K-Means Spectral Unmixing for Fluorescence Microscopy

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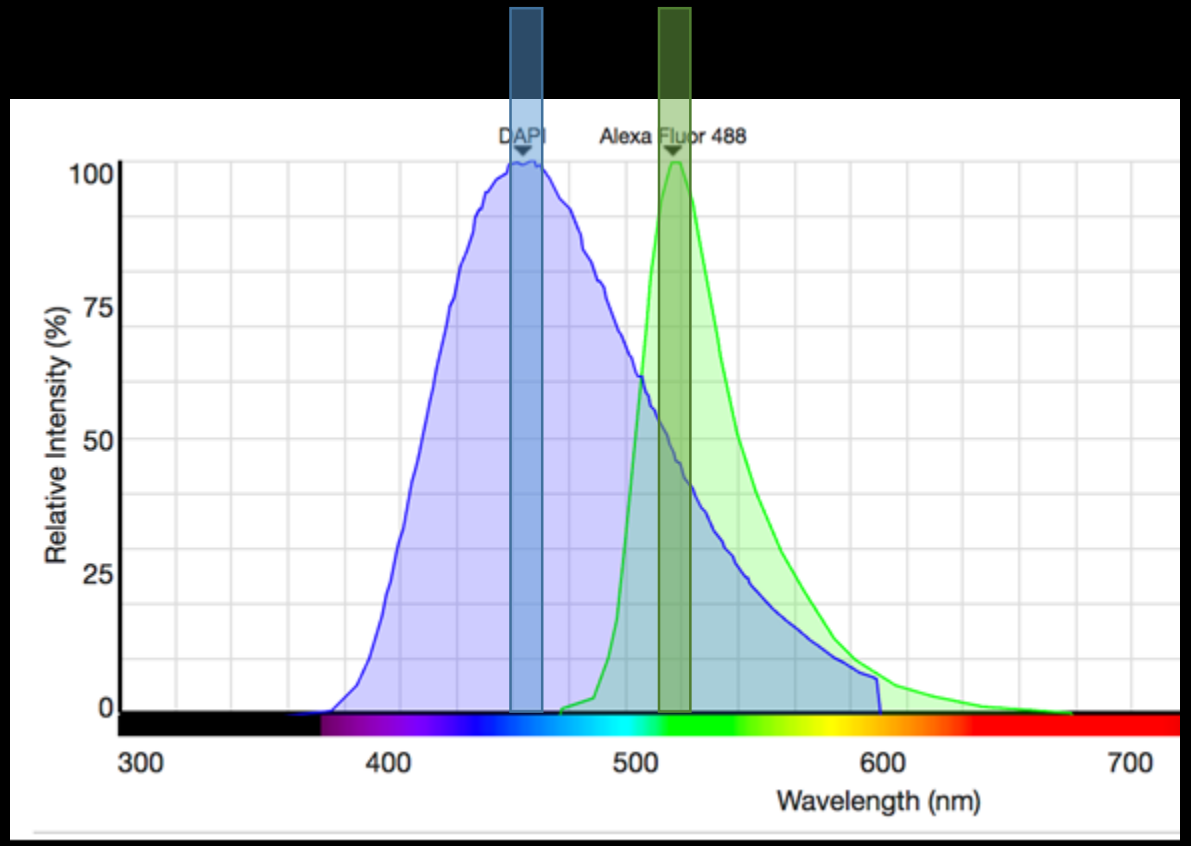
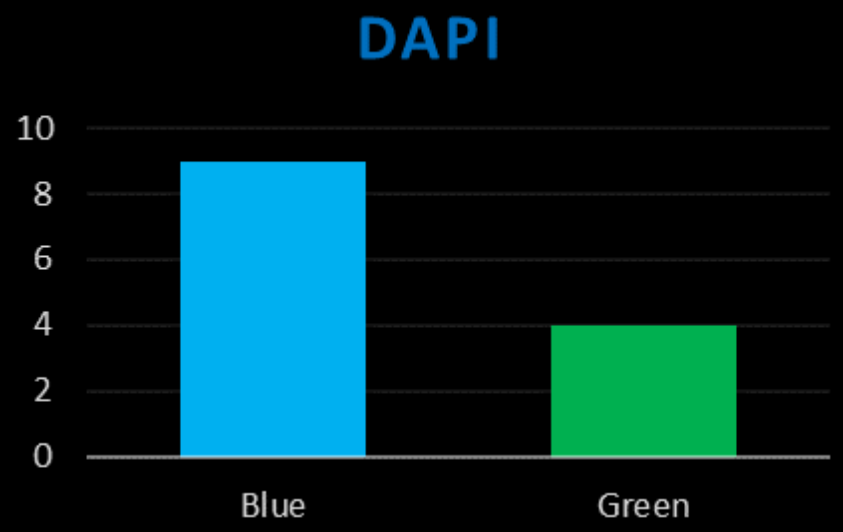
Emission spectra overlap

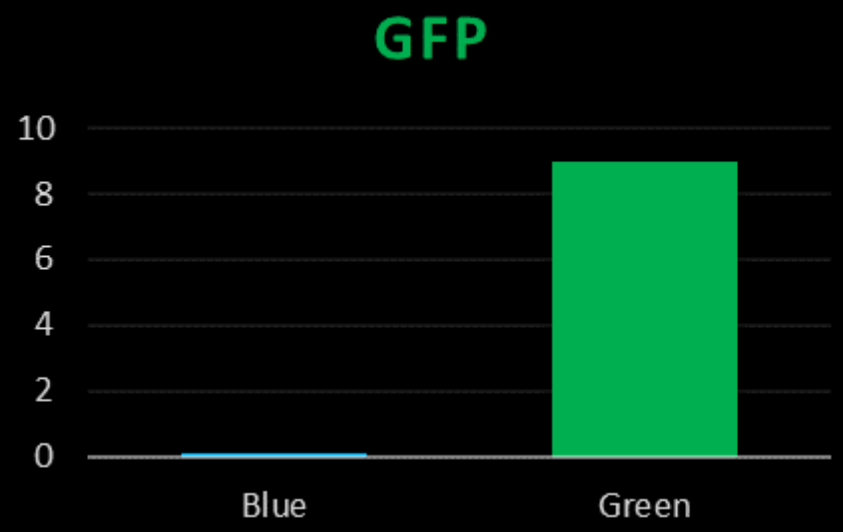


- Overlapping emission spectra of fluorophores make them show up in multiple channels.
- Different fluorophores have different intensity “signatures” in different channels.

Image source: https://www.thermofisher.com/us/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html

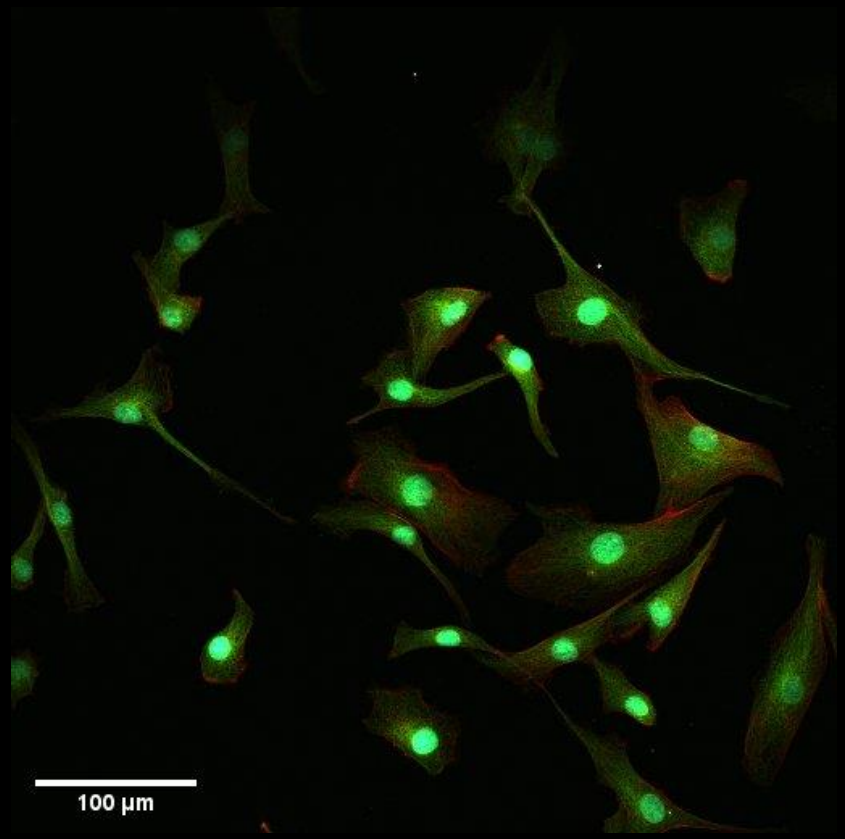
Fluorophores have Unique Signatures Across Channels





