Optiscan Five2 Spectral Unmixing Protocol

Background

There is an increasing need to analyze several fluorophores simultaneously in a single sample (1). To the eye of the observer commonly used fluorophores possess distinct colours (1, 2). However, there may be significant overlap of emission spectra between fluorophores (2). This overlap is called cross talk and occurs when fluorophores are excited using the same excitiation wavelength and have similar Stokes shifts. An example of overlapping emission spectra can be seen in Figure 1. The Five2 is equipped with a single blue 488nm-laser.   Most of the fluorophores that are excited by this laser emit green/yellow/red fluorescence, and there is an overlap of their emission spectra. Applying a spectral unmixing protocol can allow identication and separation of fluorophores with overlapping spectra, and enables collection of clean multicolour images of samples using the Five2.

The best approach to unmixing the images with crosstalk between green and red fluorophores is linear unmixing using reference spectra. For precision, reference spectra are best obtained from samples containing only the fluorophore of interest (2). They can also be acquired from mixed samples in areas where there is only the signal of interest, but this increases the risk of contamination of the signal (2). Biological specimens often have intrinsic fluorescent properties known as autofluorescence which should be accounted for when estimating the contribution of each fluorophore (3). In cases where there are no reference spectra, it may be possible to do blind unmixing by estimating the amount of crosstalk between filters. There is also an open-access Image J plugin (PoissonNMF) which may be useful for decomposition of the contribution of fluorophores in lambda stacks (4, 5, 6).
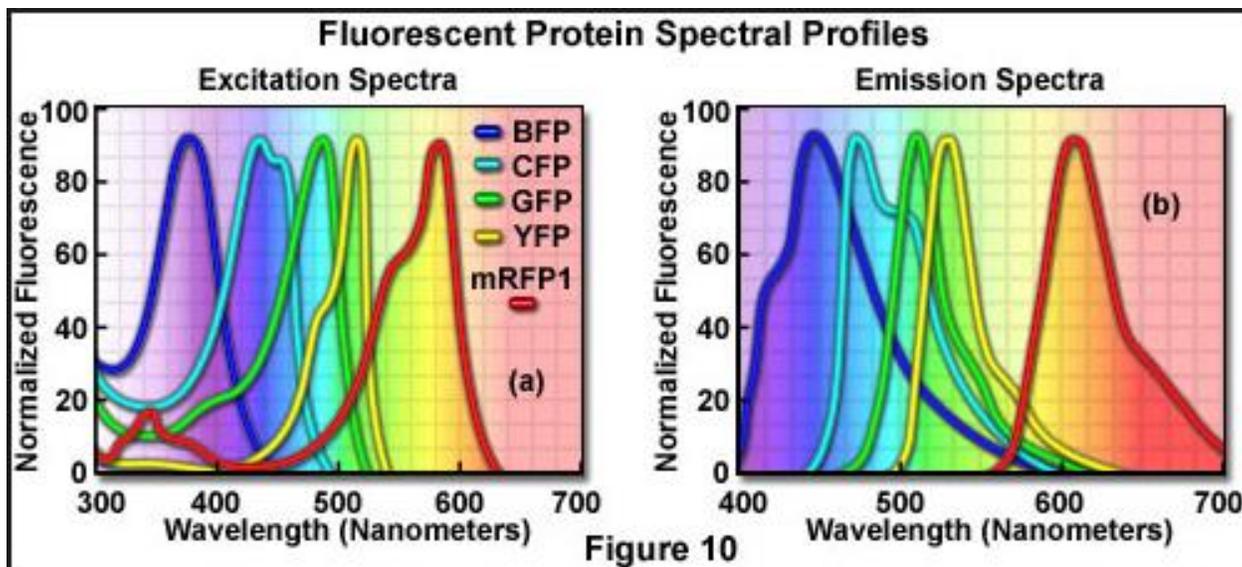

Figure 1 - Fluorescent spectral profiles for BFP, GFP, YFP and RFP.

Spectral unmixing with the FIVE2

The Five2 is equipped with a series of optical bandpass (BP 515-575, BP 515-530, BP 530-550, BP 550-575) and long pass (LP515, LP 540, LP 570) filters allowing a degree of spectral separation between green and red fluorophores. For best results, fluorophores to be imaged should be spectrally well separated but must still be able to be excited with the 488nm-laser. The green fluorophores can usually be detected with the BP 515-530 filter with no cross talk from red fluorophores. The situation is more complex when imaging red flurophores, as most green/yellow emitting flurophores have a long red tail, which shows up as cross talk, even when using the LP570 filter. Spectral unmixing can help in this situation by subtracting the red tail of the green/yellow emitter from the red flurophore image.

