

Supplemental Information: Spectral Library Construction:

A spectral library consists of pure spectra of each fluorescent label present in the sample and spectra of non-specific background signatures if any (for example, in these studies, the spectral library consists of Turquoise, Venus, and DRAQ5, as well as background spectral signatures from the cell matrix or autofluorescence, coverslip fluorescence, and reflection from the coverslip). The protocol described in this supplemental file corresponds to steps involved in construction of spectral library to perform spectral unmixing on the image data acquired in the actual manuscript titled “Measurement of Three Dimensional cAMP Distributions in Living Cells Using 4-Dimensional (x, y, z, and λ) Hyperspectral FRET Imaging and Analysis”. The Turquoise and Venus spectra for the library were acquired using HEK293 cells expressing the H188 FRET reporter. For FRET efficiency measurements, the concentration of donor and acceptor fluorophores in the sample should be equal. If single label Turquoise and Venus constructs were expressed in cells (even using equal concentrations), the expression of these fluorophores would likely not be 1:1 stoichiometry. Hence, to meet this criterion we used cells expressing the H188 FRET sensor. FRET sensors express as a single molecule in the cell and hence the concentration of Turquoise and Venus in the cells will be the same (i.e., fixed 1:1 stoichiometry). The steps involved in constructing the spectral library are described below:

1) Cell or Sample Preparation:

1.1) Seed Human Embryonic Kidney 293 cells (HEK293) on 20 mm round glass coverslips in a 6-well dish.

1.2) Maintain cells in minimal essential medium (MEM, containing 10% v/v fetal bovine serum) for 24 hours or at least cells attain 60-70% confluency at 37°C in an incubator.

1.3) Transfect three out of six wells from step 1.1 with 3 $\mu\text{g}/\mu\text{l}$ FRET biosensor (this yields 3 coverslips containing expressing cells and 3 coverslips containing control or non-expressing cells).

1.4) Incubate cells for 48 hours at 37°C in an incubator.

Note: The detailed step-by-step procedure for cell seeding and cell transfection (for steps 1.1) through 1.4)) are available in the supporting/supplemental file named “Supplemental File_Cell Culture and Transfection”.

1.5) Fix HEK293 cells expressing FRET biosensor to obtain Turquoise and Venus controls:

1.5.1) Clean the microscope slide using alcohol swab to make sure that the slide is clean and that there are no dust particles.

1.5.2) Add a drop of mounting medium on the microscope slide.

1.5.3) Gently remove the coverslip containing expressing cells using lab tweezers and place the coverslip on the microscope slide containing the mounting medium.

Note: Make sure that the face of the coverslip containing cells should be facing the microscope slide/mounting medium. Please refer to “List of Materials” for mounting medium used in these studies.

1.5.4) Allow the fixed cells to cure for 24 hours in a dark place.

1.5.5) Apply clear nail polish along the edge of the coverslip such that the coverslip is adhered to the microscope slide.

1.5.6) Allow the nail polish to dry for few minutes.

1.6) Prepare DRAQ5 single label control cell sample using non-expressing HEK293 cells.

1.6.1) Place a coverslip containing non-expressing HEK293 cells in a cell chamber.

1.6.2) Add 4 μ l of 5mM nuclear label to 800 μ l of buffer and vortex for few seconds for uniform mixing of the dye in the buffer.

1.6.3) Add the nuclear label-buffer mix to the cell chamber and incubate for 10 minutes at room temperature.

1.7) Place coverslip containing non-expressing cells in a cell chamber and cover with 800 μ l of buffer. This will be used as autofluorescence single label control.

1.8) Place a plain and clean 20mm round glass coverslip in a cell chamber. This will be used as a control to obtain spectra for coverslip fluorescence and reflection of the light.

Note: Note that fixed cell samples prepared in step 1.5 will be good for couple of weeks and can be used to image at user convenience. Sample or cell preparation outlined in steps 1.6, 1.7, and 1.8 should be prepared when the user is ready to start imaging.

2) Spectral Image Acquisition:

2.1) Turn the microscope system 30 minutes prior to imaging to allow the system warm up and to achieve optimal running conditions.

