

Supplemental Information - Cell Culture and Transfection:

Cells and cell type may vary from study to study and hence cell-specific cell culture procedures should be followed to seed and grow cells. Pulmonary microvascular endothelial cells (PMVECs) were used to study the cAMP spatial distributions shown in the manuscript titled “Measurement of Three-Dimensional cAMP Distributions in Living Cells Using 4-Dimensional (x, y, z, and λ) Hyperspectral FRET Imaging and Analysis”. This supplemental file provides detailed information on the cell seeding, cell culturing, and cell transfection protocols used.

Rat PMVECs were isolated as described previously¹ and maintained in medium (refer to “List of Materials” for details on the medium used) supplemented with 10% (vol/vol) fetal bovine serum (Gemini), 100 U/ml penicillin, and 100 μ g/ml streptomycin, pH 7.0 until cells attained 100 % confluency. Cells were seeded on 25 mm round glass coverslips for imaging as described below.

1) Cell Splitting:

1.1) Aspirate media from the dish.

1.2) Rinse cells with PBS buffer.

1.3) Add 1 ml trypsin containing 0.25 % EDTA and incubate for 5 min at 37 °C.

1.4) Add known volume of media (this volume will be used to calculate concentration of cells in later steps) containing fetal bovine serum to arrest the action of trypsin.

1.5) Resuspend cells into a conical.

1.6) Count cells using a cell counter as follows.

Note that there are several alternative ways to perform cell counting for cell culture. Here, we describe use of an automated cell counter.

1.6.1) Gently mix resuspended cells by tapping the bottom of the conical.

1.6.2) Take 10 μ l of resuspended cell media into a vial and add 10 μ l of trypan blue and mix gently using the same pipette.

1.6.3) Take 10 μ l of this mixture (cell media and trypan blue) and pipet into a disposable cell counting chamber slide.

1.6.4) Insert the slide into the cell counter.

1.6.5) Wait for 10 seconds and record the total number of viable cells. It is recommended to take picture of the cell counter screen for laboratory records.

1.6.6) Centrifuge resuspended cells (remaining cell suspension after step 1.6.2) at $135 \times g$ for 10 minutes. Make sure to note the volume of the cell suspension by reading the volume present in the conical. This is used to calculate the total number of cells present in the cell suspension.

1.6.7) Carefully remove the supernatant, so as to not disturb the cell pellet.

1.6.8) Calculate the total number of cells in the cell pellet using the recorded values. For example, if the volume of cell suspension that is centrifuged is 5 ml and total viable cell count is 1 million cells/ml (from cell counter in step 1.6.6), then the total number of cells in the cell pellet after centrifugation will be 5 million cells.

1.6.9) Resuspend the cell pellet in 1 ml of media. This establishes the working concentration of cells – as an example, 5 million cells/mL based on the above example calculation.

1.7) Plate 200,000 - 250,000 cells/well on prepared laminin coated coverslips (explained below).

1.8) Laminin Coated Coverslips:

1.8.1) Place one 25 mm round glass coverslip in each well of a six-well plate.

1.8.2) Dilute laminin in PBS buffer to make a final concentration of laminin of $5 \mu\text{g/ml}$.

1.8.3) Add 1 ml of $5 \mu\text{g/ml}$ laminin solution to each well of the six-well plate to coat the coverslips.

1.8.4) Incubate the six-well dish containing laminin coated coverslips for at least 1 hour at 37°C . Note that the laminin coated coverslips can be used even after longer incubation times.

1.8.5) Pipette off the laminin solution after ≥ 1 -hour incubation and wash 3 times with buffer (PBS) before using for cell seeding.

1.8.6) Incubate for 24 hours at 37°C or until the cells attain 70-80% confluency.

2) Cell Transfection: Transfect the cells with the FRET sensor using transfection solution, as described below.

Note: The transfection reagent used in these studies comes as solutions: Solution A and Solution B. Please refer to “Table of Materials” to see details about the transfection reagent used.

2.1) Take two test tubes (mark A and B) and add $750 \mu\text{l}$ of media to each test tube.

2.2) Add $20.5 \mu\text{l}$ solution A to test tube A and gently mix by tapping the test tube.

2.3) Add $18.3 \mu\text{l}$ ($3 \mu\text{g}/\mu\text{L}$) of FRET based biosensor plasmid (DNA) and $30 \mu\text{l}$ of solution B to test tube B and mix by tapping the tube.

Note: We have performed transfection tests with different concentrations of plasmid ranging from 2 to 5 $\mu\text{g}/\mu\text{l}$. Based on the transfection efficiency and expression levels of the probe in the cells, we selected 3 $\mu\text{g}/\mu\text{l}$ as our optimal plasmid concentration to transfect the cells. The concentration of plasmid DNA used is to attain optimum transfection in PMVECs – it may be necessary to modify this concentration for another cell line or cell type.

2.4) Mix contents in test tube A and B and incubate for 5 minutes at room temperature.

2.5) Add 250 μl of the mixture to each well of a six well dish containing cells grown on laminin-coated coverslips. Note: The volumes of media, DNA, and transfection reagent described here are sufficient to transfect 6 coverslips.

2.6) Incubate cells at 37 °C for 48 hours.

References:

1. Thompson, W. J., Ashikaga, T., Kelly, J. J., Liu, L., Zhu, B., Vemavarapu, L. & Strada, S. J. Regulation of cyclic AMP in rat pulmonary microvascular endothelial cells by rolipram-sensitive cyclic AMP phosphodiesterase (PDE4). *Biochemical Pharmacology* 63, 797–807 (2002).