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Identification of mNeonGreen as a pH-Dependent, Turn-On Fluorescent Protein Sensor for Chloride

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Abstract

Chloride-sensitive fluorescent proteins generated from laboratory evolution have a characteristic tyrosine residue that interacts with a chloride ion and π -stacks with the chromophore. However, the engineered yellow-green fluorescent protein mNeonGreen lacks this interaction but still binds chloride, as seen in a recently reported crystal structure. Based on its unique coordination sphere, we were curious if chloride could influence the optical properties of mNeonGreen. Here, we present the structure-guided identification and spectroscopic characterization of mNeonGreen as a turn-on fluorescent protein sensor for chloride. Our results show that chloride binding lowers the chromophore pK_a and shifts the equilibrium away from the weakly fluorescent phenol form to the highly fluorescent phenolate form, resulting in a pH-dependent, turn-on fluorescence response. Moreover, through mutagenesis, we link this sensing mechanism to a non-coordinating residue in the chloride binding pocket. This discovery sets the stage to further engineer mNeonGreen as a new fluorescent protein-based tool for imaging cellular chloride.

Keywords

anions; chloride binding pocket; fluorescent protein sensor; mNeonGreen; protein engineering

Since the discovery of green fluorescent protein from the jellyfish *Aequorea victoria* (avGFP), laboratory evolution has been widely used to improve and alter the properties of naturally occurring fluorescent proteins for biological imaging applications.^[1–3] Oftentimes, sensitivity to environmental factors, such as pH, molecular oxygen, and chloride, is targeted.^[1–3] Along these lines, the chloride-sensitive variant of avGFP, yellow fluorescent protein H148Q(avYFP-H148Q), is a highly explored starting point to engineer fluorescent protein sensors for chloride,^[4–13] the most abundant biologically relevant anion.^[10,14] Even though these sensors are pH-dependent and sensitive to other halides and oxyanions, they are used to image cellular chloride in different biological contexts.^[4–13] Based on the crystal structure of avYFP-H148Q bound to iodide (Figure 1A), the chloride binding pocket

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Conflict of Interest

The authors declare no conflict of interest.

consists of the residues Q69, R96, Q183, and Y203, which π -stacks with the chromophore Y66.^[6] To improve sensor properties, mutations have been made to Q69 and hydrophobic residues lining the chloride binding pocket.^[8,11–13] In general, chloride binding to avYFP-based sensors increases the pK_a of the chromophore Y66 due to electrostatic repulsion, thus shifting the chromophore equilibrium from the fluorescent phenolate form to the weakly fluorescent phenol form, resulting in a turn-off fluorescence response.^[5,6,10,13] In addition, the Y203 residue, which is essential for binding and sensing chloride, has been introduced into the avGFP variant E¹GFP to generate E²GFP with enhanced chloride sensitivity.^[6,15] Interestingly, E²GFP and its derivatives operate via a static quenching mechanism with no shifts in the chromophore equilibrium and have completely different chloride binding pockets from avYFP-H148Q in which the chloride ion directly interacts with the chromophore (Figure 1B).^[13,15–18]

To expand on this body of work, we recently identified and spectroscopically characterized the yellow fluorescent protein from the jellyfish *Phialidium* sp. (phiYFP) as a naturally occurring, turn-on fluorescent protein sensor for chloride.^[19–21] The residues in the putative chloride binding pocket of phiYFP are identical and in a similar arrangement to that observed for avYFP-H148Q (Figure 1C).^[22] Like avYFP-based chloride sensors, phiYFP is pH-dependent, and chloride binding shifts the chromophore Y66 equilibrium from the fluorescent phenolate form to the weakly fluorescent phenol form. But in the case of phiYFP, the phenol form likely undergoes an excited state proton transfer to regenerate the fluorescent phenolate form, giving rise to the turn-on fluorescence response.^[21]

