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Demaurex, Nicolas

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Calcium measurements in organelles with Ca^{2+}-sensitive fluorescent proteins

Nicolas Demaurex *

Department of Cell Physiology and Metabolism, University of Geneva, 1 rue Michel-Servet, CH-1211 Geneva 4, Switzerland

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Abstract

The recent improvement in the design and use of genetically encoded fluorescent Ca^{2+} indicators should foster major progress in three aspects of Ca^{2+} signaling. At the subcellular level, ratiometric probes with expanded dynamics are now available to measure accurately the local Ca^{2+} changes occurring within specific cell compartments. These tools will allow to determine precisely the role of organelles and of cellular microdomains in Ca^{2+} handling. At the cellular level, the permanent labeling offered by the genetic probes enables large-scale, long-term Ca^{2+} measurements with robotic multiplexing technologies such as fluorescence plate readers or automated microscopes. This opens the way to large-scale pharmacological or genetic screens based on organelle-specific functional assays. At the whole animal level, probes with improved dynamics and reduced interference with endogenous proteins will allow to generate transgenic animals bearing Ca^{2+} sensitive indicators in specific cells and tissues. With this approach, Ca^{2+} signals can be recorded in neurons, heart, and pancreas of live animals during physiological and pathological stimulations. In this chapter, I will review the progress made in the design and use of genetic Ca^{2+} indicators, and discuss how these new tools provide an opportunity to challenge several unsolved questions in Ca^{2+} signaling.

Keywords: Calcium; Signalling; GFP; Ca^{2+} probes; Endoplasmic reticulum

1. Introduction

The ability to record changes in the free Ca^{2+} concentration in living cells is fundamental to study Ca^{2+} signaling. The design more than 30 years ago of the fluorescent Ca^{2+} indicators quin2 and fura2 by Roger Tsien enabled the first quantitative measurements of the changes in the free Ca^{2+} concentrations occurring inside cells. Today, dozens of different chemical probes have been generated using this strategy (reviewed in [1]). These tools have been used to measure Ca^{2+} signals with exquisite spatio-temporal resolution, and have defined our view of the Ca^{2+} signaling circuitry, mapping the molecular mechanisms that are at the basis of cellular Ca^{2+} signals. Fluorescent dyes have a number of advantages over genetically encoded indicators: they are cheap, easy to use, have a high dynamic range, and are easy to calibrate. However, they cannot be selectively targeted to specific cellular compartments or to specific cell types within a tissue without using invasive procedures such as microinjection or pipette loading. Fluorescent dyes also leak out from cells and cannot be used for more than a couple of hours. Synthetic Ca^{2+} indicators still remain the probe of choice for short-term assays in isolated cells, but have now been surpassed by genetically encoded indicators for a number of applications [2]. Genetically encoded indicators are more difficult to use than fluorescent dyes. They require gene amplification and molecular engineering, are difficult to express in primary cells, and usually have a lower dynamics than chemical dyes. However, because the genetic encoding ensures a permanent labeling and provides a specific targeting, genetically encoded indicators are the probes of choice for long-term assays in living cells or whole organisms.

Genetically encoded indicators are usually based on fluorescent proteins (FP), which are spontaneously fluorescent

* Tel.: +41 22 379 5399; fax: +41 22 379 5338.
E-mail address: nicolas.demaurex@medecine.unige.ch.
URL: http://www.medecine.unige.ch/∼demaurex.

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without enzymes or cofactors required. Because all the information required to make a fluorescent protein is contained within its nucleic acid sequences, FP are most commonly used as reporter gene to monitor gene transcription in cells, tissues, or whole animals. FP are widely used as fusion proteins to monitor the location, appearance, degradation, aggregation, or translocation of proteins of interest, to study protein diffusion by FRAP or photoactivation, or to track organelles and cells by live imaging. In all these applications, FP are used as beacons with the hope that the exogenous protein will not interfere with cellular functions.

To use FP as biochemical sensors, the biological parameters of interest must affect the fluorescence properties of the FP indicators. Because the original FP are not sensitive to environmental factors, FP have undergone several rounds of molecular engineering to generate specific biochemical sensors. To date, FP have been engineered to monitor several biologically relevant parameters. FP mutants can detect variations in the concentration of Ca^{2+} (cameleons, pericams, G-CAMPs), in cellular or organellar pH (pHluorins, AlpHi), in redox potential (roGFP), in cAMP and cGMP, in phosphoinositides, as well as changes in the membrane potential or in the activity of specific kinases or phospholipases.

Most efforts have been devoted to the design of Ca^{2+} indicators, because of the importance of Ca^{2+} in cell physiology and because of the complexity of the intracellular Ca^{2+} circuitry. Thanks to the work of several groups, there are now a great variety of genetically encoded Ca^{2+} indicators, which can be divided into two groups based on their structure: double barrel probes, such as cameleons, made of two FP connected by a Ca^{2+}-sensitive linker (cameleons, troponeons, and D1) and single barrel probes, such as pericams, composed of a single FP bearing a Ca^{2+}-dependent inserted sequence (Camgaroos, pericams and G-CAMPs). The probes also differ by the nature of the module used as a Ca^{2+} sensor, which was originally based on calmodulin (in all the "cam" probes), and more recently on troponin C (troponeons) or on a redesigned artificial module (D1).

1.1. Cameleons

Cameleons are tandem repeats of GFP mutants with overlapping excitation/emission spectra linked together by a Ca^{2+} sensor based on calmodulin (CaM), a glycylglycine linker, and the CaM binding peptide of myosin light chain kinase M13 [3]. In this design, Ca^{2+} binding promotes the reversible association of CaM and M13, promoting fluorescence resonance energy transfer between the two GFP variants. The first probes generated, yellow cameleons (YC), used cyan and yellow fluorescent proteins (CFP and YFP) as FRET donor and acceptors. The reversible changes in FRET can be detected as changes in the yellow over cyan emission fluorescence, and the probes function as ratiometric emission Ca^{2+} indicators such as indo-1.

