Elimination of environmental sensitivity in a cameleon FRET-based calcium sensor via replacement of the acceptor with Venus

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Abstract

Genetically encoded sensors are becoming a powerful tool for investigating cellular signaling pathways and, potentially, signaling in vivo. Many sensors use changes in fluorescence resonance energy transfer (FRET) between donor and acceptor variants of GFP separated by a ligand binding domain sensitive to a particular signaling pathway. Accurate measurements require that sensors be insensitive to extraneous intracellular environmental factors. We have found that the responsiveness of the Ca2+ sensor, cameleon YC6.1, varies linearly with the resting YFP/CFP emission ratio in the cell. However, cells expressing responsive or non-responsive sensor can easily be segregated by determining a resting YFP/CFP ratio cutoff for the sensor. This environmental sensitivity has been eliminated by replacing EYFP with Venus to produce a new cameleon we have designated VC6.1. Measurements show that VC6.1 has a greater dynamic range than YC6.1 and better environmental resistance. We also show that YC6.1 is inactivated by persistent activation of the IP3 pathway following expression of constitutively active Gq, while VC6.1 is not. The stability of VC6.1 may make it well suited to studies utilizing mixed cell populations such as those encountered in vivo.

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1. Introduction

Green fluorescent protein (GFP) has become an invaluable tool for cellular imaging [1]. Cellular processes can be visualized in real time through the use of GFP-based sensors for specific intracellular constituents [2,3]. These indicators are well suited to in situ and in vivo experiments due to the fact that they are genetically encoded and can be specifically expressed in cells of interest. The creation of genetically encoded fluorescent protein sensors for intracellular Ca2+ has the potential to enable studies of calcium signaling in intact tissues and live animals which are difficult or impossible with the widely used chemically synthesized fluorescent indicators [4,5]. Genetically encoded sensors can be expressed in cells, tissues and whole animals by transfecting DNA or mRNA encoding the protein into the cells by using electroporation or adenovirus. Permanent expression can be achieved by making transgenic organisms that express the indicator in vivo, thus eliminating the difficulty of delivering the sensor to the desired target cells. Furthermore, tissue-specific promoters can restrict expression of the sensor to a defined subset of cells and protein targeting signals can further restrict expression to defined cellular compartments. Finally, the prospects for long-term imaging are likely to be greatly improved. During prolonged confocal imaging of Ca2+, laser excitation of fluorescent reporter groups attached to the Ca2+ chelator, BAPTA, in chemically synthesized indicators [6,7] has been shown to release reactive oxygen species that cause cellular toxicity and spontaneous [Ca2+] oscillations [8]. However, GFP-based Ca2+ indicators should be largely immune to these effects, as the protein-enshrouded chromophore is isolated from the cellular environment.

The first protein-based Ca2+ indicators made use of the Ca2+-sensitive photoprotein, aequorin, from the jellyfish Aequorea victoria [9]. However, a major disadvantage of this
protein is that it needs a cofactor, coelenterazine, to generate light in a Ca\(^{2+}\) dependent and irreversible reaction. GFP, which is found in association with aequorin in nature, absorbs blue light and re-emits photons of longer green wavelengths without the need for cofactors. The fact that the chromophore in GFP is generated by cyclization and oxidation of amino acids in the protein itself means that mutations of the protein can result in changes in the fluorescent properties of the protein [10]. Mutagenesis of GFP led to the creation of several variants that absorb and emit light of different wavelengths, thus enabling them to be used for FRET [11]. This important development permitted the engineering of the first FRET-based genetically encoded Ca\(^{2+}\) sensor. By inserting calmodulin (CaM) and the Ca\(^{2+}\)-CaM binding M13 myosin light chain kinase peptide (MLCKp) between two different GFP variants with overlapping emission and excitation spectra, Miyawaki et al. demonstrated that the binding of Ca\(^{2+}\) resulted in a measurable increase in FRET between donors (EBFP/ECFP) and acceptors (EYFP/EGFP) [4]. The original cameleons, cameleon-2 and yellow cameleon-2 (YC2), have since undergone significant improvement through the introduction of mutations and modifications that have resulted in significantly improved Ca\(^{2+}\) sensors (Fig. 1). For example, the original cameleons were highly sensitive to pH changes within physiological ranges, so pH-related artifacts in Ca\(^{2+}\) measurements could be quite common. It was found that introduction of the Q69K mutation into “standard” EYFP, thus introducing two mutations (V68L/Q69K) in the original cameleon (YC2), resulted in a cameleon designated YC2.1 that exhibited decreased pH-sensitivity by lowering the pK\(_a\) of EYFP from 6.9 to 6.1 [12]. It was later discovered that replacement of Q69 with Met rather than Lys resulted in a further lowering of the pK\(_a\) to 5.7 while reducing the halide sensitivity, increasing the photostability, and improving expression at 37°C, thus resulting in an improved cameleon designated YC2.3 [13]. Miyawaki et al. also demonstrated that overexpression in cells does not significantly impact CaM-dependent signaling other than through the inevitable increase in cytoplasmic buffering of Ca\(^{2+}\) [12]. One of the latest cameleons to be developed, YC6.1, offers an improved dynamic range of FRET within the physiologically relevant range of cytoplasmic Ca\(^{2+}\) concentrations as measured in HeLa cells [14]. In contrast to YC2.1 and YC2.3, in which the whole CaM domain binds to the MLCK peptide, YC6.1 contains a split CaM domain which comes together to bind the CaM-dependent kinase kinase peptide (CKKp). Structural differences in the binding of the two different linking regions between ECFP and EYFP were proposed to result in a larger change in the distance between the fluorescent proteins in the presence and absence of Ca\(^{2+}\), thus increasing

