

Protocol

Imaging Synaptic Inhibition with the Genetically Encoded Chloride Indicator Clomeleon

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Several techniques are available to image excitatory processes in the brain, but synaptic inhibition has remained largely invisible. Most synaptic inhibition in the brain arises from transmembrane fluxes of chloride ions (Cl^-), so imaging intracellular Cl^- concentration ($[\text{Cl}^-]_i$) is, in principle, a natural way to visualize the spatiotemporal dynamics of inhibition. This protocol describes the use of Clomeleon, a genetically encoded indicator of Cl^- , as a tool for monitoring synaptic inhibition. It outlines procedures that can be used to image neuronal $[\text{Cl}^-]_i$ in brain slices prepared from Clomeleon transgenic mice. With only minor adjustments, the same procedures should be suitable for imaging from cultured cells as well.



MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

Artificial cerebrospinal fluid (ACSF)
Chloride solution (see Step 8)
Clomeleon transgenic mice, 2–3-wk-old
Gluconate or F^- (see Step 7)
Isoflurane
Nigericin
Tributyltin (an OH^-/Cl^- antiporter)

Equipment

Dissection tools

Imaging setup for epifluorescence or two-photon imaging

An upright epifluorescence microscope (e.g., Nikon's Eclipse E600FN or equivalent) equipped with a mercury lamp, a 515-nm dichroic mirror, a filter wheel, and excitation (440 ± 10 nm) and emission filters (485 ± 15 nm for cyan fluorescent protein [CFP]; 530 ± 15 nm for yellow fluorescent protein [YFP]) is used to image Clomeleon fluorescence. Fluorescence excitation is produced by 200-msec-long light pulses (0.5 Hz) and fluorescence emission is collected alternately at each wavelength with a cooled charge-coupled device (CCD) camera with on-chip multiplication gain control (Cascade 512B, Photometrics).

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Clomeleon fluorescence also can be imaged with a two-photon microscope (Ultima IV, Prairie Technologies) using 860-nm laser light (Chameleon Ultra II, Coherent) and a ×40 water-immersion objective (LUMPLFL40xW/IR/0.80, Olympus) with total output power <50 mW. In this case, a fluorescence filter cube with an emission beam splitter (71007, Chroma) is used for fluorescence emission.

Incubation chamber

Incubator preset to 37°C

Vibratome

METHOD

Slice Preparation

Hippocampal slices are prepared from transgenic mice using conventional methods (see, e.g., Berglund et al. 2006).

1. Anesthetize the mice with isoflurane.
2. Decapitate the animals. Immerse the skulls in chilled ACSF.
3. Working quickly, remove the brains. Keep the brains in chilled ACSF.
4. Using a vibratome, cut 250- μ m-thick sagittal sections of the hippocampus. Transfer sections to an incubation chamber containing ACSF.
5. Incubate the slices for 30 min at 37°C before imaging with epifluorescence or two-photon imaging.

See the Discussion for an example of how the preparation can be used.

Calibration Procedure

The Clomeleon fluorescence emission ratio can be converted into $[Cl^-]_i$ (Berglund et al. 2009), as is described briefly here.

6. Incubate brain slices in situ at pH 7.1 in the presence of tributyltin (Inglefield and Schwartz-Bloom 1997; Berglund et al. 2006).
Because pH-dependent exchangers such as the Cl^-/HCO_3^- exchanger (Krapf et al. 1988) can change $[Cl^-]_i$, the K^+/H^+ ionophore nigericin is also added to clamp the intracellular pH to the value of the extracellular pH. Under these conditions, tributyltin equilibrates $[Cl^-]_i$ with $[Cl^-]_o$.
7. Incubate the slices with solutions of F^- or gluconate to determine the values when Clomeleon is Cl^- -bound (R_{min}) or Cl^- -free (R_{max}).
8. To calculate the effective Cl^- dissociation constant of Clomeleon (K_d'), incubate slices with two known concentrations of Cl^- (50 and 134 mM).
9. Calculate the $[Cl^-]_i$ using the equation $[Cl^-]_i = K_d' \times [(R_{max} - R)/(R - R_{min})]$.

