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Measuring cGAMP production in living single cells using a dedicated FRET reporter

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We use this protocol and it's working

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Abstract

This protocol outlines a workflow to generate, validate and image cell lines stably expressing a cGAMP FRET reporter in order to measure cGAMP production in live single cell assays. We detail how to generate lentiviral particles, transduce MCF10A cell lines and validate the most appropriate clones using FRET live single cell imaging. Finally, we provide advice on how to best image our cGAMP FRET reporter despite low levels of expression and challenging imaging conditions.

Materials

Cells:
HEK293FT
MCF10A

Plasmids:
Lentiviral plasmid for the cGAMP reporter
Lentiviral plasmid for the cGAMP-blind mutant reporter
psPAX2
pMD2.G

HEK293T media:
DMEM (Life) supplemented 10% Fetal Bovine Serum (Thermo) with no antibiotics

MCF10A media:
DMEM/F12 (Life) supplemented with 5% (v/v) horse serum (Thermo Fisher Scientific), 20 ng/ml EGF (Sigma-Aldrich), 0.5 µg/ml hydrocortisone (Sigma-Aldrich), 100 ng/mL cholera toxin (Sigma-Aldrich), 10 µg/mL insulin (Sigma-Aldrich) and 1% penicillin-streptomycin (Gibco)

4x lentivirus concentrator solution:
Dissolve 80g PEG-8000 and 14g NaCl in 80 mL MilliQ water and 20 mL of PBS 10X (pH 7.4).
Mix with gentle stirring until the solids are fully dissolved then adjust pH to 7.0~7.2 and final volume to 200mL.
Sterilize by filtering through 0.2 µm pores and store at 4ºC
Tip: as the solution is extremely thick, you might prefer to only filter the volume strictly necessary on step 11 at the time it is required.

Miscelanous reagents:
SiR-DNA (Spirochrome)
Lipofectamine 2000 (Thermo)
diABZI compound 3 (Invivogen)

Produce lentiviral particles to generate cell lines stably expressing the cGAMP FRET reporter

5d 1h 45m

1 On day 0, seed HEK293FT in DMEM + 10% FBS with no antibiotics in T75 flasks so that they have ~50% confluency the day after. Incubate in an incubator at 37ºC 5% CO2.

2 On day 1, prepare two tubes, containing either:
Tube 1: 1.5 mL optimem + 60 µL lipofectamine 2000
Tube 2: 1.5 mL optimem + 24 µg DNA (cGAMP reporter lentiviral plasmid:Pax2:MD2G with a **4:2:1 ratio**)

1d

3 *You can also prepare two other tubes to generate lentiviral particles for the mutant cGAMP-blind reporter:*
Tube 1: 1.5 mL optimem + 60 µL lipofectamine 2000
*Tube 2: 1.5 mL optimem + 24 µg DNA (Mutant cGAMP-blind reporter lentiviral plasmid:Pax2:MD2G with a **4:2:1 ratio**)*
You will need to have another flask of HEK293FT cells. Every subsequent steps would need to be doubled for the mutant reporter construct. As the mutant cGAMP-blind reporter is an important biological and technical control, we advise to generate cell lines expressing it.

*

4 Incubate the tubes at room temperature for 5 min

5m

5 Mix the two tubes, and incubate for a further 20 minutes at room temperature

20m

6 Add the mix dropwise to the HEK293FT cells, and mix gently by swirling the flask. Incubate the flask in an incubator at 37ºC 5% CO2.

7 On day 2, change the media for fresh media.

1d

8 On day 3, collect the media in a 15 mL falcon tube and replace the media for fresh media. Spin the collected media at 800 g for 10 min to pellet debris and floating cells. Keep only the supernatant.

1d 0h 10m

9 In preparation of future steps, prepare the 4x lentiviral concentrator solution from **Production of Vesicular Stomatitis Virus G Glycoprotein (VSV-G) Pseudotyped Retroviral Vectors** [**Ref 1**]. Briefly:

9.1 Dissolve 80g PEG-8000 and 14 g NaCl in 80 mL MilliQ water and 20 mL of PBS 10X (pH 7.4).

9.2 Mix with gentle stirring until the solids are fully dissolved then adjust pH to 7.0~7.2 and final volume to 200mL.

9.3 Sterilize by filtering through 0.2 µm pores and store at 4ºC
Tip: as the solution is extremely thick, you might prefer to only filter the volume strictly necessary on step 11 at the time it is required.

10 On day 4, collect the media a second time in a 15 mL falcon tube. 1d 0h 10m
Spin the collected media at 800 g for 10 min to pellet debris and floating cells. Keep only the supernatant.

11 Pool the media collected on days 3 and 4 into a 50 mL falcon tube. Add the homemade lentiviral concentrator solution (1 volume of filtered concentrator for 3 volumes of supernatant). Seal the falcon tube tightly with parafilm so it doesn't leak and put it on a rotating wheel overnight at 4ºC. 1d
The lentiviral particles will slowly bind to the PEG in the concentrator solution.