The discovery of phiYFP inspired us to ask if a fluorescent protein with a different chloride binding pocket and potentially different properties could have evolved in Nature. Through a Protein Data Bank (PDB) search,^[23] we uncovered the crystal structure of the tetrameric yellow fluorescent protein *lanYFP* from the cephalochordate *Branchiostoma lanceolatum* with two folding mutations, V171A and N174T, bound to chloride (Figure 1D).^[24] Like avYFP-H148Q and phiYFP, the chloride binding pocket in *lanYFP*-V171A/N174T is near the chromophore but is made up of the residues H62, R88, T153, Y175, and a highly ordered water molecule that is stabilized by hydrogen bonds from M143 and R195.^[24] To note, R195 is spatially located where Y203 is in avYFP-H148Q, E²GFP, and phiYFP, thus removing any hydrogen bonding interactions with chloride and π -stacking with the chromophore Y66.^[6,15,22,24] This could not be determined from the multiple sequence alignment alone, owing to the low sequence identity of these proteins (Figure S1 in the Supporting Information). Monomerization of *lanYFP* to mNeonGreen, the brightest green fluorescent protein reported to date, does not eliminate the chloride binding pocket.^[24,25] When crystallized under basic conditions, mNeonGreen is not bound to chloride due to carboxylation of K143, but when crystallized under acidic conditions, mNeonGreen is clearly bound to chloride at 70% occupancy by the residues H62, R88, S153, T173, and Y175 (Figures 1E and 1F).^[24] These structural observations suggest that chloride could influence the optical properties of mNeonGreen, but this has not yet been described. Herein, we show that mNeonGreen is a pH-dependent, turn-on fluorescent protein sensor for chloride that uniquely operates, independently of a fused fluorescent protein, by shifting the chromophore equilibrium from the weakly fluorescent phenol form to the highly fluorescent

phenolate form. Anion selectivity combined with structure-based mutagenesis of R195 in the chloride binding pocket further confirm the chloride sensing mechanism.

Consistent with that previously reported, apo-mNeonGreen has one major absorption band that corresponds to the phenolate form of the chromophore at 505 nm, which undergoes conversion to the phenol form at 400 nm as a function of pH (Figure S2).^[25,26] Based on this data, we determined that mNeonGreen, in the absence of chloride, has a pK_a of 5.7, which is consistent with previous studies.^[25,26] Here, we find that this chromophore equilibrium is also affected by chloride. The chromophore pK_a decreases to 4.7 in the presence of 25 mM chloride, with a large absorbance change observed at pH 4.5 ($\epsilon_{505} = 10,021 \text{ M}^{-1}\cdot\text{cm}^{-1}$, $\epsilon_{505+\text{Cl}} = 26,884 \text{ M}^{-1}\cdot\text{cm}^{-1}$; Figure S2). Encouraged by these results, we further evaluated the absorbance and fluorescence properties of mNeonGreen as a function of chloride in 50 mM sodium acetate buffer at pH 4.5. Addition of chloride shifts the absorbance from 400 nm to 505 nm, towards the phenolate form of the chromophore (Figure 2A). With $\lambda_{\text{ex}} = 495 \text{ nm}$, apo-mNeonGreen has a single emission peak at 520 nm ($\Phi = 0.18$, Figure S3). We note that with $\lambda_{\text{ex}} = 400 \text{ nm}$, apo-mNeonGreen has an emission maximum at the same wavelength (Figure S4). This has been attributed to the phenolate form of the chromophore, so $\lambda_{\text{ex}} = 400 \text{ nm}$ was not further investigated in our study.^[26] Upon the addition of 25 mM chloride, the fluorescence intensity increases by about 21-fold with no shift in the emission maximum ($\Phi = 0.40$, Figures 2B and S3). The apparent K_d for chloride binding to mNeonGreen is $9.8 \pm 0.3 \text{ mM}$ ($\eta_H = 0.72 \pm 0.01$, Figure S5). However, we do note that the chromophore equilibrium and the response to chloride can be affected by the buffer and buffer molarity (Figures S6 and S7).

