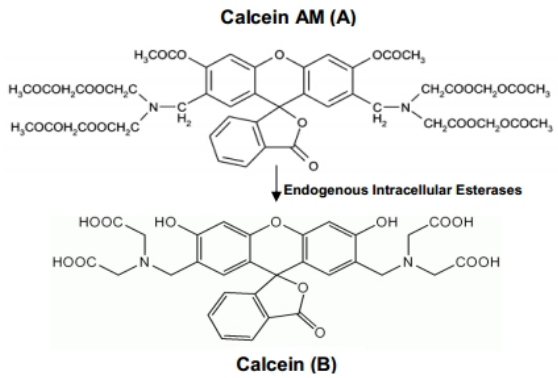
BIOLUMINOR

Calcein-AM

The Calcein-AM provides a simple, rapid and accurate method to measure cell viability and/or cytotoxicity. Calcein-AM (structure A) is a non-fluorescent, hydrophobic compound that easily permeates intact, live cells. The hydrolysis of Calcein-AM by intracellular esterases produces calcein (structure B), a hydrophilic, strongly fluorescent compound that is well-retained in the cell cytoplasm. Cells grown, preferably in black-walled plates, can be stained and quantified in less than two hours.



Features:

- ♦ suitable for proliferating and non-proliferating cells
- ♦ ideal for both suspension and adherent cells
- ♦ non-radioactive microplate
- ♦ rapid (no solubilization step as in an MTT assay)
- ♦ ideal for high-throughput assays
- ♦ better retention and brightness compared to other fluorescent compounds (i.e. fluorescein)
- ♦ useful in a variety of studies, including: cell adhesion, chemotaxis, multidrug resistance, cell viability, apoptosis, and cytotoxicity
- ♦ adaptable to a wide variety of techniques, including: microplate assays (described here), immunocytochemistry, flow cytometry, and in vivo cell tracking

Photophysical properties of Calcein-AM

Fig. 1 shows that the optimal fluorescence excitation of Calcein is at 490 nm while the maximal fluorescence emission intensity is at 515 nm.

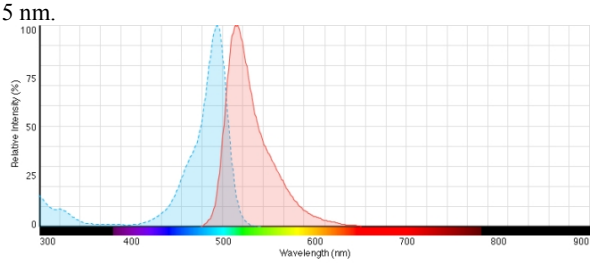


Fig. 1 Fluorescence excitation and emission spectra of Calcein in pH 9 buffer. The fluorescence emission spectrum was obtained using *λex*@490 nm, and the fluorescence excitation spectrum was scanned using *λem*@515 nm.

Materials Required but Not Provided

1. Fluorescence plate reader equipped with a 490 nm excitation filter and a 520 nm emission filter.
2. Pipettors and tips.
3. Black-walled culture plates. Depending on cell type and density, it may be possible to use transparent plates. However, background fluorescence may significantly reduce assay sensitivity. See the manufacturer's recommendations for your fluorometer and empirically test the use of transparent plates with your system.
4. Cell culture media, supplies, and centrifuge equipped to handle microplates (centrifuge able to handle microplates is ideal but optional, see Section VI, suspension cell protocol).
5. Anhydrous DMSO.
6. Equipment to desiccate at -20 C.

Reagent Preparation

1. 1X Calcein AM DW (Dilution/Wash) Buffer

Dilute the 10X Calcein AM DW Buffer to 1X before use. For each 96-well microplate, use 5 ml of 10X Calcein AM DW buffer and 45 ml of deionized sterile H_2O.

2. Calcein AM

The molecular weight of Calcein AM is 995 grams per mole. Resuspend the dehydrated pellet of one tube (50 g) in 25 μ l of anhydrous DMSO to make a 2 mM Calcein AM Stock Solution. Return the unused portion of the Calcein AM Stock Solution to storage at -20 C under desiccation. Immediately prior to use, dilute the Calcein AM Stock Solution in 1X Calcein AM DW Buffer to a 2X Calcein AM Working Solution, preparing enough for all wells using 50 l per well at the appropriate concentration. For example, for one 96-well microplate using a 1M final concentration of Calcein AM: dilute 5 l of the Calcein AM Stock Solution in 5 ml of 1X Calcein AM DW Buffer to make a 2 M (2X) Calcein AM Working Solution.

Diluted Calcein AM must be used immediately, as it will hydrolyze to Calcein in solution. Note that the final concentration of the Calcein AM will need to be empirically determined for different cell types and/or experimental conditions; ranges of 1 M to 10 M have been reported.