2.2) Open image acquisition software and load camera and system parameters as detailed in manuscript protocol section 2 for image acquisition. It may be helpful to open a prior saved image and reuse the camera and device settings.

2.3) Place the microscope slide containing fixed expressing HEK293 cells (from step 1.5) on the microscope objective stage.

2.4) Select a field of view with expressing cells using the wide-field fluorescence mode and eyepiece.

2.5) Change to A1 or confocal mode and click the XY button on the A1 plus settings window to acquire one XY plane image (this yields the spectrum of FRET signal containing donor and acceptor signals).

2.6) Save the acquired spectral image.

2.6.1) Click “File” on the top of the acquisition software window.

2.6.2) Click “Save As” under the drop-down list on the file menu.

2.6.3) Click the “folder symbol” in the pop-up window called “Save As Image” to browse and select the folder to save the image in.

2.6.4) Give the image an appropriate filename in the box next to “File name”.

2.6.5) Click save.

2.7) Open the A1 settings window and check the box corresponding to the 488 nm laser (acceptor excitation wavelength) and uncheck the box corresponding to the 405 nm laser line.

2.8) Click OK on the A1 settings window.

2.9) Set the 488 nm laser intensity at 2%.

2.10) Acquire one XY image by clicking the XY button on the A1 plus settings window and save the image as mentioned in steps 2.6.1 – 2.6.2.

Note: This should yield an acceptor signal excited using the acceptor excitation wavelength (i.e., the pure acceptor spectrum).

2.11) Switch the laser line to 514 nm as explained in step 2.7 and set the laser intensity to 100%.

2.12) Click live and expose the sample to 514 nm irradiation until the acceptor signal is completely photobleached.

Note: The time taken for complete photobleaching of acceptor may vary from microscope to microscope depending on the power at the stage, age of the laser and/or type of the microscope system. In our studies, the acceptor fluorophore was completely photobleached (no acceptor signal seen when excited using 488 or 405 nm lasers) in ~ 1.5 hour.

2.13) Switch the laser back to 405 nm and acquire one XY image by clicking the XY button and then save the image as explained in steps 2.6.1 – 2.6.2.

Note: This should yield a donor signal excited using the donor excitation wavelength and in the absence of the acceptor (i.e., the pure donor spectrum). Note that when the acceptor signal is completely photobleached in step 2.12, a pure donor spectral signature is obtained from the same concentration of donor fluorophore as was obtained from the acceptor fluorophore in step 2.10.

2.14) Remove the microscope slide from the stage.

2.15) Place the cell chamber containing single label control for nuclear dye (as prepared in the step 1.6) on the microscope objective stage.

2.16) Open A1 settings menu by clicking the gear symbol on the A1 Plus setting window and check the boxes corresponding to 405 nm and 561 nm laser lines.

2.17) Click “OK”.

2.18) Acquire XY image by clicking on “XY” icon on “A1 Plus settings” window (this yields the pure DRAQ5 spectrum).

2.19) Save the image using an appropriate file name as explained in steps 2.6.1 – 2.6.5.

2.20) Place the sample prepared in step 1.8 (cell chamber containing plain coverslip without cells) on the microscope stage.

2.21) Acquire XY image and save the image using an appropriate file name as explained in steps 2.6.1 – 2.6.5. (this yields a non-specific background spectral signatures resulting from coverslip fluorescence (424 nm peak) and from reflection of the light (505 nm peak)).

2.22) Place the cell chamber prepared in step 1.7 (cell chamber containing coverslip with non-expressing cells) on the microscope stage.

2.23) Acquire and save XY image as explained in steps 2.6.1 – 2.6.5 (this yields a spectral image to obtain cell/matrix autofluorescence spectrum).

3) Extract Spectral Information:

3.1) Create a new individual folder corresponding to images saved in steps 2.6, 2.10, 2.13, 2.18, 2.21, and 2.23.

3.2) Export nd2 files into individual tiff files as described in manuscript protocol steps 3.1 (refer to “Table of Materials” for the software used for image acquisition and data exportation).