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**Fig. 1.** Diagram of the cameleon Ca\(^{2+}\) sensors designed to measure cytosolic Ca\(^{2+}\) levels. The various cameleons are presented in the order that they were created from oldest to most recent. The FRET donor and acceptor fluorescent proteins are shown to size in blue and green/yellow, respectively. The Ca\(^{2+}\)-binding calmodulin (purple) and peptide (red) domain sits between the donor and acceptor. Mutations from native GFP are marked by their position and amino acid change.
the change in the YFP/CFP emission ratio upon binding of Ca^{2+}.

Despite the decreased pH-sensitivity of YC2.1 and YC6.1, we have found that the cellular environment can still significantly impact the Ca^{2+} responsiveness of these sensors. In this report we demonstrate that it is critical to determine the relationship between the resting YFP/CFP emission ratio and the responsiveness of the sensor in the experimental setup prior to conducting experiments in order to eliminate artifacts introduced by non-functional FRET-based sensors. These problems can largely be overcome by the introduction of a much more stable variant of YFP known as Venus[15,16]. This has been demonstrated by the creation of an improved cameleon we have designated Venus cameleon 6.1 (VC6.1). This novel cameleon is much more resistant to environmental inactivation than is YC6.1 and displays a larger dynamic range in response to Ca^{2+}, thus giving it several advantages over YC6.1 for use in vivo.

2. Methods

2.1. Materials

Plasmid encoding YC6.1 was obtained from Mitsuhiko Ikura at the University of Toronto. Venus was created through site directed mutagenesis of Clontech pEYFP with the following primers: ccggagctacctgaagctgatcgcaccaccaaccg (F486L), ctctcgataacctgaagctgatcgcaccaccaaccg (F64L), gctctcgataacctgaagctgatcgcaccaccaaccg (F183C). The constitutively active mutant of αv, designated αvAG, was created using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions using the KdVTA calculated from WEBMAX-CLITE v1.15. Imaging was carried out with a Nikon Diaphot microscope equipped with an Optical Insights Microimager to enable simultaneous imaging of the donor (CFP) and acceptor (YFP), respectively. Images were acquired and analyzed with Northern Eclipse software (Empix Imaging Inc.). The maximal response of the indicator was determined as detailed in the Molecular Probes product information. The percent increase in FRET upon agonist exposure as calculated from WEBMAX-CLITE v1.15. Imaging was carried out with a Nikon Diaphot microscope equipped with an Optical Insights Microimager to enable simultaneous imaging of the donor (CFP) and acceptor (YFP), respectively. Images were acquired and analyzed with Northern Eclipse software (Empix Imaging Inc.). The maximal response of the indicator was determined as detailed in the Molecular Probes product information.