12 The day after, spin the falcon tube 1600 g for 1 h at 4ºc to pellet the lentiviral particles. A pellet should be visible. 1h

13 Carefully discard the supernatant and resuspend the pellet in 1 mL sterile PBS. The particles can be either used immediately or stored at –80ºC.

Transduce MCF10A cells with the cGAMP reporter lentiviral particles 1w 1d

14 On day 0, seed MCF10A cells in a T25 flask in DMEM/F12 supplemented with 5% (v/v) horse serum (Thermo Fisher Scientific), 20 ng/ml EGF (Sigma-Aldrich), 0.5 µg/ml hydrocortisone (Sigma-Aldrich), 100 ng/mL cholera toxin (Sigma-Aldrich), 10 µg/mL insulin (Sigma-Aldrich) and 1% penicillin-streptomycin (Gibco), so that it reaches 50% confluence on Day 1.

15 On day 1, add 500 µL of the cGAMP reporter (or mutant cGAMP-blind reporter) lentiviral particle solution from step 13 dropwise to the T25 flask and mix gently by swirling. 1d

16 Expand the T25 flask for a week or more. 1w
Tip: In our experience, the reporter expresses at levels too low to be reliably detected by basic tissue culture fluorescent microscopes, as their illumination power and camera sensitivity tends to be limited and they rarely have optimised filters for detecting CFP and YFP fluorescence. Do not be surprised if you don't see fluorescence in that context.

17 FACS sort the CFP and YFP double positives against the non expressing parental MCF10A WT cells. Collect a 96 well plate of single cell clones, as well as the bulk population. As the reporter could potentially alter the downstream STING signalling pathway, we recommend selecting the lower expressing third of the CFP+YFP+ cells.

Validate the selected clones or bulk population by measuring FRET in single cell live imaging assays

2d

18 On Day 0, seed the selected MCF10A clones in a 96 wells imaging plate to have 80%~90% confluence on day 2. We seed directly in imaging MCF10A media, consisting in DMEM/F12 with **no** phenol red, supplemented as in Step 14.

Tip 1: We use 96 well uClear plates from greiner (655090). We have tried a similar product from Perkin-Elmer (96 well phenoplates 6055302) but experienced issues with high autofluorescence in the CFP channel. Alternatively, we recommend the IBIDI 8 well µSlide with #1.5 polymer coverslip. Any glass chambered coverslip should work as well, but you may need to coat it before seeding cells.

*Tip 2: If using another cell line than MCF10A with different media requirements, we recommend using media **without** phenol red in order to reduce autofluorescence. In our work and depending on the cell line, we use either DMEM/F12 or DMEM without phenol red (but still supplemented with the required components for your cell line to grow properly). Note that imaging media with reduced riboflavin are commercialized as low autofluorescence media, but we can unfortunately not comment on their efficacy as we do not use them.*
Alternatively, if your cell media is supplemented with 10% FBS, you may consider reducing it to 5% FBS. In our experience, proliferation should be minimally affected by the reduced serum percentage, while Signal to Noise ratio will be massively improved by the drop in media autofluorescence.

Tip 3: We usually directly seed the cells in their imaging media as changing media might be stressful, but note that changing media soon before imaging will improve the signal to noise ratio, which might be important if your cells express low levels of the fluorescent reporter and the camera struggle with detection.

Tip 4: If your microscope is not equipped with CO2, you can use L15 media without phenol red if your cells tolerate it. We have not tested it with MCF10A or the cGAMP reporter though, so cannot comment on it.

19 On day 1, treat the cells either with:
- Increasing doses of the STING agonist diABZI to directly bind and activate the reporter. We generally try 0 nM, 10 nM, 100 nM and 1000 nM.

1d

- Or by transfecting either 200 ng DNA (or transfection reagent only control) to stimulate cGAS and look at activation of the reporter from the cGAMP produced by the cell in response to the transfected DNA.

Note that it is critical to mirror these experiments with cell line(s) stably expressing the mutant cGAMP-blind reporter, as the latter should not respond to the treatment. The cGAMP-blind mutant reporter is a key biological and technical control showing that any response you may see in the WT reporter is indeed due to cGAMP binding, and not to any other biological or technical artifact. Such artifacts could include a drug treatment being autofluorescent in the CFP or YFP range, the cells becoming autofluorescent in the CFP or YFP range following treatment but independently of the FRET reporter, or any technical issue in your imaging pipeline.

20 On day 3, two to three hours before imaging, add 200 nM final concentration of the live cell far-red nuclear dye SiR-DNA to each well. You may use up to 1 µM SiR-DNA, but note that multiple days of treatment and/or higher doses of SiR-DNA may induce DNA damage and alter proliferation. 1d

21 Proceed with imaging (see below).

Imaging conditions and microscope setup

22 **Microscope, illumination source, filters and mirrors**
We are using a Zeiss Axio Observer Z1 equipped with a Yokogawa CSU-X1 spinning disk unit. For the camera we have been using either a Hamamatsu Flash4 or a Photometrics Prime95b. The system is environment controlled to have 37 ºC and 5% CO2.

