Seeing the light: calcium imaging in cells for drug discovery

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Many ion channels and G protein-coupled receptors (GPCRs) alter the calcium concentration of mammalian cells on activation. To find functional ligands for these targets, high-throughput (HT) calcium assays are required to screen large chemical libraries. This review will compare fluorescent and luminescent imaging as technology platforms for developing HT calcium assays.

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▼ Calcium is involved in many cellular processes (such as adhesion, gene expression and motility) and its concentration within the cell is carefully controlled[1–3]. While the cytosolic calcium level of quiescent cells is low (10–100 nM), stimulation can raise this concentration to 500–1000 nM and activate important calcium sensors such as calmodulin and troponin C (Ref. 4). The cell has several different mechanisms for regulating calcium entry and removal from the cytosol. Opening of voltage-gated, ligand-gated or store-operated calcium channels, or the release of calcium from intracellular stores, will result in a rapid increase in calcium concentration. Activation of G protein-coupled receptors (GPCRs) and receptor tyrosine kinases stimulates the production of inositol 1,4,5-triphosphate ($InsP_3$), which binds to $InsP_3$ receptors located on the endoplasmic reticulum to release stored calcium into the cytosol.

These calcium 'on' mechanisms are balanced by pump mechanisms, such as the plasma membrane ATPase and the sarcoplasmic/endoplasmic ATPase pumps, or the plasma membrane Na^+–Ca^{2+} exchange mechanism, which function to quickly return calcium levels to their low resting state concentration. The challenge for developing high-throughput (HT) calcium assays, therefore, is to rapidly measure these transient changes in calcium concentration, or convert the transient changes into sustained responses that can be measured at a later time.

Fluorescence-based assays

Fluorescent indicators of calcium were originally developed to study changes in intracellular calcium levels by fluorescence microscopy, flow cytometry or fluorescence spectroscopy. These fluorescent probes are structurally related to the calcium chelators ethylene glycol-bis(β-aminoethyl ether) tetracetic acid (EGTA) and 1,2-bis-(2-aminophenoxy) ethane tetracetic acid (BAPTA), and show a rapid spectral change on binding to calcium, which makes them ideal for kinetic calcium measurements.

Dual-wavelength ratiometric dyes

Fluorescent calcium dyes can be classified functionally as single-wavelength or dual-wavelength ratiometric dyes. For the single wavelength dyes, a change in calcium concentration causes the fluorescent intensity of the dye to vary, but the emission and excitation peaks remain fixed. By contrast, the dual wavelength ratiometric dyes exhibit a shift in fluorescence intensity and peak maxima in the presence of calcium. For the calcium ratiometric dye fura-2, the excitation peak shifts between the bound calcium (335 nm) and the free calcium (362 nm) forms of the dye[5]. The ratio of fluorescence intensity at these two excitation wavelengths can be used to correct for sample variations in cell number or efficiency of dye loading. Ratiometric calcium dyes have significant advantages for quantitative measurement of intracellular calcium, but they are not widely used for HTS. One reason is that they require UV light for excitation, which can result in significant autofluorescence of cells, assay plates, or compounds, and can photolyze many photosensitive caged compounds. The energy of a UV lamp is low compared to an argon laser, which makes it difficult to measure small changes in calcium levels. Another disadvantage is that building a HT fluorescence instrument that

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delivers distinct excitation wavelengths almost simultaneously can be costly and complex.

Single-wavelength dyes

By contrast, the single wavelength dyes have proven to be the most commonly used dyes for HT calcium assays[6]. Examples of these dyes include Fluo-3 (Refs 7,8), Calcium Green-1 and Oregon Green 488 BAPTA-1 (all from Molecular Probes, Eugene, OR, USA). The equilibrium dissociation constants of these dyes for calcium ranges from 200 to 400 nM, which makes them ideal for measuring physiological changes in calcium concentration. These dyes are not ratiometric, but the intensity of fluorescence is directly proportional to the calcium concentration. Thus, a single wavelength excitation source and single wavelength emission detector make the instrumentation requirements simpler compared with the ratiometric dyes. These dyes also have the advantage of being excited in the visible region (488 nM) where autofluorescence is less of a concern.

