Detection and quantification of intracellular signalling using FRET-based biosensors and high content imaging.

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Running title: High content imaging analysis of intracellular signalling
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Summary

Förster Resonance Energy Transfer (FRET) biosensors represent invaluable tools to detect the spatiotemporal context of second messenger production and intracellular signalling that cannot be attained using traditional methods. Here, we describe a detailed protocol for the use of high content imaging in combination with FRET biosensors to assess second messenger production and intracellular signalling in a time-effective manner. We use four different FRET biosensors to measure cAMP levels, kinase (ERK and PKC) and GTPase activity. Importantly, we provide the protocols to express and measure these sensors in a variety of model cell lines and primary dorsal root ganglia neurons.

Key Words: cell signalling, Förster Resonance Energy Transfer, biosensors, high content imaging, extracellular regulated kinase, protein kinase C, cyclic AMP, Rac1.

1. Introduction

The study of complex signalling systems in living cells is now possible due to a growing range of biosensors designed to alter Förster Resonance Energy Transfer (FRET) signals upon activation of specific signalling pathways [1]. These FRET biosensors represent a major advance over traditional population-based assays, as they allow visualisation of the spatial and temporal dynamics of intracellular signalling in real time and in live cells. Their
use is further facilitated by the fact they are genetically encoded and require very simple optics and software for analysis. For example, FRET sensors have been described that detect changes in cAMP levels, specific kinase activity or GTPase activity [2-7]. Importantly, these biosensors can be targeted to subcellular compartments (nucleus, mitochondria, cytosol, plasma membrane), allowing the detection of distinct signalling events with subcellular resolution (Fig 1).

[Fig 1 near here]

The use of FRET biosensors to visualise signalling at the single cell level also provides the opportunity to discriminate the responses of individual cells within a mixed cell population (for example in primary neuronal cultures). Therefore the use of such sensors provides an additional level of information that would otherwise be lost in population-based signalling assays, where the total response reflects a composite of many individual responses (Fig 2).

[Fig 2 near here]

Up until recently, FRET reporters were typically used in combination with imaging systems that have very limited throughput. Although these systems are highly sensitive, their use of entails lengthy acquisition sessions. Recent advances in high content image acquisition and analysis provide a unique opportunity to assess second messenger production and intracellular signalling in a more time-effective manner. Here, we describe protocols for the use of high content imaging to investigate intracellular signalling in model cell lines and primary neuronal cultures using FRET biosensors. We also provide macros for semi-automated analysis of the FRET measurements, and additional details that should be considered at each stage of the experimental design.

2. Materials
2.1. Cell culture and transfection

1. Dulbecco’s Modification of Eagle’s Medium (DMEM).

2. Foetal Calf Serum (FCS).

3. Roswell Park Memorial Institute-1640 medium (RPMI).

4. N1 neuronal supplement: 0.5 mg/mL recombinant human insulin, 0.5 mg/mL human transferrin (partially iron-saturated), 0.5 μg/mL sodium selenite, 1.6 mg/mL putrescine, and 0.73 μg/mL progesterone.

5. Ca\(^{2+}/\)Mg\(^{2+}\)-free Hank’s Balanced Salt Solution (CMF-HBSS): 137 mM NaCl, 5.3 mM KCl, 0.44 mM KH\(_2\)PO\(_4\), 4.1 mM NaHCO\(_3\), 0.34 mM Na\(_2\)HPO\(_4\), pH 7.4. Autoclave and store at 4°C. Add glucose at 1 g/L prior to use.

6. Cell line growth media: DMEM, 5% FCS (see Note 1).

7. Primary neuronal culture growth media: DMEM, 10% FCS, 1% antibiotic-antimycotic solution, 1% N1 neuronal supplement (see Note 2).

8. Collagenase Type IV.

9. Dispase II.

10. Digestion buffer: 2 mg/mL Collagenase Type IV, 2 mg/mL Dispase II in CMF-HBSS. Make 500 μL per mouse in 1.6 mL microtubes.

11. Sterile Phosphate Buffered Saline (PBS) Mg\(^{2+}/\)Ca\(^{2+}\)-free: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH\(_2\)PO\(_4\), 8 mM Na\(_2\)HPO\(_4\), pH 7.4.

12. PBS/EDTA: sterile PBS with 0.5 mM EDTA.

13. Laminin (1 mg/mL).

14. Poly-D-Lysine (PDL). Prepare a stock solution of 1 mg/mL PDL in sterile ultra pure water. Filter sterilise, aliquot and store at -20°C (see Note 3).
15. Dissection instruments: scissors (straight, ≥ 25 mm cutting edge), forceps (e.g. Dumont #5), spring scissors (e.g. Noyes 14 mm cutting edge, straight), spring scissors (e.g. Vannas 3 mm cutting edge, curved).


17. Nucleofection system and reagents. Lonza 4D-Nucleofector™ system and P3 Primary Cell 4D- Nucleofector™X Kit have been used for this protocol.

18. Bunsen burner.

19. Glass Pasteur pipettes and rubber bulb (see Note 4).

20. Water bath.

21. Incubator (37°C, 5% CO₂/air).

22. Cell culture hood.

23. Black, optically clear PDL-coated 96-well plates (ViewPlate, PerkinElmer). For neuronal cultures, use a half-area black, optically clear 96-well plate to increase cell density. To coat plates, dilute PDL to 50 μg/mL in PBS and incubate wells with 50 μL diluted PDL for 10 min. Rinse wells twice with 100 μL of PBS (see Notes 3 and 5).

24. 150 mM NaCl (filter sterilised).

25. Polyethylenimine (PEI), linear molecular weight ~25000 Da. Prepare a stock solution of 1 mg/mL PEI in 150 mM NaCl. Filter sterilise, aliquot and store at -20°C (see Note 6).

26. DNA constructs: receptor/protein of interest, FRET biosensors. A complete list of available FRET biosensors can be viewed at www.fret.lif.kyoto-u.ac.jp/e-phogemon/unifret.htm. The FRET biosensors routinely used in our laboratories are detailed below (see Table 1).

2.2. FRET detection and image analysis
1. Hank’s Balanced Salt Solution (HBSS): 137 mM NaCl, 5.3 mM KCl, 1.3 mM CaCl₂, 0.41 mM MgSO₄, 0.5 mM MgCl₂, 0.44 mM KH₂PO₄, 4.1 mM NaHCO₃, 0.34 mM Na₂HPO₄, pH 7.4. Autoclave and store at 4°C. Add glucose at 1 g/L prior to use.

2. Stock solution of drugs and vehicle control.

3. Stock solution of the positive control appropriate to the FRET biosensor to be used (see Table 2 and Note 7).

4. GE Healthcare INCell 2000 version 4-10590 (INCell software version 6.01_21986) with FRET module (see Note 8).

5. Image analysis software. The macros in this protocol use the FIJI distribution of ImageJ [8].

6. GraphPad Prism analysis software.

3. Methods

3.1. Cell culture

3.1.1. Cell lines

1. HEK293 cells are grown in DMEM, 5% FCS in culture flasks or dishes. Cells should be kept in incubators with a humidified atmosphere containing 5% CO₂ at 37°C and passaged once they reach 80-90% confluency.