The original YC comprised probes of different affinities, ranging from 1 to 300 μM (YC2, YC3 and YC4), but all probes suffered from a poor dynamic range and were sensitive to pH and to chloride ions. YC were subsequently made pH resistant by YFP mutagenesis (YC2.1 and YC3.1 [4]) and brighter by replacing YFP with citrine (YC3.3 [5]) or with Venus, a YFP mutant with fast and efficient maturation (YC2.12 and VC 6.1 [6] [7]). Although these cameleons were greatly improved over the original design, they still displayed insufficient signal-to-noise ratio when targeted to organelles.

To further increase the dynamic range of cameleons, Nagai et al. generated several circularly permuted YFP mutants in an attempt to optimize the relative orientation of the two FRET partners [8]. By introducing new termini into the surface-exposed loop regions of the β-barrel of Venus, they generated several YC variants with improved dynamics. The best construct, YC 3.6, which contains a YFP circularly permutated at position 173 (173cpVenus), matures efficiently, is resistant to acidification, and displays a monophasic Ca^{2+} sensitivity with a K_d of 250 nM. The cp173Venus cameleon has much better dynamics than all previous cameleons when expressed in cells, with an impressive 5.6-fold increase in ratio between R_{min} and R_{max}. The expanded dynamic range of YC3.6 is a clear advantage to image Ca^{2+} dynamics in cells and tissues with adequate spatiotemporal resolution.

1.2. Pericams

Pericams are based on a circular permutated GFP mutant (cpGFP). In this construct, the GFP β barrel has been cut open and the original N and C termini linked together to create new termini located close to each other along the side of the β barrel [9]. Amazingly, the permutated FP retain the ability to form a chromophore, but the permutation renders the chromophore more accessible to the solvent pH. Because protonation of residues surrounding the chromophore alter its ionization state, the fluorescence of all cpGFP is highly pH sensitive. This property has been exploited to detect Ca^{2+} by inserting calmodulin at a central site on a circularly permutated YFP (camgaroos [9]) or by grafting a Cam/M13 pair on its N and C termini (pericams [10]). In pericams, Ca^{2+} binding opens and closes a "cleft" in the YFP β barrel, promoting titration of the chromophore and causing large changes in fluorescence. Because pericam probes are intrinsically pH sensitive, the Ca^{2+} signals cannot be accurately measured in compartments whose pH varies. Pericams are brighter in alkaline compartments, and their pH sensitivity has been exploited to measure the pH of mitochondria [11]. Three types of pericams were generated with distinct spectral properties: probes whose fluorescence increases with calcium (flash pericam), decrease with calcium (inverse pericam) or undergoes a shift in excitation wavelength in a Ca^{2+}-dependent manner (ratiometric pericam). While the flash and inverse pericam behave as single wavelength indicators, opposite changes in Ca^{2+} are detected at two alternating excitation wavelengths in ratiometric pericam, and the probes function like fura-2. The ratiometric pericam are so far the only probes besides FRET-based YC that enables ratiometric

measurements, a property that is critical for quantitative Ca^{2+} measurements.

1.3. New Ca^{2+} sensors

The Ca^{2+} sensing module contained in all "cam" probes is problematic, because it can interact with the cell signaling machinery. The CaM domain contained within cameleons, camgaroos, pericams, and G-CAMPs can interact with numerous calmodulin-binding proteins involved in cell regulation, and endogenous calmodulin can interact with the sensor's CaM binding peptide, rendering the probe nonfunctional. To generate a more neutral Ca^{2+}-binding module, Nicolas Heim and Olivier Griesbeck designed troponeons, based on troponin C, the Ca^{2+} sensor of cardiac and skeletal muscle [12]. Troponin C is a dumbbell-shaped Ca^{2+}-binding protein with two globular heads that only binds a reduced subset of endogenous proteins and whose only known function is to regulate muscle contraction. Fragments from chicken skeletal muscle or human cardiac troponin C were inserted between CFP and citrine to generate S-troponeon and C-troponeon, respectively. Interestingly, the indicators responded to Ca^{2+} changes without the need of additional binding peptides such as M13, using only troponin C as Ca^{2+} sensor. The troponeons are FRET-based indicators with spectral properties, pH sensitivity, and Ca^{2+} responses similar to YC, and with Ca^{2+} affinities ranging from 0.5 to 1.2 μM in the original constructs to 30 μM in a low-affinity mutant. The troponeons retained a full dynamic range when expressed at the plasma membrane by fusion with GAP43, synaptobrevin, or Ha-Ras whereas yellow cameleons bearing the same targeting sequences had a reduced dynamic range [12]. The troponeons thus performed better than cameleons when targeted to a specific subcellular domains.

The group of Roger Tsien went a step further by designing an artificial Ca^{2+} binding module that would not interact with endogenous proteins. To this aim, they replaced four basic residues in the CaM binding peptide with acidic residues, generating a MLCK mutant with 10,000-fold reduced affinity for native CaM. They then made corresponding mutations in the CaM module, reversing three acidic residues by basic residues to generate a mutated CaM with higher affinity to the MLCK mutant than for native CaM. The redesigned CaM/MLCK pair was inserted in a citrine cameleon (YC3.3) and the resulting probe, dubbed D1 for design 1, exhibited FRET responses of magnitude similar to original cameleons. As expected, the FRET response of D1 was not affected by the addition of CaM, while the response of YC3.3 decreased by 60% at high CaM concentrations. Because the response of the redesigned cameleon is not perturbed by native calmodulin, this construct should be better tolerated in vivo and provides a significant improvement over all the other indicators. In addition, the cameleon bearing the artificial module had faster Ca^{2+} dissociation kinetics and a Ca^{2+} affinity of 60 μM that nicely matched the Ca^{2+} concentrations of the ER, making it an ideal probe for Ca^{2+} imaging in this compartment. Because they alleviate the potential problem of calmodulin interactions, both troponeons and D1 might be used as an alternative when calmodulin-based indicators fail to target efficiently or exhibit reduced dynamics in specific cell compartments. In addition, the dual emission YC are ideally suited for in vivo applications using two-photon confocal microscopy, as only a single excitation wavelength is required.

