Review TRENDS in Pharmacological Sciences Vol.26 No.4 April 2005

Techniques: High-throughput measurement of intracellular Ca^{2+} – back to basics

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High-throughput screening techniques continue to provide important tools to the pharmaceutical industry for the efficient identification of drug leads. However, high-throughput techniques are now being exploited to address a variety of pharmacological and cellular signaling research questions, including the regulation and role of intracellular Ca^{2+} in a plethora of biological systems. Although an awareness of specific assay conditions is crucial for reliable and reproducible measurements of intracellular free Ca^{2+} whichever system of study is used, the complex temporal nature of Ca^{2+} signals has posed some unique limitations for its measurement in high-throughput mode. Progress in high-throughput design has overcome many of these limitations and will complement other technical approaches to understanding the underlying regulation and role of intracellular Ca^{2+}.

The Ca^{2+} signal: a target for high-throughput technologies

The process of drug discovery and biomedical research has been revolutionized by high-throughput technologies, including DNA microarrays [1], proteomics [2] and high-throughput X-ray crystallography [3]. In drug discovery, high-throughput is required to cope with the vast numbers of compounds generated by techniques such as combinatorial chemistry [4], and to compress the time needed for data collection and data analysis.

Pharmaceutical companies have adopted the measurement of intracellular Ca^{2+} for high-throughput screening [5]. In this article, we will discuss the recent developments in high-throughput technologies that make measurement of intracellular Ca^{2+} more attractive for basic research.

Challenges and opportunities for high-throughput measurement of the Ca^{2+} signal

Why does Ca^{2+} matter?

Ca^{2+} is an essential regulator of a variety of biological processes [6]. Much of the ability of the Ca^{2+} signal to control a diverse array of pathways is due to tight temporal control of the amplitude and location of free Ca^{2+} levels within the cell. Changes in Ca^{2+} homeostasis have been reported in a variety of diseases, including hypertension and heart failure [7], and mutations in Ca^{2+} transporters cause human genetic disorders such as Hailey–Hailey disease and Darier disease [8,9]. Using high-throughput methodology to measure the Ca^{2+} ion should rapidly advance our understanding of Ca^{2+} homeostasis and Ca^{2+}-regulated pathways. Applications in pharmacological research are listed in Table 1.

The nature of the Ca^{2+} signal

Signaling events that remain sustained for extended periods and can be fixed or detected by gene reporter assays [10,11] do not require high temporal resolution. However, this is not the case for the regulation of intracellular Ca^{2+} in living cells. The complex temporal nature of the Ca^{2+} signal means that there is a crucial need for high temporal resolution measurements, starting in the sub-second range. Whether the Ca^{2+} signal is activated through a G-protein-coupled receptor (GPCR) or membrane depolarization, the measured intracellular Ca^{2+} level is a reflection of the Ca^{2+} homeostatic processes in operation at that particular time. For example, following the activation of GPCRs, the observed changes in intracellular Ca^{2+} are determined by: (i) the release of Ca^{2+} from sarco/endoplasmic reticulum stores by inositol-(1,4,5)-trisphosphate [Ins(1,4,5)P_3]; (ii) the enhanced entry of Ca^{2+} across the plasma membrane; and (iii) mechanisms to extrude and re-sequester Ca^{2+} after GPCR activation [12]. It is now technically feasible to use high-throughput techniques to dissect the observed Ca^{2+} signal and investigate specific effects on Ca^{2+} release and Ca^{2+} entry mechanisms (e.g. [13]). Importantly, this high-throughput approach is only feasible if

Table 1. Non-drug-screening pharmacology studies that use high-throughput assessment of intracellular Ca^{2+}[a]

Description	Refs
Receptor pharmacology characterization and functional screening of stably transfected clonal cell lines	[37–39]
Ca^{2+} homeostasis and signal transduction mechanisms	[13,36,40–42]
Ca^{2+} channel pharmacology	[43–45]
'De-orphanizing' and study of orphan G-protein-coupled receptors	[46–48]

[a]See [24–26,49,50] for alternative high-throughput methods.

