Supplementary Information

Unusual Photophysics of Geranic Acid Deep Eutectic Solvents

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Materials

The human mesenchymal stem cell line (hMSC) was a gift from the Mao Research Group at the Johns Hopkins University, Baltimore, MD, USA. Fetal bovine serum (FBS), phosphate-buffered saline (PBS), bovine serum albumin (BSA), glycine, and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich. The Dulbecco's modified Eagle medium (DMEM), TrypLE express enzyme, and trypan blue solution were obtained from Thermo Fischer Scientific. M-PER™ mammalian protein extraction reagent, prestained protein ladder, BSA FITC conjugate (BSA-FITC), goat anti-mouse IgG (H+L) highly cross-adsorbed secondary antibody Alexa Fluor™ plus 555 (IgG AF 555), Bolt™ 4 to 12%, bis-tris, 1.0 mm mini protein gels (15-well), nitrocellulose membrane (0.45 µm, 8 × 8 cm), rabbit glyceraldehyde 3-phosphate dehydrogenase (GAPDH) polyclonal antibody (pAb), SuperSignal™ West Pico PLUS stable peroxide, SuperSignal™ West Pico PLUS luminol/enhancer, tris-glycine transfer buffer, Bolt™ antioxidant, Bolt™ MES SDS running buffer, Bolt™ LDS sample buffer, and Bolt™ reducing agent were purchased from Invitrogen™. Tris-buffered saline (TBS) 10 X pH 7.4 was acquired from Quality Biological. mCherry fluorescent protein (purity > 80%) was obtained from antibodies-online.com. Ab189819 Membrane Fluidity kit and goat pAb to rabbit IgG conjugated with horseradish peroxidase (HRP) was purchased from Abcam. Tween 20 was obtained from Research Products International. Pierce™ BCA protein assay kit and Hoechst 33342 solution (20 mM) were bought from Thermo Fisher Scientific. The 4% (w/v) paraformaldehyde solution was acquired from Alfa Aesar. The anti-mCherry rabbit monoclonal antibody (mAb) was obtained from Cell Signaling Technology. All solutions and dispersion were prepared with Milli-Q ultrapure water. Fluorescence grade rhodamine 101, and 200-proof anhydrous ethanol were obtained from Sigma Aldrich.

The choline and geranic acid (CAGE) at different molar ratios (1:1, 1:2, 1:3, and 1:4 of choline to geranic acid) were prepared according to previous protocols[1] using geranic acid and choline bicarbonate 80 wt% solution in water from Sigma-Aldrich. CAGE was characterized by ^{1}H and ^{13}C NMR spectroscopic

using Bruker Avance II 400 MHz NMR spectrometer (number of scans = 16), as presented in a previous publication (the NMR spectra of CAGE matched previously published spectra from the group).[3] The pH of the CAGE aqueous solutions was measured by the SevenCompact S220 pH/Ion benchtop meter from Mettler Toledo, calibrated with buffers with pH 4.01, 7.00, and 10.01.

Fluorescence Spectroscopy

Samples were prepared 1 h before the experiments using: (i) CAGE 1:1, 1:2, 1:3, and 1:4 solutions (at 12.5, 25, and 50 mM), choline bicarbonate ([Ch]Bic) 50 mM, and geranic acid (GA) 50 mM; (ii) mCherry (5 μg/mL, 180 nM) in water and the CAGE solutions; (iii) BSA-FITC (2.5 μg/mL, 38 nM) in water and the CAGE, [Ch]Bic, GA; and (iv) IgG AF 555 (1.6 μg/mL, 10 nM) in water and CAGE 1:3 and 1:4 at 50 mM. The fluorescence spectra were obtained on a spectrofluorometer (RF-6000 from Shimadzu) at 25 °C. The range for the excitation (λ_{Ex}) and emission wavelengths (λ_{Em}) varied for each protein and solution (mCherry: λ_{Ex} 240–620 nm, λ_{Em} 300 –700 nm; IgG AF 555: λ_{Ex} 450–600 nm, λ_{Em} 540 –680 nm; BSA-FTIC, and CAGE: λ_{Ex} 240–550 nm, λ_{Em} 300–650 nm). Their excitation (BW_{Ex}) and emission (BW_{Em}) bandwidth were also adjusted for every set of samples to guarantee better resolution (mCherry: BW_{Ex} 5 nm, BW_{Em} 3 nm; all other samples: BW_{Ex} 3 nm, BW_{Em} 5 nm). The intervals for the λ_{Ex} were 2 nm and 1 nm for λ_{Em}, with a scan speed of 6000 $nm.min^{-1}$, and high sensitivity.[4] Data is presented in fluorescence intensity (FI) in arbitrary units (a.u).

