FDSS7000EX FDSS/μCELL

Evaluation of a new intracellular calcium fluorescent reagent using the FDSS6000

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Introduction

G protein-coupled receptors (GPCRs) are trans-membrane spanning proteins that transduce an extracellular signal (ligand binding) into an intracellular event (G protein activation). The diversity of functions of GPCRs is related to the wide range of ligands recognized, ranging from small molecules (bioamines) to peptides and proteins (eg. chemokines). As a consequence, these receptors are involved in many physio-pathological conditions, which has led them to be an attractive class of targets for drug development.

Institut de Recherches Servier has examined a new calcium fluorescent no wash assay kit from DiscoveRx Corp. for intracellular calcium release measurement using a Hamamatsu FDSS6000 imaging based plate reader. An aminergic and a peptidergic receptor were used as models to explore the potential of this new calcium probe, which was characterized in both agonist and antagonist modes, in different experi-mental designs corresponding to various HTS conditions.

In the present Application Note, the fluorescence spectral properties of DiscoveRx Calcium assay reagent are evaluated, and are compared with the parameters of the FDSS6000 calcium assay filter set. Data obtained in several experimental conditions are presented, and allow the assessment of DiscoveRx Calcium Assay reagent and Hamamatsu FDSS6000 reader as an appropriate environment for running GPCR calcium release functional HTS.

Some of the data presented herein were presented at the 3rd European FDSS Users Meeting, held the first of June 2006 at Hamamatsu France.

Principle of calcium dyes

Calcium probes are usually related to the Fluo-3 or Fluo-4 series, which consist in a compound with low fluorescence, until calcium ions are chelated and induce a high fluorescence of the probe. These compounds are generally used as acetomethoxyl derivatives, which are unable to bind calcium but which are cell permeable. Once loaded into the cells, cytoplasmic esterases activate the probe by releasing the acetoxymethyl group.
The probe can then chelate intracellular calcium, and produce a fluorescence which is proportional to the calcium concentration. Probe leakage is avoided by the use of probenecid, an inhibitor of membrane transporters (Fig. 1).

Model probe: Fluo-3 / Fluo-4
Acetomethoxy probes

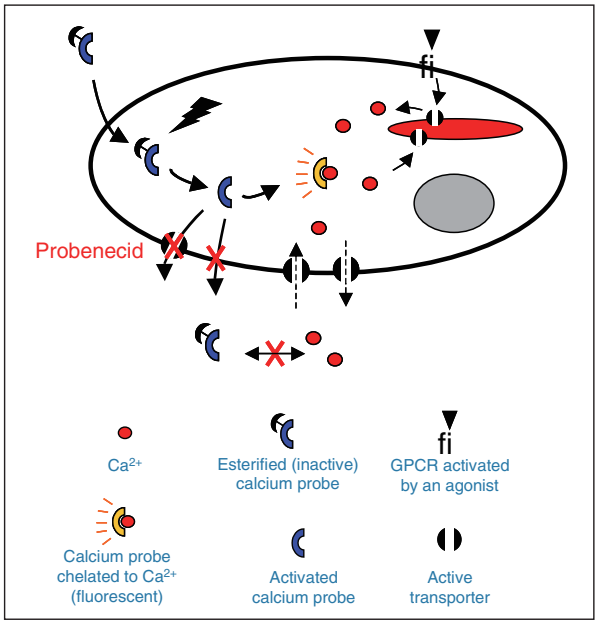


Fig. 1: Principle of calcium dye cell uptake and metabolism

Challenges

- Cell permeability of the esterified probe
- Intracellular retention of the activated probe
- High quantum yield of the fluorophore upon Ca^{2+} binding
- Low optical quenching of the extracellular esterified probe
- Low fluorescence background

Low fluorescence background can be addressed by washing out extracellular dye in excess, which is time consuming. Alternatively, homogenous calcium assay reagents have been developed to specifically address this issue and avoid washing steps. It is the case for instance of DiscoveRx Calcium No Wash Assay kit (DRx Ca NW), which was assessed in the experi-ments described herein.