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Available online 2 March 2005

 0165-6147/$ - see front matter doi:10.1016/j.tips.2005.02.002

the full dynamics of the Ca^{2+} response can be recorded with sufficient temporal resolution.

Measuring Ca^{2+} ions

Since their introduction, Ca^{2+}-sensitive fluorescent dyes [14] have been used mostly in cuvette- or microscope-based applications. In all cases the basic technical approach is the same: there is a light source to excite the Ca^{2+} indicator, a sample chamber to hold the cells of interest, and a detector to monitor the emission fluorescence of the indicator. A chief influence on Ca^{2+} measurement is the nature of the sample compartment. Cuvette-based systems enable an averaged Ca^{2+} signal to be collected from a large population of cells. Microscope-based systems enable the Ca^{2+} signal to be studied in individual cells or in groups of cells and, with perfusion of the bathing solution, there is often more control over the conditions under which the cells are studied. High-throughput technologies based around microplate readers offer a sample chamber that can be considered a compromise between cuvette- and microscope-based approaches. For example, one might consider a 96-well microplate as equivalent to 96 cuvettes.

The challenge for microplate readers and Ca^{2+} measurements

There are many challenges for performing detailed Ca^{2+} signaling studies with microplate readers in high-throughput mode: (i) microplate readers need to accommodate many different Ca^{2+}-sensitive fluorescence probes, which can vary widely in their affinities for Ca^{2+} and their spectral properties [15]; (ii) the nature of the Ca^{2+} signal means that an extensive time-course has to be recorded with sufficient temporal resolution; and (iii) it must be possible to make modifications to the cell environment during data acquisition. For high-throughput assessment of the Ca^{2+} signal, all of these factors must be addressed simultaneously in all wells of the microplate.

High-throughput assessment of intracellular Ca^{2+} – the breakthrough

Many of the early microplate readers accomplished the challenges laid out above. Most were able to excite and collect fluorescence signals over a wide spectral range, and some were able to make limited solution additions during data acquisition. However, their major problem was that these measurements could only be made on one well at a time. Practically, this presents many experimental problems, and cannot be considered high-throughput for most Ca^{2+} studies. For example, the measurement, in 96 microplate wells, of a Ca^{2+} transient lasting 30 s would take 48 min from the first measurement to the last measurement. Such an extended period is unfavorable for measuring intracellular Ca^{2+} because problems could arise as a result of time-dependent events such as Ca^{2+} probe sequestration and leakage, or a general decrease in cell viability. All such factors can interfere with the measurement of cytosolic free Ca^{2+} and invalidate comparison of responses between wells [15,16]. Thus, single-well measurement microplate readers can only be regarded as a medium-throughput device for Ca^{2+} measurements.

The breakthrough in the 'marrying' of high-throughput technology with Ca^{2+} signaling studies occurred following two key developments not featured on previous fluorescence microplate readers: (i) simultaneous illumination and detection of fluorescence in all wells; and (ii) addition of multiple separate test solutions simultaneously in all wells by means of robotic devices. The first high-throughput screening instrument for Ca^{2+} was termed a fluorescence imaging plate reader (FLIPR®) and could simultaneously acquire an image of an entire microplate. However, image resolution was insufficient to resolve subcellular compartments or even individual cells; thus, the term 'imaging' in this context is different from that associated with fluorescence digital imaging of intracellular free Ca^{2+} [17]. The high-throughput approach using FLIPR® enables the simultaneous measurement of Ca^{2+} signals in microplates with 96- (Figure 1), 384- and, more recently, 1536-well formats [18] in the time normally taken to complete one measurement using previous techniques. We consider the 96-well format optimal for complex Ca^{2+} studies when solution preparation and data analysis are taken into account. Figure 2 illustrates the application of high-throughput technology with the parallel characterization of the biphasic intracellular Ca^{2+} signal initiated either by receptor activation [with acetyl-methacholine (MeCh)] or the sarco/endoplasmic Ca^{2+}-ATPase (SERCA) pump inhibitor thapsigargin (TG). By varying the concentration of extracellular Ca^{2+}, one can compare and contrast the pharmacological characteristics of the Ca^{2+}-entry process activated by MeCh and TG while, at the same time, performing crucial controls in parallel. In this case, the controls monitor the effect of different extracellular Ca^{2+} concentrations in the absence of cell activation. With the dataset of Figure 2 as an example, Table 2 summarizes the strengths and weaknesses of cuvette-, microscope- and microplate-based Ca^{2+} measurements.

