Montana Molecular
Fluorescent Biosensors for Live Cell Discovery

Green Fluorescent Diacylglycerol (DAG) Assay

2/25/16

Overview

The green fluorescent DAG sensors are robust fluorescent protein-based sensors for measuring diacylglycerol changes in live mammalian cells. The following protocol is optimized for HEK 293 cells [Graham FL, 1977] in a 96-well plate and is appropriate for live-cell imaging and screening on automated fluorescence plate readers. This protocol can be adjusted and optimized for many different types of cells, including primary cultures, iPSC-derived cells, and pancreatic islet cells, and is easily scaled for 384 well format.

The vector carrying these sensors is a modified baculovirus. In mammalian cells, BacMam expresses only the fluorescent sensor and is a BSL-1 reagent. If you want to measure DAG in cells other than HEK 293, then the IU/mL needed to transduce a well of HEK 293 cells can be used as a starting point for assay optimization in other cell types. We recommend that you take the time to do a dilution series in your cells, to optimize in your particular cells and fluorescence detection system.

Relevant Products

Product	Description	Promoter	Recommended Use
D0300G	Green Downward DAG	CMV	Fluorescence imaging and plate reader assay (Z' > 0.8)
U0300G	Green Upward DAG	CMV	Fluorescence imaging and Plate reader assay (Z' > 0.8)

Materials included

- DAG sensor BacMam ~ $1x10^7$ IU/mL in TNM-FH Insect Culture Medium (Allele Biotech product #ABP-MED-10001).

  Green fluorescent sensor that changes in response to increases in DAG. Downward sensors decrease in fluorescence following activation of a Gq-coupled GPCR. Upward sensors increase following activation of a Gq-coupled GPCR. IU/mL are units that produce expression in mammalian cells, as distinct from plaque forming units (PFU), that for baculovirus, would be measured in insect cells.

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

  Sodium Butyrate maintains BacMam expression in HEK293 cells. Other HDAC inhibitors such as Trichostatin A (TSA) or Valproic acid (VPA) may be substituted.

- M1 muscarinic acetylcholine receptor BacMam in TNM-FH Insect Culture Medium (Allele Biotech product #ABP-MED-10001).

  A Gq-coupled GPCR in a BacMam vector, provided as a positive control for the purpose of assay optimization. Separately expresses a red fluorescent protein that is targeted to the nucleus.

- Carbachol 25 mM in H_2O

  Carbachol can be used to stimulate Gq signaling through the positive control, the M1 muscarinic acetylcholine receptor.

Storage

Baculovirus stock should be stored at 4°C and protected from light. Avoid freeze/thaw cycles.

Additional materials not supplied

- Greiner CellCoat (#655946) is our preferred plate for this assay.
- Dulbecco's Phosphate Buffered Saline (DPBS) available from VWR [Dulbecco, R. and Vogt, M. 1957].

BioSafety Considerations

BacMam does not replicate in mammalian cells and expresses only the fluorescent sensor. While it should be handled carefully, in a sterile environment, it is classified as a Biosafety Level 1 (BSL-1) reagent. This product is for research use only and is not recommended for use or sale in human or animal diagnostic or therapeutic products.

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.

About these Assays

Many cell surface receptors couple to the heterotrimeric G protein Gq, which in turn activates Phospholipase C (PLC). PLC produces two different second messengers, DAG and IP_3. IP_3 causes an increase in Ca^{2+}. This coordinated increase of both DAG and Ca^{2+} triggers the activation of conventional protein kinase C (cPKCs) to phosphorylate many different protein targets. Live cell assays that measure increases in Ca^{2+} have been used to detect this pathway, but a rise in Ca^{2+} is an ambiguous signal: there are other signaling pathways that produce increases in Ca^{2+}. This green fluorescent assay for DAG can be used to unambiguously resolve PLC pathway activation, and it can be combined with a red fluorescent sensor for Ca^{2+} , to better understand the kinetics of these coordinated, parallel signaling processes [Tewson P, et.al. 2012].