Channel bleed-through contaminates image data

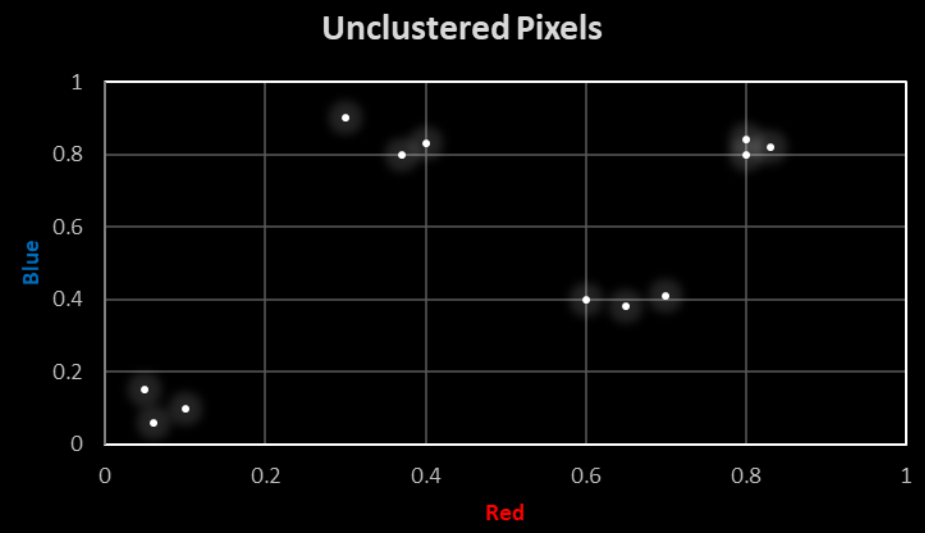
Nuclei (DAPI) Microtubules (BODIPY) F-actin (Texas Red)

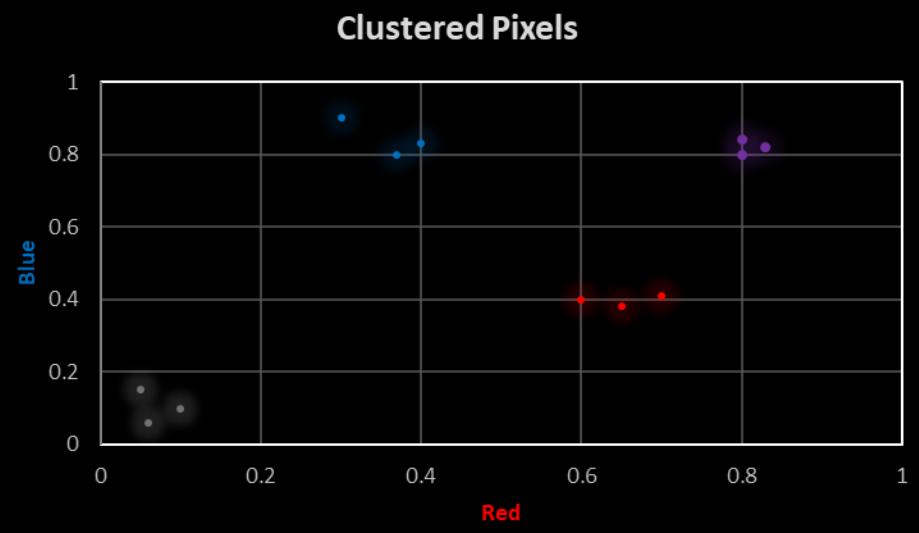


- Nuclei show up in both blue and green channels making the green channel mixes with both nuclei and microtubules information
- Blue channel only has nuclei, while green channel has both nuclei and microtubules.
- Fluorophores have different intensity “signatures” in different channels.

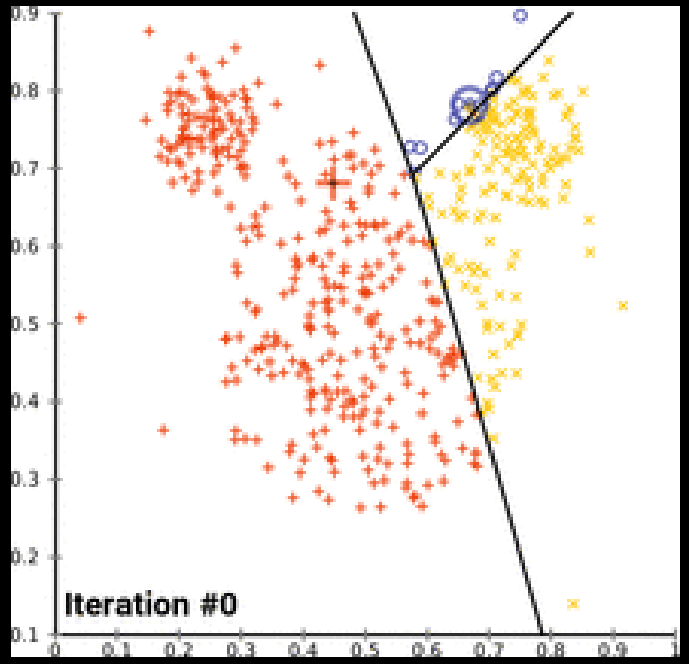
Pixel intensities group into clusters for different fluorophores

More colors exist than channels to detect them
Individual pixels are grouped into K=4 different clusters based on their intensities in each color channel





K-Means Clustering algorithm

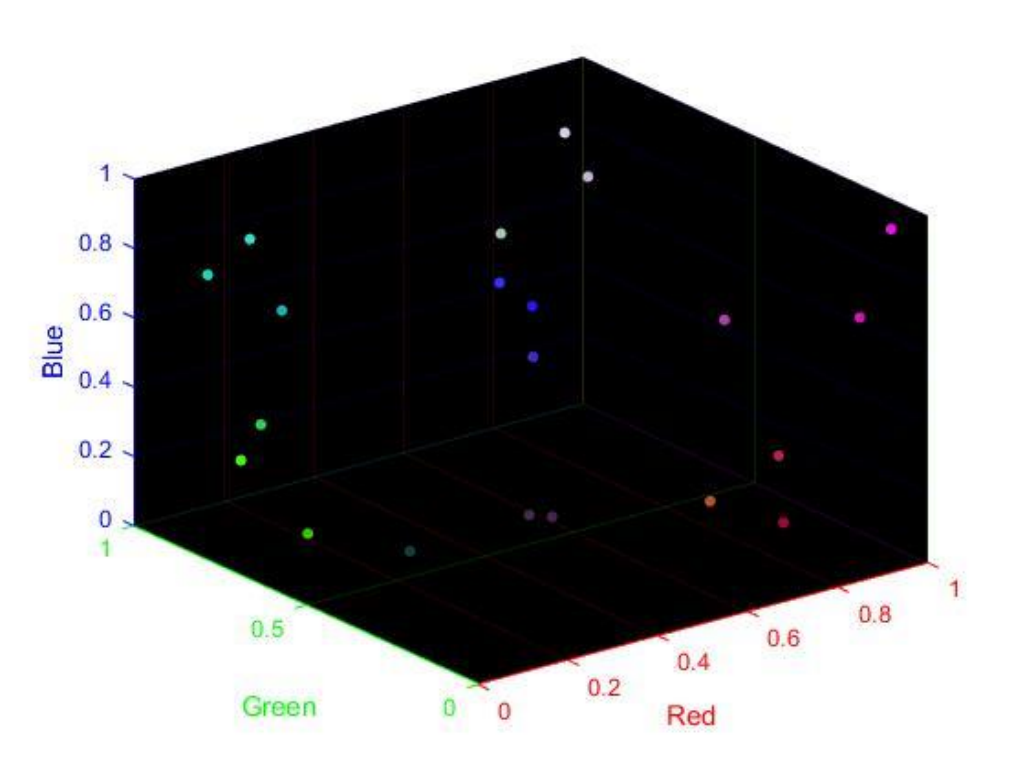


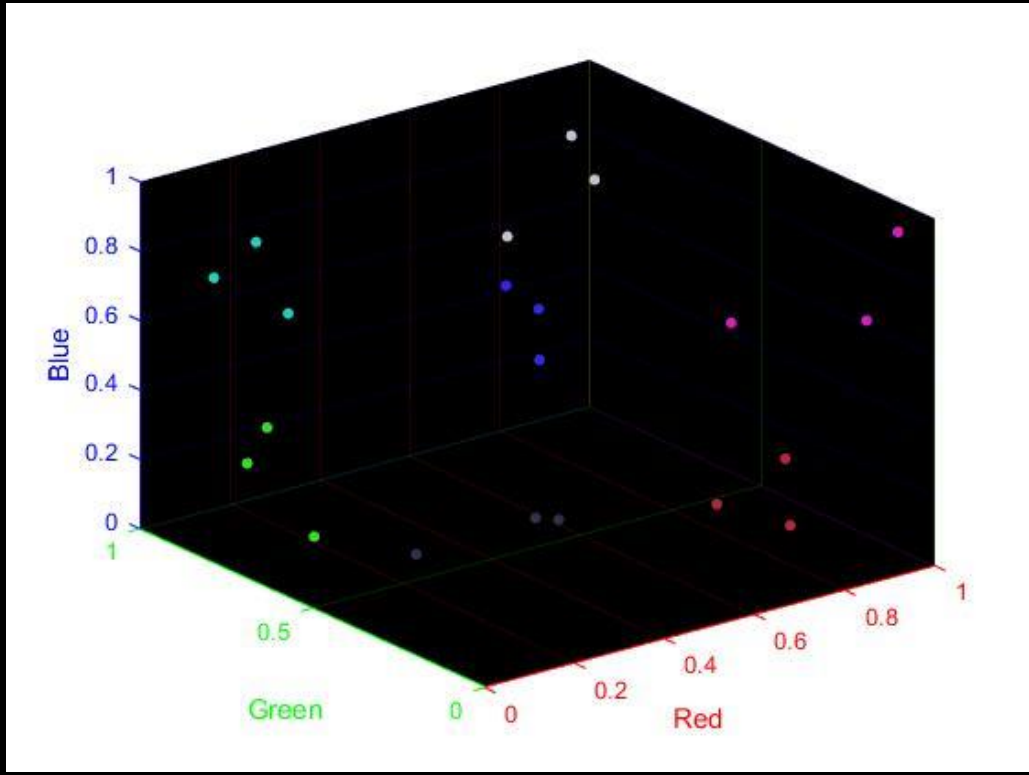
K-Means algorithm:

