Spectral Imaging

Biology 177

February 14th, 2017

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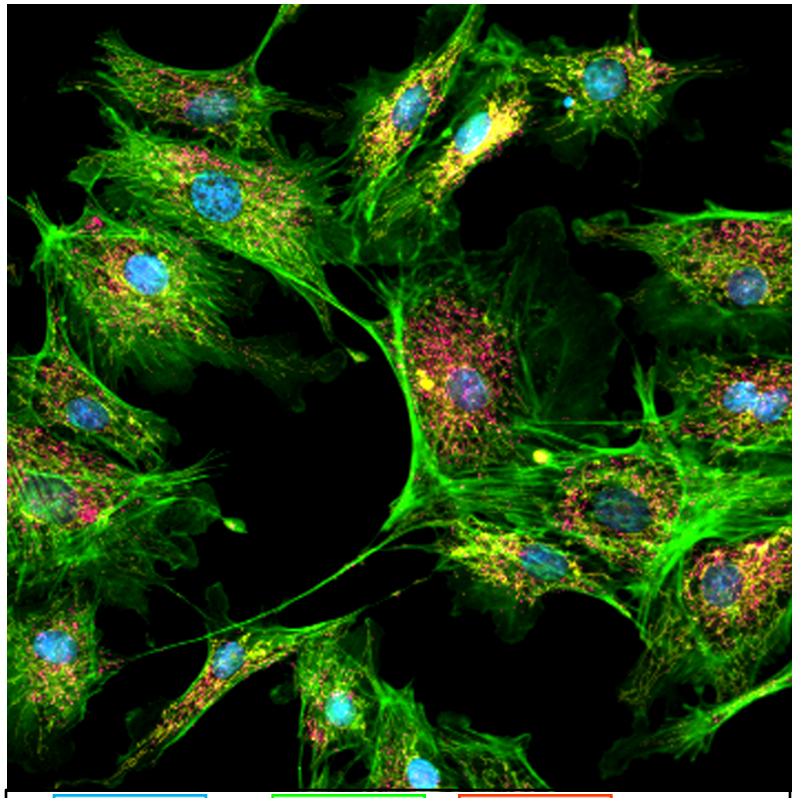
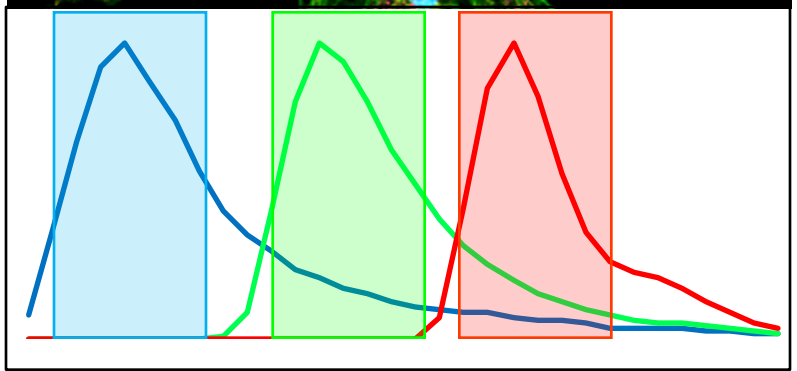
Spectral imaging: what is it and why should I use it?

Why spectral imaging?

Conventional

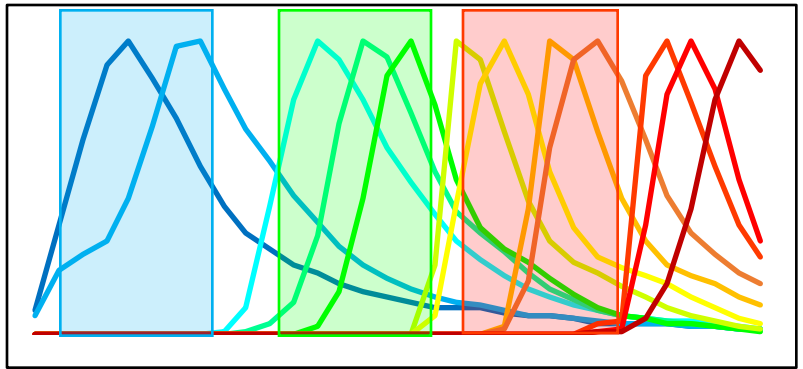
BPAE Cells - 3 Colors



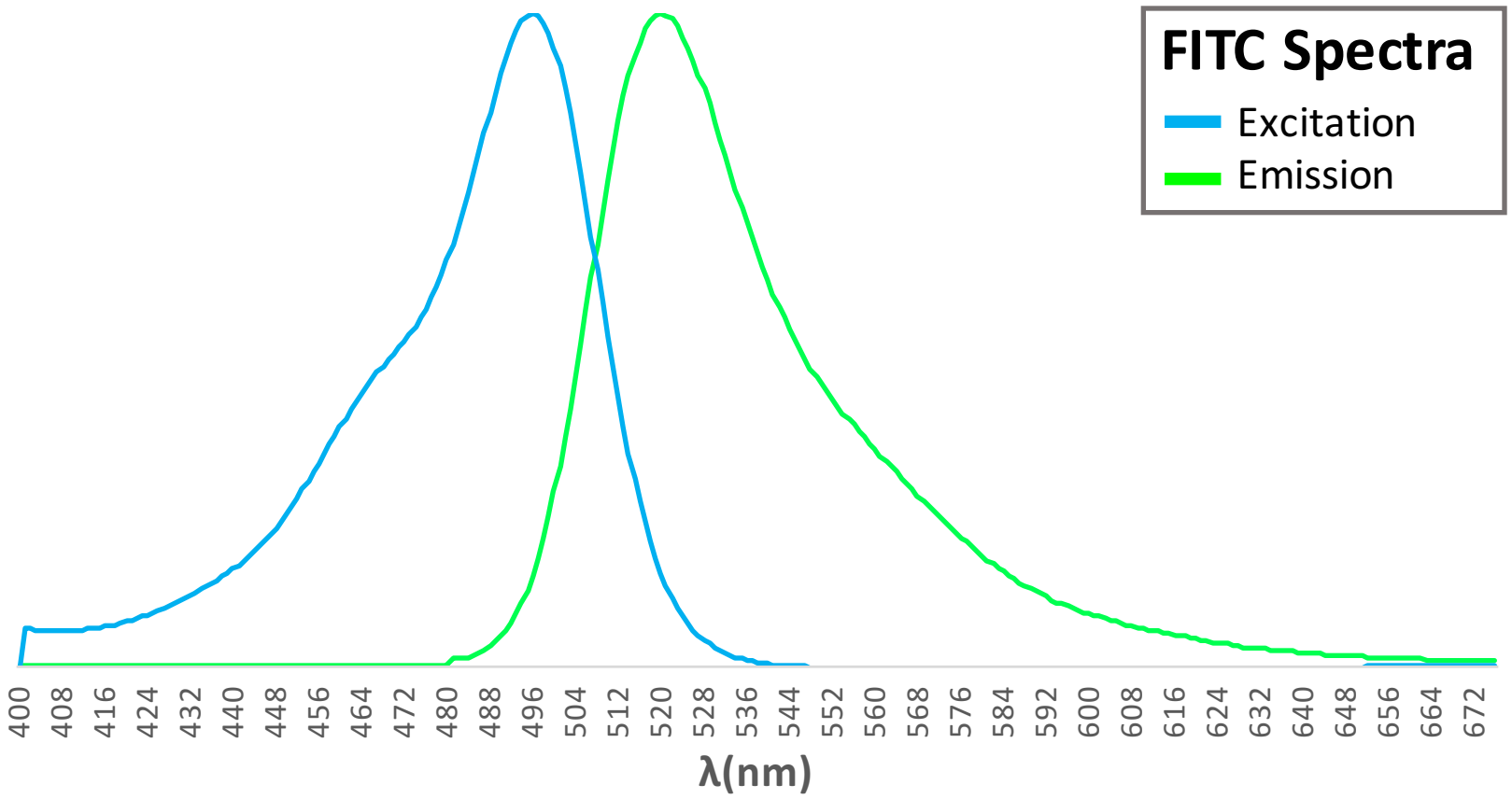


Spectral

Oral Plaque Biofilm - 12 Colors



Fluorescence spectra



How do we collect spectral datasets?