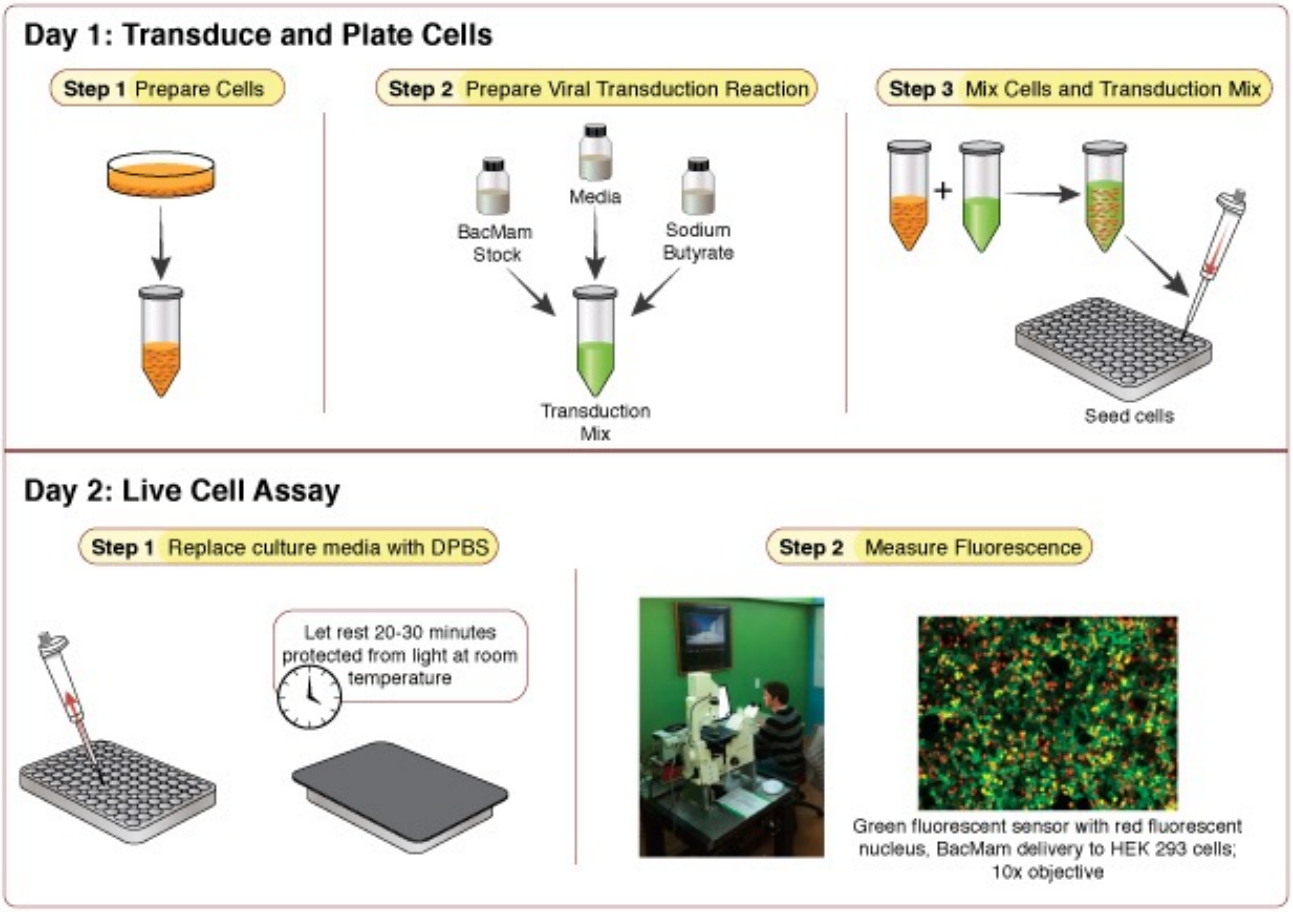
Depending on the kit, sensor fluorescence either increases or decreases in response to activation. One can imagine applications where an assay that decreases in fluorescence would have advantages, for example, if background fluorescence is very high, the fluorescence from the sensor can be more easily separated from background fluorescence.

Related Products

Product	Sensor Description	Promoter	Recommended Use
U0200G	Green Upward cADDis cAMP	CMV	Fluorescence imaging and plate reader assay (Z' > 0.85)
D0200G	Green Downward cADDis cAMP	CMV	Fluorescence imaging and plate reader assay (Z' > 0.85)
U0200R	Red Upward cADDis cAMP	CMV	Fluorescence imaging only
U0300R	Red Upward DAG	CMV	Fluorescence imaging and Plate reader assay (Z' > 0.5)
D0300R	Red Downward DAG	CMV	Fluorescence imaging and Plate reader assay (Z' > 0.7)
U0600R	Red GECO Ca^{2+}	CMV	Fluorescence imaging and Plate reader assay (Z' > 0.5)

Protocol for Use

The following 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 and determine cell count.
- Prepare a dilution of cells at your desired concentration.* 100 uL 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.

* 450,000 cells/mL works well for HEK293 cells

Example:

For **96** wells (1 plate)

100 uL cell suspension (450,000 cells/mL) per well.