2.2. Cell culture and transfection

HEK 293 and COS7 cells were cultured in DMEM containing 4.5 g/L glucose and l-glutamine supplemented with 10% fetal bovine serum and gentamycin. Cells were plated onto 22 mm square glass coverslips in 6-well cell culture plates where the coverslips had been preincubated in serum containing medium for at least 24 h to promote cell attachment. After 24 h, transfections were carried out with 1 μg of total plasmid DNA using FuGENE 6 Transfection Reagent (Roche) according to the manufacturer’s protocol.

2.3. Imaging

Twenty-four to 48 h after transfection with YC6.1 or VC6.1 the cells were imaged during constant perfusion of saline (140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 2 mM MgSO4, 10 mM HEPES, 10 mM N-glucose and 6 mM sucrose) or saline with 100 μM ATP during agonist exposure. Solutions were heated to 37°C with a multi-line solution inline heater and switched using a perfusion valve control system (Warner Instrument Company). To load cells with fluo-4 a stock solution of 2 mM fluo-4 AM was diluted 1:1 with Pluronic F-127 and then added to the cells at 1 μM final concentration for 30 min followed by de-esterification for 30 min. For calcium calibrations 100 mM K2H2EGTA and 100 mM K4CaEGTA were mixed 1:10 with saline containing (154 mM NaCl, 5.5 mM KCl, 2.2 mM MgSO4, 11 mM HEPES and 11 mM 2-deoxy-d-glucose) for a final pH of 7.35 and mixed together at various ratios to give a range of free Ca^{2+} concentrations. Cells were permeabilized with 10 μM ionomycin and equilibrated in the EGTA/CaEGTA solution while taking measurements every 30 s to determine the fluorescence plateau. The concentration of free Ca^{2+} was determined as detailed in the Molecular Probes product information. The percent increase in FRET upon agonist exposure as calculated from WEBMAX-CLITE v1.15. Imaging was carried out with a Nikon Diaphot microscope equipped with an Optical Insights Microimager to enable simultaneous imaging of the donor (CFP) and acceptor (YFP), respectively. Images were acquired and analyzed with Northern Eclipse software (Empix Imaging Inc.). The maximal response of the indicator was determined as detailed in the Molecular Probes product information.

3. Results and discussion

During an evaluation of the FRET-based Ca^{2+} sensor, cameleon YC6.1, for use in Ca^{2+} imaging it was discovered that the resting YFP/CFP emission ratio had a profound effect on the responsiveness of the sensor to increases in intracellular Ca^{2+} concentration, ([Ca^{2+}]i). Upon exposure of cells to agonists that increased [Ca^{2+}]i, it appeared that cells possessing a resting YFP/CFP emission ratio less than 1.0 exhibited a much smaller increase in YFP/CFP ratio following
agonist induced increases in [Ca\(^{2+}\)], than did cells possessing a resting ratio greater than 1.0 (Fig. 2). This seemed almost counterintuitive because a higher resting ratio would seem to provide less range for an increase in FRET to raise the ratio, whether the increased ratio was due to a higher resting Ca\(^{2+}\) concentration or some other cell-specific effect on the function of the sensor. However, if EYFP is not properly folded or is otherwise compromised, this could explain the lower resting ratio and the inability of calcium binding to increase the amount of FRET occurring. Examination of the low ratio cells in the images shows high levels of intracellular fluorescence in regions next to the nuclei that could be misfolded protein concentrated in the Golgi regions of the cells. Several environmental variables seemed to influence the resting ratio, including such things as the level of sensor expression, estimated by relative CFP fluorescence, and cell type. CFP fluorescence was used as an indicator of relative expression level between cells because its fluorescence intensity was more representative of the level of expression of the indicator, as YFP tended to decrease in fluorescence at high expression levels. Highly expressing cells were more likely to have lower ratios and some cell types had a greater fraction of cells with low resting ratios than did others. To quantify the effect that the resting emission ratio has on the Ca\(^{2+}\) responsiveness of these sensors, cameleon YC6.1 was transiently expressed in HEK 293 and COS7 cells. The Ca\(^{2+}\) mobilizing agonist ATP was used to trigger a transient [Ca\(^{2+}\)] i increase in the cells. The maximum change in the ratio of YFP to CFP fluorescence was plotted versus the resting YFP/CFP ratio (Fig. 3A). This showed a linear relationship between the resting ratio and [Ca\(^{2+}\)] i response measured, in which an increasing ratio resulted in a higher maximum response. Most of the cells possessed resting ratios between 1.2 and 1.0 and the sensors displayed significant responses to increases in [Ca\(^{2+}\)]. However, the sensors in cells with resting ratios below 1.0 were almost completely insensitive to [Ca\(^{2+}\)]. To determine the ratio at which YC6.1 begins to respond well to Ca\(^{2+}\), the cumulative response of all the cells was plotted versus the resting YFP/CFP ratio (Fig. 3B). The data from each of the cell lines showed that YC6.1 started to respond normally beginning at a resting ratio of about 1.1. The magnitude of the response as well as the resting ratio appeared to reach effective maximums at 60% and 1.6 respectively, thus producing an effective bounding box on the responsiveness (Fig. 3A). Although cells beyond these points have been observed they are very rare.

