The endoplasmic reticulum (ER) and mitochondria accumulate Ca\(^{2+}\) within their lumens to regulate numerous cell functions. However, determining the dynamics of intraorganellar Ca\(^{2+}\) has proven to be difficult. Here we describe a family of genetically encoded Ca\(^{2+}\) indicators, named calcium-measuring organelle-entrapped protein indicators (CEPIA), which can be utilized for intraorganellar Ca\(^{2+}\) imaging. CEPIA, which emit green, red or blue/green fluorescence, are engineered to bind Ca\(^{2+}\) at intraorganellar Ca\(^{2+}\) concentrations. They can be targeted to different organelles and may be used alongside other fluorescent molecular markers, expanding the range of cell functions that can be simultaneously analysed. The spatiotemporal resolution of CEPIA makes it possible to resolve Ca\(^{2+}\) import into individual mitochondria while simultaneously measuring ER and cytosolic Ca\(^{2+}\). We have used these imaging capabilities to reveal differential Ca\(^{2+}\) handling in individual mitochondria. CEPIA imaging is a useful new tool to further the understanding of organellar functions.
The endoplasmic reticulum (ER) and mitochondria are membrane-bound intracellular organelles in eukaryotic cells that carry out vital functions. Both ER and mitochondrial membranes display Ca\(^{2+}\)-transporting molecules whose function is to import Ca\(^{2+}\) into the lumen against the concentration gradient. This uphill Ca\(^{2+}\) transport is mediated in the ER membrane by sarco(endo)plasmic reticulum Ca\(^{2+}\) ATPase (SERCA) and in the inner mitochondrial membrane by the mitochondrial Ca\(^{2+}\)-unipporter (MCU). The organelar membranes also feature molecules that allow Ca\(^{2+}\) to exit from the organelles to the cytosol: inositol 1,4,5-trisphosphate receptors and ryanodine receptors in the ER, and the Na\(^{+}\)/Ca\(^{2+}\) and H\(^{+}\)/Ca\(^{2+}\) exchangers in mitochondria. Thus, the intraluminal Ca\(^{2+}\) concentration in the organelles is tightly regulated, and may exceed that of cytosol by a large factor. Ca\(^{2+}\) levels within the ER profoundly affect organelle function, and overload or depletion causes ER stress. Within the mitochondrial matrix, Ca\(^{2+}\) concentration regulates the rate of ATP production, and abnormal concentrations can lead to cell death or autophagic degradation of the mitochondria.

ER and mitochondrial structures are constantly being reorganized and close contacts form between the two types of organelles. These contact sites have recently been shown to be involved in diverse functions, including lipid biosynthesis, mitochondrial biogenesis and the transfer of Ca\(^{2+}\) (refs 5–8). Both types of organelle are also involved in the regulation of cytosolic Ca\(^{2+}\) concentrations. Release of Ca\(^{2+}\) from the ER regulates contraction, fertilization, development, secretion and synaptic plasticity. In addition, the luminal Ca\(^{2+}\) concentration regulates Ca\(^{2+}\) influx across the plasma membrane. Following a release of Ca\(^{2+}\) from the ER, STIM1, which is present in the ER membrane, functions as a Ca\(^{2+}\) transducer; it signals to the plasma membrane to activate the store-operated Ca\(^{2+}\) entry mechanism (SOCE). SOCE is mediated by the molecular complex that includes Orai1, and is important for the activation of various cell functions, the best-studied example of which is the immune response. In contrast to the active role of the ER in Ca\(^{2+}\) signalling, mitochondria have been considered to act as a passive Ca\(^{2+}\) buffer. However, recent results suggest that they may also have an active role as a source of Ca\(^{2+}\) in the regulation of cytosolic Ca\(^{2+}\) concentrations.

Although the importance of the ER and mitochondria as Ca\(^{2+}\)-handling organelles is unequivocal, the mechanism by which organelar Ca\(^{2+}\) concentrations regulate cellular processes remains elusive. New methods to dissect organellar Ca\(^{2+}\) dynamics are expected to facilitate such studies. While small molecular Ca\(^{2+}\) indicators cannot be precisely targeted to the organelles, limiting their use in living cells, genetically encoded Ca\(^{2+}\) indicators (GECIs) can be targeted to organelles with the addition of appropriate tags. Making use of this capability, GFP-based GECh13–23 and aequorin (a Ca\(^{2+}\)-sensitive photoprotein)24 have been used to measure intraorganellar Ca\(^{2+}\) concentrations. FRET-type GFP-based GEChs were first used to measure intraluminal Ca\(^{2+}\) concentration in the ER and were applied to different cell types. This type of indicators uses wide visible wavelength bands for excitation and emission, often limiting the simultaneous use of other fluorescent molecules. Subsequently, innovative modifications of the GFP molecule have yielded single-wavelength-excitation GEChs with various affinities to Ca\(^{2+}\) for ER and mitochondrial Ca\(^{2+}\) imaging. Aequorin emits dim light, and simultaneous measurement with brighter fluorescence signals is not possible with most fluorescence microscopes. Although we have a wide variety of indicators, simultaneous Ca\(^{2+}\) imaging of the ER and mitochondria has not been carried out, and improvement in the spatiotemporal resolution of organellar Ca\(^{2+}\) is expected to enhance our understanding of intraorganellar Ca\(^{2+}\) dynamics.

For these reasons, and to study the functional interaction between the ER and mitochondria, a new type of GECIs with higher spatiotemporal resolution was required.

This study reports on the generation of new organellar Ca\(^{2+}\) indicators that allow simultaneous imaging of two subcellular compartments with high spatiotemporal resolution. They are optimized in terms of Ca\(^{2+}\) affinity and dynamic range for organellar Ca\(^{2+}\) imaging and come in colour variants for simultaneous measurement of multiple signals when they are used in appropriate combinations. Using them, intraorganellar Ca\(^{2+}\) concentrations can be imaged at unprecedented spatiotemporal resolution. To illustrate the utility of the approach, we demonstrate high spatiotemporal resolution imaging of ER and mitochondrial Ca\(^{2+}\) dynamics in living cells; we show the quantitative relationship between the ER Ca\(^{2+}\) concentration and the extent of STIM1 puncta formation in the regulation of SOCE; and we show that inhomogeneity in mitochondrial Ca\(^{2+}\) responses can be observed during apparently homogenous ER and cytosolic Ca\(^{2+}\) changes, which suggest that there is a mechanism to regulate the influx of Ca\(^{2+}\) into mitochondria.

The new indicators described in this work will be valuable for further study of the roles of the ER and mitochondria, and of their functional interactions.