An important note: The concentration of fluorophores may need to be balanced to enable adequate signal at the detector through each filter. Datasets with strong spectral overlap may result in a noisy image. This may be improved using frame averaging (7).
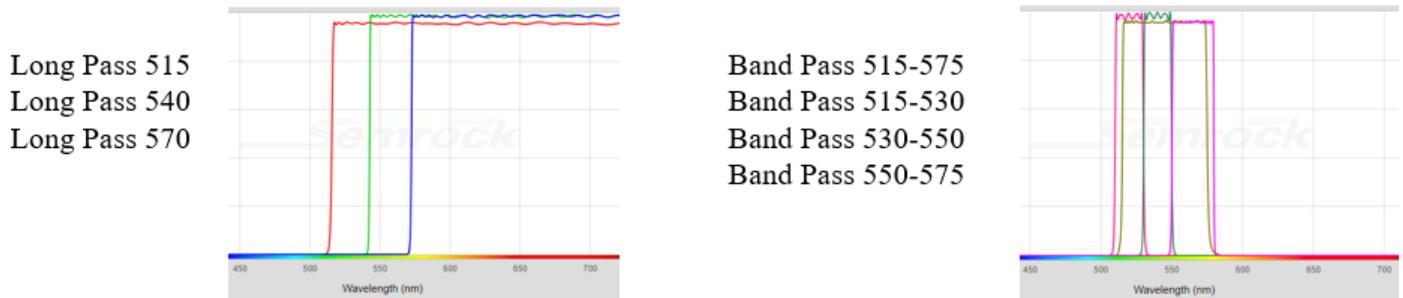

Figure 2- Five2 Standard Emission Filter Spectra

Each sample will be unique in the number of labelled structures and the density of fluorophores to be measured. Below are some guidelines for how to obtain images that contain two fluorophores using the Optiscan Five2 Viewnvivo. The guidelines are based on the use of free open access Image J software and include instructions for optimizing filter selection, signal to noise ratio, and post-processing of images. When examining large data sets these processes can all be automated by creating a macro in Image J. If the structures are morphologically well-defined thresholding of the image may reveal that there is no cross talk between fluorophores using well-separated filters and depending on the end goal of analysis spectral separation without post-processing may be enough.

Guidelines for optimal separation of fluorophores with the Five 2

- Fluorophores selected should be spectrally well separated
- Ideally, the structures being examined should be morphologicaly easy to distinguish.
- Where possible, reference samples should be obtained for each fluorophore.
    - Images of background autofluorescence in each channel should be obtained. This can be acquired before administration of dye or in an area without positive labelling.
    - The images of each fluorophore should be obtained with each of the filters used. This will give the exact amount of bleed through (+background) in each filter for the dye.
    - This can then be used to subtract from the new images once both dyes are present.
- Consider the movement of the sample. Frame-to-frame stability is critical when sequential images are captured with multiple filters.

The following website may be useful for choosing appropriate fluorescent tags for dual labelling experiments. Some candidates are also provided below in the table.

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

Table 1: Examples of dyes that can be used together for spectral unmixing

Filter: BP 515-530	Filter: LP 570
GFP	RFP (ds red)
Fluorescein or (FITC)	TRITC
Alexa 488	Rhodamine B
CY2	Alexa 555, 568
	Rhodamine red-X
Qdot 525	Qdot 625, Qdot 705

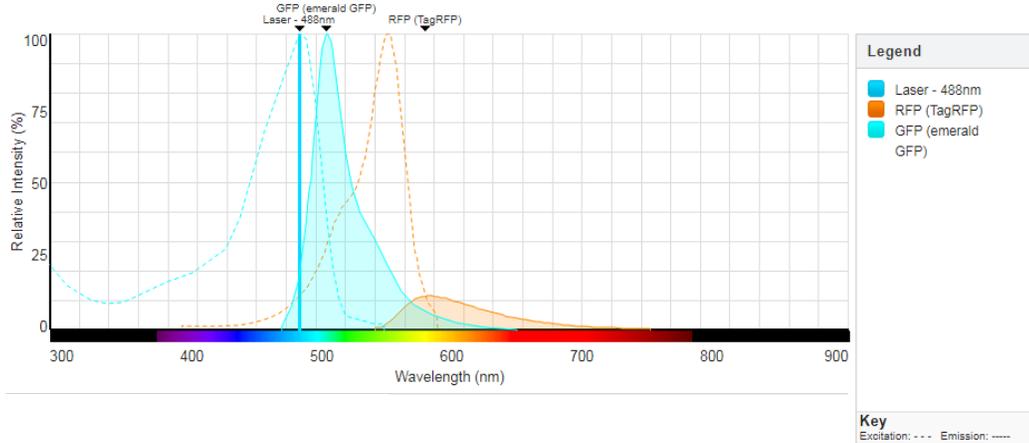**Example 1: Clean signal on channel 1, cross talk on channel 2**

Figure 3- 488nm excitation of GFP and RFP gives uncontaminated GFP signal until around 550nm.