DISCUSSION

Background

Clomeleon is a fusion protein consisting of CFP and YFP, joined by a flexible 24-amino acid linker (Fig. 1A; Kuner and Augustine 2000). Because of the close spatial proximity of the two fluorophores, fluorescence resonance energy transfer (FRET) occurs. Thus, exciting the CFP donor causes the YFP acceptor to be excited and to emit yellow fluorescence. Binding Cl^- to YFP quenches the yellow fluorophore (Jayaraman et al. 2000) and decreases the degree of FRET. Thus, measuring the ratio of emission of YFP relative to that of CFP (while exciting CFP) provides an absolute measure of the internal Cl^- concentration ($[Cl^-]_i$). Imaging of the fluorescence lifetime of the CFP donor is another way to use Clomeleon to monitor changes in $[Cl^-]_i$ (Jose et al. 2007).

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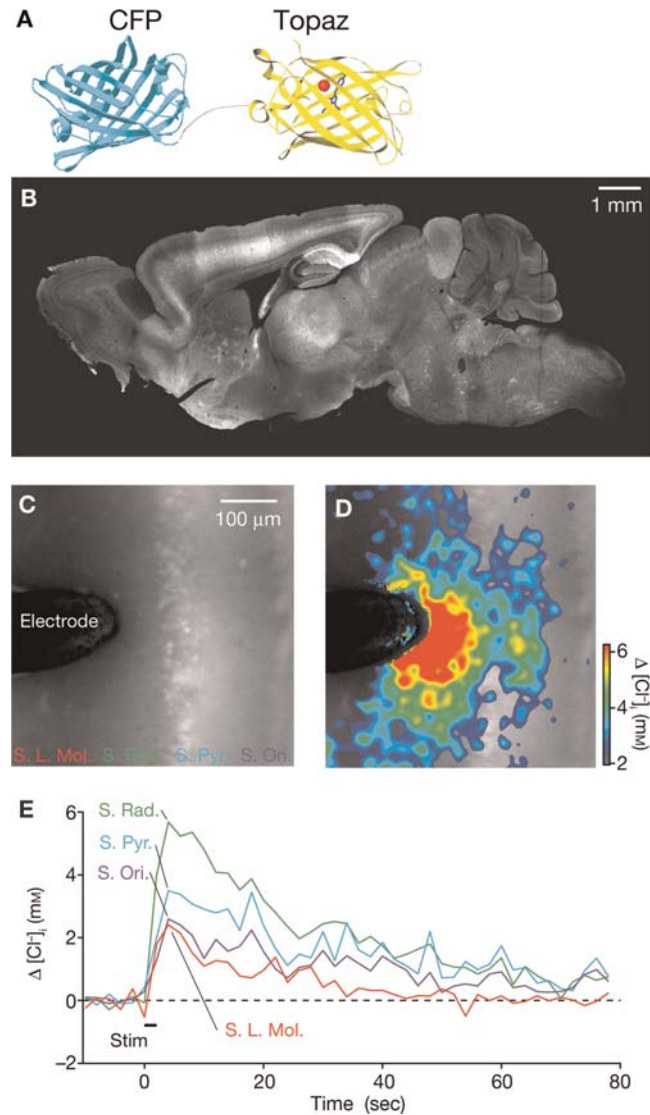


FIGURE 1. (A) Structure of Clomeleon. CFP (cyan) and Topaz (yellow) are connected by a short linker (gray). Shown inside Topaz are the halide-binding site (red) and the nearby chromophore (blue). (B) Expression of Clomeleon in the brain of an adult *thy1::Clomeleon* mouse. The image shows YFP fluorescence in a paramedial sagittal section of fixed brain from a CLM11 mouse. (C) Image of YFP Clomeleon fluorescence (grayscale) expressed in CA1 pyramidal cells from a CLM1 hippocampal slice. (D) Cl⁻ changes (pseudocolor scale) of the same areas shown in (C) in response to a train of electrical stimuli delivered through the stimulating electrode (left side of image). The Cl⁻ image was acquired 4 sec after the stimulus. S. L. Mol., *stratum lacunosum moleculare*; S. Rad., *stratum radiatum*; S. Pyr., *stratum pyramidale*; S. Ori., *stratum oriens*. (E) Time course of changes in [Cl⁻]_i calculated from each layer. Duration of the 2-sec stimulus train (Stim) is indicated by a bar.

Clomeleon imaging has found a wide range of applications, both *in vitro* and *in vivo* (Berglund et al. 2008). Such applications are by no means limited to the brain; any cellular system that permits expression of exogenous DNAs or RNAs, or introduction of a recombinant protein that has been expressed in some other way, is appropriate for Clomeleon-based Cl⁻ imaging (Lorenzen et al. 2004). The specific goals of the work described here are to document the ability to target Clomeleon expression to neurons in the mouse brain and to use Clomeleon to measure [Cl⁻]_i in these neurons.

