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Use of Genetically-encoded Calcium Indicators for Live Cell Calcium Imaging and Localization in Virus-infected Cells

Jacob L. Perry^{1,*}, Nina K. Ramachandran^{1,*}, Budi Utama², and Joseph M. Hyser¹

¹Department of Molecular Virology and Microbiology and Alkek Center for Metagenomic and Microbiome Research, Baylor College of Medicine, Houston, TX, 77030

²Shared Equipment Authority, Rice University, Houston, TX, 77030

Abstract

Calcium signaling is a ubiquitous and versatile process involved in nearly every cellular process, and exploitation of host calcium signals is a common strategy used by viruses to facilitate replication and cause disease. Small molecule fluorescent calcium dyes have been used by many to examine changes in host cell calcium signaling and calcium channel activation during virus infections, but disadvantages of these dyes, including poor loading and poor long-term retention, complicate analysis of calcium imaging in virus-infected cells due to changes in cell physiology and membrane integrity. The recent expansion of genetically-encoded calcium indicators (GECIs), including blue and red-shifted color variants and variants with calcium affinities appropriate for calcium storage organelles like the endoplasmic reticulum (ER), make the use of GECIs an attractive alternative for calcium imaging in the context of virus infections. Here we describe the development and testing of cell lines stably expressing both green cytoplasmic (GCaMP5G and GCaMP6s) and red ER-targeted (RCEPIA_{er}) GECIs. Using three viruses (rotavirus, poliovirus and respiratory syncytial virus) previously shown to disrupt host calcium homeostasis, we show the GECI cell lines can be used to detect simultaneous cytoplasmic and ER calcium signals. Further, we demonstrate the GECI expression has sufficient stability to enable long-term confocal imaging of both cytoplasmic and ER calcium during the course of virus infections.

Keywords

GCaMP5G; GCaMP6s; RCEPIA_{er}; endoplasmic reticulum; rotavirus; enterovirus; respiratory syncytial virus

Address correspondence to: Joseph M. Hyser, MS: BCM385, One Baylor Plaza, Houston, TX 77030-3411, joseph.hyser@bcm.edu, PH: 713-798-4514, FAX: 713-798-3586.

* Authors contributed equally to this work

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1. Introduction

Calcium (Ca^{2+}) is a ubiquitous eukaryotic secondary messenger involved in many cellular signal transduction pathways that control critical cellular processes. The importance of Ca^{2+} signaling and control of intracellular Ca^{2+} levels is highlighted by the large number of Ca^{2+} channels, pumps, transporters, buffering proteins, and Ca^{2+} -responsive signaling proteins evolved to transduce these signals (1). As obligate intracellular parasites, viruses have evolved a variety of mechanisms to exploit host cell Ca^{2+} signaling, which provides a replication advantage and is often an underlying cause of virus pathogenesis (2–4). The specific changes in Ca^{2+} homeostasis are different between different viral systems, but in general viruses often cause an elevation in the concentration of cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_c$) in conjunction with either depletion of endoplasmic reticulum Ca^{2+} ($[\text{Ca}^{2+}]_{\text{ER}}$) stores [e.g., rotavirus (RV)] or elevation of $[\text{Ca}^{2+}]_{\text{ER}}$ leading to disruption in Ca^{2+} signaling between the ER and mitochondria (e.g., hepatitis B virus) (5–8). More recent studies have demonstrated that virus-induced Ca^{2+} signaling can activate cellular processes, such as store-operated Ca^{2+} entry (SOCE), fluid secretion to cause diarrhea and serotonin release to cause vomiting, activation of the inflammasome leading to proinflammatory cytokine release, induction of the autophagy pathway, and disruption of the host cell's cytoskeleton (9). To characterize the roles Ca^{2+} signaling plays in virus replication and pathogenesis for these viruses, virologists have capitalized on the development of live cell Ca^{2+} imaging technology to track changes in Ca^{2+} levels during infection and to determine the molecular mechanisms used by viruses to induce these signals.

1.1. Live cell calcium imaging technology

Ca^{2+} signals are regulated both in terms of magnitude (how large was the Ca^{2+} flux) and time (how long does the flux last). In order to study both aspects of Ca^{2+} signaling, a wide range of fluorescent Ca^{2+} indicators have been developed to enable real time observations of changes in Ca^{2+} levels [reviewed in (10)]. All Ca^{2+} indicators are based upon fluorescent molecules that bind Ca^{2+} ions and have different spectral properties between the Ca^{2+} -free and the Ca^{2+} -bound states. There are two classes of fluorescent Ca^{2+} indicators: [1] small molecule dyes that are loaded into target cells and [2] genetically-encoded Ca^{2+} indicators (GECIs) that are engineered fluorescent proteins that are expressed within target cells. Both classes of indicators are briefly reviewed below.

1.1.1. Small molecule dyes—Small molecule fluorescent Ca^{2+} indicators were first developed in the early 1980s and are a mainstay for live-cell Ca^{2+} imaging by researchers due to their availability and versatility (11). There is a large variety of indicators to choose from, including: [1] single-wavelength dyes: Fluo-4 (and similar derivatives, Fluo-2, Fluo-8), [2] dual excitation dyes: Fura-2 (typically used for microscopy), and [3] dual emission dyes: Indo-1 (typically used for flow cytometry). The popularity of small molecule dyes is mainly due to their being readily available from several commercial sources, and relative ease for intracellular loading of the acetoxymethyl ester (AM)-modified versions.

Many Ca^{2+} imaging studies of virus infections have used the small molecule Ca^{2+} indicator dyes, primarily to track elevation of $[\text{Ca}^{2+}]_c$ over time using Fura-2 or Fluo-4 (12–14). We

have also used Indo-1 and Fluo-2 dyes to monitor $[Ca^{2+}]_c$ in cells expressing wild-type or mutant versions of the RV viroporin (viral ion channel) nonstructural protein 4 (NSP4) by flow cytometry (15, 16). However, there are several drawbacks to Ca^{2+} indicator dyes, including poor loading into some cell types, a tendency to become compartmentalized within subcellular organelles, and having poor retention or being subject to active efflux over time. Additionally, photobleaching that occurs over time limits the number of viable indicators that can be used, as they must be highly photostable. These potential abnormalities in dye loading/retention and dye photobleaching can be exacerbated due to the cytopathic effects of virus infection, which led us to explore GECIs as alternative indicators for Ca^{2+} imaging in virus-infected cells.