Assay Protocol

SUSPENSION CELLS

1. Grow cells at varying densities (1000-500,000 cells per ml) in appropriate medium in black-walled plates and treat according to experimental protocol (varying amounts of proliferative or toxic compounds, etc.). Alternatively, cells can be grown in transparent plates, and transferred to black-walled plates for reading (See Section IV, Note 3). For conversion of RFU to cell number, the range of cell concentrations needed for a standard curve may need to be optimized to ensure the best dynamic range. (See Section VII, page 4 for further discussion.)
2. Centrifuge at 250 x g for 5 min. with a centrifuge

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equipped to handle microplates. Alternatively, transfer cells to microfuge tubes for centrifugation and return to the plate to read.
3. Carefully discard the media supernatant and add 100 µl of 1X Calcein AM DW Buffer.
4. Centrifuge at 250 x g for 5 min.
5. Remove the 100 µl of 1X Calcein AM DW Buffer and replace with 50 µl of fresh 1X Calcein AM DW Buffer. It is important to remove any carry-over media in the supernatant, as phenol red and serum will interfere with the sensitivity of the assay.
6. Add 50 µl of freshly diluted 2X Calcein AM Working Solution to each well (see Section V, Step 2 above).
7. Incubate for 30 minutes at 37 ℃ under CO_2 (or normal cell growth conditions).
8. Record fluorescence using a 490 nm excitation filter and a 520 nm emission filter. The fluorescence intensity is proportional to the number of viable cells (see Figure 1).

ADHERENT CELLS

1. Seed cells at varying densities (1000-500,000 cells per ml) in appropriate me dium in black-walled microplates and treat according to experimental protocol (varying amounts of proliferative or toxic compounds, etc.). Transparent plates may also be used to ensure cell adherence but background fluorescence may reduce assay sensitivity (see the manufacturer's recommendations for your fluoro meter). The optimal range of cell number may need to be optimized to ensure the best dynamic range. (See Section VII, below, for further discussion.)
2. Discard the media supernatant and add 100 µl of 1X Calcein AM DW Buffer.
3. Remove the 100 µl of 1X Calcein AM DW Buffer and replace with 50 µl of fresh 1X Calcein AM DW Buffer. It is important to remove any carry-over media, as phenol red and serum will interfere with the sensitivity of the assay.
4. Add 50 µl per well of freshly prepared 2X Calcein AM Working Solution (see Section V, item 2 for preparation).
5. Incubate for 30 minutes at 37 °C under CO2 (normal culture conditions).
6. Record fluorescence using 490 nm excitation filter and a 520 nm emission filter. The fluorescence intensity is proportional to the number of viable cells (see Figure 1 below).

SAMPLE EXPERIMENTAL RESULTS

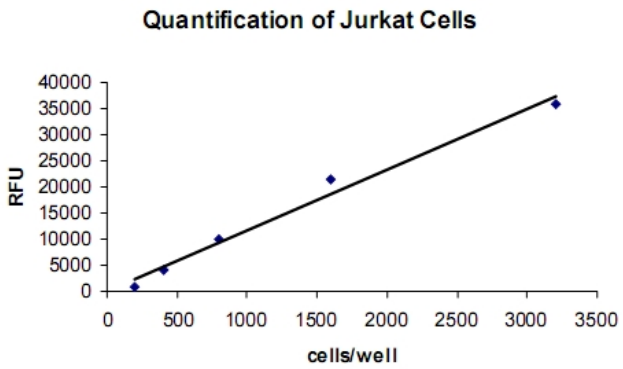

Figure 1. Calcein AM Quantification of Jurkat Cells
Jurkat cells were grown in RPMI supplemented with 10% FBS, washed with 1X Calcein AM DW Buffer, and counted using Trypan blue and a hemocytometer. Cells were serially diluted in a black-walled microplate and then incubated with 1 µ M Calcein AM for 30 minutes at 37 ℃ under 5% CO_2. Fluorescence values were obtained using a 485 nm excitation filter and a 520 nm emission filter in a BMG Laboratories' FluoStar Optima Fluorometer with a gain setting of 1600.

Standardization

There are two options for the reference system: measure relative differences or compare absolute cell number. To monitor relative changes in cell number in the same cell type it is not necessary to calibrate the system. Data may be presented as the percent change in fluorescence intensity relative to an experimental control. To calibrate using cell number, determine the cell number in a sample and plate out dilutions in triplicate covering a range of 1 x 103 cells per mL to 5 x 105 cells per ml in 50 µl of medium. Perform the standard assay. Determine averages of triplicate values and plot data as cell number per well vs. fluorescence intensity.

To calibrate fluorescence values across microplates, the same gain setting must be used. Refer to the manufacturer's instructions for your fluorometer.

Troubleshooting

Problem	Action
Low fluorescence values	-Increase concentration of Calcein AM used. -Check health of cells during incubation with Calcein AM (using Trypan blue, etc.). -Incubate plate in the dark.
Poor triplicates:	-Ensure no bubbles present in wells. -Pipet cells accurately. -Check accuracy of pipettor. -Ensure no loss of cells during wash steps.
High background:	-Use black-walled plates. -Use Calcein AM DW Buffer. -Use freshly diluted Calcein AM. -Increase washes to ensure media removal. -Shorten incubation time with Calcein AM. -Decrease number of cells per well.

References

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