In an attempt to make YC6.1 more resistant to the environmental variables that were causing it to become non-functional, several previously described mutations (F46L/F64L/M153T/V163A/S175G) were introduced by site-directed mutagenesis to change EYFP into Venus [15]. These mutations have been shown to dramatically improve the stability and fluorescence of EYFP. The resulting cameleon containing Venus in place of EYFP was designated VC6.1. The structure of this new sensor with its mutations is depicted in Fig. 1. The Ca\(^{2+}\) binding and performance of VC6.1 were compared to those of YC6.1 in situ following transient expression in cells. Exposure of the cells to 100 \(\mu\)M ATP for 3 s resulted in a 37% increase in the YFP/CFP ratio for both YC6.1 and VC6.1 (Fig. 4A). To obtain the FRET dynamic range for VC6.1 compared to YC6.1 the \(R_{\text{max}}\) and \(R_{\text{min}}\) emission ratios were measured after sustained perfusion with ionomycin (1 \(\mu\)M) and EGTA (2 mM) and then ionomycin (1 \(\mu\)M) and CaCl\(_2\) (5 mM). This gave \(R_{\text{max}}\) and \(R_{\text{min}}\) values of 1.2 and 1.9 for YC6.1 and 1.7 and 3.6 for VC6.1 resulting in dynamic ranges of 1.6 and 2.1, respectively. It is possible that the inclusion of cells with low resting ratios had a larger effect on one sensor than the other. In a separate set of experiments cells were exposed to ionomycin and zero Ca\(^{2+}\) and then 39 \(\mu\)M Ca\(^{2+}\) to determine the physiological range of sensor response. Using the results shown in Fig. 3B, non-responsive cells with resting ratios below 1.1 were excluded from the analysis. This resulted in dynamic ranges that were more similar: 2.2 for YC6.1 and 2.6 for VC6.1, though VC6.1 still displayed a significant 18% increase in dynamic range over YC6.1 (Fig. 4B). The larger dynamic range of VC6.1 suggests that it could exhibit a larger change in emission ratio than does YC6.1 when responding to agonist-induced increases in [Ca\(^{2+}\)]. As already discussed, the EYFP in YC6.1 exhibits low fluorescence in some cells, thus leading to a low
Fig. 3. Relationship between the resting YFP/CFP emission ratio and the [Ca$^{2+}$]$_i$ responsiveness of YC6.1. The resting YFP/CFP emission ratio of YC6.1 was measured in individual HEK 293 and COS7 cells transiently expressing the sensor. Intracellular calcium levels were transiently increased by bath application of 100 μM ATP (3 s) and the maximal change in YFP/CFP emission was calculated for each cell. (A) The maximal measured change in [Ca$^{2+}$]$_i$ for each cell increases linearly as the resting ratio increases. (B) Plot of the cumulative maximal response vs. the resting ratio with a linear regression curve fit (dashed line: HEK 293 cells, solid line: COS7 cells) showing the resting ratio above which the indicator is Ca$^{2+}$ responsive.