2. MCF7 cells are grown in DMEM, 5% FCS and 10 μg/mL insulin in culture flasks or dishes. Cells should be kept in incubators with a humidified atmosphere containing 5% CO₂ at 37°C and passaged once they reach 80-90% confluency.

3.1.2. Dorsal root ganglion neurons isolation and culture (see Note 9)

This protocol has been optimised for use with dorsal root ganglion (DRG) neurons from adult mice (see Note 10) and utilises the Lonza 4D-Nucleofector™ system in combination
with Nucleovette™ strips (a summary of the advantages and disadvantages of various delivery methods in primary neuronal cultures is provided by Zeitelhofer et al. [9]).

1. Kill mouse using method approved by institutional animal ethics committee (e.g. cervical dislocation).
2. Clean dorsal skin with 70% ethanol. Decapitate mouse and cut skin caudally along the vertebral column using scissors.
3. Remove vertebral column starting from the cranial end. Lift as you cut and ensure that viscera are not attached. Trim skeletal muscle and ribs (see Note 11).
4. Place tissue onto a petri dish. Open spinal canal from the cranial end by cutting halfway down the transverse face on either side. Open approximately 1 cm of the spinal column using the larger Noyes spring scissors (see Note 12).
5. Harvest DRGs using the dissection microscope. Remove and discard the spinal cord. DRG should be visible in pockets on either side at the base of the vertebrae. Isolate the ganglia by grasping the associated root with forceps (#5) and pulling gently. This will move the ganglion slightly out of the pocket. Use the small curved Vannas spring scissors to cut the nerve located behind the ganglion while still pulling on the root. Place the extracted ganglion in a 35 mm diameter petri dish containing cold CMF-HBSS on ice. Harvest all DRG along the spine unless the experiment requires the collection of specific levels only.
6. Once collected, trim any residual roots and attached connective tissue from the isolated ganglia. This will result in a cleaner neuronal culture.
7. Place the cleaned DRG into a 1.6 mL microtube containing 500 μL pre-warmed digestion buffer. Incubate at 37°C in a water bath or incubator.
8. After 30 min, triturate using the largest diameter Pasteur pipette (~5 times). Push the pipette tip to the bottom of the tube and take care not to lose any content from the tube. The solution should now appear cloudy. Return tube to 37°C.

9. After 15 min, repeat the trituration step above using the medium diameter pipette.

10. After 10 min, repeat the trituration step using the smallest diameter pipette.

11. Remove laminin solution from the PDL-coated cell culture plates (see Note 5) and allow the plates to air dry in the cell culture hood.

12. Transfer digests to a 15 mL tube containing 5 mL DMEM and centrifuge (500 x g, 5 min). Aspirate supernatant and resuspend the pellet in 5 mL DMEM. Repeat this washing step three times to remove any residual digestive enzymes.

13. Proceed to Section 3.2.3.

### 3.2. Transfection

#### 3.2.1. Introduction

1. To investigate the signalling of heterologously expressed receptors, co-transfection of the receptor and FRET biosensor of interest is required. Alternatively, if the protein of interest is endogenously expressed, transflect only the FRET biosensor of interest.

2. The transfection method depends on the cell line used for each study. Most model cell lines (including HEK293, CHO, COS, HeLa cells) can be transfected using inexpensive methods such as PEI or calcium phosphate. If these transfection methods are unsuccessful, more expensive methods such as Lipofectamine2000 or nucleofection may be required.

#### 3.2.2. Cell transfection using PEI
1. Seed the cells into a 96-well black, optically clear PDL-coated plate in culture medium at a density which achieves 50% confluency in 24 h.

2. Incubate the cells in a 37°C incubator in a humidified atmosphere containing 5% CO₂ for 24 h.

3. Prepare microtubes containing 50-100 ng of DNA (see Note 13) in sterile 150 mM NaCl to a total volume of 10 μL per well.

4. In separate tubes, prepare a solution of PEI in sterile 150 mM NaCl to a final volume of 10 μL per well. The ratio of DNA:PEI should be optimised for each cell line, but an initial ratio of 1:6 is recommended. For example, 50 ng of DNA would require 300 ng of PEI for a 1:6 ratio.

5. Add the diluted PEI solution (step 4) to the diluted DNA solution (step 3), mix thoroughly with a vortex, and incubate for 10-30 min at room temperature.

6. Change the media of the cells. Add 200 μL of fresh DMEM with 5% FCS to each well.

7. Add 20 μL/well of the DNA:PEI mix to the cells.

8. Incubate the cells in a 37°C incubator in a humidified atmosphere containing 5% CO₂.

9. Experiments are performed 48 h after transfection following serum restriction of the cells by incubation overnight in DMEM with 0.5% FCS (see Notes 14 - 17)

3.2.3. Cell transfection by nucleofection

1. Following the final wash (see step 12 in Section 3.1.2.), resuspend pellet in 20 μL P3 solution containing 600 ng plasmid (see Note 18).

2. Transfer digests from each mouse to individual wells in the Nucleocuvette™ strip and nucleofect (program DC104 according to manufacturer’s instructions).
3. Immediately, add pre-warmed RPMI medium (150 μL) to fill each well. Place the strip in the incubator for 10 min to allow neurons to recover.

4. Transfer solution to a 1.6 mL microtube and add complete media to bring the total volume to 100 μL per well (e.g. add an additional 430 μL medium to seed neurons into 6 wells of a half-area 96-well plate). Seed neurons into a PDL/laminin pre-coated black, optically clear half-area 96-well plate, and incubate in a 37°C incubator in a humidified atmosphere containing 5% CO₂.

5. Experiments are performed after 48 h in vitro following serum restriction of the cells by incubation overnight in DMEM with 0.5% FCS.

3.3. FRET detection using a high content imager

This protocol is based on the use of the widefield high content imager INCell 2000. However, the steps described below should also be applicable to other high content imagers.

3.3.1. Equilibration of cells

1. Add 1 g/L glucose to stock HBSS and pre-warm to 37°C.
2. Aspirate serum-restricted cell growth medium and gently add 160 μL pre-warmed complete HBSS to each well (see Note 19).
3. If pre-treating cells with inhibitors, add 140 μL pre-warmed complete HBSS to each well, then 20 μL of inhibitor (diluted in HBSS) to the desired final concentration.
4. Equilibrate cells in HBSS at 37°C without CO₂ for 30 min (see Note 20).

3.3.2. Instrument set up: generating an Acquisition Protocol using the GE INCell 2000 with FRET module

2. Plate/Slide: select the appropriate plate template from the drop-down list (see Note 21).

3. Objective: select the 40x objective (see Note 22).

4. Microscopy: select the number of wavelengths to be collected and the appropriate polychroic filter set (Quad); list and define the wavelengths in the order they are to be acquired (see Table 3 and Notes 23-25).

5. Focus: select the ‘Refocus at each timepoint’ option. Focus should be optimised at the commencement of each experiment (see Section 3.3.3).