Materials and Methods

Spectral Properties of the DiscoveRx Calcium No Wash Dye

Resuspended CHO-aR cells were loaded with the DiscoveRx Ca NW Dye, lysed with 0.1 % Triton X-100 and incubated with 10 mM $CaCl_2$ before fluorescence spectra recording using a HITACHI F2000 spectro-fluorimeter. The excitation spectrum was recorded with full spectrum emission (Fig. 2) and the emission spectra were recorded with excitation at different wavelengths (Fig. 3).

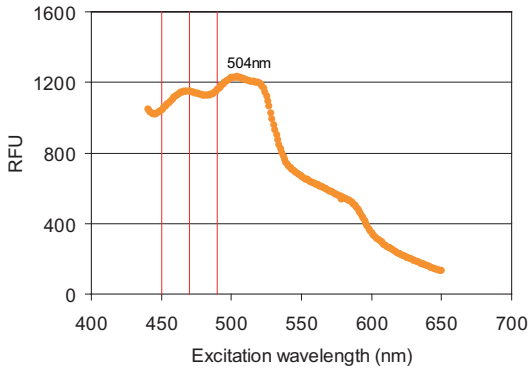


Fig. 2: Excitation spectrum of DiscoveRx Ca NW reagent. Dashed lines, 450-490 bandpass of FDSS6000 (450-490) excitation filter.

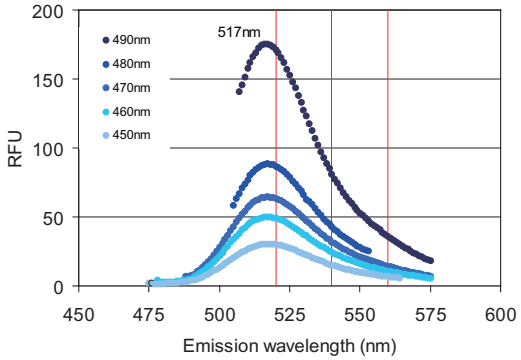


Fig. 3: Emission spectra of DiscoveRx Ca NW reagent. Dashed lines, 520-560 nm bandpass of FDSS6000 540 nm AF40 emission filter.

The Hamamatsu Fluo3/4 excitation filter fits well with the range of excitation wavelength of DiscoveRx Ca NW reagent yielding the highest fluorescence emission. Besides, the upper part of the emission spectrum of the probe is covered by the emission filter. Hence, the properties of the FDSS6000 excitation and emission filters combine good sensitivity and low diffusion when used with DiscoveRx Ca NW reagent.

Measurement conditions

The calcium release measurements were performed using the above filter set, provided by Hamamatsu as the Fluo3/4 Option.
We used an excitation bandpass filter (450-490 nm) and an emission filter at 540 nm AF40. We used to separate the excitation and emission lights for this application a 495 nm LP dichroic mirror. We performed the assay here with a standard exposure time of 0.207 s, with sensitivity set at 4 and binning (4 × 4) selected for the camera parameters. Calcium response was recorded as a kinetic during 120 seconds.

How to perform the assay

CHO (Chinese Hamster Ovary) cells stably expressing a G protein-coupled receptor (aminergic receptor, aR or peptidergic receptor, pR) were plated 24 h before the assay (15,000 cells/well in 40 µL), into 384-blackwell clear bottom culture plates in 10 % FCS + penicillin /Streptomycin antibiotics 1 % Ham F12 medium.

Cells were loaded for intracellular calcium flux measurement with a calcium no wash kit assay solution (DiscoveRx Calcium No Wash Kit, #90-0080L) supplemented with 2.5 mM probenecid to avoid calcium dye leakage from the cells. The cells were thereafter incubated at 37 °C or room temperature for 1 h in 5 % CO2 atmosphere. The agonist was added after 10 s of baseline acquisition. For antagonist studies, the compounds to be tested were added 10 min before the addition of the reference agonist. Increase of intracellular Ca^{2+} concentration was monitored using the FDSS6000 detection system in the above conditions.

Kinetic Profile of calcium release

An agonist measurement was performed according to the conditions described above. A typical set of kinetic records is presented in Fig. 4 (8 points concentration range of the agonist).