Taken together, our data indicate that chloride binding favors the conversion of the phenol form to the highly fluorescent phenolate form of the chromophore, which is in contrast to avYFP-H148Q and phiYFP in the ground state.^[5,6,8,21] We speculate that this unique sensing mechanism could be linked to the fact that mNeonGreen has more ionizable residues in the chloride binding pocket that could be positively charged in the presence of chloride at pH 4.5, thus stabilizing both the phenolate form of the chromophore and the coordination complex.^[24] Moreover, the lack of a clear proton transport pathway from the bulk solution to protonate the phenolate form of the chromophore could also contribute to these observations.^[24–26] Of note, avYFP-H148Q variants with positively charged residues in the chloride binding pocket or an altered proton transport pathway have lower chromophore pK_a values and enhanced chloride binding but retain the same sensing mechanism as avYFP-H148Q described above.^[12]

We next tested the fluorescence response of mNeonGreen to chloride from pH 4 to pH 8 (Figure 3A). Unsurprisingly, little change is observed at pH > 5.5 because the chromophore is largely in the phenolate form. Even though at pH 5.5 the phenol form of the chromophore exists, the turn-on fluorescence response is the greatest at pH 4.5. Based on these results, we evaluated the anion selectivity of mNeonGreen at pH 4.5 (Figure 3B, Figures S8–S12, Table S1). The fluorescence intensity increases in the presence of bromide (15-fold, $K_d = 1.8 \pm 0.2 \text{ mM}$), iodide (11-fold, $K_d = 1.7 \pm 0.4 \text{ mM}$), nitrate (7-fold, $K_d = 2.5 \pm 0.4 \text{ mM}$), and dihydrogen phosphate (2.5-fold, $K_d = 79 \pm 15 \text{ mM}$). However, at higher concentrations, these anions quench the fluorescence to varying degrees (Figure 3B). Only

fluorescence quenching is observed in the presence of hydrogen sulfate (72%, $K_d = 0.9 \pm 0.2$ mM). From the absorbance spectra, the effects of these anions are also seen in the chromophore equilibrium, indicating that these anions bind near the chromophore Y66 like chloride but could interact with residues in the binding pocket differently due to differences in size, shape, and charge density (Figures S8–S12). In comparison to mNeonGreen, phiYFP and avYFP/GFP-based chloride sensors are sensitive to halides and nitrate but have little to no response to phosphate and sulfate.^[4–8,15,21]

Finally, to confirm the chloride-sensing mechanism, we targeted the mNeonGreen chloride binding pocket for mutagenesis. Based on the crystal structures of mNeonGreen and avYFP-H148Q, we introduced the R195Y mutation (Figures 1A and 1F).^[6,24] As described above, R195 does not directly interact with the chloride ion and is spatially located where Y203 is in avYFP-H148Q (Figure 1F). We hypothesized that the R195Y mutation would destabilize the hydrogen bonding network with K143 but would introduce hydrogen bonding with chloride and possibly π -stacking with the chromophore Y66, thus affecting the sensing mechanism. Like wild-type mNeonGreen, the chromophore Y66 in apo R195Y can undergo conversion from the phenolate form (415 nm) to the phenol form (490 nm) as a function of pH ($pK_a = 5.3$; Figure S13). However, the chromophore pK_a increases to 5.6 in the presence of 200 mM chloride, with a large absorbance change observed at pH 4.5 ($\epsilon_{490} = 12,687 \text{ M}^{-1}\cdot\text{cm}^{-1}$, $\epsilon_{490+\text{Cl}} = 8,546 \text{ M}^{-1}\cdot\text{cm}^{-1}$; Figure S13). At this pH, titration with chloride decreases the absorbance at 490 nm and shifts the absorbance at 415 nm to 395 nm, with no shift in the chromophore equilibrium (Figure 4A). With $\lambda_{\text{ex}} = 490$ nm, the phenolate form has a single emission peak at 520 nm ($\Phi = 0.019$) that is quenched by 56% upon the addition of chloride ($\Phi = 0.010$; Figure 4B and Figure S3). Introduction of the R195Y mutation also decreases the affinity for chloride ($K_d = 26 \pm 4$ mM, $\eta_H = 0.75 \pm 0.05$; Figure S14). Similarly, fluorescence quenching is observed in the presence of bromide (42%, $K_d = 20 \pm 4$ mM), iodide (83%, K_d not determined), nitrate (35%, $K_d = 12 \pm 2$ mM), and hydrogen sulfate (12%, $K_d = 6.4 \pm 2$ mM), but interestingly no response to dihydrogen phosphate is observed (Figure 4C, Figure S15–S19, Table S2). The apparent K_d for iodide could not be determined due to weak binding or collisional quenching (Figure S16). Taken together, these data show that even though R195 does not directly interact with chloride in the wildtype, it is a key residue that contributes to the sensing mechanism and selectivity. More importantly, a tyrosine residue is not needed to π -stack with the chromophore Y66 for sensitivity to chloride and other anions. Clearly the properties of R195Y are different from wild-type mNeonGreen, but interestingly, are similar to that previously reported for E²GFP described above.^[15] Additional studies will be required to determine if mNeonGreen-R195Y operates via a static quenching mechanism or has a completely different chloride binding pocket like E²GFP.