Note that the excitation efficiency of RFP at 488nm is low, and there needs to be significant RFP expression to get detectable RFP emission. A worked example of how to unmix the red fluorescence signal from the green fluorescence is provided with later in the document
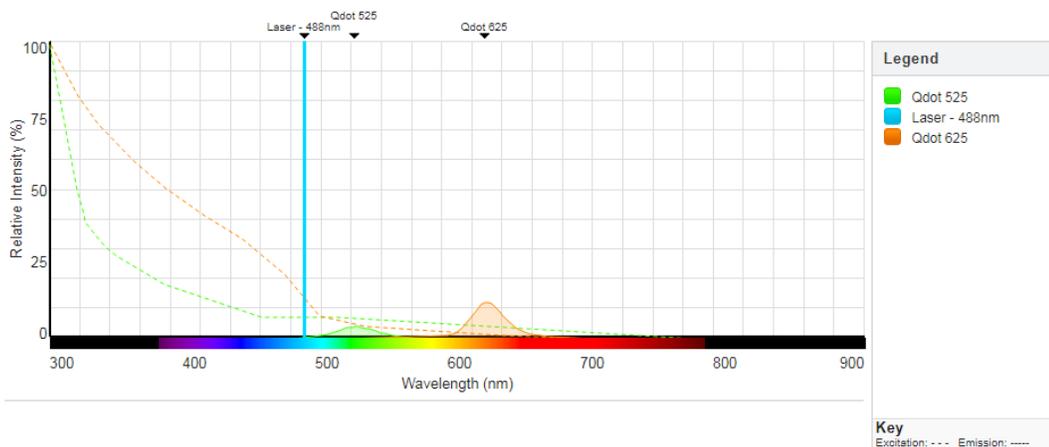
Example 2: Clean signal on both channels.

Figure 3- 488nm excitation of Qdot 525 and Qdot 625 are not spectrally overlapping. Signal from Qdot 525 can be collected through the BP515-575 filter. Signal from QDot 625 can be collected through the LP570 filter. These images can be merged and no spectral unmixing is required.