Absorbance spectra

The absorbance spectra of CAGE 1:1, 1:2, 1:3, and 1:4 at 12.5, 25, and 50 mM, [Ch]Bic and GA at 50 mM were acquired at 25 °C in the UV-Vis Spectrophotometer UV-2600 from Shimadzu from wavelengths (λ) 200 to 800 nm.

Dynamic light scattering

The radius Z-average size (nm) and polydispersity index (PdI) of CAGE 1:1, 1:2, 1:3, and 1:4 solutions (at 12.5, 25, and 50 mM) were evaluated by dynamic light scattering (DLS) in the Zetasizer Nano S from Malvern Panalytical. The parameters for this experiment followed the manufacturer's generic latex measurement standard operating procedure. The instrument optimized each sample's conditions, including accumulations, before collecting the data. In addition to the Z-average size and PdI, the equipment also provided a quality report indicating whether each sample was suitable for DLS.

Transmission electron microscopy

A 50 mM CAGE 1:3 dispersion in water was imaged by transmission electron microscope (TEM). TEM was performed at room temperature in a JEOL JEM-1400 transmission electron microscope series 120 kV. For TEM, 20 µL of 50 mM CAGE 1:3 with no stain was pipetted into a copper carbon-coated grid (300 mesh). Then, the excess solution was removed with filter paper, and the sample was left to dry at room temperature overnight.

Cell Culture

The hMSC cell line was cultivated in 10 mL of DMEM supplemented with 10 % (v/v) FBS in T75 flasks until 80 % confluence (7-9 days, changing medium every two days) at 37 °C in a cell incubator with high humidity and under a 5 % CO_2 atmosphere. After reaching 80 % confluence, the cells were dissociated using TrypLE Express from Gibco™, following the manufacturer's instructions. The live cells (cell viability of at least 90 %) were then counted by dye exclusion test using Trypan Blue Solution 0.4 % (1:1 of dye per volume of cell suspension) in EVE™ Automated Cell Counter from NanoEnTek. The cells were then

cultivated in transparent 6-well plates (300,000 cells per well) for fluorescence cell imaging and protein extraction or seeded in 96-well black microplates (10,000 cells per well) for membrane fluidity assay 24 h before starting the treatments for each experiment.

Fluorescence microscopy of fixed cells

We prepared solutions containing mCherry (3.36 μg/mL, 0.116 nM) in DMEM and in different concentrations of 50.0 mM of CAGE-1:4 in DMEM. After incubating the mCherry-CAGE solutions for 10 min at room temperature, 2 mL of each sample was used to treat the hMSC cells for 2 h at 37 °C. We also tested the effect of adding mCherry without CAGE 1:4 (control). After the treatments, the cells were fixed with 4% (w/v) paraformaldehyde for 15 min, and their nuclei were stained with Hoechst 33342 solution (1 μg/ml) for 5-10 min at room temperature. The cells were then imaged by fluorescence microscopy with an EVOS M7000 Imaging System from Invitrogen™. The light, exposure, and gain for each channel of the microscope (DAPI – $\boldsymbol{\lambda}_{Ex}$ 357, $\boldsymbol{\lambda}_{Em}$ 447 nm, RFP – $\boldsymbol{\lambda}_{Ex}$ 531, $\boldsymbol{\lambda}_{Em}$ 593 nm, and transmittance) were the same for all samples to allow comparison. The merged pictures of DAPI and RFP channels were also obtained. The background of DAPI, RFP, and merged figures were adjusted in Celleste 6.0, maintaining constant values for each channel to allow sample comparison.