Limitations for basic research applications and how they are being overcome

Apart from cost, some aspects of fluorescence plate reader design impact on the Ca^{2+} signaling study approach, and remain a focus for improvement.

Choice of Ca^{2+} indicator

In general, there are two approaches for measuring Ca^{2+} indicators: single wavelength detection and 'ratiometric' detection.

The first widely used high-throughput platform for Ca^{2+} measurements (FLIPR®) used single wavelength excitation in the visible range. This limited wavelength selection, confined the choice of Ca^{2+} indicator to those with laser excitation wavelengths of 488 nm, including Fluo-3 and Fluo-4 [15]. In general, a disadvantage of using single wavelength measurements is that it presents the problem of confusing Ca^{2+}-dependent changes with signal artifacts that have nothing to do with the Ca^{2+} ion. However, high-throughput platforms can contend with this problem because of the ability to perform multiple

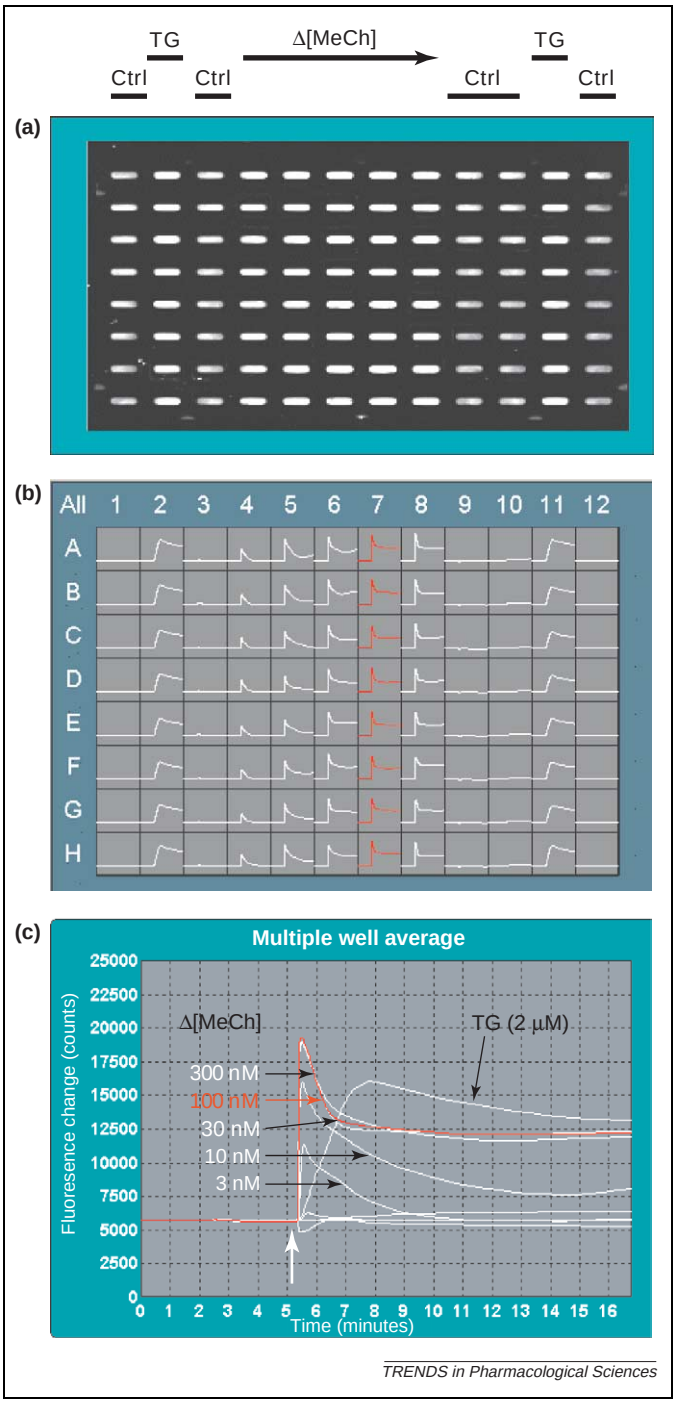

Figure 1. Example of data output of a Ca^{2+} flux assay using CHO-M1 cells in a 96-well plate format. CHO-M1 cells (Chinese hamster ovary cells overexpressing the acetylcholine muscarinic M_1 receptor) were loaded with the visible wavelength indicator for Ca^{2+} Fluo-4 and fluorescence was detected using a fluorometric imaging plate reader [FLIPR384; Molecular Devices (http://www.moleculardevices.com)]. Cells were treated [added at arrow (c)] with the sarco/endoplasmic Ca^{2+}-ATPase inhibitor thapsigargin (TG: 2 μM) and different concentrations of the G-protein-coupled receptor (GPCR) agonist acetyl-methacholine (MeCh: 3, 10, 30, 100, 300 nM), all in the presence of 1.8 mM extracellular Ca^{2+}. [Control (Ctrl) indicates addition of buffer only.] **(a)** A fluorescence image captured by the CCD camera at a single time point is shown. In this image, a 'mask' is used between the microplate and camera to enhance the separation of signals between wells (hence the slit appearance). Only information from these discrete areas of the CCD camera chip are recorded and represent fluorescence intensity information for each well. **(b)** A time-course plot of fluorescence intensity changes for each well after they have been corrected for dye loading variability and solution addition artifacts is shown. Red lines indicate data for 100 nM MeCh. **(c)** Average traces for each condition are overlaid and displayed.