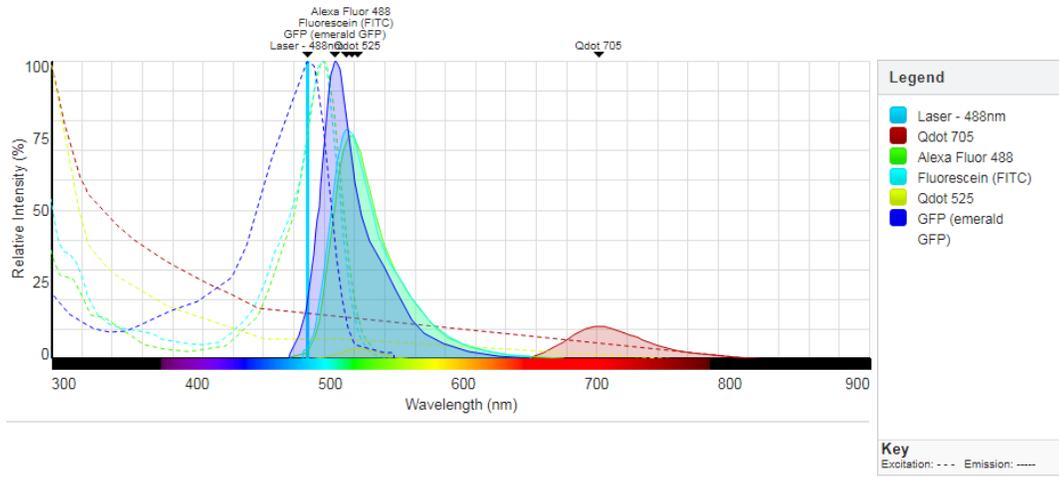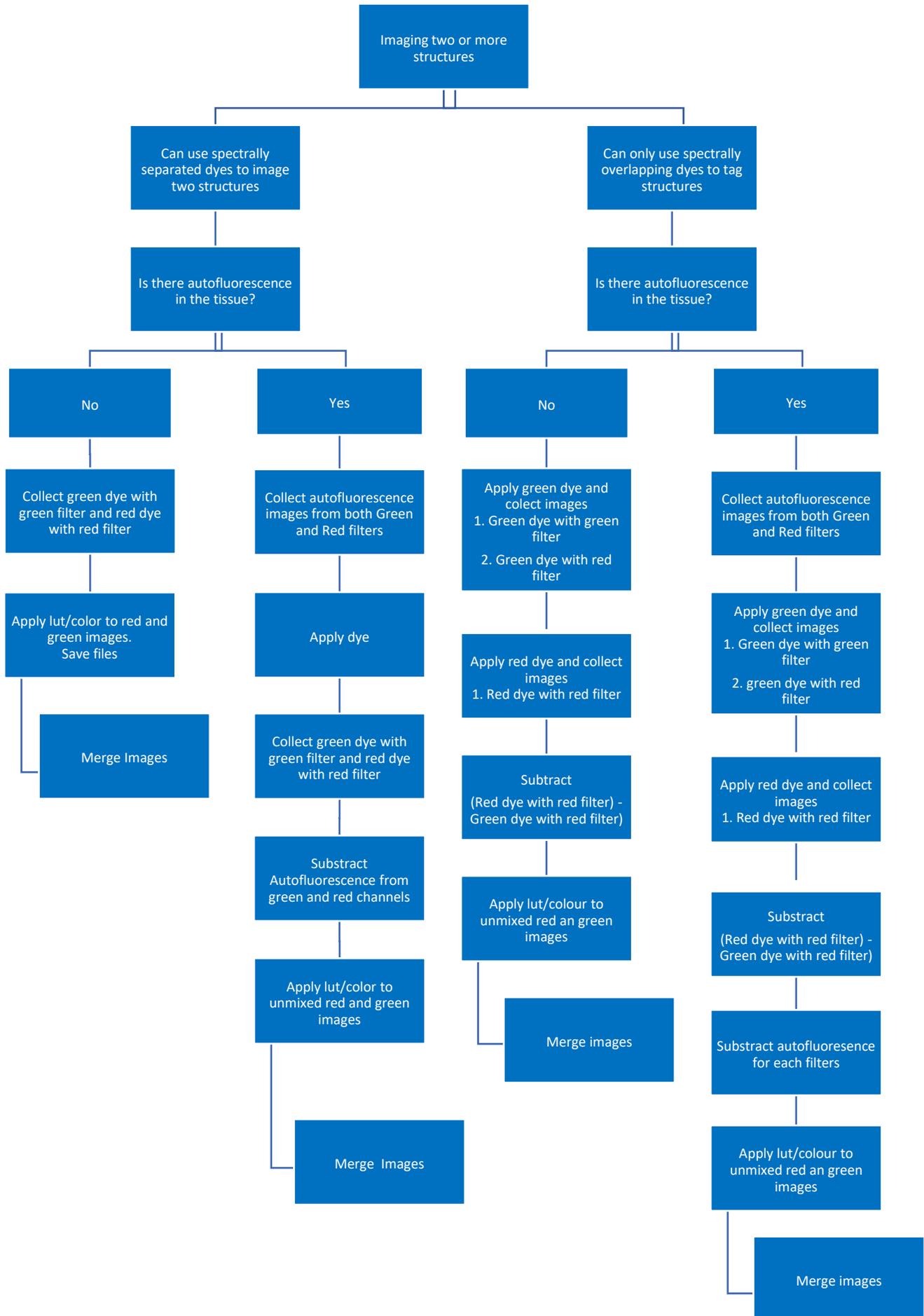**Example 3: Spectral unmixing with custom filters**

Figure 4- Alexa Fluor, Fluorescein(FITC), GFP and qdot 525 shown as examples of green/yellow light emitting dye and Qdot705 as red/far-red. This example shows non-overlapping signal on both channels, but will suffer from cross talk using standard Five2 filter set. Addition of a custom band-pass filter transmitting light in the red and infrared part of the spectrum can enable imaging of desired fluorophores with no cross talk.

Note: Custom filter options can be added on request if there is a need to detect fluorescence in the red and far red part of the spectrum.