2. Ca^{2+} imaging in cellular compartments

One great advantage of genetic indicator is that they can be targeted to specific cellular microdomains by molecular engineering. Successful functional expression has been obtained for several compartments, such as the endoplasmic reticulum [3,13], the nucleus [3], the Golgi [5], mitochondria [10,14], and the plasma membrane [12,15]. Unfortunately, this property could not be fully exploited with the original cameleons and pericams, because the probes exhibited a reduced dynamic range when expressed in subcellular compartments. In extreme cases, the Ca^{2+} dependency appeared to be completely abolished. The difficulty to achieve a specific targeting in some organelles was another major problem. The newly designed indicators have expanded dynamics and improved targeting efficiency, but these constraints still limit the resolution that can be achieved in organelle Ca^{2+} imaging.

2.1. Endoplasmic reticulum

The ER is the main intracellular Ca^{2+} store, and the Ca^{2+} released from the ER during signaling is a major component of the Ca^{2+} signals that regulate contraction, secretion, ATP production, and gene transcription. A physiological decrease in the free ER Ca^{2+} concentration, $[Ca^{2+}]_{ER}$, triggers the opening of store-operated Ca^{2+} channels [16,17] while a pathological increase in $[Ca^{2+}]_{ER}$ can lead to apoptosis [18,19]. Because of the importance of ER Ca^{2+} in cell physiology, much efforts have been devoted to develop probes that would accurately measure changes in $[Ca^{2+}]_{ER}$. YC probes with low Ca^{2+} affinity were the first developed, and enabled dynamic measurements of $[Ca^{2+}]_{ER}$ under both physiological and pathological conditions (reviewed in [20]. Unfortunately, these probes do not allow accurate $[Ca^{2+}]_{ER}$ measurements because their affinity is either too high ($YC3_{ER}$, K_d 3 μM) or too low ($YC4_{ER}$, K_d 220 μM). In addition, the probes are very difficult to use quantitatively because of their small dynamic range (40%) and pH sensitivity. This lack of precision led to some controversy. The antiapoptotic protein Bcl-2 was initially reported to decrease $[Ca^{2+}]_{ER}$ based on $YC4_{ER}$ [13] and aequorin measurements [21], but other reports indicated that Bcl-2 either does not decrease or increased $[Ca^{2+}]_{ER}$ (reviewed in [22]). More recently, ER-targeted YC were used to study Ca^{2+}-induced Ca^{2+} release in MIN6 pancreatic beta-cells [23], but the role of CICR in pancreatic islet beta-cells was subsequently challenged, because pH changes might have interfered with the $YC4_{ER}$ measurements [24].

Attempts to use $YC3_{ER}$ to measure $[Ca^{2+}]_{SR}$ in primary cultures of rat cardiac ventricular myocytes were also only partly successful, because $YC3_{ER}$ could not follow the rapid change in $[Ca^{2+}]_{SR}$ but only responded to slow and large change in $[Ca^{2+}]_{SR}$ induced by caffeine or thapsigargin [25].

The recently developed $D1_{ER}$ YC fills a gap in the Ca^{2+} sensitivity and allows more accurate measurements of $[Ca^{2+}]_{ER}$. The K_d of 60 μM of the D1 cameleon is just slightly below the free Ca^{2+} levels of the ER at rest, making this probe ideal for ER Ca^{2+} measurements. Using $D1_{ER}$, Palmer et al. were able to resolve oscillations in $[Ca^{2+}]_{ER}$, which could not be measured with the earlier cameleons. They were also able to follow both increases and decreases in $[Ca^{2+}]_{ER}$ during stimulation, and to study quantitatively the effects of the antiapoptotic protein Bcl-2 on the Ca^{2+} leak rate of the ER. Fig. 1 shows an example of the responses obtained with $D1_{ER}$ during stimulation of HeLa cells. The ratio decreased reproducibly during small and large $[Ca^{2+}]_{ER}$ decreases evoked by Ca^{2+} mobilizing agonists, and returned rapidly to basal values at the end of the stimulation protocol. The rapid and robust ratio responses of the $D1_{ER}$ probe is a major improvement as it allows to use multiple stimulation protocols, and the optimized Ca^{2+} affinity of the probe allows to resolve small changes in $[Ca^{2+}]_{ER}$ occurring during physiological stimulations.