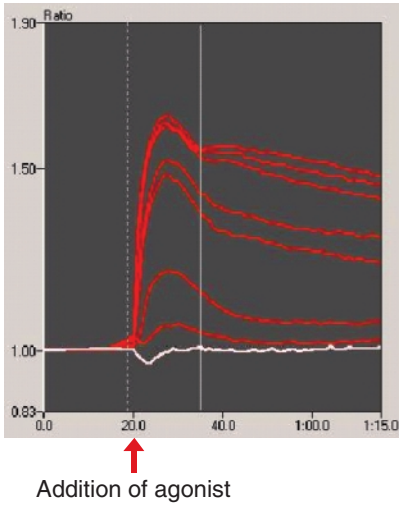


Fig. 4: Kinetic profile of the activation of CHO-aR by a dose-response of the reference agonist. Total record time is 120 s.

Pipeting conditions (I) induced no injection artifact, a pattern observed with both aminergic and peptidergic receptor expressing cell lines.

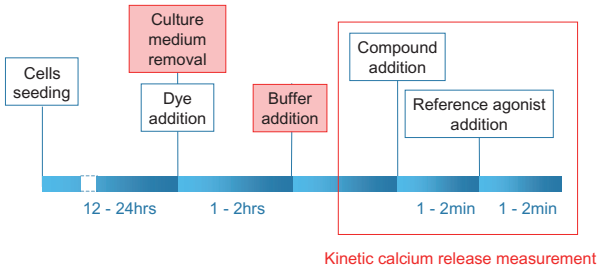


Fig. 5: General experimental procedure.

Dye loading: incubation temperature and time

Methods

Dye loading mode: Cells in 40 µL culture medium, 20 µL removed before addition of 20 µL dye solution

Sample addition: 20 µL

Z' calculation: Intraplate, 384 format, 192 points with buffer, 192 points with agonist @ [c] = 10 x EC50

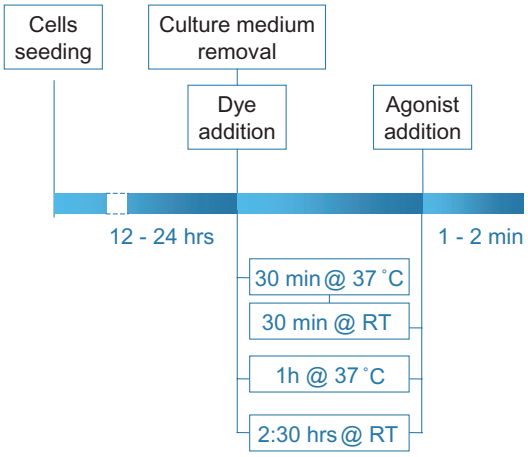


Fig. 6: Dye loading temperature experimental procedure.

Results

One of the recommended dye loading conditions is to incubate the cells at 37 °C for 30 min, followed by an incubation at room temperature for an additional 30 min. In this study, we show that with the peptidergic receptor (CHO-pR), incubation of the cells for 1h at 37 °C yields a better reproduceability of the assay, as seen with the z' determination (Fig. 7). The aminergic receptor (CHO-aR) shows a similar but more moderate difference. Interestingly, these results are not directly dependent on the efficiency of dye loading, since the aminergic receptor shows a greater increase in fluorescence ratio after dye loading at 37 °C, as compared with the peptidergic receptor (Fig. 8).