Types of Spectral detection

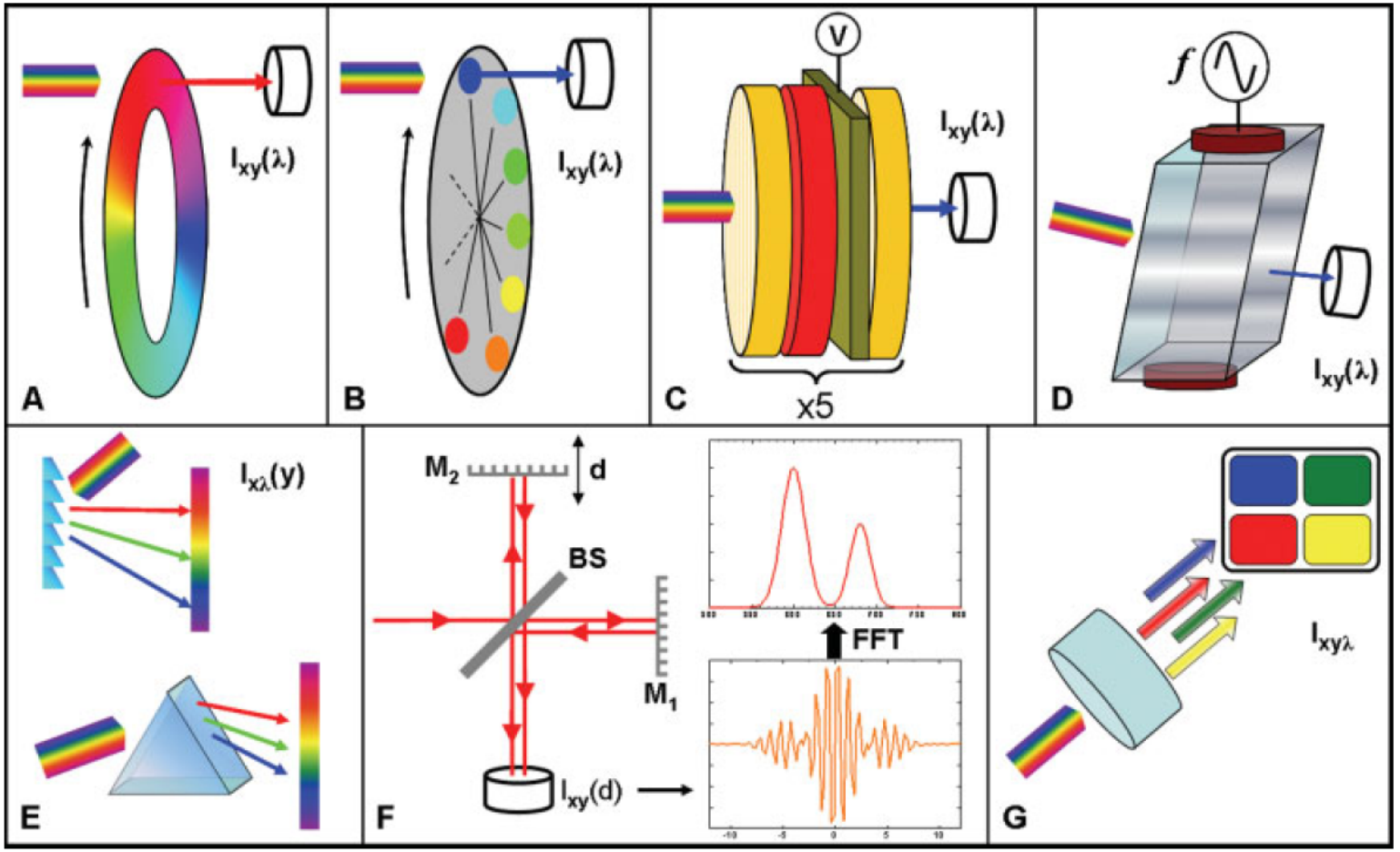
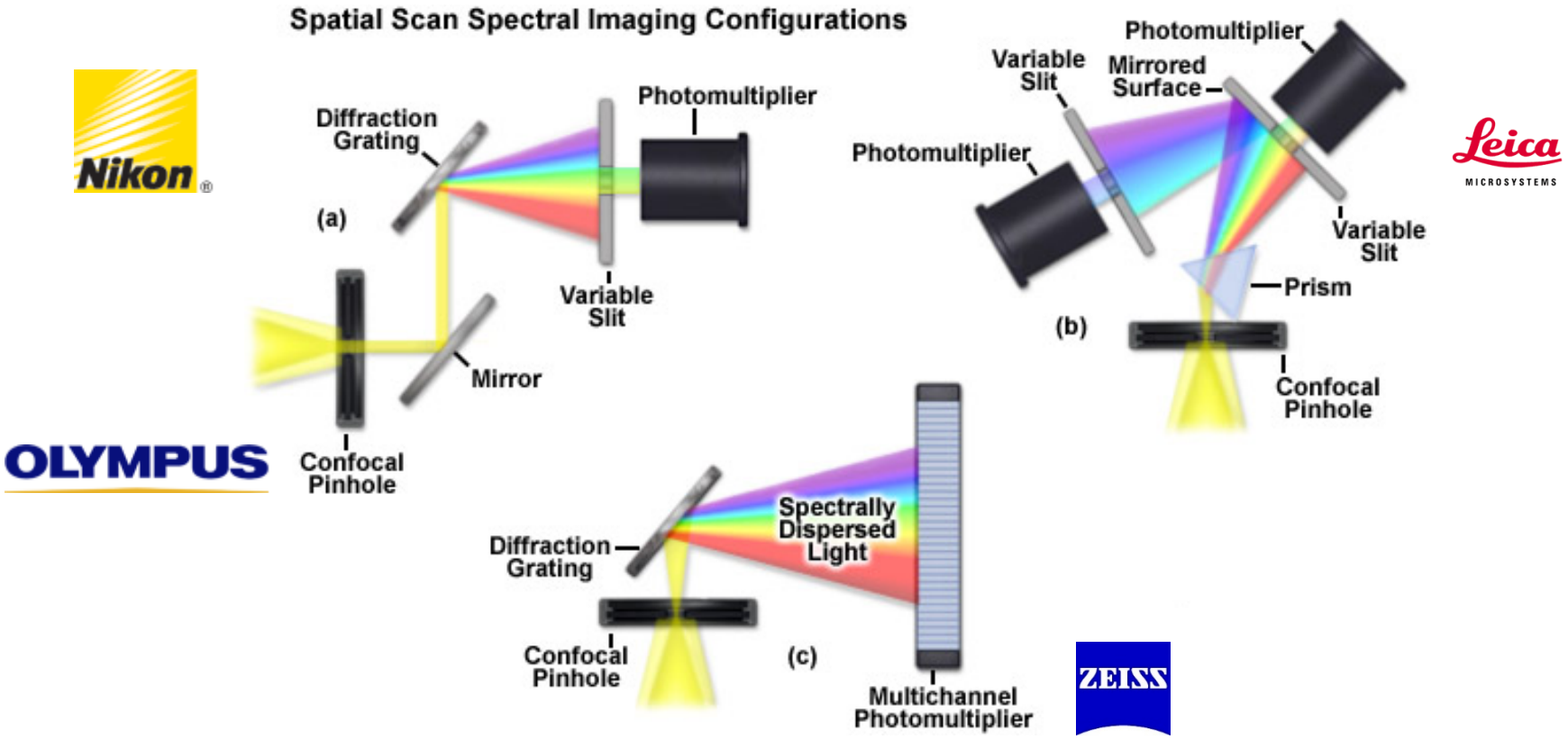


FIG. 3. Various methods of spectral imaging systems. They can be divided into four main methods: wavelength-scan (**A–D**), spatial scan (**E**), time scan (**F**) and "compromise" methods (**G**). In wavelength-scan methods, the whole image is measured one wavelength at a time. This can be realized 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.