Results

Development of ER Ca\(^{2+}\) indicator. We began measuring ER Ca\(^{2+}\) dynamics based on a lead variant of GCaMP2 (cfGCaMP2, see Methods), whereby fluorescence intensity increased 5.1-fold upon binding of Ca\(^{2+}\) with a K\(_d\) of 0.67 \(\mu\)M (Supplementary Table 1). Since ER Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{ER}\)]) is assumed to reach the sub-millimolar range, cfGCaMP2 was engineered to reduce its Ca\(^{2+}\) binding affinity by a factor of \(\sim 1,000\). We searched for low Ca\(^{2+}\) affinity variants guided by extensive structure–function analyses based on site-directed mutagenesis in the calmodulin domain (See Methods). From among the 58 variants that were generated in our search we selected one with 31D/F92W/E104D/D133E substitutions that had a low Ca\(^{2+}\) affinity (K\(_d\) = 368 \(\mu\)M) and a large dynamic range (F\(_{max}/F_{min}\) = 4.2) (Fig. 1a and Supplementary Fig. 1a,b). After attaching ER localization and retention signal sequences, the low Ca\(^{2+}\) affinity variant was expressed in HeLa cells. The engineered indicator protein colocalized with an ER marker (Supplementary Fig. 1c). Upon addition of thapsigargin, an inhibitor of SERCA, a large reduction in fluorescence intensity was noted (Fig. 1c). Also, oscillatory fluorescence intensity decreased in response to histamine, which generates cytosolic Ca\(^{2+}\) oscillations due to release of Ca\(^{2+}\) from the ER via IP\(_3\)Rs (Fig. 1d). Thus, the indicator successfully reports [Ca\(^{2+}\)\(_{ER}\)] dynamics. We designated Calcium-measuring organelle-Entrapped Protein IndicAtor 1 in the ER (CEPIA1er).

Multi-coloring of CEPIA. We generated colour variants of CEPIA, based on recently developed cytosolic Ca\(^{2+}\) indicators: R-GECO1 (red fluorescence), G-GECO1.1 (green fluorescence), and GEM-GECO1 (ratiometric blue/green fluorescence). After rigorous structure–function assessment (see Methods), we obtained R-CEPIA1er (K\(_d\) = 565 \(\mu\)M, F\(_{max}/F_{min}\) = 8.8), G-CEPIA1er (K\(_d\) = 672 \(\mu\)M, F\(_{max}/F_{min}\) = 4.7), and GEM-CEPIA1er (K\(_d\) = 558 \(\mu\)M, R\(_{max}/R_{min}\) = 21.7; Fig. 1a and Supplementary Table 1). In vitro characteristics of these CEPIA variants, compared with those of original GECO, are summarized in Table 1 and Supplementary Fig. 2a–d.

We expressed the colour variants of CEPIA with ER-targeting peptides. The engineered variants, compared with those of original GECO, are summarized in Table 1 and Supplementary Fig. 2a–d.
Figure 1 | Characterization of CEPIA. (a) In vitro \( \Delta \Delta F/F_0 \) titration curves of CEPIA1er (black solid), G-CEPIA1er (green solid), R-CEPIA1er (magenta solid), GEM-CEPIA1er (blue solid) compared with cifCaMP2 (black dotted), G-GECKO1.1 (green dotted), R-GECKO1 (magenta dotted), GEM-GECKO1 (blue dotted) and DIER (orange). Fitted Hill plot curves are shown. Putative ranges of \( \text{Ca}^{2+} \) affinity of CEPIA variants within the ER, we carried out stepwise titration experiments in permeabilized HeLa cells. Absolute \( \text{[Ca}^{2+}]_{\text{ER}} \) was estimated from the ratio of the blue to green fluorescence intensities (middle and bottom). (c) Comparison of the responses of D1ER, CEPIA1er, R-CEPIA1er and GEM-CEPIA1er to thapsigargin (3\( \mu \)M)-induced depletion of \( \text{Ca}^{2+} \) in HeLa cells (\( n = 12-31 \), mean ± s.e.m.; ***\( P < 0.001 \)). (d) \( \Delta \Delta F/F_0 \) dynamics in response to histamine (10\( \mu \)M) measured with DIER, CEPIA1er, G-CEPIA1er, R-CEPIA1er and GEM-CEPIA1er (\( n = 14-103 \), mean ± s.e.m.; ***\( P < 0.001 \)). (e) Representative traces of the histamine-induced \( \text{Ca}^{2+} \) oscillations with a high signal-to-noise ratio. (f) Cell type-specific variations of absolute \( \text{[Ca}^{2+}]_{\text{ER}} \) measured with GEM-CEPIA1er. Box plots for \( \text{[Ca}^{2+}]_{\text{ER}} \) in a variety of cell types before and after agonist stimulation (10\( \mu \)M histamine for HeLa cells, 30\( \mu \)M ATP for HEK cells and cultured astrocytes and 100\( \mu \)M bradykinin for BHK cells; \( n = 4-19 \)) were shown. Absolute \( \text{[Ca}^{2+}]_{\text{ER}} \) after agonist stimulation indicated the minimum value reached within 30 s after agonist application. The horizontal line within the box represents the median value, the upper and lower edges of the box represent 75 and 25% values and the whiskers represent the total range.

detected \( \text{Ca}^{2+} \) oscillations with a high signal-to-noise ratio (Fig. 1b–e and Supplementary Fig. 3a,b). In HeLa cells, G-CEPIA1er showed superior performance in signal amplitude over CEPIA1er (Fig. 1d). To examine whether pH changes had any effect on the signal of CEPIA indicators (Supplementary Fig. 2c), we monitored pH dynamics in the ER using a pH sensor, enhanced yellow fluorescent protein (EYFP)29.30. There was no significant change in the fluorescence intensity of ER-localized EYFP (Supplementary Fig. 4a–d), verifying that the CEPIA responses are not due to pH changes in the ER. To measure the \( \text{Ca}^{2+} \) affinity of CEPIA variants within the ER, we carried out \( \text{Ca}^{2+} \) titration experiments in permeabilized HeLa cells. Stepwise changes in \( \text{Ca}^{2+} \) concentration elicited dose-dependent fluorescence intensity changes in the presence of ionomycin to.
make the ER membrane permeable to Ca\(^{2+}\) (Supplementary Fig. 3c). The \(K_d\) values determined within the ER were almost equivalent to those measured in vitro (Supplementary Fig. 3d). Thus, we succeeded in expanding the hues of CEPIA variants.

**Table 1 | Properties of CEPIA variants.**

| Probe          | \(Ca^{2+}\) | \(\varepsilon (mM^{-1} cm^{-1})\) \((\lambda_{ABs})\) | \(\lambda_{ems}^*\) | \(\Phi (\lambda_{ems})^\dagger\) | Brightness \(^\dagger\) \((mM^{-1} cm^{-1})\) | \(pK_a\) | Dynamic range \(^\ddagger\) | \(K_d\) for \(Ca^{2+}\) | Hill coefficient |
|----------------|-------------|---------------------------------|-----------------|------------------------------|---------------------------------|----------|-----------------|-----------------|-----------------|
| G-CEPIA1er     | 54          | 1.87                            | 30              | 0.12                         | 0.106                            | 636      | 0.3             | 1.70            | 0.04            |
| G-GECO1.1      | 54          | 1.87                            | 30              | 0.12                         | 0.106                            | 636      | 0.3             | 1.70            | 0.04            |
| R-CEPIA1er     | 54          | 1.87                            | 30              | 0.12                         | 0.106                            | 636      | 0.3             | 1.70            | 0.04            |
| R-GECO1        | 54          | 1.87                            | 30              | 0.12                         | 0.106                            | 636      | 0.3             | 1.70            | 0.04            |
| GEM-CEPIA1er   | 54          | 1.87                            | 30              | 0.12                         | 0.106                            | 636      | 0.3             | 1.70            | 0.04            |
| GEM-GECO1      | 54          | 1.87                            | 30              | 0.12                         | 0.106                            | 636      | 0.3             | 1.70            | 0.04            |

\(^\dagger\)\(\lambda_{EMS}, \lambda_{ABs}, \Phi, \phi, \gamma, \) and \(2\) are the maximum wavelength of absorption, fluorescence excitation and fluorescence emission spectra, respectively.