General Spectral Unmixing Protocol

Imaging two or more structures

- **Can use spectrally separated dyes to image two structures**
  - **Is there autofluorescence in the tissue?**
    - **No**
      - Collect green dye with green filter and red dye with red filter
      - Apply lut/color to red and green images. Save files
      - Merge Images
    - **Yes**
      - Collect autofluorescence images from both Green and Red filters
      - Apply dye
      - Collect green dye with green filter and red dye with red filter
      - Substract Autofluorescence from green and red channels
      - Apply lut/color to unmixed red and green images
      - Merge Images

- **Can only use spectrally overlapping dyes to tag structures**
  - **Is there autofluorescence in the tissue?**
    - **No**
      - Apply green dye and colect images
        1. Green dye with green filter
        2. Green dye with red filter
      - Apply red dye and collect images
        1. Red dye with red filter
      - Subtract
        (Red dye with red filter) - Green dye with red filter)
      - Apply lut/colour to unmixed red an green images
      - Merge images
    - **Yes**
      - Collect autofluorescence images from both Green and Red filters
      - Apply green dye and collect images
        1. Green dye with green filter
        2. green dye with red filter
      - Apply red dye and collect images
        1. Red dye with red filter
      - Substract
        (Red dye with red filter) - Green dye with red filter)
      - Substract autofluoresence for each filters
      - Apply lut/colour to unmixed red an green images
   - Merge images

Step by step guideline for spectral unmixing and creation of a 2 colour image of two fluorophores:

1. Determine which filters best match the emission spectra of they dyes you wish to image.

2. Optimise fluorophore concentrations to get good signal through the selected filters. (Laser power and gain can be adjusted to get good signal on both channels if required, but it is better if you do not need to).

3. Using the settings determined above, capture images from an area that is absent of positive staining to determine background autofluorescence through each filter. Collect images from both filters. Save autofluorescence images with identifiable names. e.g (NAME)_Autofuorescence_BP515, (NAME)_Autofluorescence_LP570

4. Apply the green dye. Using the settings determined above, capture images through Bp515 and Lp570 filters. Save both files with identifieable names. e.g. (NAME)_Green_BP515 and (NAME)_Green_LP570

5. Apply the red dye and collect images using the settings determined above. Collect image with LP570 filter only. Save file as (NAME)_Red_LP570

6. Substract Green fluorescence from the LP570 filter to collect unmixed image of red fluorescence from the LP570 filter. Eg. {(NAME)_Red_LP570} – {(NAME)_Green_BP515}. Save file as (NAME) Red_LP570_Unmixed

    Covert all RGB files to 8-bit tiff: Image>Type>8-bit
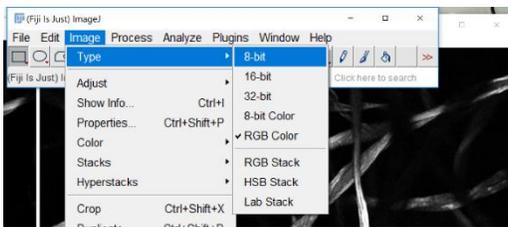
    Subtract green fluorophore from the red channel (LP570): Process> image calculator
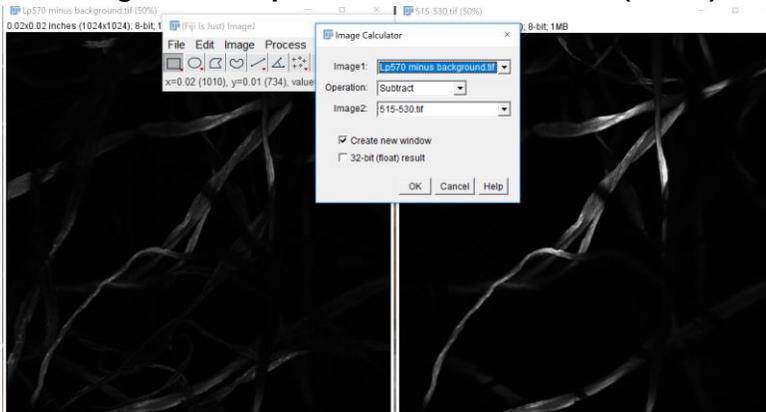

    Note: As seen above the red fluorophore should not contribute to the signal detected with the BP 515-530 filter. Therefore, subtraction of red fluorophore from the green channel isn't critical

7. Substract autofluorescence from the Green and red unmixed images following the same functions described in step 6. Save files as (Name)_Green_BP515_pure and Red_LP570_unmixed_pure

