Montana Molecular
Fluorescent Biosensors for Live Cell Discovery

Ratiometric Cell Stress Assay

About this Assay

The cell stress sensor is a genetically-encoded fluorescent biosensor that produces very bright fluorescence when the cell endures endoplasmic reticulum (ER) stress or undergoes the unfolded protein response (UPR). This version of the assay is designed for ratiometric measurements using the green fluorescent sensor and a constitutively expressed red fluorescent protein. Red and green fluorescent signals are both localized to the nucleus. Red fluorescence indicates all cells expressing the sensor, and green fluorescence indicates cells undergoing the stress response. The BacMam vector carrying these sensors is a modified baculovirus (BSL-1), which can be used for delivery to, and expression in, a wide variety of mammalian cell types including primary cultures.

A broad host of both chemical compounds and genetic mutations induce ER-mediated cell stress, making this biosensor a useful tool to study the effects of toxic compounds and stress-inducing mutations that are associated with disease. The sensor is based on splicing of the XBP1 RNA, mediated by the ER protein IRE1. This splicing removes an intron and results in the translation of the mNeon green fluorescent protein.

Overview

The following protocol is optimized for measuring cell stress responses on a 96-well plate of living cells. It has been validated in live HEK 293 cells as well as in iPSC-derived cardiomyocytes and neurons. Assay fluorescence can be detected on live-cell imaging systems, automated fluorescence plate readers or fluorescence microscopes. For use in iPSC-derived or adherent cells, see Suggestions for Assays in Adherent Cells section.

Relevant Products

Product	Description	Stress Pathway Detected	Promoter	Recommended Use
U0901G	Green Upward Cell Stress Sensor with constitutive red fluorescent protein for ratiometric measurement.	Endoplasmic Reticulum (ER) and ER Unfolded Protein Response	CMV	Fluorescence imaging and plate reader assay (Z' > 0.78)

Materials in the Kit

- Cell stress sensor BacMam ≅ 5 x10^{10} VG/mL in ESF 921 Insect Cell Culture Medium (Expression Systems #96-001-01).

  Green fluorescent sensors that increase in fluorescence intensity in response to ER and cellular stress. VG/mL is the number of viral genes per milliliter.

- Sodium Butyrate (Sigma Aldrich product # B5887) 500 mM in H_2O.

  Sodium Butyrate is added to the culture to maintain BacMam expression. Other HDAC inhibitors may work as well.

- Thapsigargin (Cayman Chemical product # 10522) dissolved in DMSO, diluted to 100 µM in in H_2O.

  Thapsigargin is a SERCA pump inhibitor that induces high levels of ER stress. It is used as the positive control when conducting the cell stress assay.

Storage

BacMam stocks should be stored at 4°C and protected from light. Avoid repeated freeze/thaw cycles.

Additional Materials not Supplied

- Greiner CellCoat (#655946) is our preferred 96-well plate available from VWR.
- Dulbecco's Phosphate Buffered Saline (DPBS) available from VWR [Dulbecco, R. and Vogt, M.1957].
- Optional: FluoroBrite DMEM media (ThermoFisher Scientific product # A1896701) supplemented with 10% FBS and 4 mM GlutaMAX.

BioSafety Considerations

BacMam is the modified baculovirus, Autographa californica, AcMNPV, polyhedron minus strain. Baculovirus is pseudotyped to infect mammalian cells, but it cannot replicate in the cells and its genome is silent in mammalian cells. While it should be handled carefully, in a sterile environment, it is classified as a BSL 1 reagent. The NIH Guidelines for Research on Recombinant DNA Molecules should be consulted for laboratory safety procedures.

ESF 921™ Insect Cell Culture Medium is a complete serum-free, protein-free medium. ESF 921™ contains L-Glutamine and Kolliphor® P188 (Pluronic F68).