\(^\ddagger\)\(pK_a\), and quantum yield (\(\phi\), \(\gamma\), \(\phi\)).

\(^\dagger\)\(\text{Dynamic range indicates the ratio of the maximum to minimum fluorescence intensity (}\text{F}_{\max}/\text{F}_{\min}\) (or fluorescence ratio (}\text{R}_{\max}/\text{R}_{\min}\).\)

\(*\)\(\text{Hill coefficient}\)

**Estimation of \([Ca^{2+}]_{ER}\).** Using ratiometric measurement of GEM-CEPIA1er, we estimated \([Ca^{2+}]_{ER}\) in intact resting cells as varying between 620 and 860 \(\mu\)M in HeLa cells, HEK293A cells, BHK cells and cultured astrocytes; this decreased to 310–570 \(\mu\)M upon stimulation with agonists (Fig. 1f). The range of \([Ca^{2+}]_{ER}\) underlines the need to increase the indicator’s \(K_d\) to > 100 \(\mu\)M to provide faithful measurements of ER \(Ca^{2+}\) dynamics, and explains the difficulty in imaging ER \(Ca^{2+}\) dynamics using D1ER, which has a \(K_d\) of \(\sim 60 \mu\)M (ref. 19; Fig. 1a,c,d). Although CEPIA indicators had relatively high Hill coefficients \((n = 1.4–2.0,\) Table 1), the relationship between \(\Delta P/F_{\max}\) (or \(\Delta R/R_{max}\)) and changes in \([Ca^{2+}]_E\) is not highly distorted within the physiologial \([Ca^{2+}]_{ER}\) range (Supplementary Fig. 2e).

**Subcellular ER \(Ca^{2+}\) dynamics visualized with CEPIA.** We next examined whether CEPIA indicators are capable of detecting ER \(Ca^{2+}\) dynamics at subcellular resolution. Agonists often induce \(Ca^{2+}\) waves, which propagate throughout the cell after initiation in focal regions \(^{31,32}\). The wave is generated by the regenerative release of \(Ca^{2+}\) from the ER, and it has been predicted that this mechanism creates an ‘inverse \(Ca^{2+}\) wave’ within the ER. However, this prediction has not been tested using GECIs in live cells. We used G-CEPIA1er imaging at a high frame rate (10–30 frames s\(^{-1}\)) to visualize inverse \(Ca^{2+}\) waves in the ER. Local decreases in \([Ca^{2+}]_{ER}\) were observed, initiating at the tips and propagating to the perinuclear region in HeLa cells (Fig. 2a and Supplementary Movie 1). The time courses measured at two subcellular locations indicated a wave-like propagation of decreasing \([Ca^{2+}]_{ER}\) (Fig. 2bc). The speed of these waves was 60.8 ± 3.2 \(\mu\)m s\(^{-1}\) (mean ± s.e.m.), which matches cytosolic \(Ca^{2+}\) waves with or without CEPIA expression (Fig. 2d). Similar observations were made with R-CEPIA1er. Thus, CEPIA indicators have high spatiotemporal resolution.

We next examined whether CEPIA can be applied to intact tissue preparations. To do so, \(Ca^{2+}\) dynamics were elucidated in the neuronal ER in response to synaptic inputs to Purkinje cell dendrites in cerebellar slice preparations. G-CEPIA1er was expressed in Purkinje cells by Sindbis virus and was imaged with a two-photon microscope. G-CEPIA1er expression was observed throughout the dendrites and into spines, matching the distribution of the ER in Purkinje cells (Fig. 3a). In response to parallel fibre stimulation, which induces \(Ca^{2+}\) release from the ER by activating the metabotropic glutamate receptor \(^{33}\), we observed a long-lasting decrease in G-CEPIA1er fluorescence intensity (Fig. 3b and Supplementary Movie 2). The ER \(Ca^{2+}\) dynamics could be visualized at the level of single spines (Fig. 3c). This response was not due to any pH change in the ER, because parallel fibre stimulation had no effect on pH within the ER measured by ER-targeted EYFP (Supplementary Fig. 4e–g). Thus, subcellular ER \(Ca^{2+}\) imaging using CEPIA is applicable to tissue preparations that have retained three-dimensional structure, which often requires two-photon excitation.

**Simultaneous imaging of \(Ca^{2+}\) dynamics in the ER and cytosol.** For simultaneous imaging of \(Ca^{2+}\) dynamics in the ER and cytosol, we used the ratiometric small-molecule \(Ca^{2+}\) indicator, fura-2 (excitation: 340–380 nm), together with G-CEPIA1er or R-CEPIA1er. \(Ca^{2+}\) signals were a mirror image of the cytosolic \(Ca^{2+}\) oscillations (Supplementary Fig. 5a,b). Although G-CEPIA1er and R-CEPIA1er are weakly excited at the excitation wavelengths of fura-2 (Supplementary Figs 2a and 6a), they had very little effect on the fura-2 fluorescence ratio (Supplementary Fig. 6b). Thus, it is possible to use G-CEPIA1er and R-CEPIA1er with fura-2. Either G-CEPIA1er or GEM-CEPIA1er can be co-expressed with R-GECO1 for simultaneous imaging of the ER and cytosolic \(Ca^{2+}\) dynamics (Supplementary Fig. 5c–f and Supplementary Movie 3). Reversal of the colours is also possible, and ER \(Ca^{2+}\) imaging using R-CEPIA1er can be simultaneously carried out with cytosolic \(Ca^{2+}\) imaging using G-GECO1 or GEM-GECO1 (Supplementary Fig. 5g,h). Spectral bleed-through was minimal (< 3%; Supplementary Fig. 6a). These results show the versatility of CEPIA as an ER \(Ca^{2+}\) indicator.

**Imaging of SOCE.** SOCE is a ubiquitous process to replenish ER \(Ca^{2+}\) by inducing \(Ca^{2+}\) influx from the extracellular space \(^{10}\), and has been shown to play an important role in the activation of immune cells \(^9\). SOCE has been previously analysed by measuring the increase in cytosolic \(Ca^{2+}\) concentration ([\(Ca^{2+}\)]\(_{cyt}\)) after store depletion by SERCA inhibitors, such as thapsigargin and...
cyclopiazonic acid (CPA). However, it remains unclear whether SOCE induces an increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) under physiological conditions. Simultaneous \(\text{Ca}^{2+}\) imaging in the ER and cytosol using G-CEPIA1er and fura-2, respectively, provides a direct means to analyse \(\text{Ca}^{2+}\) dynamics during SOCE.