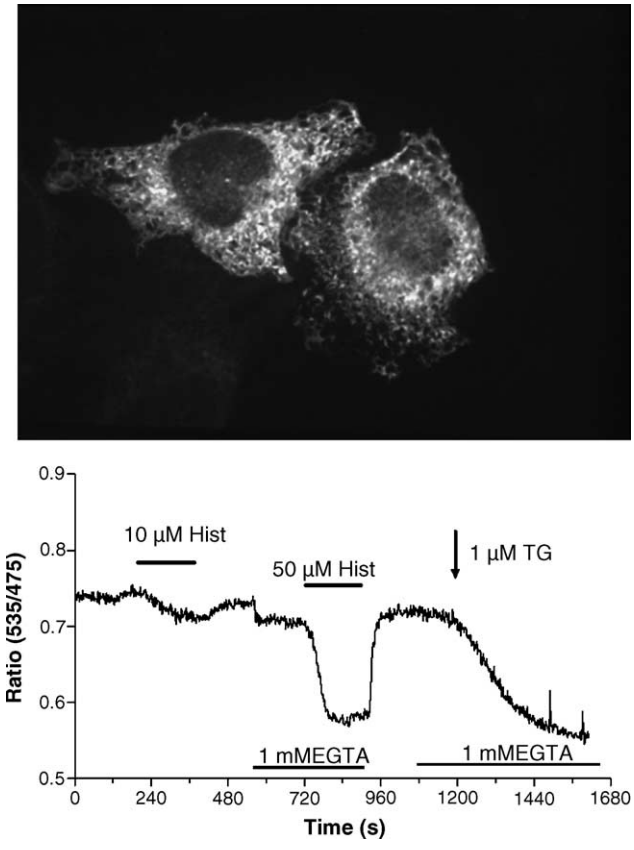

Fig. 1. $[Ca^{2+}]_{ER}$ measurements with $D1_{ER}$. The ER-targeted cameleon probe $D1_{ER}$ with redesigned Ca^{2+} binding module was expressed in HeLa cells. Top panel: $D1_{ER}$ fluorescence imaged on a spinning wheel confocal microscope using 457 nm excitation and 535 nm emission (FRET channel). Fluorescent cells exhibited a reticular pattern typical of the ER. Bottom panel: ratio changes measured on a wide-field microscope with a CCD camera, using 430 nm excitation and alternate 480/535 nm emission. Cells were stimulated sequentially with increasing concentrations of histamine, initially in Ca^{2+} containing and then in Ca^{2+} free medium. Note the rapid recovery of $[Ca^{2+}]_{ER}$ levels upon washout of the agonist and readmission of Ca^{2+}.

2.2. Mitochondria

Mitochondria are Ca^{2+} handling organelles whose importance in Ca^{2+} signaling became fully appreciated following the development of genetically encoded Ca^{2+} indicators. Mitochondria were known to take up and release Ca^{2+} for a long time, but the affinity of mitochondrial Ca^{2+} transporters was so low that their physiological role was considered negligible. The demonstration with targeted aequorin that the free Ca^{2+} concentration in the mitochondrial matrix, $[Ca^{2+}]_{mit}$, varies rapidly inside cells led to a new appreciation of the role of mitochondria in Ca^{2+} signalling [26], reviewed in [27]. Although much has been learned with aequorin, $[Ca^{2+}]_{mit}$ imaging measurements are highly desirable because of the complex and dynamic nature of mitochondria in living cells. Unfortunately, aequorin measurements are limited to cell populations because the number of photons emitted by the photoprotein is low, and $[Ca^{2+}]_{mit}$ imaging with genetic indicators had to await the development of mitochondrial-targeted Ca^{2+}-sensitive FP.

The first Ca^{2+}-sensitive FP to be targeted to mitochondria were the YC series [14]. Measurements with $YC2_{mit}$ and $YC3_{mit}$ revealed that mitochondria prevent the Ca^{2+} depletion of the ER [14], and that mitochondria buffer subplasmalemmal Ca^{2+} elevations during agonist stimulation [28]. Unfortunately, YC are very difficult to target to mitochondria and exhibit a reduced dynamic range when targeted to these organelles. In a study comparing different mitochondria–targeted FP, ratiometric pericam was found to be superior than YC2 and Camgaroo-2, which suffered from severe mistargeting and photoconversion, respectively [29]. Although the pH sensitivity of the RP probe renders its calibration problematic, the improved dynamics and more efficient targeting of RP_{mit} allowed to follow beat-to-beat $[Ca^{2+}]_{mit}$ transients in spontaneously beating ventricular myocytes from neonatal rats [30]. RP_{mit} has since been used to show that mitochondria relay Ca^{2+} from plasma membrane channels to the ER [31], and to study the effect of mitochondrial fragmentation on the Ca^{2+} propagation within interconnected mitochondria [32,33].

Although RP targeted better than camgaroos and cameleons to the mitochondria, a substantial portion of the probe still failed to enter the mitochondrial matrix and remained located in the cytosol. The targeting efficiency of all three types of Ca^{2+}-sensitive FP was improved by using two tandem repeats of the addressing sequence [34]. Again, the best results were obtained with RP, but the nature of the addressing sequence was also found to be important. The best targeting was achieved with the first 36 amino acid of the

subunit VIII of human cytochrome oxidase (mt8), a sequence that contains nine amino acids of the mature protein. A shorter peptide comprising the first 12 amino acid of the subunit IV of yeast cytochrome oxidase (mt4) was less effective. Interestingly, the duplication of the signal peptide not only improved targeting but also improved the performance of RP, increasing its dynamic range by three-fold and reducing its apparent Ca^{2+} affinity from 11 to 2 μM. The improved Ca^{2+} responses reflected the differential processing of the targeted proteins, the duplicated mt8 signal peptide being cleaved upon import into mitochondria while the duplicated mt4 signal peptide is retained after translocation within the mitochondrial matrix [34]. As a result, the mitochondrial Ca^{2+} responses measured with RP bearing a duplicated mt8 peptide (2mt8RP) were larger than the responses measured in the same cells with a RP probe targeted to the nucleus. 2mt8RP is the best mitochondrial probe so far and its improved targeting efficiency, increased dynamics, and higher Ca^{2+} affinity makes it a promising tool to study the role of mitochondria in Ca^{2+} handling.