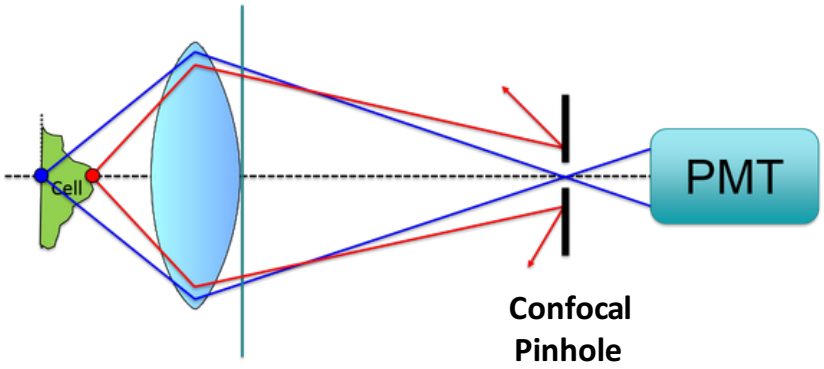
Garini et al, Cytometry Part A, 2006

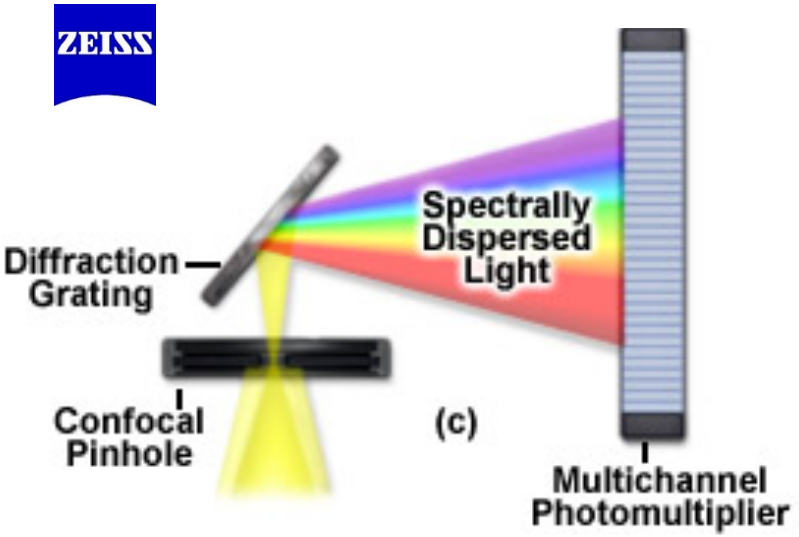
Spectral imaging methods: Spatial-scan

- 3 Different ways used by microscope companies



Conventional vs spectral detection

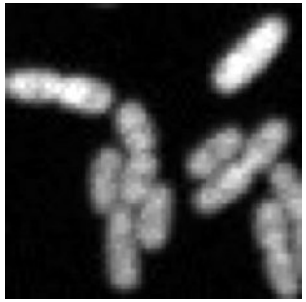


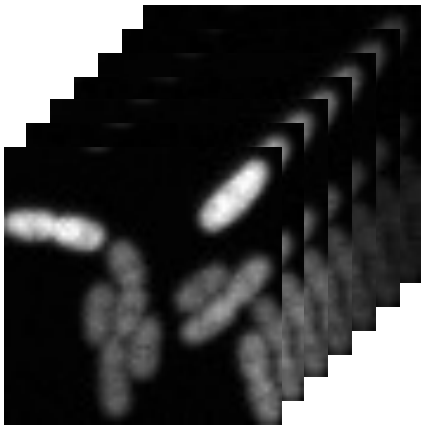


1 Channel
Sum of gated wavelengths

32 Possible Channels
Each a portion of gated wavelengths

480:540nm =

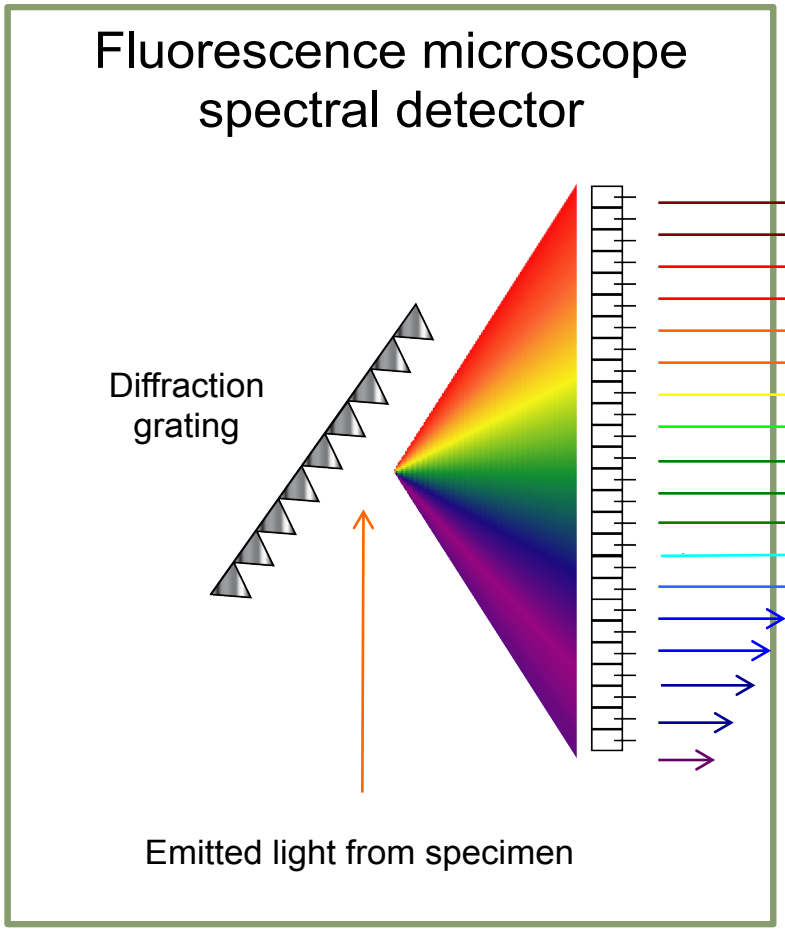




= 480nm
= 490nm
= 500nm
= 510nm
= 520nm
= 530nm
= 540nm

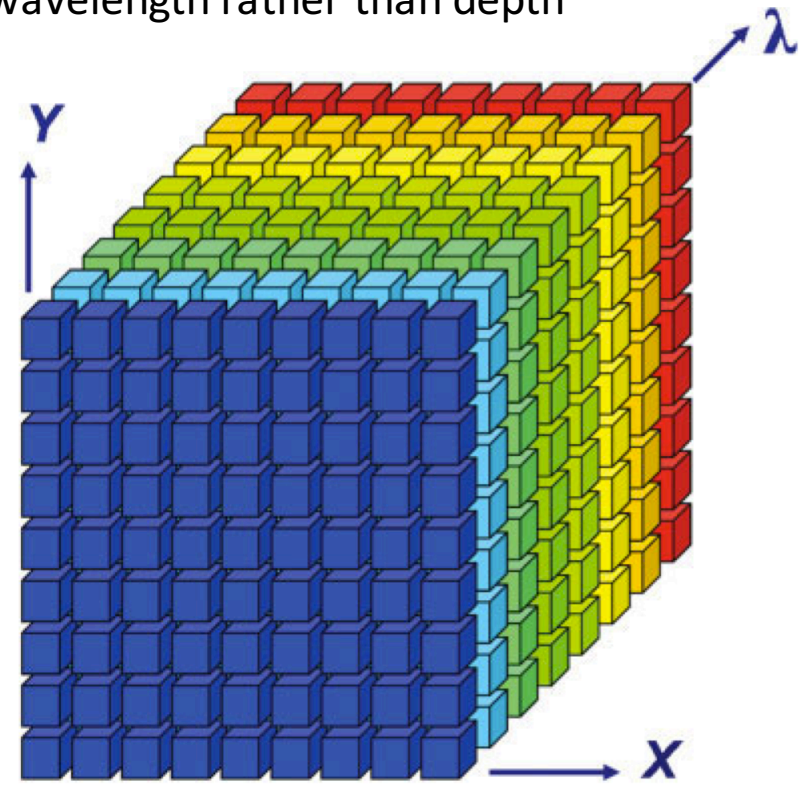
λ stack

Spectral detection



Dataset: λ stack

