Brilliant Calcium Gold *Flex*

Table 1	Package Contents			
Label	Name	Volume	Containers	Storage
Reagent A	Fluo-Gold AM (50 µg)	Dry	10	-20° C
Reagent B	DMSO	225 µL	1	-20° C *
Reagent C	DySolv	4 mL	1	4° C
Reagent D	10X Assay Buffer	20 mL	1	4° C
Reagent E	TRS	4 mL	1	4° C
Reagent F	Probenecid Solution	4 mL	1	4° C

**Hygroscopic DMSO should be stored at -20° C with desiccant pack to prevent the solvent from becoming wet. Wet solvent causes difficulties with the solubility of the dye. Use the DMSO within 6 months of receipt.*

Description

For the last 25 years, fluorescence-based measures of Ca^{2+} flux have brought about the discovery of small-molecule modulators of a host of ion channels, transporters, GPCRS and other targets of interest for both drug discovery and basic research.

ION Biosciences Brilliant Calcium Gold Assay is a total assay solution for multi-well plate-based, high-throughput measurements of changes in intracellular Ca^{2+} mediated through a wide-variety of plasma membrane and intracellular calcium channels and transporters. The kit includes our Fluo-Gold dye (Ex/Em: 524/552 nm), which is responsive to intracellular Ca^{2+} dynamics. Fluo-Gold's spectral properties enables multiplexing with GFP-expressing cells or other green-fluorescent indicators, and will help mitigate false positives caused by auto-fluorescent compounds within a library. ION Biosciences Brilliant Calcium Gold Assay is also useful for investigating numerous effectors of ion channels and transporters including G protein-coupled receptors, lipid kinases and protein kinases.

Brilliant Calcium Gold *Flex* provides all the reagents necessary for use as a wash or no-wash assay with adherent or non-adherent cells. The optional use of a probenecid solution and an extracellular background masking solution offers the ultimate in compatibility for cells types which are difficult to load with fluorescent Ca^{2+} indicators (e.g. Chinese Hamster Ovary, CHO cells) and when performing assays in complete, serum-containing cell culture medium is desired.

Laboratory Procedures

Getting Started

Before you begin, make sure that you have all the additional reagents and materials you will need for the successful completion of your experiment. While the ION Brilliant Calcium Gold Assay package contains all the reagents you will need to prepare your cells for testing, your experiments will likely include other reagents which are not included in your ION Brilliant Calcium Gold Assay package. Notably compounds to be tested are not included, neither are buffers and solvents for the dissolution of those compounds. The Brilliant Calcium Gold Assay package also does not contain reagents necessary for cell culture.

Laboratory Procedures

Getting Started

In addition to reagents, a fluorescence plate reader that is capable of providing excitation at ~530 nm and collecting emission at ~550 nM is required. Ideally this plate reader will be able to collect kinetic data at an interval of once per second (1 Hz). Examples of plate readers of this type are the WaveFront Panoptic, Hamamatsu FDSS, Molecular Devices FLIPR and Molecular Devices FlexStation.

Wash Method – Adherent Cells

The instructions given below are for one, 384-well microplate. Certain aspects of the instructions may need to be altered, as appropriate, for multiple microplates or other assay formats (e.g. 96-well microplates or non-adherent cells). The Fluo-Gold AM and Fluo-Gold AM -containing solutions should be protected from direct light.

1. Add 20 µL DMSO (Reagent B) to the tube containing Fluo-Gold AM (Reagent A)
2. Vortex until Reagent A is fully dissolved.
3. Add appropriate volume of water (Table 2) to a 15 mL centrifuge tube.
4. Add 1 mL of 10X Assay Buffer (Reagent D) to tube from step 3.
5. Add 200 µL of DySolv (Reagent C) to the tube from step 4.
6. If desired add 200 µL of Probenecid Solution (Reagent F) to the tube from step 5.