2.3. Plasma membrane

Ca^{2+} changes below the plasma membrane play a dominant role in the control of secretion and of ion channel activity. The subplasmalemmal Ca^{2+} levels, $[Ca^{2+}]_{pm}$, are affected by the activity of Ca^{2+} channels, pumps, and exchangers at the plasma membrane and the cortical domain thus constitutes a Ca^{2+} compartment distinct from the cytosol. Because a huge Ca^{2+} gradient is actively maintained across the plasma membrane, the $[Ca^{2+}]_{pm}$ levels are thought to be higher than the bulk cytosolic Ca^{2+} levels most of the time. Accordingly, long lasting subplasmalemmal domains of high Ca^{2+} were initially reported in a7R5 cells with a low affinity aequorin [35]. Standing subplasmalemmal Ca^{2+} gradients, however, were not observed with membrane-targeted troponeons in primary hippocampal neurons [12]. In this study, both high affinity and low affinity troponeons were fused to the amino terminal portion of neuromodulin (aka GAP43) to direct their expression to the plasma membrane. Similar $[Ca^{2+}]$ values were measured in the cytosol and beneath the plasma membrane with the two sets of probes, both at rest and during depolarization. Interestingly, the low-affinity troponeon was not saturated upon maximal depolarization of hippocampal neurons with KCl, indicating that the peak $[Ca^{2+}]_{pm}$ values did not exceed 10 μM under these extreme conditions.

In contrast to the study in neurons, $[Ca^{2+}]_{pm}$ levels higher than $[Ca^{2+}]_{cyt}$ levels were measured in HeLa cells using the probe with expanded dynamic range, YC3.6, which was targeted to the plasma membrane by fusing its C-terminus to the membrane anchor sequence CAAX of Ki-Ras [8]. The $YC3.6_{pm}$ probe was distributed to the cell periphery and in filipodial structures when expressed in HeLa cells, and allowed highly quantitative measurements of $[Ca^{2+}]_{pm}$. The resting $[Ca^{2+}]_{pm}$ levels were slightly higher than the basal cytosolic levels, consistent with the existence of standing microdomains of high $[Ca^{2+}]_{pm}$, and long-lasting $[Ca^{2+}]_{pm}$ changes were also observed in filipodial structures [8]. Fig. 2A and B show the typical staining pattern obtained with the $YC3.6_{pm}$ probe in HeLa cells: fluorescence is observed almost exclusively at the cell periphery and the cells appear empty on the cross section images. The filipodia and lamellipodia are strongly fluorescent, and the cells have a very hairy appearance on the wide-field and volume-reconstructed images. As shown in Fig. 2C and D, the responses obtained with $YC3.6_{pm}$ and with fura-2 during the same stimulation protocol are very different: the $YC3.6_{pm}$ response evoked by thapsigargin is minimal, while responses elicited by Ca^{2+} readmission and removal are much more transient than with fura-2. Thus, the $[Ca^{2+}]_{pm}$ responses measured with $YC3.6_{pm}$ can be temporally and functionally separated from the $[Ca^{2+}]_{cyt}$ responses recorded with a synthetic indicator loaded in the cytosol.

Recent studies indicate that the spatio-temporal pattern of $[Ca^{2+}]_{pm}$ changes is complex and highly regulated. A YC2 fused to focal adhesion kinase was generated to measure simultaneously local $[Ca^{2+}]_{pm}$ changes and focal adhesion sites dynamics in U87 astrocytoma cells [36]. The FAK-YC2 revealed that local $[Ca^{2+}]_{pm}$ elevations correlated spatially and temporally with the disassembly of focal adhesion sites. In another study, rapid changes in $[Ca^{2+}]_{pm}$ were observed in endothelial cells using YC2.1 fused to GAP43 [15]. The subplasmalemmal Ca^{2+} waves were spatially distinct from the cytosolic Ca^{2+} waves and changes in the extracellular Ca^{2+} concentration had immediate effects on $[Ca^{2+}]_{pm}$ but little effects on $[Ca^{2+}]_{cyt}$ [15]. Interestingly, increases in $[Ca^{2+}]_o$ elicited subplasmalemmal Ca^{2+} waves that originated in localized regions and propagated within a very shallow region of ~0.2 μM beneath the plasma membranes of endothelial cells [15]. Because of its expanded dynamic range and efficient targeting, the YC3.6 pm probe offers great potential to study the dynamics of these local subplasmalemmal Ca^{2+} microdomains.

3. High-throughput Ca^{2+} measurements

The improved brightness and dynamics of the new probes enables high-throughput, long-term calcium measurements using multiplate fluorescence readers or automated microscopes. High throughput screening methods are highly desirable for drug discovery, genetic screen, and for bioactivity and toxicity tests. Although fast and quantitative screening of cells can be achieved with fluorescent dyes, the loading procedure is not reliable and cannot be easily automated. Genetically encoded indicators eliminate this problem, because cells can be made permanently fluorescent by integrating the probe cDNA into the host genome. Using targeting sequences, cell lines bearing the probes targeted to specific organelles can also be generated.

Fig. 3 shows an example of multiple simultaneous Ca^{2+} recordings with a ratiometric pericam probe located in the

