Spectral Imaging

Bi177

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Fluorescence spectra
Why spectral imaging?

**Conventional**
BPAE Cells - 3 Colors

**Spectral**
Oral Plaque Biofilm - 12 Colors
How do we collect spectral datasets?
Types of Spectral detection

Fig. 3. Various methods of spectral imaging systems. They can be divided into four main methods: wavelength-scan (A–D), spatial scan (E), time scan (F) and “compromise” methods (G). In wavelength-scan methods, the whole image is measured one wavelength at a time. This can be realized using either a circular variable filter (A), a set of filters (B), a liquid crystal variable filter (C) or an acousto-optic variable filter (D). Spatial-scan methods use a dispersion element, either a grating or prism (E) and the image has to be scanned along at least one axis. There are also confocal microscopes that use a dispersive element and scan the image point by point. In time-scan method (F), the whole image is measured after passing through an interferometer (or other optical elements). In order to calculate the spectrum at each pixel a mathematical transformation has to be carried out, for example, a Fourier transform. In “compromise” methods (G) only a few spectral ranges are measured and the FOV is limited, but the measurement is fast.

Garini et al, Cytometry Part A, 2006
Spectral imaging methods: Spatial-scan

- 3 Different ways used by microscope companies
**Conventional vs spectral detection**

1 Channel
Sum of gated wavelengths

\[
480:540\text{nm} = \lambda\text{ stack}
\]

32 Possible Channels
Each a portion of gated wavelengths

\[
= 480\text{nm} \\
= 490\text{nm} \\
= 500\text{nm} \\
= 510\text{nm} \\
= 520\text{nm} \\
= 530\text{nm} \\
= 540\text{nm}
\]
Spectral detection

Fluorescence microscope
spectral detector

Diffraction
grating

Emitted light from specimen

Dataset: $\lambda$ stack
Like a Z-stack, but each slice represents wavelength rather than depth

Spectral Image Data Cube

Practical Considerations

Multiple Excitations

Single excitations/
Balancing emissions

Single excitations/
Balancing emissions/
Lots of fluorophores
Problem: Overlap
Solution: Spectral Imaging

![Image of spectral imaging with wavelengths 640nm to 700nm]
Result: **Spectral Unmixing**
How do we unmix these datasets?
Input: λ stack

Output: unmixed images

Atto633

Atto655
We can make a formula where the summed pixel intensity across lambda (S) needs to be divided up into each reference output image (R1 and R2).

\[ S(\lambda) = \%_1 * R1(\lambda) + \%_2 * R2(\lambda) \]

We need to calculate these variables (\%_1 and \%_2) such that the intensity is CLOSEST to (least different from) the reference curves.

To be least different, we need to solve this formula for \%_1 and \%_2 so that we get the smallest value possible.

\[ S(\lambda) - \%_1 * R1(\lambda) - \%_2 * R2(\lambda) = \text{minimum} \]

Since we have multiple variables to solve for simultaneously, we need to use some fancy linear algebra and matrix math.

At its core, unmixing algorithms can perform a least squares analysis to test each possible % value to get this function to its minimum.

\[ S^*[S(\lambda) - [\%_1 * R1(\lambda) + \%_2 * R2(\lambda)]]^2 \]
**Least squares function**

\[ S^* [S(\lambda) - [\%_1 R_1(\lambda) + \%_2 R_2(\lambda)]]^2 \]

Compares the measured spectrum with all possible mixtures of reference spectra, and solves for the minimal difference between measured and reference spectra.

Results:
Values for \( \%_1 \) and \( \%_2 \) that tell you what proportion of your measured value belongs in each output file.

Number of references must = number for fluorophores in the image.
Input: $\lambda$ stack

Output: unmixed images

<table>
<thead>
<tr>
<th>640nm</th>
<th>650nm</th>
<th>660nm</th>
<th>670nm</th>
<th>680nm</th>
<th>690nm</th>
<th>700nm</th>
</tr>
</thead>
</table>

Atto633

Atto655
Input: \( \lambda \) stack

Summed pixel gray value: 150
\[ %_1 = 0.90 \]
\[ %_2 = 0.10 \]

\[ \text{Atto633} = 150 \times 0.90 = 135 \]
\[ \text{Atto655} = 150 \times 0.10 = 15 \]

Output: unmixed images
Input: \( \lambda \) stack

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<tr>
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</thead>
<tbody>
<tr>
<td><img src="image1.jpg" alt="Image" /></td>
<td><img src="image2.jpg" alt="Image" /></td>
<td><img src="image3.jpg" alt="Image" /></td>
<td><img src="image4.jpg" alt="Image" /></td>
<td><img src="image5.jpg" alt="Image" /></td>
<td><img src="image6.jpg" alt="Image" /></td>
<td><img src="image7.jpg" alt="Image" /></td>
</tr>
</tbody>
</table>

Summed pixel gray value: 200

\%

\[
\%_1 = 0.05 \\
\%_2 = 0.95
\]

\[
\text{Atto633} = 200 \times 0.05 = 10 \\
\text{Atto655} = 200 \times 0.95 = 190
\]

Output: unmixed images
Input: λ stack

Output: unmixed images

Over and over

and over

and over

Atto633

Atto655
Test - Unmixing 10 fluorophores

10 tubes *L. buccalis*
10 Probes added

Reference slides
- Ref1
- Ref2
- Ref3
- Ref4
- Ref5
- Ref6
- Ref7
- Ref8
- Ref9
- Ref10

Mix labeled cells

Mix slide
<table>
<thead>
<tr>
<th>Ref1</th>
<th>Ref2</th>
<th>Ref3</th>
<th>Ref4</th>
<th>Ref5</th>
<th>Ref6</th>
<th>Ref7</th>
<th>Ref8</th>
<th>Ref9</th>
<th>Ref10</th>
</tr>
</thead>
</table>

References Lambda Stacks

Measured Reference Curves
Can we unmix more than fluorophore spectra?
Removing Autofluorescence

Unmixed channels + AF          AF          Unmixed Channels - AF
History of the Zeiss spectral detector

• Where did the idea of a multichannel detector come from?
• Collaboration between the Jet Propulsion Laboratory, Scott Fraser’s lab here at Caltech and Zeiss
History of the Zeiss spectral detector

- Zeiss META had 8 channel detector
- Replaced by 32 channel Quasar detector
Learning More

Introduction to spectral imaging and linear unmixing
http://zeiss-campus.magnet.fsu.edu/articles/spectralimaging/introduction.html

Interactive spectral unmixing tutorial
http://zeiss-campus.magnet.fsu.edu/tutorials/spectralimaging/linearunmixing/indexflash.html

Spectral Database
http://www.spectra.arizona.edu/