simultaneous Ca^{2+} assessments under identical conditions on the same microplate [19]. This means reduced variance from differences in Ca^{2+} probe loading and plating density, and the ability to incorporate and observe suitable experimental controls in parallel (e.g. Figure 2). There are also several software corrections that can be employed to take into consideration fluorescence signal variations that arise from solution additions and variations in dye loading.

The problems experienced by single wavelength indicators are significantly minimized by 'ratiometric' Ca^{2+} indicators, a capability made possible by extending wavelength selection of high-throughput platforms into the UV range [20,21]. Ratiometric dyes such as Fura-2 are often preferred by researchers because they have reduced sensitivity to signal artifacts such as differences in the concentration of intracellular probe, cell thickness and number [15,17], and they enable more accurate calibration.

The development of Ca^{2+}-sensitive fluorescent proteins has presented new opportunities for measuring Ca^{2+} ions particularly in subcellular domains [22] using fluorescence energy transfer (FRET) [23]. Indeed, FRET has been used successfully on high-throughput platforms for the ratiometric assessment of membrane potential [24]. Luminescence detection of Ca^{2+} signals is also suited to high-throughput protocols [25,26].

The high-throughput platforms summarized in Table 3 represent the latest instruments that provide great flexibility in Ca^{2+} measurement. All provide multiple wavelength selections for both excitation and emission, enabling single wavelength, ratiometric and FRET-based methodology to be considered. Extending the range and number of wavelength selections is also advantageous to measuring multiple fluorescent probes simultaneously (where spectra are not significantly overlapping). For example, it could be possible to assess simultaneous intracellular free Ca^{2+} and Na^{+} [27] or pH [28] and potentially Ca^{2+} in different compartments [29,30].