For Research Use Only. Not recommended for use or sale in human or animal diagnostic or therapeutic products. This product contains no substances which at their given concentration are considered to be hazardous to health, however we recommend handling with care. Wear impervious gloves and eye protection when handling. Do not injest.

Warranty

Materials are provided without warranty, express or implied. End user is responsible for making sure product use complies with applicable regulations. No right to resell products or any components of these products is conveyed.

Protocol for Use

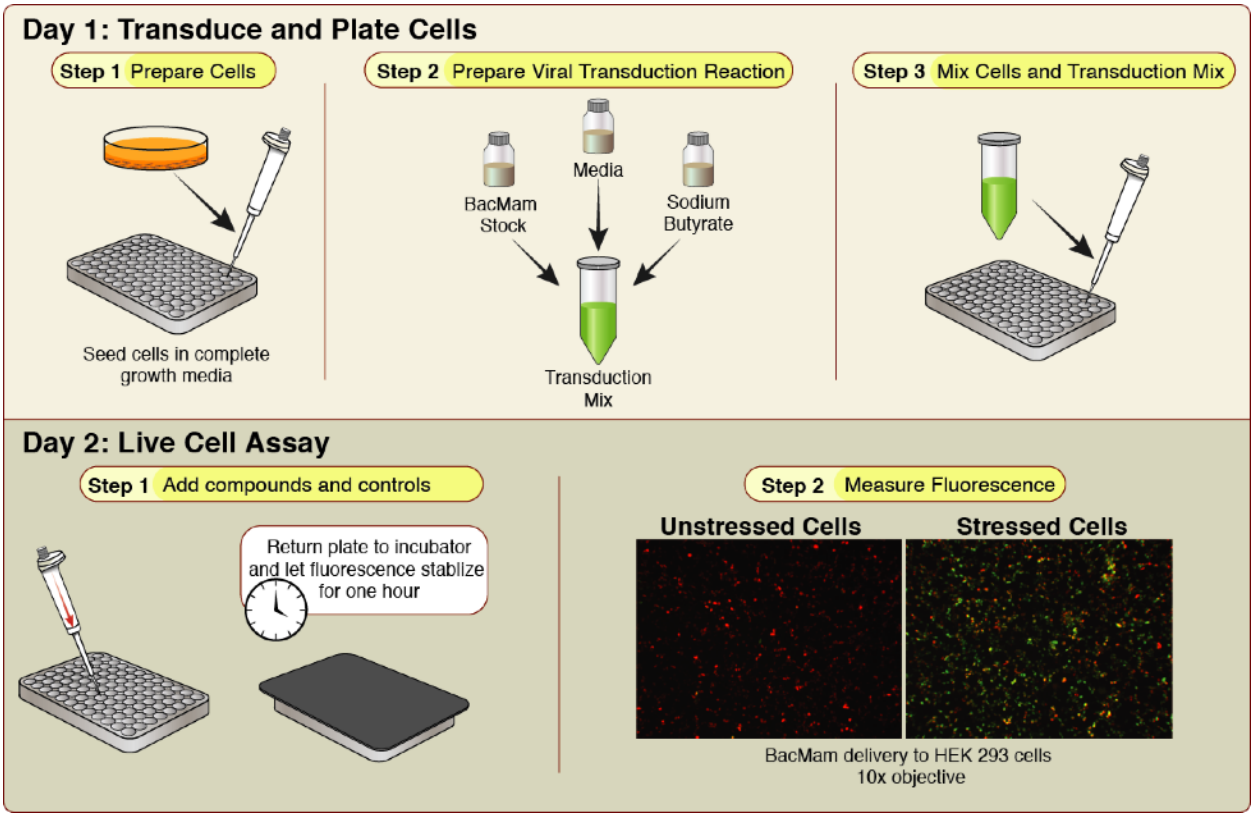
This protocol is optimized for rapidly dividing, immortalized cell lines. However, the protocol can be adjusted for transducing non-dividing adherent cells such as neurons, islets, cardiomyocytes, and iPSC-derived lines. We recommend that you take the time to optimize the assay for your particular cell type. See our Suggestions for Adherent Cells following this protocol.