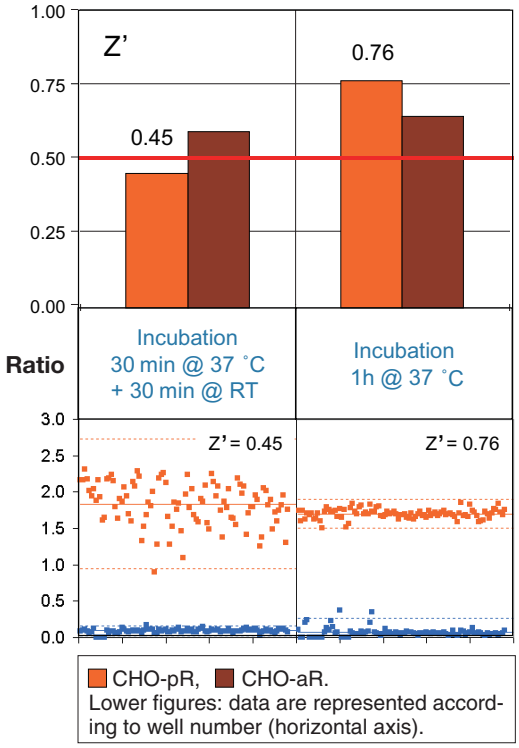


Fig. 7: z' factor obtained in agonist mode after dye loading at different temperatures.

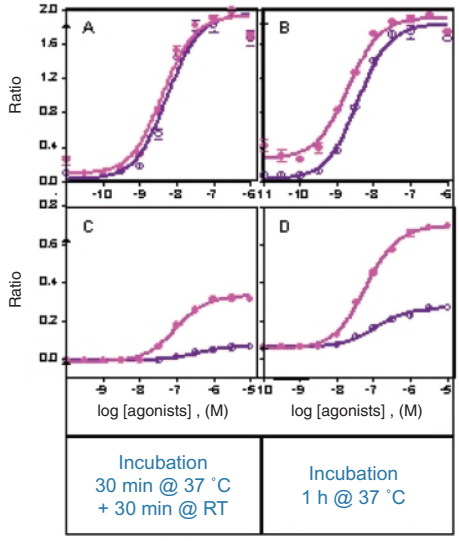
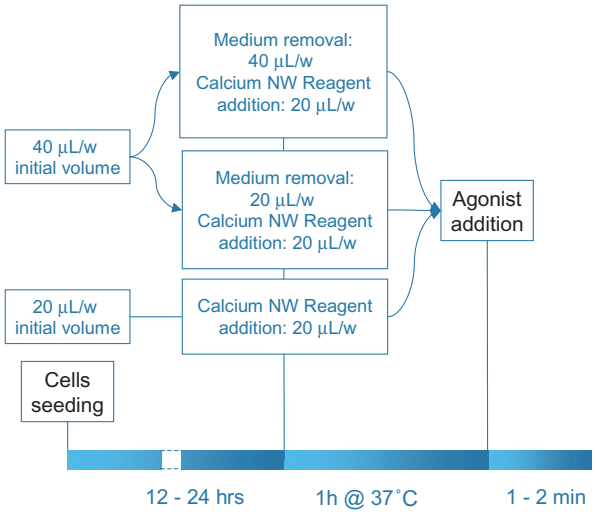


Fig. 8: Pharmacological study of agonists on cells expressing a peptidergic receptor (CHO-pR, panels A and B) or an aminergic receptor (CHO-aR, panels C and D). Figures represent the effect of two agonists for each receptor (light and dark pink). Note that one of the compounds for CHO-aR is a partial agonist (dark pink). Experiments were conducted in triplicates. Error bars represent SEM values.

Dye loading: with or without medium removal

Methods

Dye incubation: 1 h@37 °C
Sample addition: 20 µL
Z' calculation: Intraplate, 384 format, 192 points with buffer, 192 points with agonist @ [c] = 10 x EC50



Results

HTS assay development generally consists in reducing a protocol to its vital steps, hence the tendency to reduce washing steps in heterogeneous formats. The purpose is both to simplify robotics, and to avoid pipeting above the cells, an operation sometimes deleterious to the cell monolayer, and hence to the quality of the data. The Calcium No Wash assay kit was evaluated here for its performance when used in the presence of culture medium, which is usually withdrawn by a pipeting step.

Interestingly, results in Fig. 9 show no change in z' values when cells are cultured in half the volume of medium before direct addition of Calcium No Wash reagent (third panel) as compared with standard conditions with complete medium removal (first panel). On the other hand, a protocol consisting in removing half of the medium (Fig. 9, second panel) does not seem to compromise run on between the deleterious effect of the pipeting step above the cells, and the decrease in signal due to the remaining medium in the assay solution, which can be observed on the concentration range of reference compounds in Fig. 10.