100 uL cells x **110 (96** wells + 10% scale) = **11000 uL** 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 uL of the BacMam stock with 0.6 uL of the 500 mM stock solution of sodium butyrate* and 24.4 uL of the complete culture media for your cells, for a total volume of 50 uL. Mix gently.

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

Example:

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

Single Well	*Master Mix*
20 uL Sensor	x **110** = 2200 uL
5 uL Receptor Control	x **110** = 550 uL
0.6 uL 500 mM Sodium Butyrate	x **110** = 66 uL
24.4uL Complete Media	x **110** = 2684 uL
50 uL total volume	x 110 = **5500 uL** transduction mix (96 wells)

Step 3) Mix Cells and Transduction Mix from above.

- Mix Tube A and Tube B (100 uL tube A + 50 uL tube B). Mix gently and seed 150 uL 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.
- Incubate for ≈ 24 hrs under normal cell growth conditions, protected from light.

Example:

96 wells needed (1 plate)

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

Day 2 MEASURING FLUORESCENCE

- Cells are now ready for assay. Prior to measuring fluorescence, replace culture media with DPBS. Wash gently so as not to dislodge cells. Cover the cells and allow them to rest at room temperature in DPBS for 25-30 minutes before measuring fluorescence. Experiments are performed at 25°C using standard GFP excitation and emission wavelengths.
- Add 50uM carbachol to activate a set of wells transduced with control receptor.
- When monitoring the green fluorescence emitted by the sensor, a change in fluorescence intensity will be observed after addition of compounds that increase levels of DAG in the cell.

Suggestions for Assays in Adherent Cells

The protocol above is optimized for rapidly dividing immortalized cells. However, these 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. For expression in rare cell types, or specific cells in mixed cultures, Cre-dependent and specific promoter systems are available for many of our sensors.

- 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 100uL culture media per well), prepare a transduction solution by mixing 25 uL of the BacMam stock with 24.4 uL of DPBS and 0.6 uL of the 500 mM stock solution of sodium butyrate for a total volume of 50 uL. Mix the solution gently.
- Sensor 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.
- Prepare a dilution series of transduction reactions by varying the amount of BacMam. For example, a range of 10 uL to 80 uL , adjusting the amount of DPBS accordingly.
- Add the transduction reaction directly to the 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 4-8 hours (6 hours is optimal).
- Aspirate transduction solution and add 100 uL complete growth medium with sodium butyrate at a concentration of 1-2 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.

Detecting Fluorescence

Fluorescence Properties

This sensor is constructed with the very bright, mNeon green fluorescent protein [6]. We recommend Chroma's Catalog set #49003 for optimal results.