In closing, we have presented the structure-guided identification and spectroscopic characterization of mNeonGreen, an engineered monomer of the yellow fluorescent protein *IanYFP* from the cephalochordate *B. lanceolatum*, as a turn-on fluorescent protein sensor for chloride. Our results show that chloride binding lowers the chromophore pK_a and shifts the equilibrium away from the weakly fluorescent phenol form to the highly fluorescent phenolate form. Moreover, through mutagenesis, we link this unique sensing mechanism to R195, a non-coordinating residue in the chloride binding pocket. Overall, our study

showcases how natural and laboratory evolution of fluorescent proteins can not only teach us how to build chloride coordination spheres but also provide unexplored starting points to create new fluorescent protein-based tools for chloride and other anions. To this end, efforts are currently underway to shift the operational pH and improve the selectivity of mNeonGreen for imaging cellular chloride.

Experimental Section

General:

All reagents and chemicals were purchased from Sigma-Aldrich, VWR International (Radnor, PA), or Thermo Fisher Scientific unless stated otherwise.

Sequence alignment, percent identity matrix, and crystal structures:

The sequence alignment and percent identity matrix was generated using the Clustal Omega software v1.2.4 and the Clustal Omega software v2.1, respectively.^[27] The crystal structures of avYFP-H148Q (PDB ID: 1F09), E²GFP (PDB ID: 2O24), phiYFP (PDB ID: 4HE4), *Ian*YFP (PDB ID: 5LTQ), and mNeonGreen (PDB IDs: 5LTR and 5LTP) in Figure 1 were acquired from the Protein Data Bank^[23] (www.rcsb.org) and images were generated using MacPyMol v1.7 (Schrödinger, LLC, New York City, NY).

Plasmid construction and site-directed mutagenesis:

The gene-encoding wild-type mNeonGreen (UniProt ID: A0A1S4NYF2) was codon optimized for *Escherichia coli* K12 and cloned between the NdeI and BamHI restriction sites with an N-terminal polyhistidine-tag and a C-terminal stop codon into the pET-28b(+) vector (Figure S20, GenScript, Piscataway, NJ). The polymerase chain reaction (PCR) to generate the plasmid-encoding mNeonGreen-R195Y was carried out as follows: 1.5 µL of the 10 µM forward and reverse primers (Table S3, Sigma-Aldrich), 1 µL of the 10 ng/µL mNeonGreen template plasmid, 0.5 µL of 10 mM deoxynucleotides stock solution, 1 µL of DMSO, 5 µL of 5× Phusion GC buffer, and 0.25 µL of Phusion DNA polymerase (Phusion High Fidelity PCR Kit, catalogue no. M0530; New England Biolabs, Ipswich, MS) were combined and diluted to a final volume of 25 µL with autoclaved water. The PCR reaction conditions are listed in Table S4. Following PCR, the template DNA was degraded with DpnI (1 µL; catalogue no. R0176L; New England Biolabs) for 2 h at 37 °C, isolated with agarose gel electrophoresis, extracted, and purified with the Zymoclean Gel DNA Recovery Kit (catalogue no. D4002; Zymo Research, Irvine, CA). Ten microliters of the purified PCR product was recircularized with 10 µL of Gibson Assembly Master Mix (catalogue no. E2611L; New England Biolabs) for 1 h at 50 °C. The resulting product was isolated with the DNA Clean & Concentrator Kit (catalogue no. D4004; Zymo Research) and transformed into *E. coli* 10G ELITE Electrocompetent Cells (catalogue no. 600520; Lucigen, Middleton, WI), plated onto Luria Broth (LB; catalogue no. L24040500, Research Products International, Mount Prospect, IL) agar (catalogue no. 214030; BD Biosciences, San Jose, CA) plates containing 50 µg/mL kanamycin sulfate, and incubated for 16–18 h at 37 °C (New Brunswick Innova 42R Shaker; catalogue no. M13350014; Eppendorf, Hamburg, Germany). Single clones were picked into 2xYT (5 mL) containing kanamycin sulfate (50 µg/mL) and incubated overnight at 37 °C with shaking at 250 rpm. The following day, the

