

# Application Notes

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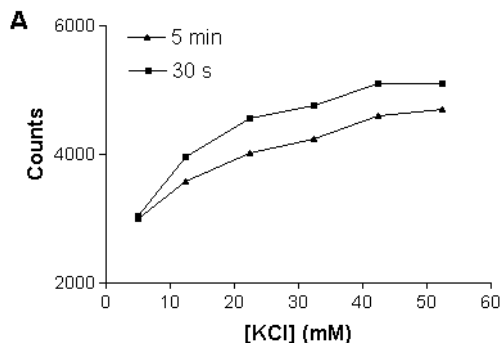
Calcium measurements on  
Wallac VICTOR<sup>2</sup> plate readers

## Calcium measurements on Wallac VICTOR<sup>2</sup> plate readers

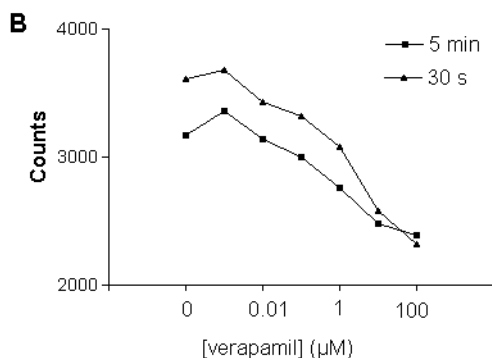
The development of fluorescent Ca<sup>2+</sup> indicators has revolutionized the determination of intracellular Ca<sup>2+</sup> levels in living cells. In the simplest of cases, Ca<sup>2+</sup> signals can be divided into two distinct types of events. One mechanism is influx from the extracellular space via ion channels, typically voltage-gated Ca<sup>2+</sup> channels (VGCC) or Ca<sup>2+</sup>-permeable ionotropic receptors such as Kainate/AMPA or NMDA-type glutamatergic receptors. Another mechanism is activation of G-protein coupled receptors (GPCR) coupled to inositol-phospholipid breakdown, leading both to release from intracellular Ca<sup>2+</sup> stores and influx through Ca<sup>2+</sup> channels in the plasma membrane. The kinetics of these events can be very different. In the case of Ca<sup>2+</sup> influx through ion channels, such as the NMDAR or L-type VGCC, cytosolic Ca<sup>2+</sup> increases over tens of seconds to reach a steady-state plateau, which often persists for minutes. In the case of GPCR-mediated Ca<sup>2+</sup> signalling, Ca<sup>2+</sup> levels peak seconds after activation and decay in tens of seconds. This poses different demands on the measuring equipment used to assay these events, i.e. end-point or kinetic measuring capability. In the present study we wanted to test the

performance of Wallac 1420 VICTOR<sup>2</sup> multilabel plate readers in Ca<sup>2+</sup> assays in microplates for two typical applications: activation of L-type Ca<sup>2+</sup> channels by K<sup>+</sup>-induced depolarization and activation of P2<sub>Y</sub> purinergic GPCRs by ATP. We also tested the performance of the Wallac VICTOR<sup>2</sup>V™ plate reader in multiwavelength measurements of intracellular Ca<sup>2+</sup>.

- Whole plate end-point Ca<sup>2+</sup> assays for VGCC or ionotropic receptors can be performed on any VICTOR<sup>2</sup> instrument.
- Kinetic Ca<sup>2+</sup> assays for GPCRs in 96-well format can be performed on VICTOR<sup>2</sup> or VICTOR<sup>2</sup> V instruments equipped with an injector.
- Multiwavelength and/or ratiometric Ca<sup>2+</sup> assays can be performed on the VICTOR<sup>2</sup>V plate reader



**Fig. 1A.** RIN insulinoma cells cultured in 96-well plates and loaded with Fluo-4, were treated with increasing concentrations of KCl. The plate was measured immediately after addition (30 s) and after 5 min. The excitation/emission filter pair 485/535 nm was used with 0.1 s measuring time per well.



**In fig. 1B** the cells were first treated with increasing concentrations of the VGCC blocker Verapamil. After incubating with the antagonist for 5 min, 50 mM KCl was added to the plate and the fluorescence of individual wells was measured after 30 s and 5 minutes. The excitation/emission filter pair 485/535 nm was used with 0.1 s measuring time per well.