In conclusion, the Calcium No Wash assay kit can be used in a no wash procedure both for the delivery of the reagent itself, and for the culture medium, which showed here on our two model cell lines that it had no impact on data quality.

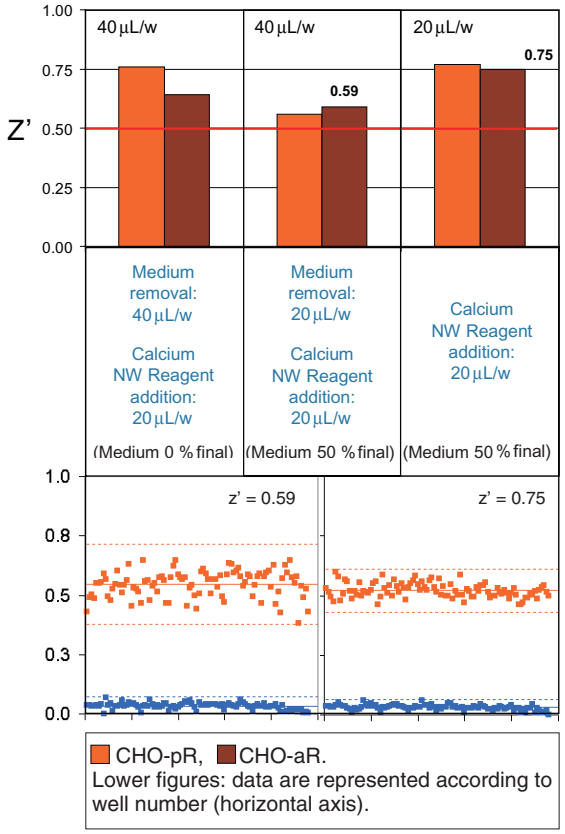


Fig. 9: z' factor obtained in agonist mode after dye loading at different temperatures.

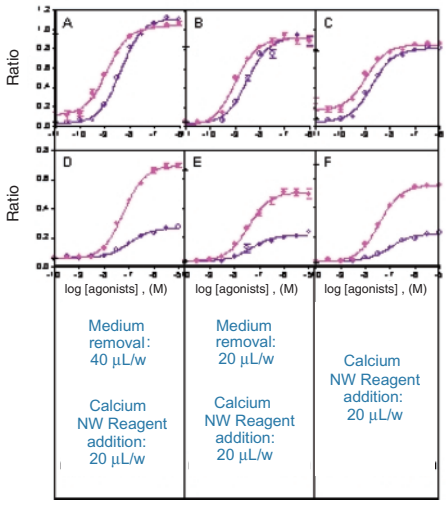


Fig. 10: Pharmacological study of agonists on cells expressing a peptidergic receptor (CHO-pR, panels A to C) or an aminergic receptor (CHO-aR, panels D to F). Figures represent the effect of two agonists for each receptor (light and dark pink). Note that one of the compounds for CHO-aR is a partial agonist (dark pink). Experiments were conducted in triplicates. Error bars represent SEM values.

Cell density

Methods

Dye incubation: 1 h@37 °C
Dye loading: 20 µL on 20 µL medium
Sample addition: 20 µL
Z' calculation: Intraplate, 384 format, 192 points with buffer, 192 points with agonist @ [c] = 10 x EC50

Results

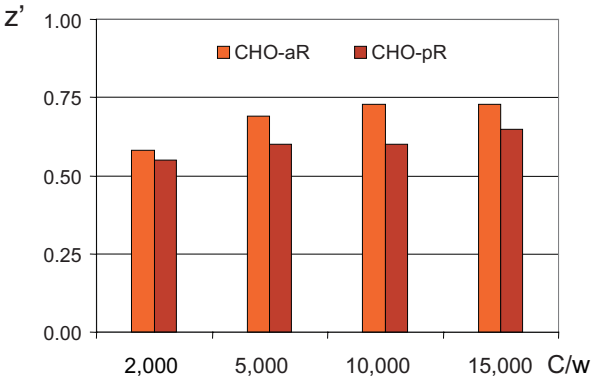


Fig. 11: z' factor obtained in agonist mode after cell seeding at different densities.