plasmids were isolated using the QIAprep Spin Miniprep Kit (catalogue no. 27106; Qiagen, Hilden, Germany) and verified by sequencing (Eurofins Scientific, Louisville, KY).

Large-scale protein expression and purification:

Plasmids encoding the wild-type mNeonGreen and mNeonGreen-R915Y were freshly transformed into *E. coli* EXPRESS BL21(DE3) Competent Cells (catalogue no. 60300; Lucigen, Middleton, WI) for large-scale protein isolation. Single colonies were picked into 2xYT medium (7 mL), containing kanamycin sulfate (50 µg/mL), in culture tubes (catalogue no. 1496132; Thermo Fisher Scientific) and incubated overnight at 37 °C with shaking at 250 rpm. The following day, 6 mL of the overnight culture was diluted into 2xYT (600 mL), containing kanamycin sulfate (50 µg/mL), in 2 L baffled flasks and incubated for 2 h at 37 °C with shaking at 250 rpm. Once the OD₆₀₀ reached ≈0.6–0.8, protein expression was induced with isopropyl β-D-thiogalactopyranoside (IPTG; 1 mM, catalogue no. I2481C50; Gold Biotechnology, Olivette, MO) and incubated for 20–22 h at 37 °C with shaking at 250 rpm. The next day, the cells were collected by centrifugation for 25 min at 3,000g and 4 °C (5810 R, catalogue no. 2231000382; Eppendorf, Hamburg, Germany), resuspended in Tris buffer (25 mL, 20 mM, pH 7.5) containing sodium chloride (200 mM), magnesium chloride (5 mM), and deoxyribonuclease I (30 µg/mL), and stored at –20 °C until further use. On the day of protein purification, the cell pellet was thawed for 30 min at room temperature, vortexed, and lysed using sonication at 35% amplitude, 15 s pulse on, and 45 s pulse off for 5 min, followed by ultracentrifugation at 18,000g (Optima XPN, catalogue no. A99839; Beckman Coulter, Brea, CA) for 25 min at 4 °C. A 1-mL nickel nitrilotriacetic acid (Ni-NTA) affinity column (HisTrap, catalogue no. 17-5248-02; GE Healthcare, Little Chalfont, UK) was equilibrated with 5 column volumes (CV) of Tris running buffer (50 mM, pH 7.5) containing sodium chloride (100 mM) and imidazole (10 mM), by using the NGC Quest 10 Chromatography System (catalogue no. 7880001; Bio-Rad Laboratories, Hercules, CA). The clarified lysate was loaded into a 90-mL sample loop (DynaLoop 90, catalogue no. 7500476; Bio-Rad Laboratories, Hercules, CA) and loaded onto the equilibrated Ni-NTA column with a 1 mL/min flow rate. The column was then washed with Tris running buffer (12 CV, 50 mM, pH 7.5) containing sodium chloride (100 mM) and imidazole (10 mM). The histidine-tagged protein was eluted over 20 CV with a 0–100% gradient of the Tris running buffer described above and Tris elution buffer (50 mM, pH 7.5) containing sodium chloride (100 mM) and imidazole (500 mM). The fractions with an absorbance at 505 nm were combined, and the buffer was exchanged three to five times into 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES, 20 mM, pH 7.5) with an EMD Millipore Amicon Ultra-15 Centrifugal Filter Unit with a 10 kDa molecular weight cut-off (catalogue no. UFC901024; MilliporeSigma, Burlington, MA) and stored at 4 °C until further use. At least two batches of wild-type mNeonGreen and mNeonGreen-R195Y were independently purified and tested.