For FRET experiments, we are using the following lasers:
- 445 nm 75 mW for the CFP excitation
- 514 nm 40 mW for a YFP emission control

The emission filters are as follow:
- Semrock 482/35 for CFP emission
- Semrock 542/27 for YFP emission

For the dichroic mirror used on our system for FRET experiments, it is:
- ZT440/514/561/640rpc from Chroma

We usually do sequential CFP then YFP images using one camera, but we also tried doing simultaneous CFP and YFP acquisition using a beamsplitter ending on two independent cameras. For that we use the following mirrors and filters, in addition to the above filters:

- 509 dichroic mirror
- 483/32 filter on the CFP path
- 525 longpass on the YFP path

In addition to measuring FRET signal, we use the far-red Sir-DNA dye in our experiments to help detect and segment cells. For that we use:

- 640 nm laser
- Dichroic ZT440/514/561/640rpc from Chroma
- And Semrock 440/521/607/700 for the emission filter

23 **Imaging parameters**

Our MCF10A cells express the reporter at low levels, so the fluorescence is low and can be tough to image properly. For endpoint experiments (not timelapse), we put the laser at max power because potential
phototoxicity is not an issue. For timelapses, we reduce power to 10~20% in order to minimize impact on cells' viability. We use 2×2 binning for increased detection and expose each of the FRET channels for 600ms. For reproducibility, we use the same acquisition parameters for all FRET channels (CFPex CFPem, CFPex YFPem and YFPex YFPem).

We typically experienced activation in only a small fraction of responders when cells were treated with DNA damaging agents, and therefore it matters more to have many cells imaged, than to have high resolution. Therefore, we use 20x magnification to have as many cells as
possible in each field, and we image a high number of fields per condition (>40). 20x is a good compromise between larger field of view, without sacrificing too much imaging quality.
In cases where you expect to see widespread activation across all cells, six to a dozen fields of view per conditions should be sufficient, depending on cell confluence.

We use a Flash4 camera for endpoint live imaging assays, but this is for consistency with older experiments rather than for imaging quality. We advise on using the most sensitive camera available to you.
For time lapses, we use the more sensitive Prime95b camera either in a single camera or dual camera acquisition setup. The higher sensitivity of the Prime95b allows to reduce illumination power and photodamage, which is critical for long term time lapse assays.

Image analysis

24 We perform segmentation, tracking and pixel value quantification using custom python scripts. We use python scripts based on Pandas and Numpy to process the resulting numerical data, then matplotlib and seaborn to generate graphs. **All code we use for image analysis is available from our original publication**, but can be reproduced in any

image analysis software of your choosing such as, for example, Fiji, Cell Profiler or Harmony.

Briefly, we use the nuclear signal provided by the SiR-DNA dye to segment nuclei, then generate a ~3px ring, 3 px away from each nucleus perimeter to obtain a cytosolic region. As the reporter is slightly more abundant in the cytosol, resulting in brighter signal, we measure the FRET shift in the cytosolic compartment. As both the reporter and its ligand are soluble, and considering the hour long timescale of cGAMP production relative to the comparatively fast diffusion speed of small molecules such as cGAMP, calculating the FRET ratio as the median in the cytosol of pixel-per-pixel CFPexCFPem and CFPexYFPem intensity ratio is almost strictly equivalent to calculating FRET as the ratio of the median cytosolic intensities measured in either channel. The image analysis scripts we provide in our original publication will measure median pixel intensity values in the CFPexCFPem, CFPexYFPem and YFPexYFPem channels for each cell, and allow you to calculate the FRET ratio either pixel-per-pixel (slower) or by calculating the ratio of CFPexYFPem median intensity divided by CFPexCFPem median intensity (faster). If you are writing the analysis pipeline for your own image analysis software suite, the latter is easier to implement while being equivalent in accuracy.

Despite being derived from a clone, we notice variability in the expression of the reporter (estimated here by the median YFPexYFPem signal). In our experience, lower values of reporter expression tends to give less reliable FRET ratio measurements, as the CFPexCFPem signal gets closer to the background noise level, resulting in a skewed FRET ratio. To avoid this, we filter expressing cells based on minimal reporter expression. In our imaging setup, we empirically determined a value of ~1500 au of YFPexYFPem signal as an optimal threshold of reporter expression to obtain robust FRET measurements. This threshold will need to be adjusted to your specific imaging modalities.

In addition, we recommend filtering out debris. In our scripts, we implement this by adding a threshold based on nucleus area (as debris particles usually are smaller than primary nuclei) as well as a threshold on SiR-DNA intensity (as debris can sometimes be brighter compared to primary nuclei). All these need to be adjusted empirically to your image acquisition setup. We implement the filtering at the last step, right before generating graphs.

Protocol references

1. Lo, H.-L. and Yee, J.-K. (2007), Production of Vesicular Stomatitis Virus G Glycoprotein (VSV-G) Pseudotyped Retroviral Vectors. Current Protocols in Human Genetics, 52: 12.7.1-12.7.11. https://doi.org/10.1002/0471142905.hg1207s52