8. Assign LUT:
   LUT>[Select green or red]
   Assign green pseudocolor to the '(Name)_Green_BP515_pure' and red pseudocolor to '(Name)_Red_LP570_unmixed_pure' image
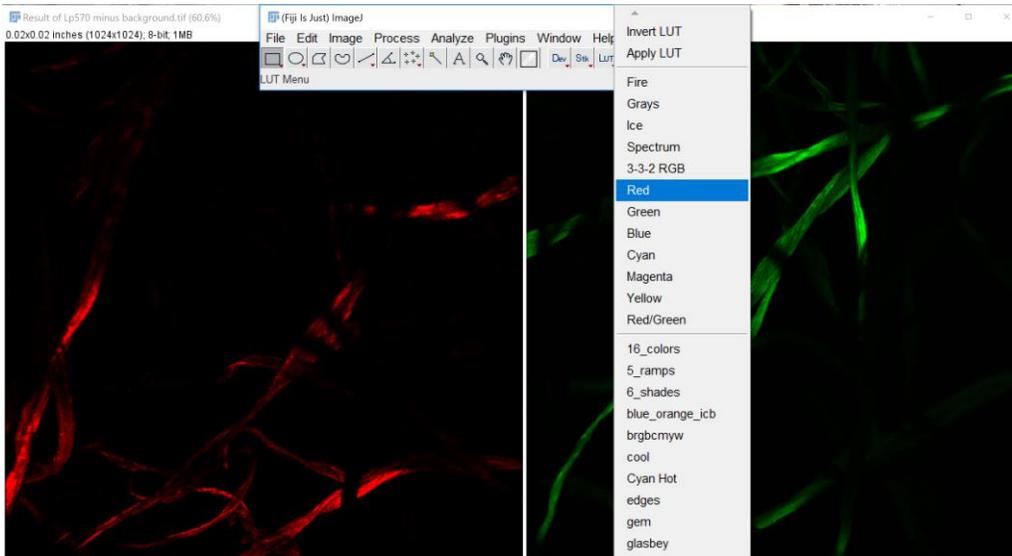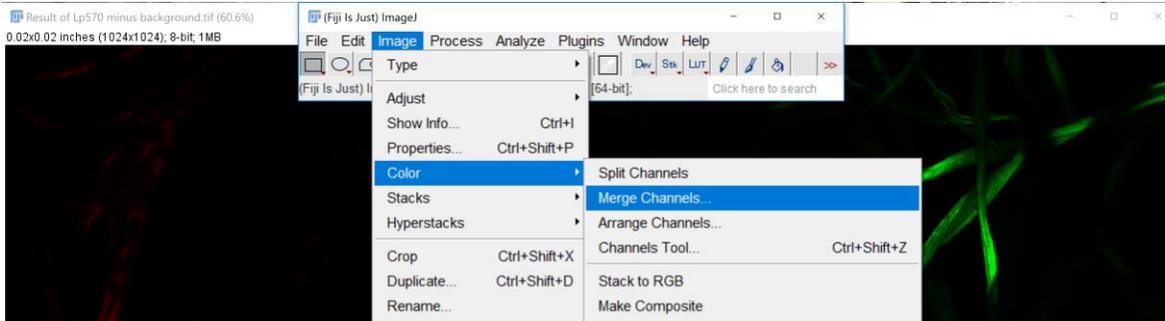