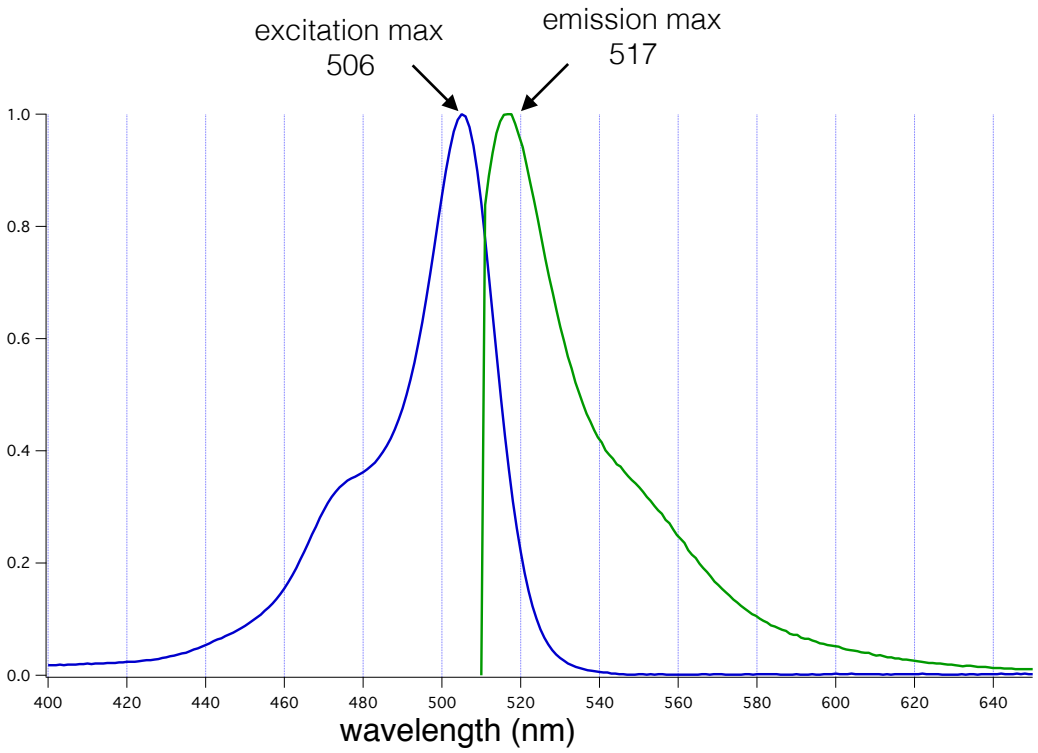


Figure 1. Absorption and emission properties of the mNeon green fluorescent protein plotted as a function of wavelength.

Timing

This is a live cell assay, and unlike many assays that measure accumulation of an analyte over time, this sensor detects, in real time, the DAG level in the living cell. Changes in DAG can occur quite rapidly, so the application of drug and resulting changes should be captured as quickly as possible. The best possible experimental setup involves capturing the fluorescence from a well of cells first, before the addition of any compound, and then again a second or so after the compound addition. Sampling the fluorescence at intervals of 2 to 5 seconds provides a good measurement of the response. The maximal response is reached at ~60 seconds after the addition of the agonist.

Assay Performance Considerations

Virus Titer

Typically, viruses are quantified in terms of plaque forming units (PFU). In the case of BacMam, PFU would be a measurement of the viruses that are capable of transducing an insect cell, the natural host. Since mammalian cell expression is the application of interest for these sensors, we

quantify infectivity by measuring the units (IU/mL) that produce expression in mammalian cells. While this correlates with PFU, IU/mL is a direct measurement of transduction in mammalian cells. We make this measurement by transducing HEK 293 cells with serial dilutions of the virus and then counting the fluorescent cells. Check the label on the BacMam stock to find IU/mL.

Level of Sensor Expression

To optimize the assay for your particular cell type, it is important to titrate the amount of virus used in the transduction. Too little virus will produce variable results, particularly if the sensor expression levels are low and difficult to detect on your instrument. Figure 2 summarizes BacMam titration in HEK 293 cells coexpressing the hM1 receptor control and the Green Upward DAG sensor. The hM1 receptor is activated by carbachol, to increase levels of DAG.

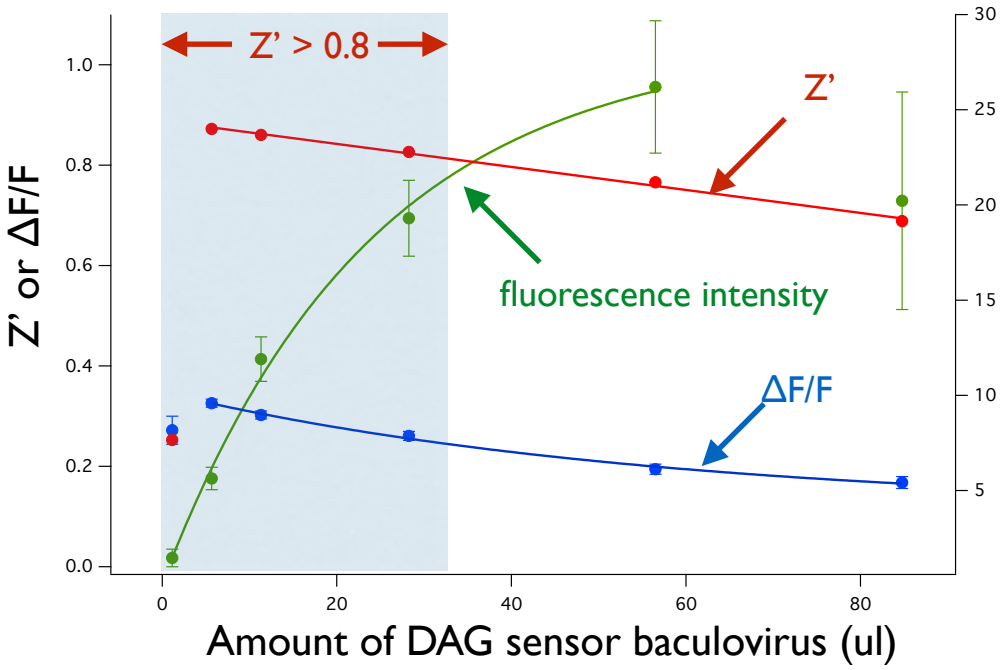


Figure 2. As the amount of virus added to the wells increases, so does the baseline fluorescence, plotted in green. Although the change in fluorescence when the sensor is activated also increases with more virus, it quickly reaches the maximum possible change shown in blue.