Like a Z-stack, but each slice represents wavelength rather than depth

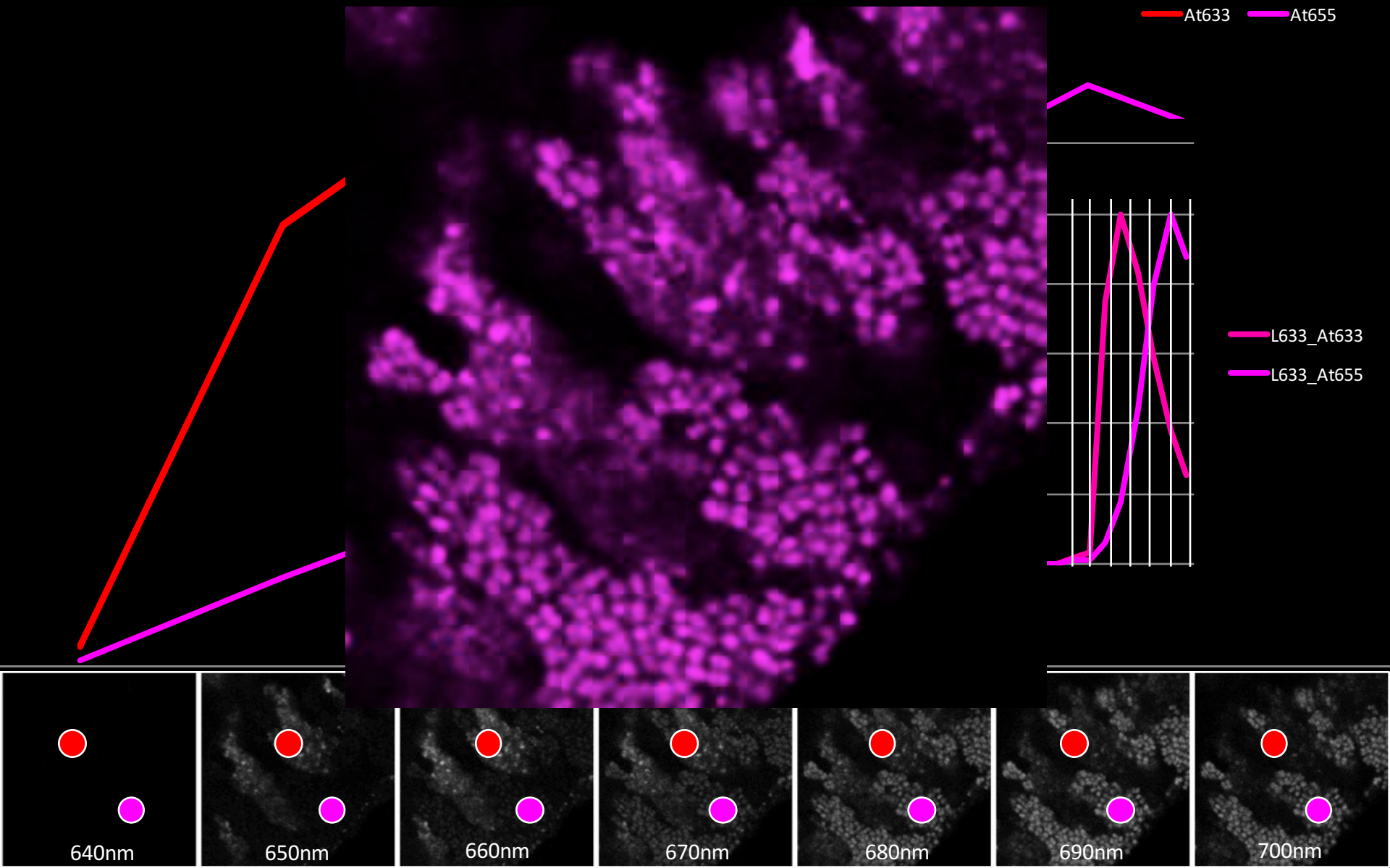


Spectral Image Data Cube

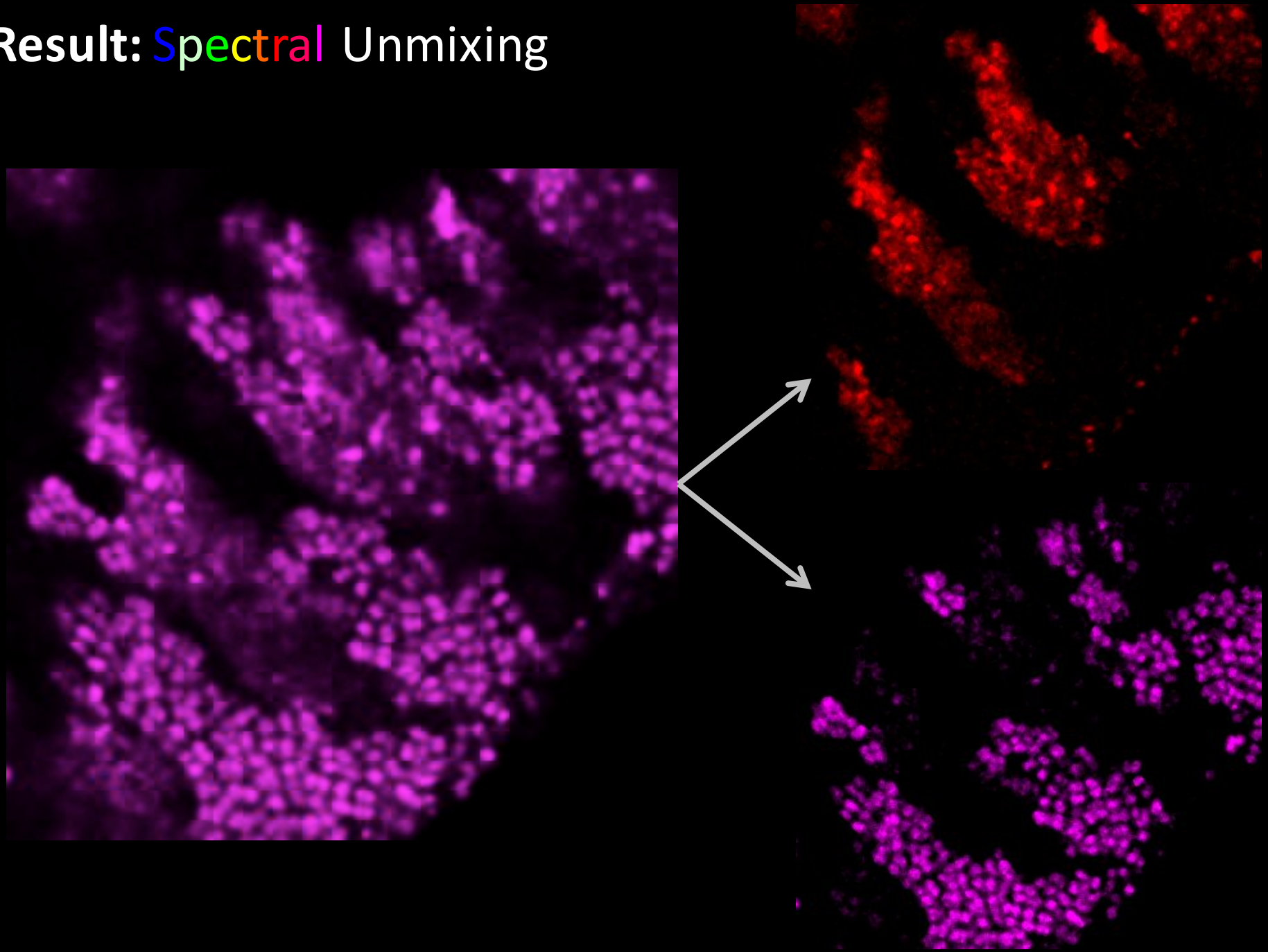
Garini Y, Young IT, McNamara G. 2006. *Cytometry*. **69A**:735-747.

Problem: Overlap

Solution: Spectral Imaging

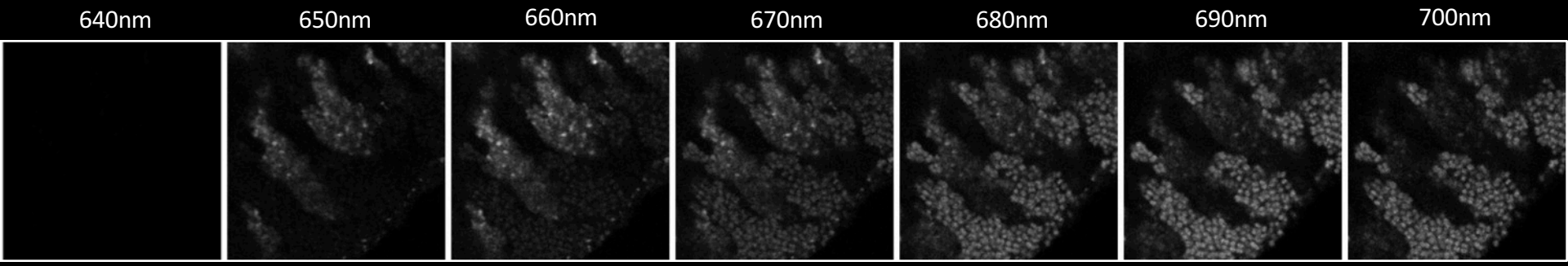


Result: Spectral Unmixing



How do we unmix these datasets?

Input: λ stack



Output: unmixed images

Linear unmixing

- 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

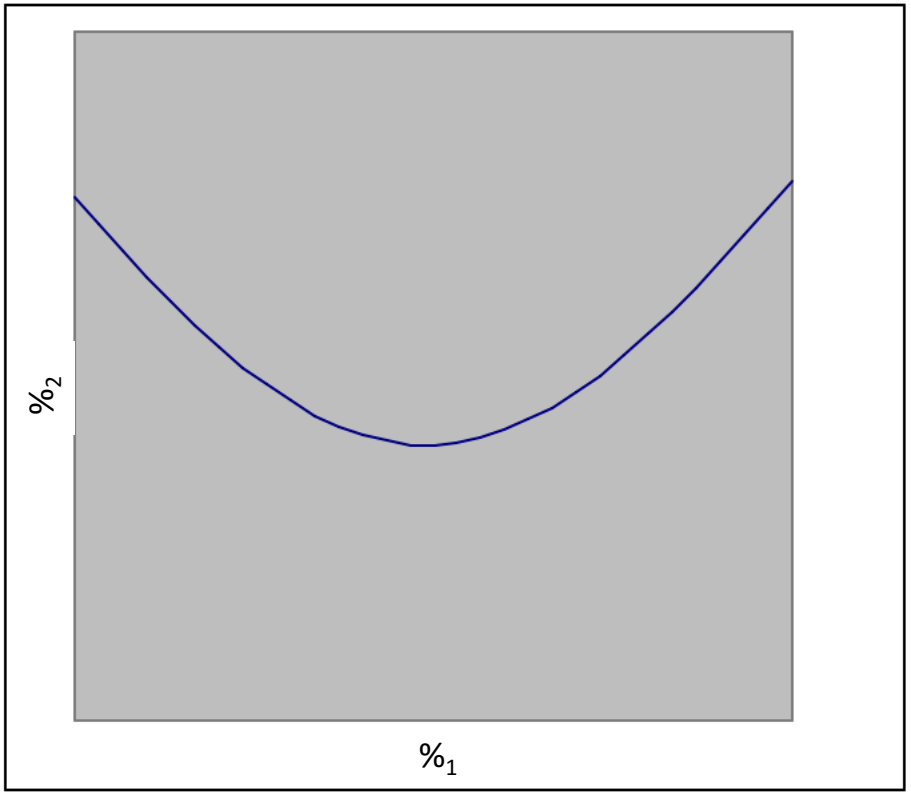
$$S(\lambda) - \%_1 * R1(\lambda) - \%_2 * R2(\lambda) = \text{minimum}$$