We found that stimulation of HeLa cells with histamine in the absence of extracellular \(\text{Ca}^{2+}\) induced an increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) and concomitant partial depletion of \([\text{Ca}^{2+}]_{\text{ER}}\) (Fig. 4a, black lines). Removal of histamine resulted in a shift of \([\text{Ca}^{2+}]_{\text{cyt}}\) to \([\text{Ca}^{2+}]_{\text{ER}}\) in the continued absence of extracellular \(\text{Ca}^{2+}\). When \(\text{Ca}^{2+}\) was reintroduced to the extracellular space (‘\(\text{Ca}^{2+}\) add back’), a recovery of \([\text{Ca}^{2+}]_{\text{ER}}\) toward the pre-stimulation level was seen. During ER refilling with \(\text{Ca}^{2+}\), the increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) was extremely small (\(\Delta [\text{Ca}^{2+}]_{\text{cyt}} = 4.8 \pm 0.6 \text{nM; Fig. 4c,d}\) as compared with the large \([\text{Ca}^{2+}]_{\text{cyt}}\) increase (241 ± 14 nM) observed after SERCA blockade by CPA or thapsigargin (Fig. 4a and Supplementary Fig. 5i). ER refilling was blocked by Gd\(^{3+}\), an Orai1 inhibitor (Fig. 4b). When STIM1 was extrinsically expressed in HeLa cells, the \([\text{Ca}^{2+}]_{\text{cyt}}\) and \(\text{Ca}^{2+}\) recovery rate during ‘\(\text{Ca}^{2+}\) add back’ were significantly

**Figure 2** | Wave-like propagation of ER \(\text{Ca}^{2+}\) release visualized with G-CEPIA1er. (a) Time-lapse images of wave-like decrease in the ER \(\text{Ca}^{2+}\) concentration visualized with G-CEPIA1er. Perfusion of 10 μM histamine was started at 0 s. Scale bar, 20 μm. (b) Time course of ER \(\text{Ca}^{2+}\) dynamics along the white line in a. (c) Comparison of ER \(\text{Ca}^{2+}\) dynamics in two regions of interest in a. The fluorescence intensity was normalized by the initial intensity. Black line: region 1; green line: region 2. (d) The velocity of ER \(\text{Ca}^{2+}\) wave measured with G-CEPIA1er (n = 23, mean ± s.e.m.) or R-CEPIA1er (n = 20). For comparison, the velocity of cytosolic \(\text{Ca}^{2+}\) wave measured with fluo-4 in cells without (n = 8) or with R-CEPIA1er expression (Fluo-4 + R-CEPIA1er; n = 6). There was no significant statistical difference among these values (P = 0.93, one-way ANOVA).

**Figure 3** | Activity-dependent ER \(\text{Ca}^{2+}\) dynamics in cerebellar Purkinje cells visualized with G-CEPIA1er. (a) G-CEPIA1er-expressing Purkinje cells in the cerebellar slice. Scale bars, 50 μm (left) and 5 μm (right). (b) PF-induced ER \(\text{Ca}^{2+}\) dynamics in the dendrites of Purkinje cells. Time course of mean \(\Delta F/F_0\) within the white circle (indicated in the left image) indicates fluorescence decrease upon PF inputs (10 stimuli at 100 Hz, grey line). The pseudo-colour image that is the average of 10 consecutive frames (indicated as magenta in the time course of \(\Delta F/F_0\)) show local dynamics of luminal \(\text{Ca}^{2+}\). Scale bar, 10 μm. (c) PF-induced response in single spine of Purkinje cells. Representative time course of \(\Delta F/F_0\) within a spine indicated by the arrow. PF inputs (10 stimuli at 100 Hz, gray line) elicited ER \(\text{Ca}^{2+}\) release within the spine. Scale bar, 2 μm.
Figure 4 | Visualization of ER Ca$^{2+}$ dynamics during SOCE. (a,b) Ca$^{2+}$ dynamics in the ER (lower panels) and cytosol (upper panels) during SOCE. After histamine (10 μM)-induced Ca$^{2+}$ release in the absence of extracellular Ca$^{2+}$, SOCE was induced by ‘Ca$^{2+}$ add back’, the reintroduction of Ca$^{2+}$ in the extracellular solution (black). To evaluate the contribution of SERCA-dependent Ca$^{2+}$ uptake by the ER, CPA was applied as indicated to the extracellular solution (a, magenta). The ER Ca$^{2+}$ refilling was inhibited by Gd$^{3+}$ (10 μM), an inhibitor of Orai, during ‘Ca$^{2+}$ add back’ (b, magenta). (c) Magnified [Ca$^{2+}$]$_{cyt}$ traces during ‘Ca$^{2+}$ add back’ in the upper panel of b. (d) Changes in the ER Ca$^{2+}$ refilling rate and [Ca$^{2+}$]$_{cyt}$ in response to ‘Ca$^{2+}$ add back’ with or without Gd$^{3+}$ application. Left, the slope of linear fitting to the G-CEPIA1r fluorescence change during the intervals $T_1$ to $T_3$ in b (lower panel) was obtained, and the indicated differences are shown. Right, the average [Ca$^{2+}$]$_{cyt}$ during the intervals $T_1$, $T_2$ and $T_3$ in b (upper panel) and c was obtained, and the indicated differences are show. n = 35 for control and 42 for Gd$^{3+}$ (mean ± s.e.m.). ***$P<0.001$.

CEPIA imaging simultaneous with other fluorescent molecules. Simultaneous time-lapse imaging of [Ca$^{2+}$]$_{ER}$ and other cellular processes should provide valuable information. We examined the possibility of simultaneously measuring [Ca$^{2+}$]$_{ER}$ and the subcellular distribution of STIM1, which senses the ER luminal Ca$^{2+}$ concentration and, upon ER Ca$^{2+}$ depletion, translocates to the subplasma membrane region to oligomerize$^{10}$. Accumulating evidence suggests that the oligomerization of STIM1, which forms as a punctate structure of fluorescent protein-tagged STIM1, is a key determinant of SOCE$^{37}$.

We measured [Ca$^{2+}$]$_{ER}$ using GEM-CEPIA1r and simultaneously imaged STIM1 dynamics using mCherry-STIM1 (Fig. 5a,b and Supplementary Movie 4). Upon addition of histamine in the absence of extracellular Ca$^{2+}$, [Ca$^{2+}$]$_{ER}$ began to decrease in an almost linear fashion. The formation of STIM1 puncta was considerably delayed, being observed only after [Ca$^{2+}$]$_{ER}$ fell below 600 μM. Thereafter, puncta formation proceeded alongside further decreases in [Ca$^{2+}$]$_{ER}$. When Ca$^{2+}$ was added back to the extracellular solution after histamine washout, [Ca$^{2+}$]$_{ER}$ refilling began and the mCherry-STIM1 puncta decomposed. The relationship between [Ca$^{2+}$]$_{ER}$ and puncta formation/dissociation exhibits considerable hysteresis. The difference of $K_{1/2}$ between puncta formation and dissociation was also observed using G-CEPIA1er and mCherry-STIM1 (Supplementary Fig. 5k,l). These observations indicate that, as far as [Ca$^{2+}$]$_{ER}$-dependent puncta formation of STIM1 is concerned, there is a threshold-like behaviour and suggest the existence of a mechanism that produces the delay in the dissociation of STIM1 puncta. Thus, CEPIA enables quantitative analysis of relationship between [Ca$^{2+}$]$_{ER}$ and STIM1 dynamics.