DAY 1 TRANSDUCE AND PLATE CELLS

Step 1) Prepare cells (Tube A)

- Detach cells from flask using standard trypsinization protocol. Resuspend cells in complete culture media or FluoroBrite media and determine cell count.
  - Note: The assay will work in either standard media or FluorBrite media, however as FluoroBrite media is formulated to reduce background fluorescence signals and signal to noise ratios are generally improved using FluoroBrite media. Alternatively, if only a single time point the media can be exchanged for DPBS before analysis.
- Prepare a dilution of cells at your desired concentration*. 100 µL of this cell resuspension will be required for a single well in a 96-well plate, so prepare enough of the dilution to seed the desired number of wells in the plate. Let cells sit in hood and move on to preparation of the viral transduction reaction.

* 480,000 cells/mL works well for HEK293 cells.



Example:

For **96** wells (1 plate)

100 µL cell suspension (480,000 cells/mL) per well.

100 µL cells x **110 (96** wells + 10% scale) = **11000 µL** cell suspension.

- When preparing the master mix, scale up by 10-15% to avoid coming up short. To seed a 96-well plate, multiply amounts in Step 1 and Step 2 by 110-120.

Step 2) Prepare Viral Transduction Reaction (Tube B)

- Prepare a 500 mM stock solution of sodium butyrate in sterile water (in your kit).
- For each transduction reaction (i.e. one well in a 96-well plate), prepare the transduction solution by mixing 25 µL of the Sensor BacMam stock with 0.6 µL of the 500 mM stock solution of sodium butyrate*, and 24.4 µL of the complete culture media for your cells, for a total volume of 50 µL. Mix gently.

* Concentration of sodium butyrate should be 6 mM in this step. Following Step 3, final concentration of sodium butyrate will be 2 mM.

Example:

96 wells (1 plate). The number of wells desired, in bold, must correspond to the number in Step 1 above.

Single Well	*Master Mix*
25 µL Sensor	x **110** = 2750 µL
0.6 µL 500 mM Sodium Butyrate	x 110 = 66 µL
24.4 µL Complete Media	x **110** = 2684 µL
50 µL total volume	x 110 = **5500 µL** transduction mix (96 wells)

Step 3) Mix Cells and Transduction Mix from above.

- Mix Tube A and Tube B (100 µL tube A + 50 µL tube B). Mix gently and seed 150 µL of mix per well on the 96-well plate.
- Cover plate with aluminum foil to protect from light and incubate at room temperature for 30 minutes. This step is important to maintain even distribution of cells throughout the well and minimize edge effects.
- Incubate ≈ 16-24 hrs under normal cell growth conditions, protected from light.

Example:

96 wells needed (1 plate)

Single Well	*Master Mix*
100 µL cell suspension	x 110 = 11000 µL
50 µL transduction reaction	x 110 = 5500 µL
150 µL Total Volume per well	x110 = 16,500 µL total reaction volume

DAY 2 MEASURING FLUORESCENCE

- Prepare positive control compound. Add 50 µL of 4 µM thapsigargin diluted in buffer of choice from the 100 µM stock to a set of wells. This creates a 1 µM concentration of thapsigargin in 200 µL of media/buffer.
- Add desired compounds to each well, return plate to incubator and allow the fluorescence to equilibrate for 1 hour. Reserve a few wells to add thapsigargin, your positive control compound.
- After 1 hour take an initial fluorescent reading or image. Experiments are performed using standard GFP excitation and emission wavelengths.
- Read or image the fluorescence intensity from the plate at reasonable time points after the addition of compounds. Cells treated with thapsigargin will begin to increase in fluorescence as soon as 3 hours after initial treatment and will reach a peak intensity 7 hours after treatment (See Figure 2).
- When monitoring the green fluorescence emitted by the sensor, either the change in fluorescence intensity over time or the absolute fluorescent intensity can be measured.
- Different compounds induce cellular stress at different rates. Thus, we suggest taking multiple fluorescence measurements over a 24-48 hour period.
- If a single endpoint measurement is required, the media can be exchanged for DPBS after the desired incubation time followed by either image or plate reader analysis.