SDS-PAGE, Coomassie staining, protein concentration, and extinction coefficient determination:

The purity of wild-type mNeonGreen and mNeonGreen-R195Y was tested by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a previously reported procedure (Figure S21).^[21] The ThermoScientific Pierce Coomassie (Bradford) Protein Assay Kit (catalogue no. PI23200; Thermo Fisher Scientific, Waltham, MA) was used to

determine the protein concentrations. To determine the extinction coefficient, purified mNeonGreen and mNeonGreen-R195Y in HEPES buffer (20 mM, pH 7.5) were diluted to 7 and 14 μM , respectively, in sodium acetate buffer (50 mM, pH 4.5) or sodium acetate buffer (50 mM, pH 4.5) containing sodium chloride (28.5 or 228.5 mM) in a 0.2 cm $\times$ 1 cm quartz cuvette (0.4 mL; catalogue no. 115F1040; Hellma USA, Plainview, NJ). Absorbance spectra were collected from 300–650 nm with a 5-nm step size using the Agilent Cary 7000 Universal Measurement Spectrophotometer (catalogue no. G687364000, Agilent, Santa Clara, CA). The Beer-Lambert law ($A = \epsilon lc$) was used where the path length (l) is 1 cm. The extinction coefficient of wild-type mNeonGreen at pH 4.5 for the 505 nm absorbance peak in the absence and presence of 25 mM chloride is $10,021 \pm 399 \text{ M}^{-1}\cdot\text{cm}^{-1}$ and $26,884 \pm 1538 \text{ M}^{-1}\cdot\text{cm}^{-1}$, respectively. The extinction coefficient of mNeonGreen-R195Y at pH 4.5 for the 490 nm absorbance peak in the absence and presence of 200 mM chloride is $12,687 \pm 931 \text{ M}^{-1}\cdot\text{cm}^{-1}$ and $8,546 \pm 92 \text{ M}^{-1}\cdot\text{cm}^{-1}$, respectively. The average of six technical replicates with standard error of the mean is reported.

Chromophore pK_a determination of wild-type mNeonGreen and mNeonGreen-R195Y:

To determine the chromophore pK_a in the absence and presence of sodium chloride, purified wild-type mNeonGreen and mNeonGreen-R195Y in HEPES buffer (20 mM, pH 7.5) were diluted to 7 and 14 μM , respectively, in sodium acetate buffer (50 mM, pH 3.5 to 8.5) or sodium acetate buffer containing sodium chloride (28.5 mM or 228.5 mM). Trace amounts of concentrated sulfuric acid or sodium hydroxide were used to adjust the solution pH. Absorbance spectra were collected from 300–650 nm with a 5-nm step size on a plate reader (Spark 10M, catalogue no. 30086376; Tecan, Männedorf, Switzerland). The pH was plotted versus the average absorbance values for wild-type mNeonGreen and mNeonGreen-R195 at 505 nm and 490 nm, respectively, in KaleidaGraph v4.5 (Synergy Software, Reading, PA) and fitted to the Henderson-Hasselbach equation to determine the chromophore pK_a . The average of six technical replicates with standard error of the mean is reported (Figures S2 and S13).

Chloride titration and determination of the apparent dissociation constant (K_d) for wild-type mNeonGreen and mNeonGreen-R195Y:

Purified wild-type mNeonGreen and mNeonGreen-R195Y in HEPES buffer (20 mM, pH 7.5) were diluted to 56 and 112 μM , respectively, in sodium acetate buffer (50 mM, pH 4.5) and further diluted to 7 and 14 μM , respectively, with buffer only or buffer containing sodium chloride (1.1, 3.5, 7.1, 14.3, 18, 23, 28.5, 57, 114.3, and 228.5 mM). Absorbance spectra were collected from 300–650 nm with a 5-nm step size on the plate reader. Wild-type mNeonGreen was excited at 495 nm, and the emission was collected from 510–650 nm with a 5-nm step size, 30 flashes, and gain of 65 on the plate reader. mNeonGreen-R195Y was excited at 490 nm, and the emission was collected from 505–650 nm with a 5-nm step size, 30 flashes, and gain of 75 on the plate reader. The average of six technical replicates with standard error of the mean is reported (Figures 2 and 4).