Intercellular heterogeneity of mitochondrial Ca$^{2+}$ signalling. We examined whether CEPIA indicators can be utilized for Ca$^{2+}$ imaging in mitochondria. To cover the broad range of mitochondrial Ca$^{2+}$ concentrations (0.05–300 μM)$^{18,38,39}$, three CEPIA variants with different Ca$^{2+}$ affinities were selected from the library: CEPIA2 ($K_d = 0.16$ μM), CEPIA3 (11 μM) and CEPIA4 (59 μM; Fig. 6a, Supplementary Fig. 2f and Supplementary Table 1). These indicators were expressed in HeLa cells by adding the mitochondrial localization signal sequence$^{40}$ (CEPIA2mt, CEPIA3mt and CEPIA4mt) to their coding sequences. Expression colocalized with MitoTracker Red and was distinct from the ER (Fig. 6b).

Histamine application to mobilize ER Ca$^{2+}$ induced a rapid increase in mitochondrial Ca$^{2+}$ levels followed by a slow decay (Fig. 6c; black line). This mitochondrial Ca$^{2+}$ signal was abolished when the mitochondrial membrane potential was dissipated by carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (Fig. 6c; magenta line).

Only a subpopulation of cells showed a mitochondrial Ca$^{2+}$ response, and the fraction of responding cells decreased with the decreasing affinities of the CEPIAmt (Fig. 6d,e and Supplementary Fig. 7a). Enhancement of mitochondrial Ca$^{2+}$, either by extrinsic expression of MCU or by suppression of Na$^+$/Ca$^{2+}$ exchanger-mediated mitochondrial Ca$^{2+}$ clearance by CGP-37157, resulted in a striking increase in the fraction of cells displaying Ca$^{2+}$ signals. Under these conditions, sustained or oscillatory mitochondrial responses were observed in a large fraction of cells, and obvious saturation of CEPIAmt was
observed in many cells (Fig. 6d and Supplementary Fig. 7a). We conclude that CEPIA3mt successfully reports mitochondrial Ca\(^{2+}\) signals, and that mitochondrial Ca\(^{2+}\) dynamics during agonist stimulation have considerable cell-to-cell variation.

**Subcellular heterogeneity of mitochondrial Ca\(^{2+}\) response.** In a fraction of cells, agonist stimulation elicited a mitochondrial Ca\(^{2+}\) response throughout the cell. In the remaining cells, only a subpopulation of mitochondria responded with a Ca\(^{2+}\) increase. For example, in the cell shown in Fig. 7a–c and Supplementary Movie 5, mitochondrial Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{mt}}\)) measured with CEPIA3mt increased in region 2; the size of the increase is three times greater than that averaged over the entire cell. In contrast, there was no increase in [Ca\(^{2+}\)]\(_{\text{mt}}\) in region 1. To exclude the possibility of regional variation in the CEPIA3mt response, we applied a Ca\(^{2+}\) ionophore, ionomycin. The same response was observed in both regions 1 and 2 (Supplementary Fig. 7b,c). Subcellular heterogeneity in mitochondrial Ca\(^{2+}\) dynamics was also visualized by CEPIA2mt (Supplementary Fig. 7d–f). These results indicate that there is a considerable subcellular variation in the capacity of mitochondria to take up Ca\(^{2+}\).

We tested a possible role for the ER in this inhomogeneous mitochondrial Ca\(^{2+}\) response, since it has been postulated that ER Ca\(^{2+}\) release is important for mitochondrial Ca\(^{2+}\) responses\(^5\). To this end, simultaneous Ca\(^{2+}\) imaging of the cytosol, ER and mitochondria was performed using GEM-GECO1, G-CEPIA1er and R-GECO1mt, respectively. Agonist stimulation induced a marked increase in [Ca\(^{2+}\)]\(_{\text{mt}}\) in region 2 but not in adjacent mitochondria, including those in region 1. In contrast to the inhomogeneous mitochondrial response, [Ca\(^{2+}\)]\(_{\text{ER}}\) and [Ca\(^{2+}\)]\(_{\text{cyt}}\) showed similar responses in regions 1 and 2 (Fig. 7d–f and Supplementary Movie 6). We also examined the subcellular heterogeneity in mitochondrial pH or membrane potentials (Ψ\(_{\text{m}}\)) as a potential mechanism of heterogeneous mitochondrial Ca\(^{2+}\) responses\(^6\). Simultaneous measurement of [Ca\(^{2+}\)]\(_{\text{mt}}\) and pH\(_{\text{mt}}\) indicated that inhomogeneous Ca\(^{2+}\) responses are not due to pH changes (Supplementary Fig. 7g–j). Neither did we find any heterogeneity in Ψ\(_{\text{m}}\) corresponding to mitochondrial Ca\(^{2+}\) responses (Supplementary Fig. 8). These results suggest that the inhomogeneous mitochondrial Ca\(^{2+}\) response is generated by heterogeneity in the strength of ER-mitochondrial coupling or in the regulation of Ca\(^{2+}\) uptake protein rather than in the amount of Ca\(^{2+}\) released from the local ER or in pH or Ψ\(_{\text{m}}\).

**Discussion**

Ca\(^{2+}\) concentrations within the ER and mitochondria control cytosolic Ca\(^{2+}\) dynamics and regulate cell functions including ER stress and cell death\(^2,4\). Methods such as CEPIA that obtain direct information about intracellular Ca\(^{2+}\) dynamics are therefore of great importance. The merits of CEPIA indicators for imaging ER or mitochondrial Ca\(^{2+}\) concentrations can be summarized as follows. First, they have very high dynamic range and spatiotemporal resolution. This has allowed, for instance, the first imaging of an inverse Ca\(^{2+}\) wave and synaptic activity-dependent ER Ca\(^{2+}\) dynamics in neurons. Second, CEPIA allows simultaneous Ca\(^{2+}\) imaging in the ER, mitochondria and cytosol with subcellular resolution. Third, CEPIA indicators are applicable to many cell types including intact neurons. Fourth, ratiometric measurement is possible with GEM-CEPIA1er, whereby intracellular Ca\(^{2+}\) concentrations can be determined independent of the indicator’s expression level. Fifth, the high signal-to-noise ratio and one-wave length measurement with G-CEPIA1er and R-CEPIA1er reduce the technical difficulty of organellar Ca\(^{2+}\) imaging and obviates the
requirement for a special imaging apparatus. Thus, CEPIA-mediated imaging methods should have broad utility in many cell biological studies. Indeed, work described in the paper addresses important cell functions.