Suggestions for Assays in Adherent Cells

The protocol above is optimized for rapidly dividing immortalized cells. However, the cell stress assays are compatible with screening primary cultures and iPSC-derived lines, where the cells are plated before transduction. Specific details of the protocol will vary by cell type, so it is important to take the time to titrate BacMam for optimal results.

- Prepare a 500 mM stock solution of sodium butyrate in sterile water.
- For each transduction reaction (i.e. one well in a 96-well plate, containing 100 µL culture media per well), prepare a transduction solution by mixing 25 µL of the cell stress sensor BacMam stock with 24.4 µL of DPBS, and 0.6 µL of the 500 mM stock solution of sodium butyrate for a total volume of 50 µL. Mix the solution gently.
- Expression and cell health can be controlled by titrating the virus, so it is worth taking the time to optimize the assay for your particular cell type. Cell culture media may be used in place of DPBS.
- Prepare a dilution series of transduction reactions by varying the amount of BacMam. For example, a range of 10 µL to 50 µL , adjusting the amount of DPBS accordingly.
- Add the transduction reaction directly to plated cells (no aspiration of cell medium necessary). Gently rock the plate 4-5 times in each direction to mix throughout the well. Incubate the cells under normal growth conditions (5% CO_2 and 37°C), protected from light, for at least 4 hours, overnight is preferable.
- Aspirate transduction solution and add 100 µL complete growth medium with sodium butyrate at a concentration of 3-6 mM. Return cells to normal growth conditions for approximately 18-22 hrs before measuring fluorescence as described above. If cells will not tolerate a full media exchange, partial media exchanges can be done.

Fluorescence Detection

We use fluorescence microscopes or plate readers including the Biotek Synergy MX, or Lionheart FX, and BMG CLARIOstar. Our customers have reported good results for our fluorescent biosensor assays on:

- Hamamatsu FDSS
- Molecular Devices FLIPR and Flexstation
- Perkin Elmer Enspire

Figure 1A. Absorption and emission properties of the mNeon green fluorescent protein plotted as a function of wavelength.