1.1.2. Genetically-encoded calcium indicators—GECIs are engineered hybrids between a fluorescent protein and a specific Ca^{2+} binding moiety, such that the spectral properties of the fluorescent protein are modified upon Ca^{2+} binding. There are three classes of GECIs: [1] bioluminescent sensors based on the aequorin protein, [2] Förster resonance energy transfer (FRET) based two-color fluorescent protein indicators, and [3] single fluorescent protein-based indicators. While there are many varieties of these GECIs [reviewed in (10, 17)], the function of both single color and the FRET based sensors are based on a similar mechanism, which uses a Ca^{2+} responsive element [typically calmodulin (CaM) and the M13 CaM-binding peptide] inserted into a fluorescent protein. In the case of the FRET based sensors, the CaM-M13 motif is inserted between CFP/YFP FRET pairs, such that upon Ca^{2+} binding the CaM conformational change brings CFP and YFP closer together resulting in increased FRET efficiency. In the case of the single color sensors, CaM and M13 are inserted at opposite termini of a circularly permuted (and therefore destabilized) fluorescent protein. Upon Ca^{2+} binding the N- and C- termini associate, stabilizing the chromophore and increasing the fluorescence emission (17).

The most commonly used GECIs used for imaging the relatively low Ca^{2+} levels found in the cytoplasm (~100 nM basal Ca^{2+}) are the green fluorescent protein (GFP) based GCaMPs and the related G-GECO1 variants (18, 19). These GECIs have low K_D values (GCaMP5G, 0.45 μ M; GCaMP6s, 0.144 μ M; and G-GECO1, 0.75 μ M) suitable for measuring cytosolic Ca^{2+} dynamics (~100 – 1000 nM) during Ca^{2+} signaling (1). These have been successfully used for Ca^{2+} imaging in cell lines, as well as *in vivo* in worms, flies, and mice (20). Plasmid vectors of popular green GECIs are available on Addgene, and commercially available adenovirus vectors (Vector BioLabs, Malvern, PA) or adeno-associated virus vectors (University of Pennsylvania Vector Core) have been made. Red-shifted (RCaMP1, RGECO1, and O-GECO) variants have been developed and are available through Addgene (21). Fewer blue-shifted GECIs also have been developed, and they have narrower dynamic ranges (~2–8 fold) than the green and red GECIs (19). Finally, the potential of GECIs for both *in vitro* and *in vivo* Ca^{2+} imaging has led to the rapid development and specialization of different GECIs, including optimization of the indicators' affinity for Ca^{2+} and addition of subcellular targeting motifs to make them appropriate for Ca^{2+} storage organelles (Ca^{2+} ranges between 100–1000 μ M) such as the ER and mitochondria (CEPIA). Several ER-targeted GECIs have recently been developed (K_D ~600 μ M) and are available as both green (GCEPIAer) and red-shifted (RCEPIAer) counterparts (22–24).

We have previously used transient expression GCaMP5G, a green fluorescent protein (GFP)-based GECI, to measure changes in $[Ca^{2+}]_c$ induced by RV NSP4 (8). Further, several previous studies have used ER or Golgi-targeted GECIs to examine Ca^{2+} in cells expressing the picornavirus 2B viroporin protein (25–27). However, the use of GECIs to monitor Ca^{2+} dynamics in virus-infected cells has not been reported. Thus, we have developed lentiviral vectors and methods to establish cell lines that stably express both cytoplasmic green (GCaMP5G or GCaMP6s) and ER-targeted red (RCEPIA_{er}) GECIs. The chosen GECIs were previously developed and characterized in cell lines and some cases for *in vivo* imaging (18, 20, 22). These cell lines we have developed enable simultaneous measurements of both cytoplasmic and ER Ca^{2+} levels, and can be used to investigate virus-induced changes in Ca^{2+} signaling, which we validate using three different viruses (RV, poliovirus and respiratory syncytial virus). Finally, the stable expression of these GECIs enables the use of confocal microscopy for long-term live cell Ca^{2+} imaging during the course of virus infections.

2. Materials and methods

General methods for the development of the GECI cell lines are outlined below and specific reagents are listed in Table 1. In general, the reagents listed have worked well for us, but likely can be substituted with similar reagents at the investigators discretion.

2.1. Cell lines and culture conditions

MA104 cells (African Green Monkey kidney cell line, ATCC CRL-2378.1) and HEK293FT cells were a kind gift from Dr. Mary Estes, Baylor College of Medicine. HeLa cells were a kind gift from Rick Lloyd, Baylor College of Medicine. Cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum, MEM non-essential amino acids, and penicillin/streptomycin at 37°C in 5% CO₂.

2.2. Vector development, cloning and preparation

Vectors pGP-CMV-GCaMP5G (Addgene plasmid #31788) and pGP-CMV-GCaMP6s (Addgene plasmid #40753) were kind gifts from Dr. Douglas Kim. The vector pCMV-RCEPIA_{er} (Addgene plasmid # 58216) was a kind gift from Masamitsu Iino. Lentivirus vectors were generated by subcloning the GCaMP5G or GCaMP6s genes into pLVX-puro and RCEPIA_{er} into pLVX-IRES-Hygro using standard molecular cloning procedures. Expression vectors were propagated in DH5α or NEB 5α™ *E. coli* and lentivirus vectors were propagated in either Stellar™ or Stbl2™ *E. coli* according to the manufacturer's instructions. Maxipreps were produced by inoculating a 5 mL LB broth culture, supplemented with either ampicillin or kanamycin as appropriate, with a single colony from a LB-agar plate streak out, and grown overnight at 37°C with shaking in 15 mL tubes with loose caps. The 5 mL culture was used to inoculate 200 mL of LB broth (with ampicillin or kanamycin) and again grown overnight at 37°C with shaking. Plasmids were purified using the Hi-Speed Plasmid Maxi Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Vectors for lentivirus packaging used were psPAX2, a gift from Didier Trono (Addgene plasmid #12260), and a pCMV-VSV-G, a gift from Bob Weinberg (Addgene plasmid #8454). These were propagated by transforming Stbl2 *E. coli* and plated on LB-agar plates with ampicillin according to the manufacturer's protocol. Well isolated colonies were directly inoculated into 200 mL LB with ampicillin and incubated at 37°C overnight with shaking. Plasmids were purified using the Hi-Speed Plasmid Maxi Kit (Qiagen) according to the manufacturer's instructions. Often yields of these plasmids are quite low, so we have found use of freshly transformed Stbl2 (or similar) *E. coli* critical for higher yields.