Table 2 Dye Loading Solution

Label	Name	Method A	Method B
Reagent A	Fluo-Gold AM Solution	20 µL	20 µL
Reagent C	DySolv	200 µL	200 µL
Reagent D	10X Assay Buffer	1 mL	1 mL
Reagent F	Probenecid Solution*	-	200 µL
	Water	8.8 mL	8.6 mL
	Total	10 mL	10 mL

**Probenecid may be included in the Dye Loading Solution to aid dye retention. This may be particularly important in certain cell lines (e.g. CHO cells). However, caution is advised when using Probenecid as it may have undesirable effects on assay performance for the target of interest.*

7. Add 20 µL of Fluo-Gold AM Solution from step 2 to the tube from step 6.
8. Briefly vortex the tube from step 7 to mix.
9. Remove the cell-culture medium from the 384-well microplate containing the cells of interest.
10. Add 20 µL per well of the Dye Loading Solution from step 8 to the microplate from step 9.

Table 3	Wash Solution				
Label	Name	Method A	Method B	Method C	Method D
Reagent D	10X Assay Buffer	1 mL	1 mL	1 mL	1 mL
Reagent E	TRS*	-	200 µL	-	200 µL
Reagent F	Probenecid Solution	-	-	200 µL	200 µL
Reagent H	Water	9 mL	8.8 mL	8.8 mL	8.6 mL
	Total	10 mL	10 mL	10 mL	10 mL

**TRS contains a membrane-impermeant dye useful for masking extracellular fluorescence. Caution is advised when using TRS or other extracellular masking solutions as they may have undesirable effects on assay performance for the target of interest.*

12. Prepare Wash Solution in a 15 mL centrifuge tube by adding the appropriate amounts of water, 10X Assay Buffer (Reagent D) and other components if desired as shown in Table 3.
13. Briefly vortex the tube from step 12 to mix.
14. Remove Dye Loading Solution from microplate in step 11.
15. Add 20 µL per well of the Wash Solution prepared in step 13 to the microplate from step 14.
16. Transfer the washed, dye-loaded, cell-containing microplate from step 15, along with an additional microplate containing a stimulus solution of interest, to a kinetic-imaging plate reader (e.g. WaveFront Panoptic, Hamamatsu FDSS, Molecular Devices FLIPR or Molecular Devices FlexStation).
17. Acquire data using an excitation wavelength of ~530 nm, an emission wavelength of ~550 nm and an acquisition frequency of 1 Hz. Begin data acquisition and after 10 seconds, add appropriate volume of the stimulus solution to the cell-containing plate and continue data acquisition for an additional 90 seconds**.

***The timing of and volume of stimulus solution addition may vary. Some experiments may include the addition of other solutions to the cell-containing microplate prior to the addition of the stimulus solution. In these cases, the volume of the stimulus solution addition should be altered to account for the additional volume of solution in the cell-containing*

No-wash Method – Adherent Cells

1. Add 20 µL DMSO (Reagent B) to the tube containing Fluo-Gold AM (Reagent A)
2. Vortex until Reagent A is fully dissolved.

Table 4 Dye Loading Solution

Label	Name	Method A	Method B
Reagent A	Fluo-Gold AM Solution	20 µL	20 µL
Reagent C	DySolv	400 µL	400 µL
Reagent D	10X Assay Buffer	1 mL	1 mL
Reagent E	TRS*	400 µL	400 µL
Reagent F	Probenecid Solution**	-	400 µL
	Water	8.2 mL	7.8 mL
	Total	10 mL	10 mL

**TRS contains a membrane-impermeant dye useful for masking extracellular fluorescence. Caution is advised when using TRS or other extracellular masking solutions as they may have undesirable effects on assay performance for the target of interest.*

***Probenecid may be included in the Dye Loading Solution to aid dye retention. This may be particularly important in certain cell lines (e.g. CHO cells). However, caution is advised when using Probenecid as it may have undesirable effects on assay performance for the target of interest.*

3. Add appropriate volume of water (Table 4) to a 15 mL centrifuge tube.
4. Add 1 mL of 10X Assay Buffer (Reagent D) to tube from step 3.
5. Add 400 µL of DySolv (Reagent C) to the tube from step 4.
6. Add 400 µL of TRS (Reagent E) to the tube from step 5.
7. If desired add 400 µL of Probenecid Solution (Reagent F) to the tube from step 6.
8. Add 20 µL of Fluo-Gold AM Solution from step 2 to the tube from step 7.
9. Briefly vortex the tube from step 8 to mix.
10. Add 20 µL per well of the Dye Loading Solution from step 9 to the cell-containing microplate. Do not remove the cell culture medium.