For the K_d , the wild-type mNeonGreen and mNeonGreen-R195Y emission spectra were integrated from 510–650 nm and 505–650 nm, respectively, using the *trapz* function in MATLAB R2017a (MathWorks, Natwick, MA). The apparent K_d was determined by

plotting the chloride concentration $[Cl^-]$ versus the relative emission response $[F = (F_{obs} - F_{min}) / (F_{max} - F_{min})]$ in KaleidaGraph in which F_{obs} is the integrated area of the observed emission, and F_{min} and F_{max} are the integrated emission of the 0 mM and the highest $[Cl^-]$, respectively. Since fluorescence quenching was observed at higher chloride concentrations, the concentration with the largest turn-on fluorescence response was used to determine the K_d . The apparent K_d was calculated using the following Equation 1:

$$F = [Cl^-] / (K_d + [Cl^-]) \quad (1)$$

The Hill coefficient was also determined by plotting $\log [(F_{min} - F_{obs}) / (F_{obs} - F_{max})]$ vs. $\log [Cl^-]$ to a linear fit model ($y = mx + b$). The average of six technical replicates with standard error of the mean is reported (Figures S5 and S14).

Fluorescence quantum yield determination for mNeonGreen and mNeonGreen-R195Y:

The fluorescence quantum yields of wild-type mNeonGreen and mNeonGreen-R195Y were determined in the presence and absence of chloride, respectively. Serial dilutions of the purified proteins were prepared in sodium acetate buffer (50 mM, pH 4.5) or sodium acetate buffer (50 mM, pH 4.5) with sodium chloride (25 or 200 mM). Serial dilutions of fluorescein in sodium hydroxide (100 mM, $\Phi = 0.92$)^[28] were used as a reference standard. Absorbance spectra were collected from 300–650 nm with a 5-nm step size. Excitation was provided at 488 nm, and the emission was collected from 505–650 nm with a 5-nm step size, 30 flashes, and gain of 75 on the plate reader. The absorbance values for fluorescein were plotted versus the integrated emission from 505–650 nm to generate a standard curve ($R^2 > 0.99$). The wild-type mNeonGreen and mNeonGreen-R195Y absorbance values at 505 and 490 nm, respectively, were plotted versus the integrated emission from 505–650 nm to generate linear plots ($R^2 > 0.99$). The wild-type mNeonGreen and mNeonGreen-R195Y fluorescence quantum yields were calculated by using Equation 2:

$$\Phi_{FP} = (\text{slope}_{FP} / \text{slope}_{ref}) (\eta_{FP}^2 / \eta_{ref}^2) \quad (2)$$

in which FP is the protein sample, ref. is the standard, and η is the refractive index for water.^[29] The average of six technical replicates with standard error of the mean is reported (Figure S3).

pH profile for wild-type mNeonGreen:

Purified wild-type mNeonGreen in HEPES buffer (20 mM, pH 7.5) was diluted to 7 μ M in sodium acetate buffer (50 mM, pH 4, 4.5, 5, and 5.5), 2-(*N*-morpholino)ethanesulfonic acid buffer (MES; 50 mM, pH 6 and 6.5), and HEPES buffer (50 mM, pH 7 and 8), or sodium chloride (28.5 mM) in the corresponding buffers. Absorbance spectra were collected from 300–650 nm with a 5-nm step size on the plate reader. Wild-type mNeonGreen was excited at 495 nm, and the emission was collected from 510–650 nm with a 5-nm step size, 30 flashes, and gain of 60. Wild-type mNeonGreen emission spectra were integrated from 510–

650 nm and 505–650 nm, respectively, using the *trapz* function in MATLAB. The average of six technical replicates with standard error of the mean is reported (Figure 3A).

Anion selectivity of wild-type mNeonGreen and mNeonGreen-R195Y:

Purified wild-type mNeonGreen and mNeonGreen-R195Y in HEPES buffer (20 mM, pH 7.5) were diluted to 56 and 112 μ M, respectively, in sodium acetate buffer (50 mM, pH 4.5) and further diluted to 7 and 14 μ M, respectively, with buffer only or buffer containing sodium bromide, sodium iodide, sodium nitrate, sodium dihydrogen phosphate, or sodium hydrogen sulfate (3.5, 7.1, 14.3, 18, 23, 28.5, 57, 114.3, and 228.5 mM). Absorbance spectra were collected from 300–650 nm with a 5-nm step size on the plate reader. Wild-type mNeonGreen was excited at 495 nm, and the emission was collected from 510–650 nm with a 5-nm step size, 30 flashes, and gain of 60 on the plate reader. mNeonGreen-R195Y was excited at 490 nm, and the emission was collected from 505–650 nm with a 5-nm step size, 30 flashes, and gain of 75 on the plate reader. The average of six technical replicates with standard error of the mean is reported (Figures S8–S12 and S15–S19).