2.3. Lentivirus packaging

We have used a variety of systems for packaging of VSV-G pseudotyped lentivirus vectors, including our in-house core lab (Vector Development Core Lab, BCM, Houston, TX), commercial services (Cyagen Bioscience Inc.), and doing our own packaging within the lab. All of these have been successful, and we have included our protocol below. We now prefer producing our own vectors; however, optimizing high titer lentivirus vector yield may not be cost effective for labs that do not routinely use lentivirus or retrovirus vectors.

For packaging of the GECI lentivirus vectors, we have used both the Clontech Lenti-X system according to the manufacturer's instructions and the second generation psPAX2/VSV-G system (available through Addgene), which is presented below. We have used both Lipofectamine 2000 and polyethylenimine (PEI) with good results, and the choice of transfection reagent is likely not critical.

Protocol

1. Split confluent HEK293FT cells and seed $\sim 5 \times 10^6$ cells per 10-cm plate in 10 mL DMEM supplemented with 10% FBS and penicillin/streptomycin.
2. At 24 hours post seeding, the cells should be ~ 80 – 90% confluent to proceed with packaging.
3. To a sterile Eppendorf tube, add the following reagents, then mix by inverting the tube 5 times and incubate 10 minutes at room temperature.

a.	Opti-MEM	600 μ L
b.	psPAX2	7 μ g
c.	VSV-G	3 μ g
d.	GECI vector	14 μ g
e.	PEI (1ug/mL)	36 μ L

4. Without removing the media on the 10-cm plate, distribute the mixture drop-wise to the plate, tilt the plate to mix, and incubate at 37°C for 48 hours. Fluorescence should be detected by 24 hours post transfection and the transfection efficiency should be between 70–90% to expect high vector titers.

5. At ~48 hours post transfection, remove the media and filter through a 0.45 μm polyethersulfone (PES) filter, aliquot into single use vials and store at -80°C . Avoid freeze/thaw cycles, as this decreases viral titers.

A variety of methods can be used to determine the titer of the viral stocks, expressed as transduction units (TU) per mL. We frequently use Lenti-X GoStix as a rapid measure of good versus poor yields. Rough estimates of the titer are determined by transducing fresh HEK293FT cells, and determining the percent transduction by flow cytometry.

2.4. Transfection and/or transduction for stable GECI cell lines

We first generated stable lines expressing cytoplasmic GCaMPs, with GCaMP5G for MA104 (made prior to the release of GCaMP6s) and GCaMP6s for HeLa using lentivirus transduction (see protocol below). Then we introduced RCEPIA_{er} by lentivirus transduction (MA104) and selected with hygromycin or plasmid transfection (HeLa) and selected with G418 (500 $\mu\text{g/mL}$). Since transfection protocols are common, they are not reviewed here. Our lentivirus transduction protocol is presented below, including special considerations for making GECI cell lines since they are functional biosensors and therefore much more dim than fluorescently labeled proteins.

Protocol

1. Seed MA104 or HeLa at $\sim 5 \times 10^5$ cells/well in a 6-well plate in 3 mL DMEM supplemented with 10% FBS and penicillin/streptomycin. Incubate overnight.
2. Plan transductions for a multiplicity of infection (MOI) of ~ 10 –20. Higher MOIs can be used for difficult to transduce cells, but excess transduction could be detrimental to the cells, particularly due to the over-expression of the Ca^{2+} binding moieties in the GECIs. If the virus titer is low, it is better to use fewer cells (and therefore a smaller dish size, such as 5×10^4 cells/well in a 24-well plate) or to concentrate the virus, rather than doing a low MOI infection.
3. Mix DMEM + 10% FBS, lentivirus stock. Then, add 8 $\mu\text{g/mL}$ polybrene. It is best to keep the inoculum volume as low as possible (1.5–2 mL for 6-well plate).
4. Remove the maintenance media from the cells and distribute the inoculum drop-wise onto the cells. Incubate overnight.
5. Remove the inoculum and replace with fresh 10% FBS-DMEM. Monitor for GECI expression from 48–72 hours post-transduction.
 - a. GECIs are not as bright as standard fluorescent proteins, particularly GCaMP5G/6s because basal cytoplasmic Ca^{2+} is maintained to low levels. Thus, expression is not as obvious as GFP/RFP positive controls early after transduction.
 - b. When examining new transductions for fluorescence, it is helpful to replace the normal DMEM with phenol red-free media because the autofluorescence from phenol red masks the dim GCaMP signal. We commonly use FluoroBrite DMEM (LifeTechnologies), which is specialized high glucose DMEM for live-cell imaging.

6. Once fluorescence is detected (typically ~72 hours post-transduction), cells should be split 1:2 and selection drug added (GCaMP6s: 5 ug/mL puromycin; RCEPIAer: 100 ug/mL hygromycin). It is important to determine the minimal drug concentration needed for each cell line. Continue to grow cells with drug selection until the line is established.
7. If non-fluorescent/low fluorescent cells persist, then additional rounds of transduction can be performed or FACS sorting can be used to enrich for the desired expression levels, keeping in mind this is still a mixed population of cells. If a cloned cell line is desired, dilution cloning can be used to generate single-cell cloned sublines. However, it is important to note that lentivirus integration could inactivate genes important for the replication of some viruses and cloned cell lines should be compared to the parental cell line for normal virus replication prior to extensive studies.

The cell lines reported here were developed as follows:

MA104-GCaMP5G/RCEPIAer cells: We initially transduced with GCaMP5G lentivirus and after puromycin selection used FACS to enrich for the positive cells prior to transduction with the RCEPIAer-expressing lentivirus vector. Two rounds of RCEPIAer transduction (MOI 10) and selected with puromycin and hygromycin B to generate the reasonable RCEPIAer signal. Finally, this cell line was enriched for the top 50% RCEPIAer expressing cells by FACS. This step is particularly important for improving the uniformity of expression.