Although the single wavelength calcium indicators have been available for the past decade, their use in HTS was limited by a lack of suitable detection instruments. A sensitive fluorescent plate reader with an on-line injector and photomultiplier tube (PMT) can measure fluorescent calcium responses for each well separately, but this is usually insufficient for today's HTS demands[9]. A parallel system for measuring the kinetic calcium responses of a 96-well plate was made possible with the introduction of the Fluorometric Imaging Plate Reader (FLIPR) (Molecular Devices, Sunnyvale, CA, USA)[10].

A unique feature of FLIPR is its ability to read fluorescent calcium signals of all 96 wells simultaneously, with kinetic updates in the 0.5–1.0 s range. The optics of FLIPR include an argon laser and a cooled charge-coupled device (CCD) camera. Cells are grown attached or centrifuged to the bottom of the assay wells, and compounds are added using a 96-channel pipettor that is integrated into the FLIPR. The laser light is directed to the underside of clear-bottom wells using a patented system that limits the depth of the excitation beam to approximately 200 μm from the bottom of the well[11]. This small optical slice limits the background fluorescence from the extracellular dye. The CCD camera captures the fluorescence from all 96 wells simultaneously at regular intervals, and the kinetic data is stored on a computer. The introduction of FLIPR was a major advance for HT calcium assays because it is an image-based instrument capable of making kinetic measurements of many calcium oscillations. Improvements to FLIPR's throughput have led to the recent introduction of a 384-well plate version.

Luminescence-based assays

It can be argued that, despite the advantages offered by fluorescence, the future of HT calcium assays is in luminescence-based screening. This is because, unlike fluorescence-based assays, cells and assay plates are not autoluminescent. The background signal of a luminescent assay, therefore, is largely determined by the electronic background of the detection system. Luminescence can be measured by a PMT or imaged by a CCD camera. A sufficiently cooled CCD camera can image a very low luminescent signal. For example, the CCD camera of the Affymax luminescent imager is cryogenically cooled to −100°C, and can measure 1–2 photons-per-hour (unpublished results). It also contains a telecentric lens, which ensures that light is collected evenly from all the wells of an assay plate.

There are several commercial luminescent imaging systems now available for HTS. The Chemiluminescence Imaging Plate Reader (CLIPR) is similar to the Affymax design and is available from Molecular Devices. Other luminescent imaging systems include LEADseeker (Amersham Pharmacia Biotech, Piscataway, NJ, USA), Wallac ViewLux (PerkinElmer Life Sciences, Turku, Finland) and NorthStar (PE Biosystems, Foster City, CA, USA). Each system is unique with regard to sensitivity, optical properties and robotic applications.

Another advantage of luminescent assays is the ease of miniaturization. An HT fluorescent imaging assay requires the excitation light to be directed to all wells simultaneously, and the background fluorescence can be minimized by confocal imaging or optical sectioning. Neither requirement is necessary for luminescent imaging. Thus, as the density of the assay wells increases to meet the ever-increasing demands of HTS, there could be significant cost savings to building a screening platform based on luminescence rather than fluorescence.

This article will now describe two luminescent calcium assays: the photoprotein aequorin, and a luciferase reporter gene under the control of a calcium-regulated promoter.

Aequorin

The photoprotein aequorin is composed of the 21 kDa apoaequorin protein bound to the prosthetic group coelenterazine and molecular oxygen. It is produced, along with green fluorescent protein (GFP), by the jellyfish *Aequorea victoria*. Aequorin is a calcium-binding protein and contains three EF-hand calcium-binding sites. Binding of calcium to the three sites causes aequorin to undergo a conformational change resulting in the oxidation of coelenterazine to coelenteramide. The relaxation of coelenteramide from the excited state to the ground state results in the emission of blue light (470 nm). In the jellyfish, this blue light is transferred to GFP by fluorescence resonance energy transfer (FRET), and green light is emitted as GFP relaxes back to its ground state.

Aequorin has been used as a calcium indicator for more than three decades[12]. Its advantage for HTS includes a large dynamic range because of the low background levels associated with

luminescent detection. This large dynamic range is useful for HTS assays, especially when screening for antagonist ligands that inhibit a ligand-induced signal.

Early studies with aequorin required the photoprotein to be microinjected into cells. Cloning of the apoaequorin gene has made it possible to express the gene in a variety of cell types and to reconstitute the aequorin complex by incubating the cells with coelenterazine. The cloned gene can also be targeted to specific intracellular locations such as the cytoplasm, nucleus, mitochondria and endoplasmic reticulum by fusing the gene to specific targeting sequences[13,14]. This makes it possible to measure calcium concentrations at specific cellular sites, which is not possible with fluorescent dyes because uptake of the dyes is nonspecific. The dyes can also alter calcium homeostasis because they accumulate to high concentrations (20–200 μM) and can buffer the calcium concentration[15]. By contrast, the expression levels of aequorin are much lower (100 nM).