1. Initialize K semi-random cluster centroids or “means”
2. Assign data points to the cluster with the closest mean
3. Compute the new mean of each cluster
4. Iterate steps 2 and 3 until convergence

https://en.wikipedia.org/wiki/K-means_clustering#/media/File:K-means_convergence.gif

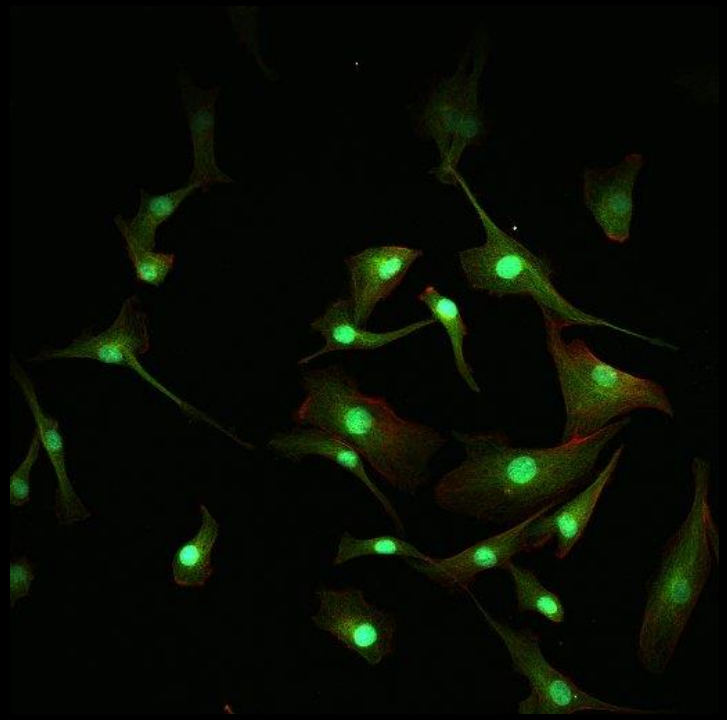
K-Means Clustering works for any number of channels