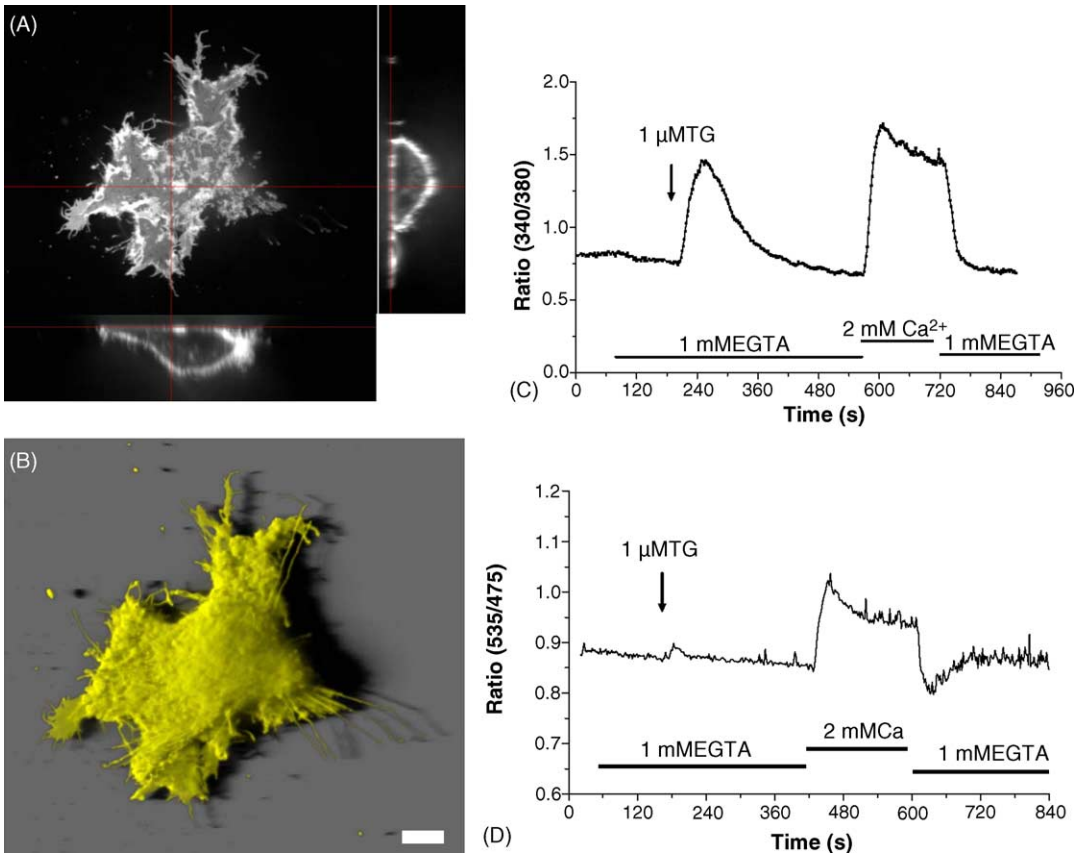

Fig. 2. Subplasmalemmal Ca^{2+} measurements with $YC3.6_{pm}$. (A) Cross-section view of a HeLa cell expressing the plasma membrane targeted cameleon probe $YC3.6_{pm}$, imaged on a spinning wheel confocal microscope using 488 nm excitation and 535 nm emission. The location of the XY, XZ, and YZ sectioning planes within the stack comprising 53 adjacent, 0.4 nm thick z sections is indicated by the red lines. Fluorescence was observed at the cell periphery as well as in filipodial and lamellipodial structures. (B) 3D reconstruction of the cell shown in (A). (C) $[Ca^{2+}]_{cyt}$ changes measured with fura-2 during ER Ca^{2+} release and store-operated Ca^{2+} entry. Cells were stimulated with thapsigargin to induce ER store depletion and Ca^{2+} was readmitted transiently to the extracellular medium. (D) Changes in $[Ca^{2+}]_{pm}$ recorded with $YC3.6_{pm}$ during the same protocol. Changes in ratio fluorescence were measured as in Fig. 1. Note the minimal $[Ca^{2+}]_{pm}$ change during thapsigargin addition and the transient responses during Ca^{2+} readdition and removal.

cytosol. HeLa cells clones stably expressing the RP probe were maintained in culture for six months and clones that retained a bright and homogenous cytosolic fluorescence were isolated and grown on 96-well plates. The readout was performed on a 96-well multiplate fluorescence reader, by measuring RP fluorescence at two excitation wavelengths in six parallel wells. As shown in Fig. 2, the temporal resolution of 0.7 ratio/s is sufficient to resolve Ca^{2+} transients evoked by agonists, and the ratio responses elicited by an identical dose of agonist in the six parallel wells are very reproducible. Because the responses are measured simultaneously in multiple wells, a full dose-response (12 concentrations) can be obtained in 10 min.

This approach can in theory be applied to specific Ca^{2+} signalling compartments such as the endoplasmic reticulum, mitochondria, and the plasma membrane. FP targeted to all these compartments exist already, but a bright, stable, and specific labeling is not easily achieved with targeted probes, as discussed above. In addition, a very efficient targeting is required in all the cells in such a population-based readout. Given the huge volume of the cytosol, even a minor fraction of the indicator retained in the cytosol can contaminate the signal originating from the desired compartment. Conversely, given the comparatively small volume of organelles, the fluorescence of correctly addressed probes might be too dim to measure. Although stable clones with bright, specific targeting can be generated and used for large-scale functional and pharmacological assays, transient expression is often preferred for functional genetic screens to ensure that all fluorescent cells express the transgene studied. In our hands, all the transiently expressed YC or RP probes tested were either incorrectly targeted or too dim to enable measurements on the fluorescence plate reader. Fortunately, the brightness of the YC3.6, together with the improved targeting conferred by the 2mt8 signal sequence and the lack of toxicity of the D1 Ca^{2+}-sensing module offers new opportunities for large-scale fluorescence measurements in organelles. With high throughput optical recordings, functional genetic screens could be designed to identify unknown proteins involved in the regulation of local Ca^{2+} signals. For instance, the molecular