In summary, high-throughput platforms capable of assessing multiple fluorophores offer great flexibility to pharmacology research laboratories seeking to resolve the complexity of cellular signaling and the inter-relationships between pathways.

No-wash Ca^{2+} dyes

For convenience and simplification of high-throughput protocols, 'no-wash' Ca^{2+} indicators were formulated to incorporate extracellular fluorescence quenchers [31,32] and avoid the need to wash away extracellular Ca^{2+} indicators after loading. No-wash protocols are of great use in drug screening where a vast number of compounds are screened daily using high-density microplates (1536 wells). Although the use of no-wash Ca^{2+} dyes could be desirable in a basic research setting, it is still feasible to use 'wash-away' Ca^{2+} dyes with 96-well microplates and most research applications would not require ultra-high-throughout techniques involving 384- or 1536-well plates [13]. Importantly, use of a no-wash dye would be redundant when different experimental conditions have to be set before the experiment can begin.

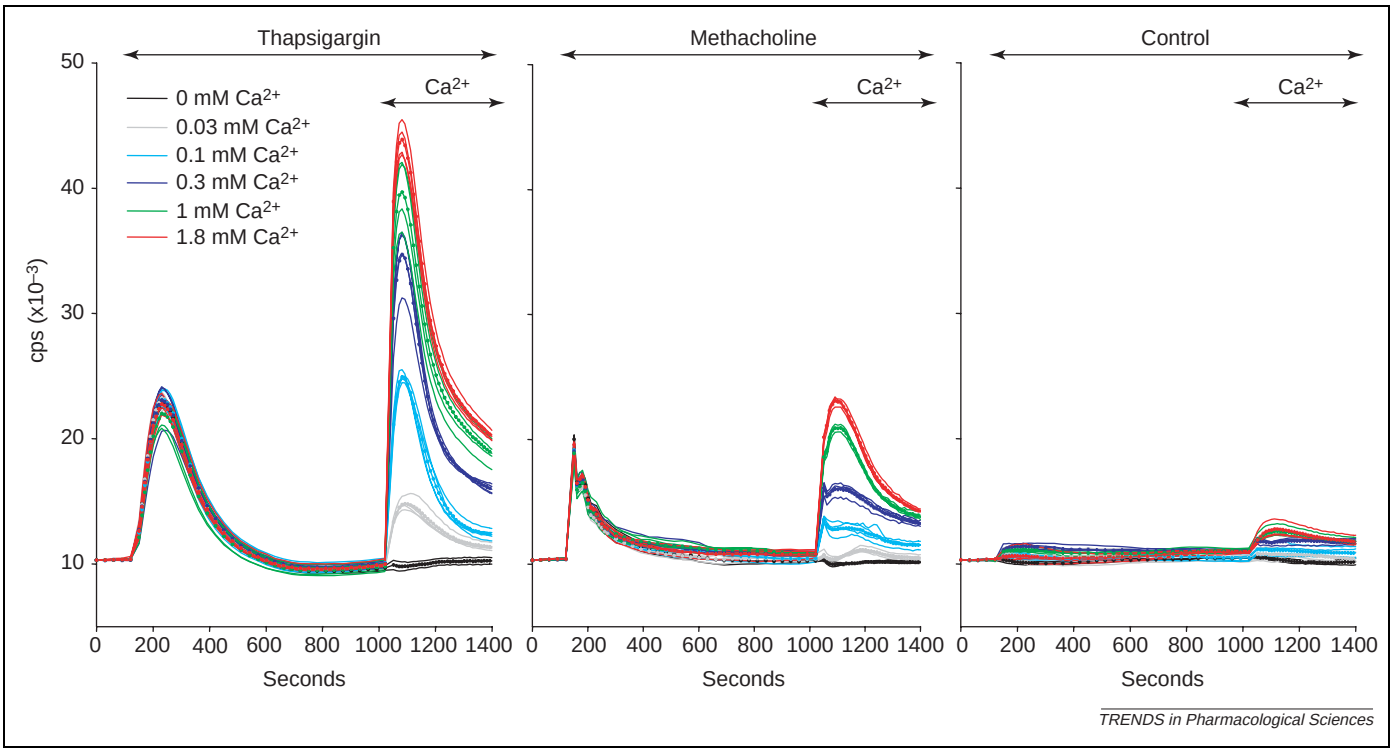

Figure 2. Example of the use of high-throughput technology to characterize the Ca^{2+}-entry process in HEK293 cells. Ca^{2+} measurements were performed on Fluo-4-loaded HEK293 cells with a FLIPR384. Cells were treated with the sarco/endoplasmic Ca^{2+}-ATPase inhibitor thapsigargin (TG: 2 μM), the G-protein-coupled receptor (GPCR) agonist acetyl-methacholine (MeCh: 300 μM) or control solution in Ca^{2+}-free buffer, followed by the addition of various concentrations of extracellular Ca^{2+}. All data were acquired simultaneously from 48 wells on one 96-well microplate (half the plate capacity) with replicates and averages (symbols) for each condition shown (triplicate for TG and MeCh, duplicate for control). The total duration of the experiment (dye loading plus Ca^{2+} measurement) was ~70 min. Accumulating a similar dataset using a conventional fluorescence microscope-based imaging or another low-throughput technique would probably take 56 h (48×70 min), effectively seven 8-h days. Abbreviation: cps, counts per second.

It should be noted that the formulation of 'no-wash dyes' are proprietary, and the identity of all constituents are not identified. Under these conditions, it is our opinion that the use of such 'no wash' dyes be avoided because the identity and pharmacological properties of the components are unknown, and might have the potential to alter pathways involved in Ca^{2+} signaling or other biological processes.