6. Plate Heater: select the ‘Use plate heating’ option, and set to 37°C.

7. Time Series: select the ‘Acquire time series’ option, and ‘look walk look’ mode (acquires images from each selected well within the specified time period). Specify the starting time point, the time interval (in seconds) and the number of time points to be acquired. For example, start at time 0, with interval of 60 seconds, and acquire 21 time points. This protocol will acquire an image every minute for 20 minutes (see Note 26).

3.3.3. Stimulation of cells and image acquisition

1. Pre-heat GE INCell 2000 to 37°C, switch on the lamp, and open the FRET biosensor Acquisition Protocol (see Section 3.3.2).

2. Highlight the wells to be acquired using the Plate/Slide Viewer, and select a region of interest (ROI, designated by the red square) within a well (see Notes 27 and 28).

3. Define the optimal focus strategy for the acquisition. Within the Focus panel in the Protocol Designer, select the ‘Laser autofocus’ option then ‘Auto Offset’. Adjust the calculated offset and exposure time as required (see Note 29).
4. Collect a ‘Baseline’ FRET time series: using an interval of 60 seconds, acquire 5 time points. For time 0 select the brightfield wavelength only, for times 1-4 select FRET and donor wavelengths only.

5. Stimulate the same cells with vehicle or ligand of interest, and collect a ‘Stimulated’ FRET time series: using an interval of 60 seconds, acquire 21 time points. Select FRET and donor wavelengths only (see Note 30).

6. Stimulate the same cells with the appropriate positive control (see Section 2.2) for 10 min. Collect a ‘Positive’ FRET time series: using an interval of 60 seconds, acquire 4 time points. Select FRET and donor wavelengths only (see Note 31).

3.4. Image analysis

3.4.1. Introduction

Pre-processing and image analysis can be performed in many different ways using a large number of image analysis environments. For this protocol, pre-processing and image analysis is performed using three in-house macros that run in the FIJI distribution of ImageJ (see Notes 32 and 33). The first (Stack Creator v2.0) combines all image files into one hyperstack per field of view (i.e. the hyperstack contains the baseline, stimulated and positive control time points from both the donor and FRET channels) (see Note 34). Once the images are combined by the Stack Creator macro they can be processed by the two FRET Analysis macros. The first (FRET Analysis – Cell Markup v1.0) requires the user to manually select cells and define the background. The second (FRET Analysis – Batch Analyse v1.0) batch processes the hyperstacks and automatically calculates the background corrected FRET ratio, F/F₀ and F/Fₘₐₓ values for all fields and timepoints. This macro also saves an overview image of each field as a record of the cells that were
analysed. A breakdown of the macros is provided below. Final data is visualised using GraphPad Prism v6.0 (Fig. 3).

[Link to macros here]

3.4.2. Automated stacking and image alignment using the Stack Creator v2.0 macro

1. Move all brightfield images from the baseline time series into a new directory.
2. Open the Stack Creator v2.0 macro using FIJI, and press Run.
3. Follow the prompts to choose the directories that contain the baseline, stimulated and positive control time series, and a new output directory in which to save the final hyperstacks.
4. Define the settings for Stack Creator by entering the total number of wells scanned, the number of fields scanned per well, the well from which to start processing (see Note 35), and the number of baseline/stimulated/positive time points captured within each series.
5. If the donor channel is listed first within the directory, select the check box.
6. For automatic alignment of the images within the hyperstack, select the check box.
7. Select OK.

3.4.3. Defining ROIs using the FRET Analysis – Cell Markup v1.0 macro

1. Open the FRET Analysis – Cell Markup v1.0 macro using FIJI, and press Run.
2. Follow the prompt to choose the directory that contains the hyperstack generated by the Stack Creator macro.
3. Follow the prompt to define the background by drawing a circle in an area without any cells, and press OK.
4. Follow the prompt to mark out all cells of interest (see Notes 36-38), then press OK.
5. Repeat for all hyperstacks within the selected directory.
3.4.4. Data analysis using the FRET Analysis – Batch Analyse v1.0 macro

1. Open the FRET Analysis – Batch Analyse v1.0 macro using FIJI, and press Run.
2. Follow the prompt to choose the directory that contains the hyperstack generated by the Stack Creator macro and a sub-directory containing the ROIs generated by the FRET Analysis – Cell Markup macro.
3. Follow the prompt to define the experimental settings by entering the number of baseline/stimulated/positive control time points, and whether the data should be output as FRET/donor or donor/FRET (see Note 39). Press OK.
4. Once the macro has finished the batch analysis, graph the F/F₀ values for all ROIs to visualise responses from the entire cell population using GraphPad Prism.
5. To be included in further analysis, cells must meet the following criteria. First, cells must demonstrate at least a 5-10% change in F/F₀ (F/F₀ value greater than 1.05-1.10) following addition of the positive control. Second, the change in F/F₀ following addition of the positive control should not exceed 200% (F/F₀ value of 2).
6. Group F/Fₘₐₓ values for all cells using GraphPad Prism (see Note 40 and Fig.3)

[Fig 3 near here]

3.4.5. Stack Creator v2.0 – Macro Function Details

1. **Initial Setup.** Values that will be needed later in the macro must first be created and set to zero. These values include: the file separator (to avoid issues in moving between operating systems), the total number of files to process, the total number of baseline frames, the total number of stimulated frames, and the total number of positive frames (see Note 32).
2. **Locating Files.** The source directories for the files must be defined. The macro will ask the user to select the source directories for the baseline, stimulated and positive control images, and to select a directory within which to save the resulting hyperstacks (see Note 41).

```javascript
//Macro to merge individual image planes into stacks from three separate directories. //directories contain baseline frames, stimulated frames and positive control frames

//store global variables for later use
//fs for file separator (allows cross compatibility between mac and pc)
//fn for total file number
//bc for total baseline frame number
//sc for total stimulate frame number
//pc for total positive stimulation frame number
var fs = File.separator;
var fn = 0;
var bc = 0;
var sc = 0;
var pc = 0;

//ask user to select baseline, stimulated, positive and output directory lists
dir1 = getDirectory("Choose Baseline Source Directory ");
list1 = getFileList(dir1);
Array.sort(list1);

dir2 = getDirectory("Choose Stimulated Source Directory ");
list2 = getFileList(dir2);
Array.sort(list2);

dir3 = getDirectory("Choose Positive Source Directory ");
list3 = getFileList(dir3);
Array.sort(list3);

dir4 = getDirectory("Choose Output Directory");
```

3. **Defining Experimental Conditions.** To correctly parse all the captured files, the user must define the conditions of image capture, including: the total number of wells; the number of fields of view per well; which well to process first (see step 4); the number of time frames captured for baseline, stimulated and positive control time series; whether the first file in the directory list is from the donor channel or the FRET channel; and whether an auto-alignment should be performed to correct for any drift and cell movement throughout the time series.
4. Starting at a Designated Well. There are instances when the user may wish to
analyse a defined subset of wells. This option allows the user to begin processing at
any well within the directory, ignoring all wells preceding this point during acquisition.