HeLa-GCaMP6s/RCEPIAer cells: We initially transduced with GCaMP6s lentivirus and selected with puromycin. No FACS enrichment was required after the initial GCaMP6s transduction and selection. RCEPIAer was introduced using plasmid transfection by Lipofectamine 2000 according to the manufacturer's protocol. Cells were selected with puromycin and G418 and then double-positive cells were enriched using FACS.

2.5. Live cell Ca^{2+} imaging

Once developed, the GECI-expressing cell lines have to be tested using known Ca^{2+} agonists to demonstrate expected fluorescence responses from the GECIs upon cell stimulation. Once proper GECI performance is validated, experiments comparing mock and virus-infected cells can be performed.

2.5.1. Validation and Ca^{2+} imaging of GECI cell lines—There are a number of commonly used Ca^{2+} agonists that work for testing GECI responses in most cells, including ATP (30–200 μM), histamine (30–100 μM), and carbachol (30–100 μM). Further, it may be important to determine the dynamic range of each GECI for each given cell type, since resting Ca^{2+} levels vary between different cell lines. Calibrating GCaMP5G/GCaMP6s can be performed using a Ca^{2+} ionophore (e.g., ionomycin) and Ca^{2+} -free or high Ca^{2+} buffers (17). However, in our hands 10 μM A23187 (a Ca^{2+} ionophore) in high Ca^{2+} Ringer's buffer (20 mM Ca^{2+} final) caused an irreversible loss of RCEPIAer signal, suggesting this GECI is not stable under these conditions (data not shown). The group that developed RCEPIAer performed *in vivo* Ca^{2+} calibration of RCEPIAer in HeLa cells by first permeabilizing the

plasma membrane with 150 μM β -escin and then adding various Ca^{2+} -containing buffers in the presence of 3 μM ionomycin and 3 μM thapsigargin (22).

Below are general principles for setting up Ca^{2+} imaging experiments using example treatments typical for studies of RV and picornaviruses; however, the details of the treatment will be determined by the goal of the experiments. There are many suitable options for microscope/light source, culture plates and perfusion setup, so only a few details are presented here. A number of helpful reviews on the general principles and equipment for Ca^{2+} imaging are available (17, 28). It is important to note that while these GECIs can be imaged using standard GFP and mCherry filter sets, these may not provide the optimal signal-to-noise ratio needed for a given experiment. We found this to be true for RCEPIAer on our confocal microscope setup due to a relatively narrow emission filter, consistent with the spectrum reported by the creators of RCEPIAer (22). Thus, depending on the resolution needed for the purpose of an experiment, filters should be customized for the GECI being used if possible. Alternatively, GECI expression can be optimized by additional lentivirus transduction and/or FACS enrichment for better signal (see above).

2.5.2. Virus infections—Cells were seeded into black optical bottom 96-well plates (Greiner Bio-One, Monroe, NC) ~24–48 hours prior to infection at 30,000 cells/well for MA104-GCaMP5G/RCEPIAer or 50,000 cells/well for HeLa-GCaMP6s/RCEPIAer. Cells were washed using serum free FluoroBrite DMEM supplemented with 15 mM HEPES, sodium pyruvate and penicillin/streptomycin, then infected with RV (SA114F), poliovirus (PV; poliovirus type I, Mahoney strain), or respiratory syncytial virus (RSV; A/Tracy) at an MOI=10, except for those presented in Figure 3 where the MOI is indicated. Infections were done in a 30–50 μL final volume per well for 1 hour. For perfusion experiments, the cells were washed with FluoroBrite, 100 μL added to each well and incubated at 37°C. Imaging is typically performed at ~6–8 hours post-infection (hpi) for RV and 4–5 hpi for PV. For long-term imaging, after the 1 hour infection, 70 μL of FluoroBrite alone or for the Ca^{2+} channel blocker studies in the presence of 0.1% DMSO, or 50 μM 2-APB or 10 μM KB-R7943 (dissolved in DMSO) was added to bring the volume to 100 μL per well.

2.5.3. Calcium imaging with buffer perfusion—Proper data collection is the most critical aspect of high quality Ca^{2+} imaging experiments. While imaging of single color GECIs does not require very rapid filter changes, simultaneous two color imaging of different GECIs does require reasonably rapid filter switching. We use a Zeiss AxioObserver A1 inverted fluorescence microscope (on an anti-vibration air table) with a Sutter DG5 Illumination and Filter box. For dual GCaMP5G/6s and RCEPIAer imaging, we use the Zeiss filter set 56 HE. The excitation filters (GCaMP5G/6s: 470/27; RCEPIAer: 556/25) are placed in the DG5 filter box. The dichroic cube holds the beam splitter (DFT 490 + 575) and dual bandpass filter (DBP 512/30 + 630/98). Our scope is equipped with a CoolSNAP CCD camera (Photometrics Scientific Inc) and computer control of the DG5 filter box and data acquisition is done by MetaFluor software (Molecular Devices). Images for GCaMP5G and GCaMP6s used 200 millisecond (ms) snaps (Gain = 1, Binning = 3) and RCEPIAer used 500 ms snaps (Gain = 1, Binning = 3) using a 40 $\times$ water immersion objective. However, many other suitable microscopes, lamps, and acquisition/analysis

software are available (28). For buffer perfusion, we use a simple gravity feed system composed of 60 mL syringes (with plungers removed) clamped onto a ring-stand with tubing fed into a multiline manifold (Warner Instruments, Hamden, CT), and suction from the plate/well is through a standard vacuum trap.

For treatment of cells with ATP to induce a Ca^{2+} signal, cells were perfused for 3–5 minutes in normal Ringer's buffer [160 mM NaCl, 4.5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES, pH 7.4 using 1M NaOH] or FluoroBrite DMEM prior to image acquisition and image acquisition was started with normal Ringer's buffer perfusion to measure basal fluorescence for 100–200 seconds. Then perfusion was changed to Ringer's buffer containing ATP (MA104 cells: 250 μM ; HeLa cells 30 μM) for ~100–150 seconds. Data was analyzed using ImageJ (29) by normalizing fluorescence intensity of selected regions of interest (ROIs) to the initial intensity value (F/F_0), and graphs were produced using Excel (Microsoft, Redman, WA) or GraphPad Prism (GraphPad Software, La Jolla, CA). All experiments were performed at least in triplicate with 3 or more replicates per condition.