Western blot

The hMSC were treated with DMEM (blank), mCherry at 3.36 μg/mL (0.116 nM) in DMEM (positive control), and mCherry at 3.36 μg/mL (0.116 nM) in CAGE 1:3 or CAGE 1:4 at 50 mM for 2 h at 37 °C. The proteins of each sample were extracted using 500 μL of M-PER™ Mammalian Protein Extraction Reagent for 5 min incubation at room temperature with gentle agitation. The cell debris was removed by 10 min centrifugation at 14,000 × g. Then, the samples were concentrated to around 100 μL using a vacuum concentrator for 1 h. The total proteins of the concentrated samples were quantified using a BCA protein

assay kit. For the western blot, 40 µg of protein per sample was denatured at 70 °C for 10 min with LDS sample buffer and reducing agent. Then, samples were loaded to the 4-12% Bis-Tris 1.0 mm electrophoresis gel and ran at 200 V for 22 min in MES SDS running buffer with 0.25 % (v/v) antioxidant in the Mini Gel Tank from Invitrogen™. The proteins from the gel were then transferred to a nitrocellulose membrane (0.45 µm) using 10 V for 1 h in the same equipment in a tris-glycine transfer buffer. The membrane was then incubated in blocking buffer (5 % (w/v) BSA in TBST) for 2 h under gentle agitation, and a primary rabbit anti-mCherry mAb (1:1,000) was added for 1 h, followed by a secondary anti-rabbit pAb conjugated to HRP (1:20,000). The membrane was treated with a 1:1 peroxide and luminol/enhancer solution. The membrane was imaged for chemoluminescence in the Invitrogen™ iBright™ 1500 Imager, and the bands were quantified in the iBright™ Analysis Software, keeping the same area for all bands. After mCherry, we also evaluated GAPDH as a loading control after stripping the membrane with Restore Stripping Buffer (Thermo Fisher Scientific) based on the buffer manufacturer's protocol.

Membrane Fluidity Assay

The hMSC were treated with DMEM (control) and CAGE 1:4 at 50 mM for 2 h at 37 °C (*n* = 10). The membrane fluidity of the cells was evaluated using the lipophilic pyrene probe (pyrene decanoic acid (PDA) from Ab189819 Membrane Fluidity kit), following the manufacturer's recommendations for plate adherent cells. PDA fluorescence was measured by exciting at 350 nm and taking emission values at 400 nm (monomer) and 470 nm (excimer) in a Thermo Scientific™ Varioskan™ LUX multimode microplate reader. The relative membrane fluidity was calculated as the excimer-to-monomer fluorescence ratio.

Quantum yield calculation

The quantum yield of IgG AF 555 in MilliQ water or 3.125 mM CAGE-1:3/water mixture was determined at described previously[5] using Rhodamine 101 in ethanol as the standard with QY = 0.915.[6]

The absorbances of different solutions of IgG AF 555 at 553 nm in MilliQ water or 3.125 mM CAGE-1:3/water were kept between 0–0.08. The samples were excited at 553 nm and the integrated fluorescence intensity measured from 563 to 580 nm. A plot of the integrated fluorescence intensity as a function of absorbance gave the slope from a linear fit. The QY was obtained using the equation:

$$QY_{sample} = QY_{standard}\frac{(\eta_{sample})^2 \ m_{sample}}{(\eta_{standard})^2 m_{standard}}$$

where QY_{sample} is the quantum yield of the sample (IgG AF 555 at 553 nm in MilliQ water or 3.125 mM CAGE-1:3/water); $QY_{standard}$ is the quantum yield of the standard (rhodamine 101); m_{sample} is the slope of the linear fit for the integrated fluorescence intensity of the sample (IgG AF 555 at 553 nm in MilliQ water or 3.125 mM CAGE-1:3/water); $m_{standard}$ is the is the slope of the linear fit for the integrated fluorescence intensity of the standard (rhodamine 101); η_{sample} and $\eta_{standard}$ is the refractive index of the solvent for the sample and standard, respectively. The refractive index of ethanol, milliQ water and 3.125 mM CAGE-1:3/water was measured in-house using Huanyu monocular digital refractometer.

Statistical analysis

Different groups were compared for statistical differences by unpaired t-test (n = 10), considering $p < 0.05$ in GraphPad Prism 8.0.1.