   // if starting from a well other than the first one, other time values need to be adjusted
   appropriately
   if (startWell>1){
       bc = (startWell-1)*baseCapture;
       sc = (startWell-1)*stimulateCapture;
       pc = (startWell-1)*positiveCapture;
       fn = startWell*(baseCapture*2);
   }

5. Generation of Hyperstacks. The macro automatically processes all files in the
selected directories. This uses one of two sections of code, depending on whether
the FRET or donor images are listed first within the directory. The first version
assumes the donor images are first and is run under the If(donorFirst==1) logic. The
second version assumes the FRET images are first, and is run under the
if(donorFirst==0) logic. The value for donorFirst is set by the user in the initial
configuration dialog by the “Is the Donor Channel First in the File List?” check box
(see Note 42). To generate the combined hyperstacks, the macro first creates a
series of blank “dummy” images, into which it then copies each captured image
within the time series.
Batch Processing the Hyperstacks. The baseline images for the donor channel are processed first. This part of the code is looped depending on the number of baseline images that were entered in the initial configuration dialog. The end result is a stack of baseline images for the donor channel with one slice per time point.

This is repeated for the stimulated and positive control donor channel images, then the whole process is repeated for the FRET channel images.

Correcting for Incorrect name Sorting. Loading files through the Array commands puts them in a non-natural order. If there are no leading zeros on single digit numbers they will be loaded in the order 1,10,11,12,13,14,15,16,17,18,19,2,20,21 for example. To correct this the created stacks are reordered to be in the correct order. The code example here will only process up to 30 timepoints. This code is only applied to the stimulate capture series as it is the only one that has enough timepoints to have issues.
//correct order of time points of the Donor Stack if greater than 9 captured
if (stimulateCapture>=18&&stimulateCapture<=19){
  stimulateEnd=stimulateCapture-10;
  sub1Start=1;
  sub1End=1;
  sub2Start=2;
  sub2End=sub2Start+stimulateEnd;
  sub3Start=sub2End+1;
  sub3End=stimulateCapture;
  selectWindow("Stimulate Stack - Donor");
  run("Make Substack...", " slices="+sub1Start+-"+sub1End);
  rename("Sub 1");
  selectWindow("Stimulate Stack - Donor");
  run("Make Substack...", " slices="+sub2Start+-"+sub2End);
  rename("Sub 2");
  selectWindow("Stimulate Stack - Donor");
  run("Make Substack...", " slices="+sub3Start+-"+sub3End);
  rename("Sub 3");
  run("Concatenate...", " title=[Concatenated Stacks] image1=[Sub 1] image2=[Sub 3] image3=[Sub 2]");
  selectWindow("Stimulate Stack - Donor");
  close();
  selectWindow("Concatenated Stacks");
  rename("Stimulate Stack - Donor");
}

if (stimulateCapture>=20&&stimulateCapture<=29){
  stimulateEnd=stimulateCapture-20;
  sub1Start=1;
  sub1End=1;
  sub2Start=2;
  sub2End=13;
  sub3Start=12;
  sub3End=12;
  sub4Start=13;
  sub4End=sub4Start+stimulateEnd;
  sub5Start=sub4End+1;
  sub5End=stimulateCapture;
  selectWindow("Stimulate Stack - Donor");
  run("Make Substack...", " slices="+sub1Start+-"+sub1End);
  rename("Sub 1");
  selectWindow("Stimulate Stack - Donor");
  run("Make Substack...", " slices="+sub2Start+-"+sub2End);
  rename("Sub 2");
  selectWindow("Stimulate Stack - Donor");
  run("Make Substack...", " slices="+sub3Start+-"+sub3End);
  rename("Sub 3");
  selectWindow("Stimulate Stack - Donor");
  run("Make Substack...", " slices="+sub4Start+-"+sub4End);
  rename("Sub 4");
  selectWindow("Stimulate Stack - Donor");
  run("Make Substack...", " slices="+sub5Start+-"+sub5End);
  rename("Sub 5");
  run("Concatenate...", " title=[Concatenated Stacks] image1=[Sub 1] image2=[Sub 3] image3=[Sub 5] image4=[Sub 2] image5=[Sub 4]");
  selectWindow("Stimulate Stack - Donor");
  close();
  selectWindow("Concatenated Stacks");
  rename("Stimulate Stack - Donor");
}
8. **Merging Donor and FRET Channel Stacks.** The donor and FRET channel stacks are merged into a single hyperstack with channel 1 representing the donor stack and channel 2 representing the FRET stack (see Note 43). If selected in the initial configuration, an automatic alignment using a translation model in the StackReg plugin is performed. The alignment can compensate for movement of cells or slight inaccuracies in the return of the well by the high content imager throughout the time series (see Note 44).

```
//create one stack incorporating baseline, stimulate and positive stacks of donor images
run("Concatenate...", " title=[Concatenated Stacks] image1=[Baseline Stack - Donor] image2=[Stimulate Stack - Donor] image3=[Positive Stack - Donor] image4=[-- None --]");
rename("[Donor Stack]");

//create one stack incorporating baseline, stimulate and positive stacks of FRET images
run("Concatenate...", " title=[Concatenated Stacks] image1=[Baseline Stack - FRET] image2=[Stimulate Stack - FRET] image3=[Positive Stack - FRET] image4=[-- None --]");
rename("[FRET Stack]");

//if auto-alignment was selected carry it out
if(doAlign==1){
    selectWindow("Composite");
    run("Hyperstack to Stack");
    run("StackReg", "transformation=Translation");
    run("Stack to Hyperstack...", "order=xyzt(default) channels=2 slices="+(baseCapture+stimulateCapture+positiveCapture)+" frames=1 display=Color");
}

//merge donor and fret channels into a single hyperstack
run("Merge Channels...", "c1=[Donor Stack] c2=[FRET Stack] create");
```

9. **Naming Files.** The name of the final hyperstack is generated from the first complete file name in the baseline directory (e.g. A -1(fld 1 wv CFP - CFP- time 1 - 0 ms)). Final hyperstacks are saved by well name and field number (e.g. A - 1 - fld 1).

```
//determine well name from file name
forName = indexOf(list1[fi], "(");
if (forName==6){
    wellName = substring(list1[fi], 0, 6);
    fieldName = substring(list1[fi], 11, 12);
    wellID = wellName+- fld"+fieldName;
}
if (forName==5){
    wellName = substring(list1[fi], 0, 5);
    fieldName = substring(list1[fi], 10, 11);
    wellID = wellName+- fld"+fieldName;
}
```

10. **Saving the Final Hyperstacks.** The final hyperstack is saved into the output directory selected by the user.
11. Garbage Collection. Once the final hyperstack is saved, the macro frees all memory used by FIJI (see Note 45).

```java
//save final hyperstack into the user defined output directory
saveAs("Tiff", dir4+fs+wellID);

//close all open images
run("Close All");
```