Influx of Ca\(^{2+}\) from the extracellular space to the cytoplasm through SOCE is found in many types of cells\(^9\),\(^44\). The inhibition of SERCA (an ER Ca\(^{2+}\) pump) by pharmacological means, such as thapsigargin and CPA, to deplete ER Ca\(^{2+}\) has been the standard method to study the SOCE mechanism\(^9\). Now, using CEPIA, we have studied SOCE under physiological conditions, without SERCA inhibition. This revealed that [Ca\(^{2+}\)]\(_{\text{cyt}}\) is held constant during SOCE activation in HeLa cells, although [Ca\(^{2+}\)]\(_{\text{ER}}\) is sufficiently low to induce significant [Ca\(^{2+}\)]\(_{\text{cyt}}\) increase when SERCA is inhibited. These results indicate that the balance between the SOCE and SERCA activities is important for the capacity of SOCE to induce sustained increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\). In HeLa cells, SERCA activity is sufficiently high to take up Ca\(^{2+}\) influx through SOCE. However, the balance can differ between cell types, and if it favors Ca\(^{2+}\) influx there may be a large increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) during SOCE activation. Indeed, we found that the balance favors Ca\(^{2+}\) influx in Jurkat T cells, being consistent with the importance of SOCE in immune cells\(^9\).

SOCE is a remarkable mechanism, in which [Ca\(^{2+}\)]\(_{\text{ER}}\) regulates the Ca\(^{2+}\) permeability in the plasma membrane. The ER Ca\(^{2+}\) transducer of SOCE is STIM1, which senses [Ca\(^{2+}\)]\(_{\text{ER}}\) and translocates to the subplasmalemmal ER domain to form punctate structures and recruits Orai1 for Ca\(^{2+}\) influx through the plasma membrane. The steady-state [Ca\(^{2+}\)]\(_{\text{ER}}\) dependence of the amplitude of ionic current carried by SOCE (I\(_{\text{CRAC}}\)) and the subplasmalemmal translocation of STIM1 has been quantified\(^45\). I\(_{\text{CRAC}}\) and STIM1 translocation were found to be nonlinear functions of [Ca\(^{2+}\)]\(_{\text{ER}}\) with a Hill coefficient of \(~4\) and a K\(_{1/2}\) of \(~200\) \(\mu\)M. This is consistent with the dissociation constant of the Ca\(^{2+}\)-binding domain (EF-SAM) of STIM1 measured with a \(45\)Ca\(^{2+}\)-binding assay (K\(_d\) \(\approx\) \(250\) \(\mu\)M)\(^46\). STIM1 deoligomerization for the termination of SOCE is thought to be mediated by ER Ca\(^{2+}\) refilling\(^10\). Comparisons of the [Ca\(^{2+}\)]\(_{\text{ER}}\) dependence of STIM1 puncta formation and dissociation have shown that K\(_{1/2}\) of STIM1 puncta dissociation is lower than that of formation\(^42\)\(^48\). However, in these studies, STIM1 dynamics and [Ca\(^{2+}\)]\(_{\text{ER}}\) had to be measured in separate cells, because both signals were measured using FRET between YFP and CFP. We found that extrinsic expression of STIM1 significantly increases both [Ca\(^{2+}\)]\(_{\text{ER}}\) and ER Ca\(^{2+}\) refilling rate during SOCE. Thus, the comparison between cells with and without STIM1 expression requires caution. To circumvent this problem, we simultaneously measured STIM1 puncta formation/dissociation and [Ca\(^{2+}\)]\(_{\text{ER}}\) in the same cells. Both STIM1 puncta formation and dissociation were highly nonlinear functions of [Ca\(^{2+}\)]\(_{\text{ER}}\). Furthermore, the K\(_{1/2}\) of [Ca\(^{2+}\)]\(_{\text{ER}}\) for puncta dissociation was greater than that for formation (530 and 350 \(\mu\)M for dissociation and formation, respectively). These results indicate that the STIM1

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Figure 6 | Intercellular heterogeneity of mitochondrial Ca\(^{2+}\) imaging visualized with CEPIA. (a) Ca\(^{2+}\) titration curves of three mitochondria-targeted CEPIA variants (CEPIA2mt, K\(_d\) = 160 \(n\)M, F\(_{\text{max}}\)/F\(_{\text{min}}\) = 1.7; CEPIA3mt, 11 \(\mu\)M, 1.6; CEPIA4mt, 56 \(\mu\)M, 1.5). The measurements were performed at pH 8.0. (b) Representative images of HeLa cells expressing CEPIA2mt (left) co-stained with MitoTracker Red (middle). The merged images are shown in the right panels. The areas within the white boxes were expanded (lower). Scale bars, 10 \(\mu\)m (upper) and 2 \(\mu\)m (lower). (c) Time courses of mitochondrial Ca\(^{2+}\) response using CEPIA2mt in HeLa cells prestimulated with FCCP (magenta) or DMSO (black). Representative trace of 11 cells for vehicle only (DMSO) and 15 cells for FCCP. (d) Representative traces of mitochondrial Ca\(^{2+}\) dynamics upon stimulation with histamine (10 \(\mu\)M) in HeLa cells. To enhance mitochondrial Ca\(^{2+}\) signal, rat MCU was extrinsically expressed (+ MCU, blue) or the inhibitor of Na\(^{+}\)/Ca\(^{2+}\) exchanger CGP-37157 (10 \(\mu\)M) was applied (+ CGP, magenta). (e) The percentage of cells with mitochondrial Ca\(^{2+}\) responses upon histamine application measured with CEPIA2–4mt among control HeLa cells, MCU-expressing cells (+ MCU) and CGP-37157 (10 \(\mu\)M)-pretreated cells (+ CGP).
The deoligomerization process is not a simple reversal reaction of STIM1 oligomerization.

Within neurons the ER forms a continuous network throughout the cell (described as a ‘a neuron within a neuron’ that produces slowly propagating regenerative Ca\(^{2+}\) signals using a conduction system based on IP3R and RyR\(^{49,50}\). However, neuronal ER Ca\(^{2+}\) dynamics have previously been estimated only indirectly, by cytosolic Ca\(^{2+}\) imaging. We have imaged Ca\(^{2+}\) dynamics in the neuronal ER, in response to synaptic inputs to Purkinje cell dendrites in cerebellar slice preparations. Neuronal ER Ca\(^{2+}\) dynamics are important for physiological functions such as synaptic plasticity\(^{50}\), but also for pathological states including neurodegenerative diseases\(^{4,51}\). Thus, the application of CEPIA to intact neurons should provide a new imaging modality to analyse brain function.