The studies described here use lines of transgenic mice that express Clomeleon under the control of a neuron-specific promoter, *thy1* (Feng et al. 2000). The transgene has been used to produce several

lines of transgenic mice, each of which expresses Clomeleon in specific subsets of neurons (Berglund et al. 2006). These mosaic patterns of expression are heritable and are therefore found in all individuals of a given line. One of the lines expresses Clomeleon within CA1 pyramidal cells in the hippocampus, as well as in neurons of many other brain regions (Fig. 1B). We are in the process of making these mice commercially available via the Jackson Laboratory (www.jax.org).

Example of Application

To determine whether chronically expressed Clomeleon could report $[Cl^-]_i$ changes associated with synaptic inhibition, changes in Clomeleon signals were measured in response to electrical stimulation (800 μA for 1 sec at 20 Hz) via a concentric bipolar metal electrode placed in the *stratum lacunosum moleculare* of hippocampal slices from transgenic mice. All measurements were made in the presence of 3 mM kynurenic acid (a glutamate antagonist) to prevent activation of excitatory synapses (Fig. 1C). A train of stimuli produced a transient increase in $[Cl^-]_i$ of ~ 3 mM (Fig. 1D), which returned to baseline within 30–50 sec (Fig. 1E). The Cl^- transient was almost completely blocked by 10 μM of the GABA_A receptor antagonist gabazine, indicating that the transient was the result of activation of GABA_A receptors. These results indicate that Clomeleon can report changes in neuronal $[Cl^-]_i$ associated with synaptic inhibition. By measuring $[Cl^-]_i$ over many (e.g., >20) cells, Clomeleon can detect $[Cl^-]_i$ transients of ~ 200 μM caused by only five inhibitory postsynaptic potentials (Berglund et al. 2006). In addition, Clomeleon allows detection of gradients in $[Cl^-]_i$ associated with synaptic activity (Fig. 1E).

Not all FRET-based indicator proteins are suitable for two-photon microscopy. However, two-photon excitation spectra of cerebellar granule cells in slices prepared from Clomeleon transgenic mice (Fig. 2A) showed that emission of both CFP and YFP was brightest when excited by 860-nm light (Fig. 2B). The YFP/CFP emission ratio was also relatively high at 860 nm (Fig. 2B). To determine

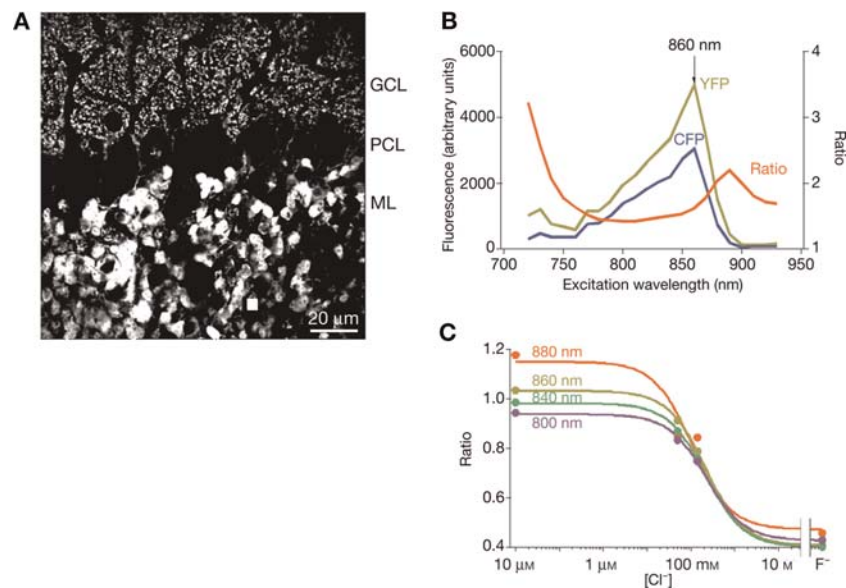


FIGURE 2. (A) A two-photon image of YFP fluorescence in the cerebellar cortex, showing expression of Clomeleon exclusively in granule cells. Both the molecular layer (ML) and granule cell layer (GCL) are fluorescent, indicating the presence of Clomeleon in granule cell bodies/dendrites and parallel fibers. Note that Purkinje cell bodies do not express Clomeleon, as indicated by the absence of YFP fluorescence in the Purkinje cell layer (PCL). (B) Two-photon excitation spectrum of Clomeleon. Both the CFP and YFP fluorescences of Clomeleon were brightest when excited by 860-nm light. The YFP/CFP emission ratio (Ratio) was also relatively high at 860 nm. (C) Cl^- titration curves for Clomeleon measured at various excitation wavelengths. $[Cl^-]_i$ was clamped at $[Cl^-]_o$ using a Cl^-/H^+ antiporter/ionophore cocktail, as described in the text. Using an 860-nm excitation light, the dynamic range of Clomeleon was nearly maximal.