3.4.6. FRET Analysis – Cell Markup v1.0 – Macro Function Details

1. Initial Setup. The file separator character must be defined to maintain compatibility between operating systems.

```java
//Macro to manually mark out cells for automated analysis of FRET Ratios
//fs for file separator (allows cross compatibility between mac and pc)
fs=File.separator;
```

2. Locating Files. The source directory for the files must be defined. This directory must contain the hyperstacks that were generated using the Stack Creator macro. The macro also creates a directory in which to save the user defined background and cell regions of interest (ROI) within the selected source directory (see Note 41).

```java
//ask user to select source directory that contains stacks generated by the stack creator macro
dir=getDirectory("Select Source Directory");
list=getFileList(dir);
Array.sort(list);

//create directory to store cell ROIs
roidir=dir+fs+"ROIs";
File.makeDirectory(roidir);
```

3. Looping for all Files. The code that defines the background and cell ROIs is looped for all stacks in the source folder.

```java
//Loop code for all tif images in the selected directory
for(r=0;r<list.length;r++){
    fileName=dir+list[r];

    //only process tif images
    if (endsWith(fileName, ".tif")){
        open(fileName);
        nameStore=getTitle();
        run("Select None");
    }
```
4. **Set Up for User Input.** The macro first clears any existing ROIs from the image, and adjusts the contrast to optimise the fluorescent signal.

```plaintext
//check for any existing ROIs and delete them
anyROIs=roiManager("count");
if(anyROIs>0){
    roiManager("Deselect");
    roiManager("Delete");
}

//auto contrast both the donor and FRET channels to make visualisation easier
Stack.setChannel(1);
run("Enhance Contrast", "saturated=0.30");
Stack.setChannel(2);
run("Enhance Contrast", "saturated=0.30");
```

5. **Defining the Background.** The background fluorescence intensity in both the donor channel and FRET channel must be measured. The user is asked to define a region of background (containing no cells) and the background ROI is saved in the ROI directory.

```plaintext
//select the freehand tool and ask the user to define the background
setTool("Freehand");
waitForUser("Draw a region on the background");
roiManager("Add");

//background ROI is saved into the ROIs directory under the image name
roiManager("Save", roidir+fs+nameStore+" - Background.zip");
roiManager("Deselect");
roiManager("Delete");
run("Select None");
```

6. **Defining Cells of Interest.** The user is asked to outline any cells required for subsequent analysis by the FRET Analysis - Batch Analyse macro. Using the freehand tool, draw around each cell to be analysed and add to the ROI Table (see Notes 36-38). These ROIs are saved into the ROIs folder.

```plaintext
//select the freehand tool and ask the user to outline all the cells to be analysed
setTool("Freehand");
roiManager("Show all with labels");
waitForUser("Mark out Cells of Interest\nPress the T Key to add them to the ROI Manager\n\nPress OK when complete");

//marked cell ROIs are saved into the ROI directory
roiManager("Save", roidir+fs+nameStore+" - Cells.zip");
roiManager("Deselect");
roiManager("Delete");
run("Select None");
close(nameStore);
```
3.4.7. FRET Analysis – Batch Analyse v1.0 – Macro Function Details

1. **Initial Setup.** The file separator character must be defined to maintain compatibility between operating systems.

```
//Macro to batch analyse the FRET Ratio, F/F0 and F/FMax values of cells marked out with the FRET Analysis - Cell Markup macro
//fs for file separator (allows cross compatibility between mac and pc)
fs=File.separator;
```

2. **Locating Files.** The source directory for the files must be defined. This directory must contain the hyperstacks that were generated using the Stack Creator macro, and a sub-directory containing the ROIs generated using the FRET Analysis – Cell Markup macro. The macro also creates a directory in which to save the overview images of the analysed cells within the selected source directory (see Note 41).

```
//ask the user to select the source image directory
dir=getDirectory("Select Source Directory");
list=getFileList(dir);
Array.sort(list);

//locate the ROI directory and create an overview directory to save overview images to
roidir=dir+fs+"ROIs";
overviewSave=dir+fs+"Overview";
File.makeDirectory(overviewSave);
```

3. **Defining Experimental Conditions.** To correctly analyse the data, the user must define the number of time points captured for baseline, stimulated and positive control time series, and whether the FRET ratio should be calculated as donor/FRET or FRET/donor (see Note 39).

```
//create a dialog box to collect capture conditions to aid in calculations
//also ask user to select the type of FRET calculation
Dialog.create("FRET Analysis Settings");
Dialog.addNumber("Enter the Number of Baseline FramesCaptured", 0);
Dialog.addNumber("Enter Number of Stimulated Frames Captured", 0);
Dialog.addNumber("Enter Number of Positive Frames Captured", 0);
items = newArray("Donor/FRET", "FRET/Donor");
Dialog.addRadioButtonGroup("Analysis Type", items, 2, 1, "Donor/FRET");
Dialog.show();
baseCapture = Dialog.getNumber();
stimulateCapture = Dialog.getNumber();
positiveCapture = Dialog.getNumber();
analysisType = Dialog.getRadioButton();
totalTime=baseCapture+stimulateCapture+positiveCapture;
```
4. **Data Logging.** A customised table will be created for the analysed data with the following column titles: image name, cell number, time point, background intensity donor, background intensity FRET, raw donor intensity, raw FRET intensity, corrected donor intensity, corrected FRET intensity, FRET ratio, background ratio, F/F₀, F/F₉₅₄.

```plaintext
//define measurements required - only mean intensity is needed
run("Set Measurements...", "mean redirect=None decimal=3");

//create a custom table to log the data into
tableTitle=\"[FRET Analysis]\";
if(isOpen("FRET Analysis")){
    print("Table already open");}
else{
    run("Table...", "name=tableTitle+ width=1500 height=250");
    print(tableTitle, "\tHeadings:Image Name\tCell Number\tTime Point\tBackground Intensity Donor\tBackground Intensity FRET\tRaw Donor Intensity\tRaw FRET Intensity\tCorrected Donor Intensity\tCorrected FRET Intensity\tFRET Ratio\tBackground Ratio\tF/F₀\tF/F₉₅₄");
}
```

5. **Batch Analysis.** The code will be looped for all files in the selected directory. At the start of each loop the hyperstack is opened and the cell ROIs are loaded to count the number of cells to be analysed.

```plaintext
//enter batch mode to speed up processing time
setBatchMode(true);

//batch process for all files in the selected directory
for(r=0;r<list.length;r++){
    fileName=dir+list[r];
    //only process tif files
    if (endsWith(fileName, ".tif")){
        open(fileName);
        nameStore=getTitle();
        run("Select None");
        //clear any ROIs if present
        anyROIs=roiManager("count");
        if(anyROIs>0){
            roiManager("Deselect");
            roiManager("Delete");
        }
        //load the selected cells ROIs to count them and determine how many cells to loop for
        roiManager("Open", roidir+fs+nameStore+" Cells.zip");
        numberCells=roiManager("Count");
        roiManager("Deselect");
        roiManager("Delete");
        run("Select None");
    }
}
```