2.5.4. Long-term live cell confocal Ca^{2+} imaging—For long-term confocal microscopy, cells were seeded into optical bottom 96-well plates and infected as described above. All of our live cell confocal imaging was performed using a Nikon A1-Rsi inverted confocal microscope with 40 $\times$ objective (NA 0.95, Nikon Instruments, Melville, NY) in a unidirectional multi-track mode without averaging. Cells were maintained in a humidified, 5% CO_2 environmental chamber at 37°C (OKOLab, Pozzuoli, Italy). A 488 nm Argon laser was used for GCaMP5G and GCaMP6s imaging with a 500–550 nm emission filter. A 561 nm diode laser was used for imaging RCEPIAer with a 570–620 nm emission filter. Laser intensities were set at 5% of the maximum output power (1.6 mW for 488 nm and 2.2 mW for 561 nm). The pinhole was set at 5 AU to get ~5 μm optical slices and time-lapse images were collected for 14–15 hours at 2–3 minute interval (depending on the number of samples included in a given run). The more narrow emission filter on the confocal microscope is less optimal for RCEPIAer and resulted in somewhat lower signal resolution; however we needed to use this configuration in order to perform the GCaMP/RCEPIAer dual imaging experiments.

To determine the optimal emission range for RCEPIAer, we used the Nikon Spectral detector and found that a wider red-shifted emission (605–745 nm) provided better RCEPIAer resolution. Still images of RCEPIAer in Figures 1 and 4 were taken using a similar setup as described above; except the laser power was set to 10% of the maximum output power, the pinhole was set to 2 AU, and 4 frames averaged per image. For images obtained using the Spectrum Detector, the RCEPIAer emission was collected from 605–745 nm, which was more optimal for RCEPIAer than the 570–620 nm emission filter. All images were collected and processed using Nikon NIS-Element (Nikon Instruments, Japan) and saved in 12-bit format. Exported TIF files were analyzed using ImageJ (29).

3. Results

3.1. GCaMP/RCEPIAer cell lines

Our goal in developing the stable GCaMP/RCEPIAer double-positive cell lines was to take advantage of the ability to target different colored GECIs to the cytoplasm or ER, and then perform simultaneous Ca^{2+} measurements of both subcellular localizations during virus replication. Using lentivirus transduction and/or plasmid transfection, we have generated and characterized two different cell lines: MA104-GCaMP5G/RCEPIAer and HeLa-GCaMP6s/RCEPIAer. In both cell lines, GCaMP5G and GCaMP6s were localized to the cytoplasm (Figure 1A and C) and RCEPIAer had a reticular distribution consistent with proper localization to the ER (Figure 1B and D). Initially, the MA104 GCaMP5G/RCEPIAer cell line had relatively low RCEPIAer signal in the majority of cells, with expression good enough for imaging on only about 30–50% of the cells (Figure 1B). To increase the uniformity and resolution, we enriched for double positive cells with the 50% brightest RCEPIAer expression by FACS. This increased the resolution of RCEPIAer and uniformity of strong expression (Figure 1F) compared to the unsorted cells (Figure 1E). Additionally, using a Spectral detector, we found that an emission filter with a wider/red-shifted emission range than standard tetramethylrhodamine isothiocyanate (TRITC)/mCherry filter cubes was better for imaging RCEPIA, with the best results being from ~690–700 nm. However, since standard cubes are more widely available and spectral detector capabilities are not compatible with two-color imaging, we performed imaging with the standardly available filter cubes (see the Materials and Methods section). Next, we performed live-cell Ca^{2+} imaging to detect concomitant changes in cytoplasmic and ER Ca^{2+} in uninfected and virus-infected cells. First, cells were equilibrated to FluoroBrite DMEM and then treated with ATP (250 μM for MA104, 30 μM for HeLa) by perfusion (Figure 2A–D). ATP treatment of MA104-GCaMP5G/RCEPIAer cells induced a strong increase in GCaMP5G with irregularly spaced oscillations and this was paired with an initial sharp decrease in RCEPIAer fluorescence and smaller magnitude decreases corresponding to the GCaMP5G oscillations (Figure 2A, arrows). A similar response was observed for ATP treatment of HeLa-GCaMP6s/RCEPIAer cells; however, the Ca^{2+} oscillations are more regular in HeLa cells than in MA104 cells (Figure 2B).

3.2. Ca^{2+} imaging of virus-infected GCaMP/RCEPIAer cells

Both RV and PV deplete ER Ca^{2+} stores through expression of viroporins (RV NSP4 and PV 2B proteins) that target the ER and this reduces PLC/IP3-dependent Ca^{2+} signaling (9). We tested the response to ATP in RV-infected MA104-GCaMP5G/RCEPIAer and poliovirus-infected HeLa-GCaMP6s/RCEPIAer cells and the results of a representative experiment are shown in Figure 2. As expected, RV-infected MA104 cells had a blunted and delayed response to ATP with no detectable oscillations (Figure 2C). The relative GCaMP5G and RCEPIAer response was significantly lower in RV-infected than mock cells (Figure 2E). PV-infected HeLa cells also had a severely blunted ATP response with small irregular oscillations (Figure 2D). Significantly lower GCaMP6s and RCEPIAer changes were detected from PV-infected than from mock cells (Figure 2F).