- 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

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

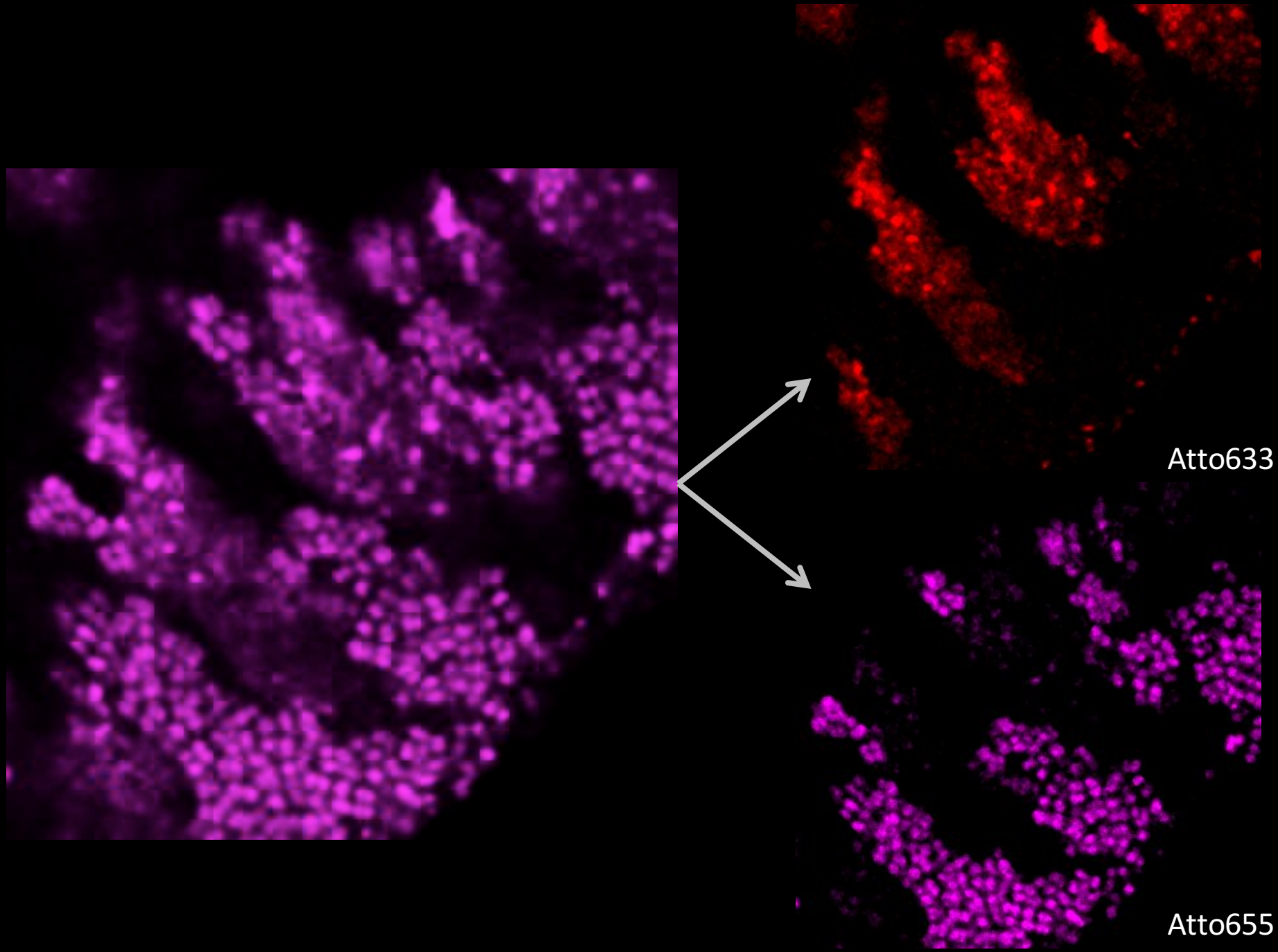


Compares the measured spectrum with all possible mixtures of reference spectra, and solves for the minimal difference between measured and reference spectra

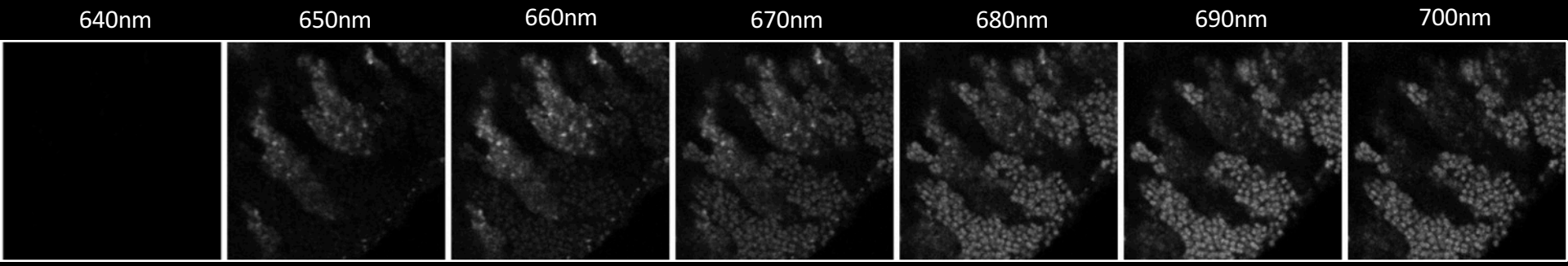
Results:
Values for $\%_1$ and $\%_2$ that tell you what proportion of your measured value belongs in each output file

Number of references must = number for fluorophores in the image.

```
parfor i = 1:N
    channels(:,i) = lsqnonneg(refSpectra, lambdaStack(:,i));
end
```