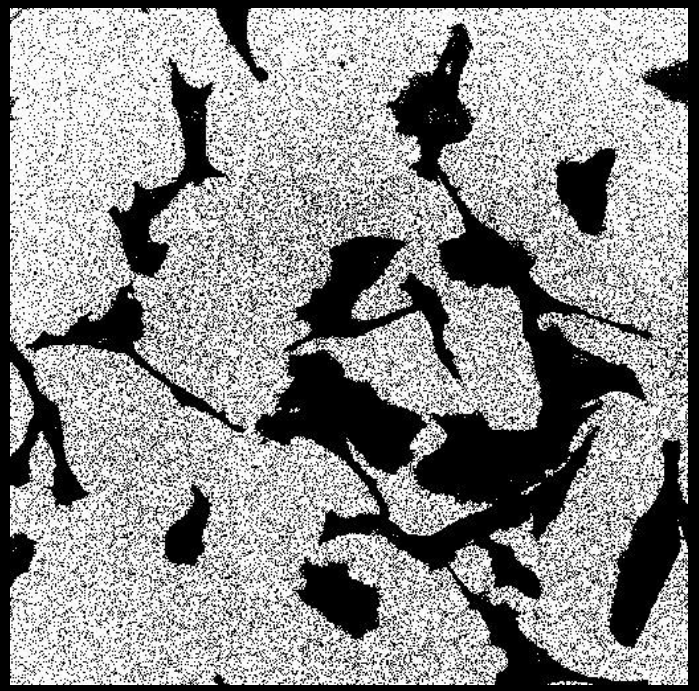


Background as a cluster to remove background noise

Original Image

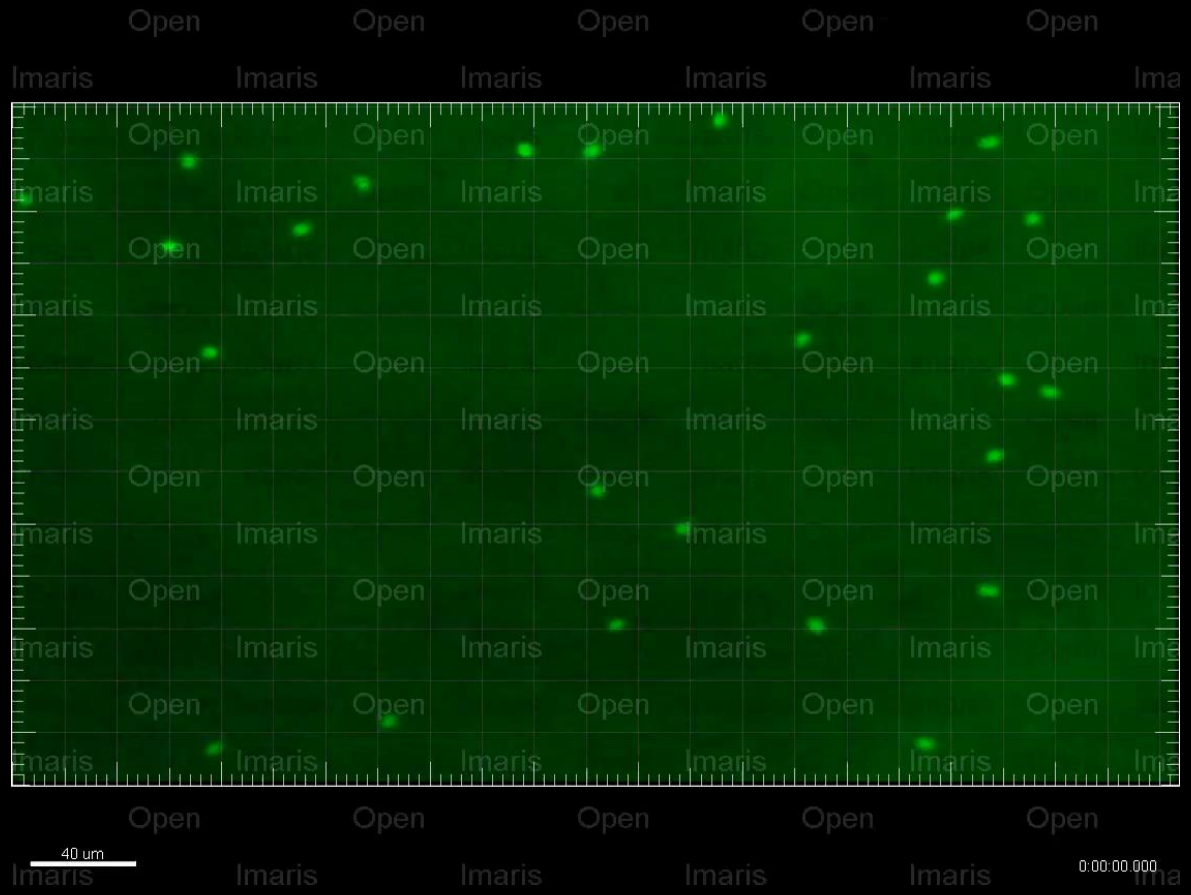


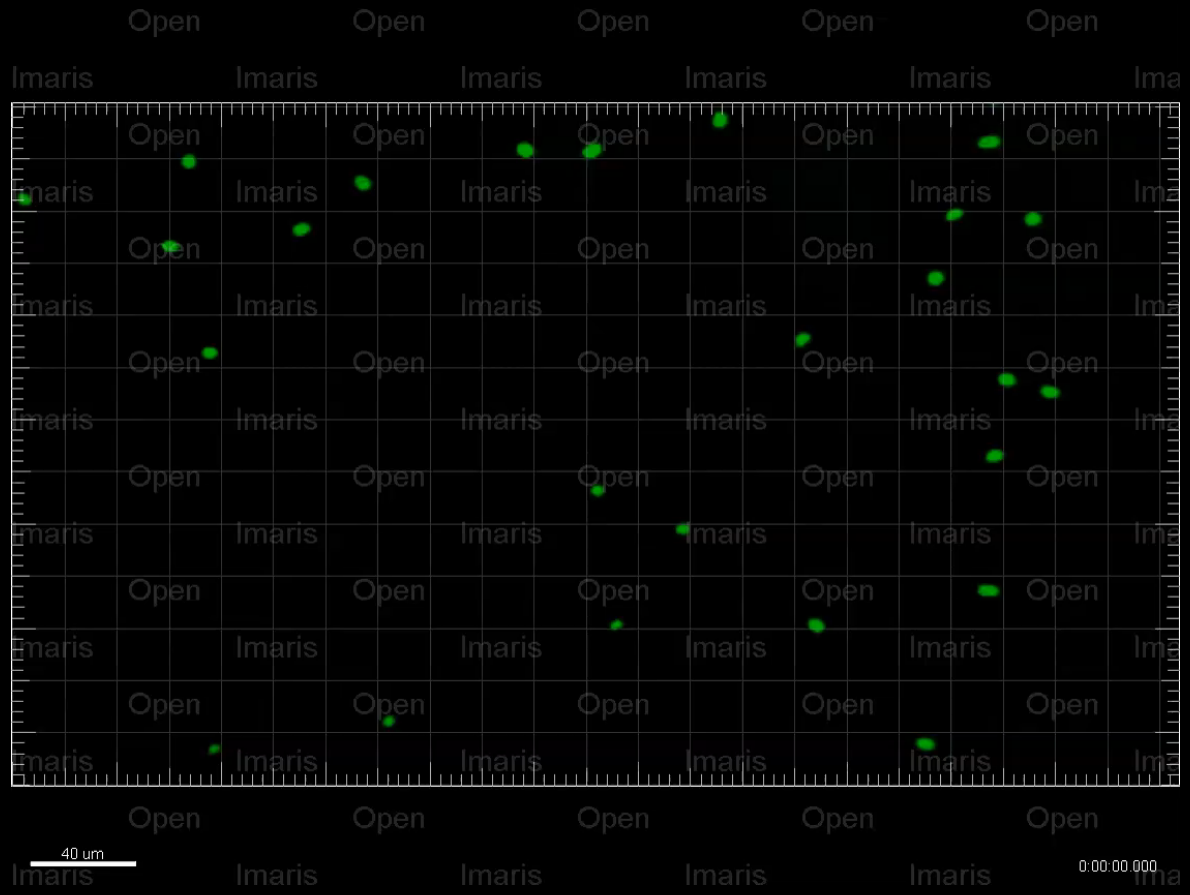
Background Cluster



Pixels with low intensities group together into a cluster than can be removed

Example: Background Removal on Algae





Example 1: BPAE Cell Fixed Slide

Original Image

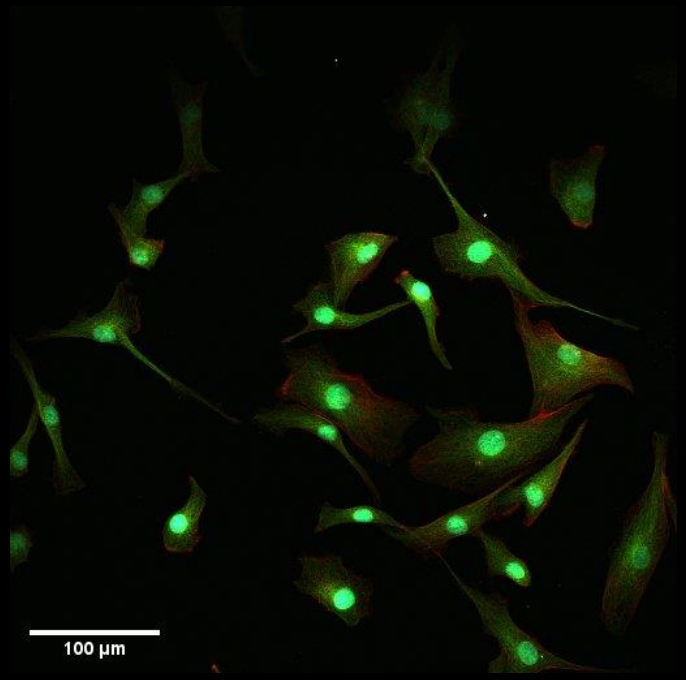
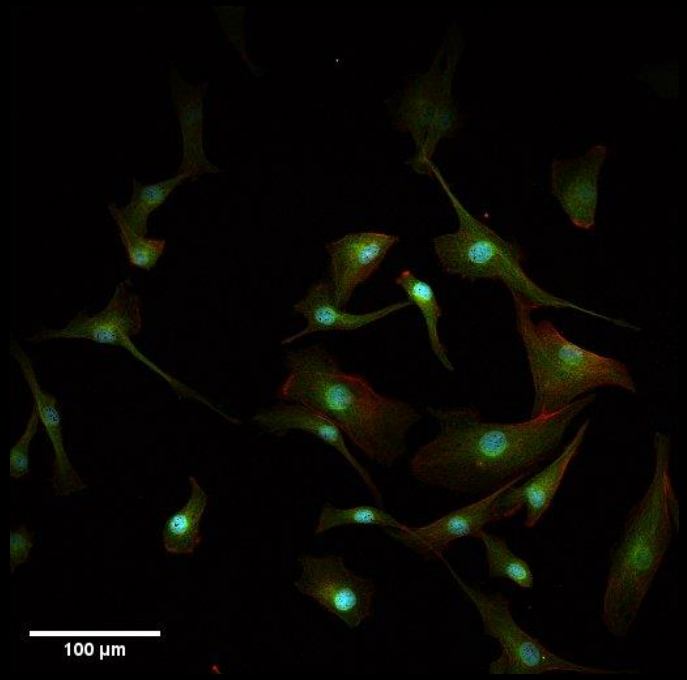


Image unmixed with K - means

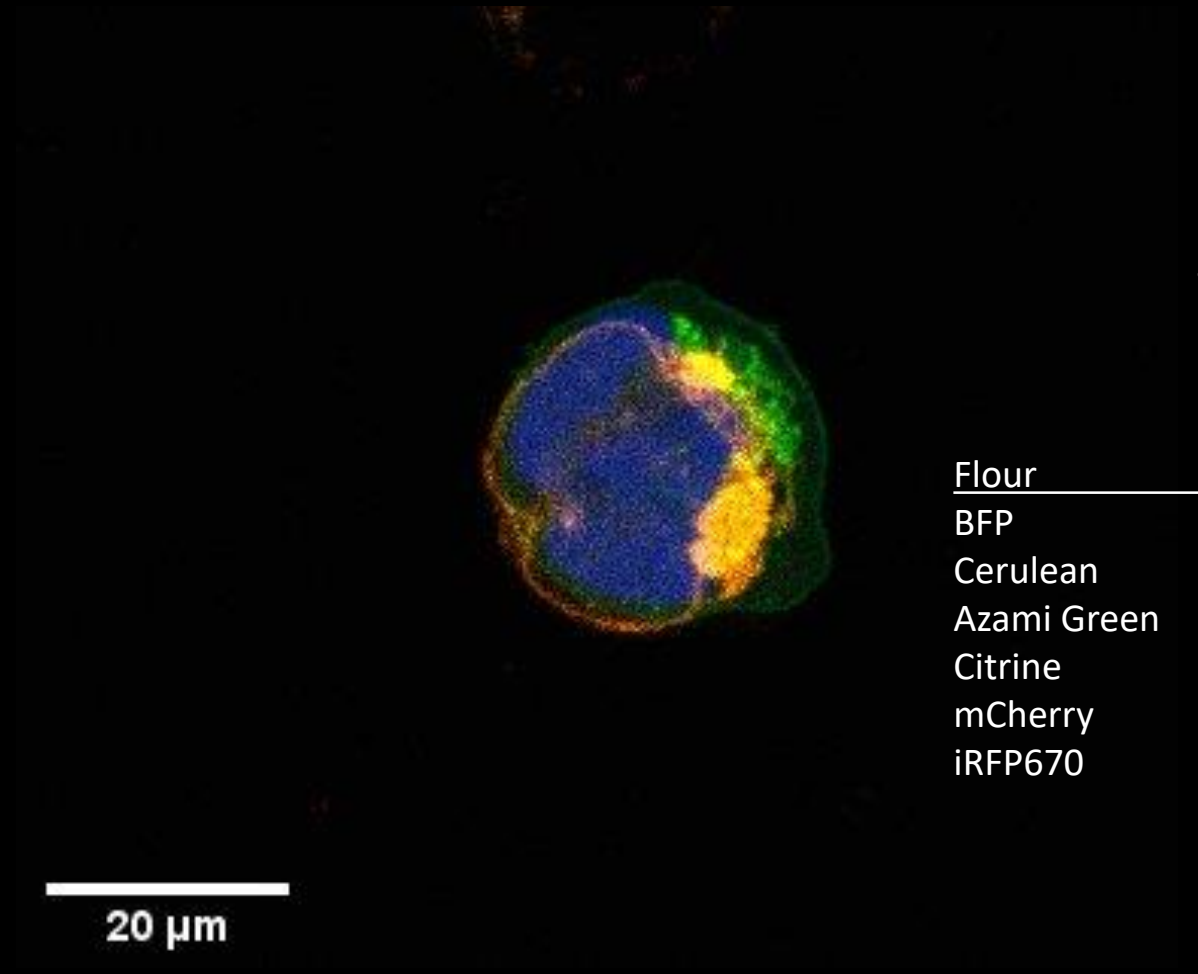


K-means is able to separate out the nuclei from the green channel.