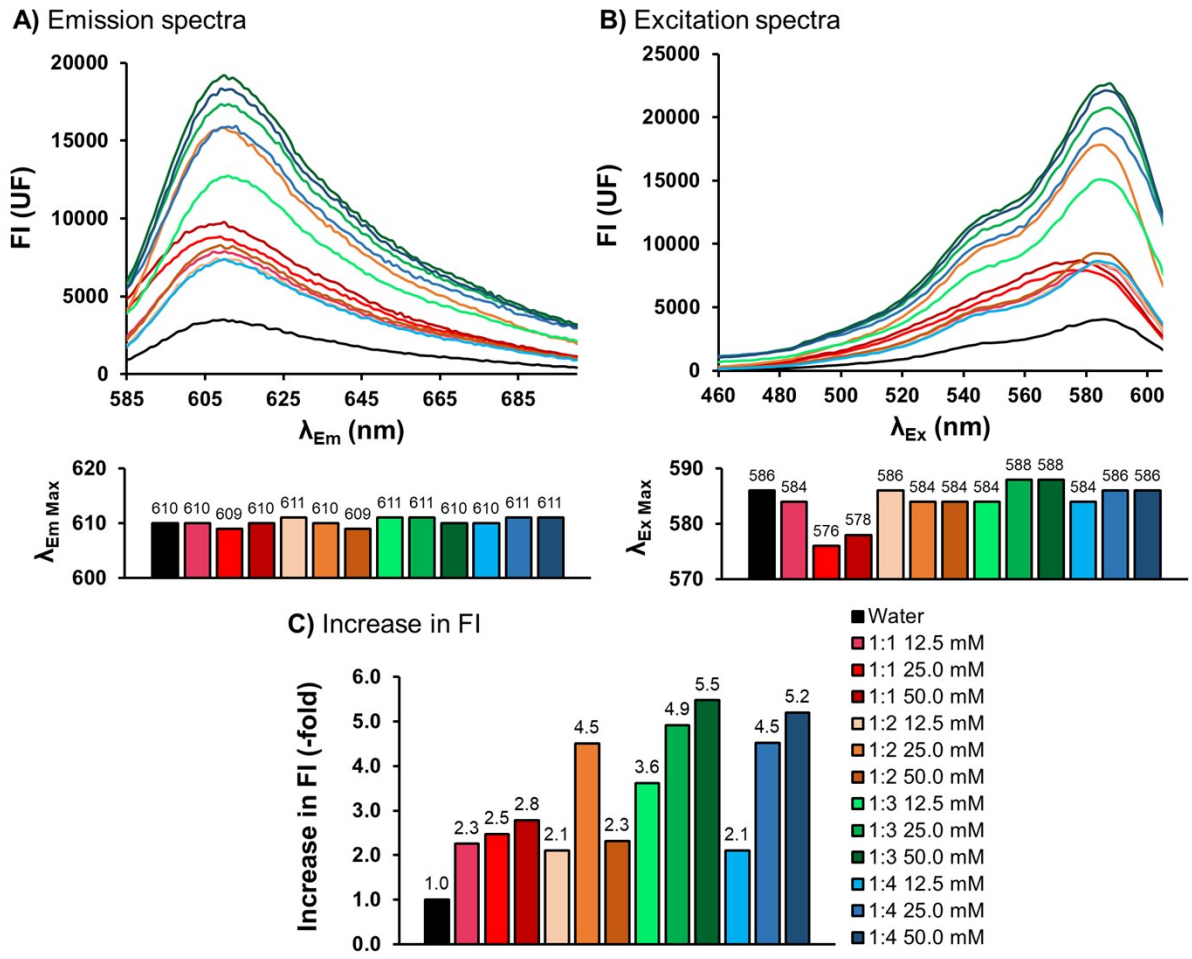

Figure S1. Effect of CAGEs on mCherry fluorescence. A) Emission spectra at λ_{ex} 570 nm and $\lambda_{Em\ Max}$, **B)** Excitation spectra at λ_{em} 620 nm and $\lambda_{Ex\ Max}$ of mCherry (180 nM) at different CAGE solutions at 25 °C after 1 h incubation. Data presented in fluorescence intensity (FI) in arbitrary units.