11. Incubate the microplate containing the cells and Dye Loading Solution for 30 - 60 minutes at 37° C in a cell culture incubator.
12. Transfer the dye-loaded, cell-containing microplate from step 11, along with an additional microplate containing a stimulus solution of interest, to a kinetic-imaging plate reader (e.g. WaveFront Panoptic, Hamamatsu FDSS, Molecular Devices FLIPR or Molecular Devices FlexStation).
13. Acquire data using an excitation wavelength of ~530 nm, an emission wavelength of ~550 nm and an acquisition frequency of 1 Hz. Begin data acquisition and after 10 seconds add appropriate volume of the stimulus solution to the cell-containing plate and continue data acquisition for an additional 90 seconds**.

***The timing of and volume of stimulus solution addition may vary. Some experiments may include the addition of other solutions to the cell-containing microplate prior to the addition of the stimulus solution. In these cases, the volume of the stimulus solution addition should be altered to account for the additional volume of solution in the cell-containing*

Example Results

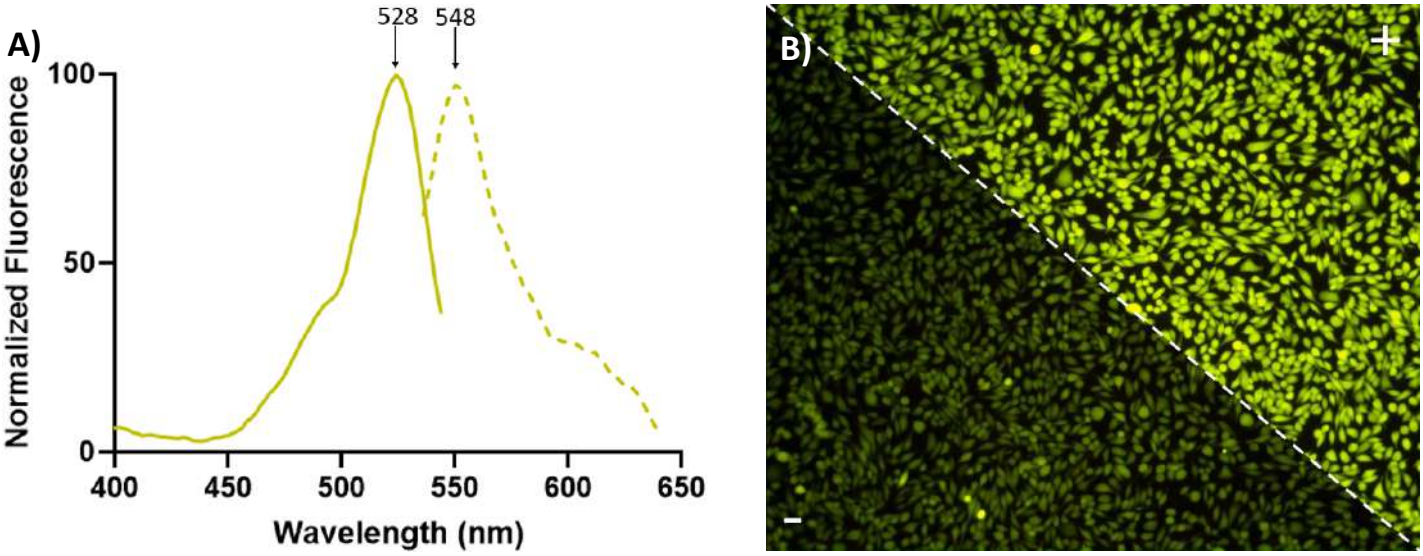