6. **Calculation of FRET ratios.** Once the number of cells to be analysed is defined, each cell is processed using two very similar sets of code. The first allows
calculation of the average baseline and positive control FRET ratios. The second can then calculate the F/F₀ (FRET ratio / average baseline FRET ratio) and F/Fₘₐₓ values ( [FRET ratio – average baseline FRET ratio] / [average positive control FRET ratio – average baseline FRET ratio] ) for each time point.

```plaintext
// Loop for the number of cells selected
for (i=0; i<numberCells; i++) {
    // Rezero the average baseline FRET and average positive FRET values
    averageBaselineFRET=0;
    averagePositiveFRET=0;

    // Loop for all time points
    for (j=0; j<totalTime; j++) {
        // Load the background ROI and measure its intensity in the donor and FRET channels
        roiManager("Open", roidir+fs+nameStore+" - Background.zip");
        roiManager("Select", 0);
        Stack.setSlice(j+1);
        Stack.setChannel(1);
        run("Measure");
        donorBackgroundInt=getResult("Mean");
        Stack.setChannel(2);
        run("Measure");
        FRETBackgroundInt=getResult("Mean");

        // Clear background ROI
        roiManager("Deselect");
        roiManager("Delete");
        run("Select None");

        // Load the cells ROIs and select one for processing.
        roiManager("Open", roidir+fs+nameStore+" - Cells.zip");
        roiManager("Select", i);

        // Select the correct timepoint to measure
        Stack.setSlice(j+1);

        // Measure the raw intensity of the donor and FRET channels
        Stack.setChannel(1);
        run("Measure");
        rawDonorInt=getResult("Mean");
        Stack.setChannel(2);
        run("Measure");
        rawFRETInt=getResult("Mean");

        // Subtract the background values from donor and FRET values
        correctedDonorInt=rawDonorInt-donorBackgroundInt;
        correctedFRETInt=rawFRETInt-FRETBackgroundInt;

        // Calculate Corrected FRET Ratio based on analysis type selected by user
        if (analysisType=="Donor/FRET"){
            FRETRatio=correctedDonorInt/correctedFRETInt;
        }
        if (analysisType=="FRET/Donor"){
            FRETRatio=correctedFRETInt/correctedDonorInt;
        }

        // Calculate the background ratio between the channels
        backgroundRatio=donorBackgroundInt/FRETBackgroundInt;

        // If at the correct time point (baseline or positive) store the FRET ratio for future calculation
        if ((j+1)<=baseCapture){
            averageBaselineFRET=averageBaselineFRET+FRETRatio;
        }
        if ((j+1)>baseCapture+stimulateCapture){
            averagePositiveFRET=averagePositiveFRET+FRETRatio;
        }

        // Advance to the next time point in preparation for the next loop
    }
}
```
Stack.setSlice(j+2);

//clear any ROIs
    roiManager("Deselect");
    roiManager("Delete");
    run("Select None");
}

before looping again calculate the average Baseline and Positive FRET ratios
    averageBaselineFRET=averageBaselineFRET/baseCapture;
    averagePositiveFRET=averagePositiveFRET/positiveCapture;

loop for all cells calculating F0 and FMax as average baseline and positive FRET ratios are known
for(j=0; j<totalTime; j++)
{

The second set of code allows for calculation of $F/F_0$ and $F/F_{Max}$.

//calculate the $F_0$ and $F_{Max}$ ratio values
    fZero=FRETRatio/averageBaselineFRET;
    fMax1=FRETRatio-averageBaselineFRET;
    fMax2=averagePositiveFRET-averageBaselineFRET;
    fMax=fMax1/fMax2;

//calculate the background intensity ratio
    backgroundRatio=donorBackgroundInt/FRETBackgroundInt;

//advance to the next time point in preparation for the next loop
    Stack.setSlice(j+2);
}

7. Logging Data. The calculated data is logged into the customised table.

//Log all measured and calculated values out to the table
    print(tableTitle,
        nameStore+"\t"+(i+1)+"\t"+(j+1)+"\t"+donorBackgroundInt+"\t"+FRETBackgroundInt+"\t"+rawDonorInt+"\t"+rawFRETInt+"\t"+correctedDonorInt+"\t"+correctedFRETInt+"\t"+FRETRatio+"\t"+backgroundRatio+"\t"+fZero+"\t"+fMax);

//clear any ROIs
    roiManager("Deselect");
    roiManager("Delete");
    run("Select None");
}

//clear the results table for the next run
    run("Clear Results");
}

8. Overview images. To keep a record of which cells were analysed, the first time frame of the FRET channel is copied and the cell ROIs are loaded. This image is then flattened and saved as a RGB tiff into the Overview directory.

//create a copy of the first time point FRET image and auto contrast it
    run("Duplicate...", "title=Temp duplicate channels=2 slices=1");
    run("Enhance Contrast", "saturated=0.30");

//Load the cell ROIs onto it, flatten the image and save it out to the overview directory
    roiManager("Open", roidir+fs+nameStore+" - Cells.zip");
    roiManager("Show all with labels");
    run("Flatten");
    saveAs("Tiff", overviewSave+fs+nameStore+" - Overview");
    close(nameStore);
    close("Temp");

9. Garbage Collection. The macro frees memory used by FIJI (see Note 45).

//run java garbage collection to free up used memory
    wait(2000);
    call("java.lang.System.gc");
    wait(2000);
10. **Saving Logged Data.** The custom table is saved as an excel file into the source directory and all other tables are closed.

```r
//save the completed table out to the selected source directory as an xls file
selectWindow("FRET Analysis");
saveAs("Text", dir+fs+"FRET Analysis.xls");

//close the custom and results tables
selectWindow("FRET Analysis");
run("Close");
selectWindow("Results");
run("Close");
```
4. Notes

1. Addition of Penicillin/Streptomycin antibiotics to DMEM, 5% FCS after transfection is recommended.

2. For primary neuronal cultures, antimycotic solution is recommended (25 μg/mL Amphotericin B).

3. Most cell lines adhere well to PDL-coated plates. However, primary neuronal cultures require PDL and laminin-coated plates. Once plates are coated with PDL, incubate with 50 μL of 50 μg/mL laminin for the duration of the DRG isolation protocol. Remove laminin and air-dry plates after DRG trituration, and prior to nucleofection (see Step 11 “3.1.2. Dorsal root ganglion neurons isolation and culture”).

4. Using a Bunsen burner, insert the tip of a glass Pasteur pipette into the flame and rotate. Monitor the diameter of the tip opening, which will be reduced over time. Make pipettes with small, medium or large diameter bores. These will be used for sequential trituration of suspensions with the aim to yield single cell suspensions.

5. Coating of plates with PDL can be performed in bulk. Extra plates can be stored at 4°C for 3-4 weeks.

6. PEI stock solution requires agitation, heating (60-70°C) and neutral pH (pH 7) to dissolve. Adjust pH using 1 M HCl or 1 M NaOH.

7. Individual cells exhibit large variation in basal activity and in their relative capacity to maximally induce a signalling mediator. Therefore, in addition to measuring, for example, the response to receptor activation, the baseline and maximal response capacity must also be determined for every cell. Different stimuli are used to
determine the maximal response capacity of a cell, subject to the signalling pathway under investigation (see Table 2).