Note the lack of background noise in the unmixed version as the background cluster is easily removed

Nuclei (DAPI) Microtubules (BODIPY) F-actin (Texas Red)

Example 2: Cultured “Colorful” T Cells



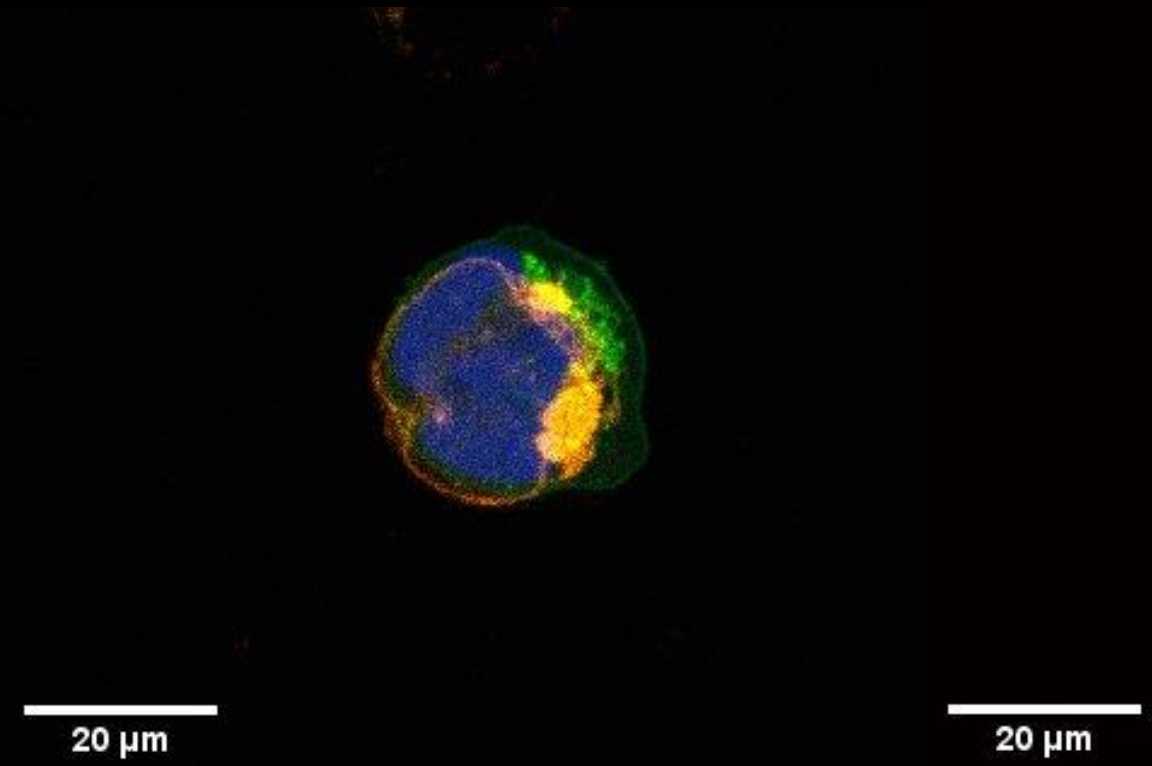
Lasers: MaiTai 840nm, Insight 1050nm,
Sequential Scan
Cubes: BFP/GFP, nRed/fRed Cubes

Flour	Structure	Image Color	Wavelength (Excitation, Emission, Cube)		
BFP	Nucleus	Blue	(840nm,	420-500nm,	BFP)
Cerulean	Membrane	Green	(840nm,	465-505nm,	GFP)
Azami Green	Mitochondria	Green	(1050nm,	496-536nm,	GFP)
Citrine	Golgi	Orange	(840nm,	515-565nm,	GFP)
mCherry	ER	Red	(1050nm,	605-635nm,	nRed)
iRFP670	Peroxisomes	Yellow	(1050nm,	650-675nm,	fRed)

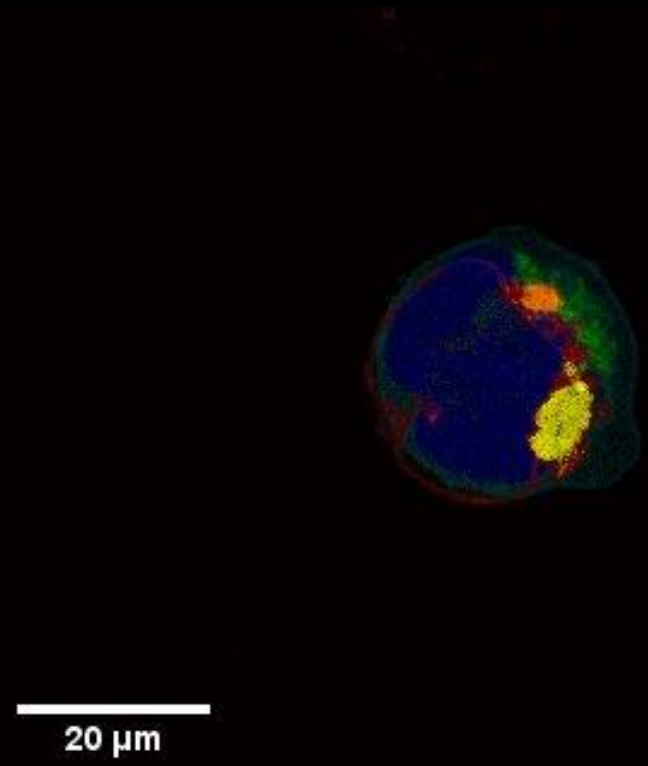
Images courtesy of David Oleksyn, Program for Advanced Immune Bioimaging
Colorful 840-1050 BFP-GFP cube-high qual-3

Example 2: Cultured “Colorful” T Cells

Original



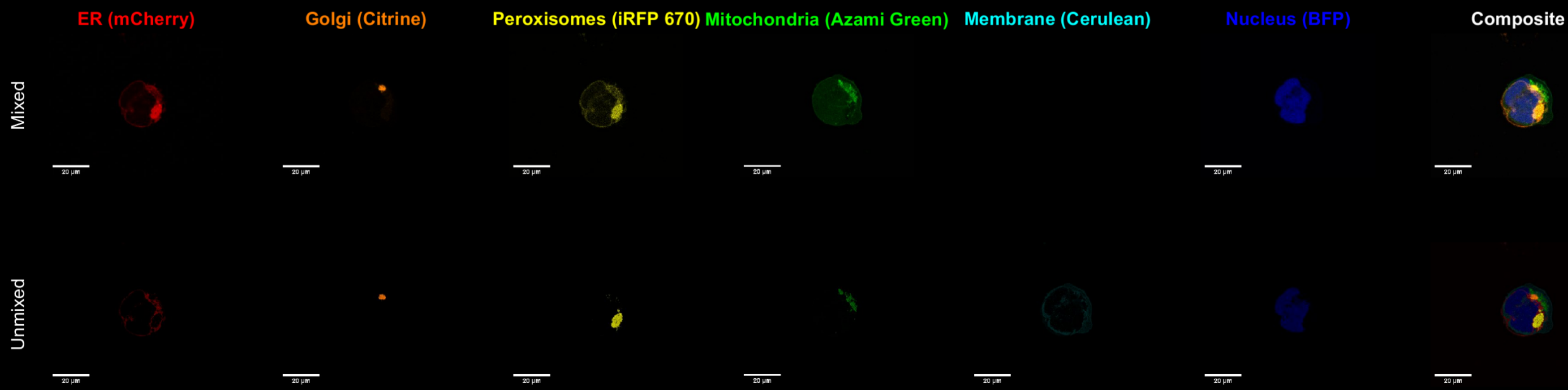
Unmixed



K-means generalizes well in situations with more fluorophores and channels such as this example with 6 fluorophores and 5 channels

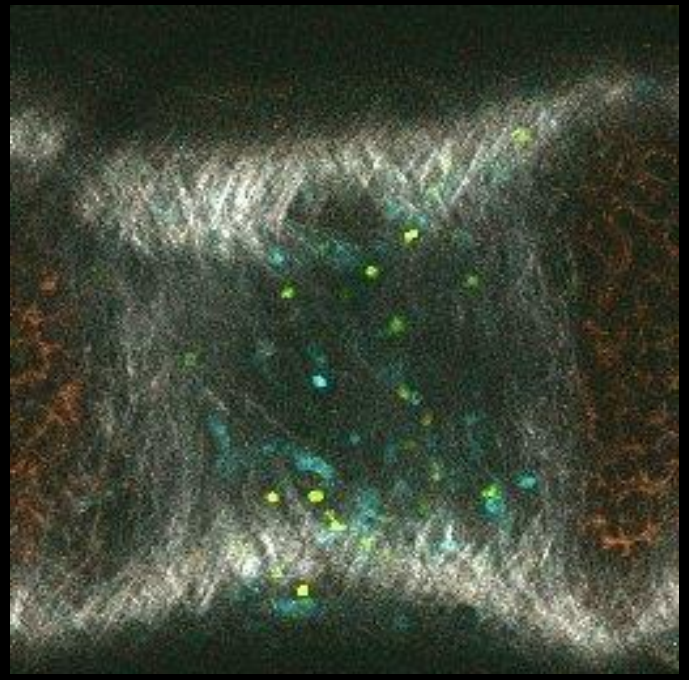
Images courtesy of David Oleksyn, Program for Advanced Immune Bioimaging
Colorful 840-1050 BFP-GFP cube-high qual-3