Aequorin assays have been validated for many GPCRs and calcium channels, and the dose responses created by these assays are similar to the values obtained using fluorescent calcium dyes[16,17]. The significant challenges for adapting aequorin assays to HTS are the flash kinetics and low quantum yield of the reaction. A typical aequorin signal in mammalian cells occurs within 30 s because the product of the oxidation reaction, coelenteramide, remains bound to apoaequorin and the protein is consumed. Even so, the intensity of the aequorin flash is proportional both to the fraction of total protein consumed and to the calcium concentration. The assay is sufficiently sensitive that it can be calibrated and used to measure the absolute calcium concentration of cells with the same degree of accuracy as fura-2 measurements[15].

The quantum yield of aequorin, defined as the number of photons per reacting molecule, is only 0.12–0.20, and is less than for GFP (0.72–0.85)[18]. To measure the low light of aequorin, a PMT is often used because it is a sensitive and inexpensive technology and can measure kinetic changes in the aequorin signal[13–16]. The PMT is not, however, ideal for HTS because each PMT can only measure the light output of a single assay well at any one time. An added complexity of the aequorin assay is that compounds or cells must be dispensed immediately prior to measuring luminescence. An instrument containing an array of six PMTs, six injectors and a detection time of 30 s would still require 32 min to assay a single 384-well plate, which is too long to screen a large library efficiently. The ideal aequorin instrument, therefore, would be based on CCD imaging. It would contain an integrated system for dispensing compounds to all wells of an assay plate, as does the FLIPR, but would have a more sensitive CCD camera for measuring aequorin luminescence. An instrument such as this, however, is not yet commercially available.

Calcium-regulated reporter genes

Reporter gene assays rely on unique signal transduction pathways that are controlled by second messengers, such as calcium. The nuclear factor of activated T cells (NFAT) and cAMP response-element binding protein (CREB) are examples of transcription factors that become activated when there is a rapid rise in the intracellular calcium concentration[19,20]. On activation, these factors bind to unique sites on promoters and stimulate transcription. Reporter genes are constructed by fusing a calcium-regulated promoter to the coding sequence of an enzyme that catalyzes a luminescent or fluorescent reaction such as firefly luciferase, β-lactamase or β-galactosidase[21,22]. Compared with aequorin, the luminescent signal of firefly luciferase is amplified, bright (quantum yield is >0.88) and linear over a wide range of enzyme concentrations[21].

Calcium-reporter gene assays convert a transient change in calcium concentration into a sustained luminescent readout. For example, binding of an agonist to a $G\alpha_q$-coupled GPCR will cause release of calcium from intracellular stores, but the cytosolic calcium concentration might only remain elevated for several minutes before the ATPase pumps return the calcium to its resting-state concentration. However, this might be enough time for a transcription factor, such as NFAT, to become activated and translocate into the nucleus to stimulate transcription. The synthesis of luciferase, therefore, might occur several hours after the calcium spike. A unique example using a Cre recombinase reporter gene under the control of the NFAT promoter showed that a change in calcium concentration can cause cells to undergo DNA recombination and express luciferase indefinitely[23].

Reporter gene assays, therefore, enable compound addition and signal detection to occur at separate stations. This is advantageous for HTS because it greatly simplifies the screening process and instrument design. The fluorescent dye and aequorin assays require detection and compound dispensing to be integrated because these assays are kinetic measurements of changes in the calcium concentration. Although reporter genes are an indirect and endpoint measurement of calcium levels, the level of gene expression is proportional to the calcium concentration[24–26]. The dose-response curve of a calcium reporter gene assay is often similar to that obtained using a fluorescent dye (Fig. 1).