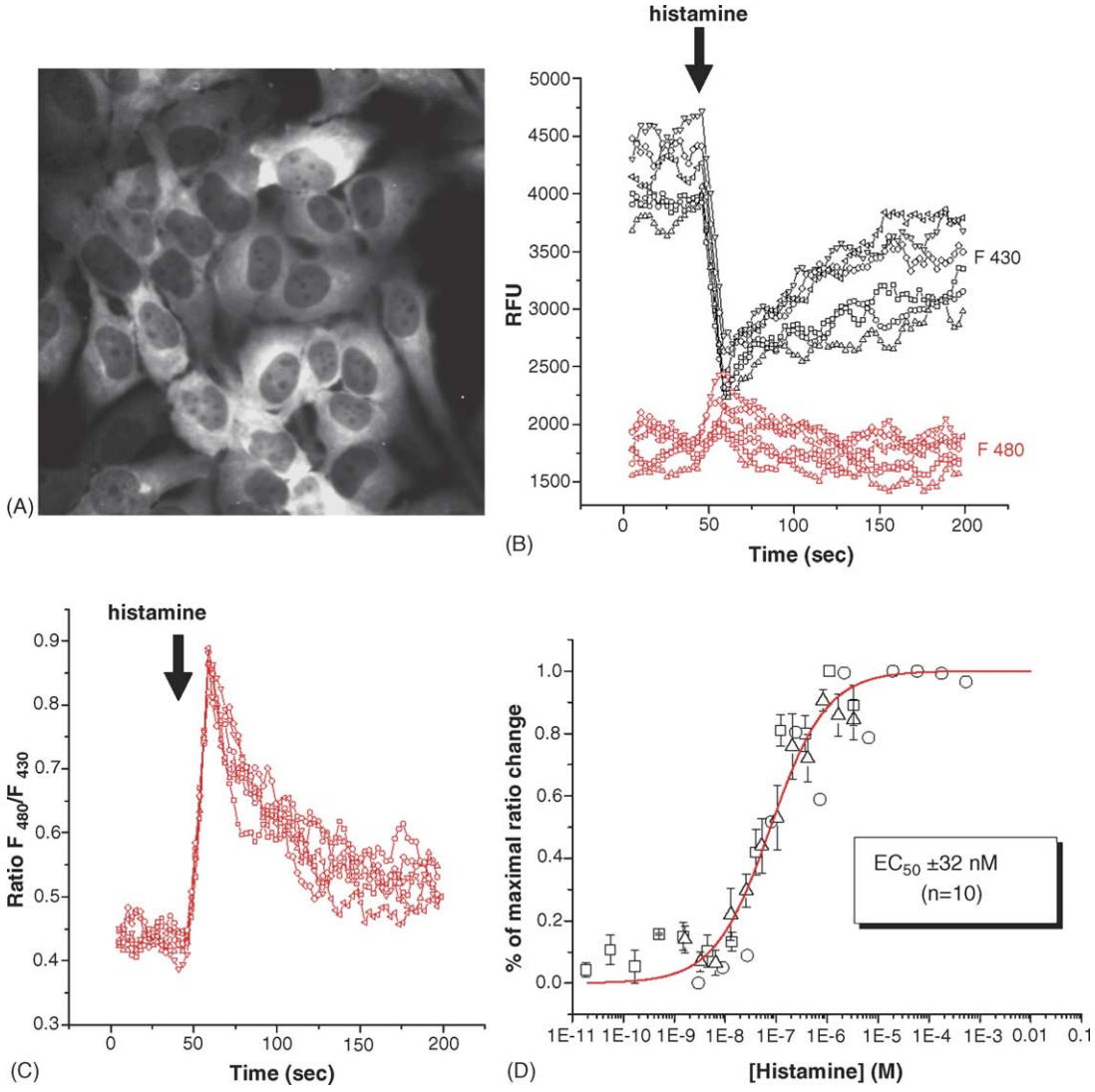

Fig. 3. High-throughput Ca^{2+} measurements with RP3.1. HeLa cell lines expressing the RP3.1 probe were generated using self-inactivating HIV-based lentiviral vectors that allow stable integration of the probe cDNA into the host cell genome. Clones displaying homogenous cytosolic fluorescence were isolated and maintained in culture for six months. (A) Wide-field fluorescence image of HeLa cells stably expressing $RP3.1_{cyt}$. (B) Changes in RP fluorescence measured at λ_{ex} = 430 and 480 nm on a 96-well plate fluorescence reader (FlexstationTM). Addition of histamine induced opposite changes in RP fluorescence at 430 and 480 nm. (C) Changes in ratio fluorescence evoked by the addition of a supramaximal dose of histamine (10 μM). The responses measured simultaneously in six parallel wells were highly reproducible. Temporal resolution: 1 ratio/1.4 s. (D) Aggregated data from 10 experiments (12 concentrations each, readout time: 10 min). The average half maximal concentration of histamine, determined by fitting a Hill equation to the 10 datasets, was 73 ± 32 nM.

identity of the Ca^{2+} transporters located on the membrane of mitochondria is still unknown. With efficient and specific functional assays, whole genome siRNA or cDNA screen could be used to identify such elusive protein(s).

4. In vivo studies

The ultimate advantage of genetic Ca^{2+} indicators is the possibility to perform functional Ca^{2+} measurements in transgenic animals. However, attempts with the first generation of probes were not satisfactory, and initial studies were restricted to fly [37,38] and worms [39]. Attempts from several laboratories to express fluorescent Ca^{2+} probes in mammals were not successful, probably because of interactions with endogenous Ca^{2+}-binding proteins. Measurements in brain slices indicated that Ca^{2+} responses in situ are complex and depend on the particular indicator [40]. FRAP analysis in individual dendritic spines from pyramidal neurons revealed that circularly permuted indicators (GCaMP, Camgaroo2, and Inverse Pericam) had motilities similar to GFP and did not associate notably with immobile CaM-binding proteins. During electrical stimulations, the indicators responded poorly at low action potential frequencies compared with synthetic Ca^{2+} indicators but