3.3. Long-term live cell calcium imaging

3.3.1. Rotavirus-induced calcium signaling—Unlike small molecule dyes, GECI technology enables stable indicator expression and therefore the potential to image changes in cytoplasmic and ER Ca^{2+} throughout an entire virus infection. To test this we mock treated or RV infected MA104-GCaMP5G/RCEPIAer cells at different multiplicities of infection (MOI= 0.5, 1, 5, 10, and 20) and imaged them 2.5 minute intervals from ~2–14 hpi by confocal microscopy. The cells were maintained in a humidified, 5% CO_2 and 37°C environmental chamber throughout the entire imaging run. For each experiment the infection was performed in duplicate and experiments have been performed at least twice. Representative analyses are presented below and movies are available in the online Supplemental material. Mock infected cells showed normal growth throughout the entire time course with only a few GCaMP5G cytoplasmic or RCEPIAer Ca^{2+} transients (Supplemental Movie 1, MA104_Mock). In contrast, RV-infected cells show numerous Ca^{2+} transients beginning as early as 2.5 hpi and substantial elevation in GCaMP5G signal between 4–6 hpi that lasts until cell death. Transients of reduced RCEPIAer were observed and coincided with the appearance of the GCaMP5G Ca^{2+} transients (Supplemental movie 2, MA104_RV_MOI10). The high degree of cell movement and shape change made accurate single-cell region-of-interest (ROI) analysis impossible, so we instead graphed the average GCaMP5G signal for the entire field-of-view (Figure 3A). Mock infected cells showed only a few, small magnitude Ca^{2+} transients, one large Ca^{2+} transient (~12 hr.), and a shallow steady-state increase in GCaMP5G signal up to 10 hpi, perhaps representing an increase in the number of cells in the field-of-view from continued cell division. In all RV-infected cells, a steady-state elevation in $[\text{Ca}^{2+}]_c$ occurred by 6 hpi with strong Ca^{2+} transients observed even earlier (Figure 3A and Supplemental movie 3, MA104_RV_MOIs). To measure the number and magnitude of early (2–6 hpi) Ca^{2+} transients more accurately, we reanalyzed the images with an array of 100 square ROIs from ~2–6 hpi. The ROI array consists of a 10×10 array, with each ROI being 51×51 pixels, such that this encompassed the entire field-of-view. The number and magnitude of the Ca^{2+} transients increased with a higher RV dose (Figure 3B), which showed a clear dose-dependence (Figure 3C). Finally, although RCEPIAer had a relative weak signal in the majority of cells, we were able to observe concomitant changes in GCaMP5G and RCEPIAer Ca^{2+} during spontaneous Ca^{2+} transients in mock-infected cells (Figure 3D). Further, the early Ca^{2+} transients involved ER Ca^{2+} release since GCaMP5G spikes were concomitant with RCEPIAer drops (Figure 3E). Later during infection there was a decrease in RCEPIAer signal and loss of strong correlation between GCaMP5G and RCEPIAer signal changes. In a subset of cells, we observed that the elevation of $[\text{Ca}^{2+}]_c$ (Figure 4A versus 4C and 4E versus 4G) correlated with a re-organization of the diffuse ER localized RCEPIAer protein (Figure 4B and 4F) into discrete puncta (Figure 4D and 4H). In a separate experiment using the sorted MA104 GCaMP5G/RCEPIAer cells, we were able to observe RCEPIAer localized to ring-like structures (Figure 4I–J), which are morphologically similar to the ER-derived membranes around RV replication complexes (called viroplasms) (30); however, since this was not seen in all infected cells (even at high MOIs) it is also possible this is related to RV-induced disruption in ER-Golgi trafficking (31). Alternatively, and a possible caveat for the use of GECIs in all viral systems, is the recent identification of a CaM binding site on the RV structural protein VP6 (32), which could cause sequestration of RCEPIAer. At the resolution

used for these experiments, we were not able to determine whether the diffuse and punctate RCEPIAer displayed different responses to Ca^{2+} transients, which would indicate they are distinct pools within the cell.

3.3.2. GECI calcium imaging for drug screening—Several pathways for Ca^{2+} influx through the plasma membrane are predicted to be activated by RV, including store-operated Ca^{2+} entry (SOCE) and activation of the sodium/calcium exchanger (NCX) in reverse mode (NCX_{rev}), which results in calcium influx into the cytoplasm (8, 13). Next, we used this long-term live cell Ca^{2+} imaging method to assess the effect of Ca^{2+} channel blockers that antagonize these Ca^{2+} entry pathways on RV-induced Ca^{2+} signaling. Cells were mock treated or RV-infected (MOI 10), as described above, and then at 1 hpi treated with the non-specific SOCE blocker 2-APB (50 μM) or the NCX_{rev} blocker KB-R7943 (10 μM) and imaged as before (Supplemental movie 4, MA104_RV_Ca2+Blockers). GCaMP5G fluorescence in mock-infected cells (Figure 5A, top) treated with either 2-APB (dark grey dashed line) or KB-R7943 (light grey dashed line) was lower than cells treated with DMSO alone (black dashed line), as would be expected for inhibition of potential Ca^{2+} entry pathways. Treatment of RV-infected cells with 2-APB (dark grey line) or KB-R7943 (light grey line) showed substantial decreases in the steady-state elevation in $[\text{Ca}^{2+}]_c$ (Figure 5A, bottom), with 2-APB treatment being much more effective than KB-R7943. Further, treatment with 2-APB delayed the onset and reduced the magnitude of RV-induced Ca^{2+} transients between 2–6 hpi (Figure 5B). KB-R7943 treatment caused a minor reduction in the magnitude of the early Ca^{2+} transients but did not delay the onset of these signals (Figure 5C).

3.3.3. Long-term Ca^{2+} imaging of poliovirus and respiratory syncytial virus—We next sought to determine whether these GECI cell lines would be useful to monitor Ca^{2+} signaling induced by other viruses. We tested PV infection of the HeLa-GCaMP6s/RCEPIAer cells and RSV infection of the MA104-GCaMP5G/RCEPIAer cells using the long-term live cell confocal microscopy method described above. HeLa-GCaMP6s/RCEPIAer cells were mock treated or PV infected at an MOI 10 and imaged at 2:45 minute intervals from ~1.5 – 15 hpi. Mock infected HeLa-GCaMP6s/RCEPIAer cells displayed a very high basal level of Ca^{2+} , spontaneous fluxuations in $[\text{Ca}^{2+}]_c$ (Supplemental movie 5, HeLa_Mock), as well as reticular distribution of RCEPIAer (Figure 6A & B). In PV-infected cells (Figure 6D–E), the $[\text{Ca}^{2+}]_c$ fluxuations became erratic between 3–4 hpi (Figure 6F, Supplemental movie 6, HeLa_PV), which is concomitant with the rearrangement of the RCEPIAer signal into punctate structures (Figure 6E). By 6–10 hpi, apoptotic cell death can be observed; however, in many cells small magnitude subcellular Ca^{2+} transients can be observed.

