Protocol for *SubstrateUnmixing*

Required software

1. MATLAB: https://www.mathworks.com/products/matlab.html
    a. NOTE: Add the Image Processing Toolbox upon installation.

2. FIJI (ImageJ): https://fiji.sc/

Unmixing in MATLAB

1. We recommend create a directory for each experiment. This folder will contain input images, the unmixing algorithm, and output images.

2. Inputs are "luminescent.TIF" images from IVIS. There should be one image for each substrate. We recommend that these images be renamed to the name of the substrate that was added (i.e., "AkaLumine.TIF").

3. Edit the following code depending on the number of substrates that are being unmixed. To edit code, copy the entire text highlighted in yellow and add to the appropriate line in the algorithm. The # symbol should be changed to reflect the change in input. For example, if adding a 4th enzyme-substrate pair, change # = 4. Code highlighted in green represents examples of three component unmixing.

    a. *Line 17 - Image Input:* Add or delete ",imread('filename.TIF')" for each substrate that is being unmixed. The filename should exactly match the name of the input image, and should be listed in order of substrate addition during the experiment.

```
luc_images = {imread('4Br-Luc.TIF'),imread('D-Luc.TIF'),imread('AkaLumine.TIF')};
```

    b. *Line 23 - ROI Coordinates:* Add or delete "luc#_X = %%; luc#_Y = %%;" for each substrate that is being unmixed. Input a number between 1-256 in %%. The specific values will be edited in later steps.

```
luc1_X = 30; luc1_Y = 80;
luc2_X = 70; luc2_Y = 80;
luc3_X = 110; luc3_Y = 80;
```

    c. *Line 34 - Array Containing ROI Coordinates* - Add or delete "; luc#_X luc#_Y" based on the number of substrates that are being unmixed.

```
ROI_coord = [luc1_X luc1_Y; luc2_X luc2_Y; luc3_X luc3_Y];
```

    d. *Line 134 - Normalized, pre-unmixed output images* Add or delete the line "dlmwrite('Pre-Unmix 1.txt',luc_normalized{1},'delimiter','\t');" based on the number of substrates being unmixed.

```
dlmwrite('Pre-Unmix 1.txt',luc_normalized{1},'delimiter','\t');
```

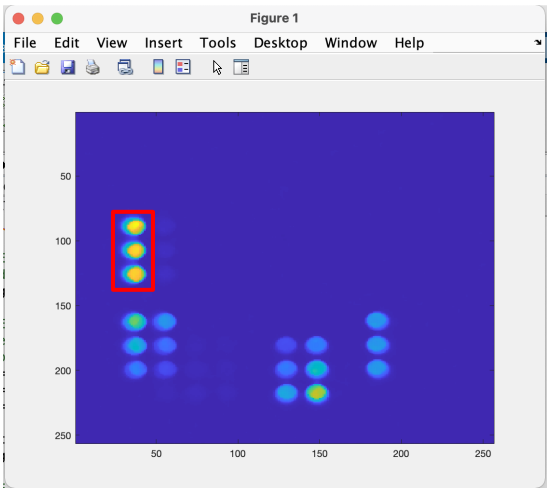
```
dlmwrite('Pre-Unmix 2.txt',luc_normalized{2},'delimiter','\t');
dlmwrite('Pre-Unmix 3.txt',luc_normalized{3},'delimiter','\t');
```

e. *Line 141 - Unmixed Output Images* - Add or delete the line "dlmwrite('Luciferase #.txt',unmix{#},'delimiter','\t');" based on the number of substrates being unmixed.

```
dlmwrite('Luciferase 1.txt',unmix{1},'delimiter','\t');
dlmwrite('Luciferase 2.txt',unmix{2},'delimiter','\t');
dlmwrite('Luciferase 3.txt',unmix{3},'delimiter','\t');
```

4. Next, you will assign an ROI to positive control wells. Run the algorithm (Editor > Run) in order to load inputs into the algorithm. An output should appear of three images that can be discarded.
   a. Note: If the algorithm repeatedly outputs "Warning: Matrix is singular to working precision," pause the run (Editor > Pause) and then select "Quit Debugging." This error occurs due to incorrect positioning of the ROI.

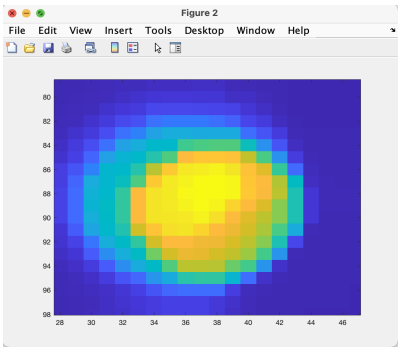
5. In the command window, load the normalized, pre-unmixed image using "figure; imagesc(luc_normalized{#})" Change the number # based on which image you would like to load. For example, if you would like to visualize the image acquired after adding the first substrate, # = 1. Below is an example of the output of this command. Highlighted in red is the ROI of interest.