3.3) Extract donor spectrum: This can be done using several image analysis software platforms. ImageJ software is used in these studies.

3.3.1) Open image analysis platform (“ImageJ” is used in these studies).

3.3.2) In the main menu, select File → Import → Image Sequence.

3.3.3) Browse to the folder containing the exported .tiff image files (exported in step 3.2) for the spectral image that was acquired using 405 nm excitation. This opens an image stack (each slice in the stack represents an emission wavelength of the spectral image).

3.3.4) Draw regions of interest using the rectangular selection tool (or other shape tool if desired).

Note: Make sure that the regions of interest selected contain sufficient signal intensity and that they do not contain oversaturated pixels (for examples, see Figure 3, A and B in the manuscript). Note that Specify Selection tool may also be used to accurately define the size of a rectangular ROI.

3.3.5) In the main ImageJ window select Analyze → Tools → ROI Manager.

3.3.6) Within the ROI Manager, click ADD. This adds the information of the X and Y coordinates of the ROI to the ROI Manager.

3.3.7) Hover to more in the ROI Manager and click Save.

3.3.8) Select a destination folder and save the regions of interest for further use (if needed) or for documentation (recommended).

3.3.9) In the main ImageJ window select Image → Stacks → Plot Z-Axis Profile.

3.3.10) Click “List” on the pop-up window. This displays the set of values in a new window called Plot Values.

3.3.11) Copy the values from this list.

Note that the Y-axis represents the mean intensity value at a given emission wavelength (X-axis).

3.3.12) Open spreadsheet software, such as Microsoft Excel, and paste the intensity values as a column. Save the Excel file with a file name such as “Spectral Library”.

3.3.13) Create a heading (name) row that contains “Wavelength (nm)” and the endmember names of the spectral library (in this case in the 1st row enter “Wavelength (nm)”, in the 2nd row enter “Turquoise”, in the 3rd row “Venus”, in the 4th row “DRAQ5”, in the 5th row “Background @424nm”, in the 6th row “Background @510nm”, and in the 7th row “Autofluorescence”).

3.3.14) Enter the wavelength information (i.e., 414 – 724 with 10nm intervals excluding wavelength number 564 nm) in the 1st column corresponding to wavelength.

3.3.15) Within the “Plot Values” window on ImageJ, copy the values in the second column and paste these values into the spreadsheet for the column corresponding to Turquoise.

3.4) Extract the acceptor spectrum:

3.4.1) Open the *.tiff image file sequence that was exported in step 3.2 corresponding to the spectral image of the H188 FRET sensor acquired using 488 nm excitation (for the Venus spectrum).

3.4.2) Copy the region(s) of interest that were created and saved in steps 3.3.8 and apply to the Venus image stack. If the ROI manager is closed, open it again and load the saved ROIs (Analyze → Tools → ROI Manager → Open).

3.4.3) Repeat steps 3.3.9 and 3.3.11 and paste the values into the spreadsheet column corresponding to Venus.

Note that these values correspond to the Venus emission spectrum when excited using a 488 nm laser. However, for linear unmixing and further FRET efficiency calculations, it is necessary to correct the Venus spectrum to units that would be obtained as if Venus were excited using the same acquisition settings as those used to measure the Turquoise spectrum (i.e., the 405 nm

laser). To correct the Venus spectrum obtained with 488 nm laser excitation back to similar intensity units as would be expected with 405 nm laser excitation perform the following steps:

3.5) Test the laser linearity of the confocal system using either a laser power meter or a fiber-coupled spectrometer with integrating sphere calibrated to a NIST traceable light source (refer to “List of materials” for the details).

3.5.1) Collect the absolute irradiance of the 405 nm laser line and the 488 nm laser line over a range of illumination intensities (e.g., 0%, 5%, 10%, 20%, 30%, 40%, ..., 100%).

3.5.2) Plot the integrated absolute irradiance (units of mW or total photons) detected (Y-axis) at each laser intensity (X-axis) for each laser line. For the laser platform on the Nikon A1R, we have found that the laser response with intensity setting is linear (this should be the case for most laser platforms on most confocal microscopes).