Next we observed RSV-infected MA104-GCaMP5G/RCEPIAer cells at ~24–38 hpi, a time during which syncytia formation and cell death occur (Supplemental movie 7, MA104_RSV). MA104-GCaMP5G/RCEPIAer cells were infected at an MOI 10 and imaged at 2 minute intervals from ~24–38 hpi. We observed greater GCaMP5G signal, indicating elevated $[\text{Ca}^{2+}]_c$, as well as Ca^{2+} flux during cell fusions events (Figure 6G–I) first into the cytoplasm (GCaMP5G, upper panels) and then into the ER (RCEPIAer, lower

panels). Single cell analysis was performed on a cell during the process of fusion with a multinucleated giant cell. We found an ~8 min delay between the increase in $[Ca^{2+}]_c$ and elevation in $[Ca^{2+}]_{ER}$ (Figure 6J). By approximately 28 hpi the steady-state $[Ca^{2+}]_c$ begins to increase rapidly until ~34 hpi when large localized Ca^{2+} transients begin that lead to massive Ca^{2+} flux immediately preceding cell death (Supplemental movie 7).

4. Discussion

The recent work to develop more versatile GECIs, including greater dynamic range, color variants and Ca^{2+} affinities, which are appropriate for Ca^{2+} storage organelles, has opened the possibility to establish stable cell lines and knock-in animals for real-time monitoring of Ca^{2+} signaling (20). Similarly, GECI technology is an attractive alternative to small molecule Ca^{2+} dyes for live cell Ca^{2+} imaging during virus infections. Here we describe the establishment and validation of stable GECI cell lines expressing both green cytoplasmic and red ER-localized GECIs and then used three different viruses that have been previously characterized to exploit Ca^{2+} homeostasis during virus replication (RV, PV, and RSV) to validate the use of GECIs in virus-infected cells (5, 14, 33). Using GECIs we are able to detect simultaneous cytoplasmic and ER Ca^{2+} fluxes induced by application of ATP, a Ca^{2+} agonist, and diminished responses in RV or PV-infected cells, similar to previous studies using Ca^{2+} dyes (25, 34).

GECIs are engineered biosensors based on fluorescent proteins and therefore exhibit greater long-term stability in cells than small molecule dyes, which enables long-term Ca^{2+} imaging experiments to be conducted during the course of virus infections and a more ‘hands-off’ approach for observing naturally developing Ca^{2+} signals. Using long-term live Ca^{2+} confocal microscopy imaging, we observed that RV induced a dose-dependent increase in both the steady-state $[Ca^{2+}]_c$ and the number/magnitude of Ca^{2+} transients. Interestingly, even in cells infected with an MOI = 0.5, where only 20–30% of cells are expected to be infected, we observed numerous strong Ca^{2+} transients (Figure 3A & C) supporting the existence of a RV-induced paracrine signal that functions through a Ca^{2+} signaling pathway. The RV enterotoxin, which is a cleavage product of RV NSP4 that is secreted from RV-infected cells, is one possible candidate for such a paracrine signal because it binds to a receptor and elicits a phospholipase-C (PLC)-dependent Ca^{2+} transient from uninfected cells (35, 36). However, since typical PLC-dependent agonists (e.g., ATP, Figure 2) elicit reduced Ca^{2+} responses from RV-infected cells, the RV-induced Ca^{2+} transients observed in the long-term live cell imaging runs may also involve non-PLC-dependent signaling pathways (15). In addition to viral Ca^{2+} agonists, previous studies have identified SOCE and NCX_{rev} as important host Ca^{2+} entry pathways exploited by RV to elevate $[Ca^{2+}]_c$. We found that both SOCE blocker 2-APB and NCX_{rev} blocker KB-R7943 decreased the steady-state elevation in $[Ca^{2+}]_c$ and magnitude of spontaneous Ca^{2+} transients observed in RV-infected MA104-GCaMP5G/RCEPIAer cells, similar to a previous study using small molecule Ca^{2+} dyes (13). While 2-APB is a blocker of the SOCE channel Orai1, this drug also targets other aspects of the SOCE pathway, including STIM1 puncta formation (37). Both activation of STIM1 and Ca^{2+} entry through SOCE have been observed in RV-infected cells, thus reduction of Ca^{2+} signaling by 2-APB using the GECI cell lines is consistent with previous

studies; however, the ability to observe the Ca^{2+} signaling dynamics provides new information about when and how the Ca^{2+} signals manifest (8, 13).

Finally, we observed PV- and RSV-infected cells to demonstrate that GECl cell lines/technology can be applied to other viral systems, and other cell lines (e.g., HeLa cells). Of note, each of the viruses presented in this study showed dramatically different Ca^{2+} signaling phenotypes. For example RV caused an increase in Ca^{2+} transients but RSV elevated $[\text{Ca}^{2+}]_c$ with few Ca^{2+} transients until just prior to cell death, and PV infection disrupted the normal Ca^{2+} oscillations of HeLa cells. Future studies using these GECl-expressing cell lines, as well as additional lines developed with other GECl varieties, will be able to further dissect the mechanisms by which viruses exploit ER Ca^{2+} signaling (10, 22, 23).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Genetically encoded calcium indicators (GECIs) are now available in both green and red-shifted variants and designed for simultaneous imaging of cytoplasmic and ER Ca^{2+} signals.
- GECIs have distinct advantages over traditional Ca^{2+} indicator dyes for long term and organellar Ca^{2+} imaging, particularly for imaging the Ca^{2+} dynamics during virus infections.
- We used lentivirus transduction to establish stable cell lines (HeLa and MA104) expressing cytoplasmic GCaMP (green) and ER-targeted RCEPIA_{er} (red) GECIs.
- These cell lines enable real-time dynamics of virus-induced Ca^{2+} signaling to be observed in multiple viral systems (rotavirus, poliovirus and respiratory syncytial virus)
- Using GECI-expressing cell lines to observe how Ca^{2+} signaling manifests during virus replication will provide key insights into viruses exploitation of Ca^{2+} signaling

Figure 1. Stable GECl cell lines

Confocal microscopy of the established MA104 (**A & B**) and HeLa (**C & D**) cell lines for basal GCaMP5G (**A**), GCaMP6s (**C**) and RCEPIAer (**B & D**). Enrichment in RCEPIAer expressing cells by FACS is shown for unsorted (**E**) and sorted (**F**) cells. RCEPIAer signal was also improved using a wider filter emission range (605–745 nm) in both the unsorted (**G**) and sorted (**H**) cells.