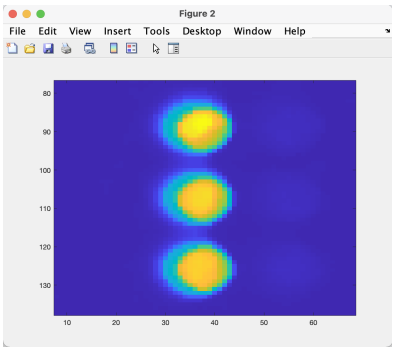


6. To identify the coordinate of the ROI, scroll to zoom into the well or population of interest. MATLAB will automatically scroll toward the location of the cursor. Below includes example inputs based on the image from step 5. The first image is the top well of the highlighted ROI. The second image is the entire highlighted ROI.

   a. Record the y-coordinate of the top of the well and the x-coordinate of the left of the well. Input these coordinates into %% on line 11 (luc#_X = %%; luc#_Y = %%;).

   b. Record the height and width of the desired ROI on line 15. This should reflect the entire highlighted region from step 5. Note that these values will be the same for all images.

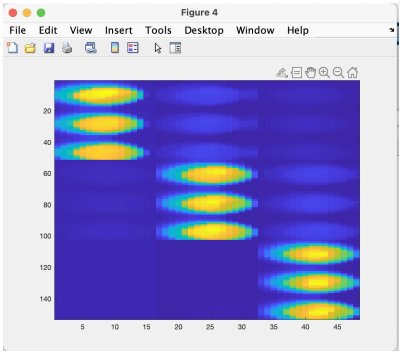




luc1_X = 25; luc1_Y = 80;

ROI_width = 20;
ROI_height = 55;

7. Repeat step 5 & 6 for each image in the series. There should be a unique ROI for each positive control population. For example, if performing three-component unmixing, there should be three unique ROIs.
    a. Note: To check the pre-unmixed ROI, input "figure; imagesc(Preunmix_ROI)" into the command line (this must be done after the algorithm is run with the correct inputs). Below is an example output of this command. Each column represents an ROI, and each row represents an image in the series.



8. Run the algorithm to perform unmixing (Editor > Run). The outputs of the algorithm are listed below:

    a. Unmixed images will appear to verify efficacy of unmixing.

    b. Image files (.txt) of unmixed images will appear in the folder created in step 1. These can be opened in FIJI (image processing will be described later in the protocol).

    c. The condition number for inversion (cond) value will appear in the command window. This value represents the sensitivity of the unmixing reference related to input data (such as ROI coordinates). Ideal cond = 1. This value can be used as a reference to approximate efficacy of unmixing.

Image Processing in FIJI

1. Unmixed images can be imported into FIJI: File > Import… > Text Image. Unmixed images will be imported as 32-bit with a maximum pixel intensity of 1 (this value might be higher if there are parts of your image that are brighter than the reference).

2. Some images may have high background due to negative pixel values in unmixed wells. Adjust the visible pixel values with Image > Adjust > Brightness/Contrast. A B&C box will open. Select “Set” and set “Minimum displayed value” = 0 and “Maximum displayed value” = 1. Check the box to propagate to all other open images to apply these changes to all unmixed images.

3. Convert outputs to 16-bit images for quantification using Image > Type > 16-bit. This will change the pixel values to 0-65536.

4. It is recommended that the adjusted images are saved as .TIF files for easy access.

To create a false color composite:

1. Go to Image > Color > Merge Channels…. A dialog box will open where you can assign the color of each channel.
    a. Note: If you want to include a photograph (e.g., 96-well plate or mouse), import a photograph.TIF file into FIJI. Change the size to match the unmixed images (Image > Adjust… > Size ... , then input 256 x 256). When making your composite, input that image into the gray channel.

2. You can view individual channels with the channels tool. Go to Image > Color > Channels Tool… and a box will appear. In the dropdown menu, select “Color” to view individual channels. False colors can be changed by selecting “More >>” and selecting a new color. Custom LUTs can be applied by selecting “More >>” > Edit LUT...

3. Save the composite image and each individual channel as .JPEG or .PNG. It is also recommended to save the composite as a .TIF file (this file can be opened and edited in FIJI again at a later time).