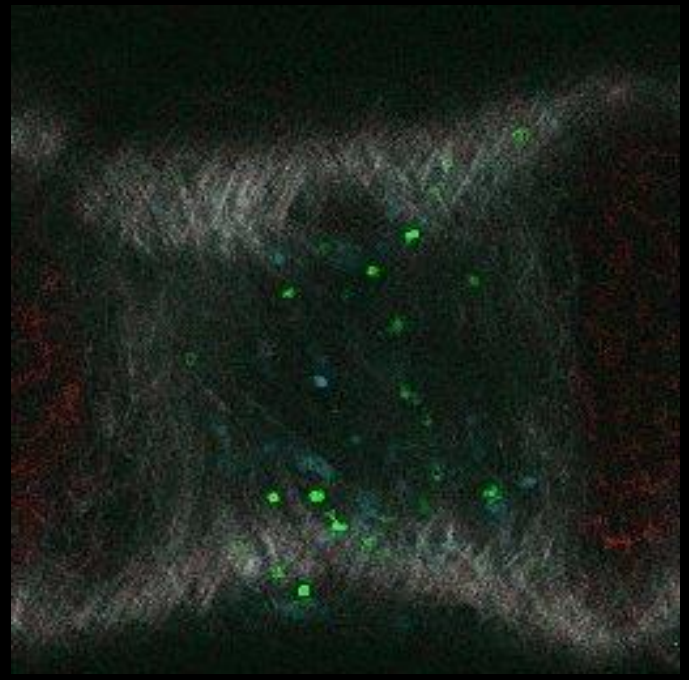
Cultured “Colorful” T Cells Channel Breakdown



Images courtesy of David Oleksyn, Program for Advanced Immune Bioimaging
Colorful 840-1050 BFP-GFP cube-high qual-3

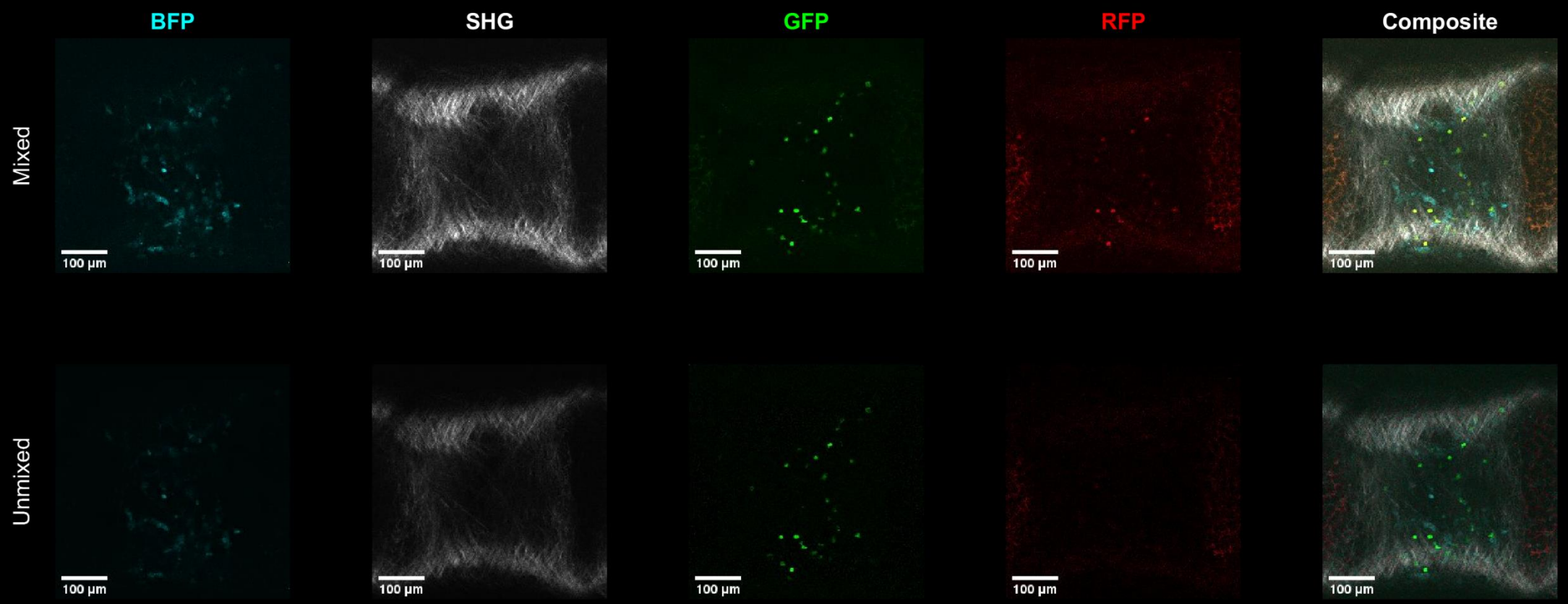
Example 3: In Vivo Mouse Trachea Sample





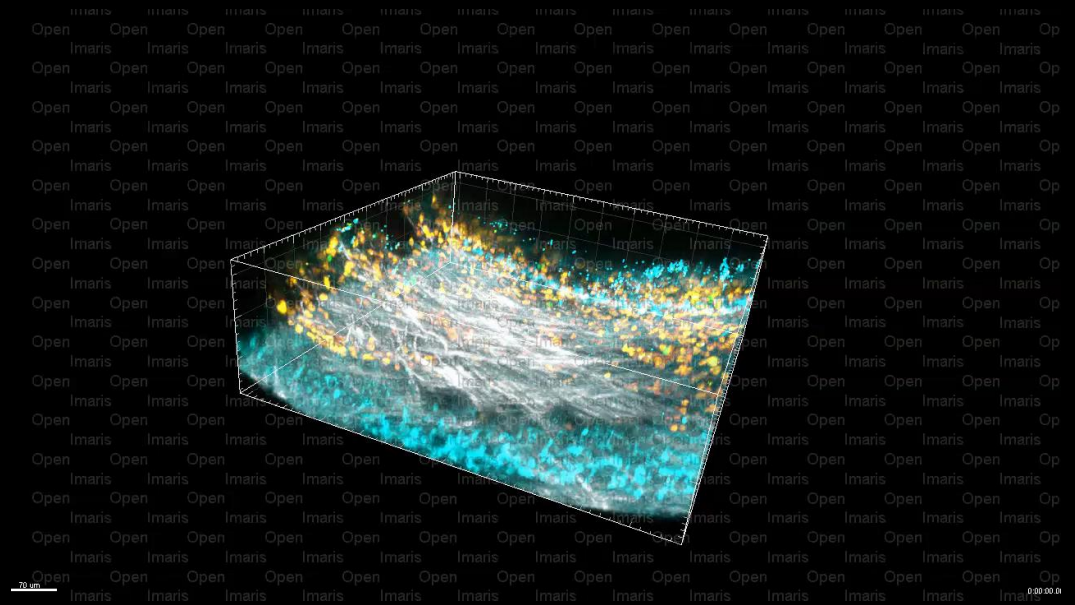
Images courtesy of Kris Lambert and Emma Reilly, Program for Advanced Immune Bioimaging
090618 D7 Rex3 MHC NT-1

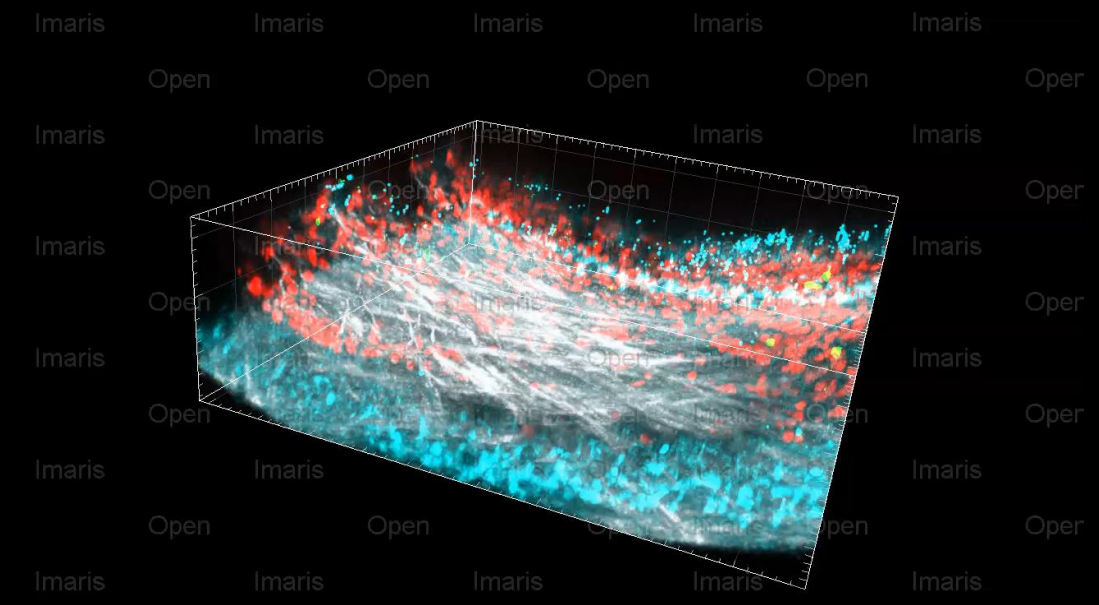
In Vivo Mouse Trachea Channel Breakdown



Images courtesy of Kris Lambert and Emma Reilly, Program for Advanced Immune Bioimaging
090618 D7 Rex3 MHC NT-1