produced robust signals in response to high-frequency trains of action potentials [40]. A more detailed comparison of the performance of 10 different genetic calcium-sensitive indicators was performed in *Drosophila* [41]. The indicators were expressed in the entire nervous system using the neuronal Gal-4 promoter and in vivo responses were recorded in presynaptic boutons of the *Drosophila* larval neuromuscular junction. The boutons were identified by the fluorescence of the indicators and Ca^{2+} responses elicited by electrical stimulation of up to 80 Hz were compared. The response characteristics of the 10 indicators differed considerably: no changes in fluorescence were observed during electrical stimulation with Flash-Pericam and Camgaroos, and only small changes in fluorescence (<10%) were observed with YC, Inverse-Pericam, and troponeons. The largest and fastest fluorescence responses (18%) were observed with GCaMP1.6, a circular permutated variant, but this probe exhibited rapid photobleaching. In contrast, the smaller fluorescence signals of the cameleons and troponeons were more photostable and reproducible and nearly identical signals were recorded during repetitive stimulations, indicating that these probes are superior for in vivo measurements.

Using two-photon microscopy of YC2 acutely expressed in mouse skeletal muscle, the group of Tullio Pozzan measured both the cytosolic and the mitochondrial Ca^{2+} spikes occurring during muscle contraction in vivo [42]. The YC2 cDNA was introduced into the tibialis anterior muscle by electroporation, and 3 weeks later 50% of the fibers exhibited a strong cytosolic YC2 signal. The YFP/CFP ratio was stable in the absence of stimulation despite substantial specimen movements, demonstrating that the ratiometric procedure corrected well for movement artifacts in live anesthetized animals. The YC2 ratio increased by 40% during tetanic stimulation with an extracellular electrode, and allowed to resolve the fast $[Ca^{2+}]_{cyt}$ transients elicited by low frequency stimulation. Next, a YC2 bearing a duplicated mitochondrial targeting sequence (2mtYC2) was used to image $[Ca^{2+}]_{mit}$ changes in vivo. More than 90% of the fluorescence colocalized with the mitochondrial marker TMRM and the ratio changes were abolished by the mitochondrial uncoupler CCCP, indicating that 2mtYC2 reported $[Ca^{2+}]_{mit}$ changes in vivo. The $[Ca^{2+}]_{mit}$ spikes could be elicited by low frequency stimulation and lagged the $[Ca^{2+}]_{cyt}$ spikes by ~10–20 ms, demonstrating that mitochondria accumulate Ca^{2+} during single twitches in fast skeletal muscle.

Last year, the group of Winfried Denk reported the first in vivo functional imaging of genetic Ca^{2+} indicators in transgenic mice [43]. Using inverse pericam and camgaroo-2 placed under the control of the tetracycline promoter, they generated stable transgenic mouse lines that expressed functional Ca^{2+} indicators in the nervous system. The success rate was low: only 5 out of 36 transgenic mice could be imaged, and none of the YC lines generated were functional. The indicators were not expressed in every cell, and a substantial fraction of the fluorescent Ca^{2+}-sensitive proteins were found to be immobile or located in aggregates. Nonetheless, the brightness of the fluorescent proteins allowed in vivo two photon imaging of neurons located deep in the brain in adult anesthetized mice. Robust signals could be recorded in 8–12 weeks old mice, indicating that the indicators do not loose activity after expression in living animals for long periods. The fluorescent neurons had normal electrophysiological properties, but the fluorescence changes observed in vivo were much smaller than those seen in cultured cells. The responses ranged from 8% with wide-field CCD imaging to 50% with two photon imaging, which was clearly better because it effectively rejected the contaminating signal from non-stimulated cells. The responses evoked by synaptic stimulation in acute brain slices were spatially heterogenous, the changes reaching 100% in localized hotspots. In live mice imaged through the intact skull, fluorescence changes of 8% were observed in the olfactory bulb during stimulation with odorants, and each odour evoked a unique activity map. The highly reproducible signals detected in vivo confirm that genetically encoded Ca^{2+} indicators can be used to image the activity from populations of neurons in living animals.

Shortly after, transgenic mice expressing the membrane-targeted probe $YC3.6_{pm}$, which has expanded dynamics, were generated and used to image $[Ca^{2+}]_{pm}$ changes in hippocampal brain slices [8]. The probe was placed under the control of the β-actin promoter and one line exhibited bright fluorescence in the brain, enabling fast (100 Hz) imaging of neuronal Ca^{2+} activity. In acute slices, $[Ca^{2+}]_{pm}$ transients were reproducibly evoked in the CA1 region by tetanic stimulation of the Schaffer collaterals. Again, the responses were small (3%), but in this case the neuronal signals were diluted not only by contaminating signals from out of focus cells but also by the signals from glial cells since the probe expression was not restricted to neurons. In addition, endogenous CaM or CaM-binding proteins might interfere with the CaM-based $YC3.6_{pm}$ probe and reduce its dynamics. Because they alleviate the potential problem of calmodulin interactions, the newly designed troponeons and D1 probes might increase the success rate of obtaining transgenic animals expressing functional Ca^{2+} indicators.

In summary, the last few years have seen remarkable progress in the design and use of genetically encoded Ca^{2+} indicators. The existing probes have been systematically evaluated for their performance in various applications. New probes with increased dynamics have been generated that do not interfere with endogenous proteins, and improved targeting sequences have been designed to address the probes more specifically to organelles. These new indicators will allow faster and more accurate Ca^{2+} imaging, both at the cellular level and at the whole animal level. Efficient and specific functional assays can now be developed for whole genome genetic screen of Ca^{2+} transporters located in organelles or in Ca^{2+} microdomains, and two photon imaging of transgenic animals expressing Ca^{2+}-sensitive fluorescent proteins has been validated as a robust approach to image Ca^{2+} activity in vivo.

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