Accumulating evidence suggests that Ca\(^{2+}\) dynamics in mitochondria are involved in the regulation of cell physiology and pathology, including autophagy, cell death, ATP synthesis, mitochondrial morphology and neurodegenerative diseases\(^{2,8}\). Mitochondria constantly undergo fusion and fission for the maintenance of functions\(^{52}\). Thus, subcellular inhomogeneity in mitochondrial functions is of great importance. Intra-cellular inhomogeneity in mitochondrial Ca\(^{2+}\) dynamics was first proposed based on mitochondria-targeted aequorin measurements\(^{53}\), although these measurements did not have spatial resolution, and the inhomogeneity was inferred from the partial consumption of aequorin. Later, mitochondria-targeted GECIs were used to image the subcellular mitochondrial Ca\(^{2+}\) response following agonist-induced ER Ca\(^{2+}\) release\(^{17,18,23}\). These studies revealed that, after a rapid increase in [Ca\(^{2+}\)]\(_{\text{mt}}\) in response to agonist stimulation, there is a considerable variation in the decay time course among the subpopulation of mitochondria within the cell, and a few minutes after the agonist stimulation only a minor subpopulation of mitochondria retained Ca\(^{2+}\) signals.

Figure 7 | Subcellular heterogeneity of mitochondrial Ca\(^{2+}\) response. (a) Fluorescence image of a CEPIA3mt-expressing HeLa cell. The area within the white box was expanded. Scale bars, 10 \(\mu\)m (left) and 2 \(\mu\)m (right). (b) Time courses of mitochondrial Ca\(^{2+}\) signal during 10 \(\mu\)M histamine application within the two regions of interest shown in (a). Time course averaged over the entire cell was also shown (Global). Averaged fluorescence images at resting state (\(T_1\), left blue box) and after histamine application (\(T_2\), right blue box) were indicated in (c). (c) Upper, averaged fluorescence images at resting state (\(T_1\), left) and after histamine application (\(T_2\), right) as indicated in (b). Lower, time-dependent changes in the fluorescence intensity were shown in pseudo-colour. Scale bar, 2 \(\mu\)m. (d) The fluorescence images of a HeLa cell expressing G-CEPIA1er (green) and mitochondria-localized R-GECO1 (R-GECO1mt, magenta). The area within the white box was expanded. Scale bars, 5 \(\mu\)m (left) and 1 \(\mu\)m (right). (e) Time courses of Ca\(^{2+}\) signal in the mitochondria, ER and cytosol in a HeLa cell stimulated with 10 \(\mu\)M histamine within the two regions of interest indicated in (d). Time courses averaged over the entire cell were also shown (Global). (f) The images of R-GECO1mt (magenta) and G-CEPIA1er (green) at three time points were shown. Representative images of six cells. Scale bar, 1 \(\mu\)m.
the increase in \([\text{Ca}^{2+}]_{\text{mt}}\) (refs 17,54,55). Our results showed that there is considerable intercellular and intracellular inhomogeneity in mitochondrial \(\text{Ca}^{2+}\) responses after agonist-induced \(\text{Ca}^{2+}\) release from the ER through IP_3Rs, despite the fact that consistent cytosolic \(\text{Ca}^{2+}\) increases were observed. These results are consistent with the observation using a small molecular \(\text{Ca}^{2+}\) indicator^4. Furthermore, simultaneous \(\text{Ca}^{2+}\) imaging of the ER and mitochondria did not show any inhomogeneous ER \(\text{Ca}^{2+}\) release. The inhomogeneity of the resting \(\text{Ca}^{2+}\) also did not correlate with the inhomogeneity of \(\text{Ca}^{2+}\) signal. These findings indicate that mitochondrial \(\text{Ca}^{2+}\) responses involve more than simple, passive uptake of cytosolic \(\text{Ca}^{2+}\); there must be a regulatory mechanism for \(\text{Ca}^{2+}\) uptake. One possibility is that recently identified MCU^2 and its associate proteins such as mitochondrial calcium uptake 1 (MICU1)^35, Mitochondrial Calcium Unipporter 1 (MCUR1)^36, MCU regulatory subunit (MCUb)^37 and essential MCU regulator (EMRE)^38 are inhomogeneously distributed, allowing regulated in a subcellular region-specific manner. Another possibility is that \(\text{ER}\)-mitochondria tethering proteins such as mitofusin 2 (ref. 8) are distributed in a distinct manner. Nevertheless, the other region-specific transfer of \(\text{Ca}^{2+}\) from the ER to mitochondria at the ER-mitochondrial junction^6.7. Further study using CEPIA will provide clues to the mechanism coupling mitochondria and the ER.

We created 58 variants of cfGCaMP2 and studied how their fluorescence intensity related to \(\text{Ca}^{2+}\) levels. This work sheds light on the structure–function relationship of CaM-based indicators. Previous studies have reported that highly conserved glutamate residues at \(\text{Z}\) position in each EF-hand motif are key determinants of the \(\text{Ca}^{2+}\) sensitivity of CaM-based \(\text{Ca}^{2+}\) indicators^11,13,59. Here we further established that the substitution of glutamate with a non-acidic amino acid (E31A or E31Q) reduces not only the \(\text{Ca}^{2+}\) binding affinity but also the dynamic range of the indicator. In contrast, conservative substitutions to aspartate reduced the \(\text{Ca}^{2+}\) binding affinity, as illustrated in Fig. 1a, cyan and dark blue circles. These findings suggest that acidic residues in \(\text{Z}\) positions of each EF-hand motif are key determinants of the \(\text{Ca}^{2+}\) binding affinity, which led to the increase in \(\text{Ca}^{2+}\) signals. In particular, we found that \(\text{Ca}^{2+}\) binding affinity and dynamic range are synergistically reduced when acidic substitutions are combined. This result suggests that acidic substitutions are essential. Accordingly, we created a variant where we replaced the CaM domain of G-GECO1.1 with CEPIA1 (Fig. 1a and Supplementary Table 1), which we designated R-CEPIA1. We then examined whether combinations of \(\text{Z}\) substitutions with acidic residues had a synergetic effect on the \(\text{Ca}^{2+}\) binding affinity and dynamic range.

**Development of CEPIA indicators.** \(\text{Ca}^{2+}\) affinity of cfGCaMP2 is primarily determined by calmodulin (CaM), which has four EF-hand motifs. We first tried the amino-acid substitutions that were used to reduce \(\text{Ca}^{2+}\) affinity of the FRET-type \(\text{Ca}^{2+}\) indicator, D1ER^5, but the \(K_{\text{d}}\) of cfGCaMP2 increased only to 14.5 \(\mu\text{M}\) (Supplementary Fig. 1a, grey circle). Amino-acid substitutions at \(\text{Z}\) position (12th amino-acid residues) of the EF-2 and EF-4 