Table 2. Comparison of possible methods for the measurement of intracellular free Ca^{2+} in adherent cell lines

Parameter	Cuvette	Microscope	Microplate	
			Single-well analysis	Multi-well analysis
Analysis	1 cuvette (population of cells)	1 field (single cell or population of cells)	1 well (population of cells)	96, 384 1536 wells (population of cells)
Cell preparation	Suspension (most often)	Adherent (most often)	Adherent or suspension	Adherent or suspension
Automation options	Restricted	Limited	Moderate	Advanced
Throughput	Low	Low	Moderate	High
Potential advantages	High temporal resolution	High temporal and spatial resolution; perfusion enables rapid changes in the extracellular environment and removal of agonists	High temporal resolution; automation	High temporal resolution; automation; test solution applications and data analysis in parallel
Disadvantages	Limited ability to remove reagents during testing; time-consuming	Time-consuming	Limited or no ability to remove reagents during testing; delay between first and last well measurements compromises comparisons when many wells are assessed	Limited or no ability to remove reagents during testing
Approximate time (including loading and additional tasks) to perform 25-min experiment shown in Figure 2 (assuming 96 measurements)	112 h (14 working days)	112 h (14 working days)	41 h	1.2 h

Table 3. Fully automated microplate readers capable of simultaneous (≥96) well assessment and flexible wavelength selection

Model	Manufacturer	URL	Notes
FLIPR^{TETRA}	Molecular Devices	http://www.moleculardevices.com	96-, 384- and 1536-well reading and additions; light emitting diode (LED) excitation source; three reagent plates for addition of pharmacological agents
Cell Lux	Perkin Elmer	http://www.perkinelmer.com	96 wells, xenon excitation lamp source, UV excitation; minimum of two reagent plates for addition of pharmacological agents
FDSS6000	Hamamastsu	http://www.usa.hamamatsu.com	96- and 384-well read and addition, xenon lamp excitation source; three reagent plates for addition of pharmacological agents

Data analysis

High-throughput techniques generate large datasets, a problem that has beset the drug industry in their endeavor to increase their throughput and move to 1536-well formats. Feature recognition and data mining are thus important for the effective use of this technology. However, when handling and analyzing temporally complex Ca^{2+} signals such as those shown in Figure 2, the current proprietary software approaches provided on high-throughput platforms are insufficient for basic research needs and alternative methods must be considered.

