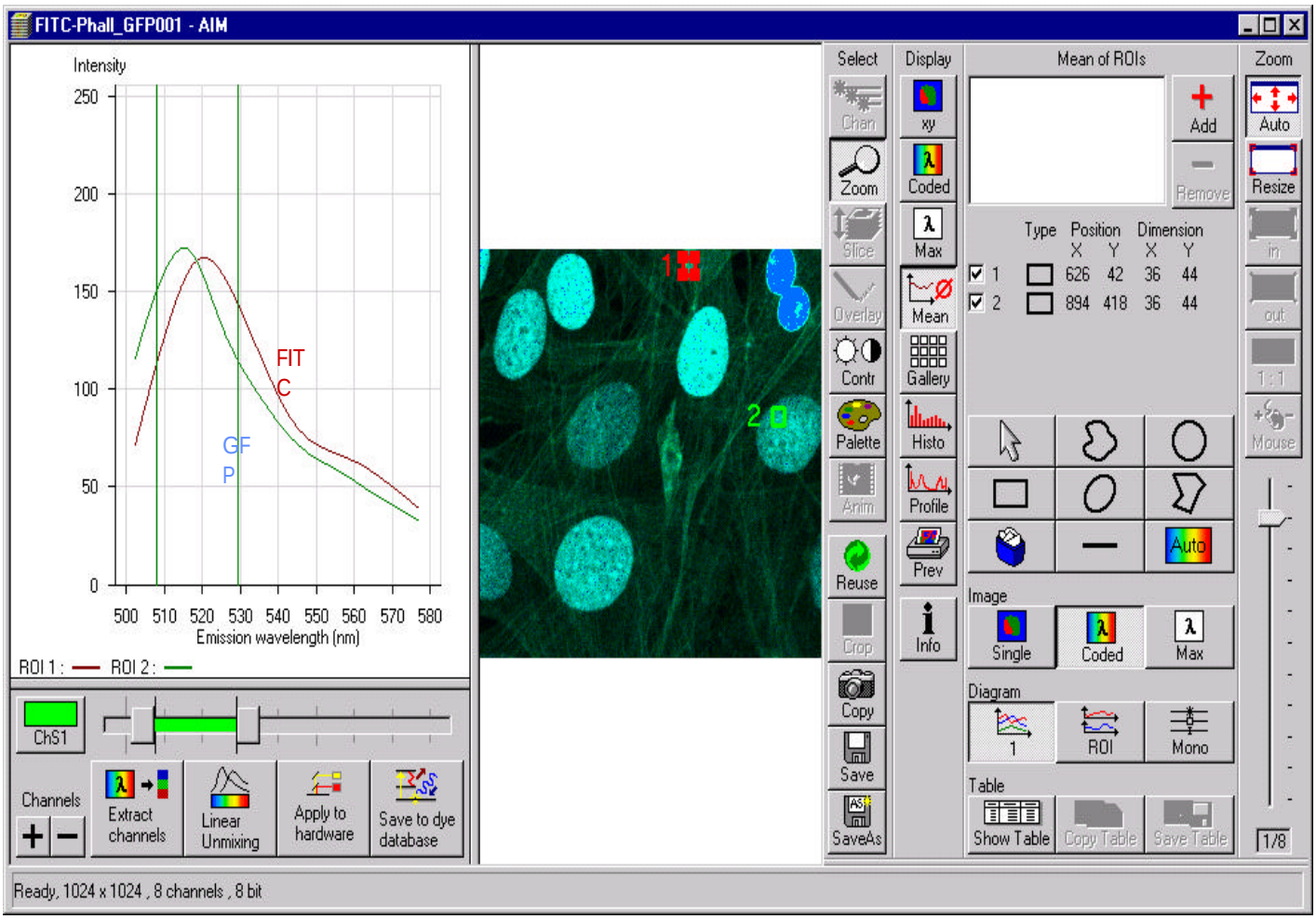
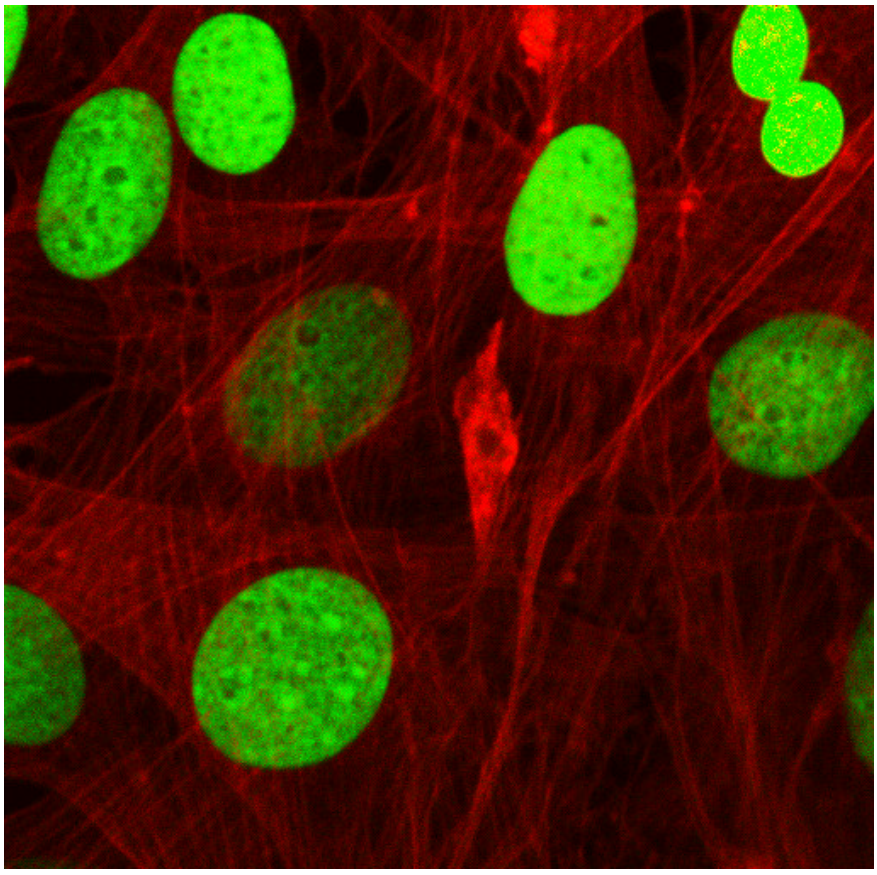
GFP and FITC (Distance of emission peaks 7nm)

Cultured fibroblasts expressing a GFP-Histone2B fusion protein, actin filaments stained with FITC-phalloidin

Through the eyepieces!



Reference Spectra



UNMIXED RESULT