the sensitivity of Clomeleon to Cl^- , the Clomeleon emission ratio was measured at different excitation wavelengths and at different $[\text{Cl}^-]$ values (Fig. 2C). Half-maximal quenching of Clomeleon occurred at 120 μM $[\text{Cl}^-]$, irrespective of excitation wavelength. Thus, excitation at 860 nm is optimal for Clomeleon-based measurements of $[\text{Cl}^-]_i$. These results indicate that chronically expressed Clomeleon is suitable for two-photon imaging of $[\text{Cl}^-]_i$ (Duebel et al. 2006). This permits in vivo measurement of $[\text{Cl}^-]_i$ in the brains of Clomeleon transgenic mice (Berglund et al. 2009) and is being used to image tonic inhibition of granule cells by ambient GABA in vivo, as well as in slices in vitro (Lee et al. 2010).

Advantages and Limitations

Clomeleon offers two distinct advantages over existing Cl^- indicator dyes. The first is that Clomeleon can be targeted to specific cells via genetic targeting. Using specific promoters, stereotaxic delivery of viruses, and/or Cre recombinase techniques, Clomeleon expression already has been targeted to a variety of neuronal types (Berglund et al. 2008), and it is very likely that such applications will expand in the future. Second, Clomeleon is ratiometric and, therefore, permits determination of absolute $[\text{Cl}^-]$ within the cells of interest. In addition, unlike other FRET-based indicator proteins, Clomeleon does not require a conformational change between the units of the FRET pair because quenching of YFP by Cl^- is sufficient to induce a change in the YFP/CFP emission ratio. Thus, Clomeleon permits efficient two-photon excitation by Ti:sapphire lasers, enabling studies in visible light-sensitive retinas (Duebel et al. 2006) and in the mouse brain in vivo (Berglund et al. 2008).

There are three significant limitations when using Clomeleon to image $[\text{Cl}^-]_i$.

- Clomeleon is sensitive to protons, with lower pH increasing the affinity of Clomeleon for Cl^- (Kuner and Augustine 2000). In practice, interference by protons is minimal when $[\text{Cl}^-]_i$ is low, as is the case under most physiological conditions. In an experimental paradigm in which higher $[\text{Cl}^-]_i$ and/or significant pH changes are expected, the internal pH of the cells under investigation should be measured by conventional, organic pH indicator dyes so that the known relationship between pH and Clomeleon affinity (Kuner and Augustine 2000) can be used to calibrate Clomeleon responses accurately (Pond et al. 2006; Berglund et al. 2009).
- Intense illumination of Clomeleon causes differential bleaching of CFP and YFP, leading to a decrease in the Clomeleon emission ratio. This artifact can incorrectly indicate an increase in $[\text{Cl}^-]_i$. In practice, this problem can be avoided by minimizing the intensity and duration of Clomeleon excitation and by using highly sensitive CCD cameras or photomultiplier tubes.
- The affinity of Clomeleon for Cl^- is not optimal, being $\sim 90 \mu\text{M}$ (Kuner and Augustine 2000), whereas $[\text{Cl}^-]_i$ in mature neurons is in the order of 5 μM (Berglund et al. 2006). This means that the sensitivity of Clomeleon for detecting synaptic inhibition is limited, particularly in neurons in which the membrane potential is not voltage clamped and the changes in $[\text{Cl}^-]_i$ associated with synaptic inhibition are $\ll 1 \mu\text{M}$ (Berglund et al. 2006). This limitation can be overcome by signal averaging over many cells and/or trials (Berglund et al. 2006), although the ultimate solution is to reengineer Clomeleon to improve its Cl^- binding properties.

Despite these limitations, the utility of Clomeleon for measuring $[\text{Cl}^-]_i$ makes this indicator a valuable tool for analysis of the spatiotemporal dynamics of inhibitory networks in living brain tissue.

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