Figure 1. Fluo-Gold spectral properties. A) Spectral data was collected from CHO K1 cells loaded with Fluo-Gold for 30 minutes. Data was acquired with a BioTek® Cytation 5 plate reader. Maximum excitation is at 528 nm and maximum emission is at 548 nm. B) Cells were imaged using a Zeiss Axiovert 25 Inverted Phase Contrast Microscope, before (-) and after (+) the addition of 100nM ionomycin, a calcium ionophore. (Ex: 517/20 nm, Em: 575/59 nm).

Example Results

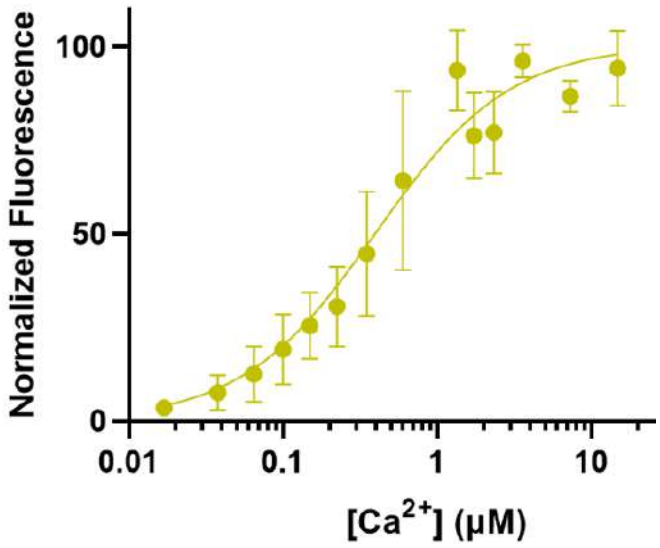

Figure 2. Saturation binding curve. Fluo-Gold titrated in calcium calibration buffers. Fluo-Gold has a slightly lower calcium affinity (K_d ~400 nM) than Fluo-4.

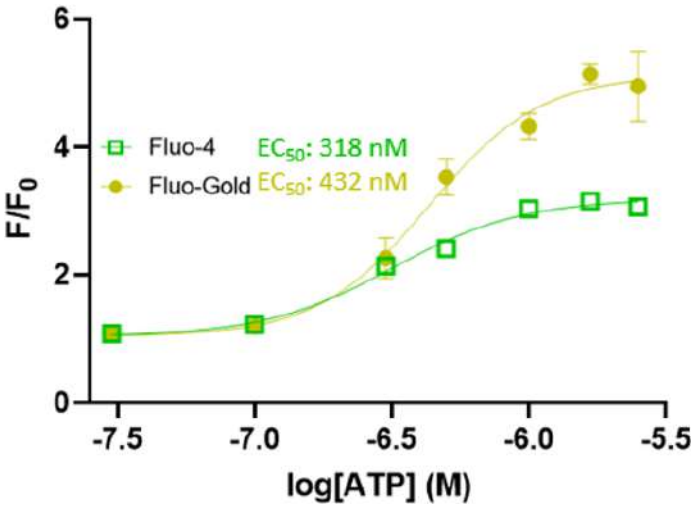

Figure 3. Concentration response curve of ATP in GFP-expressing CHO cells. ATP activates endogenously expressed purinergic (P2X7) receptors, which leads to increased intracellular calcium. Fluo-4 fluorescence (Ex: 485 nm, Em: 525 nm, Cutoff: 515 nm) and Fluo-Gold fluorescence (Ex: 517 nm, Em: 546 nm, Cutoff: 530 nm) were recorded at ~1 Hz using a Molecular Devices FlexStation® plate reader. Error bars represent standard deviation (n=3). Fluo-Gold displays a larger dynamic range and is better suited for assays using GFP-expressing cells.