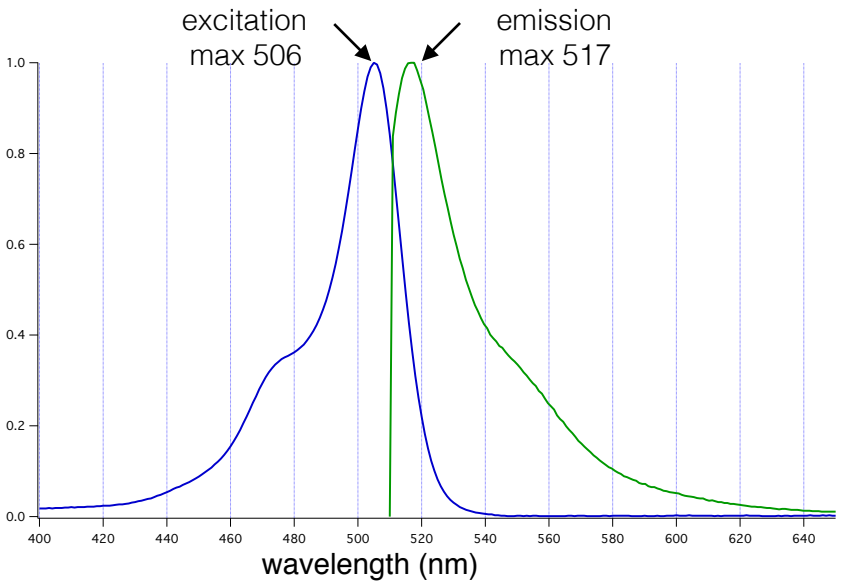
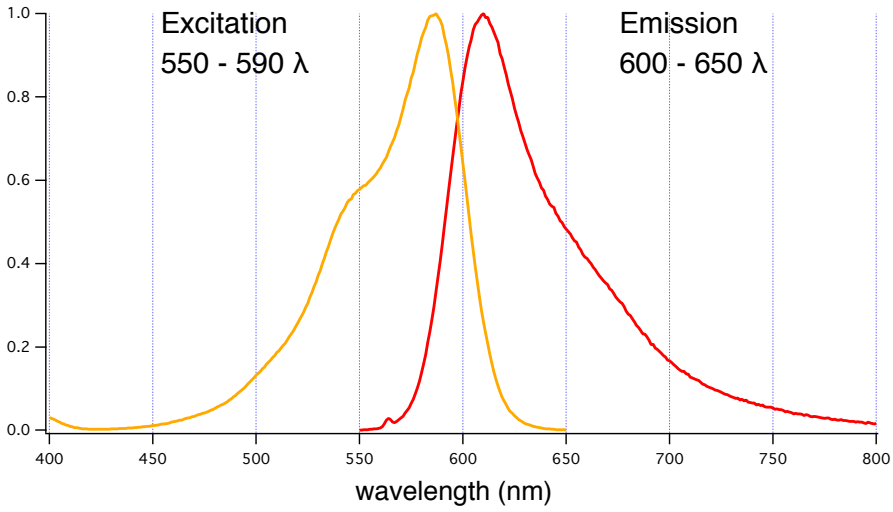


Figure 1B: Absorption and emission properties of red fluorescent protein plotted as a function of wavelength.



Fluorescence Properties

The fluorescent signal is produced by the mNeon green fluorescent protein [6] and SantakaRFP. We recommend Chroma's Catalog set #49003 for optimal results imaging mNeon Green. Preferred excitation and emission wavelengths for mNeon Green are 485/528 and preferred excitation and emission wavelengths for SantakaRFP are 558/603.

Timing

The cell stress assay is a live cell assay, allowing detection over short and long time intervals. For best results, be sure to analyze fluorescence between 0 and 1 hour after addition of stress inducing compounds and at multiple time points up to 48 hours after to capture changes cellular stress levels. In Figure 2, fluorescence was captured from cells at 1 hour after compound addition then sampled at regular intervals. The maximal response for the positive control, thapsigargin is reached at 7 hours after the addition of the drug.

Assay Performance Considerations

How we measure the infectivity of the viral stock.

Typically, viruses are quantified in terms of plaque forming units (PFU). In the case of BacMam, this would be a measurement of the viruses that are capable of transducing an insect cell, the natural host. Since mammalian cell expression is our goal for this assay, we quantify infectivity by measuring viral genes (VG) per milliliter (mL) of the BacMam stock. Viral samples are prepared to release genomic DNA, then multiple dilutions of the preparation are run in qPCR against a standard curve to generate an average titer for each viral stock. Check the label on the tube to find VG/mL for your cAMP sensor stock. For difficult to transduce cultures, as us about our purified stocks.

Level of sensor expression

It is important to optimize the amount of virus used in transduction for your particular cell type. Too little virus will produce variable results, particularly if the sensor expression levels are low. In the case of HEK 293 cells, the baseline fluorescence goes up as you add more virus, and when a particular threshold is reached, the absolute change in sensor fluorescence, as well as the Z' for the assay, becomes constant.

Trouble Shooting

Are your cells fluorescent?

Different types of promoters drive expression in mammalian cells. The CMV promoter is effective in many cell lines, but not all. If you have a particular promoter you know works best in your cells, let us know. Twenty four hours after transduction, you should see bright green fluorescent cells with an epifluorescence microscope. If using a plate reader, reserve a few control wells with untransduced cells. The transduced wells on the plate should be significantly more fluorescent than untransduced cells on the same plate.