Fig. 4. Function of a cameleon with Venus in place of EYFP. YC6.1 and VC6.1 were transiently expressed in HEK 293 or COS7 cells and their responsiveness to [Ca$^{2+}$]$_i$ signals was determined. (A) [Ca$^{2+}$]$_i$ responsiveness of all COS7 cells expressing VC6.1 and YC6.1 visualized by agonist exposure (100 μM ATP, 5 s) and determination of $R_{min}$ and $R_{max}$ by treatment with 1 μM ionomycin and zero or 5 mM Ca$^{2+}$. (B) The mean $R_{min}$ and $R_{max}$ at zero Ca$^{2+}$ and 39 μM Ca$^{2+}$ determined for HEK 293 cells with resting emission ratios greater than 1.1. Error bars are ±1 S.D.

showed that there were almost no non-responsive cells to the left of the line as there were with YC6.1 (Fig. 5B). Although no cells displayed a resting emission ratio below 1.3, suggesting that the sensor should be fully functional, there were still cells that did not show an increase in [Ca$^{2+}$], following agonist treatment. These non-responsive cells were cells that expressed extremely high levels of VC6.1 and presumably were non-responsive due to buffering of intracellular Ca$^{2+}$ by the sensor (arrow in Fig. 5A). This buffering hypothesis is supported by the observation that prolonged exposure to agonist eventually led to an increase in fluorescence in cells that were non-responsive to a 3 s exposure (unpublished observation). Examination of the responsive and non-responsive cells indicated that there is a relationship between expression...
level and responsiveness. To examine this relationship the maximal response was plotted versus the level of expression as measured by the intensity of CFP fluorescence (Fig. 5C). This revealed a relationship between expression level and maximal response for YC6.1 and VC6.1 with increasing expression resulting in a decrease in the resting YFP/CFP emission ratio of YC6.1 but not VC6.1. This can clearly be seen in Fig. 5D, where the resting ratio of YC6.1 falls significantly in cells with higher CFP fluorescence while the ratio remains constant for VC6.1 at the same levels of expression. These results demonstrate that the resting YFP/CFP emission ratio of YC6.1 is not indicative of the basal Ca\textsuperscript{2+} level. The effect of increased expression level and possibly other environmental factors on inactivation of YFP fluorescence overwhelms any effect of basal Ca\textsuperscript{2+}. Because VC6.1 appears to be resistant to these effects it suggests that it should be much more effective at comparing resting Ca\textsuperscript{2+} levels.

To ensure that the calcium binding properties of the cameleon had not been altered by use of Venus instead of EYFP, the in situ Ca\textsuperscript{2+} binding of the sensors was examined. Because cells normally regulate cytosolic calcium levels and this interferes with calibration, cells were depleted of ATP by replacement of D-glucose with 2-deoxy-D-glucose, removal of sucrose, and addition of rotenone (10 \mu M) as previously described [18]. EGTA and CaEGTA buffers were mixed at various ratios to produce solutions with free calcium levels ranging from 130 nM to 39 \mu M supplemented with 10 \mu M of the Ca\textsuperscript{2+} ionophore ionomycin to permeabilize the cells. The EC\textsubscript{50} determined from the response curves indicated an apparent Ca\textsuperscript{2+} binding affinity in nM of 1290 ± 96 for VC6.1 which was not significantly different from that determined for YC6.1 (1130 ± 140) or fluo-4 (1080 ± 93). This in situ binding affinity for fluo-4 is similar to that determined previously [18].