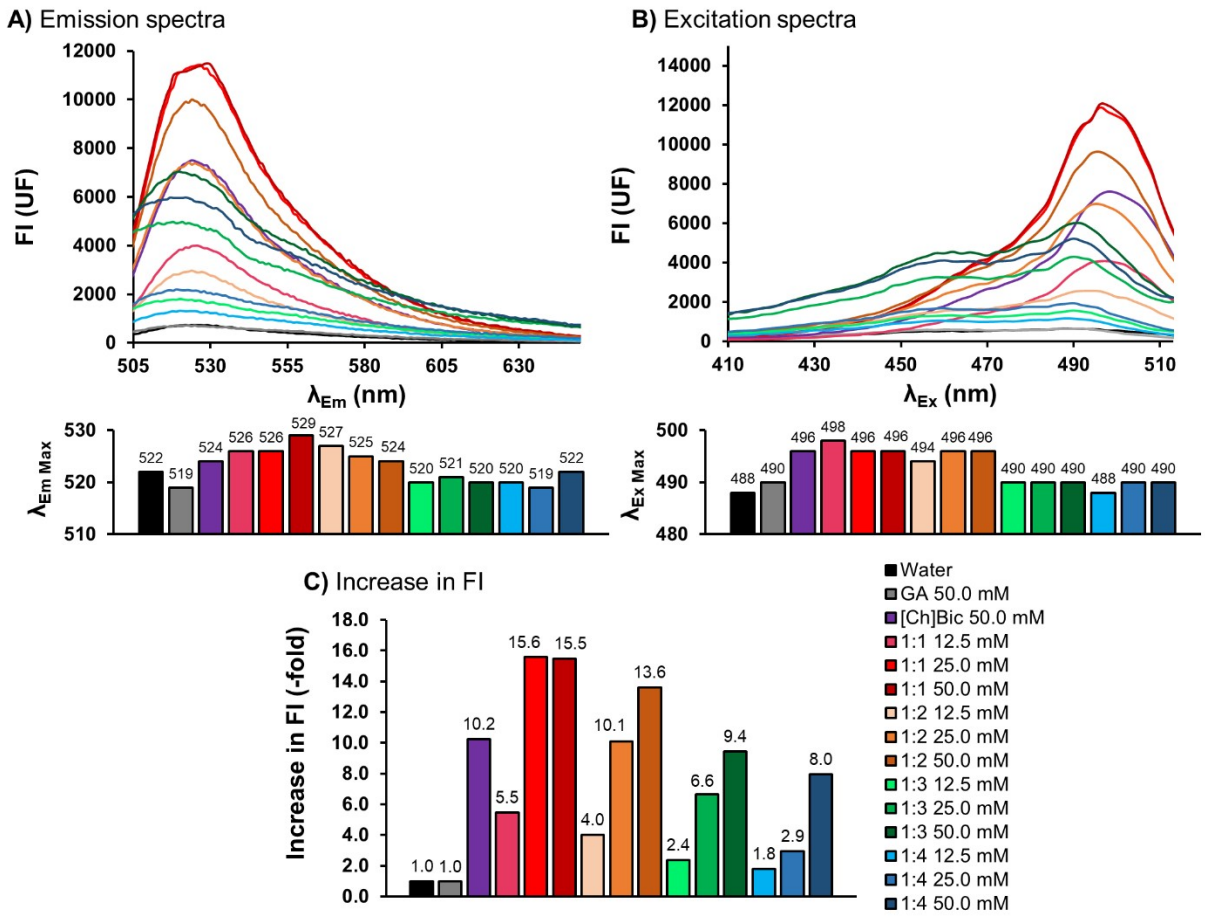

Figure S2. Effect of CAGEs on BSA-FITC fluorescence. A) Emission spectra at λ_{ex} 490 nm and **B)** Excitation spectra at λ_{em} 535 nm of BSA-FITC (38 nM) at different CAGE solutions at 25 °C after 1 h incubation. Data presented in fluorescence intensity (FI) in arbitrary units.