An HDAC inhibitor may be used to boost expression of the sensors. While BacMam transduction alone will result in sensor expression, sodium butyrate or another HDAC inhibitor such as valproic acid or trichostatin A (TSA) may help maintain the level of expression over time [Kost, T. et. al. 2007]. If cells look unhealthy, use lower concentrations or no HDAC inhibitor.

Finally, the type of cell culture media used in your experiment can affect the transduction efficiency of BacMam. Our assays have been validated in EMEM, DMEM, and F12K culture media.

Is the positive control working?

In cells that are expressing the sensor, addition of the positive control compound, thapsigargin, will result in increased green fluorescence within 7 hours. Both the green sensor and constituitively active red label are localized to the nucleus to simplify segmentation for high content imaging applications.

A serial dilution series of the sensor with a constant amount of thapsigargin positive control can be used to find the optimal expression for your cell type. It is important to determine the kinetics of the response and to set your instrument to measure in the appropriate time frame while avoiding oversampling.

Ratiometric Analysis

The two-color or ratiometric cell stress biosensor is a useful tool for determining ER-mediated cell stress on both image-based and plate reader-based systems. Discussed here are important aspects of the two-color stress sensor to be considered when analyzing data generated using this biosensor.

Changes in protein expression

Expression of mutant genes or treatment of cells with exogenous compounds can alter protein expression. Moreover, activation of cell stress can also alter protein expression. The constitutively expressed, nuclear localized red fluorescent protein can serve as an indicator of changes in overall protein expression. The green stress-induced fluorescence can be compared to the red fluorescence to normalize for changes in overall protein expression. However, as many compounds or mutations can alter cellular expression levels over different time frames it is important to explore changes in protein expression and cell stress activation over a range of time frames. Examples of chemically and genetically induced changes to protein expression levels and cell stress activation are shown below in Figure 2.

Plate reader analysis

When analyzing the cell stress sensor on a standard fluorescence plate reader either green fluorescence intensity or green/red ratio fluorescence can be used to determine cellular stress.

Image Analysis

When using an imaging-based approach to detect cell stress the same fluorescence intensity measurements of green or green/red ratio fluorescence can be used. However, another method that can be used with imaging and high content based analysis is the percent of stressed cells in the region of interest.In this readout, all cells within a region of interest expressing the biosensor are marked in red, while only the stressed cells within the region express green fluorescence. Dividing the number of cells expressing both green and red fluorescence by all cells expressing red fluorescence gives the fraction of stressed cells within the imaged region. This type of measurement does not rely on fluorescent intensities that may be altered by overall protein expression changes and may be useful when analyzing a number of different stress inducing compounds that alter general protein expression. Importantly, both intensity based and percentage based quantifications of cell stress produce highly similar results as shown in Fig 3.