9. If unmixed red image is dim compared to green, adjust brightness of the unmixed red image.
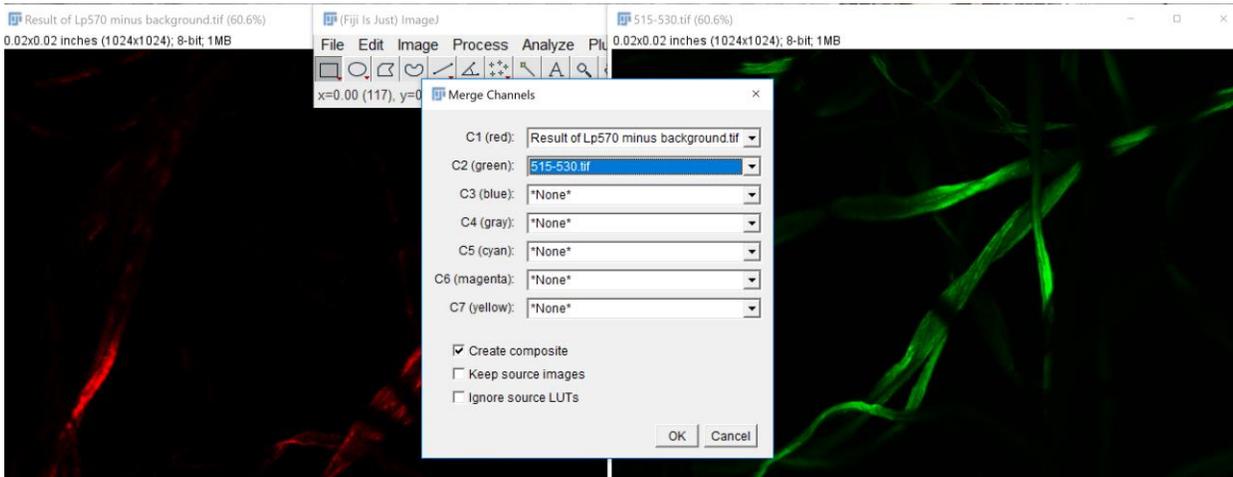   **Image>adjust> brightness/contrast**
   Use auto fuction to set image J defined optimum brightness or drag the scale to set the brightness of your choice.
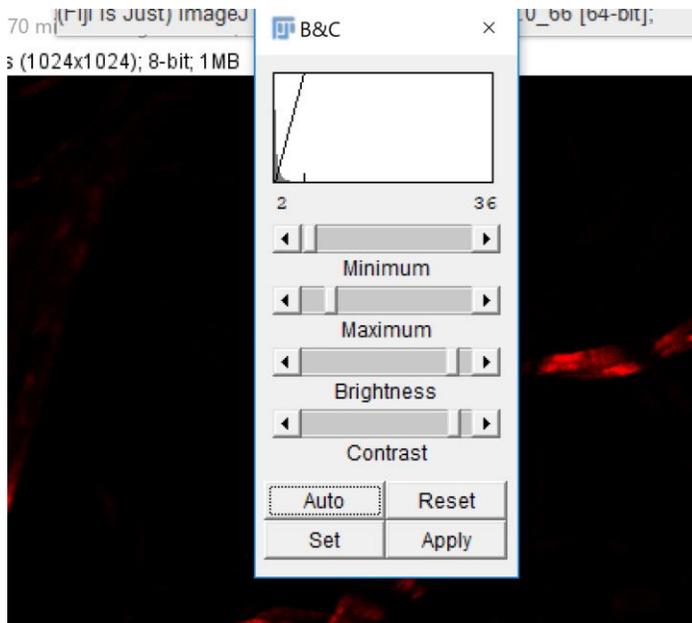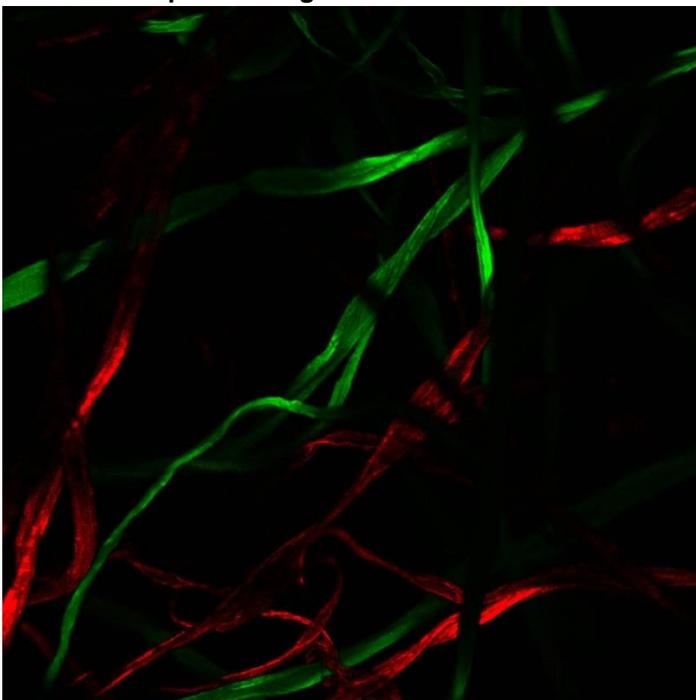
10. Merge channels to get a composite image
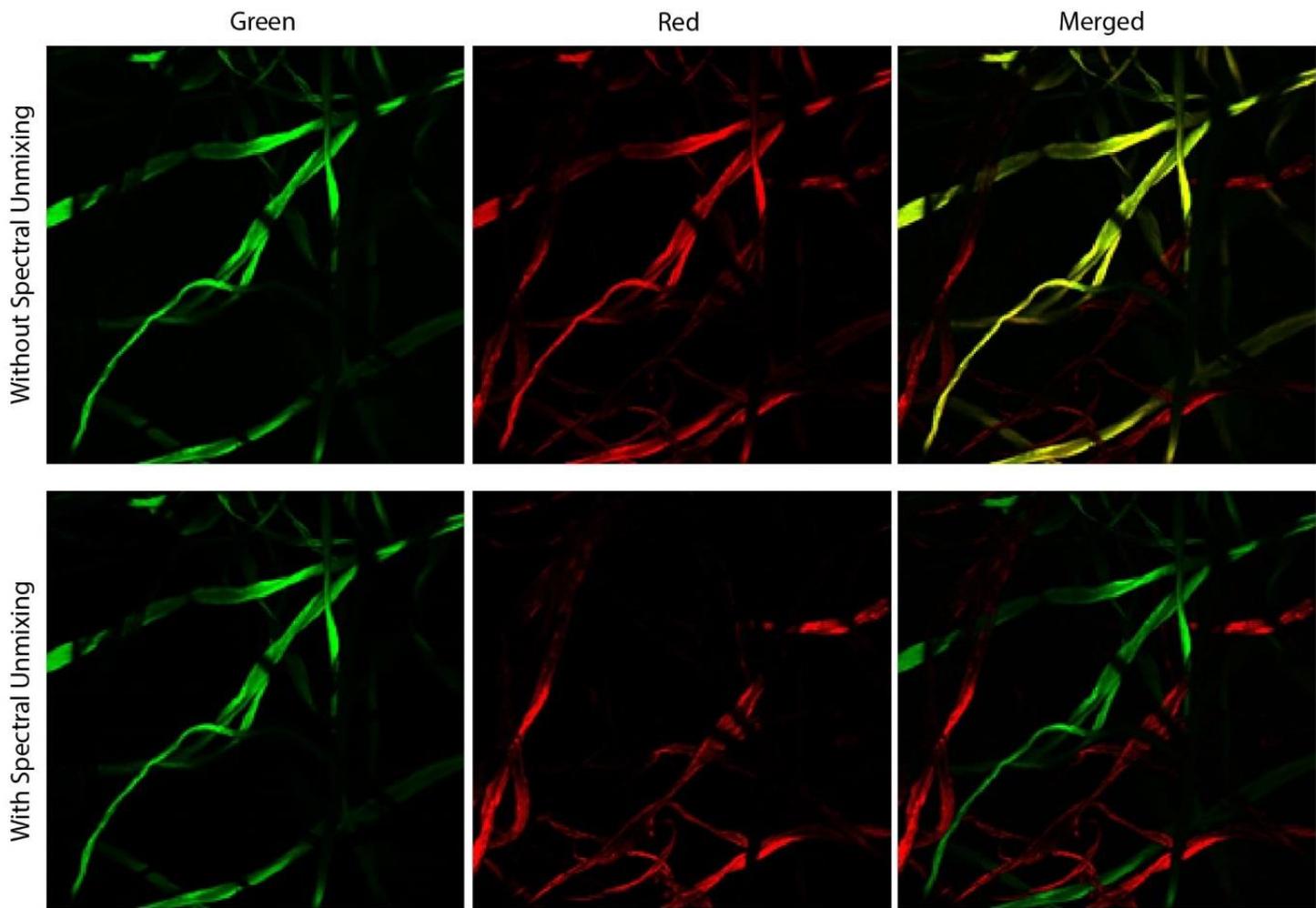**Image>colour>merge channels>[Select channels]**