### Materials and methods

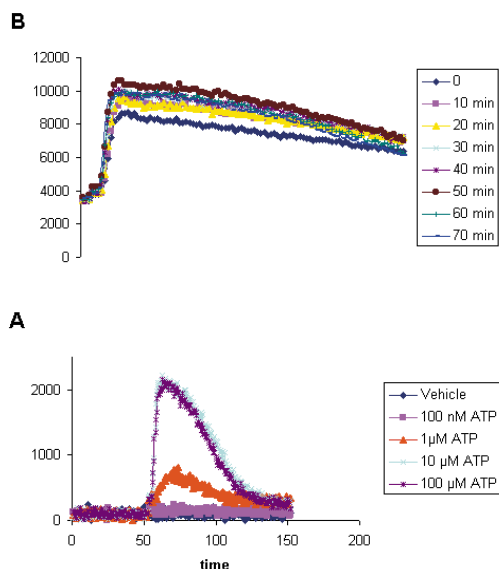
Cells were cultured in RPMI-1640 (Gibco) medium supplemented with 10 % FBS (Gibco) and penicillin/streptomycin (Gibco), plated at a density of 100 000 (RIN) and 50 000 (CHO) cells per well in Costar white 96-well plates, and used 48 hours after plating. Prior to experiments, cells were loaded in the culture medium above containing

2.5 mM probenecid, 4.5 µM Fluo-4 AM, Fura-2 AM (Molecular Probes) and 0.01 % pluronic acid (Molecular Probes) at 37°C in a CO<sub>2</sub> incubator for 30-40 min. Probenecid is an inhibitor of the multidrug resistance transporter, which otherwise will transport loaded dye out of the present cell types. Plates were washed three times with HBSS (Hank's basal saline solution, Gibco) containing 20 mM HEPES (Sigma), 1 mM Ca<sup>2+</sup>, 1 mM Mg<sup>2+</sup> and 2.5 mM probenecid. The same medium was used in all experiments and to dilute test compounds. ATP, Probenecid, Suramin and Verapamil were purchased from Sigma.

### End-point calcium assays

RIN rat insulinoma cells which endogenously express L-type VGCC were used in these experiments on a VICTOR2 instrument without injectors. We wanted to determine whether one can measure slow influx type Ca<sup>2+</sup> responses as an "end-point" assay. All additions to the assay plate were made outside of the instrument, which mean that the minimum time to acquire the first data point after challenging the cells is 30 s. When measuring a whole plate, the key question is for how long the signal persists over time. The data in fig. 1A show that the dose-response relationship to KCl persists for 5 minutes, with only a slight decrease in total signal span.

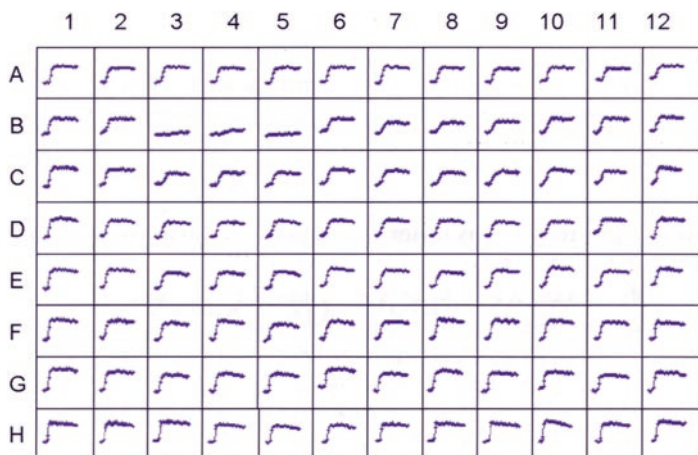
The dose-response relationships of the L-type VGCC blocker Verapamil, shown in fig. 1B, demonstrate that they remain similar from 30 s to 5 min. Note that the data shown in the figures are raw data from one single well per data point, with no well normalization or multiwell averaging etc. Test substances and activator (ligand) can all be added outside of the instrument, manually or using a liquid handling system. The plate can easily be read before each addition, in order to monitor non-specific effects of effectors.



**Fig 2.** Kinetic fluorescence measurements in CHO cells cultured in 96-well plates loaded with Fluo-4 plotted as counts over time. In fig. 2A the cells were challenged with increasing concentrations of the P<sub>2</sub><sub>Y</sub> receptor-agonist, ATP. At 50 s, the agonist (50  $\mu$ l) was added via the injector to individual wells each containing 150  $\mu$ l of buffer, and the fluorescence signal was monitored for a further 100 s. An upward deflection of the curve represents an increase in the intracellular Ca<sup>2+</sup> concentration. The excitation/emission filter pair 485/535 nm was used in the experiments, with 0.1 s measuring time per data point and 2 measurements/s. The injector speed was set at 5. In fig. 2B the wells were challenged with 100  $\mu$ M ATP at different time points to test the stability of the response in a loaded plate over a prolonged period of time.