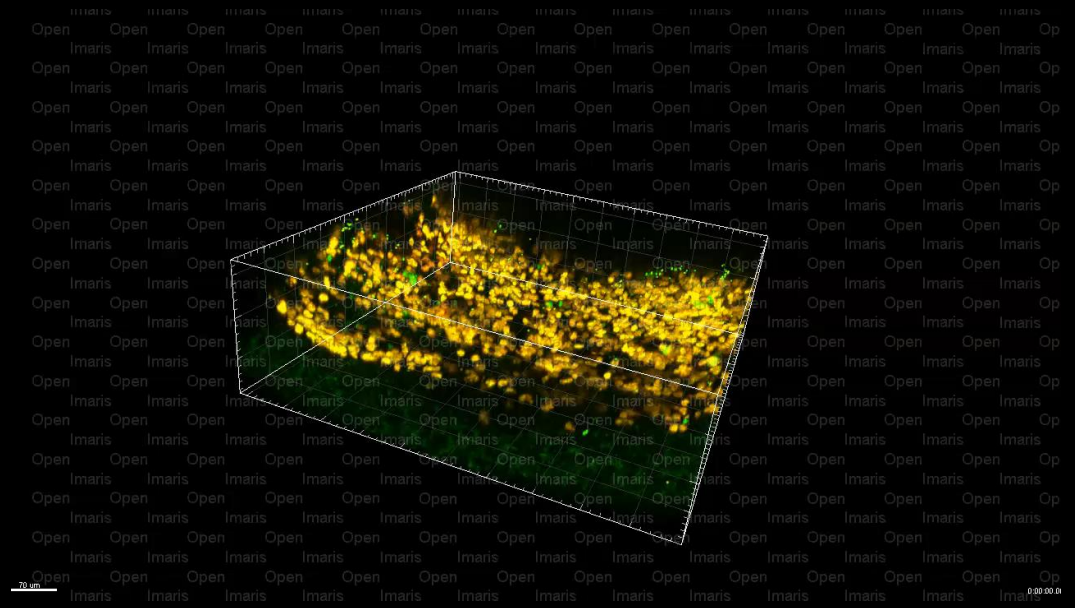
Unmixing Works for 3D Videos

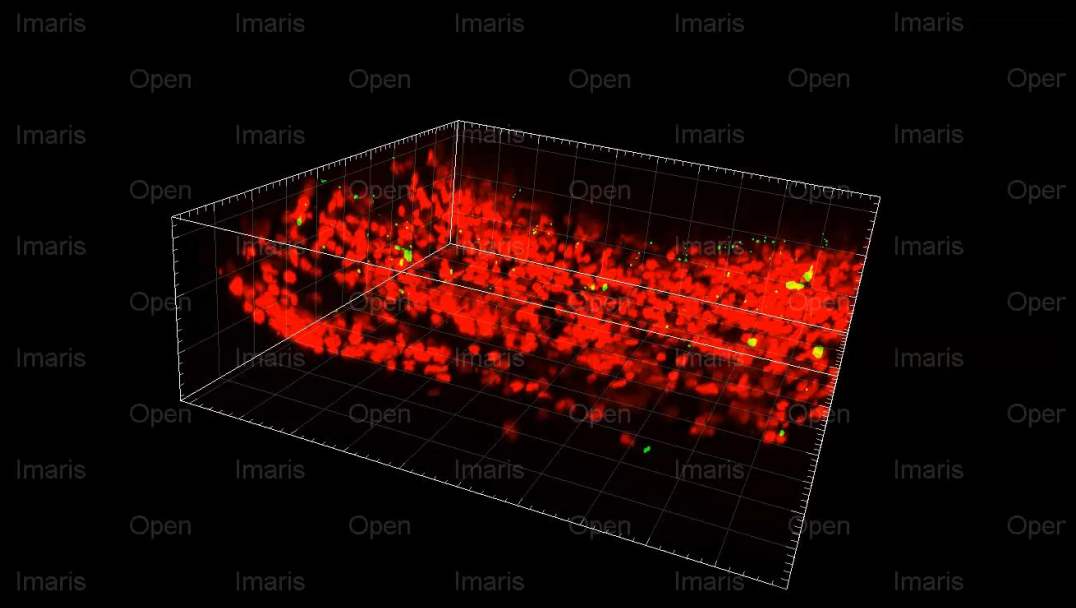




Images courtesy of Kris Lambert and Emma Reilly, Program for Advanced Immune Bioimaging
181106_D7_OTI TdT_OTII GFP_Ecad_L_0001

Unmixing Works for 3D Videos





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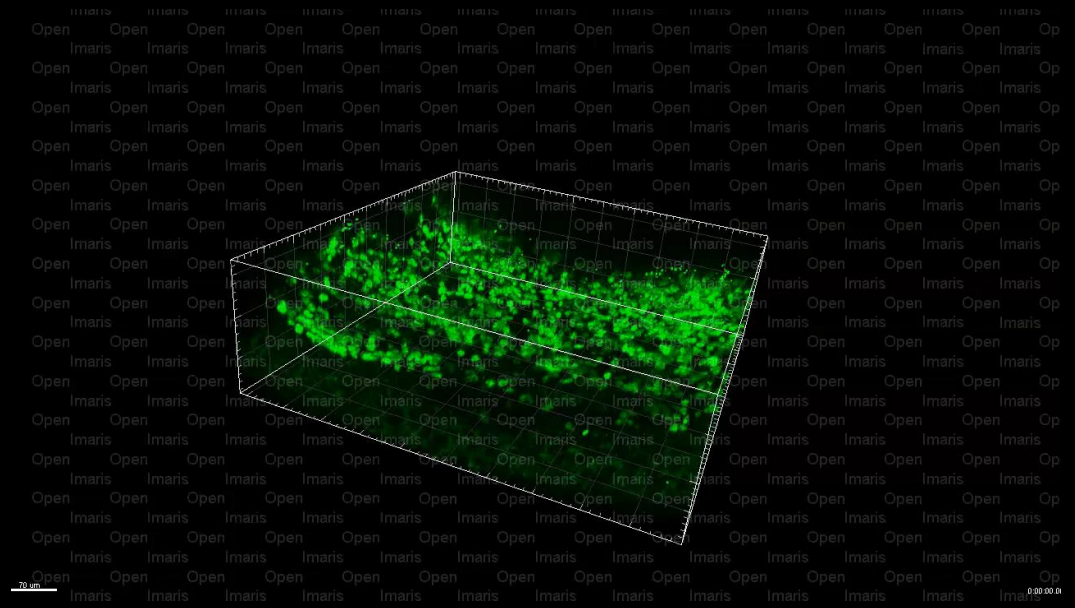
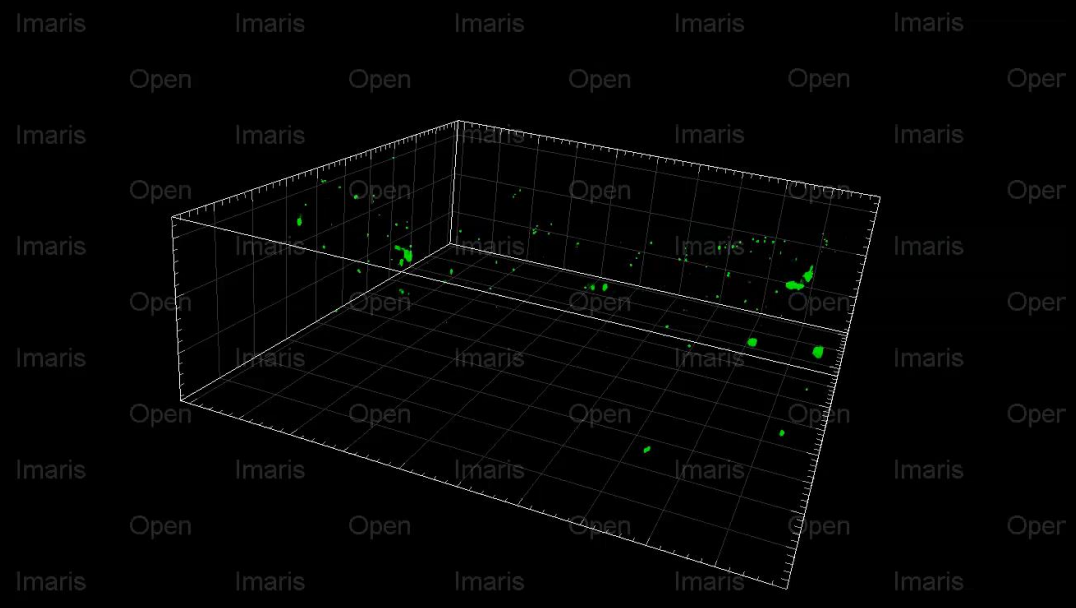
Unmixing Works for 3D Videos