8. This protocol is focused on the use of the specified high content imager. However, any high content imager can be used, provided that appropriate adjustments to FRET detection protocols are performed.

9. DRG isolation may take up to 3.5 hr: 1 hr to prepare reagents (coating plates, preparation of digestion buffer and fire-polishing of pipettes), 20-40 min to isolate DRGs of one mouse, 1 hr of enzymatic and mechanical digestion and 30 min for nucleofection and plating.

10. Our protocol is based on adult C57BL/6J mice, 6-8 weeks, male or female.

11. Ribs can be used as landmarks if specific spinal levels are required.

12. This step can be performed by pinning down the vertebral column onto a silicone elastomer-lined dish and immersing the column in cold CMF-HBSS.

13. The total amount of DNA used will depend on cell type, and whether cells are to be transfected with FRET biosensor alone or co-transfected with receptor/protein of interest and FRET biosensor. For most model cell lines (HEK293, CHO, COS, HeLa), good expression is achieved using 90 ng/well FRET biosensor alone, or 55 ng/well receptor/protein of interest and 40 ng/well FRET biosensor. For MCF7 cells, good expression is achieved using 200 ng/well FRET biosensor, or 100 ng/well receptor/protein of interest and 200 ng/well FRET biosensor.

14. A modified transfection protocol can be used for cells that are difficult to transfect, such as the MCF7 cell line. In this case, cells are transfected and seeded into the 96-well plate at the same time, to increase penetrance of the transfection mix. The DNA:PEI solution is incubated at room temperature for 10-30 min, cells are diluted in DMEM with 5% FCS and insulin to achieve 70% confluency, then mixed with the
DNA:PEI solution immediately prior to seeding. Each well has a final volume of 220 μL (200 μL cell dilution, 20 μL DNA:PEI solution). Experiments are performed 48 h after transfection and seeding.

15. It is important to keep the final concentration of NaCl relatively low so as not to disturb the osmolarity of the cell growth medium.

16. Serum restriction medium for MCF7 cells should contain 10 μg/mL insulin.

17. Significant variation in activation can be observed between individual cells within a population (see Introduction). As such each cell is considered a ‘unique’ repeat, and only one well is collected per experimental condition within each biological repeat. Experiments should be repeated with at least 2-3 independent transfections on different days.

18. The nucleofection solution is cytotoxic after extended periods of time.

19. For DRG cultures, two washes with HBSS are recommended to remove cellular debris.

20. Re-check pH of HBSS prior to use. Standard fluorescent proteins (e.g. YFP) are very sensitive to changes in pH, and addition of acidic solutions will alter the FRET ratio irrespective of FRET biosensor activation. Experiments in systems that experience local changes in pH (e.g. activity induced changes in local calcium concentrations in neurons) will need to use modified FRET sensors with pH-insensitive fluorescent proteins [10, 11].

21. It is critical to choose the correct plate, as the recorded dimensions dictate the autofocus of the instrument. If a plate is not listed, manually enter plate dimensions (available from many Supplier’s websites) into the Plate/Slide Manager and/or use the Laser Autofocus Trace option to check plate parameters.
22. The 40x objective should be suitable for most experiments. If transfection efficiency is low or if the cells are large (i.e. DRG neurons, MCF7 cells) either the 20x or 10x objectives can be used. Aim to capture at least 50 cells per field of view.

23. The INCell 2000 does not support simultaneous imaging of two emission channels following a single excitation. As images are captured sequentially, it is important to capture the FRET image prior to capture of the donor image. Other high content imagers do support simultaneous detection of two emission channels (e.g. Perkin Elmer Opera).

24. Exposure times will change depending on the objective selected, and the transfection efficiency of the FRET biosensors. These settings should be optimised at the commencement of each experiment (see Section 3.3.3).

25. In the INCell 2000, the polychroic filters included in the FRET module are optimised for the following filter pairs: Quad3 for CFP, YFP, dsRed, Cy5; and Quad4 for DAPI, FITC, dsRed and Cy5.

26. A Time Series can be further customised by deselecting individual time points or wavelengths. For example, while a reference brightfield image is useful, acquisition of this wavelength throughout the entire experiment is unnecessary and extends the acquisition time of every well.

27. Within a single well, multiple fields of view can be acquired as defined within the Plate/Slide Viewer window. This is especially useful when acquiring FRET data from DRG neurons or cells with low transfection efficiency.

28. The number of wells that can be imaged within a single time series will depend on exposure time, well layout, number of fields of view per well, and the interval of the time series. If wells are in close proximity, a 1 min interval will allow capture of up to 15 wells, whereas a 20 sec interval will allow capture of up to 5 wells. For all high
content imagers, a motorised stage is required to capture more than one well in a given run. The temporal resolution will be limited by how fast this stage can (accurately) move between wells.

29. Ensure the image is not saturated by checking the %Optimum value below the image (this should not exceed ~80%). Exposure times should be as short as possible to minimise the exposure to excitation light that can induce phototoxic damage. The balance between exposure time and intensity of emitted light will be governed by several factors: i) **Detectors**: more sensitive detection systems will record more emitted light and therefore allow shorter exposure times; and ii) **Filters, shutters and light sources**: these processes may take up to an extra 500 ms to close/turn off following the designated exposure time. So while the camera acquisition may be short, the length of time the cells are exposed to light may be much longer. Faster termination of the excitation source can be achieved through the use of filter wheels, fast shutters or LED light sources.

30. The INCell 2000 takes approximately 20 seconds to start an image acquisition. To minimise the time between drug addition and acquisition of the first image, eject the plate with the ‘Stimulate’ Acquisition Protocol window open (and the Base Image Folder for image stack storage pre-defined), add drugs simultaneously from a compound plate using a multi-channel pipette, gently replace plate and ‘Run Protocol’.

31. The field of view, focus and exposure settings must be identical for the acquisition of all three time series (baseline, stimulated and positive control), to allow for the calculation of FRET ratios in the final, merged time series following image alignment (see Section 3.4.1).
32. The raw macro code is presented in the format it appears in the FIJI script editor. Different colours represent the different functions of the code: Green indicates comments and notes that are for information purposes only and do not run as code; Blue indicates global system commands; Yellow indicates FIJI/ImageJ commands; and Magenta indicates text strings for names and command configuration.

33. The macros will work in the standard FIJI or ImageJ distribution (ImageJ may need the StackReg plugin installed). Computer performance does not limit the macros. A standard data set (e.g. total of 28 time points) requires less than 2GB of RAM. CPU speed will only affect the speed at which the macros are completed.

34. This macro is designed for data captured using a GE INCell 2000 high content imager in the format of one donor channel and one FRET channel in three sets – baseline, stimulated and positive control. It requires all files (baseline, stimulated and positive control) to be in separate folders. The macro is adaptable to different numbers of time points, wells and fields of view.