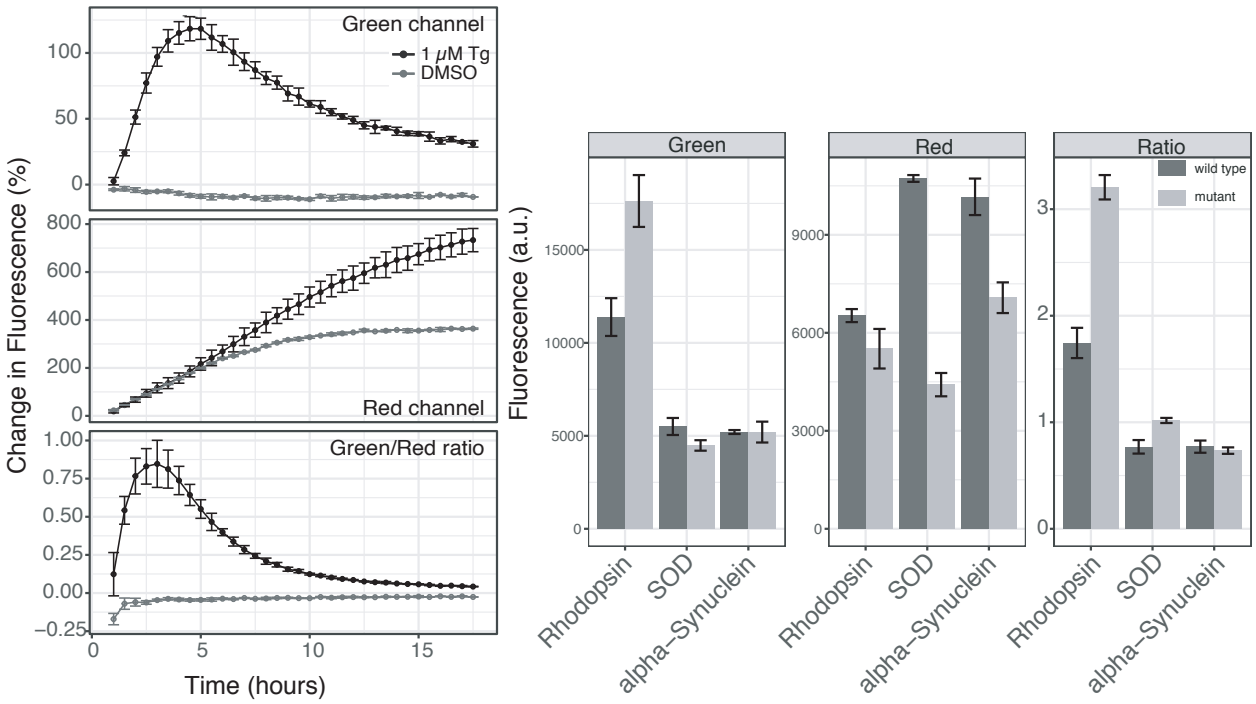


Figure 2: Monitoring changes in protein expression and cell stress with the two-color cell stress biosensor. Left: HEK293T cells transduced with the two-color cell stress biosensor and treated with either DMSO (control) or 1 μM thapsigargin (Tg). Green and red fluorescence monitored for 24 hours on a BioTek Synergy MX plate reader. Note that stress induction, indicated by an increase in green fluorescence, appears within 60 minutes, and begins to decrease by 7 hours. Long term treatment with thapsigargin also changes protein expression levels. Note that red fluorescence increases at similar rates for both DMSO and thapsigargin treated cells until about 7 hours when the stress response activated by thapsigargin begins to recover. After this point, general protein expression is upregulated in cells treated with thapsigargin. Using a green/red fluorescence ratio normalizes for this change in protein expression. Right: HEK293T cells transduced with the two-color cell stress biosensor and either wild type or mutant versions of three proteins: rhodopsin, SOD, and alpha-synuclein. Note the rhodopsin mutant with a significant increase in stress indicated by green fluorescence. In contrast, both SOD and alpha-synuclein mutants significantly decreased protein expression indicated by the red channel. The ratio of green/red fluorescence controls for the decrease in protein expression and allows for detection of cellular stress in the SOD mutant compared to its wild type control. All data are potted as as mean ± SD, n=3-6 wells.

