Spectral Imaging
Bi227
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Spectral imaging: what is it and why should I use it?
Fluorescence spectra

FITC Spectra
- Blue: Excitation
- Green: Emission

λ (nm)

400 408 416 424 432 440 448 456 464 472 480 488 496 504 512 520 528 536 544 552 560 568 576 584 592 600 608 616 624 632 640 648 656 664 672
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-scanning 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.
Spectral imaging methods: Spatial-scan

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

1 Channel
Sum of gated wavelengths

480:540nm =

32 Possible Channels
Each a portion of gated wavelengths

= 480nm
= 490nm
= 500nm
= 510nm
= 520nm
= 530nm
= 540nm

λ stack
Spectral detection

Fluorescence microscope spectral detector

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

Problem: Overlap
Solution: Spectral Imaging
Result: Spectral Unmixing
How do we unmix these datasets?
Input: $\lambda$ stack

Output: unmixed channels

Atto633

Atto655
Linear unmixing

Summed pixel intensity across lambda (S) needs to be divided up into each reference output image (R1 and R2):

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

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

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 of fluorescent signatures in the sample.
Input: λ stack

640nm  650nm  660nm  670nm  680nm  690nm  700nm

Output: unmixed images

Atto633  Atto655
Input: λ stack

Output: unmixed images

Summed pixel gray value: 150

%₁ = 0.90
%₂ = 0.10

Atto633 = 150 * 0.90 = 135
Atto655 = 150 * 0.10 = 15
Input: $\lambda$ stack

<table>
<thead>
<tr>
<th>640nm</th>
<th>650nm</th>
<th>660nm</th>
<th>670nm</th>
<th>680nm</th>
<th>690nm</th>
<th>700nm</th>
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<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
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<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
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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: \( \lambda \) 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
Can we unmix more than fluorophore spectra?
Removing Autofluorescence

Unmixed channels + AF

AF

Unmixed Channels - AF
Spectral Summary

1. Use spectrally separated fluorophores when you can, if not possible, spectral imaging and unmixing!

2. Methods: generating spectra by selectively imaging one wavelength at a time, or imaging a range of wavelengths simultaneously.

3. Can be used for separating highly overlapping spectra and removing unwanted autofluorescence.

4. Reference library for unmixing must equal number of fluorophores in sample.

5. Unmixing:
   
   **Input**: Lambda stack, references
   
   **Output**: One channel per reference, each containing a percent of it’s contribution of original measured pixel.
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/