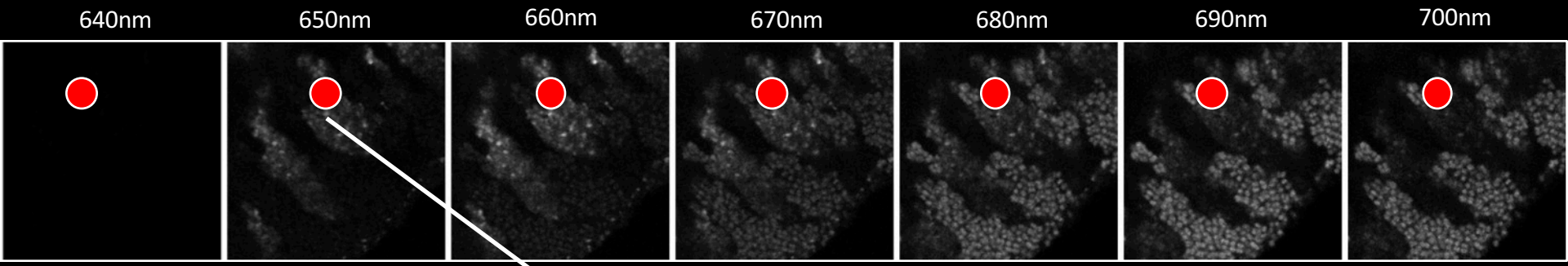

Atto633
Atto655

Input: λ stack



Output: unmixed images

Input: λ stack



Summed pixel gray value: 150

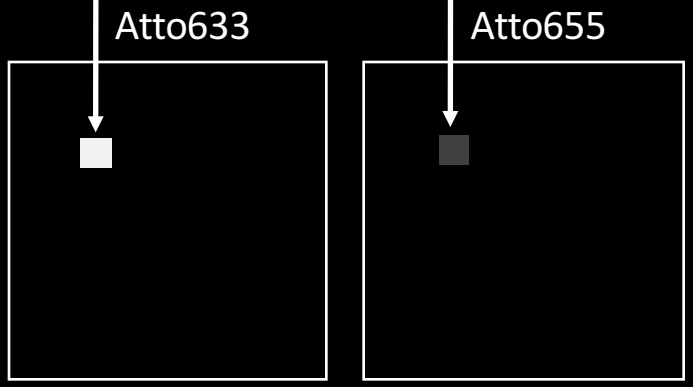
$\%_1 = 0.90$

$\%_2 = 0.10$

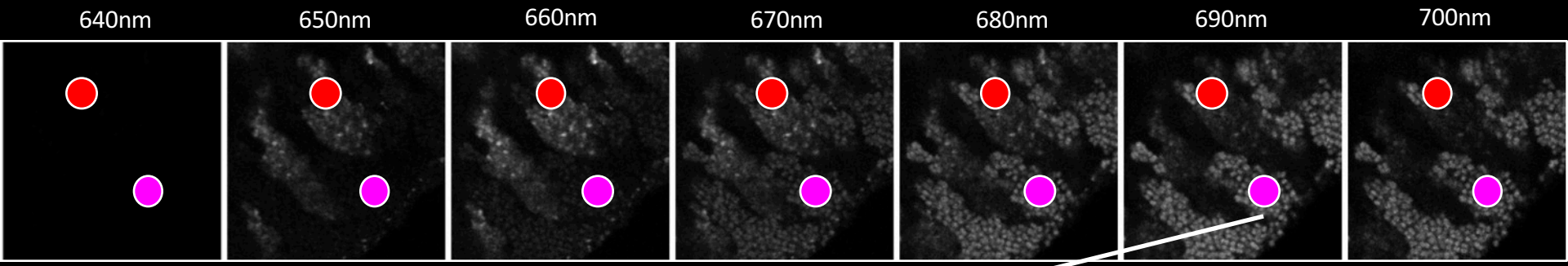
Atto633 = 150*0.90 = 135

Atto655 = 150*0.10 = 15

Output: unmixed images



Input: λ stack



Summed pixel gray value: 200

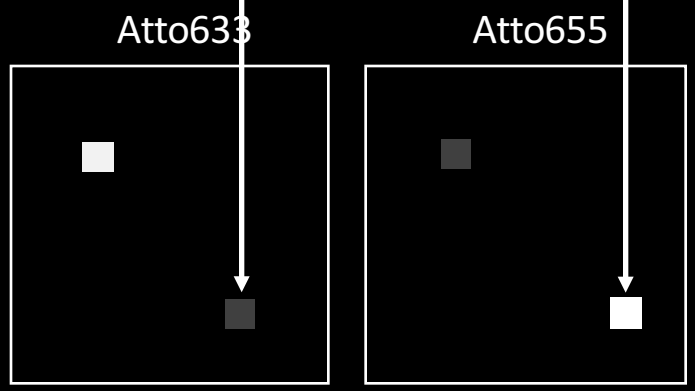
$\%_1 = 0.05$

$\%_2 = 0.95$

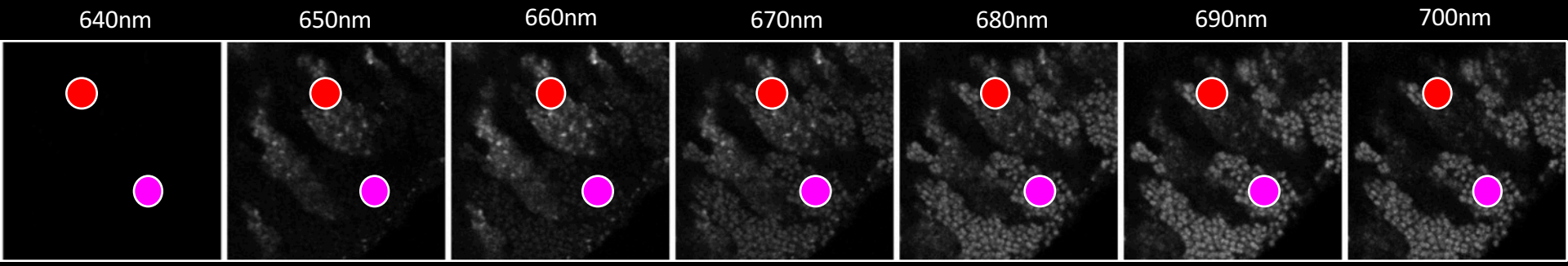
Atto633 = 200*0.05 = 10