Level of receptor expression

The magnitude of the sensor response can be affected by the level of receptor expression in your cells. We have found that low levels of receptor expression often produce the largest signals, while high levels of receptor expression often produce smaller responses. This is consistent with the observation that over-expression of some GPCRs can change the levels of second messengers due to low levels of spontaneous activity.

Validated Instruments

- Biotek Synergy MX
- Biotek Cytation
- Molecular Devices FLIPR
- Molecular Device Flexstation
- Perkin Elmer Enspire
- BMG Pherastar - in progress

Our green fluorescent sensors under CMV promoter control have been validated on several automated fluorescence plate readers. However, sensors under synapsin or weaker promoters, may be limited to detection to imaging systems. In this section, we summarize some of the considerations in the use of various types of fluorescence detection instruments and the trade offs between these systems.

The simplest format: one drug, one sensor, one time point.

By following the sample protocol described on the previous pages, you can expect to record a robust response on standard fluorescence plate readers. Fluorescence may be recorded sequentially from each well before adding drug, and again after adding the drug. The response to the receptor and agonist controls provided in your kit occurs over a few minutes. While this protocol is simple, the drawback is that it does not capture the kinetics of the response, only the fluorescence before and after the addition of drug.

Two channel format: one drug, two sensors, one time point.

By combining green and red sensors, one can simultaneously record two limbs of second messenger signaling. In this case, standard fluorescence plate readers can be used, but they need the optics necessary to collect two different channels of fluorescence, which will involve specific filter sets and either two detectors or very fast filter switching.

Capturing the kinetics, one well at a time.

High content imaging systems offer excellent opportunities for capturing kinetic responses. In the models that have onboard liquid handling, fluorescence can be recorded before and after the addition of vehicle, and then continuously recorded after addition of the drug. This provides kinetic data, but each well in the plate is recorded over relatively long time frames. Confocal systems are not recommended, as the signal outside of the focal plane is eliminated in these systems.

Capturing the kinetics in parallel, multi-well recording.

There are instruments that can collect fluorescence data from all of the wells of a plate simultaneously and have automated liquid handling. These are perfectly suited for recoding the kinetics of the response from every well. Because the recordings are all done in parallel, the speed of the assay is considerably faster than a single channel instrument. An example of such an instrument is the Molecular Devices “Flex Station” and “FLIPR” series of plate readers. Our green fluorescent assays have been validated on these instruments.

Trouble Shooting

If you encounter problems, there are several steps that you can consider when troubleshooting.

Are the cells fluorescent?

Different types of promoters drive expression in mammalian cells. The CMV promoter in our BacMam vectors is an effective promoter in many cell lines, but not all. Twenty four hours after transduction, you should see bright green fluorescent cells in a typical epifluorescence microscope, or the transduced wells in a 96 well plate should be significantly more fluorescent than untransduced cells in wells on the same plate.

HDAC inhibitors are important to expression of the sensors. While BacMam transduction alone will initially generate low levels of sensor expression, sodium butyrate or another HDAC inhibitor such as VPA or trichostatin A (TSA) will generate optimal levels of sensor expression and maintain this level of expression [Kost, T. et. al. 2007]. If cells look unhealthy, lower concentrations of HDAC inhibitor may be used. This may improve cell health, but it will also reduce sensor expression.

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?

If the cells are expressing the sensor, and fluorescence is detectable on your instrument, then a good positive control is included in this kit. Adding 5 uL of the M1 muscarinic acetylcholine receptor virus to a set of positive control wells will ensure that a Gq-coupled receptor is present. You can double check to make sure the receptor is expressed by examining cells in a fluorescent microscope with filters for red fluorescence. You should see the green sensor fluorescence throughout the cell, and red nuclear fluorescence that marks the cells that also express the receptor control.

Addition of carbachol will then generate a change in fluorescence. If it does not, then it is important to use this positive control to optimize three aspects of your assay. First, a serial dilution series of the sensor with a constant amount of receptor virus can be used to optimize for your cells, receptor, and instrumentation. Second, find the amount of virus sufficient to transduce all of the cells in the well. Third, it is important to determine what the kinetics of the response is and whether your assay and instrument are capable of making the measurements in that time frame.

Contact Us

After following the suggested protocol and trouble shooting steps, if you still have questions or feedback, contact us at info@montanamolecular.com.

Bibliography

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