A key statistical parameter that is used to describe the suitability of an assay for high-throughput screening is the Z-factor [33]. Although this is an important assay indicator, it might not be the most appropriate way of validating assay protocols used in basic research for Ca^{2+} measurements. As described above, the ability in high-throughput mode to perform multiple replicates and controls in parallel makes the high-throughput approach attractive for Ca^{2+} studies, even if a Z-factor would indicate otherwise.

Does high-throughout assessment mark the end of other Ca^{2+} measurement techniques?

High-throughput methods for the assessment of Ca^{2+} homeostasis are part of an integrated research approach rather than a potential replacement to current methods of assessing intracellular Ca^{2+}. The ability to examine Ca^{2+} in individual cells enables the assessment of a diverse array of important processes such as Ca^{2+} waves, the interaction between cells in a mixed population, and Ca^{2+} in cellular compartments and microdomains [17]. Moreover, imaging also has a place in optimizing high-throughput assays, such as the optimization of fluorescence Ca^{2+} indicator loading conditions and/or the selection of Ca^{2+} indicators for optimal loading [15,16].

High content screening: a place in Ca^{2+} signaling research?

There have also been developments in high content screening, a process whereby fluorescent images are obtained in a microplate environment at a resolution capable of resolving single cells and subcellular compartments (see [34] for an extensive review). Such equipment has been modified or adapted to enable 'kinetic' studies, where images are acquired before and after physiological stimuli with high temporal resolution. These protocols have been used to assess intracellular Ca^{2+} in single cells [34]. Although such studies potentially allow for automation of processes previously restricted to fluorescence digital imaging, only one well can be imaged at a time and as discussed above this would limit the application of Ca^{2+} imaging in high-throughput mode. Indeed, where high temporal resolution is required and the Ca^{2+} signal must be assessed as described in Figure 2, current high-throughput imaging techniques can be regarded as being equivalent to the moderate throughput described for 'single-well' analysis (Table 2). However, studies where Ca^{2+} changes are slow and sustained (e.g. during cytotoxic events) would be more appropriate for such high-throughput assessment [34]. Recent progress in developing fluorescent proteins to detect Ca^{2+} in sub-regions of the cell [22] also have an impact on Ca^{2+} signaling research, and might be ideally suited for high-throughput imaging techniques.

High content screening, if combined with genuine high-throughput assessment of intracellular Ca^{2+}, offers a potential high-throughput way to further assess the role of Ca^{2+} in a variety of physiological pathways. High content screening potentially enables (often simultaneously) the assessment of processes as diverse as nuclear morphology, mitochondrial membrane potential, receptor translocation, mitosis, cell mobility and neurite extension [34–36]. Although unlikely to be an option for most basic pharmacology research laboratories at present, the correlation of high-throughput Ca^{2+} signaling data and data from high content screening will enable the role of Ca^{2+} signaling to be probed at new levels of complexity and detail [36].

Concluding remarks

The transformation by high throughput of a linear experimental protocol to one that is parallel has a tremendous impact on the pace of biological research, and pharmaceutical companies have been quick to take advantage and foster the development of high-throughput technologies.

Certainly, the ability to technically perform complex Ca^{2+} studies in intact, adherent cells can only facilitate the ability of researchers to probe basic pharmacology questions, and improve our understanding of the complexity between Ca^{2+} signaling and specific cellular processes.

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