The use of NFAT reporter genes for assaying GPCRs has been studied in lymphoid and nonlymphoid cells[27]. However, it is unclear how useful NFAT is for measuring rapid calcium responses, because NFAT-mediated gene expression is dependent on the magnitude, duration and frequency of the calcium oscillation[24–26]. For targets such as fast-inactivating calcium channels that produce a small change in calcium concentration, the aequorin and fluorescent dye assays might be preferable. Reporter gene assays also cannot measure the kinetics of a calcium response, which can be useful for determining the mode of

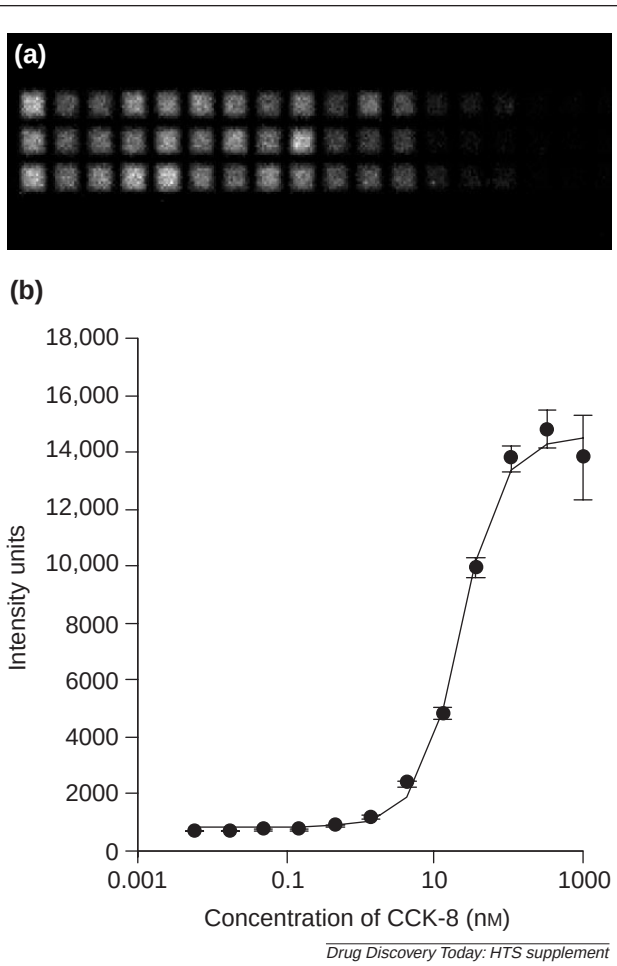

Figure 1. An 864-well luminescent nuclear factor of activated T cells (NFAT) reporter gene assay for the cholecystokinin 1 (CCK_1) receptor, a $G\alpha_q$-coupled G protein-coupled receptor (GPCR). Approximately 10^4 cells of a CHO-K1 clone transfected with the CCK_1 receptor and NFAT-luciferase reporter genes were dispensed into wells of an 864-well plate and incubated with various concentrations of the ligand CCK-8 for 4 h. The luciferase substrate reagent LucLite (Packard, Meriden, CT, USA) was added to each well and the plate was imaged for 2.5 min using a cryogenically cooled, scientific grade, 512 × 512 CCD camera (Photometrics, Tucson, AZ, USA). The luminescence intensity of each well was determined by summing the total number of pixel counts corresponding to each well (IPLab Spectrum software; Scanalytics, Vienna, VA, USA). Shown is (a) the image and (b) the corresponding dose-response curve (EC_{50} = 22 nM) for CCK-8.

action of a compound. Thus, reporter gene assays might be most useful in primary screening applications, followed by the information-rich kinetic assays as a secondary screening tool.

Conclusions

Today, there is a variety of assays and instruments available for HT imaging of calcium. The choice depends on the target, the size of the library to be screened and the cost. Fluorescent dye assays are widely used for HTS. By contrast, luminescent imaging of calcium is a new technology that potentially offers significant advantages such as a simpler instrument design and a larger signal dynamic range. However, the first CCD-based aequorin instrument needs to be developed and rigorously compared to the widely used FLIPR system for HTS. For some targets, such as GPCRs, it might be desirable to switch from a fluorescent assay to a simple luciferase reporter gene assay, provided that an endpoint calcium assay is judged to be reliable for primary screening. For all of these HTS technologies, the major bottleneck in miniaturization will be developing precise liquid handling capabilities.

For drug development and optimization, the future of calcium imaging could be to measure the calcium responses of whole animals. New technologies, such as a calcium-sensitive contrast agent for magnetic resonance imaging (MRI) and *in vivo* calcium reporter genes, could soon provide unique perspectives on the actions of drugs and the physiology of calcium-dependent processes[28].

