Spectral Unmixing on the LSM 780

Spectral unmixing is a method for the complete separation of overlapping emission spectra. It is used with specimens labelled with more than one fluorescent dye or which are inherently autofluorescent and exhibit both excitation and emission crosstalk.

Options:

1) **Channel unmixing** – if the emission spectra of fluorescent markers overlap only slightly, the signals can be separated by channel unmixing…
2) **Spectral unmixing** – if the emission spectra of fluorescent markers are known to overlap a lot but have not been previously defined…
3) **Online fingerprinting** – if the emission spectra of fluorescent markers overlap a lot and the spectra have been previously defined…

1) Channel Unmixing

- **Smartest Line** on Smart Setup.
- Select **Channel mode** in the Light Path tab.
- Select appropriate lasers and dichroic filters.
- View sample in **Live** mode.
- Set laser power and gain for each channel as normal. Avoid Saturation (no red pixels on range indicator).

Ideally:

- Select the optimal frame size.
- Scan at a slower speed of ≤ 5.
- Increase averaging to ≥ 4.
- Select 12 bit grey scale.

- Acquire image.
- Then view the image in the **Unmixing** tab.
- Select **Autofind ACE** and input the number of dyes (components) you are looking for.

*ACE = Automatic Component Extraction.
*The number of extractable components cannot be higher than the number of acquired channels.
*This strategy will not work if fluorophores are completely co-localised, it will only work if there are some pixels in the image which are only stained with one fluorophore.

- Select the sample-specific fluorescence dye reference spectra from the **Spectra Database**.

Other options:
Auto scale: this balances the intensity of the unmixed channels to equal levels.
Display Channel with Residuals: this generates an additional channel for emitted light which does not strictly fall into any channel. *Selecting Display Channel with Residuals can help remove autofluorescence from an image.
Weighted unmixing: this results in spectral channels with high noise: signal ratio contributing less to the unmixing result. *Selecting Weighted unmixing increases the time it takes to perform unmixing.
Multichannel Unmixing: When this option is chosen the unmixing algorithm is applied to a multichannel image (up to 10 channels) without the use of reference spectra.
Remove **Background**: You can indicate a background region by drawing on your image, and then selecting this region on the dropdown menu.

- Click **Linear unmixing** to create the unmixed image. This will be created in a new image tab with the suffix "_Linear unmixing". The unmixed image contains a channel for each component with a reference spectrum.
- View in the **Split** tab to see all dyes separately.

2) Spectral Unmixing

Ideally:

- Use single stain controls – this can improve the accuracy of this technique, enabling the complete unmixing of dyes which even have the same emission maxima. These need to be acquired and saved in the **Spectra Database**.
- Ensure in the work-up of the antibody-fluorochrome concentrations in your sample preparation that the brightness of each is similar as opposed to having one very bright dye and one very dim dye.

- Select in **Lambda mode** in the Light Path tab.
- Set resolution to 8.9 nm between wavelengths on the scan settings.
- View the Lambda stack in the Gallery tab.

*The Lambda stack is a series of images of the intensity of light emitted at different wavelengths as detected by the GAsP detector. Therefore, the data allow deducing an emission spectrum for each pixel corresponding to the emission spectrum of a specific dye.

- View sample in **Live** mode in the Gallery tab.
- Select 'palette' to get range indicator on all images in the Lambda stack.
- Set laser power for each laser and the gain (master) for all channels (there is only one gain that can be adjusted here).
- Acquire the Lambda stack.
- Perform **Linear unmixing** on the **Unmixing** tab.

*Splits in emission wavelengths, which create the appearance of multiple peaks in emission spectra are due to the filters which block the excitation wavelengths.

- View the unmixed image.
- You can save the unmixing of single stain controls or ROI containing specific fluorophores as references in the spectra database and then use these saved configurations to do online fingerprinting of a multi-stained sample.

3) <u>Online fingerprinting</u>

- Load saved configurations from the reference database.
- Ensure you are using the same objective, lasers, and filters as used when the saved configurations were created.
- Set laser power for each channel and gain for all channels (there is only one gain).
- Select **Online fingerprinting** mode in the Light Path tab.
- View sample in **Live** mode in the Gallery tab.
- Select 'palette' to get range indicator on all images in the Lambda stack.
- Set laser power for each laser and the gain (master) for all channels (there is only one gain that can be adjusted here).
- Perform spectral unmixing in real time.
- This helps to reduce the size of an image.
- White indicates areas which are saturated.