Atto655 = 200*0.95= 190

Output: unmixed images



Input: λ stack



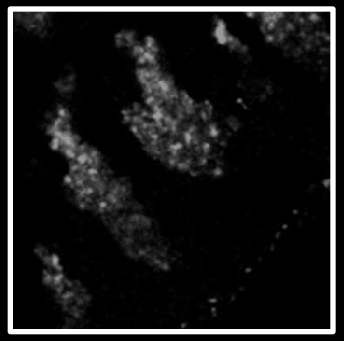
Over and over

and over

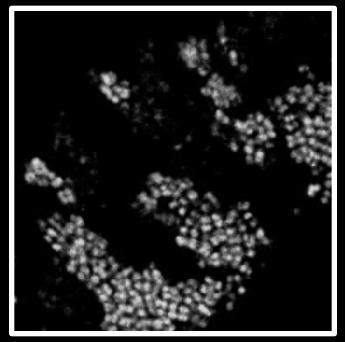
and over

Output: unmixed images

Atto633

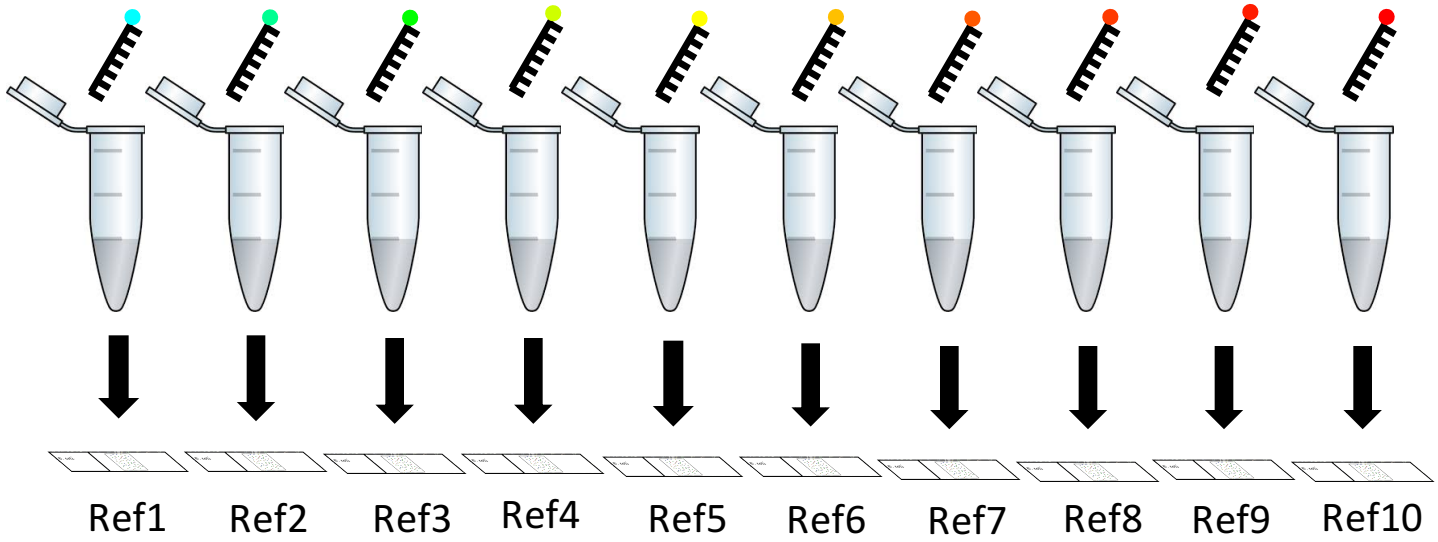


Atto655



Test - Unmixing 10 fluorophores

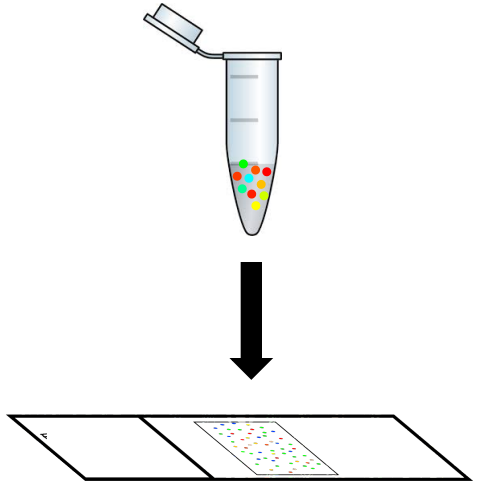
10 tubes *L. buccalis*
10 Probes added



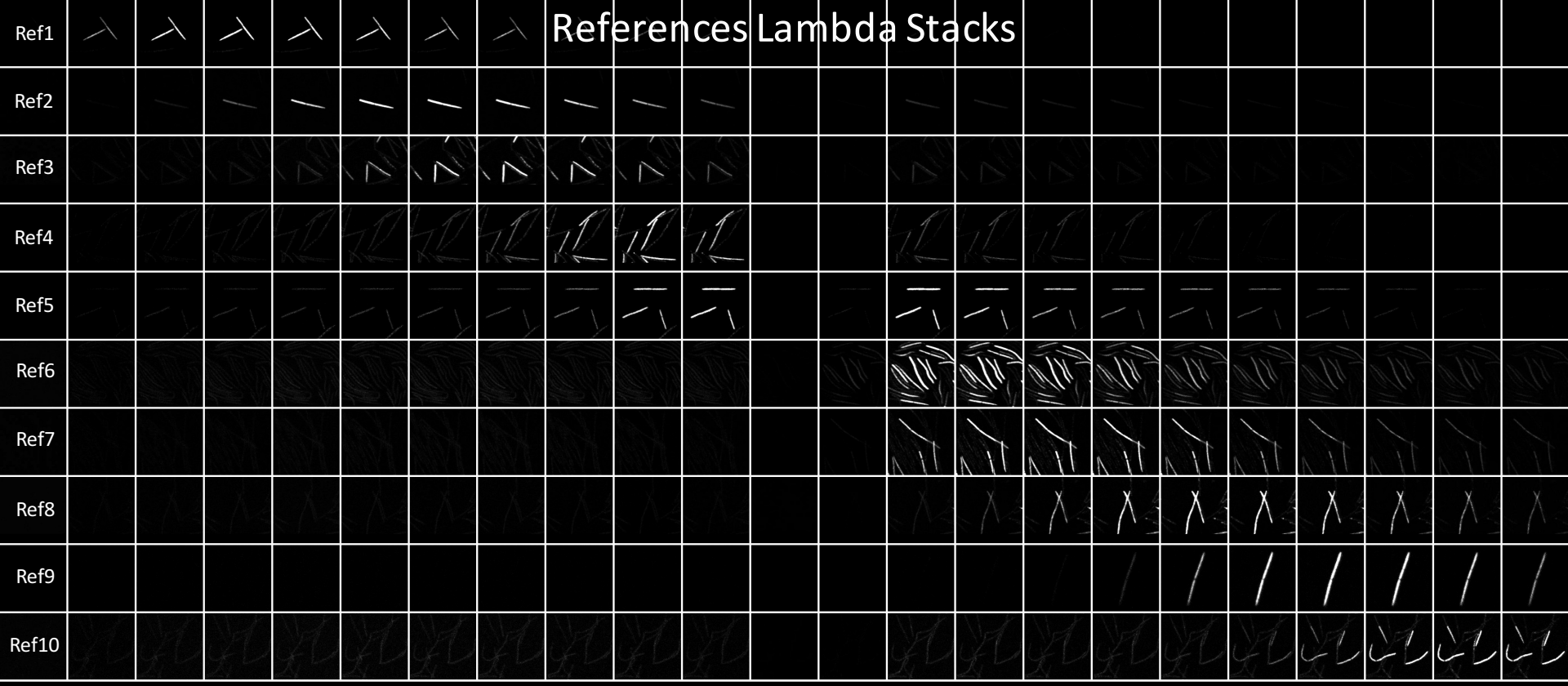
Reference slides

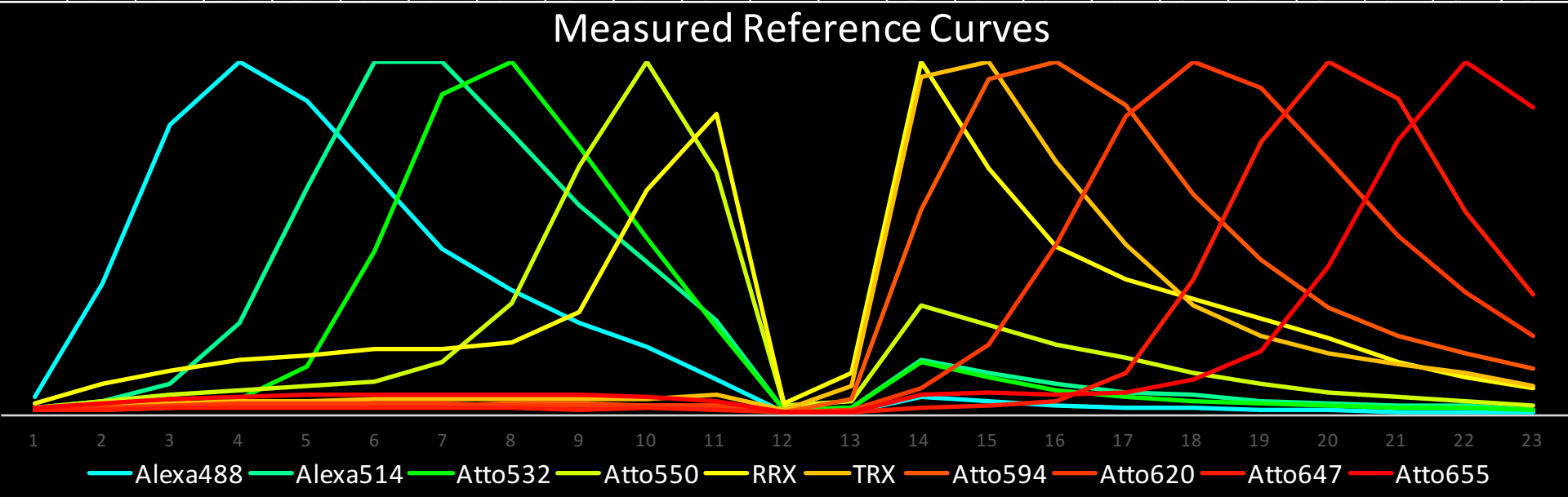
Ref1 Ref2 Ref3 Ref4 Ref5 Ref6 Ref7 Ref8 Ref9 Ref10