35. Enter “1” to start processing from the first well in the time series list, enter “2” to start processing from the second well in the time series list (this will ignore well 1), enter “3” to start processing from the third well in the time series list (this will ignore wells 1 and 2), and so forth.

36. Check that the images in the time series are aligned. If the stack is not well aligned, manually use an alternative plugin that allows selection of a defined image region to use during the alignment (e.g. cvMatch Template plugin available at https://sites.google.com/site/qingzongtseng/template-matching-ij-plugin).

37. Only select cells with the appropriate FRET biosensor localisation and good morphology. Include within the selection cells with low levels of fluorescence; the FRET ratio change within these cells is generally much greater than cells with high
fluorescence (these cells typically have increased levels of non-specific FRET).

While it may be tempting to select the cell with a high and bright signal, in most cases larger FRET changes are usually obtained from a cell with a mid to low level of expression.

38. When analysing primary neuronal cultures, it is likely that only a subset of neurons will endogenously express the protein of interest. To identify specific neuronal subtypes, staining of neurons using neurochemical markers (e.g. isolectin B4, CGRP, neurofilament 200) upon completion of the experiment is recommended. Subsequent ROI selection and FRET analysis of only positively stained cells will more accurately reflect the response of the selected population.

39. Depending on the biosensor the FRET ratio can be calculated as either ‘FRET/donor’ or the inverse ‘donor/FRET’ where donor = average intensity of donor fluorophore upon donor excitation, and FRET = average intensity of acceptor upon donor excitation. For example, increased ERK or Rac1 activity causes an increase in FRET within the EKAR and the RaichuEV-Rac1 biosensors; calculating the FRET/donor ratio represents this increase. In contrast, increases in cAMP or PKC activity cause a decrease in FRET within the Epac2-camps or the CKAR biosensors; calculating the inverse donor/FRET ratio allows a corresponding graphical representation of this increase.

40. It is important to consider that individual cells may display distinct temporal profiles within the same well. It is therefore recommended to plot and analyse the responses at a single cell level before plotting the overall ‘cell population’ response detected in a single well (this overall response will be a composite of the individual responses within the well). Such analysis can deliver important information about the signalling processes under consideration (Fig. 2).
41. The Array.sort command ensures correct file order across all operating systems.

42. If the donor channel is not listed first in the source directories, the macro runs a nearly identical code instead. In this alternative code, the macro builds the FRET channel stack first and then the donor channel stack. However, the final combined stack is still in the format of channel 1 = donor, channel 2 = FRET.

43. A single hyperstack makes all subsequent data management and analysis easier. It avoids the need to store and handle individual images (i.e. for each time point) or multiple stacks (i.e. one stack per channel) for each experiment.

44. The automatic alignment adds extra processing time and requires more RAM. The plugin can sometimes generate erroneous data by aligning different cells (e.g. if the image contains few cells, or moving cells that are highly fluorescent). Check the alignment of images within the hyperstack carefully. If the stack is not well aligned, manually use an alternative plugin that allows selection of a defined image region to use during the alignment (e.g. cvMatch Template plugin available at https://sites.google.com/site/qingzongtseng/template-matching-ji-plugin).

45. Image processing in FIJI is carried out in the Java environment. Images that are opened and subsequently closed do not always release the memory they used. This is not a problem when the data set is small, but can result in errors/crashing with large data sets.
5. References


10.1091/mbc.E11-01-0072


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### Tables

#### Table 1. List of FRET biosensors used in this protocol

<table>
<thead>
<tr>
<th>Target</th>
<th>Name</th>
<th>FRET pair</th>
<th>Localisation</th>
<th>Lab</th>
<th>References</th>
<th>Addgene Plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP</td>
<td>Epac2-camps</td>
<td>CFP/YFP</td>
<td>cytosol</td>
<td>MJ Lohse</td>
<td>[7]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>plasma membrane</td>
<td>DMF Cooper</td>
<td>[12, 13]</td>
<td></td>
</tr>
<tr>
<td>ERK</td>
<td>EKAR</td>
<td>Cerulean/Venus</td>
<td>cytosol</td>
<td>K Svoboda</td>
<td></td>
<td>18679</td>
</tr>
<tr>
<td>PKC</td>
<td>CKAR</td>
<td>CFP/YFP</td>
<td>cytosol</td>
<td>AC Newton</td>
<td>[5]</td>
<td>14860</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>plasma membrane</td>
<td>AC Newton</td>
<td></td>
<td>14862</td>
</tr>
<tr>
<td>Rac1</td>
<td>RaichuEV-Rac1</td>
<td>CFP/YFP</td>
<td>Rac1 localisation</td>
<td>M Matsuda</td>
<td>[15, 16]</td>
<td></td>
</tr>
</tbody>
</table>

#### Table 2. List of positive controls

<table>
<thead>
<tr>
<th>Target</th>
<th>FRET Biosensor</th>
<th>Positive Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP</td>
<td>Epac2-camps</td>
<td>10 μM forskolin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 μM 3-isobutyl-1-methylxanthine (IBMX)</td>
</tr>
<tr>
<td>ERK</td>
<td>EKAR</td>
<td>200 nM phorbol 12,13-dibutyrate (PDBu)</td>
</tr>
<tr>
<td>PKC</td>
<td>CKAR</td>
<td>200 nM PDBu</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1x phosphatase inhibitor cocktail</td>
</tr>
<tr>
<td>Rac1</td>
<td>RaichuEV-Rac1</td>
<td>1 μM isoprenaline</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 ng/mL epidermal growth factor (EGF)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AlF₄⁻ (10 μM AlCl₃, 10 mM NaF) [17]</td>
</tr>
</tbody>
</table>

#### Table 3. Microscopy settings for CFP/YFP and GFP/RFP FRET biosensors

<table>
<thead>
<tr>
<th>FRET Pair</th>
<th>Order</th>
<th>Excitation filter</th>
<th>Emission filter</th>
<th>Exposure</th>
<th>Polychroic</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFP/YFP</td>
<td>1</td>
<td>Brightfield</td>
<td>DAPI</td>
<td>0.3</td>
<td>Quad3</td>
</tr>
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Figure Captions

Figure 1: Subcellular targeting of FRET biosensors expressed in model cell lines and primary neuronal cultures. (A) Plasma membrane targeted cAMP biosensor (pmEpac2-camps) expressed in HEK293 cells, (B) nuclear ERK biosensor (nucEKAR) expressed in HEK293 cells, (C) cytosolic ERK biosensor (cytoEKAR) expressed in dorsal root ganglia neurons and (D) Rac 1 biosensor (RaicuEV-Rac1) expressed in MCF7 cells. Scale bars represent 10 µm (A, B) or 100 µm (C, D).

Figure 2: Single cell analysis allows discrimination between different cell populations depending on signalling kinetics. A) within the same image, cells can elicit different responses (e.g. transient versus sustained), that cannot be detected when using traditional population assays (B).

Figure 3: Workflow of the steps to calculate FRET ratios from images. A) Stacking images in one file, B) determine the region of interest (ROI) and background, C) plot $F/F_0$ for each individual cell and D) group $F/F_{Max}$ values for all cells.