3.5.3) Fit a linear trendline to the data for each laser response and make note of the trendline equation. This equation can be used for comparing laser intensities at any setting (see below).

3.5.4) Using laser trendline equations, calculate total number of photons measured at 2% and at 8% laser intensities of 405 and 488 nm laser lines.

3.5.5) Correct the Venus emission intensity obtained at each wavelength when excited using the 488 nm laser (in step 3.4) to obtain the Venus emission intensity that would be expected if excited using a 405 nm laser using the following equation (Beer-Lambert’s Law):

$$I_{Venus_{405\text{ nm}@8\%}} = I_{Venus_{488\text{ nm}@2\%}} * \left(\frac{A_{Venus@405}}{A_{Venus@488}} \right) * \left(\frac{\#of\ Photons_{405\text{ nm}@8\%}}{\#of\ Photons_{488\text{ nm}@2\%}} \right)$$

where, I is emission intensity (at each emission wavelength) and A is absorbance measured using spectrofluorimeter at specified wavelengths.

Note: We set the 405 nm laser line to 8% laser intensity during image acquisition. However, to avoid oversaturated pixels, we set the 488 nm laser line to 2% intensity for acquiring Venus image. So, when correcting the Venus image from 488 nm to 405 nm, the values of the two laser lines at the two different illumination intensity settings were used by calculating the intensity values from the linear trendlines for each laser.

3.5.6) Create a new column in the excel spread sheet to paste the corrected Venus spectral data.

3.6) Open the sequence corresponding to the DRAQ5 control image, draw regions of interests and extract the spectral information of selected regions by repeating steps 3.3.9 and 3.3.11. Paste the spectral data that corresponds to pure DRAQ5 to the “DRAQ5” column of the spreadsheet created in steps 3.3.12 – 3.3.13.

3.7) Repeat steps 3.3.9 and 3.3.11 to extract spectral data corresponding to two different background spectral signatures: 424 nm (select a region of interest with peak intensity at 424 nm) and 510 nm (select a region of interest with peak intensity at 510 nm). Extract the average

spectrum from each region and paste them in the columns corresponding to “424 nm Background” and “510 nm Background” of the excel sheet created in step 3.3.12.

Note: Two different signal patterns and spectral signatures were observed when imaging the sample blank (plain coverslip). These signatures have been described here as background signatures with a peak intensity of either 424 nm or 510 nm. Both of these signatures may be extracted from the same control image that was acquired using plain coverslip.

3.8) Extract spectral data corresponding to the “autofluorescence” from the image acquired using control cells (from step 2.23) and paste the spectral information in the column corresponding to “Autofluorescence” of the spreadsheet created in step 3.3.12.

3.9) Normalize each spectrum in the spectral library, EXCEPT for the Venus spectrum, to a peak value of unity by dividing each spectrum by the value at the highest intensity wavelength (e.g., peak normalized to a value of one).

3.10) Normalize the Venus spectrum with respect to the peak value of the Turquoise spectrum (Venus and Turquoise spectral data are both normalized to the peak signal intensity of Turquoise).

3.11) Create the spectral library as a MATLAB variable. An example spectral library is provided on the University of South Alabama BioImaging and BioSystems website, under the Resources tab (<https://www.southalabama.edu/centers/bioimaging/resources.html>)

3.11.1) Open programming software (refer to “List of materials” for the details on programming software used).

3.11.2) Create a new variable called ‘Library’.

3.11.3) Copy and paste the normalized data (step 3.9 and 3.10) into the ‘Library’ variable.

3.11.4) Create a new variable called ‘EndMember_Name’ and enter the names of each endmember in the same order in which the spectral information is pasted into Library file.

3.11.5) Save the Library and EndMember_File together with the filename “Library.mat” or similar that can be easily identified (add a date or experiment number to the file name if desired).

3.12) Open a new variable called ‘Wavelengths’ and enter wavelength numbers corresponding to channels in the spectral image as a signal column.

3.13) Save the wavelength variable as “Wavelengths.mat” or a similar file name and place the file in the same folder in which the library file is saved.