Cell densities of 2000-5000 cells per well are com-patible with quality HTS, the latter conditions yielding slightly better reproducibility due to an increase in the intensity of fluorescence (data not shown).

Additional results

In addition to the data presented here several other experiments allowed further evaluation of the DiscoveRx Calcium No Wash assay kit.

The reagent was evaluated in antagonist measure-ments using the CHO-aR and CHO-pR cell lines. Taken together the results correlated well with those presented above with the measurements in the agonist mode.

Additional dye loading conditions were investigated, including increased incubation times at room temperature. With the two cell lines used here, dye loading time of 2:30 hrs at room temperature proved to be as efficient as 1h at 37 °C.

Conclusion

DiscoveRx No Wash Calcium Dye was for its compatibility with FDSS6000 for HTS.

- The dye displayed no or a few injection artefacts
- The dye yielded good reproducibility of basal and maximal values in HTS conditions
- The dye sensitivity allows seeding at 2000-5000 C/w (384w)

Problems in a wash protocol include:

- During washing, cells can be removed from the plates
- Spontaneous Calcium flux in the negative control cells after buffer addition
- Any incomplete washing might result in a significant drop in signal upon addition of test compound
- Washer dependent variations in residual volumes added

Advantages of DiscoveRx Calcium No Wash Kit Compared to the other no wash reagents

- Better dynamic range
- Reduced well-to-well variation
- Rapid protocol with less hands on time than a usual wash protocol, this leads to a higher throughput
- Easy to use with adherent and non-adherent cells
- Reduced spontaneous calcium flux and minimal perturbation of the cells
- Wide range of applications for GPCR targets and Cachannels

Troubleshooting

1. Fluorescence drop after

Usually lowering the dispensing speed solves the problem.

Adding volumes greater than recommended may increase the initial decrease in fluorescence. In order to solve this point, it might be necessary to adjust the volume of the compound solution. We typically recommend to use a ratio of 1:4 or 1:5 between the initial volume in the well and the volume added. In these conditions, the sample added should mix by itself because of the spontaneous turbulences due to pipeting. For instance, in a 96-well plate add 15-25 μL in an initial volume of 75 μL, or in a 384-well format add 5-6 μL into 25 μL.

Note:
If the final concentration of the loading buffer is decreased it may result in a decreased response of the assay. If you do just one addition, please add the recommended volume.

2. Serum-sensitive cells

In some cases, cells are sensitive to serum. As mentioned in the DiscoveRx protocol, remove the culture medium to avoid this problem. If you cannot, try to work at low serum concentration for overnight incubation after cell seeding (eg. use 1 % FCS instead of 10 % FCS).

3. Concerns with DMSO

The plate controls should always contain the same final concentration of DMSO than the concentration in the wells dedicated to assay compounds. This DMSO concentration, however, can induce a calcium flux. If so, use loading buffer adjusted for the same DMSO concentration as what is present in the sample volume added during the experiment. In any case, it is not recommended to use more than 1 % DMSO final. You can reduce the effect of calcium flux using the option call "plate shaking" on the FDSS 6000 (II)

4. Smaller response than

This could be due to sticky compounds, which will stick to the tips or trays. In this case use 1 % BSA in all compound buffer diluents. In addition, a pre-soak of the tips in buffer with 1 % BSA or triturated with compound plate first might help.

Authors

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References

I) Pipeting conditions : Volume: 20 μl/ Aspirate: 10 μl/s - High : 0 mm/
Dispense: 15 μl/s -High : 2 mm
II) FDSS Application Note_No3 : Effect of Plate Mixing, Fluid Addition Height and speed on Reducing Addition Artifacts and Negative Control Drift Using the FDSS6000.

Consumables

Calcium No Wash (CaNW) Assay Kit,Order number : 90-0080L (20 plates) and 90-0080XL (100 plates)

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