For the K_d , the wild-type mNeonGreen and mNeonGreen-R195Y emission spectra were integrated from 510–650 nm for salt all concentrations using the *trapz* function in MATLAB. The apparent K_d for each anion was determined as in Equation 1. Since fluorescence quenching was observed at higher anion concentrations, the concentration with the largest turn-on fluorescence response was used to determine the K_d values. The average of six technical replicates with the standard error of the mean is reported (Figures S8–S12 and S15–S19, Tables S1 and S2).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Comparison of the anion binding sites in the crystal structures (left) and chromophores (right) of A) *avYFP-H148Q* with iodide (PDB ID: 1F09), B) *E²GFP* with chloride (PDB ID: 2O24), C) *phiYFP* (PDB ID: 4HE4), D) *IanYFP* with chloride (PDB ID: 5LTQ), E) *mNeonGreen* crystallized at pH 8.0 where KCX143 corresponds to a carboxylated lysine residue (PDB ID: 5LTR), and F) *mNeonGreen* with chloride crystallized at pH 4.5 (PDB ID: 5LTP). Chromophores are shown as sticks in yellow, and residues within 4 Å of the known or predicted anion binding sites are shown as sticks in gray with oxygen atoms in red and nitrogen atoms in blue. Labels correspond to the single letter amino acid code and residue

number. The iodide and chloride ions and water are shown as spheres in purple, green, and red, respectively.

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Figure 2.

Spectroscopic characterization of mNeonGreen in 50 mM sodium acetate buffer at pH 4.5 with chloride. A) UV/Vis and B) fluorescence spectra of 7 μ M mNeonGreen in the presence of 0 (bold), 1, 3.1, 6.3, 12.5, 16, 20, and 25 mM (red) chloride. Arrow direction corresponds to increasing chloride concentrations. Excitation was provided at 495 nm, and the emission was collected from 510–650 nm. The average of six technical replicates with standard error of the mean is reported.

Figure 3.

The pH profile and anion selectivity of mNeonGreen. A) Fluorescence response of 7 μ M mNeonGreen to 0 mM (black bar) and 25 mM (gray bar) chloride from pH 4–8. Spectra were acquired in 50 mM sodium acetate buffer (pH 4, 4.5, 5, and 5.5), 50 mM MES buffer (pH 6 and 6.5), and 50 mM HEPES buffer (pH 7 and 8). B) Fluorescence response of 7 μ M mNeonGreen to 0 mM (F_0 , black bar), 12.5, 25, 50, 100, and 200 mM (white bar) chloride, bromide, iodide, nitrate, dihydrogen phosphate, and hydrogen sulfate in 50 mM sodium acetate at pH 4.5. The sodium salt was used for each anion tested. Excitation was provided at 495 nm, and the emission was integrated from 510–650 nm. The average of six technical replicates with standard error of the mean is reported.

Figure 4.

Spectroscopic characterization of mNeonGreen-R195Y in 50 mM sodium acetate buffer at pH 4.5. A) UV/Vis and B) fluorescence spectra of 14 μ M mNeonGreen-R195Y in the presence of 0 (bold), 12.5, 25, 50, 100, and 200 mM (red) chloride. Arrow direction corresponds to increasing chloride concentrations. Excitation was provided at 490 nm, and the emission was collected from 505–650 nm. C) Fluorescence response of 14 μ M mNeonGreen-R195Y to 0 mM (F_0 , black bar), 12.5, 25, 50, 100, and 200 mM (white bar) chloride, bromide, iodide, nitrate, dihydrogen phosphate, and hydrogen sulfate. The sodium salt was used for each anion tested. Excitation was provided at 490 nm, and the emission was integrated from 510–650 nm. The average of six technical replicates with standard error of the mean is reported.