During experiments using co-expression of various mutants of the G\textsubscript{q} G protein \alpha-subunit it was found that expres-
sion of constitutively active mutants containing the R183C mutation resulted in significant reductions in the YFP/CFP resting ratio of YC6.1. It is possible that such an effect is due to direct protein–protein interactions between the expressed G protein and the sensor or to the resulting elevation in IP3 and [Ca2+]i. To help discriminate between these possibilities we utilized a previously characterized A2G mutation that results in myristoylation of the G protein and persistent plasma membrane localization [17]. This greatly reduced the possibility of the G protein remaining in the cytoplasm where it could physically interfere with the function of the sensor. Constitutively active and membrane localized sαAGRC was transiently expressed with YC6.1 to provide chronic stimulation of the IP3-mediated Ca2+-release pathway in cells. This led to a 30% decrease in the average YFP/CFP resting ratio of YC6.1 from 1.35 to 0.95, thus making it impossible to measure intracellular Ca2+ because the indicator had become inactivated by the fall in the resting ratio below the 1.1 ratio cut-off as defined previously (Fig. 6A). The observed reduction in the resting ratio was always due to a decrease in the fluorescence from EYFP. When the new Venus containing cameleon (VC6.1) was used in the same assay it showed only an 11% reduction in its resting ratio when comparing cells expressing sαAGRC with control cells not expressing the G protein (Fig. 6A). Artificially increasing intracellular Ca2+ by bathing the cells in ionomycin (1 μM) with CaCl2 (5 mM) demonstrated that VC6.1 was able to detect the increase in Ca2+ while YC6.1 was almost entirely non-functional (Fig. 6B). The increase in [Ca2+]i caused the emission ratio to increase from 1.5 to 3.4 for a full dynamic range of 1.93, whereas YC6.1 increased only from 0.99 to 1.3 for a dynamic range of just 0.31. Furthermore, the time it took YC6.1 to undergo this change in ratio was significantly longer than for VC6.1. Thus, VC6.1 appears to be far more resistant to environmental inactivation than does YC6.1 and more accurately reflects both the resting [Ca2+]i level and any transient increases.

Previous work in Hela cells showed that high expression (500 μM) of cameleons would result in slow recovery of responses back to baseline but did not eliminate or inhibit [Ca2+]i responses [12]. However, in this study we have found that environmental factors such as high expression of the sensor can lead to a decrease in the resting YFP/CFP emission ratio of the cameleon, YC6.1. Decreases in this resting ratio below a defined threshold inevitably resulted in a sensor that was non-responsive to increases in intracellular Ca2+. Working under the hypothesis that the environmental sensitivity of YC6.1 was due to problems with EYFP, the acceptor was changed to the more stable Venus variant. As expected, this modification of YC6.1 to form VC6.1 resulted in a sensor that was resistant to inactivation due to the sensitivity of YFP. This was highlighted by the ability of VC6.1 to still respond under conditions such as constitutive elevation of IP3 that caused the inactivation of the EYFP-based cameleon, YC6.1.

Although we have not tested any other FRET-based indicators that use GFP variants as donors and acceptors it is likely that any which use EYFP or other environmentally sensitive fluorescent protein as an acceptor are susceptible to the same problems documented here for cameleon YC6.1. As such, it will be critical for anyone using such indicators in live cells to determine the relationship between the resting YFP/CFP emission ratio and the responsiveness of the sensor in their experimental setup as was done for YC6.1 (Fig. 3A). This will give a threshold resting ratio that can be used to exclude cells that do not contain properly functioning sensor prior to the collection or analysis of data. Such a threshold will be dependent upon the properties of the sensor as well as the configuration of the imaging setup as the excitation wavelength and the specifications of the emission filters will influence this number. Sensors such as VC6.1 that use Venus should be immune to these problems because the fluorescence appears to be so stable. However, it was found that high levels of sensor expression can in fact buffer Ca2+ so well that the indicator blocks induction of [Ca2+]i transients, it will be very important to keep expression of the sensor as low as possible. 

**Fig. 6.** Effect of constitutive elevation of IP3 on the function of cameleons. YC6.1 and VC6.1 were transiently expressed in HEK 293 cells along with a constitutively active G protein subunit, sαAGRC. The resting YFP/CFP emission ratio and responsiveness of the sensors was determined. (A) Average resting YFP/CFP emission ratio of YC6.1 and VC6.1 in cells with and without transient expression of sαAGRC. Error bars are ± 1 S.D. (B) [Ca2+]i responsiveness of YC6.1 and VC6.1 in cells transiently expressing sαAGRC after artificially increasing intracellular Ca2+ levels through application of 1 μM ionomycin and extracellular calcium.
possible. In fact, the responsiveness of VC6.1 increased as expression of the sensor decreased (Fig. 5C). These findings demonstrate that care must be taken in the use of sensors that utilize EYFP as acceptors for FRET. Although EYFP is much more resistant to pH than were previous YFP variants it can still be inactivated by environmental conditions found in vivo. However, by using the analysis method presented in this study it is relatively simple to eliminate cells containing sensor that is not functioning optimally. In addition, it is possible to eliminate this particular problem through the use of the Venus variant of YFP rather than the standard EYFP as demonstrated by the creation of the much more stable cameleon, VC6.1.

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