## Methods

**Gene construction.** For creating a CEPIA library, we cloned cfGCaMP2 (GCaMP2 with amino-acid substitutions of M36L in CaM, and N105Y and E124V in circular permuted enhanced GFP (pEGFP)) with ER retention signal sequence (SEKDEL) into a bacterial expression vector, pET70b (Novagen, USA), using primers 1 and 2 (Supplementary Table 2). G-GECO1.1, R-GECO1 and GEM-GECO1 were also cloned into pET7b by using pET7b and primers 3 and 4. The CaM sequences in G-GECO1.1, R-GECO1 and GEM-GECO1 were swapped with that in cfGCaMP2 variants using primer 4–7. Site-directed mutagenesis was performed by PCR using primers 8–36. For mammalian expression of CEPIA variants in the ER and mitochondria, we cloned CEPIA into pCMV/myc/ER and pCMV/myc/mito vector (Invitrogen, USA) using primers 3, 4 and 37–39. For mitochondrial \(\text{Ca}^{2+}\) imaging, we enhanced the specificity of mitochondrial localization by attaching the mitochondria targeting sequences in tandem^50 to CEPIA–4amt, R-GECO1mt and GEM-GECO1mt using primers 40–47. To enhance protein expression, GEM–CEPIA1er was cloned into the CAG promoter-containing vector, pCS, using primers 48 and 49. To express G-CaPIA1er in the Purkinje cells, G-CaPIA1er was cloned into pSinRep5 (Invitrogen) using primers 50–53. To localize EGFP and mCherry in the ER, EGFP and mCherry were cloned into pDNA3 D1ER^19 to attach calreticulin signal sequence using primers 54–59. To construct EYFP-er, EYFP was cloned from pDNA3-YCter^13 into pCMV/myc/ER vector using primers 60 and 61. To construct SypHer-dmito, we added an amino-acid substitution of C199S (ref. 62) in pHyper-dmito (Evrogen, Russia) using primers 62 and 63. To construct mCherry-STIM1, STIM1 was cloned from pApuro-GFP-STIM1 (ref. 63) into pShuttle2 vector using primers 60 and 64–66.

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buffer containing 0 or 1 mM EGTA at pH 7.2. The Ca\(^{2+}\) concentration carried over from the recombinant protein samples was estimated to be 2 μM. In the presence of 1 mM EGTA, the \([Ca^{2+}]_{\text{free}}\) at pH 7.2 was calculated as follows:

\[
\frac{[Ca^{2+}]_{\text{free}}}{[Ca^{2+}]_{\text{total}}} = \left( \frac{-D + (4 \times K_d) + (4 \times D)^2}{2} \right) \cdot \frac{100}{100 - 100},
\]

where \(D = [EGTA] - [Ca^{2+}]_{\text{free}}\cdot K_d\).

\([Ca^{2+}]_{\text{free}}\) was calculated as:

\[
[Ca^{2+}]_{\text{free}} = \frac{[Ca^{2+}]_{\text{free}}}{[Ca^{2+}]_{\text{total}}} \cdot [Ca^{2+}]_{\text{total}} = 150.5 \text{ nM}.
\]

To perform in situ Ca\(^{2+}\) titration of CEPIA, we permeabilized the plasma membrane of HeLa cells with 150 μM β-escin (Nacalai Tesque, Japan) in a solution containing (in mM) 140 KCl, 10 NaCl, 1 MgCl\(_2\) and 20 HEPES (pH 7.2). After 4 min, we applied various Ca\(^{2+}\) concentrations in the presence of 0.1 mM iomomycin and 3 μM thapsigargin and estimated the maximum fluorescent intensity (\(R_{\text{max}}\)) and minimum fluorescent intensity (\(R_{\text{min}}\)), dynamic range (\(R_{\text{max}}/R_{\text{min}}\)) and Hill coefficient (\(n\)).

\[\frac{[Ca^{2+}]_{\text{free}}}{[Ca^{2+}]_{\text{total}}} \cdot [Ca^{2+}]_{\text{total}} = 150.5 \text{ nM}.
\]

Animal experiments. All animal experiments were carried out in accordance with the regulations and guidelines for institutional Animal Care and Use Committee at The University of Tokyo and were approved by the institutional review committee of the Graduate School of Medicine, The University of Tokyo.

Cell culture. HeLa cells, HEK293A cells, BHK cells and astrocytes were cultured on collagen-coated dishes in DMEM supplemented with 10% foetal bovine serum (FBS), 200 U/ml penicillin and 200 μg/ml streptomycin (P/S) at 37°C in a humidified CO\(_2\) incubator (5% CO\(_2\)). For cytosolic transient calcium imaging, the cells were transfected using Lipofectamine 2000 (Invitrogen). Subsequently, the cells were seeded on collagen-coated dishes in DMEM supplemented with 10% foetal bovine serum, and the loading buffer containing 0 or 1 mM EGTA at pH 7.2. The Ca\(^{2+}\) concentration carried over from the recombinant protein samples was estimated to be 2 μM. In the presence of 1 mM EGTA, the \([Ca^{2+}]_{\text{free}}\) at pH 7.2 was calculated as follows:

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prepared[9]. Mice were anaesthetised with diethyl ether and decapitated. The brain was removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) bubbled with 95% O2/5% CO2. Slices were cut using a microslicer (PRO 7, Dosaka EM, Japan). The slices were incubated in a holding chamber containing ACSF bubbled with 95% O2 and 5% CO2 at 35 °C for 1 h and then returned to 23 °C. ACSF for slicing and incubation contained (in mM) 125 NaCl, 2.5 KCl, 2 CaCl2, 1 MgSO4, 1.25 NaH2PO4, 26 NaHCO3 and 20 glucose. Slices were transferred to a recording chamber under a microscope, and continuously perfused with ACSF supplemented with 10 μM bicusculine (Tocris Bioscience, UK) and 10 μM NBQX (Tocris Bioscience) to block inhibitory postsynaptic potentials and accompanying Ca2+ influxes. ACSF was bubbled with 95% O2 and 5% CO2.

Imaging was carried out with a two-photon microscope (TSC MP5, Leica) equipped with a water-immersion objective (×25, NA 0.95, HCS IR APO, Leica) and a Tissapphire laser (MatTai DeepSee; Spectra Physics, USA). Excitation wavelength was 900–920 nm for both G-CEPIA1 and EYFP-er. Data were acquired with time-lapse XY-scan mode (8 Hz) and analysed using ImageJ software. Fluorescence intensities were corrected for background fluorescence by measuring a non-fluorescent area. When necessary, photobleaching was corrected for using a linear fit to the fluorescence intensity change. For the focal stimulation of parallel fibres, square pulses (0.1 ms) were applied through stimulation pipettes (3–6 μm tip diameter) filled with ACSF. The stimulation intensity was adjusted within 4–5 V to induce G-CEPIA1er signals with a range of ~20 μm in diameter. For EYFP-er, the stimulation intensity was fixed at 5 V. Experiments were carried out at room temperature.

Statistics. Two-tailed Student’s t-tests were performed to determine the significance if not stated otherwise.

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**Acknowledgements**

We thank the following researchers for providing the plasmid vectors: T. Nagai for R-GECO1, R. Tsien for D1ER and D. Ino for pCIS and MCU. We also thank Y. Kawashima for technical assistance. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology, Japan and Takeda Science Foundation.

**Author contributions**

J.S. and Y.O. performed experiments and analysed the data; J.S., K.K. and M.I. designed the experiment. J.S., K.K. and M.I. wrote the paper; K.I. and M.O. generated the cfGaMP2.

**Additional information**

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

**Competing financial interests:** The authors declare no competing financial interests.

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**How to cite this article:** Suzuki, J. et al. Imaging intraorganellar Ca\(^{2+}\) at subcellular resolution using CEPIA. *Nat. Commun.* 5:4153 doi: 10.1038/ncomms5153 (2014).

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