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References

1 Carafoli, E. (1987) Intracellular calcium homeostasis. *Annu. Rev. Biochem.* 56, 395–433

2 Tsien, R.W. and Tsien, R.Y. (1990) Calcium channels, stores and oscillations. *Annu. Rev. Cell Biol.* 6, 715–760

3 Bootman, M.D. and Berridge, M.J. (1995) The elemental principles of calcium signaling. *Cell* 83, 675–678

4 Berridge, M.J. *et al.* (1998) Calcium – a life and death signal. *Nature* 395, 645–648

5 Grynkiewicz, G. *et al.* (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260, 3440–3450

6 González, J.E. *et al.* (1999) Cell-based assays and instrumentation for screening ion-channel targets. *Drug Discovery Today* 4, 431–439

7 Minta, A. *et al.* (1989) Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. *J. Biol. Chem.* 264, 8171–8178

8 Kao, J.P. *et al.* (1989) Photochemically generated cytosolic calcium pulses and their detection by fluo-3. *J. Biol. Chem.* 264, 8179–8184

9 Lin, K. *et al.* (1999) Rapid measurements of intracellular calcium using a fluorescence plate reader. *BioTechniques* 2, 318–322

10 Schroeder, K.S. and Neagle, B.D. (1996) FLIPR: a new instrument for accurate, high throughput optical screening. *J. Biomol. Screening* 1, 75–80

11 Schroeder, K. and Neagle, B. (1994) Method and apparatus for quantitative fluorescence measurements. US 5355215

12 Ridgway, E.B. and Ashley, C.C. (1967) Calcium transients in single muscle fibers. *Biochem. Biophys. Res. Commun.* 29, 229–234

13 Montero, M. *et al.* (1995) Monitoring dynamic changes in free Ca^{2+} concentration in the endoplasmic reticulum of intact cells. *EMBO J.* 14, 5467–5475

14 De Giorgi, F. *et al.* (1996) Targeting aequorin and green fluorescent protein to intracellular organelles. *Gene* 173, 113–117

15 Brini, M. *et al.* (1995) Transfected aequorin in the measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]c$). A critical evaluation. *J. Biol. Chem.* 270, 9896–9903

16 Stables, J. *et al.* (1997) A bioluminescent assay for agonist activity at potentially any G-protein-coupled receptor. *Anal. Biochem.* 252, 115–126

17 Maeda, A. *et al.* (1996) Generation of cell transfectants expressing cardiac calcium ion channel and calcium indicator protein aequorin. *Anal. Biochem.* 242, 31–39

18 Kendall, J.M. and Badminton, M.N. (1998) *Aequorea victoria* bioluminescence moves into an exciting new era. *Trends Biotechnol.* 16, 216–224

19 Rao, A. *et al.* (1997) Transcription factors of the NFAT family: regulation and function. *Annu. Rev. Immunol.* 15, 707–747

20 Sheng, M. *et al.* (1991) CREB: a Ca^{2+}-regulated transcription factor phosphorylated by calmodulin dependent kinases. *Science* 252, 1427–1430

21 Bronstein, I. *et al.* (1994) Chemiluminescent and bioluminescent reporter gene assays. *Anal. Biochem.* 219, 169–181

22 Zlokarnik, G. *et al.* (1998) Quantitation of transcription and clonal selection of single living cells with β-lactamase as reporter. *Science* 279, 84–88

23 Mattheakis, L.C. *et al.* (1999) Expression of Cre recombinase as a reporter of signal transduction in mammalian cells. *Chem. Biol.* 6, 835–844

24 Dolmetsch, R.E. *et al.* (1997) Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration. *Nature* 386, 855–858

25 Dolmetsch, R.E. *et al.* (1998) Calcium oscillations increase the efficiency and specificity of gene expression. *Nature* 392, 933–936

26 Li, W. *et al.* (1998) Cell-permeant caged $InsP_3$ ester shows that Ca^{2+} spike frequency can optimize gene expression. *Nature* 392, 936–941

27 Boss, V. *et al.* (1996) Induction of NFAT-mediated transcription by G_q-coupled receptors in lymphoid and non-lymphoid cells. *J. Biol. Chem.* 271, 10429–10432

28 Li, W. *et al.* (1999) A calcium-sensitive magnetic resonance imaging contrast agent. *J. Am. Chem. Soc.* 121, 1413–1414