Select channels

Result = composite image

Comparison of spectrally mixed and unmixed images from the same site.

References:

1.   Zimmermann T. Spectral imaging and linear unmixing in light microscopy. Adv Biochem Eng Biotechnol. 2005;95:245-65.

2.   Zimmermann T, Marrison J, Hogg K, O'Toole P. Clearing up the signal: spectral imaging and linear unmixing in fluorescence microscopy. Methods Mol Biol. 2014;1075:129-48.

3.   Shirakawa H, Miyazaki S. Blind spectral decomposition of single-cell fluorescence by parallel factor analysis. Biophys J. 2004;86(3):1739-52.

4.   Hartig SM. Basic Image Analysis and Manipulation in ImageJ. Current Protocols in Molecular Biology. 2013;102(1):14.5.1-.5.2.

5.   Neher RA, Mitkovski M, Kirchhoff F, Neher E, Theis FJ, Zeug A. Blind source separation techniques for the decomposition of multiply labeled fluorescence images. Biophys J. 2009;96(9):3791-800.

6.   PoissonNMF [ImageJ Documentation Wiki] [Internet]. Imagejdocu.tudor.lu. 2019 [cited 12 June 2019]. Available from: https://imagejdocu.tudor.lu/plugin/analysis/poissonnmf/start

7.   Conchello JA, Lichtman JW. Optical sectioning microscopy. Nat Methods. 2005;2(12):920-31.