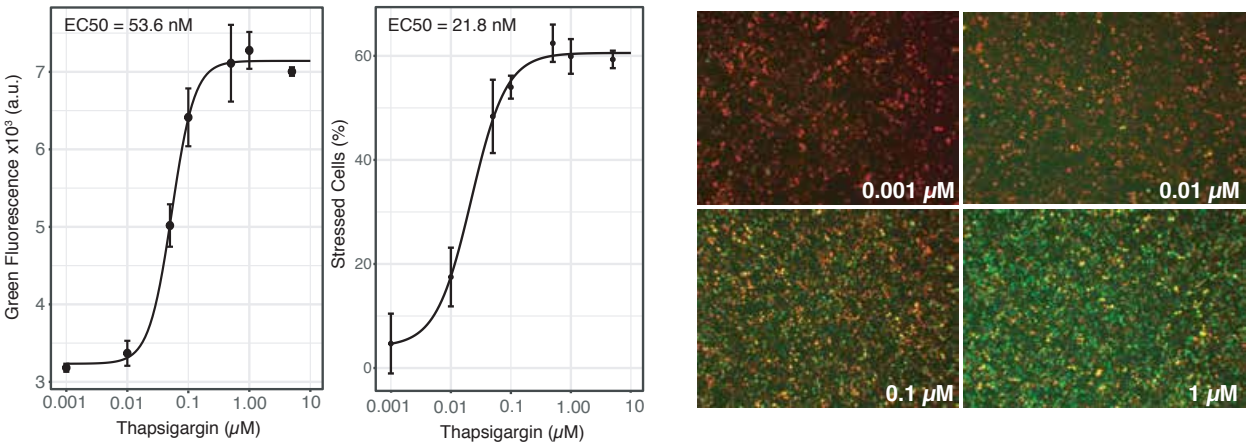


Figure 3: Plate reader and image based analysis of the two-color cell stress biosensor. Left: EC50 values determined in HEK293T cells for the ER stress inducing compound thapsigargin using either green fluorescence intensity from plate reader-based measurements (left) or percent stressed cells within a region of interest of an image (right). Note that the EC50 values determined for thapsigargin are highly similar between the plate reader and image based analysis. Data are plotted as mean ± SD, n=3 wells. Right: Images of HEK293T cells treated with different concentrations of thapsigargin. Note the dose dependent increase in the percent of cells expressing the green stress marker in each image.

Contact Us

If you have any questions about the protocols described here, or if you have ideas about how we can improve these tools or the protocol, then we want to hear from you. Your feedback is extremely valuable. Please send an email to info@montanamolecular.com, and we'll respond as quickly as we can.

References

1. Iwawaki, Takao, Ryoko Akai, Kenji Kohno, and Masayuki Miura. 2003. “A Transgenic Mouse Model for Monitoring Endoplasmic Reticulum Stress.” Nature Medicine 10 (1). Nature Publishing Group: 98–102.

2. Rong, Juan, Ian Pass, Paul W. Diaz, Tram A. Ngo, Michelle Sauer, Gavin Magnuson, Fu-Yue Zeng, et al. 2015. “Cell-Based High-Throughput Luciferase Reporter Gene Assays for Identifying and Profiling Chemical Modulators of Endoplasmic Reticulum Signaling Protein, IRE1.” Journal of Biomolecular Screening 20 (10). SAGE Publications: 1232–45.

3. Graham FL, Smiley J, Russell WC, Nairn R: Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol 1977, 36(1):59-74.

4. Dulbecco R and Vogt M: Plaque formation and isolation of pure lines with poliomyelitis viruses. The Journal of experimental medicine 1954.

5. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC: Green fluorescent protein as a marker for gene expression. Science 1994.

6. Kost T, Condreay J, Ames R, Rees S, Romanos M: Implementation of BacMam virus gene delivery technology in a drug discovery setting. Drug Discovery Today 2007, 12(9-10):396-403.

7. Tewson PH, Martinka S, Shaner N, Hughes TE, Quinn AM: New DAG and cAMP sensors optimized for live cell assays in automated laboratories. Journal of Biomolecular Screening 2015.

8. Shaner, N.C., Lambert, G.G., Chammas, A., Ni, Y., Cranfill, P.J., Baird, M.A., Sell, B.R., Allen, J.R., Day, R.N., Israelsson, M., Davidson, M.W., & Wang, J. (2013) "A bright monomeric green fluorescent protein derived from Branchiostoma lanceolatum." Nature Methods, May;10(5):407-9. doi: 10.1038/nmeth.2413.