### Assays on GPCRs with fast kinetics

In experiments on CHO cells, which endogenously express a P<sub>2</sub><sub>Y</sub> purinergic receptor, the kinetics of the Ca<sup>2+</sup> response is faster. We therefore used a VICTOR<sup>2</sup> V HTS instrument with an injector for these experiments. A VICTOR<sup>2</sup> with injector would work just as well for this study. Fig. 2A shows a dose-response relationship to the ligand ATP added via the injector. Note that the curves shown represent data readout from one well, with no normalization or averaging. In the experiments with the injector, the whole time-course of the event is determined one well at a time. For screening purposes it is important to test whether the cells show a similar response to the ligand over the time the plate is read. Fig. 2 B demonstrates that the response to ATP remains similar over 70 minutes in the same plate, i.e. considerably longer than the reading time for a plate. This enables experiments such as the whole-plate screening experiment shown in fig. 3. The baseline level was 3950 (SD  $\pm$  240) counts. Control wells responded with a signal of 8400 (SD  $\pm$  510) and the three wells treated with 500  $\mu$ M of the P<sub>2</sub><sub>Y</sub> antagonist Suramin with 4020 (SD  $\pm$  360) counts (98 % inhibition). The total reading time per well (16 s in the present experiment) can be optimized further depending on the kinetics of the particular response.



**Fig. 3.** Kinetic fluorescence measurements as in fig. 2 for a whole 96-well plate plotted as counts over time. 100  $\mu$ M ATP was added to all wells as a volume of 50  $\mu$ l using the injector. In wells B3 – B5 500  $\mu$ M of the antagonist Suramin was added prior to experiment, and the Suramin titration series was continued down in columns 3-5: wells C3-C5 had 50  $\mu$ M, wells D3-D5 5  $\mu$ M etc. down to wells G3-G5 with 5nM. To wells H3-H5 vehicle buffer was added. A 0.1 s measuring time per data point gave approximately 2 measurements/s, and the wish to monitor for 16 s (30 repeats) per well resulted in a total reading time of 26 min per plate. The fluorescence was measured using the filter pair 485/535 nm.

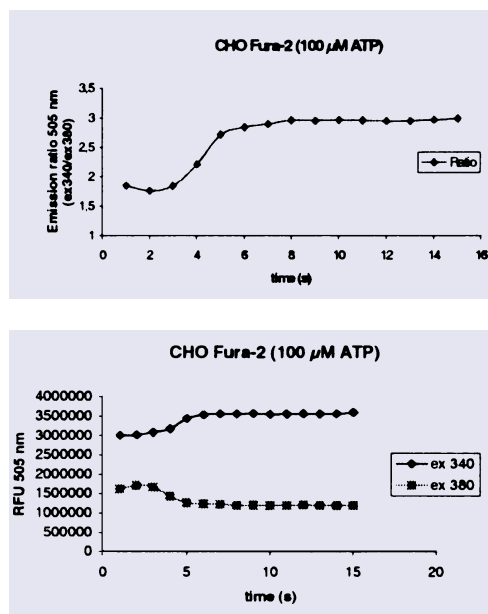


Fig. 4. CHO cells cultured in clear bottom white 96 well plates and loaded with 4  $\mu$ M Fura-2 AM at 37 $^{\circ}$ C for 40 min. The graph on the left shows raw data from one well of a 96 well plate measuring fluorescence emission at 510 nm with excitation alternating between 340 and 380 nm every 0.5 s on the VICTOR<sup>2</sup>V<sup>TM</sup>. The effector, ATP, was added through the VICTOR integral injector. An increase in intracellular Ca<sup>2+</sup> is reflected by a fluorescence increase when exciting at 340 nm and a corresponding decrease with excitation at 380 nm. Data from these dual wavelength dyes are usually presented as a ratio of the two excitation wavelengths, as seen on the right. The ratio is independent of both intracellular dye concentration and the number of cells in the well, and can be calibrated to units of intracellular Ca<sup>2+</sup> activity, enabling real comparison between different experiments, cell types etc. The fast filter changer capability of the VICTOR<sup>2</sup>V<sup>TM</sup> enables the use of several dyes monitoring different cellular functions for each experiment.

### Multiwavelength cellular assays

A limitation of cell-based fluorescent assays in microplates today is the necessity of using single wavelength light sources. In many cases much more information could be gathered through the use of ratiometric indicators or indeed multiple probes in one experiment. The new VICTOR<sup>2</sup>V<sup>TM</sup> series does multi wavelength and/or ratiometric Ca<sup>2+</sup> analysis with sub second time resolution and integral addition of effectors.

### Products Available

Wallac 1420 VICTOR<sup>2</sup>

Wallac 1420 VICTOR<sup>2</sup> V, including all five leading measurement technologies

Wallac 1420-251 injector unit



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