<Instruction>

Journal : Nature communications

Title : PICASSO allows ultra-multiplexed fluorescence imaging of spatially overlapping proteins without reference spectra measurements

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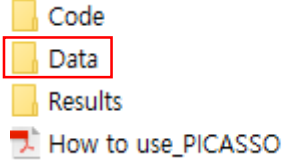
- **Minimum requirements of the code**
  - ✓ To run this code, MATLAB (Mathworks) should be installed. In addition, MATLAB Image Processing Toolbox and Wavelet Toolbox are required to run unmixing code.
  - ✓ We have tested the code with MATLAB R2020b running on Windows 10.

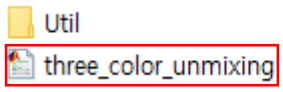
- **Demo image**
  - ✓ A demo input image is in the 'Data' folder, named '3color_data.tif'.
  - ✓ The demo input image was acquired by a single excitation laser but within three different spectral ranges from a mouse brain slice labelled with three preformed rabbit antibody complexes (PV-CF488A ,Neun-ATTO514, GFAP-ATTO532).
  - ✓ Simply running the code ('three_color_unmixing.m') will generate an unmixed image, named '3color_data_unmixed.tif' in the 'Results' folder.

- **How to modify the code to unmix other images**
  - ✓ Input files should be a tif format images, having three channels where the spectral detection range includes the corresponding fluorophore's emission peak (input *IMG*1, input *IMG* 2, and input *IMG* 3 shown in Fig. 3a).

    1. Copy your input image files to the 'Data' folder

    2. Open the code in the 'Code' folder

    3. Change the 'filename' as the name of your input image file

```
imgPath = '../Data/';
filename = '3color_data.tif';
```

4. **Run the unmixing code**

5. **Resultant unmixed images are saved in the 'Results' folder, named '[filename]_unmixed.tif'.**

6. **Input mixed images and unmixed images both can be displayed in individual channel mode and composite mode through the imageJ software program, as shown below.**