Mix labeled cells



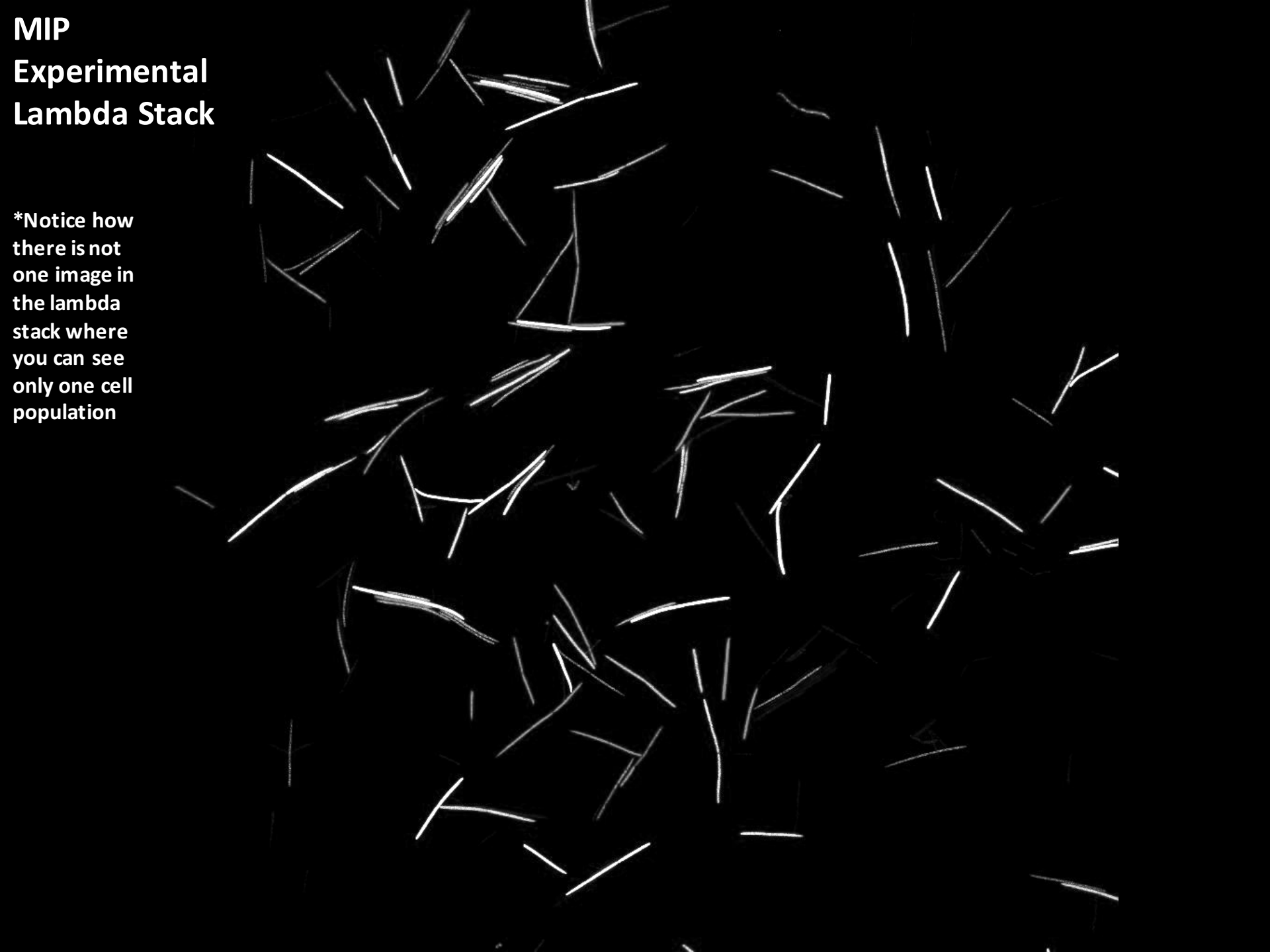
Mix slide

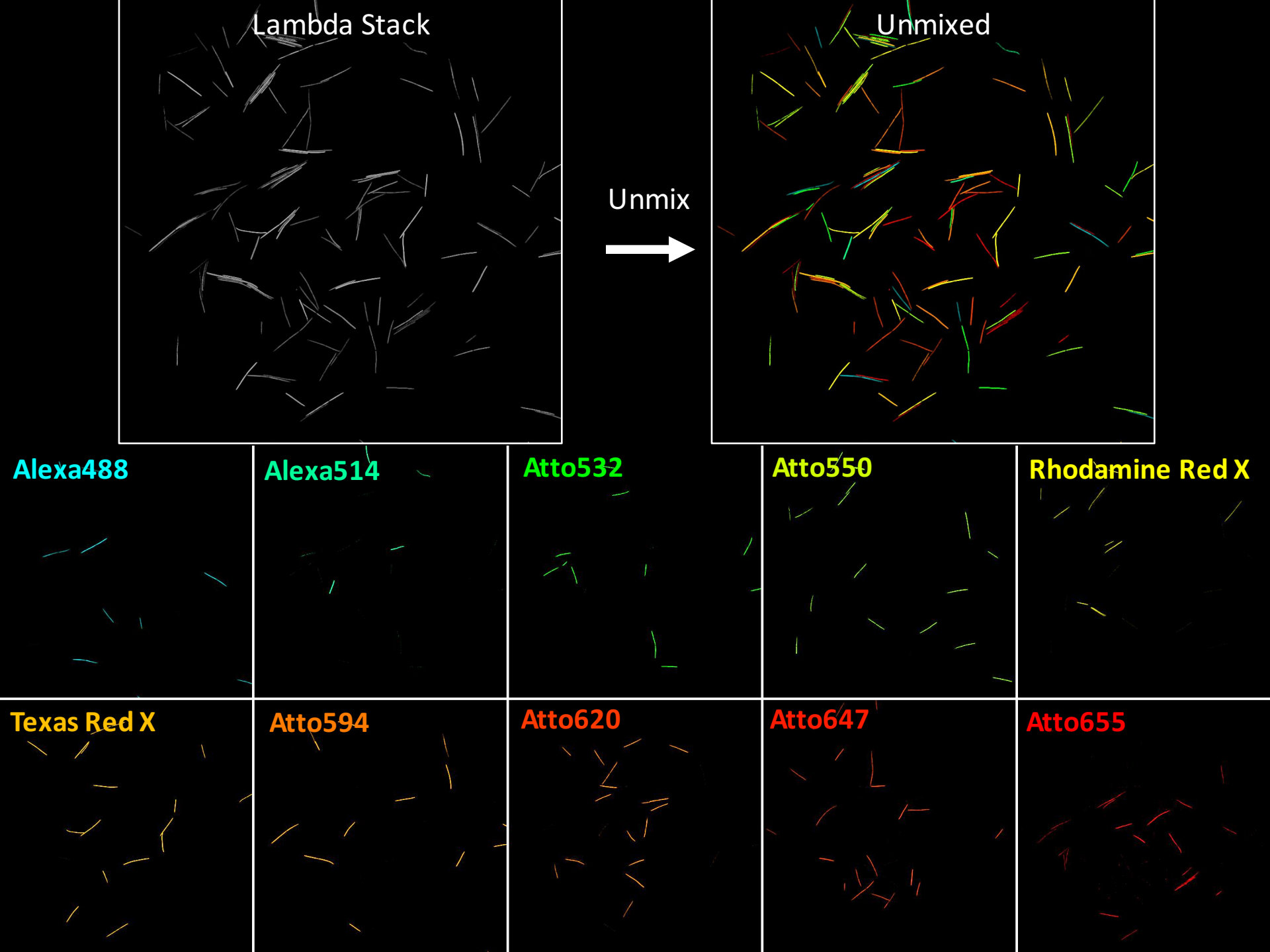

References Lambda Stacks
Ref1
Ref2
Ref3
Ref4
Ref5
Ref6
Ref7
Ref8
Ref9
Ref10


Measured Reference Curves
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
Alexa488
Alexa514
Atto532
Atto550
RRX
TRX
Atto594
Atto620
Atto647
Atto655

MIP Experimental Lambda Stack

*Notice how there is not one image in the lambda stack where you can see only one cell population

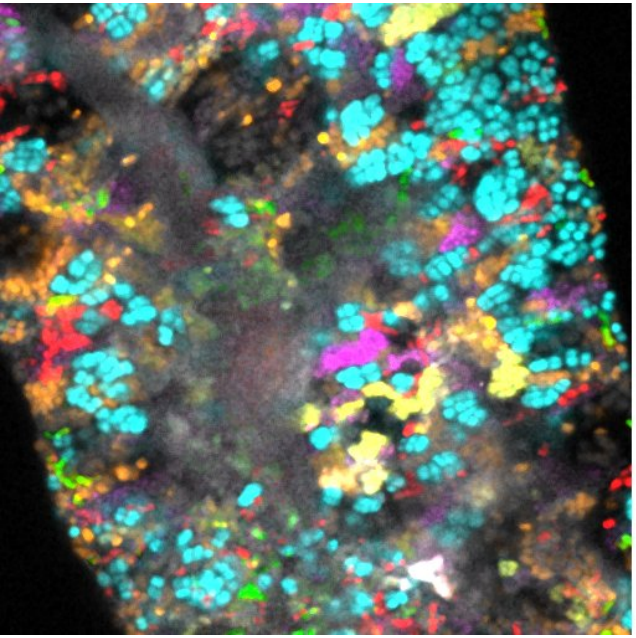



Lambda Stack
Unmix
Unmixed
Alexa488
Alexa514
Atto532
Atto550
Rhodamine Red X
Texas Red X
Atto594
Atto620
Atto647
Atto655

Can we unmix more than fluorophore spectra?

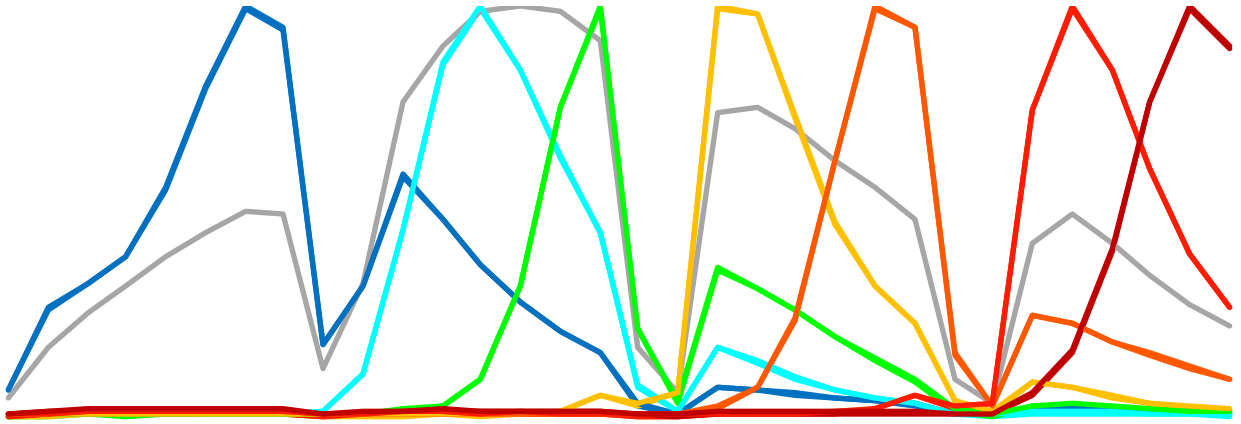
Removing Autofluorescence

Unmixed channels + AF



AF

Unmixed Channels - AF



Spectral Summary

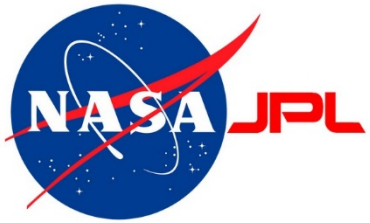
1. Use spectrally separated fluorophores when you can, if not possible, spectral imaging and unmixing!
2. Methods: generating spectra by selectively imaging one wavelength at a time, or imaging a range of wavelengths simultaneously.
3. Can be used for separating highly overlapping spectra and removing 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.

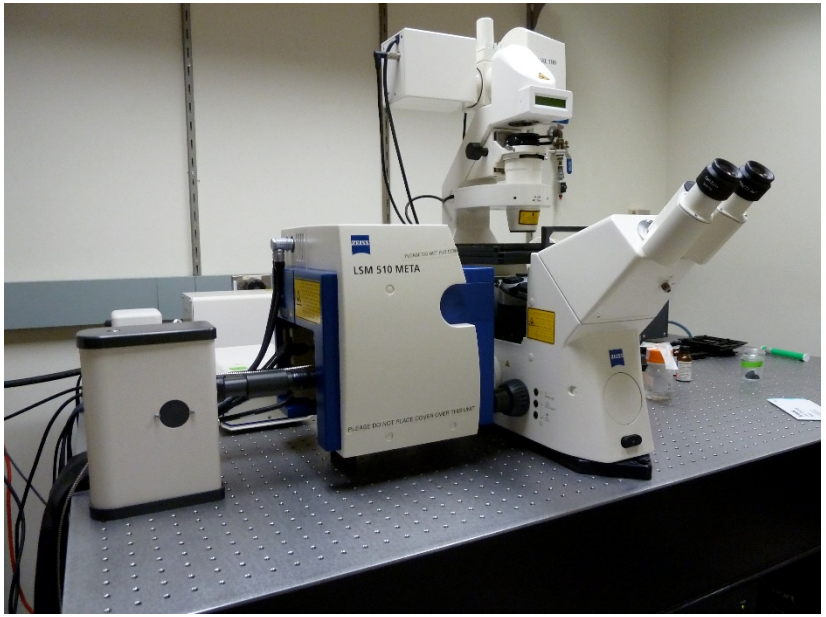
History of the Zeiss spectral detector

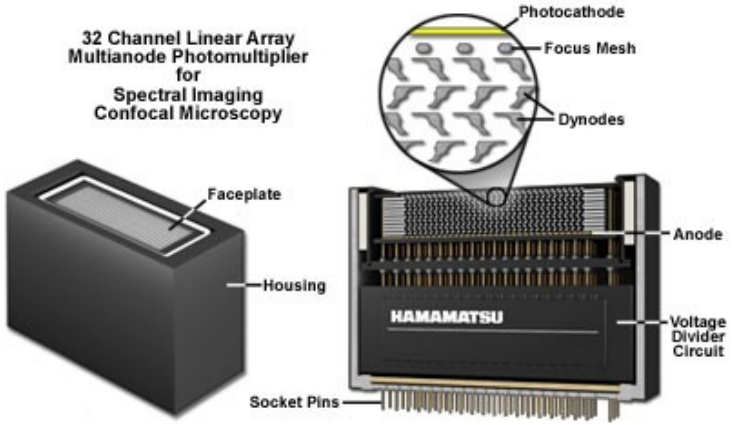
- Where did the idea of a multichannel detector come from?
- Collaboration between the Jet Propulsion Laboratory, Scott Fraser's lab here at Caltech and Zeiss



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 unmxing

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/