Figure 2. Evaluation of cytoplasmic and endoplasmic reticulum Ca^{2+} responses in virus-infected GECI-expressing cells

A–D. MA104-GCaMP5G/RCEPIAer (**A & C**) or HeLa-GCaMP6s/RCEPIAer (**B & D**) were mock treated (**A & B**) or infected with RV (**C**) or PV (**D**). Cells were perfused with FluoroBrite DMEM and then treated with ATP for ~150 sec. Cytoplasmic Ca^{2+} increases paired with co-incident ER Ca^{2+} decreases are indicated by arrows. **E.** Relative change in GCaMP5G (left) or RCEPIAer (right) fluorescence after 250 μM ATP stimulation in mock (N=34) or RV-infected (N=44) cells showed delayed and reduced ATP responses in RV-infected cells. **F.** Relative change in GCaMP6s (left) or RCEPIAer (right) fluorescence after 30 μM ATP stimulation in mock (N=40) or PV-infected (N=56) showed reduced and dysregulated ATP-induced oscillations in PV-infected cells. **P < 0.0001. Each experiment was performed in triplicate with 3 replicates per condition.

Figure 3. Long-term live cell Ca²⁺ imaging during rotavirus infection

A. Relative GCaMP5G fluorescence of RV-infected MA104 cells from ~2–14 hpi. Cells were infected with the indicated MOI in duplicate. Traces represent the average fluorescence intensity from the field of view of one of the two wells. **B.** The GCaMP5G fluorescence intensity from 2–6 hpi of mock treated cells (top), or cells infected with different MOIs (as indicated) were analyzed using a 10×10 ROI array. Ca²⁺ transients are observed as spikes in the individual traces. **C.** The percent of ROIs from the 10×10 array analyses in panel B are graphed as a function of the MOI. Values are shown with the mean $\pm$ the standard error of the mean. **D.** Relative GECI fluorescence of concomitant cytoplasmic/ER Ca²⁺ transients in mock (D) or RV-infected (E) MA104 cells detected with GCaMP5G (black line) and

RCEPIA_{er} (grey line), respectively. Data are representative of two experiments with at least two replicates per MOI in each experiment.

Figure 4. Changes in RCEPIAer localization during rotavirus infection

Examples (A–D and E–H) of RCEPIAer redistribution into discrete puncta during the long-term imaging of RV infection. The images were taken during the long-term confocal microscopy runs. GCaMP5G (A & C, E & G) shows the increase in fluorescence as the infection progresses. RCEPIAer signal is diffuse in the ER at 5–6 hpi (B and F) but moves into discrete puncta structures (D and H, arrows) by 7–8 hpi. RV-induced puncta were observed as ring-like structures at ~7 hpi in the sorted MA104 GCaMP5G/RCEPIAer cells (I & J, arrows).

Figure 5. Calcium channel blockers reduce rotavirus-induced Ca^{2+} signals

A. Average traces from a representative well for mock (top) or RV-infected (bottom) cells treated with DMSO (black), 2-APB (dark grey), and KB-R7943 (light grey). **B–C.** RV-induced Ca^{2+} transients were measured by plotting the relative GCaMP5G fluorescence from RV-infected (2–6 hpi) cells mock treated (solid lines) or treated with 50 μM 2-APB (**B**, dashed lines) or 10 μM KB-R7943 (**C**, dashed lines). Each experiment was performed at least 2 times with 2–3 replicates per drug treatment.

Figure 6. Long-term Ca^{2+} imaging of poliovirus and respiratory syncytial virus-infected cells
A–E. Confocal images of mock (**A–B**) or poliovirus-infected (**D–E**) HeLa-GCaMP6s/RCEPIAer cells. Cytoplasmic GCaMP6s (**A** and **D**) and ER-localized RCEPIAer (**B–E**). **A** 10×10 ROI array was used to examine relative GCaMP6s changes for $[\text{Ca}^{2+}]_c$ in mock (**C**) and PV-infected (**F**) cells. **G–I.** Sequence of confocal images of RSV-infected MA104-GCaMP5G/RCEPIAer cells highlighting a cell fusion event (arrow head). **J.** Analysis of the cell fusion illustrating a spike in $[\text{Ca}^{2+}]_c$ (GCaMP5G, green) precedes the elevation in

$[\text{Ca}^{2+}]_{\text{ER}}$ (RCEPIA $_{\text{er}}$, red). Each long-term imaging experiment was performed twice with at least 3 replicates per infection.

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Table 1

Reagents used for development of the GECI cell lines.

Tissue Culture		
Name of Reagent	Manufacturer	Catalog Number
Corning® 100mm TC-Treated Culture Dish	Corning	430167
Dulbecco's Modified Eagle's Medium (DMEM) high glucose	Sigma-Aldrich	D6429
Fetal Bovine Serum	LifeTechnologies	16000-044
0.25% Trypsin EDTA	LifeTechnologies	25200-056
1M HEPES buffer (15mM final)	LifeTechnologies	15630080
Penicillin/Streptomycin solution	LifeTechnologies	15240-062
Imaging		
Name of Reagent	Manufacturer	Catalog Number
96-well µ-clear Optiplate	Greiner Bio-One	655090
FluoroBright DMEM	LifeTechnologies	A18967-01
16% Paraformaldehyde	ThermoScientific (Pierce)	PI28906
adenosine triphosphate (ATP)	Sigma Aldrich	A9187-1G
DMSO	Sigma Aldrich	D8418-250ML
2-Aminoethoxydiphenyl borate	Tocris	1224
KB-R7943 mesylate	Tocris	1244
Lentivirus Packaging and Transduction		
MAX Efficiency Stbl2 Competent <i>E. coli</i>	LifeTechnologies	10268-019
Stellar Competent <i>E. coli</i>	Clontech	636766
HiSpeed Plasmid Maxi Kit (25)	Qiagen	12663
Poly-D-Lysine	Cultrex	3439-100-01
Opti-MEM	LifeTechnologies	11058-021
Lipofectamine 2000	LifeTechnologies	11668-019
Polyethylenimine (PEI)	Polyscience Inc.	23966-2
Polybrene	Santa Cruz Biotech	sc-134220
Puromycin dihydrochloride	LifeTechnologies	A1113802
Hygromycin B	LifeTechnologies	10687010
Vectors		
Constructs	Source	Addgene No.
pGP-CMV-GCaMP5G	Addgene	31788
pGP-CMV-GCaMP6s	Addgene	40753
pCMV-RCEPIAer	Addgene	58216
pLVX-Puro	Clontech	632164
pLVX-IRES-Hyg	Clontech	632182
psPAX2	Addgene	12260
pCMV-VSV-G	Addgene	8454