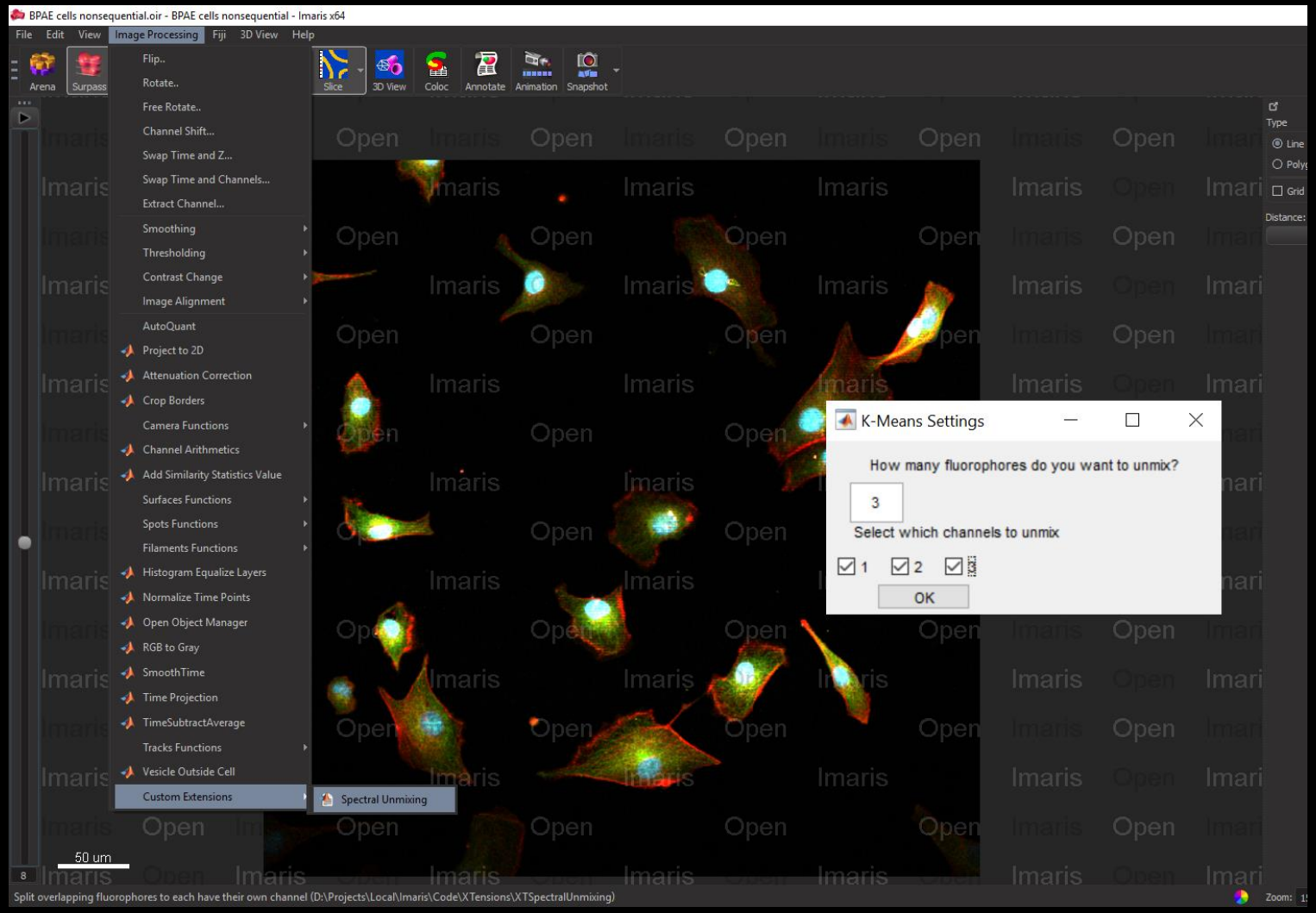
Images courtesy of Kris Lambert and Emma Reilly, Program for Advanced Immune Bioimaging
181106_D7_OTI TdT_OTII GFP_Ecad_L_0001

Imaris XTension

We made an XTension to perform k-means unmixing inside of Imaris

Instructions on running this and other XTensions are available in the resources section of the Multiphoton Core Website

The XTension is available on GitHub



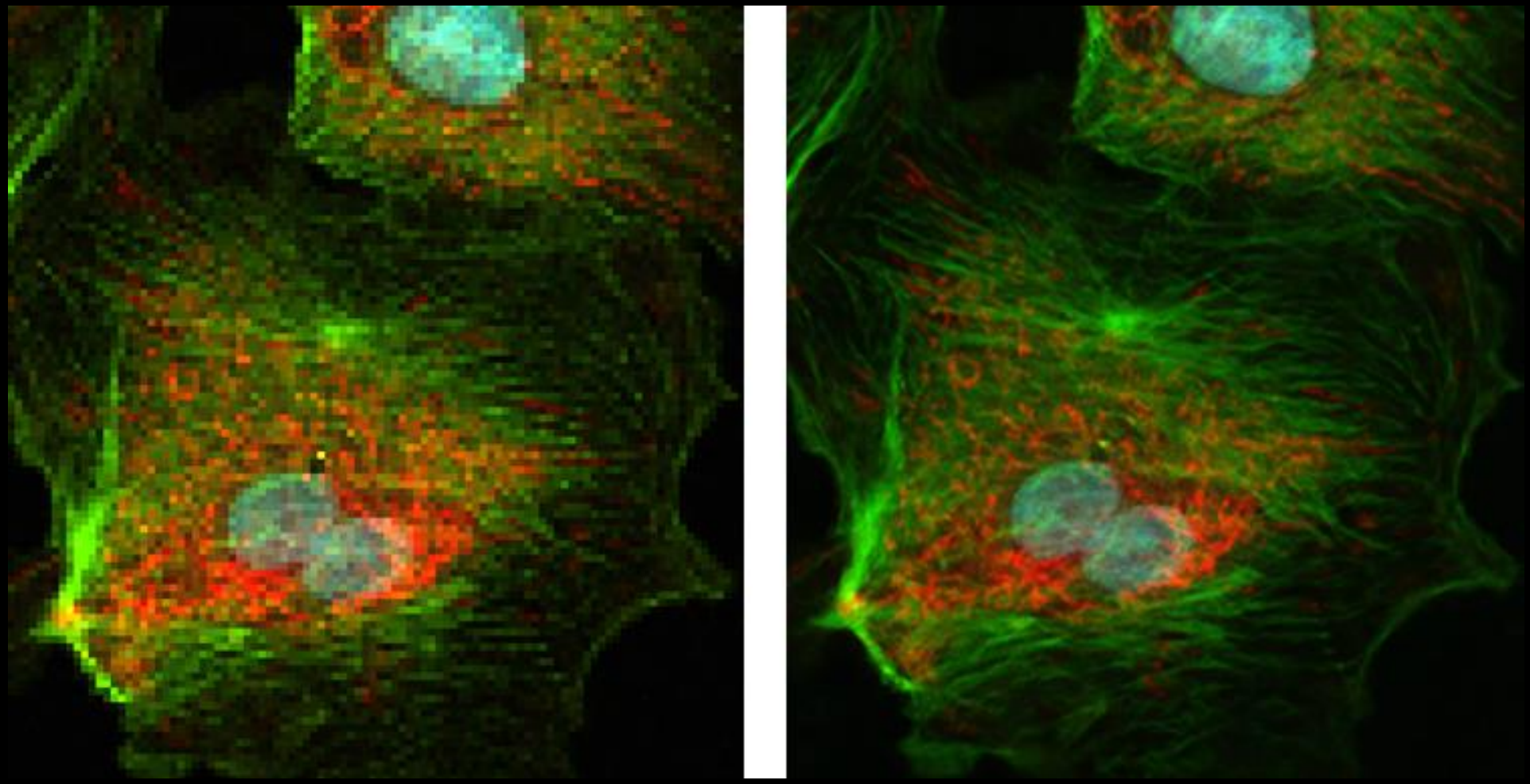
Overview of Unmixing Methods

Method	Blind?	Uses Spatial Relations?	Key Limitations
K-means	Yes	No	May lose effectiveness with very high dimensional data (8+ fluorophores)
SIMI	No	No	Needs emission spectra, assumes one fluorophore per pixel
Cornell	No	No	Needs excitation and emission spectra (particularly hard for multiphoton)
Non-negative Matrix Factorization	Yes	No	No solution when fluorophores outnumber channels, struggles to converge with more than 3 fluorophores
In-Silico Labeling	No	Yes	Requires labeled datasets
Autoencoder Clustering	Yes	Optional	Requires training time

Future Work

- Improved performance and functionality of k-means XTension
- Addition of other spectral unmixing methods
- Requests accepted
- Super resolution through machine learning

Super Resolution through Machine Learning



Questions?