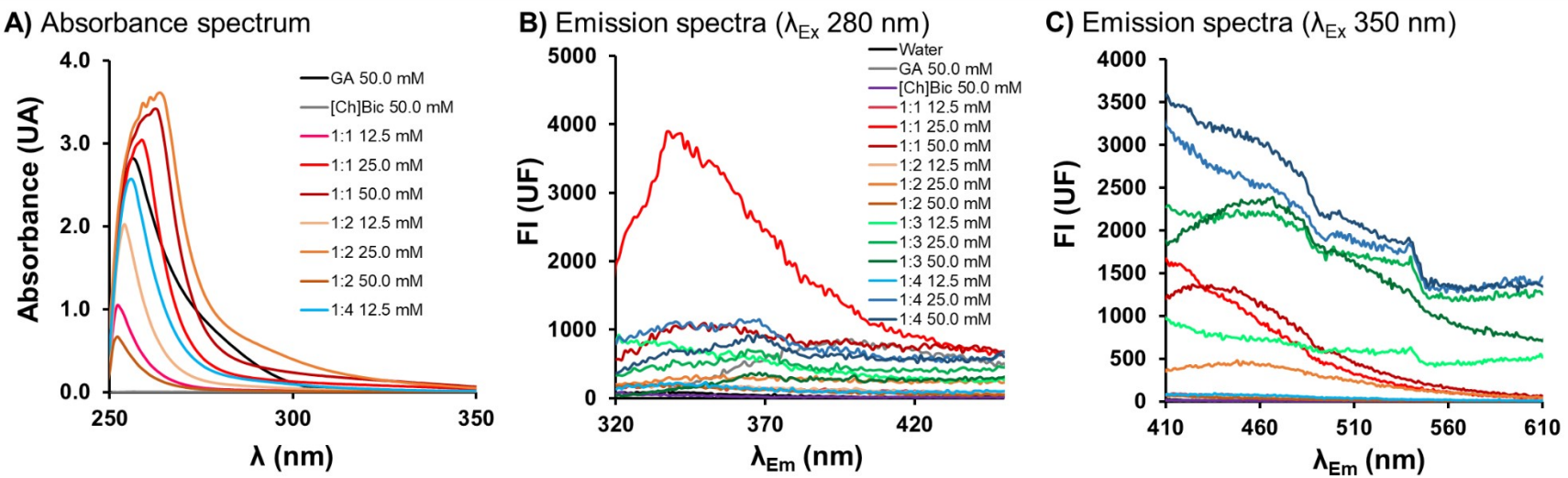

Figure S3. Properties of CAGE solutions. A) Absorbance spectra of CAGEs 1:1 and 1:2 at 2.5, 25, and 50 mM. **B)** Emission spectra at λ_{ex} 280 nm and **C)** at λ_{Ex} 350 nm of CAGE solutions at 25 °C. Data presented in fluorescence intensity (FI) in arbitrary units.

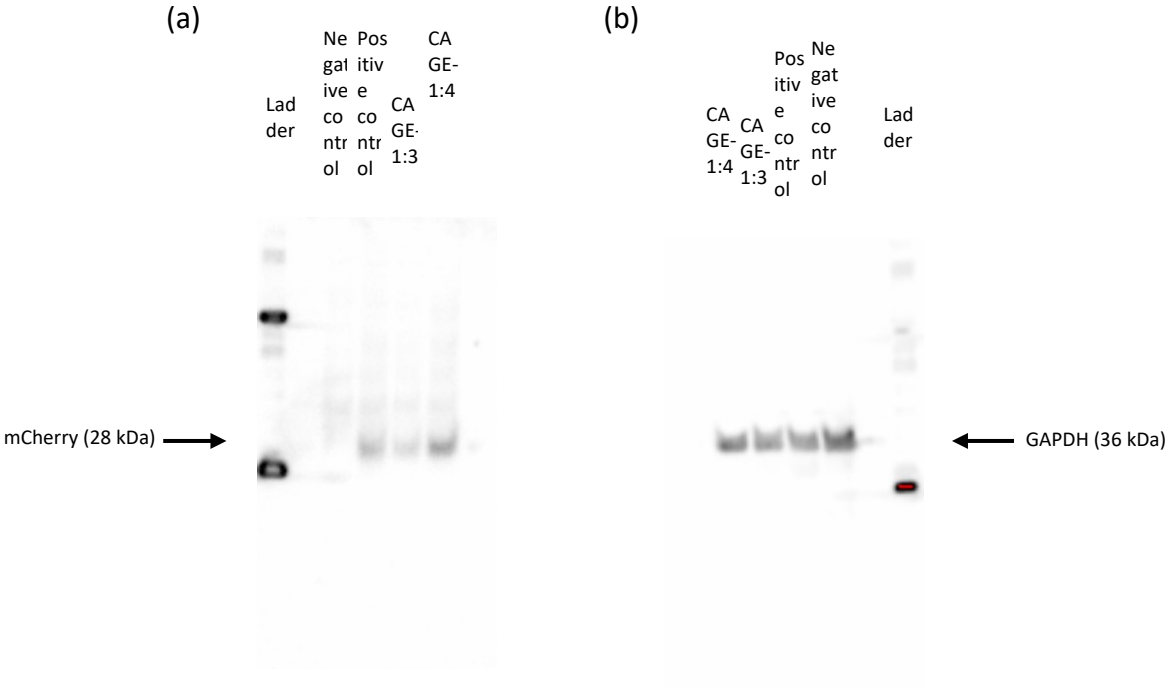

Figure S4. Western blot band of (a) mCherry and (b) GAPDH, loading control.

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