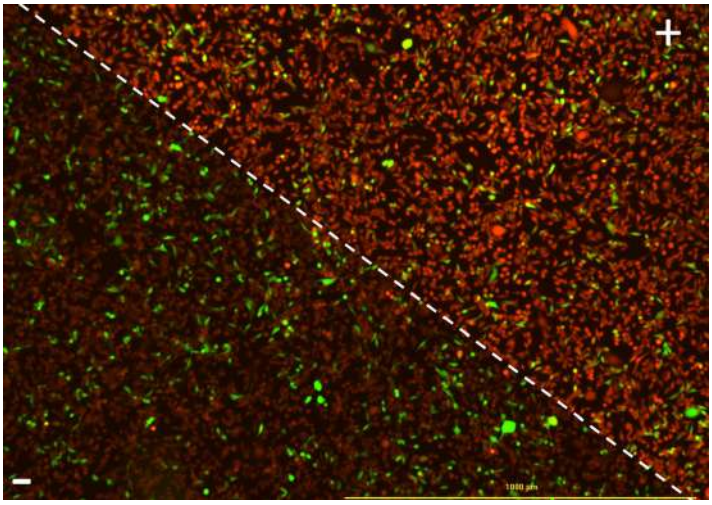

Figure 4. Calcium mobilization in GFP-expressing cells loaded with Fluo-Gold. Representative fluorescence images of polyclonal, GFP-expressing CHO cells loaded with Fluo-Gold for ~30 minutes. Images were acquired before (-) and after (+) addition of the ionomycin (100 nM) with a BioTek® Cytation equipped with a GFP filter cube (Ex: 469/35 nm, Em: 529/39 nm), Propidium Iodide filter cube (Ex: 531/40 nm, Em: 647/57 nm), and 4X objective. Fluo-Gold fluorescence appears red because a propidium iodide filter cube was used to capture the images.

Example Results

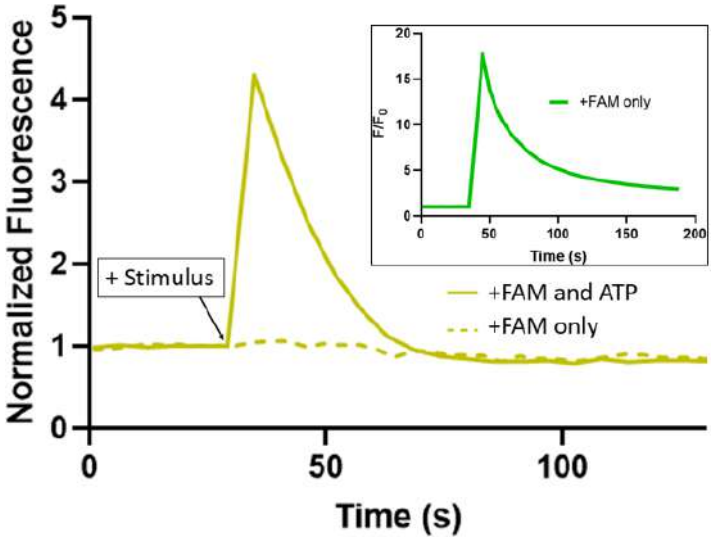

Figure 5. Fluo-Gold minimizes compound interference from green fluorescent compounds. CHO K1 cells were loaded with Fluo-Gold for 30 minutes. Fluorescence for Fluo-Gold (Ex: 540 nm, Em: 580 nm, Cutoff: 570 nm) was recorded at ~1 Hz using a Molecular Devices FlexStation®. Baseline fluorescence was collected for 30 seconds before the addition of 5 µM carboxyfluorescein (FAM), a highly fluorescent compound, and 5 µM FAM + 1.7 µM ATP. ATP activates endogenously expressed P2X7 receptors, which leads to increased intracellular calcium. FAM fluorescence does not disrupt Fluo-Gold signal. Inset) Fluorescence profile of 5 µM FAM addition when using acquisition settings for Fluo-4 (Ex: 485 nm, Em: 525 nm, Cutoff: 515 nm).